An Investigation Into The Inhibition Of Interferon Action By Fowlpox Virus Proteins

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

Elizabeth Clare Pollitt, BSc. (Leeds).
Department of Microbiology and Immunology,
University of Leicester.

January 1997

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

An Investigation Into The Inhibition Of Interferon Action By Fowlpox Virus Proteins

By

Elizabeth Clare Pollitt

This work is concerned with the ability of poxviruses to overcome the antiviral state induced by interferons. A major part of the antiviral state induced by interferons is mediated by PKR, a serine/threonine protein kinase activated by dsRNA which phosphorylates eIF-2α, thereby inhibiting translation initiation.

Many viruses interfere with the PKR response. Vaccinia virus (VV), like all poxviruses, replicates in the cytoplasm where it is directly exposed to the action of PKR (and 2'-5'A synthetase). In order to replicate it must overcome the action ot interferons and PKR (and 2'-5'A synthetase). VV encodes proteins which bind interferons and encodes two proteins known to interfere with PKR: E3L, encodes a dsRNA-binding protein and K3L, encodes a homologue of eIF-2α.

The investigation has shown that an avian poxvirus, fowlpox virus (FPV), is resistant to more than 8 U/ml chicken IFN. FPV is capable of rescuing an IFN-sensitive virus, Semliki Forest virus (SFV) from the effects of chicken IFN. FPV is also capable of promoting the replication of SFV in the presence or absence of IFN. These results suggest that FPV does make antagonists of IFN.

Biochemical assays were used to test IFN-treated avian cell extracts for the ability to phosphorylate known substrates of PKR. Major changes occur in the phosphorylation profile of CEF treated with IFN. Poly IC-binding proteins from IFN-treated CEF are capable of phosphorylation of histone proteins and phosphorylation of mammalian eIF-2α peptides. These data suggest the presence of a protein(s) with properties similar to PKR. Attempts to clone an avian homologue of PKR from a commercial chicken cDNA library, proved unsuccessful.

A FPV homologue of E3L does not lie between the homologues of E2L and E4L. Southern blot analysis of FPV DNA suggested that homologues of E3L and K3L exist in a 5.3 kbp restriction fragment. The appropriate fragment has been cloned and sequenced. The sequence shows that FPV has potential ORFs to encode homologues of VV E10R, E11L, O1L, I1L, I2L, and I3L but no ORF for E3L, K3L or O2L could be found.

An Investigation Into The Inhibition Of Interferon Action By Fowlpox Virus Proteins

This thesis is dedicated to the inspirational Mais Davison and Meg Pollitt

Acknowledgments

I am indebted to Dr. Shaun Heaphy and Dr. Mike Skinner for their support, advice and input throughout this project. I would like to thank everybody in Lab 136 and G1A for their invaluable technical advice and patience:

Andy "Grump" Lear
Ben "Fluffy Bunny" Gale
Mal "Jammy Git" Haddrick
Jacquie "Save the World" Russell
Peter "Where's the Vodka?"
Pankash "Must be Monday, I'm Not Here"
Sarah "Mad Woman" Rogan
Phil "A Flash of Lightning Streaking Across The Sky" Green
Denise "Trypanosomiasis" Boulanger
Warren "Ranger" Thomas
Steve "Ordering" Laidlaw
Cliff "Hamster Killer" Luke
Arif "Panic" Anwar

A grateful acknowldegment of:
Dr. J. Tartaglia of Virogenetics, Inc., for providing the vaccinia virus mutants.
F. Puttock and S. Duggan, for CEF and HeLa cells.
Karen Mawditt and everbody in PNACL, for oligonucleotide synthesis.
Stuart (PNACL), for running automated sequencing gels.
BBSRC and IAH, for their financial and administrative support of the project.

A big thank you for the team of friends and family who delivered on a personal, financial and resource front - Moth, Bill, Snoopy, Hammy, YAF, Cliff & Gail, Sarah, Gary & Lucy, and Steve.

A final thank you to Paul for assistance with my lifestyle, for listening to the whinging and moaning, and for not understanding any of this.

Table of Contents

List of Figures and Tables

1. INTRODUCTION

2. MATERIALS AND METHODS

3. EVIDENCE FOR INHIBITION OF INTERFERON ACTION BY FOWLPOX VIRUS

4. ANALYSIS OF EXTRACELLULAR PROTEINS PRODUCED BY FOWLPOX VIRUS INFECTED CHICK EMBRYO FIBROBLASTS

5. BIOCHEMICAL TESTS FOR PKR ACTIVITY IN AVIAN CELLS

6. PROBING FOR AN AVIAN HOMOLOGUE OF PKR BY HYBRIDIZATION AND PCR

7. CLONING AND SEQUENCING OF A GENOMIC SEGMENT OF FOWLPOX VIRUS DNA WHICH HYBRIDIZES TO VACCINIA VIRUS E3L AND K3L DNA

Chapter 1

Introduction

1. INTRODUCTION

1.1 A History of Interferon Studies

In 1957 Isaacs and Lindenmann described the release of a new factor, from chick chorio-allantoic membranes incubated with heat-inactivated influenza virus. They showed that this new factor was able to induce interference with complete influenza virus in fresh pieces of membrane, and called this factor interferon.

1.1.1. Cloning of Interferons

Interferons are now known to be a family of regulatory glycoproteins which have been characterized and divided into two groups. Type I, interferons (pH 2 stable): α (leukocyte) and β (fibroblast), which are induced by virus infection and type II (immune or acid-labile), interferon γ, which is produced by T-lymphocytes and natural killer cells when they are stimulated by antigen or mitogen (for reviews see Samuel, 1988, Samuel, 1991).

Human fibroblast interferon was cloned in 1979 by Taniguchi *et al* and sequenced in 1980 (Taniguchi *et al.*,) as was human leukocyte interferon (Mantei *et al.*, 1980). A comparison of human fibroblast and leukocyte interferons showed that they are structurally similar. A chicken interferon gene has been cloned and sequenced (Sekellick *et al.*, 1994). The gene has amino acid identity with mammalian IFNs of: alpha 24%, beta 20%, and gamma 3%. The gene has been classified as a type I interferon on the basis of predicted secondary structure (Sekellick *et al.*, 1994). The chicken IFN-γ gene encodes a protein of 145 amino acids with 32 % homology to human IFN-γ and highly conserved motifs that are present in all mammalian IFN-γ (Digby & Lowenthal, 1995).

1.1.2. Interferons, Cytokines and the Immune Response

T-lymphocytes can be divided into two groups, $CD4^+$ helper cells and $CD8^+$ cytotoxic cells. When an antigen is recognized in association with MHC-II (found only on the surface of immune system cells), $CD4^+$ T-cells release cytokines and other factors to attract inflammatory cells to the site of infection. $CD4^+$ T-cells also aid antibody production by B-cells and recruit $CD8^+$ T-cells. $CD8^+$ T-cells kill cells when they recognize antigen in association with MHC-I (found on all cells in the body except red blood cells) and also release antiviral cytokines.

IFN-γ is predominantly produced by a subset of $CD4^+$ T-cells known as Th1 cells. Th1 cells secrete interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor β (TNF-β) (Mosmann *et al.*, 1986) which stimulate cellular immunity. Th2 cells secrete IL-4, Il-5 and IL-10 (Mosmann *et al.*, 1986) which result in antibody production by B cells. The production of Th1 cytokines is increased by response to intracellular pathogens and is associated with macrophage activation and IgG production (Belardelli, 1995). Th2 cytokine production is predominantly induced by allergic reactions and helminth infections, and is mainly associated with IgE, eosinophil and mast cell production (Belardelli, 1995). The cytokines produced by the subsets of Th cells regulate each other's functions (Seder & Paul, 1994), as shown in Fig. 1.1 (Belardelli, 1995). Th cell subsets are derived from a common precursor which secretes high levels of IL-2 but little IL-4 or IFN-γ, differentiation of which can be affected by many factors (Belardelli, 1995).

Monocytes/macrophages produce TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12 and type I IFNs which can affect the differentiation of T cells towards Th1 or Th2 cells. Macrophages are the first producers of type I IFNs in response to infection (De Maeyer & De Maeyer-Guignard, 1988). IFN-β is expressed by resting (or unstimulated) macrophages (Gessani *et al.*, 1989) and is thought to play a role in macrophage differentiation (Vogel & Fertsch, 1984) as well as maintaining an antiviral state in macrophages (Vogel & Fertsch, 1987).

IFN-γ plays a major role in the regulation of immune responses, particularly by affecting the differentiation process of Th cells. IFN-γ inhibits the proliferation of Th2 cells, pushing differentiation towards a Th1 response (Gajewski & Fitch, 1988). IFN-γ also stimulates macrophage activation (Le *et al.*, 1983, Nathan *et al.*, 1984). Many of the effects of IL-12, resulting in the Th1 response are caused by production of IFN-γ by T cells and natural killer cells (NK) (Belardelli, 1995).

Type 1 IFNs (α and β) have a variety of effects. Type I IFNs can affect antibody production, although whether this results in inhibition or increased production depends on the dose and time of IFN addition (Siegel & Vilcek, 1986, Sonnenfeld, 1984). Type I IFNs enhance NK cell activity (Lindahl *et al.*, 1971), enhance macrophage activity (Vogel & Friedman, 1984) and increase MHC class I expression (Gresser, 1984).

IFN-α modulates the immunoglobulin isotype selection process, specifically inhibiting IgE production (Finkelman *et al.*, 1991). IFN-α inhibits Ag-induced proliferation of Th2 cells and cytokine production of TH2 cells (Parronchi *et al.*, 1992). IFN-α can increase the proportion of Th1 cells expressing IFN-γ and reduce the suppressive effects of IL-4 on IFN-γ production (Brinkmann *et al.*, 1993). IFN-α is thought to act as a modulator of allergic inflammation by inhibiting the Ag-induced recruitment of eosinophils and Th cells into tissue (Nakajima *et al.*, 1994).

1.1.3. Other Activities of Interferons

It has become increasingly clear that interferons play a role in many cell processes as well as having antiviral activity. Interferons have been found to inhibit cell motility (Brouty-Boye & Zetter, 1980), cell multiplication, and have pronounced effects on the cytoskeleton (Pfeffer *et al.*, 1980). Interferons have also been found to have antitumour activity (Gresser & Tovey, 1978). It is believed that the variety of responses to interferon are due to the cascade of intracellular signals prompted by interferon binding to the receptor (Lengyel *et al.*, 1995, Levy, 1995). It is known that more than 30 genes are transcriptionally activated by the cascade.

1.1.4. IFN and Double-Stranded RNA

In 1967 whilst searching for an IFN inducer, Field *et al* (1967) found that synthetic polynucleotide polymers, particularly poly IC, were capable of inducing IFN in rabbits in microgram amounts, and rendering mice and cell cultures resistant to viral infection. This and other similar reports lead to the idea that a viral nucleic acid may be responsible for IFN induction.

Protein synthesis was found to be inhibited when dsRNA was added to a cell free system (Ehrenfield & Hunt, 1971). Protein synthesis was also found to be decreased in interferon-treated vaccinia virus (VV)-infected mouse L-cells when VV RNA was detectable (Metz & Esteban, 1972). A decrease in EMC RNA translation occurred on interferon treatment of encephalomyocarditis virus (EMC) infected L-cells (Freidman *et al.*, 1972). Several groups then demonstrated that the inhibition of protein synthesis did not require the addition of dsRNA in cells infected with virus (Kerr *et al.*, 1974).

These and other reports led to the discoveries of two interferon-induced, dsRNA binding proteins, 2'-5'A synthetase and PKR.

Fig. 1.1. Diagram to Show the Effects of IFNs on the Th Response

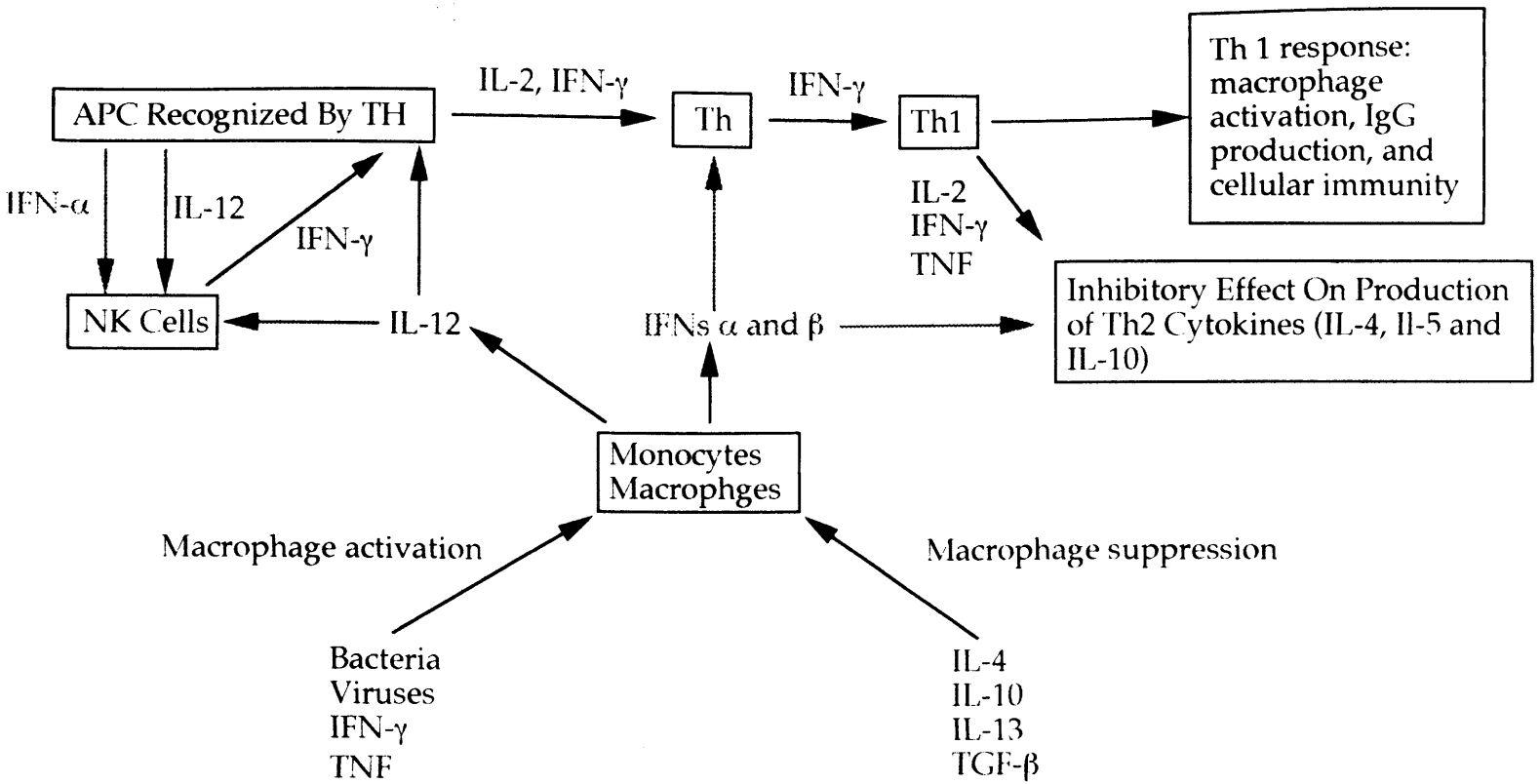


Fig. 1.1. Diagram to Show the Effects of IFNs on the Th Response
The diagram is reproduced from Belardelli, 1995.

1.2. 2′-5′A Synthetase

1.2.1. Action of 2'-5'A Synthetase

2′-5′A synthetase is an IFN induced, dsRNA binding protein which catalyses the 2′-5′ linkage of adenosine oligomers (pp{A2′p 5′A}$_n$, where n>2) from ATP. The linked oligomers are known as 2'-5'A. A latent cellular endoribonuclease, RNase L which preferentially cleaves ssRNA, is activated by binding 2'-5'A which leads to dimerization of RNase L (Cole *et al.*, 1996). The active endonuclease is a homodimer thought to preferentially cleave mRNA bound to dsRNA. The mechanism enabling RNase L to distinguish between viral and cellular mRNA is not fully understood (Nilsen & Baglioni, 1979).

The action of 2-5A is limited by a phosphodiesterase which cleaves 2′-5′ linked oligonucleotides, thus decreasing activation of RNase L. The phosphodiesterase also degrades the CCA terminus of tRNA, reducing the ability of tRNA to accept amino acids and causing inhibition of mRNA translation (Schmidt *et al.*, 1979).

1.2.2. Forms of 2'-5'A Synthetase

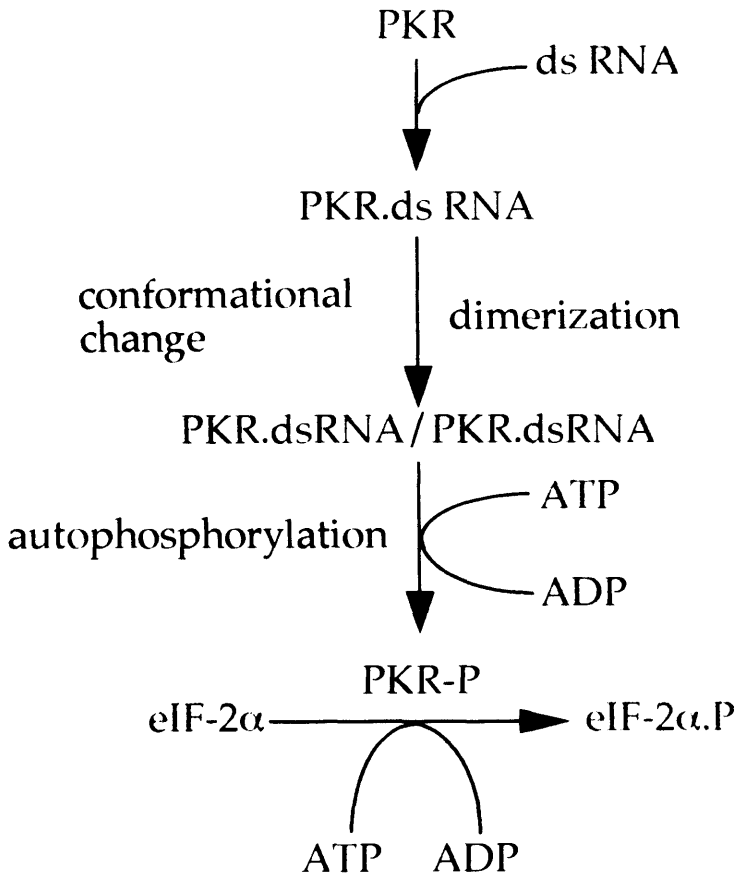
2'-5'A synthetase has been detected in many mammalian tissues (Chebath *et al.*, 1987, Hovanessian *et al.*, 1987a), and in chicken fibroblasts (Ball, 1979). Several forms of the enzyme exist in human cells: a 40-46 Kd protein, 69/71 Kd protein and a 100 Kd protein.

p40 and p46 vary only at the carboxy terminus as both are encoded by the same gene but variable splicing between the 5th and 6th exons leads to the size difference (Benech *et al.*, 1985a, Benech *et al.*, 1985b, Saunders *et al.*, 1985).

p69 and p71 are identical in amino acids 1 to 683, which contains two homologous, adjacent domains also found in p40 and p46. p69 and p71 are associated with the cell membrane (Hovanessian *et al.*, 1987b, Hovanessian *et al.*, 1988, Marié & Hovanessian, 1992). The p69, p71 and p100 forms of the protein are thought to derive from a different gene from that encoding the p40 and p46 forms (Marié & Hovanessian, 1992).

p100 is thought to play a role in RNA splicing (Sperling *et al.*, 1991) as it is associated with ribosomes. p100 and p69 are not usually expressed at similar levels in the same human cell types (Hovanessian *et al.*, 1988). p100 tends to form lower orders of oligonucleotides where as p69 often forms higher oligomers (Hovanessian *et al.*, 1987b).

Fig. 1.2. Schematic Diagram to Show Activation of PKR



1.3. PKR

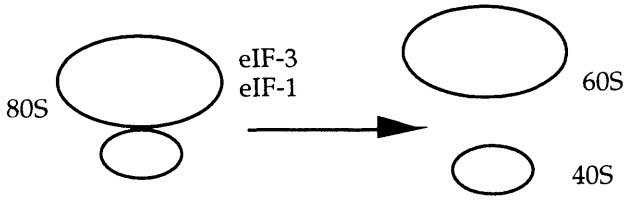
1.3.1. Introduction to PKR

PKR is the interferon-induced (Laurent *et al.*, 1985) double-stranded RNA-activated protein kinase (Lebleu *et al.*, 1976, Roberts *et al.*, 1976a). PKR is a serine/threonine kinase, with the protein kinase domains described by Hanks (Hanks *et al.*, 1988). When activated by dsRNA, PKR dimerizes (Langland & Jacobs, 1992, Ortega *et al.*, 1996, Patel *et al.*, 1995, Wu & Kaufman, 1996), becomes phosphorylated and phosphorylates the α subunit of elongation initiation factor 2, eIF-2 (Samuel, 1979). An overview of PKR activation is shown in Fig. 1.2.

1.3.2. Effects of eIF-2α Phosphorylation

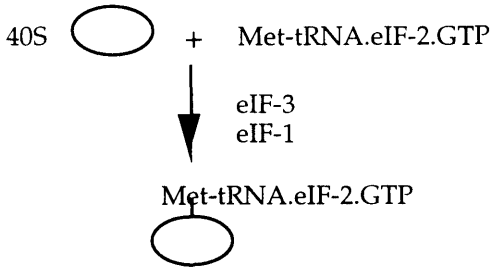
Phosphorylation of eIF-2α leads to the inhibition of translation initiation. Translation initiation can be divided into four steps (Hershey, 1993) :
1) The 80S ribosome dissociates into 60S and 40S subunits (aided by eIF-3 and eIF-1 which alter the equilibrium of association).

Fig. 1.3a. Step 1 of Translation Initiation



2) The free 40S subunits binds to initiator Met-tRNA,(a complex of Met-tRNA with eIF-2 and GTP) in a reaction stabilised by eIF-3 and eIF-1A.

Fig. 1.3b. Step 2 of Translation Initiation



3) mRNA binds to the 40S ribosome and the start codon AUG is found by one of two mechanisms:

a) Scanning: The mRNA is recognized by the cap-binding initiation factor eIF-4F. eIF-4F consists of three subunits: eIF-4E which recognizes the m^7G cap, eIF-4A which is an RNA-dependent ATPase, and the γ subunit. Together they have helicase activity and reduce the secondary structure at the 5' end of the mRNA. When the 40S ribosomal subunit binds Met-tRNA, it attaches at 5' end of the mRNA and scans through the UTR (untranslated region) to the initiation codon, AUG. The 40S ribosome subunit becomes stably bound to the mRNA when the anticodon of the tRNA interacts with the AUG of the mRNA.

b) Internal binding: This mechanism involves cap-independent binding of the 40S ribosome to an internal structural feature in the 5' UTR sequence of the mRNA, known as IRES (internal ribosome entry sequence). It is not commonly found in cells but is often found at the 5' end of picornaviral RNAs. The ribosome may bind near to the AUG or some distance upstream, in which case it scans to the start codon (Jackson *et al.*, 1990).

Fig. 1.3c. Step 3 of Translation Initiation (Scanning Mechanism)

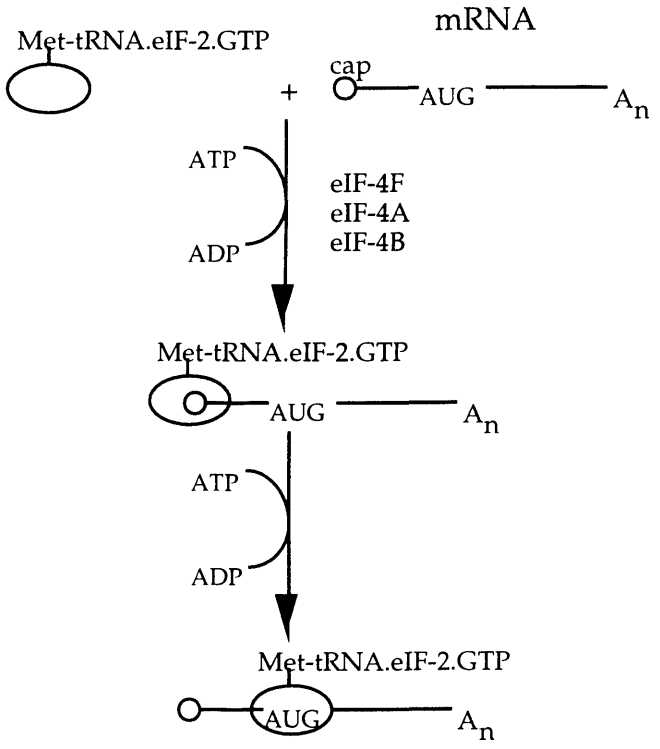


Fig. 1.3d. Step 4 of Translation Initiation

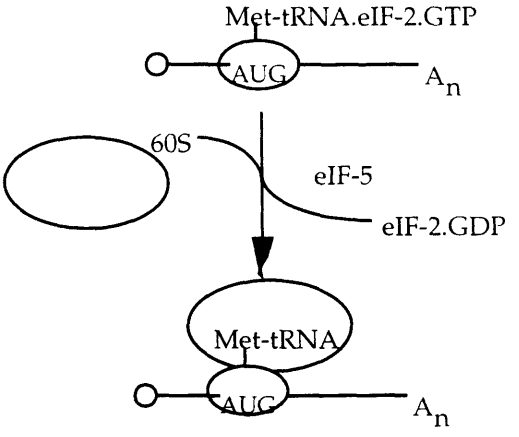


Fig. 1.3e. Recycling of eIF-2

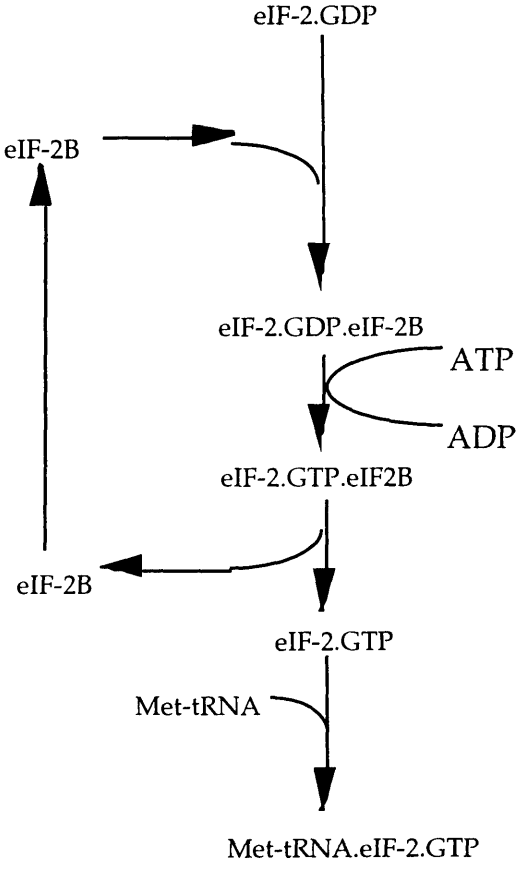


Fig. 1.4. Pathway of Translation Initiation

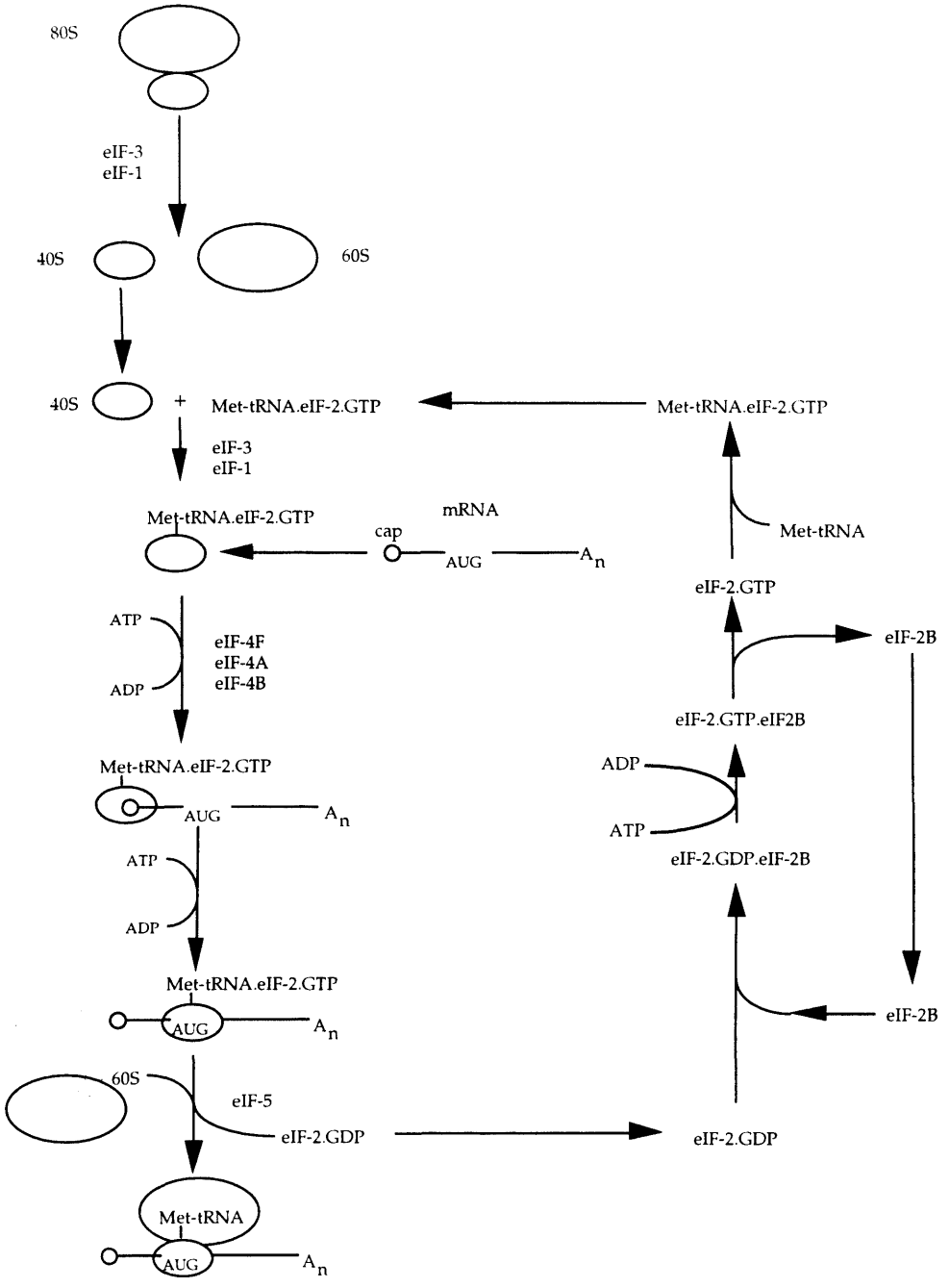


Diagram to show translation initiation, Fig. 1.4 is taken from Hershey, 1993.

4) GTP in the 40S complex is hydrolysed to GDP in an eIF-5-dependent reaction, allowing the 60S ribosome subunit to join. Further rounds of initiation can only occur if the GDP of eIF-2.GDP is exchanged for GTP. The exchange reaction requires catalysis by eIF-2B (the guanine exchange factor) as eIF-2 has a higher affinity for GDP than for GTP. If the α subunit of eIF-2 is phosphorylated, the exchange of GDP for GTP is not efficient and a stable complex of eIF-2, GDP and eIF-2B is formed. eIF-2B is present at a very low molar concentration in the cell (20% of eIF-2 levels) and so is effectively sequestered to the inactive complex. The exchange of GDP for GTP is prevented and further rounds of translation initiation cannot occur (Hershey, 1993).

1.3.3. Site of eIF-2α Phosphorylation

The site of eIF-2α phosphorylation by PKR is thought to be Ser^{51} (Samuel, 1979). A peptide of eIF-2α (45-56 aa) can be phosphorylated by PKR at Ser^{51}, but surrounding sequences are also thought to be important (Mellor & Proud, 1991). Mutations in eIF-2α which affect Ser^{51} prevent phosphorylation by PKR but mutations of Ser^{48} have no effect on the phosphorylation of eIF-2α by PKR (Pathak *et al.*, 1988).

1.3.4. Eukaryotes and PKR

PKR homologues have been found in cells of several species. Human (Meurs *et al.*, 1990) and murine (Feng *et al.*, 1992) PKR have been cloned and sequenced. Amino acid identity between human and mouse PKR is 61 % (Feng *et al.*, 1992). Rat PKR has also been cloned (Mellor *et al.*, 1994) and has 61 % amino acid identity with human PKR, and 72 % amino acid identity with murine PKR. The amino acid sequence and domains of human PKR are shown in Fig. 1.5 and is taken from Feng *et al.*, 1992.

In *Saccharomyces cerevisiae* PKR can substitute for GCN2, the yeast eIF-2 kinase which controls the translation of GCN4 (Mellor *et al.*, 1994). GCN4 activates the transcription of amino acid biosynthetic genes to overcome amino acid starvation. PKR (and the other eIF-2 kinase HRI, heme-regulated inhibitor of translation) can stimulate the translation of GCN4. High expression levels of PKR in the yeast lead to a slow-growth phenotype due to the inhibition of protein synthesis. Expression of a point-mutated form of eIF-2 in the yeast eliminates the slow-growth phenotype (Chong *et al.*, 1992).

Fig. 1.5. Diagram to Show Domains of Human PKR and Amino Acid Sequences Used for Primer Design

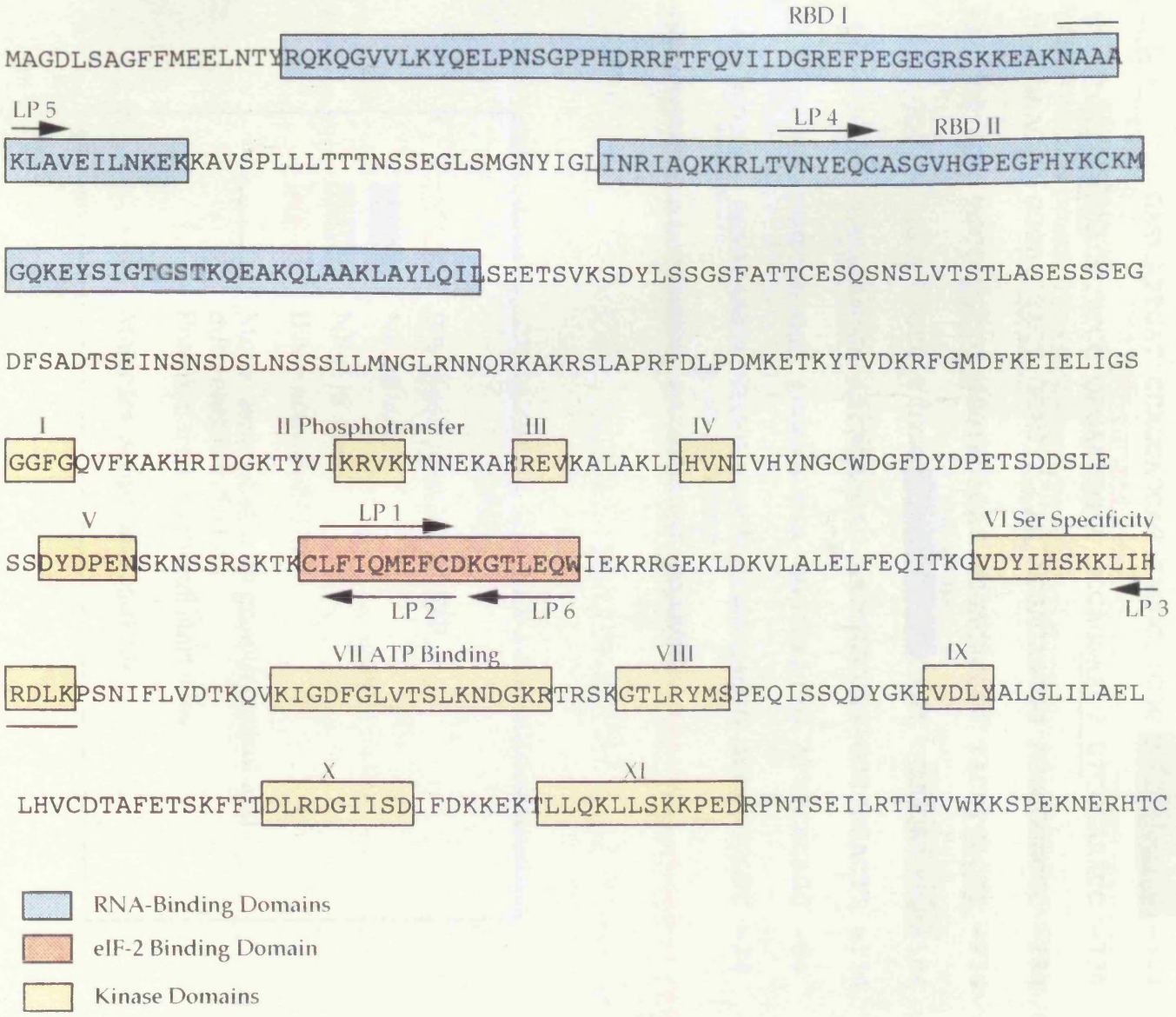
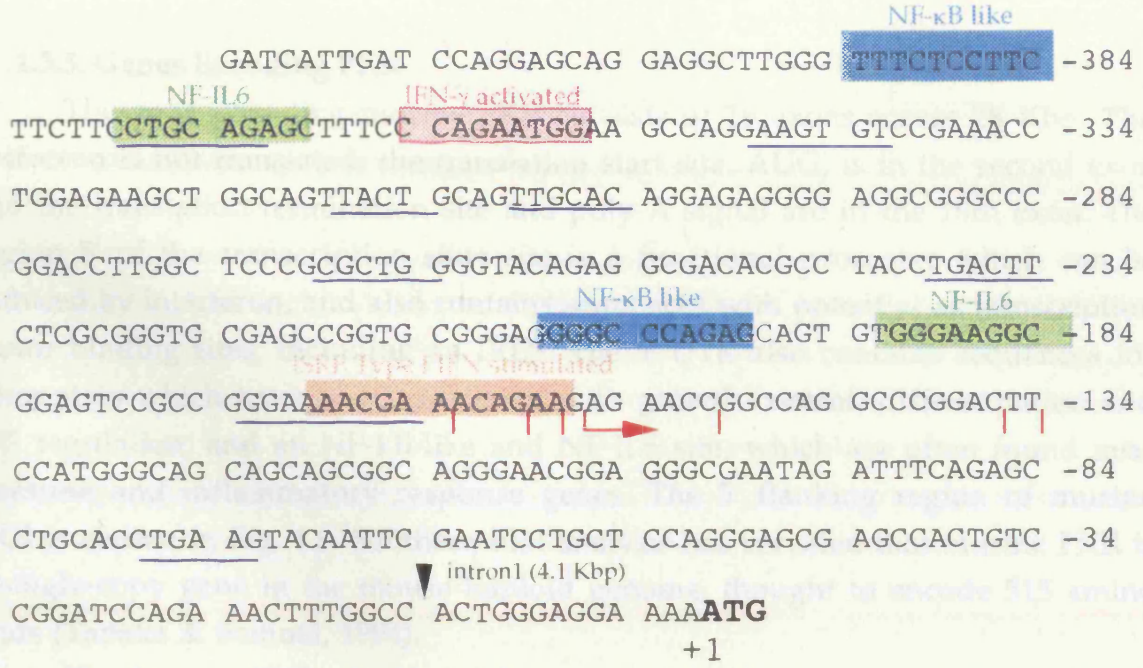


Fig. 1.6. Diagram to Show Promoter Regions in the 5' Region of the Mouse PKR Gene



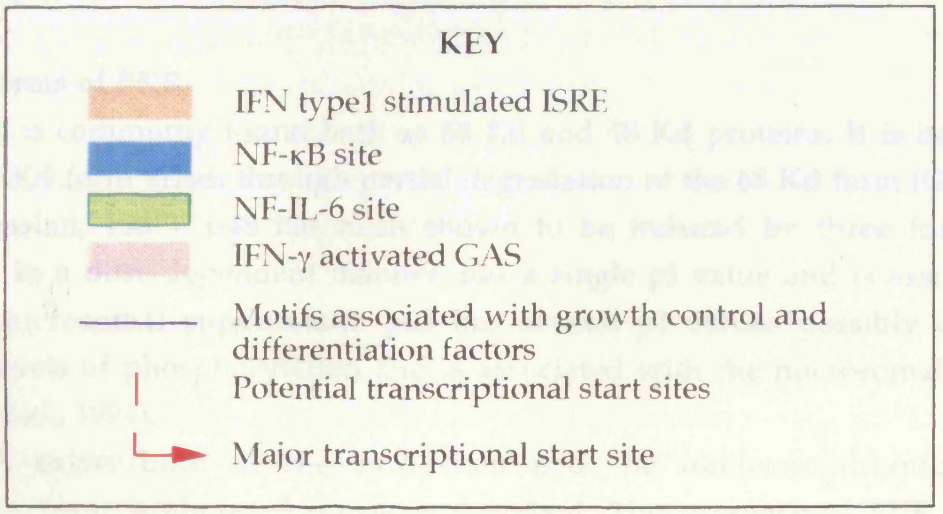


Fig. 1.6 Diagram To Show Promoter Regions in the 5' Region of the Mouse PKR Gene
The diagram is taken from tanaka and samuel, 1994 and shows the nucleotide sequence 5' of the translation start site of mouse PKR. The translation start site ATG is shown in bold and marked +1

Plant PKR has been identified as a 68 Kd protein which is phosphorylated to a greater extent in extracts from viroid-infected tissues, and found to be similar to PKR (Hiddinga *et al.*, 1988, Langland *et al.*, 1995).

1.3.5. Genes Encoding PKR

The gene encoding murine PKR consists of 16 exons across 28 Kbp. The first exon is not translated; the translation start site, AUG, is in the second exon and the translation termination site and poly A signal are in the 16th exon. The region 5' of the transcription start site is a functional promoter which can be induced by interferon, and also contains sequences with potential as transcription factor binding sites, including an IRES. The 5' UTR also contains sequences for promoters which have been associated with growth control, differentiation and cell regulation, and an NF-kB-like and NF-IL6 site, which are often found near immune and inflammatory response genes. The 5' flanking region of murine PKR is shown in Fig. 1.6. Southern blot analysis has revealed that murine PKR is a single-copy gene in the mouse haploid genome, thought to encode 515 amino acids (Tanaka & Samuel, 1994).

The human PKR gene has been mapped to chromosome region 2p21-22. Abnormalities of this region are often associated with acute myelogenous leukaemia (Hanash *et al.*, 1993).

1.3.6. Forms of PKR

PKR is commonly found both as 68 Kd and 48 Kd proteins. It is believed that the 48 Kd form arises through partial degradation of the 68 Kd form (Galabru & Hovanessian, 1987). p48 has been shown to be induced by three forms of interferon, in a dose-dependent manner, has a single pI value and is associated with the microsomal supernatant. p68 has several pI values possibly due to different levels of phosphorylation and is associated with the microsomal pellet (Kadereit *et al.*, 1994).

PKR exists both in the cytoplasm and the nucleus, although the cytoplasmic form is almost 5 times as abundant. The presence of PKR in the nucleus of cells may indicate a role for PKR in cell-cycle control (Jeffrey *et al.*, 1995).

1.3.7. Chemical Inhibitors and Activators of PKR

PKR can be activated by polyanionic substances other than dsRNA. Activation of PKR by heparin is reversible. It has been suggested that heparin activation of PKR occurs through a different mechanism to activation by dsRNA (Patel *et al.*, 1994). Activation by heparin can be inhibited by antithrombin III, a heparin binding protein. Antithrombin III is able to inhibit activation of PKR but not substrate phosphorylation once PKR is autophosphorylated (Hovanessian & Galabru, 1987).

Both autophosphorylation of PKR and substrate phosphorylation by PKR can be inhibited *in vitro* and *in vivo* by the addition of 2-aminopurine. 2-aminopurine is an ATP analogue and is thought to bind to the kinase at the ATP binding sites. The binding of ATP and 2-aminopurine are thought to be competitive as 2-aminopurine inhibition of PKR can be reversed by addition of higher concentrations of ATP (Hu & Conway, 1993).

1.3.8. Domains of PKR

PKR has two distinct kinase activities, autophosphorylation and substrate phosphorylation. Autophosphorylation is thought to occur through an intermolecular phosphorylation event, when two molecules of PKR bind to dsRNA and become associated (Langland & Jacobs, 1992, Ortega *et al.*, 1996, Patel *et al.*, 1995a). However, intramolecular phosphorylation events are difficult to rule out. The critical step in activation of PKR is thought to be dsRNA-binding (Wu & Kaufman, 1996). Many studies have been carried out on the RNA-binding domain of the kinase, to characterize the interactions which occur.

1.3.8.1 RNA-Binding Domain

PKR can be activated by low concentrations of dsRNA but high concentrations inhibit activation (Galabru *et al.*, 1989). Amino acids 1-171 contain two copies of a dsRNA-binding domain rich in highly basic residues (Green & Mathews, 1992), known as R_1 (55-75 aa) and R_2 (145-166 aa). When murine PKR was cloned, sequence alignment was used to identify conserved domains in the N-terminal region of the protein sequence (Feng *et al.*, 1992).

The 1-170 aa region is capable of binding dsRNA and deletions of either aa 1-34, aa 129-170 or aa 55-75 prevent dsRNA binding (Patel & Sen, 1992).

Although 2'-5'A synthetase is able to bind dsRNA, there is little or no similarity in primary or predicted secondary structure between the two IFN-induced proteins.

Site directed mutagenesis indicates that Gly^{57} and Lys^{60} of PKR are absolutely essential for dsRNA binding and that mutations in Lys^{64} and Leu^{75} reduce dsRNA binding by 90% (Barber *et al.*, 1995).

R_1 (aa 55-75) alone is sufficient for dsRNA binding, but R1 and R2 together bind dsRNA more tightly (McCormack *et al.*, 1994). R_1 and R_2 may have distinct functions in RNA-binding, as although R_1 appears to play a more important role in RNA binding, R_2 is thought to have a more flexible structure. Both domains have been predicted to contain a C-terminus α helix, which when disrupted by mutation does not bind RNA efficiently (Green *et al.*, 1995).

Mutations of either amino acids 38, 39, 58, 60 or 61 are deficient in dsRNA-binding and can act as transdominant inhibitors of PKR. These mutants are thought to act by sequestering dsRNA and so preventing, or reducing the extent, of PKR autophosphorylation and activation (Barber *et al.*, 1995). The active form of PKR is now thought to be a dimer, and the dominant negative interference exhibited by dsRNA-binding domain mutants might be due to the formation of defective heterodimers (Romano *et al.*, 1995).

Double-stranded RNA of less than 30 bp is unable to activate PKR but can prevent activation of PKR by longer RNAs when present at high concentrations (Manche *et al.*, 1992). Small RNAs do not fully occupy the dsRNA-binding site of PKR and so the conformational change required to activate PKR does not occur. Although dsRNA of more than 30 bp is able to bind and activate PKR, dsRNA of 85 bp provides the most efficient activation of PKR. Even dsRNA of 85 bp prevents activation of PKR at high concentrations, possibly by preventing molecules of PKR associating. PKR is able to interact with 11 bp of dsRNA but activated PKR can protect 80 bp. When the PKR RNA-binding site is fully occupied a conformational change occurs (Manche *et al.*, 1992).

Recently, the NMR structure of the dsRNA-binding domains of *Drosophila* Staufen protein and ribosomal protein S have been elucidated and have an α–β–β–β–α structure (Bycroft *et al.*, 1995). It is likely that the RNA-binding domain of PKR adopts a similar structure.

1.3.8.2. Catalytic Domain

The catalytic domain of PKR contains all the conserved motifs (I-XI), consistent with the protein kinase family (Hanks *et al.*, 1988). The domains of PKR are highlighted in Fig. 1.5.

PKR expression may be autoregulated by catalytically active PKR (Thomis & Samuel, 1992) at the level of mRNA translation (Barber *et al.*, 1993) as PKR can be activated by binding its own mRNA (Thomis & Samuel, 1993).

A catalytically inactive mutant of PKR is phosphorylated in the presence of wt PKR, indicating that autophosphorylation could be an intermolecular event (Thomis & Samuel, 1993). Lys^{296} is critical to the catalytic function of PKR (Lee *et al.*, 1993). Catalytic mutants of PKR exert a dominant negative effect over wt PKR (Sharp *et al.*, 1993b). The dominant negative effect may be due to the formation of autophosphorylation incompetent heterodimers of mutant and wt PKR. Mutants of the catalytic domain can reverse the inhibition of protein synthesis and reduce eIF-2α phosphorylation, as can mutants of the dsRNA-binding domain. However higher levels of the catalytic mutants are required for complete shut-down of PKR (Sharp *et al.*, 1993b).

The sequence LFIQEFCDK 361-370 aa is thought to be the eIF-2α binding domain or recognition sequence and so be essential for PKR function (Feng *et al.*, 1992).

1.3.9. Cellular Inhibitors of PKR

Several cellular inhibitors of PKR exist:

1.3.9.1. La Antigen

The La antigen is a 47 Kd protein found in the nucleus and cytoplasm. Antibodies to the La antigen are associated with autoimmune diseases, such as systemic lupus erythematosis and Sjogren's syndrome. La antigen is known to have DNA and RNA unwinding activity and to interact with a variety of RNA molecules. La antigen can inhibit activation of PKR and thus the phosphorylation of eIF-2α (Xiao *et al.*, 1994). Inhibition is reversed by high concentrations of dsRNA, and the inhibiting activity only applies to PKR prior to activation. As the La antigen is capable of converting dsRNA to ssRNA, it has been suggested that La inhibits PKR by binding and unwinding dsRNA (Xiao *et al.*, 1994).

1.3.9.2. p58

PKR is inhibited in influenza infected cells (Lee *et al.*, 1990). Influenza infection inhibits autophosphorylation of PKR and phosphorylation of eIF-2α, but inhibition is not caused by protease or phosphatase activity. The 58 Kd protein responsible is a cellular protein and not a viral-encoded protein. The 58

Kd cellular protein does not act through degradation or sequestration of PKR and is present in infected and uninfected cells in equal amounts (Lee *et al.*, 1992). It has been proposed that virus infection releases p58 from an inactive complex with a repressor, Ip58, enabling p58 to block PKR activation through a direct interaction (Polyak *et al.*, 1996) with the ATP-binding region of PKR (Gale *et al.*, 1996). In uninfected cells, the dissociation of p58 from Ip58 is thought to be controlled by specific cell signals (Lee *et al.*, 1992).

p58 has been cloned, sequenced and expressed (Lee *et al.*, 1994) and is predicted to be a 504 aa hydrophilic protein which is highly conserved in many mammalian species (Korth *et al.*, 1996b). p58 has nine repeats of a 34 aa motif, referred to as a tetratricopeptide repeat (TPR). TPR motifs are found in a range of proteins which mediate protein-protein interactions in mitosis, transcription and RNA synthesis (Lamb *et al.*, 1995). p58 has homology with the *E.coli* DnaJ family of proteins (also tetracopeptides) which function during heat-shock (Lee *et al.*, 1994).

p58 may have oncogenic potential through the down-regulation of PKR. NIH 3T3 cells expressing p58 have faster growth rates, higher saturation densities and anchorage-independent growth. When inoculated into mice these cells produced tumours (Barber *et al.*, 1994). The p58 gene has been mapped to chromosome 13 band q32. Aberrations in chromosome 13 have been identified in a variety of human cancers, but particularly in acute leukaemia (Korth *et al.*, 1996a).

1.3.9.3. p15

Another cellular inhibitor of PKR has been reported in the mouse cell line, 3T3 F442 (Judware & Petryshyn, 1991). Cells cultured in conditions non-permissive for differentiation into adipocytes have lower levels of PKR activity than cells cultured in conditions permissive for differentiation into adipocytes. This is due to an inhibitor of PKR, which is present in reduced amount in cells incubated at conditions suitable for differentiation. It has been suggested that PKR may be an important signal in controlling growth arrest
prior to differentiation into adipocytes. The 15 Kd protein purified from the cells does not have protease or phosphatase activity and can reversibly inhibit PKR (Judware & Petryshyn, 1992). p15 is thought to prevent ATP binding to PKR through direct interaction with PKR (Judware & Petryshyn, 1992).

1.3.10. PKR Involvement in Other Cellular Functions

PKR may be involved in a variety of cell processes other than translation initiation.

1.3.10.1. PKR and Heat-Shock

Heat-shock causes the cessation of DNA replication, RNA splicing, hormonal responses and protein synthesis. The precise mechanisms have not yet been elucidated but it is known that many cellular proteins become aggregated and inactive under heat-shock conditions (Dubois *et al.*, 1991).

It has been reported that PKR is affected by heat-shock in HeLa cells, becoming aggregated as a thick ring around the nucleus and co-localizing with the 70 Kd heat-shock protein (Dubois *et al.*, 1991). During cell recovery, PKR resolubilizes and becomes active again. Disruption of the cytoskeleton does not affect the insolubility of PKR during heat-shock. In thermotolerant cells (previously heat-shocked), PKR does not become insoluble under heat-shock conditions (Dubois *et al.*, 1991).

1.3.10.2. PKR as a Tumour Suppressor

PKR may play a role in tumour suppression and cell proliferation. Expression of PKR mutants in cells can lead to transformation of the cells and tumours in mice (Koromilas *et al.*, 1992, Meurs *et al.*, 1993).

Human PKR maps to chromosome region 2p21-22 (Hanash *et al.*, 1993). Abnormalities in 2p21-22 are predominantly found in patients with acute myelogenous leukaemia. High levels of PKR expression have also been associated with survival in patients with squamous cell carcinomas of the head and neck (Haines *et al.*, 1993).

Expression of PKR in HeLa cells induces rapid cell death, a characteristic of apoptosis (Lee & Esteban, 1994). Thus, it has been suggested that tumour suppression by PKR may function through apoptosis (Lee & Esteban, 1994). However, expression of mutant eIF-2, lacking Ser^{51} (therefore not phosphorylated by PKR), can also cause transformation of cells. Thus transformation by mutants of PKR may occur through inhibition of eIF-2 phosphorylation (Donzé *et al.*, 1995).

Activation of PKR may be the mechanism of tumour suppression by the 3' UTR of tropomyosin RNA (Davis & Watson, 1996). Interferon regulatory factor 1 (IRF-1) which activates type 1 IFN and interferon-inducible genes also has

tumour suppressor activity which is thought to be mediated by PKR (Beretta *et al.*, 1996, Kirchhoff *et al.*, 1995).

Despite the *in vitro* evidence to suggest that PKR may have tumour suppressor function, the *in vivo* evidence, in which PKR-knockout mice do not develop tumours, suggests that tumour suppression may not be a role of PKR *in vivo* or that mechanisms exist to compensate for any depletion of PKR (Yang *et al.*, 1995).

1.3.10.3. PKR in Signal Transduction

NF-κB is a multisubunit transcription factor, consisting of p50, p65 and Rel proteins. NF-κB is located in the cytoplasm and must translocate to the nucleus in order to function as a transcription factor. There is evidence to suggest that a constitutive nuclear pool of NF-κB exists (Dobranzski *et al.*, 1994). NF-κB is thought to exist as a latent heterodimer with its inhibitor, I-κB. Phosphorylation of IkB is thought to be a means of NF-κB regulation and degradation (Thanos & Maniatis, 1995, Williams, 1995). However, there are some data to suggest that I-κB phosphorylation alone is not sufficient to release NF-κB and its activity (Alkalay *et al.*, 1995). I-κB phosphorylation may mark it for degradation, releasing NF-kB which is able to target specific genes for transcription (DiDonato *et al.*, 1995).

PKR is able to phosphorylate I-κB, and release NF-κB DNA-binding activity (Kumar *et al.*, 1994). As IFN-β has an NF-kB binding site in its promoter region, it has been suggested that PKR may regulate IFN-β induction (Kumar *et al.*, 1994) as well as its own transcription (Tanaka & Samuel, 1994).

1.3.10.4. Platelet-Derived Growth Factor (PDGF)

PDGF (platelet-derived growth factor) can stimulate DNA synthesis and mitosis in some fibroblast cells. PKR may play a role in signal transduction by PDGF, as dsRNA and activated PKR can induce the transcription of the same genes (c-*myc*, c-*fos*, *JE*) that are expressed immediately upon induction by PDGF. An inhibitor of PKR, 2-aminopurine, blocks the induction of the c-*myc*, c-*fos*, and *JE* genes by dsRNA (Mundschau & Faller, 1995).

1.3.10.5. Interleukin 3

Interleukin 3 (IL-3) stimulates cell growth through a receptor signalling pathway. The mechanism of growth stimulation is at present unknown but there is some evidence that IL-3 may decrease the level of activated PKR, and thus

lower levels of eIF-2α phosphorylation (Ito *et al.*, 1993, Ito *et al.*, 1994a). Inhibition of PKR by IL-3 may involve a 97 Kd phosphotyrosine protein which associates with PKR following IL-3 receptor binding (Ito *et al.*, 1994b).

1.3.10.6. Immune System

PKR may play a role in induction and splicing of TNF-α mRNA (Jarrous *et al.*, 1996). PKR may also regulate IFN-α induction (Der & Lau, 1995) and the macrophage tumouricidal response triggered by LPS and type I IFNs (Gusella *et al.*, 1995).

1.4. Viruses and PKR

PKR can be activated by viral dsRNA and limits host infection by shutting-down protein synthesis, preventing viral proteins from being formed and ensuring cell death. Many viruses have evolved mechanisms to overcome the protein synthesis inhibition caused by PKR (for reviews see Katze, 1992, Katze, 1993, Katze, 1995, Mathews, 1993, Samuel, 1991).

1.4.1. Adenovirus VAI RNA

Adenoviruses are common causes of acute respiratory disease. They have icosahedral symmetry and are 60-90 nm in diameter. The genome consists of a linear dsDNA molecule of 30-38 Kbp with inverted terminal repeats and a covalently bound protein at the 5' end.

Adenoviruses encode two distinct virus-associated (VA) RNAs. The major species, VAI RNA is a 160 nucleotide RNA which is synthesized by RNA polymerase III and accumulates late in adenovirus infection, at 10^8 molecules per cell. The minor species VAII is also about 160 nucleotides long and accumulates to 10^7 molecules per cell. Both RNAs have a high degree of secondary structure. VAI consists of 2 long imperfectly base-paired duplexes, separated by a short central stem-loop.

VAI RNA can inhibit PKR activation, but not eIF-2 phosphorylation by activated PKR. Activation of PKR in adenovirus infected cells, in the absence of VAI RNA, is due to dsRNA, at least some of which is viral (Maran & Mathews, 1988). The action of VAI RNA is not due to PKR degradation (Katze *et al.*, 1987). VAI RNA inhibits PKR activation in a dose-dependent manner and although PKR has a lower affinity for VAI RNA than for poly IC (a synthetic dsRNA), VAI

RNA can not be displaced by poly IC (Galabru *et al.*, 1989). These data imply that the interaction of VAI RNA with PKR is not reversible.

Fig. 1.7. Secondary Structure of VAI RNA

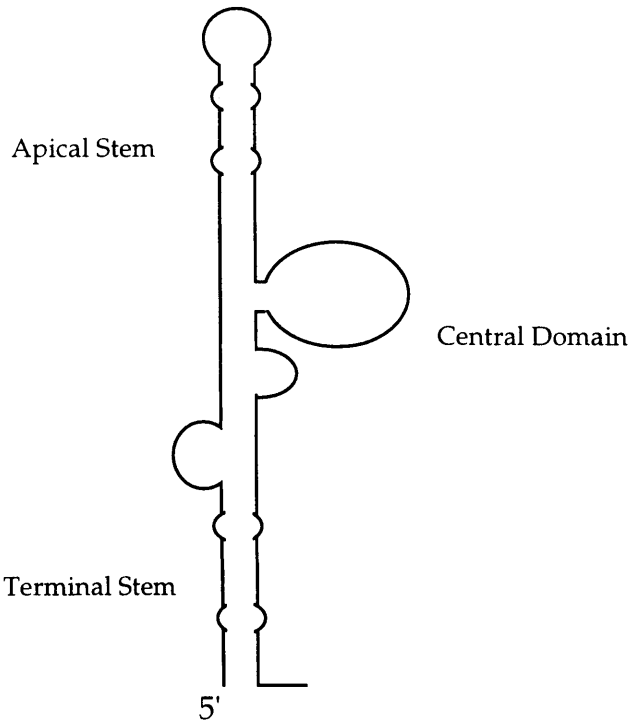


Diagram of VAI secondary structure, Fig. 1.7, is taken from Mathews & Shenk, 1991.

The partial double-stranded nature of VAI may enable it to bind and block activation of PKR by dsRNA (O'Malley *et al.*, 1986). Studies with an adenovirus mutant in VAI have indicated that VAII RNA may be able to substitute for VAI but does not overcome translation inhibition by PKR as effectively as VAI (Mathews & Shenk, 1991). It has been suggested that VAI RNA is too small to activate PKR but is sufficiently large to bind to the kinase and prevent dsRNA from binding (Clemens *et al.*, 1994). The major interactions between PKR and VAI RNA are thought to be electrostatic and areas outside of the dsRNA-binding domains are important for the interaction (Clarke *et al.*, 1994).

Mutations in the apical stem, terminal stem and central domain of VAI RNA have been tested *in vitro* for their effects on PKR binding ability (Clarke *et al.*, 1994). Over half a turn of intact apical stem is required for interaction with

PKR and there is a relationship between a mutant's ability to bind PKR and its ability to function *in vitro* and *in vivo*. The structure of the stem loop is critical for PKR binding (Ghadge *et al.*, 1994). The lack of activation of PKR by the mutants is not due to imperfect base-pairing in the duplexes, as mutant VAI RNA with perfectly base-paired duplexes fails to activate PKR and blocks activation of PKR better than wt VAI RNA (Ghadge *et al.*, 1994).

Although VAI RNA binds but does not activate PKR, it is capable of binding and activating 2'-5'A synthetase. Mutations in VAI which remove the mismatches in the stems enhance 2'-5'A synthetase activation but not PKR activation (Desai *et al.*, 1995).

A study of 54 VAI RNA mutants with single base substitutions in the central domain has shown that many lost the inhibitory activity whilst retaining significant secondary structure, indicating that single bases and not just secondary structure are important in PKR binding and activation inhibition (Rahman *et al.*, 1995).

1.4.2. Retroviruses

1.4.2.1. HIV

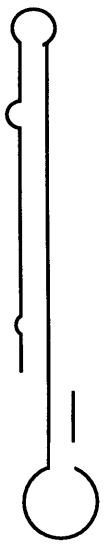
HIV is a member of the *Retroviridae* family. The virion is spherical, 80-120 nm in diameter, and contains 2 molecules of single-stranded positive sense RNA, each of about 10 Kbp. Reverse transcriptase, a viral enzyme, synthesizes a linear viral DNA, the viral RNA is degraded and a second strand of DNA is formed. Sequences in the LTR enable the DNA to integrate into the host genome. Transcription is regulated by sequences in the LTR, a polyprotein is produced by translation and is then cleaved by a viral protease to provide the proteins necessary for viral assembly (Davis *et al.*, 1980, Singleton & Sainsbury, 1987).

HIV TAR RNA (transactivation responsive RNA) is a highly structured RNA of about 160 nucleotides which forms a stem-bulge-loop structure due to extensive base-pairing. TAR binds the viral trans-acting protein, Tat. TAR is present at the 5' UTR of all HIV mRNAs and transcription is regulated by its interaction with Tat. TAR RNA also exists as a small RNA (60 nucleotides) in the cytoplasm of the infected cell, possibly arising through nuclease degradation of HIV transcripts back to a nuclease resistant core (Clemens *et al.*, 1994).

HIV TAR RNA can be bound by the N-terminal dsRNA-binding region of PKR (McCormack *et al.*, 1992). However, there is some debate as to the effects of

TAR binding to PKR. Some studies suggest that TAR activates PKR while other suggest that TAR inhibits PKR activation.

Fig. 1.8. Secondary Structure of HIV TAR RNA



The diagram of the secondary structure of HIV TAR RNA, Fig. 1.8, is taken from Clemens *et al.*, 1994.

In some reports it has been proposed that TAR binds to PKR which becomes activated and translation is inhibited. HIV-1 infection of CEM cells is reported to result in a decrease in PKR late in infection. HeLa cells stably expressing TAR have reduced levels of PKR compared with IFN-treated cells (Roy *et al.*, 1990). Messenger RNA from TAR-expressing cell lines can activate PKR and TAR-containing RNAs are able to complex with purified PKR, although a synthetic dsRNA can compete out TAR.PKR complexes. Both PKR binding and activation are reported to be dependent on the stem loop structure of the TAR RNA (Roy *et al.*, 1991). Chemically synthesized TAR is also capable of activating PKR and 2'-5'A synthetase (Maitra *et al.*, 1994) and TAR mutants which have a disrupted secondary structure are unable to activate either enzyme.There are reports that the binding of TAR to PKR inhibits PKR activation. It has been suggested that the activation of PKR by TAR is due to contaminating dsRNAs

from *in vitro* transcription (Benech *et al.*, 1985a, Benech *et al.*, 1985b, Saunders *et al.*, 1985), although experiments with chemically synthesized TAR seem to disprove this hypothesis. Purified TAR is reported to inhibit PKR activation when present at high concentrations, as does dsRNA (Gunnery *et al.*, 1990). It has also been reported that TAR blocks PKR inhibition of translation in a cell-free system. Mutation studies indicate that 14 bp of TAR stem are required to inhibit PKR in cell-free translation. As truncation of TAR to 68 nucleotides led to loss of PKR inhibition, it has been suggested that cytoplasmic TAR does not inhibit PKR *in vivo*, but that TAR at the 5' UTR of mRNAs does still inhibit PKR *in cis* (Gunnery *et al.*, 1992).

A synthetic peptide of Tat is reported to be able to inhibit the activation of PKR by TAR (Judware *et al.*, 1993). This could be by binding to TAR and thus sequestering it from PKR but Tat is thought to interact directly with PKR (McMillan *et al.*, 1995).

A TAR RNA binding protein (TRBP) has been isolated from HeLa cells (Gatignol *et al.*, 1993). TRBP contains a lysine-rich RNA-binding domain which has homology with PKR and other dsRNA-binding proteins. As a peptide the lysine rich domain (247-267 aa) is able to bind TAR with a similar affinity to complete TRBP (Gatignol *et al.*, 1993). TRBP inhibits PKR autophosphorylation and eIF-2α phosphorylation. It has been proposed that TRBP is a cellular regulatory protein which binds RNAs with a specific secondary structure in order to control PKR activation (Park *et al.*, 1994). TRBP dimerizes with itself and PKR in a yeast two-hybrid assay (Cosentino *et al.*, 1995).

1.4.2.2. HTLV-1

Human T-cell leukaemia virus type 1 Rex-response element (Rex-RE) is a highly structured RNA sequence and target for the 27 Kd Rex protein, essential in HTLV-1 regulation. Binding of Rex-RE by Rex induces expression of unspliced and singly spliced mRNAs encoding structural proteins of HTLV-1. The Rex-RE synthesized *in vitro* is capable of activating 2'-5'A synthetase in a dose-dependent manner. Rex-Re also activates PKR at low concentrations but inhibits PKR activation when present at high concentration. Addition of poly IC dissociates Rex-RE from both PKR and 2'-5'A synthetase. It has been suggested that binding of Rex-RE by Rex may prevent activation of 2'-5'A synthetase and PKR, and thus allow the production of mature virions (Mordechai *et al.*, 1995).

1.4.3. Epstein-Barr Virus (EBV)

Epstein-Barr virus (EBV) which causes infectious mononucleosis (glandular fever) and has been associated with several tumours, such as Burkitt's lymphoma and nasopharyngeal carcinoma, is a member of the *Gammaherpesvirinae* . The virus is 120-200 nm in diameter, and contains a central core of DNA wound around a protein structure, within an icosahedral capsid. The capsid is surrounded by tegument proteins and the whole is enclosed in a lipoprotein envelope. The 154 Kbp dsDNA linear genome of EBV has a 60% G+C content and has terminal and internal terminal repeats. EBV mainly infects B-lymphocytes but can also infect the epithelium of the nasopharynx even though these cells do not have the receptor for EBV. Infected B-cells are often immortalised, and the virus can persist in a latent form in the lymphocytes (Davis *et al.*, 1980, Singleton & Sainsbury, 1987).

EBERs (Epstein-Barr encoded RNAs) are abundant RNAs encoded by EBV, with similarity to VAI RNAs of adenoviruses. EBERs are of a similar size to VAI, both are transcribed by RNA pol III and have a similar degree of secondary structure.

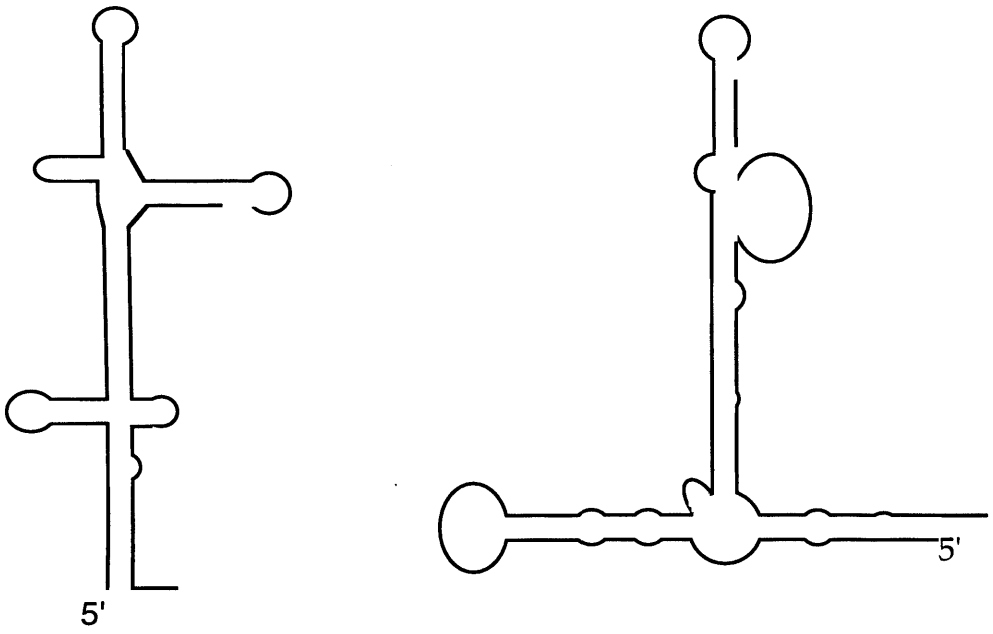
EBERs can partially substitute for VAI RNA in adenovirus deletion mutants (Bhat & Thimmappaya, 1983). EBERs are able to bind to the RNA-binding domain of PKR and it is thought that they may function in a similar way to VAI as they can complement VAI RNA function in lytic growth of adenovirus (Bhat & Thimmappaya, 1985).

Both EBER-1 and EBER-2 are present in the nucleus and the cytoplasm of infected interphase Raji cells (Burkitt lymphoma cells). In the cytoplasm the EBERs are reported to co-localize with PKR. In cells undergoing mitosis, the EBERs localize around the chromosomes, but PKR is still distributed in the cytoplasm (Schwemmle *et al.*, 1992).

High concentrations of EBER-1 can block the activation of PKR, and are able to form a stable complex with PKR *in vitro*. (Clarke *et al.*, 1990). The binding of EBER-1 to PKR is dependent on the secondary structure of the RNA (Clemens *et al.*, 1994).

An eIF-2 phosphorylation assay demonstrated that EBER-1 inhibits activation of purified PKR. EBER-2 also regulates PKR and can compete with EBER-1 to bind PKR. It has been proposed that the EBERs may be involved in the transformation of B-lymphocytes as PKR is known to have tumour suppressor function (Sharp *et al.*, 1993a).

Fig. 1.9. Secondary Structure of EBER-1 and EBER-2



EBER-1 EBER-2

The diagram of the secondary structures of EBER-1 and EBER-2, Fig. 1.9, is reproduced from Clemens *et al.*, 1994 and Glickman *et al.*, 1988.

1.4.4. *Reoviridae*

The *Reoviridae* (respiratory enteric orphan viruses) are a family of non-enveloped viruses of 60-80 nm in diameter which infect the respiratory and gastrointestinal tracts of many animals, usually without causing disease.
The virions of reoviruses contain a segmented genome of 10 different linear dsRNA molecules, of three size classes, a total of 46 Kbp. Reoviruses have been divided into types depending on their ability to agglutinate red blood cells (Davis *et al.*, 1980, Singleton & Sainsbury, 1987).

1.4.4.1. Reovirus

The reovirus σ3 protein, encoded by small RNA 4 (S4 or segment 10), is expressed early in infection, is associated with the capsid and is able to bind dsRNA. Reovirus type 1 infected cells contain an inhibitor of PKR. The inhibition of PKR can be overcome by adding 100 times as much dsRNA as is needed to activate PKR in uninfected cell extracts. It has been suggested that the dsRNA-binding activity of σ3 is required for PKR inhibition as activation of partially purified PKR can be inhibited by purified σ3 (Imani & Jacobs, 1988). The RNA-binding domain of σ3 is thought to be an 85 aa region from 234-297 aa, containing a repeated basic amino acid motif (Miller & Samuel, 1992). Some of the basic residues in this region are vital for dsRNA-binding (Denzler & Jacobs, 1994, Wang *et al.*, 1996). Both repeated amino acid motifs are involved in dsRNA-binding (Mabrouk *et al.*, 1995).

Expression of the S4 gene which encodes σ3 is able to stimulate translation, although *in vivo* σ3 is thought to be complexed with protein μ1c, and thus may not be freely available to bind dsRNA and inhibit PKR activation (Giantini & Shatkin, 1989). Reovirus S1 mRNA (1463 nucleotides) is an activator of PKR whereas S4 mRNA which encodes σ3 (1196 nucleotides) does not activate PKR. It has been proposed that inhibition of translation by PKR is selective for mRNAs possessing the activator region present in S1 (Henry *et al.*, 1994).

1.4.4.2. Rotavirus

Rotaviruses are a major cause of gastroenteritis in humans and animals and are classified into groups on the basis of serology. Little is known about the non-structural proteins of rotaviruses. However, porcine group C rotavirus NSP3 protein has homology with dsRNA-binding proteins. A NSP3 clone expressed *in vitro* and in COS cells, produces proteins of 45, 38 and 8 Kd. The 38 Kd and 8 Kd proteins are thought to be cleavage products of the 45 Kd protein. The dsRNA-binding motif is present in the 45 Kd and 8 Kd proteins and the NSP3 products are capable of inhibiting PKR activation when expressed in COS cells (Langland *et al.*, 1994).

1.4.5. *Picornaviridae*

The *Picornaviridae* is a family of small (22-33 nm in diameter) icosahedral viruses, all of which contain a single strand of positive-sense RNA. The RNA has a 3' poly A sequence and is covalently bound to a viral protein, VPg, at the 5' end which is not capped. Translation of the viral RNA occurs at an internal ribosome entry site, IRES (Davis *et al.*, 1980, Singleton & Sainsbury, 1987).

It has been known for some time that poliovirus actively shuts down host cell protein synthesis through the degradation of the eIF-4F subunit by the viral protease Vp2A. Vp2A is required for proteolytic cleavage of precursor polyproteins but is also thought to cleave the p220 subunit of eIF-4F, the cap-binding initiation factor. It is thought that the cleavage of eIF-4F prevents translation occurring through the cap-dependent scanning mechanism, and thus confers an advantage to the viral RNAs which are translated by a cap-independent mechanism involving internal binding of the ribosome to specific sequences in the RNA (Ehrenfield, 1982, Etchison *et al.*, 1982, Sonenberg, 1990, Sonenberg, 1987). This mechanism does not however, remove the need to prevent eIF-2 phosphorylation by PKR. PKR is highly phosphorylated and activated during poliovirus infection, and eIF-2 is phosphorylated in poliovirus infected cells. However, PKR is significantly degraded and levels of the kinase lowered in poliovirus infected cells, possibly through the action of a cellular protease (Black *et al.*, 1989).

1.4.6. Influenza Virus

Discussed earlier p58, see section 1.3.9.2.

1.5. The *Poxviridae*

The *Poxviridae* is a family of complex DNA viruses, which replicate in the cytoplasm of the host cell, and consist of a large virion containing; a genome of a single linear dsDNA molecule, covalently closed at the termini, and enzymes involved in mRNA synthesis.

The poxvirus, variola virus is the causative agent of smallpox which was eradicated in 1977 by a World Health Organisation campaign of vaccination (reviewed Fenner, 1990) using vaccinia virus.

The *Poxviridae* can be divided into two subfamilies, the *Chordopoxviridae* which infect vertebrates and the *Entemopoxviridae* which infect insects. The *Chordopoxviridae* have been divided into 8 genera on the basis of antigenic relatedness and host range (Moss, 1990).

Genera	Prototypical member
Orthopoxvirus	vaccinia
Parapoxvirus	orf
Avipoxvirus	fowlpox
Capripoxvirus	sheepox
Lepripoxvirus	myxoma
Suipoxvirus	swinepox
Molluscipoxvirus	molluscum contagiosum
Yatapoxvirus	tanapox

The restriction maps of members within a group are usually similar although there are some exceptions (racoon pox and tatera pox of the *Orthopoxviruses*).

Despite vaccinia virus (VV) being the most extensively studied of the *Poxviridae*, its precise origins remain unknown, although several hypotheses have been proposed. Here knowledge gained from studies of VV will be used as a general model for poxvirus replication.

1.5.1. Structure

Several forms of VV exist. The two infectious forms are IMV, intracellular mature virus, which are thought to remain cell-associated and EEV, extracellular enveloped virus, which is released from the cell.

IMV has been thought of as having an oval or brick shaped biconcave core, covered by an outer membrane of surface tubule elements. Two structures of unknown function inhabit the cavities of the core and are known as lateral bodies. A recent study has suggested that IMV is a dense core surrounded by a thick surface domain limited by two membranes (Dubochet *et al.*, 1994). EEV is thought to differ from IMV by the presence of a third layer of membrane, the envelope.

The cores contain the viral genome and many viral encoded proteins, some of which are involved in transcription (Moss, 1990). Four major structural proteins make up the greater part of the core weight (Goebbel *et al.*, 1990, Johnson *et al.*, 1993).

1.5.2. Genome

The large linear dsDNA molecules are terminally linked by hairpins (Geshelin & Berns, 1974). Hairpins can exist in two isomeric forms, inverted and complementary to each other (Baroudy *et al.*, 1982). Telomere target sequences exist next to the hairpins and are thought to play a vital role in the resolution of concatomers in DNA replication (Merchlinsky & Moss, 1989). The ends of the genome are inverted terminal repeats (ITRs).

The genes of VV are closely packed with little intergenic DNA (Goebbel *et al.*, 1990, Smith *et al.*, 1991a), do not contain introns, are transcribed from both strands of DNA and have short promoters.

The central region is most conserved amongst *orthopoxviruses* and contains essential genes, including those encoding for enzymes involved in DNA replication as well as structural proteins. The ends of the genome are much more variable and tend to contain genes non-essential for replication (at least *in vitro*), often involved in pock formation, host range and viral virulence.

The vaccinia virus (Copenhagen strain) genome consists of 191,636 bp, predicted to encode 263 proteins of 65 amino acids or more (Goebbel *et al.*, 1990, Johnson *et al.*, 1993). The variola virus (Bangladesh 1975 strain) genome consists of 186,102 bp, predicted to encode 187 proteins of at least 65 amino acids (Massung *et al.*, 1994). The molluscum contagiosum virus (subtype 1) genome consists of 190,289 bp and is predicted to encode 163 proteins (Senkevich *et al.*, 1996).

1.5.3. Life Cycle

The life cycle of VV has been reviewed recently (Moss, 1990) and is summarised here.

1.5.3.1. Entry And Uncoating

The mechanisms of attachment and penetration of the *Poxviridae* remain unclear, partially due to the complications arising from the existence of two forms of the virus. Most studies of VV have been performed with IMV and antibodies to several proteins have been shown to be able to neutralize the infectivity of IMV but not infection by EEV. It is thought that EEV plays a greater role in the extracellular spread of virus in natural infection, while IMV may be involved in cell-cell spread of the virus (Appleyard *et al.*, 1971, Boulter, 1969, Boulter & Appleyard, 1973, Payne & Kristensson, 1985).

At one time it was thought that the epidermal growth factor receptor acted as receptor for VV by binding vaccinia growth factor protein (VGF), but this has since been disproved (Hugin & Hauser, 1994). Thus, the mechanism by which poxviruses enter a cell remains unknown.

Uncoating occurs in two stages. The first stage, which begins soon after penetration, does not require RNA or protein synthesis and results in viral cores being released into the cytoplasm. The second stage requires some viral transcription and protein synthesis and results in the release of the genome from the cores into the cytoplasm, and susceptibility of the genome to DNase.

1.5.3.2. Transcription

Transcription of the viral genome occurs as a regulated cascade. The apparatus involved in transcription is mainly virus encoded, but at least one cellular protein, a nuclear cell protein, is required (Rosales *et al.*, 1994b).

Transcription of early mRNAs occurs in the cores after their release into the cytoplasm and thus is resistant to inhibitors of protein synthesis and replication. The early mRNAs are capped, polyadenylated, and have short 5' UTRs (Boone & Moss, 1977, Kates & Beeson, 1970, Wei & Moss, 1975) some of which are polyA leader sequences (Ahn *et al.*, 1990b, Ink & Pickup, 1990). Early gene promoters have been characterized (Davison & Moss, 1989, Rohrman *et al.*, 1986, Schuman & Moss, 1988, Yuen & Moss, 1987). Transcription of early genes requires the DNA-dependent polymerase (Ahn *et al.*, 1990a, Ahn *et al.*, 1990c, Amegadzie *et al.*, 1992, Amegadzie *et al.*, 1991, Broyles & Moss, 1986, Broyles & Pennington, 1990, Quick & Broyles, 1990), the RNA polymerase-associated protein, RAP94 (Ahn *et al.*, 1994, Ahn *et al.*, 1992, Kane & Shuman, 1992) and VV early transcription factor VETF (Broyles & Fesler, 1990, Gershon & Moss, 1990).

Intermediate genes are transcribed once DNA replication starts. Transcription of intermediate genes requires the capping enzyme, the VV intermediate transcription factor 1, VITF-1, VITF-2 and the RNA polymerase (Rosales *et al.*, 1994a, Vos *et al.*, 1991a, Vos *et al.*, 1991b). The intermediate mRNAs have poly(A) leader sequences (Baldick & Moss, 1993) and the promoters have been characterized (Baldick *et al.*, 1992).

Late genes encode many structural proteins and enzymes which are packaged into the virion. Transcription of late genes requires DNA replication and protein synthesis. Late mRNAs are capped and have a 5' poly(A) leader sequence of 35 nucleotides which is not virally encoded but is thought to be produced by VV poly(A) polymerase (Cooper *et al.*, 1981, Lu & Bablanian, 1996, Mahr & Roberts, 1984, Yuen & Moss, 1987). Late gene transcription requires 3 transcription factors which are encoded by intermediate genes (Keck *et al.*, 1990)

1.5.3.3. DNA Replication

Within the infected cell, DNA replication occurs at sites in the cytoplasm which are known as virus factories and starts after the second uncoating step when the genome is released into the cytoplasm (Traktman, 1990). Many virus encoded enzymes are required, including a DNA polymerase, thymidine kinase, thymidylate kinase, ribonucleotide reductase, dUTPase, serine/threonine protein kinase, a topoisomerase and a nicking-joining enzyme (Banham & Smith, 1992, Beaud *et al.*, 1984, Broyles, 1993, Earl *et al.*, 1986, Hruby & Ball, 1982, Hruby *et al.*, 1983, Hughes *et al.*, 1991, Lakritz *et al.*, 1985, Lin *et al.*, 1992, Reddy & Bauer, 1989, Shaffer & Traktman, 1987, Shuman *et al.*, 1989, Shuman & Moss, 1987, Slabaugh *et al.*, 1988, Smith *et al.*, 1989a, Tengelsen *et al.*, 1988, Traktman *et al.*, 1984, Weir & Moss, 1983).

Although the precise details of DNA replication are not yet known, it is thought to occur through a process involving self-priming and the production of concatomers which are then resolved into monomers (DeLange & McFadden, 1986, DeLange & McFadden, 1990, DeLange *et al.*, 1986). A diagram of poxvirus genome replication is shown in Fig. 1.10.

Fig. 1.10. Diagram to Show Mechanism of Poxvirus Genome Replication

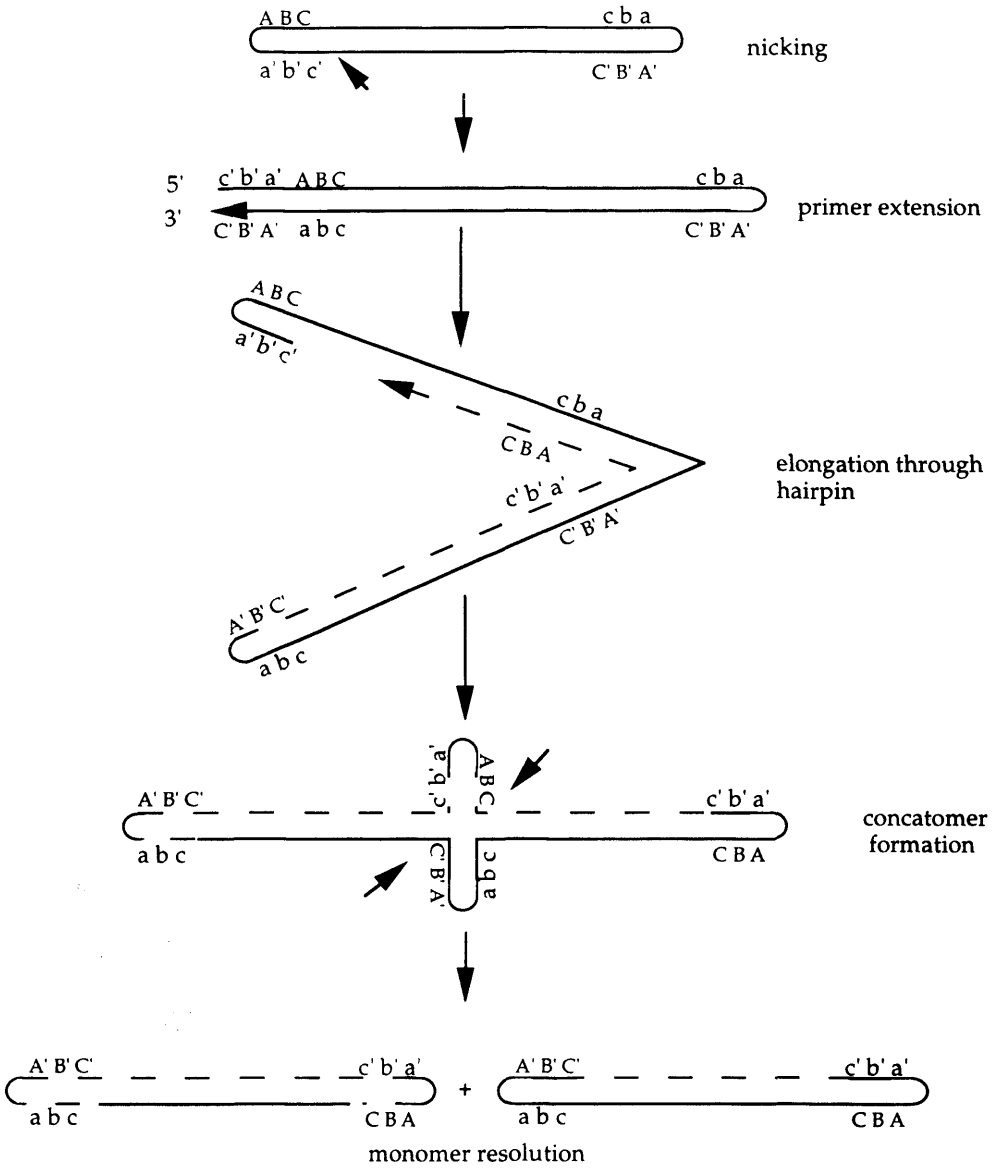


Fig. 1.10. Diagram to Show Mechanism of Poxv irus Genome Replication

The model for poxvirus replication. Inverted repeats at the termini are labelled a b c or the complementary A B C. In mature termini the hairpin regions are not fully complementary as one strand has extra bases, shown by the prime ('). Replication starts with a single nick near the terminal region which enables strand displacement and elongation of the newly formed 3' end. The complementarity of the elongated strand enables it to fold back on itself and prime further elongation. Elongation progresses through the hairpin terminus, creating a tail to tail genomic dimer. Synthesis of longer concatomers has been detected. The inverted repeats in the concatomer junction are thought to enable resolution of the concatomers through splitting of the cruciform.

