The Genetic Basis for the Attenuation of the Sabin Type 3 Poliomyelitis Vaccine, P3/Leon/12a$_1$b

by

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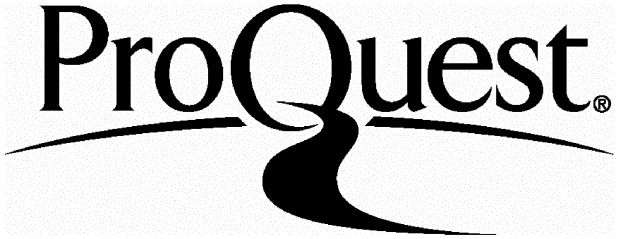

To My Parents,

With Thanks.

STATEMENT

Primer extension sequencing of poliovirus recombinants was performed by Dr. D. Evans and G. Dunn in the Division of Viral Products at the N.I.B.S.C., Hampstead, London. Neurovirulence assays were performed by Dr. D. Magrath, Dr. F. Taffs and S. Marsden at the N.I.B.S.C. All other work was conducted by myself in the Department of Microbiology at the University of Leicester between December 1982 and November 1985.

All the work presented in this thesis is original unless acknowledged by references in the text. This work has not been submitted for any other degree in this or any other University.

Signed: G.D. Westrop. Dated: 6.7.86.

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ABSTRACT

Complete nucleotide sequences have been derived for the Sabin type 3 vaccine, P3/Leon/12a$_1$b, and its neurovirulent progenitor, P3/Leon/37 (Stanway et al., 1983, 1984). These studies revealed 10 base substitution mutations which must account for the attenuated and temperature sensitive phenotypes of the vaccine. Complete DNA copies of the genomes of P3/Leon/12a$_1$b and P3/Leon/37 were constructed, each within the prokaryote vector, pAT 153. The full-length cDNA clones were shown to be infectious following transfection of human epithelial cells. Virus rescued from the cDNA clone of P3/Leon/12a$_1$b could not be distinguised from reference preparations of the Sabin type 3 vaccine in standard assays for neurovirulence and temperature sensitivity. By the same criteria, virus rescued from the cDNA clone of P3/Leon/37 was shown to be identical to the parental strain. To determine the genetic basis for the attenuated phenotype, a series of inter-strain poliovirus recombinants were constructed via cDNA in-vitro. Attenuation results from the concerted effect of two independent point mutations. The first is a C-U substitution at position 472 in the 5' non-coding region of the viral genome. The second is a C-U substitution at position 2034 which results in a serine to phenylalanine substitution in VP3. The VP3 mutation confers a temperature sensitive phenotype. This appears to be the only temperature sensitive mutation in the vaccine.

ABBREVIATIONS

A	Adenine
amp	Ampicillin
amp^r	Ampicillin resistant
BCIG	5-bromo-4-chloro-3-indolyl-B-D-galactoside
bp	Base pairs
C	Cytosine
cDNA	Copy DNA
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	D,L-dithiothreitol
E.coli	Escherichia coli
EDTA	Diaminoethanetetra-acetic acid
EtBr	Ethidium bromide
G	Guanine
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethane sulfonic acid
IPTG	Isopropyl-B-D-thio-galacto-pyranoside
IPV	Inactivated poliovirus vaccine
kb	Kilobases
L	Luria medium
mRNA	Messenger RNA
OPV	Oral poliovirus vaccine
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl phosphate
SSC	Saline sodium citrate

T	Thymine
TBE	Tris-borate-EDTA
TE	Tris-EDTA
tet	Tetracycline
tet^r	Tetracycline resistant
Tris	Tris(hydroxymethyl)methylamine
ts	Temperature sensitive
u	Units
U	Uracil
UV	Ultraviolet
vRNA	Virus RNA
v/v	Volume for volume
w/w	weight for weight

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CHAPTER ONE

Introduction

1.1 General Introduction

Sporadic cases of persisting paralytic illness have been recorded since antiquity. Poliomyelitis was not, however, clearly defined as a clinical entity until the beginning of the 20th century when an abrupt increase in the incidence of the disease occurred in the urban, industrialised regions of Northern Europe and the United States. The transition from the endemic pattern of the relatively uncommon infantile paralysis to epidemic poliomyelitis presented one of the most formidable public health problems of recent times. An alarming trend towards epidemics of increasing frequency and severity was observed throughout the developed world during the first half of this century. Very little could be done to treat or even to control the disease until prophylactic vaccination became a viable option in the late 1950s.

The Salk vaccine, a formalin inactivated virus preparation, was introduced in 1955. A dramatic reduction in the incidence of the disease was achieved over the next six years. In spite of this remarkable achievement, the Salk vaccine was largely replaced by the Sabin live attenuated vaccine when it became available in 1961. For a number of reasons, the Sabin vaccine was considered to be superior. Consistently higher levels of serum antibody are induced in recipients, providing a

potentially longer lasting immunity. Also, replication of the vaccine strains in the alimentary tract, the primary site for multiplication of the virus, leads to a strong secretory IgA response. Intestinal immunity appears to block the circulation of wild-type strains (Yourke et al., 1979).

The Sabin vaccine is still preferred by most countries. Its continued use has lead to the virtual eradication of the disease in the developed world. Poliomyelitis, however, remains a serious concern. The disease is endemic throughout the third world. Surveys of residual lameness attributable to poliomyelitis indicate that the annual incidence of the disease in these countries may be as high as 5-10 cases per 1,000 population. Such a rate greatly exceeds that observed in the United States at the end of the pre-vaccine era (Robbins et al., 1984).

Also, in those countries where the Sabin vaccine is in use, a low, residual level of the disease has persisted. Most of these rare cases are temporally associated with administration of the vaccine. The Sabin vaccine is administered as a trivalent vaccine, consisting of attenuated strains of each of the three serotypes. Poliovirus types 2 and 3 are more frequently implicated as the cause of vaccine associated paralysis than type 1 (Assad and Cockburn, 1982). There is now good evidence that the type 3 strain and probably the type 2 strain, may revert to neurovirulence during replication in recipients of the vaccine or their contacts (Cann et al., 1984).

The lowest estimates puts the rate of vaccine associated paralysis at 1 case in 11 million doses (Melnick, 1982). However, this estimate takes no account of the number of doses administered to susceptible individuals, the number of booster doses given and the number of doses discarded (OPV is distributed in the UK in 10 dose vials with instructions to discard any vaccine not used at the end of each clinic). In the recent WHO study (Assad and Cockburn, 1982) 46/52 (89%) of cases of vaccine associated paralysis occurred after administration of the first dose of vaccine, 5/52 (9%) after

the second dose and 1/52 (2%) after the third dose. Thus the true risk of vaccine associated disease is probably closer to 1 case per 0.5-1 million doses administered (CDR, 1982).

By relative standards, the Sabin vaccine is one of the safest vaccines currently in use. However the evidence of genetic instability points to the continued need for surveillance of new vaccine preparations. Also the rarity of vaccine associated disease is offset by the severity of the illness and the permanence of the resulting disability.

The type 3 vaccine strain, P3/Leon/12a$_1$b, is the strain most frequently associated with complications in recipients. P3/Leon/12a$_1$b was derived empirically by serial rapid passage of a virulent clinical isolate in tissue culture (Sabin et al., 1954; Sabin and Boulger, 1973). The aim of the work presented for this thesis is to characterize the genetic changes responsible for the attenuated phenotype of P3/Leon/12a$_1$b. In the short term this work may lead to a much needed <u>in-vitro</u> assay. The current monkey neurovirulence assay is expensive, cumbersome and can yield ambiguous results. In the longer term, identification of regions of the genome important for maintaining virulence is a vital step towards a better understanding of the pathogenicity of poliovirus. It is hoped that such knowledge could be used to design a safer, more stabley attenuated vaccine strain.

1.2. Picornaviral Taxonomy.

The picornaviruses are a family of mammalian viruses causing a wide range of diseases. The name is derived from the prefix "pico", meaning small and The possession of an RNA genome. All picornaviruses share common structural and morphogenic properties. A single stranded, positive sense RNA genome of approximately 2.7 x 10^6 molecular weight, is enclosed in an icosahedral protein capsid. The capsids are composed of sixty sub-units each of which contains four non-identical polypeptide chains (Putnak and Phillips, 1981). The RNA genome is polyadenylated at its 3′ terminus (Fellner, 1979) and has a small, viral coded protein (VPg) covalently attached to the 5′ terminus (Nomoto et al., 1976).

The picornaviruses are currently classified into four genera on the basis of stability to acid pH, buoyant density and genome structure (see Table 1.1). A number of plant and insect viruses have many characteristics in common with picornaviruses but are not presently included in the familly.

The enteroviruses are primarily viruses of the gastro intestinal tract but may also replicate in other tissues such as nerve, liver, pancreas and muscle. Most enteroviral infections are subclinical, although some are associated with gastro-intestinal disorders or, more clinically significant, heart or central nervous system involvement. Over seventy distinct antigenic types of enteroviruses have been identified. These have been divided into a number of groups including the human polioviruses, the coxsackieviruses and the echoviruses. Newly identified enteroviruses are now numbered sequentially from 68, irrespective of former groupings.

The rhinoviruses are the major causative agents of the upper respiratory tract infections collectively known as the common cold. The disease is one of the most common virus infections of humans and is of considerable economic importance. The genus is composed of 115 human and 2 bovine serotypes.

The aphthoviruses cause the extremely contagious foot-and-mouth disease of cattle. Although rarely fatal, the disease does cause serious loss of condition and is therefore of significant economic importance. The disease is characterized by vesicular eruptions on the mouth, tongue, muzzle and hooves. The aphthoviruses show a wide host range infecting most cloven-hooved animals.

The cardioviruses, commonly infecting mice, comprise the smallest genus of picornaviruses. Cardioviruses have been isolated from the blood and stools of humans in whom they may cause febrile illness with central nervous system involvement.

Table 1.1.

Classification of the Picornaviridae.

FAMILY: PICORNAVIRIDAE

Main characteristics

Nucleic acid: composed of one infectious, positive sense ssRNA, mol.wt. 2.5 x 10^6. 5′ terminus is covalently linked to a protein, VPg (mol. wt. approx 2,400). The 3′ terminus is polyadenylated.
Protein: Four major polypeptides.
Lipid: None.
Carbohydrate: none.
Physico-chemical properties: Mol.wt. 8-9 x 10^6, buoyant density in CsCl 1.33-1.45 g/ml.

GENUS ENTEROVIRUS. Type species: poliovirus type 1

Main characteristics

Stable at acid pH; buoyant density 1.33-1.34g/ml.

Members

Human polioviruses types 1-3
Human coxsackieviruses A1-22, 24
Human coxsackieviruses B1-6
Human echoviruses 1-9, 11-27, 29-34

Table 1.1. contd.

Human enteroviruses 68-72
Murine polioviruses
Simian enteroviruses 1-18
Porcine enteroviruses 1-8
Bovine enteroviruses 1-7

GENUS: RHINOVIRUS. Type species: Human rhinovirus 1A

Main characteristics:
Unstable below pH 5-6; buoyant density 1.38-1.42g/ml
Members:
Human rhinoviruses 1A, 1B, 2-115
Bovine rhinoviruses 1-2

GENUS: APHTHOVIRUS Type species: FMDV type O

Main characteristics:
Unstable below pH 5-6; Buoyant density 1.38-1.42; poly(C) tract of variable length about 400 bases from the 5′ terminus of vRNA.
Members:
FMDV O
FMDV A
FMDV C
FMDV SAT 1-3
FMDV Asia

GENUS: CARDIOVIRUS

Main characteristics:
Unstable at pH below 5-6 in the presence of 0.1M halide; buoyant density 1.33-1.34; poly (C) tract of variable length (80-250 bases from 5′ terminus of vRNA.
Members:
Encephalomyocarditis (EMC) virus
Mengovirus
Murine encephalomyelitis (ME) virus

1.3. Structure of Picornavirions

Picornaviruses are small (22-30nm), unenveloped RNA containing viruses. The virion is a spherical particle with a sedimentation coefficient of 150S. A protein capsid contains one molecule of single-stranded, positive-sense RNA. Varion RNA serves as a functional message in eukaryotic cell-free systems and is infectious following transfection of susceptible cells.

1.3.1. Virion architecture

X-ray diffraction patterns of crystallised poliovirus have 5:3:2 symmetry, implying that the virion is an icosahedral particle composed of 60 or N.60 identical assymetric subunits (Finch and Klug, 1959). On the basis of this observation and extrapolating from estimates for virion mass of 6.7×10^6 and RNA mass of 2×10^6, these authors presented a 60 subunit model for poliovirus in which the fundamental repeating morphological unit was a polypeptide with a predicted diameter of 60-65A and a molecular weight of 80×10^3. However, it was later demonstrated, using SDS polyacrylamide gel electrphoresis, that the poliovirus capsid was composed of not one, but several distinct polypeptide species (Maizel, 1963).

Electrophoretic profiles of the capsid proteins of representative enteroviruses (Summers et al., 1965; Crowell and Philipson, 1971), cardioviruses (Rueckert et al., 1969), rhinoviruses (Medappa et al., 1971; Korant et al., 1972) and apthoviruses (Brown, 1978) are remarkably similar, suggesting that, in spite of the marked differences in acid stability and buoyant density between the genera, all picornaviruses share fundamental features of virion structure. The picornavirus capsid is composed of equimolar amounts of at least four major polypeptide species named in order of increasing electrophoretic mobility: VP1 or alpha (Mol. wt 35.5 +/- 1.5 x 1 0^3),

VP2 or β (mol.wt. 30.0 +/- 1.0 x 10^3), VP3 or γ (mol.wt. 25.0 +/- 2.0 x 10^3) and VP4 or δ (mol.wt. 8.0 +/- 2.0 x 10^3) (Putnak and Philips, 1981). Comparison of the molecular weights of the four capsid polypeptides with the protein mass of the virion indicates that there are 60 copies of the major capsid proteins in the virion. Highly purified preparations of most picornaviruses also contain an additional polypeptide, VP0, at a level equivalent to one or two copies per virion. VP0 is the uncleaved precursor of VP2 and VP4 (Kitamura et al., 1981).

Attempts to determine how these proteins could be assembled into an icosahedral particle have been made using controlled degradation (with physical and chemical agents), surface labeling and chemical cross-linking. Several different models have been proposed (Dunker and Rueckert, 1971; Talbot and Brown, 1972; Philipson et al., 1973)

Thermal dissociation of cardioviruses such as ME virus or mengovirus leads to the release of infectious RNA and the formation of homogeneous 13-14S substructures containing equimolar proportions of VP1, VP2 and VP3. VP4 and VP0 form an insoluble precipitate under the conditions used (Dunker and Rueckert, 1971). The 13-14S particles can be further degraded by treatment with 2M Urea to yield homogeneous 5S substructures composed of a single copy each of VP1, VP2 and VP3. Although neither of these species has a direct parallel within infected cells, it was proposed that the 5S particle represents the fundamental morphological subunit of the mature virion. In this scheme five such particles or protomers are linked by hydrophobic interactions (disrupted by 2M Urea) into aggregates or pentamers (14S particles). A single pentamer is located at each of the twelve vertices of a simple icosahedron. Each 5S particle has an estimated molecular mass of 86 x 10^3 and a diameter of 68A (Mak et al., 1974), in good agreement with the earlier prediction based on X-ray diffraction data. The results of studies using protein cross-linking agents in conjunction with controlled degradation suggest that, for mengovirus, VP1-VP2 bonds

form the primary stabilising contacts between neighbouring pentamers and that VP1-VP1 bonds are involved in protomer-protomer interactions within pentamers (Horden et al., 1979).

Acid dissociation of apthoviruses yields 12S particles composed of three copies each of VP1, VP2 and VP3. This result is consistent with the protomer model if it is assumed that the acid labile bonds are aligned along the boundaries of the twenty icosahedral faces (Talbot and Brown, 1972). However, the products of analogous treatments of the acid stable enteroviruses are qualitatively different.

Treatment of coxsackievirus B3 with alkaline urea results in the release of VP4, probably as a monomer, and the formation of 5S substructures composed of VP2 and 20S substructures containing VP1 and VP3 (Philipson et. al., 1973). Controlled alkaline degradation of poliovirus type 1 leads to the stepwise release of VP4 and VP2 (Katagiri et al. 1971). These results suggest that the enteroviruses, at least, are composed of a stable matrix of VP1 and VP3. A close association between VP1 and VP3 has been confirmed, for poliovirus type 1, by treatment of virions with bifunctional cross-linking reagents.

Chemical modification of intact poliovirions by lactoperoxidase catalysed iodination of surface directed tyrosine residues (Beneke et al., 1977) or acetylation of amide groups with acetic anhydride (Lonberg-Holm and Butterworth 1976) have shown that VP1 is prominently expressed on the surface of the virion. VP2 and VP3 were less accessible for surface labelling than VP1. VP4 could not be modified in intact virions and was therefore assumed to occupy an internal location. Similar results have been obtained for foot-and-mouth-disease virus (Talbot et al.,1973), mengovirus (Lund et al., 1977), and bovine enterovirus (Carthew and Martin, 1974).

The three dimensional structure of the Mahoney strain of poliovirus type 1 has recently been solved to high resolution using X-ray crystalography (Hogle et al., 1985). The conformation of capsid proteins

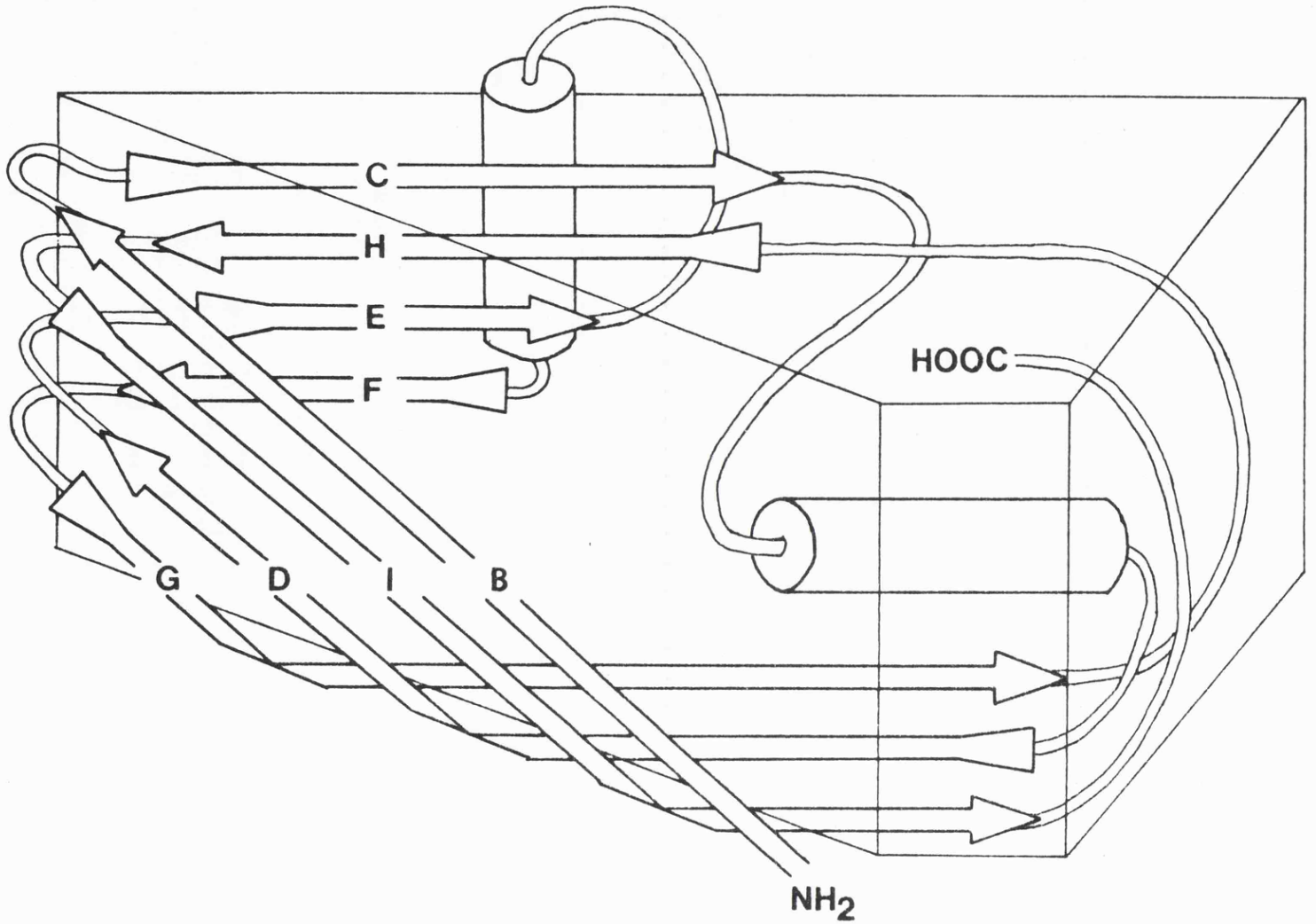

C
H
E
F
G
D
I
B
HOOC
NH2

Fig. 1. 1.

Core Structure of Poliovirus Capsid Proteins.

Simplified diagram showing the structurally conserved core of the poliovirus capsid proteins, VP1, VP2 and VP3. The core is composed of an eight-stranded, anti-parallel beta-barrel flanked by two alpha-helices. Strands G, D, I and B make up a large, twisted beta-sheet which forms the front and bottom surfaces of the beta-barrel. Strands C, H, E and F make up a smaller beta-sheet which forms the back surface of the beta-barrel. The strands forming the front and back surfaces are joined by short connecting loops so that the barrel is wedge-shaped.

VP1, VP2 and VP3 are remarkably similar. Each is composed of a common core structure consisting of an eight stranded anti-parallel β-barrel with two flanking α-helices. Four strands (B, D, I and G in fig 1.1.) make up a large, twisted β-sheet which forms the front and bottom surfaces of the β-barrel. Four shorter strands (C, H, E and F) make up a flatter β-sheet which forms the back surface of the β-barrel. The strands forming the front and back surfaces of the β-barrel are joined at one end by short connecting loops so that the overall structure is wedge shaped.

Each of these capsid proteins have unique N-terminal and C-terminal extensions. They also have different sets of internal insertions. The largest insertion of VP1 (residues 207-237 from the N-terminus) connects strand G at the back of the β-barrel with strand H at the bottom. The largest insertion of VP2 (residues 127-185) connects strand E at the back of the β-barrel with the flanking α-helix. The most significant insertion of VP3 (residues 53-69) connects the N terminal extension with strand B at the bottom of the β-barrel. The C-terminal extensions and internal insertions are located at the top of the β-barrel. The N-terminal extensions are located at the bottom of the β-barrel.

In contrast to the compact structures obtained for the other three capsid proteins, VP4 has a more extended conformation. VP4 is similar in position and conformation to the N-terminal strands of VP1 and VP3. It is best considered as the detatched N-terminal extension of VP2 rather than as an independent capsid protein.

Fig. 1.2. shows the position and orientation of the three major capsid proteins at the icosahedral surface. The subunits indicated show extensive inter-protein interactions. These form a unit which probably corresponds to the protomer substructure identified by degradation studies of cardioviruses (Dunker and Rueckert, 1971). The VP1 subunit of the protomer is positioned so that the thin end of its wedge-shaped β-barrel is directed towards the five-fold axis of symmetry. The thin ends of the VP2

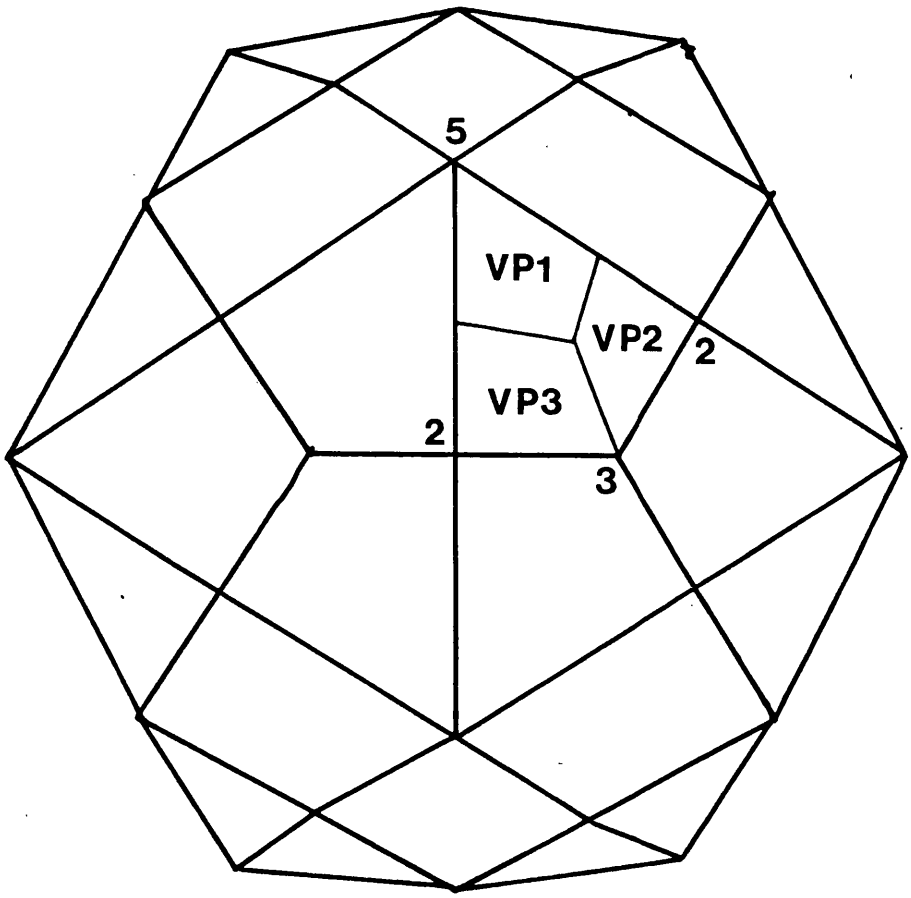

5
VP1
VP2
2
VP3
2
3

Fig. 1. 2.