1.5.3.4. Assembly

Assembly begins in the virus factories. The first stage is the formation of crescent-shaped membranes. Until recently it was believed that these membranes are formed *de novo* , it is now known that they are derived from the cisternae of the intermediate compartment, located between the endoplasmic reticulum and the Golgi (Sodeik *et al.*, 1993) and are two tightly opposed membranes. The crescents become associated with areas of the factories, granular viroplasm, which they enclose forming immature virions (IV). The IV gain an electron-dense core and mature into oval IMV particles, partly through processing of core proteins (Moss & Rosenblum, 1973). Several proteins are known to play a role in the packaging of core proteins and DNA into the IMV but the precise details of the mechanism remain unknown (Baldick & Moss, 1987, Kao & Bauer, 1987, Kao *et al.*, 1981, Moss *et al.*, 1969, Sodeik *et al.*, 1994, Wilcock & Smith, 1994).

Some of the IMV gain another two membranes from the trans-Golgi network to form the four membraned intracellular enveloped virus (IEV) (Schmelz *et al.*, 1994). IEV are transported to the edges of the cell, a process which may involve the cytoskeleton (Cudmore *et al.*, 1995, Hiller *et al.*, 1981, Hiller *et al.*, 1979, Stokes, 1976). The IEV outer membrane fuses with the cell membrane and the triple membraned EEV is released.

EEV is thought to be important in the dissemination of the virus (Appleyard *et al.*, 1971, Boulter, 1969, Boulter & Appleyard, 1973, Payne & Kristensson, 1985) and it is the form against which protective antibodies are produced.

1.6. Poxviridae and the Antiviral Activity of Interferon

An early study of IFN and viruses (Youngner *et al.*, 1972) showed that coinfection of VV and vesicular stomatitis virus (VSV), enables VSV to replicate in IFN-treated RK cells. It was suggested that VV contains or produces a product which enables the rescue of VSV from the effects of IFN.

It has since become apparent that the *Poxviridae* have evolved several mechanisms to overcome the antiviral effects of interferon. These include the production of soluble receptors for IFN, virus-encoded proteins able to down-regulate the inhibition of translation by PKR, and enzymes which inhibit the 2'-5' polyA synthetase pathway.

Although the importance of these mechanisms has been demonstrated *in vitro*, several studies have shown that despite these mechanisms IFN may protect against poxvirus infection *in vivo* (Jacoby *et al.*, 1989, Muller *et al.*, 1994, Rodriguez *et al.*, 1991).

1.6.1. Virus-Encoded Receptors

Soluble glycoprotein receptors for IFN and other cytokines are produced by many poxviruses (for reviews see McFadden & Graham, 1994, Smith, 1993) and are thought to bind to interferons and cytokines, preventing them from binding to the cell receptors. The presence of these virus-encoded receptors in many poxviruses possibly indicates the importance of interferons and cytokines in fighting poxvirus infections.

A VV gene, B18R, encodes a protein which acts as a soluble receptor, has a high affinity for human IFN-α but also binds to rabbit, bovine, rat and mouse type I IFNs, reflecting the broad host range of the virus (Symons *et al.*, 1995).

Vaccinia, cowpox and camelpox viruses also produce soluble receptors for IFN-γ. The virus-encoded receptors are unlike the natural receptors, which are highly species specific, in that they are able to bind human, bovine and rat IFN-γ. In VV the receptor is encoded by open reading frame (ORF) B8R (Alcami & Smith, 1995). Myxoma virus also encodes an IFN-γ receptor although it specifically binds rabbit IFN-γ (Upton *et al.*, 1992).

1.6.2. Antagonists of PKR

Co-infection of VV and VSV in IFN-treated cells has no effect on VSV RNA synthesis but VSV protein synthesis is increased. This was taken as an indication that VV infection is able to inhibit PKR (Whitaker-Dowling & Youngner, 1983). Shortly afterwards it was demonstrated that PKR is inhibited in VV infected cells, even late in infection when viral dsRNA is present (Rice & Kerr, 1984). It has since become apparent the inhibition of PKR by VV is due to the products of ORFs E3L and K3L.

1.6.2.1. E3L

The VV E3L gene product was originally known as specific kinase inhibitory factor (SKIF), present early in infected mouse L cells, able to inhibit phosphorylation of eIF-2α, and interact with dsRNA in a stoichiometric manner (Whitaker-Dowling & Youngner, 1984). SKIF inhibition of PKR can be reversed by addition of dsRNA and does not affect phosphorylation of eIF-2α by HRI (Akkaraju *et al.*, 1989).

Purification of SKIF revealed a protein of 25 Kd, which is present at highest levels 5 h post-infection (pi) and co-purifies with PKR inhibition (Watson *et al.*, 1991). Protein sequencing of chymotryptic fragments of p25 revealed that p25 (SKIF) is encoded by E3L and has homology with the dsRNA-binding domain of PKR (Chang *et al.*, 1992). Amino acids at the C-terminus of the protein, in the area with homology with PKR, are required for dsRNA-binding and PKR inhibition (Chang & Jacobs, 1993).

Fig. 1.11. Homology Between E3L and PKR
Adapted from Chang *et al.*, 1992

32	NSGPPHDRRFTFQVIIDGREFPEGEGRSKKEAKNAAAKLAVEIL	75	PKR
	GP + F V IDGR F ++G+SK++AKN AAKLAV L		
139	SVGPSNSPTFYACVDIDGRVFDKADGKSKRDAKNNAAKLAVDKL	182	E3L

As well as existing in the cytoplasm, particularly in virus factories, p25 can also be found in the nucleus of infected cells. The nuclear localisation of the protein is abolished by treatment of cells with RNase, but has led to the idea that p25 may play more than one role in VV infection (Yuwen *et al.*, 1993). The ability to restore PKR inhibited replication is associated with the ability of E3L to bind dsRNA and nuclear localisation of the protein is unimportant in replication competence (Chang *et al.*, 1995). Any role the nuclear localisation of E3L may play remains unknown at present. Each E3L protein contains only 1 copy of the dsRNA-binding motif at the C-terminus. It is thought that E3L forms dimers in cells which together can act as a high affinity dsRNA-binding pocket, and that the N-terminus may play a role in dimerization of E3L proteins (Ho & Shuman, 1996).

1.6.2.2. K3L

Complete sequencing of VV (Goebbel *et al.*, 1990) revealed an ORF with 28% homology to eIF-2α over an 87 amino acid region, including Ser^{51}, the site of PKR phosphorylation, although the Ser is not conserved in K3L. K3L deletion mutants show increased sensitivity to IFN (Beattie *et al.*, 1991).

The 10.5 Kd product of K3L inhibits eIF-2α phosphorylation, and PKR activation, without becoming phosphorylated itself, suggesting that K3L may inhibit PKR phosphorylation of eIF-2α by acting as a pseudosubstrate of PKR (Davies *et al.*, 1992). K3L binds PKR tightly and is also able to prevent HRI phosphorylation of eIF-2α (Carroll *et al.*, 1993). K3L is also able to interact with the kinase catalytic domain of GCN2, the yeast eIF-2α kinase (Qian *et al.*, 1996).

Fig. 1.12. Homology Between K3L and eIF-2α
Adapted from Beattie *et al.*, 1991

```
K3L             MLAFCYSLPNAGDVIKGRVYE-KDYALYIYLFDYPHSEA-              38
                   + +++P+++DV++ +V +  + + Y+ L++Y + E+
eIF-2a     MPGLSCRFYQHKFPEVEDVVMVNVRSIAEMGAYVSLLEYNNIEGM              45

K3L          ILAESVKMHMDRYVEYRDKLVGKTVKVKVIRVDYTKGYIDVNYKRMCRHQ        87
             IL +++++++ R ++ +   +G++  V VIRVD +KGYID++ +R+++++
eIF-2a       ILLSELSRRRIRSIN-KLIRIGRNECVVVIRVDKEKGYIDLSKRRVSPEEAIKCEDK....
                   *
                Ser 51
```

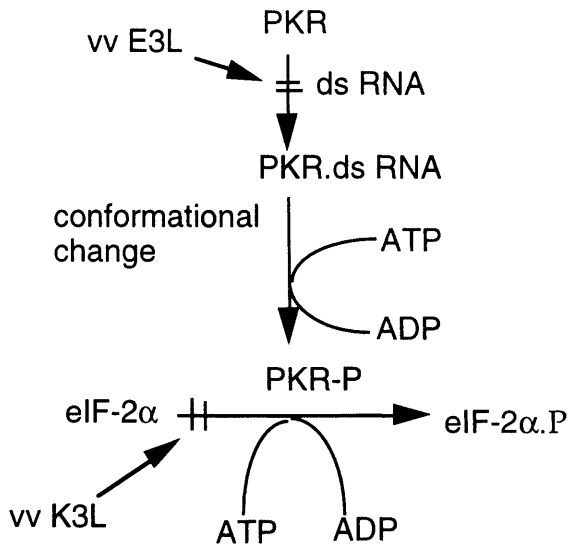
E3L inhibits PKR 50-100 times better than K3L. E3L interferes with dsRNA binding by PKR, K3L does not and the effect of both on translation is not synergistic (Davies *et al.*, 1993).

Both E3L and K3L are detectable 30 minutes post-infection but, whereas K3L expression is undetectable by 3 h p.i., E3L can be detected up to 4 h p.i. (Beattie *et al.*, 1995b). $K3L^-$ mutants of VV are sensitive to IFN by 30 minutes p.i., but $E3L^-$ mutants do not become sensitive to IFN until 2 h p.i. This information implies that K3L is the first mechanism to inhibit the effects of IFN.

2'-5' polyA synthetase is active in both VV $E3L^-$ and VV $K3L^-$ infected cells. It is thought that E3L may be able to decrease the activity of 2'-5' polyA synthetase by binding RNA and preventing activation of the enzyme but how K3L affects 2'-5' polyA synthetase is not known. It has been proposed that K3L

is required for maximal expression of VV proteins, including those involved in the evasion of 2'-5' polyA synthetase (Beattie *et al.*, 1995b). E3L$^-$ mutants are unable to replicate in Vero and HeLa cells but are replication competent in RK cells and primary CEF.

Fig. 1.13. Where E3L and K3L Act in the PKR Pathway



1.6.3. Inhibition of 2'-5' PolyA Synthetase

An early study of VV and IFN showed that VV is resistant to IFN in mouse L cells and HeLa cells. IFN of 400 U/ml has little effect on protein synthesis or virus yield (20,000-200,000 U/ml are required for inhibition of VV replication) and high levels (5 mM) of 2'-5'A are found, although HeLa cells naturally have high levels of 2'-5'A. Thus it has been proposed that high levels of 2'-5'A have little effect on VV replication. Cleavage of RNA by RNase L is delayed relative to 2'-5'A accumulation in VV infected cells, although the 2'-5'A and induction of RNase L are not defective (Rice *et al.*, 1984).

The inhibition of the 2'-5'A synthetase system is thought to be due to virus mediated ATPase and phosphatase, as the inhibition of the 2'-5'A synthetase system is associated with degradation of ATP and dephosphorylation of 2'-5'A (Paez & Esteban, 1984).

1.6.4. Other Factors in VV inhibition of Interferon

The nucleoside triphosphate phosphohydrolase 1 (NPH-1), a DNA-dependent ATPase and possible DNA or RNA helicase, may play a role in the resistance of VV to IFN through the disruption of the 2'-5'A synthetase system. Indeed a decrease in levels of a reporter gene mRNA has been demonstrated with a temperature-sensitive (*ts*) mutant of NPH-1 at the non-permissive temperature late in infection (Diaz-Guerra *et al.*, 1993).

1.7. Poxviridae and Other Areas of the Immune System

The *Poxviridae* have developed many mechanisms to counteract other areas of the immune system.

1.7.1. T-Cells

See 1.1.2. Several studies have suggested that T-cells play a role in the host defence against poxvirus infection. Ectromelia virus and VV infections are enhanced in mice treated with anti-thymocyte serum (Blanden, 1970, Hirsch *et al.*, 1968). Also during the smallpox eradication program, patients with deficiencies in cellular immune functions often developed a progressive VV infection (Fulginiti *et al.*, 1968).

$CD8^+$ T-cells are produced early in infection before antibody production, and are important in clearing infections, in a process which does not require $CD4^+$ T-cells (Buller *et al.*, 1987). $CD8^+$ T-cells are capable of killing virus-infected cells *in vitro* but *in vivo* they may help draw TNF-α and IFN-γ to the site of infection (Ramsay *et al.*, 1993).

Poxviruses are capable of disrupting MHC-I expression and thus the presentation of viral antigens to $CD8^+$ T-cells (reviewed in (McFadden & Kane, 1994). MHC-I expression is upregulated by TNF-α and IFN-γ (Wallach *et al.*, 1982). One mechanism poxviruses use to lower MHC-I expression is to encode receptors for IFN-γ and TNF-α which sequester IFN-γ and TNF-α.

Both myxoma virus and VV have been shown to be able to reduce the expression of MHC-I (Boshkov *et al.*, 1992, Brutkiewicz *et al.*, 1992). VV is also capable of inhibiting the presentation of specific peptides to $CD8^+$ T-cells, possibly by interfering in the proteolytic processing of viral antigens (Townsend *et al.*, 1988), a process which was thought to involve viral-encoded serine protease inhibitors (serpins) (Smith *et al.*, 1989b). However, this seems to be specific for certain antigens.

1.7.2. Tumour Necrosis Factors

TNF-α and TNF-β are produced by activated macrophages and B-cells, and are able to mediate the immune response in many ways on binding to cellular receptors (reviewed Beutler & Cerami, 1989). MHC-I and MHC-II expression is increased by TNF-α (Fruend *et al.*, 1990, Johnson & Pober, 1990). TNF-α can also kill virus-infected cells and protect against virus infection by inducing the antiviral state (Mestan *et al.*, 1986, Wong & Goeddel, 1986).

TNF-α can protect against poxvirus infection (Sambhi *et al.*, 1991). However Shope fibroma virus (SFV) encodes a secreted protein which has amino acid similarity to the cellular TNF receptor (Smith *et al.*, 1991b) and can bind TNF-α and TNF-β, preventing interaction with the cellular receptor. CPV, myxoma virus and rabbit fibroma virus encode a similar protein (Hu *et al.*, 1994). The myxoma virus protein with homology to the TNF receptor has been associated with virulence (Upton *et al.*, 1991).

VV has genes similar to TNF receptors but they are fragmented (Howard *et al.*, 1991, Smith *et al.*, 1991b, Upton *et al.*, 1991).

1.7.3. Complement

The complement system consists of around 20 serum proteins which when activated attracts phagocytes to the site of infection, coats invading micro-organisms to enhance phagocytosis, and forms a membrane spanning complex which enables lysis of infected cells or micro-organisms.

Complement can be activated by two pathways: the classical pathway which requires an antibody-antigen interaction, and the alternative pathway in which no antibody is required.

The alternative pathway is known to play a role in protection against fowlpox virus (FPV) infection. Cobra venom factor decreases the activation of complement by the alternative pathway. Treatment of 1-3 day old chicks with cobra venom factor leads to an increase in the numbers of viral progeny and a decrease in the inflammatory response (Ohta *et al.*, 1986).

VV IMV neutralization *in vitro* is enhanced by the classical pathway in the presence of antibody (Leddy *et al.*, 1977).

Vaccinia virus encodes two proteins with amino acid similarity to complement regulatory proteins. One is the EEV envelope glycoprotein gp42, which may protect EEV from complement proteins by binding complement factors or may enable the virus to enter cells through an interaction with

complement receptors (Engelstad & Smith, 1993). The other is a secreted 35 Kd protein which has similarity to C4b-binding protein and can prevent activation of the classical pathway by binding C4b, and the alternative pathway by binding C3b (Isaacs *et al.*, 1992, Kotwal *et al.*, 1990, McKenzie *et al.*, 1992).

1.7.4. Interleukin-1

Poxviruses produce receptors for other cytokines, in particular IL-1β is targeted. IL-1 is produced by monocytes and macrophages in response to infection or tissue damage and is involved in the regulation of the immune response.

Both VV and cowpox produce glycoprotein receptors for IL-1β (Spriggs *et al.*, 1992). Deletion of the gene which encodes the receptor has been shown to increase illness and mortality in mice, thus it has been suggested that the blockage of IL-1β reduces the acute phase response to VV infection (Alcami & Smith, 1992).

In order for mature, active Il-1β to be released from a cell, the immature protein must be cleaved, in a process which requires interleukin-1 converting enzyme (ICE) (Mosely *et al.*, 1987). CPV crmA protein, an early intracellular protein, inhibits ICE *in vitro* and prevents processing of IL-1β to its active, mature form (Ray *et al.*, 1992). VV encodes a protein with homology to CPV crmA protein (Kotwal & Moss, 1989, Smith *et al.*, 1989b).

1.7.5. Apoptosis

Apoptosis is the process of programmed cell death. Characteristics of apoptosis include shrinkage of the cell and nucleus, condensation of chromatin, fragmentation of genomic DNA, and the formation of membrane-bound apoptotic bodies.

Apoptosis can be triggered by internal metabolic disturbances, such as those resulting from viral infection, or by external stimuli such as the removal of a growth factor or the binding of specific ligands to cell receptors.

ICE, the interleukin-1 converting enzyme is thought to play a role in apoptosis as apoptosis can be induced by overexpression of ICE (Miura *et al.*, 1993). Apoptosis can also be induced by TNF binding to the cell (Enari *et al.*, 1995, Tewari & Dixit, 1995).

CPV crmA overexpression leads to inhibition of ICE and also apoptosis (Enari *et al.*, 1995, Miura *et al.*, 1993, Tewari & Dixit, 1995). It is also thought that inhibition of PKR by E3L can prevent PKR-induced apoptosis (Lee & Esteban, 1994).

1.7.6. Steroid Hormones

Steroid hormones are important in regulating the immune system. Immunosuppression can be achieved by glucocorticoids and prostaglandins can mediate inflammation. Both VV, FPV and MCV encode an enzyme which is important in steroid hormone synthesis, 3β-hydroxysteroid dehydrogenase and is involved in virus virulence (Moore & Smith, 1992, Senkevich *et al.*, 1996, Skinner *et al.*, 1994). The role of this enzyme in virulence is not yet understood and it is not yet known how it may affect the immune system.

1.7.7. Areas of the Immune System Effective Against Poxviruses

Several areas of the immune system have been demonstrated to be effective against poxviruses.

1.7.7.1. The Acute Inflammatory Response

The acute inflammatory response is the localised infection caused by infection or tissue injury. Chemoattractants are released by infected cells and macrophages at the infection site, resulting in increased blood flow to the area, allowing inflammatory cells, neutrophils and macrophages to permeate the infected or damaged site. This process results in the phagocytosis or destruction of damaged or infected cells.

The acute inflammatory response plays a role in controlling early poxvirus infection. A correlation has been shown between the strength of the early inflammatory response and poxvirus replication (Blanden, 1971, Dvorak & Hirsch, 1971, Ohta *et al.*, 1986).

1.7.7.2. Natural Killer Cells (NK)

NK cells are large granular lymphocytes which lyse virus-infected and tumour cells in an antigen non-specific mechanism which occurs prior to the cytotoxic lymphocyte response. NK cells are activated by IFN-α and kill cells by producing IFN-γ.

Several studies have shown that NK cells play a part in the defence against poxvirus infection (Jacoby *et al.*, 1989, Karupiah *et al.*, 1990, Stitz *et al.*, 1985).

1.7.7.3. Antibodies

Antibodies to viral antigens enhance the uptake of virus by phagocytic cells, aid in antigen-dependent cell-mediated cytotoxicity and can activate the classical complement pathway.

Immune serum protects rabbits from challenge with a lethal dose of rabbit poxvirus (Boulter *et al.*, 1971). Antibodies are capable of neutralizing IMV and EEV *in vitro*, but only antibodies against EEV envelope proteins are capable of protecting against VV infection *in vivo* (Appleyard *et al.*, 1971, Boulter *et al.*, 1971, Payne, 1980, Turner & Squires, 1971).

1.8. Fowlpox Virus (FPV)

FPV is the prototype of the *Avipoxviruses*.. FPV is unlike VV in that FPV infection does not inhibit host cell protein synthesis. FPV infection stimulates the uptake of nucleic acid precursors and allows cellular DNA, RNA and protein synthesis to continue for at least 48 h p.i. FPV DNA synthesis is thought to start 12-24 h p.i. and continues for 72-96 h. FPV transcription is biphasic, with early genes being transcribed before DNA replication and late transcription occuring only after the synthesis of new viral DNA (Webster & Granoff, 1994).

1.8.1. FPV Disease

FPV is a slow-spreading pox disease of chickens, which is characterised by cutaneous lesions or diptheritic lesions of the gastro/respiratory tract (Tanizaki *et al.*, 1989, Tripathy & Cunningham, 1984). The disease causes decreased egg production and weight loss and in some cases (particularly the diptheritic form) can lead to 50 % flock mortality. An effective attenuated vaccine is available. FPV infection of chick scalps produces epithelial hyperplasia with an increase in cell numbers of 250 % or more (Webster & Granoff, 1994).

The structure of FPV particles is very similar to the structure of VV particles. Brick-shaped fowlpox virus EEV particles leaving a chick embryo fibroblast (CEF) cell are shown in Fig. 1.14. The dense core and lateral bodies of a fowlpox virus particle can be seen in Fig. 1.15. Maturing virus particles within the CEF cell can also be seen in Fig. 1.15. The brick shaped biconcave core of an FPV particle, covered by an outer membrane of surface tubule elements, can be seen in Fig. 1.16.

Fig. 1.14. Electron Micrograph of a FPV Infected Chick Embryo Fibroblast

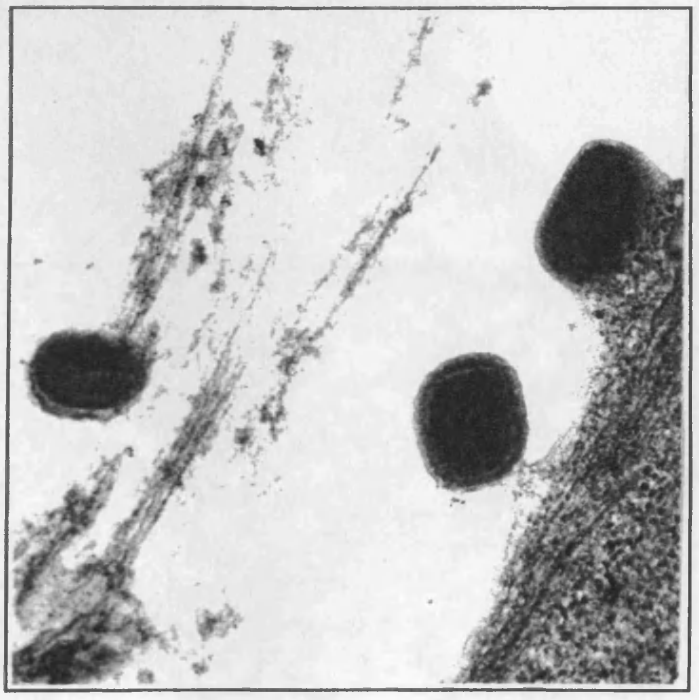


Fig. 1.14. Electron Micrograph of a FPV Infected Chick Embryo Fibroblast.
CEF cells were infected with FPV. Sections were taken and fixed with glutaraldehyde (2.5 % glutaraldehyde in 0.1 M phosphate buffer). Electron microscopy was performed with a magnification of 68 K. Photographs kindly provided by Dr. D. Boulanger.

Fig. 1.15. Electron Micrograph of a FPV Infected Chick Embryo Fibroblast

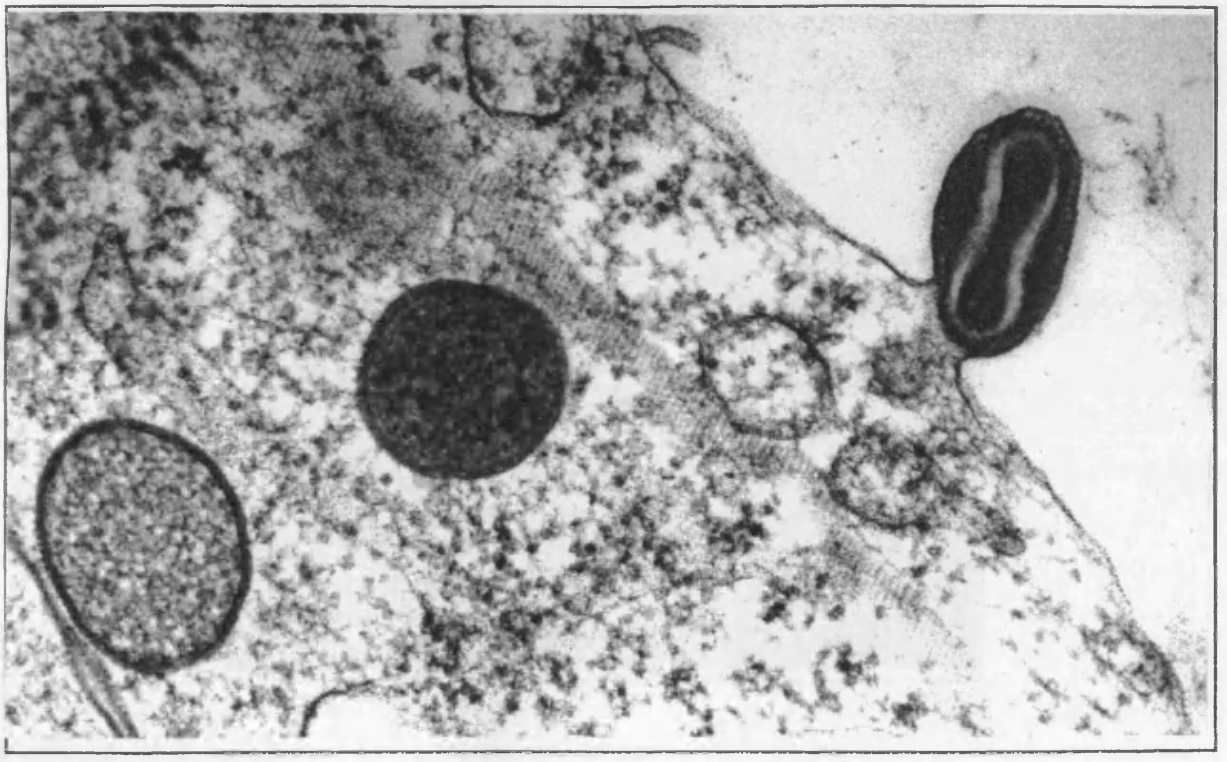


Fig. 1.15. Electron Micrograph of a FPV Infected Chick Embryo Fibroblast.
CEF cells were infected with FPV. Sections were taken and fixed with glutaraldehyde (2.5 % glutaraldehyde in 0.1 M phosphate buffer). Electron microscopy was performed with a magnification of 109 K. Photographs kindly provided by Dr. D. Boulanger.

Fig. 1.16. Electron Micrograph of FPV

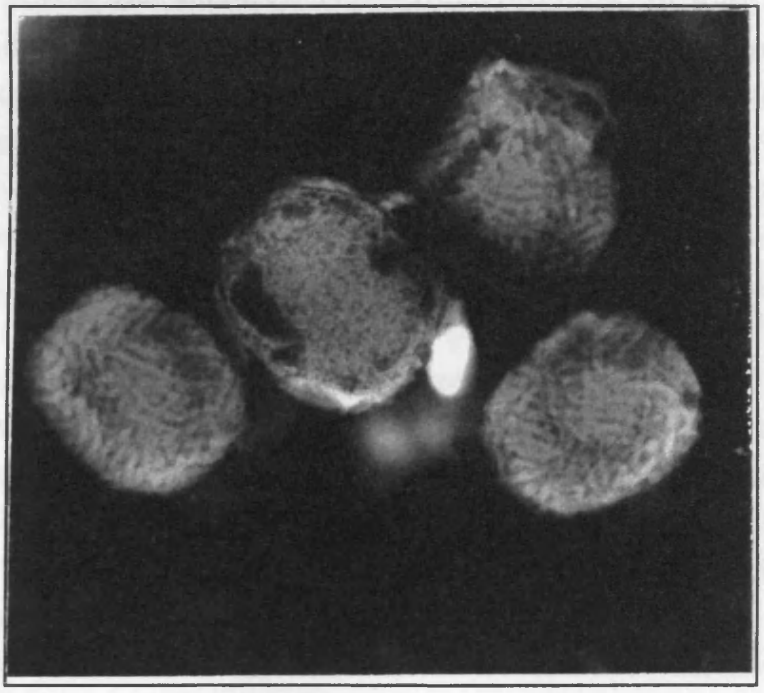


Fig. 1.16. Electron Micrograph of FPV.
Purified FPV was negatively stained with 2% PTA . Electron microscopy was performed with a magnification of 86 K. Photographs kindly provided by Dr. D. Boulanger.

1.8.2. FPV Vaccines

Much of the recent interest in the poxviruses focuses on their potential as vaccine vectors.

Foreign genes can be introduced to VV by homologous recombination and expressed (Mackett *et al.*, 1982, Panicali & Paoletti, 1982). However vaccination with VV has problems associated with it. Firstly, a significant proportion of the world population have already been exposed to VV during the smallpox eradication program. Secondly, VV can cause human disease as was demonstrated during the smallpox eradication program. In individuals with a cell-mediated immune system deficiency, progressive vaccinia often occurred and usually resulted in death. Eczema sufferers developed a condition known as eczema vaccinatum (see Fenner *et al.*, 1989) and in otherwise healthy individuals, a systemic infection of vaccinia, or neurological complications sometimes arose which could lead to death or neurological damage.

Avipoxviruses are able to express recombinant antigens quite efficiently, but unlike VV they are only able to replicate productively in avian cells. In some mammalian cells *avipoxviruses* are able to initiate replication and express early and late genes but the replication is abortive, possibly due to a block in the assembly/packaging process (Somogyi *et al.*, 1993). *Avipoxvirus* vaccines are able to induce protective immunity against live challenge (Baxby & Paoletti, 1992, Taylor *et al.*, 1991, Taylor *et al.*, 1988) and thus show potential as mammalian vaccine vectors.

FPV has been successfully used as a recombinant vaccine vector in the protection against several important chicken diseases, such as Newcastle disease virus (NDV) (Boursnell *et al.*, 1990, Taylor *et al.*, 1992), and avian influenza virus (ALV) (Beard *et al.*, 1991), and has shown some potential as a vaccine vector against infectious bursal disease virus, IBDV (Bayliss *et al.*, 1991).

Much of the current work involving the *avipoxviruses* is focused on vaccine research.

1.8.3. The FPV Genome

FPV is one of the largest viruses known, with particle dimensions of 100 x 250 x 300 nm and a genome of 300 Kbp. Unlike VV, only a small proportion of the genome has been sequenced.

Different strains of the virus have different genome sizes, FP9 254 Kbp, FPV-M (the vaccine strain) 309 Kbp, and FPV-M3 (a derivative of FPV-M) 299 Kbp (Coupar *et al.*, 1990). The inverted terminal repeats of FP9 are 10 Kbp (Tomley *et al.*, 1988). The terminal regions of the FPV genome contains an AT-rich region which consists of units of 32 bp or 56 bp repeats, although the terminal region is unique (Campbell *et al.*, 1989).

The promoter elements of some FPV genes, consisting of an AT-rich region are similar to those of VV early genes, with a 16 bp critical region, separated from the 7 bp purine-containing initiation region by an 11 bp T region (Tomley *et al.*, 1988). However a bidirectional promoter of 42 bp has also been described which is able to produce both early/late, and late mRNAs in the opposite orientation. The early and late mRNAs from the early/late promoter sequence start at the same TAAAT sequence and have no 5' polyA leader sequence. The late mRNA from the TAAAT sequence of the opposite strand has a 26 bp leader sequence (Kumar & Boyle, 1990).

The DNA polymerase of FPV has been sequenced and the predicted amino acid sequences of it and VV DNA polymerase have high levels of conservation. The conservation occurs in certain regions, possibly indicating functional domains (Binns *et al.*, 1987).

The proteins encoded by genes of FPV often have homology to proteins encoded by VV genes but the genes are often found in a different arrangement or different genomic locations (Mockett *et al.*, 1992).

A region of FPV with homology to VV genes D6-A1, contains homologues of D6, D7, D9, D10, D11, D12, D13 and A1 but not of D8. Instead, a gene with no similarity to any known poxvirus gene is in the place of D8 and is transcribed from the opposite strand. The D9 homologue contains frame-shifts at the amino terminus and so is thought to be non-functional (Binns *et al.*, 1990).

A 3.1 Kbp fragment of FPV which hybridizes to the VV region containing the TK gene, contains 6 ORFs but not the FPV TK gene (Drillien *et al.*, 1987). The TK gene of FPV is found in a 5.5 Kbp EcoRI fragment and has homology with VV TK at the nucleotide level, is predicted to be a 20.380 Kd protein and 6 amino acids larger than VV TK (Boyle *et al.*, 1987).

A 10.5 Kbp Hind III fragment of FPV which hybridises to VV HindIII-D has 10 ORFs, 5 of which have homology to potential VV ORFs but they are arranged differently (Tartaglia *et al.*, 1990).

An 11.2 Kbp near-terminal BamHI fragment of FPV contains 20 ORFs, 3 of which are predicted to have homology to *orthopoxvirus* genes; VV 42 Kd early protein, VV 32.5 Kd host range protein, and CPV 38 Kd crmA protein (Tomley *et al.*, 1988). FPV has acquired a gene related to human deoxycytidine kinase, although its function in the poxvirus is not yet understood (Koonin & Senkevich, 1993).

1.8.4. Fowlpox Virus and Interferon

A study of FPV and IFN found that FPV was 40 times less sensitive to chicken IFN in CEF than VV in a plaque inhibition assay and that FPV was a poor inducer of IFN, although small amounts were detected 96 hours p.i. (Asch & Gifford, 1970). However there is no information available on the mechanisms of FPV resistance to IFN, or on the similarities these mechanisms may have with the mechanisms of VV resistance to IFN.

1.9. Aims of Project

Little is known about avian effectors of the interferon response or of viral antagonists. The thesis presented here describes experiments to address the questions:

1) What is the IFN phenotype of FPV ?
2) Is FPV like VV in ability to rescue IFN-sensitive virus from the effects of IFN ?
3) Do avian cells contain a protein with similar function to PKR ?
4) Can an avian homologue of PKR be cloned and sequenced ?
5) Does FPV possess homologues of VV E3L and K3L ?
6) Does FPV produce secreted cytokine receptors ?

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1. Abbreviations, Bacteria, Viruses and Oligonucleotides

2.1.1. List of Abbreviations

AMPS	Ammonium persulphate
CEF	Chick embryo fibroblasts
CIP	Calf intestinal phosphatase
DMF	Dimethyl formamide
DTT	Dithiothreitol
EDTA	Diaminoethanetatra-acetic acid
FPV	Fowlpox virus
HEPES	N-[2-hydroxyethyl]piperazine
IFN	Interferon
IPTG	Isopropyl β-D-thiogalactopyranoside
MOI	Multiplicity of infection
MOPS	3-[N-morpholino]propanesulfonic acid
NBT	Nitroblue tetrazolium
NIBSC	National Institute of Biological Standards and Controls
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonylfluoride
PVP	Polyvinyl pyrrolidone
SDS	Sodium dodecyl sulphate
SFV	Semliki Forest virus
TEMED	N,N,N',N'-Tetramethylethylethylenediamine
VV	Vaccinia Virus
X-gal	5-Bromo-4-chloro-indoyl-β-D galactopyranoside
X-phos	5-Bromo-4-chloro-3-indoyl-phosphate

2.1.2. Bacterial Strains, Plasmids and Media

Table 2.1. Bacterial Strains

E.coli strain	Source	Genotype
JM109	Promega	*rec A1, sup E44, end A1, hsd R17 , gyr A96, rel A1, thi, D(lac-proAB), (r_k^-,m_k^+), [F', traD36, proAB, lac Iq ZDM15]*
XL1-Blue	Stratagene	*D(mcrA)183, D(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA, gyrA96, relA1, lac, [F' proAB, lacIqZDM15, Tn10 (Tet^r)]*
Y1090	Clontech	*F' D(lacU 169), $proA^+$, D(lon), araD139, strA, supF, hsdR, [trpC22::Tn10(tet^r)],(pMC9), mcrB*

Table 2.2. Plasmids

Plasmid	Manufactuer	Comments
pBS KS^+	Stratagene	Derived from pUC19, F1 origin, β-galactosidase selection, T3 and T7 promoters, ampicillin resistant
pET 16b	Novagen	Derived from pBR322, ColE1 origin, T7 promoter, ampicillin resistant
pGem 3ZF	Promega	F1 origin, T7 and SP6 promoters, β-galactosidase selection, ampicillin resistant

Table 2.3. Bacterial Media

Media	Contents	Per litre
LB broth	Bacto-tryptone	10 g
	Bacto-yeast extract	5 g
	NaCl	10 g
2xTY broth	Bacto-tryptone	16 g
	Bacto-yeast extract	10 g
	NaCl	5 g

All media were prepared with distilled water and were sterilized at 15 psi for 20 min. Antibiotics were added at the appropriate concentration.

To make agar, 15 g bacteriological agar (Oxoid) was added per litre of broth. To make NZY top agar, 7 g of agar was added per litre of broth.

2.1.3. Viruses, Eukaryotic Cells and Media

2.1.3.1. Viruses

Fowlpox virus FP9 is a thrice plaque purified isolate, derived from a wild strain, HP 1 which has been passaged once in eggs and 444 times in CEF.

Vaccinia virus SB-1 is the WR strain of VV which was obtained from G. Smith, Oxford. VV SB-1 is a thymidine kinase, TK^- mutant of VV WR strain with a gene encoding IBV (infectious bronchitis virus) spike protein inserted in the TK locus.

Semliki Forest virus stock was obtained from Dr. Alan Morris, Interferon Laboratory, Warwick University. The stock had been grown on neonatal mouse brain, infected intracerebrally with SFV.

2.1.3.2. Cells

Primary CEF were provided by F. Puttock and S. Duggan (tissue culture lab, I.A.H., Compton), and were produced from Rhode Island Red chickens.

Secondary CEF were produced by treating primary CEF monolayers with trypsin, washing in growth medium and reseeding.

OU2 cells are a chemically transformed CEF derived cell line.

2.1.3.3. Media

CEF Growth Medium:

1 x 199 medium with Earle's Salts (Gibco BRL), 10 % tryptose phosphate broth (Difco), 0.22 % (w/v) sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma), and 8 % foetal calf serum.

Manipulation Medium, 1 x MEM:

1 x MEM Eagle modified with Earle's Salts (ICN FLOW), 0.176 % sodium bicarbonate, 2 mM L-glutamine (ICN FLOW), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma).

OU2 Growth Medium:

1 x MEM Eagle modified with Earle's Salts (ICN FLOW), 5 % foetal calf serum, 10 % tryptose phosphate broth (Difco), 0.22 % (w/v) sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma), and 2 % chick serum.

All media were warmed to 37 ^{o}C before use.

2.1.4. Oligonucleotides

Table 2.4. Standard Sequencing Oligonucleotides

Primer	Sequence 5'-3'	Synthesized	Position
λ forward	GGTGGCACGACTCCTGGA GCC	Genosys	34 bp 5' to EcoR1 site in λ
λ reverse	GACACCAGACCAACTGG TAATG	Genosys	41 bp 3' to EcoR1 site in λ
40	GTAAAACGACGGCCAG	Leic Uni	-20 pBS
41	CAGGAAACAGCTATGAC	Leic Uni	+20 pBS

Table 2.5. Degenerate Oligonucleotides for PCR and Probing of cDNA Library

Primer	Sequence 5'-3'	Synthesized	Position
LP1	TSTTYATYCARATGGART TYTGYGA	Leic Uni	361-369 aa human PKR (LFIQMEFCD)
LP2	TCRCARWAYTCCATYTG RWKAA	Leic Uni	complementary to 361-369 aa human PKR (LFIQMEFCD)
LP3	GGYTTSARRTCNCKRTGR AT	Leic Uni	complementary to 409-416 aa human PKR (LIHRDLKP)
LP4	GTBAAYTAYGARCARTG YG	Leic Uni	116-122 aa human PKR (VNYEQCA)
LP5	AACGCCGCCGCCAAGCTS GCYGTB	Leic Uni	65-72 aa human PKR (NAAAKLAV)
LP6	CCACTGCTCCAGGGTSCC YTTRTC	Leic Uni	complementary to 369-376 aa human PKR (DKGTLEGW)

IUB Group Codes:
R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, V=G+A+C, N=A+C+G+T

Table 2.6. Oligonucleotides for PCR of FPV DNA and VV DNA and Probing of Southern Blots

Primer	Sequence	Synthesized	Position
E2L	5'AGGTTTAAATAAATATATCTTATCAACACAGAA 3'	Leic Uni	FPV homologue of VV E2L, 55-65 aa
E4L	5'GTACTTAAAGAAAAATCTACTATAAGATGC 3'	Leic Uni	FPV homologue of VV E4L, 150-160 aa
E3L-1	5'TTRATDGCDGCGCARACRATYTCDGCRTC 3'	Leic Uni	VV E3L 11-19 aa
E3L-2	5'AARGCNGCNAAYAAYAARGCNGA 3'	Genosys	complementary to VV E3L 169-176 aa
E3L-3	5'ATGGCNGAYGTNATYATYGA 3'	Leic Uni	complementary to VV E3L 80-86 aa
K3L-1	5'CCNAAYGCNGGNGAYGTNATHAARGGN 3'	Genosys	VV K3L 7-15 aa
K3L-2	5'TCRATGTANCCYTTDGTGTARTCVACVCKRATVAC 3'	Leic Uni	complementary to VV K3L 67-77 aa
K3L-3	5'GTNAARATGCAYATGGA 3'	Leic Uni	VV K3L 44-49 aa
E3L Init	5'CGGGATCCCGATGTCTAAGATCTATATTGACG 3'	K. Mawdit	VV 51483- bp, E3L initiation codon with 5' RE site
E3L Stop	5'GGAATTCCTCAGAATCTAATGATGACG 3'	K. Mawdit	VV 50914 bp E3L termination codon with 5' RE site
K3L Init	5'CAAGATGCTTGCATTTTGTTATTCG 3'	K.Mawdit	VV bp, K3L initiation codon with 5' RE site
K3L Stop	5'TAAAAATTATTGATGTCTACACATCC 3'	K.Mawdit	VV bp, K3L termination codon with 5' RE site

2.2. Biological Assays of FPV Resistance to IFN

2.2.1. Growth of Primary Chick Embryo Fibroblasts (CEF)

Primary CEF were seeded in the appropriate flask or plate at 1 x 10^6 cells/ml in 1 x 199 medium with Earle's Salts (Gibco BRL), 10 % tryptose phosphate broth (Difco), 0.22 % (w/v) sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma), and 8 % newborn calf serum. The cells were incubated at 37 ^oC, 5 % CO_2 until confluent, usually 20 h.

All plastic used in tissue culture was obtained from Nunclon.

2.2.2. Production of Interferon

The method used was essentially that of Yoshida and Marcus, 1990. Primary CEF in 175 cm^2 flasks (Nunclon) were grown for 8-11 days at 37 ^oC, 5 % CO_2, without a medium change or other disturbance. The cell layers were then washed twice with serum free 1 x MEM medium; 1 x MEM Eagle modified with Earle's Salts (ICN FLOW), 0.176 % sodium bicarbonate, 2 mM L-glutamine (ICN FLOW), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma). The cells were then incubated at 37 ^oC, 5 % CO_2 for a further 12 h in 20 ml of serum free 1 x MEM, containing either 0 ng/ml, 50 ng/ml, 500 ng/ml polyriboinosinic acid : polyribocytidylic acid, poly IC (Sigma) or 2 U/ml chicken interferon standard (NIBSC). The medium was aspirated and centrifuged for 10 min at 800 g (MSE Mistral 3000), stored at -70 ^oC and assayed for interferon activity. Cytoplasmic extracts were prepared from the cells.

2.2.3. Growth of Semliki Forest Virus from Stock

The seed stock of SFV was provided by Dr. Alan Morris, Interferon Laboratory, Warwick University.

Secondary CEF and OU2 (CEF cell line) were grown to confluency in 80 cm^2 flasks. The cells were infected with 1.4 x 10^7 pfu (multiplicity of infection, moi 10) of the seed stock SFV in 1 x MEM and incubated at 37 ^oC, 5 % CO_2 for 1 h with occasional shaking. The inoculum was then removed and replaced

with 20 ml 1 x MEM, 2 % newborn calf serum and incubation continued at 37 ^{o}C, 5 % CO_2.

After 2 days of incubation, when the cells were lysing and lifting from the monolayer, the medium was removed and the cell debris pelleted by centrifugation at 1,500 rpm for 15 min (MSE Mistral 3000). The supernatant was aliquoted and stored at -20 ^{o}C.

2.2.4. Preparation of Viral DNA

Small flasks (T25) of CEF were infected with virus at a moi of 10 in 1 x MEM (1 x MEM Eagle modified with Earle's Salts (ICN FLOW), 0.176 % sodium bicarbonate, 2 mM L-glutamine (ICN FLOW), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 U/ml nystatin (Sigma). The cells were incubated at 37 ^{o}C, 5 % CO_2 until the cells could be shaken from the flask. The cells were pelleted by low speed centrifugation and resuspended in 600 µl of PBS.

The cells were freeze/thawed (-70 ^{o}C/37 ^{o}C) three times and an equal volume of 2 x TEM (1 % Triton X-100, 40 mM EDTA, 70 mM β-mercaptoethanol) was added. The cell debris was pelleted by low speed centrifugation (3000 rpm) for 3 min. The supernatant was transferred to a clean tube and the virus cores were pelleted by high speed centrifugation (14000rpm) for 15 min. The pellet was resuspended in 100 µl of digestion buffer (10 mM Tris, pH 8, 1 mM EDTA, 5 mM β-mercaptoethanol, 150 µg/ml proteinase K, 200 mM NaCl, 1 % SDS) and incubated at 50 ^{o}C for 30 min. The DNA solution was extracted twice with phenol/chloroform/isoamyl alcohol (50:48:2) and ethanol precipitated. The DNA was stored either as a dry pellet or under ethanol at -20 ^{o}C.

2.2.5. Titre of Viruses

Primary CEF were seeded at 5 x 10^8 or 1 x 10^6 in 60 mm Petri dishes (Nunclon) and allowed to grow to confluency. The virus was 10 fold serially diluted in phosphate buffered saline, PBS (36 mM NaCl, 0.9 mM KCl, 10.2 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2). The growth medium was removed from the cells, and 0.5 ml of each of the virus dilutions were applied to duplicate dishes. The dishes were incubated at 37 ^{o}C for 1 h with occasional shaking.

Agarose overlay was prepared by mixing equal volumes of melted sterile 2 % LMP agarose and pre-warmed 2 x MEM (2 x MEM Eagle modified with Earle's Salts (ICN FLOW), 0.1352 % sodium bicarbonate, 4 mM L-glutamine (ICN FLOW), 200 U/ml penicillin, 200 µg/ml streptomycin, 50 U/ml nystatin (Sigma)). The inoculum was removed from the dishes with a 10 ml pipette and 3 ml of the agarose overlay was applied gently to each plate and allowed to set. The plates were incubated at 37 ^{o}C, 5 % CO_2. SFV infected dishes were incubated for 2 days, FPV infected plates were incubated for 4 days and VV infected dishes were incubated for 2 days.

After the appropriate incubation time, 3 ml of fix solution (10 % formaldehyde in PBS) was applied directly to the agarose overlay. The overlay was flicked off with a sterile toothpick and the cells treated again with fix solution for 10 min. The cells were stained for 10 min with gentian violet stain (1 % gentian violet, 20 % ethanol in PBS), washed with water and the plaques counted.

2.2.6. Interferon Assays

Interferon assays (Katze & al, 1991) were used to establish the concentration of IFN produced by poly IC treatment of CEF, by comparison with the IFN standard (National Institute of Biological Standards and Controls, NIBSC) and also to establish the relative IFN sensitivity of SFV, FPV and VV.

IFN was provided as a desiccated sample which was dissolved in water to provide a stock solution of 80 international units (IU)/ml which was stored at -20 ^{o}C. This stock solution was diluted to 8 IU/ml in 1 x MEM before use in the IFN assay.

The IFN solution was serially 2 fold diluted in 125 µl of 1 x MEM. The growth medium was aspirated from confluent primary CEF. Medium alone was placed in the cell control wells and the virus control wells and 100 µl of the diluted IFN solutions were applied to the wells. The plate was incubated at 37 ^{o}C, 5 % CO_2 for 16 h.

The IFN solutions were flicked off the cells and replaced with either 1 x 10^5 pfu/well SFV, 1 x 10^7 pfu/well FPV or 2 x 10^3 pfu/well VV. The cells were incubated at 37 ^{o}C, 5 % CO_2 for the appropriate time for each virus; SFV

infected plates were incubated for 2 days, FPV infected plates were incubated for 4 days and VV infected plates were incubated for 2 days.

Cells were stained by one of the three following methods:

Gentian Violet:

Fix solution (10 % formaldehyde in PBS) was placed on the cells for 10 min and removed by gently inverting the plate. The stain solution (1 % gentian violet, 20 % ethanol in PBS) was gently applied to the cells and left on for 10 min. The plates were washed gently with water and dried.

Neutral Red:

Three ml of a stock solution (0.3 % neutral red in water) was added to 100 ml of medium. The medium was added to the cells and incubated for 4 h at 37 ^{o}C, 5 % CO_2. The cells were then washed with water and allowed to dry.

Amido Black:

The cells were fixed (10 % formaldehyde, 9 % acetic acid, 0.1 M NaOAc) for 10 min, stained (0.05 % amido black, 9 % acetic acid, 0.1 M NaOAc) for 10 min, washed in water and dried.

2.2.7. Rescue of SFV from the Effects of IFN by FPV

The method used was essentially that of Whitaker-Dowling and Younger, 1983, however the plaque morphology and the incubation time required to see FPV and SFV plaques is so different that an inhibitor of FPV late events was not considered necessary.

Primary CEF were seeded in 60 mm petri dishes at 1 x 10^6 cells/ml and allowed to grow to confluency. The growth medium was removed and replaced with 1 x MEM, 0.5 % NBCS, with or without 4 IU/ml of IFN. The cells were incubated 37 ^{o}C, 5 % CO_2 for 16 h.

The appropriate dishes were then infected with FPV (FP9) at 2 x 10^7 pfu/dish (moi of 10) in serum-free 1 x MEM. The virus was allowed to absorb by incubation at 37 ^{o}C, 5 % CO_2 for 1 h with occasional shaking. The inoculum was removed and replaced with 3 ml of serum-free 1 x MEM and incubation was carried out for a further 3 h.

The appropriate dishes were then infected with SFV at 2 x 10^7 pfu/dish (moi of 10) in serum-free 1 x MEM. The virus was allowed to absorb by incubation at 37 ^oC, 5 % CO_2 for 1 h with occasional shaking. The inoculum was removed and replaced with 3 ml of serum-free 1 x MEM and incubation was carried out for 16 h.

The supernatants and cells were removed from the dishes and freeze-thawed (-70 ^oC, 37 ^oC) three times to release the virus. The virus was titred in triplicate, as in 2.2.5.

Dishes	Interferon Treatment	FPV Infection	SFV Infection
1-2	+	+	-
3-4	+	-	+
5-6	+	+	+
7-8	-	+	-
9-10	-	-	+
11-12	-	+	+

2.3. Analysis of Cytoplasmic Extracts for Biochemical Activities Associated with PKR

2.3.1. Production of Cytoplasmic Extracts

Primary CEF were seeded at 1 x 10^6 cells/ml in 175 cm^2 and grown to confluency. The confluent cell monolayers were then treated with IFN solutions, or infected with FPV (moi of 5-10) and incubated at 37 ^oC, 5 % CO_2.

Preparation of cytoplasmic extracts was carried out essentially as in the method of Rice and Kerr, 1984.

The cells were washed twice with phosphate buffered saline, PBS (136 mM NaCl, 0.9 mM KCl, 10.2 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2). The cells were harvested with a rubber policeman and 1.5 ml of PBS, and pelleted in an eppendorf tube by centrifugation at 13,000 rpm (MSE Microcentaur) for 30 sec. The cells were lysed by adding 2 volumes of cytoplasmic extract buffer (0.15 M NaCl, 10 mM Tris, pH 7.9, 1.5 mM $MgCl_2$, 0.65 % Nonidet P-40) and

vortexing. Cell debris was pelleted by centrifugation at 13,000 rpm (MSE Microcentaur) for 30 sec, the supernatant was removed and stored at -70 ^{o}C.

2.3.2. Production of Human PKR

Human PKR was produced by *in vitro* transcription and translation using the T7 Coupled TNT Rabbit Reticulocyte Lysate System (Promega) and pGem (Novagen) cloned human PKR.

A 50 µl reaction was set up with 25 µl of TNT Rabbit Reticulocyte Lysate, 2 ml TNT Reaction Buffer, 1 µl TNT T7 RNA Polymerase, 1 µl of 1 mM Amino Acid Mixture, 4 µl ^{35}S methionine (1,000 Ci/mmol, 10mCi/ml, DuPont), 1 µl RNasin ribonuclease inhibitor, and 1 µg pGem.PKR. The reaction was incubated at 30 ^{o}C for 90 min.

2.3.3. Autophosphorylation of Cell Extracts

The method used was essentially that of Rice and Kerr, 1984. Reactions consisted of 5 µl of cell extract, 100 µM ATP, 1 µCi ^{32}P (3,000 Ci/mmol, DuPont), 10 mM MgOAc, in a final reaction volume of 10 µl. To some reactions 0.01 µg of poly IC (Sigma) was added.

The reactions were incubated at 30 ^{o}C for 15 min and then stored at -20 ^{o}C, until analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE).

2.3.4. Partial Purification of Cell Extracts

The method uses poly IC bound agarose to purify dsRNA-binding proteins from the cell extracts and is essentially that of Patel and Sen, 1984 .

Twenty five µl of the poly IC agarose beads (Pharmacia) were washed three times in 500 µl of binding buffer (20 mM HEPES pH 7.5, 0.3 M NaCl, 5 mM MgOAc, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride (PMSF), 0.5 % nonidet P-40, 10 % glycerol). The poly IC agarose beads were resuspended in 25 µl of binding buffer, mixed with 25 µl of the cell extract and incubated at 30 ^{o}C for 30 min with occasional mixing.

The beads were washed twice with wash buffer (20 mM HEPES pH 7.5, 0.5 M NaCl, 5 mM MgOAc, 1 mM dithiothreitol, 1 mM PMSF, 0.5 % nonidet P-40, 10 % glycerol), once with binding buffer and were resuspended in 21 µl of binding buffer. Seven µl of the poly IC agarose-bound cell extracts were used in the autophosphorylation reaction as in 2.3.3. and analysed by SDS PAGE.

2.3.5. Phosphorylation of Histone Proteins

Cell extracts were bound to poly IC agarose beads and used in the autophosphorylation reaction as in 2.3.3. with 2 μg of calf thymus histones (Sigma).

2.3.6. Phosphorylation of eIF-2α Peptides

The eIF-2α peptide (NIEGMILLSELSRRRIRSIN) and the pseudopeptide (NIEGMILLSELARRRIRSIN) were provided by M. Clemens.

Five μl of poly IC agarose bound cell extract was mixed with peptide buffer (20 mM Tris, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10 % glycerol, 5 mM 2-mercaptoethanol), 3 μCi ^{32}P dATP (5,000 Ci/mmol, DuPont), and 2 mM peptide in a final volume of 10 μl, and incubated at 30 ^{o}C for 15 min.

The samples were mixed with an equal volume of peptide sample buffer (1 % sodium dodecyl sulphate (SDS), 8 M urea, 1 % 2-mercaptoethanol, 0.01 M H_3PO_4 adjusted to pH 6.8 with Tris base), boiled and run on an oligopeptide gel.

2.3.7. Oligopeptide Gels

The Swank and Munkres, (1971) method was used to analyse the peptides.

Acrylamide-Bisacrylamide: 12.5 g of acrylamide and 1.25 g of bisacrylamide were dissolved in 50 ml of water and filtered.

Gel Buffer Stock: 1 % SDS, 1 M H_3PO_4 adjusted to pH 5 with Tris base.

Reservoir Buffer Stock: 0.1 % SDS, 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris base.

The resolving gels were prepared with 15 ml acrylamide, 3 ml gel buffer stock, 14.4 g of urea, made up to 30 ml with water and 0.3 ml of 6 % AMPS and 30 μl of TEMED. The resolving gel was poured between 20 x 18 cm plates, with the appropriate spacers or in the Mini Protean II (Biorad) system. The gel was overlaid with water and allowed to set.

The stacking gels were prepared in the same way but with only 1.875 ml of acrylamide. The water was removed from the top of the resolving gel and the stacking gel was poured on top. The appropriate comb was placed in the stacking gel and the gel was allowed to set.

The samples were boiled for 5 min in an equal volume of sample buffer and loaded onto the gels. The gels were run in reservoir buffer at 80 V for 16 h, then the plates were separated and the gel soaked in Coomassie blue stain (0.1 % Coomassie Blue G250, 10 % methanol, 10 % acetic acid) for 20 min. The gels were destained by soaking in 10 % methanol, 10 % acetic acid for several hours, then sealed in bags and exposed to film.

2.3.8. SDS Polyacrylamide Gel Electrophoresis

30 % Acrylamide Mix: 290 g of acrylamide and 10 g of bisacrylamide were dissolved in 1 l of water. The solution was deionised by mixing with monobed resin, then filtered and stored in a darkened bottle.

10 % Resolving Gel: 10 ml of 30 % acrylamide mix, 7.5 ml Tris, pH 8.8, 0.3 ml 10 % SDS, 0.3 ml 10 % AMPS and 12 µl of TEMED were made up to 30 ml with water.

12 % Resolving Gel: 12 ml of 30 % acrylamide mix, 7.5 ml Tris, pH 8.8, 0.3 ml 10 % SDS, 0.3 ml 10 % AMPS and 12 µl of TEMED were made up to 30 ml with water.

15 % Resolving Gel: 15 ml of 30 % acrylamide mix, 7.5 ml Tris, pH 8.8, 0.3 ml 10 % SDS, 0.3 ml 10 % AMPS and 12 µl of TEMED were made up to 30 ml with water.

5 % Stacking Gel: 1.7 ml of 30 % acrylamide mix, 1.25 ml Tris, pH 6.8, 0.1 ml 10 % SDS, 0.1 ml 10 % AMPS and 10 µl of TEMED were made up to 10 ml with water.

Loading Buffer: 50 mM Tris pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol.

The resolving gel was poured between 20 x 18 cm plates, with the appropriate spacers or in the Mini Protean II (Biorad) system. The gel was overlaid with water and allowed to set.

The water was removed from the top of the resolving gel and the stacking gel was poured on top. The appropriate comb was placed in the stacking gel and the gel was allowed to set.

The samples were boiled for 5 min in an equal volume of loading buffer and loaded onto the gels. The gels were run in tris-glycine buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1 % SDS) at 150 V for 2-5 h, then the plates were separated and the gel soaked in Coomassie blue stain (0.1 % Coomassie

Blue G250, 10 % methanol, 10 % acetic acid) for 20 min. The gels were destained by soaking in 10 % methanol, 10 % acetic acid for several hours, then dried down onto paper under vacuum, or sealed in bags and exposed to film.

2.4. Immunological Methods

2.4.1. Labelling Proteins with ^{35}S Methionine

Primary CEF in 25 cm^2 flasks were virus infected or interferon treated for the appropriate periods. The medium was removed and replaced with 3 ml of serum-free, methionine-free medium (Sigma), and incubated at 37 ^oC, 5 % CO_2 for 2 h. The medium was removed and replaced again with 3 ml serum-free, methionine-free medium, containing 1.85 MBq ^{35}S methionine (1,000 Ci/mmol, 10mCi/ml, DuPont) per ml for a further 2 h incubation at 37 ^oC, 5 % CO_2.

The supernatants were filtered through 0.2 µm filters and concentrated in Centriprep 10 (Amicon) and Microcon 30 (Amicon) columns.

The cells were removed from the flasks in PBS and lysed in cytoplasmic extract buffer as in 2.3.1.

2.4.2. Radio-Immuno Precipitation Assay (RIPA)

Protein A Sepharose CL-4B (Pharmacia) was made to a 10 % slurry in 0.1 M sodium phosphate (pH 8.1). Fifty µl of the slurry was washed twice in sodium phosphate buffer and 2 µl of the monoclonal was added and incubated at 4 ^oC for 1 h on a rotating mixer. The slurry was washed twice in sodium phosphate buffer and 20 ml of unlabelled control cell extract was added and incubated with the sepharose for 16 h at 4 ^oC. The sepharose was pelleted by brief centrifugation and the supernatant removed.

Meanwhile, 50 µl of labelled cell extract was mixed with 50 µl of protein A sepharose slurry at 4 ^oC for 16 h. The sepharose was pelleted and the supernatant collected.

The pre-cleared labelled cell extract was mixed with the sepharose-bound monoclonal at 4 ^oC for 3h. The sepharose was washed five times with RIPA I buffer (0.15 M NaCl, 0.05 M Tris, pH 7.2, 1 % Triton-X-100, 0.1 % SDS, 1

% sodium deoxycholate) and twice with RIPA II buffer (0.15 M NaCl, 0.05 M Tris, pH 7.2, 1 % Triton-X-100). The samples were analysed by SDS PAGE.

2.4.3. Western Blotting

2.4.3.1. Transfer of Proteins to Nitrocellulose

Samples were run on SDS polyacrylamide gels and soaked in western blotting buffer (25 mM Tris, 250 mM Glycine, 20 % methanol) for 30 min. The gel holder was opened and placed in a tray of western blotting buffer. Four pieces of 3MM and 1 piece of Hybond-C nitrocellulose (Amersham) were cut to size and soaked in western blotting buffer. Two pieces of wetted 3MM were placed on top of the fibrous pad of the gel holder. The gel was placed on top of the 3MM, with the nitrocellulose on top of the gel. Air bubbles between the gel and nitrocellulose were removed and the other two pieces of 3MM were placed on top of the nitrocellulose. The fibrous pad of the holder was placed on top of the 3MM paper, air bubbles were removed and the holder was closed. The gel holder was placed in the buffer tank, containing western blot buffer and orientated so that the nitrocellulose was towards the positive electrode. The proteins were transferred to the nitrocellulose by electrophoresis at 30 V for 16 h at 4 ^{o}C.

2.4.3.2. Antibody Probing

After transfer, the nitrocellulose was rinsed in water and stained with Ponceau S (0.2 % Ponceau S in 7 % acetic acid) to enable visualization of markers.

The nitrocellulose was blocked by incubation in TN buffer (10 mM Tris, pH 7.5, 500 mM NaCl), with 3 % milk powder for at least 30 min. The nitrocellulose was then incubated with the appropriate dilution of the first antibody in TTN (10 mM Tris, pH 7.5, 500 mM NaCl, 0.05 % Tween) for 2 h.

The nitrocellulose was washed three times in water for 5 min each wash, then with TTN for 20 min. This series of washes was repeated 3 times and then the nitrocellulose was incubated with the appropriate dilution of the second antibody (alkaline phosphatase conjugated) for 2 h. The series of washes was repeated and the alkaline phosphatase substrate prepared using the Pierce kit by mixing 10 ml of 0.1 M Tris with 1 ml of X-phos (5-bromo-4-

chloro-3-indolyl-phosphate, 2.5 g/l) and 1 ml of NBT (nitrobluetetrazolium, 5 g/l). The substrate was placed on the nitrocellulose and gently shaken for 10 min. When the colour had developed sufficiently, the nitrocellulose was washed twice with water, allowed to dry and stored in the dark.

2.5. Analysis of FPV Extracellular Proteins

2.5.1. Production and Concentration

The following protocol was used for both labelled and non-labelled virus supernatants, and is essentially that of Martinez-Pomares *et al*, (1995). Primary CEF were seeded in 175 cm^2 flasks and allowed to grow to confluency. The cells were washed twice with serum free 1 x MEM. The cells were infected with 5 x 10^6 pfu/ml FP9 (moi of 5). After 12 h incubation at 37 ^oC, 5 % CO_2, the inoculum was removed. The cells were washed twice with serum-free 1 x MEM and 10 ml of serum-free 1 x MEM was applied to the cells. The cells were incubated at 37 ^oC, 5 % CO_2 for a further 12-24 h. The medium was removed from the cells and filtered through a 0.2 μm filter to remove extracellular virus. The medium was concentrated 25-75 fold using Centriprep 10 and Microcon 30 spin columns (Amicon), before being analysed.

2.5.2. Analysis of Glycosylation State

^{35}S methionine labelled FPV supernatants were concentrated 30 fold. The supernatants were mixed with an equal volume of one of the three following buffers and heated to 100 ^oC for 3 min.

Buffer 1: 50 mM EDTA, 0.8 mM PMSF in 50 mM sodium phosphate buffer, pH 6.1.

Buffer 2: 50 mM EDTA, 0.8 mM PMSF, 0.2 % SDS, 1 % Triton-X-100 in 50 mM sodium phosphate buffer, pH 6.1.

Buffer 3: 50 mM EDTA, 0.8 mM PMSF, 0.2 % SDS, 1 % Triton-X-100, 2 % β-mercaptoethanol in 50 mM sodium phosphate buffer, pH 6.1.

Once the samples had cooled, 0 or 0.4 U of endoglycosidase F/N-glycosidase F (Boehringer Mannheim Biochemica) were added. The samples were incubated at 37 ^oC for 20 h then analysed by SDS PAGE.

2.5.3. Inhibition of Glycosylation

FPV infected cells were labelled with ^{35}S methionine as in 2.4.1. The methionine free medium was supplemented with 0.5 or 5 µg/ml of tunicamycin (Sigma) at both the pre-labelling and labelling stages. The medium was processed in the usual way and analysed by SDS PAGE.

2.5.4. Lectin Analysis

Concentrated FPV medium was run on SDS polyacrylamide gels in both native and denaturing conditions. The proteins were blotted onto nitrocellulose membrane as in 2.4.3.1. The blotted proteins were probed with biotinylated lectins (Vector Laboratories) according to the manufacturer's protocol.

The nitrocellulose was incubated in TTBS (0.1 % Tween 20, 100 mM Tris, 0.9 % NaCl pH 7.5) for at least 30 min with gentle agitation. The nitrocellulose was then incubated in TTBS with 20 µg/ml of the biotinylated lectin for 1 h with gentle shaking. The blot was washed three times in water for 5 min each wash, then with TTN for 20 min. This series of washes was repeated 3 times.

The nitrocellulose blot was then incubated in a 1/400 dilution of alkaline phosphatase conjugate for 1 h. The series of washes was repeated and the alkaline phosphatase substrate prepared using the Pierce kit by mixing 10 ml of 0.1 M Tris with 1 ml of X-phos (5-bromo-4-chloro-3-indolyl-phosphate, 2.5 g/l) and 1 ml of NBT (nitrobluetetrazolium, 5 g/l). The substrate was placed on the nitrocellulose and gently shaken for 10 min.

2.5.5. Concanavalin A Purification

FPV extracellular proteins were partially purified by binding to concanavalin A (Con A)-sepharose.

Con A-sepharose (Pharmacia) was washed in 10 volumes of binding buffer (20 mM Tris, pH 7.5, 0.5 M NaCl). A column of 0.5-1 ml of washed Con A sepharose was set up in a 1 ml syringe as in 2.1.10.3. The column was washed three times by applying 0.5 ml of binding buffer to the column and allowing it to flow through. The sample was applied to the column which was then washed six times with binding buffer. The bound proteins were eluted by applying 500 µl washes of 0.3 M methyl-α-D glucose. The eluted

fractions were concentrated using Microcon 30 spin columns and analysed by SDS PAGE.

2.6. DNA Methods

2.6.1. Ethanol Precipitation

For each volume of nucleic acid-containing solution, 0.1 volumes of 3M NaOAc and 2.5 volumes of ethanol were added. The mixture was incubated at -20 °C for 20 minutes and centrifuged at high speed to pellet the nucleic acid (13000 rpm, benchtop MSE Microcentaur, 15000 rpm SS34 rotor in Beckman Sorval RC-5B). The ethanol was removed and the nucleic acid pellet was carefully washed with 70 % ethanol before being air-dried and resuspended in the appropriate buffer.

2.6.2. Analysis of Nucleic Acid Concentration

Spectrometric analysis of nucleic acid concentration was carried out using a Pharmacia Ultraspec III and a 50-200 µl quartz cuvette.

For double-stranded DNA, 1 A_{260} unit = 50 µg/ml

For single-stranded DNA, 1 A_{260} unit = 40 µg/ml

For oligonucleotides, 1 A_{260} unit = 30 µg/ml

2.6.3. Restriction Digestion of DNA

DNA digestion was performed with 1-5 units of enzyme per µg of DNA. The reactions were set up in volumes of 10-100 µl with the reaction buffer recommended by the enzyme manufacturer (predominantly Gibco BRL and New England Biolabs) and were incubated at 37 °C for 2-16 h.

2.6.4. Dephosphorylation of 5' Ends of DNA

0.25 units of calf intestinal phosphatase (CIP) (Promega) were added per µg of digested vector DNA. Incubation was carried out at 37 °C for 30 minutes in the buffer provided by the manufacturer (1x buffer: 50 mM Tris, pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine). The enzyme was then inactivated by heating at 75 °C for 10 minutes. The protein was extracted with

phenol (equilibrated with 1M Tris pH 7.5): chloroform: isoamyl alcohol (50:48:2) and the DNA was ethanol precipitated.

2.6.5. Ligation Reaction

10-100 µg of vector DNA and a three fold molar excess of insert DNA were ligated in a final reaction volume of 10 µl, containing 1 mM ATP, 1 unit of T4 ligase (Promega) and the manufacture's buffer (1x concentration: 66 mM Tris, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, pH 7.5). Incubation was carried out at 4 °C or 15 °C for 16 h.

2.6.6. Transformation of *E.coli*

2.1.6.1. Preparation of Competent Cells for Heat-Shock Transformation

A single colony of *E.coli* (strain JM109, or XL1-blue) was inoculated into 10 ml of 2xTY medium (1.6 % tryptone, 1 % yeast extract, 85 mM NaCl, pH 7.4) and grown overnight at 37 °C in a shaking incubator. Five ml of the overnight culture was used to inoculate 250 ml of 2xTY in a 2l flask which was shaken at 37 °C for 2-3 h or until an OD_{600} of 0.7 (mid log phase) was reached.

The cells were then chilled on ice for 20 min, transferred to cold 250 ml tubes and pelleted by centrifugation at 5,000 rpm for 5 min at 4 °C (Beckman Sorval RC-5B Centrifuge, GSA rotor). The supernatant was removed and the pellet of cells was gently resuspended in 125 ml of ice-cold 10 mM NaCl. The cells were then repelleted as before, resuspended in 62.5 ml of ice-cold 30 mM $CaCl_2$ and incubated on ice for 20 min. The cells were pelleted once more and resuspended in 10 ml of ice-cold 30 mM $CaCl_2$, containing 15 % glycerol. The cell suspension was aliquoted into cold eppendorf tubes, snap frozen in a dry ice and alcohol bath and stored at -70 °C.

2.6.6.2. Heat-Shock Transformation

The frozen competent cells were thawed slowly on ice, 150 µl of competent cells were added to each 10 µl ligation mix and incubated on ice for 10 min. The cells were then heat-shocked at 42 °C for 2 min, were returned to ice for 10 min and 200 ml of 2xTY was added. The mix was then incubated at 37 °C for 30 min and 150 µl were plated onto 2xTY agar plates containing 100

µg/ml ampicillin (or other appropriate antibiotic), to select for ampicillin-resistant, transformed colonies, and were incubated at 37 °C for 16 h.

If β-galactosidase expression was used to identify recombinant plasmids, the plates were pre-spread with 100 µl of 100 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 50 µl of 2 % 5-bromo-4-chloro-indoyl-β–D galactopyranoside (X-gal) in dimethylformamide (DMF).

2.6.6.3. Preparation of Competent Cells for Electroporation

A single colony of *E.coli* (strain JM109, or XL1-blue) was inoculated into 10 ml of 2xTY medium (1.6 % tryptone, 1 % yeast extract, 85 mM NaCl, pH 7.4) and grown overnight at 37 °C in a shaking incubator. Five ml of the overnight culture was used to inoculate 250 ml of 2xTY in a 2l flask which was shaken at 37 °C for 2-3 h or until an OD_{600} of 0.7 (mid log phase) was reached.

The cells were then chilled on ice for 30 min, transferred to cold 250 ml tubes and pelleted by centrifugation at 3,500 rpm for 15 min at 4 °C (Beckman Sorval RC-5B Centrifuge, GSA rotor). The supernatant was removed and the pellet of cells was slowly and gently resuspended in 250 ml of ice-cold sterile water. The cells were then repelleted as before and resuspended in 150 ml of ice-cold water. The cell suspension was pelleted once again in a 50 ml tube at 5,800 rpm (Beckman Sorval RC-5B Centrifuge, SS34 rotor), resuspended in 500 µl ice-cold 10 % glycerol, aliquoted into cold eppendorf tubes, snap frozen in a dry ice and alcohol bath and stored at -70 °C.

2.6.6.4. Transformation by Electroporation

Electroporation was carried out according to the manufacturer's instructions using an Invitrogen Electroporator II.

Competent cells were thawed on ice whilst the cuvettes (0.1 or 0.2 cm gap) and DNA ligation mix were chilled. Forty µl of the competent cells were mixed with 2 µg of DNA and placed in the cuvette. The electroporater was set at 50 µF capacitance and 150 Ohm resistance. The power supply to the electroporater was provided by a power pack (LKB Bromma), which was set at 1500 V, 25 mA, 25 W. The cuvette was placed in the elecroporater, which was allowed to charge before the cuvette was pulsed. Immediately following

pulsing 1 ml of SOC was added to the cuvette, mixed with the cells before being transferred to a sterile bijoux and incubated at 37 ^{o}C for 1 h.

Plating was carried out as in 2.1.6.2. although the efficiency of electroporation is such that dilution of the transformed cells in SOC was sometimes required prior to plating.

2.6.7. Preparation of Plasmid DNA

2.6.7.1. Caesium Chloride Preparation of Plasmid DNA

A 10 ml overnight culture, grown from a single colony, was used to inoculate 500 ml of 2xTY or LB, containing the appropriate antibiotic, which was incubated for 16 h in a shaking incubator. The cells were pelleted by centrifugation at 5,000 rpm for 5 min at 4 ^{o}C (Beckman Sorval RC-5B Centrifuge, GSA rotor). The supernatant was poured off and the pellet allowed to drain, before being resuspended in 5.5 ml of GTE (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA). 11 ml of NaOH/SDS solution (0.2M NaOH, 1 % SDS) was added, mixed by gentle shaking and incubated on ice for 10 min. 8 ml of KAc/HAc (3M/2M) was added, mixed by gentle shaking and incubated on ice for 10 min. The tubes were centrifuged at 15,000 rpm, 4 ^{o}C for 30 min (SS34 rotor in RC-5B). The supernatant (28 ml) was removed, 17 ml of isopropanol added and incubated at room temperature for 15 min. The tubes were then centrifuged for 20 min at 10,000 rpm and 4 ^{o}C to pellet the DNA.

The pellet was resuspended in 2 ml of TE (10 mM Tris, pH 8, 1 mM EDTA) and 2.4 g of CsCl was added. The tubes were spun and the supernatant removed to ultracentrifuge tubes (Sorvall). The volume was made up with CsCl/TE solution (1.2 g/ml) and 50 µl of EtBr (10 mg/ml). The tubes were sealed and spun for 16 h at 50,000 rpm (Beckman Optima TL Ultracentrifuge).

The plasmid band was removed by side puncture with a needle and syringe and the EtBr was extracted several times with isopropanol saturated with NaCl and water. The DNA solution was diluted with 3 volumes of TE and 2 final volumes of ethanol were added. The DNA was precipitated by centrifugation at 13,000 rpm for 30 min and washed with 70 % ethanol before being dried and resuspended.

2.6.7.2. Preparation of Plasmid DNA by Quiagen Column

The protocol was carried out as described by the manufacturer, using Quiagen tip 500s.

A 10 ml overnight culture, grown from a single colony, was used to inoculate 250 ml of 2xTY or LB, containing the appropriate antibiotic, which was incubated for 16 h in a shaking incubator. The cells were pelleted by centrifugation at 5,000 rpm for 5 min at 4 ^{o}C (Beckman Sorval RC-5B Centrifuge, GSA rotor). The supernatant was poured off and the pellet allowed to drain, before being resuspended in 10 ml of P1 buffer (100 µg/ml RNase A, 50 mM Tris, 10 mM EDTA, pH 8.0). 10 ml of buffer P2 (200 mM NaOH, 1 % SDS) was added, mixed gently and incubated at 25 ^{o}C for 5 min. Ten ml of buffer P3 (3 M KAc, pH 5.5) was added, mixed and incubated on ice for 20 min. The mixture was then centrifuged at 16,000 rpm (SS34 rotor) for 30 min and the clear supernatant removed.

The Quiagen tip 500 was equilibrated by applying 10 ml of QBT (750 mM NaCl, 50 mM MOPS, 15 % ethanol, 0.15 % Triton X-100, pH 7.0) and allowing it to run through the column. The supernatant was applied to the equilibrated column and allowed to run through. The column was washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS, 15 % ethanol, pH 7) and the DNA was eluted with 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris, 1 mM EDTA, pH 8). The DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuging at 15,000 rpm 4 ^{o}C for 30 min.

2.6.7.3. Small Scale Preparation of Plasmid DNA by Alkaline Lysis

The method described by Sambrook *et al*, (1989) was used. A single colony was inoculated into 10 ml of 2xTY broth and incubated in a shaking 37 ^{o}C incubator for 16 h. The culture was pelleted in a 1.5 ml eppendorf tube by centrifugation at 13,000 rpm for 30 sec. The supernatant was removed and the pellet was resuspended in 100 µl of solution 1 (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA pH 8). The cells were lysed by addition of 200 µl of solution 2 (0.2 M NaOH, 1 % SDS) and incubated at 25 ^{o}C for 5 min. Proteins were precipitated by addition of 150 µl of solution 3 (3 M KOAc, 11 % acetic acid) and incubated at 25 ^{o}C for 5 min. The tube was centrifuged at 13,000 rpm for 5 min and the supernatant was transferred to a clean tube. Any remaining protein was removed by phenol/chloroform extraction. The DNA was

ethanol precipitated, washed in 70 % ethanol and resuspended in the appropriate buffer.

2.6.8. Agarose Gels

2.6.8.1. Resolution of DNA Fragments

DNA samples were mixed with 0.2 volumes of 5 x DNA loading buffer (4 ml glycerol, 1.6 ml 0.5 M EDTA, 0.4 ml 1 M Tris, pH 7.5, 0.4 ml water, 0.002 g bromophenol blue, 0.002 g xylene cyanol) and then resolved by electrophoresis through agarose gels (in the range of 0.3 % to 2 %) prepared using 1 x TBE buffer (0.9 M Tris, boric acid, 0.002 M EDTA) and containing a final concentration of 0.5 μg/ml ethidium bromide. Electrophoresis was performed in 1 x TBE buffer in minigel electrophoresis tanks (7.5 cm x 8.5 cm gel) for 1-2 h at 70 V or in large electrophoresis tanks (20.5 x 19.5 cm gel) for 16 h at 35 V. One to two μg of DNA markers (ϕx 174 or λ Hind III, Gibco BRL) were electrophoresed beside the DNA samples to act as size markers. The DNA was visualized using an ultra-violet (UV) transilluminator (Ultraviolet Products Inc.) and photographed (Polaroid MP4 Land Camera or Mitsubishi Video Copy Processor)

2.6.8.2. Isolation of DNA Fragments

DNA fragments were resolved by electrophoresis through low melting point (LMP) agarose (Sigma) TBE gels and the appropriate DNA fragment was located by ethidium bromide staining. The DNA was excised from the gel as a thin slice using a scalpel blade and then extracted from the agarose using β-agarase I (Biolabs) or GELase (Cambio Ltd). The slice of DNA-containing agarose was weighed and placed in an eppendorf tube with 0.1 volumes of the manufacturer's buffer (Biolabs: 10 mM BisTris pH 6.5, 1 mM Na_2EDTA). The agarose was melted by incubation at 65 ^oC for 10 min and then cooled to 40 ^oC. Once cooled 1 unit of the enzyme was added per 200 μl of 1 % agarose and incubated at 40 ^oC for 1-16 h. Following digestion of the agarose, 0.1 volumes of 3M NaOAc was added, incubated at 0 ^oC for 15 min and centrifuged at 13,000 rpm (MSE Microcentaur) for 15 min to pellet any undigested carbohydrates. The DNA-containing supernatant was placed in a clean eppendorf and 2 volumes of isopropanol were added. The mixture was

incubated at -20 °C for 30 min and centrifuged at 13,000 rpm for 15 min to precipitate the DNA. The pellet was washed with 70 % ethanol and dried before being resuspended in the appropriate buffer.

2.6.9. Sequencing of DNA

2.6.9.1. DNA Sequencing with Sequenase

DNA was sequenced using the dideoxy-chain termination method of Sanger *et al*, (1977) and the Sequenase Version 2.0 sequencing kit according to the manufacturer's instructions (Amersham/USB).

Double-stranded DNA, 5-10 µg was made up to 50 µl with water and denatured by addition of an equal volume of denaturation solution (0.4 M NaOH, 0.4 mM EDTA) and incubating at 25 °C for 5 min. The DNA was ethanol precipitated, dried and resuspended in the appropriate volume of water.

The oligonucleotide primer (0.5-1 pmol) was annealed to the denatured DNA in 10 µl of 1 x reaction buffer (40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) by heating to 65 °C for 2 min and cooling slowly to 25 °C. One µl of 0.1 M DTT, 2 µl of diluted labelling mix (dGTP, dCTP and dTTP each at 1.5 µM), 0.5 µl [α^{35}S]-dATP (0.37 MBq/µl) (3000 Ci/mmol, DuPont or Amersham) and 2 µl of diluted Sequenase enzyme were added to the annealed template and the reaction was incubated for 2-5 min at 25 °C to allow labelling to occur. Meanwhile, 2.5 ml of each of the dideoxyribonucleoside 5'-triphosphate ddATP, ddCTP, ddGTP, and ddTTP termination mixes (50 mM NaCl, 80 µM dNTPs and 8 µM of ddATP, ddCTP, ddGTP, and ddTTP) were placed into separate wells of a round bottomed 96 well plate and warmed to 37 °C. Three and a half µl of the labelling mix were added to each of the terminator wells and incubated for 3-5 min at 37 °C to allow termination to occur. The reactions were stopped by adding 4 µl of stop solution (95 % formamide, 20 µM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) and stored on ice or at -20 °C before resolution by denaturing polyacrylamide gel electrophoresis.

2.6.9.2. Denaturing Polyacrylamide Gel Electrophoresis

Eight percent denaturing polyacrylamide gels (8 % acrylamide, 1 X TBE, 44 % (w/v) urea, 0.0075 % (v/v) ammonium persulphate (AMPS), 0.003 %

(v/v) N,N,N',N'-tetramethylethylenediamine, TEMED) were cast between siliconized glass plates (38 x 20) using 0.5 mm spacers and 2-5 mm combs to form the wells. The gels were warmed for 30 min at 2,500 V, 37 W with 1 x TBE in the upper buffer tank and 0.5 x TBE in the lower buffer tank. The samples were denatured by heating to 80 ^{o}C for 2 min and cooling on ice, the urea was dispelled from the wells and 2.5 µl of each sample was loaded into a well. Electrophoresis was performed at 2,500 V, 37 W for 1-3 h then 0.5 volumes of 3M NaOAc were added to the lower buffer tank and electrophoresis was continued for a further 1 h. The gel was fixed for 20 min in 10 % acetic acid, 10 % ethanol and dried at 80 ^{o}C under vacuum for 1 h (Savant, Stratech Scientific) and exposed to film.

2.6.9.3. Automated Cycle Sequencing

Automated cycle sequencing was carried out using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). Nine and a half µl of terminator premix was mixed with 1 µg of CsCl prepared DNA and 3.2 pmol of oligonucleotide primer, and overlaid with 40 µl of mineral oil in a 0.5 ml eppendorf. The mix was heated to 96 ^{o}C for 1 min and then cycled to 96 ^{o}C for 30 sec, 50 ^{o}C for 15 sec, 60 ^{o}C for 4 min for 25 cycles and cooled to 4 ^{o}C in a PCR machine (Programmable Dri-Block, Techne). The mineral oil was dissolved by addition of 100 µl of chloroform, 80 µl of water was added and 100 µl of phenol:chloroform:water (68:18:14) was added to extract the excess terminators. The tubes were centrifuged 13,000 rpm for 10 min and the aqueous phase removed to a clean tube. The DNA was ethanol precipitated, washed in 70 % ethanol and dried.

The samples were processed by the Leicester University sequencing service and read using Seqed on a Macintosh computer.

The sequence was initially checked and edited using Seqed, version 1.0.3. from Applied Biosystems Inc. Assembly of the sequences was carried out using the Sequence Assembly Program (SAP), version 4.0, May 1991, written by Roger Staden. Sequences were analysed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service.

2.6.10. Hybridization

2.6.10.1. Southern Blot (Southern, 1975)

DNA fragments were separated by agarose gel electrophoresis, and visualized to ensure that the DNA was fully digested. Gels were photographed with a ruler alongside the size markers to ensure that the positions of the markers could be identified. Gels were rinsed in water and soaked in 0.2 M HCl for 15-30 min to nick DNA fragments of greater than 15 Kbp and ensure their efficient transfer. Gels were then rinsed in water and soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 min. The gel was rinsed in water once again and soaked in neutralization solution (1.5.M NaCl, 0.5 M Tris, pH 7.2, 0.001 M EDTA) for 30 min. The gels were then soaked in 20 x SSC (3M NaCl, 0.3M sodium citrate) for 15 min. DNA was transferred to Hybond-N membrane (Amersham) by capillary action using 20 x SSC.

Whatmann 3MM was soaked in 20 x SSC and placed on an inverted gel tray, with the ends forming a wick into a tray of 20 x SSC. The gel was placed on the soaked paper and the membrane placed on top, expelling all air bubbles. Three pieces of Whatmann 3MM wetted in 20 x SSC were placed on top with a stack of dry Whatmann 3MM and paper towels on top. A glass plate was placed at the top of the stack with a weight on top and was left for 16 h.

Following transfer the blot was dismantled and the membrane was rinsed in 6 X SSC to remove any pieces of agarose, and air-dried. The DNA was covalently bound to the membrane using a UV Stratalinker (120000 µjoules) (Stratagene) and the membrane was stored at 4 ^{o}C until hybridization.

2.6.10.2. Colony Blots and Plaque Lifts

Plates were incubated at 4 ^{o}C for 1h before being blotted. Hybond-N (Amersham) nylon membrane was placed on the surface of the plate for 2 min and the orientation was marked. The membrane was lifted off the plate and placed DNA-side up on top of 3 sheets of Whatmann 3MM paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH). After 5 min the membrane was removed and placed on Whatmann 3MM paper which had

been soaked in neutralization solution (1.5.M NaCl, 0.5 M Tris, pH 7.2, 0.001 M EDTA). After 3 min the membrane was removed and placed for a further 3 min on fresh 3MM which had been soaked in neutralization solution. The membrane was then air-dried and the DNA was covalently bound to the membrane using a UV Stratalinker (120000 µjoules) (Stratagene).

2.6.10.3. Oligonucleotide Probes

Membranes were incubated in filtered hybridization solution containing 6 x SSC, 2 x Denhartdt's solution (0.02 % bovine serum albumin (BSA) (Fraction V, sigma), 0.02 % Ficoll type 400, Pharmacia, 0.02 % polyvinylpyrolidone, PVP), 0.25 % SDS, and 100 mg/ml denatured heterologous DNA, for at least 1 h at 30-55 ^{o}C in a hybridization oven (Appligene).

The oligonucleotide probe was prepared by end-labelling the oligonucleotide. Equal molar amounts of oligonucleotide and [γ-^{32}P] dATP (3000 Ci/mmol, DuPont or Amersham) were mixed with 1 U/µl of T4 kinase (Gibco BRL) or polynucleotide kinase (Promega) in the appropriate reaction buffer (1x T4 kinase buffer: 50 mM Tris, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine, pH 8.2). The reaction mix was incubated at 37 ^{o}C for 30 min.

The probe was purified from unincorporated nucleotides by ethanol precipitation, or by spin column chromatography as follows. A small wad of glass wool was placed in a 1 ml syringe and pushed to the bottom with the plunger to form a tight plug. The syringe was filled with a suspension of Sephadex G-50 (Pharmacia) pre-swollen in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and centrifuged at 1,500 rpm for 3 min in a (MSE Mistral 3000), supported in a 10 ml conical disposable centrifuge tube. Sephadex was added until a bed volume of 0.8 ml was obtained. The column was then washed twice with 500 µl of TE by centrifugation. A 1.5 ml eppendorf tube was placed at the bottom of the column, the labelled probe was added to the top and the column centrifuged. The eluate was collected in the eppendorf tube and stored at -20 ^{o}C or added to the hybridization solution and membrane.

Hybridization was carried out at 30-55 ^{o}C overnight in a hybridization oven (Appligene). After hybridization the filters were washed twice with 2 x SSC, 0.1 % SDS, and twice with 1 x SSC, 0.5 % SDS for 30 min at 30-55 ^{o}C each

time. The filters were wrapped in saran wrap or cling film and exposed to autoradiography film at -70 °C.

2.6.10.4. Double-Stranded DNA Probes (ECL)

Hybridization was carried out using the ECL direct nucleic acid labelling and detection systems (Amersham) according to the manufacturer's instructions. The system enables the direct labelling of DNA with horseradish peroxidase, by cross-linking the enzyme to single-stranded DNA with glutaraldehyde. The detection system uses the reduction of hydrogen peroxide by horseradish peroxidase to oxidise luminol and produce blue light.

The hybridization buffer contained manufacturer's ECL gold buffer, 5 % manufacturer's blocking agent and 0.5 M NaCl which was mixed and incubated at 42 °C for 1 h before use. The membrane was incubated in the hybridization buffer at 42 °C in a hybridization oven (Appligene) for at least 30 min before addition of the probe.

The probe was produced using 300 ng of DNA. The DNA was diluted to 10 ng/µl, denatured by boiling for 5 min and snap cooling on ice for 5 min. The denatured DNA was mixed with one volume of the manufacturer's labelling mix and one volume of glutaraldehyde and incubated at 37 °C for 10 min. The labelled probe was then added to the hybridization solution and membrane. Hybridization was carried out at 42 °C for 4-16 h.

After hybridization the membrane was washed twice in 0.4 % SDS, 0.5 x SSC at 55 °C for 10 min and twice in 2 x SSC at 25 °C for 5 min. The detection reagents were then mixed and placed on the membrane for 1 min. The membrane was blotted with paper towels to remove excess detection fluid, wrapped in saran wrap and exposed to film for 1 min-2 h.

2.6.11. Amplification of DNA

2.6.11.1. Oligonucleotide Primers

Oligonucleotide primers were synthesised by K. Mawditt (I.A.H. Compton) and PNACL (University of Leicester) as a liquid sample. Primers were purified by ethanol precipitation and the concentration determined by measuring the OD_{260} (1 OD_{260}= 30 µg/ml oligonucleotide primer). Additional primers were obtained from Genosys.

2.6.11.2. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify sequences of DNA and was performed in sterile 0.5 ml eppendorf tubes in a Programmable Dri-Block, Techne. Reactions were performed in volumes of 25, 50 or 100 µl using 0.05 U/µl Taq DNA polymerase (Promega). Reactions contained 2.5 µM each primer, 200 µM each dATP, dCTP, dGTP, dTTP, 0.75-5 mM $MgCl_2$, 1 x reaction buffer (50 mM KCl, 10 mM Tris, pH 9.0, 0.1 % Triton-x-100) and 20-100 ng template DNA. The reaction mix was overlaid with mineral oil and cycled in the PCR machine. The DNA was amplified by 25-40 cycles, of denaturation at 94 ^{o}C for 0.5 min, annealing of the primers to the DNA at 30-55 ^{o}C for 1 min and elongation at 72 ^{o}C, allowing 1 min for every Kb of DNA to be amplified. The reactions were held at 72 ^{o}C for 10 min at the end of all the cycles to ensure complete elongation. If 40 cycles of amplification were carried out, a further 0.05 U/µl Taq were added at the 20th cycle.

2.6.11.3. Cloning PCR Products

Taq polymerase adds a single deoxyadenosine to the 3'-end of PCR products which can be used to clone the PCR product in to a vector with a 5' T-overhang.

Vector DNA was fully digested with a blunt cutting restriction enzyme (such as Sma I). The vector DNA was phenol extracted to remove the restriction enzyme and ethanol precipitated. The blunt cut vector DNA (20 mg) was then incubated at 70 ^{o}C for 2 h with 0.75 mM $MgCl_2$, 2 mM dTTP, 1 x manufacturer's reaction buffer, 0.04 U/µl Taq DNA polymerase (Promega) in a 25 µl reaction, overlaid with mineral oil.

After incubation the vector was stored at -20 ^{o}C before being used directly in ligation reactions.

2.6.12. Screening of cDNA Library

A λ gt11 5' stretch library of chicken lung cDNA was purchased from Clontech.

Five µl of the cDNA library was diluted in 1 ml of lambda dilution buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.035 M Tris, pH 7.5, 2 % gelatin). Fifty µl of the diluted library was mixed with 1 ml of *E.coli* Y1090 (OD_{600} 0.7) for 10 min

at 25 °C. The phage and bacteria were then shaken at 37 °C for 30 min, mixed with 15 ml of LB top agar and plated on 14 cm diameter LB plates (100 μg/ml ampicillin).

The plates were incubated at 37 °C overnight then blotted and probed. Plaques that were positive after hybridization were picked from the plate using a sterile Pasteur pipette, placed in 1 ml of lambda dilution buffer and stored at 4 °C until the next round of plating and hybridization

2.7. RNA Work

2.7.1. Isolation of Messenger RNA

Messenger RNA was purified from control and interferon treated cells using the Micro-Fast Track Kit (Invitrogen).

Cells were washed in PBS and pelleted in an eppendorf. The pellet (5 x 10^6 cells) was resuspended in 1 ml of Micro-Fast Track Lysis buffer and the suspension passed through a 21 gauge needle. The suspension was incubated at 45 °C for 20 min with shaking to digest proteins and RNAs. The eppendorf was spun to pellet any insoluble material and the supernatant was removed. The NaCl concentration was adjusted to 0.5 M by adding 63 μl of 5 M NaCl and the DNA was sheared by passing the solution through a 21 gauge needle several times.

An oligo (dT) cellulose tablet was added to each lysate, and allowed to swell for 2 min. The eppendorf was gently shaken to disperse the tablet and placed on a rocking platform for 20 min. The oligo (dT) cellulose was then pelleted and the supernatant was removed. The oligo (dT) cellulose was washed several times in the manufacturer's binding buffer, until the supernatant was clear and then resuspended in 0.3 ml of binding buffer.

The sample was placed in a spin column and centrifuged briefly. The column was washed with the manufacturer's binding buffer three times and the non-polyadenylated RNA was removed by mixing the cellulose with the manufacturer's low salt wash buffer and centrifugation. The column was placed in a sterile RNase-free eppendorf, 100 μl of the manufacturer's elution buffer was mixed with the cellulose and the eppendorf was centrifuged briefly. The elution was repeated.

The RNA was precipitated by adding 10 µl of glycogen at 2 mg/ml, 30 µl of 2 M NaOAc and 600 µl of ethanol and incubating at -70 °C until solid. The RNA was stored under ethanol at -70 °C. Before use, the RNA was centrifuged at high speed, washed in 70 % ethanol and resuspended in RNase-free water.

2.7.2. Analysis of RNA

2.7.2.1. Electrophoresis of RNA

A denaturing formamide gel (17 cm x 20 cm) was prepared by dissolving 2 g of NA agarose in 124 ml of water and allowing it to cool. In a fume hood, 40 ml of 5 x running buffer (0.1 M MOPS pH 7, 40 mM NaOAc, 5 mM EDTA pH 8), 36 ml of formaldehyde and 8 µl of ethidium bromide (10 mg/ml) were added to the agarose, mixed and poured over a 2 % supporting base gel. The gel was allowed to set for 1 h.

The RNA sample and the RNA ladder (Gibco BRL) were prepared by adding 2 µl of 5 x running buffer, 3.5 µl of formaldehyde and 10 µl of deionised formamide. The samples were heated to 56 °C for 15 min and cooled on ice. Two µl of ficoll loading dye (0.25 % bromophenol blue, 15 % ficoll in water) was added to the samples before they were loaded.

The gel was run in 1 x running buffer and ethidium bromide for 10 min at 120 V and then at 24 V for 16 h. The buffer was recirculated using a pump. Following electrophoresis the gel was examined by UV illumination.

2.7.2.2. Transfer of RNA to Nylon Membrane

After electrophoresis, a piece of nylon membrane (Hybond-N, Amersham) slightly larger than the gel was soaked in running buffer (1.87 g/l NaH_2PO_4, 4.08 g/l Na_2HPO_4). Six pieces of 3MM paper were cut to the gel size and soaked in running buffer. Four were placed on the positive plate of the electroblot apparatus. The membrane was placed on top of the 3MM paper, with the gel on top of the membrane. The supporting base of the gel was removed and the gel covered with the other two pieces of 3MM. The gel assembly was clamped shut and placed in a tank of running buffer. The buffer was continuously mixed with a magnetic stirrer and run at 200 mA overnight.

After transfer the apparatus was taken apart and the nylon membrane removed. The wells were marked and the RNA covalently bound to the membrane using a UV Stratalinker (120,000 µjoules, Stratagene). The size ladder was cut from the membrane and soaked in 0.04 % methylene blue in 3 M NaOAc, pH 5.2 for 15 min then rinsed in water. The membrane was stored at 4 ^{o}C until it was probed.

2.7.2.3. Probing RNA Blots with DNA Probe

Hybridization solution was made up with 15 ml deionised formamide, 6 ml 50 % dextran sulphate, 3 ml water, 1.74 g NaCl and 6 ml of filtered 5 x TP (1 % polyvinyl pyrrolidone PVP, 1 % bovine serum albumin BSA, 1 % ficoll, 0.1 % sodium pyrophosphate, 5 % SDS, 250 mM Tris, pH 7.5). The hybridization solution was preheated at 42 ^{o}C until the sodium chloride had dissolved. The membrane was placed in a hybridization bottle and 5-10 ml of hybridization solution was added. The bottle was placed in a rotating hybridization oven at 35 ^{o}C for 3 h.

2.7.2.4. Production of a DNA Probe

The Rediprime DNA labelling system (Amersham) was used to produce the probe. This system uses random nonamer primers and klenow to label the DNA.

The DNA fragment was diluted to 25 ng in 45 µl of water. The DNA was denatured by boiling for 5 min and briefly centrifuged. The denatured DNA was added to the manufacturer's labelling mix and flicked gently to reconstitute the labelling mix. The tube was centrifuged, 5 µl of ^{32}P dCTP (3000 Ci/mmol, DuPont or Amersham) was added and mixed by pipetting. The mix was incubated at 37 ^{o}C for 10 min then 5 µl of 0.5 M EDTA was added to stop the reaction.

2.7.2.5. Purification of a DNA Probe

The probe was purified from unincorporated nucleotides using a G100 Sephadex (Pharmacia) column. The column was set up as in 2.6.10.4 but using a drawn out Pastuer pipette. The probe was applied to the top of the column which was then washed through 15-20 times with 200 µl of TE pH 8. The

washes were allowed to flow through under gravity and the fractions were collected in eppendorfs.

To check the efficiency of the labelling reaction, 3 µl of each fraction was placed in a scintillation vial with 5 ml of scintillation fluid and counted in a scintillation counter (Minamaxi 4000, United Technologies).

2.7.2.6. Hybridization and Washes

The probe was heat denatured at 100 ^{o}C for 5 min, and mixed with 166 µl of 5 x TP and 416 µl of deionized formamide per 5 ml of hybridization solution. The probe was added to the membrane in the bottle and incubated at 35 ^{o}C overnight.

The membrane was washed in 2 x SSC, 1 % SDS at 35 ^{o}C for 10 min. The washes were carried out three times before the membrane was wrapped in saran and exposed to film.

Chapter 3

Evidence for Inhibition of Interferon Action by Fowlpox Virus

3. EVIDENCE FOR INHIBITION OF INTERFERON ACTION BY FOWLPOX VIRUS

Vaccinia virus (VV) is resistant to interferon and is able to rescue interferon sensitive viruses such as vesicular stomatis virus (VSV), from the effects of interferon. Although it has been reported that FPV is resistant to chicken IFN in CEF, it is not known whether FPV has the same mechanisms for IFN resistance as VV. Interferon assays were set up to confirm that FPV is resistant to IFN. SFV rescue experiments were performed in order to further characterise the interferon phenotype of FPV.

3.1. Interferon Assays

3.1.1 Interferon Assays

Interferon assays (Clemens *et al.*, 1985) are based on the ability of interferon to inhibit the replication of a virus. Briefly, cells are treated with interferon and then infected with a virus. If the virus is sensitive to the amount of interferon the cells have been treated with, then no virus CPE will be visible. If, however, the virus is resistant to the amount of interferon the cells have been treated with, then CPE will be visible.

Interferon assays were set up in primary CEF, using chicken IFN. An interferon sensitive virus, Semliki Forest virus (SFV) was used as a positive control for interferon sensitivity. VV was used as a negative control for interferon sensitivity as it is IFN-resistant in mammalian cells. However, the IFN phenotype of VV has not been determined for avian cells and avian IFN. It was expected that FPV would be resistant to IFN, as has previously been reported (Asch & Gifford, 1970).

Three different stains were used to ensure that the stains did not affect the results and to ensure that the results were clear. Gentian violet and amido black colour all the cells present, whereas neutral red is only taken up by living cells.

The assays shown here were assessed by eye. It is possible to assess IFN assays by spectrophotometry at 550 nm using amido black dye extracted from the cell monolayers (Clemens *et al.*, 1985). Spectrophotometry was not used for the final experiments as trials with this method did not produce repeatable results, possibly due to problems with stain extraction.

One unit of interferon is defined as the amount of interferon which results in a 50 % CPE (or a 50 % reduction in plaque numbers) with an

interferon sensitive virus (usually VSV). VSV was not used in these experiments as it can infect man, resulting in influenza-like symptoms, and the Ministry of Agriculture Food and Fisheries (MAFF) have placed restriction on its use in this country. SFV, however, is thought to be non-pathogenic in man and its use is not restricted by MAFF (Singleton & Sainsbury, 1987).

3.1.2. Results of Interferon Assays

The 50 % CPE for SFV was obtained with chicken IFN at 1U/ml, Fig. 3.1a. The 50 % CPE was expected to occur at 1 U/ml of chicken IFN as one unit of interferon is defined as the amount of interferon which results in a 50 % CPE with an interferon sensitive virus.

The different stains do not appear to have any effect on the result but CPE is shown most clearly by gentian violet staining, Fig. 3.1a.

The 50 % CPE for VV was obtained with chicken IFN at between 4 and 8 U/ml, Fig. 3.1b. Some CPE was evident at 8 U/ml of IFN. Thus VV is relatively resistant to chicken IFN in CEF. The VV infected cell sheets appear to stain darker than non-infected cell sheets, Fig. 3.1b, indicating that the infected cell sheets may be thicker than non-infected cell sheets. This could be due to the effects of the VV epidermal growth factor.

FPV induces CPE with 8 U/ml of chicken IFN (Fig. 3.1c). Thus FPV is resistant to more than 8 U/ml of chicken IFN. FPV is more resistant to chicken IFN than is VV and much more resistant to chicken IFN than is SFV. Higher concentrations of chicken IFN were not tested, because the partially purified IFN used in this experiment did not allow this to be done easily.

The results of the IFN assays show that:

- SFV is sensitive to chicken IFN in CEF
- VV is more resistant to chicken IFN in CEF than SFV
- VV is less resistant to chicken IFN in CEF than FPV.
- FPV is resistant to more than 8 U/ml of chicken IFN in CEF.

FPV resistance to the effects of chicken IFN may be due to trans-acting factors such as those known to be active in VV.

Some of the FPV infected cell sheets seen in Fig. 3.1c appear to be lifting away from the plastic. The uninfected cell sheets appear to be firmly fixed to the plastic so the age of the cells and the staining process were not considered to be factors in the cell lifting. To check that the cell lifting was due to virus

Fig. 3.1a. Resistance of Semliki Forest Virus to Chicken Interferon

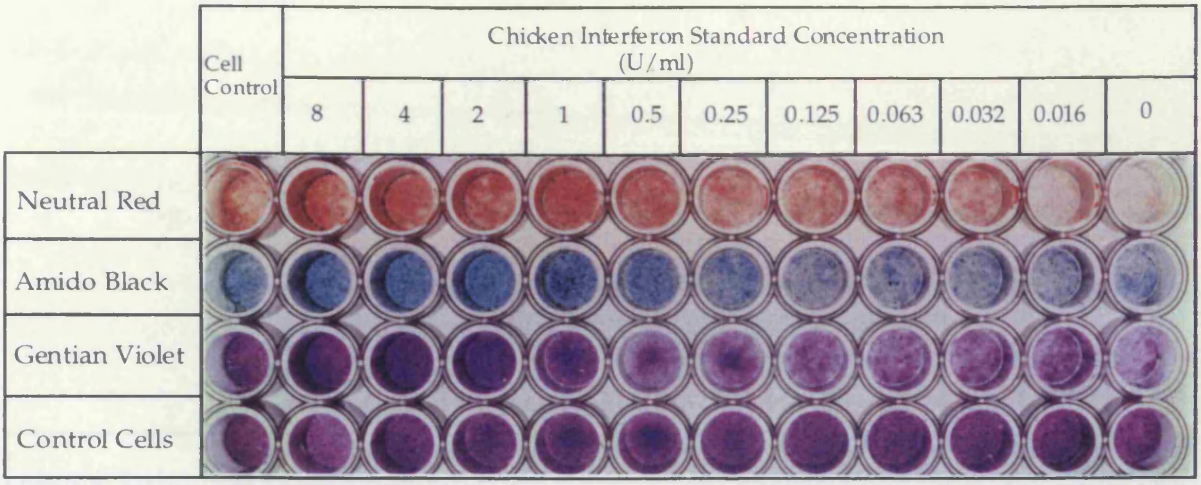


Fig. 3.1a. Resistance of Semliki Forest Virus to Chicken Interferon

CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 1 x 10^5 pfu/well semliki forest virus (SFV). The plates were incubated at 37 ^{o}C, 5 % CO_2. for 2 days. The cells were then stained with either neutral red, amido black or gentian violet.

Fig. 3.1b. Resistance of Vaccinia Virus to Chicken Interferon

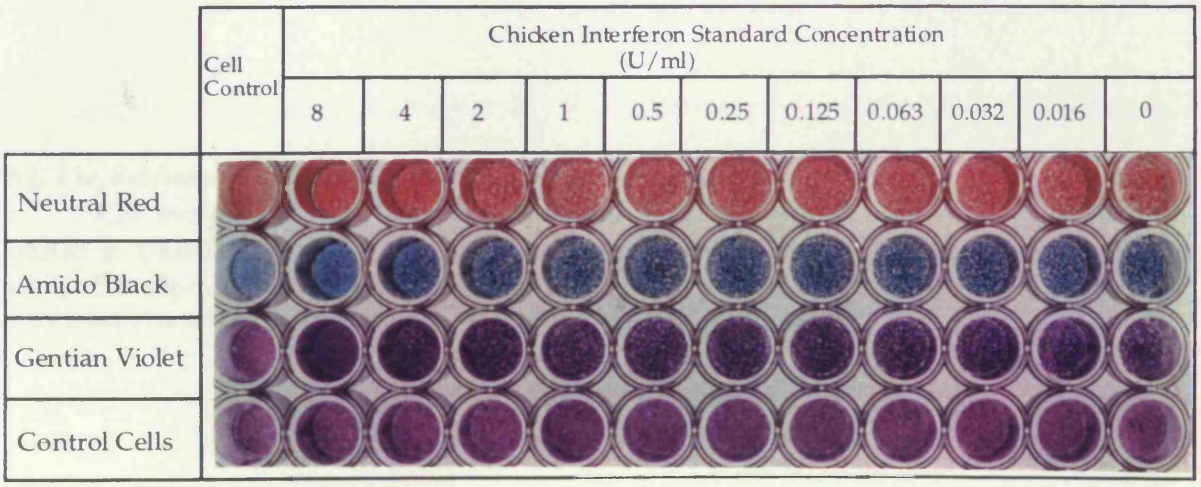


Fig. 3.1b. Resistance of Vaccinia Virus to Chicken Interferon

CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 2 x 10^3 pfu/well vaccinia virus (VV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 2 days. The cells were then stained with either neutral red, amido black or gentian violet.

Fig. 3.1c. Resistance of Fowlpox Virus to Chicken Interferon

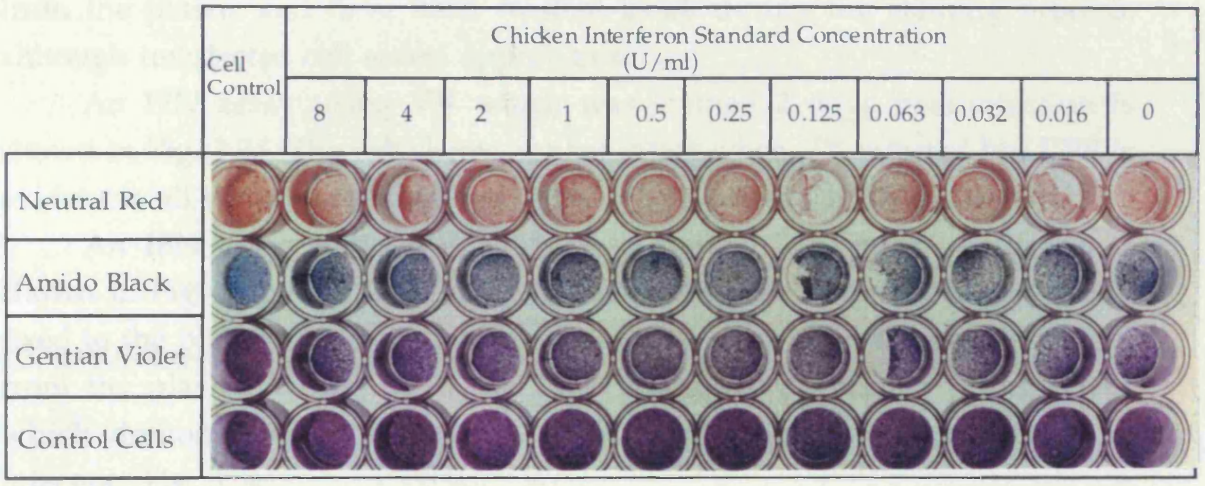


Fig. 3.1c. Resistance of Fowlpox Virus to Chicken Interferon

CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 1 x 10^7 pfu/well fowlpox virus (FPV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 3 days. The cells were then stained with either neutral red, amido black or gentian violet.

CPE (and not any contamination), IFN assays were carried out with FPV and VV and incubated for longer periods of time before staining, Figs. 3.2 and 3.3.

An IFN assay using FPV, which was stained 3 days post-infection, is shown in Fig. 3.2a. Two of the infected cell sheets have completely lifted away from the plastic and one infected cell sheet has partially lifted away. All remaining infected cell sheets show CPE.

An IFN assay using FPV, which was stained 4 days post-infection is shown in Fig. 3.2b. All the infected cell sheets have completely lifted away from the plastic and have been washed away during the staining process, although uninfected cell sheets appear intact.

An IFN assay using VV which was stained 2 days post-infection is shown in Fig. 3.3a. The cell sheets appear intact when VV infected but CPE is evident in all wells, particularly wells treated with less than 4 U/ml of IFN.

An IFN assay using VV which was stained 3 days post-infection is shown in Fig. 3.3b. The uninfected cell sheets appear to be intact and firmly fixed to the plastic, but some infected cell sheets have almost completely lifted from the plastic. Lifting of the cell sheets appears to occur in those wells which demonstrated the greatest CPE when stained at earlier times in infection, Fig. 3.3a.

The data shown in Figs. 3.2 and 3.3 indicate that the lifting of FPV infected cell sheets seen in Fig. 3.1 is a direct result of FPV infection, and correlates with CPE. Uninfected wells do not exhibit cell-lifting, and cell-lifting in infected wells is more pronounced at later times post-infection. Both poxviruses have an effect on the cell monolayers, which results in the infected cell sheet losing its ability to adhere to the plastic. This could be due to shut-down of host protein synthesis caused by the poxviruses, preventing the production of proteins involved in cell adhesion. It has also been suggested that VV IEV induce the formation of actin tails, similar to those seen with bacterial intracellular pathogens (Cudmore *et al.*, 1995). The actin tails induced by IEV are thought to be the means of cell to cell transfer of this form of VV. Thus poxvirus infection may interfere with the natural distribution of cytoskeletal proteins and so interfere with cell adhesion.

Fig. 3.2a. Interferon Assay with a Three Day Fowlpox Virus Infection

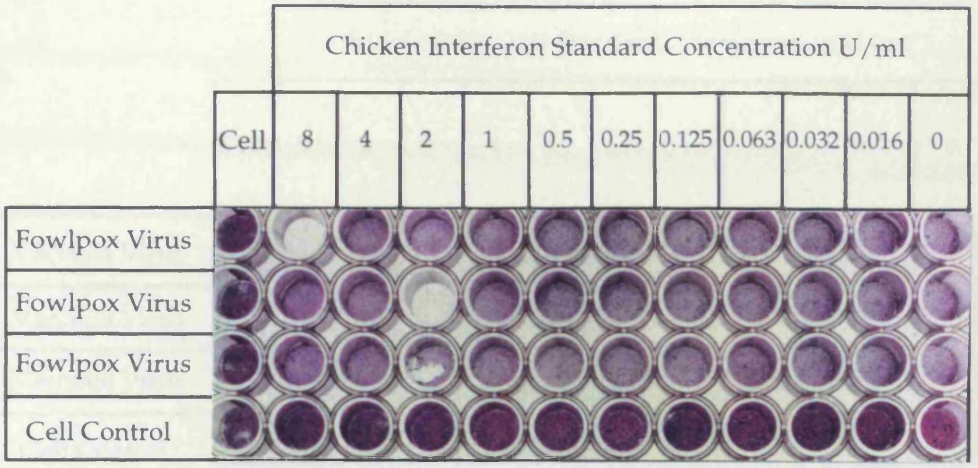


Fig. 3.2a. Interferon Assay with a Three Day Fowlpox Virus Infection

Triplicate wells of CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 1 x 10^7 pfu/well fowlpox virus (FPV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 3 days. The cells were then stained with gentian violet.

Fig. 3.2b. Interferon Assay with a Four Day Fowlpox Virus Infection

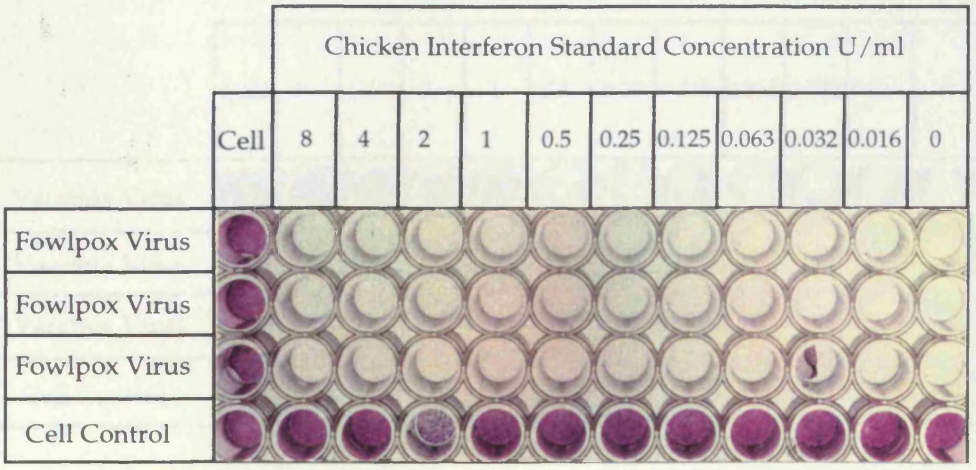


Fig. 3.2b. Interferon Assay with a Four Day Fowlpox Virus Infection

Triplicate wells of CEF monolayers were treated for 16h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 1 x 10^7 pfu/well fowlpox virus (FPV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 4 days. The cells were then stained with gentian violet.

Fig. 3.3a. Interferon Assay with a Two Day Vaccinia Virus Infection

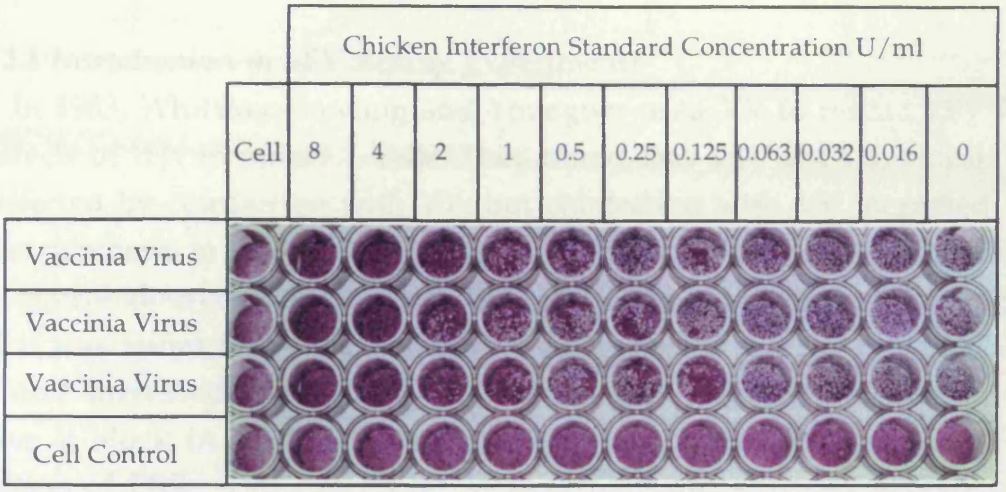


Fig. 3.3a. Interferon Assay with a Two Day Vaccinia Virus Infection

CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 2 x 10^3 pfu/well vaccinia virus (VV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 2 days. The cells were then stained with gentian violet.

Fig. 3.3b. Interferon Assay with a Three Day Vaccinia Virus Infection

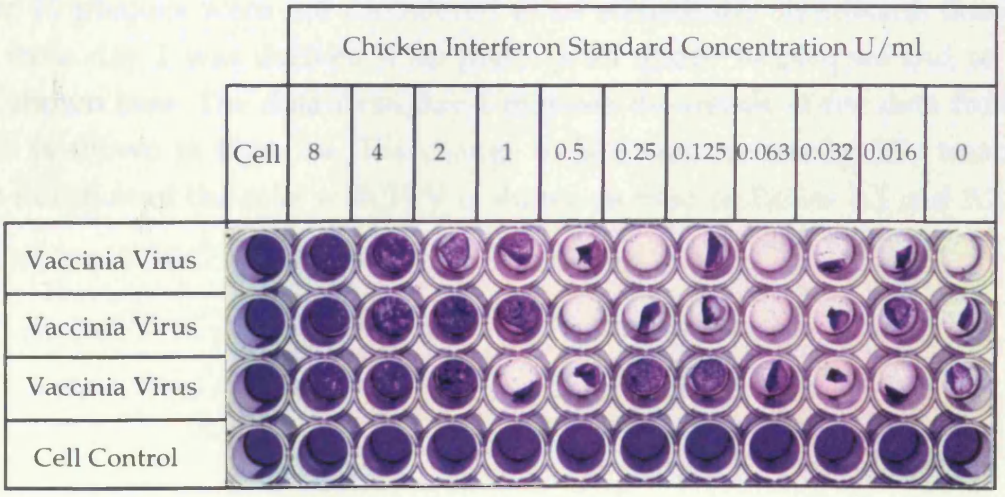


Fig. 3.3b. Interferon Assay with a Three Day Vaccinia Virus Infection

CEF monolayers were treated for 16 h at 37 ^{o}C, 5 % CO_2 with chicken IFN (standard) diluted in DMEM, 0.5 % FCS. The IFN was removed and cells were infected with 2 x 10^3 pfu/well vaccinia virus (VV). The plates were incubated at 37 ^{o}C, 5 % CO_2 for 3 days. The cells were then stained with gentian violet.

3.2. Rescue of Semliki Forest Virus from the Effects of Interferon by Fowlpox Virus

3.2.1 Introduction to SFV Rescue Experiments

In 1983, Whitaker-Dowling and Youngner used VV to rescue VSV from the effects of IFN in mouse L cells. They found that VSV RNA synthesis was not affected by coinfection with VV, but coinfection with VV increased VSV protein synthesis in IFN-treated cells and inhibited PKR. In a later experiment with encephalomyocarditis virus, EMC, (Whitaker-Dowling & Youngner, 1986) it was found that coinfection with VV increased the yield of EMC 1000 fold, and increased EMC RNA and protein synthesis. The rescue did not involve a block in the 2'-5'A synthetase pathway but correlated with VV inhibition of PKR.

A similar experiment was set up to determine whether FPV is able to rescue SFV from the effects of interferon, and thus show whether FPV contains factors that are able to overcome IFN inhibition of SFV. SFV was used as the interferon sensitive virus. It has been shown, in Fig. 3.1a, that SFV is sensitive to chicken interferon in CEF.

The experiment was carried out in duplicate, on two different days and virus was harvested at 16 hours and 22 hours post SFV infection. Plates with under 10 plaques were not considered to be statistically significant. Some of the data from day 1 was derived from plates with under 10 plaques and so has not been shown here. The data from day 1 matches the trends of the data from day 2, which is shown in Figs. 3.4. The change in SFV titre caused by IFN treatment or prior infection of the cells with FPV is shown as ratio in Tables 3.1 and 3.2.

Fig. 3.4a. Graph to Show Rescue of SFV at 16 Hours Post-Infection

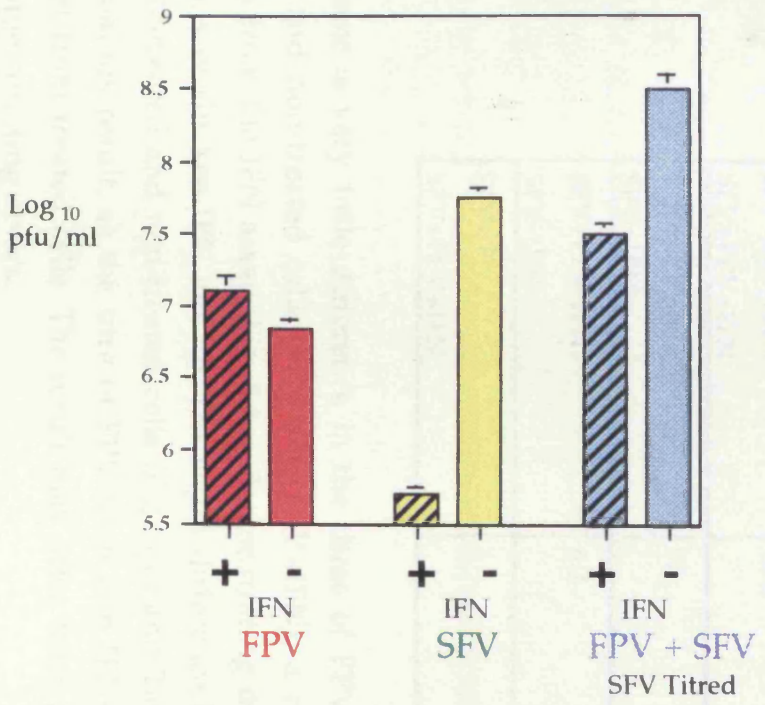
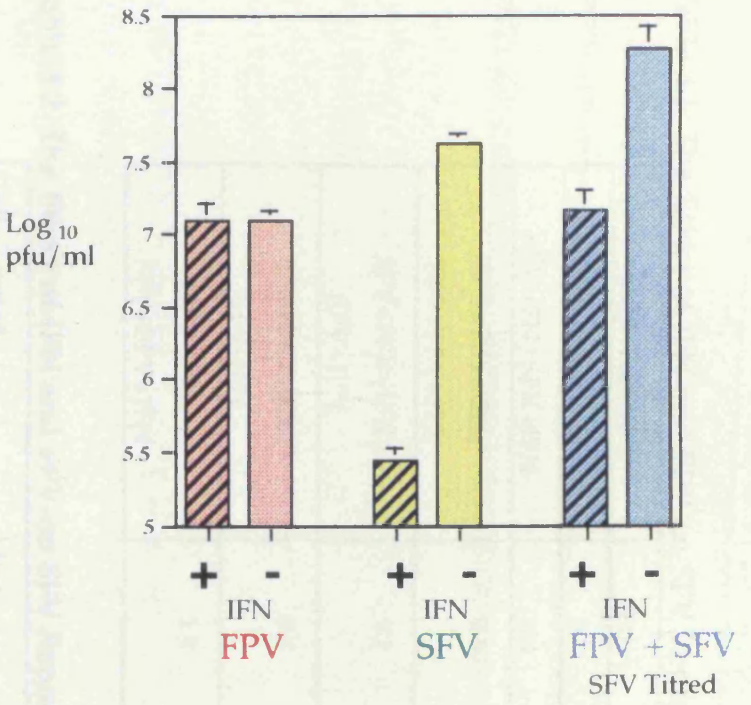


Fig. 3.4b. Graph to Show Rescue of SFV at 22 Hours Post-Infection



Figs. 3.4. FPV Rescue of SFV from the Effects of IFN.
The method used was essentially that of Whittaker-Dowling and Younger, 1983 . Dishes of primary CEFs were treated with serum-free 1 x MEM with or without 4 IU/ml of IFN. The cells were incubated 37°C, 5% CO_2 for 16 h. The appropriate dishes were infected with FPV (FP9) at 2 x 10^7 pfu/dish (moi of 10) in serum-free 1 x MEM. After 1 h the inoculum was removed and replaced with 3 ml of serum-free 1 x MEM and incubation was carried out for a further 3 h. The appropriate dishes were then infected with SFV at 2 x 10^7 pfu/dish (moi of 10) in serum-free 1 x MEM. After 1 h the inoculum was removed and replaced with 3 ml of serum-free 1 x MEM and incubation was carried out for 16 h. The supernatants and cells were removed from the dishes and freeze-thawed (-70 °C, 37°C) three times to release the virus. The virus was titred in quadruplicate, the mean calculated and plotted on the graph. The error bars above the mean show log mean+standard deviation - log mean. Where error bars are not shown, the error is too small to be seen on the graph.

3.2.2. Results of SFV Rescue Experiment

Table 3.1. The Effect of IFN and FPV on SFV Replication after 16 h

Cells Treated	Ratio
FPV-IFN/FPV+IFN	0.7
SFV-IFN/SFV+IFN	114
SFV+FPV-IFN/ SFV+FPV+IFN	9.6
SFV+FPV+IFN/ SFV+IFN	62
SFV+FPV-IFN/ SFV-IFN	5.2
SFV-IFN/ SFV+FPV+IFN	1.8

Table 3.2. The Effect of IFN and FPV on SFV Replication after 22 h

Cells Treated	Ratio
FPV-IFN/FPV+IFN	1
SFV-IFN/SFV+IFN	150
SFV+FPV-IFN/ SFV+FPV+IFN	18.6
SFV+FPV+IFN/ SFV+IFN	50
SFV+FPV-IFN/ SFV-IFN	6.2
SFV-IFN/ SFV+FPV+IFN	3

There is very little difference in the titres of FPV obtained from IFN-treated and non-treated cells (FPV-IFN/FPV+IFN), a result which confirms the data from the IFN assay (Fig. 3.1c), demonstrating that FPV is resistant to the effects of chicken IFN in CEF. The slight difference between titres of FPV from IFN-treated and non-treated cells (Fig. 3.4a and Table 3.1) appears to be an anomalous result, as the titre of FPV from non IFN-treated cells is lower than that from treated cells. The result may arise from pipetting inaccuracies and plaque counting errors.

The titre of SFV from non IFN-treated cells (SFV-IFN) is more than 100 fold higher than the titre of SFV from IFN-treated cells (SFV+IFN), as shown in Figs. 3.4 and Tables 1 and 2. These data confirm that SFV is sensitive to chicken IFN in CEF.

FPV infection of IFN-treated cells, prior to SFV infection, (SFV+FPV+IFN) (Figs. 3.4), resulted in an SFV titre:

- Only 10-20 fold lower than the titre of SFV from non IFN-treated FPV-infected cells (SFV+FPV-IFN).
- More than 50 fold higher than the SFV titre from SFV infected IFN-treated cells (SFV+IFN).
- Only 2-3 fold lower than the titre of SFV from SFV infected non IFN treated cells (SFV-IFN).

Prior infection of IFN-treated cells with FPV, partially removed the block to SFV replication caused by IFN treatment of the cells. FPV has the same effect on IFN inhibition of SFV replication as VV has on IFN inhibition of EMC and VSV replication. Thus FPV may have mechanisms similar to those of VV to overcome the effects of IFN. Experiments were not carried out to determine whether FPV rescue of SFV is due to increased RNA synthesis or increased protein synthesis or both. However the results from VV rescue of EMC and VSV indicate that FPV rescue of SFV may be due to inhibition of PKR and/or the 2'-5'A pathway.

When cells which had not been IFN-treated were infected with FPV prior to SFV infection (SFV+FPV-IFN), the resulting SFV titre (Figs. 3.4) was:

- 5-6 fold higher than the SFV titre from non-IFN treated cells which were SFV infected (SFV-IFN)

Coinfection with FPV appears to enable SFV to grow to higher levels than in the absence of FPV. This may be because SFV infection of a cell activates PKR as EMC does (Rice *et al.*, 1985), and PKR is inhibited by FPV infection, or alternatively may be due to a FPV homologue of VV growth factor.

When plates were infected with both FPV and SFV, only the SFV titre was determined and no chemical blocking agents of FPV replication were used. This was possible because SFV plaques are easily visible on CEF after only a 2 day incubation whereas FPV plaques require at least a 4 day

Fig. 3.5a. FPV Plaques Four Days Post-Infection

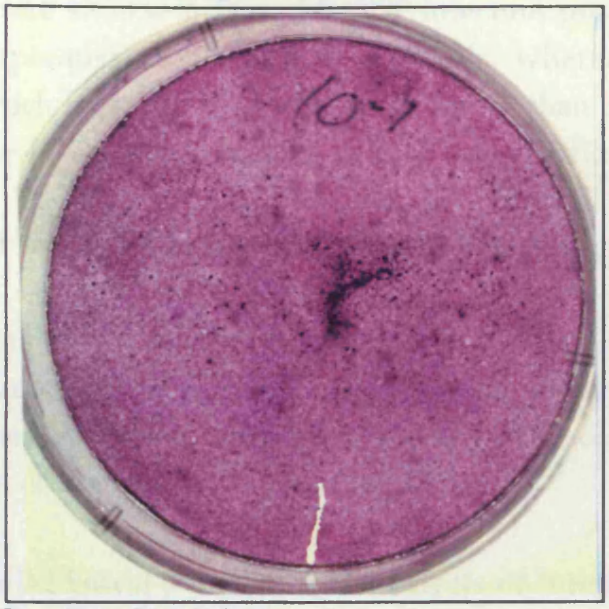


Fig. 3.5a. FPV Plaques Four Days Post-Infection

Confluent primary CEF were infected with 0.5 ml of FPV diluted in MEM without serum, and incubated for 1 hour at 37 ^{o}C, 5 % CO_2. The inoculum was removed and the cells were overlaid with a prewarmed solution of 1 % LGT agarose in 1 x MEM. The cells were incubated at 37 ^{o}C, 5 % CO_2 for 4 days before the overlay was removed and the cells fixed and stained with gentian violet.

Fig. 3.5b. SFV Plaques Two Days Post-Infection

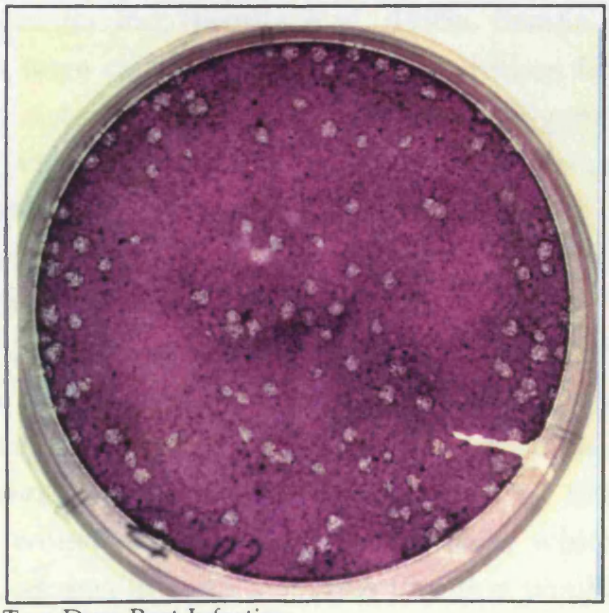


Fig. 3.5b. SFV Plaques Two Days Post-Infection

Confluent primary CEF were infected with 0.5 ml of SFV diluted in MEM without serum, and incubated for 1 hour at 37 ^{o}C, 5 % CO_2. The inoculum was removed and the cells were overlaid with a prewarmed solution of 1 % LGT agarose in 1 x MEM. The cells were incubated at 37 ^{o}C, 5 % CO_2 for 2 days before the overlay was removed and the cells fixed and stained with gentian violet.

incubation to be visible. The plaque morphology of the two viruses are also very different and are shown in Figs. 3.5. SFV infection produces large clear, irregularly edged plaques as a result of cell lysis, whereas FPV produces smaller plaques which look like cell dense areas rather than cell clear areas.

The cell dense FPV plaques may be caused by activation of the cells by a protein similar to VVEGF. Even if FPV and SFV did not require different incubation times, it would be easy to differentiate between the plaques of each virus.

There are areas of cell density in the centre of all the dishes, which could be due to aggregation of the cells when they are first seeded. The clear lines at the top of the dishes are due to damage to the cell sheet occurring when the agarose overlay was removed.

3.3. Rescue of Semliki Forest Virus from the Effects of Interferon by Vaccinia Viruses with Deletion Mutations in E3L and K3L

3.3.1 Introduction

VV expresses two proteins, E3L and K3L that are thought to be involved in the inhibition of PKR by VV (see 1.6.2.). Vaccinia viruses with deletion mutations of E3L and K3L, (vP1080 and vP872), were obtained from Dr. Tartaglia of Virogenetics Inc. (Beattie *et al.*, 1995a, Beattie *et al.*, 1995b). SFV rescue experiments were carried out in CEF with chicken IFN and the mutant vaccinia viruses to determine if one or both of these genes are involved in VV rescue of RNA viruses, and also to provide an indication of whether FPV homologues of these genes are required for the rescue of SFV by FPV. The change in virus titre caused by IFN treatment or coinfection with a VV is shown as ratio in Tables 3.4, 3.5 and 3.6.

No chemical blocking agent was used to inhibit late expression of the vaccinia viruses as the plaque morphology of SFV and the vaccinia viruses is clearly different, as can be seen in Fig. 3.6. However passing the samples through a 0.2 μm was found to reduce the titre of VV by 100 fold, as the virus is 200 x 170 nm, whilst reducing the titre of SFV, which is 50-70 nm in diameter by 2 fold, as seen in Table 3.3. Thus filtration would be suitable if the viruses had a similar plaque morphology.

Fig. 3.6. Plaque Morphology of Vaccinia Virus E3L and K3L Mutant Viruses and Semliki Forest Virus

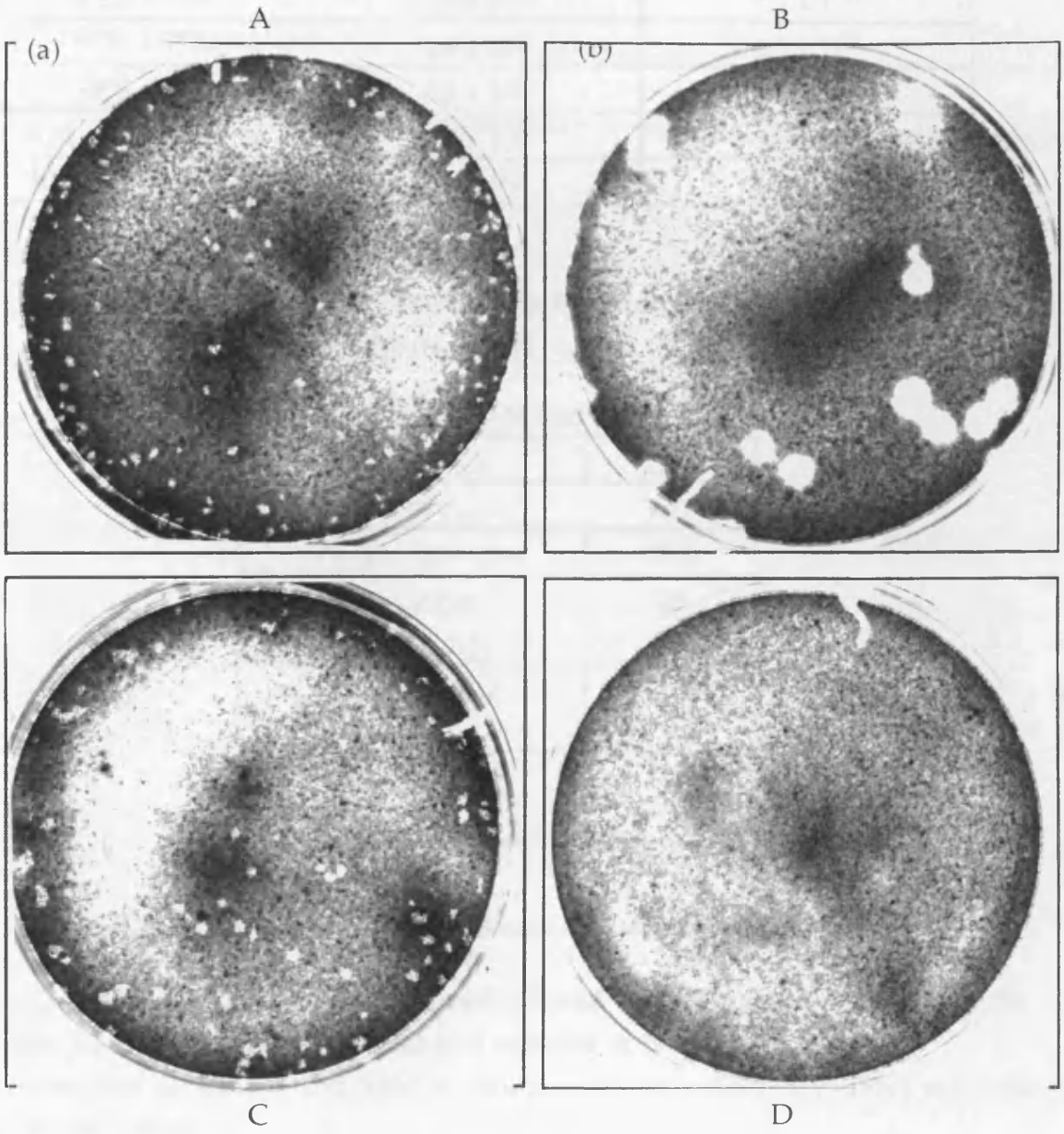


Fig. 3.6. Plaque Morphology of Vaccinia Virus E3L and K3L Mutant Viruses and Semliki Forest Virus.

Confluent primary CEF were infected with 0.5 ml virus diluted in MEM without serum, and incubated for 1 hour at 37 ^{o}C, 5 % CO_2. The inoculum was removed and the cells were overlaid with a prewarmed solution of 1 % LGT agarose in 1 x MEM. The cells were incubated at 37^{o}C, 5 % CO_2 for 2 days before the overlay was removed and the cells fixed and stained with gentian violet.

A	VV E3L- mutant	B	SFV
C	VV K3L- mutant	D	No virus

Table 3.3. The Effect of Filtration on Virus Titre

Sample	- 0.2 μm Filtration	+ 0.2 μm Filtration
K3L$^-$ + IFN	1.8×10^6	4.5×10^3
K3L$^-$ - IFN	1.0×10^7	1.1×10^4
SFV + IFN	1.9×10^5	4.5×10^5
SFV - IFN	6.6×10^6	4.5×10^6
K3L$^-$ +SFV + IFN	3.2×10^6	3.0×10^6
K3L$^-$ +SFV - IFN	2.4×10^7	1.5×10^7

3.3.2. Ability of Vaccinia Viruses with Deletion Mutations in E3L and K3L to Rescue SFV from the Effects of IFN

Table 3.4. The Effect of IFN and VV on SFV Replication

Cells Treated	Ratio
VV-IFN/VV+IFN	1.77
SFV-IFN/SFV+IFN	33.6
SFV+VV-IFN/ SFV+VV+IFN	29
SFV+VV+IFN/ SFV+IFN	18
SFV+VV-IFN/ SFV-IFN	15.8
SFV-IFN/ SFV+VV+IFN	1.8

VV (WR) replication is reduced 1.7 fold in IFN-treated cells, (Fig. 3.7a, Table 3.3.4). SFV replication is 33 fold reduced in IFN-treated cells.
Coinfection of wt VV and SFV in IFN-treated cells (SFV+VV+IFN) resulted in an SFV titre:

- 29 fold lower than the titre of SFV from non-IFN treated, VV-infected cells (SFV+VV-IFN).
- 18 fold higher than the SFV titre from SFV infected, IFN-treated cells (SFV+IFN).
- Only 1.8 fold lower than the titre of SFV from SFV infected, non IFN treated cells (SFV-IFN).

The results demonstrate that VV is capable of rescuing SFV replication from the effects of IFN in CEF.

When cells which had not been IFN-treated were infected with VV prior to SFV infection (SFV+VV-IFN), the resulting SFV titre (Figs. 3.4) was:

- 15 fold higher than the SFV titre from non-IFN treated cells which were SFV infected (SFV-IFN).

Coinfection with wt VV also appears to enable SFV to grow to higher levels than in the absence of VV. This may be because SFV infection of a cell activates PKR as EMC does (Rice *et al.*, 1985), which is inhibited by VV infection, or may be due to VV growth factor or some other mechanism.

Table 3.5. The Effect of IFN and a VV E3L$^-$ Mutant on SFV Replication

Cells Treated	Ratio
E3L$^-$-IFN/E3L$^-$+IFN	1.9
SFV-IFN/SFV+IFN	33.6
SFV+E3L$^-$-IFN/ SFV+E3L$^-$+IFN	7.4
SFV+E3L$^-$+IFN/ SFV+IFN	22
SFV+E3L$^-$-IFN/ SFV-IFN	4.8
SFV-IFN/ SFV+E3L$^-$+IFN	1.5

Replication of the E3L- mutant of VV is reduced 1.9 fold in IFN-treated cells, Fig. 3.7b, Table 3.5. SFV replication is 33 fold reduced in IFN-treated cells. Coinfection of the E3L- mutant of VV and SFV in IFN-treated cells (SFV+E3L$^-$+IFN) resulted in an SFV titre:

- 7.4 fold lower than the titre of SFV from non-IFN treated, VV-infected cells (SFV+E3L$^-$-IFN).
- 22 fold higher than the SFV titre from SFV infected, IFN-treated cells (SFV+IFN).
- Only 1.5 fold lower than the titre of SFV from SFV infected, non IFN treated cells (SFV-IFN).

Fig. 3.7a. Graph to Show Rescue of SFV by Wild Type VV

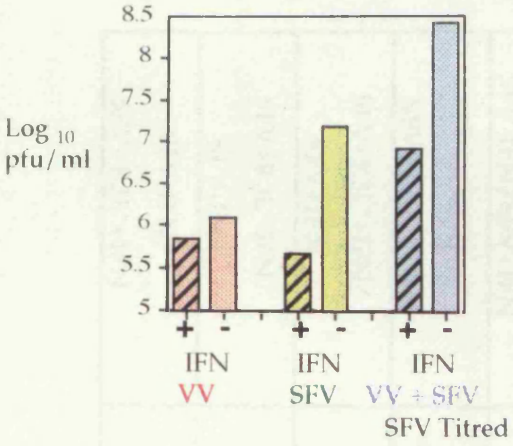


Fig. 3.7b. Graph to Show Rescue of SFV by VV E3L⁻ Mutant

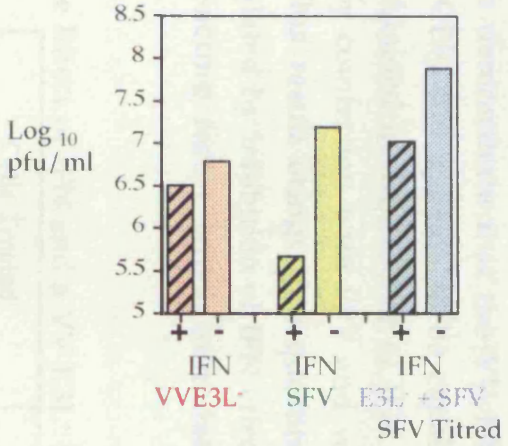
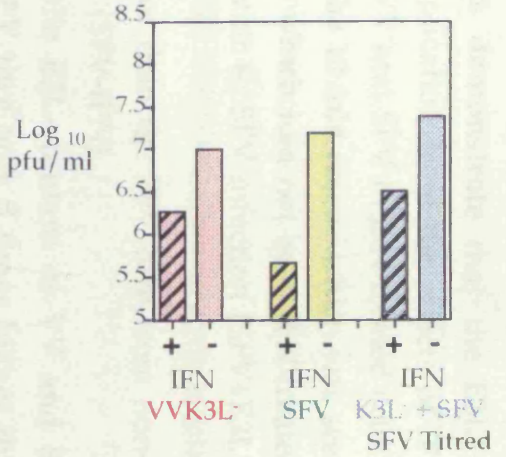


Fig. 3.7c. Graph to Show Rescue of SFV by VV K3L⁻ Mutant



Figs. 3.7. VV Rescue of SFV

The method used was essentially that of Whittaker-Dowling and Younger, 1983. Dishes of primary CEFs were treated with 1 x MEM, 0.5% FCS with or without 2.8 IU/ml of IFN. The cells were incubated 37°C, 5% CO_2 for 24 h. The appropriate dishes were infected with VV (WR strain) at 2×10^7 pfu/dish (moi of 5), VV E3L⁻ at 3.3×10^7 pfu/dish (moi of 10), or VV K3L⁻ at 4.2×10^7 pfu/dish (moi of 12) in 1 x MEM, 0.5% FCS. After 1 h the inoculum was removed and replaced with SFV at 3.4×10^7 pfu/dish (moi of 10), 1 x MEM, 0.5%FCS. After 1 h the inoculum was removed and replaced with 3 ml of 1 x MEM, 0.5% FCS and incubation was carried out for 16 h. The supernatants and cells were removed from the dishes and freeze-thawed (-70°C, 37°C) three times to release the virus. The virus was titred in triplicate, the mean calculated and plotted on the graph. The error bars above the mean show log mean+standard deviation - log mean. Where error bars are not shown, the error is too small to be seen on the graph.

The results demonstrate that the E3L$^-$ mutant of VV is capable of rescuing SFV replication from the effects of IFN in CEF. Coinfection of the E3L- mutant of VV and SFV in IFN-treated cells resulted in a 22 fold rescue of SFV, instead of the 18 fold rescue which was seen with wt VV.

When cells which had not been IFN-treated were infected with the E3L$^-$ mutant of VV prior to SFV infection (SFV+E3L$^-$-IFN), the resulting SFV titre (Figs. 3.4) was:

- 4.8 fold higher than the SFV titre from non-IFN treated cells which were SFV infected (SFV-IFN)

Coinfection of the E3L- mutant of VV and SFV in non IFN-treated cells resulted in an SFV titre only 5 times higher than that of SFV in non-treated cells alone, instead of the 15 fold difference which was seen with wt VV coinfection.

The results demonstrate that the VV E3L$^-$ mutant is not inhibited by chicken IFN in CEF, and appears to be capable of rescuing SFV to similar levels as those obtained in the absence of IFN. However, the extra growth of SFV conferred by coinfection with FPV and wt VV is not seen with the VV E3L$^-$ mutant. This result brings into question the ability to separate SFV replication stimulated by inhibition of IFN effector pathways from that of any other VV trans-acting factor which stimulates cell growth and/or virus replication.

Table 3.6. The Effect of IFN and a VV K3L$^-$ Mutant on SFV Replication

Cells Treated	Ratio
K3L$^-$-IFN/K3L$^-$+IFN	5.5
SFV-IFN/SFV+IFN	33
SFV+K3L$^-$-IFN/ SFV+K3L$^-$+IFN	7.7
SFV+K3L$^-$+IFN/ SFV+IFN	6.8
SFV+K3L$^-$-IFN/ SFV-IFN	1.5
SFV-IFN/ SFV+K3L$^-$+IFN	4.9

Replication of the K3L- mutant of VV is reduced 5.5 fold in IFN-treated cells, (Fig. 3.7c, Table 3.6). SFV replication is 33 fold reduced in IFN-treated cells. Coinfection of the K3L- mutant of VV and SFV in IFN-treated cells (SFV+K3L$^-$+IFN) resulted in an SFV titre:

- 7.7 fold lower than the titre of SFV from non-IFN treated, K3L$^-$-infected cells (SFV+K3L$^-$-IFN).
- 6.8 fold higher than the SFV titre from SFV infected, IFN-treated cells (SFV+IFN).
- 4.9 fold lower than the titre of SFV from SFV infected, non IFN treated cells (SFV-IFN).

The results show that the ability of the K3L- mutant of VV to rescue SFV replication from the effects of IFN, is significantly lower (at 6.8 fold rescue) than the ability of wt VV (at 18 fold rescue) and the E3L$^-$ mutant (at 22 fold rescue) to rescue SFV replication from the effects of IFN.

When cells which had not been IFN-treated were infected with the K3L- mutant of VV prior to SFV infection (SFV+K3L$^-$-IFN), the resulting SFV titre (Figs. 3.4) was:

- 1.5 fold higher than the SFV titre from non-IFN treated cells which were SFV infected (SFV-IFN).

Coinfection of the K3L- mutant of VV and SFV in non IFN-treated cells, resulted in an SFV titre only 1.5 times higher than that of SFV in non-treated cells alone, instead of 15 times higher as was seen with wt VV and 5 times higher which was seen with the E3L$^-$ mutant of VV.

The data imply that although VV E3L may play a part in the rescue of SFV from the effects of IFN in mammalian cells, K3L appears to play a greater part in the VV mediated rescue of SFV from the effects of IFN in CEF. It has been reported that the VV E3L$^-$ mutant is sensitive to mammalian IFN in L929 cells and debilitated in its ability to rescue VSV from the effects of IFN in L929 cells (Beattie *et al.*, 1995a). As the same mutant virus was used in the experiment shown here and those of Beattie *et al*, the difference in the results may be due to differences in the cells and IFN used.

3.4. Assessment of FPV Mutant Phenotype by SFV Rescue

SFV rescue experiments were performed with FPV mutants to determine whether the mutations could affect the ability of FPV to rescue SFV. The FPV mutants used in these experiments had deletions in genes

Fig. 3.8a. Rescue of SFV by FPV

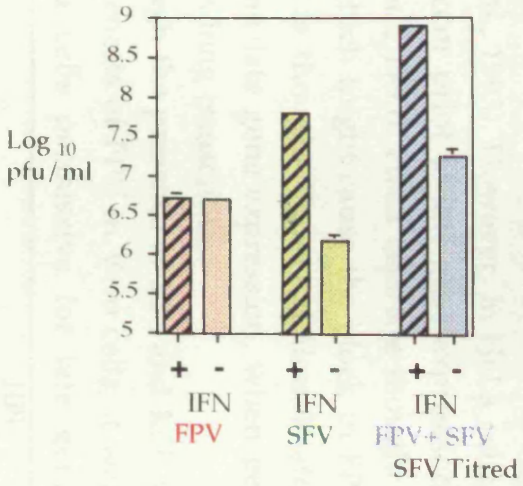


Fig. 3.8b. Rescue of SFV by FP-ank2 Mutant

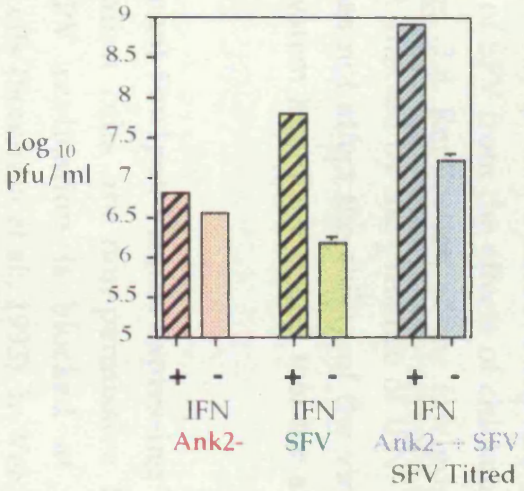
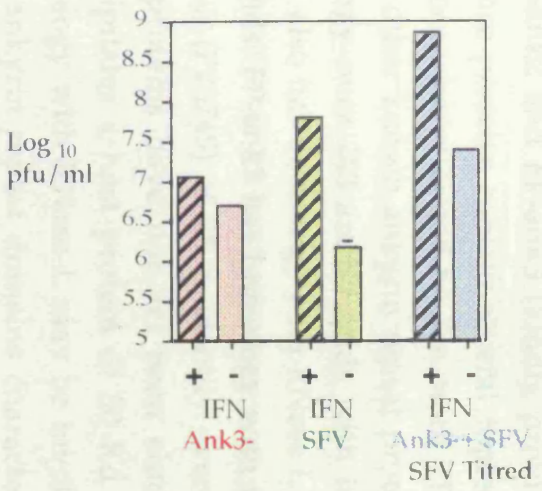


Fig. 3.8c. Rescue of SFV by FP-ank3 Mutant



Figs. 3.8. Ank Mutant Rescue of SFV

The method used was essentially that of Whittaker-Dowling and Younger, 1983. Dishes of primary CEFs were treated with 1 x MEM, 0.5% FCS with or without 2.5 IU/ml of IFN. The cells were incubated 37°C, 5% CO_2 for 24 h. The appropriate dishes were infected with FPV at 1×10^7 pfu/dish (moi of 5), FPV-ank2$^-$ at 1×10^7 pfu/dish (moi of 5), or FPV-ank3$^-$ at 1×10^7 pfu/dish (moi of 5) in 1 x MEM, 0.5% FCS. After 1 h the inoculum was removed and replaced with SFV at 2×10^7 pfu/dish (moi of 10), 1 x MEM, 0.5%FCS. After 1 h the inoculum was removed and replaced with 3 ml of 1 x MEM, 0.5% FCS and incubation was carried out for 16 h. The supernatants and cells were removed from the dishes and freeze-thawed (-70°C, 37°C) three times to release the virus. The virus was titred in triplicate, the mean calculated and plotted on the graph. The error bars above the mean show log mean+standard deviation - log mean. Where error bars are not shown, the error is too small to be seen on the graph.

encoding FP-ank2 and FP-ank3 (kindly provided by Dr. W Thomas and S. Laidlaw). These proteins contain several copies of the 33 amino acid ankyrin repeat sequence, characterized by the motif TPLH. The FP-ank proteins have homology to other known ankyrin repeat proteins. For example, FP-ank2 has 28 % homology over 263 amino acids with human ankyrin brain variant 1 (Q01484) and also has homology with RNase L (Q 00653) of 28 % identity over 123 amino acids. FP-ank3 has homology with the B-cell lymphoma 3-encoded protein, BCL-3 (P20749) of 25 % identity over 169 amino acids. Functions of the FP-ank proteins have not yet been elucidated, although FP-ank2 co-immunoprecipitates a host protein of 50 Kd. It was thought that FP-ank2, having homology with RNase L may be involved in inhibition of the effects of IFN. The ankyrin repeat domains characterized so far are thought to be involved in protein-protein interactions (Blank *et al.*, 1992). All information kindly provided by personal communication, Dr. M Skinner.

Rescue of SFV from the effects of chicken IFN by FPV FP-ank mutants is shown in Figs. 3.8. Replication of the fowlpox viruses with Ank2 and Ank3 deleted is not affected by the presence of IFN. The deletion of Ank2 and Ank3 from FPV does not affect the ability of the virus to rescue SFV. However the SFV rescue system remains a useful tool for analysing the phenotype of FPV mutants.

3.5. Recombinant Fowlpox Viruses Expressing VV E3L and VV K3L

Mammalian cells are non-permissive for productive FPV infection. However, FPV replication is blocked at different points in different mammalian cells (Somogyi *et al.*, 1993). In Vero cells, FPV early and late genes are expressed, and immature virus particles are formed; the block in productive infection is thought to be in morphogenesis of the virus particles (Somogyi *et al.*, 1993). However, in HeLa cells, the block in FPV replication is thought to occur prior to late gene expression, with little genome replication (Somogyi *et al.*, 1993). HeLa cells are thought to contain high levels of 2'-5'A synthetase which might cause the block in FPV replication (Rice *et al.*, 1984). However, it is thought that viral double-stranded RNA is predominantly formed during late gene expression, when genes from opposing strands are transcribed as long transcripts.

Although the presence of E3L and K3L would not be expected to rescue the morphogenesis of FPV in Vero cells, it was thought that E3L or K3L may render HeLa cells permissive for late gene expression. Thus, two FPV

recombinants, designed to express either VV E3L or VV K3L under their own promoters, were created using a vector permitting insertion in the ITR and GPT selection. Recombinant viruses were selected, plaque purified and grown, although the recombinant viruses were not tested for E3L and K3L expression. HeLa cells were infected with the recombinant viruses or wt FPV, cells were harvested at several time-points post-infection and the proteins were analysed by SDS-PAGE. Expression of FPV late genes could not be detected in HeLa cells infected with wt virus or either of the recombinants.

3.6. Discussion

FPV is resistant to more than 8 U/ml of chicken IFN in CEF, VV is resistant to 4 U/ml of chicken IFN in CEF and SFV is sensitive to less than 1 U/ml of chicken IFN in CEF. A previous study of FPV and IFN found that FPV was 40 times less sensitive to chicken IFN in CEF than VV in a plaque inhibition assay, and that FPV was a poor inducer of IFN, although small amounts were detected 96 hours p.i. (Asch & Gifford, 1970). Our results have shown that FPV is at least 2 times less sensitive to chicken IFN in CEF than VV. However, as high levels of IFN were not used here, the end-point of FPV resistance to IFN could not be elucidated. We were also unable to detect IFN in the medium taken from CEF infected with FPV at times up to 48 h.

Youngner *et al* (1972) reported that both VV and VSV were sensitive (71 % and 78 % inhibited by 1 U IFN, by plaque reduction assay) to chicken IFN in CEF. The difference in these results and the data shown here, may reflect the different methods used, or the amounts of virus and source of IFN used. It was also found that VV was resistant to 1000 U of rabbit IFN in RK13 cells, but VSV was sensitive to just 1 U of rabbit IFN in RK13 cells (Youngner *et al.*, 1972). Their results suggest that a virus may be sensitive to IFN in the cells of one species whilst being resistant to IFN in the cells of another species, implying that:

- There are differences between cells of different species in the mechanisms or molecules responsible for virus inhibition by IFN
- Virus encoded mechanisms to overcome IFN are cell specific.

The result of the IFN assay using FPV was confirmed by the ability of the virus to rescue SFV from the effects of IFN in chicken cells. The rescue of SFV by FPV was as efficient as the rescue of EMC by VV in mouse L cells

(Whitaker-Dowling & Youngner, 1986). Whitaker-Dowling and Youngner (1983, 1986) found that rescue of VSV and EMC by VV in L cells was due to increased protein synthesis, enabled by a VV inhibitor of PKR, and not due to a block in the 2'-5'A synthetase pathway, although VV does have an ATPase and a phosphatase which block activation of 2'-5'A synthetase (Paez & Esteban, 1984). Thus, it is possible that the rescue of SFV by FPV is due to inhibition of PKR by a FPV encoded protein, although as experiments to ascertain levels of SFV RNA and protein synthesis were not performed, it is not possible to rule out any effects caused by a block in the 2'-5'A synthetase pathway.

Whitaker-Dowling and Youngner (1983, 1986) found that coinfection with VV dramatically stimulated VSV protein synthesis, whilst only slightly increasing VSV mRNA synthesis. However, coinfection of VV and EMC, stimulated viral RNA and protein synthesis to similar levels. It was proposed that the variation was due to the different mechanisms of replication of the positive-stranded EMC and negative-stranded VSV. The positive-stranded EMC is completely dependent on protein synthesis, but the negative-stranded VSV can produce primary transcripts even when protein synthesis is blocked. SFV is a positive-stranded RNA virus and member of the *Togaviridae*, so it is probable that protein synthesis and viral RNA synthesis are stimulated to the same extent on coinfection with VV, although this has not been tested experimentally.

Inhibition of PKR by VV is mediated through two gene products, E3L and K3L (Akkaraju *et al.*, 1989, Carroll *et al.*, 1993, Chang *et al.*, 1992, Davies *et al.*, 1993, Whitaker-Dowling & Youngner, 1984). To determine whether the rescue of SFV by FPV could be due to homologues of one or both of these genes, vaccinia viruses with deletions in each of these genes were obtained (Dr. Tartaglia of Virogenetics Inc.) and used in SFV rescue experiments.

It was found that the VV mutant with E3L deleted (vP1080) was as capable of rescuing SFV from the effects of IFN as wt VV. The VV mutant with K3L deleted (vP1080) was less capable of rescuing SFV from the effects of IFN than wt VV or the E3L$^-$ mutant. The result implies that K3L may be more efficient than E3L in inhibiting avian effectors of the IFN response. By analogy, the result also implies that the rescue of SFV by FPV may be due to the presence of a homologue of K3L, although other FPV encoded factors may be responsible.

E3L is thought to be a more potent inhibitor of PKR than K3L in a COS cell transfection system (Davies *et al.*, 1993). The results shown here may indicate that this is not the case in CEF, assuming that an avian homologue of PKR is at least partially responsible for the reduction in SFV replication in IFN-treated cells.

The 2'-5'A synthetase pathway is active in mouse L929 cells infected with the VV K3L$^-$ mutant but not in cells infected with the wt VV (Beattie *et al.*, 1995b). The authors hypothesised that K3L is required for optimal protein expression of factors that allow VV to evade the 2'-5'A synthetase pathway. Thus, the reduced rescue of SFV by the VV K3L$^-$ mutant may be due to activation of the 2'-5'A pathway which is not inhibited by the ATPase and phosphatase (Paez & Esteban, 1984) when K3L is not present, rather than being directly due to deficient inhibition of PKR.

The VV E3L$^-$ virus did not have a reduced ability to rescue SFV from the effects of chicken IFN in CEF, although the same virus was reported to be sensitive to IFN and reduced in its ability to rescue VSV from the effects of IFN in L929 cells (Beattie *et al.*, 1995a). Both RNase L and PKR were found to be active in L929 cells infected with the E3L$^-$ mutant (Beattie *et al.*, 1995a). The contrast in these results may be due to, cell-specific differences in the mechanisms of anti-viral activity induced by IFN, or differences in the levels of PKR and 2'-5'A synthetase in these cells. This hypothesis is supported by a report in which the E3L$^-$ virus has a host range phenotype, being able to replicate normally in CEF, and RK-13 cells but not in Vero or HeLa cells; whereas the K3L$^-$ mutant is able to replicate normally in Vero cells (Chang *et al.*, 1995) . A phenotype for the K3L$^-$ virus in HeLa cells has not been reported. IFN-treatment of Vero and HeLa cells was found to further reduce the replication of the E3L$^-$ virus (Chang *et al.*, 1995). High levels of 2'-5'A exist in non IFN-treated HeLa cells, although VV is capable of replicating normally in these cells (Rice *et al.*, 1984). CEF have been shown to have 2'-5'A synthetase (Ball, 1979). Treatment of CEF with 125 U/ml of IFN was shown to result in a 3700 fold increase in 2'-5'A synthetase activity whereas treatment of mouse L cells with 500 U/ml of IFN resulted in a 180 fold increase in 2'-5'A synthetase activity. Both cell types displayed similar 2'-5'A synthetase activity when they were not IFN-treated (Ball, 1979). As yet an avian homologue of PKR has not been identified or studied.

A study of the 2'-5'A system and PKR in animals, plants and lower organisms found that RNase L is readily detectable (by binding assay) in

extracts from mammalian cells and reptile tissue, but was present only in trace amounts in amphibian tissues and was not detectable in fish tissue, insect cells, plants, slime moulds or bacteria (Cayley *et al.*, 1982). The same study found that 2'-5'A synthetase was present in reptilian extracts at a lower level than in extracts from mouse L cells and was not detectable in amphibian extracts. PKR was not detected in any of the reptilian or amphibian extracts or in plant extracts, although a plant homologue of PKR (Langland *et al.*, 1995, Langland *et al.*, 1996a, Langland *et al.*, 1996b) and a homologue of PKR in yeast (Dever *et al.*, 1993) have since been described. This information supports the hypothesis that the differences in the rescue of SFV by the E3L$^-$ virus seen here and the rescue of VSV by the E3L$^-$ mutant in L929 cells (Beattie *et al.*, 1995a), may be due to differences in the relative and absolute levels of PKR and 2'-5'A synthetase in the cells.

Chapter 4

Analysis of Extracellular Proteins Produced by Fowlpox Virus Infected Chick Embryo Fibroblasts

4. ANALYSIS OF EXTRACELLULAR PROTEINS PRODUCED BY FOWLPOX VIRUS INFECTED CHICK EMBRYO FIBROBLASTS

4.1 Proteins Secreted from FPV Infected Cells

It is known that secreted glycoprotein receptors for interferons and other cytokines are encoded by many poxviruses (for reviews see McFadden & Graham, 1994, Smith, 1993). The secreted glycoprotein receptors are thought to bind to cytokines, including interferons, preventing the cytokines from binding to the cell receptors and thus interfering with the host defence mechanisms. To determine if this mechanism is part of the FPV defence against interferons, experiments were undertaken to identify and characterise any proteins secreted by FPV-infected cells. A better way to undertake this study would be to use chicken cytokine binding as an assay for FPV encoded cytokine receptors. However, very little is known about chicken cytokines, and the information available suggests that they are significantly different from mammalian cytokines (Digby & Lowenthal, 1995, Sekellick *et al.*, 1994). Cloning of chicken IFNs was reported only late into the project and were not available at the time these experiments were performed.

Pulse labelling with ^{35}S methionine was used to visualise proteins produced at various times after FPV infection, and also to distinguish proteins produced during the pulse from proteins produced before the pulse. The labelled supernatants from IFN-treated cells were analysed by SDS-PAGE to show proteins which are produced on interferon induction or viral infection. All protein sizes suggested in the following experiments have been estimated by comparison with known markers, within confidence limits of + or - 20 Kd.

Many extracellular proteins are synthesised *de novo* in untreated uninfected cells, Fig. 4.1, lane C.

Cells labelled at 6-8 hours after interferon treatment produce fewer extracellular proteins than non-treated cells, although proteins of approximately 116 Kd, 66 Kd and 36 Kd are clearly visible. The proteins of 116 Kd and 66 Kd are produced by untreated cells. The diffuse 36 Kd band appears to consist of two proteins, both of which are also visible in the supernatants of untreated cells.

Fig. 4.1. Analysis of Pulse-Labelled Proteins Secreted by FPV-Infected Cells

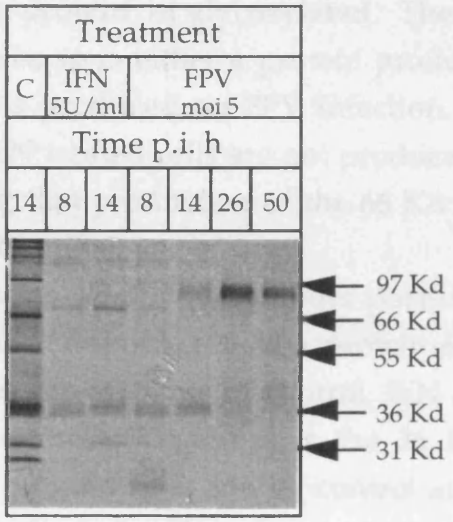


Fig. 4.1. Analysis of Pulse-Labelled Proteins Secreted by FPV-Infected Cells.

Primary CEF in 25 cm^2 flasks were infected with FPV (moi 5) or interferon treated (16U/flask) for the indicated times. Control cells were not FPV-infected or IFN-treated. The medium was removed and replaced with 3 ml of serum-free, methionine-free medium (Sigma), and incubated at 37 ^oC, 5 % CO_2 for 2 h. The medium was removed and replaced again with 3 ml serum-free, methionine-free medium, containing 1.85 MBq 35S methionine (1,000 Ci/mmol, 10 mCi/ml, DuPont) per ml for a further 2 h incubation at 37 ^oC, 5 % CO_2.

The supernatants were filtered through 0.2 µm filters and concentrated in Centriprep 10 (Amicon) and Microcon 30 (Amicon) columns. The concentrated samples were analysed on a 12 % non-reducing acrylamide gel which was run at 150 V for 1 hour. The gel was Coomassie blue stained and dried before being exposed to film for 3 days.

Labelling carried out at a later time after IFN treatment shows the same protein bands, but the 66 Kd protein appears to be produced in greater quantities. There is also a faint band at 31 Kd which may be the same as the protein produced by control cells and cells labelled at the earlier time after IFN treatment.

Cells labelled at 6-8 hours after FPV infection appear to produce the same proteins as cells labelled 6-8 hours after IFN treatment, although a diffuse band of well under 31 Kd is also produced. The fuzziness of this band may indicate that this protein is glycosylated. The 31 Kd protein is not produced by control cells, so is either a protein produced by FPV itself, or a cellular protein which is produced on FPV infection. The 66 Kd and 31 Kd proteins produced by IFN treated cells are not produced by cells infected with FPV, possibly indicating that production of the 66 Kd and 31 Kd proteins are induced or up-regulated by IFN.

FPV infected cells labelled at 12-14 hours post-infection only appear to produce two extracellular proteins, one is a protein of approximately 80 Kd which is not seen in the supernatants of control, IFN treated or early in FPV infected cells. The other protein present is the 36 Kd protein(s) which is produced early in FPV infected cells and by control and IFN treated cells. By 24-26 hours post FPV infection, only a strong but diffuse band at 80 Kd is visible. The 80 Kd protein is still being produced between 48 and 50 hours post FPV infection. The diffuse nature of the band at 80 Kd may indicate that this is a glycoprotein.

The small 31 Kd protein produced at 6-8 hours after FPV infection and the 80 Kd protein which is produced at 12-50 hours post-infection, are not seen in any of the other samples. These proteins are either cellular proteins, production of which is induced by FPV infection or are FPV encoded proteins.

4.2. Analysis of the Glycosylation State of the Extracellular 80 Kd Protein Produced by FPV Infected Cells

The band indicating a protein of approximately 80 Kd produced 12-50 hours post FPV infection is diffuse, possibly indicating that it is glycosylated. The interferon and cytokine receptors produced by *Orthopoxviruses* are thought to be glycoproteins (McFadden & Graham, 1994, Smith, 1993) thus the glycosylation state of the 80 Kd protein was analysed.

One way to study the glycosylation state of a protein is to use enzymes which remove the oligosaccharide from the protein. An alternative is to use antibiotics which block the glycosylation process. Both of these approaches were used to analyse the glycosylation state of the 80 Kd extracellular protein produced by FPV infected cells.

The enzyme used was a commercial mixture of endoglycosidase F and N-glycosidase F. Endoglycosidase F removes the oligosaccharide chain from the protein, leaving the first glucosamine, thus affecting only N-linked glycoproteins. N-glycosidase F removes the first glucosamine from asparagine which is left as an aspartate residue, thus affecting only N-linked glycoproteins. The results are shown in Fig. 4.2.

In buffers lacking β-mercaptoethanol, the FPV extracellular protein is approximately 80 Kd, Fig. 4.2. When the protein was incubated with endoglycosidase F and N-glycosidase F, in the presence or absence of SDS, the size of the protein was reduced to approximately 55 Kd, indicating that the protein is glycosylated. The multiple bands visible on the film when the protein had been incubated with the enzymes, indicate that deglycosylation was not complete.

In the presence of β-mercaptoethanol and SDS, but absence of endoglycosidase F and N-glycosidase F, the 80 Kd protein was reduced to approximately 27 Kd, indicating that the 80 Kd protein consists of multiple subunits which are held together by disulphide bonds.

When the 80 Kd protein was reduced to the smaller (27 Kd) form by boiling in a buffer containing β-mercaptoethanol and SDS, and then incubated with endoglycosidase F and N-glycosidase F, the protein appeared as a band of 18 Kd. Thus, all the 27 Kd subunits of the protein contain N-linked oligosaccharides.

These results suggest that the extracellular protein of approximately 80 Kd, produced by FPV infected cells 12-50 h p.i., is a multimeric glycoprotein and that all of the subunits are glycosylated.

In another experiment, the antibiotic tunicamycin was used to block core glycosylation. Tunicamycin prevents the transfer of N-acetylglucosamine from UDP to the dolichol phosphate carrier, thus inhibiting N-linked glycosylation. The results are shown in Fig. 4.3.

Fig. 4.2. Removal of the Oligosaccharide from the 80 Kd Extracellular Protein using Endoglycosidase F and N-Glycosidase F

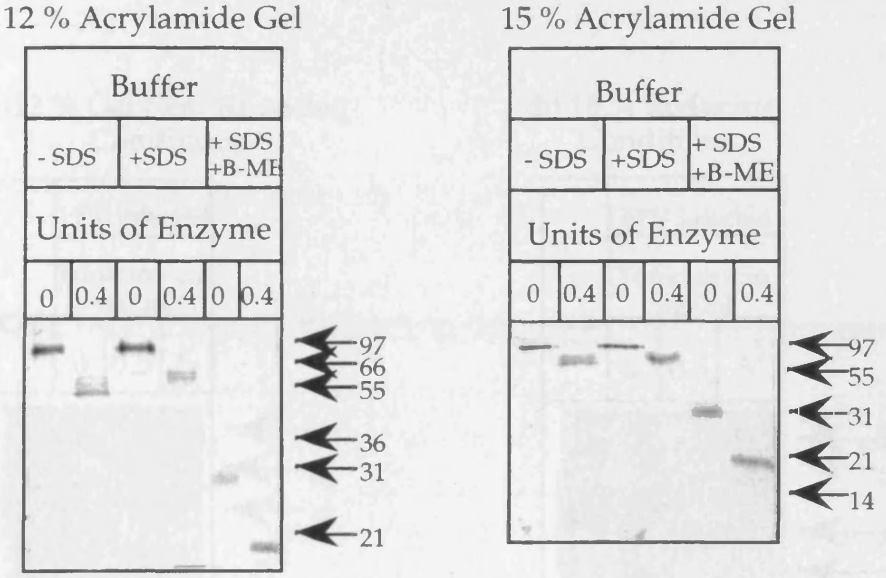


Fig. 4.2. Removal of the Oligosaccharide from the 80 Kd Extracellular Protein using Endoglycosidase F and N-Glycosidase F

^{35}S methionine labelled FPV supernatants (25-27 hours and 48-50 hours) were concentrated 30 fold. The supernatants were mixed with an equal volume of one of the three following buffers and heated to 100 ^{o}C for 3 min.

Buffer 1: 50 mM EDTA, 0.8 mM PMSF in 50 mM $NaPO_4$ pH 4.1.

Buffer 2: 50 mM EDTA, 0.8 mM PMSF, 0.2 % SDS, 1 % Triton-X-100 in 50 mM $NaPO_4$ pH 4.1.

Buffer 3: 50 mM EDTA, 0.8 mM PMSF, 0.2 % SDS, 1 % Triton-X-100, 2 % β-mercaptoethanol in 50 mM $NaPO_4$ pH 4.1.

Once the samples had cooled, 0 or 0.4 U of endoglycosidase F/N-glycosidase F mixture (Boehringer Mannheim Biochemica) were added. The samples were incubated at 37 ^{o}C for 20 h then analysed by SDS PAGE a) 12 % b) 15 % and exposed to film.