Orientation of the Poliovirus Capsid Proteins on the Surface of the Virion.

Diagram showing the position and orientation of the poliovirus capsid proteins on the icosahedral surface of the virion. Each capsid protein is folded into a wedge-shaped beta-barrel. The closed end of the VP1 barrel is located near the 5-fold axis of symmetry. The closed ends of the VP2 and VP3 barrels alternate around the 3-fold axis. The proteins indicated probably represent a 'protomer' subunit generated by cleavage of a single P1-1a precursor molecule.

and VP3 β-barrels alternate around the three-fold axis of symmetry.

The packing and orientation of the capsid proteins are such that two prominent sets of surface projections are produced. A pronounced outward tilt at the closed ends of the VP1 β-barrel results in the formation of a ribbed peak at the five-fold axis of symmetry. The upper surface of the VP1 β-barrel forms the slope of the peak and the broad moat that surrounds it. The uppermost three loops of the B-barrel (residues 96-104, 245-251 and 142-152) are exposed at the summit. The top loop (residues 96-104) twists away from the surface so that it is particularly well exposed. The outward tilts at the closed ends of VP2 and VP3 are less well pronounced. The outermost two loops (residues 72-75 and 240-244 in VP2 and residues 75-81 and 196-206 of VP3) are exposed at the surface forming a plateau at the three-fold axis. The plateau is broadened by two sets of outward projections. The larger projection is formed by the internal insertion in VP2 (residues 127-185) and by residues 207-237 and 271-295 of VP1.

The interior surface of the capsid is formed by the bottom surfaces of the β-barrels of VP1, VP2 and VP3, by VP4 and by the N-terminal extensions of VP1, VP2 and VP3. Extensive interactions between VP1 and VP3 are seen within protomers. The N-terminal extensions also form a network which links 5-fold related protomers to form pentamers. The N-termini of five subunits of VP3 intertwine about the five-fold axis forming a five stranded tube of extremely twisted parallel β-sheet structure. The viral RNA appears to be spatially disordered within the interior of the virion.

The high resolution structure of human rhinovirus type 14 (Rossmann et al., 1985) is very similar to that obtained for poliovirus type 1. This is consistent with the high degree of sequence homology observed between these two viruses. The positions and interactions of the individual capsid proteins have implications for virion antigenicity and morphogenesis which will be discussed in section 1.4.2 and section 1.5.5. respectively

1.3.2. Genome Organisation.

The picornavirus genome is a single stranded, unsegmented molecule of 2.6 x 10^6 molecular weight, or approximately 7,400 nucleotides in length. Complete nucleotide sequences have been derived for representative strains of all three serotypes of poliovirus (Racaniello and Baltimore, 1981a; Kitamura et al., 1981; Stanway et al., 1983; Toyoda et al., 1984), and human rhinovirus type 14 (Stanway et al., 1985). The sequence of the coding region has been determined for EMCV (Palmenberg et al., 1984) and FMDV type $A^1_0$61 (Carrollet al., 1984). The genome structure is analogous to that of a typical eukaryotic messenger RNA and can be considered as three functional sections:

1) a 5′ non-coding region comprising 10-15% of the molecule

2) a coding region comprising approximately 90% of the genome

3) a 3′ non-coding region of between 30 and 80 bases

1) The 5′ non-coding region. Unlike all eukaryotic mRNAs so far examined, picornavirus genomes do not possess a m^7GpppG cap structure at the 5′ end (Nomoto et al., 1976). Instead, these viruses have a small, basic, viral-coded protein (VPg) covalently linked to the 5′ terminal uridylate via a phosphdiester bond (Petterson et al., 1977; Nomoto et al., 1977a,b; Sangar et al., 1977; Rothberg et al., 1978). VPg is not required for translation and has recently been implicated in the initiation of transcription where it is believed to serve as a primer for the viral coded RNA polymerase (see section 1.5.4.).

The 5′ non-coding regions of aphthoviruses and cardioviruses contain an internal poly(C) tract which is not present in enteroviruses or rhinoviruses (Brown et al., 1974; Porter et al., 1974). In the

aphthoviruses this tract occurs approximately 400 nucleotides from the 5′ terminus and varies between 100 and 200 nucleotides in length (Harris and Brown, 1977; Rowlands et al., 1978; Black et al., 1979). In EMCV the poly(C) tract occurs approximately 150 nucleotides (Porter et al., 1974). The function of these poly(C) tracts is not known. There is some speculation that they may be involved in encapsidation of the genome.

The 5′ non-coding region of the poliovirus genome is unusually long and could conceivably encode one or more small peptides not yet identified. In poliovirus type 1, eight AUG codons were observed upstream of the translational start at N741 (Kitamura et al., 1981; Racaniello and Baltimore, 1981). Three of these were conserved in poliovirus type 2 and 3, at N318, N455 and N584 (Toyoda et al., 1984). However the length of the reading frames they establish was not conserved. Furthermore the peptides encoded show little homology. It is therefore unlikely that peptides which function in viral replication are produced from this 5′ region.

In poliovirus type 1 there is a stable hairpin within the 5′ terminal 85 nucleotides (Larsen et al., 1981). Only one mutation was observed between poliovirus type 1 and 3 in the first 54 nucleotides (N22 in poliovirus type 1) and this did not affect the stability of the hairpin loop (Stanway et al., 1983). Poliovirus type 2 shows two differences from poliovirus type 1 in this region, and one of these differences (N28 in poliovirus type 1) reduces the stability of the hairpin loop (Toyoda et al., 1984).

2) <u>The major open reading frame.</u> Early studies of protein synthesis in poliovirus infected HeLa cells revealed that a greater number of proteins were produced than could be accounted for by the total coding capacity of the genome (Summers et al., 1965). However, pulse chase experiments and tryptic peptide analysis soon revealed a precursor/product relationship between certain viral proteins (Jacobson et al., 1970). Following treatment of cells with zinc (Butterwoth and Korant, 1974), amino

acid analogues (Jacobson and Baltimore, 1968) or protease inhibitors (Korant, 1972), a large protein of approximately 220 x 10^6 molecular weight was detected. This protein (NVPOO) represented 90% of the coding capacity of the genome.

These results were consistent with a model in which translation was initiated at a single site near the 5′ terminus, resulting in the synthesis of a precursor polyprotein from which functional viral specific proteins were derived by proteolytic cleavage. Nucleotide sequence data has confirmed that picornaviral genomes contain a single, long open reading frame (Kitamura et al., 1981; Racaniello and Baltimore., 1981a; Stanway et al., 1983; Carrollet al., 1984; Palmenberg et al., 1984).

The relative gene order of the products of proteolytic cleavage was determined for poliovirus by pulse chase experiments carried out in the presence of pactomycin, an inhibitor of the initiation of translation. The basis of this technique was that the amount of radiolabel incorporated into polypeptides, various times after the inhibition of translation, would depend on the distance from the translational start. These studies revealed that viral capsid proteins mapped at the N-terminus of the polyprotein and the catalytic functions, such as the viral polymerase and the protease activities mapped at the carboxy terminus.

Fig. 1.3. represents the biochemical map produced for poliovirus by combining the results of pactomycin mapping (Rekosh et al., 1970; Summers and Maizel, 1971; Taber and Baltimore, 1971; Butterworth, 1973), tryptic peptide analysis of the relationship between precursor and mature proteins (Jacobson and Baltimore, 1968; Jacobson et al, 1970; Rueckert et al, 1979), partial sequencing of polypeptides (Semler et al., 1981a,b; Larsen et al., 1982; Emini et al., 1982) and nucleotide sequence studies (Kitamura et al., 1981., Racaniello and Baltimore., 1981; Stanway et al., 1983; Toyoda et al., 1984).

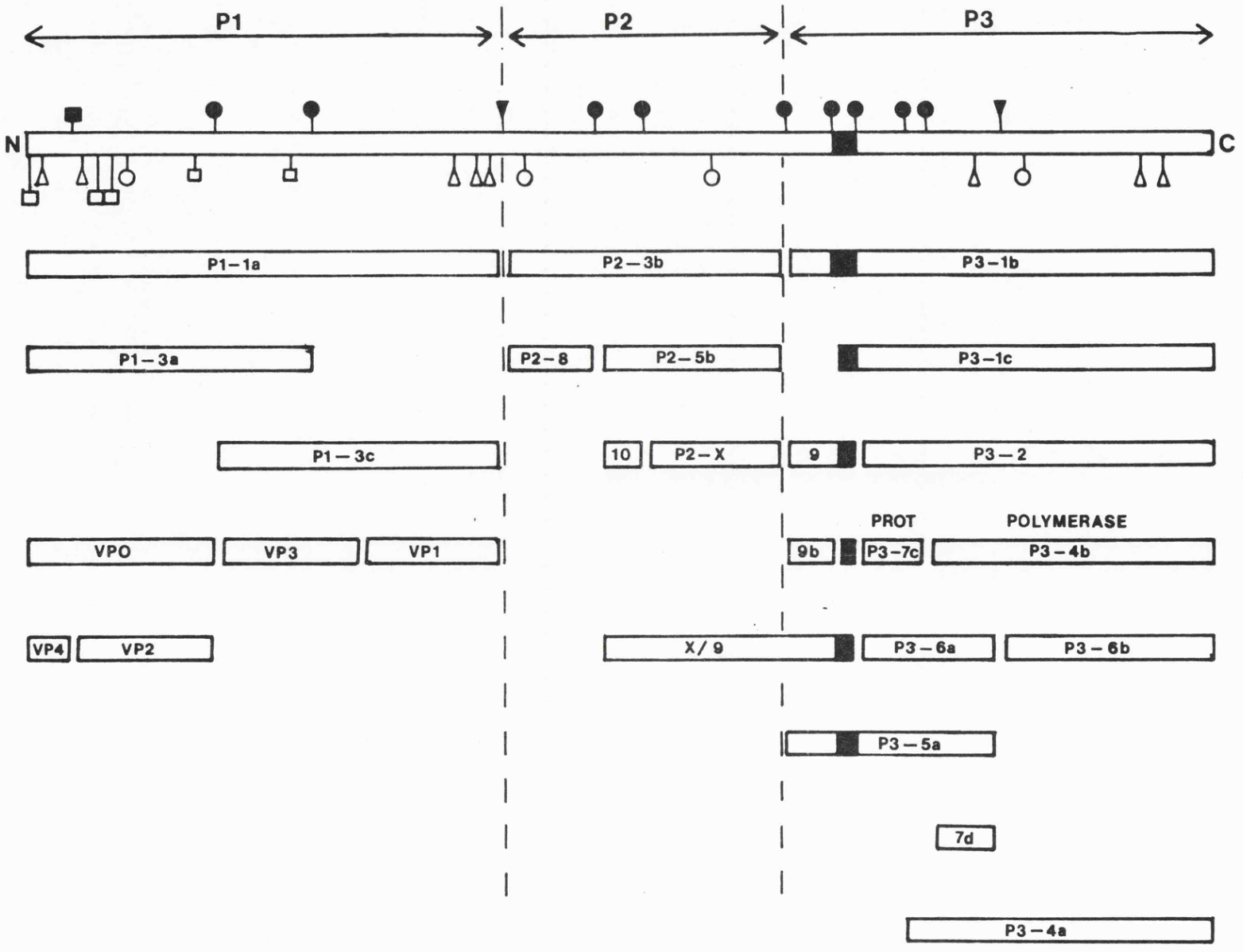

P1
P2
P3
N
C
P1–1a
P2–3b
P3–1b
P1–3a
P2–8
P2–5b
P3–1c
P1–3c
10
P2–X
9
P3–2
PROT
POLYMERASE
VPO
VP3
VP1
9b
P3–7c
P3–4b
VP4
VP2
X/9
P3–6a
P3–6b
P3–5a
7d
P3–4a

Fig. 1. 3.

Protein Processing Map of Poliovirus.

The polyprotein is divided into three regions (P1, P2 and P3). Amino acid pairs (sites) known to be cleaved are indicated by filled symbols, apparently uncleaved sites by open symbols.

Glutamine Glycine (Q.G)

Tyrosine Glycine (Y.G)

Asparagine Serine (N.S)

The numbers given refer to estimated molecular weights (in SDS PAGE). Figure from Pallansch et al., 1984.

The biochemical maps derived for HRV 14, FMDV and EMCV are similar to that of poliovirus, but the proteolytic processing does show some differences. Notably, translation of the FMDV polyprotein occurs at two alternative initiation sites upstream of the capsid encoding region, creating leader peptides (Beck et al., 1983). The EMCV polyprotein also possesses a 5′ leader sequence (Palmenberg et al., 1984).

3) The 3′ non-coding region. The termination codon of the major open reading frame is followed by a short, untranslated region. The length of this region is variable between genera, but is highly conserved in length and sequence between closely related viruses. The 3′ non- coding region of poliovirus type 1 is 72 nucleotides in length and is totally conserved in poliovirus type 3 (Stanway et al., 1983). Two nucleotide differences were observed between poliovirus types 1 and 2 (Toyoda et al., 1984).

Picornaviral genomes possess a 3′ terminal poly(A) tract of 50-100 nucleotides (Fellner, 1979) which is required for infectivity (Spector and Baltimore, 1974). Removal of poly(A) from the viral RNA had no effect on translation, suggesting a role for the poly(A) tract in transcription (Spector et al., 1975).

Unlike eukaryotic mRNAs, picornavirus genomes (with the exception of EMCV) do not contain the universal polyadenylation signal, 5′ AAUAAA 3′, at the 3′ terminus. The observation that the -ve sense strand of the replicative intermediate has a 5′ poly(U) tract suggests that the poly(A) tract of the +ve sense strand is template coded

1.4. Antigenicity and Neutralisation of Picornaviruses.

The human polioviruses are currently classified into three stable serotypes (types 1, 2 and 3) on the basis of their neutralisation reactions with immune sera. Each serotype is defined by the inability of antisera raised against the other two serotypes to completely neutralise infectivity. Serologically distinct strains within the serotypes have been identified using specific antisera prepared by cross absorption with heterologous strains (van Wezel and Hazendonk, 1979) or, more recently, using monoclonal antibodies (Ferguson et al., 1982).

The antigenicity of foot and mouth disease viruses and the human rhinoviruses have also been studied in great detail. There are seven serotypes of FMDV (types A, O, C, SAT1, SAT2, SAT3 and Asia1) and over 115 serotypes of human rhinovirus. Antigenic variation is also more marked and clinically significant in the foot and mouth disease viruses than the polioviruses.

1.4.1. D and C antigenicity.

Supernatant media harvested from poliovirus infected cells contain two antigenically distinct populations of particles (Mayer et al., 1957; Minor et al., 1980). Intact infectious virions sedimenting at 155S in sucrose gradients show D antigenicity. Empty capsids or procapsids, sedimenting at 80S, express an alternate set of surface determinants designated C antigen.

Following treatment with heat (56°C), U.V. irradiation or chemical agents infectious virions lose their D antigenicity and acquire C antigenicity. The antigenic shift following mild denaturation can be considered a general property of picornavirions (Rueckert, 1976). It is accompanied by loss of ability to adsorb to host cells, a decrease in the

sedimentation rate and elution of VP4 from the particle. The internal location of VP4 in intact virions (Hogle et al., 1985; Rossmann et al., 1985) suggests that loss of VP4 involves a profound conformational change. The observation that surface labelling of denatured virions labels VP2 and VP3 rather than VP1 supports this conclusion (Longberg-Holm and Butterworth, 1976). A similar conformational shift is observed during natural infection. Particles with C antigenic character which lack infectivity are eluted from cells following adsorption at physiological temperature (Joclik and Darnell, 1961; Longberg-Holm et al., 1975). These particles (A particles) also lack VP4.

The antigenic differences between infectious poliovirions and empty capsids have been investigated using monoclonal antibodies. Ferguson et al. (1984) produced a large number of hybridoma cell lines secreting monoclonal antibodies raised against infectious particles of several poliovirus type 3 strains. These were assayed for virus neutralising activity and their ability to bind purified D and C antigen in an autoradiographic single radial diffusion assay. Monoclonal antibodies which reacted specifically with D antigen or C antigen were identified. There was also evidence for common antigenic determinants between infectious virions and empty capsids.

Only a proportion of the antibodies which bound purified virus particles were able to neutralise infectivity. Eight out of nine antibodies which reacted with both C and D antigen neutralised a wide range of type 3 strains. Twenty-five antibodies bound exclusively to D antigen and nineteen of these had neutralising activity, although often at low titre. The D specific antibodies generally reacted with only a narrow range of type three strains.

It has generally been assumed that antibodies raised against C antigen are non-neutralising. It was therefore surprising that one of twenty-five C specific antibodies showed neutralising activity. Detailed

studies on this antibody suggested that the mechanism of neutralisation was unusual in that it did not prevent the initial infection of cells but acted at a late stage in infection, possibly inhibiting the release of infectious virus from cells. Blondel et al. (1983) isolated a neutralising antibody raised against poliovirus type 1 C antigen. This antibody bound infectious virions and denatured particles in an immune precipitation assay. Again it was unusual in that it also bound purified VP1.

1.4.2. Characteristics of the Antigenic Determinants of Picornaviruses

A number of approaches have been used to identify the antigenic determinants involved in the neutralisation of picornaviruses. Studies involving immunisation with purified capsid proteins have, in general, been of limited use because the conformation of the individual capsid proteins and their relationship to each other within the virion are major factors in the antigenicity of picornaviruses (Thorpe et al., 1982; Ferguson et al., 1984). Nonetheless, early studies with FMDV revealed that VP1 purified from infectious virions was sufficiently immunogenic to induce a significant neutralising antibody response in animals, whereas the other three viral polypeptides had no such activity (Laporte et al., 1973; Bachrach et al., 1975). Similarly immunisation of cattle with VP1 expressed from cloned cDNA as a fusion protein in *E.coli* could provide protective immunity (Kleid et al., 1981).

In contrast with the results obtained with foot and mouth disease virus, the induction of neutralising antibody has been reported for each of the three major capsid proteins (VP1, VP2 and VP3) of poliovirus type 1 (Chow and Baltimore, 1982; Wiegers and Dernick, 1983; Dernick et al., 1983; van der Marel et al, 1983). However the titre of neutralising antibody obtained in each case was very low. Attempts to further define the neutralising antigens of picornavirions have involved more sophisticated techniques.

Strohmaier et al. (1982) prepared specific fragments from the purified VP1 of foot and mouth disease virus O1K using chemical or enzymic cleavage. The fragments were identified by sequencing the termini and tested for immunogenicity in mice. The results indicated that at least two antigenic sites were located in the carboxy terminal third of VP1, between residues 146-154 and 201-213.

The availabilty of the complete nucleotide sequences of representative strains of each of the three serotypes of poliovirus and several serotypes and subtypes of FMDV has allowed the identification of potential antigenic sites. Regions of high local hydrophilicity can be predicted from primary sequence by the method of Hopp and Woods (1981). It has been argued that such regions are more likely to be exposed at the virion surface and may therefore be available for interaction with the immune system. Alternatively specific sites have been implicated by evidence of high sequence variation between serotypes or subtypes on the grounds that some of the differences observed could be the result of immune selection. Chemically synthesised peptides corresponding to regions of potential immunogenicity have been tested for the ability to induce neutralising antibody, following linkage to a suitable carrier protein.

For FMDV this approach has been quite successful. Synthetic peptides spanning amino acid residues 141-160, 200-213 (Bittle et al, 1982) and 144-159 (Pfaff et al., 1982) of VP1 each stimulated a high titre neutralising antibody response. These sites had previously been implicated by the more empirical approach of Strohmaier et al. (1982). Peptides corresponding to other theoretically promising regions did not induce a measurable level of neutralising antibody.

The same approach has been used to identify antigenic sites of poliovirus type 1. Several peptides have been shown to induce neutralising antibodies in animals (Emini et al., 1983c; Emini et al., 1984; Jameson et al., 1985; Chow et al., 1985). These map to six separate regions of VP1.

Table 1.2.

Antigenic Sites of Poliovirus Identified using Synthetic Peptides.

A. Peptides Inducing as Neutralising Antibody Response

Site	Peptide	Reference	Location in intact virion
1	VP1 61-80	Chow et al., 1985	Deeply buried
2	VP1 86-103	Chow et al., 1985	Exposed
	VP1 91-109	Chow et al., 1985	
	VP1 100-109	Chow et al., 1985	
	VP1 93-103	Chow et al., 1985	
3	VP1 141-147	Jameson et al., 1985	Exposed
4	VP1 161-173	Emini et al., 1984	Partially exposed
	VP1 165-172	Jameson et al., 1985	
	VP1 161-181	Chow et al., 1985	
5.	VP1 182-201	Chow et al., 1985	Deeply buried
6	VP1 222-241	Chow et al., 1985	Exposed

B Peptides Inducing a `Priming´ Response

Site	Peptide	Reference	Location in intact virion
7	VP1 11-17	Emini et al., 1983c	Deeply buried
8	VP2 162-173	Emini et al., 1984	Exposed

An additional site of VP1 and a site of VP2 have been implicated by the ability of peptides to `prime´ animals for a high titre neutralising antibody response following subsequent challenge with a sub-immunising dose of infectious virions (Emini et al., 1983c; Emini et al., 1984; Jameson et al., 1985). The results of these studies are summarized in table 1.2.

It should be noted that the methods used in these studies were more sensitive than standard clinical assays (Domok and Magrath, 1979). The synthetic peptides like the purified structural proteins are very poorly immunogenic. This approach has failed to identify any sequence which is capable of inducing high titres of neutralising antibody against poliovirus. Interpretation of these results is further complicated by the observation that several of the proposed antigenic sites (indicated in table 1.2.) are deeply buried in the interior of the virion (Hogle et al., 1985; Rossman et al., 1985). This suggests that peptides can assume conformations in solution which resemble completely unrelated antigenic sites or that antibodies made against free peptides can react during breathing of the particle.

The observation that viral variants resistant to specific monoclonal antibodies can be obtained at high frequency has allowed the development of an alternative strategy for the definition of antigenic sites of poliovirus. Mutants resistant to specific antibodies were obtained at a frequency of -2.9 to -5.0 log10 PFU per wild type PFU (Minor et al., 1983).

213 mutants derived from P3/Leon/37, the virulent progenitor of the poliovirus type 3 vaccine strain, could be assigned to 16 groups on the basis of the pattern of their neutralisation reactions with a panel of 12 neutralising monoclonal antibodies (Minor et al., 1983; Evans et al., 1983; Minor et al., 1985). Each mutant was resistant to at least three and often eight or more antibodies other than the one used to select it and groups of mutants existed which were resistant to any given pair of antibodies. These

results suggested that the 12 monoclonal antibodies recognised functionally distinct epitopes of a single antigenic site.

The T1 oligonucleotide fingerprints of viruses from three of the mutant groups showed one spot to be altered. The sequence of this oligonucleotide was determined and assigned to a position within the viral RNA encoding VP1 by comparison with the previously determined sequence of a type 3 poliovirus (Stanway et al., 1983). Representative strains from each mutant group were sequenced in this region, directly from the viral RNA by primer extension (Evans et al., 1983; Minor et al., 1985). Single amino acid substitutions were observed for viruses from each mutant group in the sequence 91 to 100 residues from the N-terminus of VP1.

Subsequently it was shown that 129 mutants derived from the Sabin type 3 vaccine strain, P3/Leon/12a1b, could be assigned to 15 mutant groups (Minor et al., 1985). Viruses from 12 of the 15 groups contained single amino acid substitutions located between residues 89 to 100 of VP1. In all cases where the same amino acid substitutions were observed in mutants derived from P3/Leon/37 and mutants derived from P3/Leon/12a1b identical patterns of resistance were obtained. This provided convincing evidence that the mutations observed conferred resistance to neutralisation.

The region 89 to 100 residues from the N-terminus of VP1 was designated antigenic site 1. Evidence for a second independent antigenic site was obtained using a highly strain specific monoclonal antibody. Monoclonal antibody 138 neutralised P3/Leon/12a1b but not P3/Leon/37 or other strains (Ferguson et al., 1982). It was shown that mutants of P3/Leon/12a1b resistant to members of the panel of 12 monoclonal antibodies were fully sensitive to 138. Conversely mutants resistant to 138 were sensitive to all monoclonal antibodies directed against site 1. The site recognised by 138 was assigned to a position towards the C-terminus of VP1 using recombinant polioviruses constructed via cDNA for this thesis (see Chapter 6) and by sequencing variants resistant to 138. These results

implied that 138 recognised a strain specific site, designated site 2, located 285 to 287 residues from the N-terminus of VP1. Of 26 neutralising monoclonal antibodies generated for this study 25 were directed against site 1 and only 1 recognised site 2. Site 1 was therefore considered to be immunodominant.

Emini et al. (1983) identified 7 distinct neutralisation epitopes of the Mahoney strain of poliovirus type 1 using a panel of neutralising monoclonal antibodies. These were assigned to two independent antigenic sites of VP1 on the basis of the binding of the antibodies to synthetic peptides in ELISA (Emini et al., 1983b). These were located between residues 70 to 80 and 93 to 103 from the N-terminus of VP1. Variants resistant to the type 1 monoclonal antibodies were sequenced through the capsid encoding region (Diamond et al., 1985). Single amino acid substitutions were observed for each variant but in no case did the mutations map within the proposed antigenic site. These authors concluded that mutations conferring resistance need not occur within the site involved in the binding of neutralising antibody. It was argued that mutations distant from the antigenic site could affect its capacity to bind neutralising antibody by inducing a conformational change.

The neutralising antigens of poliovirus are to some extent conformational antigens in that they are not entirely determined by primary sequence (Thorpe et al., 1982; Ferguson et al., 1984). Mutations affecting the quarternary organisation of the capsid proteins could indeed alter the presentation of an antigenic site. However such changes would probably have a pleiotropic effect on viral replication. It is therefore unlikely that this would be the most frequent mechanism by which a virus escapes neutralisation. Furthermore it has recently been shown that one of the proposed antigenic sites of VP1 (residues 70 to 75) is deeply buried within the virion and probably in association with the RNA (Hogle et al., 1985; Rossman et al.,1985).

Mutations conferring resistance to the type 1 monoclonal antibodies were observed in VP1 (residues 221-223), in VP2 (residue 270) and VP3 (residues 60 and 71). Each of these sites is exposed on the surface of the virion. The interpretation that these mutational loci represent the sites to which the antibodies bind now seems more likely.

Minor and co-workers have recently extended their work to poliovirus types 1 and 2 (P. D. Minor, personal communication.). Poliovirus type 2 is similar to poliovirus type 3 in that mutations conferring resistance to five out of seven type 2 specific monoclonal antibodies are clustered within the region 96 to 102 residues from the N-terminus of VP1. The results obtained with poliovirus type 1 are in good agreement with those reported by Diamond et al. (1985). By the criteria used for the type 3 mutants, monoclonal antibodies raised against poliovirus type 1 fell into four distinct groups. Subsequent sequencing studies revealed that one group has mutations in VP3 (at residues 58, 59, 60 and 71) and a second group has mutations in VP1 at residues 220 and 222) or in VP2 at residues 169 and 170). The mutations in the remaining groups have not yet been characterized but they are known to be outside VP1. The antigenic structure of poliovirus type 1 is therefore strikingly different to poliovirus type 2 and type 3.

Using an approach analogous to that of Minor et al. (1983), antigenic variants of human rhinovirus type 14 have been isolated and characterised (Sherry and Rueckert, 1984; Scherry et al., 1985). Four major antigenic sites were identified. These were defined by mutations in VP1 (residues 93 and 95), VP3 (residues 72,79 and 78), VP2 (residues 158-162) and VP1 (residues 83, 89, 138 and 139). The first of these sites corresponds to site 1 of poliovirus type 3 and the second site corresponds to the VP3 site of poliovirus type 1.

1.4.3 Neutralisation.

Dulbecco et al. (1956) observed that the kinetics of neutralisation of poliovirus was first order. This result was interpreted in terms of a critical site mechanism in which binding of a single antibody to any one of a number of critical sites is sufficient to inactivate infectivity of the virus particle. It was subsequently shown that the pI (isoelectric pH) of poliovirus is lowered from 7.0 to between 4.0 and 5.5 after denaturation of virus by mild heating or after treatment with neutralising antibody (Mandel 1976, 1978). Mandel (1978) hypothesised that binding of neutralising antibody induced a cooperative transition in the orientation of the capsid subunits, stabilising the virions in a biologicaly inactive conformation.

Recently it was reported that the pI shift could also be induced by neutralising monoclonal antibodies (Emini et al., 1983; Icenogle et al., 1983). First order neutralisation kinetics were observed but an average of four bound antibodies were required to neutralise the virus. Treatment of virion-antibody complexes with papain to generate monovalent F(ab) fragments resulted in reversal of neutralisation. Neutralisation could be restored by treatment of the latter complexes with anti-mouse IgG. These results suggested that bivalent attatchment of antibody is essential for neutralisation.

Individual neutralising monoclonal antibodies which do not conform to this model have been isolated (Icenogle et al., 1983; Emini et al., 1983; Brioen et al 1983). The mechanism of neutralisation by one of these antibodies has been investigated (Brioen et al., 1983). This antibody caused polymerisation of the virions as observed by sucrose gradient centrifugation and electron microscopy. Monomeric virions remaining after neutralisation retained full infectivity whilst the specific infectivities of dimers, trimers and higher polymers decreased in that order.

1.5. The Infectious Cycle.

1.5.1 Viral Penetration.

The initial event in picornaviral infection is the adsorption of virus to specific cellular receptors located at the cell surface. This interaction is mediated by a virion attatchment protein (VAP) occurring in multiple copies on the capsid surface. One VAP binds to a complementary structure on the external surface of the plasma membrane (the cellular receptor). Adsorption is reversible under certain salt and pH conditions. The virus then penetrates the cell membrane and becomes inaccessible to neutralising antibody. Virus may be recovered at this stage by dissociation of the virus-receptor complex with non ionic detergents. Penetration is followed by modification of the virion which leads to eclipse of infectivity and facilitates the release of viral RNA or uncoating. Viral eclipse is defined as an irreversible step which occurs at 37°C and is characterized by loss of ability to recover infectious particles (Fenwick and Cooper, 1962; Holland, 1962). Uncoating of the viral genome is distinguished by attainment of RNase sensitivity of the viral RNA (Joklik and Darnell, 1961) and loss of photosensitivity of virus labelled with acridine dyes (Schaffer and Hackett, 1963).

The successful identification of the components of bacteriophages, adenoviruses and enveloped viruses which are involved in receptor recognition has stimulated investigation of the functional topology of picornaviruses. Any one of the four capsid proteins of picornaviruses could function as the virion attachment protein. Early studies implicated VP4 because this protein was lost during in-vitro conversion of picornavirions to sub-viral particles which were unable to adsorb to susceptible host cells. However it was subsequently shown that VP4 occupies an internal location in the native virus. These studies revealed that VP1 is

prominently expressed on the surface of the virion. Treatment of aphthoviruses with trypsin destroys their ability to attatch to host cells. Since only VP1 is cleaved by this treatment it may be considered to be the attachment protein.

The 3-dimensional structures of human rhinovirus type 14 and the Mahoney strain of poliovirus type 1 have recently been solved to high resolution using X-ray crystallography (Rossman et al., 1985; Hogle et al., 1985). One of the most prominent features of the capsid surface is a 25A deep `moat' or `canyon' which surrounds each of the twelve pentamer vertices. It has been argued that this structure is involved in receptor recognition (Rossman et al., 1985). The proposed receptor binding site may be protected from immune selection because antibody molecules would have difficulty in entering the canyon. A protected sialic acid binding site has been observed in the neuraminidase spike of influenza virus (Colman et al., 1983; Varghese et al., 1983). Viruses which attach to different receptors (see below) would be expected to show variation in the residues lining the canyon. It is noteworthy that the carboxy terminal ends of VP1 and VP3 which line the canyon are among the least conserved regions of picornaviruses.

Concanavalin A, a lectin which is able to bind D-glucose and D-mannose pyranosides, has been shown to interfere with the replication of coxsackievirus B3 (Krah and Crowell, 1985), HRV and poliovirus (Lonberg-Holm, 1975). The tertiary structure of concanavalin A (Argos et al., 1980) is very similar to VP1 of HRV 14 and poliovirus type 1. It has therefore been hypothesised that the lectin competes with these viruses for a specific polysaccharide involved in receptor recognition. The functional sugar binding site of concanavalin A has been identified (Argos et al., 1980). The homologous region of VP1 of HRV 14 corresponds to an antigenic site which lies on the rim of the canyon and encompasses amino acids 89-139 from the N-terminus

Table 1.3.

Classification of Picornaviruses into Receptor Groups

Based on Interference of Viral Attachment

Virus	Interference of attachment of virus immunotypes				
	Polio 1-3	Cox B1-6	Cox A21	HRV-14	HRV-2
Polio 1-3	+	-	-	-	-
Cox B1-6	-	+	-	-	-
Cox A21	-	-	+	+	-
HRV-14[1]	-	-	+	+	-
HRV-2[2]	-	-	-	-	+

1. HRV-14 also inhibits attachment of HRV-3, -5, -15, -39, -41 and -51.
2. HRV-2 also inhibits attachment of HRV-1A and HRV-1B

The importance of cellular receptors as major determinants of susceptibility of cells to picornaviruses was first demonstrated by Holland and co-workers (Holland and McLaren, 1959; Holand et al 1959a,b; Mclaren et al, 19691 Holland and Hoyer, 1962). These studies revealed that cells not normally susceptible to poliovirus could support a single round of infection following transfection with viral RNA whereas infection with intact virus needed the presence of specific receptors. The observation that virus in high titre could saturate the available receptors on a susceptible cell population has allowed classification of picornaviruses into specific receptor groups (Crowell and Landau, 1983). It has been shown that the three serotypes of poliovirus compete for the same receptor. Similarly the six group B coxsackieviruses compete for a single receptor which is distinct from the poliovirus receptor. The human rhinoviruses have been classified into two additional receptor groups (see table 1.3). It is of interest that HRV 14 and coxsackievirus A21 compete for the same receptor (Lonberg-Holm et at, 1976). These two viruses cause identical upper respiratory tract infections and may therefore share a common tissue tropism mediated by the virus receptor interaction.

Virus saturation experiments have shown that the number of receptor sites per cell varies from about $3x10^3$ for poliovirus (Lonberg-Holm and Philipson, 1974) to about 10^5 for coxsackievirus B3 (Crowell, 1966). About 10^4 to 10^6 sites were found for human rhinoviruses (Lonberg-Holm and Korant, 1972). Attempts to characterise picornaviral receptors have involved definition of those enzymic treatments, physical conditions and chemical reagents that inactivate or inhibit the adsorption of virus (Crowell and Landau, 1983). These suggest that the cellular receptors for picornaviruses are integral membrane glycoproteins. Receptors for polioviruses and group B coxsacki viruses are inactivated by trypsin and chymotrypsin respectively. Regeneration of cellular receptors is prevented by treatment

with inhibitors of cellular protein synthesis such as puromycin and actinomycin D (Levitt and Crowell, 1967). Furthermore the capacity of receptors to bind virus is greatly inhibited by conditions that control the ionic charges of proteins. Evidence that the receptors are glycoproteins is provided by the observation that concanavalin A competes with coxsackievirus B3 for receptors on HeLa cells (Krah and Crowell, 1985). The lectin also interferes with the infection of human rhinoviruses and polioviruses (Lonberg-Holm et al., 1975).

Polyclonal antisera which specifically inhibited the adsorption of the group B coxsackieviruses was prepared by immunisation of rabbits with HeLa cells (Axler and Crowell, 1968). Indirect immunofluorescence assays with acetone fixed and unfixed HeLa cells revealed only surface fluorescence indicating that the receptors for the group B coxsackieviruses were confined to the external surface of the cell. Similar results have been reported with monoclonal antibodies directed against the cellular receptors for poliovirus (Minor et al., 1984). The isolation of such highly specific monoclonal antibodies may facilitate the purification of picornavirus receptors by affinity chromatography of solubilised membrane fractions.

The normal cellular functions of picornavirus receptor proteins have not been determined. It is noteworthy that Hep 2c cells survive repeated serial passage in medium containing monoclonal antibodies directed against the poliovirus receptor. Similarly inactivation of cellular receptors by enzyme treatment does not result in cell death. Receptors for poliovirus are encoded on chromosome 19 (Miller at al., 1974). This rules out the possibility that the poliovirus receptor is the major histocompatability antigen as was suggested previously.

Recent studies suggest that the cellular receptor for poliovirus belongs to a class of membrane proteins which is involved in an important and general pathway by which cells take up nutritional and regulatory

molecules from the extracellular fluid. Biologicaly important macromolecules known to be taken up receptor mediated endocytosis include certain polypeptide hormones (such as insulin and epidermal growth factor) and plasma transport proteins (such as low density lipoprotein) (Goldstein et al., 1979; Brown et al., 1983; Helenius et al., 1983). These proteins interact as ligands with specific receptors at the cell surface. Internalisation of the ligand is coupled to receptor binding. The receptor bound proteins enter the cell through a specialised region of the cell membrane known as a coated pit whose principal distinguishing feature is a coating of clathrin on its intracellular surface. Receptors for epidermal growth factor (EGF) are evenly distributed over the entire membrane surface. Binding of EGF to its receptor induces migration of the EGF-receptor complex to the coated pit. Receptors for low density lipoprotein (LDL) are pre-clustered in that they are predominantly located in coated pits. Internalisation of the ligand into the cell results in the formation of coated vesicles. These vesicles loose their clathrin coat and fuse with intracellular vesicles to form endosomes. The internalised ligand may then be delivered to secondary lysosomes, the golgi sytems or directly into the cytoplasm. The release of ligands from endosomes or lysosomes may be dependent on the low pH of these vesicles. Exposure of cells to weak bases such as chloroquine, monesin and NH_4Cl raises the intra-endosomal and intra-lysosomal pH above 6.0. Under these conditions the endocytosed particles are entrapped in endosomes and/or lysosomes (Marsh et al., 1982; Tartakoff, 1983).

Certain viruses including Semliki Forest virus, vesicular stomatitis virus and adenovirus have been shown to enter cells by receptor mediated endocytosis. Transmission electron microscopy of ultra thin sections of poliovirus infected Hep 2c cells revealed that poliovirus also exploits this pathway (Zeichardt et al.,1985). Following adsorption at $0^{\circ}C$ virus particles could be detected evenly distributed at the cell surface.

When the temperature of such infected cells was shifted to 37°C for one minute most poliovirus particles were located in clathrin coated pits. Entry of poliovirus into cells via coated vesicles and endosomes could be observed by increasing the incubation period.

Treatment of cells with chloroquine, NH_4Cl or monesin prior to infection inhibited replication of poliovirus (Zeichardt et al., 1985; Madshus et al., 1984) The protection was fully overcome by exposing the cells to pH 5.5 and lower (Madshus et al., 1984b). The results of these studies indicated that the step dependent on the low pH of the endosomes and/or lysosomes was uncoating of the viral RNA.

One of the most conspicuous features of the virus-cell interaction is perhaps the least understood. Following adsorption at 0°C, most of the virions which become cell associated are eluted as non-infectious particles (A-particles) when the temperature is raised to 37°C. This phenomenon has been observed for polioviruses (Joklik and Darnell, 1961), echoviruses (Rosenworth and Eggers, 1979), coxsackieviruses (Crowell et al., 1971) and rhinoviruses (Noble and Lonberg-Holm, 1973). A particles sediment more slowly than intact virions in sucrose gradients, lack VP4 and are insensitive to RNase despite containing a full complement of RNA. Faliure to detect A particles following adsorption of aphthovirus (Brown et al., 1962; Cavanagh et al., 1978) or cardioviruses (Hall and Rueckert, 1971) may reflect inherent differences in the bonding interactions of the constituent capsid proteins which renders the A particle too unstable to be detected.

It has been suggested that A particles are an intermediate on the pathway leading to uncoating of the viral genome. A particles have lipophilic properties that may facilitate fusion with the endosomal and/or lysosomal membranes (Lonberg-Holm et al, 1976b)

1.5.2 Shut-Off of Host Macromolecular Synthesis.

Poliovirus infection of HeLa cells results in a rapid inhibition of host cell DNA, RNA and protein synthesis (Franklin and Baltimore, 1962). The mechanism of the shut off of host cell protein synthesis has been intensively studied. No effect is observed for the first 30 minutes which is the time required for adsorption, penetration and uncoating. The rate of protein synthesis then declines reaching a minimum at 2-2.5 hours post infection. At this time almost no polysomes are detectable. A second burst of protein synthesis then occurs, which represents the synthesis of viral specific proteins (Summers et al., 1965).

Early studies revealed that shut off of host cell protein synthesis required expression of the viral genome (Penman and Summers, 1965; Borgert et al., 1971) but was independent of replication of viral RNA (Penman and Summers, 1965). During the period of polysome disaggregation, the elongation rates of the fraction of remaining polysomes was normal (Summers and Maizel, 1967). This implied that the cause of the declining rates of protein synthesis was a block at the initiation of translation. Direct measurement of initiation complex formation demonstrated that 80S complexes containing ribosomes, mRNA and met $tRNA_f^{met}$ were not formed in infected cells during the shut off period (Ehrenfeld and Manis, 1979). In vitro studies indicated that the mRNA failed to bind to the 40S subunit suggesting that the inhibited step preceded the 60S junction reaction (Brown and Ehrenfeld 1980).

Cellular mRNA isolated from poliovirus infected cells was structurally intact (Lebowitz and Penman, 1971; Koschel, 1974; Fernandoz-Munoz and Darnell, 1976) and was capable of stimulating protein synthesis in vitro (Kaufman et al., 1976). It was subsequently shown that the ribosomal salt wash from poliovirus infected cells, which contains the protein synthesis initiation factors, was unable to stimulate translation

identified as eIF-4A by immune precipitation and tryptic peptide analysis (Edery et al., 1983). The 24kd subunit could be crosslinked specifically to the 5′ end of capped mRNA, confirming its identity with 24-CBP. Very little is known about the 220kd polypeptide. An antigen with identical electrophoretic mobility was detected in uninfected cell lysates using polyclonal antisera raised against eIF-3 (which contained eIF-4F). This antigen, p220, was not present in infected cells although antigenically related polypeptides of 100kd and 130kd could be detected (Etchinson et al., 1982). These authors suggested that proteolytic cleavage of the 220kd subunit may cause the dissociation and inactivation of eIF-4F in poliovirus infected cells.

The mechanism of shut off of host cell protein synthesis may be distinct for different groups of picornaviruses. Extracts of EMC or mengovirus infected cells were equally active in the translation of both cellular and viral mRNAs (Svitkin et al., 1974; Lawrence and Thatch, 1974). However when saturating amounts of viral RNA and cellular mRNA were added simultaneously to a cell free translation system, the viral RNA was preferentially translated (Lawrence and Thatch, 1974; Abreu and Lucas-Lenard, 1976). This competition could be relieved by adding partially purified preparations of eIF-4B, suggesting that eIF-4B may be in limiting amounts in the translation system such that the viral RNA and the cellular mRNA compete for the available initiation factor (Golini et al., 1976). Measurements of RNA binding to eIF-4B by retention on nitrocellulose filters demonstrated that EMC RNA has a greater affinity for this initiation factor than globin mRNA or other capped mRNAs (Baglioni et al., 1978).

Very little is known about the mechanism of virus induced inhibition of host cell nuclear functions. Inhibition of host cell RNA synthesis was first characterized in mengovirus infected L cells, where cellular RNA synthesis decreased to less than 10% of the initial level by

one hour after infection (Baltimore and Franklin, 1962). This inhibition was paralleled by a decrease in the activity of DNA dependent RNA polymerase in isolated nuclei (Baltimore and Franklin, 1962) and in a crude DNA-protein complex (Holland, 1962). By measuring α-amanatin sensitive transcription in nuclei isolated from EMC and mengovirus infected cells it was shown that transcription by RNA polymerase II was rapidly inhibited (Aprilelli and Penhoet, 1974; Schwartz et al., 1974). Polymerase I and III activity was also inhibited but at a slower rate

The electrophoretic mobility of polymerase II subunits purified from infected and uninfected cells were identical. Also when the RNA polymerases from infected cells were solubilisied and fractionated, all three RNA polymerase activities could be detected as assayed under conditions that required only non specific initiation (Apriletti and Penhoet, 1974, 1978; Schwartz et al 1974). These results suggested that the cause of the shut off host cell RNA synthesis was a block in the initiation of transcription. Using a cell free transcription assay that gave specific initiation on exogenously added DNA, Crawford et al. (1981) demonstrated that at least one factor needed for transcription was deficient in extracts from poliovirus infected cells.

1.5.3. Translation and Post-Translational Processing.

Between 2-4 hours post infection the levels of viral RNA and virally encoded proteins increase and accounts for a large proportion (>40%) of the host cell synthesis (Baltimore, 1969). It is thought that a single initiation event occurs, at position N741 in poliovirus type 1. Nucleotide sequencing studies revealed that there were several initiation codons upstream of the translational start (see section 1.3.1.).Thisisat variance with the favoured model for initiation of translation whereby the 40S ribosomal subunit binds at the 5′ terminus and migrates in the 3′

direction until it reaches the first AUG. The 60S ribosomal subunit then binds to form the 80S initiation complex (Kozak, 1978). Recent work suggests that the sequences flanking the initiation codon are important for its selection by the 40S ribosomal subunit (Kozak, 1981, 1984).

From a comparison of the 5′ non-coding regions of over 200 cellular and 50 viral mRNAs a consensus sequence CCACCAUGGG was proposed for eukaryotic initiation sites. The most conserved features were a purine (usually adenosine) at position -3 and a guanosine at position +4. The prediction that adenosine at -3 is required for efficient initiation was confirmed by introducing point mutations in a cloned preproinsulin gene and monitoring its expression in vivo (Kozak, 1984b).

Analysis of the 5′ non-coding sequences of poliovirus types 1, 2 and 3 revealed a consensus sequence in poliovirus type 3 at nucleotide positions 474-482 (UAACCAUGG). However this region is not conserved in poliovirus types 1 and 2. Liu et al. (1984) demonstrated that insertion of an upstream AUG in a favourable sequence context could severely depress the expression of a cloned gene for hepatitis B surface antigen. However this effect was suppressed if, as is the case of poliovirus type 3, the upstream AUG was closely followed by an in phase termination codon.

The nascent polyprotein is proteolytically cleaved during translation of poliovirus RNA (Baltimore 1969; Rueckert, 1976). Partial amino acid sequencing of poliovirus infected cell proteins has enabled a map of the cleavage sites used in proteolytic prosessing to be constructed (Kitamura et al., 1981; Larsen et al., 1982; Semler et al., 1981a,b; Semler et al., 1982). Twenty-seven virus specific proteins, representing 95% of the total methionine incorporated into poliovirus infected cells, can be accounted for by 12 cleavage sites. Cleavages occur at 9 glutamine-glycine (Q.G) pairs, 2 tyrosine-glycine (Y.G) pairs and one asparagine-serine (N.S) pair.

In EMC virus a proteolytic activity was associated with a small polypeptide, p22 (Palmenberg et al., 1979). Hanecak et al. (1982) demonstrated that monospecific antisera raised against the corresponding poliovirus protein, P3-7c, inhibited cleavage at Q.G sites in a poliovirus cell-free translation system. The tyrosine glycine (Y.G) cleavages are not inhibited by antibodies directed against P3-7c and are therefore carried out by an alternative proteolytic activity. Examination of the poliovirus nucleotide sequence revealed that some Q.G pairs do not form substrates for P3-7c so additional factors must be involved in the selection of cleavage sites by the viral protease. Similarly only a small proportion of Y.G pairs are utilised in proteolytic processing. Cleavage at the N.S dipeptide generates VP2 and VP4 from the precursor polypeptide VPO. The cleavage represents the final step in morphogenesis and appears to coincide with addition of the viral RNA to the procapsid to form the mature virion. Cleavage at this site is probably autocatalytic (see section 1.5.5).

The processing pathways of rhinoviruses, cardioviruses and aphthoviruses are very similar to those of enteroviruses. However, some differences in the specificities of the viral proteases have been observed. Cleavages attributed to the viral specific proteases of EMCV and HRV 14 occur predominantly at Q.G dipeptides. However the proteases encoded by these viruses appear to be less stringent than that of poliovirus because additional dipeptides are recognised. These are Q.T and Q.A for HRV 14 (Stanway et al., 1984) and Q.S for EMCV (Palmenberg et al., 1984). The FMDV protease recognises a wider range of sequences, with no apparent preference for any one dipeptide (Carroll et al., 1984).