Fig. 4.3. Inhibition of Glycosylation of the 80 Kd Extracellular Protein using Tunicamycin

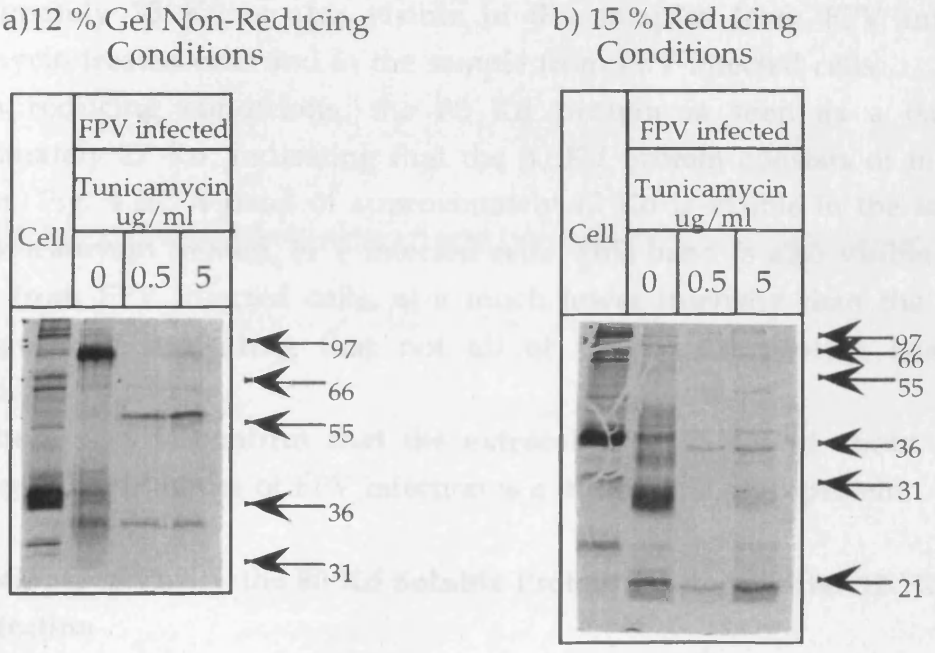


Fig. 4.3. Inhibition of Glycosylation of the 80 Kd Extracellular Protein using Tunicamycin

FPV infected cells (moi 5) were labelled with ^{35}S methionine at 22-24 hours post-infection. The methionine free medium was supplemented with 0.5 or 5 mg/ml of tunicamycin (Sigma) at both the pre-labelling and labelling stages. The medium was filtered and concentrated 250 fold before being run on SDS acrylamide gels a) 12 % gel, SDS loading buffer b) 15 % gel, β-mercaptoethanol loading buffer and exposed to film.

The dominant bands which are synthesised *de novo* in non-treated, uninfected cells are the two bands of just under 36 Kd, Fig. 4.3a. The control cells also produce other proteins, including two of approximately 80 Kd. The FPV infected cells produce a protein of 80 Kd which is seen as a dark diffuse band. When the FPV infected cells were treated with tunicamycin during labelling, the dominant band was seen at approximately 55 Kd and appears sharper, although the tunicamycin did not completely block glycosylation as a trace of the 80 Kd band is still visible. Thus Fig. 4.3a supports the conclusions based on Fig. 4.2a and indicates that the 80 Kd protein produced from 12 hours of FPV infection is a glycoprotein with a 55 Kd protein component. A band of approximately 33 Kd is also visible in the samples from FPV infected, tunicamycin treated cells and in the sample from FPV-infected cells.

In reducing conditions, the 80 Kd protein is seen as a band of approximately 27 Kd, indicating that the 80 Kd protein consists of multiple subunits, Fig. 4.2b. A band of approximately 17 Kd is visible in the samples from tunicamycin treated, FPV infected cells. This band is also visible in the sample from FPV infected cells, at a much lower intensity than the 27 Kd band, possibly indicating that not all of the 17 Kd protein has been glycosylated.

These results confirm that the extracellular protein of about 80 Kd produced after 12 hours of FPV infection is a multimeric glycoprotein.

4.3. Attempts to Purify the 80 Kd Soluble Protein Produced after 12 Hours of FPV Infection

Attempts were made to purify the 80 Kd soluble protein, in order that N-terminal sequencing could be carried out to provide information on amino acid content of the protein. This would enable oligonucleotide probes to be synthezised and used to identify the gene.

Lectins are non-enzymatic proteins which can bind to specific carbohydrate groups and so can be used to purify glycoproteins. As specific lectins bind specific carbohydrate groups, lectin analysis can also provide information on the carbohydrate content of a glycoprotein. Biotinylated lectins were used to probe a blot of the supernatant from FPV infected cells, in which the 80 Kd protein produced by FPV infected cells after 12 hours was in its reduced 27 Kd form. The experiment aimed to find a lectin suitable for

Fig. 4.4. Analysis of the Extracellular Proteins from FPV Infected Cells using Biotinylated Lectins

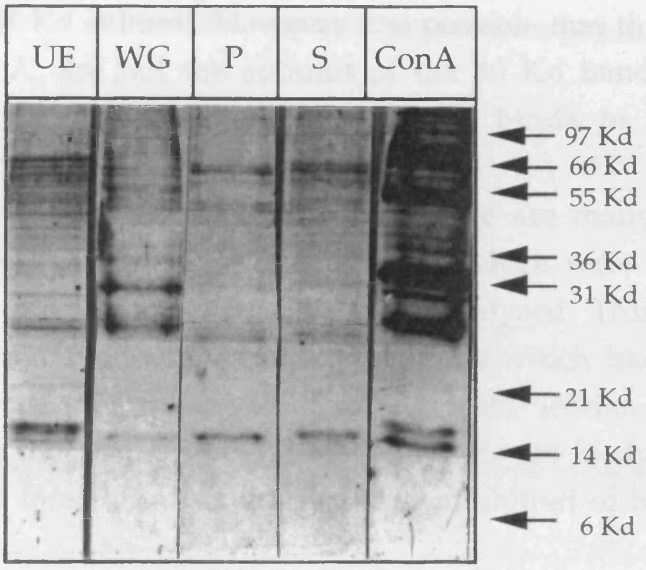


Fig. 4.4. Analysis of the Extracellular Proteins from FPV Infected Cells using Biotinylated Lectins.

FPV infected cells (moi 5) were washed three times with medium at 19 hr p.i., the medium was replaced and the cells were incubated 37 ^{o}C 5 % CO_2 until 25 h p.i. The medium was removed, filtered through 0.2 µm filter and concentrated 50 fold in Centriprep 10 (Amicon) and Microcon 30 (Amicon) columns. Ten µl were mixed with an equal volume of SDS-Page loading buffer, containing β-mercaptoethanol and run on a 15 % SDS Page gel. The proteins were blotted onto nitrocellulose membrane and probed with biotinylated lectins (Vector Laboratories) according to the manufacturer's protocol.

The nitrocellulose was incubated in TTBS (0.1 % Tween 20, 100 mM Tris, 0.9 % NaCl pH 7.5) for at least 30 min with gentle agitation. The nitrocellulose was then incubated in TTBS with 20 mg/ml of the biotinylated lectin for 1 h with gentle shaking. The blot was washed three times in water for 5 min each wash, then with TTN for 20 min. This series of washes was repeated 3 times.

The nitrocellulose blot was incubated in a 1/400 dilution of alkaline phosphatase conjugate for 1 h. The series of washes was repeated and the alkaline phosphatase substrate prepared using a Pierce kit by mixing 10 ml of 0.1 M Tris with 1 ml of X-phos (5-bromo-4-chloro-3-indolyl-phosphate, 2.5 g/l) and 1 ml of NBT (nitrobluetetrazolium, 5 g/l). The substrate was placed on the nitrocellulose and gently shaken for 10 min.

UE = Ulex Europaus Agglutinin I
WG= Wheat Germ Agglutinin
P= Peanut Agglutinin
S= Soybean Agglutinin
Con A = Concanavalin A

purifying the 80 Kd protein of interest from other proteins of a similar size. The results are shown in Fig. 4.4.

Bands of approximately 27 Kd are seen in all lanes of Fig. 4.4, but are most clearly visible in the lane probed with Concanavalin A (Con A), indicating that Con A may be the lectin most suitable for purification of the 80 Kd protein or the 27 Kd subunit. However it is possible that the bands seen in Fig. 4.4, lane Con A, are not the subunit of the 80 Kd band but are other proteins of a similar molecular weight. Con A binds to terminal α-D-mannopyranosyl and α-D-glucopyranosyl residues.

It is also clear from using lectins that there are many glycoproteins present in the supernatant of FPV infected cells which were not seen when the supernatants from ^{35}S labelled cells were analysed. This indicates that washing the cells did not remove all the proteins which had already been synthesized, and demonstrates that probing with lectins is a sensitive technique. Many of the proteins bound by the lectins are likely to be encoded by the cells as FPV infection does not result in inhibition of host cell protein synthesis.

Attempts were made to use a Con A-sepharose column to purify the 80 Kd protein and the 27 Kd subunits from other proteins in the supernatant of FPV infected cells Figs. 4.5a and 4.5b.

The Con A-bound proteins start to be eluted from the column during the first wash with 0.3 M methyl-α-D glucose, Fig. 4.5a. The Con A-bound proteins are mainly concentrated in washes 3 and 4 although some protein is present in all washes. The major protein bound by the Con A column is a protein of approximately 31 Kd, possibly a serum protein, but two proteins of 60-70 Kd are also bound and a diffuse band can also be seen at about 80 Kd. The diffuse 80 Kd band appears equally intense in the non-purified sample. Thus, although Con A appears to bind to a protein of 80 Kd, the interaction appears to be weak and it is possible that this is not the 80 Kd protein of interest.

The 31 Kd protein is not reduced by β–mercaptoethanol, Fig. 4.5b. A band of around 27 Kd, possibly the 80 Kd protein under reducing conditions, is visible below the 31 Kd band. It is not possible to be certain that this band is a subunit of the 80 Kd protein, but it is apparent that the 31 Kd protein would interfere with any attempt to use Con A to purify the reduced form of the 80 Kd protein.

Fig. 4.5a. Binding of Proteins from FPV Infected Cell Supernatants to a Con A-Sepharose Column

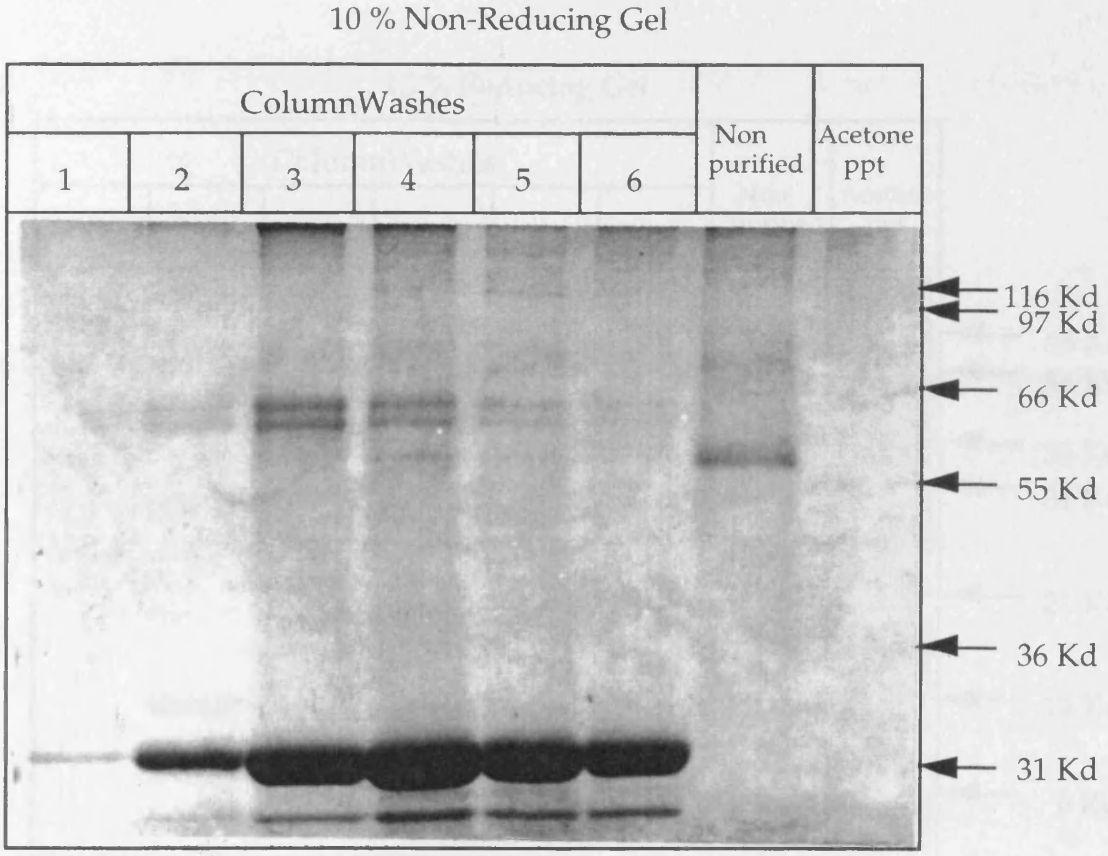


Fig. 4.5a. Binding of Proteins from FPV Infected Cell Supernatants to a Con A-Sepharose Column

Con A sepharose (Pharmacia) was washed in 10 volumes of binding buffer (20 mM Tris/HCl pH 7.5, 0.5 M NaCl). A column of 0.5-1 ml of washed Con A sepharose was set up in a 1 ml syringe with a glass wool stopper. The column was washed three times by applying 0.5 ml of binding buffer to the column and allowing it to flow through. The sample of supernatant from FPV infected cells was concentrated 10 fold and applied to the column which was then washed six times with binding buffer. The bound proteins were eluted by applying 500 µl washes of 0.3 M methyl-α-D glucose. The eluted fractions 1-6 were concentrated using Microcon 30 spin columns and analysed alongside a non-purified sample and a sample which had been acetone precipitated, by Coomassie blue staining of a 10 % gel run under non-reducing conditions.

Fig. 4.5b. Binding of Proteins from FPV Infected Cell Supernatants to a Con A-Sepharose Column

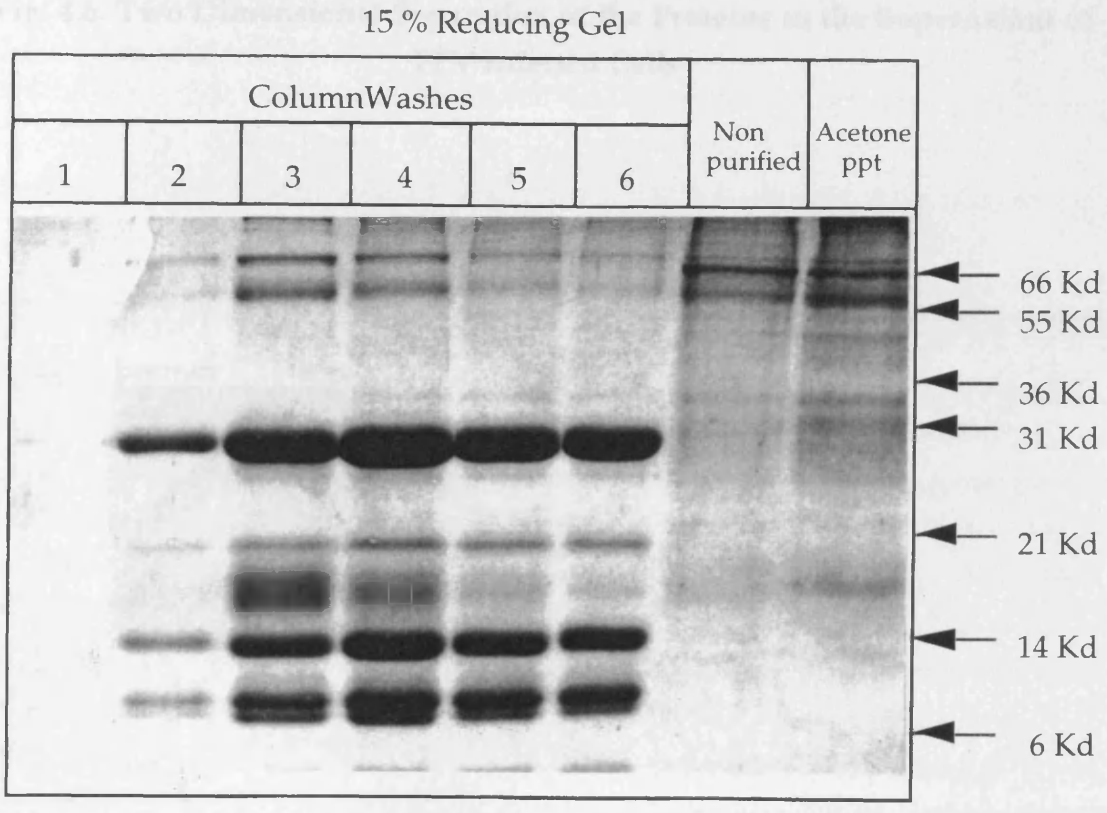


Fig. 4.5b. Binding of Proteins from FPV Infected Cell Supernatants to a Con A-Sepharose Column
Con A sepharose (Pharmacia) was washed in 10 volumes of binding buffer (20 mM Tris/HCl pH 7.5, 0.5 M NaCl). A column of 0.5-1 ml of washed Con A sepharose was set up in a 1 ml syringe with a glass wool stopper. The column was washed three times by applying 0.5 ml of binding buffer to the column and allowing it to flow through. The sample of supernatant from FPV infected cells was concentrated 10 fold and applied to the column which was then washed six times with binding buffer. The bound proteins were eluted by applying 500 µl washes of 0.3 M methyl-α-D glucose. The eluted fractions 1-6 were concentrated using Microcon 30 spin columns and analysed alongside a non-purified sample and a sample which had been acetone precipitated by Coomassie blue staining of a 15 % reducing gel.

Fig. 4.6. Two Dimensional Separation of the Proteins in the Supernatant of FPV Infected Cells

Fig. 4.6. Two Dimensional Separation of the Proteins in the Supernatant of FPV Infected Cells

Primary CEF in 25 cm^2 flasks were infected with FPV (moi 5) or interferon treated (16U/flask) for the appropriate periods. The medium was removed at 22 h p.i. and replaced with 3 ml of serum-free, methionine-free medium (Sigma), and incubated at 37 ^oC, 5 % CO_2 for 2 h. The medium was removed and replaced again with 3 ml serum-free, methionine-free medium, containing 1.85 MBq ^{35}S methionine (1,000 Ci/mmol, 10 mCi/ml, DuPont) per ml for a further 2 h incubation at 37 ^oC, 5 % CO_2.

The supernatant was filtered through a 0.2 µm filter and concentrated in Centriprep 10 (Amicon) and Microcon 30 (Amicon) columns. The concentrated sample was analysed on a 10 % non-reducing acrylamide gel which was run at 150 V for 1 hour. The appropriate track of the gel was cut out, then incubated in β-mercaptoethanol loading buffer for 45 min at 25 ^oC and placed at the top of a 15 % gel, surrounded by 0.5 % LMP agarose. Following electrophoresis, the gel was dried and exposed to film over night.

These results show that even if Con A does bind to the 80 Kd protein of interest, the interaction is weak. When this experiment was repeated using silver staining (data not shown), which can detect smaller amounts of protein than Coomassie blue staining, several proteins of around 80 Kd which had been bound to Con A were revealed. Thus, Con A is not suitable for purification of the 80 Kd protein seen in Fig. 4.1.

As the 80 Kd protein of interest can be reduced into a smaller 27 Kd form, a two-dimensional gel approach was used to separate the protein of interest from the other proteins in the supernatants. A gel was run under non-reducing conditions, the track was removed, incubated in β-mercaptoethanol loading buffer and then placed at the top of a second gel. These results are shown in Fig. 4.6.

Two bands which appear to be derived from the 80 Kd protein can be seen on the reducing gel. These 27 Kd and 55 Kd bands are subunits of the 80 Kd protein. These two bands indicate that the reduction of the 80 Kd protein was not complete; previously reduction (Fig. 4.2) resulted in only one band. The lower of the two bands, 27 Kd may be the monomer(s) form of the protein whilst the upper 55 Kd form could be a dimer of the subunits. Thus, the 80 Kd protein may be a trimer of different polypeptide species, or possibly a tetramer of one or more polypeptide species. None of the other proteins appear to have been reduced by treatment with β-mercaptoethanol and thus migrate as they did on the first gel. The result shows that this two-dimensional approach is suitable for purification of the subunit form of the 80 Kd protein of interest from other proteins in the sample. However, when unlabelled sample was used, the bands were not visible by Coomassie blue staining, and attempts to scale-up the system proved unsuccessful due to aggregation of concentrated protein samples under non-reducing conditions. Thus N-terminal sequencing and further analysis of the 80 Kd protein was not possible.

4.4. Discussion

I have shown, that an 80 Kd soluble protein is secreted by FPV infected CEF from 12-50 hours post infection, although whether this protein is encoded by a viral or cellular gene has not been determined. Analysis of the

glycosylation state of the protein revealed that it consists of a 55 Kd polypeptide with the remainder of the molecular mass consisting of carbohydrate groups. Reduction of the glycosylated form of the protein using β-mercaptoethanol resulted in a 27 Kd protein. Thus, the 80 Kd soluble protein produced by FPV infected cells is a multimeric glycoprotein. The available data suggest that the 80 Kd protein is a trimer of subunits of approximately 27 Kd, although further experimentation would be required to confirm this hypothesis. No data is available to indicate whether the subunits are all of one polypeptide species or are different polypeptides. Two subunit forms of the protein are visible in Fig. 4.6 under reducing conditions, suggesting that if the 80 Kd form is a trimer, it is likely to consist of different polypeptide species, as a homotrimer would dissociate into monomers and not produce an intermediate form.

Lectin purification of the 80 Kd protein and the 27 Kd subunit proved unsuitable as did a two-dimensional gel approach. The function of the 80 Kd protein and the location of the gene encoding it remain unknown.

Many soluble proteins have been reported in a variety of poxviruses. The first to be identified was the T2 gene of Shope Fibroma virus (SFV) (Smith *et al.*, 1991b). The T2 ORF was predicted to encode a 34 Kd protein however, when the T2 ORF was expressed using a plasmid in COS cells, a protein of 58 Kd was produced. The difference in predicted and actual molecular weight was thought to be due to glycosylation as the predicted protein contained 4 potential glycosylation sites. The 58 Kd protein was found to be able to bind human TNF-α and TNF-β, and prevent TNF-α and TNF-β from binding to their natural TNF receptors. VV contains an ORF with homology to the TNF receptor of SFV but the VV ORF is discontinuous (Howard *et al.*, 1991). Myxoma virus and malignant fibroma virus encode T2 homologues, deletion of the copies of T2 from myxoma virus resulted in the loss of a 52-56 Kd band and reduced viral disease in rabbits (Upton *et al.*, 1991). Cowpox virus *crmB* gene produces a 48 Kd product which is able to bind TNF-α and TNF-β (Hu *et al.*, 1994). All the genes mentioned above are found in the inverted terminal repeats of the DNA of the viruses and thus are present in two copies.

Vaccinia and cowpox viruses also encode a secreted IL-1 binding protein (Spriggs *et al.*, 1992). Supernatants from VV infected CV-1 cells were found to contain a 33 Kd protein which was capable of binding murine IL-1β.

A VV ORF (B15R) which had 30 % aa identity to the type II IL-1 receptor and was able to specifically bind mIL-1β when expressed in CV-1-EBNA cells. A similar ORF with similar properties was identified in cowpox virus. The VV B15R encoded IL-1 receptor was found to differ from the cellular counterpart in that it could bind IL-1β but not IL-1α (Alcami & Smith, 1992).

A homologue of the IFN–γ receptor was originally identified in myxoma virus. The M-T7 ORF was found to encode a secreted protein of 37 Kd which had sequence homology with the human and mouse receptor for IFN-γ and was found to bind specifically to rabbit IFN-γ (Upton *et al.*, 1992). Soluble receptors for IFN-γ are now known to be secreted by vaccinia (B8R gene), cowpox, rabbitpox, buffalopox, elephantpox and camelpox viruses and have a broad species specificity in contrast to the cellular receptors (Alcami & Smith, 1995).

The B18R gene of VV encodes a 60-65 Kd glycoprotein which has a high affinity for IFN-α of a broad range of species and is a member of the immunoglobulin superfamily (Symons *et al.*, 1995).

As well as encoding a range of soluble cytokine receptors, VV encodes a soluble glycoprotein growth factor, VGF, of 23-25 Kd. VGF is thought to stimulate metabolic activity and proliferation of uninfected cells so that the virus can replicate efficiently as it spreads to these cells (Smith, 1993). VV also secretes a 35 Kd protein which has similarity to C4b-binding protein and can prevent activation of the classical pathway by binding C4b, and the alternative pathway by binding C3b (Isaacs *et al.*, 1992, Kotwal *et al.*, 1990, McKenzie *et al.*, 1992).

It is likely that FPV encodes a homologue of an avian cytokine receptor or a growth factor, although it has not been determined whether the 80 Kd protein secreted by FPV infected CEF 12-50 h p.i. is able to bind any cytokine(s). Avian cytokines other than IFNs, have not been cloned or characterized as yet, although assays exist for IL-6, IL-8, TGF-β, stem cell factor and a TNF-like activity (personal communication, Dr. P. Kaiser). The first chicken interferon gene to be cloned, sequenced and analysed, displayed little sequence homology to mammalian IFNs and has been classified as a type I IFN on the basis of its predicted secondary structure (Sekellick *et al.*, 1994). The chicken IFN-γ gene is predicted to encode a protein of 145 amino acids. The predicted chicken IFN-γ protein contains the highly conserved motifs that are present in all mammalian IFN-γ proteins and has 32 % aa identity with equine and human

IFN-γ proteins (Digby & Lowenthal, 1995). Thus, it was decided that an approach to characterize the binding property of the secreted 80 Kd protein of FPV, using mammalian cytokines would be unfeasible.

The cytokine receptors of the *Orthopoxviruses* described earlier are encoded by genes within the terminal regions of the virus genome, and it is possible that any FPV genes encoding cytokine receptors are also contained in these regions. An 11.2 Kb BamHI fragment from the near-terminal region of the FPV genome has been sequenced and analysed (Tomley *et al.*, 1988). Twenty ORFs were predicted including homologues of the VV 42 Kd early gene and 32.5 Kd host range gene, and a homologue of the cowpox virus 38 Kd red pock gene, but the exact physical locations of these genes varied between VV and FPV and the level of conservation was lower than between genes in the central regions of the genome.

Chapter 5

Biochemical Tests for PKR Activity in Avian Cells

5. BIOCHEMICAL TESTS FOR PKR ACTIVITY IN AVIAN CELLS

Little is known about the effects of IFN on avian cells, although CEF are known to express 2'-5'A synthetase activity (Ball, 1979). The only existing report of an avian PKR suggests that two proteins of 67 Kd and 38 Kd are phosphorylated by PKR (Ball, 1979). However, the data to support this was not published.

It was decided that it was important to try to confirm the existence of a PKR-like activity in avian cells. Although I have demonstrated that FPV rescues SFV from the inhibitory effects of IFN in CEF, this does not enable one to draw any conclusions about the existence of avian PKR. However, I have also demonstrated that a VV mutant with E3L deleted is able to rescue SFV from the inhibitory effects of IFN in CEF. This may imply that any avian PKR is not inhibited by competition for dsRNA by VV E3L. Alternatives are, that avian PKR is not active under the conditions being studied, or that avian PKR does not exist.

VV E3L and K3L are thought to inhibit PKR activity by two distinct mechanisms, see 1.6.2 (Davies *et al.*, 1993), thus experiments were also designed to provide information on any inhibition of PKR activity by FPV factors, which could indicate the presence of FPV homologues of E3L and K3L.

5.1. Biochemical Assays for PKR Activities

Human PKR binds polyanionic molecules such as RNA and poly IC, an RNA analogue (Rice & Kerr, 1984). Binding results in autophosphorylation and activation of PKR (Hovanessian & Kerr, 1979, Rice & Kerr, 1984). PKR is then able to phosphorylate other protein substrates including histone proteins (Roberts *et al.*, 1976b) and eIF-2α (Rice & Kerr, 1984). If a similar biochemical activity were detected in avian cells, it would provide circumstantial evidence for the existence of avian PKR. If FPV infection were able to reduce this biological activity, it would provide circumstantial evidence for the existence of viral antagonist activities. Such activities could be analogous to the E3L and K3L proteins of VV.

Cell extracts were prepared from CEF cells treated with varying doses of IFN for 24 h. Cell extracts were also prepared from CEF infected with FPV for periods of 1-24 h. Samples were incubated with γ-^{32}P in the presence or absence of poly IC. The samples were analysed by SDS polyacrylamide gel electrophoresis and either Coomassie blue stained or exposed to film.

5.2. Assay of Cell Extracts for Kinase Activity

The Coomassie blue stained proteins in the samples are shown in Figs. 5.1a and 5.1b. The unlabelled protein profiles of all the samples are similar, indicating that there are no large variations in the relative abundance of protein species. Thus differences in the phosphorylated protein profiles described below can not be explained by differences in sample recovery, proteolysis or other sampling artefacts.

All of the cell extracts contain a number of phosphoproteins and thus kinase activity, Fig 5.2a. There are subtle differences in the profiles of phosphoproteins in the samples from untreated and 1 U/ml IFN-treated cells. In the untreated cell extracts the major phosphoproteins appear as bands of approximately 120 Kd, 100 Kd, 60 Kd and 56 Kd. In cell extracts from cells treated with 1 U/ml of IFN, the major phosphoproteins are approximately 40 Kd and 35 Kd.

Samples from cells treated with 2 U/ml of IFN and 8 U/ml chicken IFN have the same profile of phosphoproteins as cells treated with 1 U/ml of chicken IFN.

Cells infected with FPV for 1h have the same phosphoprotein profile as IFN-treated cells. This may indicate that FPV rapidly induces IFN synthesis, or the kinase associated with IFN induction, or that infectious FPV virus particles contain phosphoproteins.

The presence of poly IC does not affect the phosphorylation profile at any time point.

The results from cells infected with FPV for 6, 12 and 24 h are shown in Fig. 5.2b. CEF infected with FPV for 6 hours contain phosphoproteins of approximately 120 Kd, 100 Kd, 80 Kd, 55 Kd and 40 Kd. CEF infected with FPV for 12 hours have a phosphoproteins of approximately 120 Kd, 100 Kd, 80 Kd and 55 Kd, a similar phosphoprotein profile to untreated cells. The dominant bands are the 100 Kd and 55 Kd bands as in the untreated cells. This could indicate that FPV infection is able to reverse the effect of IFN activation on these kinase activities. Such a reversal could be due to the synthesis of viral antagonistic proteins.

Fig. 5.1a. Coomassie Blue Stain of Cell Extract Proteins

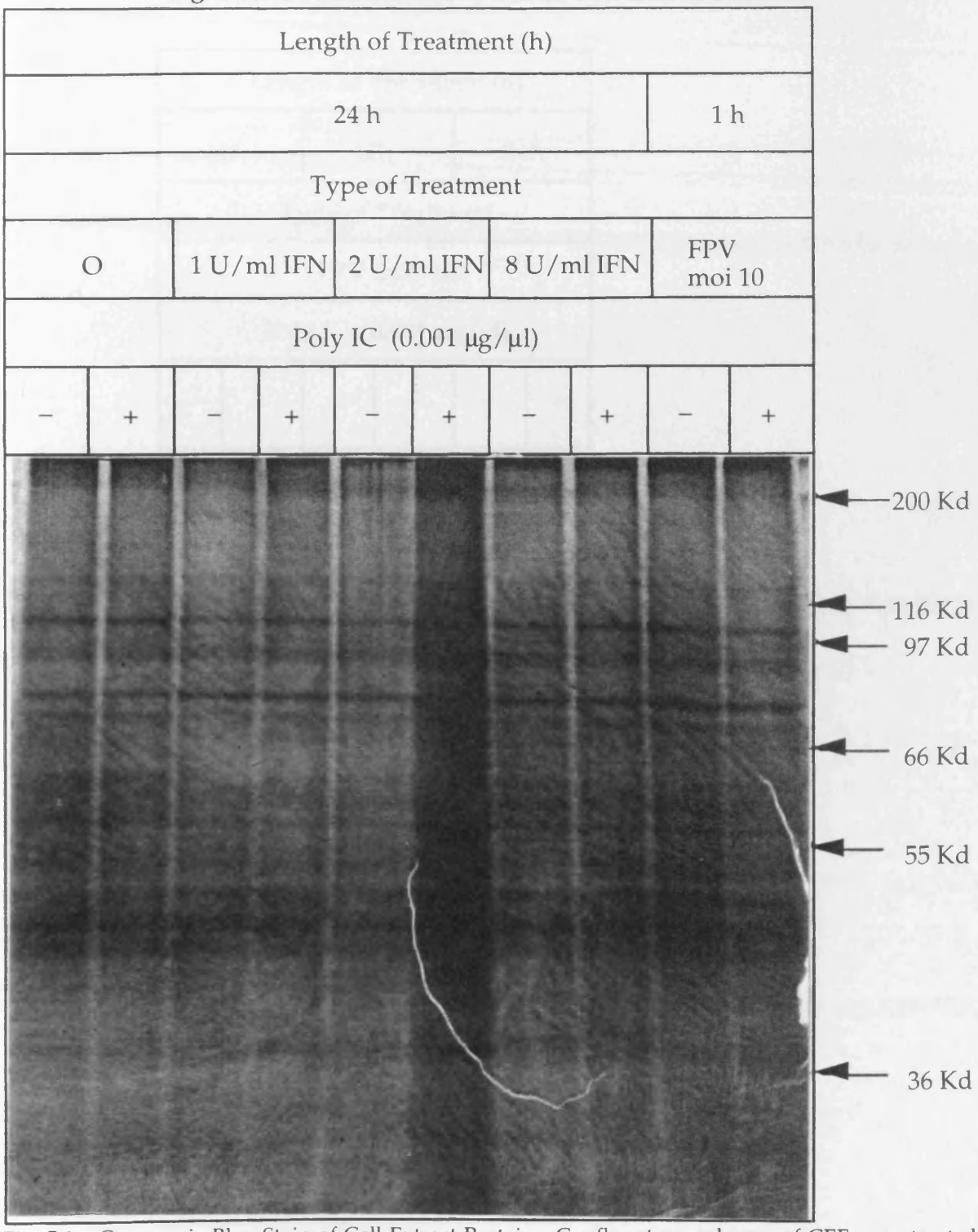


Fig. 5.1a. Coomassie Blue Stain of Cell Extract Proteins. Confluent monolayers of CEF were treated with IFN (range of concentrations), or infected with FPV (moi of 5-10) and incubated at 37 ^{o}C, 5 % CO_2 for the times shown. Cell extracts were prepared as in 2.3.1. Extracts (8 µl) were incubated at 30 ^{o}C for 15 minutes in the presence (+) or absence (-) of 1 ng/µl poly IC with 100 µM cold ATP, 0.37 MBq ^{32}P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 10 % SDS-PAGE, which was Coomassie blue stained.

Fig. 5.1b. Coomassie Blue Stain of Cell Extract Proteins

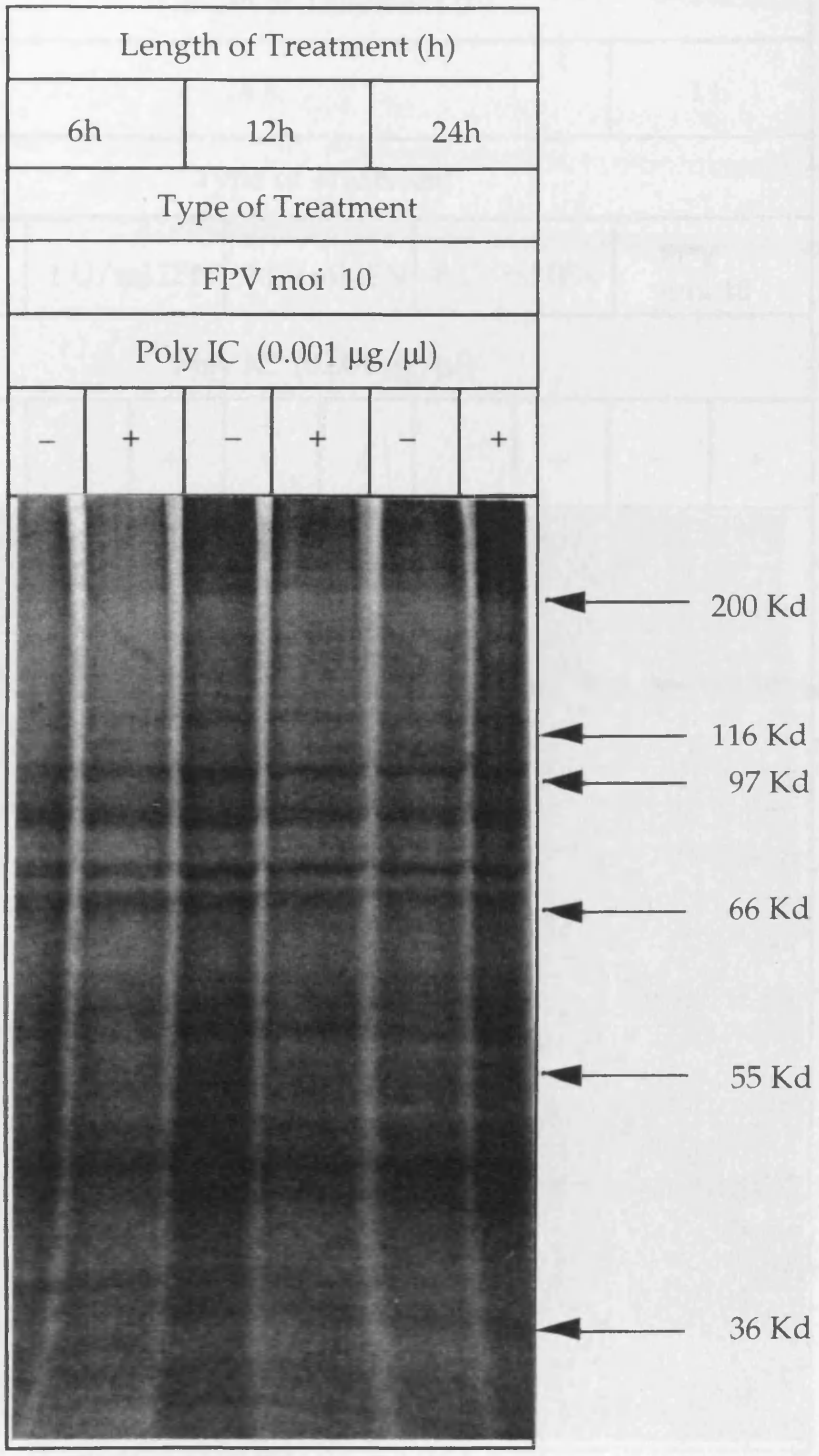


Fig. 5.1b. Coomassie Blue Stain of Cell Extract Proteins. Confluent monolayers of CEF were infected with FPV (moi of 5-10) and incubated at 37 ^{o}C, 5 % CO_2 for the times shown. Cell extracts were prepared as in 2.3.1. Extracts (8 µl) were incubated at 30 ^{o}C for 15 minutes in the presence (+) or absence (-) of 1 ng/µl poly IC with 100 µM cold ATP, 0.37 MBq ^{32}P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 10 % SDS-PAGE, which was Coomassie blue stained.

Fig. 5.2a. Autoradiograph of Kinase Assay of Cell Extracts

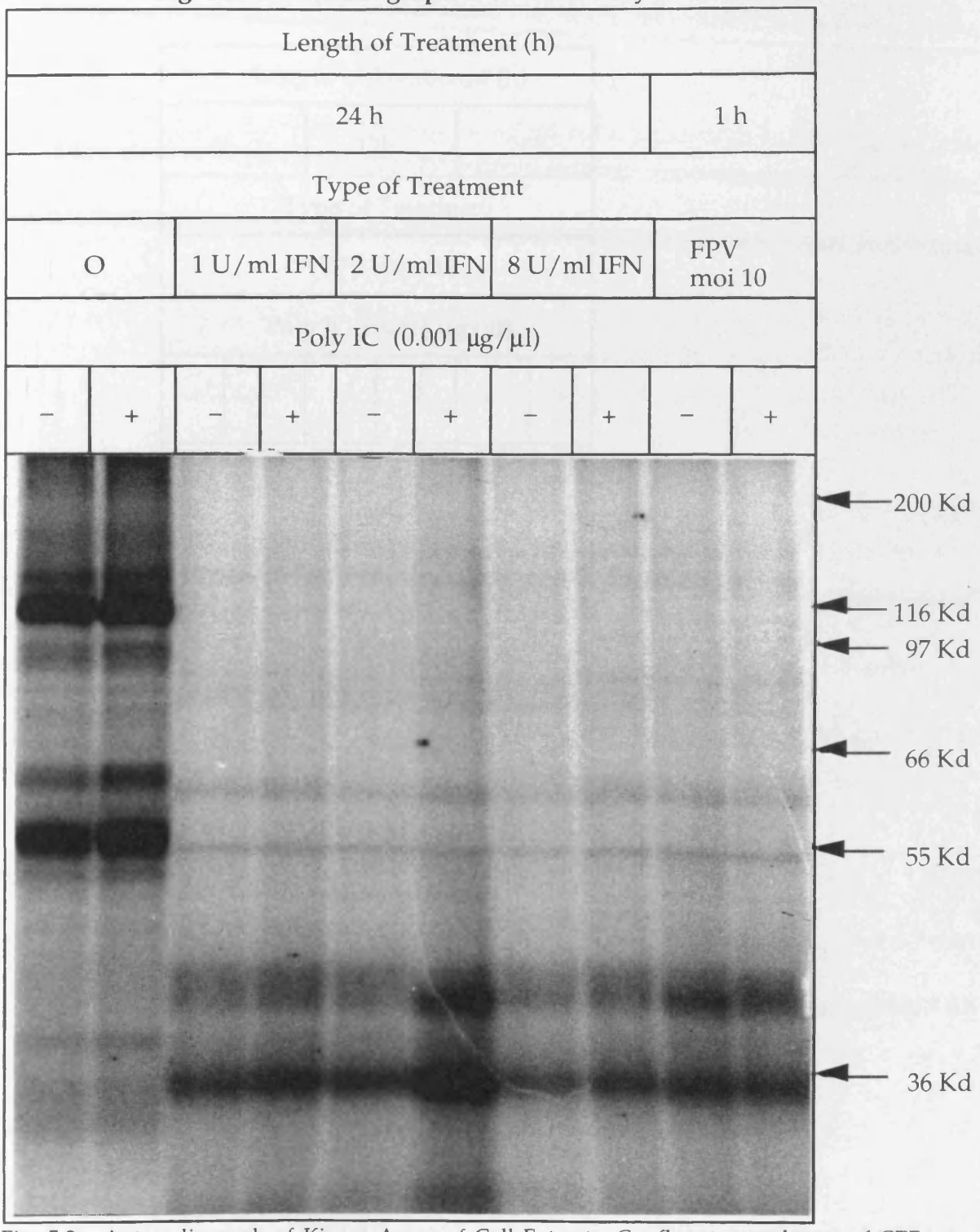


Fig. 5.2a. Autoradiograph of Kinase Assay of Cell Extracts. Confluent monolayers of CEF were treated with IFN (range of concentrations), or infected with FPV (moi of 5-10) and incubated at 37 ^{o}C, 5 % CO_2 for the times shown. Cell extracts were prepared as in 2.3.1. Extracts (8 µl) were incubated at 30 ^{o}C for 15 minutes in the presence (+) or absence (-) of 1 ng/µl poly IC with 100 µM cold ATP, 0.37 MBq ^{32}P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 10 % SDS-PAGE, which was Coomassie blue stained, dried and exposed to film.

Fig. 5.2b. Autoradiograph of Kinase Assay of Cell Extracts

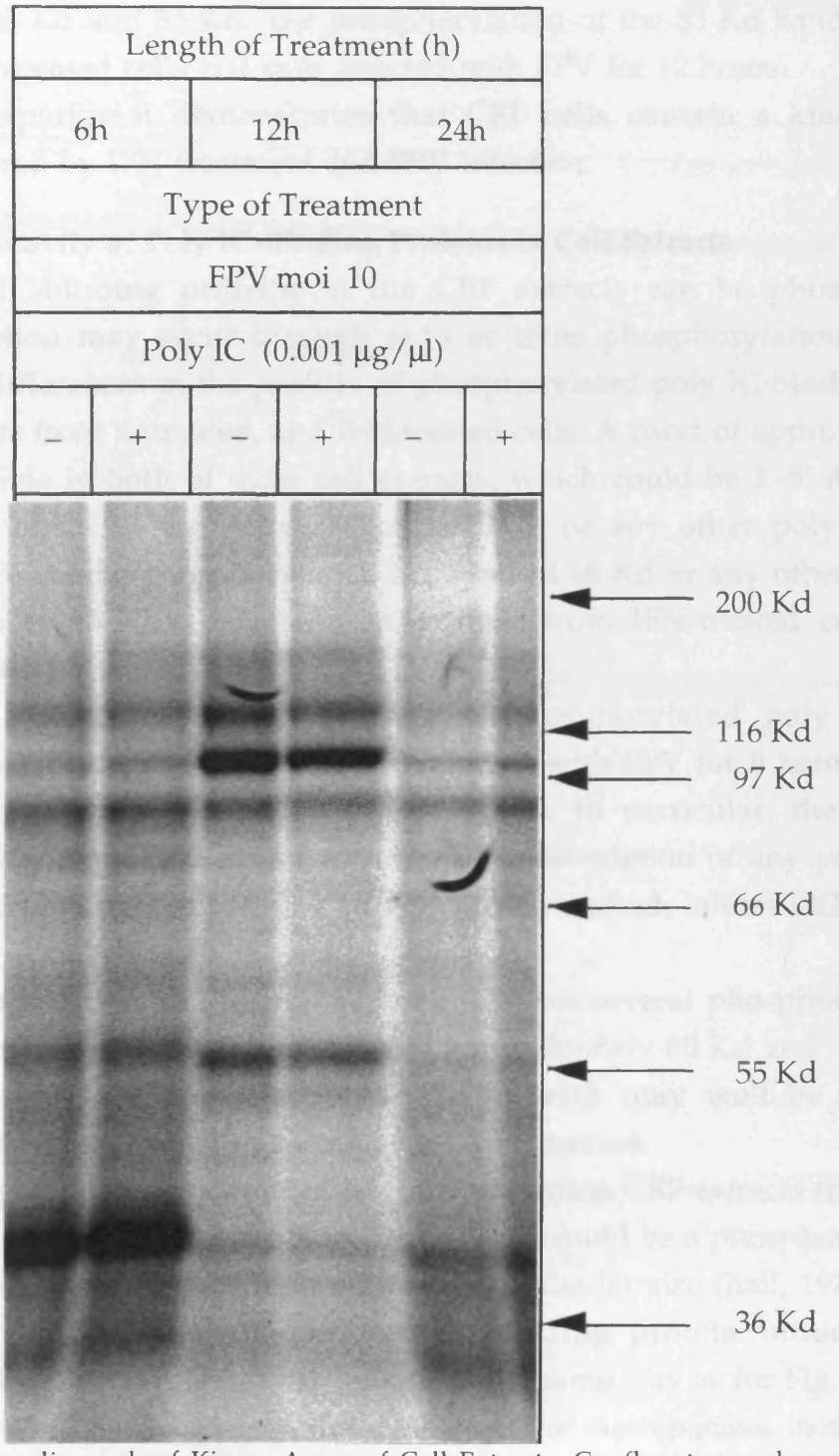


Fig. 5.2b. Autoradiograph of Kinase Assay of Cell Extracts. Confluent monolayers of CEF were infected with FPV (moi of 5-10) and incubated at 37 ^{o}C, 5 % CO_2 for the times shown. Cell extracts were prepared as in 2.3.1. Extracts (8 µl) were incubated at 30 ^{o}C for 15 minutes in the presence (+) or absence (-) of 1 ng/µl poly IC with 100 µM cold ATP, 0.37 MBq ^{32}P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 10 % SDS-PAGE, which was Coomassie blue stained, dried and exposed to film.

CEF infected with FPV for 24 hours contain phosphoproteins of 120 Kd, 100 Kd, 80 Kd, 40 Kd and 35 Kd. The phosphorylation of the 55 Kd band is reduced relative to untreated cells and cells infected with FPV for 12 hours.

The experiment demonstrates that CEF cells contain a kinase activity which is altered by IFN treatment and FPV infection.

5.3. Kinase Activity of Poly IC-Binding Proteins in Cell Extracts

Poly IC-binding proteins in the CEF extracts can be phosphorylated. Phosphorylation may occur through auto or trans phosphorylation. There are few visible differences in the profiles of phosphorylated poly IC-binding proteins in the extracts from untreated, and IFN-treated cells. A band of approximately 53-57 Kd is visible in both of these cell extracts, which could be 2'-5' A synthetase (Ball, 1979) which is also activated by dsRNA, or any other poly IC-binding protein which can be phosphorylated. No band of 68 Kd or any other size which could be an avian PKR, is visible in extracts from IFN-treated cells but not untreated cells.

There are no visible differences in phosphorylated poly IC-binding proteins in samples prepared from CEF infected with FPV for 8 hours and those prepared from IFN-treated or non-treated cells. In particular, there does not appear to be a significant decrease in the phosphorylation of any protein band, which would indicate the presence of FPV proteins which inhibit PKR or another kinase.

CEF infected with FPV for 26 hours contain several phosphorylated poly IC-binding proteins, including proteins of approximately 60 Kd and 66 Kd which are not present in other samples. These proteins may well be part of the replication machinery of FPV, such as RNA polymerase.

A poly IC-binding protein of 55 Kd is present in CEF extracts treated with 2 U/ml IFN and 8 U/ml IFN, Fig 5.3b. This protein could be a phosphorylated form of 2'-5'A synthetase which is reported to be of a similar size (Ball, 1979), or could represent avian PKR or another poly IC-binding protein induced by IFN treatment. The experiment was carried out in the same way as for Fig. 5.3a but the cell extracts were produced on a different day. The discrepancies in the results of Figs. 5.3a and 5.3b could be due to differences in the CEF, differences in the preparation of the cell extracts or differences in the washing of the poly IC agarose beads.

Fig. 5.3a. Autoradiograph to Show Kinase Activity of Poly IC-Binding Proteins from Cell Extracts

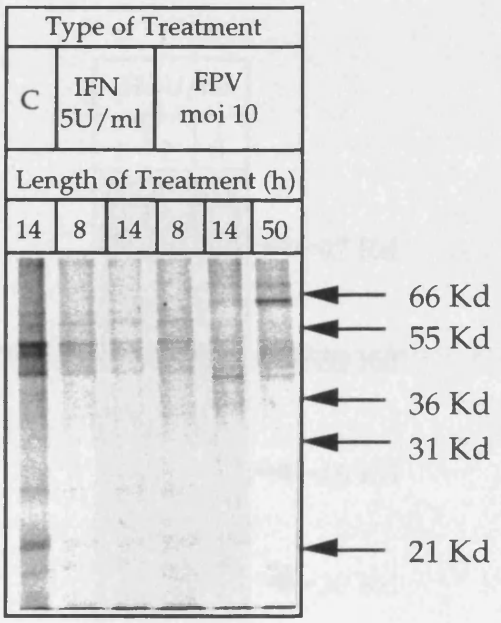


Fig. 5.3a. Autoradiograph to Show Kinase Activity of Poly IC-Binding Proteins from Cell Extracts

Poly IC agarose beads (25 µl) were washed three times in 500 µl of binding buffer. The poly IC agarose beads were resuspended in 25 µl of binding buffer, mixed with 25 µl of the cell extract and incubated at 30 ^{o}C for 30 min with occasional mixing.

The beads were washed twice with wash buffer, once with binding buffer and were resuspended in 21 µl of binding buffer. Poly IC agarose-bound cell extracts (7 µl)were incubated at 30 ^{o}C for 15 minutes with 100 µM cold ATP, 0.37 MBq 32P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 12 % SDS-PAGE, which was Coomassie blue stained, dried and exposed to film.

Fig. 5.3b. Autoradiograph to Show Kinase Activity of Poly IC-Binding Proteins from Cell Extracts

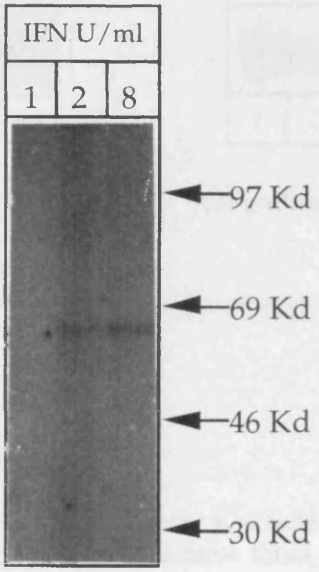


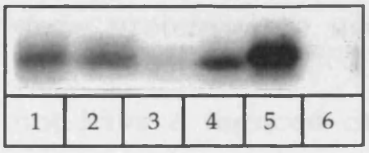
Fig. 5.3b. Autoradiograph to Show Kinase Activity of Poly IC-Binding Proteins from Cell Extracts

Cells were treated with IFN (range of concentration) for 12 h. Poly IC agarose beads (25 µl) were washed three times in 500 µl of binding buffer. The poly IC agarose beads were resuspended in 25 µl of binding buffer, mixed with 25 µl of the cell extract and incubated at 30 ^{o}C for 30 min with occasional mixing.

The beads were washed twice with wash buffer, once with binding buffer and were resuspended in 21 µl of binding buffer. Poly IC agarose-bound cell extracts (7 µl) were incubated at 30 ^{o}C for 15 minutes with 100 µM cold ATP, 0.37 MBq 32P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 10 % SDS-PAGE, which was Coomassie blue stained, dried and exposed to film.

Fig. 5.4. Phosphorylation of Histone Proteins by Poly IC-Binding Proteins

Coomassie Stain of Phosphorylated Histones



Autoradiograph of Phosphorylated Histones

Fig. 5.4 . Phosphorylation of Histone Proteins by Poly IC-Binding Proteins

Poly IC agarose beads (25 µl) were washed three times in 500 µl of binding buffer. The poly IC agarose beads were resuspended in 25 µl of binding buffer, mixed with 25 µl of the cell extract and incubated at 30 ^{o}C for 30 min with occasional mixing. The beads were washed twice with wash buffer, once with binding buffer and were resuspended in 21 µl of binding buffer. Poly IC agarose-bound cell extracts (7 µl) were incubated at 30 ^{o}C for 15 minutes with 0.2 µg/µl calf thymus histone proteins, 100 µM cold ATP, 0.37 MBq ^{32}P dATP (6000 Ci/mmol), 10 mM magnesium acetate. The samples were analysed by 12 % SDS-PAGE, which was Coomassie blue stained, dried and exposed to film.

Cell extracts from:

Lane 1 Control (medium only) 12 h
Lane 2 1 U/ml IFN 12 h
Lane 3 8 U/ml IFN 12h
Lane 4 FPV (moi 10) for 12h
Lane 5 FPV (moi 10) for 24h
Lane 6 No cell extract

5.4. Ability of Poly IC-Binding Kinase(s) to Phosphorylate Histone Proteins

The poly IC-binding proteins in samples from control, IFN-treated and FPV infected CEF, were able to phosphorylate calf thymus histones, Fig 5.4. Cells treated with 8 U/ml of IFN for 12 h appear to have a reduced ability to phosphorylate histones. There was no apparent increase in ability to phosphorylate histones by samples from IFN-treated cells. The differences in the levels of histone phosphorylation could be due to slight experimental inconsistencies, such as in the washing of the poly IC agarose, or in the levels of the kinase(s) responsible for the phosphorylation. Coomassie blue staining indicates that similar concentrations of histone protein were used in each sample.

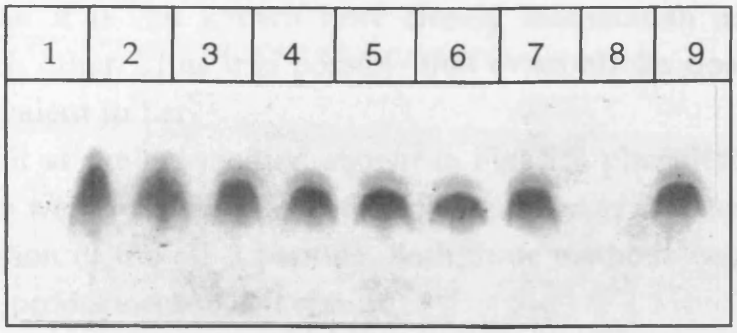
Extracts from FPV infected CEF do not have a reduced capability to phosphorylate histone proteins. In fact, cells infected with FPV for 24 h appear to have an increased ability to phosphorylate histones compared to the control. Thus, the presence of FPV proteins, such as a homologue of E3L which can inhibit activation of PKR (Beattie *et al.*, 1995a, Davies *et al.*, 1993), may be questionable. It is also possible that the phosphorylation seen in Fig. 5.4 is due to the presence of kinase(s) other than PKR, which are able to bind poly IC and phosphorylate histones. Although at present there is no other known dsRNA-activated kinase, there is another kinase which uses eIF-2α as substrate, the heme-regulated eIF-2 alpha kinase , HRI (Chen & London, 1995, De-Haro *et al.*, 1985).

5.5. Ability of Poly IC-Binding Kinase(s) to Phosphorylate Mammalian eIF-2α Peptide

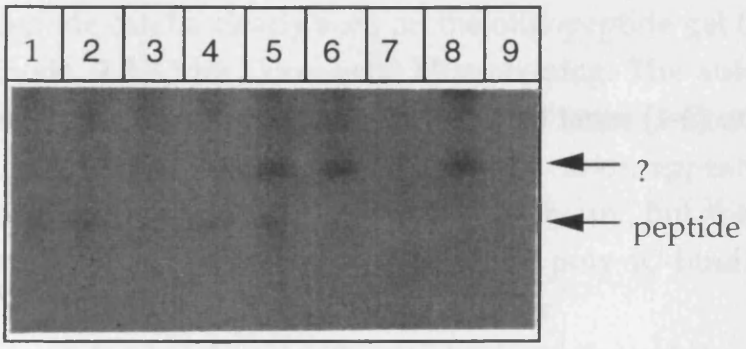
5.5.1. Mammalian eIF-2α Peptides

Peptides of mammalian eIF-2α were used in this experiment as the chicken eIF-2α has not yet been cloned or sequenced. The peptides used were similar to those described by Mellor and Proud (1991), and were obtained from Professor M. J. Clemens, Division of Biochemistry, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School. Both the substrate peptide (NIEGMILLSELSRRRIRSIN) and the pseudo-substrate peptide in which Ser^{51} was replaced with Ala (NIEGMILLSELARRRIRSIN), were phosphorylated by poly IC-binding proteins from cell extracts (data not shown). Both substrate and pseudo-substrate peptides contain serine residues other than Ser^{51} and so may be phosphorylated by any Ser/Thr kinase.

Fig. 5.5. Phosphorylation of Mammalian eIF-2α Peptide by Poly IC-Binding Proteins



Coomasie Stain of Peptide Gel



Autoradiograph of Peptide Gel

Fig. 5.5. Phosphorylation of Mammalian eIF-2α Peptide by Poly IC-Binding Proteins

Poly IC agarose beads (25 µl) were washed three times in 500 µl of binding buffer. The poly IC agarose beads were resuspended in 25 µl of binding buffer, mixed with 25 µl of the cell extract and incubated at 30 ^{o}C for 30 min with occasional mixing. The beads were washed twice with wash buffer, once with binding buffer and were resuspended in 21 µl of binding buffer. Poly IC agarose bound cell extract (5 µl) was mixed with peptide buffer, 3 µCi ^{32}P dATP (5,000 Ci/mmol, DuPont), and 2 µM of substrate peptide in a final volume of 10 µl, and incubated at 30 ^{o}C for 15 min as in 2.3.6.

The samples were mixed with an equal volume of peptide sample buffer, boiled and run on a 15 % oligopeptide gel as in 2.3.7 (Swank & Munkres, 1971). The gel was Coomassie blue stained, sealed in polythene sheeting and exposed to film.

Lane 1 Extract from untreated cells 16 h
Lane 2 Extract from cells treated with 2.6 U/ml IFN for 16h
Lane 3 Extract from cells infected with FPV (moi 10) for 16 h
Lane 4 Extract from cells treated with 1.3 U/ml IFN 16 h
Lane 5 Extract from cells infected with FPV (moi 10) for 24h
Lane 6 Extract from cells infected with FPV (moi 10) for 53 h
Lane 7 No cell extract
Lane 8 No peptide, extract from cells treated with 2.6 U/ml IFN 16h
Lane 9 No ATP, extract from cells treated with 2.6 U/ml IFN 16h

It is possible that avian PKR phosphorylates a different site of the mammalian eIF-2α peptides, and not Ser^{51}. The chicken eIF-2α has not yet been cloned and sequenced, so it is not known how closely mammalian and chicken eIF-2α resemble each other. Thus it is possible that avian eIF-2α does not have a serine residue equivalent to Ser^{51}.

As well as the gel method shown in Fig. 5.5, phosphocellulose paper and spin columns were used with a scintillation counter in an attempt to quantify the phosphorylation of the eIF-2 peptide. Both these methods were unsatisfactory as they did not produce consistent results.

5.5.2. Assay for Phosphorylation of Mammalian eIF-2α Peptides by Poly IC-Binding Kinase(s)

The eIF-2α peptide can be clearly seen on the oligopeptide gel (described in Materials and Methods, 2.3.7.) by Coomassie blue staining. The autoradiograph shows bands representing phosphorylated peptide in all lanes (1-6) other than the controls (lanes 7, 8, and 9). The intensity of these bands varies, appearing stronger in samples from cells infected with FPV for 24 and 53 hours, but the intensity is relatively low in all lanes. All the samples contained poly IC-binding proteins capable of phosphorylation of the eIF-2 substrate peptide.

In lanes 5, 6, and 8 a band is clearly visible above that of the peptide and may be present at a lower intensity in other lanes, as is the even higher molecular weight band which can be seen in lane 1-6 and 8. Lane 8 is a control without peptide and the band is still present and as intense as in lanes 5 and 6 (extracts from FPV infected CEF at 24 hours and 53 hours pi). The bands of higher molecular weight than the peptide are not present in the absence of cell extract or ATP (lanes 7 and 9). It is possible that these band represents 2'-5' linked units of labelled ATP which have been produced by 2'-5'A synthetase present in the cell extracts, although experiments to test this hypothesis were not carried out. It is possible that the bands of higher molecular weight than the peptide were responsible for the inconsistencies in scintillation counting that arose when phosphocellulose paper and spin columns were used.

Fig. 5.6. Autoradiograph of Radio Immuno Precipitation Assay with PKR Monoclonal

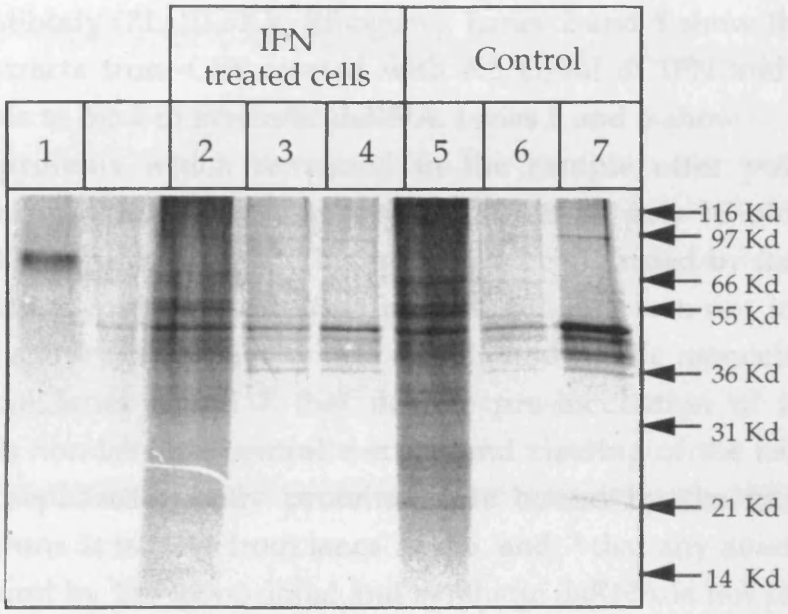


Fig. 5.6. Autoradiograph of Radio Immuno Precipitation Assay with PKR Monoclonal

The pre-cleared ^{35}S labelled cell extracts were mixed with the sepharose-bound monoclonal (71/10 PKR, Ribogene) at 4 ^{o}C for 3h. The sepharose was washed five times with RIPA I buffer and twice with RIPA II buffer. The samples were analysed by 12 % SDS PAGE, alongside poly IC agarose bound and non bound proteins from the same cell lysate.

Lane 1	RIPA with human PKR (*in vitro* translated)
Lanes 2+5	Poly IC agarose bound proteins
Lanes 3+6	Non poly IC agarose bound proteins
Lanes 4+7	RIPA
Lanes 2-4	CEF treated 5.3 U/ml IFN 14 h
Lanes 5-7	Control, CEF treated medium 14 h

5.6. Binding of Cell Extract Proteins to a Monoclonal Antibody which Recognises Human PKR

The monoclonal antibody used was 71/10 against PKR (Ribogene). The antibody was originally produced by Laurent *et al* (1985).

Fig. 5.6, lane 1 shows that *in vitro* translated human PKR was bound by the monoclonal antibody (71/10 PKR, Ribogene). Lanes 2 and 5 show the ^{35}S labelled proteins in extracts from CEF treated with 5.3 U/ml of IFN and control cells, which were able to bind to synthetic dsRNA. Lanes 3 and 6 show the labelled proteins which remained in the sample after poly IC-binding proteins had been removed. It is apparent that not all poly IC-binding proteins were removed using this process. This may have been caused by insufficient poly IC, alternatively it could indicate that poly IC binding was not totally specific. Lanes 4 and 7 show the proteins which were bound by the monoclonal antibody. It is clear from lanes 4 and 7 that despite pre-incubation of the protein A sepharose with non-labelled control extracts and clearing of the labelled extracts with poly A sepharose, many proteins were bound by the monoclonal and sepharose mixture. It is clear from lanes 3, 4, 6, and 7 that any avian PKR present which was bound by the monoclonal and synthetic dsRNA is not present in large quantities, as bands of the expected size (68 Kd) are very weak in intensity. It is possible that avian PKR is not recognised by the monoclonal antibody which binds the N-terminus of human PKR, although the monoclonal antibody is reported to be able to bind to plant PKR (Langland *et al.*, 1995).

5.7. Discussion

Evidence has been shown here that avian cell extracts contain proteins which are capable of phosphorylation, and that different proteins are phosphorylated by extracts from control and IFN-treated CEF. These kinase activities appear to be unaffected by the presence or absence of the synthetic dsRNA, poly IC.

Extracts from CEF infected with FPV for 1 hour showed the same phosphorylation profile as extracts from IFN-treated cells. Extracts from CEF infected with FPV for 12 hours had a profile of phosphorylated proteins similar to extracts from untreated, non-infected CEF. Thus it appears that FPV induces IFN or the effector pathways of IFN within 1 hour of infection. By 12 hours of

FPV infection the virus either no longer induces these pathways or is able to overcome the effects of IFN or the pathways induced by IFN.

The poly IC-binding proteins from cell extracts were partially purified by binding to poly IC agarose. The poly IC-bound proteins were capable of phosphorylation, phosphorylation of histone proteins and phosphorylation of mammalian eIF-2α peptides. Although these activities suggest the presence of a protein(s) with properties similar to PKR, the protein(s) responsible for these activities could not be identified. The activities associated with PKR did not seem to be reduced in extracts from FPV infected CEF. Thus extracts from FPV infected CEF do not appear to contain inhibitors of PKR, such as E3L and K3L. It has been reported that the product of VV E3L accumulates to its highest levels at 5h post-infection (Watson *et al.*, 1991), although a more recent study found that E3L product was expressed 0.5-4 hour post infection and K3L was expressed 0.5-3 hours post infection (Beattie *et al.*, 1995b). Extracts were obtained from FPV CEF at later times post-infection to take into account the slower growth rate of FPV. It is possible that inhibition of PKR was not seen in extracts from FPV infected CEF because the extracts were taken when inhibitor was not produced.

Many proteins bound by poly IC were phosphorylated in all extracts. A poly IC-binding protein of 55 Kd was seen in the extracts from IFN-treated avian cells. It is possible that this is the 55 Kd avian protein identified by Ball (1979) as co-purifying with 2'-5'A synthetase activity. Bands were also seen on an oligopeptide gel (details given in 2.3.7) which may have been 2'-5' linked oligomers of ATP produced by 2'-5'A synthetase, although this possibility was not tested. PKR is known to be ribosome associated in mammalian cells (Samuel, 1979), whereas 2'-5'A synthetase activity is located in the post-ribosomal supernatant fraction of chicken cells (Ball, 1979). Thus it would be possible to separate the two proteins by fractionation. However, this was not performed on the cell extracts used in these experiments as there was some concern that protease activity could affect the results.

Rice and Kerr (1984) performed kinase assays in the presence and absence of dsRNA on HeLa cell extracts obtained by the same method as used here. Phosphorylation of the 69 Kd human PKR was clearly visible in extracts from control and IFN-treated cells when dsRNA was present. PKR was also visible when recovered from the cell extracts by binding to poly IC cellulose. The results shown here, coupled with the information above suggests that if there is an

avian equivalent of PKR, as is suggested by the phosphorylation results, it is less abundant in CEF extracts than is the human protein in HeLa cell extracts.

Roberts *et al* (1976b) used the post mitochondrial supernatant fractions from mouse L-cells to demonstrate phosphorylation of histones by PKR. The phosphorylation of the histones was greatest when incubated with dsRNA and cell extracts from IFN-treated cells. I have tried to replicate this experiment in avian cells and found that there was no visible difference in the phosphorylation of histones by extracts from IFN-treated and non-treated cells which could not be accounted for by inconsistencies in the loading of the gel.

It has been reported that avian PKR phosphorylates two proteins, of 67 Kd and 38 Kd (Ball, 1979). However the data to support this was not published. On an evolutionary scale, birds are more closely related to reptiles and amphibian than mammals (Davison *et al.*, 1996). One study of the 2'-5'A system and PKR in animals, plants and lower organisms found that PKR was not detectable in any of the tested reptilian, amphibian or plant extracts (Cayley *et al.*, 1982). However a plant homologue of PKR (Langland *et al.*, 1995, Langland *et al.*, 1996a, Langland *et al.*, 1996b) has since been described. Thus it is possible (although unlikely) that avian cells do not contain a protein homologous to PKR. A more realistic scenario is that any avian homologue of PKR differs from the human protein at the amino acid level as do the chicken IFNs (Digby & Lowenthal, 1995, Sekellick *et al.*, 1994). Another, equally valid alternative, is that avian PKR is not involved in the IFN response and is therefore regulated differently.

In conclusion, CEF contain a kinase(s) with the ability to bind poly IC, phosphorylate histones and phosphorylate a peptide of mammalian eIF-2α. The activity of the kinase(s) was not increased by addition of poly IC nor was the kinase(s) induced by IFN-treatment of the cells or immunoprecipitated with a PKR monoclonal. Thus, it was not possible to determine whether or not the kinase activities seen in the experiments is an avian PKR. Nor is it therefore possible to determine whether or not FPV expresses inhibitors of PKR, such as homologues of VV E3L and K3L.

Chapter 6

Probing for an Avian Homologue of PKR by Hybridization and PCR

6. PROBING FOR AN AVIAN HOMOLOGUE OF PKR BY HYBRIDIZATION AND PCR

6.1. PCR of a Chicken Lung cDNA Library

A lambda gtll 5' stretch cDNA library of chicken lung (purchased from Clontech, CL1014b), was used in these experiments. The library of chicken lung cDNA was chosen because, of the few libraries available, it was considered the most likely to contain significant levels of PKR cDNA.

PCR was performed on the cDNA library, using degenerate primers designed to amino acid sequences of functional domains conserved between human and mouse PKR (Feng *et al.*, 1992). These include domains such as the RNA binding domain and the eIF-2α binding domain. Conservation between human and murine PKR and functional domains are shown in Fig. 6.1. The degeneracy of the primers was designed to take into account both the degeneracy of the genetic code and the preferred codon usage of chickens.

Table 6.1. Oligonucleotide Primers for PCR of the cDNA Library

Primer	Sequence 5'-3'	Position
LP1	TRTTYATYCARAT GGARTTYTGYGA	361-368 aa human PKR (LFIQMEFC), eIF-2α binding domain
LP2	TCRCARAAYTCCA TYTGRWKAA	complementary to 362-369 aa human PKR (FIQMEFCD), eIF-2α binding domain
LP3	GGYTTSARRTCNC KRCGRAT	complementary to 410-417 aa human PKR (IHRDLKP), kinase domain VI
LP4	GTBAAYTAYGAR CARTGYG	116-122 aa human PKR (VNYEQCA), RNA binding domain II

IUB Group Codes For Degeneracy:
R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, V=G+A+C, N=A+C+G+T

The amino acid sequence and domain structure of human PKR are shown in Fig. 6.2. The positions of sequences used to design primers are also indicated. The primers were used in pairs:

Primer pair A = LP1 + LP3

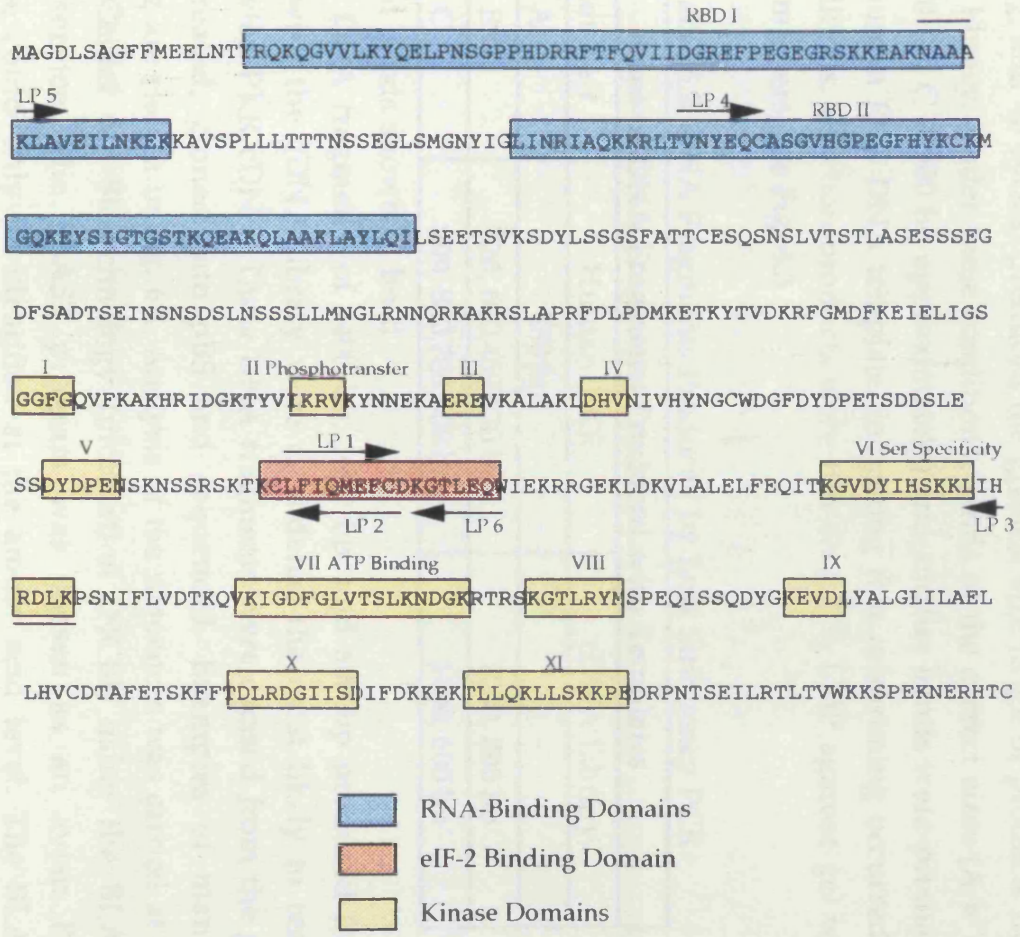
Primer pair B = LP2 + LP4

Primer pair C = LP3 + LP4

Fig. 6.1. Diagram to Show Domains of Human and Murine PKR

Fig. 6.1 Diagram to Show Domains of Human and Murine PKR
Alignment of the amino acid sequences of human and murine PKR is taken from Feng *et al*, 1992. Kinase domains shown are described by Hanks *et al*, 1988 and Meurs *et al* 1990. RNA-binding domains are described by Green and Mathews, 1992.

Fig. 6.2. Diagram to Show Domains of Human PKR and Amino Acid Sequences Used for Primer Design



Under stringent conditions (45-50 ^{o}C annealing temperature) all three primer pairs produced DNA fragments of the correct/expected sizes (A = 180 bp, B= 700 bp. C = 900 bp approximately) when used with the human PKR plasmid template. Under the same conditions but with the cDNA library as template, primer pairs B and C produced very few DNA fragments which were all of small sizes (400-500 bp).

Low stringency conditions (with an annealing temperature of 35 ^{o}C, 1 mM $MgCl_2$, and 40 cycles) produced the greatest size range of products from the cDNA library. Under these conditions, bands of the correct sizes (A = 180 bp, B = 700 bp, C = 900 bp approximately) and smaller bands were produced with the human PKR DNA template, indicating that mispriming occurred in these conditions. Reaction products were run on a 2 % LMP agarose gel with DNA size markers, see Fig. 6.3.

Table 6.2. DNA Fragments Produced by Low Stringency PCR

Sizes of DNA Fragments Produced with Templates		
Primer Pair	Human PKR	cDNA Library
A	**180** bp	-
B	**720**, 600,400,230 bp	**1000, 800** bp
C	**900**, 800,700,500 bp	**1400**, 600 bp

* Dominant bands shown in **bold**

The DNA fragments of 1400 bp, 1000 bp, and 800 bp produced from reactions with the cDNA library were considered the most likely to result from an avian PKR cDNA. These DNA fragments were excised from the gel, GELase treated, cloned into pBS and sequenced. Examples of manual sequencing are shown in Fig. 6.4. Analysis of the sequences was carried at the National Center for Biotechnology Information (NCBI) using the BLAST network service. The BLAST program was chosen as an avian PKR homologue could only be identified at the amino acid level. The BLAST program translates the query sequence into all six reading frame before comparing the results with sequences in the databases. The results of the analysis of the 1000 bp, 1400 bp and 800 bp clones are shown in Appendix 6.1.

The analysis revealed that none of the cloned sequences showed any similarity to PKR at the amino acid level. The 1000 bp fragment is most likely to be derived from lambda (p03772 in the SwissProt database), having 91 %

Fig. 6.3. The Products of a PCR Reaction using the cDNA Library and Human PKR DNA as Templates

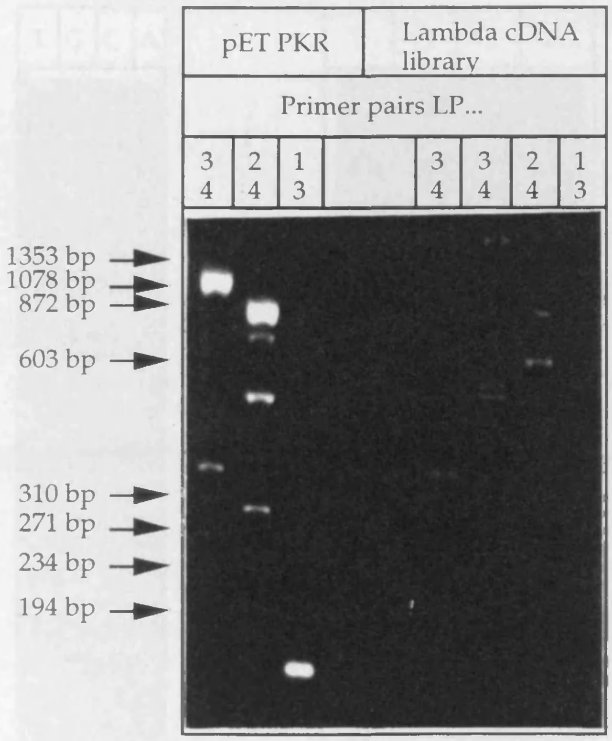


Fig. 6.3. The Products Of A PCR Reaction Using The cDNA Library And pET PKR Control As Templates

PCR reactions were performed in volumes of 25 µl using 0.05 U/µl Taq DNA polymerase (Promega). Reactions contained 2.5 µM each primer, 200 mM each dATP, dCTP, dGTP, dTTP, 1 mM $MgCl_2$, 1 x reaction buffer, 2 µl of the cDNA library and 50 ng PKR template DNA. The reaction mix was overlaid with mineral oil and cycled in the PCR machine. The DNA was amplified by 40 cycles, of denaturation at 92 ^{o}C for 1 min, annealing of the primers to the DNA at 35 ^{o}C for 1 min and elongation at 72 ^{o}C for 1 min. Taq polymerase, 0.05 U/µl was added again at the end of the 20 th cycle. The reactions were held at 72 ^{o}C for 10 min at the end of all the cycles to ensure complete elongation.

Ten µl of each reaction was mixed with loading buffer and electrophoresed on a 2 % agarose gel. The gel was stained with ethidium bromide and visualised by U.V. illumination.

Fig. 6.4. Examples of Autoradiographs Showing Manual DNA Sequencing

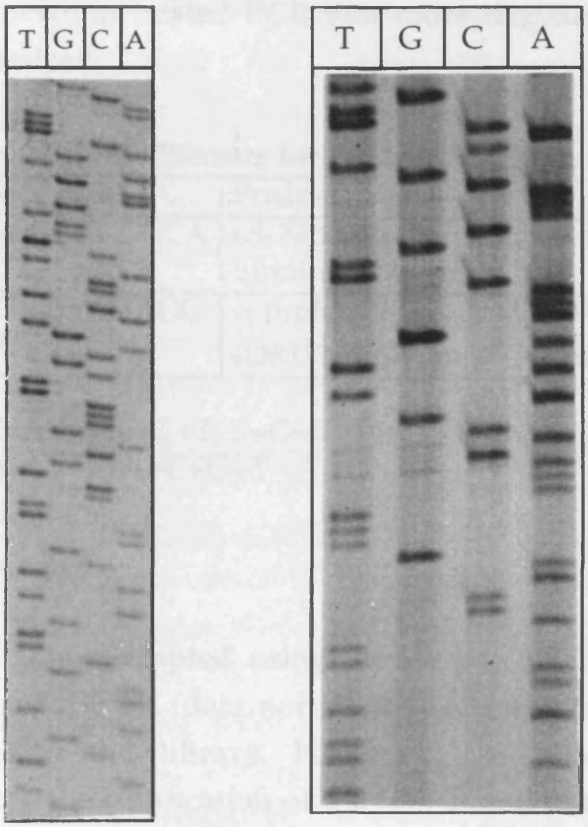


Fig. 6.4. An Example Of Autoradiographs Showing Manual DNA Sequencing

DNA was sequenced using the dideoxy-chain termination method of Sanger *et al*, 1977 and the Sequenase Version 2.0 sequencing kit according to the manufacturer's instructions (Amersham/USB). The reactions were denatured by heating to 80 ^{o}C for 2 min and run on 8 % denaturing polyacrylamide gels Electrophoresis was performed at 37 W for 1-3 h then 0.5 volumes of 3 M NaOAc were added to the lower buffer tank and electrophoresis was continued for a further 1 h. The gels were fixed for 20 min in 10 % acetic acid, 10 % ethanol and dried at 80 ^{o}C under vacuum for 1 h and exposed to film.

amino acid identity with the lambda serine/threonine protein phosphatase over 35 amino acids. The 800 bp fragment appears to be most closely related to a bacterial isoleucine tRNA ligase (SYEXI in the PIR database), with a 53 % amino acid identity over 30 amino acids. The 1400 bp fragment appears to be most closely related to a *Homo sapiens* furin gene, with 40 % amino acid identity over 25 amino acids.

In order to perform nested PCR two more degenerate primers were designed and synthesized.

Table 6.3. Oligonucleotide Primers for Nested PCR of the cDNA Library

Primer	Sequence 5'-3'	Position
LP5	AACGCCGCCGCCA AGCTSGCYGTB	65-72 aa human PKR (NAAAKLAV) RNA binding domain I
LP6	CCACTGCTCCAGG GTSCCYTTRTC	complementary to 369-376 aa human PKR (DKGTLEGW) eIF-2α binding domain

IUB Group Codes:
R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, V=G+A+C, N=A+C+G+T

Primer pair D = LP3 + LP5

Primer pair E = LP4 + LP6

Nested PCR was attempted using primer pair D followed by E, and resulted in a band of 750 bp (data not shown) when used with the human PKR DNA and with the library. However, the possibility of cross-contamination during the purification of the first round products was high so the product was not cloned and sequenced. This was justified by repetitions of the experiment which failed to produce bands when the cDNA library was used as template.

Products of PCR reactions using primer pair C were run on an agarose gel which was blotted onto nylon membrane and probed with radiolabelled LP2 (eIF-2 binding domain). A 900 bp product and larger bands from the reaction with human PKR DNA hybridized to LP2. Two DNA fragments of approximately 900 bp, derived the reaction with the cDNA library, also hybridized to LP2. The larger of the two 900 bp fragments derived from the reaction with the library was judged because of its size to result from cross-contamination with human PKR. However it was decided that the smaller 900 bp fragment could represent avian PKR. Thus, the two 900 bp DNA

fragments from the library reaction were separated, cloned, sequenced and analysed by FASTA of the Wisconsin GCG package.

The larger of the two DNAs (900+) had a 96 % nucleotide identity with human PKR over 118 bp and thus was considered to be human PKR DNA resulting from cross-contamination. A lower level of conservation would be expected for an avian PKR due to differences in codon usage in chickens and humans. The 4 % of non-matching nucleotides may have arisen during PCR with Taq which has a high error rate (1:3000 bases) and no proof-reading mechanism, or could be due to poor sequence reading.

The smaller of the two DNAs (900-) had a 67 % nucleotide identity to *Salmon salar Nhe I* repeat element over 118 bp when analysed at the DNA level. Analysis at the National Center for Biotechnology Information (NCBI) using the BLAST network service revealed that the smaller 900 bp DNA fragment had a low level of amino acid homology with several kinases, see Appendix 6.2. However when the DNA was sequenced in the reverse direction and analysed at the amino acid level, there was no homology to any RNA-binding protein as would be expected if the DNA represented an avian PKR homologue. The match with β-galactosidase (Cloning vector pBBR1MCS-2), lac Z, is present because the sequencing included the end of the cloning vector.

6.2. Screening of a Chicken Lung cDNA Library for PKR

The cDNA library was screened using a succession of probes. These probes included radiolabelled degenerate oligonucleotides, LP4 (RNA binding domain PKR) and LP2 (eIF-2α binding domain PKR), and also chemically labelled (ECL) DNA covering 20-416 aa human PKR and DNA covering of 1-227 aa (the RNA binding domains) of human PKR.

The library was initially plated at a high density so that all clones would be represented. The high density plating prevented picking of individual plaques so initially areas containing many plaques were picked and then replated so that individual plaques could be isolated. Initial probing of the complete library with LP1 (eIF-2α binding domain) and LP4 (RNA binding domain) identified 96 areas containing positive plaques. These plaque areas were picked, DNA was produced and dotted onto a membrane which was then probed with human PKR (20-416 aa PCR product). This identified 4 areas of plaques to be positive. The positive plaque areas were replated and probed

Fig. 6.5. Autoradiograph of Plaques Probed with ECL Labelled Human PKR DNA

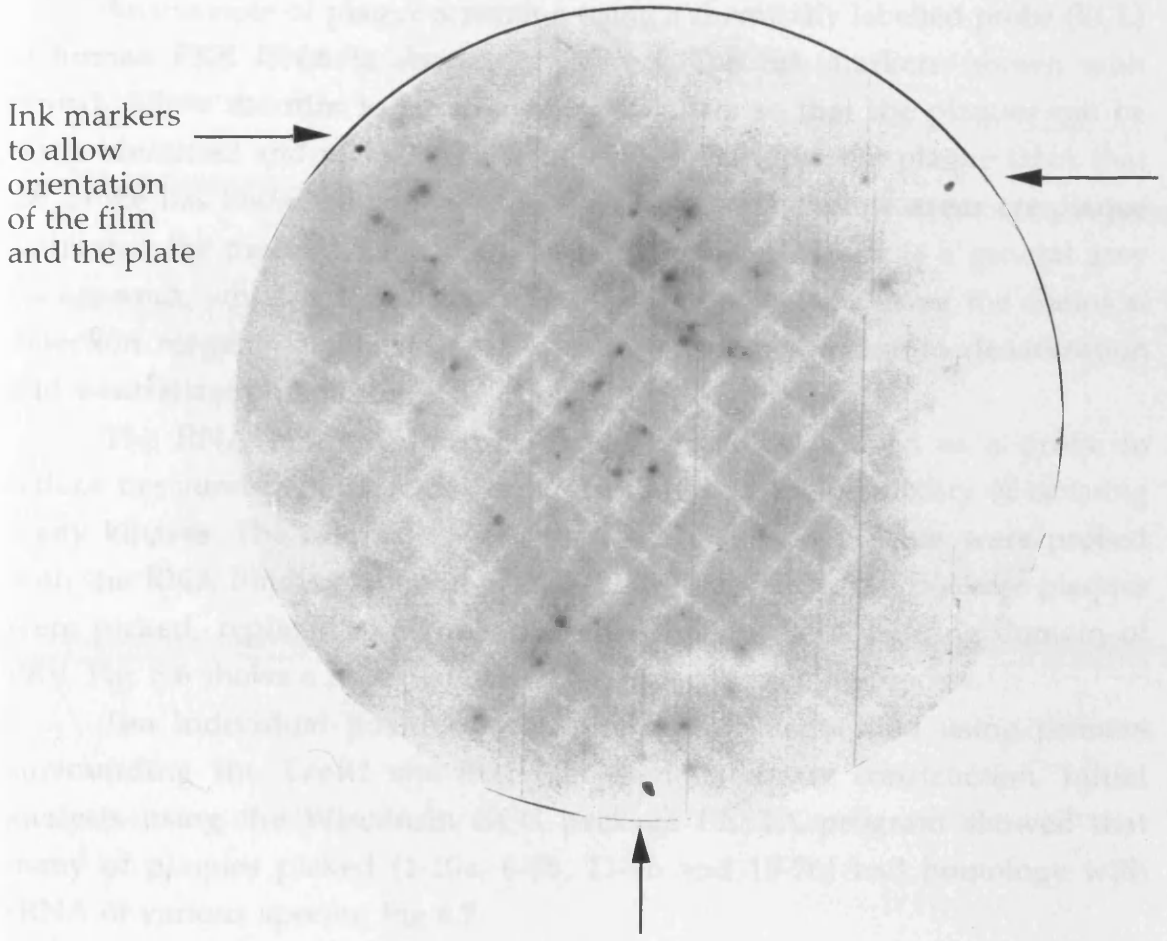


Fig. 6.5. Autoradiograph Of Plaques Probed With Chemically Labelled Human PKR DNA

Plaques were blotted on to nylon membrane. The membrane was soaked in denaturation solution for 5 min, in neutralization solution twice for 3 min, then air-dried and the DNA was covalently bound to the membrane using UV illumination. Hybridization was carried out using the ECL direct nucleic acid labelling and detection systems. The hybridization buffer contained manufacturer's ECL gold buffer, 5 % manufacturer's blocking agent and 0.5 M NaCl which was mixed and incubated at 42 ^{o}C for 1 h before use. The membrane was incubated in the hybridization buffer at 42 ^{o}C in a hybridization oven (Appligene) for at least 30 min before addition of the probe.