1.5.4. Viral Transcription.

Synthesis of picornavirus RNA occurs in the cytoplasm and does not require the nucleus or DNA dependent RNA polymerase activity (Follett et al., 1975). The rate of viral RNA synthesis is exponential early in infection but becomes linear at the end of the eclipse phase when the viral particles first appear. Poliovirus infected cells contain a number of virus specific RNA molecules.

a) Single-stranded +ve sense RNA. The bulk of the RNA synthesised in infected cells has the same base composition as genomic RNA (Baltimore and Franklin, 1962) and is intrinsically infectious (Alexander at al., 1958). These molecules may function as a messenger for virus directed protein synthesis, as a template for the replication of viral RNA or as a substrate for packaging in the formation of progeny virions. Poliovirus mRNA differs from genomic RNA only in the absence of the covalently bound VPg at the 5′ terminus. This is believed to be the result of post transcriptional cleavage of VPg from newly made RNA by a cellular enzyme (Ambros et al., 1978).

b) Single stranded -ve sense RNA. Replication of viral RNA is a two step transcriptional process. Genomic RNA serves as a template for the synthesis of a complementary (-ve sense) strand which has a 5′ poly(U) tract covalently attached to VPg. RNA strands of the original polarity (+ve sense) are then synthesised using the -ve sense strand as a template. Single stranded negative sense RNA forms only a minor fraction of the RNA found in poliovirus infected cells, indicating that virus directed transcription is strongly assymetric. The mechanism by which -ve sense RNA is preferentialy transcribed is not known.

c) Double stranded RNA. A small fraction of virus induced RNA is in a double stranded form. This RNA consists of an intact +ve sense strand hydrogen bonded to a full length -ve sense strand. Like viral RNA, double

stranded RNA is infectious, but is now generally believed to have no direct role in viral replication.

d) Replicative Intermediate (RI) RNA. RNase treatment of RI reveals that the stucture is partially single stranded and partially double stranded RNA. The structure is composed of one full length -ve sense strand associated with nascent +ve sense strands. Pulse chase experiments have shown that between 5.5 and 6.5 nascent strands are present per -ve strand template (Baltimore et al., 1969; Nomoto et al., 1977b). VPg is covalently linked to the 5′ ends of the nascent +ve strands.

The absence of an RNA dependent RNA polymerase activity in host cells implies that the activity is viral specific. A soluble template dependent RNA polymerase (Replicase) has been purified from cytoplasmic extracts of infected cells (Dasgupta et al., 1979; Flanegan and van-Dyke, 1979). The replicase co-purified with an activity which could transcribe poly(A) in the presence of an oligo(U) primer (Flanegan and Baltimore, 1977). Further purification resulted in the loss of the replicase activity although the oligo(U) dependant poly(U) polymerase activity was retained. The highly purified preparations contained a single virus specific protein designated P3-4b or p63, together with traces of other proteins (Tuschall et al., 1982; van-Dyke and Flanegan, 1980). Antibodies raised against a synthetic peptide corresponding to the carboxy terminal region of P3-4b were shown to inhibit the replicase and poly(U) polymerase activities in vitro (Baron and Baltimore, 1982).

Purified P3-4b could synthesise full length copies of poliovirus RNA and various other polyadenylated RNAs, but only when oligo(U) was added to the reaction (Tuschall et al., 1982; van-Dyke et al., 1983; Baron and Baltimore, 1982). Thus unlike many RNA polymerases, the poliovirus RNA polymerase is a primer dependent enzyme. The requirement for a primer could be eliminated by adding a protein contained in the high salt wash of host cell ribosomes (Dasagupta et al., 1980). The `host factor′ was identified

as a cytoplasmic protein of molecular weight 67kd which could be retained on a column of P3-4b, indicating a physical relationship with the enzyme. Partially purified P3-4b is active on polioviral RNA without the requirement for oligo(U) primer or host factor. This activity is inhibited by anti-host factor antibody, which implies that partially purified forms of P3-4b contain sufficient amounts of host factor to mediate the initaiation of RNA synthesis (Dasgupta et al, 1982).

As discussed previously, the RNA genome of poliovirus is covalently linked at its 5′ end to a small, virus specific protein (VPg). VPg is attached to the 5′ ends of newly synthesised +ve and -ve strands (Nomoto et al., 1977; Petterson et al., 1978) and to the nascent +ve strands of the replicative intermediate (Flanegan et al., 1977; Nomoto et al., 1977; Petterson et al., 1978). Based on these findings, it was proposed that VPg may act as a primer for the initiation of RNA synthesis. It was subsequently shown that anti-VPg antibody could specificaly inhibit host factor stimulated transcription of poliovirus RNA by P3-4b, whereas the oligo(U) primed copying of viral RNA was not affected by the antibody (Baron and Baltimore, 1982; Morrow and Dasgupta, 1983). These antibodies detected no free VPg in infected cells but specifically immunoprecipitated three larger polypeptides with VPg determinants. (Baron and Baltimore, 1982; Semler et al., 1982; Takegami et al,1982). The smallest of these (P3-9) is an abundant product of proteolytic processing. P3-9 is a membrane bound protein, a property consistent with a role as the precursor of VPg in the membrane associated RNA replication complex. Tagegami et al. (1983) demonstrated that a crude membrane fraction isolated from poliovirus infected cells could synthesise a nucleotidyl protein identified as VPg-pU-pU. The same species has been isolated from poliovirus infected cells (Crawford and Baltimore, 1983).

A model has been proposed in which the initiation of RNA synthesis is preceded by the linking of pU to the tyrosine residue within the hydrophilic carboxy terminal region of P3-9. This modification may lead to a conformational change which activates the Q.G cleavage site such that VPg is cleaved from P3-9 by the viral protease, P3-7c. The newly formed VPg-pU or VPg-pU-pU could now function as a primer for the primer dependent RNA polymerase, P3-4b. This is an attractive model because the structure at the 5′ end of both the +ve and the -ve strand is VPg-pU-pU. The correct 5′ end would therefore be generated on each strand without need for further processing.

1.5.5. Morphogenesis.

The early/late control of expression of virus specific proteins exhibited in the majority of viral systems is not a feature of the picornavirus infectious cycle. The mode of translation were all proteins are derived from a single polyprotein precludes temporal control of the synthesis of the different viral proteins. However, some degree of control may occur at the level of post-translational processing. Butterworth and Korant (1972) studied the kinetics of the synthesis and cleavage of EMCV infected cell proteins. The half-lives of individual precursor polypeptides differed from one another suggesting that the accessibility of cleavage sites to the protease(s) affected the rates of processing of individual precursor molecules. The observation that some of the Q.G dipeptides and most of the Y.G dipeptides in the polyprotein are not recognised as cleavage sites supports this conclusion. The kinetics of the interactions between virus components in the infected cell may also provide control mechanisms (Baltimore, 1969)

The assembly of virus particles is thought to proceed via the pathway outlined in Fig. 1.4. Cleavage of the capsid precursor polypeptide

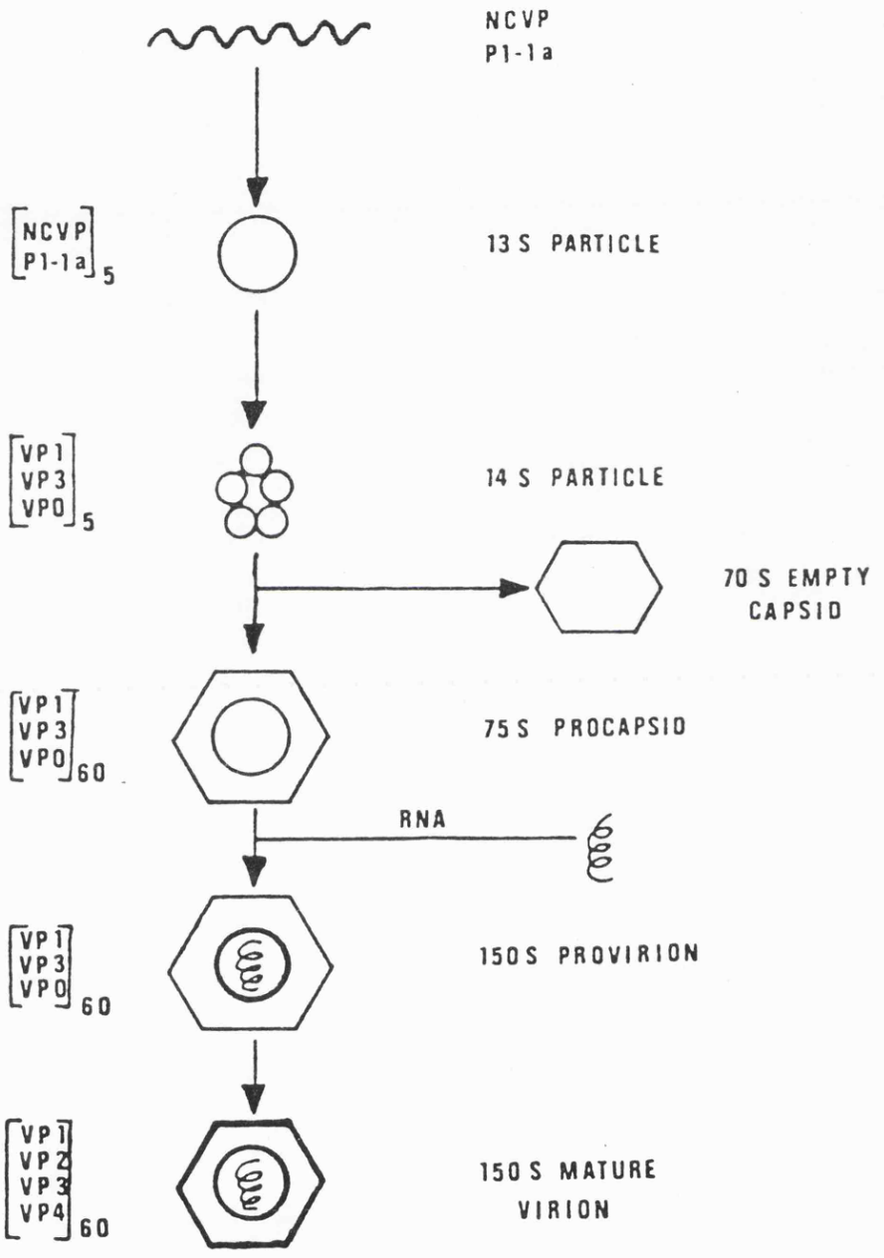
(P1-1a) from the nascent polyprotein occurs before translation is completed. This cleavage occurs at a Y.G dipeptide and is catalysed by an activity distinct from the virus specific protease, P3-7c. P1-1a may undergo pentamerization to form a 13S particle (McGreggor et al., 1975,1977). Each P1-1a molecule is then cleaved by the viral protease, forming a 14S subviral particle composed of five copies each of VP0, VP1 and VP3. Alternatively individual P1-1a molecules may be cleaved to form protomers (VP0-VP1-VP3), which then aggregate to form the 14S particle. In the infected cell the 14S particles polymerise into an empty capsid species called the procapsid (Philips et al., 1968). The final step of morphogenesis is the cleavage of VP0 into VP2 and VP4 which is thought to occur simultaneously with encapsidation of the viral RNA.

In the presence of cytoplasmic extracts of infected cells, isolated 14S particles form empty capsid like structures in vitro (Philips et al, 1968; Philips, 1969). It was later shown that 14S particles could self-assemble in the absence of exogenous factors. Unlike extract-mediated assembly, the latter reaction was highly dependent upon the concentration of 14S particles. Self assembled empty capsids can be differentiated from extract assembled empty capsids and procapsids formed in infected cells by their isoelectric point, trypsin sensitivity, antigenicity and sedimentation coefficient (Putnak and Philips, 1982). These studies suggested that correct assembly of 14S subunits into procapsids is a process catalysed by a factor present in infected cells. The nature of the assembly promoting or morphopoietic factor has not been determined.

High resolution X-ray crystallography of crystallised poliovirus and human rhinovirus has lead to a greater understanding of the interactions between the constituent capsid proteins of picornavirions (Hogle et al., 1985; Rossman et al., 1985). The extensive interactions between 5-fold related protomers is consistent with the proposal that the 14S subviral particle isolated from poliovirus infected cells represents an intermediate

Fig 1. 4.

Morphogenesis of Poliovirus



in the morphogenic pathway. The N-termini of five subunits of VP3 intertwine about the 5-fold axis to form a five-stranded tube of extremely twisted, anti-parallel β-sheet. This structure may direct the formation of the 14S pentamer and should contribute to its stability once formed.

The N and C termini generated by processing of P1-1a to form VP0, VP1 and VP3 are located on opposite surfaces of the pentamer. The separation of these termini and the extensive interactions of the N-terminal strands of VP3 suggests that cleavage of P1-1a occurs prior to pentamer formation.

Thus assembly of picornaviruses proceeds from protomers of VP1, VP3 and VP0, via 14S pentamers of five protomers to mature virions. The final stage involves inclusion of the genomic RNA into procapsids with simultaneous cleavage of VP0 into VP2 and VP4. The proximity of Ser 10 in VP2 with the carboxy terminal end of VP4 suggests that cleavage of VP0 is autocatalytic (Rossman te al., 1985). Nucleotide bases of RNA could act as proton acceptors in the autocatalysis. Thus insertion of RNA into the empty capsid could trigger the cleavage of VP0.

1.6. The Genetics of Picornaviruses.

1.6.1 Recombination.

The high level of recombination exhibited by RNA viruses with segmented genomes is undoubtedly the result of independent re-assortment of segments in doubly infected cells. Picornaviruses appear to be unique amongst the RNA viruses in that their replication strategy allows the exchange of genetic information between RNA molecules. Recombination was first suggested for poliovirus when it was shown that infection of cells with a mixture of inhibitor sensitive mutants of poliovirus resulted in an enhanced yield of resistant progeny which were genetically stable (Hirst, 1962, Ledinko, 1963).

Early attempts to produce a genetic map of poliovirus were hampered by pleiotropism or covariation, multiple mutation events, high spontaneous reversion frequencies and `leaky' mutations where some of the wild-type character persisted under restrictive conditions. Despite these problems, linear genetic maps were eventually obtained for both poliovirus (Cooper, 1969, 1977) and FMDV (Lake et al., 1975; McCahon et al., 1977) in which the recombination frequencies were approximately additive. The observation that mutations could be correlated with changes in electrophoretic mobility of infected cell proteins allowed the alignment of the genetic and biochemical maps of FMDV. Genetic loci within the left-hand half, the middle and the right-hand half of the genetic map appeared to correlate with respective physical locations near the 5′ end (King and Newman, 1980; King et al., 1980), middle (Saunders and King, 1980) and 3′ end of the genome (Lowe et al., 1981).

Conclusive evidence of recombination in picornaviruses was obtained by investigating the inheritance of unselected, biochemical markers. Recombination between different sub-type strains of FMDV was

demonstrated by electrofocusing virus induced proteins and RNase T1 fingerprinting of genomic RNAs (King et al., 1982). The inheritance of T1 oligonucleotides and viral polypeptides by putative recombinants indicated that their genomes were generated by a single cross-over between the parental genomes. Using the same techniques, recombination was subsequently shown to occur between poliovirus types 1 and 3 (Tolskaya et al., 1983; Agol et al., 1984).

Although there is no evidence for inter-molecular recombination in RNA viruses other than picornaviruses, several examples of intra-molecular rearrangements of RNA molecules have been well-characterised. These include specific rearrangements such as splicing of nuclear RNAs and the apparently non-specific result in sub-genomic deletion mutants of RNA viruses designated defective interfering (DI) particles. Splicing is known to involve breakage and reunion of RNA molecules catalysed by nuclear endonuclease and ligase activities. Lazzarini et al. (1981) reviewed the structure of DI particle genomes and concluded that these particles were probably generated by aberrant replication of viral RNA. In this model the RNA polymerase jumps from one region of the template to another without terminating replication.

In theory, either mechanism could be involved in genetic recombination. Mapping of the cross over sites in a large number of FMDV recombinants by electrofocussing of virus induced proteins and RNase T1 fingerprinting, revealed that crossovers occurred in 12 distinct regions of the genome (A. King, personal communication). These results imply that recombination is a general rather than a site specific phenomenom. This tends to rule out an involvement of nuclear splicing functions in picornaviral recombination, because these activities are known to be sequence specific.

1.6.2. Mutation Frequency of RNA Genome Replication.

Errors in the replication of DNA chromosomes vary considerably, but they average as low as one false nucleotide incorporated per 10^{-8} to 10^{-11} nucleotides polymerised (Fowler et al., 1974; Kunkel and Loeb, 1980; Meselson et al., 1981). This is in part due to the proof-reading activities of DNA polymerases which excise mis-matched bases and use the information encoded in the methylated template strand to re-copy the excised region (Kornberg, 1980). Post-replicative DNA repair mechanisms also contribute to the stability of DNA genomes. No error correcting mechanisms have yet been found associated with enzymes that synthesise RNA or use RNA as a template. As a result, synthesis of RNA is an intrinsically error-prone process with estimated mutation frequencies of between 10^{-3} and 10^{-4} (Eigan, 1981).

In a definitive study of the bacteriophage, Qβ, Domingo at al. (1978) demonstrated that 15% of clones plaque-purified from a multiply passaged population showed T1 fingerprints which deviated from that of the RNA of the total population. The differences could be attributed to one or, less frequently, two or more nucleotide transitions. Since only 10% of the RNA sequence could be analysed by this technique, the results suggested that each viable phage genome differs from the 'average' sequence of the total population by one or two mutations.

Several deviant clones were tested by growth competition against the uncloned population. The proportion of variant phage, determined by finger-print analysis, was reduced to about 5% of the original value following twenty passages at high multiplicity. It was proposed that populations of Qβ achieve a state of dynamic equilibrium in which wild-type virus predominates. Viable mutants arise with high frequency on the one hand and are strongly selected against on the other.

High spontaneous mutation frequencies have been observed for the RNA viruses of plants and animals. For example, temperature sensitive (ts)

mutants of NDV could be isolated from clonal pools with a frequency of 2% (Tippis and Batt, 1976). Similarly, Flammond (1970) estimated the spontaneous mutation frequency of ts mutants of VSV Indiana to be 2.3%. These high frequencies may be a refelection of the ability of ts mutants to occur in most, if not all viral genes. An independent estimate of RNA genome mutation can be obtained by using monoclonal antibodies to select for antigenic variants. The advantage of this technique is that monoclonal antibodies monitor nucleotide changes at defined locations in small sections of the genome. Portner et al. (1980) compared the rates of mutation to monoclonal antibody resistance for three unrelated RNA viruses, namely, influenza, VSV and Sendai. The mutation frequency, calculated by dividing the titre of the virus escaping neutralisation by the titre before neutralisation, was the same for all three viruses, that is about $10^{-4.5}$. Monoclonal antibodies have also been used to determine a major antigenic site for poliovirus neutralisation (Minor et al., 1983). These studies confirm that the average frequency with which resistant mutants appear under monoclonal antibody selection is 10-4

1.7. The Problem of Poliomyelitis.

1.7.1. Historical Aspects.

The human poliovirus was identified as the causative agent of poliomyelitis by Lansteiner and Popper in 1909. Over the next forty years knowledge of poliomyelitis progressed alongside developments in other fields, particularly the new science of immunology. It was shown that all isolates of poliovirus could be classified into three antigenic types (see Melnick, 1982). Virus of one type was not effectively neutralised by antibody raised against the other two. To be complete, immunological protection had to be gained against not one but three types of virus.

During the 1930s and 1940s, work tended to concentrate on treatment of the disease. The work of Elizabeth Kenny demonstated the benefits of physiotherapy for those suffering poliomyelitis paralysis (see Paul, 1971). The cerebral cortex is generally unaffected by the disease process (Bodian, 1949). It is therefore possible to relearn patterns of motor activity which were formally regulated by damaged brainstem functions. Physiotherapy may assist in this process and counteract the effects of muscular wasting. The development of the iron lung enabled poliomyelitis victims who would have died of suffocation, following paralysis of respiratory muscles, to survive.

Although very important, such measures could only reduce the severity of the damage caused by the disease. It became obvious that the only route towards successful control of poliomyelitis had to be based on prophylactic vaccination.

Developments in the field of tissue culture and growth of polioviruses in-vitro facilitated the development of two effective anti-poliomyelitis vaccines. The first of these was a formalin inactivated vaccine (IPV) developed by Jonas Salk, which was introduced in 1955. The live-attenuated vaccine (OPV), based on avirulent strains developed by Albert Sabin, became available for widespread use in 1961 (Sabin and Boulger, 1973). The successful use of these vaccines in controlling poliomyelitis is discussed in section 1.7.5.

1.7.2. The Epidemiology of Poliomyelitis.

In the developing countries, in the absence of vaccination, poliomyelitis has continued to be a disease of infancy that is seen only rarely (i.e. the endemic pattern). Serological surveys conducted within these countries have shown that more than 90% of children of three or more years of age possess antibody to at least one serotype of poliovirus (Schonberger et al., 1981). In addition it is found that there are between 100 and 200 sub-clinical infections for each paralytic case.

This situation is thought to have existed in Northern Europe and the United States up until the start of the 19th Century. The shift from the endemic to the epidemic pattern of poliomyelitis was probably related to changes in socioeconomic conditions occurring at that time. Primitive sanitation conditions ensured the widespread circulation of poliovirus which is transmitted by the faecal-oral route. As a result, most individuals experienced their first, immunising infections during infancy. With increased resources for household and

community hygiene, the opportunities for infection of the very young were reduced. Increasing numbers of persons encountered poliovirus for the first time in later childhood or early adult life, when infections are more likely to take a paralytic form (Johnson, 1982).

During the first half of this century, poliovirus epidemics increased almost yearly in size and severity. In addition, a drift in the age distribution of the disease was observed. In the United States, at the end of the pre-vaccine era, the average annual incidence of poliomyelitis was between 5 and 10 cases per 100,000 population (Melnick, 1982). One third of the cases were in persons over 15 years of age. This was in marked contrast to the first of the major epidemics when over 80% of the cases were in children under 5 years of age.

1.7.3. The Pathology of Poliomyelitis.

The primary site for multiplication of polioviruses is the human alimentary tract. The initial infection is usually asymptomatic, but may be accompanied by low grade fever, headaches, sore-throat or nausea. Excretion of poliovirus in stools reaches a peak at 5 to 6 days post-infection, and has normally ceased after 2 to 3 weeks. Virus can also be isolated form the oropharynx and mesentary during the early stages of infection. Replication of the virus in the gut stimulates high levels of serum and secretory antibody (Bodian and Paffenbarger, 1954; Horstmann et al., 1954). In 99% of cases the infection is terminated at this stage and the only lasting consequence is a protective antibody response against polioviruses of the same serotype. However, in the remaining 1%

of cases, a systemic infection may follow, usually with some form of neurological involvement.

A brief 2 to 3 day remission follows the initial multiplication of the virus and then headache stiffness and muscle pains are quickly followed by the onset of paralysis, a process which is complete within 24 hours. Flaccid paralysis tends to affect legs rather than arms and is freqeuntly assymetric. In 10 to 15% of patients, there is also some bulbar paralysis which may lead to death following cardio-respiratory failiure (see Paul, 1971).

Histologically, the effects of poliovirus on the CNS consists of inflammation and neuronal destruction of the anterior horns of the spinal cord, the bulbar motor nuclei, reticular formation, thalamus and motor cortex (Bodian, 1949). It is generally considered that nerve cell injury must reach a certain threshold of severity before a clinical effect is observed (Bodian, 1949, Beswick and Coid, 1961, Beswick et al., 1964).

Although the portal of entry of poliovirus into the body has been established to be the faecal-oral route, the way in which the virus enters the CNS is not known. The initial multiplication of the virus seems to be associated with lymphatic tissues (Peyer's patches in the small intestine, mesenteric lymph nodes and adenoidal tissue in the throat) and infection of the CNS is preceded by viraemia (Bodian and Paffenbarger, 1954; Horstmann et al., 1954; Sabin, 1956). The precise way in which these events are related is not clear.

Several factors are known which predispose infected patients to paralysis. Undoubtedly, the major factor is the degree of neurovirulence of the strain involved (Bodian, 1949;

Sabin, 1956). Host factors include increasing age (Johnson, 1982) and physical activity at the time of the onset of paralysis (Horstmann, 1950).

The major defence against poliomyelitis involves humoral immunity rather than cell mediated immunity. Initial protection against infection involves the production of secretory antibodies in the saliva and the gut (Johnson, 1982). Serum antibody may play a role in modifying the course of infection during the viraemia phase (Sabin, 1956; Melnick, 1982). Once CNS involvement occurs, the T-cell response is dominant in clearing virus (Johnson, 1982). However, damage progresses so rapidly (i.e. within hours) that it is impossible to halt the disease at this stage.

1.7.4. Development of the Salk and Sabin Vaccines.

Two competing types of poliovirus vaccine were developed almost simultaneously in the late 1950s: the inactivated poliovirus vaccine (IPV), which was administered by intramuscular injection, and the live attenuated poliovirus vaccine, which was administered orally (OPV). These were commonly called the Salk and the Sabin vaccines respectively after the scientists who played a leading role in their development.

The discovery by Enders et al. (1949, 1954) that polioviruses could be propagated in cultures of human epithelial tissue was a vital step towards the development of both types of vaccine. The presence of virus could be recognised by a microscopically visible cytopathic effect, allowing the quantity of virus in culture fluds to be

determined. It was also possible to test serum for the presence of antibody against the virus. These new techniques were used to evaluate methods of inactivating poliovirus while maintaining antigenicity. Such treatments included heat, phenol, U.V. light and electron bombardment. Formalin inactivation was eventually shown to be the best method for production of an inactivated poliovirus vaccine (IPV).

At the same time, a quantitative monkey neurovirulence assay was developed. Early studies were complicated by the observation that brain-stem neurons proved to be less susceptible than the lower motor neurons of the spinal cord. It was also recognised that neurovirulence was not an `all or none' character that was present or absent in a given strain. Rather a spectrum of activity was observed, ranging from high to low. The neurovirulence of any given strain could be measured quantitatively by correlating the incidence of paralysis or the development of neuronal lesions with the concentration of virus determined by cytopathic effect in tissue culture (Sabin, 1956).

Serial passage of the Brunhilde strain of poliovirus type 1 in-vitro led to a marked reduction in neurovirulence for monkeys (Enders et al., 1954). This observation facilitated the development of attenuated strains of all three serotypes of poliovirus (Sabin et al., 1954; Sabin and Boulger, 1975). It is noteworthy that passage in-vitro had no effect on neurovirulence as long as single or small numbers of virus particles were used to initiate cultures and a large number of replication cycles were permitted (Sabin et al., 1954). Avirulent variants could be isolated only when large inocula (10^5 to 10^6 $TCID_{50}$) were used together with rapid passage (24

Table 1.4.

Origin of the Sabin Vaccine Strains.

Type 1	
P1/Mahoney/41	14 passages in vivo (monkeys)
(faecal isolate,	2 passages in vitro (monkey testicle)
healthy children)	
P1/Monk14 T2	24 passages in vitro (monkey testicle)
	18 passages in vitro (monkey kidney)
	10 alternate passages:-
	5 in vivo (intradermally in monkeys)
	5 in vitro (monkey kidney)
P1/LS-c	5 passages in vitro (monkey kidney)
	3 plaque purifications (monkey kidney)
	2 passages in vitro (preparative) (monkey kidney)

P1/LS-c, 2ab : Sabin type 1 vaccine strain

Type 2	
P2/P712/56	4 passages in vitro (monkey kidney)
(faecal isolate,	3 plaque purifications (monkey kidney)
healthy children)	1 passage in vivo (orally in chimpanzees)
	3 plaque purifications (monkey kidney)

P2/P712,Ch,2ab : Sabin type 2 vaccine strain

Type 3	
P3/Leon/37	21 passages in vivo (intracerebrally in monkeys)
(isolate from fatal	8 passages in vitro (monkey testicle)
paralytic case)	
	39 passages in vitro (monkey kidney)
	3 plaque purifications (monkey kidney)
	3 passages in vitro (preparative) (monkey kidney)

P3/Leon/12a_1b : Sabin type 3 vaccine strain

hour intervals). Such conditions would allow the selection of any faster growing variants, suggesting that attenuation was the result of adaptation to growth in-vitro.

The procedures used to derive the Sabin attenuated vaccine strains are summarised in Table 1.4. The derivation of the type 1 and type 3 vaccine strains were very similar in that both were segragated by serial rapid passage of a well-characterised virulent strain in-vitro (Sabin and Boulger, 1975). The most suitable candidate for a type 2 vaccine strain was a naturally occurring strain isolated from the stool of a healthy child.

Quantitative studies had shown that the nervous system of lower monkeys (rhesus and cynomolgus monkeys) was more susceptible to polioviruses than that of primates (chimpanzees and, on the basis of epidemiological data, man). It was therefore argued that the attenuated strains, each of which showed only residual neurotropism for the lower monkeys, would be safe for administration to humans.

1.7.5. Vaccination Against Poliomyelitis.

Towards the end of the pre-vaccine era, the annual rate of paralytic poliomyelitis in the United States was between 5 and 10 cases per 100,000 population. The formalin inactivated poliovirus vaccine (IPV) was introduced in 1955. A marked reduction in the incidence of poliomyelitis was achieved, in some years reaching as low as 0.5 cases per 100,000 population. However this still meant that significant number of cases were occurring annually. In 1960, for example, more than 2,500 paralytic cases were recorded. There were also

considerable problems with the potency of different IPV preparations. In a study of several thousand paralytic cases, 17% were in children who had received three injections of the vaccine (Melnick, 1982).

IPV was largely replaced by the live attenuated vaccine (OPV) when it became available in 1961. The incidence of paralytic disease continued to fall. Between 1970 and 1979, the average number of paralytic cases in the United States was 17. This has meant case rates ranging from 0.2 to 0.02 per million population (Melnick, 1982).

The live attenuated vaccine strains are amongst the safest of such vaccines. However, rare cases of vaccine associated paralysis do occur. In a recent WHO survey in 10 developed countries, 281 paralytic cases were recorded over a 10 year period (Assad and Cockburn, 1982). Of these, 122 (43%) were vaccine associated, 52 in vaccine recipients and 70 in household contacts. This is equivalent to a risk of 1 case per million children receiving the vaccine (the estimated risk varied between 0.5 and 3.4 per million in different countries).

Of the recipient cases, 2/52 (4%) were associated with type 1, 8/52 (15%) were associated with type 2 and 17/52 (33%) were associtaed with type 3 (in 21/52 cases, more than one serotype was isolated). All the type 2 and type 3 strains were vaccine like on the basis of biochemical and serological tests. However, both type 1 strains were wild-type, and probably represent chance infections. 12 of the type 3 strains were characterised by T1 oligonucleotide mapping (Minor, 1982). The maps of all 12 strains were isolated from the central nervous system of fatal cases. These findings therefore provide strong evidence that the Sabin type 3 vaccine strain can cause poliomyelitis.

1.7.6. Attenuation.

Although the Sabin vaccines have been in use for over twenty years the actual basis for their attenuation has never been established. Early studies attempted to correlate physical properties of the virus particle with attenuation or virulence. It was observed that the plaquing efficiency under acid overlay showed some correlation with virulence (Vogt et al., 1957). Other workers noted a differential affinity between attenuated and neurovirulent strains for compounds such as calcium phosphate (Hodes et al., 1960), cellulose resin (Woods and Robins, 1961) or aluminium hydroxide (Koza et al., 1963). Differences have also been observed in the degree to which various strains aggregate in conditions of low ionic strength (Totsuka et al., 1978). The Sabin type 1 vaccine is more labile in the presence of SDS than the virulent Mahoney strain from which it was derived (Young and Moon, 1975). Kew et al. (1980) were able to demonstrate amino acid changes between Sabin type 1 and Mahoney in three of the four capsid proteins. Thes results suggest that attenuated strains have altered physical and particularly surface properties, a conclusion strengthened by the observation that there are minor antigenic differences between Sabin type 1 and Mahoney (van Wezel and Hazendonk, 1979).

For none of the above physical changes has it been possible to demonstrate a cause and effect relationship with attenuation. However, the possibility that attenuation involves physical changes to the surface of the virus particle is an attractive model. Sabin suggested that neurovirulence of a poliovirus strain may be determined " by its specific interaction with neuronal target cells" (Sabin 1956). Mutations giving rise to a reduced affinity of the virus for nerve cells at the level of receptor binding would therefore possess an attenuated phenotype. The possibility that the Sabin vaccine strains are attenuated simply because they have aquired random temperature sensitive (ts) mutations has also been

considered (Lwoff, 1959; Sabin, 1961). Certainly all the vaccine strains show a reduced capacity for growth at 40°C. However it has not been possible to demonstrate a direct correlation between temperature sensitivity and attenuation (Sabin, 1961).

Growing concern over the stability of the Sabin vaccine strains has generated new interest in the molecular basis for attenuation. Application of the techniques of molecular cloning and rapid sequence analysis has lead to the characterization of the genetic changes induced during the attenuation of the Sabin type 1 and Sabin type 3 vaccines. Comparison of the complete nucleotide sequence of the Sabin type 1 vaccine strain, LSc/2ab, with the previously determined sequence of the neurovirulent parent strain, Mahoney, revealed that 57 base substitution mutations are distributed throughout the genome (Nomoto et al., 1982). Of these, 21 result in amino acid substitutions in the viral polyprotein. 12 of the 21 amino acid substitutions are located in the region of the viral genome encoding the capsid proteins. Of particular interest is a cluster of five amino acids changes within 20 amino acid residues in the middle of the VP1 polypeptide. This cluster of changes tends to support the model for attenuation based on the idea that the reduced neurotropism of attenuated strains is the result of reduced affinity of the virus for nervous tissue at the level of receptor binding. When mapped onto the high resolution structure of Poliovirus type 1, the cluster of mutations are located near a site implicated in receptor recognition (Hogle et al., 1985; Rossmann et al., 1985).

A similar strategy was used to investigate the genetic basis for the attenuation of the Sabin type 3 vaccine strain. A more precise characterisation of the genetic changes responsible for attenuation was achieved by the careful selection of strains for nucleotide sequence analysis (Stanway et al., 1984; Cann et al., 1984). The three strains of type 3 poliovirus used in this study represent a direct genealogic lineage.

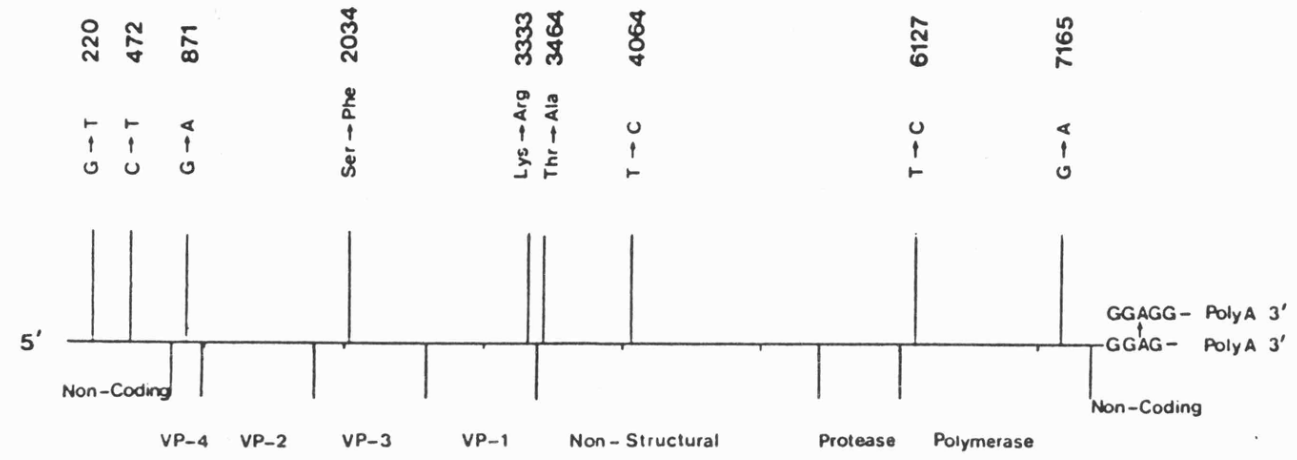
P3/Leon/37 is the neurovirulent progenitor of the Sabin type 3 vaccine strain (Sabin et al., 1954; Sabin and Boulger, 1983). P3/Leon/12a^{1}b is the Sabin type 3 vaccine itself. P3/WHO/119is a virus isolated from the CNS of a fatal case of paralytic poliomyelitis associated with administration of the vaccine (Minor, 1982). Each of these strains was plaque purified in Hep 2c cells and virus from a single plaque was used to initiaite stocks on which all subsequent experiments were performed.

Ribonuclease T1 fingerprints of the RNA genomes of these viruses were shown to be identical. Although the technique directly examines only approximately 15% of the genome, identical fingerprints from RNA molecules of this size suggests an overall homology in base sequence of >99%. This degree of homology suggests that random mutational drift between the strains is minimal, and therefore observed changes in nucleotide sequence have a high probability of relating directly to neurovirulence.

The plaque purified viruses were characterised for their reaction in the WHO monkey neurovirulence assay. All animals inoculated with P3/Leon/12a$_{1}$b survived free from paralysis throughout the 22 days of the test. The mean lesion score, a measure of the level of virus specific damage was 0.72. On the basis of this result the virus would be acceptable for use as a vaccine. P3/Leon/37 and P3/WHO/119 were both highly neurovirulent, causing paralysis in all animals tested and death in 5/6 and 6/6 animals respectively. The phenotypes of the plaque purified isolates were therefore identical to that of the pools from which the plaques were derived.

Complete nucleotide sequences for each of these strains were derived from cloned cDNA using the di-deoxy chain termination method. To ensure accuracy, all regions of the cDNA were sequenced at least twice. At each position where a nucleotide difference was detected between P3/Leon/12a$_{1}$b and its progenitor or the revertant, P3/WHO/119, the sequence was checked in both orientations. Only ten base substitution mutations

Leon → Sabin



Sabin → 119

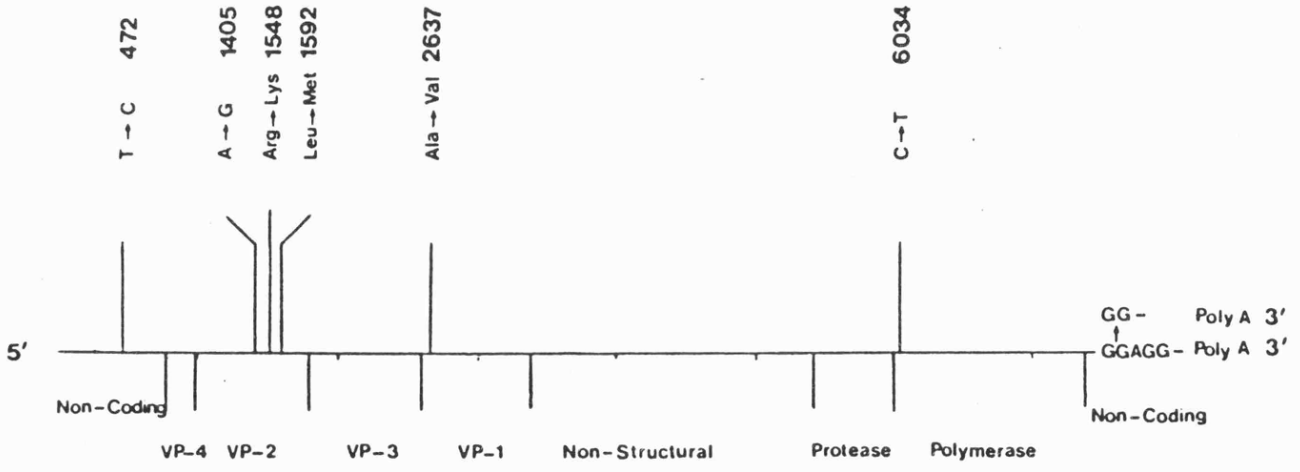


Fig. 1. 5.

Nucleotide Sequence Comparison of Attenuated and Virulent Strains of Poliovirus Type 3.

Comparison of the genomic sequences of the attenuated Sabin type 3 vaccine strain, its virulent progenitor, P3/Leon/37 (top panel), and a virulent revertant strain, P3/WHO/119 (bottom panel). The positions of nucleotide differences between the virulent strains and the attenuated vaccine strain are indicated and any amino acid substitutions which result are identified. For example the alteration in sequence at 2034 generated in deriving the Sabin strain from P3/Leon/37 changes a serine in the capsid protein VP3 of the Leon strain to a phenylalanine in the capsid protein VP3 of the Sabin strain. Base changes which did not produce amino acid changes are also indicated.

distinguish the type 3 vaccine strain from its neurovirulent progenitor (see Fig. 1.5.). Only three of these mutations result in amino acid substitutions in viral specific proteins. The most drastic of these is a serine to phenylalanine substitution in the structural protein VP3. The lysine to arginine substitution in VP1 and the threonine to valine change in P2-3b, a non-structural protein of undefined function, are chemically more conservative.

It is significant that 8 out of 10 of the Sabin-specific mutations are conserved in P3/WHO/119, providing convincing evidence that P3/WHO/119 is a bone fide revertant of the vaccine (Cann et al., 1984). Seven additional changes have occurred between the Sabin vaccine strain and the revertant. Again, only three amino acid substitutions were observed. Two of these changes are located close together in VP2 and one change is in VP1.

Taken as a whole, the sequence data suggest that there are three possible genetic bases for the attenuation of the Sabin type 3 vaccine strain. Firstly, a mutation C-U at N472 in the 5′ non-coding region is implicated by direct back mutation in the revertant P3/WHO/119. A mutation A-G at N7432 in the 3′ non-coding region may also be significant. This position represents the first A residue of the template coded poly (A) tract. It is therefore not clear whether the mutational event is a base substitution or an insertion. In Fig 1.6. the mutation is shown as a base substitution which reverts by direct back mutation in P3/WHO/119. The vaccine revertant is not entirely wild-type in this region, however, as an additional mutation, A-G, has occurred in the adjacent position (N7431).

The third mechanism is perhaps the more predictable; that is a functional change induced in a virus specific protein by an amino acid substitution. Any one or combination of the three amino acid substitutions observed between the vaccine and its progenitor could be involved in attenuation. Here the mechanism of reversion would be suppression because

Fig 1.6.

Nucleotide Sequence at 3′ Terminus of Attenuated and Virulent strains of Poliovirus type 3

P3/Leon/37	UUAAUUCGGAGAAAAA(A)$_n$
P3/Leon/12a$_1$b	UUAAUUCGGAGGAAAA(A)$_n$
P3/WHO/119	UUAAUUCGGAAAAAAA(A)$_n$

each of the mutations has been conserved in P3/WHO/119. Also, the three potential suppressor mutations in the revertant are located in structural proteins. This tends to preclude a role in attenuation for the Sabin-specific mutation in P2-3b because it is difficult to envisage a model in which a mutation in a non-structural protein could be suppressed by a mutation in a structural protein.

The major objective of the work carried out for this thesis was to distinguish amongst the possible genetic bases for the attenuation of Sabin type 3. The work involved the construction of recombinant genomes between the parent and the vaccine via infectious cDNA in-vitro and the rescue of virus by transfection. Each of the above Sabin-specific mutations were isolated using conserved restriction endonuclease sites in the poliovirus cDNA. By subjecting the series of inter-strain recombinants to neurovirulence assay, the significance of the individual Sabin-specific mutations to attenuation was determined.

CHAPTER TWO

The construction of full-length cDNA clones of the poliovirus type 3 Vaccine strain, P3/Leon/12a$_1$b, and its neurovirulent progenitor, P3/Leon/37.

2.1 Introduction.

In 1981, Racaniello and Baltimore demonstrated that a full-length cDNA of poliovirus type 1, cloned into the prokaryote vector, pBR322, was infectious following transfection of susceptible cells. Although it was not possible to produce a dose response, a comprehensive series of controls was used in this study. The results suggested that infectious RNA molecules are transcribed from the cDNA in-vivo. The mechanism of this transcription event was not known, although the authors suggested that the host transcription apparatus may recognise a promoter-like sequence in the vector. Alternatively, integration into the host genome may be required to place the cDNA under the control of a cellular promoter.

Whatever the mechanism, the initial transcript would be expected to contain non viral sequences at the 5′ and 3′ termini. Racaniello and Baltimore (1982) therefore examined the termini of genomic RNAs purified from cDNA derived virus. The sequence of the first 38 nucleotides from the 5′ end was determined and shown to be identical to wild-type virus. The cDNA derived virus was polyadenylated at the 3′ end and a T1 oligonucleotide representing nucleotides 7419-7434 was unaltered in the T1 RNase fingerprint suggesting that the 3′ end of the cDNA derived virus

was very similar or identical to the 3′ end of wild-type virus. These results imply that some sort of processing event occurs in transfected cells. Recent studies suggest that the first two nucleotides of the poliovirus genome are not template coded, but are derived from a species VPg-pU-pU which serves as a primer for the viral RNA replicase (Tagegami et al., 1983). If this model is correct, accurate processing could be achieved following replication of a primary transcript.

This chapter describes the construction of full-length cDNAs of the poliovirus type 3 vaccine strain, P3/Leon/12a$_1$b, and its neurovirulent progenitor, P3/Leon/37. The cDNA was cloned into the PstI site of pAT 153, a high copy number derivative of pBR322. The possibility of obtaining infectious virus from these cDNA clones was investigated by transfection of human epithelial cells using the modified calcium phosphate co-precipitation technique.

2.2 The origin of sub-genomic cDNA clones of P3/Leon/12a$_1$b

Overlapping cDNA clones representing the entire genome of the poliovirus type 3 vaccine strain, P3/Leon/12a$_1$b, were prepared in E.coli using the RNA.cDNA hybrid cloning technique (Cann et al., 1983). The advantage of this technique is that cDNA clones corresponding to the 5′ terminus can be isolated. Viral RNA was prepared from a plaque purified isolate of P3/Leon/12a$_1$b. The purified RNA was used as a template for cDNA synthesis by AMV reverse transcriptase using oligo dT as a primer. dC tailed RNA.CDNA hybrids were annealed to dG tailed pAT 153 and transformed into E.coli strain JA221, which were plated out in the presence of tetracycline. Replacement of the viral RNA strand with DNA and repair of the homopolymeric regions to produce a ds cDNA recombinant occurs in-vivo and is a function of the host cell metabolism. Recombinants were identified by their sensitivity to ampicillin.

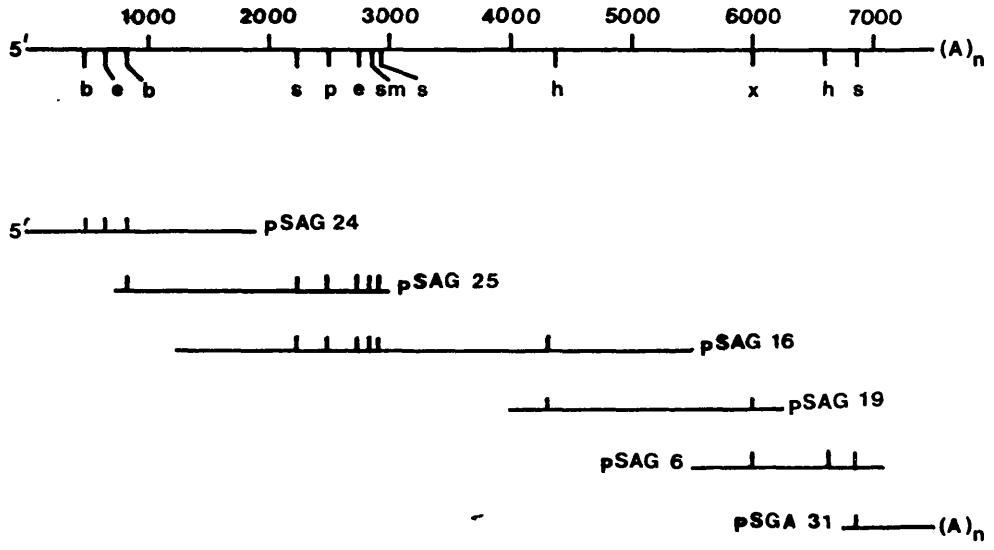

1000
2000
3000
4000
5000
6000
7000
5'
(A)n
b e b
s p e sm s
h
x
h s
5'
pSAG 24
pSAG 25
pSAG 16
pSAG 19
pSAG 6
pSGA 31
(A)n

Fig. 2. 1.

Summary of Sub-genomic cDNAs Used to Construct pOLIO SABIN.

Sub-genomic cDNA clones were obtained from P3/Leon/12a$_1$b by the hybrid cloning technique (Cann et al., 1983). A subset of overlapping cDNA clones (b) were selected for the construction of a complete cDNA copy on the basis of a composite restriction endonuclease map (a) derived by the method of Smith and Birnsteil (1976).

Amp^s Tet^r transformants were characterised by colony hybridisation experiments using the method of Grunstein and Hogness (1975). Clones containing cDNA corresponding to the 3′ region of the genome were identified using a 3′ enriched probe prepared from a reverse transcription reaction containing sub-optimal ammounts of dATP. Clones containing cDNA corresponding to regions towards the 5′ end of the genome were identified using cDNA probes prepared from a reverse transcription reaction in which DNase I treated, sheared, salmon-sperm DNA was used as a primer. Strongly positive colonies were isolated and grown in 10ml cultures. Plasmid DNA prepared by a rapid isolation technique was digested with restriction endonucleases. Representative plasmids showing overlapping hexameric restriction sites were characterized further by the restriction mapping method of Smith and Birnsteil (1976). Fig. 2.1. shows the structure of the sub-genomic cDNA clones used to construct a full-length cDNA of P3/Leon/12a_1b, pOLIO SABIN.

2.3. The construction of pOLIO SABIN.

Sub-genomic cDNAs of P3/Leon/12a_1b were obtained from Dr. A. Cann. The work described in this section was carried out in collaboration with R. C. Mountford. The procedure used to construct a full-length cDNA clone of P3/Leon/12a_1b is outlined in Fig. 2.2 and Fig. 2.3. pSR 1, a recombinant clone containing the 1382 nucleotides from the 3′ terminus, was produced using restriction fragments generated by PstI and SphI double digestion of pSGA 31 and pSAG 6. The digestion products were separated by electrophoresis on a 1% agarose gel and the fragments indicated in Fig. 2.2. were electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. These fragments were ligated using T4 DNA ligase and the products of ligation were analysed by agarose gel electrophoresis. A linear fragment of the appropriate length was identified and electroeluted.