The probe was produced using 300 ng of a PCR product of human PKR DNA. The DNA was diluted to 10 ng/µl, denatured by boiling for 5 min and snap cooling on ice for 5 min. The denatured DNA was mixed with one volume of the manufacturer's labelling mix and one volume of glutaraldehyde and incubated at 37 ^{o}C for 10 min. The labelled probe was then added to the hybridization solution and membrane. Hybridization was carried out at 42 ^{o}C for 4-16 h.

After hybridization the membrane was washed twice in 0.4 % SDS, 0.5 x SSC at 55 ^{o}C for 10 min and twice in 2 x SSC at 25 ^{o}C for 5 min. The detection reagents were mixed and placed on the membrane for 1 min. The membrane was blotted with paper towels to remove excess detection fluid, wrapped in saran wrap and exposed to film for 1 min-2 h.

again with human PKR, approximately 90 % of the resulting plaques were positive.

An example of plaque screening using a chemically labelled probe (ECL) of human PKR DNA is shown in Fig. 6.5. The ink markers, shown with arrows, allow the film to be aligned to the plate so that the plaques can be easily identified and picked. The small dark spots represent plaque DNA that the probe has bound, therefore 'positive plaques'. The white areas are plaque DNA that the probe did not bind, 'negative plaques'. There is a general grey background, which may be the result of a long exposure using the chemical detection reagents or due to overexposure of the membrane to denaturation and neutralization solutions.

The RNA binding domain of human PKR was used as a probe to reduce the number of plaques involved and reduce the possibility of isolating many kinases. The original 96 and the PKR-positive 4 plaques were probed with the RNA binding domain of PKR (1-227 aa PKR). Five positive plaques were picked, replated and probed again with the RNA binding domain of PKR. Fig. 6.6 shows a flow diagram of the library screening process.

Ten individual positives were picked and sequenced using primers surrounding the *EcoRI* site that was used in library construction. Initial analysis using the Wisconsin GCG package FASTA program showed that many of plaques picked (1-10a, 6-9b, 11-9b and 15-7b) had homology with rRNA of various species, Fig 6.7.

The sequences were also analysed at the amino acid level at the National Center for Biotechnology Information (NCBI) using the BLAST network service to confirm the results. The most significant hits from BLAST analysis are shown in Appendix 6.3. All of the sequences that showed homology to ribosomal RNAs when analysed at the DNA level, had relatively low homology with any known amino acid sequences in the database and thus are likely to be chicken ribosomal RNA genes. Some sequences had similarity to known kinases when analysed at the amino acid level but in all cases the amino acid conservation was low and there was no homology to any of the PKR proteins. Few of the sequences analysed had homology to any known chicken genes. This probably reflects how few chicken genes are currently known rather than any problem with the library.

Fig. 6.6. Flow Diagram of the Library Screening Process

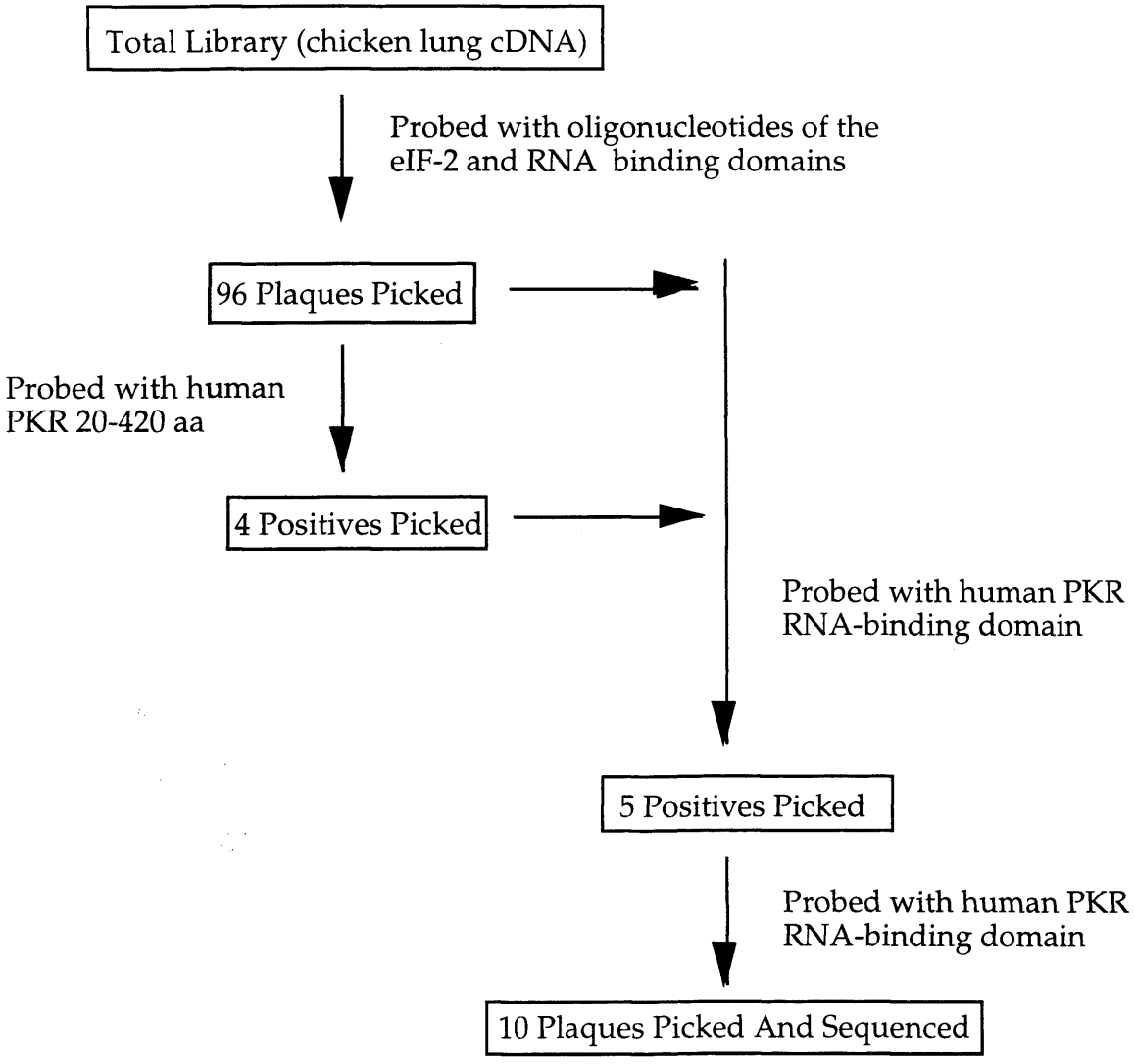


Fig. 6.7. DNA Level Analysis of Plaque Sequences

Plaque	Homology With	% Homology
1-10a Rev	Ribosomal 28S RNA (Gorilla)	92 % /189 bp
6-9b Rev	rRNA (*T. chloroplast*)	60 % /101 bp
11-9b Rev	18S rRNA (*Rhinobatos lentiginous*)	96 % /179 bp
15-7b For	Mitochondrial genome rRNA (*P. anserina*)	94 % /179 bp

Fig. 6.7. DNA Level Analysis Of Plaque Sequences

Plaques picked were grown and the DNA extracted. Manual sequencing was carried out and the sequences analysed by the FASTA program of the Wisconsin package, Genetics Computer Group, Inc. Only the highest homology for each sequence is shown here.

6.3. Northern Blot of Poly A RNA from IFN-Treated CEF and Total RNA from U937 Cells Probed with Human PKR DNA

Messenger RNA was isolated from IFN-treated CEF, approximately 5 μg was run on a denaturing formamide gel, blotted onto nylon membrane and probed with human PKR DNA. Total RNA from human U937 cells (20 μg), a monocyte-like cell line (kindly provide by J. Russell), was added to the gel as a control. A blot of various amounts of human PKR DNA was used as a quality and quantity control for the probe. Human PKR DNA (10 pg) was easily visible after probing and an overnight exposure.

The autoradiographs of a northern blot of poly A RNA from IFN-treated CEF and total RNA from U937 cells, probed with human PKR DNA after a 3 day exposure and a 7 day exposure are shown in Fig. 6.8. After a 3 day exposure of the blot to film no bands are visible in lane 1 (poly A RNA from IFN-treated CEF). A band of approximately 2.5-3.0 Kb is easily visible in lane 2 (total RNA from U937 cells) as are two faint bands of approximately 4.4 and 9.5 Kb.

The 7 day exposure was carried out to ensure that any weak signals in lane 1 could be visualised. After a 7 day exposure of the blot to film, a faint band of between 7.5 and 9.5 Kb is visible in lane 1. The band in lane 1 does not extend across the whole width of the lane and so is likely to be non-specific binding of the probe. Several bands are visible in lane 2 after a 7 day exposure and the band of 2.5-3.0 Kb is the most intense. The other bands are of approximately 10 Kb, 4.4 Kb, 2.1 Kb and 7 Kb. The 4.4 Kb and 2.1 Kb bands probably represent ribosomal RNA which could be seen on ethidium bromide staining and UV illumination of the gel prior to blotting. The 10 Kb band could represent a splicing intermediate or alternatively non-specific binding of the probe, as hybridization was carried out at a low stringency of 35 ^{o}C. Human PKR probes have been reported to bind to two RNA species of 2.5 Kb and approximately 6 Kb (Thomis *et al.*, 1992). Thus it is probable that the bands of 2.5-3.0 Kb and approximately 7 Kb represent mRNA of human PKR.

Fig. 6.8. Autoradiograph of a Northern Blot of CEF Poly A RNA and U937 Total RNA Probed with Human PKR

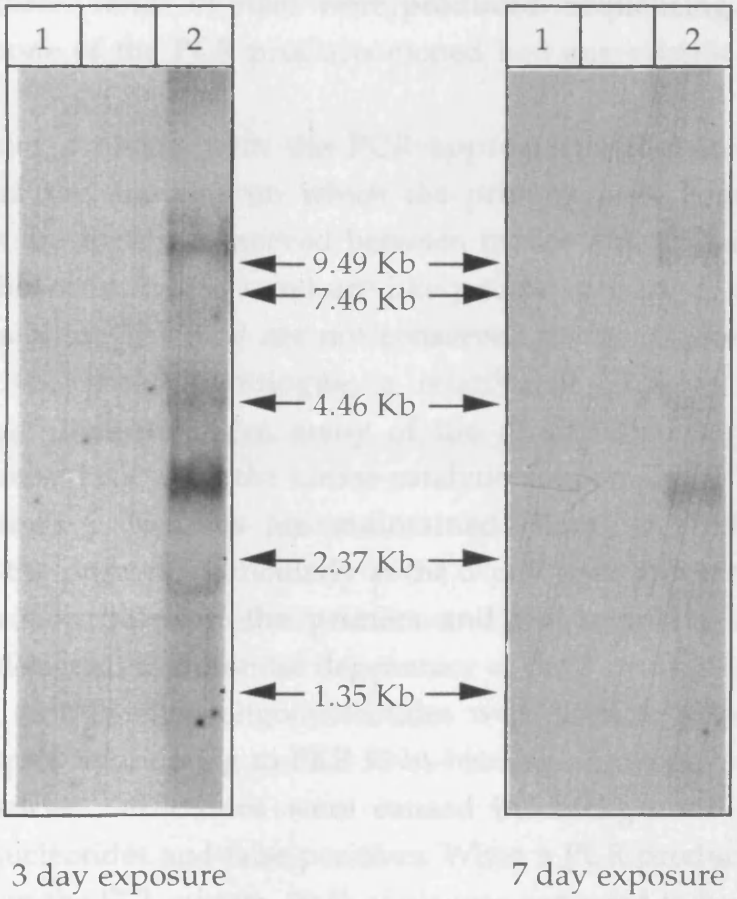


Fig. 6.8. Autoradiograph Of A Northern Blot Of CEF Poly A RNA And U937 Total RNA Probed With Human PKR.

Poly A RNA was purified from interferon treated CEF using the Micro-Fast Track Kit (Invitrogen). Five µg of the RNA was run on a denaturing formamide gel alongside 20 µg of total RNA from human U937 cells. The PKR probe was produced using the Rediprime DNA labelling system (Amersham) and heat denatured at 100 ^{o}C for 5 min. The probe was added to the membrane in the bottle and incubated at 35 ^{o}C overnight.

The membrane was washed in 2 x SSC, 1 % SDS at 35 ^{o}C for 10 min. The washes were carried out three times before the membrane was wrapped in Saran (Dow) and exposed to film.

Lane 1 5 µg poly A RNA from IFN-treated CEF
Lane 2 20 µg total RNA from human U937 cells

6.4. Discussion

PCR conditions of high and low stringency were tested using the cDNA library and human PKR control DNA. It was found that at high temperatures only small DNAs were produced from the library with degenerate primer pair LP3/LP4, and avian PKR was expected to produce DNA of a larger size (as does the human PKR control DNA). Under lower stringency conditions many DNAs of a greater range of sizes were produced. Sequencing and analysis showed that none of the PCR products cloned had any significant similarity with PKR.

The major problem with the PCR approach is that it relies on the conservation of the domains on which the primers were based. Although these domains are highly conserved between mouse and human PKR (Feng *et al.*, 1992) (shown in Fig. 6.1) and are likely to be conserved in avian PKR, there is a possibility that they are not conserved to the degree required for PCR. Indeed the yeast homologue, a relative of PKR, is a functional homologue and does not have many of the domains conserved between human and mouse PKR, only the kinase catalytic subdomains I, II, VI and VII and eIF-2α-binding domains are maintained (Ramirez *et al.*, 1992). The degeneracy of the primers, particularly at the 3' end may prevent a strong and specific interaction between the primers and the template, although the primers were designed to minimise degeneracy at the 3' ends.

Initially, end-labelled oligonucleotides were used to screen the cDNA library for plaques hybridizing to PKR RNA-binding sequences and PKR eIF-2 binding sequences. Difficulties were caused by background due to non-incorporated nucleotides and false positives. When a PCR product of 20-420 aa PKR was used in the ECL system, 90 % of plaques appeared to be positive. This may be because this probe picked up all kinase and RNA binding type sequences, but temperature constraints imposed by the ECL system prevented an increase in stringency. The PKR RNA-binding domain produced by PCR was used to reduce the numbers of plaques involved. It was apparent after analysis that the screening processes undertaken had not removed all false positives. The cDNA library contained rRNA clones, presumably a result of incomplete separation of mRNA from the total RNA and priming with random primers as well as oligo dT (Clontech). The screening process relied heavily on the assumed/hoped for conservation of sequences between PKR and any avian PKR, and as discussed earlier there is a possibility that these sequences may not be conserved to the level required for the methods used.

Analysis of the DNA sequences was used to rule out obvious false positives and known clones. Analysis of the protein sequences translated from the DNA was considered the best method to search for PKR sequence similarities in the cDNA library clones. The highly conserved eIF-2 binding domain indicative of PKR is several hundred bases from the ends of the human PKR gene, and so a lot of sequencing may be required to reach these areas and identify a clone as being avian PKR. None of the plaques picked and sequenced had any similarity to PKR, although complete sequencing was not carried out.

It is possible that any clone expressing functional PKR might be lost during the first plating of the library as a functional PKR might inhibit protein synthesis and the replication of the clone. However, any functional protein produced would have to interact and phosphorylate the *E.coli* specific initiation factor 2 (Singleton & Sainsbury, 1987) to inhibit protein synthesis. Production of a functional protein is unlikely as post-translational modifications might be required to produce a functional protein. Also the library was constructed so that proteins were expressed as part of a β-galactosidase fusion protein (Clontech) which might interfere with the tertiary structure of the protein and so inhibit function.

The expression library could have been screened using a monoclonal recognizing PKR. For this approach to be successful, the epitope of human PKR (N-terminus) recognized by the monoclonal must be conserved in chicken PKR. Although the monoclonal recognises plant PKR (Langland *et al.*, 1995) previous experiments involving the monoclonal antibody and proteins from CEF (see section 4.6) indicated that this approach was unlikely to work on avian PKR.

IFN-α/β are thought to be produced by most cell types, except embryonal carcinoma cells (Harada *et al.*, 1990). A cDNA library of chicken lung (Clonetech), was used in these experiments as it was thought that the lung was likely to be a site of IFN production caused by infection. In retrospect, the choice of a chicken lung cDNA library may have been inappropriate as infection and stimulation of IFN production in the lung tissue used to make the library could not be guaranteed. Chicken IFN has since been cloned from a cDNA library made from primary chick embryo cells (Sekellick *et al.*, 1994). To overcome this potential problem RNA was extracted from IFN-treated CEF and probed with human PKR DNA.

When total RNA from human U937 cells was probed with human PKR DNA, a band of 2.5-3.0 Kb was easily visible after a 3 day exposure. This result is consistent with a previous report of human PKR mRNA species of 2.5 Kb and approximately 6 Kb (Thomis *et al.*, 1992). Thus it is likely that the 2.5-3.0 Kb RNA species seen in Fig. 6.8 represents human PKR mRNA. After a 7 day exposure, bands corresponding to rRNA and other non-specific interactions with human RNA species were visible on the autoradiograph.

No bands were visible after a 3 day exposure of poly A RNA from IFN-treated CEF probed with the human PKR DNA probe, although a faint band of 7.5-9.5 Kb was visible after a 7 day exposure. As the band did not extend the full width of the gel lane, it is thought that the band represents a non-specific interaction of the probe and not an avian PKR RNA species. The size of this band is also different from all other reported PKR transcripts. Murine cells are reported to contain 3 RNA species of 2.5, 4 and 6 Kb, which were identified with human PKR cDNA probes (Icely *et al.*, 1991). Rat PKR mRNA is thought to be 4.4 Kb species (Mellor *et al.*, 1994) and plant PKR mRNA a 2.5 Kb transcript (Langland *et al.*, 1995).

This experiment relied upon conservation at the nucleotide level between human PKR DNA and any avian RNA species. A mammalian PKR cDNA was successfully used to detect the 2.5 Kb mRNA in plants (Langland *et al.*, 1995). However, it is possible that any homologue of PKR in avian cells is a functional homologue, similar to GCN 2 of yeast (Dever *et al.*, 1993, Ramirez *et al.*, 1992, Zhu *et al.*, 1996), and thus avian PKR RNA might not have much similarity to human PKR DNA.

No control was included for the quality of the RNA extracted from CEF due to the small amounts of RNA available after polyA selection. Thus, the experiment might have been better carried out with total RNA from IFN-treated CEF, as then the rRNA species could provide information on the quality of the RNA present. An alternative would be to reprobe the blot with DNA from a known chicken gene.

In conclusion, I have been unable to identify any avian homologue of PKR by PCR of a chicken lung cDNA library, screening of the chicken cDNA library or by probing poly A RNA from IFN-treated CEF with human PKR. It seems likely that DNA sequence conservation between human and avian PKR is too low for an avian PKR to be identified using any of these methods.

Chapter 7

Cloning and Sequencing of a Genomic Segment of Fowlpox Virus DNA which Hybridizes to Vaccinia Virus E3L and K3L DNA

7. CLONING AND SEQUENCING OF A GENOMIC SEGMENT OF FOWLPOX VIRUS DNA WHICH HYBRIDIZES TO VACCINIA VIRUS E3L AND K3L DNA

7.1. PCR of FPV DNA for a FPV Homologue of VV E3L

In VV, E3L exists between the genes E2L and E4L. Random M13 clones of FPV had shown amino acid homology to VV E2L and E4L, and hydridized to the BamHI-I fragment (10 Kbp) of the FPV genome (personal communication, Dr. M. Skinner). It was thought that an FPV homologue of E3L may be present on this DNA fragment, in an analogous location to VV E3L. Accordingly, the areas of homology, shown in Fig. 7.1 were used to design primers to enable PCR of FPV E3L. PCR was carried out on both total FPV DNA and a pBR322 clone of FPV BamHI-I fragment, Fig. 7.2.

A DNA fragment of approximately 500 bp was produced by PCR of both total FPV DNA and pMB 280 DNA (a clone of FPV BamHI-I) with primers designed to FPV homologues of E2L and E4L, Fig. 7.2. From the E2L primer to the start of E2L, there are approximately 190 bp, and from the E4L primer to the end of E4L there are approximately 320 bp, Fig. 7.1. Thus if the FPV homologues of E2L and E4L are the same size as the VV genes, a total of 510 bp of the PCR product only accounts for the E2L and E4L genes. VV E3L is 570 bp. Thus it seems likely that any FPV homologue of VV E3L does not exist between the FPV homologues of E2L and E4L.

The sizes of E2L and E4L genes of FPV are not known. There are approximately 130 bp from the E2L primer to the end of the known region of FPV homology with VV E2L and approximately 145 bp from the E4L primer to the end of the known region of FPV homology with VV E4L, Fig. 7.1. Thus if the FPV homologues of E2L and E4L are only the size of the known homology, a total of 275 bp would account for the E2L and E4L portions of the PCR product, leaving approximately 225 bp which could be an FPV homologue of E3L. A 225 bp E3L gene only allows for an FPV E3L protein of 75 amino acids and the VV protein is 190 amino acids (Goebbel *et al.*, 1990), 43 of which have homology to the RNA-binding domain of PKR and so are

Fig. 7.1. DNA and Amino Acid Sequences of VV E2L, E3L and E4L and Areas of FPV M13 Clone Homology


```
ACA ATA TCA TCT ATA GTT TAG GTT ACC TCG ATG TCA AGA ACC GAA TTT GTC TAT ATC AAA
 T   I   T   S   I   L   D   L   P   A   V   T   R   P   K   F   L   Y   L   K

AAG ACC TTG TTT AAG ATG TTG TAA TAA TAT TTC CTG AAA CCC ATC TAT TCA CCC TAC TTT
 E   P   V   F   E   V   V   N   N   Y   L   V   K   P   L   Y   T   P   H   F

AGG ATA AAA TTA ATT ACG ATA GCG TAA CAG GAG CAC GTT TAT AGG TTT GCG AAA ACA CTA
 G   I   K   I   L   A   I   A   N   D   E   H   L   Y   G   F   A   K   T   I

TCA TAC CGT AAG TAA CAG ATC TTT GCG AGA TGC TTA TAG ACA CTG TCT ATA GTA GAA ATC
 T   H   C   E   N   D   L   F   A   R   R   I   D   T   V   S   I   M   E2L

TCT TAT ATG ATC AGC GCA ATT ATC ATG ATG TTA AAC ATA AAA AAT TAG ATA GAG TTA TTT

TTT TAA TTA TAC ATA CTA AGT TAC ATA TTG ATT TGA TGA TTG ACA ATA ACT ATT GAT CTT

AGT CTT AGA TTA CTA CTG CAT TGG TTC TTC AAA TAG ATG ACG GTT AAA TCG ACG TAA TAA
 *   F   R   I   I   V   Y   G   L   L   K   D   V   A   L   K   A   A   N   N

AAA TCG TAG AGC AAA TCT AAA AGG TAG ACG GAA TAG CTT ATG AGA AGG CAG CTA CAG ATG
 K   A   D   R   K   S   K   G   D   A   K   D   F   V   R   G   D   I   D   V

TGT CCG TAT TTT ACA TCC TCT CAA TGA TCC GGG TTG ACT AAG TTA TGC TTT TCT GGT TAG
 C   A   Y   F   T   P   S   N   S   P   G   V   S   E   I   R   F   S   W   D

AGA GAA TCA ATA AAC CGT CAT GAG TAA TTA TTA CCA CTG TCC CAA TCG TAG AAA GGT TAG
 R   K   T   I   Q   C   Y   E   N   I   I   T   V   P   N   A   D   K   W   D

TTA TTA AAA AAA TCG GCC TTA TTG TAG TAG TTT TCT GAA TAC TAG GAG AGA GTA ACT AAA
 I   I   K   K   A   P   I   V   D   D   F   S   K   H   D   E   R   M   S   K

AAG CGC CCT ATG TAG TAG ATA ATA CTG CAG TCG GTA TCG TAG TCG TAG GCC GAA TAG GCG
 E   R   S   V   D   D   I   I   V   D   A   M   A   D   A   D   P   K   D   A

GAG GCA ACA GTA TTT GGT TGC TCC TCC TTA TAG CAG CCT CGA CAT GTG GTA TCG TGA TGC
 E   T   T   M   F   W   R   P   P   I   D   D   S   S   Y   V   M   A   S   R

AAC TTC TAG CAT GTC TCG AAA TAA TTG AAG AGC GAA GAG GTA TAA TTC AAC AGA TCA ATC
 Q   L   D   Y   L   A   K   N   V   E   R   K   E   M   N   L   Q   R   T   L

AAC ACG TCG TCA TCG AGG AAG CTA AGG TTA CAA AAA TTA TCG GCG TGT GTG TTA GAG ACG
 Q   A   A   T   A   G   E   I   G   I   N   K   I   A   A   C   V   I   E   A

CAG TCT TGC GAG CAG TTA TAT CTA GAA TCT GTA AAA ATC TCT CTT GAT TGT GTT GGT CGT
 D   S   R   E   D   I   Y   I   K   S   M   E3L
```

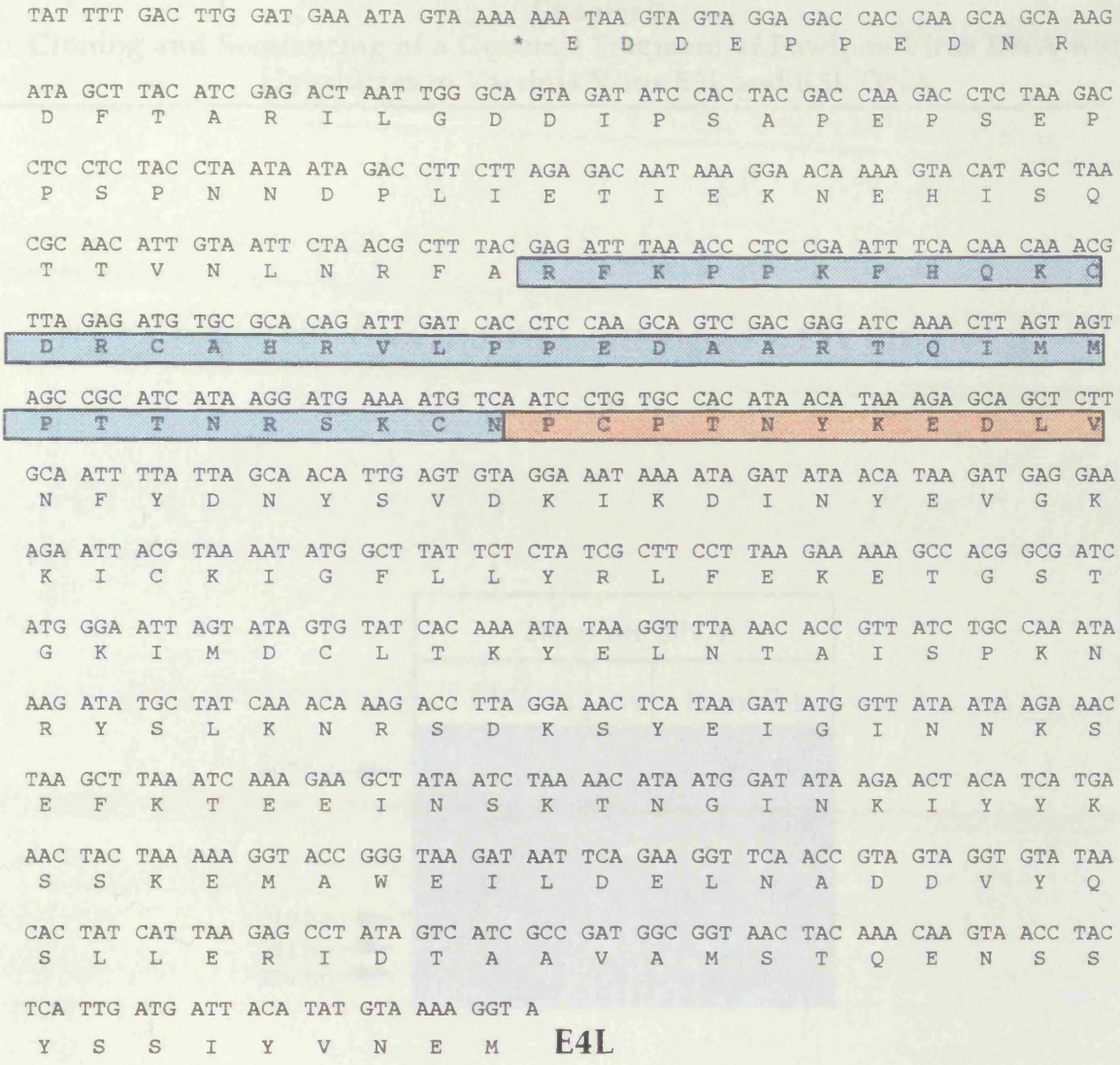

TAT TTT GAC TTG GAT GAA ATA GTA AAA AAA TAA GTA GTA GGA GAC CAC CAA GCA GCA AAG
* E D D E P P E D N R
ATA GCT TAC ATC GAG ACT AAT TGG GCA GTA GAT ATC CAC TAC GAC CAA GAC CTC TAA GAC
D F T A R I L G D D I P S A P E P S E P
CTC CTC TAC CTA ATA ATA GAC CTT CTT AGA GAC AAT AAA GGA ACA AAA GTA CAT AGC TAA
P S P N N D P L I E T I E K N E H I S Q
CGC AAC ATT GTA ATT CTA ACG CTT TAC GAG ATT TAA ACC CTC CGA ATT TCA CAA CAA ACG
T T V N L N R F A R F K P P K F H Q K C
TTA GAG ATG TGC GCA CAG ATT GAT CAC CTC CAA GCA GTC GAC GAG ATC AAA CTT AGT AGT
D R C A H R V L P P E D A A R T Q I M M
AGC CGC ATC ATA AGG ATG AAA ATG TCA ATC CTG TGC CAC ATA ACA TAA AGA GCA GCT CTT
P T T N R S K C N P C P T N Y K E D L V
GCA ATT TTA TTA GCA ACA TTG AGT GTA GGA AAT AAA ATA GAT ATA ACA TAA GAT GAG GAA
N F Y D N Y S V D K I K D I N Y E V G K
AGA ATT ACG TAA AAT ATG GCT TAT TCT CTA TCG CTT CCT TAA GAA AAA GCC ACG GCG ATC
K I C K I G F L L Y R L F E K E T G S T
ATG GGA ATT AGT ATA GTG TAT CAC AAA ATA TAA GGT TTA AAC ACC GTT ATC TGC CAA ATA
G K I M D C L T K Y E L N T A I S P K N
AAG ATA TGC TAT CAA ACA AAG ACC TTA GGA AAC TCA TAA GAT ATG GTT ATA ATA AGA AAC
R Y S L K N R S D K S Y E I G I N N K S
TAA GCT TAA ATC AAA GAA GCT ATA ATC TAA AAC ATA ATG GAT ATA AGA ACT ACA TCA TGA
E F K T E E I N S K T N G I N K I Y Y K
AAC TAC TAA AAA GGT ACC GGG TAA GAT AAT TCA GAA GGT TCA ACC GTA GTA GGT GTA TAA
S S K E M A W E I L D E L N A D D V Y Q
CAC TAT CAT TAA GAG CCT ATA GTC ATC GCC GAT GGC GGT AAC TAC AAA CAA GTA ACC TAC
S L L E R I D T A A V A M S T Q E N S S
TCA TTG ATG ATT ACA TAT GTA AAA GGT A
Y S S I Y V N E M
E4L

Fig. 7.2. PCR of FPV DNA with Primers Designed to FPV E2L and E4L

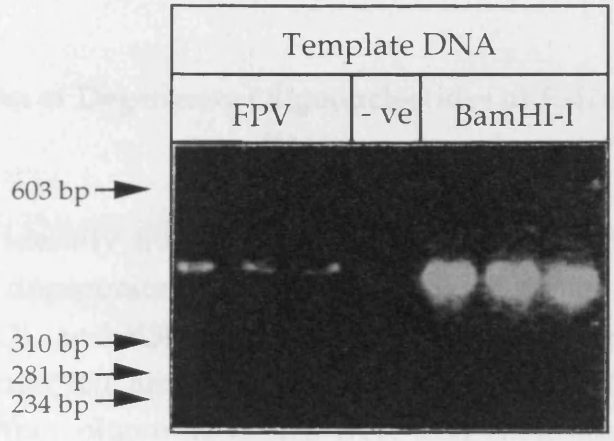


Fig. 7.2. PCR of FPV DNA with Primers Designed to FPV E2L and E4L

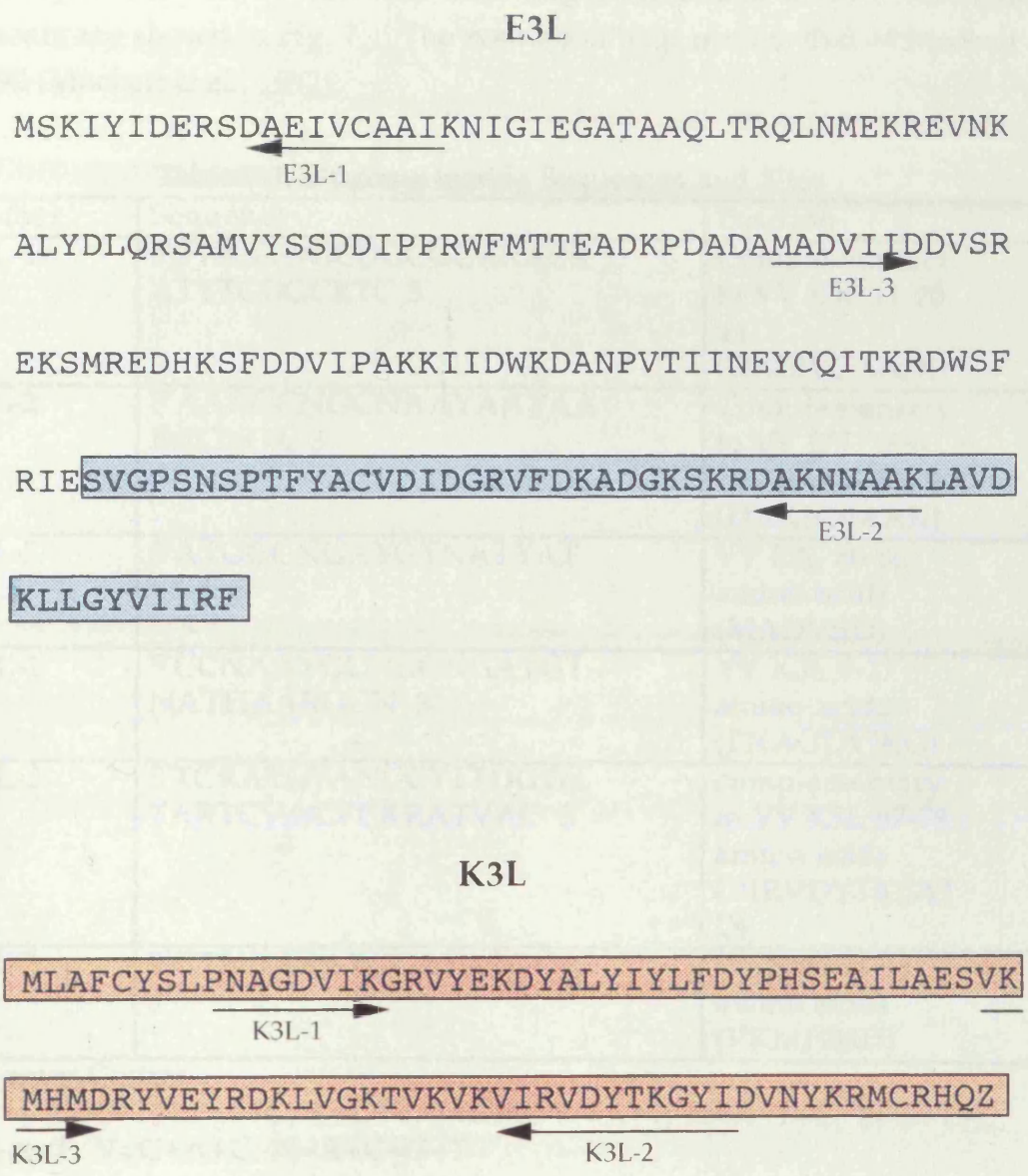
PCR was carried out using FPV DNA or DNA from a pBR322 clone of FPV BamHI-I and primers designed to FPV homologues of VV E2L and E4L. PCR reactions of 25 µl were set up, containing 2.5 mM of each primer, 200 mM each dATP, dCTP, dGTP, dTTP, 1-5 mM $MgCl_2$, 1 x reaction buffer and 20 ng pBR322/BamHI-I DNA or 100 ng FPV DNA. The reaction mix was overlaid with mineral oil and cycled in the PCR machine. The DNA was amplified using 25 cycles of 92 ^{o}C for 1 min, 35 ^{o}C for 1 min and 72 ^{o}C for 1 min. The reaction products were run on a 1.5 % agarose TBE gel.

thought to be vital for function (Chang & Jacobs, 1993, Chang *et al.*, 1992). The variola virus homologue of E3L encodes a protein of 192 amino acids (Massung *et al.*, 1994). Thus, it seems unlikely that the gene order of E2L-E3L-E4L is conserved in FPV, although FPV may have a homologue of VV E3L in an alternative genomic location. Molluscum contagiosum virus, MCV, is predicted to encode homologues of both VV E2L and E4L, but not a homologue of E3L (Senkevich *et al.*, 1996), MCV60315. Between the MCV genes which encode homologues of E2L and E4L exists a gene which is predicted to encode a secreted protein, which contains a signal peptide, and an immunoglobulin domain similar to Xenopus class I histocompatibility antigen.

7.2. Hybridization of Degenerate Oligonucleotides of E3L and K3L to FPV DNA

In order to identify the genomic location of any FPV homologues of VV E3L and K3L, degenerate oligonucleotides were designed to amino acid sequences in VV E3L and K3L. Some of the oligonucleotides were chosen to include highly conserved amino acid sequences such as the RNA-binding domain of E3L. Other oligonucleotides were chosen to include amino acid sequences with few codon choices to minimise the degeneracy of the oligonucleotide. The amino acid sequences of VV E3L and K3L, and the amino acid sequences used to design oligonucleotides are shown in Fig. 7.3. Although only the C-terminus region of VV E3L is thought to be directly involved in RNA-binding (Chang & Jacobs, 1993), amino acid sequences outside this region are thought to be involved in the nuclear localization of the protein (Yuwen *et al.*, 1993) and thus may play a role in other functions of E3L. For these reasons oligonucleotides were designed to amino acid sequences both within, and outside of the RNA-binding domain of E3L, Fig. 7.3. The VV K3L protein has 28 % homology to eIF-2α, over all 87 amino acids (Goebbel *et al.*, 1990) and thus oligonucleotides were designed to amino acid sequences in several areas of the protein.

Fig. 7.3. The Amino Acid Sequences of VV E3L and K3L and Amino Acid Sequences Used for Oligonucleotide Design



The primers were used as probes on a Southern blot of digested FPV DNA. Hybridization of E3L and K3L oligonucleotides to FPV restriction fragments are shown in Fig. 7.4. The restriction map used is that of Mockett *et al*, 1992 (Mockett *et al.*, 1992).

Table 7.1. Oligonucleotide Sequences and Sites

Primer	Sequence	Position
E3L-1	5'TTRATDGCDGCGCARACR ATYTCDGCRTC 3'	Complementary to VV E3L 11-20 aa (DAEIVCAAIK)
E3L-2	5'AARGCNGCNAAYAAYAA RGCNGA 3'	Complementary to VV E3L 169-176 amino acids (DAKNNAAK)
E3L-3	5'ATGGCNGAYGTNATYAT YGA 3'	VV E3L 80-86 amino acids (MADVIID)
K3L-1	5'CCNAAYGCNGGNGAYGT NATHAARGGN 3'	VV K3L 9-17 amino acids (PNAGDVIKG)
K3L-2	5'TCRATGTANCCYTTDGTG TARTCVACVCKRATVAC 3'	complementary to VV K3L 67-78 amino acids (VIRVDYTKGYI D)
K3L-3	5'GTNAARATGCAYATGGA 3'	VV K3L 44-49 amino acids (VKMHMD)

IUB Group Codes:
R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+T, H=A+T+C, B=G+T+C, D=G+A+T, V=G+A+C, N=A+C+G+T

The E3L oligonucleotides, shown in Table 7.2, hybridized to two different regions of the FPV genome, BamHI fragments A and B, PstI fragments B and F, and Nco I fragments P and Y. The K3L oligonucleotides hybridized to two different regions of the FPV genome, BamHI fragments N, G and Q, Pst I fragments A and D/E, and Nco I fragments J and K/L. As the

Fig. 7.4. Hybridization of E3L and K3L Oligonucleotides to FPV Restriction Enzyme Fragments

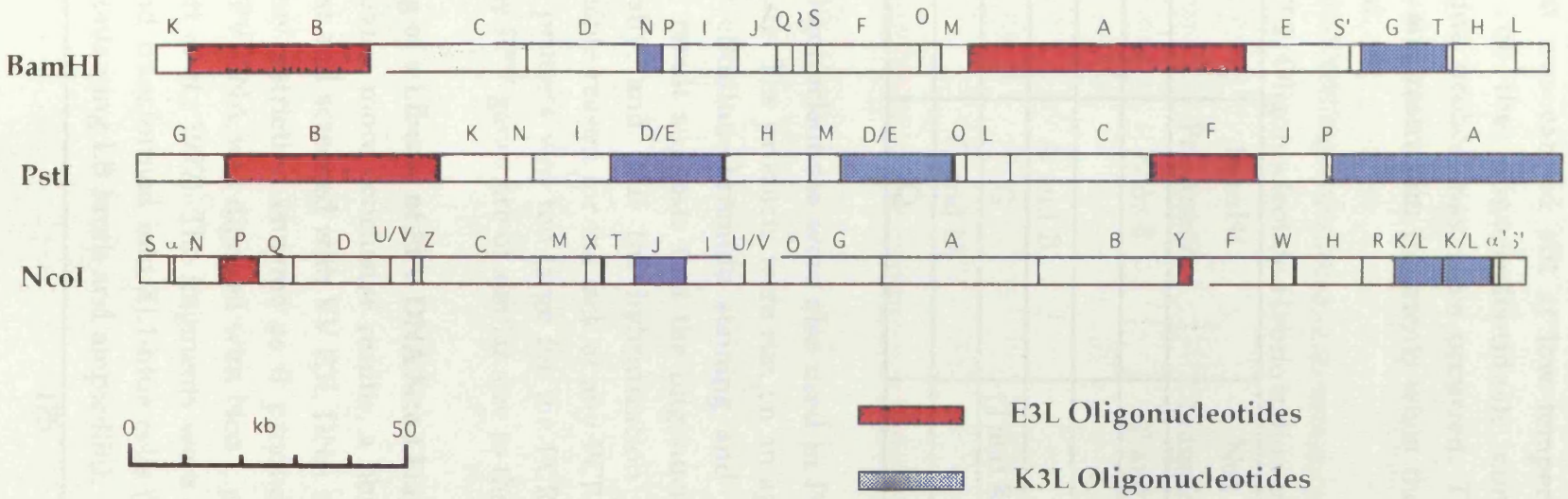


Fig. 7.4. Hybridization of E3L and K3L Oligonucleotides to FPV Restriction Enzyme Fragments.
FPV DNA was digested with BamHI, Nco I or Pst I, and run on a 0.5 % agarose gel. The gel was soaked in 0.2 M HCl for 30 min, denaturation solution for 30 min and neutralization solution for 30 min before being capillary blotted on to nylon membrane.The oligonucleotide probe was prepared by end-labelling the oligonucleotide. Equal molar amounts of oligonucleotide and [g-32P] dATP were mixed with 1 U/ml of T4 kinase in the appropriate reaction buffer. The reaction mix was incubated at 37 °C for 30 min. Hybridization was carried out at 24-32 °C overnight in a hybridization oven . After hybridization the filters were washed twice with 2 x SSC, 0.1% SDS, and twice with 1 x SSC, 0.5 % SDS for 30 min at 24-32 °C each time. The membrane was wrapped in saran wrap or cling film and exposed to autoradiography film at -70 °C. The restriction fragment(s) bound by the oligonucleotide probe were calculated by size.

hybridization was carried out at low temperatures (due to the level of degeneracy of the oligonucleotides), non-specific binding of the oligonucleotide probes may have occurred. The oligonucleotides did not hybridize to any restriction fragments when the stringency of hybridization was increased.

Table 7.2. Oligonucleotide Hybridization to Restriction Fragments

Oligonucleotide	BamHI Fragments	Nco I Fragments	Pst I Fragments
E3L-1	A or B	P and Y	B and F
E3L-2	-	-	-
E3L-3	A and B	-	-
K3L-1	G	J and K/L	A and D/E
K3L-2	G and N	-	-
K3L-3	Q	-	-

The oligonucleotides were also used in PCR reactions with FPV DNA as the template. The products were run on an agarose gel but no bands were visible after ethidium bromide staining and U.V. illumination (data not shown). The result suggests that the oligonucleotides bound to FPV DNA non-specifically, and thus the hybridization results may be misleading. Another possible reason for the lack of any PCR products is that the distance between the primers was too large for the PCR conditions used, but this is unlikely if the FPV genes are of similar size to the VV E3L and K3L genes.

7.3. Screening of a Library of FPV DNA Nco I Fragments

To provide more conclusive results, a library of FPV Nco I fragments was produced and screened with VV E3L DNA and VV K3L DNA. Nco I was chosen as the restriction enzyme as it produces a large number of small fragments. FPV DNA was digested with Nco I, producing fragments of 0.9-30 Kbp (Mockett *et al.*, 1992). The fragments were ligated into the Nco I site of pGem 5zf and transformed into XL1-blue cells (colonies were picked into 96 well plates containing LB broth and ampicillin).

It was calculated that Nco I fragments of up to 12 Kbp would be included in the library, based on the size of insert that can be ligated into pGem 5zf. Thus, Nco I fragments A-G, representing approximately 50 % of the genome were excluded from the library.

The results of oligonucleotide hybridization to FPV DNA, Fig. 7.4, suggested that FPV homologues of E3L and K3L exist in Nco I fragments P, J and K/L. All of these fragments are under 12 Kbp and so should have been represented in the library.

The library colonies were picked from the 96 well plates, grown, blotted onto nylon membrane and probed with VV E3L DNA or VV K3L DNA which was produced by PCR. The positive colonies (12 with each probe) were grown and the DNA extracted. The DNA was digested with Nco I to release the insert, run on a gel, southern blotted and probed again with VV E3L DNA or VV K3L DNA. The aim of this approach was to remove the possibility of false positives arising from hybridization of the probe to vector DNA. Clones 2G5 and 1C12, containing a 5.3 Kbp insert (Nco I-T), were found to be positive after screening with both VV E3L and VV K3L DNA.

To check the results and the insert sizes, the clones and VV E3L DNA and VV K3L DNA were used as probes on a Southern blot of restriction digested FPV DNA.

Restriction enzyme digested FPV DNA is shown in Fig. 7.5a. Restriction enzyme fragments of FPV DNA hybridized with VV K3L DNA are shown in Fig. 7.5b. VV K3L DNA hybridizes with FPV BamHI fragments of approximately 4.3 and 3.1 Kbp (N and P), a Nco I fragment of approximately 5.3 Kbp (T) and a Pst I fragment of approximately 20 Kbp (D/E). The same fragments also hybridized with clones 2G5 and 1C12 and VV E3L DNA, confirming that both clones contain a 5.3 Kbp insert, Nco I-T (all data not shown).

Fig. 7.5a. Restriction Enzyme Fragments of FPV DNA

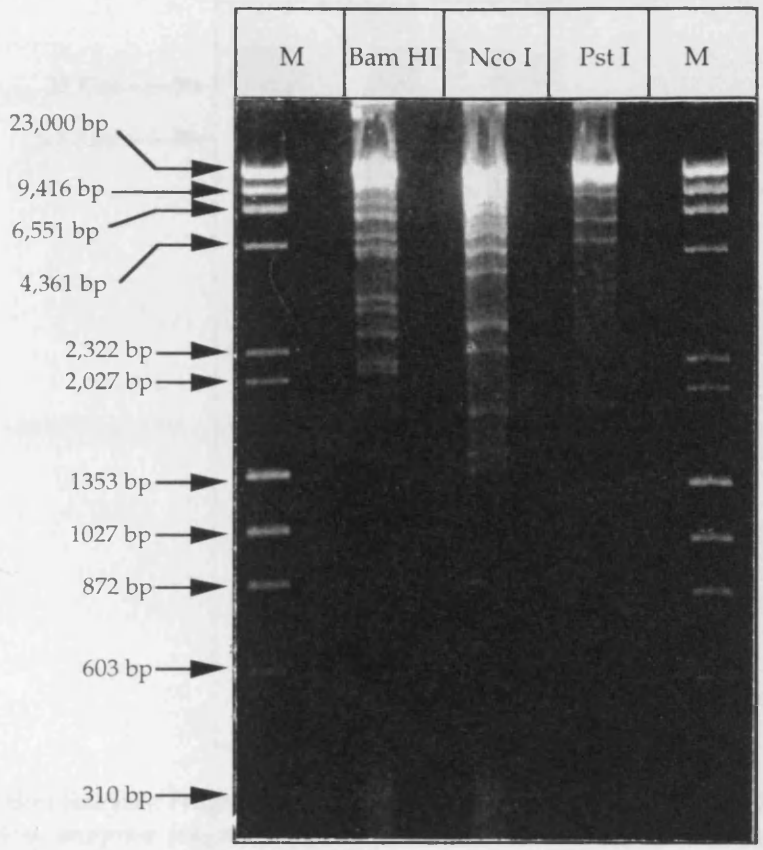


Fig. 7.5a. Restriction Enzyme Fragments of FPV DNA

Restriction enzyme digests of a final volume of 50 µl were set up with 5 µg of FPV DNA and 50 U of restriction enzyme in the appropriate buffer. The reactions were incubated at 37 ^{o}C for 16 h. The digested DNA was run alongside marker DNA (λ Hind III and ϕx 174 Hae III) on a 0.6% agarose TBE gel at 35 V for 16 h. The gel was ethidium bromide stained and visualized by U.V. illumination.

Fig. 7.5b. Restriction Enzyme Fragments of FPV DNA Probed with VV K3L DNA

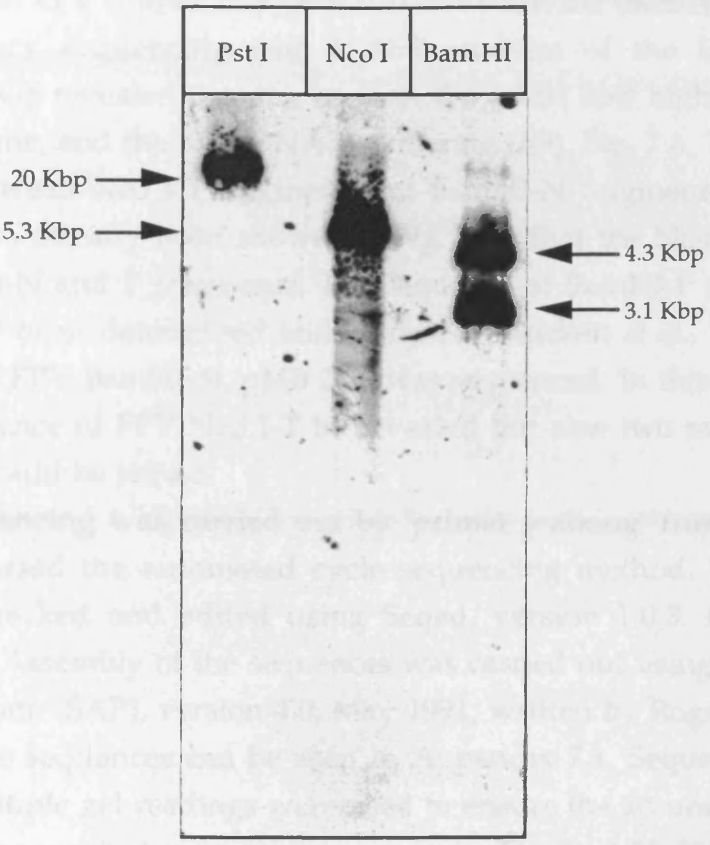


Fig. 7.5b. Restriction Enzyme Fragments of FPV DNA Probed with VV K3L DNA

Restriction enzyme fragments of FPV DNA were blotted on to nylon membrane. Hybridization was carried out using the ECL direct nucleic acid labelling and detection systems. The hybridization buffer contained manufacturer's ECL gold buffer, 5 % manufacturer's blocking agent and 0.5 M NaCl which was mixed and incubated at 42 ^{o}C for 1 h before use. The membrane was incubated in the hybridization buffer at 30 ^{o}C in a hybridization oven for 4 h before addition of the probe.

The probe was produced using 300 ng of a PCR product of VV K3L DNA. The DNA was diluted to 10 ng/µl, denatured by boiling for 5 min and snap cooling on ice for 5 min. The denatured DNA was mixed with one volume of the manufacturer's labelling mix and one volume of glutaraldehyde and incubated at 37 ^{o}C for 10 min. The labelled probe was then added to the hybridization solution and membrane. Hybridization was carried out at 30 ^{o}C for 4-16 h.

After hybridization the membrane was washed twice in 0.4% SDS, 0.5 x SSC at 55 ^{o}C for 10 min and twice in 2 x SSC at 25 ^{o}C for 5 min. The detection reagents were mixed and placed on the membrane for 1 min. The membrane was blotted with paper towels to remove excess detection fluid, wrapped in Saran (Dow) and exposed to film for 1 min-2 h.

7.4 Sequencing of FPV Clones 2G5 (Nco I-T) and pMB 282 (BamHI-N)

Preliminary sequencing and BLAST analysis of the E3L and K3L positive clone 2G5 revealed that the ends of the insert had highest homology with VV O1L gene, and the FPV DNA polymerase (E9), Fig. 7.6. The ORFs and relationship between Nco I-T fragment and BamHI-N fragments can be seen in Fig. 7.9. It has already been shown, in Fig. 7.5b that the Nco I-T fragment overlaps BamHI-N and P fragments. The sequence of BamHI-P and BamHI-D have previously been determined and analysed (Mockett *et al.*, 1992), Thus, a pBR322 clone of FPV BamHI-N, pMB 282, was sequenced. In this way not only would the sequence of FPV Nco I-T be revealed but also two major blocks of FPV sequence could be joined.

The sequencing was carried out by 'primer walking' from the ends of the insert and used the automated cycle sequencing method. The sequence was initially checked and edited using Seqed, version 1.0.3. from Applied Biosystems Inc. Assembly of the sequences was carried out using the Sequence Assembly Program (SAP), version 4.0, May 1991, written by Roger Staden. The alignment of the sequences can be seen in Appendix 7.1. Sequencing of both strands and multiple gel readings were used to ensure the accuracy of the final consensus sequence which was used in analysis. The first 30-50 bases of each gel reading, and areas with large numbers of unread bases were excluded from the gel readings used in the alignment, again to ensure accuracy. In a small area, from 3275-3350 bp, only one sequence has been used in the alignment, Appendix 7.1. The chromatogram and sequence of this gel reading have been very carefully checked against the ends of surrounding gels (not used in the alignment) to ensure that the sequence over this region is correct.

Fig. 7.6. Analysis of Sequences of Clone 2G5 (Nco I-T)

Sequence of clone 2G5 with forward primer

PIR1:DJVZFP

DNA-directed DNA polymerase (2.7.7.7) fowlpox virus (strain HP444)

Length = 988

```
 Score = 366 (178.7 bits), Expect = 9.1e-48, P = 9.1e-48
 Identities = 72/88 (81%), Positives = 79/88 (89%), Frame = -3

Query:   379  VVNLLIQTRGQQEIPSLILQSLETDLISEFTPNREFEKYLLSRKHHNNPKSATHPNFELV  200
             ++ LL+Q++GQQEI  LILQSLETD+ISEFT NREFEKYLLSRKHHNN KSATH NFELV
Sbjct:   834  LMKLLMQSKGQQEITRLILQSLETDMISEFTHNREFEKYLLSRKHHNNYKSATHSNFELV  -2564

Query:   199  KRPNLENTAQIEIGERSYYIYICDISLP  116
             KR NLENT +IEIGER YYIYICDISLP
Sbjct:   894  KRYNLENTEKIEIGERYYYIYICDISLP  -2648

 Score = 115 (57.5 bits), Expect = 1.2e-22, Poisson P(2) = 1.2e-22
 Identities = 27/50 (54%), Positives = 30/50 (60%), Frame = -2

Query:   533  LXXQPGKKNPAMEYLATPSRGPKPIRVPGGPSETRRAVALSHKHLTQDIK  384
             L  Q  KK   ++YLA    G KPIRV  G SETRR VAL HKH+ Q  K
Sbjct:   782  LILQSKKKYTTIKYLANYKPGDKPIRVNKGTSETRRDVALFHKHMIQRYK  -2377
```

Sequence of clone 2G5 with reverse primer

PIR2:D42510

O1L protein - vaccinia virus (strain Copenhagen)

Length = 666

```
 Score = 51 (25.3 bits), Expect = 32., P = 1.0
 Identities = 15/56 (26%), Positives = 24/56 (42%), Frame = +3

Query:   276  GKLPPKYYNFGKGLDLNNILAFGREIIQLNDLKKLIMRIPLLPDWFTDVISVKKNT  443
             G LP   Y      D+ N+  F  EII + D  K +     + ++   V    KN+
Sbjct:    40  GLLPKSLYLEAINSDILNVRFFPPEIINVTDIVKALQNSCRVDEYLKAVSLYHKNS  287

 Score = 46 (23.6 bits), Expect = 0.089, Poisson P(2) = 0.085
 Identities = 9/27 (33%), Positives = 15/27 (55%), Frame = +1

Query:   163  LYSKKVRKSIRKFIRPGLNFDLLHEKH  243
             +Y +  RK++ K I   LN + +  KH
Sbjct:     3  MYPEFARKALSKLISKKLNIEKVSSKH  87
```

Fig. 7.6. Analysis Of Sequences Of Clone 2G5

Sequences were analysed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service which translates the DNA sequence in all 6 frames and then searches the databases for similar proteins. The top of the three sequences is the experimental (query) sequence. The bottom sequence (subject) is the library sequence. The middle sequence shows amino acids which are identical in both the experimental and library sequences and + signs represent conservative changes between the two sequences.

7.5 Analysis of the Sequence of FPV BamHI-N

Analysis of the consensus sequence was carried out using the BLAST network service at the National Centre for Biotechnology Information (NCBI), PILEUP of the Wisconsin GCG package and CAD Gene version 2.0 from Genetic Technology Corporation. The nucleotide sequence of 4367 bp from pMB 282 (FPV BamHI-N) is shown in Fig. 7.7, with translation of major open reading frames (ORFs) shown using the single letter amino acid code. An ORF map of the sequence, BamHI-N is shown in Fig. 7.8. The sequence exhibits typical poxvirus characteristics, with tightly packed genes. The 3' ends of ORF 1 and ORF 2 overlap as do the 5' end of ORF 2 and the 3' end of ORF 3, another characteristic of poxvirus genes.

7.5.1. Homology of ORFs to Poxvirus Proteins

The predicted translation products were compared against a database of all published sequences, and several homologies were noted with poxvirus genes, which are shown in Table 7.3.

ORF 4, shown only in Fig. 7.7, is a small open reading frame, predicted to encode 34 amino acids, which does not exhibit homology to any known poxvirus genes. ORF 4 exists between the FPV homologues of VV O1 and I1, a region which in VV encodes O2, a protein of 108 amino acids with homology glutaredoxin proteins (Ahn & Moss, 1992, Goebbel *et al.*, 1990, Massung *et al.*, 1994). No evidence could be found to suggest that ORF 4 is a truncated form of O2 or that ORF 4 had disrupted O2. It thus appears that the FPV BamHI-N sequence does not encode a homologue of VV O2. It is unlikely that ORF 4 of only 34 amino acids (approximately 4 Kd) encodes a functional protein as most poxvirus proteins are larger than this (Goebbel *et al.*, 1990) Thus ORF 4 has not been shown in further diagrams. ORF 4 possesses the late gene promoter motif, TAAAT, at the 5' end of the gene, which indicates that ORF 4 is expressed as a late gene (after DNA replication) if it is expressed at all. It is possible that ORF 4 has a homologue in VV which has been overlooked in the analysis of the DNA sequence of VV which was only carried out on open

Table 7.3. Homology of Predicted Open Reading Frames with Poxvirus Genes

FPV Sequence	Homology with	Amino Acid Identity	Amino Acid Conservation
ORF 1 (E10)	Variola virus (Ind) E10	53 %	72 % /89 aa
	Vaccinia virus (Cop) E10	50 %	72 % /89 aa
	ORF virus E10	42 %	80 % /22 aa
	African Swine Fever virus	23 %	42 % /69 aa
ORF 2 (E11)	Variola virus (Ind) E11	38 %	63 % /104 aa
	Vaccinia virus (Cop) E11	37 %	64 % /104 aa
ORF 3 (O1)	Vaccinia virus (Cop) O1	28 %	52 % /433 aa
	Variola virus (Ind) Q1 (O1 homologue)	29 %	52 % /433 aa
	Vaccinia virus B19R	30 %	48 % /70 aa
ORF 4	No homology to poxviruses		
ORF 5 (I1)	Vaccinia virus (Cop) I1	53 %	73 % /307 aa
	Variola virus (Ind) K1L (I1 homologue)	51 %	73 % /307 aa
	Vaccinia virus 36 Kd late protein	51 %	73 % /293 aa
ORF 6 (I2)	Vaccinia virus I2	29 %	53 % /64 aa
	Variola virus (Ind) K2L (I2 homologue)	29 %	53 % / 64 aa
ORF 7 (I3)	Fowlpox virus (HP444) I3	100 %	100 % /77 aa
	Variola virus K3L (I3L homologue)	38 %	68 % /80 aa
	Vaccinia virus (Cop) I3	40 %	69 % /75 aa

Table 7.3. Homology of Predicted Open Reading Frames With Poxvirus Genes

The predicted translation products were compared against a database of all published sequences using the BLAST network service at the National Centre for Biotechnology Information (NCBI). Homology with poxvirus genes are shown. Amino acid identity is the number of identical amino acids in the two sequences whereas amino acid conservation is the number of identical amino acids and amino acids which differ but are conservative changes.

Fig. 7.7. Consensus Sequence of FPV BamHI-N and Open Reading Frames

ORF 1 (E10)

```
    1/1                                          31/11
M   D   P   R   Y   W   G   R   S   F   G   I   V   I   F   I   I   I   T   K   F
ATG GAT CCT AGA TAC TGG GGA CGC AGC TTT GGG ATA GTT ATA TTT ATT ATA ATT ACC AAG TTT
TAC CTA GGA TCT ATG ACC CCT GCG TCG AAA CCC TAT CAA TAT AAA TAA TAT TAA TGG TTC AAA

61/21                                        91/31
K   H   D   I   E   T   C   K   R   H   L   Y   N   I   C   K   A   L   P   R
AAA CAC GAT ATA GAA ACT TGC AAA CGT CAC TTG TAT AAC ATA TGT AAA GCA TTA CCG CGT
TTT GTG CTA TAT CTT TGA ACG TTT GCA GTG AAC ATA TTG TAT ACA TTT CGT AAT GGC GCA

121/41                                       151/51
 A   E   C   K   D   H   A   L   Q   A   I   Q   K   N   N   I   M   S   S   N
GCT GAA TGT AAG GAC CAT GCT CTA CAG GCA ATA CAG AAG AAT AAT ATT ATG TCA AGC AAC
CGA CTT ACA TTC CTG GTA CGA GAT GTC CGT TAT GTC TTC TTA TTA TAA TAC AGT TCG TTG

181/61                                       211/71
 D   I   N   Y   I   Y   S   S   F   I   S   L   Y   N   N   L   V   F   N   P
GAT ATT AAT TAT ATA TAC TCC TCT TTC ATA AGT CTT TAC AAC AAT CTA GTG TTT AAT CCA
CTA TAA TTA ATA TAT ATG AGG AGA AAG TAT TCA GAA ATG TTG TTA GAT CAC AAA TTA GGT

241/81                                       271/91
E   R   C   I   D   I   K   K   V   K   K   L   I   *
GAA AGA TGT ATA GAT ATA AAA AAA GTA AAA AAA CTA ATT TAG TAA TAC CTA GAA TTA TCA
CTT TCT ACA TAT CTA TAT TTT TTT CAT TTT TTT GAT TAA ATC ATT ATG GAT CTT AAT AGT
                                             *   Y   Y   R   S   N   D   S

301/101                                      331/111
GAT CTA ACT TTA ACA ATG TAA CCC GTT TTT GGC TTT ACA GAA ATA CTC ATA TCG TCA GTA
CTA GAT TGA AAT TGT TAC ATT GGG CAA AAA CCG AAA TGT CTT TAT GAG TAT AGC AGT CAT
  R   V   K   V   I   Y   G   T   K   P   K   V   S   I   S   M   D   D   T   I

361/121                                      391/131
ATA GTA AAA CAA ATA GAA GTT ATT TCT GAG GAA GTA TAG TCC GTG CTA GCT AGT TCT TTA
TAT CAT TTT GTT TAT CTT CAA TAA AGA CTC CTT CAT ATC AGG CAC GAT CGA TCA AGA AAT
  T   F   C   I   S   T   I   E   S   S   T   Y   D   T   S   A   L   E   K   S

421/141                                      451/151
CTG AAT GTA TAA TAA GTA TTA TCT ACT GGA GTT AGT TCT CCT TTA TCG TAT TTA TAT GTA
GAC TTA CAT ATT ATT CAT AAT AGA TGA CCT CAA TCA AGA GGA AAT AGC ATA AAT ATA CAT
  F   T   Y   Y   T   N   D   V   P   T   L   E   G   K   D   Y   K   Y   T   F

481/161                                      511/171
AAT AAT GTT GTA TCC TTA ACT ACT ACA TAG AAC GTA GAT TCT TTT AGT TTT ATA TAT TTC
TTA TTA CAA CAT AGG AAT TGA TGA TGT ATC TTG CAT CTA AGA AAA TCA AAA TAT ATA AAG
  L   T   T   D   K   V   V   V   Y   F   T   S   E   K   L   K   I   Y   K   K

541/181                                      571/191
TTT ATA ACC GAT ATG AAA TAT TTA ACC GCT CTA TCT ACC TCA CAC TTG GTT CTT AAA GAT
AAA TAT TGG CTA TAC TTT ATA AAT TGG CGA GAT AGA TGG AGT GTG AAC CAA GAA TTT CTA
  I   V   S   I   F   Y   K   V   A   R   D   V   E   C   K   T   R   L   S   K
```

```
601/201                                          631/211
TTT TTT GGT GGA ATA TCA TAA TAG AGT TTT ACT CTT TCT GAC TCT GAT TCT AAA AGA ATG
AAA AAA CCA CCT TAT AGT ATT ATC TCA AAA TGA GAA AGA CTG AGA CTA AGA TTT TCT TAC
                    *   L   L   T   K   S   K   R   V   R   I   R   F   S   H
  K   P   P   I   D   Y   Y   L   K   V   R   E   S   E   S   E   L   L   I   N

661/221                                          691/231
TTT ACT AAT TCC ATT TAA GTT ATT AAA AAT GGT TAT TAA TAG TTT CAT TAA TAT TCC GTA
AAA TGA TTA AGG TAA ATT CAA TAA TTT TTA CCA ATA ATT ATC AAA GTA ATT ATA AGG CAT
K   S   I   G   N   L   N   N   F   I   T   I   L   L   K   M   L   I   G   Y
  V   L   E   M
            ORF 2 (E11)

721/241                                          751/251
GGT TTT GAA AGG TAC AAC ATA GTT ATT GAT TAG GTT TTC TAA TTC ACA ACA AAT ATT CAT
CCA AAA CTT TCC ATG TTG TAT CAA TAA CTA ATC CAA AAG ATT AAG TGT TGT TTA TAA GTA
T   K   F   P   V   V   Y   N   N   I   L   N   E   L   E   C   C   I   N   M

781/261                                          811/271
TTC ATT AAC GTA TAC ATT TTT ATA CAG TTT TGG TGA TAT ACC AAA TCC TTT TAA GCA TCT
AAG TAA TTG CAT ATG TAA AAA TAT GTC AAA ACC ACT ATA TGG TTT AGG AAA ATT CGT AGA
E   N   V   Y   V   N   K   Y   L   K   P   S   I   G   F   G   K   L   C   R

841/281                                          871/291
ATA TAA TAA CAT TTC TAA AAA CGA TCT TAA ATT ATA GCT TGT TTT TTG ATA TCT GAT AAT
TAT ATT ATT GTA AAG ATT TTT GCT AGA ATT TAA TAT CGA ACA AAA AAC TAT AGA CTA TTA
Y   L   L   M   E   L   F   S   R   L   N   Y   S   T   K   Q   Y   R   I   I

901/301                                          931/311
TAT CAA AGT AAA AAT ATA TTC GAA AAT AAC ATT AGC TAG TAT TAC TTT TTT AAT GAA GTT
ATA GTT TCA TTT TTA TAT AAG CTT TTA TTG TAA TCG ATC ATA ATG AAA AAA TTA CTT CAA
I   L   T   F   I   Y   E   F   I   V   N   A   L   I   V   K   K   I   F   N

961/321                                          991/331
TAT TCC ATA AAG TTT TAT TCG ACT ATA GTC TAA TAG TTT ATA TAT ATC TAT ATT ATC TAT
ATA AGG TAT TTC AAA ATA AGC TGA TAT CAG ATT ATC AAA TAT ATA TAG ATA TAA TAG ATA
I   G   Y   L   K   I   R   S   Y   D   L   L   K   Y   I   D   I   N   D   I

1021/341                                         1051/351
ATG TTC AAA ATT ATT ATT AAT ATT TTC GTC CTT GTT TTT GAG ATA ATT TAT GTT ATC GGC
TAC AAG TTT TAA TAA TAA TTA TAA AAG CAG GAA CAA AAA CTC TAT TAA ATA CAA TAG CCG
H   E   F   N   N   N   I   N   E   D   K   N   K   L   Y   N   I   N   D   A

1081/361                                         1111/371
TTT TAT ATT CTT TGT AGC CAT CAT AAA CCT CGT GAA ATC TAC TTT ACA CGA ATC ATC TAT
AAA ATA TAA GAA ACA TCG GTA GTA TTT GGA GCA CTT TAG ATG AAA TGT GCT TAG TAG ATA
K   I   N   K   T   A   M   M   F   R   T   F   D   V   K   C   S   D   D   I

1141/381                                         1171/391
AAG ATA CAT CAA AGG ACT CCA TAG CGA GTT AGT ATT CAG AAA ATT GAT ATC TAT AAT ACC
TTC TAT GTA GTT TCC TGA GGT ATC GCT CAA TCA TAA GTC TTT TAA CTA TAG ATA TTA TGG
L   Y   M   L   P   S   W   L   S   N   T   N   L   F   N   I   D   I   I   G

1201/401                                         1231/411
TTT CTT GGC GGT AGA AAA TAA GAA ACT TGC TAG AGT CAA TAA TCC TAT ATC ATT ATT ATC
AAA GAA CCG CCA TCT TTT ATT CTT TGA ACG ATC TCA GTT ATT AGG ATA TAG TAA TAA TAG
K   K   A   T   S   F   L   F   S   A   L   T   L   L   G   I   D   N   N   D
```

```
1261/421                                        1291/431
GAA AGT ACT ATT TTT AGA CAT ATA ATC TAA CAG TTT ATC GCT ATA CAT TAC ATA ATC TAC
CTT TCA TGA TAA AAA TCT GTA TAT TAG ATT GTC AAA TAG CGA TAT GTA ATG TAT TAG ATG
F   T   S   N   K   S   M   Y   D   L   L   K   D   S   Y   M   V   Y   D   V

1321/441                                        1351/451
CGG GTG ATA TTG AAA CTT TAT TAA TAC AGA ATA TAA ATT ACC TAG ATC AAA ACA CTT AGC
GCC CAC TAT AAC TTT GAA ATA ATT ATG TCT TAT ATT TAA TGG ATC TAG TTT TGT GAA TCG
P   H   Y   Q   F   K   I   L   V   S   Y   L   N   G   L   D   F   C   K   A

1381/461                                        1411/471
ATT TTC TAA ACA AAC TAC TTG TTT CTG TAA CAT GTT TAG AGC ATC CAT CTT TAC CTC ATT
TAA AAG ATT TGT TTG ATG AAC AAA GAC ATT GTA CAA ATC TCG TAG GTA GAA ATG GAG TAA
N   E   L   C   V   V   Q   K   Q   L   M   N   L   A   D   M   K   V   E   N

1441/481                                        1471/491
GGA CGA TGA CGA GTC AAG TAT GTA TTT CCC TAT AGC ATA GTT ATA TTT AAA ATC ATT TAA
CCT GCT ACT GCT CAG TTC ATA CAT AAA GGG ATA TCG TAT CAA TAT AAA TTT TAG TAA ATT
S   S   S   S   D   L   I   Y   K   G   I   A   Y   N   Y   K   F   D   N   L

1501/501                                        1531/511
TAG TAC TTT CAT AGT TTG TTT ATT ATC TAA ATA CTT GAA TGA TTC TTT TAC AAC ATA TTT
ATC ATG AAA GTA TCA AAC AAA TAA TAG ATT TAT GAA CTT ACT AAG AAA ATG TTG TAT AAA
L   V   K   M   T   Q   K   N   D   L   Y   K   F   S   E   K   V   V   Y   K

1561/521                                        1591/531
TTC TTT AGA ATC TTG TTT ATA AGT AAT AAC GGG AAA TAC TTT TTG AGG ATA CAT ACT ATT
AAG AAA TCT TAG AAC AAA TAT TCA TTA TTG CCC TTT ATG AAA AAC TCC TAT GTA TGA TAA
E   K   S   D   Q   K   Y   T   I   V   P   F   V   K   Q   P   Y   M   S   N

1621/541                                        1651/551
ACA ATA ATA TAG ATG CCA TCT AAA CGC ATT TAC AAA CAA CTC TGA CTT TTT TTT GTA TAT
TGT TAT TAT ATC TAC GGT AGA TTT GCG TAA ATG TTT GTT GAG ACT GAA AAA AAA CAT ATA
C   Y   Y   L   H   W   R   F   A   N   V   F   L   E   S   K   K   K   Y   I

1681/561                                        1711/571
ATC TAT ATA ACG GCA GAT AAA TTT AGT TTC ATC CTC CGT TAT GTT TAG AAA CAA GTA CTT
TAG ATA TAT TGC CGT CTA TTT AAA TCA AAG TAG GAG GCA ATA CAA ATC TTT GTT CAT GAA
D   I   Y   R   C   I   F   K   T   E   D   E   T   I   N   L   F   L   Y   K

1741/581                                        1771/591
ATC TAT GTA TAG AGG TAT ATC TAT CCA TTC AAA CAA CGC ATA AAA GTA TAT ACC AAA TTC
TAG ATA CAT ATC TCC ATA TAG ATA GGT AAG TTT GTT GCG TAT TTT CAT ATA TGG TTT AAG
D   I   Y   L   P   I   D   I   W   E   F   L   A   Y   F   Y   I   G   F   E

1801/601                                        1831/611
ATC TCT AAG TTT ATC GGG TGG TAA TTG TGT TGA TAT CCA TTT ATT CAA GCA AGG TGA TAT
TAG AGA TTC AAA TAG CCC ACC ATT AAC ACA ACT ATA GGT AAA TAA GTT CGT TCC ACT ATA
D   R   L   K   D   P   P   L   Q   T   S   I   W   K   N   L   C   P   S   I

1861/621                                        1891/631
ATC GGG TAA TCT ATC CTT AAT GAT AGT AAT TAT TGA TTC TAT AAA ATC TTT TTT TGA TTT
TAG CCC ATT AGA TAG GAA TTA CTA TCA TTA ATA ACT AAG ATA TTT TAG AAA AAA ACT AAA
D   P   L   R   D   K   I   I   T   I   I   S   E   I   F   D   K   K   S   K

1921/641                                        1951/651
TAC TAT TCC ATT GAC ATA ATC AAT AAT ATC ATC ATC GGG ATA CTT CTT TAT CAA ATA TAT
ATG ATA AGG TAA CTG TAT TAG TTA TTA TAG TAG TAG CCC TAT GAA GAA ATA GTT TAT ATA
V   I   G   N   V   Y   D   I   I   D   D   D   P   Y   K   K   I   L   Y   I
```

```
1981/661                                        2011/671
AAT TTT ATC AAC ATC AGT TAC TTG ATA TAG GGA TGA ATG AAC CGG TGA TAT AGA AAA TTT
TTA AAA TAG TTG TAG TCA ATG AAC TAT ATC CCT ACT TAC TTG GCC ACT ATA TCT TTT AAA
I   K   D   V   D   T   V   Q   Y   L   S   S   H   V   P   S   I   S   F   K

2041/681                                        2071/691
TTC TTC CAT ATA GAG AAC GCT GTG AAT AGG TGT TTC TAT TCT CTT GTA TAG CTC TTT CAC
AAG AAG GTA TAT CTC TTG CGA CAC TTA TCC ACA AAG ATA AGA GAA CAT ATC GAG AAA GTG
E   E   M   Y   L   V   S   H   I   P   T   E   I   R   K   Y   L   E   K   V

2101/701                                        2131/711
GTT TTC TGC ACT AGT GTT TTC AAA AAT ATA TTT TAC TTC ATC TAG AGA AAA TGG AGT ACC
CAA AAG ACG TGA TCA CAA AAG TTT TTA TAT AAA ATG AAG TAG ATC TCT TTT ACC TCA TGG
N   E   A   S   T   N   E   F   I   Y   K   V   E   D   L   S   F   P   T   G

2161/721                                        2191/731
CGG TAT AAC TAA AGA GCT GTT AAG CAA TGC TAT TTC AAC AGG TGT TTT TAT TCT GTT ATC
GCC ATA TTG ATT TCT CGA CAA TTC GTT ACG ATA AAG TTG TCC ACA AAA ATA AGA CAA TAG
P   I   V   L   S   S   N   L   L   A   I   E   V   P   T   K   I   R   N   D

2221/741                                        2251/751
TAT TAT ATT ACG TAT ATC TGA AAG AGA TAC CAT ATT AGA GTT AAT AAG TTT ATT CAC AAT
ATA ATA TAA TGC ATA TAG ACT TTC TCT ATG GTA TAA TCT CAA TTA TTC AAA TAA GTG TTA
I   I   N   R   I   D   S   L   S   V   M   N   S   N   I   L   K   N   V   I

2281/761                                        2311/771
AGC AGC ATC CGA TAA TAA GTA TTT TTT ATG ACA CGA TAT TAC ATC GGT AAA ACA GTC TGG
TCG TCG TAG GCT ATT ATT CAT AAA AAA TAC TGT GCT ATA ATG TAG CCA TTT TGT CAG ACC
A   A   D   S   L   L   Y   K   K   H   C   S   I   V   D   T   F   C   D   P

2341/781                                        2371/791
TAA AAG AGG TAG TCT CAT AAT CAG TTT TTT TAA GTC ATT AAG TTG TAT TAT TTC GCT ATC
ATT TTC TCC ATC AGA GTA TTA GTC AAA AAA ATT CAG TAA TTC AAC ATA ATA AAG CGA TAG
L   L   P   L   R   M   I   L   K   K   L   D   N   L   Q   I   I   E   S   D

2401/801                                        2431/811
AAA CGC TAG TAT ATT GTT TAA ATC CAG ACC TTT AGC AAA GTT ATA ATA TTT TGG GGG TAA
TTT GCG ATC ATA TAA CAA ATT TAG GTC TGG AAA TCG TTT CAA TAT TAT AAA ACC CCC ATT
F   A   L   I   N   N   L   D   L   G   K   A   F   N   Y   Y   K   P   P   L

2461/821                                        2491/831
TTT AAC GAA TAT ATT GTT AAT TAT TAA TCG GCG TCC GTG TTT CTC ATG TAA TAA GTC GAA
AAA TTG CTT ATA TAA CAA TTA ATA ATT AGC CGC AGG CAC AAA GAG TAC ATT ATT CAG CTT
K   V   F   I   N   N   I   I   L   R   R   G   H   K   E   H   L   L   D   F

2521/841                                        2551/851
GTT TAA ACC TGA ACG AAT AAA TTT GCG TAT GGA TTT TCT AAC TTT CTT AGA ATA GAG ATT
CAA ATT TGG ACT TGC TTA TTT AAA CGC ATA CCT AAA AGA TTG AAA GAA TCT TAT CTC TAA
N   L   G   S   R   I   F   K   R   I   S   K   R   V   K   K   S   Y   L   N

2581/861                                        2611/871
CGC CAT CTC AGA CAA GAT GAC AAG TAG TGA TTA TAT AAT TAT AAA TTT ATA TTT CCA TTT
GCG GTA GAG TCT GTT CTA CTG TTC ATC ACT AAT ATA TTA ATA TTT AAA TAT AAA GGT AAA
A   M
```

ORF 3 (O1)

```
2641/881                                        2671/891
CGT ATC TAC GTG ACT TCA CGT TCG ACA TAG AAG ATC CAT GGT TTC AAA TAG TGA TAG CTG
GCA TAG ATG CAC TGA AGT GCA AGC TGT ATC TTC TAG GTA CCA AAG TTT ATC ACT ATC GAC
      *   T   V   E   R   E   V   Y   F   I   W   P   K   L   Y   H   Y   S   Y
```

Chapter 7
Cloning and Sequencing of a Genomic Fragment of Fowlpox Virus DNA which Hybridizes to Vaccinia Virus E3L and K3L DNA

```
2701/901                                    2731/911
TAT AAC GTG CAA ATG AAG AAC ATA ATT AAA AAA AGT ACT GTT ACT ATC ATT TAT TCT ACA
ATA TTG CAC GTT TAC TTC TTG TAT TAA TTT TTT TCA TGA CAA TGA TAG TAA ATA AGA TGT
  L   T   C   I   F   F   M   I   L   F   L   V   T   V   I   M   *   E   V   T
                                                                ORF 4

2761/921                                    2791/931
GTA TTT TCT ATT TTC ACA GTA GAT ATA GTT AGG TAT TCT ATT ACC TTA GAT AAA TTA ATT
CAT AAA AGA TAA AAG TGT CAT CTA TAT CAA TCC ATA AGA TAA TGG AAT CTA TTT AAT TAA
  N   E   I   K   V   T   S   I   T   L   Y   E   I   V   K   S   L   N   I   K


2821/941                                    2851/951
TTC TCG TTA TCT TTG ATA TAC TCT GAT ATG GAG CAT AAA AAC TGG TCG TTA AAC TTT TTA
AAG AGC AAT AGA AAC TAT ATG AGA CTA TAC CTC GTA TTT TTG ACC AGC AAT TTG AAA AAT
  E   N   D   K   I   Y   E   S   I   S   C   L   F   Q   D   N   F   K   K   S

2881/961                                    2911/971
GAA TAC TTA TCT ATT TGA TTA GAA ATC GAC GAT ACC AAA AGA CTG TGT TTC GTA TAG ATA
CTT ATG AAT AGA TAA ACT AAT CTT TAG CTG CTA TGG TTT TCT GAC ACA AAG CAT ATC TAT
  Y   K   D   I   Q   N   S   I   S   S   V   L   L   S   H   K   T   Y   I   F

2941/981                                    2971/991
AAA TGG TCT ATA ACT ATA GTT TCT ATG TTC AGT ATT TTA GCG ATA TTA TCA TCT TTT AGA
TTT ACC AGA TAT TGA TAT CAA AGA TAC AAG TCA TAA AAT CGC TAT AAT AGT AGA AAA TCT
  H   D   I   V   I   T   E   I   N   L   I   K   A   I   N   D   D   K   L   I

3001/1001                                   3031/1011
ATA ACG TAT ATT TTT GAA TCG TCG GGT TTC GTA GCT TTA ATA AAA TAT TCG TAC AAT CGA
TAT TGC ATA TAA AAA CTT AGC AGC CCA AAG CAT CGA AAT TAT TTT ATA AGC ATG TTA GCT
  V   Y   I   K   S   D   D   P   K   T   A   K   I   F   Y   E   Y   L   R   A

3061/1021                                   3091/1031
GCA CGT GAT ATA ATA TTA TCT TTA ACT TGA GGA CAT TTA AAT AGT CTA TTT TTA AAT ACA
CGT GCA CTA TAT TAT AAT AGA AAT TGA ACT CCT GTA AAT TTA TCA GAT AAA AAT TTA TGT
  R   S   I   I   N   D   K   V   Q   P   C   K   F   L   R   N   K   F   V   D

3121/1041                                   3151/1051
TCC AGA AGT TCA TCG GAT ATC ATA TAA AAT GTA TCT TTA ATA GTA TTT TTC ATA TCC ATA
AGG TCT TCA AGT AGC CTA TAG TAT ATT TTA CAT AGA AAT TAT CAT AAA AAG TAT AGG TAT
  L   L   E   D   S   I   M   Y   F   T   D   K   I   T   N   K   M   D   M   S

3181/1061                                   3211/1071
GAT ATA GCT GAA TTC TTA ATA AGC TGA TTG ATA ATA TCC TCT GTT TTA TTT TGT TCT AAG
CTA TAT CGA CTT AAG AAT TAT TCG ACT AAC TAT TAT AGG AGA CAA AAT AAA ACA AGA TTC
  I   A   S   N   K   I   L   Q   N   I   I   D   E   T   K   N   Q   E   L   Y

3241/1081                                   3271/1091
TAA TAA GTA TGA CCT TTA AAC TCA AAT CTA TCG GCA TTT GTT CTC AAT CTA TTT GCT AAA
ATT ATT CAT ACT GGA AAT TTG AGT TTA GAT AGC CGT AAA CAA GAG TTA GAT AAA CGA TTT
  Y   T   H   G   K   F   E   F   R   D   A   N   T   R   L   R   N   A   L   V

3301/1101                                   3331/1111
ACC TTA ACG TCT AGT ACG TCT ATA TTG AGA CTA GAA TTT TCA TCT ATT CTG GCC AAT ATT
TGG AAT TGC AGA TCA TGC AGA TAT AAC TCT GAT CTT AAA AGT AGA TAA GAC CGG TTA TAA
  K   V   D   L   V   D   I   N   L   S   S   N   E   D   I   R   A   L   I   E
```

```
3361/1121                                   3391/1131
TCT TTT TCT ATA TCT AGA AGA ACT AGT TCA TCG TGT TTA CCC CTT TTA AGT TTG CCA TTT
AGA AAA AGA TAT AGA TCT TCT TGA TCA AGT AGC ACA AAT GGG GAA AAT TCA AAC GGT AAA
  K   E   I   D   L   L   V   L   E   D   H   K   G   R   K   L   K   G   N   E

3421/1141                                   3451/1151
TCA AAG TAA TTA TTT TCT ACT AAA CTA CAT ATC AAA CTA TTC AGT ACA CCA TCT TTA TAT
AGT TTC ATT AAT AAA AGA TGA TTT GAT GTA TAG TTT GAT AAG TCA TGT GGT AGA AAT ATA
  F   Y   N   N   E   V   L   S   C   I   L   S   N   L   V   G   D   K   Y   G

3481/1161                                   3511/1171
CCC TTT AGC CCA AAC TTA GAC ACA AAG TTC TTA TTT ATG AGA TCT AGA GGA ATT CTA TTC
GGG AAA TCG GGT TTG AAT CTG TGT TTC AAG AAT AAA TAC TCT AGA TCT CCT TAA GAT AAG
  K   L   G   F   K   S   V   F   N   K   N   I   L   D   L   P   I   R   N   E



3541/1181                                   3571/1191
TCG GGT TTT TTA GTT TGT GCC TTC TTT TTA GGA GAA TTC TTT TTA GCG GCT AGT TCA TCT
AGC CCA AAA AAT CAA ACA CGG AAG AAA AAT CCT CTT AAG AAA AAT CGC CGA TCA AGT AGA
  P   K   K   T   Q   A   K   K   K   P   S   N   K   K   A   A   L   E   D   I

3601/1201                                   3631/1211
ATA GCT TCA GCT ATT TTC GTA GTC AGA TAT GAC TTT AAA GCT TTA GCG CTA ATA CTA TTA
TAT CGA AGT CGA TAA AAG CAT CAG TCT ATA CTG AAA TTT CGA AAT CGC GAT TAT GAT AAT
  A   E   A   I   K   T   T   L   Y   S   K   L   A   K   A   S   I   S   N   L

3661/1221                                   3691/1231
AGA ACA AGT TGA TCA AAT TGC TCC ATT TAA TCT TAA CAC ATT AAC TTT AAA TAA AGA AAC
TCT TGT TCA ACT AGT TTA ACG AGG TAA ATT AGA ATT GTG TAA TTG AAA TTT ATT TCT TTG
  V   L   Q   D   F   Q   E   M           *   C   M   L   K   L   Y   L   F   T
                          ORF 5 (I1)

3721/1241                                   3751/1251
GTA AAG ACC ACT ATG CAT ACT ACA AAT ATA ATA AAA ATT GTT AGC CAT GAT AAT TGT TTG
CAT TTC TGG TGA TAC GTA TGA TGT TTA TAT TAT TTT TAA CAA TCG GTA CTA TTA ACA AAC
  F   V   V   I   C   V   V   F   I   I   F   I   T   L   W   S   L   Q   K   S

3781/1261                                   3811/1271
CTT TCT TTT TTA CCA GTT AGC ACT GTC ATT ATT GTA TTG ATA AAA TCC TCA AAA TCA GAA
GAA AGA AAA AAT GGT CAA TCG TGA CAG TAA TAA CAT AAC TAT TTT AGG AGT TTT AGT CTT
  E   K   K   G   T   L   V   T   M   I   T   N   I   F   D   E   F   D   S   D

3841/1281                                   3871/1291
TCA TTT GAT TCC AGA AAA ACA CCG TAT GTA CCT GTA AAT AGC TTT TCC ATT TAG TTT ACG
AGT AAA CTA AGG TCT TTT TGT GGC ATA CAT GGA CAT TTA TCG AAA AGG TAA ATC AAA TGC
  N   S   E   L   F   V   G   Y   T   G   T   F   L   K   E   M   *   N   V   N
                                                          ORF 6 (I2)

3901/1301                                   3931/1311
TTA AAT AAT GAT CGT TCA TCA TAT TCA TCT GTT TCT TTT ATT TTG TAA CCA CCT CTC CTG
AAT TTA TTA CTA GCA AGT AGT ATA AGT AGA CAA AGA AAA TAA AAC ATT GGT GGA GAG GAC
  F   L   S   R   E   D   Y   E   D   T   E   K   I   K   Y   G   G   R   R   N

3961/1321                                   3991/1331
TTA CCA TCA GAT CTT TCT TCT AAA ATT CCA GAT ACA CCG TCT GAT TCA TAT TCA AAC TCT
AAT GGT AGT CTA GAA AGA AGA TTT TAA GGT CTA TGT GGC AGA CTA AGT ATA AGT TTG AGA
  G   D   S   R   E   E   L   I   G   S   V   G   D   S   E   Y   E   F   E   G
```

```
4021/1341                                   4051/1351
CCC GAT TCA TCC TCT ATA TCA TCG TCT CCG GAG GTT ACG ACG CTG ATT ACT TCT CCG TTA
GGG CTA AGT AGG AGA TAT AGT AGC AGA GGC CTC CAA TGC TGC GAC TAA TGA AGA GGC AAT
  S   E   D   E   I   D   D   D   G   S   T   V   V   S   I   V   E   G   N   T

4081/1361                                   4111/1371
GTA TCT ATC AGA GTT TTC CTA CAA ACT TTT TCC ATA ATA AGC TTT TTC AAG CTA AAG CTT
CAT AGA TAG TCT CAA AAG GAT GTT TGA AAA AGG TAT TAT TCG AAA AAG TTC GAT TTC GAA
  D   I   L   T   K   R   C   V   K   E   M   I   L   K   K   L   S   F   S   I

4141/1381                                   4171/1391
ATT TTA CAC ATT ACC GCA TTT TCG AAA AAT ATA GAA GCA ACT AGT ACA GCT TTA ACC ATC
TAA AAT GTG TAA TGG CGT AAA AGC TTT TTA TAT CTT CGT TGA TCA TGT CGA AAT TGG TAG
  K   C   M   V   A   N   E   F   F   I   S   A   V   L   V   A   K   V   M   Q

4201/1401                                   4231/1411
TGA GGA TCT TTC TTC GAT AGA TGT TCT ACT TCG CTG GAT AGA GTT TTT CTA CCA GCT ACT
ACT CCT AGA AAG AAG CTA TCT ACA AGA TGA AGC GAC CTA TCT CAA AAA GAT GGT CGA TGA
  P   D   K   K   S   L   H   E   V   E   S   S   L   T   K   R   G   A   V   E

4261/1421                                   4291/1431
TCC TTG TTG TTT CTC GTA TAT TCT ATT ATA GTA GAT GAC ATT TTA TTT TTA TTA ATG TAT
AGG AAC AAC AAA GAG CAT ATA AGA TAA TAT CAT CTA CTG TAA AAT AAA AAT AAT TAC ATA
  K   N   N   R   T   Y   E   I   I   T   S   S   M   K   N   K   N   I   Y   I

4321/1441                                   4351/1451
ATA TAA CCA TCT TTA AAT GTT TTT TCA TCC ATA GTA TTT TCC GGA TC
TAT ATT GGT AGA AAT TTA CAA AAA AGT AGG TAT CAT AAA AGG CCT AG
  Y   G   D   K   F   T   K   E   D   M   T   N   E   P   D
                                                    ORF 7 (I3)
```

Fig. 7.7. Consensus Sequence of FPV BamHI-N and Open Reading Frames
Analysis of the consensus sequence was carried out using the BLAST network service at the National Centre for Biotechnology Information (NCBI), Pileup of the Wisconsin GCG package and CAD Gene version 2.0 from Genetic Technology Corporation. The nucleotide sequence of 4367 bp from pMB 282 (FPV BamHI-N) is shown with translation of major open reading frames (ORFs) shown using the single letter amino acid code.