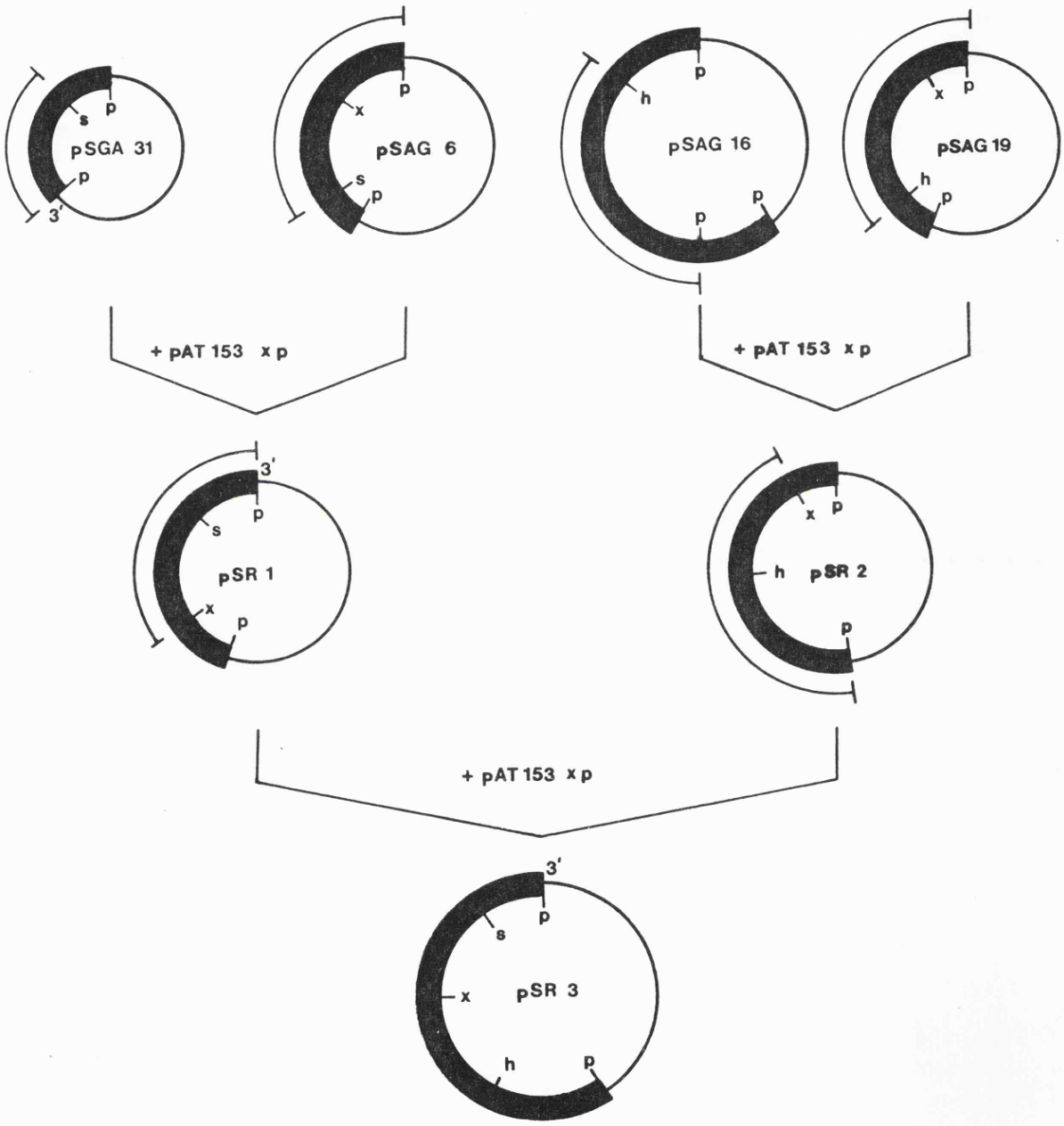

pSGA 31
s
p
p
3'
pSAG 6
p
x
s
p
pSAG 16
p
h
p
p
pSAG 19
x
p
h
p
+ pAT 153 x p
+ pAT 153 x p
pSR 1
3'
p
s
x
p
pSR 2
x
p
h
p
+ pAT 153 x p
pSR 3
3'
p
s
x
h
p

Fig. 2. 2.

The Construction of pSR3, a cDNA Clone Representing the 3' Terminal 4,700 Nucleotides of P3/Leon/12a$_1$b.

Poliovirus cDNA was cloned into the PstI site of pAT 153. Plasmid sequences are shown as single lines and poliovirus cDNA as heavy lines. Barred lines indicate the restriction fragments used at each step in the construction (see text for details). 3' corresponds to the 3' terminus of the poliovirus genome.

h = HindIII, p = PstI, s = SphI, X = XhoI.

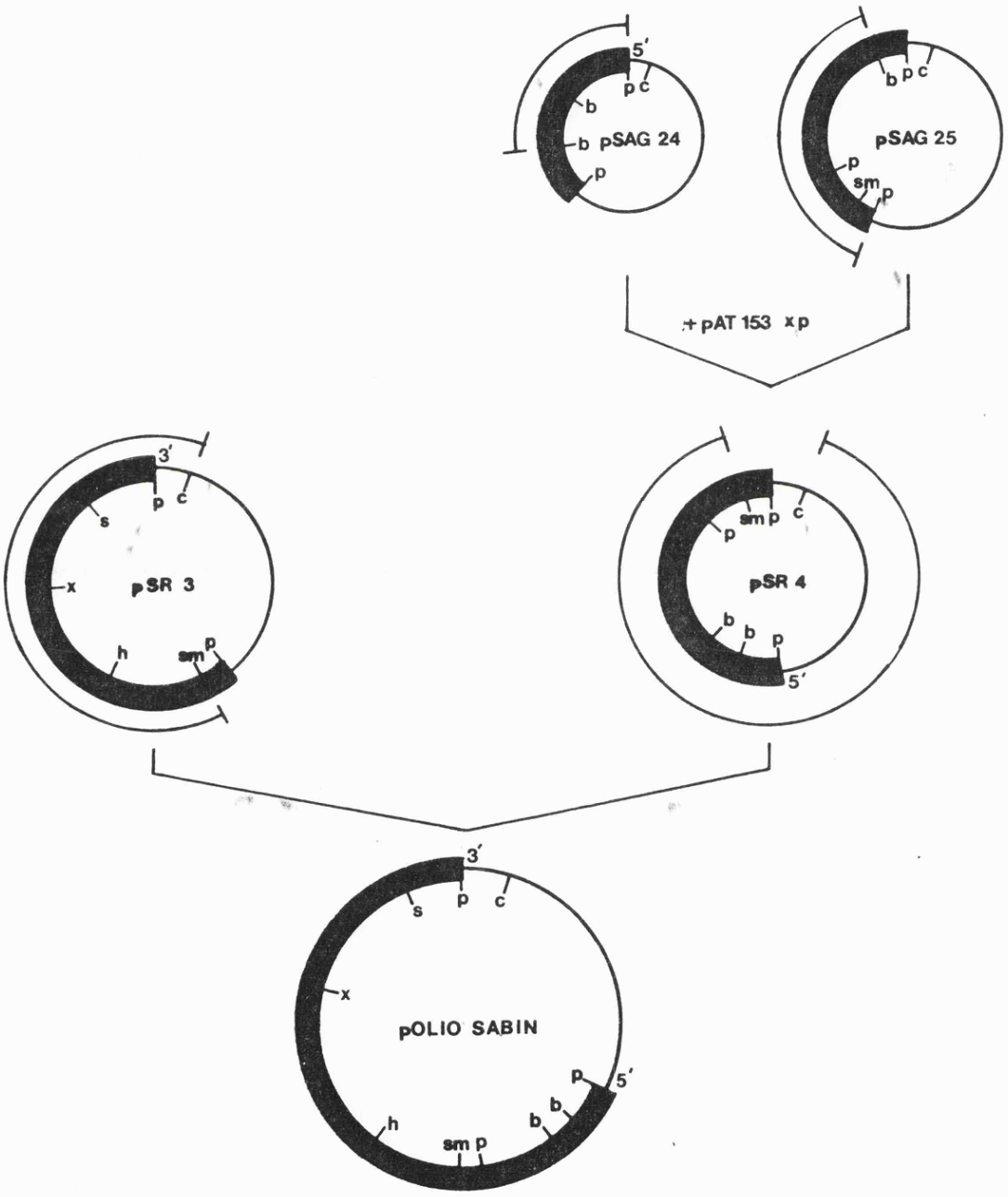

5'
p c
b
b pSAG 24
p
b p c
pSAG 25
p
sm
p
+ pAT 153 x p
3'
p c
s
x
pSR 3
h
sm
p
p
sm p
c
pSR 4
b
b
p
5'
3'
s
p
c
x
pOLIO SABIN
p
5'
h
b
b
sm p

Fig. 2. 3.

The Construction of pOLIO SABIN, a Clone Containing a Full-length cDNA of P3/Leon/12a$_1$b.

Sub-genomic cDNAs were cloned into the PstI site of pAT 153. Plasmid sequences are shown as single lines and poliovirus cDNA as heavy lines. Barred lines indicate the restriction fragments used at each step in the construction (see text for details). 3' and 5' correspond to the termini of the poliovirus genome.

b = BamHI, c = ClaI, h = HindIII, p = PstI, s = SphI, sm = SmaI, x = XhoI.

The purified fragment was then ligated into PstI digested, phosphatase treated pAT 153. Competent E.coli JA221 cells were transformed with the ligation mixture and plated out in the presence of tetracycline. Plasmid DNA was prepared from 10ml overnight cultures of tetracycline resistant transformants using the rapid isolation technique. Recombinant plasmids were identified on the basis of size by agarose gel electrophoresis, using supercoiled pAT 153 as a size marker. The insert could be ligated into the vector in either of two orientations. The orientation of the insert with respect to plasmid sequences was considered to be important for infectivity because it had been suggested that a promoter-like sequence in the vector was required for transcription of cDNA in transfected cells (Racaniello and Baltimore, 1981). A recombinant containing the insert in the correct, `infectious cDNA', orientation was identified by restriction mapping.

pSR 2 was constructed in a manner analogous to that used for pSR 1, i.e. by ligating PstI-HindIII fragments from pSAG 16 and pSAG 19 into PstI digested, phosphatase treated pAT 153. Similarly, pSR 3, a recombinant containing the 3' terminal 4824 nucleotides of the genome, was constructed using PstI-XhoI fragments of pSR 1 and pSR 2. pSR 4, a recombinant containing the 2764 nucleotides from the 5' terminus was constructed using fragments isolated from a PstI and BamhI double digest of pSAG 25 and a PstI complete and BamhI partial digest of pSAG 24. The fragments indicated in Fig. 2.3 were ligated into PstI digested, phosphatase treated pAT 153. Finally, a full-length clone, designated pOLIO SABIN, was constructed using the unique SmaI site in the poliovirus cDNA and the unique ClaI site at N23 in the vector. pSR 3 and pSR 4 were digested with ClaI and SmaI and the appropriate fragments were ligated together. Recreation of the restriction sites used in the construction was confirmed at every step, therefore pOLIO SABIN contains a complete cDNA copy of the P3/Leon/12a_1b genome.

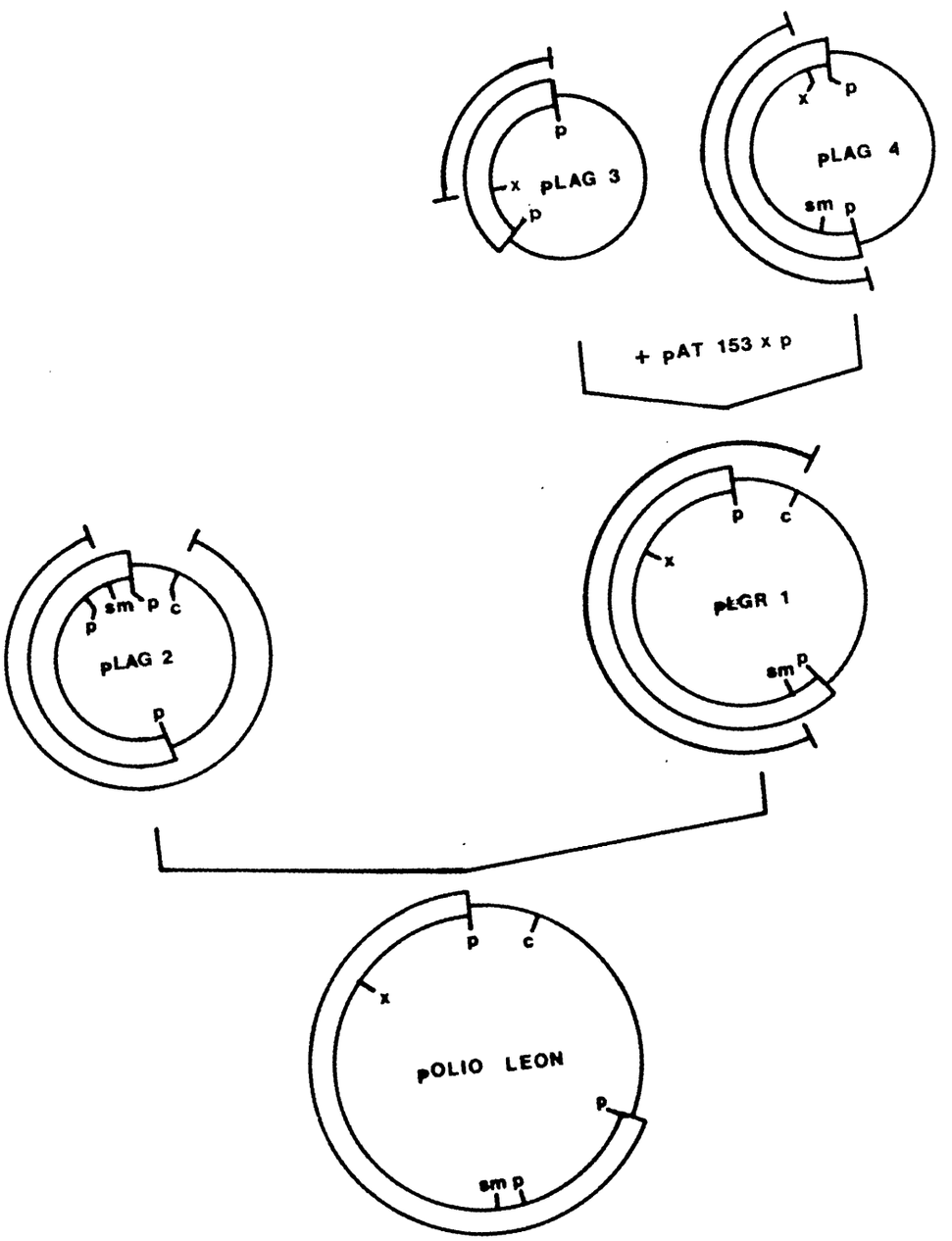

p
x
pLAG 3
p
x
p
pLAG 4
sm
p
+ pAT 153 x p
p
sm
p
c
pLAG 2
p
p
c
x
pLGR 1
sm
p
p
c
x
POLIO LEON
p
sm
p

Fig. 2. 4.

The Construction of pOLIO LEON, a Clone Containing a Full-lengthcDNA of P3/Leon/37.

Plasmid sequences are shown as single lines, poliovirus cDNA as double lines. Barred lines indicate the restriction fragments used at each step in the construction (see text for details). 5' and 3' correspond to the termini of the poliovirus genome.

c = ClaI, p = PstI, s = SmaI, x = XhoI.

2.4. The construction of pOLIO LEON.

The work described in this section was carried out by Dr. G. Stanway. Overlapping cDNA clones, representing the entire genome of a plaque purified isolate of P3/Leon/37, were obtained using the RNA.cDNA hybrid cloning technique described in section 2.1. Fig. 2.4. shows the procedure used to constuct a full-length cDNA clone of P3/Leon/37. pGLR 1 was constructed using fragments produced by PstI and XhoI double digestion of pLAG 3 and pLAG 4. The fragments indicated in Fig. 2.4. were ligated into PstI digested, phosphatase treated pAT 153. The complete copy, pOLIO LEON, was constructed using the SmaI site in the poliovirus cDNA and the ClaI site at N23 in the vector, as described for pOLIO SABIN. It was confirmed that the PstI XhoI and SmaI sites used in the construction were recreated. Hence pOLIO LEON contains a complete copy of the P3/Leon/37 genome.

2.5. Preparation of plasmid DNA for transfection

One problem encountered during the course of this study was the instability of plasmids containing full-length poliovirus cDNAs during passage in E.coli. Spontaneous deletion mutants were frequently generated following storage of clones as streak cultures at +4°C This is suprising because the host strain used throughout this study, E.coli JA221, is a RecA mutant. However, several intra-plasmidic recombination pathways have been identified which are independent of a functional recA gene product (Laban and Cohen, 1981; Jones et al., 1982; Cohen and Laban., 1983). These pathways are site specific with the recombinational event occurring within regions of internal homology. It is noteworthy that plasmid molecules containing a direct repeat of only 7 nucleotides may form a substrate for intra-plasmidic recombination (Cohen and Laban, 1983).

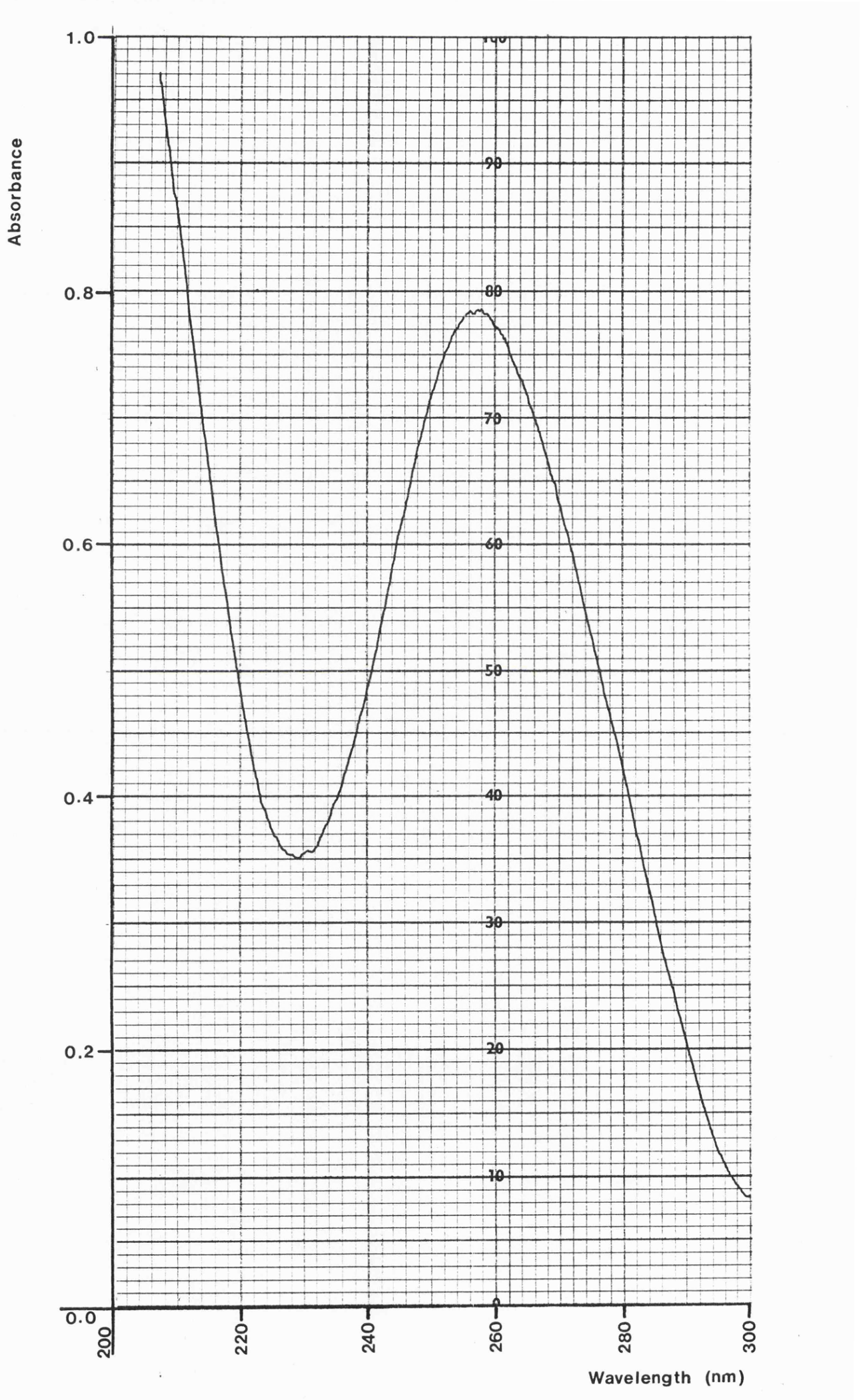

Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
100
90
80
70
60
50
40
30
20
10
0
200
220
240
260
280
300
Wavelength (nm)

Fig. 2. 5.

Yield of Plasmid DNA Obtained From a 1 Litre Culture of Transformed Cells.

Plasmid DNA was prepared by the cleared lysate method and resuspended in a total volume of 1 ml. The absorbance of a 100X dilution was determined over the range 200nm - 300nm. An absorbance$_{260}$ of 1.00 is equivalent to a DNA concentration of 50ug/ml.

Abs_{260} = 0.75 (100X dilution).

DNA concentration = 0.75 X 0.05 X 100 = 3.75mg/ml.

The frequency with which deletion mutants of the poliovirus cDNA clones were isolated suggests that some form of selection pressure is operating. Both the original plasmid and the deletion product belong to the same incompatability group. As a result any selective advantage for the replication and maintenance of the deletion product would lead to a rapid change in the plasmid population following intra-plasmidic recombination (Laban and Cohen, 1981). The basis of this selective advantage could be the reduced size of the deletion product, although other authors have speculated that fortuitous expression of the poliovirus gene products could be detrimental to the host cell (Semler et al., 1984).

In order to minimise the amount of time and effort wasted, plasmid DNA was routinely prepared on a large scale. Up to 4mg of plasmid DNA could be obtained from a 1 litre culture of cells using the `cleared-lysate' method described in chapter 10 (see Fig. 2.5). Each new preparation of plasmid DNA was examined by restriction endonuclease mapping for evidence of deletion mutation before aliquoting for storage as an ethanol precipitate at -20°C.

2.6. Transfection of Hep 2c monolayers with poliovirus cDNAs.

Three full-length poliovirus cDNAs were available for transfection studies. pVR106, a complete cDNA copy of the Mahoney strain of poliovirus type 1, was the kind gift of Vincent Racaniello. pVR106 had previously been shown to be infectious following transfection of HeLa cell monolayers (Racaniello and Baltimore, 1981). pOLIO SABIN is a recombinant plasmid containing a full-length cDNA of P3/LEON/12a_1b, the Sabin attenuated poliovirus type 3 vaccine strain. pOLIO LEON is a recombinant plasmid containing a full-length cDNA copy of P3/LEON/37, the neurovirulent progenitor of P3/LEON/12a_1b. The construction of pOLIO SABIN and pOLIO LEON was described in sections 2.2 and 2.3 of this chapter.

Hep 2c is a human epithelial cell-line derived from a carcinoma of the pharynx. The `Cincinnati´ strain is permissive for poliovirus types 1, 2 and 3 and is currently the preferred cell-line for characterization of the Sabin attenuated vaccine strains in-vitro (Domok and Magrath, 1979). The cell-line is maintained by continuous passage in MEM (Eagle) supplemented with 5% NCS.

The method of transfection used throughout this study was the modified calcium phosphate co-precipitation technique (Parker and Stark, 1979). This method involves the direct application of DNA to sub-confluent monolayers of cells, in the form of a calcium phosphate/DNA co-precipitate. Following 20 minutes adsorption at 37°C the precipitate is diluted 1:10 with fresh medium, and the monolayers are incubated for a further 4 hours at 37°C. This is followed by a post-incubation treatment with glycerol, which enhances the uptake of the calcium phosphate/DNA co-precipitate. The cells are then washed three times in fresh medium before applying virus growth medium consisting of MEM supplemented with 2% FCS. The transfected plates are then incubated at 35°C, which is the permissive temperature for the Sabin vaccine strains.

The sensitivity of sub-confluent monolayers of Hep 2c to increasing concentrations of glycerol was investigated in a preliminary series of experiments. Monolayers survived a three minute exposure to 20% glycerol in Hepes buffered saline without visible damage to cells. At higher concentrations however, an increasing proportion of cells failed to recover from the osmotic shock. 20% glycerol was therefore chosen as the appropriate concentration for transfection.

The conditions for transfection of Hep 2c monolayers described in chapter 8 were established using the infectious cDNA clone, pVR106. Observations made during the course of this study have shown that important parameters are:-

1. The nature of the calcium phosphate/DNA co-precipitate. The precipitate is prepared by mixing equal volumes of DNA, diluted in 2M $CaCl_2$ and Hepes buffered saline containing phosphate, under conditions of continuous agitation. The character of the precipitate is determined by the pH of the Hepes buffered saline prior to addition of the $CaCl_2$. At pH 7.1, a very fine precipitate develops during a 20 minute incubation at room temperature. No infectivity was observed using the coarser, flocculent precipitates formed at higher pH.

2. The concentration of the DNA. The optimum concentration for transfection was determined to be 20ug DNA per 2 x 10^6 cells. Transfection was not generally reproducible at lower concentrations.

3. The condition of cell monolayers. Monolayers were seeded the day before transfection and sub-confluent monolayers (70-80% confluence) gave better results than those which were very dense at the time of addition of the calcium phosphate/DNA co-precipitate.

4. Glycerol treatment. There appeared to be an absolute requirement for the post-incubation treatment with 20% glycerol. Transfections using a 15% glycerol treatment failed to yield virus.

When sub-confluent monolayers were transfected with 20ug of the recombinant plasmid pOLIO LEON, as described in chapter 8, the first signs of cytopathic effect were observed 5 to 7 days post- transfection as plaque-like areas of rounded, refractile cells in an otherwise intact monolayer. All cells were similarly affected within 24 hours. Virus was harvested by three cycles of freeze-thawing, followed by centrifugation at 2k for 10 minutes to remove cell debris. Virus titres obtained varied between 10^5 and 10^8 PFU/ml. The cDNA transfection was otherwise completely reproducible. Similar results were obtained with pVR106.

Monolayers transfected with pOLIO SABIN, in contrast, showed no difference to control monolayers, transfected with 20ug of pAT 153, throughout a 14 day incubation at 35°C. No virus could be detected

Fig. 2. 6.

Transfection of Hep 2c Monolayers with pOLIO LEON.

Subconfluent monolayers of Hep 2c were transfected with 20ug of pOLIO LEON (B) or mock transfected with 20ug of pAT 153 (A) using the calcium phosphate co-precipitation technique. Monolayers were stained with carbol fuschin after 9 days incubation at 33°C under a 0.5% agar overlay. The specific infectivity of pOLIO LEON was estimated to be between 1 and 2 pfu/ug DNA

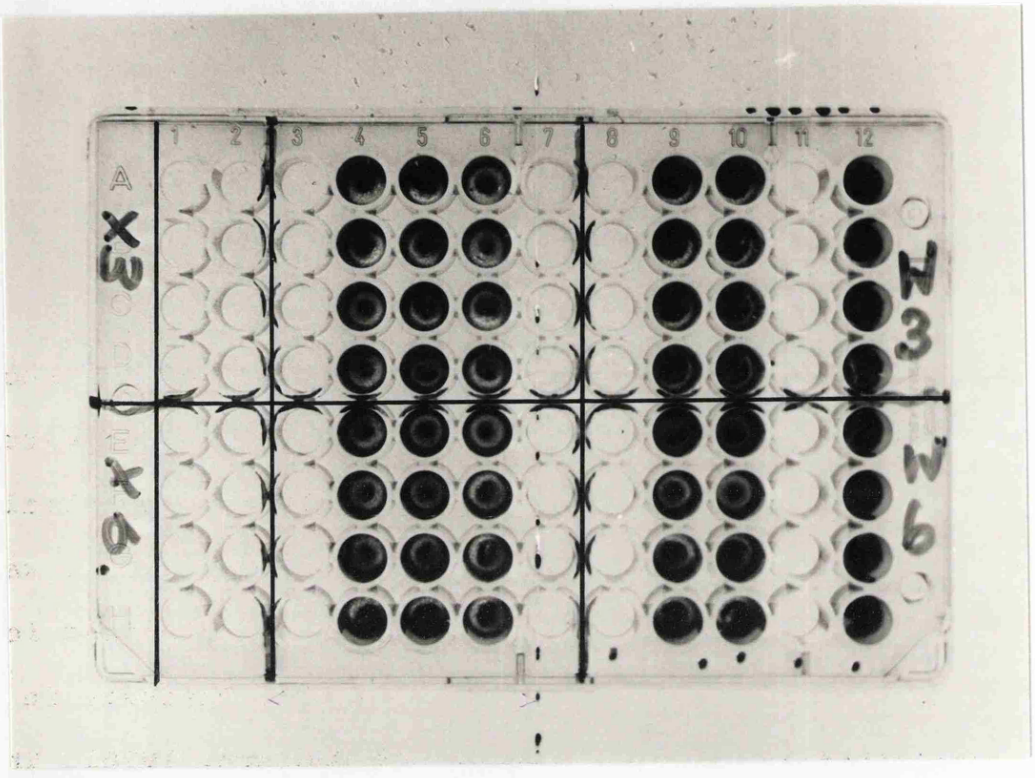
following plaque assay of supernatent media. pOLIO SABIN failed to yield virus in three subsequent transfections in which either pVR106 or pOLIO LEON were used as a positive control. pOLIO SABIN therefore appeared to be non-viable.

An attempt was made to estimate specific infectivity of pOLIO LEON by a direct plaque assay. Monolayers transfected with pOLIO LEON were covered with medium containing 10% FCS and 1% Seakem agarose. 10% FCS was required for growth of the monolayers to full confluence under medium containing agarose. The monolayers were incubated for 9 days at 35°C in an inverted position. To count plaques, the overlay was removed and the monolayers were fixed and stained with carbol fuschin. Fig. 2.6. Shows a 10cm plate transfected with 20ug of pOLIO LEON. Between 20 and 40 poliovirus plaques were obtained on each of four plates transfected. Similar numbers of plaques were obtained following transfection with 40ug and 100ug of DNA, indicating that monolayers were already saturated with DNA at 20ug per plate.

2.8 Characterisation of virus produced by cDNA transfection.

Virus recovered from Hep 2c monolayers transfected with pOLIO LEON was characterised in the following series of experiments:-

(a) Antigenicity. Neutralisation test were performed using polyclonal antisera raised against poiovirus types 1, 2 and 3. The results shown in Fig. 2.7 indicate that the DNA derived virus was neutralised by antibody raised against poliovirus type 3, but was resistant to neutralisation by antibody raised against poliovirus types 1 and 2. The DNA derived virus was therefore shown to be a type 3 poliovirus. The cDNA derived virus was designated rP3/LEON (for rescued poliovirus type 3 LEON). rP3/LEON was also shown to be resistant to a strain specific monoclonal antibody which neutralised the type 3 vaccine strain but not P3/Leon/37.



	1	2	3	4	5	6	7	8	9	10	11	12
			VIRUS ONLY	TYPE I, II & III	TYPE I & III	TYPE II & III	TYPE I & II	VIRUS ONLY	TYPE I, II & III	TYPE I & III	TYPE II & III	TYPE I & II
A												
B			10^5 $TCID_{50}$ rLEON					10^5 $TCID_{50}$ rMAHONEY				
C												
D												
E												
F			10^2 $TCID_{50}$ rLEON					10^2 $TCID_{50}$ rMAHONEY				
G												
H												

Fig. 2. 7.

Neutralisation of cDNA-derived Viruses with Type-specific Antisera.

Virus rescued from pOLIO LEON, a full-length cDNA of the Leon strain of poliovirus type 3, was designated rLEON. Virus rescued from pVR106, a full-length cDNA of the Mahoney strain of poliovirus type 1 (Racaniello and Baltimore, 1982), was designated rMahoney. rLeon and rMahoney were characterised for their reaction with type specific poliovirus antisera in a standard neutralisation assay (Domok and Magrath, 1976). The assay was performed in 96 well microtitre plates. 100ul of virus suspension containing 10^5 $TCID_{50}$ (rows A - D) or 10^2 $TCID_{50}$ (rows E - F) were incubated for 3hrs at 35°C with 100ul of the following combinations of antisera:

Row		Row		Poliovirus Antisera
3	rLeon	8	rMahoney	Type I and Type II
4	rLeon	9	rMahoney	Type II and Type III
5	rLeon	10	rMahoney	Type I and Type III
6	rLeon	11	rMahoney	Type I, Type II and Type III
7	rLeon	12	rMahoney	Virus only

10^5 Hep 2c cells were added to each well. The plates were sealed and scored for CPE following 7 days incubation at 37°C.


B
X


B
X

Fig. 2. 8.

T1 RNase Fingerprint of Virus Rescued From pOLIO LEON.

T1 RNase oligonucleotide fingerprints were prepared for virus rescued from pOLIO LEON (A) and authentic P3/Leon/37 (B). The origin (not shown) is towards the bottom left hand corner of each fingerprint. X = position of xylene cyanol FF dye marker, B = position of bromophenol blue dye marker.

(b) Analysis of viral RNA. T1 oligonucleotide fingerprint analysis of viral RNA was carried out by Dr. P.D. Minor. ^{32}P-labelled RNA was prepared from purified virions of authentic P3/Leon/37 and rP3/LEON, as described previously (Minor, 1980). The fingerprints of both viral genomes obtained after RNase T1 digestion are shown in Fig. 2.7. Examination of the fingerprints indicated complete identity of all the large oligonucleotides. This result indicates that the genome structure of rP3/LEON is very similar and probably identical to P3/Leon/37.

(c) Temperature sensitivity. The rct (reproductive capacity at different temperatures) marker test is frequently used to compare seed poliovirus strains, attenuated viruses in vaccines and poliovirus strains excreted by vaccinees. This test is based on the temperature sensitivity of the poliovirus vaccine strains which replicate poorly at the restrictive temperature of 40°C. The titre of the virus is determined at 35°C and 40°C using the $TCID_{50}$ assay described in chapter 8. The rct^{40} value is the ratio of the $\log_{10}$ titre at 35°C to the $\log_{10}$ titre at 40°C, and is considered to be a stably inherited character of any particular strain. Table 2.1 shows the results of an rct marker test which revealed that rP3/LEON is indistinguishable from P3/LEON/37. Both viruses are `wild-type' with respect to their sensitivities to temperature. 71/303, a WHO reference strain of the Sabin type 3 vaccine, was included as a temperature sensitive control.

Table 2.1.

Temperature sensitivity of rP3/LEON.

	Virus	Titre at 35°C	Titre at 40°C	Reduction in Infectivity Titre 35/40°C (Log_{10})	
Experiment 1.	P3/Leon/37	7.75	6.50	1.25	rct/40$^+$
	71/303	7.25	0.50	6.75	rct/40$^-$
	rP3/LEON	8.50	7.25	1.25	rct/40$^+$
Experiment 2.	P3/LEON/37	7.75	6.50	1.25	rct/40$^+$
	71/303	7.00	0.50	6.50	rct/40$^-$
	rP3/LEON	7.75	6.50	1.25	rct/40$^+$

2.8 Summary and conclusions.

Full-length cDNAs of the Sabin type 3 vaccine strain, P3/Leon/12a$_1$b, and its neurovirulent progenitor, P3/Leon/37, were constructed. In each case the poliovirus cDNA was inserted into the PstI site of the plasmid pAT 153. The recombinant plasmid pOLIO LEON, which contained the cDNA copy of P3/Leon/37, was shown to be infectious. When Hep 2c monolayers were transfected with pOLIO LEON, 20-40 plaques were observed per 20ug of plasmid DNA. Comparison of the RNase T1 fingerprints of RNA purified from recovered virus and RNA purified from authentic P3/Leon/37 revealed that the genome structure of these viruses were very similar and probably identical. Recovered virus also had the same properties as P3/Leon/37 with respect to antigenicity and temperature sensitivity. These results indicate that the cDNA clone pOLIO LEON generates virus that is not significantly different from the parent virus, P3/Leon/37. pOLIO LEON can therefore be used to produce directed alterations in the genome of P3/Leon/37.

In contrast to the results obtained with pOLIO LEON, the recombinant plasmid pOLIO SABIN failed to yield virus in a series of transfection experiments. Identification of an internal restriction fragment of the poliovirus cDNA which appeared to contain a lethal mutation is described in the next chapter.

CHAPTER THREE

Partial Characterisation of a Defective Region of pOLIO SABIN.

3.1. Introduction.

pOLIO SABIN, a recombinant plasmid containing a full-length cDNA copy of P3/Leon/12a$_1$b, consistently failed to yield virus following transfection of Hep 2c monolayers. The specific infectivity of pOLIO LEON was estimated to be less than 10 PFU/ug plasmid DNA, which is in good agreement with results obtained in other laboratories (Racaniello and Baltimore, 1981; Semler et al., 1984). With such low yields of virus from cDNA, it may be impossible to differentiate between a non-viable mutant and a viable mutant in which the growth of the virus is severely retarded compared to wild-type virus. However, the Sabin type 3 vaccine strain grows to high titre in tissue culture at the permissive temperature of 35°C. It was therefore concluded that pOLIO SABIN was intrinsicaly non-viable.

The complete nucleotide sequence of P3/Leon/37 was derived from the cDNA insert of pOLIO LEON using a protocol which involved sub-cloning of random-sheared fragments into M13mp9. The integrity of the recombinant plasmid, pOLIO LEON was therefore never in doubt. The complete nucleotide sequence of P3/Leon/12a$_1$b, in contrast, was derived from overlapping sub-genomic cDNA clones. Some of these proved to be unsuitable for use in the construction of the complete cDNA copy. As a result, several of the sub-genomic, cDNA clones used in the construction of pOLIO SABIN had not been sequenced. It was possible that one of these cDNAs contained a lethal mutation. Such mutations could be an artifact of the cloning technique,

such as a reverse transcriptase error or a mutation introduced during the repair of the RNA.cDNA hybrid in E.coli. In this respect it should be noted that the restriction maps of pOLIO LEON and pOLIO SABIN are identical. The mutation is unlikely to be the result of a major rearrangement of the type reported to be associated with the hybrid cloning technique (Okayama and Berg, 1982). An alternative source of aberrant cDNA could be reverse transcription of a non viable genome. As discussed in section 1.6.2., populations of RNA genomes are heterogeneous and non-viable genomes are probably generated at high frequency during normal viral replication.

The aim of the work described in this chapter was to determine why pOLIO SABIN was non-viable. This was achieved by constructing a series of reciprocal recombinants between pOLIO SABIN and pOLIO LEON and assaying these recombinant cDNAs for infectivity by transfection of Hep 2c monolayers.

3.2. The construction of SLR 1 and SLR 2.

The construction of the first pair of reciprocal recombinants is outlined in Fig. 3.1. These constructions use the unique SmaI site at N2769 in the poliovirus cDNA and the unique SalI site at N651 in the vector, pAT 153. pOLIO SABIN and pOLIO LEON were digested to completion with SmaI and SalI to generate fragments of 5.0kb and 6.1kb. SLR 1 was constructed by ligating the 5.0kb fragment of pOLIO SABIN to the 6.1kb fragment of pOLIO LEON. The resulting plasmid contained a recombinant cDNA insert in which the 5′ terminal 2769 nucleotide residues were derived from pOLIO SABIN and remainder of the genome were derived from the pOLIO LEON. SLR 2 was constructed as the genetic reciprocal of SLR 1.

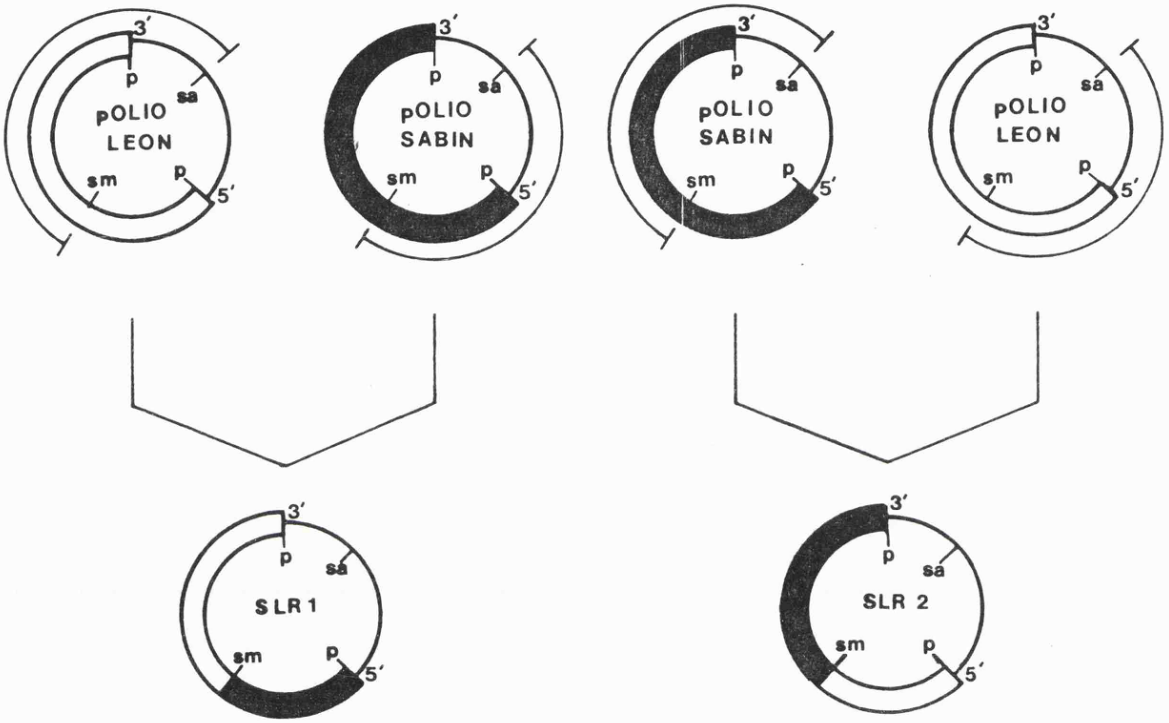

3′
p
sa
POLIO
LEON
sm
p
5′
3′
p
sa
POLIO
SABIN
sm
p
5′
3′
p
sa
POLIO
SABIN
sm
p
5′
3′
p
sa
POLIO
LEON
sm
p
5′
3′
p
sa
SLR 1
sm
p
5′
3′
p
sa
SLR 2
sm
p
5′

Fig. 3. 1.

The Construction of SLR 1 and SLR 2.

pOLIO LEON and pOLIO SABIN are recombinant plasmids containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. A pair of reciprocal recombinants were constructed between pOLIO LEON and pOLIO SABIN using the SmaI site at N2764 in the cDNA and the SalI site in the vector, pAT 153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences and filled double lines represent P3/Leon/12a$_1$b sequences. The restriction fragments used to construct SLR 1 and SLR 2 are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

p = PstI, s = SalI, sm = SmaI.

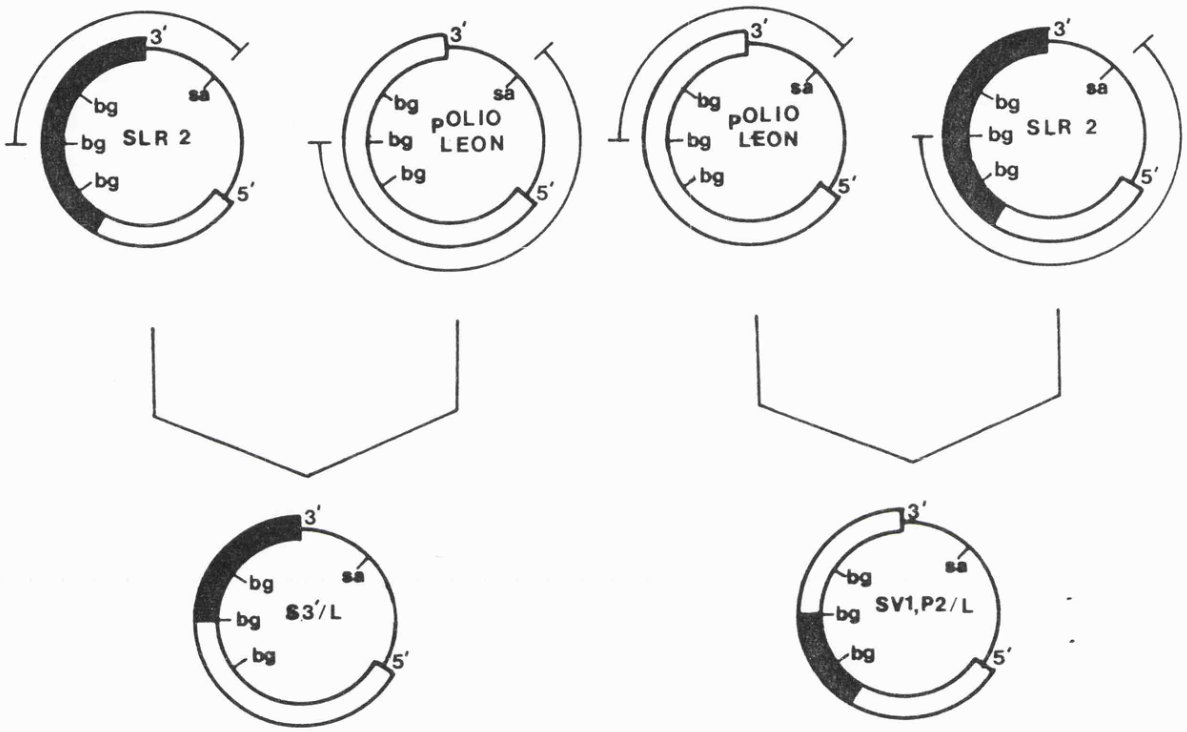
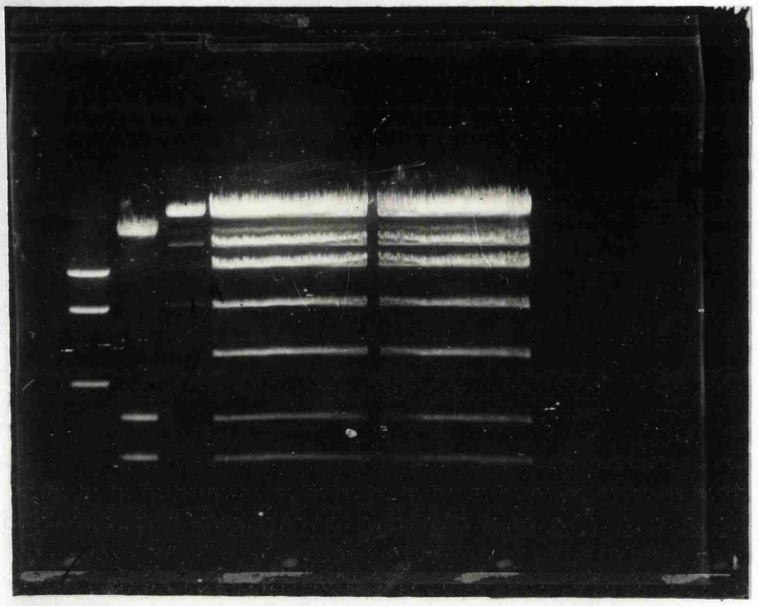

3'
bg
sa
bg
SLR 2
bg
5'
3'
bg
sa
bg
pOLIO
LEON
bg
5'
3'
bg
sa
bg
pOLIO
LEON
bg
5'
3'
bg
sa
bg
SLR 2
bg
5'
3'
bg
sa
bg
S3'/L
bg
5'
3'
bg
sa
bg
SV1,P2/L
bg
5'

Fig. 3. 2.

The Construction of S3'/L and SV1,P2/L.

pOLIO LEON and pOLIO SABIN are clones containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. SLR 2 contains a recombinant cDNA insert in which the 5' terminal 2764 residues were derived from pOLIO LEON and the remainder of the cDNA from pOLIO SABIN. A pair of reciprocal recombinants were constructed between SLR 2 and pOLIO LEON using the BglII site in the cDNA and the SalI site in the vector, pAT153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences and filled double lines represent P3/Leon/12a$_1$b sequences. The restriction fragments used to construct S3'/L and SV1,P2/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

bg = BglII, s = SalI.



SLR 2 xSal I xBgl II

M1 M2 10m 25m

5316
3703
2083
8215
1643
1244
11102
8586
7342
5412/
5699
3769
2525
1643
1244

Fig. 3. 3.

The Construction of S3'/L: Isolation of the 3.8kb BglII/SalI Fragment of SLR 2.

SLR 2, was linearised by digestion with SalI and then partially digested with BglII (1u enzyme/ ug DNA). Partial digestion products were separated by electrophoresis on a 1% agarose gel (2hrs at 100V). The products of a 10 minute digestion with BglII (10m) are shown, together with the products of a 25 minute (25m) digestion which was determined to be optimal for the isolation of the required 3.8kb fragment. pOLIO LEON digested with EcoRI (M1) or BglII (M2) were used as size markers. The diagram indicates the predicted size (bp) of each digestion product.

O = origin, B = position of bromophenol blue.

3.3. The construction of S3′/L and SV1,P2/L.

Fig. 3.2. shows the construction of a pair of reciprocal recombinants between SLR 2 and pOLIO LEON. These constructions used the BglII site at N5064 in the poliovirus cDNA and the SalI site at N651. There are two additional BglII sites in the cDNA, at N3421 and N6308. SLR 2 was digested to completion with SalI. The linearised plasmid was then partially digested with BglII. Fig. 3.3. shows the partial digestion products following electrophoresis on a 1% agarose gel for two hours. The 3.8kb fragment was electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. The second fragment used in the construction was obtained by SalI complete an BglII partial digestion of pOLIO LEON.

S3′/L was constructed by ligating the 3.8kb fragment of SLR 2 to the 7.3kb fragment of pOLIO LEON. The SalI site is located within the coding region of the tetracycline resistance gene of pAT 153. Therefore, when E.coli cells were transformed with the ligation mixture and plated out in the presence of tetracycline, only those cells containing the required plasmid were able to form colonies. S3′/L contains a recombinant cDNA insert in which the 5′ terminal 5064 nucleotides were derived from pOLIO LEON and the rest of the genome was derived from pOLIO SABIN. SV1,P2/L was constructed as the genetic reciprocal of S3′/L.

3.4. Transfection of Hep 2c monolayers with recombinant cDNAs.

Fig. 3.4. shows the structure of four recombinant cDNAs constructed between the infectious cDNA clone pOLIO LEON and the non-viable cDNA clone pOLIO SABIN. These were assayed for infectivity by transfection of Hep 2c monolayers using the calcium phosphate co-precipitation technique described in chapter 2. The results of the transfection experiments are summarized in Table 3.1.