Fig. 7.8. FPV BamHI-N Sequence and ORFs

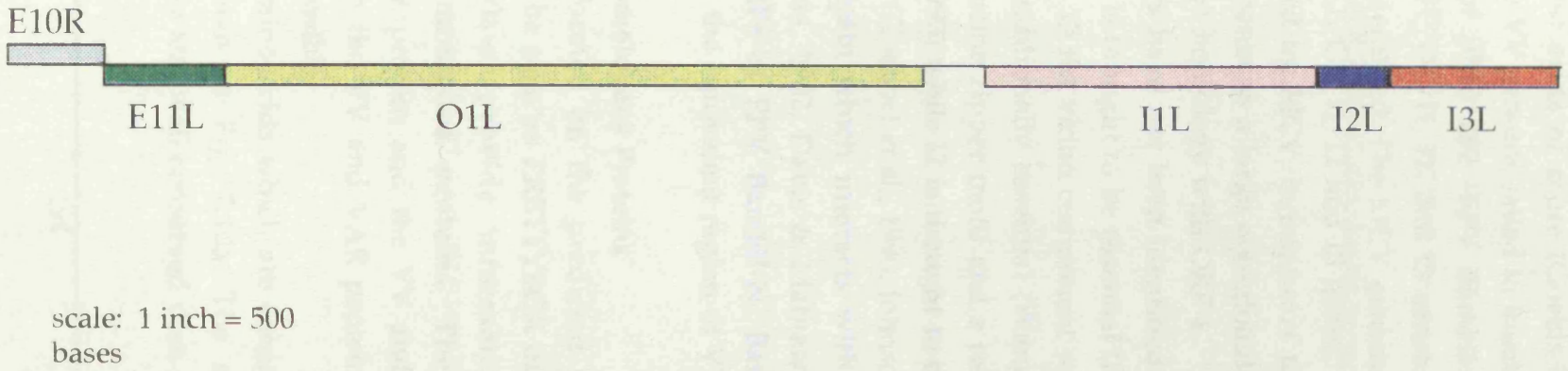


Fig. 7.8. FPV BamHI-N Sequence and ORFs
Analysis of the consensus sequence was carried out using the BLAST network service at the National Centre for Biotechnology Information (NCBI), Pileup of the Wisconsin GCG package and CAD Gene version 2.0 from Genetic Technology Corporation.

reading frames of 65 amino acids or more (Goebbel *et al.*, 1990). A BLAST search of ORF 4 against the VV genome failed to identify a homologue of ORF 4 in VV. The sequence of pMB 282 (FPV BamHI-N) appears to contain homologues of VV E10, E11, O1, I1, I2, and I3 genes. No FPV homologue of VV E3L or K3L could be identified. The MCV genome is predicted to encode homologues of VV E10, E11, O1, I1, I2 and I3 genes. A homologue of O2L is not predicted to be encoded by MCV, but appears to be replaced by a gene encoding a protein which contains a large non-globular domain (Senkevich *et al.*, 1996)MCV60315, with no homology with ORF 4.

Functions and motifs have not been identified for the products of VV E10, E11, or I1 although I1 is thought to be essential (Shchelkunov *et al.*, 1991). E11 is thought to encode a 15 Kd virion component which may be required at a threshold level or be conditionally essential (Wang & Shuman, 1996). VV O1 is thought to have a leucine zipper motif and a two part nuclear targeting sequence (Johnson *et al.*, 1993) while I2 is thought to be an acidic protein with a hydrophobic C-terminus (Goebbel *et al.*, 1990, Johnson *et al.*, 1993). VV I3 is a DNA-binding phosphoprotein which interacts with F4, the ribonucleotide small subunit (Ahn & Moss, 1992, Davis & Mathews, 1993, Slabaugh *et al.*, 1988). Comparison of ORFs of FPV BamHI-N, BamHI-P, and the partial sequence of BamHI-D with the equivalent region of VV are shown in Fig. 7.9.

7.5.2. Analysis of Homologous Proteins

PILEUPs were performed on the predicted ORFs and homologous poxvirus proteins and can be seen as PRETTYBOX diagrams in Figs. 7.10 a-f. The diagrams, Figs 7.10a-e, provide information on the degree of conservation of the homologous proteins. There is some sequence conservation between FPV protein and the VV and VAR proteins but the function of the domains in the VV and VAR proteins are not known so no positive conclusions are possible.

The domains and amino acids which are conserved in FPV and ASFV E10 homologues are shown in Fig. 7.10a. The sequence of the ASFV homologue of E10 is slightly less well conserved than that of FPV.

Fig. 7.9. Comparison of ORFs of FPV BamHI-P, N, and D with the Equivalent Region of VV

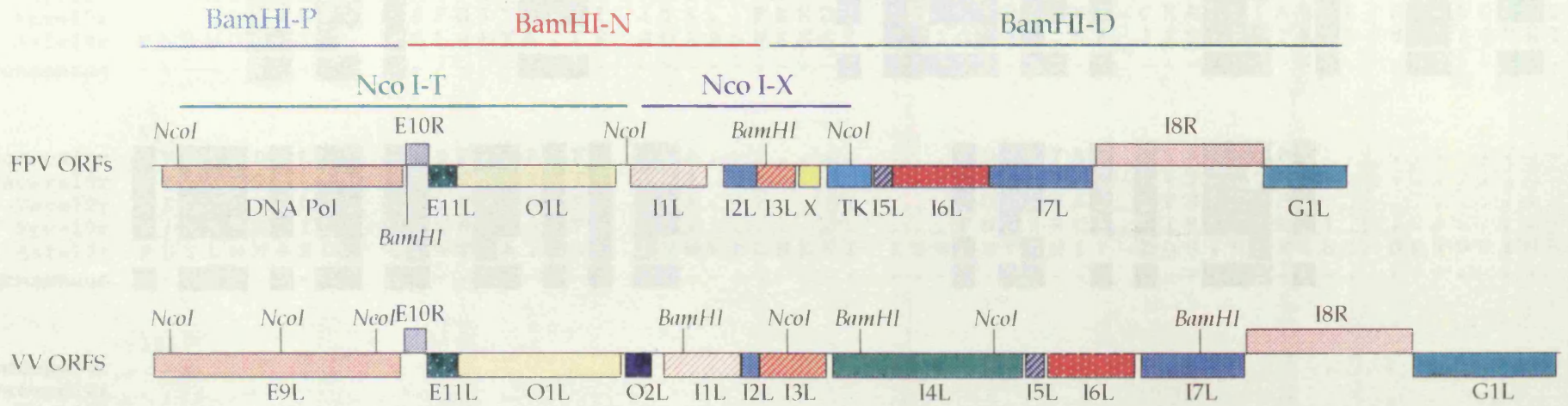


Fig. 7.9. Comparison of ORFs of FPV BamHI-P, N, and D with the Equivalent Region of VV
The sequence of pMB 282 was joined to the sequences of the surrounding fragments, BamHI-P and BamHI-D so that the ORFs of FPV homologues of E11 and I3 were complete. The equivalent region of VV is shown to enable comparison.

Fig. 7.10a. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV E10R

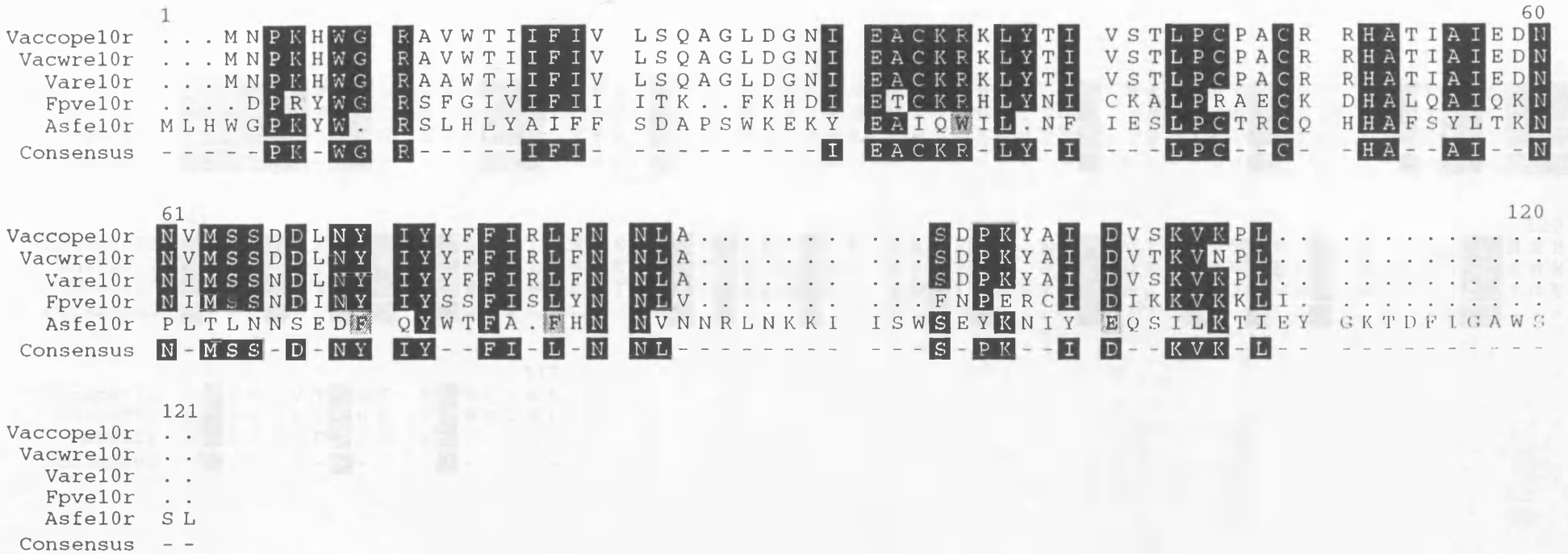


Fig. 7.10a. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV E10R
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV E10R encoded protein.
Vaccope10R = the protein encoded by Vaccinia virus (Copenhagen strain) E10R.
Vacwre10r = the protein encoded by Vaccinia virus (WR strain) E10R.
Vare10r = the protein encoded by Variola virus (India strain) E10R.
Fpve10r = the protein encoded by the Fowlpox virus homologue of VV E10R.
Asfe10r = the protein encoded by the African Swine Fever virus homologue of VV E10R.

Fig. 7.10b. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV E11L

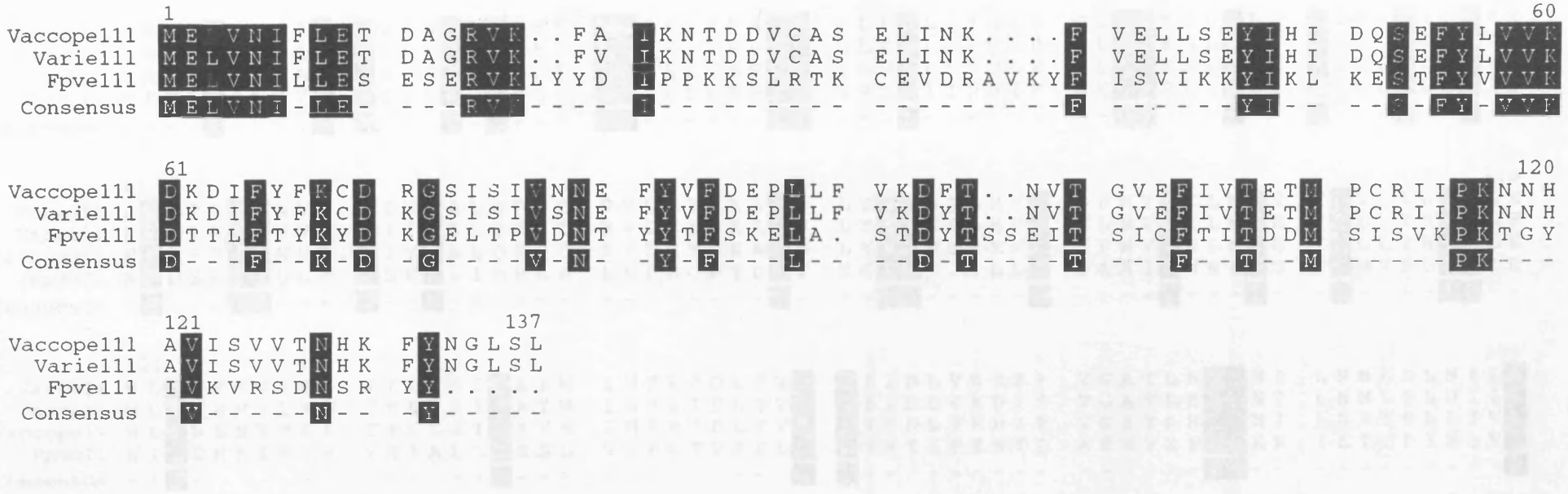


Fig. 7.10b. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV E11L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV E11L encoded protein.
Vaccope11L = the protein encoded by Vaccinia virus (Copenhagen strain) E11L.
Varie11L = the protein encoded by Variola virus (India strain) E11L.
Fpve11L = the protein encoded by the Fowlpox virus homologue of VV E11L.

Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L

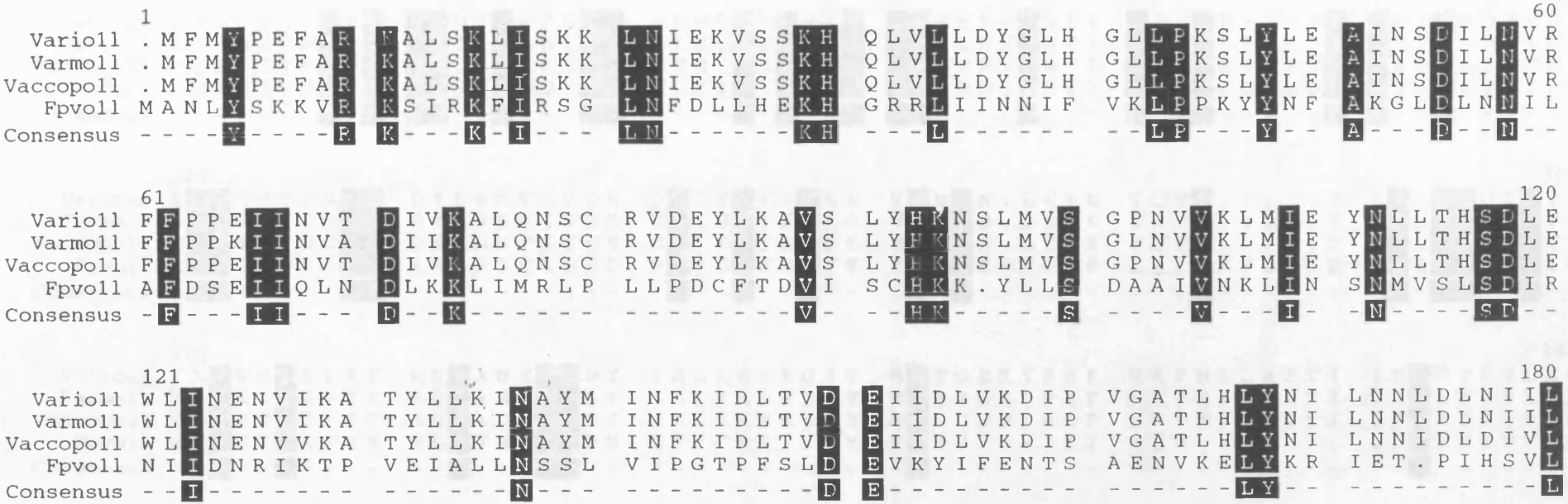


Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV O1L encoded protein.
Vario1l = the protein encoded by Variola virus (India strain) Q1L.
Varmo1l = the protein encoded by Variola virus (Major strain) Q1L.
Vaccopo1l = the protein encoded by Vaccinia virus (Copenhagen strain) O1L.
Fpvo1l = the protein encoded by the Fowlpox virus homologue of VV O1L.

Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L

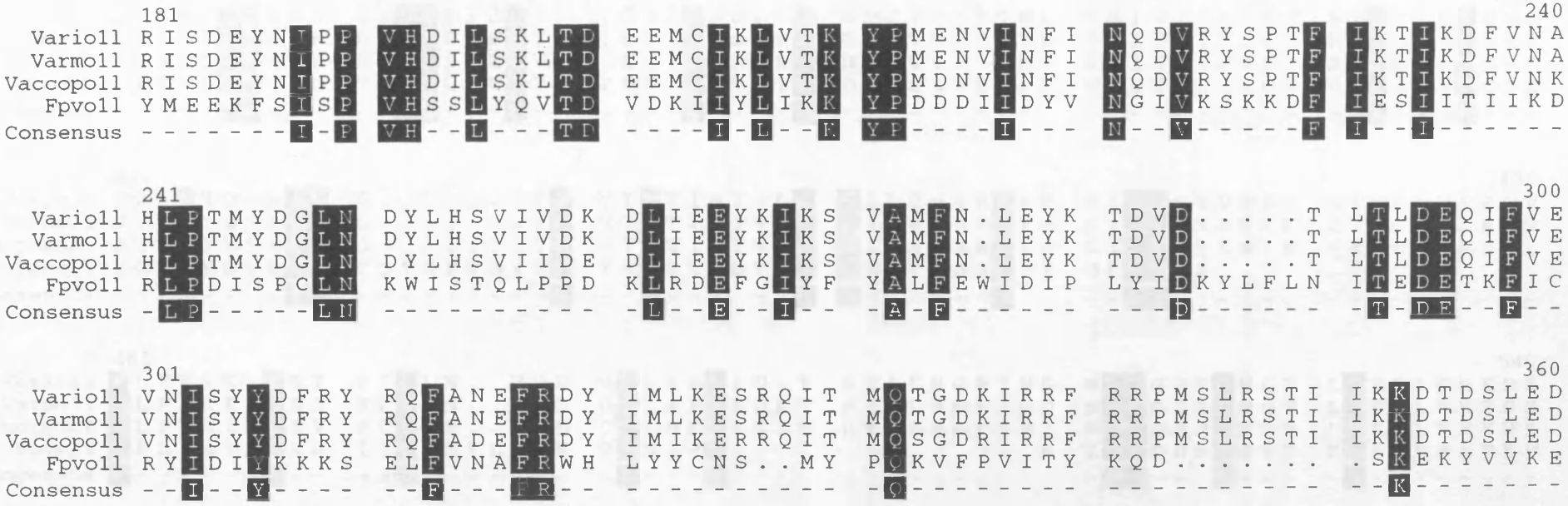


Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV O1L encoded protein.
Variol1 = the protein encoded by Variola virus (India strain) Q1L.
Varmol1 = the protein encoded by Variola virus (Major strain) Q1L.
Vaccopol1 = the protein encoded by Vaccinia virus (Copenhagen strain) O1L.
Fpvol1 = the protein encoded by the Fowlpox virus homologue of VV O1L.

Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L

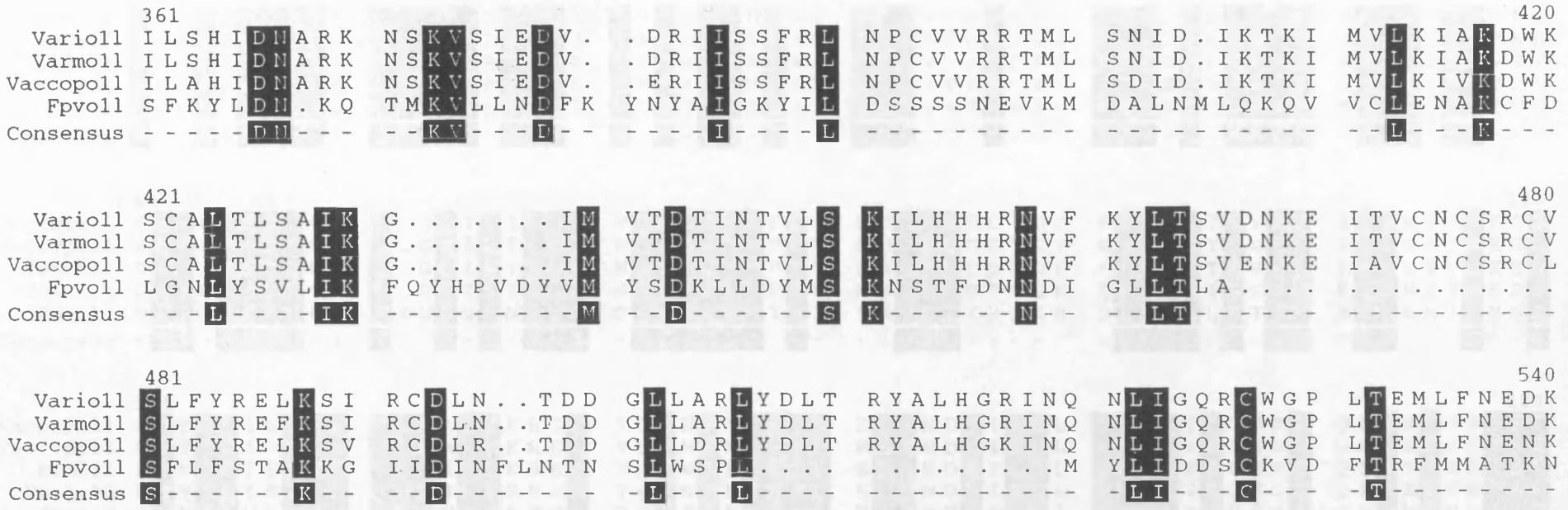


Fig. 7.10c. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV O1L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV O1L encoded protein.
Vario11 = the protein encoded by Variola virus (India strain) Q1L.
Varmo11 = the protein encoded by Variola virus (Major strain) Q1L.
Vaccopo11 = the protein encoded by Vaccinia virus (Copenhagen strain) O1L.
Fpvo11 = the protein encoded by the Fowlpox virus homologue of VV O1L.

Fig. 7.10d. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I1L

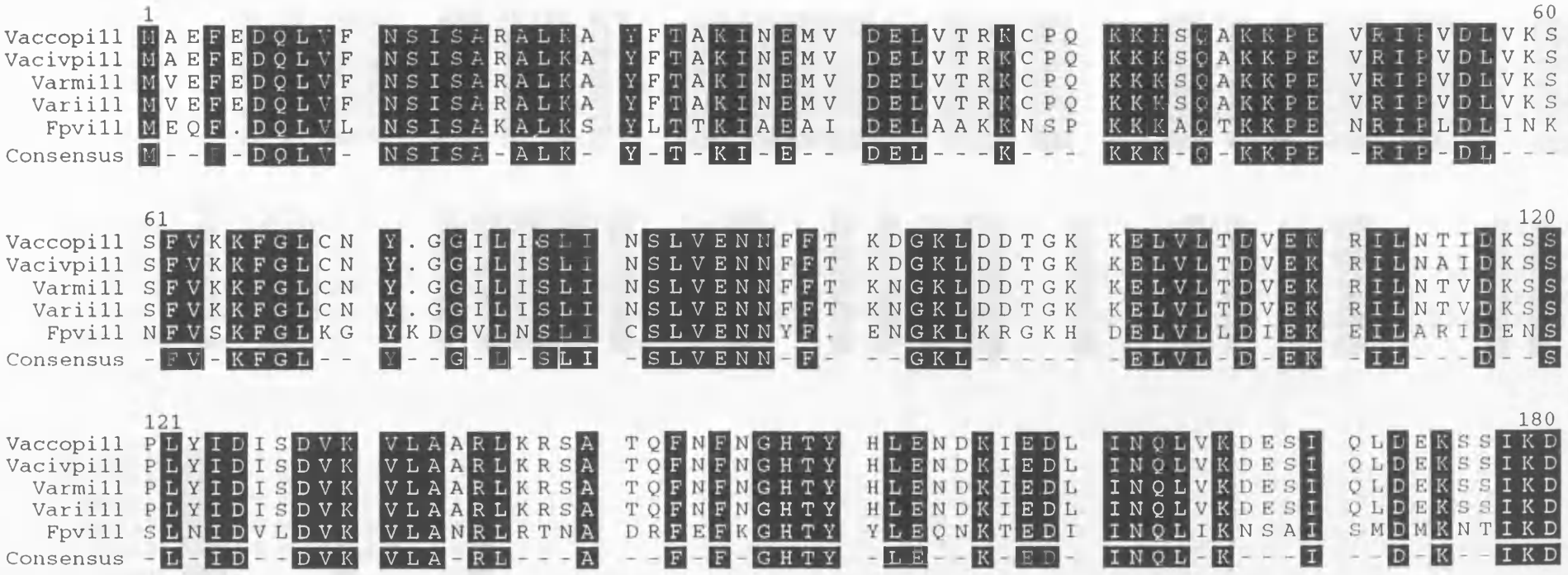


Fig. 7.10d. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I1L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV I1L encoded protein.
Vaccopi1l = the protein encoded by Vaccinia virus (Copenhagen strain) I1L.
Varivpi1l = the protein encoded by Variola virus (IVP strain) K1L.
Varmi1l = the protein encoded by Variola virus (Major strain) K1L.
Variil1 = the protein encoded by Variola virus (India strain) K1L.
Fpvil1 = the protein encoded by the Fowlpox virus homologue of VV I1L.

Fig. 7.10d. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I1L

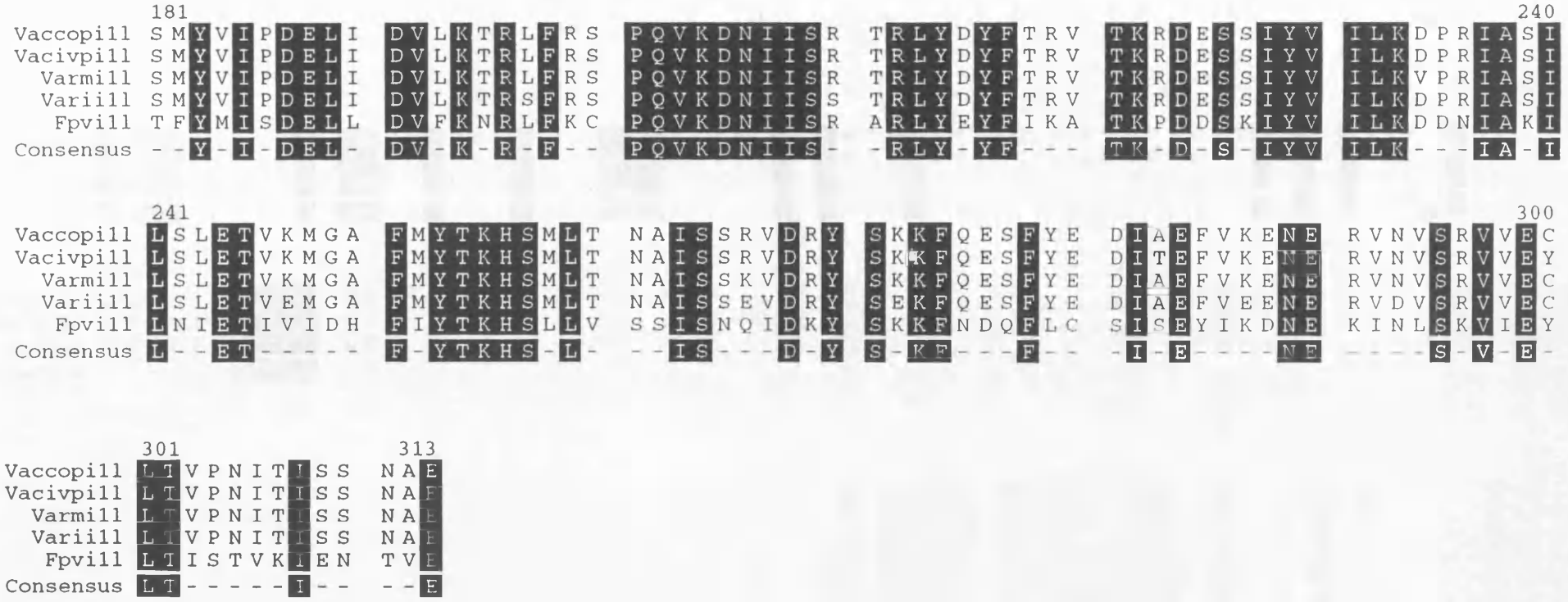


Fig. 7.10d. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I1L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV I1L encoded protein.
Vaccopi1l = the protein encoded by Vaccinia virus (Copenhagen strain) I1L.
Varivpi1l = the protein encoded by Variola virus (IVP strain) K1L.
Varmi1l = the protein encoded by Variola virus (Major strain) K1L.
Variі1l = the protein encoded by Variola virus (India strain) K1L.
Fpvi1l = the protein encoded by the Fowlpox virus homologue of VV I1L.

Fig. 7.10e. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I2L

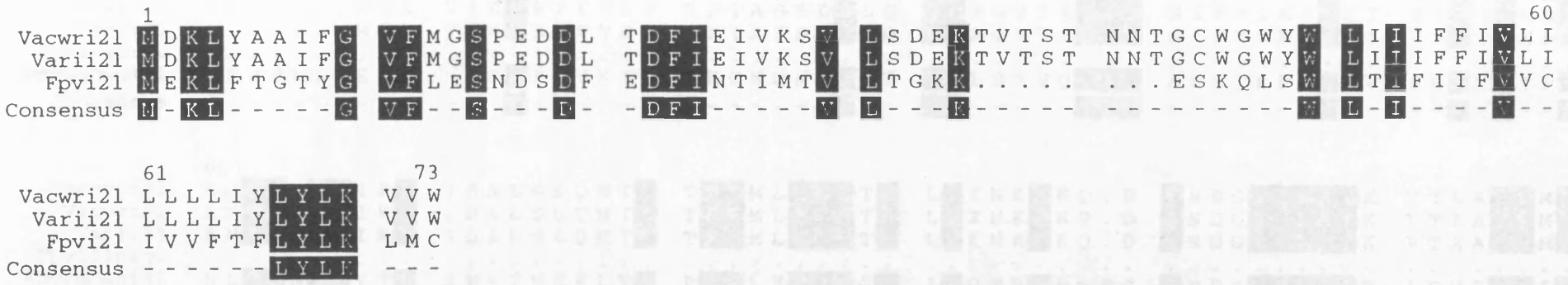


Fig. 7.10e. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I2L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV I2L encoded protein.
Vacwri2l = the protein encoded by Vaccinia virus (WR strain) I2L.
Varii2l = the protein encoded by Variola virus (India strain) K2L.
Fpvi2l = the protein encoded by the Fowlpox virus homologue of VV I2L.

Fig. 7.10f. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I3L

```
             1                                                                                    60
Vaccopi3l    .......MSK VIKKRVETSP RPTASSDSLQ TCAGVIEYAK SISKSNAKCI EYVTLNASQY
Vacwri3l     .......MSK VIKKRVETSP RPTASSDSLQ TCAGVIEYAK SISKSNAKCI EYVTLNASQY
Varii3l      .......MSK VIKKRVETSP RPTASSDSLQ TCAGVIEYAK SISKSNAKCI EYVTLNASQY
Fpvi3lprot   .......... .......... .......... .......... .......... ..........
Fpvknowni3l  MKNNLYEEKM NMSKKQVKTQ SKCNNNASRF TCLDAVQYAK ALCTKDTKIV KSVKLTPSHH
Consensus    ---------- ---K------ -------S-- TC-----YAK -------K-- --V-L--S--

             61                                                                                  120
Vaccopi3l    ANCSSISIKL TDSLSSQMTS TFIMLEGETK LYKNKSKQ.D RSDGYFLKIK VTAASPMLYQ
Vacwri3l     ANCSSISIKL TDSLSSQMTS TFIMLEGETK LYKNKSKQ.D RSDGYFLKIK VTAASPMLYQ
Varii3l      ANCSSISIKL TDSLSSQMTS TFIMLEGETK LYKNKSKQ.D RSDGYFLKIK VTAASPMLYQ
Fpvi3lprot   .......... .......... .......... .......... .......... ..........
Fpvknowni3l  NLCSNISVTL EPKYNEKLVS PFILVEGEGK IYQTRSDNFS REESYFLKIR PSVISPILHQ
Consensus    --CS-IS--L ---------S -FI--EGE-K -Y---S---- R---YFLKI- ----SP-L-Q

             121                                                                                 180
Vaccopi3l    LLEAVYGNIK HKERIPNSLH SLSVETITEK TFKDESIFI. .NKLNGAMVE YVSAG.ESSI
Vacwri3l     LLEAVYGNIK HKERIPNSLH SLSVETITEK TFKDESIFI. .NKLNGAMVE YVSTG.ESSI
Varii3l      LLEAVYGNIK HKERIPNSLH SLLVETITEK TFKDESIFI. .NKLNGAMVE YVSTG.ELSI
Fpvi3lprot   .......... ..DP...... ...ENTMDEK TFKDGYIYIN KNKMSSTIIE YTRNNKEVAG
Fpvknowni3l  MMECIYSDLG YLDP...... ...ENTMDEK TFKDGYIYIN KNKMSSTIIE YTRNNKEVAG
Consensus    --E--Y---- ---------- -----T--EK TFKD--I-I- -NK------E Y-----E---
```

Fig. 7.10f. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I3L.
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV I3L encoded protein.
Vaccopi3l = the protein encoded by Vaccinia virus (Copenhagen strain) I3L.
Vacwri3l = the protein encoded by Vaccinia virus (WR strain) I3L.
Varii3l = the protein encoded by Variola virus (India strain) K3L.
Fpvi3l = the protein encoded by the Fowlpox virus homologue of VV I3L, found in sequencing FPV BamHI-N.
Fpvknowni3l = the protein encoded by the Fowlpox virus homologue of VV I3L, found by Binns *et l*, 1992 {Binns, 1992 #162}.

Fig. 7.10f. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I3L

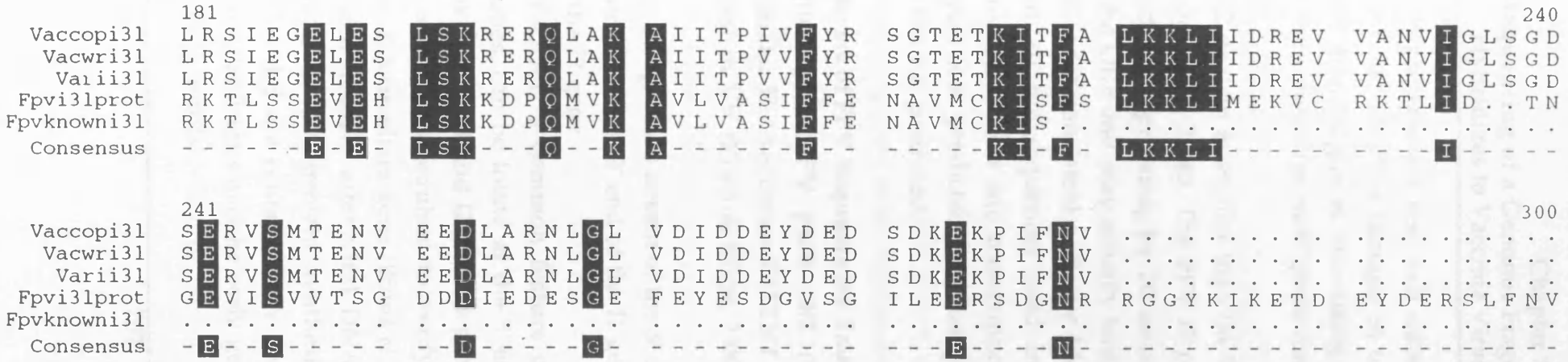


Fig. 7.10f. PRETTYBOX Diagram of Poxvirus Proteins Encoded by Homologues of VV I3L
PILEUP and PRETTYBOX of the Wisconsin GCG package were used to line up poxvirus homologues of VV I3L encoded protein.
Vaccopi3l = the protein encoded by Vaccinia virus (Copenhagen strain) I3L.
Vacwri3l = the protein encoded by Vaccinia virus (WR strain) I3L.
Varii3l = the protein encoded by Variola virus (India strain) K3L.
Fpvi3l = the protein encoded by the Fowlpox virus homologue of VV I3L, found in sequencing FPV BamH II-N.
Fpvknowni3l = the protein encoded by the Fowlpox virus homologue of VV I3L, found by Binns *et l*, 1992 [Binns, 1992 #162].

All the FPV proteins are less well conserved, relative to VV and VAR proteins, than FPV I1. VV I1 is thought to be essential (Shchelkunov *et al.*, 1991) so the FPV homologue is also likely to be essential. However the requirements of the viruses for each genes can only be determined by further experimentation.

The FPV I2 protein is smaller than the VV and VAR proteins, lacking 8 amino acids at positions 36-43. The FPV I3 protein is predicted to be larger than the VV and VAR proteins, by 20 amino acids. The FPV protein was predicted from the ORF and may actually terminate earlier than predicted and thus have a size more consistent with the VV and VAR proteins. It must be emphasised that all the sequences used in the protein comparisons are derived from predicted ORFs and transcription and translation may not start and end at the positions predicted. For example translation may start at a site down stream of the site predicted.

7.5.3. Analysis of FPV Sequence for Transcription Signals

The sequence of FPV pMB 282 (BamHI-N) was searched for transcription signals. The sequence TTTTNT is thought to be an early gene termination signal in VV (Yuen & Moss, 1987). The early termination signal was found:

1) approximately 30 bp down stream of the 3' end of the E11 gene
2) 15 bp down stream of the 3' end of the I1 gene and once within the I1 gene
3) twice within the I2 gene

The sequence TAAAT, a common feature of translation initiation sites of poxvirus late genes, can be found at the 5' ends of the I2, I1 and E11 genes. Thus it seems likely that I1 and I2 are late genes and E11 is an early/late gene although RNA analysis is required to verify these findings. The I3 gene is thought to be an intermediate gene (Binns *et al.*, 1992), a class of genes which are expressed immediately after viral DNA replication but which do not require continued *de novo* protein synthesis (Vos & Stunnenberg, 1988). Genes E10 and O1 did not contain either the early termination signal or the late promoter motif and thus may be early genes or may be under the control of unidentified FPV motifs.

Vaccinia virus transcription early promoters are thought to consist of a 16 bp critical region (AAAAgTaGAAAataTA) separated by an 11 bp less critical T-rich sequence from a 7 bp region containing a purine at which initiation usually occurs (Davison & Moss, 1989). No such FPV consensus sequence has yet been derived (personal communication Dr. M Skinner).

7.6. Discussion

PCR of FPV DNA with primers designed to FPV homologues of VV E2L and E4L resulted in a product of approximately 500 bp. The result suggests that any functional FPV homologue of VV E3L does not exist between the FPV homologues of E2L and E4L in fragment BamHI-I. It has been reported that FPV thymidine kinase (TK) gene has undergone a gene translocation relative to VV, appearing to have replaced I4, the ribonucleotide reductase large subunit gene (Binns *et al.*, 1992). It is thought that the gene translocation may have been facilitated by 15 bp repeats which flank the thymidine kinase gene (Binns *et al.*, 1992). It is possible that a transposition event may have occurred involving the FPV homologue of VV E3L, which does not exist between FPV E2L and E4L as was expected.However, the sequences flanking VV E3L do not show any sign of flanking repeats. It is equally likely that VV has acquired E3L as FPV sequence represents the ancestral poxvirus sequence. In fact, the absence of E3L from between E2L & E4L in MCV makes this more likely (Senkevich *et al.*, 1996).

In attempts to identify the genomic location of any FPV homologues of VV E3L and K3L, both degenerate oligonucleotides and the VV genes have been used as probes on FPV DNA. The E3L oligonucleotides hybridized to BamHI-B, Pst I-B, and Nco I-P fragments. Each of the K3L oligonucleotides hybridized to two different regions of the FPV genome, BamHI fragments N and G, Pst I fragments A and D/E, and Nco I fragments J and K/L. When either VV E3L or K3L DNA was used to probe a partial library of FPV Nco I fragments, clones containing a 5.3 Kbp insert (Nco I-T) were found to be positive.

In both experiments low stringency conditions (0.5 M NaCl, 50 % formamide, 30^{o}C) were used, which may have resulted in non-specific

binding of the probe to the DNA and misleading results. Low stringency conditions were required because the degree of conservation of nucleotides of homologous genes in VV and FPV may be low. It has been reported that the DNA sequence of FPV DNA polymerase, expected to be highly conserved is 60% conserved relative to the sequence of the VV gene. At the amino acid level, 48% of the amino acids encoded by the FPV gene are identical to the VV protein and a further 32% of the amino acids appear to be conservative changes (Binns *et al.*, 1987). However, levels of conservation of nucleotides in non-essential genes such as E3L and K3L (Beattie *et al.*, 1995b) may be lower than in essential genes such as the DNA polymerase (Binns *et al.*, 1990, Skinner *et al.*, 1994, Tomley *et al.*, 1988).

FASTA of the Wisconsin GCG package was used to identify the areas of homology between VV E3L and K3L DNA and FPV BamHI-N DNA, and thus attempts to show why VV E3L and VV K3L DNA hybridized to the FPV NcoI-T clone and BamHI-N and P fragments. The analysis revealed that VV E3L DNA had 59 % homology with FPV BamHI-N over a 64 bp region, and K3L DNA had homology of 81 % over a 22 bp region and also 65 % identity over another 37 bp region, shown in Appendix 7.1.

Sequencing and analysis of the pMB 282 clone (FPV BamHI-N) revealed FPV ORFs with homology to VV E10, E11, O1, I1, I2 and I3 genes. No FPV homologues of VV E3L or K3L were identified. In Southern analysis VV K3L DNA (270 bp) hybridized to both FPV BamHI-N and FPV BamHI-P, which sequencing has shown to be the sites of E10 and the DNA polymerase genes. It appears that the probes bound specifically under the conditions used but not to FPV DNA encoding homologues of E3L and K3L. Only one Nco I fragment was positive after hybridization so an alternative region for sequencing was not available. It is possible that any FPV homologues of E3L and K3L have little homology to the VV genes at the DNA level. It is also possible that the DNA of VV E3L and K3L has greater homology to the FPV BamHI-N/P junction than to any FPV homologues of the genes. FPV may not contain homologues of VV E3L and K3L at all and may rely on entirely novel mechanisms to overcome the effects of IFN. Indeed, the sequence of Canarypox has recently been analysed and no homologue of VV E3L has been

found (personal communication, Dr. E. Paoletti). The MCV genome has also recently been analysed and does not appear to encode homologues of E3L or K3L (Senkevich *et al.*, 1996).

In FPV, the O2 gene position has been replaced with ORF 4. It is possible that the O2 gene has undergone translocation and exists elsewhere in the FPV genome. It is thought that the replacement of FPV I4 with genes TK and X arose through a transposition event, involving 15 bp repeats which flank the TK/X insert (Binns *et al.*, 1992). No evidence could be found to suggest that ORF 4 is flanked by repeat regions but this may be comparable to the position in FPV where TK is 'missing'. However the possibility remains that O2 has undergone transposition. The situation may become clearer if O2 is located and analysed. In MCV the O2 position of VV is occupied by a gene which is predicted to encode a secreted protein which contains a signal peptide and an immunoglobulin domain similar to Xenopus class I histocompatibility antigen.

Comparison of the proteins encoded by FPV ORFs with the proteins of VV and VAR was undertaken. The predicted protein sequences of FPV ORFs were less conserved than the protein sequences of VV and VAR. As more poxvirus sequences become available it may be possible to identify motifs within homologous proteins and so assign functions to the proteins.

Identification of transcription signals in the ORFs was used to estimate when the FPV proteins are expressed. However, the transcription signals are based on VV research and have not been elucidated for all parts of the cascade of gene expression (Vos & Stunnenberg, 1988, Yuen & Moss, 1987). VV early terminator sequences appear to be used in FPV. Late gene promoters in FPV look similar to those in VV, although a consensus sequence for early gene promoter sequences in FPV has not yet been elucidated and so are hard to identify. However, association of a promoter or terminator consensus sequence with a gene only provides an indication of when the gene might be expressed. RNA analysis is required to genuinely establish the temporal expression of the genes.

In conclusion, FPV homologues of VV E3L and K3L are not located in fragments BamHI-N or Nco I-T which were bound by VV E3L and K3L DNA,

even though this region gave the highest hybridization signal with VV E3L and K3L probes. Unfortunately no equivalent method exists for protein homology.

Chapter 8

Conclusions

8. CONCLUSIONS

- FPV is resistant to more than 8 U/ml of IFN in CEF.
- VV is resistant to 4 U/ml of IFN in CEF.
- SFV is sensitive to less than 1U/ml of IFN in CEF.

FPV is therefore, more resistant to IFN in CEF than is VV.

- FPV is able to rescue SFV from the effects of IFN in chicken cells.
- VV is capable of rescuing SFV from the effects of IFN in CEF
- A VV mutant with E3L deleted (vP1080) was nevertheless capable of rescuing SFV in IFN-treated CEF as efficiently as was the wt virus.
- A VV with K3L deleted (vP872) had a reduced capability to rescue SFV from the effects of IFN in CEF.

K3L may be more efficient than E3L in inhibiting any avian homologue of PKR. The rescue of SFV by FPV may be due to the presence of a homologue of K3L, although other FPV encoded factors may be responsible.

- CEF extracts contain a protein(s) with the ability to phosphorylate proteins in the extracts.
- CEF extracts contain a kinase(s) which is able to bind poly IC.
- Proteins from CEF extracts which bind to poly IC are able to phosphorylate histone proteins.
- Proteins from CEF extracts which bind to poly IC are able to phosphorylate a peptide of mammalian eIF-2α.
- The activity of the kinase(s) in CEF extracts was not increased by addition of poly IC.
- The kinase(s) was not induced by IFN-treatment of the cells.
- No evidence could be found to suggest that FPV proteins reduced the activity of the kinase(s).

It is not possible to determine whether or not the kinase activity seen in these experiments is avian PKR.

- An avian homologue of PKR was not identified by PCR of a chicken lung cDNA library using primers designed against highly conserved domains of PKR

- An avian homologue of PKR was not identified by screening of the chicken cDNA library with oligonucleotides designed against highly conserved domains of PKR and human PKR DNA.
- An avian homologue of PKR was not identified by probing polyadenylated RNA from IFN-treated CEF with human PKR.

The level of conservation between mammalian and any avian PKR may not be high enough to identify an avian PKR by these methods. Avian cells may not have a homologue of mammalian PKR but this seems unlikely. Avian cells may not use the same system of regulation of expression for PKR (i.e. it may not be induced by interferon).

- An 80 Kd soluble protein is secreted by FPV infected CEF from 12-50 hours post infection.
- The protein consists of a 55 Kd polypeptide with the remainder of the molecular mass consisting of carbohydrate groups.
- In reducing conditions, the glycosylated form of the protein is a 27 Kd protein.
- The 80 Kd soluble protein produced by FPV infected cells is a multimeric glycoprotein.
- Attempts to purify the 80 Kd protein and the 27 Kd subunit using lectins proved unsuccessful as did a two-dimensional gel approach.

The function of the 80 Kd protein and the location of the gene encoding it remain unknown. The 80 Kd protein may be a soluble glycoprotein receptor which binds to IFNs or cytokines and so inhibit the host defence against the virus.

- Any functional FPV homologue of VV E3L does not exist between the FPV homologues of E2L and E4L in fragment BamHI-I
- Clones containing a 5.3 Kbp insert (Nco I-T) from a partial FPV library were hybridized by VV E3L and K3L DNA.
- Sequencing and analysis of FPV BamHI-N (which overlaps Nco I-T) revealed FPV ORFs with homology to VV E10, E11, O1, I1, I2 and I3 genes. As in MCV, the homologue of vv O2L was not present.
- No FPV homologues of VV E3L or K3L were identified by sequencing FPV BamHI-N.

FPV homologues of VV E3L and K3L have not been identified. Like MCV, FPV may have novel mechanisms to overcome the effects of IFN.

Bibliography

BIBLIOGRAPHY

Ahn, B. Y., Gershon, P. D., Jones, E. V. & Moss, B. (1990a). Identification of *rpo30*, a Vaccinia Virus RNA Polymerase Gene with Structural Similarity to a Eukaryotic Transcription Elongation Factor. *Molecular and Cellular Biology* **10**, 5433-5441.

Ahn, B. Y., Jones, E. V. & Moss, B. (1990b). Identification of a Vaccinia Virus Gene Encoding an 18-Kilodalton Subunit of RNA Polymerase and Demonstration of a 5' Poly(A) Leader on its Early Transcript. *Journal of Virology* **64**, 3019-3024.

Ahn, B. Y., Jones, E. V. & Moss, B. (1990c). Identification of the Vaccinia Virus Gene Encoding an 18-Kilodalton Subunit of RNA Polymerase and Demonstration of a 5' Poly(A) Leader on its Early Transcript. *Journal of Virology* **64**, 3019-3024.

Ahn, B.-Y. & Moss, B. (1992). Glutaredoxin Homologue Encoded by Vaccinia Virus is a Virion-Associated Enzyme with Thioltransferase and Dehydroascorbate Activities. *Proceedings of the National Academy of Science (USA)* **89**, 7060-7064.

Ahn, B. Y., Rosel, J., Cole, N. B. & Moss, B. (1992). Identification and Expression of *rpol19*, a Vaccinia Virus Gene Encoding a 19-Kilodalton DNA-Dependent RNA Polymerase Subunit. *Journal of Virology* **66**, 971-982.

Ahn, B. Y., Gershon, P. D. & Moss, B. (1994). RNA Polymerase-Associated Protein Rap94 Confers Promoter Specificity for Initiating Transcription of Vaccinia Virus Early Stage Genes. *Journal of Biological Chemistry* **299**, 833-836.

Akkaraju, G. R., Whitaker-Dowling, P., Younger, J. S. & Jagus, R. (1989). Vaccinia Specific Kinase Inhibitory Factor Prevents Translational Inhibition by Double-Stranded RNA in Rabbit Reticulocytes Lysates. *Journal of Biological Chemistry* **264**, 10321-10325.

Alcami, A. & Smith, G. L. (1992). A Soluble Receptor for Interleukin-1B Encoded by Vaccinia Virus : A Novel Mechanism of Virus Modulation of the Host Response to Infection. *Cell* **71**, 153-167.

Alcami, A. & Smith, G. L. (1995). Vaccinia, Cowpox, and Camelpox Viruses Encode Soluble Gamma Interferon Receptors with Novel Broad Species Specificity. *Journal of Virology* **69**, 4633-4639.

Alkalay, I., Yaron, A., Hatzbai, A., Jung, S., Avraham, A., Gerlitz, O., Pashut-Lavon, I. & Ben-Neriah, Y. (1995). *In Vivo* Stimulation of IκB Phosphorylation is Not Sufficient to Activate NF-κB. *Molecular and Cellular Biology* **15**, 1294-1301.

Amegadzie, B. Y., Holmes, M. H., Cole, N. B., Jones, E. V., Earl, P. L. & moss, B. (1991). Identification, Sequence, and Expression of the Gene Encoding the Second Largest Subunit of the Vaccinia Virus DNA-Dependent RNA-Polymerase. *Virology* **180**, 88-98.

Amegadzie, B. Y., Ahn, B. Y. & Moss, B. (1992). Characterization of a 7-Kilodalton Subunit of Vaccinia Virus DNA-Dependent RNA Polymerase with Structural Similarities to the Smallest Subunit of the Eukaryotic RNA Polymerase II. *Journal of Virology* **66**, 3003-3010.

Appleyard, G., Hapel, A. J. & Boulter, E. A. (1971). An Antigenic Difference Between Intracellular and Extracellular Rabbitpox Virus. *Journal of General Virology* **13**, 9-17.

Asch, B. B. & Gifford, G. E. (1970). Fowlpox Virus: Interferon Production and Sensitivity. *Proceedings of the Society For Experimental Biology and Medicine.* 135, 177-179.

Baldick, C. J. & Moss, B. (1987). Resistance of Vaccinia Virus to Rifampicin Conferred by a Single Nucleotide Substitution Near the Predicted NH2 Terminus of a Gene Encoding an Mr 62,000 Polypeptide. *Virology* **156**, 138-145.

Baldick, C. J., Keck, J. G. & Moss, B. (1992). Mutational Analysis of the Core, Spacer, and Initiator Regions of Vaccinia virus Intermediate-Class Promoters. *Journal of Virology* **66**, 4710-4719.

Baldick, C. J. & Moss, B. (1993). Characterization and Temporal Regulation of mRNAs Encoded by Vaccinia Virus Intermediate-Stage Genes. *Journal of Virology* **67**, 3515-3527.

Ball, L. A. (1979). Induction of 2'-5'-Oligoadenylate Synthetase Activity and a New Protein by Chick Interferon. *Virology* **94**, 282-296.

Banham, A. H. & Smith, G. L. (1992). Vaccinia virus B1R Encodes a 34-kDa Serine/Threonine Protein Kinase that Localizes in Cytoplasmic Factories and is Packaged into Virions. *Virology* **191**, 803-812.

Barber, G. N., Wambach, M., Wong, M., Dever, T. E., Hinnesbuch, A. G. & Katze, M. G. (1993). Translational Regulation by the Interferon-Induced Double-Stranded RNA-Activated 68 Kd Protein Kinase. *Proceedings of the National Academy of Science (USA)* **90**, 4621-4625.

Barber, G. N., Thompson, S., Lee, T. G., Strom, T., Jagus, R., Darveau, A. & Katze, M. G. (1994). The 58-Kilodalton Inhibitor of the Interferon-Induced Double-Stranded RNA-Activated Protein-Kinase is a Tetratricopeptide Repeat Protein with Oncogenic Properties. *Proceedings of the National Academy of Science (USA)* **91**, 4278-4282.

Barber, G. N., Wambach, M., Thompson, S., Jagus, R. & Katze, M. G. (1995). Mutants of the RNA-Dependent Protein-Kinase (PKR) Lacking Double-Stranded-RNA Binding Domain-I can Act as Transdominant Inhibitors and Induce Malignant Transformation. *Molecular and Cellular Biology* **15**, 3138-3146.

Baroudy, B. M., Venkatesan, S. & Moss, B. (1982). Incompletely Base-Paired Flip-Flop Terminal Loops link the Two DNA Strands of the Vaccinia Virus Genome into One Uninterrupted Polynucleotide Chain. *Cell* **28**, 315-324.

Baxby, B. & Paoletti, E. (1992). Potential Use of Non-Replicating Vectors as Recombinant Vaccines. *Vaccine* **10**, 8-9.

Bayliss, C. D., Peters, R. W., Cook, J. K., Reece, R., Howes, K., Binns, M. M. & Boursnell, M. E. (1991). A Recombinant Fowlpox Virus that Expresses the VP2 Antigen of Infectious Bursal Disease Virus Induces Protection Against Mortality Caused by the Virus. *Archives of Virology* **20**, 193-205.

Beard, C. W., Schnitzlein, W. M. & Tripathy, D. N. (1991). Protection of Chickens against Highly Pathogenic Avian Influenza Virus (H5N2) by Recombinant Fowlpox Viruses. *Avian Disease* **35**, 356-359.

Beattie, E., Tartaglia, J. & Paoletti, E. (1991). Vaccinia Virus-Encoded eIF-2α Homologue Abrogates the Antiviral Effect of Interferon. *Virology* **183**, 413-422.

Beattie, E., Denzler, K. L., Tartaglia, J., Perkus, M. E., Paoletti, E. & Jacobs, B. L. (1995a). Reversal of the Interferon-Sensitive Phenotype of a Vaccinia Virus Lacking E3L by Expression of the Reovirus S4 Gene. *Journal of Virology* **69**, 499-505.

Beattie, E., Paoletti, E. & Tartaglia, J. (1995b). Distinct Patterns of Interferon Sensitivity in Cells Infected with Vaccinia K3L- and E3L- Mutant Viruses. *Virology* 210, 254-263.

Beaud, G., Sharif, A., Topa-Masse', A. & Leadr, D. P. (1984). Ribosomal Protein S2/Sa Kinase Purified from HeLa Cells Infected with Vaccinia Virus Corresponds to the B1R Protein Kinase and Phosphorylates *in Vitro* the Viral ssDNA-Binding Protein. *Journal of General Virology* **75**, 283-293.

Belardelli, F. (1995). Role of Interferons and Other Cytokines in the Regulation of the Immune Response. *APMIS* **103**, 161-179.

Benech, P., Merlin, G., Revel, M. & Chebath, J. (1985a). 3' End Structure of the Human (2'-5') Oligo A Synthetase Gene: Prediction of Two Distinct Proteins with Cell Type-Specific Expression. *Nucleic Acids Research* **13**, 1267-1281.

Benech, P., Mory, Y., Revel, M. & Chebath, J. (1985b). Structure of Two Forms of the Interferon-Induced (2'-5') Oligo A Synthetase of Human Cells Based on cDNAs and Gene Sequences. *EMBO Journal* **4**, 2249-2256.

Beretta, L., Gabbay, M., Berger, R., Hanash, S. M. & Sonenberg, N. (1996). Expression of the Protein-Kinase PKR is Modulated by IRF-1 and is Reduced in 5q-Associated Leukemias. *Oncogene* **12**, 1593-1596.

Beutler, B. & Cerami, A. (1989). The Biology of Cachectin/TNF- A Primary Mediator of the Host Response. *Annual Review of Immunology* **7**, 625-655.

Bhat, R. A. & Thimmappaya, B. (1983). Two Small RNAs Encoded by Epstein-Barr Virus can Functionally Substitute for the Virus-Associated RNAs in the Lytic Growth of Adenovirus 5. *Proceedings of the National Academy of Science (USA)* **80**, 4789-4793.

Bhat, R. A. & Thimmappaya, B. (1985). Construction and Analysis of Additional Adenovirus Substitution Mutants Confirm the Complementation of VAI RNA Function by Two Small RNAs Encoded by Epstein-Barr Virus. *Journal of Virology* **56**, 750-756.

Binns, M. M., Stenzler, L., Tomley, F. M., Campbell, J. & Boursnell, M. E. G. (1987). Identification by a Random Sequencing Strategy of the Fowlpoxvirus DNA Polymerase Gene, its Nucleotide Sequence and Comparison with Other Viral Polymerases. *Nucleic Acids Research* **15**, 6563-6572.

Binns, M. M., Britton, B. S., Mason, C. & Boursnell, M. E. G. (1990). Analysis of the Fowlpox Virus Genome Region Corresponding to the Vaccinia Virus D6 to A1 Region: Location of, and Variation in, Non-Essential Genes in Poxviruses. *Journal of General Virology* **71**, 2873-2881.

Binns, M. M., Boursnell, M. E. G. & Skinner, M. A. (1992). Gene Translocations in Poxviruses: The Fowlpox Virus Thymidine Kinase Gene is Flanked by 15 bp Direct Repeats and Occupies the Locus Which in Vaccinia Virus is Occupied by the Ribonucleotide Reductase Large Subunit Gene. *Virus Research* **24**, 161-172.

Black, T. L., Safer, B., Hovanessian, A. & Katze, M. (1989). The Cellular 68,000 Mr Protein Kinase is Highly Autophosphorylated and Activated yet Significantly Degraded During Poliovirus Infection: Implications for Translational Regulation. *Journal of Virology* **63**, 2244-2251.

Blanden, R. V. (1970). Mechanisms of Recovery from a Generalised Viral Infection: Mousepox. I. The Effects of Anti-Thymocyte Serum. *Journal of Experimental Medicine* **132**, 1035-1053.

Blanden, R. V. (1971). Mechanisms of Recovery from a Generalised Viral Infection: Mousepox. III. Regression of Infectious Foci. *Journal of Experimental Medicine* **133**, 1090-1104.

Blank, V., Kourilsky, P. & Israel, A. (1992). Nk-κB and Related Proteins: Rel/Dorsal Homologies Meet Ankyrin-Like Repeats. *Trends in Biochemical Science* 17, 135-140.

Boone, R. F. & Moss, B. (1977). Methylated 5'-Terminal Sequences of Vaccinia Virus mRNA Species Made *In Vivo* at Early and Late Times after Infection. *Virology* **79**, 67-80.

Boshkov, L. K., Macen, J. L. & McFadden, G. (1992). Virus-Induced Loss of Class I MHC Antigens from the Surface of Cells infected with Myxoma Virus and Malignant Rabbit Fibroma Virus. *Journal of Immunology* **148**, 881-887.

Boulter, E. A. (1969). Protection Against Poxviruses. *Proceedings of the Royal Society of Medicine* **62**, 295-297.

Boulter, E. A., Zwartouw, H. T., Timuss, D. H. I. & Maber, H. W. (1971). The Nature of the Immune State Produced by Inactivated Vaccinia Virus in Rabbits. *American Journal of Epidemiology* **94**, 612-620.

Boulter, E. A. & Appleyard, G. (1973). Differences Between Extracellular and Intracellular Forms of Poxviruses and their Implications. *Progress in Medical Virology* **16**, 86-108.

Boursnell, M. E., Green, P. F., Campbell, J. I., Deuter, A., Peters, R. W., Tomley, F. M., Samson, A. C., Emmerson, P. T. & Binns, M. M. (1990). A Fowlpox Virus Vaccine Vector with Insertion Sites in the Terminal Repeats: Demonstration of its Efficacy Using the Fusion Gene of Newcastle Disease Virus. *Veterinary Microbiology* **23**, 305-316.

Boyle, D. B., Coupar, B. E., Gibbs, A. J., Seigman, L. J. & Both, G. W. (1987). Fowlpox Virus Thymidine Kinase: Nucleotide Sequence and Relationship to other Thymidine Kinases. *1987* **156**, 355-366.

Brinkmann, V., Geiger, T., Alkan, S. & Heusser, C. H. (1993). Interferon Alpha Increases the Frequency of Interferon Gamma-Producing Human CD4+ T Cells. *Journal of Experimental Medicine* **178**, 1655-1663.

Brouty-Boye, D. & Zetter, B. R. (1980). Inhibition of Cell Motility by Interferon. *Science* **208**, 516-518.

Broyles, S. S. & Moss, B. (1986). Homology Between RNA Polymerases of Poxviruses, Prokaryotes and Eukaryotes: Nucleotide Sequence and Transcriptional Analysis of Vaccinia Virus Genes Encoding 147-kDa and 22-kDa Subunits. *Proceedings of the National Academy of Science (USA)* **83**, 3141-3145.

Broyles, S. S. & Fesler, B. S. (1990). Vaccinia Virus Gene Encoding a Component of the Viral Early Transcription Factor. *Journal of Virology* **64**, 1523-1529.

Broyles, S. S. & Pennington, M. J. (1990). Vaccinia Virus Gene Encoding a 30 Kd Subunit of the Viral DNA-Dependent RNA Polymerase. *Journal of General Virology* **64**.

Broyles, S. S. (1993). Vaccinia Virus Encodes a Functional dUTPase. *Virology* **195**, 863-865.

Brutkiewicz, R. R., Klaus, S. J. & Welsh, R. M. (1992). Window of Vulnerability of Vaccinia Virus-Infected Cells to Natural Killer (NK) Cell-Mediated Cytolysis Correlates with Enhanced NK Cell Triggering and is Concomitant with a Decrease in H-2 Class I Antigen Expression. *Natural Immunity* **11**, 203-214.

Buller, R. M. L., Holmes, K. L., Hugin, A., Frederickson, T. N. & Morse III, H. C. (1987). Induction of Cytotoxic T-cell Responses *in Vivo* in the Absence of CD4 Helper Cells. *Nature* **328**, 77-79.

Bycroft, M., Grünert, S., Murzin, A. G., Proctor, M. & St. Johnston, D. (1995). NMR Solution Structure of a dsRNA Binding Domain from *Drosophila* Staufen Protein Reveals Homology to the N-Terminus of Ribosomal Protein S. *The EMBO Journal* **14**, 3563-3571.

Campbell, J. I. A., Binns, M. M., Tomley, F. M. & Boursnell, M. E. G. (1989). Tandem Repeated Sequences within the terminal Region of the Fowlpox Virus Genome. *Journal of General Virology* **70**, 145-154.

Carroll, K., Elroystein, O., Moss, B. & Jagus, R. (1993). Recombinant Vaccinia Virus K3L Gene-Product Prevents Activation of Double-Stranded RNA-Dependent, Initiation Factor-2-Alpha-Specific Protein-Kinase. *Journal of Biological Chemistry* **268**, 12837-12842.

Cayley, P. J., White, R. F., Antoniw, J. F., Walesby, N. J. & Kerr, I. M. (1982). Distribution of the ppp(A2'p)nA-Binding Protein and Interferon-Related Enzymes in Animals, Plants, and Lower Eukaryotes. *Biochemical and Biophysical Research Communications* 108, 1243-1250.

Chang, H., Watson, J. C. & Jacobs, B. L. (1992). The E3L Gene of Vaccinia Virus Encodes an Inhibitor of the Interferon-Induced, Double-Stranded RNA-Dependent Protein Kinase. *Proceedings of the National Academy of Science (USA)* **89**, 4825-4829.

Chang, H. & Jacobs, B. L. (1993). Identification of a Conserved Motif that is Necessary for the Binding of Vaccinia Virus E3L Gene to Double-Stranded RNA. *Virology* **194**, 537-547.

Chang, H.-W., Uribe, L. H. & Jacobs, B. L. (1995). Rescue of Vaccinia Virus Lacking the E3L Gene by Mutants of E3L. *Journal of Virology* **69**, 6605-6608.

Chebath, J., Benech, P., Hovanessian, A., Galabru, J. & Revel, M. (1987). Four Different Forms of Interferon-Induced 2'5'-Oligo(A) Synthetase Identified By Immunoblotting in Human Cells. *Journal of Biological Chemistry* **262**, 3852-3857.

Chen, J. J. & London, I. M. (1995). Regulation of Protein-Synthesis by Heme-Regulated eIF-2-Alpha Kinase. *Trends in Biochemical Sciences* **20**, 105-108.

Chong, K. L., Feng, L., Schappert, K., Meurs, E., Donahue, T. F., Frieson, J. D., Hovanessian , A. G. & Williams, B. R. G. (1992). Human P68 Kinase Exhibits Growth Suppression in Yeast and Homology to the Translational Regulator GCN2. *EMBO Journal* **11:4**, 1553-1562.

Clarke, P. A., Sharp, N. A. & Clemens, M. J. (1990). Translational Control by the Epstein-Barr Virus Small RNA EBER-1. *European Journal of Biochemistry* **193**, 635-641.

Clarke, P. A., Pe'ery, T., Ma, Y. & Mathews, M. B. (1994). Structural Features of Adenovirus 2 Virus-Associated RNA Required for Binding to the Protein Kinase DAI. *Nucleic Acids Research* **22**, 4364-4374.

Clemens, M. J., Morris, A. G. & Gearing, A. J. H. (1985). Lymphokines and Interferons: A Practical Approach. In *The Practical Approach Series*. Edited by D. Rickwood & B. D. Homes. Oxford: IRL Press Ltd.

Clemens, M. J., Laing, K. G., Jeffrey, I. W., Schofield, A., Sharp, T. V., Elia, A., Matys, V., James, M. C. & Tilleray, V. J. (1994). Regulation of the Interferon-Inducible eIF-2-Alpha Protein-Kinase by Small RNAs. *Biochimie* **76**, 770-778.

Cole, J. L., Carroll, S. S. & Kuo, L. C. (1996). Stoichiometry of 2',5'-Oligoadenylate-Induced Dimerization of Ribonuclease L. *Journal of Biological Chemistry* **271**, 3979-3981.

Cooper, J. A., Wittek, R. & Moss, B. (1981). Hybridization Selection and Cell-Free Translation of mRNAs Encoded Within the Inverted Terminal Repetition of the Vaccinia Virus Genome. *Journal of Virology* **37**, 284-294.

Cosentino, G. P., Venkatesan, S., Serluca, F. C., Green, S. R., Mathews, M. B. & Sonenberg, N. (1995). Double-Stranded-RNA-Dependent Protein-Kinase and TAR RNA-Binding Protein Form Homodimers and Heterodimers In-Vivo. *Proceedings of the National Academy of Science (USA)* **92**, 9445-9449.

Coupar, B. E., Teo, T. & Boyle, D. B. (1990). Restriction Endonuclease Mapping of the Fowlpox Virus Genome. *Virology* **179**, 159-167.

Cudmore,S., Cossart, P., Griffiths, G., Way, M. (1995). Actin-Based Motility of Vaccinia Virus. *Nature* **378**, 636-638.

Davies, M. V., Elroy-Stein, O., Jagus, R., Moss, B. & Kaufman, R. J. (1992). The Vaccinia Virus K3L Gene Product Potentiates Translation by Inhibiting Double-Stranded RNA-Activated Protein Kinase and Phosphorylation of the Alpha Subunit of Eukaryotic Initiation Factor 2. *Journal of Virology* **66**, 1943-1950.

Davies, M. V., Chang, H. W., Jacobs, B. L. & Kaufman, R. J. (1993). The E3L and K3L Vaccinia Virus Gene-Products Stimulate Translation Through Inhibition of the Double-Stranded RNA-Dependent Protein-Kinase by Different Mechanisms. *Journal of Virology* **67**, 1688-1692.

Davis, B. D., Dulbecco, R., Eisen, H. N. & Ginsberg, H. S. (1980). Microbiology, 3rd edn, pp. 851-1275. Philadelphia: Harper International.

Davis, R. E. & Mathews, C. K. (1993). Acidic C Terminus of Vaccinia Virus DNA-Binding Protein Interacts With Ribonucleotide Reductase. *Proceedings of the National Academy of Science (USA)* **90**, 745-749.

Davis, S. & Watson, J. C. (1996). *In-Vitro* Activation of the Interferon-Induced, Double-Stranded RNA- Dependent Protein-Kinase PKR by RNA, from the 3'-Untranslated Regions of Human Alpha-Tropomyosin. *Proceedings of the National Academy of Sciences (USA)* **93**, 508-513.

Davison, A. J. & Moss, B. (1989). Structure of Vaccinia Virus Early Promoters. *Journal of Molecular Biology* **210**, 749-769.

Davison, T. F., Morris, T. R. & Payne, L. N. (1996). Poultry Immunology. In *Poultry Science Symposium Series*, pp. 149-151. Edited by B. M. Freeman. Abingdon: Carfax Publishing Company.

De-Haro, C., De-Herreros, A. G. & Ochoa, S. (1985). Protein Phosphorylation and Translational Control in Reticulocytes: Activation of the Heme-Controlled Translational Inhibitor by Calcium Ions and Phospholipid. *Current Topics in Cell Regulation* **27**, 63-81.

DeLange, A. M. & McFadden, G. (1986). Sequence-Nonspecific Replication of Transfected Plasmid DNA in Poxvirus-Infected Cells. *Proceedings of the National Academy of Science (USA)* **83**, 614-618.

DeLange, A. M. R., M., Scraba, D., Upton, C. & McFadden, G. (1986). Replication and Resolution of Cloned Poxvirus Telomeres *in Vivo* Generates Linear Minichromosomes with Intact Viral Hairpin Termini. *Journal of Virology* **59**, 249-259.

DeLange, A. M. & McFadden, G. (1990). The Role of Telomeres in Poxvirus DNA Replication. *Current Topics in Microbial Immunology* **163**, 71-92.

De Maeyer, E. & De Maeyer-Guignard, J. (1988). Macrophages as IFN Producers and Interferons as Modulators of Macrophage Activity. In *Interferons and Other Regulatory Cytokines*, pp. 194-219: John Wiley & Sons, Inc.

Denzler, K. L. & Jacobs, B. L. (1994). Site-Directed Mutagenic Analysis of Reovirus σ3 Protein Binding dsRNA. *Virology* **204**, 190-199.

Der, S. D. & Lau, A. S. (1995). Involvement of the Double-Stranded-RNA-Dependent Kinase PKR in Interferon Expression and Interferon-Mediated Antiviral Activity. *Proceedings of the National Academy of Science (USA)* **92**, 8841-8845.

Desai, S. Y., Patel, R. C., Sen, G. C., Malhotra, P., Ghadge, G. D. & Thimmapaya, B. (1995). Activation of Interferon-Inducible 2'-5'-Oligoadenylate Synthetase by Adenoviral VAI RNA. *Journal of Biological Chemistry* **270**, 3454-3461.

Dever, T. E., Chen, J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G. & Hinnebusch, A. G. (1993). Mammalian Eukaryotic Initiation Factor 2a Kinases Functionally Substitute for GCN2 Protein Kinase in the GCN4 Translational Control Mechanism of Yeast. *Proceedings of the National Academy of Science (USA)* 90, 4616-4620.

Diaz-Guerra, M., Khan, J. S. & Esteban, M. (1993). A Mutation of the Nucleoside Triphosphate Phosphohydrolase (NPH-1) Gene Confers Sensitivity of Vaccinia Virus to Interferon. *Virology* **197**, 485-491.

DiDonato, J. A., Mercurio, F. & Karin, M. (1995). Phosphorylation of IκBα Precedes but Is Not Sufficient for Its Dissociation from NF-κB. *Molecular and Cellular Biology* **15**, 1302-1311.

Digby, M. R. & Lowenthal, J. W. (1995). Cloning and Expression of the Chicken Interferon-γ Gene. *Journal of Interferon and Cytokine Research* **15**, 939-945.

Dobranzski, P., Ryseck, R. & Bravo, R. (1994). Differential Interactions of Rel-N-κB Complexes with IκBα Determine Pools of Constitutive and Inducible NF-κB. *The EMBO Journal* **13**, 4608-4616.

Donzé, O., Jagus, R., Koromilas, A. E., Hershey, J. W. B. & Sonenberg, N. (1995). Abrogation of Translation Initiation Factor eIF-2 Phosphorylation Causes Malignant Transformation of NIH 3T3 Cells. *The EMBO Journal* **14**, 3828-3834.

Drillien, R., Spehner, D., Villeval, D. & Lecocq, J. (1987). Similar Genetic Organization Between a Region of Fowlpox Virus DNA and the Vaccinia Virus HindIII J Fragment Despite Divergent Location of the Thymidine Kinase Gene. *Virology* **160:1**, 203-209.

Dubochet, J., Adrian, M., Richter, K., Garces, J. & Wittek, R. (1994). Structure of Intracellular Mature Vaccinia Virus Observed by Cryoelectron Microscopy. *Journal of Virology* **68**, 1935-1942.

Dubois, M. F., Hovanessian, A. G. & Bensuade, O. (1991). Heat Shock-Induced Denaturation of Proteins : Characterization of the Insolubilization of the Interferon-Induced P68 Kinase. *Journal of Biological Chemistry* **266**, 9707-9711.

Dvorak, H. F. & Hirsch, M. S. (1971). Role of Basophilic Leukocytes in Cellular Immunity to Vaccinia Virus Infection. *Journal of Immunology* **107**, 1576-1582.

Earl, P. L., Jones, E. V. & Moss, B. (1986). Homology Between DNA Polymerases of Poxviruses, Herpesviruses, and Adenoviruses; Nucleotide Sequence of Vaccinia Virus DNA Polymerase Gene. *Proceedings of the National Academy of Science (USA)* **83**, 3659-36663.

Ehrenfield, E. & Hunt, T. (1971). Double-Stranded Poliovirus RNA Inhibits Initiation of Protein Synthesis by Reticulocyte Lysates. *Proceedings of the National Academy of Science (USA)* **68**, 1075-1078.

Ehrenfield, E. (1982). Poliovirus-Induced Inhibition of Host-Cell Protein Synthesis. *Cell* **28**, 435-436.

Enari, M., Hug, H. & Nagata, S. (1995). Involvement of an ICE-Like Protease in Fas-Mediated Apoptosis. *Nature* **375**, 78-81.

Engelstad, M. & Smith, G. L. (1993). The Vaccinia Virus 42-kDa Envelope Protein is Required for the Envelopment and Egress of Extracellular Virus and Virus Virulence. *Virology* **194**, 627-637.

Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N. & Hershey, J. B. (1982). Inhibition of HeLa Cell Protein Synthesis Following Poliovirus Infection Correlates with the Proteolysis of a 220,000-Dalton Polypeptide Associated with Eucaryotic Initiation Factor 3 and a Cap Binding Complex. *Journal of Biological Chemistry* **257**, 14806-14810.

Feng, G., Chong, K., Kumar, A. & Williams, B. R. G. (1992). Identification of the Double-Stranded RNA-Binding Domains in the Interferon-Induced Double-Stranded RNA-Activated P68 Kinase. *Proceedings of the National Academy of Science (USA)* **89**, 5447-5451.

Fenner, F., Wittek, R. & Dumbell, K. R. (1989). The Orthopoxviruses. London: Academic Press, Inc.

Fenner, F. (1990). Poxviruses. In *Virology*, 2 edn, pp. 2133-2131. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press, Ltd.

Field, A. K., Tytell, A. A., Lampson, G. P. & Hilleman, M. R. (1967). Inducers of Interferon and Host Resistance, II. Multistranded Synthetic Polynucleotide Complexes. *Biochemistry* **58**, 1004-1010.

Finkelman, F. D., Svetic, A., Gresser, I., Snapper, C., Holmes, J., Trotta, P. P., Katona, I. M. & Gause, W. C. (1991). Regulation by Interferon α of Immunoglobulin Isotype Selection and Lymphokine Production in Mice. *Journal of Experimental Medicine* **174**, 1179-1188.

Freidman, R. M., Metz, D. H., Esteban, R. M., Tovell, D. R., Ball, L. A. & Kerr, I. M. (1972). Mechanism of Interferon Action: Inhibition of Viral Messenger Ribonucleic Acid Translation in L-Cell Extracts. *Journal of Virology* **10**, 1184-1198.

Fruend, Y. R., Dedrick, R. L. & Jones, P. P. (1990). *Cis*-acting Sequences Required for Class II Gene Regulation by Interferon γ and Tumour Necrosis Factor α in a Murine Macrophage Cell Line. *Journal of Experimental Medicine* **171**, 1283-1299.

Fulginiti, V. A., Kempe, C. H., Hathaway, W. E., Pearlman, D. S., Serber, J. O. F., Eller, J. J., Joyner, S. J. J. & Roinson, A. (1968). Progressive Vaccinia in Immunologically Deficient Individuals. In *Immunologic Deficiency Diseases in Man*, pp. 129-145. Edited by D. Bergsma. New York: National Foundation March of Dimes.

Gajewski, T. F. & Fitch, F. W. (1988). Anti-Proliferative Effect of IFN-gamma in Immune Regulation. IFN-Gamma Inhibits the Proliferation of Th2 but Not Th1 Murine Helper T Lymphocyte Clones. *Journal of Immunology* **140**, 4245-4252.

Galabru, J. & Hovanessian, A. (1987). Autophosphorylation of the Protein Kinase Dependent on Double-Stranded RNA. *Journal of Biological Chemistry* **262**, 15538-15544.

Galabru, J., Katze, M. G., Robert, N. & Hovanessian, A. G. (1989). The Binding of Double-Stranded RNA and Adenovirus VAI RNA to the Interferon-Induced Protein Kinase. *European Journal of Biochemistry* **178**, 581-589.

Gale, M., Tan, S. L., Wambach, M. & Katze, M. G. (1996). Interaction of the Interferon-Induced PKR Protein-Kinase with Inhibitory Proteins P58(Ipk) and Vaccinia Virus K3L is Mediated by Unique Domains - Implications for Kinase Regulation. *Molecular and Cellular Biology* **16**, 4172-4181.

Gatignol, A., Buckler, C. & Jeang, K. (1993). Relatedness of an RNA-Binding Motif in Human Immunodeficiency Virus Type 1 TAR RNA-binding Protein TRBP to Human P1/dsI Kinase and *Drosophila Staufen. Molecular and Cellular Biology* **13**, 2193-2202.

Gershon, P. D. & Moss, B. (1990). Early Transcription Factor Subunits are Encoded by Vaccinia Virus Late Genes. *Proceedings of the National Academy of Science (USA)* **87**, 4401-4405.

Geshelin, P. & Berns, K. I. (1974). Characterization and Localization of the Naturally Occurring Cross-Links in Vaccinia Virus. *Journal of Molecular Biology* **88**, 785-796.

Gessani, S., Belardelli, F., Pecorelli, A., Pudda, P. & Baglioni, C. (1989). Bacterial Lipopolysaccharide and Gamma Interferon Induce Transcription of Beta Interferon mRNA and Interferon Secretion in Murine Macrophages. *Journal of Virology* **63**, 2785-2789.

Ghadge, G. D., Malhotra, P., Furtado, M. R., Dhar, R. & Thimmapaya, B. (1994). *In-Vitro* Analysis of Virus-Associated RNA-I (VAI RNA) - Inhibition of the Double-Stranded RNA-Activated Protein-Kinase PKR by VAI RNA Mutants Correlates with the *in Vivo* Phenotype and the Structural Integrity of the Central Domain. *Journal of Virology* **68**, 4137-4151.

Giantini, M. & Shatkin, A. J. (1989). Stimulation of Chloramphenicol Acetyltransferase mRNA Translation by Reovirus Capsid Polypeptide σ3 in Cotransfected COS Cells. *Journal of Virology* **63**, 2415-2421.

Glickman, J. N., Howe, G. & Steitz, J. A. (1988). Structural Analyses of EBER-1 and EBER-2 Ribonucleoprotein Particles Present in Epstein-Barr Virus-Infected Cells. *Journal of Virology* **62**, 902-911.

Goebbel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P. & Paoletti, E. (1990). The Complete DNA Sequence of Vaccinia Virus. *Virology* **179**, 247-266.

Green, S. R. & Mathews, M. B. (1992). Two RNA-Binding Motifs in the Double-Stranded RNA-Activated Protein Kinase, DAI. *Genes and Development* **6**, 2478-2490.

Green, S. R., Manche, L. & Mathews, M. B. (1995). Two Functionally Distinct RNA-Binding Motifs in the Regulatory Domain of the Protein Kinase DAI. *Molecular and Cellular Biology* **15**, 358-364.

Gresser, I. & Tovey, M. G. (1978). Antitumour Effects of Interferon. *Biochimica et Biophysica Acta* **516**, 231-247.

Gresser, I. (1984). The Effect of Interferon on the Expression of Surface Antigens. In *Interferon And The Immune System*, pp. 113-132. Edited by J. Vilcek & E. De Maeyer. Amsterdam: Elsevier Science Publishing Company, Inc.

Gunnery, S., Rice, A. P., Robertson, H. D. & Mathews, M. B. (1990). Tat-Responsive Region RNA of Human Immunodeficiency Virus 1 can Prevent Activation of the Double-Stranded RNA-Activated Protein Kinase. *Proceedings of the National Academy of Science (USA)* **87**, 8687-8691.

Gunnery, S., Green, S. R. & Mathews, M. B. (1992). Tat-Responsive Region RNA of Human Immunodeficiency Virus Type 1 Stimulates Protein Synthesis *in Vivo* and *in Vitro* : Relationship Between Structure and Function. *Proceedings of the National Academy of Science (USA)* **89**, 11557-11561.

Gusella, G. L., Musso, T., Rottschafer, S. E., Pulkki, K. & Varesio, L. (1995). Potential Requirement of a Functional Double-Stranded RNA-dependent Protein-Kinase (PKR) for the Tumoricidal Activation of Macrophages by Lipopolysaccharide or IFN-Alpha-Beta, but Not IFN-gamma. *Journal of Immunology* **154**, 345-354.

Haines, G. K., Becker, S., Ghadge, G., Kies, M., Pelzer, H. & Radosevich, J. A. (1993). Expression of Double-Stranded RNA-Dependent Protein Kinase (P68) in Squamous Cell Carcinoma of the Head and Neck Region. *Archives of Otolaryngology Head and Neck Surgery* **119**, 1142-1147.

Hanash, S., Beretta, L., Barcroft, C. L., Sheldon, S., Glover, T. W., Ungar, D. & Sonenberg, N. (1993). Mapping of the Gene for Interferon Inducible Double-Stranded RNA-Dependent Protein Kinase to Chromosome Region 2p21-22 : A Site of Rearrangement in Myeloproliferative Disorders. *Genes, Chromosomes and Cancer* **8**, 34-37.

Hanks, S. K., Quinn, A. M. & Hunter, T. (1988). The Protein Kinase Family : Conserved Features and Deduced Phylogeny of the Catalytic Domains. *Science* **241**, 42-51.

Harada, H., Willison, K. & Sakakibara, J. (1990). Absence of the Type I IFN System in EC Cells: Transcriptional Activator (IRF-1) and Repressor (IRF-2) Genes are Develpomentally Regulated. *Cell* **63**, 303-312.

Henry, G. L., McCormack, S. J., Thomis, D. C. & Samuel, C. E. (1994). Mechanism of Interferon Action - Translational Control and the RNA-Dependent Protein-Kinase (PKR) - Antagonists of PKR Enhance the Translational Activity of Messenger-RNAs that Include a 161-Nucleotide Region from Reovirus-S1 Messenger-RNA. *Journal of Biological Regulators and Homeostatic Agents* **8**, 15-24.

Hershey, J. W. B. (1993). Introduction to Translational Initiation Factors and Their Regulation by Phosphorylation. *Seminars in Virology* **4**, 201-207.

Hiddinga, H. J., Crum, C. J., Hu, J. & Roth, D. A. (1988). Viroid Induced Phosphorylation of a Host Protein Related to a ds.RNA-Dependent Protein Kinase. *Science* **22**, 451-453.

Hiller, G., Weber, K., Schneider, L., Parajsz, C. & Jungwirth, C. (1979). Interaction of Assembled Progeny Pox Viruses with the Cellular Cytoskeleton. *Virology* **98**, 142-153.

Hiller, G., Jungwirth, C. & Weber, K. (1981). Fluorescence Microscopical Analysis of the Life Cycle of Vaccinia Virus in Chick Embryo Fibroblasts. *Experimental Cellular Research* **132**, 81-87.

Hirsch, M. S., Nahmias, A. J., Murphy, F. A. & Kramer, J. H. (1968). Cellular Immunity in Vaccinia Virus Infection of Mice. Anti-Thymocyte Serum Effects on Primary and Secondary Responsiveness. *Journal of Experimental Medicine* **128**, 131-132.

Ho, C. K. & Shuman, S. (1996). Physical and Functional-Characterization of the Double-Stranded-RNA Binding-Protein Encoded by the Vaccinia Virus E3 Gene. *Virology* **217**, 272-284.

Hovanessian, A. G. & Kerr, I. M. (1979). The (2'-5') Oligoadenylate (pppA2'-5'A2'-5') Synthetase and Protein Kinase(s) from Interferon-Treated Cells. *European Journal of Biochemistry* **93**, 515-526.

Hovanessian, A. G. & Galabru, J. (1987). The Double-Stranded RNA-Dependent Protein Kinase is Also Activated by Heparin. *European Journal of Biochemistry* **167**, 467-473.

Hovanessian, A. G., Galabru, J., Meurs, E., Buffet-Janvresse, C., Svab, J. & Robert, N. (1987a). Rapid Decrease in the Levels of the Double-Stranded RNA-Dependent Protein Kinase During Virus Infections. *Virology* **159**, 126-136.

Hovanessian, A. G., Laurent, A. G., Chebath, J., Galabru, J., Robert, N. & Svab, J. (1987b). Identification of 69-Kd and 100-Kd Forms of 2-5A Synthetase in Interferon-Treated Human Cells by Specific Monoclonal Antibodies. *The EMBO Journal* **6**, 1273-1280.

Hovanessian, A. G., Svab, J., Marié, I., Robert, N., Chamaret, S. & Laurent, A. G. (1988). Characterization of 69- and 100-kDa Forms of 2-5A-Synthetase From Interferon-Treated Human Cells. *Journal of Biological Chemistry* **263**, 4945-4945.

Howard, S. T., Chan, Y. S. & Smith, G. L. (1991). Vaccinia Virus Homologues of the Shope Fibroma Virus Inverted Terminal Repeat Proteins and a Discontinuous ORF Related to the Tumour Necrosis Factor Receptor Family. *Virology* **180**, 633-647.

Hruby, D. E. & Ball, L. A. (1982). Mapping and Identification of the Vaccinia Virus Thymidine Kinase Gene. *Journal of Virology* **43**, 403-409.

Hruby, D. E., Maki, R. A., Miller, D. B. & Ball, L. A. (1983). Fine Structure Analysis and Nucleotide Sequence of the Vaccinia Virus Thymidine Kinase Gene. *Proceedings of the National Academy of Science (USA)* **80**, 3411-3415.

Hu, Y. H. & Conway, T. W. (1993). 2-Aminopurine Inhibits the Double-Stranded RNA-Dependent Protein-Kinase Both *in-Vitro* and *in-Vivo*. *Journal of Interferon Research* **13**, 323-328.

Hu, F.-Q., Smith, C. A. & Pickup, D. J. (1994). Cowpox Virus Contains Two Copies of an Early Gene Encoding a Soluble Secreted Form of the Type II TNF Receptor. *Virology* **204**, 346-356.

Hughes, S. J., Johnston, L. H., DeCarlos, A. & Smith, G. L. (1991). Vaccinia Virus Encodes an Active Thymidylate Kinase that Complements a *cdc8* Mutant of *Saccharomyces Cerevisiae*. *Journal of Biological Chemistry* **266**, 20103-20109.

Hugin, A. W. & Hauser, C. (1994). The Epidermal Growth Factor Receptor is not a Receptor for Vaccinia Virus. *Journal of Virology* **68**, 8409-8412.

Icely, P. L., Gros, P., Bergeron, J. J. M., Devault, A., Afar, D. E. H. & Bell, J. C. (1991). TIK, a Novel Serine/Threonine Kinase, is Recognized by Antibodies Directed Against Phosphotyrosine. *Journal of Biologicai Chemistry* **266:24**, 16073-16077.

Imani, F. & Jacobs, B. L. (1988). Inhibitory Activity for the Interferon-Induced Protein Kinase is Associated with the Reovirus Serotype 1 σ3 Protein. *Proceedings of the National Academy of Science (USA)* **85**, 7887-7891.

Ink, B. S. & Pickup, D. J. (1990). Vaccinia Virus Directs the Synthesis of Early mRNAs Containing 5' Poly(A) Sequence. *Proceedings of the National Academy of Science (USA)* **87**, 1536-1540.

Isaacs, A. & Lindenmann, J. (1957). Virus Interference. I. The Interferon. *Proceedings of the Royal Society of London* Series B. **147**, 258-267.

Isaacs, S. N., Kotwal, G. L. & Moss, B. (1992). Vaccinia Virus Complement-Control Protein Prevents Antibody Dependent Complement-Enhanced Neutralisation of Infectivity and Contributes to Virulence. *Proceedings of the National Academy of Science (USA)* **89**, 628-632.

Ito, T., Jagus, R. & May, W. S. (1993). Interleukin-3 Stimulates Protein-Synthesis by Regulating Double-Stranded RNA-Dependent Protein-Kinase (PKR) - A Novel Signalling Pathway. *Blood* **82**, A 364-A 364.

Ito, T., Jagus, R. & May, W. S. (1994a). Il-3 Regulates PKR in a Novel Postreceptor Signalling Pathway. *Experimental Hematology* **22**, 763-763.

Ito, T., Jagus, R. & May, W. S. (1994b). Interleukin-3 Stimulates Protein-Synthesis by Regulating Double-Stranded RNA-Dependent Protein-Kinase. *Proceedings of the National Academy of Science (USA)* **91**, 7455-7459.

Jackson, R. J., Howell, M. T. & Kaminski, A. (1990). The Novel Mechanism of Initiation of Picornavirus RNA Translation. *Trends in Biological Sciences* **15**, 477-483.

Jacoby, R. O., Bhatt, P. N. & Brownstein, D. G. (1989). Evidence that NK Cells and Interferon are Required for Genetic Resistance to Lethal Infection with Ectromelia Virus. *Archives of Virology* **9**, 135-150.

Jarrous, N., Osman, F. & Kaempfer, R. (1996). 2-Aminopurine Selectively Inhibits Splicing of Tumor-Necrosis-Factor-Alpha Messenger-RNA. *Molecular and Cellular Biology* **16**, 2814-2822.

Jeffrey, I. W., Kadereit, S., Meurs, E. F., Metzger, T., Bachmann, M., Schwemmle, M., Hovanessian, A. G. & Clemens, M. J. (1995). Nuclear-Localization of the Interferon-Inducible Protein-Kinase PKR in Human-Cells and Transfected Mouse Cells. *Experimental Cell Research* **218**, 17-27.

Johnson, D. R. & Pober, J. S. (1990). Tumour Necrosis Factor and Immune Interferon Synergistically Increase Transcription of HLA Class I Heavy- and Light-Chain Genes in Vascular Endothelium. *Proceedings of the National Academy of Science (USA)* **97**, 1583-1587.

Johnson, G. P., Goebbel, S. J. & Paoletti, E. (1993). Minireview: An Update of the Vaccinia Virus Genome. *Virology* **196**, 381-401.

Judware, D. & Petryshyn, R. (1991). Partial Characterization of a Cellular Factor That Regulates the Double-Stranded RNA-Dependent eIF-2α Kinase in 3T3-F442A Fibroblasts. *Molecular and Cellular Biology* **11:6**, 3259-3267.

Judware, R. & Petryshyn, R. (1992). Mechanisms of Action of the Cellular Inhibitor of the Double-Stranded RNA-Dependent Protein Kinase From 3T3-F442A Cells. *Journal of Biological Chemistry* **267:30**, 21685-21690.

Judware, R., Li, J. & Petryshyn, R. (1993). Inhibition of the Double-Stranded RNA-Dependent Protein Kinase by a Peptide Derived From the Human Immunodeficiency Virus Type-1 Tat Protein. *Journal of Interferon Research* **13**, 153-160.

Kadereit, S., Galabru, J., Robert, N., Meurs, E. F. & Hovanessian, A. G. (1994). Characterization of an Interferon-Induced 48-kD Protein Immunologically Related to the Double-Stranded RNA-Activated Protein-Kinase PKR. *Journal of Interferon Research* **14**, 251-257.

Kane, E. M. & Shuman, S. (1992). Temperature-Sensitive Mutations in the Vaccinia Virus H4 Gene Encoding a Component of the Virion RNA Polymerase. *Journal of Virology* **66**, 5752-5762.

Kao, S. Y., Ressner, E., Kates, J. & Bauer, W. R. (1981). Purification and Characterization of a Superhelix Binding Protein from Vaccinia Virus. *Virology* **111**, 500-508.

Kao, S. Y. & Bauer, W. R. (1987). Biosynthesis and Phosphorylation of Vaccinia Virus Structural Protein VPII. *Virology* **159**, 399-407.

Karupiah, G., Coupar, B. E. H., Andrew, M. E., Boyle, D. B., Phillips, S. M., Mullerbacher, A., Blanden, R. V. & Ramshaw, I. A. (1990). Elevated Natural Killer Cell Responses in Mice Infected with Recombinant Vaccinia Virus Encoding Murine IL-2. *Journal of Virology* **144**, 290-298.

Kates, J. & Beeson, J. (1970). Ribonucleic Acid Synthesis in Vaccinia Virus. II. Synthesis of Polyriboadenylic Acid. *Journal of Molecular Biology* **50**, 19-23.

Katze, M. G., DeCorato, D., Safer, B., Galabru, J. & Hovanessian, A. G. (1987). Adenovirus VAI RNA Complexes with the 68 000 Mr Protein Kinase to Regulate its Autophosphorylation and Activity. *The EMBO Journal* **6**, 689-697.

Katze, M. G. (1992). The War Against Interferon-Induced Double-Stranded RNA-Activated Protein Kinase: Can Viruses Win? *Journal of Interferon Research* **12**, 241-248.

Katze, M. G. (1993). Games Viruses Play : A Strategic Initiative Against the Interferon-Induced Double-Stranded RNA-Activated 68,000 Mr Protein Kinase. *Seminars in Virology* **4**, 259-268.

Katze, M. G. (1995). Regulation of the Interferon-Induced PKR: Can Viruses Cope? *Trends in Microbiology* **3**, 75-78.

Keck, J. G., Baldick, C. J. & Moss, B. (1990). Role of DNA Replication in Vaccinia Virus Gene Expression: A Naked Template is Required for Transcription of Three Late *Trans*-activator Genes. *Cell* **61**, 801-809.

Kerr, I. M., Brown, R. E. & Ball, L. A. (1974). Increased Sensitivity of Cell-Free Protein Synthesis to Double-Stranded RNA After Interferon Treatment. *Nature* **250**, 57-59.

Kirchhoff, S., Koromilas, A. E., Schaper, F., Grashoff, M., Sonenberg, N. & Hauser, H. (1995). IRF-1 Induced Cell-Growth Inhibition and Interferon Induction Requires the Activity of the Protein-Kinase PKR. *Oncogene* **11**, 439-445.

Koonin, E. V. & Senkevich, T. G. (1993). Fowlpox Virus Encodes a Protein Related to Human Deoxycytidine Kinase: Further Evidence for Independent Acquisition of Genes for Enzymes of Nucleotide Metabolism by Different Viruses. *Virus Genes* **7:3**, 289-295.

Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G. & Sonenberg, N. (1992). Malignant Transformation by a Mutant of the Interferon-Inducible Double-Stranded RNA-Dependent Protein Kinase. *Science* **257**, 1685-1689.

Korth, M. J., Edelhoff, S., Disteche, C. M. & Katze, M. G. (1996a). Chromosomal Assignment of the Gene Encoding the Human 58-kDa Inhibitor (PRKRI) of the Interferon-Induced dsRNA-Activated Protein- Kinase to Chromosome 13q32. *Genomics* **31**, 238-239.

Korth, M. J., Lyons, C. N., Wambach, M. & Katze, M. G. (1996b). Cloning, Expression, and Cellular-Localization of the Oncogenic 58- kDa Inhibitor of the RNA-Activated Human and Mouse Protein-Kinase. *Gene* **170**, 181-188.

Kotwal, G. J. & Moss, B. (1989). Vaccinia Virus Encodes Two Proteins that are Structurally Related to Members of the Plasma Serine Protease Inhibitors Superfamily. *Journal of Virology* **63**, 600-606.

Kotwal, G. J., Isaacs, S. N., McKenzie, R., Frank, M. M. & Moss, B. (1990). Inhibition of the Complement Cascade by the Major Secretory Protein of Vaccinia Virus. *Science* **250**, 827-830.

Kumar, S. & Boyle, D. B. (1990). A Poxvirus Bidirectional Promoter Element with Early/Late and Late Functions. *Virology* **179**, 151-158.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J. & Williams, B. (1994). Double-Stranded RNA-Dependent Protein-Kinase Activates Transcription Factor NF-Kappa-B by Phosphorylating I-Kappa-B. *Proceedings of the National Academy of Science (USA)* **91**, 6288-6292.

Lakritz, N., Fogelsong, P. D., Reddy, M., Baum, S., Hurwitz, J. & Bauer, W. R. (1985). A Vaccinia Virus DNase Preparation Which Cross-Links Superhelical DNA. *Journal of Virology* **53**, 935-943.

Lamb, J. R., Tugendreich, S. & Hieter, P. A. (1995). Tetratrico Peptide Repeat Interactions: To TPR or Not to TPR? *Trends in Biological Sciences* **20**, 257-259.

Langland, J. O. & Jacobs, B. L. (1992). Cytosolic Double-Stranded RNA-Activated Protein Kinase is Likely a Dimer of Partially Phosphorylated Mr=66,000 Subunits*. *Journal of Biological Chemistry* **267**, 10729-10736.

Langland, J. O., Pettiford, S., Jiang, B. M. & Jacobs, B. L. (1994). Products of the Porcine Group-C Rotavirus NSP3 Gene Bind Specifically to Double-Stranded-RNA and Inhibit Activation of the Interferon-Induced Protein-Kinase PKR. *Journal of Virology* **68**, 3821-3829.

Langland, J. O., Jin, S., Jacobs, B. L. & Roth, D. A. (1995). Identification of a Plant-Encoded Analogue of PKR, the Mammalian Double-Stranded RNA-Dependent Protein-Kinase. *Plant Physiology* **108**, 1259-1267.

Langland, J. O., Langland, L. A., Browning, K. S. & Roth, D. A. (1996a). Phosphorylation of Plant Eukaryotic Initiation Factor-II by the Plant-Encoded Double-Stranded RNA-Dependent Protein-Kinase, PKR, and Inhibition of Protein-Synthesis *In-Vitro*. *Journal of Biological Chemistry* **271**, 4539-4544.

Langland, J. O., Langland, L. A. & Roth, D. A. (1996b). Polyanion Regulation of the Plant-Encoded Double-Stranded RNA- Dependent Protein-Kinase (pPKR). *Plant Physiology and Biochemistry* **34**, 521-526.

Laurent, A. G., Krust, B., Galabru, J., Svab, J. & Hovanessian, A. G. (1985). Monoclonal Antibodies to an Interferon-Induced Mr 68,000 Protein and their Use For the Detection of Double-Stranded RNA-Dependent Protein Kinase in Human Cells. *Proceedings of the National Academy of Science USA* **82**, 4341-4345.

Le, J., Prensky, W., Yip, Y. K., Chang, Z., Hoffman, T., Stevenson, H. C., Balazs, I., Sadlik, J. R. & Vilcek, J. (1983). Activation of Human Monocyte Cytotoxicity by Natural and Recombinant Immune Interferon. *Journal of Immunology* **131**, 2821-2826.

Lebleu, B., Sen, G. C., Cabrer, B. & Lengyl, P. (1976). Interferon, Double-Stranded RNA, and Protein Phosphorylation. *Proceedings of the National Academy of Science (USA)* **73**, 3107-3111.

Leddy, J. P., Simons, R. L. & Douglas, R. G. (1977). Effect of Selective Complement Deficiency on the Rate of Neutralisation of Enveloped Viruses by Human Sera. *Journal of Immunology* **118**, 28-34.

Lee, S. B., Melkova, Z., Yan, W., Williams, B. R. G., Hovanessian, A. G. & Esteban, M. (1993). The Interferon-Induced Double-Stranded RNA-Activated Human P68 Protein Kinase Potently Inhibits Protein Synthesis in Cultured Cells. *Virology* **192**, 380-385.

Lee, S. B. & Esteban, M. (1994). The Interferon-Induced Double-Stranded RNA-Activated Protein Kinase Induced Apoptosis. *Virology* **199**, 491-496.

Lee, T. G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1990). Purification and Partial Characterization of a Cellular Inhibitor of the Interferon-Induced Protein Kinase of Mr 68,000 from Influenza Virus-Infected Cells. *Proceedings of the National Academy of Science (USA)* **87**, 6208-6212.

Lee, T. G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1992). Characterization and Regulation of the 58,000 Da Cellular Inhibitor of the Interferon-Induced, RNA-Activated Protein Kinase. *Journal of Biological Chemistry* **267**, 14238-14243.

Lee, T. G., Tang, N., Thompson, S., Miller, J. & Katze, M. G. (1994). The 58,000-Dalton Cellular Inhibitor of the Interferon-Induced Double-Stranded RNA-Activated Protein-Kinase (PKR) is a Member of the Tetratricopeptide Repeat Family of Proteins. *Molecular and Cellular Biology* **14**, 2331-2342.

Lengyel, P., Choubey, D., Li, S. & Datta, B. (1995). The Interferon-Activatable Gene 200 Cluster: From Structure Toward Function. *Seminars in Virology* **6**, 203-213.

Levy, D. E. (1995). Interferon Induction of Gene Expression Through the Jak-Stat Pathway. *Seminars in Virology* **6**, 181-189.

Lin, S., Chen, W. & Broyles, S. S. (1992). The Vaccinia Virus B1R Gene Product is a Serine/Threonine Protein Kinase. *Journal of Virology* **66**, 2717-2723.

Lindahl, P., Leary, P. & Gresser, I. (1971). Enhancement By Interferon of the Specific Cytotoxicity of Sensitized Lymphocytes. *Proceedings of the National Academy of Science (USA)* **69**, 721-725.

Lu, C. X. & Bablanian, R. (1996). Characterization of Small Nontranslated Polyadenylylated RNAs in Vaccinia Virus-Infected Cells. *Proceedings of the National Academy of Science (USA)* **93**, 2037-2042.

Mabrouk, T., Danis, C. & Lemay, G. (1995). Two Basic Motifs of Reovirus Sigma-3 Protein are Involved in Double- Stranded-RNA Binding. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **73**, 137-145.

Mackett, M., Smith, G. L. & Moss, B. (1982). Vaccinia Virus: A Selectable Eukaryotic Cloning and Expression Vector. *Proceedings of the National Academy of Science (USA)* **79**, 7415-7419.

Mahr, A. & Roberts, B. E. (1984). Organization of Six Early Transcripts Synthesized from a Vaccinia Virus *Eco*RI DNA Fragment. *Journal of Virology* **49**, 497-509.

Maitra, R. K., McMillan, N., Desai, S., McSwiggen, J., Hovanessian, A. G., Sen, G., Williams, B. & Silverman, R. H. (1994). HIV-1 TAR RNA Has an Intrinsic Ability to Activate Interferon-Inducible Enzymes. *Virology* **204**, 823-827.

Manche, L., Green, S. R., Schmedt, C. & Mathews, M. B. (1992). Interactions Between Double-Stranded RNA Regulators and the Protein Kinase DAI. *Molecular and Cellular Biology* **12:11**, 5238-5248.

Mantei, N., Schwarzstein, M., Streuli, M., Panem, S., Nagata, S. & Weissmann, C. (1980). The Nucleotide Sequence of a Cloned Human Leukocyte Interferon cDNA. *Gene* **10**, 1-10.

Maran, A. & Mathews, M. B. (1988). Characterization of the Double-Stranded RNA Implicated in the Inhibition of Protein Synthesis in Cells Infected with a Mutant Adenovirus Defective for VA RNA. *Virology* **164**, 106-113.

Marié, I. & Hovanessian, A. G. (1992). The 69-kDa 2-5A Synthetase is Composed of Two Homologous and Adjacent Domains. *Journal of Biological Chemistry* **267**, 9933-9939.

Martinez-Pomares, L., Thompson, J. P. & Moyer, R. W. (1995). Mapping and Investigation of the Role in Pathogenesis of the Major Unique Secreted 35-kDa Protein of Rabbitpox Virus. *Virology* **206**, 591-600.

Massung, A. F., Liu, L., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C. & Esposito, J. J. (1994). Analysis of the Complete Genome of Smallpox Variola Major Virus Strain Bangladesh-1975. *Virology* **201**, 215-240.

Mathews, M. B. & Shenk, T. (1991). Adenovirus Virus-Associated RNA and Translational Control. *Journal of Virology* **65**, 5657-5662.

Mathews, M. B. (1993). Viral Evasion of Cellular Defence Mechanisms : Regulation of the Protein Kinase DAI by RNA Effectors. *Seminars in Virology* **4**, 247-257.

McCormack, S. J., Thomis, D. C. & Samuel, C. E. (1992). Mechanisms of Interferon Action: Identification of a RNA-Binding Domain Within the N-Terminal Region of the Human RNA-Dependent P1/eIF-2α Protein Kinase. *Virology* **188**, 47-56.

McCormack, S. J., Ortega, L. G., Doohan, J. P. & Samuel, C. E. (1994). Mechanism of Interferon Action Motif-1 of the Interferon-Induced, RNA-Dependent Protein-Kinase (PKR) is Sufficient to Mediate RNA-Binding Activity. *Virology* **198**, 92-99.

McFadden, G. & Graham, K. (1994). Modulation of Cytokine Networks by Poxvirus: The Myxoma Virus Model. *Seminars in Virology* **5**, 421-429.

McFadden, G. & Kane, K. (1994). How DNA Viruses Perturb Functional MHC Expression to Alter Immune Recognition. *Advances in Cancer Research* **63**, 117-209.

McKenzie, R., Kotwal, G. J., Moss, B., Hammer, C. H. & Frank, M. M. (1992). Regulation of Complement Activity by Vaccinia Virus Complement-Control Protein. *Journal of Infectious Diseases* **166**, 1245-1250.

McMillan, N. A. J., Chun, R. F., Siderovski, D. P., Galabru, J., Toone, W. M., Samuel, C. E., Mak, T. W., Hovanessian, A. G., Jeang, K. T. & Williams, B. R. G. (1995). HIV-1 Tat Directly Interacts With the Interferon-Induced, Double-Stranded RNA-Dependent Kinase, PKR. *Virology* **213**, 413-424.

Mellor, H. & Proud, C. G. (1991). A Synthetic Peptide Substrate for Initiation Factor-2 Kinases. *Biochemical and Biophysical Research Communications* **178**, 430-437.

Mellor, H., Flowers, K. M., Kimball, S. R. & Jefferson, L. S. (1994). Cloning and Characterization of a cDNA-Encoding Rat PKR, the Double-Stranded RNA-Dependent Eukaryotic Initiation Factor-II Kinase. *Biochimica et Biophysica Acta* **1219**, 693-696.

Merchlinsky, M. & Moss, B. (1989). Nucleotide Sequence Required for the Resolution of the Concatomer Junction of Vaccinia Virus DNA. *Journal of Virology* **63**, 4354-4361.

Mestan, J., Digel, W., Mittnacht, S., Hillen, H., Blohm, D., Moller, A., Jacobsen, H. & Kirchner, H. (1986). Antiviral Effects of Recombinant Tumour Necrosis Factor *in Vitro*. *Nature* **323**, 816-822.

Metz, D. H. & Esteban, M. (1972). Interferon Inhibits Viral Protein Synthesis in L Cells Infected with Vaccinia Virus. *Nature* **238**, 385-388.

Meurs, E., Chong, K., Galabru, J., Thomas, N. S. B., Kerr, I. M., Williams, B. R. G. & Hovanessian, A. G. (1990). Molecular Cloning and Characterization of the Human Double-Stranded RNA-Activated Protein Kinase Induced by Interferon. *Cell* **62**, 379-390.

Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G. & Hovanessian, A. G. (1993). Tumor Suppressor Function of the Interferon-Induced Double-Stranded RNA-Activated Protein-Kinase. *Proceedings of the National Academy of Science (USA)* **90**, 232-236.

Miller, J. E. & Samuel, C. E. (1992). Proteolytic Cleavage of the Reovirus σ3 Protein Results in Enhanced Double-Stranded RNA-Binding Activity: Identification of a Repeated Basic Amino Acid Motif Within the C-Terminal Binding Region. *Journal of Virology* **66**, 5347-5356.

Miura, M., Zhu, H., Rotello, R., Hartweig, E. A. & Yuan, J. (1993). Induction of Apoptosis by IL-1β-Converting Enzyme, a Mammalian Homologue of the *C.elegans* Cell Death Gene. *Cell* **75**, 653-660.

Mockett, B., Binns, M. M., Boursnell, M. E. G. & Skinner, M. A. (1992). Comparison of the Locations of Homologous Fowlpox and Vaccinia Virus Genes Reveals Major Genome Reorganization. *Journal of General Virology* **73**, 2661-2668.

Moore, J. B. & Smith, G. L. (1992). Steroid Hormone Synthesis by a Vaccinia Enzyme: A New Type of Virulence Factor. *The EMBO Journal* **11**, 1973-1980.

Mordechai, E., Kon, N., Henderson, E. E. & Suhadolnik, R. J. (1995). Activation of the Interferon-Inducible Enzymes, 2',5'-Oligoadenylate Synthetase and PKR By Human T-Cell Leukemia-Virus Type-I Rex-Response Element. *Virology* **206**, 913-922.

Mosely, B., Urdal, D. L., Prickett, K. S., Larsen, A., Cosman, D., Conlon, P. J., Gillis, S. & Dower, S. K. (1987). The Interleukin-1 Receptor Bind the Human Interleukin-1α Precursor but not the IL-1β Precursor. *Journal of Biological Chemistry* **262**, 2941-2944.

Mosmann, T. R., Cherwinsky, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986). Two Types of Murine Helper T-Cell Clone. I. Definition According to Profiles of Lymphokine Activities and Secreted Proteins. *Journal of Immunology* **136**, 2348-2357.

Moss, B., Rosenblum, E. N. & Katz, E. (1969). Rifampicin, a Specific Inhibitor of Vaccinia Virus Assembly. *Nature* **224**, 1280-1284.

Moss, B. & Rosenblum, E. N. (1973). Protein Cleavage and Poxvirus Morphogenesis: Tryptic Peptide Analysis of Core Precursors Accumulated by Blocking Assembly with Rifampicin. *Journal of Molecular Biology* **81**, 267-269.

Moss, B. (1990). Poxviruses and Their Replication. In *Virology*, 2nd edn. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press Ltd.

Muller, U., Steinhoff, U., Reis, R. F. L., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. (1994). Functional Role of Type I and Type II Interferons in Antiviral Defence. *Science* **264**, 1918-1921.

Mundschau, L. J. & Faller, D. V. (1995). Platelet-Derived Growth-Factor Signal-Transduction Through the Interferon-Inducible Kinase PKR - Immediate-Early Gene Induction. *Journal of Biological Chemistry* **270**, 3100-3106.

Nakajima, H., Nakao, A., Watanabe, Y., Yoshida, S. & Iwamoto, I. (1994). IFN-α Inhibits Antigen-Induced Eosinophil and CD4+ T Cell Recruitment into Tissue. *Journal of Immunology* **153**, 1264-1270.

Nathan, C. F., Prendergast, T. J., Wiebe, M. E., Stanley, E. R., Platzer, E., Remold, H., Welte, K., Rubin, B. & Murray, H. W. (1984). Activation of Human Macrophages. Comparison of Other Cytokines With Interferon-Gamma. *Journal of Experimental Medicine* **160**, 600-605.

Nilsen, T. W. & Baglioni, C. (1979). Mechanism for Discrimination Between Viral and Host mRNA in Interferon-Treated Cells. *Proceedings of the National Academy of Science (USA)* **76**, 2600-2604.

O'Malley, R. P., Mariano, T. M., Siekierka, J. & Mathews, M. B. (1986). A Mechanism for the Control of Protein Synthesis by Adenovirus VA RNA I. *Cell* **44**, 391-400.

Ohta, H., Yoshikawa, T., Kai, C., Yamanouchi, K., Taniguchi, H., Komine, K.-I., Ishijima, Y. & Okada, H. (1986). Effect of Complement Depletion by Cobra Venom Factor on Fowlpox Virus Infection in Chickens and Chicken Embryos. *Journal of Virology* **57**, 670-673.

Ortega, L. G., McCotter, M. D., Henry, G. L., McCormack, S. J., Thomis, D. C. & Samuel, C. E. (1996). Mechanism of Interferon Action - Biochemical and Genetic-Evidence For the Intermolecular Association of the RNA-Dependent Protein-Kinase PKR From Human-Cells. *Virology* **215**, 31-39.

Paez, E. & Esteban, M. (1984). Nature and Mode of Action of Vaccinia Virus Products that Block Activation of the Interferon-Mediated ppp(A2'p)nA-Synthetase. *Virology* **134**, 29-39.

Panicali, D. & Paoletti, E. (1982). Construction of Poxviruses as Cloning Vectors: Insertion of the Thymidine Kinase Gene from Herpes Simplex Virus into the DNA of Infectious Vaccinia Virus. *Proceedings of the National Academy of Science (USA)* **79**, 4927-4931.

Park, H. S., Davies, M. V., Langland, J. O., Chang, H. W., Nam, Y. S., Tartaglia, J., Paoletti, E., Jacobs, B. L., Kaufman, R. J. & Venkatesan, S. (1994). Tar RNA-Binding Protein is an Inhibitor of the Interferon-Induced Protein-Kinase PKR. *Proceedings of the National Academy of Science (USA)* **91**, 4713-4717.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M.-P., Macchia, D., Maggi, E., Del Prete, G. & Romagnani, S. (1992). IL-4 and IFN (Alpha and Gamma) Exert Opposite Regulatory Effects on the Development of Cytolytic Potential By Th1 or Th2 Human T Cell Clones. *Journal of Immunology* **149**, 2977-2983.

Patel, R. C. & Sen, G. C. (1992). Identification of the Double-Stranded RNA-Binding Domain of the Human Interferon-Inducible Protein Kinase. *Journal of Biological Chemistry* **267:11**, 7671-7676.

Patel, R. C., Stanton, P. & Sen, G. C. (1994). Role of the Amino-Terminal Residues of the Interferon-Induced Protein-Kinase in its Activation by Double-Stranded-RNA and Heparin. *Journal of Biological Chemistry* **269**, 18593-18598.

Patel, R. C., McMillan, N. M. J., Williams, B. R. G. & Sen, G. C. (1995). The Interferon-Inducible Double-Stranded RNA-Activated Protein Kinase Self-Associates *In Vitro* and *In Vivo*. *Proceedings of the National Academy of Science (USA)* **92**, 8283-8287.

Pathak, V. K., Schindler, D. & Hershey, J. W. B. (1988). Generation of a Mutant Form of Protein Synthesis Initiation Factor eIF-2 Lacking the Site of Phosphorylation by eIF-2 Kinases. *Molecular and Cellular Biology* **8:2**, 993-995.

Payne, L. G. (1980). Significance of Extracellular Enveloped Virus in the *in Vitro* and *in Vivo* Dissemination of Vaccinia. *Journal of General Virology* **50**, 89-100.

Payne, L. G. & Kristensson, K. (1985). Extracellular Release of Enveloped Vaccinia Virus from Mouse Epithelial Cells *in Vivo*. *Journal of General Virology* **66**, 643-646.

Pfeffer, L., Wang, E. & Tamm, I. (1980). Interferon Effects on Microfilament Organization, Cellular Fibronectin Distribution, and Cell Motility in Human Fibroblasts. *Journal of Cell Biology* **85**, 9-17.

Polyak, S. J., Tang, N., Wambach, M., Barber, G. N. & Katze, M. G. (1996). The P58 Cellular Inhibitor Complexes With the Interferon-Induced, Double-Stranded RNA-Dependent Protein-Kinase, PKR, to Regulate Its Autophosphorylation and Activity. *Journal of Biological Chemistry* **271**, 1702-1707.

Qian, W. J., Zhu, S. H., Sobolev, A. Y. & Wek, R. C. (1996). Expression of Vaccinia Virus K3L Protein in Yeast Inhibits Eukaryotic Initiation Factor-II Kinase GCN2 and the General Amino-Acid Control Pathway. *Journal of Biological Chemistry* **271**, 13202-13207.

Quick, S. D. & Broyles, S. S. (1990). Vaccinia Virus Gene D7R Encodes a 20,000-Dalton Subunit of the Viral DNA-Dependent RNA-Polymerase. *Virology* **178**, 603-605.

Rahman, A., Malhotra, P., Dhar, R., Kewalramani, T. & Thimmapaya, B. (1995). Effect of Single-Base Substitutions In the Central Domain of Virus-Associated RNA-I on its Function. *Journal of Virology* **69**, 4299-4307.

Ramirez, M., Wek, R. C., Vazquez De Aldana, C. R., Jackson, B. M., Freeman, B. & Hinnebusch, A. G. (1992). Mutations Activating the Yeast eIF-2α Kinase GCN2: Isolation of Alleles Altering the Domain Related to Histidyl-tRNA Synthetases. *Molecular and Cellular Biology* **12**, 5801-5815.

Ramsay, A. J., Ruby, J. & Ramshaw, I. A. (1993). A Case for Cytokines as Effector Molecules in the Resolution of Virus Infection. *Immunology Today* **14**, 155-157.

Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S. & Pickup, D. J. (1992). Viral Inhibition of Inflammation: Cowpox Virus Encodes an Inhibitor of the Interleukin-1β Converting Enzyme. *Cell* **69**, 597-604.

Reddy, M. K. & Bauer, W. R. (1989). Activation of the Vaccinia Virus Nicking-Joining Enzyme by Trypsinisation. *Journal of Biological Chemistry* **264**, 443-449.

Rice, A. P. & Kerr, I. M. (1984). Interferon-Mediated, Double-Stranded RNA-Dependent Protein Kinase is Inhibited in Extracts From Vaccinia Virus-Infected Cells. *Journal of Virology* **50**, 229-236.

Rice, A. P., Roberts, W. K. & Kerr, I. M. (1984). 2-5A Accumulates to High Levels in Interferon-Treated, Vaccinia Virus-Infected Cells in the Absence of Any Inhibition of Virus Replication. *Journal of Virology* **50**, 220-228.

Rice, A. P, Duncan, R., Hershey, J. & Kerr, I. (1985). The Double-Stranded RNA-Dependent Protein Kinase and 2-5A System Are Both Activated in Interferon-Treated, Encephalomyocarditis Virus-Infected HeLa Cells. *Journal of Virology* 54, 894-898.

Roberts, W. K., Clemens, M. J. & Kerr, I. M. (1976a). Interferon-Induced Inhibition of Protein Synthesis in L-Cell Extracts: An ATP-Dependent Step in the Activation of an Inhibitor by Double-Stranded RNA. *Proceedings of the National Academy of Science (USA)* **73**, 3136-3140.

Roberts, W. K., Hovanessian, A. G., Brown, R. E., Clemens, M. J. & Kerr, I. M. (1976b). Interferon-Mediated Protein Kinase and Low-Molecular-Weight Inhibitor of Protein Synthesis. *Nature* **264**, 477-480.

Rodriguez, J.-R., Rodriguez, D. & Esteban, M. (1991). Interferon Inhibits Early Vaccinia Virus Gene Expression in Infected Mice. *Virology* **185**, 929-933.

Rohrman, G., Yuen, L. & Moss, B. (1986). Transcription of Vaccinia Virus Early Genes by Enzymes Isolated from Vaccinia Virus Virions Terminates Downstream of a Regulatory Sequence. *Cell* **46**, 1029-1035.

Romano, P. R., Green, S. R., Barber, G. N., Mathews, M. B. & Hinnebusch, A. G. (1995). Structural Requirements for Double-Stranded RNA Binding, Dimerization, and Activation of the Human eIF-2 Alpha Kinase DAI in *Saccharomyces Cerevisiae*. *Molecular and Cellular Biology* **15**, 365-378.

Rosales, R., Harris, N., Ahn, B. Y. & Moss, B. (1994a). Purification and Identification of a Vaccinia Virus-Encoded Intermediate Stage Promoter-Specific Transcription Factor that has Homology to Eukaryotic Transcription Factor SII (TFIIS) and an Additional Role as a Viral RNA Polymerase Subunit. *Journal of Biological Chemistry* **269**, 14620-14627.

Rosales, R., Sutter, G. & Moss, B. (1994b). A Cellular Factor is Required for Transcription of Vaccinia Viral Intermediate-Stage Genes. *Proceedings of the National Academy of Science (USA)* **91**, 3794-3798.

Roy, S., Katze, M. G., Parkin, N. T., Edery, I., Hovanessian, A. G. & Sonenberg, N. (1990). Control of Interferon-Induced 68-Kilodalton Protein Kinase by the HIV-1 *Tat* Gene Product. *Science* **247**, 1216-1219.

Roy, S., Agy, M., Hovanessian, A. G., Sonenberg, N. & Katze, M. G. (1991). The Integrity of the Stem Structure of Human Immunodeficiency Virus Type 1 Tat-Responsive Sequence RNA is Required for Interaction with the Interferon-Induced 68,000-Mr Protein Kinase. *Journal of Virology* **65**, 632-640.

Sambhi, S. K., Kohonen-Corish, M. R. J. & Ramshaw, A. (1991). Local Production of Tumour Necrosis Factor Encoded by Recombinant Vaccinia Virus is Effective in Controlling Viral Replication *In Vivo*. *Proceedings of the National Academy of Science (USA)* **88**, 4025-4029.

Sambrook, Fritsch & Maniatis (1989). Molecular Cloning - A Laboratory Manual., 2 edn. Edited by N. Ford: Cold Spring Harbour Laboratory Press.

Samuel, C. E. (1979). Mechanism of Interferon Action: Phosphorylation of Protein Synthesis Initiation Factor eIF-2 in Interferon-Treated Human Cells by a Ribosome-Associated Kinase Processing Site Specificity Similar to Hemin-Regulated Rabbit Reticulocyte Kinase. *Proceedings of the National Academy of Science (USA)* **76**, 600-604.

Samuel, C. E. (1988). Mechanisms of Antiviral Action of Interferons. *Progress in Nucleic Acids Research and Molecular Biology* **35**, 27-65.

Samuel, C. E. (1991). Antiviral Actions of Interferon - Interferon Regulated Cellular Proteins and Their Surprisingly Selective Antiviral Actions. *Virology* **183**, 1-11.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA Sequencing With Chain-Terminating Inhibitors. *Proceedings of the National Academy of Science (USA)* **74**, 5463-5467.

Saunders, M. E., Gewert, D. R., Tugwell, M. E., McMahon, M. & Williams, B. R. G. (1985). Human 2-5A Synthetase: Characterization of a Novel cDNA and Corresponding Gene Structure. *The EMBO Journal* **4**, 1761-1768.

Schmelz, M., Sodeik, B., Ericcson, M., Wolffe, E. J., Shida, H., Hiller, G. & Griffiths, G. (1994). Assembly of Vaccinia Virus: The Second Wrapping Cisternae is Derived from the Trans Golgi Network. *Journal of Virology* **68**, 130-147.

Schmidt, A., Chernajovsky, Y., Shulman, L., Federman, P., Berissi, H. & Revel, M. (1979). An Interferon-Induced Phosphodiesterase Degrading (2'-5')Oligoisoadenylate and the C-C-A Terminus of tRNA. *Proceedings of the National Academy of Science (USA)* **76**, 4788-4792.

Schuman, S. & Moss, B. (1988). Factor-Dependent Transcription Termination by Vaccinia Virus RNA Polymerase. *Journal of Biological Chemistry* **263**, 6220-6225.

Schwemmle, M., Clemens, M. J., Hilse, K., Pfeifer, K., Tröster, H., Müller, W. E. & Bachmann, M. (1992). Localization of Epstein-Barr Virus-Encoded RNAs EBER-1 and EBER-2 in Interphase and Mitotic Burkitt Lymphoma Cells. *Proceedings of the National Academy of Science (USA)* **89**, 10292-10296.

Seder, R. A. & Paul, W. E. (1994). Acquisition of Lymphokine Producing Phenotype By CD4+ T Cells. *Annual Review of Immunology* **12**, 635-637.

Sekellick, M. J., Ferrandino, A. F., Hopkins, D. A. & Marcus, P. I. (1994). Chicken Interferon Gene: Cloning, Expression, and Analysis. *Journal of Interferon Research* **14**, 71-79.

Senkevich, T. G., Bugert, J. J., Sisler, J. R., Koonin, E. V., Darai, G. & Moss, B. (1996). Genome Sequence of a Human Tumorigenic Poxvirus: Prediction of Specific Host Response-Evasion Genes. *Science* **273**, 813-816.

Shaffer, R. & Traktman, P. (1987). Vaccinia Virus Encapsidates a Novel Topoisomerase with the Properties of a Eukaryotic Type I Enzyme. *Journal of Biological Chemistry* **262**, 9309-9315.

Sharp, T. V., Schwemmle, M., Jeffrey, I., Laing, K., Mellor, H., Proud, C. G., Hilse, K. & Clemens, M. J. (1993a). Comparative-Analysis of the Regulation of the Interferon-Inducible Protein-Kinase PKR by Epstein-Barr-Virus RNAs EBER-1 and EBER-2 and Adenovirus VA(I) RNA. *Nucleic Acids Research* **21**, 4483-4490.

Sharp, T. V., Xiao, Q. R., Jeffrey, I., Gewert, D. R. & Clemens, M. J. (1993b). Reversal of the Double-Stranded-RNA-Induced Inhibition of Protein-Synthesis by a Catalytically Inactive Mutant of the Protein-Kinase PKR. *European Journal of Biochemistry* **214**, 945-948.

Shchelkunov, S. N., Blinov, V. M., Totmenin, A. V., Chizikov, V. E. & Malygin, E. G. (1991). Molecular Biological Study of the Vaccinia Virus Genome. IV. The Late Nonstructural 36 K Protein of Vaccinia Virus is Essential For Virus Reproduction. *Molecular Biology (Mosk)* **25**, 396-404.

Shuman, S. & Moss, B. (1987). Identification of a Vaccinia Virus Gene Encoding a Type I DNA Topoisomerase. *Proceedings of the National Academy of Science (USA)* **84**, 7478-7482.

Shuman, S., Golder, M. & Moss, B. (1989). Insertional Mutagenesis of the Vaccinia Virus Gene Encoding a Type I DNA Topoisomerase: Evidence that the Gene is Non-Essential For Virus Growth. *Virology* **170**, 302-306.

Siegel, D. S. L., J. & Vilcek, J. (1986). Modulation of Lymphocyte Proliferation and Immunoglobulin Synthesis By Interferon-Gamma and "Type I" Interferons. *Cell Immunology* **101**, 380-390.

Singleton, P. & Sainsbury, D. (1987). Dictionary of Microbiology and Molecular Biology, 2nd Edition edn. Chichester: Wiley-Interscience Publication.

Skinner, M. A., Moore, J. B., Binns, M. M., Smith, G. L. & Boursnell, M. E. G. (1994). Deletion of Fowlpox Virus Homologues of Vaccinia Virus Genes Between the 3β-hydroxysteroid dehydrogenase (A44L) and DNA Ligase (A50R) Genes. *Journal of General Virology* **75**, 2495-2498.

Slabaugh, M., Roseman, N., Davis, R. & Mathews, C. (1988). Vaccinia Virus-Encoded Ribonucleotide Reductase: Sequence Conservation of the Gene for the Small Subunit and its Amplification in Hydroxyurea-Resistant Mutants. *Journal of Virology* **62**, 519-527.

Smith, G. L., DeCarlos, A. & Chan, Y. S. (1989a). Vaccinia Encodes a Thymidylate Kinase Gene: Sequence and Transcriptional Mapping. *Nucleic Acids Research* **17**, 7581-7590.

Smith, G. L., Howard, S. T. & Chan, Y. S. (1989b). Vaccinia Virus Encodes a Family of Genes with Homology to Serine Protease Inhibitors. *Journal of General Virology* **70**, 2333-2343.

Smith, G. L., Chan, Y. S. & Howard, S. T. (1991a). Nucleotide Sequence of 42 kbp of Vaccinia Virus Strain WR from Near the Right Inverted Terminal Repeat. *Journal of General Virology* **72**, 1349-1376.

Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G. & Goodwin, R. G. (1991b). T2 Open Reading Frame of Shope Fibroma Virus Encodes a Soluble Form of the TNF Receptor. *Biochemical and Biophysical Research Communications* **176**.

Smith, G. L. (1993). Vaccinia Virus Glycoproteins and Immune Evasion. *Journal of General Virology* **74**, 1725-1740.

Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machmer, C. E., van t'Hof, W., van Meer, G., Moss, B. & Griffiths, G. (1993). Assembly of Vaccinia Virus: Role of the Intermediate Compartment Between the Endoplasmic Reticulum and Golgi Stacks. *Journal of Cellular Biology* **121**, 521-541.

Sodeik, B., Griffiths, G., Ericsson, M., Moss, B. & Doms, R. W. (1994). Assembly of Vaccinia Virus: Effects of Rifampicin on the Intracellular Distribution of Viral Protein p65. *Journal of Virology* **68**, 1103-1114.

Somogyi, P., Frazier, J. & Skinner, M. A. (1993). Fowlpox Virus Host Range Restriction: Gene Expression, DNA Replication, and Morphogenesis in Nonpermissive Mammalian Cells. *Virology* **197**, 439-444.

Sonnenfeld, G. (1984). Effects of Interferon On Antibody Formation. In *Interferon And The Immune System*, pp. 85-100. Edited by J. Vilcek & E. De Maeyer. Amsterdam: Elsevier Science Publishing Company, Inc.

Sonenberg, N. (1987). Regulation of Translation by Poliovirus. *Advances in Virus Research* **33**, 175-204.

Sonenberg, N. (1990). Measures and Counter Measures in the Modulation of Initiation Factor Activities By Viruses. *The New Biologist* **2**, 402-409.

Southern, E. M. (1975). Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. *Journal of Molecular Biology* **98**, 503-517.

Sperling, J., Chebath, J., Arad-Dunn, H., Offen, D., Spann, P., Lehrer, R., Goldblatt, D., Jolles, B. & Sperling, R. (1991). Possible Involvement of (2-5) Oligoadenylate Synthetase Activity in Pre-mRNA Splicing. *Proceedings of the National Academy of Science (USA)* **88**, 10377-10381.

Spriggs, M. K., Hruby, D. E., Maliszewski, C. R., Pickup, D. J., Sims, J. E., Buller, R. M. L. & VanSlyke, J. (1992). Vaccinia and Cowpox Viruses Encode a Novel Interleukin-1-Binding Protein. *Cell* **71**, 145-152.

Stitz, L., Althage, A., Hengartner, H. & Zinkernagel, R. (1985). Natural Killer Cells vs Cytotoxic T Cells in the Peripheral Blood of Virus-Infected Mice. *Journal of Immunology* **134**, 598-602.

Stokes, G. (1976). High-Voltage Electron Microscope Study of the Release of Vaccinia Virus From Whole Cells. *Journal of Virology* **18**, 636-643.

Swank, R. T. & Munkres, K. D. (1971). Molecular Weight Analysis of Oligopeptides By Electrophoresis in Polyacrylamide Gel With Sodium Dodecyl Sulphate. *Analytical Biochemistry* **39**, 462-477.

Symons, J. A., Alcami, A. & Smith, G. L. (1995). Vaccinia Virus Encodes a Soluble Type I Interferon Receptor of Novel Structure and Broad Species Specificity. *Cell* **81**, 551-560.

Tanaka, H. & Samuel, C. E. (1994). Mechanism of Interferon Action - Structure of the Mouse PKR Gene Encoding the Interferon-Inducible RNA-Dependent Protein-Kinase. *Proceedings of the National Academy of Science (USA)* **91**, 7995-7999.

Taniguchi, T., Sakai, M., Fujii-Kuriyama, Y., Muramatsu, M., Kobayashi, S. & Sudo, T. (1979). Construction and Identification of a Bacterial Plasmid Containing the Human Fibroblast Interferon gene Sequence. *Proceedings of the Japanese Academy, Ser. B* **55 (B)**, 464-469.

Taniguchi, T., Ohno, S., Fujii-Kuriyama, Y. & Muramatsu, M. (1980). The Nucleotide Sequence of Human Fibroblast Interferon cDNA. *Gene* **10**, 11-15.

Tanizaki, E., Kotani, T., Odagiri, Y. & Horiuchi, T. (1989). Pathologic Changes in Chickens Caused by Intravenous Inoculation with Fowlpox Virus. *Avian Disease* **33**, 333-339.

Tartaglia, J., Winslow, J., Goebel, S., Johnson, G. P., Taylor, J. & Paoletti, E. (1990). Nucleotide Sequence Analysis of a 10.5 Kbp HindIII Fragment of Fowlpox Virus: Relatedness to the Central Portion of the Vaccinia Virus HindIII D Region. *Journal of General Virology* **71**, 1517-1524.

Taylor, J., Weinberg, R., Languet, B., Desmettre, P. & Paolettie, E. (1988). Recombinant Fowlpox Virus Inducing Protective Immunity in Non-Avian Species. *Vaccine* **6**, 497-503.

Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Gullemin, F., Desmettre, P. & Paolettie, E. (1991). Efficacy studies on a Canarypox-Rabies Recombinant Virus. *Vaccine* **9**, 190-193.

Taylor, J., Tartaglia, J., Richardson, G., Alkatib, G., Briedis, D., Appel, M., Norton, E. & Paolettie, E. (1992). Nonreplicating Viral Vectors as Potential Vaccines: Recombinant Canarypox Virus Expressing Measles Virus Fusion (F) and Haemaglutinatinin (HA) Glycoproteins. *Virology* **187**, 321-328.

Tengelsen, L. A., Slabaugh, M. B., Bibler, J. K. & Denis, E. H. (1988). Nucleotide Sequence and Molecular Genetic Analysis of the Late Subunit of Ribonucleotide Reductase Encoded by Vaccinia Virus. *Virology* **164**, 121-131.

Tewari, M. & Dixit, V. M. (1995). Fas- and Tumour Necrosis Factor-Induced Apoptosis is Inhibited by the Poxvirus crmA Gene Product. *Journal of Biological Chemistry* **270**, 3255-3260.

Thanos, D. & Maniatis, T. (1995). NF-κB: A Lesson in Family Values. *Cell* **80**, 529-532.

Thomis, D. C., Doohan, J. P. & Samuel, C. E. (1992). Mechanisms of Interferon Action: cDNA Structure, Expression, and Regulation of the Interferon-Induced, RNA-Dependent P1/eIF-2α Protein Kinase from Human Cells. *Virology* **188**, 33-46.

Thomis, D. C. & Samuel, C. E. (1992). Mechanism of Interferon Action - Autoregulation of RNA-Dependent P1/eIF-2-Alpha Protein-Kinase (PKR) Expression in Transfected Mammalian-Cells. *Proceedings of the National Academy of Science (USA)* **89**, 10837-10841.

Thomis, D. C. & Samuel, C. E. (1993). Mechanism of Interferon Action - Evidence for Intermolecular Autophosphorylation and Autoactivation of the Interferon-Induced, RNA-Dependent Protein-Kinase PKR. *Journal of Virology* **67**, 7695-7700.

Tomley, F., Binns, M., Campbell, J. & Boursnell, M. (1988). Sequence Analysis of an 11.2 Kilobase, Near-Terminal, BamHI Fragment of Fowlpox Virus. *Journal of General Virology* **69**, 1025-1040.

Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, M., Coupar, B., Boyle, D., Chan, S. & Smith, G. (1988). Defective Presentation to Class I-Restricted Cytotoxic T Lymphocytes in Vaccinia Virus Infected Cells is Overcome by Enhanced Degradation of Antigen. *Journal of Experimental Medicine* **168**, 1211-1224.

Traktman, P. (1990). The Enzymology of Poxvirus DNA Replication. *Current Topics in Microbial Immunology* **163**, 93-123.

Traktman, P., Sridhar, P., Condit, R. C. & Roberts, B. E. (1984). Transcriptional Mapping of the DNA Polymerase Gene of Vaccinia virus. *Journal of Virology* **49**, 125-131.

Tripathy, D. N. & Cunningham, C. H. (1984). Avipox. In *Diseases in Poultry*, pp. 524-534. Edited by M. S. Hofstad, J. H. Barnes, B. W. Calneck, M. W. Reid & H. W. Yoder. Ames: Iowa State University Press.

Turner, G. S. & Squires, E. J. (1971). Inactivated Smallpox Vaccine: Immunogenicity of Inactivated Intracellular and Extracellular Vaccinia Virus. *Journal of General Virology* **13**, 19-25.

Upton, C., Macen, J. L., Wishart, D. S. & McFadden, G. (1991). Myxoma Virus Expresses a Secreted Protein with Homology to the Tumour Necrosis Factor Receptor Gene Family that Contributes to Virus Virulence. *Virology* **184**, 370-382.

Upton, C., Mossman, K. & McFadden, G. (1992). Encoding of a Homologue of the IFN-γ Receptor by Myxoma Virus. *Science* **258**, 1369-1372.

Vogel, S. N. & Fertsch, D. (1984). Endogenous Interferon Production by Endotoxin-Responsive Macrophages Provides an Autostimulatory Differentiation Signal. *Infect. Immun.* **45**, 417-423.

Vogel, S. N. & Friedman, R. M. (1984). Interferon and Macrophage Activation and Cell Surface Changes. In *Interferon And The Immune System*, pp. 35-60. Edited by J. Vilcek & E. De Maeyer. Amsterdam: Elsevier Science Publishing Company, Inc.

Vogel, S. N. & Fertsch, D. (1987). Macrophages From Endotoxin-Hyporesponsive (Lpsd) C3H/HeJ Mice are Permissive for Vesicular Stomatitis Virus Because of Reduced Levels of Endogenous Interferon: Possible Mechanism for Natural Resistance to Virus Infection. *Journal of Virology* **61**, 812-818.

Vos, J. C. & Stunnenberg, H. G. (1988). Derepression of a Novel Class of Vaccinia Virus Genes Upon DNA Replication. *The EMBO Journal* **7**, 3487-3492.

Vos, J. C., Sasker, M. & Stunnenberg, H. G. (1991a). Promoter Melting by a Stage-Specific Vaccinia Virus Transcription Factor is Independent of the Presence of RNA Polymerase. *Cell* **65**, 105-113.

Vos, J. C., Sasker, M. & Stunnenberg, H. G. (1991b). Vaccinia Virus Capping Enzyme is a Transcription Initiation Factor. *EMBO Journal* **10**, 2553-2558.

Wallach, D., Fellous, M. & Revel, M. (1982). Preferential Effect of Gamma Interferon on the Synthesis of HLA Antigens and their mRNAs in Human Cells. *Nature* **299**, 833-836.

Wang, Q., Bergeron, J., Mabrouk, T. & Lemay, G. (1996). Site-Directed Mutagenesis of the Double-Stranded RNA Binding Domain of Bacterially Expressed σ3 Reovirus Protein. *Virus Research* **41**, 141-151.

Wang, S., Shuman, S. (1996). A Temperature-Sensitive Mutation of the Vaccinia Virus E11 Gene Encoding a 15-kDa Virion Component. *Virology* **216**, 252-257.

Watson, J., Chang, H. & Bertram, J. L. (1991). Characterization of a Vaccinia Virus-Encoded Double-Stranded RNA-Binding Protein that may be Involved In Inhibition of the Double-Stranded RNA-Dependent Protein Kinase. *Virology* **185**, 26-219.

Webster, R. G. & Granoff, A. (1994). Encyclopedia of Virology, pp. 496-503. London: Academic Press Limited.

Wei, C. M. & Moss, B. (1975). Methylated Nucleotides Block 5'-Terminus of Vaccinia Virus Messenger RNA. *Proceedings of the National Academy of Science (USA)* **72**, 318-322.

Weir, J. P. & Moss, B. (1983). Nucleotide Sequence of the Vaccinia Virus Thymidine Kinase Gene and the Nature of Spontaneous Frameshift Mutations. *Journal of Virology* **46**, 530-537.

Whitaker-Dowling, P. & Youngner, J. S. (1983). Vaccinia Rescue of VSV from Interferon-Induced Resistance: Reversible Translation Block and Inhibition of Protein Kinase Activity. *Virology* **131**, 128-136.

Whitaker-Dowling, P. & Youngner, J. S. (1984). Characterization of a Specific Kinase Inhibitory Factor Produced by Vaccinia Virus Which Inhibits the Interferon-Induced Protein Kinase. *Virology* **137**, 171-181.

Whitaker-Dowling, P. & Youngner, J. S. (1986). Vaccinia Mediated Rescue of Encephalomyocarditis Virus from the Inhibitory Effects of Interferon. *Virology* **152**, 50-57.

Wilcock, D. & Smith, G. L. (1994). Vaccinia Virus Core Protein VP8 is Required for Virus Infectivity, but not for Core Protein Processing or for INV and EEV Formation. *Virology* **202**, 294-304.

Williams, B. R. G. (1995). The Role of the dsRNA-Activated Kinase, PKR, in Signal Transduction. *Seminars in Virology* **6**, 191-202.

Wong, G. H. W. & Goeddel, D. V. (1986). Tumour Necrosis Factors α and β Inhibit Virus Replication and Synergise with Interferons. *Nature* **323**, 819-822.

Wu, S. Y. & Kaufman, R. J. (1996). Double-Stranded (Ds) RNA-Binding and Not Dimerization Correlates With the Activation of the dsRNA-Dependent Protein-Kinase (PKR). *Journal of Biological Chemistry* **271**, 1756-1763.

Xiao, Q. R., Sharp, T. V., Jeffrey, I. W., James, M. C., Pruijn, G., van Venrooij, W. J. & Clemens, M. J. (1994). The La Antigen Inhibits the Activation of the Interferon-Inducible Protein-Kinase PKR by Sequestering and Unwinding Double-Stranded-RNA. *Nucleic Acids Research* **22**, 2512-2518.

Yang, Y. L., Reis, L. F. L., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R. G., Aguet, M. & Weissmann, C. (1995). Deficient Signalling in Mice Devoid of Double-Stranded RNA-Dependent Protein-Kinase. *The EMBO Journal* **14**, 6095-6106.

Yoshida, I. & Marcus, P. I. (1990). Interferon Induction by Viruses. XX. Acid-Labile Interferon Accounts for the Antiviral Effect Induced by Poly(rI). Poly(rC) in Primary Chick Embryo Cells. *Journal of Interferon Research* **10**, 461-468.

Youngner, J. S., Thacore, H. R. & Kelly, M. E. (1972). Sensitivity of Ribonucleic Acid and Deoxyribonucleic Acid Viruses to Different Species of Interferon in Cell Cultures. *Journal of Virology* **10**, 171-178.

Yuen, L. & Moss, B. (1987). Oligonucleotide Sequence Signalling Transcriptional Termination of Vaccinia Virus Early Genes. *Proceedings of the National Academy of Science (USA)* **84**, 6417-6421.

Yuwen, H., Cox, J. H., Yewedell, J. W., Bennink, J. W. & Moss, B. (1993). Nuclear Localization of a Double-Stranded RNA-Binding Protein Encoded by the Vaccinia Virus E3L Gene. *Virology* **195**, 732-744.

Zhu, S., Sobolov, A. & Wek, R. C. (1996). Ribosome Targeting of eIF-2 Protein-Kinases, GCN2 and PKR. *FASEB Journal* **10**, 1548-1548.

Appendices

APPENDICES

Appendix 6.1. BLAST Analysis of PCR Clones

Query= 1000 bp Clone
(151 letters)

```
                                                                    Smallest
                                                                      Sum
                                                    Reading  High  Probability
Sequences producing High-scoring Segment Pairs:       Frame Score  P(N)       N

sp|P03772|PP_LAMBD   SERINE/THREONINE PROTEIN PHOSPHAT... +3   121  1.7e-14    2


sp|P03772|PP_LAMBD SERINE/THREONINE PROTEIN PHOSPHATASE. pir||Q1BP1L
            hypothetical protein C-221 (nin region) - phage lambda
gi|215160
            (J02459) Nin 221 (pept unknown;221) [Bacteriophage lambda]
            Length = 221

  Plus Strand HSPs:

 Score = 121 (55.7 bits), Expect = 1.7e-14, Sum P(2) = 1.7e-14
 Identities = 21/22 (95%), Positives = 21/22 (95%), Frame = +3

Query:    12 PFGEYEFGKPVDHQQVIWNRER 77
             PF EYEFGKPVDHQQVIWNRER
Sbjct:   143 PFDEYEFGKPVDHQQVIWNRER 164

 Score = 52 (23.9 bits), Expect = 1.7e-14, Sum P(2) = 1.7e-14
 Identities = 11/13 (84%), Positives = 12/13 (92%), Frame = +1

Query:    76 ERISNSQNGIGKK 114
             ERISNSQNGI K+
Sbjct:   163 ERISNSQNGIVKE 175
```

Query= 800 bp clone
(124 letters)

```
                                                                    Smallest
                                                                      Sum
                                                    Reading  High  Probability
Sequences producing High-scoring Segment Pairs:       Frame Score  P(N)       N

pir||SYEXI           isoleucine--tRNA ligase (EC 6.1.1... +3    80  0.0015     1
sp|P26499|SYI_METTH  ISOLEUCYL-TRNA SYNTHETASE (ISOLEU... +3    80  0.0015     1
sp|P46214|SYI_PYRFU  ISOLEUCYL-TRNA SYNTHETASE (ISOLEU... +3    71  0.022      1


pir||SYEXI isoleucine--tRNA ligase (EC 6.1.1.5) - Methanobacterium
            thermoautotrophicum gi|149728 (M59245) transfer RNA-Ile
synthetase
            [Methanobacterium thermoautotrophicum]
            Length = 1045

  Plus Strand HSPs:
```

```
 Score = 80 (35.9 bits), Expect = 0.0015, P = 0.0015
 Identities = 16/30 (53%), Positives = 18/30 (60%), Frame = +3

Query:    18 GEDDFNVGKEYGLEVAVTVDEKGYIAISAG 107
             G +DF +GKEYGL V   VDE G     AG
Sbjct:   323 GPEDFEIGKEYGLPVFCPVDEAGVFTEDAG 352


sp|P26499|SYI_METTH ISOLEUCYL-TRNA SYNTHETASE (ISOLEUCINE--TRNA LIGASE)
            (ILERS).
            Length = 1046

  Plus Strand HSPs:

 Score = 80 (35.9 bits), Expect = 0.0015, P = 0.0015
 Identities = 16/30 (53%), Positives = 18/30 (60%), Frame = +3

Query:    18 GEDDFNVGKEYGLEVAVTVDEKGYIAISAG 107
             G +DF +GKEYGL V   VDE G     AG
Sbjct:   324 GPEDFEIGKEYGLPVFCPVDEAGVFTEDAG 353


sp|P46214|SYI_PYRFU ISOLEUCYL-TRNA SYNTHETASE (ISOLEUCINE--TRNA LIGASE)
            (ILERS). gi|598367 (L37105) transfer RNA-Ile synthetase
[Pyrococcus
            furiosus]
            Length = 546

  Plus Strand HSPs:

 Score = 71 (31.9 bits), Expect = 0.023, P = 0.022
 Identities = 13/23 (56%), Positives = 18/23 (78%), Frame = +3

Query:    18 GEDDFNVGKEYGLEVAVTVDEKG 86
             GE+DF VG++YGL V   VD++G
Sbjct:   254 GEEDFEVGQKYGLPVYSPVDDQG 276


gi|1591614 (U67538) isoleucyl-tRNA synthetase [Methanococcus jannaschii]
            Length = 1039

  Plus Strand HSPs:

 Score = 67 (30.1 bits), Expect = 0.037, Sum P(2) = 0.036
 Identities = 11/23 (47%), Positives = 17/23 (73%), Frame = +3

Query:    18 GEDDFNVGKEYGLEVAVTVDEKG 86
             GE+DF VGK+Y L +    +D++G
Sbjct:   341 GEEDFEVGKKYNLPIYSPIDDEG 363

 Score = 25 (11.2 bits), Expect = 0.037, Sum P(2) = 0.036
 Identities = 4/7 (57%), Positives = 5/7 (71%), Frame = +2

Query:    86 LHSNKCW 106
             L +NK W
Sbjct:   628 LSANKVW 634
```

Query= 1400 bp clone

(136 letters)

Translating both strands of query sequence in all 6 reading frames

Database: Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR
229,924 sequences; 64,927,982 total letters.

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
gnl|PID|e4100	(X04329) furin; 1231 is 2nd base ...	+1	32	0.011	2
gi|1261996	(Z70537) viral protein 1 [Erythro...	+2	47	0.013	2
gi|1353320	(U31157) amylase [Drosophila eugr...	+2	42	0.024	2

gnl|PID|e4100 (X04329) furin; 1231 is 2nd base in codon, 1348 is 1st base in codon [Homo sapiens]
Length = 39

Plus Strand HSPs:

Score = 32 (14.7 bits), Expect = 0.011, Sum P(2) = 0.011
Identities = 5/15 (33%), Positives = 7/15 (46%), Frame = +1

```
Query:    34 PTCWISTLGKRGQTG 78
             P  W+   G+R   G
Sbjct:     2 PLIWLRAFGRRRHGG 16
```

Score = 30 (13.8 bits), Expect = 0.011, Sum P(2) = 0.011
Identities = 5/10 (50%), Positives = 6/10 (60%), Frame = +3

```
Query:    90 PSFRLVHRHP 119
             P   + HRHP
Sbjct:    26 PPAEVHHRHP 35
```

gi|1261996 (Z70537) viral protein 1 [Erythrovirus B19]
Length = 102

Plus Strand HSPs:

Score = 47 (21.6 bits), Expect = 0.013, Sum P(2) = 0.013
Identities = 11/31 (35%), Positives = 15/31 (48%), Frame = +2

```
Query:    26 DHHQPVGYQLSARGGRLVHPTSKLQTGAPTS 118
             D H+P    +   G   V P ++LQ G P S
Sbjct:    32 DLHKPGQVSVQLPGTNYVGPGNELQAGPPQS 62
```

Score = 35 (16.1 bits), Expect = 0.013, Sum P(2) = 0.013
Identities = 5/8 (62%), Positives = 7/8 (87%), Frame = +2

```
Query:    11 HGIVTDHH 34
             HG ++DHH
Sbjct:     2 HGQLSDHH 9
```

```
gi|1353320 (U31157) amylase [Drosophila eugracilis]
           Length = 145

  Plus Strand HSPs:

 Score = 42 (19.3 bits), Expect = 0.025, Sum P(2) = 0.024
 Identities = 6/15 (40%), Positives = 10/15 (66%), Frame = +2

Query:     26 DHHQPVGYQLSARGG 70
              + +QP+ Y+L  R G
Sbjct:     20 ERYQPISYKLETRSG 34

 Score = 41 (18.9 bits), Expect = 0.025, Sum P(2) = 0.024
 Identities = 9/20 (45%), Positives = 11/20 (55%), Frame = +2

Query:     65 GGRLVHPTSKLQTGAPTSKL 124
              GG   +P+SK   G P S L
Sbjct:     71 GGSTANPSSKSFPGVPYSSL 90



gi|1353308 (U31124) amylase [Drosophila varians]
           Length = 144

  Plus Strand HSPs:

 Score = 41 (18.9 bits), Expect = 0.046, Sum P(2) = 0.045
 Identities = 9/20 (45%), Positives = 10/20 (50%), Frame = +2

Query:     65 GGRLVHPTSKLQTGAPTSKL 124
              GG    P+SK   G P S L
Sbjct:     71 GGTTASPSSKSYPGVPYSSL 90

 Score = 40 (18.4 bits), Expect = 0.046, Sum P(2) = 0.045
 Identities = 6/15 (40%), Positives = 10/15 (66%), Frame = +2

Query:     26 DHHQPVGYQLSARGG 70
              + +QP+ Y+L  R G
Sbjct:     19 ERYQPISYKLVTRSG 33
```

Appendix 6.1. BLAST Analysis of PCR Clones

The clones were manually sequenced and anaysed at at the National Center for Biotechnology Information (NCBI) using the BLAST network service which translates the DNA sequence in all 6 frames and then searches the databases for similar proteins. The top of the three sequences is the experimental (query) sequence. The bottom sequence (subject) is the library sequence. The middle sequence shows amino acids which are identical in both the experimental and library sequences and + signs represent conservative changes between the two sequences. Only the most significant hits are shown here.

Reference for BLast Analysis: Gish, Warren and David J. States (1993). Identification of
protein coding regions by database similarity search. Nat. Genet. 3:266-72.
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Appendix 6.2. BLAST Analysis of the Smaller 900 bp PCR Fragment

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
sp|P24834|VE5_RHPV1	PROBABLE E5 PROTEIN.	-1	37	0.0084	3
pir||W5WLR1	E5 protein - rhesus papillomaviru...	-1	37	0.0084	3
gi|1139663	(U43400) late spliced gene [Human...	-2	37	0.14	3
sp|P15790|KC21_YEAST	CASEIN KINASE II, ALPHA CHAIN (CK...	-2	36	0.17	3
gi|1098998	(U41510) coded for by C. elegans ...	-2	42	0.27	2
gi|773414	(U23751) beta galactosidase [Clon...	+3	42	0.34	2
pir||S51428	hypothetical protein L9470.22 - y...	-1	40	0.42	2
gi|532798	(U13398) protein kinase [Saccharo...	-2	43	0.43	2
gi|1055256	(U36899) pheromone receptor VN2 [...	-2	43	0.46	2
sp|P16731|UL88_HCMVA	HYPOTHETICAL PROTEIN UL88. /pir||...	-2	31	0.46	3
gnl|PID|e191449	(X89999) 2B1 antigen gene product...	+2	45	0.55	2
sp|P38990|PAK1_YEAST	SERINE/THREONINE-PROTEIN KINASE P...	-2	43	0.63	2
gi|757507	(D38564) receptor protein kinase ...	-2	48	0.63	2
pir||JC2482	S-receptor kinase (EC 2.7.1.-) 12...	-2	48	0.63	2

```
sp|P24834|VE5_RHPV1 PROBABLE E5 PROTEIN.
           Length = 157

  Minus Strand HSPs:

 Score = 37 (17.0 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 8/22 (36%), Positives = 12/22 (54%), Frame = -1

Query:     70 FCSRVFFSNPPRSETGLQEFDI 5
              F + V F   P    + GLQ+ D+
Sbjct:     97 FFNPVAFDTPACPQCGLQQNDV 118

 Score = 31 (14.3 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 4/11 (36%), Positives = 6/11 (54%), Frame = -2

Query:    153 WVHSEDIHLFN 121
              W H + +H  N
Sbjct:      9 WSHFKPVHTLN 19

 Score = 30 (13.8 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 4/6 (66%), Positives = 5/6 (83%), Frame = -1

Query:     82 RKPPFC 65
              + PPFC
Sbjct:     35 KPPPFC 40


pir||W5WLR1 E5 protein - rhesus papillomavirus (type 1)
           Length = 157

  Minus Strand HSPs:

 Score = 37 (17.0 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 8/22 (36%), Positives = 12/22 (54%), Frame = -1

Query:     70 FCSRVFFSNPPRSETGLQEFDI 5
              F + V F   P    + GLQ+ D+
Sbjct:     97 FFNPVAFDTPACPQCGLQQNDV 118
```

```
 Score = 31 (14.3 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 4/11 (36%), Positives = 6/11 (54%), Frame = -2

Query:   153 WVHSEDIHLFN 121
             W H + +H  N
Sbjct:     9 WSHFKPVHTLN 19

 Score = 30 (13.8 bits), Expect = 0.0085, Sum P(3) = 0.0084
 Identities = 4/6 (66%), Positives = 5/6 (83%), Frame = -1

Query:    82 RKPPFC 65
             + PPFC
Sbjct:    35 KPPPFC 40



gi|1139663 (U43400) late spliced gene [Human herpesvirus 7]
           Length = 663

  Minus Strand HSPs:

 Score = 37 (17.0 bits), Expect = 0.15, Sum P(3) = 0.14
 Identities = 6/23 (26%), Positives = 11/23 (47%), Frame = -2

Query:   123 NKCFFCSIKSLFISENLRFVRAF 55
             N   FC  + + ++  L F  A+
Sbjct:    33 NSAIFCQKRFIILTPELGFTHAY 55

 Score = 33 (15.2 bits), Expect = 0.15, Sum P(3) = 0.14
 Identities = 4/10 (40%), Positives = 7/10 (70%), Frame = -2

Query:   156 IWVHSEDIHL 127
             IW H +D+ +
Sbjct:    18 IWKHEQDVKI 27

 Score = 31 (14.3 bits), Expect = 0.15, Sum P(3) = 0.14
 Identities = 6/10 (60%), Positives = 8/10 (80%), Frame = -1

Query:    34 SETGLQEFDI 5
             +E GL EFD+
Sbjct:   423 TEQGLIEFDL 432



sp|P15790|KC21_YEAST CASEIN KINASE II, ALPHA CHAIN (CK II). pir||A31564
           casein kinase II (EC 2.7.1.-) alpha chain - yeast
(Saccharomyces
           cerevisiae) gi|171324 (M22473) casein kinase II alpha subunit
           [Saccharomyces cerevisiae] gi|600013 (Z46861) casein kinase II
           alpha chain [Saccharomyces cerevisiae] gi|763311 (Z47047) Cka1p
           [Saccharomyces cerevisiae]
           Length = 372

  Minus Strand HSPs:

 Score = 36 (16.6 bits), Expect = 0.19, Sum P(3) = 0.17
 Identities = 5/8 (62%), Positives = 8/8 (100%), Frame = -2
```

```
Query:     48 VIHRDLKP 25
              ++HRD+KP
Sbjct:    191 IMHRDVKP 198

 Score = 31 (14.3 bits), Expect = 0.19, Sum P(3) = 0.17
 Identities = 7/12 (58%), Positives = 8/12 (66%), Frame = -3

Query:     89 LYPKTSVLFARF 54
              LYPK + L  RF
Sbjct:    163 LYPKLTDLEIRF 174

 Score = 29 (13.3 bits), Expect = 0.19, Sum P(3) = 0.17
 Identities = 5/9 (55%), Positives = 7/9 (77%), Frame = -2

Query:    147 HSEDIHLFN 121
              H+  IHLF+
Sbjct:    130 HANIIHLFD 138


gi|1098998 (U41510) coded for by C. elegans cDNA yk85d4.5;  Similar to
             map kinase kinase; 3beta-hsd. [Caenorhabditis elegans]
             Length = 1218

  Minus Strand HSPs:

 Score = 42 (19.3 bits), Expect = 0.32, Sum P(2) = 0.27
 Identities = 7/16 (43%), Positives = 10/16 (62%), Frame = -2

Query:    141 EDIHLFNKCFFCSIKS 94
              ED  +++  FFCS  S
Sbjct:    683 EDYEIYHTSFFCSTSS 698

 Score = 42 (19.3 bits), Expect = 0.32, Sum P(2) = 0.27
 Identities = 7/17 (41%), Positives = 11/17 (64%), Frame = -2

Query:     75 LRFVRAFSSVIHRDLKP 25
              L  ++    +IHRD+KP
Sbjct:    898 LEHLKTTHHIIHRDIKP 914


gi|773414 (U23751) beta galactosidase [Cloning vector pBBR1MCS-2]
             gi|833819 (U25059) LacZ alpha peptide [Cloning vector pBBR1MCS-
3]
             gi|833823 (U25060) LacZ alpha peptide [Cloning vector pBBR1MCS-
4]
             gi|833827 (U25061) LacZ alpha peptide [Cloning vector pBBR1MCS-
5]
             Length = 121

  Plus Strand HSPs:

 Score = 42 (19.3 bits), Expect = 0.42, Sum P(2) = 0.34
 Identities = 8/8 (100%), Positives = 8/8 (100%), Frame = +3

Query:      3 LISNSCSP 26
              LISNSCSP
Sbjct:     33 LISNSCSP 40
```

```
 Score = 31 (14.3 bits), Expect = 0.42, Sum P(2) = 0.34
 Identities = 6/11 (54%), Positives = 6/11 (54%), Frame = +2

Query:     41 WITEENARTKR 73
              W   E ART R
Sbjct:     97 WRNSEEARTDR 107



pir||S51428 hypothetical protein L9470.22 - yeast (Saccharomyces
            cerevisiae) gi|577214 (U17246) L9470.22 gene product
[Saccharomyces
            cerevisiae]
            Length = 489

  Minus Strand HSPs:

 Score = 40 (18.4 bits), Expect = 0.55, Sum P(2) = 0.42
 Identities = 6/11 (54%), Positives = 9/11 (81%), Frame = -1

Query:    157 HMGALRRHSFI 125
              HMG++R+H  I
Sbjct:    266 HMGSIRKHPLI 276

 Score = 39 (17.9 bits), Expect = 0.55, Sum P(2) = 0.42
 Identities = 9/24 (37%), Positives = 13/24 (54%), Frame = -1

Query:    103 DKVSLYIRKPPFCSRVFFSNPPRS 32
              DK  + ++KP    RV  S P +S
Sbjct:    340 DKNPMTMKKPKLNKRVLPSKPKKS 363



gi|532798 (U13398) protein kinase [Saccharomyces cerevisiae]
            Length = 658

  Minus Strand HSPs:

 Score = 43 (19.8 bits), Expect = 0.56, Sum P(2) = 0.43
 Identities = 6/11 (54%), Positives = 9/11 (81%), Frame = -2

Query:     57 FSSVIHRDLKP 25
              +  +IHRD+KP
Sbjct:    269 YQGIIHRDIKP 279

 Score = 37 (17.0 bits), Expect = 0.56, Sum P(2) = 0.43
 Identities = 10/18 (55%), Positives = 11/18 (61%), Frame = -2

Query:    114 FFCSIKSLFISENLRFVR 61
              FF  IKS  ISEN +  R
Sbjct:    174 FFTFIKSSKISENDKIKR 191

gi|1055256 (U36899) pheromone receptor VN2 [Rattus norvegicus]
            Length = 321

  Minus Strand HSPs:

 Score = 43 (19.8 bits), Expect = 0.61, Sum P(2) = 0.46
 Identities = 9/25 (36%), Positives = 13/25 (52%), Frame = -2
```

```
Query:   147 HSEDIHLFNKCFFCSIKSLFISENL 73
             H    H+F    F S+  +FIS +L
Sbjct:   137 HKPPHHIFCAMLFLSVLYMFISSHL 161

 Score = 34 (15.6 bits), Expect = 0.61, Sum P(2) = 0.46
 Identities = 6/12 (50%), Positives = 7/12 (58%), Frame = -1

Query:    67 CSRVFFSNPPRS 32
             CSR  + N P S
Sbjct:   270 CSRTMYLNDPTS 281



sp|P16731|UL88_HCMVA HYPOTHETICAL PROTEIN UL88. pir||S09852
             hypothetical protein UL88 - human cytomegalovirus (strain
AD169)
             gi|833072 (X17403) HCMVUL88 (AA 1-429) [Human cytomegalovirus]
             Length = 429

  Minus Strand HSPs:

 Score = 31 (14.3 bits), Expect = 0.62, Sum P(3) = 0.46
 Identities = 4/5 (80%), Positives = 5/5 (100%), Frame = -2

Query:   117 CFFCS 103
             CF+CS
Sbjct:    69 CFYCS 73

 Score = 31 (14.3 bits), Expect = 0.62, Sum P(3) = 0.46
 Identities = 6/10 (60%), Positives = 9/10 (90%), Frame = -3

Query:    89 LYPKTSVLFA 60
             ++P TSVL+A
Sbjct:   163 IWPGTSVLWA 172

 Score = 31 (14.3 bits), Expect = 0.62, Sum P(3) = 0.46
 Identities = 8/16 (50%), Positives = 9/16 (56%), Frame = -1

Query:    61 RVFFSNPPRSETGLQE 14
             R+  SN PRS   L E
Sbjct:   245 RLRMSNIPRSSARLLE 260



gnl|PID|e191449 (X89999) 2B1 antigen gene product [Rattus norvegicus]
             Length = 512

  Plus Strand HSPs:

 Score = 45 (20.7 bits), Expect = 0.79, Sum P(2) = 0.55
 Identities = 11/33 (33%), Positives = 17/33 (51%), Frame = +2

Query:     8 IEFLQPGFRSRWITEENARTKRRFSDIKRDFIE 106
             IE +Q    +  ITE   R K +F    ++F+E
Sbjct:   168 IELVQAADPAINITEATVRAKAQFEGAAKEFME 200

 Score = 33 (15.2 bits), Expect = 0.79, Sum P(2) = 0.55
 Identities = 7/16 (43%), Positives = 10/16 (62%), Frame = +3
```

```
Query:    93 ETLSNKRSIY*INECL 140
             E  S+ RSI  +N C+
Sbjct:   444 EETSDMRSIQDVNVCM 459


sp|P38990|PAK1_YEAST SERINE/THREONINE-PROTEIN KINASE PAK1. pir||S50632
           protein kinase PAK1 (EC 2.7.1.-) - yeast (Saccharomyces
cerevisiae)
           gi|603368 (U18916) Saccharomyces cerevisiae chromosome V
cosmids
           9781, 8198, 9115, 9981, and lambda clones 3955 and 6052.
           [Saccharomyces cerevisiae]
           Length = 1142

  Minus Strand HSPs:

 Score = 43 (19.8 bits), Expect = 0.99, Sum P(2) = 0.63
 Identities = 6/11 (54%), Positives = 9/11 (81%), Frame = -2

Query:    57 FSSVIHRDLKP 25
             +  +IHRD+KP
Sbjct:   270 YQGIIHRDIKP 280

 Score = 37 (17.0 bits), Expect = 0.99, Sum P(2) = 0.63
 Identities = 10/18 (55%), Positives = 11/18 (61%), Frame = -2

Query:   114 FFCSIKSLFISENLRFVR 61
             FF  IKS  ISEN +  R
Sbjct:   174 FFTFIKSSKISENDKIKR 191


gi|757507 (D38564) receptor protein kinase [Brassica campestris]
           prf||2106157B S-receptor kinase [Brassica rapa]
           Length = 856

  Minus Strand HSPs:

 Score = 48 (22.1 bits), Expect = 1.0, Sum P(2) = 0.63
 Identities = 8/9 (88%), Positives = 9/9 (100%), Frame = -2

Query:    48 VIHRDLKPG 22
             +IHRDLKPG
Sbjct:   648 IIHRDLKPG 656

 Score = 31 (14.3 bits), Expect = 1.0, Sum P(2) = 0.63
 Identities = 5/13 (38%), Positives = 8/13 (61%), Frame = -2

Query:   159 FIWVHSEDIHLFN 121
             ++WV + D  L N
Sbjct:    85 YVWVANRDSSLSN 97


pir||JC2482 S-receptor kinase (EC 2.7.1.-) 12 precursor - field mustard
           Length = 856

  Minus Strand HSPs:
```

```
 Score = 48 (22.1 bits), Expect = 1.0, Sum P(2) = 0.63
 Identities = 8/9 (88%), Positives = 9/9 (100%), Frame = -2

Query:     48 VIHRDLKPG 22
              +IHRDLKPG
Sbjct:    648 IIHRDLKPG 656

 Score = 31 (14.3 bits), Expect = 1.0, Sum P(2) = 0.63
 Identities = 5/13 (38%), Positives = 8/13 (61%), Frame = -2

Query:    159 FIWVHSEDIHLFN 121
              ++WV + D  L N
Sbjct:     85 YVWVANRDSSLSN 97
```

Appendix 6.2. BLAST Analysis of of the Smaller 900 bp PCR Fragment
Manual sequencing was carried out on all the picked plaques. The sequences were analysed at at the National Center for Biotechnology Information (NCBI) using the BLAST network service which translates the DNA sequence in all 6 frames and then searches the databases for similar proteins. The top of the three sequences is the experimental (query) sequence. The bottom sequence (subject) is the library sequence. The middle sequence shows amino acids which are identical in both the experimental and library sequences and + signs represent conservative changes between the two sequences. The top 12 hits are shown here

Appendix 6.3. BLAST Analysis of Plaque Sequences

1-10a Forward

```
>sp|P35574|GDE_RABIT GLYCOGEN DEBRANCHING ENZYME (4-ALPHA-
GLUCANOTRANSFERASE
            (OLIGO-1,4-1,4-GLUCANTRANSFERASE) / AMYLO-1,6-GLUCOSIDASE
(DEXTRIN
            6-ALPHA-D-GLUCOSIDASE)) (GLYCOGEN DEBRANCHER). >gi|294478
(L10605)
            amylo-1, 6-glucosidase/4-alpha-glucancotransferase [Oryctolagus
            cuniculus]
            Length = 1555

  Minus Strand HSPs:

 Score = 57 (26.2 bits), Expect = 1.3, P = 0.73
 Identities = 9/20 (45%), Positives = 14/20 (70%), Frame = -1

Query:    62 PRRGPAAGEIPLLYRFFTYP 3
             P+ GP    + PL+ R+FT+P
Sbjct:   437 PKLGPVTRKYPLVTRYFTFP 456
```

1-10a Reverse

```
>gi|607954 (U12823) hemolysin [Acanthamoeba polyphaga]
           Length = 114

  Minus Strand HSPs:

 Score = 68 (31.3 bits), Expect = 3.1e-08, Sum P(2) = 3.1e-08
 Identities = 12/15 (80%), Positives = 14/15 (93%), Frame = -2

Query:    99 MLFTWNPAPLRPSKL 55
             +LFTWN +PLRPSKL
Sbjct:     1 LLFTWNLSPLRPSKL 15

 Score = 53 (24.4 bits), Expect = 3.1e-08, Sum P(2) = 3.1e-08
 Identities = 12/18 (66%), Positives = 13/18 (72%), Frame = -2

Query:    54 SFE*LLLPPRSAPAAAPP 1
             SFE LLLPPRSA  +  P
Sbjct:    18 SFEYLLLPPRSALGSVRP 35
```

1-9a Forward

```
>pat|US|5489524|4 Sequence 4 from patent US 5489524
            Length = 26

  Plus Strand HSPs:

 Score = 123 (56.6 bits), Expect = 3.7e-11, P = 3.7e-11
 Identities = 22/23 (95%), Positives = 22/23 (95%), Frame = +1

Query:    91 IHCTAGPPTVSHSGHGVRLTCLL 159
             IHCTAGPPTVSHSGHGV LTCLL
Sbjct:     1 IHCTAGPPTVSHSGHGVPLTCLL 23

>gi|58115 (X04125) open reading frame [Cloning vector lambda gt11]
          Length = 25
```

```
  Minus Strand HSPs:

 Score = 113 (52.0 bits), Expect = 9.4e-10, P = 9.4e-10
 Identities = 21/21 (100%), Positives = 21/21 (100%), Frame = -3

Query:    65 MACPVIIIFDTRPTGNGSDRR 3
             MACPVIIIFDTRPTGNGSDRR
Sbjct:     1 MACPVIIIFDTRPTGNGSDRR 21
```

1-9a Reverse

```
>gb|I00683| Sequence 11 from Patent US 4745179
            Length = 428

  Minus Strand HSPs:

 Score = 363 (167.0 bits), Expect = 6.0e-50, Sum P(3) = 6.0e-50
 Identities = 64/64 (100%), Positives = 64/64 (100%), Frame = -1

Query:   220 PSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAEEGTWLNIDGFHM
             PSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAEEGTWLNIDGFHM
Sbjct:   282 PSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAEEGTWLNIDGFHM

Query:    40 GIGG 29
             GIGG
Sbjct:   342 GIGG 345

 Score = 46 (21.2 bits), Expect = 6.0e-50, Sum P(3) = 6.0e-50
 Identities = 9/13 (69%), Positives = 10/13 (76%), Frame = -2

Query:    39 GLVAYDSWSPSVS 1
             G+   DSWSPSVS
Sbjct:   342 GIGGDDSWSPSVS 354

 Score = 40 (18.4 bits), Expect = 6.0e-50, Sum P(3) = 6.0e-50
 Identities = 7/8 (87%), Positives = 7/8 (87%), Frame = -3

Query:   242 MLTPYVFP 219
             M TPYVFP
Sbjct:   275 MYTPYVFP 282
```

1-9b Forward

```
>pat|US|5489524|4 Sequence 4 from patent US 5489524
            Length = 26

  Plus Strand HSPs:

 Score = 136 (62.6 bits), Expect = 5.9e-13, P = 5.9e-13
 Identities = 24/25 (96%), Positives = 24/25 (96%), Frame = +1

Query:    91 IHCTAGPPTVSHSGHGVRLTCLLIC 165
             IHCTAGPPTVSHSGHGV LTCLLIC
Sbjct:     1 IHCTAGPPTVSHSGHGVPLTCLLIC 25

>gi|58115 (X04125) open reading frame [Cloning vector lambda gt11]
          Length = 25

  Minus Strand HSPs:
```

```
 Score = 113 (52.0 bits), Expect = 9.4e-10, P = 9.4e-10
 Identities = 21/21 (100%), Positives = 21/21 (100%), Frame = -3

Query:    65 MACPVIIIFDTRPTGNGSDRR 3
             MACPVIIIFDTRPTGNGSDRR
Sbjct:     1 MACPVIIIFDTRPTGNGSDRR 21
```

1-10b Forward

```
>sp|P36730|VE1_HPV52 E1 PROTEIN. >pir||S36575 envelope protein - human
            papillomavirus type 52 >gi|397041 (X74481) early protein [Human
            papillomavirus type 52]
            Length = 647

  Plus Strand HSPs:

 Score = 52 (23.9 bits), Expect = 0.17, Sum P(2) = 0.15
 Identities = 10/22 (45%), Positives = 17/22 (77%), Frame = +3

Query:    48 ILLSI*CFFKFGKNRLTMAKIL 113
             +L+ +   FK GKNRLT++K++
Sbjct:   269 VLILLLIRFKCGKNRLTVSKLM 290

 Score = 32 (14.7 bits), Expect = 0.17, Sum P(2) = 0.15
 Identities = 5/7 (71%), Positives = 6/7 (85%), Frame = +1

Query:   109 FSFKNPY 129
             F FKNP+
Sbjct:   582 FHFKNPF 588
```

11-8a Forward

```
sp|P24170|POTE_ECOLI PUTRESCINE-ORNITHINE ANTIPORTER (PUTRESCINE
            TRANSPORT PROTEIN). pir||B40839 putrescine transport protein -
            Escherichia coli gi|147332 (M64495) putrescine transport
protein
            [Escherichia coli] gi|1651299 (D90708) Putrescine-ornithine
            antiporter (putrescine transport protein) [Escherichia coli]
            Length = 439

  Plus Strand HSPs:

 Score = 237 (109.0 bits), Expect = 1.2e-25, P = 1.2e-25
 Identities = 47/47 (100%), Positives = 47/47 (100%), Frame = +1

Query:     1 TLWAFLGLESACANTDVVENPERNVPIAVLGGTLGAAVIYIVSTNVI 141
             TLWAFLGLESACANTDVVENPERNVPIAVLGGTLGAAVIYIVSTNVI
Sbjct:   199 TLWAFLGLESACANTDVVENPERNVPIAVLGGTLGAAVIYIVSTNVI 245
```

11-8a Reverse

```
>sp|P32674|PFLD_ECOLI FORMATE ACETYLTRANSFERASE 2 (PYRUVATE FORMATE-LYASE
2).
            >gi|396298 (U00006) similar to E. coli pyruvate formate-lyase
            [Escherichia coli]
            Length = 765

  Plus Strand HSPs:

 Score = 182 (81.0 bits), Expect = 3.2e-17, P = 3.2e-17
```

```
 Identities = 37/37 (100%), Positives = 37/37 (100%), Frame = +3

Query:     3 GPTAVLKSVSKLDNTLLSNGTLLNVKFTPATLEGEAG 113
             GPTAVLKSVSKLDNTLLSNGTLLNVKFTPATLEGEAG
Sbjct:   657 GPTAVLKSVSKLDNTLLSNGTLLNVKFTPATLEGEAG 693
```

11-8 b Reverse

```
gi|532413 (U10214) polyprotein [Hepatitis C virus]
            Length = 191

  Plus Strand HSPs:

 Score = 60 (27.6 bits), Expect = 0.31, P = 0.27
 Identities = 10/14 (71%), Positives = 11/14 (78%), Frame = +3

Query:    27 YPWWFTANDRLGWA 68
             YPW F AN+ LGWA
Sbjct:    81 YPWPFYANEGLGWA 94
```

11-9b Reverse

```
sp|P34055|IC11_TRIHA INDC11 PROTEIN. pir||S32624 INDC11 protein -
            fungus (Trichoderma harzianum) gi|288988 (Z2?221) INDC11
            [Trichoderma harzianum]
            Length = 339

  Plus Strand HSPs:

 Score = 48 (22.1 bits), Expect = 0.00027, Sum P(3) = 0.00027
 Identities = 10/31 (32%), Positives = 14/31 (45%), Frame = +2

Query:    47 LGFPGAARRVMGITPPDREVGIVYGRNYDGI 139
             LGF          +TP +R V + YG N    +
Sbjct:   216 LGFESFDESYSILTPKERSVVVAYGNNLSNL 246

 Score = 35 (16.1 bits), Expect = 0.00027, Sum P(3) = 0.00027
 Identities = 6/7 (85%), Positives = 6/7 (85%), Frame = +2

Query:    26 LPPEPKD 46
             LPP PKD
Sbjct:    27 LPPPPKD 33

 Score = 34 (15.6 bits), Expect = 0.00027, Sum P(3) = 0.00027
 Identities = 6/10 (60%), Positives = 7/10 (70%), Frame = +1

Query:   148 FEPRLSFLIN 177
             FEP L  L+N
Sbjct:   286 FEPELQLLLN 295
```

15-7b Forward

```
gi|695543 (Z47995) FAS soluble protein [Homo sapiens]
            Length = 86

  Plus Strand HSPs:

 Score = 38 (17.5 bits), Expect = 0.054, Sum P(2) = 0.053
 Identities = 5/8 (62%), Positives = 6/8 (75%), Frame = +1
```

```
Query:     61 GGLCHQPC 84
              G  CH+PC
Sbjct:     56 GQFCHKPC 63

 Score = 38 (17.5 bits), Expect = 0.054, Sum P(2) = 0.053
 Identities = 7/11 (63%), Positives = 9/11 (81%), Frame = +1

Query:      7 IWIVQPVVLTS 39
              IW + P+VLTS
Sbjct:      4 IWTLLPLVLTS 14
```

15-7 b Reverse

```
pir||PN0116 insulin-like growth factor S11 - soybean (fragment)
            Length = 20

  Minus Strand HSPs:

 Score = 26 (12.0 bits), Expect = 0.65, Sum P(2) = 0.48
 Identities = 5/6 (83%), Positives = 5/6 (83%), Frame = -1

Query:     62 N*ACSP 45
              N ACSP
Sbjct:      4 NGACSP 9

 Score = 25 (11.5 bits), Expect = 0.65, Sum P(2) = 0.48
 Identities = 3/4 (75%), Positives = 4/4 (100%), Frame = -2

Query:     43 LPPC 32
              +PPC
Sbjct:     12 MPPC 15



sp|P35429|HES2_RAT TRANSCRIPTION FACTOR HES-2. pir||S35037
            transcription factor HES-2 - rat gi|436245 (D14029) HES-2
[Rattus
            norvegicus]
            Length = 157

  Minus Strand HSPs:

 Score = 55 (25.3 bits), Expect = 1.8, P = 0.84
 Identities = 10/17 (58%), Positives = 11/17 (64%), Frame = -2

Query:     79 GYTQCLIELAHLLPPCS 29
              GY  CL  LA +LP CS
Sbjct:     89 GYRACLARLARVLPACS 105
```

6-10a Forward

```
pir||A46709 small proline-rich protein spr1 - human
            Length = 89

  Minus Strand HSPs:

 Score = 39 (17.9 bits), Expect = 0.45, Sum P(2) = 0.36
 Identities = 6/9 (66%), Positives = 6/9 (66%), Frame = -2
```

```
Query:    76 CHPLNAEPC 50
             CHP   EPC
Sbjct:    41 CHPKVPEPC 49

 Score = 35 (16.1 bits), Expect = 0.88, Sum P(2) = 0.59
 Identities = 5/11 (45%), Positives = 8/11 (72%), Frame = -3

Query:    45 CVPRLKKLNHP 13
             C+P+ K+  HP
Sbjct:    33 CIPKTKEPCHP 43

 Score = 33 (15.2 bits), Expect = 0.88, Sum P(2) = 0.59
 Identities = 5/10 (50%), Positives = 6/10 (60%), Frame = -2

Query:    76 CHPLNAEPCL 47
             C P   EPC+
Sbjct:    25 CQPPPQEPCI 34

 Score = 31 (14.3 bits), Expect = 0.45, Sum P(2) = 0.36
 Identities = 5/14 (35%), Positives = 8/14 (57%), Frame = -3

Query:    54 PA*CVPRLKKLNHP 13
             P  C P++ +  HP
Sbjct:    54 PEPCQPKVPEPKHP 67

 Score = 31 (14.3 bits), Expect = 6.5, Sum P(2) = 1.0
 Identities = 5/9 (55%), Positives = 5/9 (55%), Frame = -2

Query:    76 CHPLNAEPC 50
             C P   EPC
Sbjct:    33 CIPKTKEPC 41
```

6-10a Reverse

```
pir||S40989 hypothetical protein F55H2.6 - Caenorhabditis elegans
            gi|1066982 (Z27080) F55H2.6 [Caenorhabditis elegans]
            Length = 839

  Plus Strand HSPs:

 Score = 70 (32.0 bits), Expect = 0.023, P = 0.023
 Identities = 14/19 (73%), Positives = 15/19 (78%), Frame = +3

Query:     3 LVFGEDHPEMALLDNNIGL 59
             LVFGE HP MA +D NIGL
Sbjct:   655 LVFGEKHPVMAQIDANIGL 673
```

6-10b Forward

```
sp|P03701|VLOM_LAMBD OUTER MEMBRANE PROTEIN LOM PRECURSOR (ORF 206A).
            pir||MMBPL membrane protein lom - phage lambda gi|215126
(J02459)
            lom (outer host membrane;206a) [Bacteriophage lambda] gi|288850
            (X55793) lom gene product [Bacteriophage lambda] prf||1614339B
bor
            gene [Bacteriophage lambda]
            Length = 206

  Minus Strand HSPs:
```

```
 Score = 96 (44.2 bits), Expect = 3.3e-06, P = 3.3e-06
 Identities = 17/19 (89%), Positives = 19/19 (100%), Frame = -2

Query:   126 SYAMAGVAHSRWSGSTMDF 70
             +YAMAGVAHSRWSGSTMD+
Sbjct:   120 AYAMAGVAHSRWSGSTMDY 138
```

6-10b Reverse

```
gi|146062 (M38327) beta-galactosidase [Escherichia coli]
           Length = 58

  Minus Strand HSPs:

 Score = 59 (26.5 bits), Expect = 1.0e-08, Sum P(2) = 1.0e-08
 Identities = 10/10 (100%), Positives = 10/10 (100%), Frame = -1

Query:    32 DGFHMGIGGD 3
             DGFHMGIGGD
Sbjct:    22 DGFHMGIGGD 31

 Score = 47 (21.1 bits), Expect = 1.0e-08, Sum P(2) = 1.0e-08
 Identities = 8/9 (88%), Positives = 9/9 (100%), Frame = -2

Query:    61 NAEEGTWLN 35
             +AEEGTWLN
Sbjct:    12 HAEEGTWLN 20
```

Appendix 6.3. BLAST Analysis of Plaque Sequences

Manual sequencing was carried out on all the picked plaques. The sequences were analysed at at the National Center for Biotechnology Information (NCBI) using the BLAST network service which translates the DNA sequence in all 6 frames and then searches the databases for similar proteins. The top of the three sequences is the experimental (query) sequence. The bottom sequence (subject) is the library sequence. The middle sequence shows amino acids which are identical in both the experimental and library sequences and + signs represent conservative changes between the two sequences.The most significant hits only are shown here.

Appendix 6.4. Sequences of PCR Fragments and Plaques

1000 bp PCR Fragment
TTCGCAGTATTCCATTTGGAGAATACGAGTTTGGAAAGCCAGTTGATCATC
AGCAGGTAATCTGGAACCGCGAACGAACGAATCAGCAACTCACAAAACGG
GATCGGAAAGAAATCGGCGCGACACGTTCATCTTGTCATCGCAGCGTGAC

800 bp PCR fragment
TTCGTAGTACTCCATCTGGTGAAGATGACTTTAACGTTGGAAAAGAATATG
GTCTTGAAGTTGCGGTGACAGTTGACGAAAAGGGTTACATAGCAATAAGT
GCTGGTGCAGACTTTGAAGGAAT

1400 bp PCR Fragment
CCAACAGAGGCATGGAATTGTAACTGACCACCACCAACCTGTTGGATATCA
ACTCTCGGCAAGAGGGGGCAGACTGGTGCACCCGACATCCAAGCTTCAGAC
TGGTGCACCGACATCCAAGCTTCATGGAATTCGA

900- PCR Fragment Forward Sequence
GCTTGATATCGAATTCCTGCAGCCCGGTTTCAGATCGCGGTGGATTACTGAA
GAAAACGCGCGAACAAAACGGAGGTTTTCGGATATAAAGAGAGACTTTA
TCGAACAAAAGAAGCATTTATTGAATAAATGAATGTCTTCTGAGTGCACC
CATATGAAG

900- PCR Fragment Reverse Sequence
GAGCAATGCGGAGTGCAGATTGACCTTGCGTCATCTGTGGATCTGTTGGGG
CGGTATGCGAATTGGAGTGGGTCTAGGTTTTCCGCAATTAT

1-10a Plaque Forward Sequence
ACCGGGTAAGTGAAAAAACGATAGAGTAGTGGTATTTCACCGGCGGCCGG
GCCGCGGCGCGGAGTCGCGCGACC

1-10a Plaque Reverse Sequence
GGGTGGAGCCGCCGCAGGTGCAGATCTTGGTGGTAGTAGCAACTATTCAAA
CGAGAGCTTTGAAGGCCGAAGTGGAGCAGGGTTCCATGTGAACAGCATTG
AACATGGGTCAGTTCGGTCCTAAGCGATAGGCGAGCGCCTTCCGAAGGGAC
GGGCGATGGCCTCCTCCGTTACCCTCAGCCGATCG

1-10b Plaque Forward Sequence
CAAATAATCCTTTCCCAACGGTCATTGTATCATCCGATTTAAATTTGATAT
TACTTTCCATTTAATGCTTTTTCAAATTCGGAAAAAATAGGTTAACCATGG
CTAAAATTCTCCTTTAAAAATCCTTATATGTCACCATCACTTGACACGGTT
GGTTCATTCATCTCATCGGG

1-10b Plaque Reverse Sequence
GGGGCAGCCGCTATTCTCAGAAAAACGTGATGCAGATCTCCGGAAACGTCT
CGCAAACGGTTTATATCGCCAATTCAACGAAGCAAAGACATTGATGTCTCG
TCAACCTGAGGAAATGTTAGATCGGGTGAAGCAACATCATAAACTTATGG
ATCTGCACATGGACTGGCCTCAAGAGAAATGAGGTGACGCGTCCTGATAAC
AAAAATCCAACAAT

1-9a Plque Forward Sequence
GAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAATAATA
ACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTGTACTG
CCGGACCACCGACTGTGAGCCACTCCGGCCATGGCGTACGACTGACCTGCTT
ACTGA

1-9a Plaque Reverse Sequence
CGATACTGACGGGCTCCAGGAGTCGTACGCCACCAATCCCCATATGGAAAC
CGTCGATATTCAGCCATGTGCCTTCTTCCGCGTGCAGCAGATGGCGATGGCT
GGTTTCCATCAGTTGCTGTTGACTGTAGCGGCTGATGTTGAACTGGAAGTCG
CCGCGCCACTGGTGTGGGCCATAATTCAATTCGCGCGTCCCGCAGCGCAGAC
CGTTTTCGCTCGGGGAAGACGTACGGGGTTAACATGT

1-9b Plaque Forward Sequence
GAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAATAATA
ACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTGTACTG
CCGGACCACCGACTGTGAGCCACTCCGGCCATGGCGTACGACTGACCTGCTT
ACTGATTTGTA

11-8a Plaque Forward Sequence
ACGCTGTGGGCTTTTCTTGGTCTGGAGTCTGCGTGTGCGAATACTGATGTAG
TGGAAAACCCGGAACGTAATGTGCCAATCGCGGTACTCGGCGGTACGTTAG
GTGCGGCGGTGATTTATATCGTCTCCACCAACGTGATT

11-8a Plaque Reverse Sequence
CAGGGCCAACAGCGGTACTGAAGTCAGTCAGTAAGCTCGATAACACGCTGC
TGTCTAACGGTACGTTGCTGAACGTGAAATTCACTCCGGCGACCCTGGAAG
GTGAAGCAGGTTACGCAACTGGCCG

11-8b Plaque Forward Sequence
TATATAACTTCTTAATTGCATGGGATCAAGGCATAAGCCCAACCCACGTTT
TGCATACGTTCGTAGTTCCATGAACCTTGTAAGAACTGTGATCGC

11-8b Plaque Reverse Sequence
AACATTATTGGTTTGGCTGCAGGTATTATCCTTGGTGGTTCACTGCAAATG
ATCGCCTTGGTTGGGCTAACATGG

11-9 b Plaque Reverse Sequence
TTTAGTTTCAGCCTTTGCATCCATACTCCCCCCGGAACCCAAAGACTTGGGT
TTCCCGGGAGCTGCCCGGCGGGTCATGGGAATAACGCCGCCGGATCGCGAA
GTCGGCATCGTTTATGGTCGGAACTACGACGGTATCTGATCGTCTTCGAACC
TCGACTTTCGTTCTTGATTAATG

15-7b Plaque Forward Sequence
AATTAGATTTGGATTGTACAGCCAGTGGTTCTTACCAGCTCCAGCATGATC
CTTTTAGCAGGTGGATTATGTCATCAGCCTTGCAG

15-7b Plaque Reverse Sequence
ATTTATATATTAAGAATTAAAAATAACGACTACAAGGTGGGAGTAGGTG
AGCAAGCTCAATTAGACATTGGGTGTATCCC

6-10a Plaque Forward Sequence
CAGATTCAGAAAAGGGTGGTTTAACTTTTTTAAGCGAGGTACACATTAAG
CAGGGCTCTGCGTTAAGGGGATGGCAGCAGAAGGCATGCTTCCGTGAGCTT
CTTTCTCCT

6-10a Plaque Reverse Sequence
TATTGGTATTTGGGGAGGATCACCCAGAAATGGCACTCTTGGATAACAAC
ATCGGCTTGTCTCCACGGGGTTATGGAGTATAACCTGTCCCTCCGGTTCCTG
AGAAT

6-10b Plaque Forward Sequence
ACCAGTTGGTCTGGTGTCAAAAATAATAATAACCGGGCAGGCCATGTCTGC
CCGTAATTTCGGCGTAAGGAAATCCATTGTACTGCCGGACCACCGACTGTG
AGCCACTCCGGCCATGGCGTACGACTGA

6-10b Plaque Reverse Sequence
TCGTCGCCACCAATCCCCATATGGAAACCGTCGAATTCAGCCATGTGCCTTC
TTCCGCGTTGCATGCA

6-9b Plaque Reverse Sequence
TGGGTCTTCCCATGTAGATTCCAGGGGTAGGTGTGTGAGGTCTTTTTGTTAT
GGAAAAAGTCTACCCGGATTCTTCTTCCATCCAACTCCATTCCATTGGCACG
TTCCTTAGCTTCCTTAGCATCCTCACGTTTCAAGTCACAAATGCAAACCC

Appendix 7.1. Sequence Alignment

```
                          10         20         30         40         50
-1  21_2.sdn;1  AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGT-TAAG
    CONSENSUS   AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGT-TAAG

                          60         70         80         90        100
-1  21_2.sdn;1  AAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGG
    CONSENSUS   AAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGG

                         110        120        130        140        150
-1  21_2.sdn;1  CCCTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGATAAGC
    CONSENSUS   CCCTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGATAAGC

                         160        170        180        190        200
-1  21_2.sdn;1  TTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCG
    CONSENSUS   TTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCG

                         210        220        230        240        250
-1  21_2.sdn;1  AGAAAGAAATAAAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCT
    CONSENSUS   AGAAAGAAATAAAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCT

                         260        270        280        290        300
-1  21_2.sdn;1  GGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGC
    CONSENSUS   GGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGC

                         310        320        330        340        350
-1  21_2.sdn;1  GGGATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTA
-2  21_1.SDN;1                                    GTGGTGG********TA
    CONSENSUS   GGGATATCGTCCATTCCGACAGCATCGCCAGTC----TGG--------TA

                         360        370        380        390        400
-1  21_2.sdn;1  GCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACT
-2  21_1.SDN;1  GCGTCATATGTGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCAAT
    CONSENSUS   GCG--ATATG-GTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCA-T

                         410        420        430        440        450
-1  21_2.sdn;1  GTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAG
-2  21_1.SDN;1  GTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAG
-4  26_2.SDN;1              TGGCCGCCGCCCAGTCCTGCTcGCTTCGCTACttGGAG
    CONSENSUS   GTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAG

                         460        470        480        490        500
-1  21_2.sdn;1  CCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTA
-2  21_1.SDN;1  CCACTATCGACTACGCGaTCATGGcGACCACACCCGTCCTGTGGATCCTA
-4  26_2.SDN;1  CCACTATCGActACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTA
 6  83_3.SDN;1                                        CCTGTGGATCCTA
    CONSENSUS   CCACTATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTA
                                                      cloning site

                         510        520        530        540        550
-1  21_2.sdn;1  GATACTGGGGACGCAGCTTTGGGaTAGTTtAtAtttattatAATTaccaAg
-2  21_1.SDN;1  GaTAC
-4  26_2.SDN;1  GATACTGGGGACGCAGCTTTGGgATAGTTATATTTATTATAATTACCAAG
 6  83_3.SDN;1  GaTACTGGGGACGCAGCTTTGGGATAGTTATATTTATTATAATTACCAaG
 5  83_2.SDN;1            ACGCAGCTttgGGATAGTTATaTTTattATAATTACCAAG
    CONSENSUS   GATACTGGGGACGCAGCTTTGGGATAGTTATATTTATTATAATTACCAAG
```

```
                          560        570        580        590        600
 -1   21_2.sdn;1   tttaaacacgATa
 -4   26_2.SDN;1   TTTAaACACGATATAGAAACTTGCAaACGTCACTTGTATAACATATGTAA
  6   83_3.SDN;1   TTTAAACACGATATAGAAACTTGCAAACGTCACTTGTATAACATATGTAA
  5   83_2.SDN;1   TTTAAACACGATATAGAAACTTGcAAACGTCACTTGTATAACATATGTAA
      CONSENSUS    TTTAAACACGATATAGAAACTTGCAAACGTCACTTGTATAACATATGTAA

                          610        620        630        640        650
 -4   26_2.SDN;1   AGCATTACCGcgTGCTGAATGTAAGGACCATGCTCTACAGGCAATACAGA
  6   83_3.SDN;1   AGCATTACCGCGTGCTGAATGTAAGGACCATGCTCTACAGGCAATACAGA
  5   83_2.SDN;1   AGCATTACCGCgTGCTGAATGTAAGGACCATGCTCTACAGGCAATACAGA
      CONSENSUS    AGCATTACCGCGTGCTGAATGTAAGGACCATGCTCTACAGGCAATACAGA

                          660        670        680        690        700
 -4   26_2.SDN;1   AGAATAATATTATGTCAAGCAACGATATTAATTATATATACTccTCTTTC
  6   83_3.SDN;1   AGAATAATATTATGTCAAGCAACGATATTAATTATATATACTCCTCtttC
  5   83_2.SDN;1   AGAATAATATTATGTCAAGCAACGATATTAATTATATATACTCCTCTTTC
      CONSENSUS    AGAATAATATTATGTCAAGCAACGATATTAATTATATATACTCCTCTTTC

                          710        720        730        740        750
 -4   26_2.SDN;1   ATAAGTCTTTACAACAATCTAGTGTTTAATCCAGAAAGATGTATAGATAT
  6   83_3.SDN;1   ATAAGTCTTTACAACAATCTAGTGTTTAATCCAGAAAGATGTATAGATAT
  5   83_2.SDN;1   ATAAGTCTTTACAACAATCTAGTGTTTAATCCAGAAAGATGTATAGATAT
 -9   19_3.SDN;1       GTCTTTACaACAATCTaGTGTTTAATCCAGAAAGATGTATAGATAT
      CONSENSUS    ATAAGTCTTTACAACAATCTAGTGTTTAATCCAGAAAGATGTATAGATAT
                                homology with VV E3L DNA

                          760        770        780        790        800
 -4   26_2.SDN;1   AAAAAAAGTAAAAAAACTAATTTAGTAATACCTAGAATTATCAGATCTAA
  6   83_3.SDN;1   AAAAAAAGTAAAAAAACTAATTTAGtAATACCTAGAATTATCAGATCTAA
  5   83_2.SDN;1   AAAAAAAGTAAAAAAACTAATTTAGtAATACCTAGAATTATCAGATCTAA
 -9   19_3.SDN;1   AAAAAAAGTAAAAAAACTaATTTAGTAATACCTAGAATTATCAGATCTAA
-11   41_2.SDN;1                                CCTAGAATTATCAGATCTAA
      CONSENSUS    AAAAAAAAGTAAAAAAACTAATTTAGTAATACCTAGAATTATCAGATCTAA

                          810        820        830        840        850
 -4   26_2.SDN;1   CTTTAACAATGTAACCCGTTTTTGGCTTTACAGAAATACTCATATCGTCA
  6   83_3.SDN;1   CTttaaCAATGTAACCCGtTtttGGCTTTACAGAAATACTCATATCGtCA
  5   83_2.SDN;1   CTTtAACAATGTAACCCgtTTTTGGCTTTACAGAAATACTCATATCGTCA
 -9   19_3.SDN;1   CTTTAACAATGTAACCCGTTTTTGGCTTTACAGAAATACTCATATCGTCA
-11   41_2.SDN;1   CTTTAACAATGTaACCCGTTTTTGGCTTtACAGAAAtACTCATATCGTCA
 -8   19_2.SDN;1                                ACAGAAATACTCATATCGTCA
      CONSENSUS    CTTTAACAATGTAACCCGTTTTTGGCTTTACAGAAATACTCATATCGTCA

                          860        870        880        890        900
 -4   26_2.SDN;1   GTAATAGTAAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGTGCT
  6   83_3.SDN;1   GTAATAGtAAAACAAaTAGAAGTTATTtCTGAGGaAGtATaGtCCGtgCT
  5   83_2.SDN;1   GTAATAGTAAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGtGCT
 -9   19_3.SDN;1   GTAATAGTAAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGTGCT
-11   41_2.SDN;1   GTaATAGTAAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGTGCT
 -8   19_2.SDN;1   GTAATAGTaAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGTGCT
 -7   19_1.SDN;1                                                    CT
      CONSENSUS    GTAATAGTAAAACAAATAGAAGTTATTTCTGAGGAAGTATAGTCCGTGCT
```

```
                                910        920        930        940        950
 -4  26_2.SDN;1  AGCTAGTTCTTTACTGAATGTATAATAAGTATTATCTACTGGAGTTAGTT
  6  83_3.SDN;1  AGCTAGttCCTtACTGAaTGTaTaaTaAGTATTATCTACTGGAGtTAGtt
  5  83_2.SDN;1  AGCTAGTTCTTTACTGAATGTATAATAAGTATTATCTACTGGAGCTAGtt
 -9  19_3.SDN;1  AGCTAGTTCTTTACTGAATGTATAATAAGTATTATCTACTGGAGTTAGTT
-11  41_2.SDN;1  AGCTaGTTCTTTACTGAAtGTaTAATAAGTaTTATCTACTGGAGTTAGTT
 -8  19_2.SDN;1  AGCTAGTTCTTTACTGAATGTATAATAAGTAttaTCTACTGGAGTTAGTT
 -7  19_1.SDN;1  AGCTAGTTcTTTACtGaaTGTATAATAAGTaTTATCTACTGGAGTTAGTT
     CONSENSUS   AGCTAGTTCTTTACTGAATGTATAATAAGTATTATCTACTGGAGTTAGTT

                          960        970        980        990       1000
 -4  26_2.SDN;1  CTCCTTTATCGTATTTATATGTAAATAATGTTGTATCCTTAACTACTACA
  6  83_3.SDN;1  CTCCTttatCG
  5  83_2.SDN;1  CTCCTttATCgtaTTTATaTGTAAATAAtGTTGtATCCTTAACTACTACA
 -9  19_3.SDN;1  CTCCTTTATCGTATTTATATGTAAATAATGTTGTATCCttAACTACTACA
-11  41_2.SDN;1  CTCCTTTATCGTATTTATATGTAAATaATGTTGTaTCCTTAACTAcTACA
 -8  19_2.SDN;1  CTCCTTTATCGTATTTATATGTAAATAATGTTGTATCCTTAACTACTACA
 -7  19_1.SDN;1  cTCCTttatCGTATTTATATGTAAATAAtGTTGTATCCTTAACTACTACA
 23  36_2.SDN;1                                    TGttGTATCCTTAACtaCTACA
-15  46_1.SDN;1                                          TAACTACTACA
     CONSENSUS   CTCCTTTATCGTATTTATATGTAAATAATGTTGTATCCTTAACTACTACA

                        1010       1020       1030       1040       1050
 -4  26_2.SDN;1  TAGaACGTAGATTCTTTtAGTTTTATATATTTCTTTATAACCGATATGAA
  5  83_2.SDN;1  TAGAACGTaGaTTCttttagttttATaTATTTCTtTATAaCCgataTGAA
 -9  19_3.SDN;1  TAGAACGTAGATTCTTTTAGTTTTATATATTTCTTTATAACCGATATGAA
-11  41_2.SDN;1  TaGAACGTAGATTCTTtTAGTTTTATATATTTCTTTtATaACCGa
 -8  19_2.SDN;1  TAGAACGTAGATTCTTTTagTTTTATATATTTCTTTATAACCGATATGAA
 -7  19_1.SDN;1  TAGAACGTAGATTCTTTTAGTTTTATATATTTCTTTATAACCGATATGAA
 23  36_2.SDN;1  TAGAacgtagATTCTTTTAGTTTTATATATTTCTTTATAACCGATATGAA
-15  46_1.SDN;1  TAGAACGTAGaTTCTTTTAGTTTTATATATTTCTTTATAACCGATATGAA
 13  35_2.SDN;1                          TaTATATttCTTTATAACCGATATGAA
 12  35_3.SDN;1                                CTTTATAACCGATATGAA
 52  6_2.SDN;1                                       CCGATATGAA
 14  35_1.SDN;1                                            ATGAA
     CONSENSUS   TAGAACGTAGATTCTTTTAGTTTTATATATTTCTTTATAACCGATATGAA

                        1060       1070       1080       1090       1100
 -4  26_2.SDN;1  ATATTTAACC
  5  83_2.SDN;1  ATATTTAACCgCTCTATCTACCtCACACTTGGtTCttAAAGATTTTTTTG
 -9  19_3.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGtTctTaaAgaTTTTTTTG
 -8  19_2.SDN;1  ATAtTTAAC
 -7  19_1.SDN;1  ATATTTAACCGCTCTaTCTACCTCACacttGGttcttaaagAt
 23  36_2.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATttttttG
-15  46_1.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
 13  35_2.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
 12  35_3.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
 52  6_2.SDN;1   ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
 14  35_1.SDN;1  ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
-16  46_2.SDN;1                  TCTACCTCACACTTGGTTCTTAaAGAttTTTTTG
     CONSENSUS   ATATTTAACCGCTCTATCTACCTCACACTTGGTTCTTAAAGATTTTTTTG
```

```
                          1110       1120       1130       1140       1150
  5  83_2.SDN;1   GtGGAATATCAtAATAGAGtTttAC
 -9  19_3.SDN;1   GTGGg
 23  36_2.SDN;1   GtGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCtAAAAGA
-15  46_1.SDN;1   GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA
 13  35_2.SDN;1   GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA
 12  35_3.SDN;1   GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA
 52  6_2.SDN;1    GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA
 14  35_1.SDN;1   GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA
-16  46_2.SDN;1   GTGGAATATCATAATAGAGTTtTACTCTTTCTGACTCTGATTCTaAaAGA
 51  6_1.SDN;1      GGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCtAAAAGA
     CONSENSUS    GTGGAATATCATAATAGAGTTTTACTCTTTCTGACTCTGATTCTAAAAGA

                          1160       1170       1180       1190       1200
 23  36_2.SDN;1   ATGTTTACtAATtCCATTTAAGTTATTAaAAATGGTTATTAATAGTTTCA
-15  46_1.SDN;1   ATGTtTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA
 13  35_2.SDN;1   ATGTTTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA
 12  35_3.SDN;1   ATGTTTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA
 52  6_2.SDN;1    ATGTTTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA
 14  35_1.SDN;1   ATGTTTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA
-16  46_2.SDN;1   ATGTTTACTAATTCCATTTaAGTTATTaaAAATGGTTATTAATAGTTTCA
 51  6_1.SDN;1    ATGTTTACTAATTCCAttTAAGTTATTAAAAATGGTTATTAATAGTTTCA
     CONSENSUS    ATGTTTACTAATTCCATTTAAGTTATTAAAAATGGTTATTAATAGTTTCA

                          1210       1220       1230       1240       1250
 23  36_2.SDN;1   TTAAtATTCCGTAGGTTttGAAAGGTACAACaTAGTtAttGATTAGGTTT
-15  46_1.SDN;1   TTAATATTCCGTAGGTTTTGAAAGGTACAACATAGTTATTGATTAGGTTT
 13  35_2.SDN;1   TTAATAtTCCGTAGGTTTTGAAAGGTACAACATAGTTATTGATTAGGTTT
 12  35_3.SDN;1   TTAATATTCCGTAGGtTTTGAAAGGTACAACATAGTTATTGATTAGGTTT
 52  6_2.SDN;1    TTAATATTCCGTAgGTTTTGAAA
 14  35_1.SDN;1   TTAATATTCCGTAGGTTTTGAAAGGTACAACATAGTTATTGATTAGGTTT
-16  46_2.SDN;1   TTAATATTCCGTAGGTTTTGAAAGGTACAACATAGTTATTGATTAGGTTT
 51  6_1.SDN;1    TTAAtatTCCGTAGGTTTTGAAAGGTACAACATAGTTATtGATTAgGTTT
     CONSENSUS    TTAATATTCCGTAGGTTTTGAAAGGTACAACATAGTTATTGATTAGGTTT

                          1260       1270       1280       1290       1300
-15  46_1.SDN;1   TCTAATTCACAACAAATATTCATTTCATTAACGTATACATTTTTATACAG
 13  35_2.SDN;1   TCtAATTCACAACAAATATTCATTTCATTAACGTATACATTTTTATACAG
 12  35_3.SDN;1   TCTAATTCACAACAAATATTCATTTCATTAACGTATACATTTTTATaCaG
 14  35_1.SDN;1   tCTAATtCACAACAAATATTCATTTCATTAACGTATACATTTTTATACAG
-16  46_2.SDN;1   TCTAATTCACAACAAATATTCATTTCATTAACGTATACATTTTTATACAG
 51  6_1.SDN;1    TCtAATTCACAACAAATATTCaTTTCaTTAACGTATACATTTTTATACAG
 22  36_1.SDN;1                              ttcATTAACGtatacatTtttATAcaG
     CONSENSUS    TCTAATTCACAACAAATATTCATTTCATTAACGTATACATTTTTATACAG

                          1310       1320       1330       1340       1350
-15  46_1.SDN;1   TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
 13  35_2.SDN;1   TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
 12  35_3.SDN;1   TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAAC
 14  35_1.SDN;1   TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
-16  46_2.SDN;1   TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
 51  6_1.SDN;1    TTtTGGTGATATACCAAATCCTTTTAAGCAtCtATATaATAACATTTCtA
 22  36_1.SDN;1   TtTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
     CONSENSUS    TTTTGGTGATATACCAAATCCTTTTAAGCATCTATATAATAACATTTCTA
```

```
                          1360       1370       1380       1390       1400
-15  46_1.SDN;1   AAAACGATCTTAAATTATAGCTTGttTTTT
 13  35_2.SDN;1   AAAACGATCTTAAATTATAGCTTGTtTTTTGAtATCTGATA
 14  35_1.SDN;1   AAAACGATCTTAAATTATAGCTTGTtTTTTGATATCTGATAATTAT
-16  46_2.SDN;1   AAAACGATCTTAAATT
 51  6_1.SDN;1    AAaaCGATCTTAAATTATAGCTtGTTTTT
 22  36_1.SDN;1   AAAACGATCTTAAATTATAGCTTGTTTTTTGATATCTGATAATTATCAAA
 25  27_1.SDN;1        GATCTTAAATTATAGCTTGTTTTTTGATATCTGATAATTATCAAA
 26  27_3.SDN;1         ATCTTAAATTATAGCTTGTTTTTTGATATCTGATAATTATCAAA
     CONSENSUS    AAAACGATCTTAAATTATAGCTTGTTTTTTGATATCTGATAATTATCAAA

                          1410       1420       1430       1440       1450
 22  36_1.SDN;1   GTAAAAATATATTCGAAAATAACATTAGCTAGTATTACTTTTTTAATGAA
 25  27_1.SDN;1   GTAAAAATATATTCGAAAATAACATTAGCTAGTATTACTTTTTTAATGAA
 26  27_3.SDN;1   GTAAAAATATATTCGAAAATAACATTAGCTAGTATTACTTTTTTAATGAA
     CONSENSUS    GTAAAAATATATTCGAAAATAACATTAGCTAGTATTACTTTTTTAATGAA

                          1460       1470       1480       1490       1500
 22  36_1.SDN;1   GTTTAttCCATAAAGTTTTATTCGACTATAGTCTAATAGTTTATATATAT
 25  27_1.SDN;1   GTTTATTCCATAAAGTTTTATTCGACTATAGTCTAATAGTTTATATATAT
 26  27_3.SDN;1   GTTTATTCCATAAAGTTTTATTCGACTATAGTCTAATAGTTTATATATAT
 27  44_1.SDN;1         CATAAAGTTTTATTCGACtATAGTCTAATAGTTTATATATAT
 29  44_3.SDN;1               GTTTTATTCGACTATAGTCTAaTAGTTTATATATAT
 28  44_2.SDN;1                  TATTCGActaTAGTCTAATAGTTTATATATAT
     CONSENSUS    GTTTATTCCATAAAGTTTTATTCGACTATAGTCTAATAGTTTATATATAT

                          1510       1520       1530       1540       1550
 22  36_1.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTtGTTT
 25  27_1.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT
 26  27_3.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT
 27  44_1.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT
 29  44_3.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT
 28  44_2.SDN;1   CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT
     CONSENSUS    CTATATTATCTATATGTTCAAAATTATTATTAATATTTTCGTCCTTGTTT

                          1560       1570       1580       1590       1600
 22  36_1.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTtTGTAGCCATCATAaA
 25  27_1.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA
 26  27_3.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA
 27  44_1.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA
 29  44_3.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA
 28  44_2.SDN;1   TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA
     CONSENSUS    TTGAGATAATTTATGTTATCGGCTTTTATATTCTTTGTAGCCATCATAAA

                          1610       1620       1630       1640       1650
 22  36_1.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
 25  27_1.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
 26  27_3.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
 27  44_1.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
 29  44_3.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
 28  44_2.SDN;1   CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
-32  45_3.SDN;1                                              ATCAAAGGAC
     CONSENSUS    CCTCGTGAAATCTACTTTACACGAATCATCTATAAGATACATCAAAGGAC
```

```
                          1660       1670       1680       1690       1700
 22   36_1.SDN;1   TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
 25   27_1.SDN;1   TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
 26   27_3.SDN;1   TCCAtAGCGAGT
 27   44_1.SDN;1   TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
 29   44_3.SDN;1   TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
 28   44_2.SDN;1   TCCATAGCGAGTtAGTATtCAGAAAATTGATATCTATAATACCTTTCTTG
-32   45_3.SDN;1   TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
      CONSENSUS    TCCATAGCGAGTTAGTATTCAGAAAATTGATATCTATAATACCTTTCTTG
                                                homology with VV K3L DNA

                          1710       1720       1730       1740       1750
 22   36_1.SDN;1   GCGGTAGAAAATAAGAAACTTGCtAGAGTCAATAATCCTATATCATTATT
 25   27_1.SDN;1   GCGGTAGAAAATAAGAAACTTGCTAGAGTCAATAATCCTATATCATTATT
 27   44_1.SDN;1   GCGGTAGAAAATAAGAAACTTGCtAGAGTCAATAATCCTATATCATTATT
 29   44_3.SDN;1   GCGGTAGAAAATAAGAAACTTGCTAGAGTCAATAATCCTATATCATTATT
 28   44_2.SDN;1   GCGGTAGAAAATAaGAAACTTGCTAGAGTCAATAaTCCTATATCATTATT
-32   45_3.SDN;1   GCGGTAGAAAATAAGAAACTTGCTAGAGTCAATAATCCTATATCATTATT
      CONSENSUS    GCGGTAGAAAATAAGAAACTTGCTAGAGTCAATAATCCTATATCATTATT

                          1760       1770       1780       1790       1800
 22   36_1.SDN;1   ATCGAaAGTACTATTTTTAGACATATAATCT
 25   27_1.SDN;1   ATCGAAAGTACTATTTTTAGACATATAATCTAACAGTTTATCGCTATACA
 27   44_1.SDN;1   ATCGAAAGTACTATTTTTAGACATATAATCTAACAGTTTATCGCTATACA
 29   44_3.SDN;1   ATCGAAAGTACTATTTTTAGACATATAATCTAACAGTTTATCGCTATACA
 28   44_2.SDN;1   ATCGAAAGTACTATTtttAGACATATAATCTAACAGTTTATCGCTATACA
-32   45_3.SDN;1   ATCGAAAGTACTATTTTTAGACATATAATCTAACAGTTTATCGCTATACA
-31   45_2.SDN;1                              AATCTAACAGTTTATCGCTATACA
      CONSENSUS    ATCGAAAGTACTATTTTTAGACATATAATCTAACAGTTTATCGCTATACA

                          1810       1820       1830       1840       1850
 25   27_1.SDN;1   T
 27   44_1.SDN;1   TTACATAATCTACCGGGTGATATTGAAACTTTATTAATACAGAATATAAA
 29   44_3.SDN;1   TTACATAATCTACCGGGTGATATTGAAACTTTATTAATACAGAATATAAA
 28   44_2.SDN;1   TTACATAATCTACCGGGTGATATTGAAACTTTATTAATAcAGAATA
-32   45_3.SDN;1   TTACATAATCTACCGGGTGATATTGAAACTTTATTAATACAGAATATAAA
-31   45_2.SDN;1   TTACATAATCTACCGGGTGATATtGAAACTTTATTAATACAGAATATAAA
      CONSENSUS    TTACATAATCTACCGGGTGATATTGAAACTTTATTAATACAGAATATAAA

                          1860       1870       1880       1890       1900
 27   44_1.SDN;1   TTACCtAGATCAAAACACTTAGCATTTTCTAAACAAACTACTTGTTTCTG
 29   44_3.SDN;1   TTACCTAGATCAAAACACTTAGCATTTTCTAAACAAACTACTTGTTTCTG
-32   45_3.SDN;1   TTACCTAGATCAAAACACTTAGCATTTTCTAAACAAACTACTTGTTTCTG
-31   45_2.SDN;1   TTACCTAGATCAAAACACTTAGCATTTTCTAAACAAACTACTTGTTTCTG
-30   45_1.SDN;1                AAAACACTTAGCATTTTCTaAACAAACTACTtGTTTCtG
      CONSENSUS    TTACCTAGATCAAAACACTTAGCATTTTCTAAACAAACTACTTGTTTCTG

                          1910       1920       1930       1940       1950
 27   44_1.SDN;1   TAACATGTTTAGAGCATCCATCTTTAC
 29   44_3.SDN;1   TAACATGTTTAGAGCATCCATCtTTACCTCATTG
-32   45_3.SDN;1   TAACATGTTTAGAGCATCCATCTTTACCTCATTGGACGATGACGAGTCAA
-31   45_2.SDN;1   TAACATGTTTAGAGCATCCATCTTTACCTCATTGGACGATGACGAGTCAA
-30   45_1.SDN;1   TAACATGTTTAGAGCATCCATCTTTACCTCATTGGACGATGACGAGTCAA
      CONSENSUS    TAACATGTTTAGAGCATCCATCTTTACCTCATTGGACGATGACGAGTCAA

                          1960       1970       1980       1990       2000
-32   45_3.SDN;1   GTATGTATTTCCCTATAGCATAGTTATATTTAAAATCATTTAATAGTACT
-31   45_2.SDN;1   GTATGTATTTCCCTATAGCATAGTTATATTTAAAATCATTTAATAGTACT
-30   45_1.SDN;1   GTATGTATTTCCCTATAGCATAGTTATATTTAAAATCATTTAATAGTACT
      CONSENSUS    GTATGTATTTCCCTATAGCATAGTTATATTTAAAATCATTTAATAGTACT
```

```
                              2010       2020       2030       2040       2050
-32   45_3.SDN;1    TTCATAGTTTGTTTATTATCTAAATACTTGAATGATTCTTTTACAACATA
-31   45_2.SDN;1    TTCATAGTTTGTTTATTATCTAAATACTTGAATGATTCTTTTACAACATA
-30   45_1.SDN;1    TTCATAGTTTGTTTATTATcTAAATACTTGAATGATTCTTTTACaACATA
      CONSENSUS     TTCATAGTTTGTTTATTATCTAAATACTTGAATGATTCTTTTACAACATA

                              2060       2070       2080       2090       2100
-32   45_3.SDN;1    TTTTTCTTTAGAATCTTGTTTATAAGTAATAACGGGaaatACTTTTTGAG
-31   45_2.SDN;1    TTTTTCTTTAGAATCTTGTTTATAAGTAATAACGG
-30   45_1.SDN;1    TTTTTCTTTAGAATCTTGTTTATAAGTAATAACGGGaAATACTT
-35   42_3.SDN;1                                                ACTTTTTGAG
-34   42_2.SDN;1                                                 CTTTTTGAG
-33   42_1.SDN;1                                                       GAG
      CONSENSUS     TTTTTCTTTAGAATCTTGTTTATAAGTAATAACGGGAAATACTTTTTGAG

                              2110       2120       2130       2140       2150
-32   45_3.SDN;1    GATACATACTA
-35   42_3.SDN;1    GATACATACTATTACAATAATATAGATGCCATCTAAACGCATTTACAAAC
-34   42_2.SDN;1    GATACATACTATTACAATAATATAGATGCCATCTAAACGCATTTACAaAC
-33   42_1.SDN;1    GATACATACTATTACAATAATATAGATGCCATCTAAACGCATTTACAAAC
      CONSENSUS     GATACATACTATTACAATAATATAGATGCCATCTAAACGCATTTACAAAC

                              2160       2170       2180       2190       2200
-35   42_3.SDN;1    AACTCTGACTTTTTTTTGTATATATCTATATAACGGCAGATAAATTTAGT
-34   42_2.SDN;1    AACTCTGACTTTTTTTTGTATATATCTATATAACGGCAGATAAATTTAGT
-33   42_1.SDN;1    AACTCTGACTTTTTTTTGTATATATCTATATAACGGCAGATAAATTTAGT
 37   47_2.SDN;1                                     CGGcAGATAAATTTAGT
 36   47_1.SDN;1                                           AATTTAGt
      CONSENSUS     AACTCTGACTTTTTTTTGTATATATCTATATAACGGCAGATAAATTTAGT

                              2210       2220       2230       2240       2250
-35   42_3.SDN;1    TTCATCCTCCGTTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA
-34   42_2.SDN;1    TTCATCCTCCGTTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA
-33   42_1.SDN;1    TTCATCCTCCGTTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA
 37   47_2.SDN;1    ttCATCCTCCGtTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA
 36   47_1.SDN;1    ttCATCCTCCGtTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA
      CONSENSUS     TTCATCCTCCGTTATGTTTAGAAACAAGTACTTATCTATGTATAGAGGTA

                              2260       2270       2280       2290       2300
-35   42_3.SDN;1    TATCTATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA
-34   42_2.SDN;1    TATCTATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA
-33   42_1.SDN;1    TATCTATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA
 37   47_2.SDN;1    TATCTATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA
 36   47_1.SDN;1    TATCtATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA
      CONSENSUS     TATCTATCCATTCAAACAACGCATAAAAGTATATACCAAATTCATCTCTA

                              2310       2320       2330       2340       2350
-35   42_3.SDN;1    AGTTTATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
-34   42_2.SDN;1    AGTTTATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
-33   42_1.SDN;1    AGTTtATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
 37   47_2.SDN;1    AGTTTATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
 36   47_1.SDN;1    AGTTTATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
      CONSENSUS     AGTTTATCGGGTGGTAATTGTGTTGATATCCATTTATTCAAGCAAGGTGA
```

```
                          2360       2370       2380       2390       2400
-35  42_3.SDN;1  TATATCGGGTAATCTATCCTTAATGATAGTAATTATTGATTCTATAAAAT
-34  42_2.SDN;1  TATATCGGGTAATCtATCCTTAATGATAGTAATTATTGATTCTATAAAAT
-33  42_1.SDN;1  TATATCGGGTAATCTATCCTTAATGATAGTAATTATTGATTCTATAAAAT
 37  47_2.SDN;1  TATATCGGGTAATCTATCCTTAATGATAGTAATTATTGATTCTATAAAAT
 36  47_1.SDN;1  TATATCGGGTAATCTATCCTTAATGATAGTAATTATTGAtTCTATAAAAT
     CONSENSUS   TATATCGGGTAATCTATCCTTAATGATAGTAATTATTGATTCTATAAAAT

                          2410       2420       2430       2440       2450
-35  42_3.SDN;1  CTTTTTTTGATTTTACTATTCCATTGACATAATCAATAATATCATCATCG
-34  42_2.SDN;1  CTTTTTTTGATTTTACTATTCCATTGACATAATCAATAATATCATCATCG
-33  42_1.SDN;1  CTTTTTTTGATTTTACTATTCCATTGACATAATCAATAATATCATCATCG
 37  47_2.SDN;1  CTTTTTTTGATTTTACTATTCCATTGACATAATCAATAATATCATCATCG
 36  47_1.SDN;1  CTTTTTTTGATTTTACtATTCCATTGACATAATCAATAATATCATCATCG
     CONSENSUS   CTTTTTTTGATTTTACTATTCCATTGACATAATCAATAATATCATCATCG

                          2460       2470       2480       2490       2500
-35  42_3.SDN;1  GGATACTTCTTTATCAAATATATAATTTTATCAACATCAGTTACTTGATA
-34  42_2.SDN;1  GGATACTTCTTTATCAAATATATAATTTTATCAACATCAGTTACTTGATA
-33  42_1.SDN;1  GGATACTTCTTTATCAAATATATAATTTTATCAACATCAGTTACTTGATA
 37  47_2.SDN;1  GGATACtTCTtTATCAAATATATAATTTTATCAACATCAGTTACTTGATA
 36  47_1.SDN;1  GGATACTTCTTTATCAAATATATAATTTTATCAACATCAGTTACTTGATA
-39  37_3.SDN;1                              TTTTATCAACATCAGTTACTTGATa
     CONSENSUS   GGATACTTCTTTATCAAATATATAATTTTATCAACATCAGTTACTTGATA

                          2510       2520       2530       2540       2550
-35  42_3.SDN;1  TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
-34  42_2.SDN;1  TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
-33  42_1.SDN;1  TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
 37  47_2.SDN;1  TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
 36  47_1.SDN;1  TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
-39  37_3.SDN;1  TAGGGAtGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
-38  37_1.SDN;1                     TGATATAGaAAATTTTTCTTCCATATAGAGAA
     CONSENSUS   TAGGGATGAATGAACCGGTGATATAGAAAATTTTTCTTCCATATAGAGAA
                 homology to VV K3L DNA

                          2560       2570       2580       2590       2600
-35  42_3.SDN;1  CGCTGTGAATAGGTGTTTCTATTCTCTTGTATAGCTCtTtCACGTTTTCT
-34  42_2.SDN;1  CGCTGTGAATAGGTGTTTCTATTCTCTTGTATAGCTCttTCACGTTTTCT
-33  42_1.SDN;1  CGCTGTGAATAGGTGTTTCTATTCTCTTGTATAGCTCttTCACGTTTTCT
 37  47_2.SDN;1  CG
 36  47_1.SDN;1  CGCtGTGAATAGGTGTTTCTATtCTCTTGTATAGCTCTTTCACGTTTTCT
-39  37_3.SDN;1  CGCTGTGAATAGGTGTTTCTATTCTCTTGTATAGCTCTTTCACGTTTTCT
-38  37_1.SDN;1  CGCTGTGAATAGGTGttCTATTCTCTTGTATAGCTCTTTCACGTTTTCT
     CONSENSUS   CGCTGTGAATAGGTGTTTCTATTCTCTTGTATAGCTCTTTCACGTTTTCT

                          2610       2620       2630       2640       2650
-35  42_3.SDN;1  GCACTAGTG
-33  42_1.SDN;1  GCACTAG
 36  47_1.SDN;1  GCACTAg
-39  37_3.SDN;1  GCACTAGtGTTTTCAAAAATATATTTTACTTCATCTAGAGAAAATGGAGT
-38  37_1.SDN;1  GCACTAGTGTTTTCaAAaATATATTTTACTTCATCTAGAGAAAATGGaGT
     CONSENSUS   GCACTAGTGTTTTCAAAAATATATTTTACTTCATCTAGAGAAAATGGAGT

                          2660       2670       2680       2690       2700
-39  37_3.SDN;1  ACCCGGTATAACTAAAGAGCTGTTAAGCAATGCTATTTCAACAGGTGTTT
-38  37_1.SDN;1  ACCCGGTaTAACTAAAGAGCTGTTAAGCAATGCTATTTCAACAGGTGTTT
     CONSENSUS   ACCCGGTATAACTAAAGAGCTGTTAAGCAATGCTATTTCAACAGGTGTTT
```

```
                          2710       2720       2730       2740       2750
-39   37_3.SDN;1   TTATTCTGTTATCTATTATATTACGTATATCTGAAAGAGATACCATATTA
-38   37_1.SDN;1   TTATTCTGtTATCTATTATATTACGTATATCTGAAAGAGATACCATATTA
 41   48_3.SDN;1     aTTCtgttaTCTATTATATTACGTATATCTGAAAGAGATACCATATTA
 40   48_2.SDN;1     AtTCTGTtATCTATTATATTACGTATATCTGAAAGAGATACCATATTA
-42   34_2.SDN;1              ATCTATTATAttACGtaTATCTGaaAGAGATACCatATTA
-43   34_3.SDN;1                           CGTATATCTGAAAGAGAtACCATATTA
      CONSENSUS    TTATTCTGTTATCTATTATATTACGTATATCTGAAAGAGATACCATATTA

                          2760       2770       2780       2790       2800
-39   37_3.SDN;1   GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT
-38   37_1.SDN;1   GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT
 41   48_3.SDN;1   GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT
 40   48_2.SDN;1   GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT
-42   34_2.SDN;1   GAGtTaATAAGtTTATTCACAATAGCaGCATCCGATAATAAgTATTTTTT
-43   34_3.SDN;1   GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT
      CONSENSUS    GAGTTAATAAGTTTATTCACAATAGCAGCATCCGATAATAAGTATTTTTT

                          2810       2820       2830       2840       2850
-39   37_3.SDN;1   ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAGTCTCA
-38   37_1.SDN;1   ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAgTctCA
 41   48_3.SDN;1   ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAGTCTCA
 40   48_2.SDN;1   ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAGTCTCA
-42   34_2.SDN;1   AtGACACGATATTACATCGGtaAAACAGTcTGGTAAAAGAGGtAGTCTCa
-43   34_3.SDN;1   ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAGTCTCa
      CONSENSUS    ATGACACGATATTACATCGGTAAAACAGTCTGGTAAAAGAGGTAGTCTCA

                          2860       2870       2880       2890       2900
-39   37_3.SDN;1   TAATCAGTTTTTTTAAGTCATTAAGTTGTATTATTTCGCTATCA
-38   37_1.SDN;1   AAATCaGttTtTTTAAgtc
 41   48_3.SDN;1   TAATCAGTTTTTTTAAGTCATTAAGTTGTATTATTTCGCTATCAAACGCT
 40   48_2.SDN;1   TAATCAGTTTTTTTAAGTCATTAAGTTGTATTATTTCGCTATCAAACGCT
-42   34_2.SDN;1   tAatCaGtttTTTTAaGTCattAAGttgtATTatttCGctATCaAACGCT
-43   34_3.SDN;1   tAATCAGTTTTTTTAAGTCATTAAGTTGTATTATTTCGCTATCAAACGCT
      CONSENSUS    TAATCAGTTTTTTTAAGTCATTAAGTTGTATTATTTCGCTATCAAACGCT

                          2910       2920       2930       2940       2950
 41   48_3.SDN;1   AGTATATTGTTTAAATCCAGACCTTTAGCAAAGTTATAATATTTTGGGGG
 40   48_2.SDN;1   AGTATATTGTtTAAATCCAGACCTTTAGCAAAGTTATAATATTTTGGGGG
-42   34_2.SDN;1   aGTATATTGTTTAAATCCAGACCTTTAGCAAAGTTATAATATTTTGGGGG
-43   34_3.SDN;1   AGTATATTGTTTAAATCCAGACCTTTAGCAAAGTTATAATATTTTGGGGG
      CONSENSUS    AGTATATTGTTTAAATCCAGACCTTTAGCAAAGTTATAATATTTTGGGGG

                          2960       2970       2980       2990       3000
 41   48_3.SDN;1   TAATTTAACGAATATATTGTTAATTATTAATCGGCGTCCGTGTTTCTCAT
 40   48_2.SDN;1   TAATTtAACGAATATATTGTTaATTATTAATCGGCGTCCGTGTTTCTCAT
-42   34_2.SDN;1   TAATTTAACGAATATATTGTTAATTATTAATCGGCGTCCGTGTTTCTCAT
-43   34_3.SDN;1   TAATTTAACGAATATATTGTTAATTATTAATCGGCGTCCGTGTTTCTCAT
      CONSENSUS    TAATTTAACGAATATATTGTTAATTATTAATCGGCGTCCGTGTTTCTCAT

                          3010       3020       3030       3040       3050
 41   48_3.SDN;1   GTAATAAGTCGAAGTTTAAACCTGAACGAATAAATTTGCGTATGGATTTT
 40   48_2.SDN;1   GTAATAAGTCGAAGTTTAAACCTGAACGAATAAATTTGCGTATGGATTTT
-42   34_2.SDN;1   GTAATAAGTCGAAGTTTAAACCTGAACGAATAAATTTGCGTATGGATTTT
-43   34_3.SDN;1   GTAATAAGTCGAAGTTTAAACCTGAACGAATAAATTTGCGTATGGATTTT
      CONSENSUS    GTAATAAGTCGAAGTTTAAACCTGAACGAATAAATTTGCGTATGGATTTT
```

```
                          3060       3070       3080       3090       3100
 41   48_3.SDN;1   CTAACTTTCTTAGAATAGAGATTCGCCATCTCAGACAAGATGACAAGTAG
 40   48_2.SDN;1   CTAACTTTCTTAGAATAGAGATTCGCCATCTCAGACA
-42   34_2.SDN;1   CTAACTTTCTTAGAATAGAGATTCGCCATCTCAGACAAGATGACAAGTAG
-43   34_3.SDN;1   CTAACTTTCTTAGAATAGAGATTCGCCATCTCAGACAAGATGACAAGTAG
      CONSENSUS    CTAACTTTCTTAGAATAGAGATTCGCCATCTCAGACAAGATGACAAGTAG

                          3110       3120       3130       3140       3150
 41   48_3.SDN;1   TGATTATATAATTATAAATTTATATTTCCATTTCGTATCTACGTGACTTC
-42   34_2.SDN;1   TGATTATATAATTATAAATTTATATTTCCATTTCGTATCTACGTGACTTC
-43   34_3.SDN;1   TGATTATATAATTATAAATTTATATTTCCATTTCGTATCTACGTGACTTC
      CONSENSUS    TGATTATATAATTATAAATTTATATTTCCATTTCGTATCTACGTGACTTC

                          3160       3170       3180       3190       3200
 41   48_3.SDN;1   aCGTTCGACATAGAAGATCCATGGTTTCA
-42   34_2.SDN;1   ACGTTCGACATAGAAGATCCATGGTTTCAAATAGTGATAGCTGTATAACG
-43   34_3.SDN;1   ACGTTCGACATAGAAGATCCATGGTTTCAAATAGTGATAGCTGTATAACG
 44   1_1.SDN;1                                    GTGATAGCTGTATAACG
      CONSENSUS    ACGTTCGACATAGAAGATCCATGGTTTCAAATAGTGATAGCTGTATAACG

                          3210       3220       3230       3240       3250
-42   34_2.SDN;1   TGCAAATGAAGAACATAATTAAAAAAAGTACTGTTACTATCattTATTCT
-43   34_3.SDN;1   TGCAAATGAAGAACATAATTAAAAAAAGTACTGTTACTATC
 44   1_1.SDN;1    TGCAAATGAAGAACATAATTAAAAAAAGTACTGTTACTATCATTTATTCT
      CONSENSUS    TGCAAATGAAGAACATAATTAAAAAAAGTACTGTTACTATCATTTATTCT

                          3260       3270       3280       3290       3300
-42   34_2.SDN;1   ACAGTaTTTTCtATtttCACAGTAG
 44   1_1.SDN;1    ACAgTATTTTCTATTTTCACAGTAGATATAGTtAGGTATTCTATTACCTT
      CONSENSUS    ACAGTATTTTCTATTTTCACAGTAGATATAGTTAGGTATTCTATTACCTT

                          3310       3320       3330       3340       3350
 44   1_1.SDN;1    AGATAAATTAATTTtCTCGTTATCTTTGATATACTCTGATATGGAGCata
-45   14_1.SDN;1                                                   ATA
-63   14_2.SDN;1                                                     A
      CONSENSUS    AGATAAATTAATTTTCTCGTTATCTTTGATATACTCTGATATGGAGCATA

                          3360       3370       3380       3390       3400
 44   1_1.SDN;1    AAAACTGGTCGTTAAACTTTTTAGAATACTTAtCTATtTGATTAGAAATC
-45   14_1.SDN;1   AAAACtGGTCGTtAAACTTTTTaGAATACTTATCTATTtGATTAGAAATC
-63   14_2.SDN;1   AAAACTGgTCGTTAAACTTTTTAGAATACTTATCTATTtGATTAGAAATC
      CONSENSUS    AAAACTGGTCGTTAAACTTTTTAGAATACTTATCTATTTGATTAGAAATC

                          3410       3420       3430       3440       3450
 44   1_1.SDN;1    GACGATACCAAAAGACTGTGTtTCGTATAGATAAAATGGTCTATAACTAT
-45   14_1.SDN;1   GACGATACCAAAAGACTGTGTTTCGtATAGATAAAAtGGTCtATAACTAT
-63   14_2.SDN;1   GACGATACCAAAAGACTGTGTTTCGTATAGATAAAATGGTCTATAACTAT
-46   32_3.SDN;1                                                AACTAT
      CONSENSUS    GACGATACCAAAAGACTGTGTTTCGTATAGATAAAATGGTCTATAACTAT

                          3460       3470       3480       3490       3500
 44   1_1.SDN;1    AGTTtCTATGTTCAGTATtTtAGCGATATTAtCATCTTTtAGAATAACGT
-45   14_1.SDN;1   aGTTTCtATGTTCAGTATTTtaGCGATATTATCATCTTTTaGAATAACGT
-63   14_2.SDN;1   AGTTTCTATGTTCAGTATTTTAGCGATATTATCATCTTTTAGAATAACGA
-46   32_3.SDN;1   AGTTTCTATGTTCAGTATTTTAGCGATATTaTCATCTTTTAGAATAACGT
      CONSENSUS    AGTTTCTATGTTCAGTATTTTAGCGATATTATCATCTTTTAGAATAACGT
```

```
                          3510       3520       3530       3540       3550
 44  1_1.SDN;1    ATATTTgTGAATCGtcGGGTTTCGTAGCTTTAATAAAATATTCGaACAAt
-45  14_1.SDN;1   AtATTTGtGAATCGTCGGGTTTcGtaGCTTtAATAAAATATTCGAACAAT
-63  14_2.SDN;1   ATATTTGtGAATCGTCGGGTTTCGTAGCTTTAATAAAATATTCGaACAAT
-46  32_3.SDN;1   ATATTTGTGAATCGTCGGGTTTCGTAGCTTTAATAAAAtAtTCGAACAAT
     CONSENSUS    ATATTTGTGAATCGTCGGGTTTCGTAGCTTTAATAAAATATTCGAACAAT

                          3560       3570       3580       3590       3600
 44  1_1.SDN;1    CGAGCACGTGATATAATATtATCTttAACttGAGGACATTTAAATAGTCT
-45  14_1.SDN;1   CGAGCACGTGATATAATATTATCTTTAACTTGAGGACATTTAAATAGTCT
-63  14_2.SDN;1   CGAGCACGTGATATAATATTATCTTTAACTTGAGGACATTTAAATAGTCT
-46  32_3.SDN;1   CGAGCACGTGATATAATATTATCTTTAACttGAGGACATTTAAATAGTCT
     CONSENSUS    CGAGCACGTGATATAATATTATCTTTAACTTGAGGACATTTAAATAGTCT

                          3610       3620       3630       3640       3650
 44  1_1.SDN;1    ATtTTTAAATACatCCAGAAGTtCAtCGGATATCATATAA
-45  14_1.SDN;1   ATTTTTAAATACATCCAGAAGTTCATCGGATATCATATAAAATGTATCTT
-63  14_2.SDN;1   ATTTTTAAATACATCCAGAAGTTCATCGGATATCATATAAAATGTATCTT
-46  32_3.SDN;1   ATTTTTAAATACATCCAGAAGTTCATCGGATATCATATAAAATGTATCTT
-19  7_4.SDN;1                          CGGATATCATATAAAAtGTATCTT
     CONSENSUS    ATTTTTAAATACATCCAGAAGTTCATCGGATATCATATAAAATGTATCTT

                          3660       3670       3680       3690       3700
-45  14_1.SDN;1   TAATAGTATTTTTCATATCCATAGATATAGCTGAATTCTTAATAAGCTGA
-63  14_2.SDN;1   TAATAGTATTTTTCATATCCATAGATATAGCTGAATTCTTAATAAGCTGA
-46  32_3.SDN;1   TAATAGTATTTTTCATATCCATAGATATAGCTGAATTCTTAATAAGCTGA
-19  7_4.SDN;1    TAATAGTATTTTTCATATCCATAGATATAGCTGAATTCTTAATAAGCTGA
 48  33_2.SDN;1                         ATAGCTGAATTCTTAATAAGCTGA
 47  33_1.SDN;1                                                CtGA
     CONSENSUS    TAATAGTATTTTTCATATCCATAGATATAGCTGAATTCTTAATAAGCTGA

                          3710       3720       3730       3740       3750
-45  14_1.SDN;1   TTGATAATATCCTCTGTTTTATTTTGTTCTAAGTAATAAGtaTGACCTTT
-63  14_2.SDN;1   TTGATAATATCCTCTGTTTTATTTTGTTCTAAGTAATGCGTATGCCCTT
-46  32_3.SDN;1   TTGATAATATCCTCTGTTTTATTTTGTTCTAAGTAATAAGTATGACCTTT
-19  7_4.SDN;1    TTGATAATATCCTCTGTTTTATTTTGTTCTAAGTAATAAGTATGACCTTT
 48  33_2.SDN;1   TTGATAATATCCTCTGTTTTATTTtGTTCTAAGTAATAAGTATGACCTTT
 47  33_1.SDN;1   TTGATAATATCCTCTGTTTTATTTtGTTCTAAGTAATAAGTATGACCTtT
 50  23_1.SDN;1                                        AAGTATGACCttT
-17  7_1.SDN;1                                          GTATGACCTTT
-20  7_5.SDN;1                                             TGACCTTT
     CONSENSUS    TTGATAATATCCTCTGTTTTATTTTGTTCTAAGTAATAAGTATGACCTTT

                          3760       3770       3780       3790       3800
-45  14_1.SDN;1   AAACTCAA
-46  32_3.SDN;1   AAACTCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCttaA
-19  7_4.SDN;1    AAACTCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCTTAA
 48  33_2.SDN;1   AAACTCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCTTAA
 47  33_1.SDN;1   AAACtCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCTTAA
 50  23_1.SDN;1   AAACTCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCTTAA
-17  7_1.SDN;1    AAACTCAAATCTATCGGCATTtGTTCTCAATCTATttGCTAAAACCTTAA
-20  7_5.SDN;1    AAACTCAAATCTATCGGCATTTGTTCTCAATCTATttGCTAAAACCTTaA
-18  7_2.SDN;1          ATCTATCGGCATTTGTTCTCAATCTATTTGCTAAaACCTTAA
-49  25_1.SDN;1                                              ACCTTaA
     CONSENSUS    AAACTCAAATCTATCGGCATTTGTTCTCAATCTATTTGCTAAAACCTTAA
```

```
                          3810       3820       3830       3840       3850
-46   32_3.SDN;1    CGTCTaGtaCGTCT
-19   7_4.SDN;1     CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCCAAT
 48   33_2.SDN;1    CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCcAAT
 47   33_1.SDN;1    CGTCTAGTACGTCTATATTGAGACTAGAATTTTCAtCTATTCTGGC
 50   23_1.SDN;1    CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCCAAT
-17   7_1.SDN;1     CGTcTAGtACGTCTATATtGAGACTaGAATTTTCATCTATTCTGGCCAAt
-20   7_5.SDN;1     CGTCTAGtACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCCAAT
-18   7_2.SDN;1     CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGgCCAAT
-49   25_1.SDN;1    CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCCaAT
      CONSENSUS     CGTCTAGTACGTCTATATTGAGACTAGAATTTTCATCTATTCTGGCCAAT

                          3860       3870       3880       3890       3900
-19   7_4.SDN;1     ATTTCTTTTTCTATATCTAGAAGAACTAGTTCATCGTGTTTACCCCTTTT
 48   33_2.SDN;1    AtTTCTTTTTCTATATCTAGAAGAACTAGTTCATCGTGTTTACCCCTTTT
 50   23_1.SDN;1    ATTTCTTTTTCTATATCTAGAAGAACTAGTTCATCGTGTTTACCCCTTTT
-17   7_1.SDN;1     ATTTCTTTTTCtATATCTAGAAGAACTAGTTCATCGtGTTTACCCCTTTT
-20   7_5.SDN;1     ATTTCTTTTTCTATATCTaGAAGAACTAGTTCATCGTGTTtACCCCTTTT
-18   7_2.SDN;1     ATTTCTTTTTCTATATCTAGAAGaACTAGTTCATCGTGTTTACCCCTTTT
-49   25_1.SDN;1    ATTTCTTTTTCTATATCTAGAAGAACTAGTTCATCGTGTTTACCCCTtTT
-55   10_3.SDN;1                          ACTAGTTCATCGTGTTTACCCCtttT
-21   7_6.SDN;1                                TCaTCGtGTTTACCCCTTTT
      CONSENSUS     ATTTCTTTTTCTATATCTAGAAGAACTAGTTCATCGTGTTTACCCCTTTT

                          3910       3920       3930       3940       3950
-19   7_4.SDN;1     AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
 48   33_2.SDN;1    AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
 50   23_1.SDN;1    AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
-17   7_1.SDN;1     AAGTTTGCCATTTTCAAAGTaATTATTTTCTACTAAACTACATATCAAAC
-20   7_5.SDN;1     AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
-18   7_2.SDN;1     aAGtTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
-49   25_1.SDN;1    AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
-55   10_3.SDN;1    AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
-21   7_6.SDN;1     AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC
      CONSENSUS     AAGTTTGCCATTTTCAAAGTAATTATTTTCTACTAAACTACATATCAAAC

                          3960       3970       3980       3990       4000
-19   7_4.SDN;1     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
 48   33_2.SDN;1    TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
 50   23_1.SDN;1    TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-17   7_1.SDN;1     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-20   7_5.SDN;1     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-18   7_2.SDN;1     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-49   25_1.SDN;1    TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-55   10_3.SDN;1    TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
-21   7_6.SDN;1     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG
      CONSENSUS     TATTCAGTACACCATCTTTATATCCCTTTAGCCCAAACTTAGACACAAAG

                          4010       4020       4030       4040       4050
-19   7_4.SDN;1     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGTTTG
 48   33_2.SDN;1    TTCTTATTTATGAGATCTAGAGGAATTCTATtCTCGGGTTTTTTAGTTTG
 50   23_1.SDN;1    TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGTTTG
-17   7_1.SDN;1     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTT
-20   7_5.SDN;1     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGtTTG
-18   7_2.SDN;1     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGtTTG
-49   25_1.SDN;1    TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGTTTG
-55   10_3.SDN;1    TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGTTTG
-21   7_6.SDN;1     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGtttg
      CONSENSUS     TTCTTATTTATGAGATCTAGAGGAATTCTATTCTCGGGTTTTTTAGTTTG
```

```
                          4060       4070       4080       4090       4100
-19  7_4.SDN;1    TGCCTTCTTTTTAGGA
 48  33_2.SDN;1   TGCCTTCTTTTTAGgagAaTTCTTTTTAGCGGcTAGTTCATCTATAGCTT
 50  23_1.SDN;1   TGCCTTCTTTTTAGGAGAATTCTTTtTAGCGGCTAGTTCATCTATAGCTT
-20  7_5.SDN;1    tGcCTTCTTTTTAG
-18  7_2.SDN;1    TGCCTTCTTTTTAGGAGAATTCTTTTTAGCG
-49  25_1.SDN;1   TGCCTTCTTTTTAGGAGAATTCTTTTTAGCGGCTAGTTCATCTATAGCTT
-55  10_3.SDN;1   TGcCTTCTTTTTAGGAGAATTCTTTTTAGCGGCTAGTTCATCTATAGCTT
-21  7_6.SDN;1    tgccttcttTTTaGGagaaTTCTTT
-54  10_2.SDN;1   TGCCTTCTTTTtTaGGaGAATTCTTTTTAGCGGCTaGTTCATCTATAGCTT
 56  24_1.SDN;1                          CTTTTTAGCGGCtagTTCATCTAtAGCTT
 57  24_2.SDN;1                          CTTTTTAGCGGCTAGTTCATCTAtAGCTT
     CONSENSUS    TGCCTTCTTTTTAGGAGAATTCTTTTTAGCGGCTAGTTCATCTATAGCTT

                          4110       4120       4130       4140       4150
 48  33_2.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATA
 50  23_1.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCtAATACTA
-49  25_1.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA
-55  10_3.SDN;1   CAGCTATTTTCGTAGTCAGATAtGACTTTAAAGCTTTAGCGCTAATACTA
-54  10_2.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA
 56  24_1.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA
 57  24_2.SDN;1   CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA
-53  10_1.SDN;1                AGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA
     CONSENSUS    CAGCTATTTTCGTAGTCAGATATGACTTTAAAGCTTTAGCGCTAATACTA

                          4160       4170       4180       4190       4200
 50  23_1.SDN;1   TTAAGAACAAGTTGATCAAA
-49  25_1.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
-55  10_3.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
-54  10_2.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
 56  24_1.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
 57  24_2.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
-53  10_1.SDN;1   TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT
-58  3_1.SDN;1                                 TTTAATCTTAACACATTAACTt
     CONSENSUS    TTAAGAACAAGTTGATCAAATTGCTCCATTTAATCTTAACACATTAACTT

                          4210       4220       4230       4240       4250
-49  25_1.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
-55  10_3.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
-54  10_2.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
 56  24_1.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
 57  24_2.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
-53  10_1.SDN;1   TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA
-58  3_1.SDN;1    TAAATAAAGAAACGTAAAGACCACTATgCATACTACAAATATAATAAAAA
     CONSENSUS    TAAATAAAGAAACGTAAAGACCACTATGCATACTACAAATATAATAAAAA

                          4260       4270       4280       4290       4300
-49  25_1.SDN;1   TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCaGttAGCACTG
-55  10_3.SDN;1   TTGTTAGCCATGATAATTGTTTGCTTTcTTTTTTACCAGTTAGCACTGTC
-54  10_2.SDN;1   TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCAGT
 56  24_1.SDN;1   TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCAGTTAGCACTGTC
 57  24_2.SDN;1   TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCAGTTAGCACTGTC
-53  10_1.SDN;1   TTGTTAGCCATGATAATTGTTTGcTTTCTTTTTTACCAGTTAGCACTGtc
-58  3_1.SDN;1    TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCAGTTAGCACTGTC
-59  3_2.SDN;1                                                 TGTC
     CONSENSUS    TTGTTAGCCATGATAATTGTTTGCTTTCTTTTTTACCAGTTAGCACTGTC
```

```
                          4310       4320       4330       4340       4350
-55   10_3.SDN;1    attATTGTat
 56   24_1.SDN;1    ATTATTGTATTGATAAAATCCtCAAAATCAGAATCATTTGATTCCAGAAA
 57   24_2.SDN;1    ATTATTGTATTGATAAAATCCTCAAAATCAGAATCATTTGATTCCAGAAA
-53   10_1.SDN;1    attATT
-58   3_1.SDN;1     ATTATTGTATTGATAAAATCCTCAAAATCAGAATCATTTGATTCCAGAAA
-59   3_2.SDN;1     ATTATTGTATTGATAAAATCCTCaAAATCAGAATCATTTGATTCCAGAAA
-60   3_3.SDN;1                              TCaAAATCAGAATCATTtGATTCCAGAAA
      CONSENSUS     ATTATTGTATTGATAAAATCCTCAAAATCAGAATCATTTGATTCCAGAAA

                          4360       4370       4380       4390       4400
 56   24_1.SDN;1    AACACCGTATGTACCTGTAAATAGCTTTTCCATTTAGtttACGTTaaaTa
 57   24_2.SDN;1    AACACCGTATGTaCCTGTAAATAGCTTTTCCATtTAGTTTACGTTAAATA
-58   3_1.SDN;1     AACaCCGTATGTACCTGTAAATAGCTTTTCCATTTAGTTTACGTTAAATA
-59   3_2.SDN;1     AACACCGTATGTACCTGTAAATAGCTTTTCCATTTAGTTTACGTTAAATA
-60   3_3.SDN;1     AACACCGTATGTACCTGTaaaTaGCTTTTCCATTTAGTTTACGTTAAATA
      CONSENSUS     AACACCGTATGTACCTGTAAATAGCTTTTCCATTTAGTTTACGTTAAATA

                          4410       4420       4430       4440       4450
 56   24_1.SDN;1    aTGATCGTTCAtCATATTCATCTGTTTCTTTTATTTTGTAACCACCTCTC
 57   24_2.SDN;1    ATGATCGTTCATCATATTCATCTGTTTCTTTTAtTTTGTAACCACCTCTC
-58   3_1.SDN;1     ATGATCGTTCATCATATTCATCTGTTTCTTTTATTTTGTAACCACCTCTC
-59   3_2.SDN;1     ATGATCGTTCATCATATTCATCTGTTTCTTTTATTTTGTAACCACCTCTC
-60   3_3.SDN;1     ATGATCGTTCATCATATTCATCTGTTTCTTTTATTTTGTAACCACCTcTC
      CONSENSUS     ATGATCGTTCATCATATTCATCTGTTTCTTTTATTTTGTAACCACCTCTC

                          4460       4470       4480       4490       4500
 56   24_1.SDN;1    CTGTTACCATCAGATCTTTCTTCTAAAATtCCAGATACACCGTCTGATTC
 57   24_2.SDN;1    CTGTTACCATCAGATCTTTCTTCTAAAATTCC
-58   3_1.SDN;1     CTGTTACCATCAGATCTTTCTTCTAAAATTCCAGATACACCGTCTGATTC
-59   3_2.SDN;1     CTGTTACCATCAGATCTTTCTTCTAAAATTCCAGATACACCGTCTGATTC
-60   3_3.SDN;1     CTGTTACCATCAGATCTTTCTTcTAAAATTCCaGATACACCGTcTGATTC
      CONSENSUS     CTGTTACCATCAGATCTTTCTTCTAAAATTCCAGATACACCGTCTGATTC

                          4510       4520       4530       4540       4550
 56   24_1.SDN;1    ATATTCAAACTCTCCCGATTCATCCTCTATATCATCGTCTCC
-58   3_1.SDN;1     ATATTCAAACTCTCCCGATTCATCCTCTATATCATCGTCTCCGGAGGTTA
-59   3_2.SDN;1     ATATTCAAACTCTCCCGATTCATCCTCTATATCATCGTCTCCGGAGGTTA
-60   3_3.SDN;1     ATATTCAAACTCTCCCGATTCATCCTCTATATCATCGTCTCCGGAGGTTA
      CONSENSUS     ATATTCAAACTCTCCCGATTCATCCTCTATATCATCGTCTCCGGAGGTTA

                          4560       4570       4580       4590       4600
-58   3_1.SDN;1     CGACGCTGATTACTTCTCCgtTAgtATCTATCAGAgT
-59   3_2.SDN;1     CGACGCTGATTACTTCTCCGTTAGTATCTATCAG
-60   3_3.SDN;1     CGACGcTGATTACTTCTCCGTTGGTATCTATCAGAGTTTTCcTACAAACT
 62   31_2.SDN;1       CGCTGATTACTTctCCGTTAGTATCTATCAGAGTTTTCCTACAAACT
 61   31_1.SDN;1        GCTGATTACTTCTCCgTTaGTATCTATCAGAGTTTTCCTACAAACT
      CONSENSUS     CGACGCTGATTACTTCTCCGTTAGTATCTATCAGAGTTTTCCTACAAACT

                          4610       4620       4630       4640       4650
-60   3_3.SDN;1     TTTTC
 62   31_2.SDN;1    TTTTCCATAATAAGCTTTTTCAAGCTAAAGCTTATTTTACACATTACCGC
 61   31_1.SDN;1    TTTTCCATAATAAGCTTTTTCAAGCTAAAGCTTATTTTACACATTACCGC
      CONSENSUS     TTTTCCATAATAAGCTTTTTCAAGCTAAAGCTTATTTTACACATTACCGC

                          4660       4670       4680       4690       4700
 62   31_2.SDN;1    ATTTTCGAAAAATATAGAAGCAACTAGTACAGCTTTAACCATCTGAGGAT
 61   31_1.SDN;1    ATTTTCGAAAAATATAGAAGCAACTAGTACAGCTTTAACCATCTGAGGAT
      CONSENSUS     ATTTTCGAAAAATATAGAAGCAACTAGTACAGCTTTAACCATCTGAGGAT
```

```
                          4710       4720       4730       4740       4750
62   31_2.SDN;1   CTTTCTTCGATAGATGTTCTACTTCGCTGGATAGAGTTTTTCTACCAGCT
61   31_1.SDN;1   CTTTCTTCGATAGATGTTCTACTTCGCTGGATAGAGTTTTTCTACCAGCT
     CONSENSUS    CTTTCTTCGATAGATGTTCTACTTCGCTGGATAGAGTTTTTCTACCAGCT

                          4760       4770       4780       4790       4800
62   31_2.SDN;1   ACTTCCTTGTTGTTTCTCGTATATTCTATTATAGTAGATGACATTTTATT
61   31_1.SDN;1   ACTTCCTTGTTGTtTCTCGTATATTCTATTATAGTAGATGACATTTTATT
     CONSENSUS    ACTTCCTTGTTGTTTCTCGTATATTCTATTATAGTAGATGACATTTTATT

                          4810       4820       4830       4840       4850
62   31_2.SDN;1   TTTATTAATGTATATATAACCATCTTTAAATGTTTTTTCATCCATAGTAT
61   31_1.SDN;1   TTTATTAATGTaTATATAACCATCTTTAAATGTTTTTTCATCCATAGTAT
     CONSENSUS    TTTATTAATGTATATATAACCATCTTTAAATGTTTTTTCATCCATAGTAT

                          4860       4870       4880       4890       4900
62   31_2.SDN;1   TTTCCGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCA
61   31_1.SDN;1   TTTCCGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACC
     CONSENSUS    TTTCCGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCA
                    Cloning site

                          4910       4920       4930       4940       4950
62   31_2.SDN;1   CAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGAT
     CONSENSUS    CAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGAT

                          4960       4970       4980       4990       5000
62   31_2.SDN;1   CGGGCTCGCCACTTCGGGCTCATGAACGCTTGTTTCGGCGTGGGTATGGT
     CONSENSUS    CGGGCTCGCCACTTCGGGCTCATGAACGCTTGTTTCGGCGTGGGTATGGT

                          5010       5020
62   31_2.SDN;1   GGCAGCCCCGTGGCCGGGGGA
     CONSENSUS    GGCAGCCCCGTGGCCGGGGGA
```

Appendix 7.1. Sequence Alignment

Automated cycle sequencing was carried out using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). Nine and a half µl of terminator premix was mixed with 1 µg of CsCl prepared DNA and 3.2 pmol of oligonucleotide primer, and overlaid with 40 µl of mineral oil in a 0.5 ml eppendorf. The mix was heated to 96 ^{o}C for 1 min and then cycled to 96 ^{o}C for 30 sec, 50 ^{o}C for 15 sec, 60 ^{o}C for 4 min for 25 cycles and cooled to 4 ^{o}C in a PCR machine (Programmable Dri-Block, Techne). The mineral oil was dissolved by addition of 100 µl of chloroform, 80 µl of water was added and 100 µl of phenol:chloroform:water (68:18:14) was added to extract the excess terminators. The tubes were centrifuged 13,000 rpm for 10 min and the aqueous phase removed to a clean tube. The DNA was ethanol precipitated, washed in 70% ethanol and dried.

Assembly of the sequences was carried out using the Sequence Assembly Program (SAP), version 4.0, May 1991, written by Roger Staden. The cloning sites and areas of homology with VV E3L and VV K3L DNA are also shown.