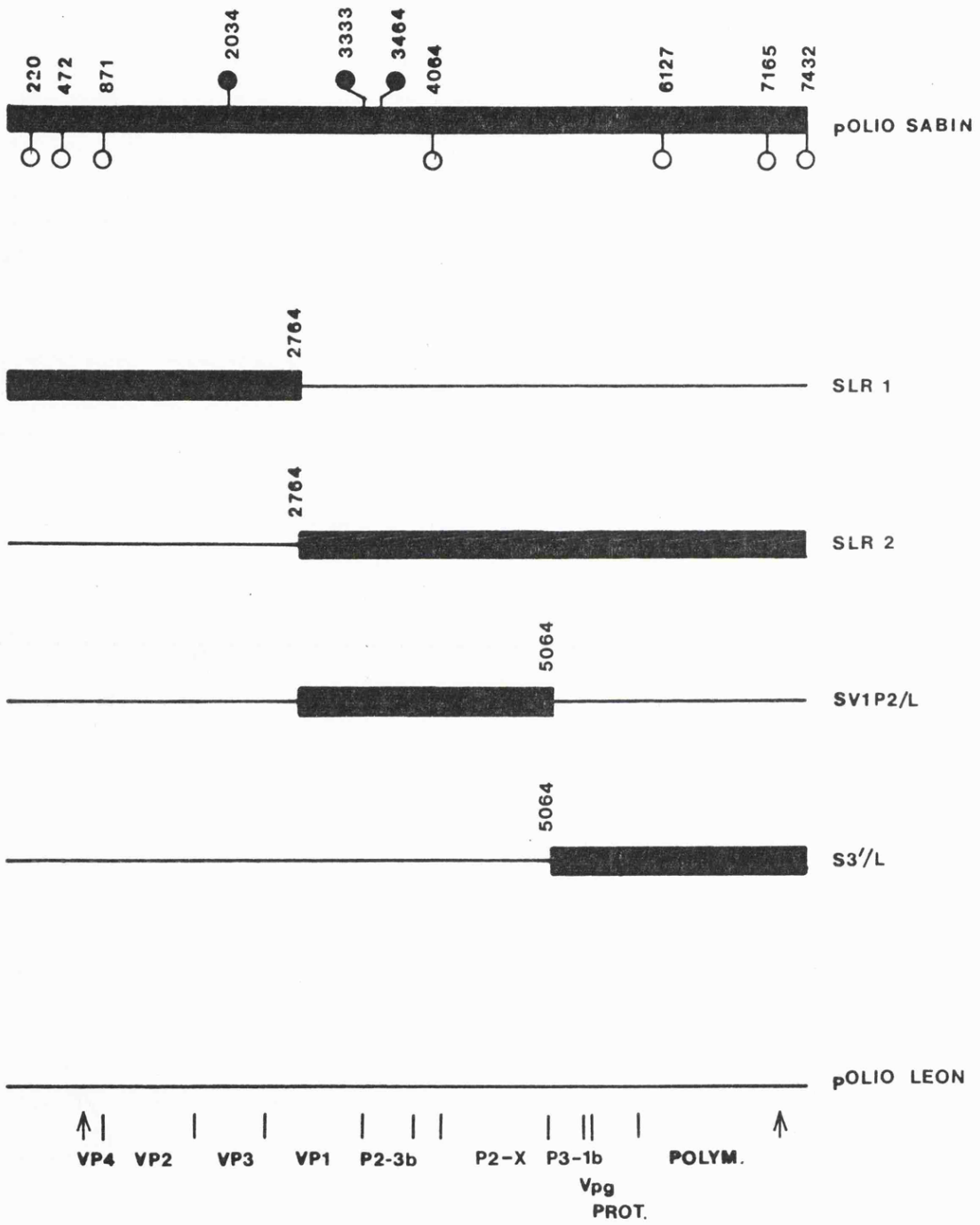

220
472
871
2034
3333
3464
4064
6127
7165
7432
pOLIO SABIN
2764
SLR 1
2764
SLR 2
5064
SV1P2/L
5064
S3'/L
pOLIO LEON
VP4
VP2
VP3
VP1
P2-3b
P2-X
P3-1b
Vpg
PROT.
POLYM.

Fig. 3. 4.

Structure of Recombinant Poliovirus cDNAs.

Single lines represent P3/Leon/37 sequences (derived from pOLIO LEON) and heavy lines represent P3/Leon/12a$_1$b sequences (derived from pOLIO SABIN). Nucleotide differences observed between P3/Leon/37 and P3/Leon/12a$_1$b are shown as open circles for the non-coding changes and filled circles for the coding changes. The positions of restriction sites used to construct the recombinants are shown in bp.

Table 3.1

Infectivity of Recombinant cDNAs.

Recombinant	Region of the genome derived from pOLIO SABIN	Infectivity following Transfection of Hep 2c monolayers.
SLR 1	5′ end - N2764	1.1×10^7 PFU/ml 3.0×10^5 PFU/ml
SLR 2	N2764- 3′ end	Non-viable
SV1,P2/L	N2764 - N5064	Non-viable
S3′/L	N5064 - 3′ end	1.2×10^7 PFU/ml 1.4×10^7 PFU/ml

pOLIO LEON was used as a positive control in each experiment and consistently yielded between 10^5 and 10^6 PFU/ml of virus. Similar titres were obtained for the recombinant SLR 1. SLR 2, in contrast, failed to yield virus in three separate transfection experiments and was therefore judged to be non-viable. These results suggested that a lethal mutation was located between residue 2764 and the 3′ terminus of the pOLIO SABIN cDNA.

SLR 2 was used to construct two further recombinants. S3′/L consistently yielded virus following transfection indicating that the region of the pOLIO SABIN cDNA from residue N5064 to the 3′ terminus was structurally intact. The reciprocal recombinant, SV1,P2/L, was shown to be non-viable in three separate transfection experiments in which pOLIO LEON had yielded virus. In this recombinant an internal restriction endonuclease fragment of pOLIO LEON, spanning residues 2764-5064, has been replaced with the corresponding region of pOLIO SABIN.

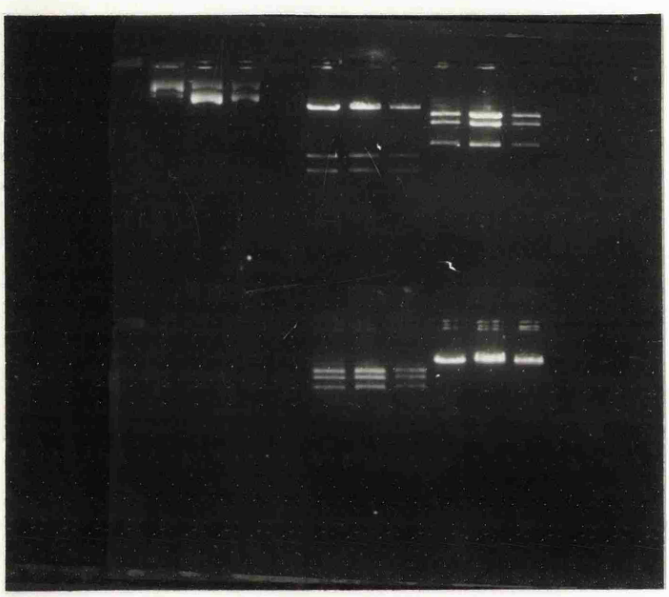


Fig. 3. 5.

Restriction Endonuclease Digestions of SLR 2 and SV1,P2/L: Comparison with pOLIO LEON.

Plasmids containing full-length cDNAs of the poliovirus genome are unstable in E.coli. To confirm that the non-viable clones, SLR 2 and SV1,P2/L, contained no gross deletions, a series of restriction endonuclease digests were performed. Restriction fragments obtained following digestion of SLR 2 and SV1,P2/L with BglII, EcoRI, PstI and SmaI were identical to those obtained following digestion of the viable clone, pOLIO LEON, with the same enzymes. In vivo deletion usually involved sequences in the 3' region of the genome, indicated by the loss of the 1.3kb BglII fragment (representing residues N5064 - N6308). The 1.3kb BglII fragment is indicated by an arrow.

	pOLIO LEON	SLR 2	SV1,P2/L
Uncut	a	b	c
X BglII	d	e	f
X EcoRI	g	h	i
X PstI	j	k	l
X SmaI	m	n	o

O = origin, B = position of bromophenol blue.

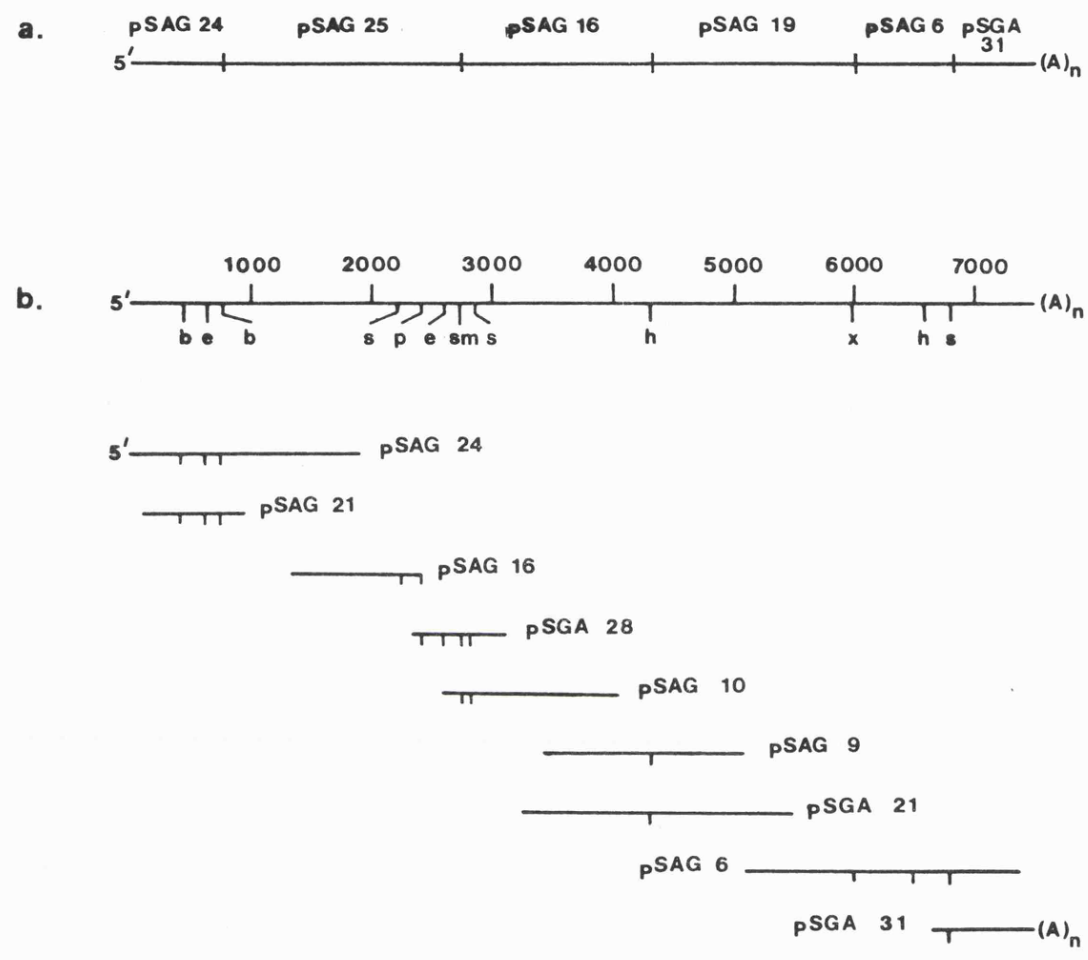

a.
pSAG 24
pSAG 25
pSAG 16
pSAG 19
pSAG 6
pSGA 31
5′
(A)n
b.
1000
2000
3000
4000
5000
6000
7000
5′
(A)n
b e b
s p e sm s
h
x
h s
5′
pSAG 24
pSAG 21
pSAG 16
pSGA 28
pSAG 10
pSAG 9
pSGA 21
pSAG 6
pSGA 31
(A)n
c.
5′
(A)n
2769
5064

Fig. 3. 6.

Summary of the Sub-genomic cDNAs Used to Derive the Complete Nucleotide Sequence of P3/Leon/12a1b.

The complete nucleotide sequence of P3/Leon/12a$_1$b was derived from a series of overlapping cDNAs cloned into the PstI site of pAT 153 (b). Many of these proved to be unsuitable for the construction of a complete cDNA copy. Note, for example, the absence of any convenient restriction endonuclease site in the overlapping regions of pSAG 24 and pSAG 16. Three additional cDNA clones were used to construct the complete cDNA copy of P3/Leon/12a$_1$b, designated pOLIO SABIN (a). These were pSAG 25, pSAG 16 and pSAG 19. pOLIO SABIN was shown to be non-viable following transfection of Hep 2c monolayers. The full-length cDNA appeared to contain a lethal mutation which was mapped to a region of the cDNA spanning residues N2769-N5064 (c). This region of the cDNA corresponds to sequences derived from pSAG 25, pSAG 16 and pSAG 19. No nucleotide sequence data were available for either of these clones.

The poliovirus cDNAs were known to be unstable in E.coli. For this reason, each of the plasmid preparations used in the transfection study was examined by restriction enzyme mapping for evidence of deletion mutations. Fig. 3.5. shows the restriction fragments obtained following digestion of SLR 2 and SV1,P2/L with EcoRI, BglII, PstI and SmaI. These were identical to those obtained for pOLIO LEON using the same enzymes. The non-viable clones therefore contained full-length poliovirus cDNAs. These digests also confirmed that the SmaI site used in the construction of SLR 2 and the BglII site used in the construction of SV1,P2/L had been recreated.

It was concluded that pOLIO SABIN contained a lethal mutation mapping between nucleotide residues 2769 and 5064. Although partial or complete nucleotide sequences were derived for several of the sub-genomic cDNA clones used in the construction of pOLIO SABIN, no nucleotide sequence data were available for this region of the cDNA (see Fig. 3.6.).

3.5. Nucleotide sequence analysis of recombinant polioviruses.

Only ten base substitution mutations were observed between P3/Leon/37 and P3/Leon/12a$_1$b. The restriction maps of pOLIO LEON and pOLIO SABIN were therefore identical. In order to confirm that viruses recovered from the recombinant cDNAs described above were bone fide poliovirus recombinants, partial nucleotide sequences were derived directly from purified viral RNA by primer extension. This was particularly important because it was not possible to demonstrate that recombination had taken place at the DNA level by restriction mapping.

Nucleotide sequences were obtained from viral RNA by the dideoxy chain termination method in an oligonucleotide primed reverse transcription reaction. The results of the nucleotide sequencing study, summarized in Table 3.2. are compatible with the genome structures of the recombinant cDNAs. For example, SLR 1 contains the 5′ terminal 2769 nucleotides of

pOLIO SABIN joined to the 3′ terminal 4664 nucleotides of pOLIO LEON. Nucleotide sequencing confirmed that mutations mapping to 5′ to the crossover site, specifically C-T at N472 and C-T at N2034 were `Sabin-like′ whereas a mutation mapping 3′ to the crossover site, A-G at N3333, was `Leon-like′. SLR 1 is therefore a bone fide poliovirus recombinant.

Table 3.2.

Nucleotide sequence analysis of recombinant polioviruses.

Virus	Base at position 472	Base at position 2034	Base at position 3333	Base at position 3464	Base at position 7165
P3/Leon/37	C	C	A	A	G
rSLR 1	T	T	A	ND	ND
rS3′/L	C	C	A	A	A
P3/Leon/12a$_1$b	T	T	G	G	A

3.7. Summary and Conclusions.

A series of reciprocal recombinants were constructed between the infectious cDNA pOLIO LEON and the non-viable cDNA, pOLIO SABIN. These were assayed for infectivity by transfection of Hep 2c monolayers. The results suggested that an internal restriction endonuclease fragment of the cDNA insert of pOLIO SABIN contained a non-viable mutation. This observation facilitated the construction of an infectious cDNA of P3/Leon/12a$_1$b, as described in the next chapter.

CHAPTER FOUR

The Construction of SABIN GDW

4.1 Introduction.

A non-viable mutation in the cDNA insert of pOLIO SABIN had been mapped to an internal SmaI/BglII restriction endonuclease fragment spanning nucleotide residues N2764-N5064. This chapter describes the construction of an infectious cDNA copy of P3/Leon/12a$_1$b by replacing the SmaI/BglII fragment of pOLIO SABIN with the corresponding region of an alternative cDNA clone.

pOLIO 119 is a recombinant plasmid containing a complete cDNA copy of P3/WHO/119, a neurovirulent revertant of P3/Leon/12a$_1$b (Stanway et al., 1984). pOLIO 119 was constructed prior to nucleotide sequencing studies. A random shearing protocol was used to sub-clone fragments of the poliovirus cDNA into M13mp9. The complete nucleotide sequence of the full-length poliovirus cDNA was then determined by the di-deoxy chain termination method. These studies revealed that only 7 base substitution mutations had occurred between P3/Leon/12a$_1$b and P3/WHO/119. Moreover the region spanning nucleotide residues N2764-N5064 was entirely conserved in P3/WHO/119 (see Fig. 4.1.). pOLIO 119 could therefore be used as a source of cDNA to replace the defective region of pOLIO SABIN.

SABIN GDW was constructed by recombination in vitro between

Sabin → 119

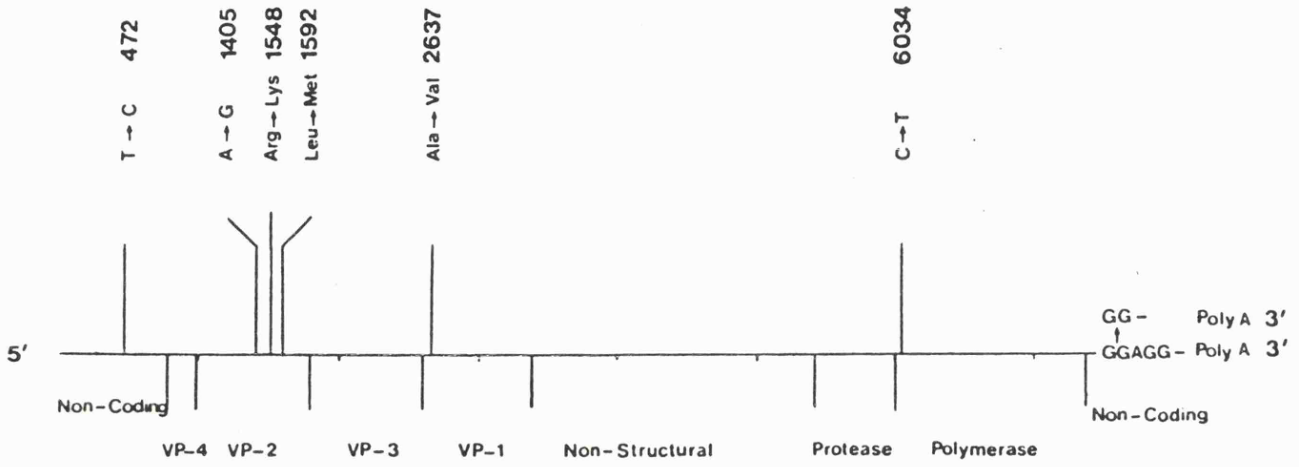

T → C 472
A → G 1405
Arg → Lys 1548
Leu → Met 1592
Ala → Val 2637
C → T 6034
5′
GG – Poly A 3′
GGAGG – Poly A 3′
Non-Coding
Non-Coding
VP-4
VP-2
VP-3
VP-1
Non-Structural
Protease
Polymerase

Fig. 4. 1.

Nucleotide Sequence Comparison of P3/Leon/12a$_1$b and P3/WHO/119.

The diagram illustrates a comparison of the RNA sequences of the Sabin type 3 vaccine, P3/Leon/12a$_1$b, and a neurovirulent revertant of the vaccine, P3/WHO/119. The positions of the nucleotide differences observed between the vaccine and the revertant are indicated and any amino acid changes which result are identified. It should be noted that the region of the vaccine strain spanning residues N2764 to N5064 is completely conserved in P3/WHO/119.

pOLIO SABIN and pOLIO 119. The genome structure of virus rescued from SABIN GDW was analysed by partial nucleotide seequencing of purified viral RNA. The biological characteristics of the cDNA derived virus was determined in the rct marker test and the WHO monkey neurovirulence assay. The results of these studies indicated that the cDNA derived virus was indistinguishable from P3/Leon/12a$_1$b.

4.2. The construction of S/119(1).

The first step in the construction of SABIN GDW was the construction of a S/119(1), a recombinant plasmid in which the 5′ terminal 2764 nucleotides were derived from pOLIO SABIN and the rest of the genome was derived from pOLIO 119. This construction used the unique SmaI site at N2764 in the poliovirus cDNA and the unique ClaI site at N23 in the vector, pAT 153.

pOLIO 119 was obtained from Dr. G. Stanway. The procedure used to construct S/119(1) is shown in Fig. 4.2. SmaI and ClaI double digestion of pOLIO SABIN generates fragments of 5.4kb and 5.7kb. It would not be possible to resolve these fragments by agarose gel electrophoresis. An additional enzyme was therefore used to digest the unwanted fragment. pOLIO SABIN was digested to completion with SmaI, ClaI and BglII to generate the 5.7kb SmaI/ClaI fragment and several smaller fragments formed by BglII digestion of the 5.4kb SmaI/ClaI fragment. Similarly, pOLIO 119 was digested with BamHI in addition to SmaI and ClaI in order to resolve the 5.4kb SmaI/ClaI fragment.

The 5.7kb fragment of pOLIO SABIN was ligated to the 5.4kb fragment of pOLIO 119. Competent E.coli JA221 were transformed with 100ng of ligated DNA and plated out in the presence of tetracycline. 23 out of 24 tet^r transformants appeared to contain full-length recombinant plasmids, as judged by agarose gel electrophoresis of plasmid DNA, using pOLIO LEON as a

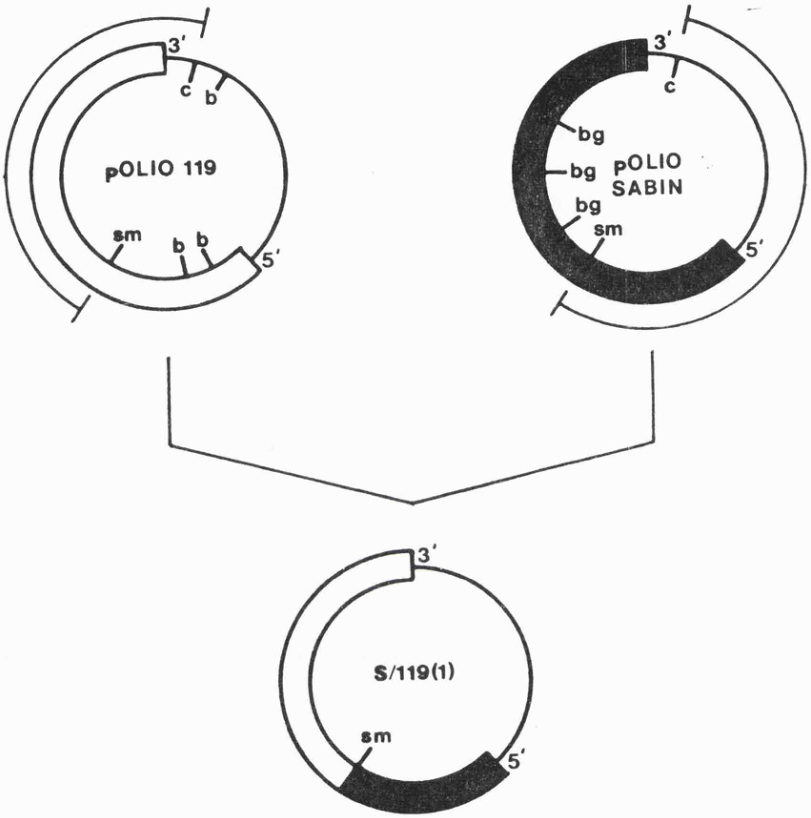

3'
c
b
pOLIO 119
sm
b
b
5'
3'
c
bg
bg
bg
pOLIO
SABIN
sm
5'
3'
S/119(1)
sm
5'

Fig. 4. 2.

The Construction of S/119(1).

pOLIO SABIN is a non-viable clone containing a full-length cDNA of P3/Leon/12a$_1$b. The non-viable lesion was mapped to a restriction endonuclease fragment spanning residues N2674 to N5064 of the cDNA. pOLIO 119 is an infectious clone containing a full-length cDNA of P3/WHO/119. The region spanning residues N2764 to N5064 is entirely conserved between P3/Leon/12a$_1$b and P3/WHO/119. An infectious cDNA clone of P3/Leon/12a$_1$b was constructed by substituting the defective region of pOLIO SABIN with the corresponding region of pOLIO 119. The first step was the construction of S/119(1) in which the 5' terminal 2769 residues of the cDNA were derived from pOLIO SABIN and the remainder of the cDNA from pOLIO 119 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Filled double lines represent P3/Leon/12a$_1$b sequences and open double lines represent P3/WHO/119 sequences. The restriction fragments used to construct S/119(1) are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

b = BamHI, bg = BglII, c = ClaI, sm = SmaI

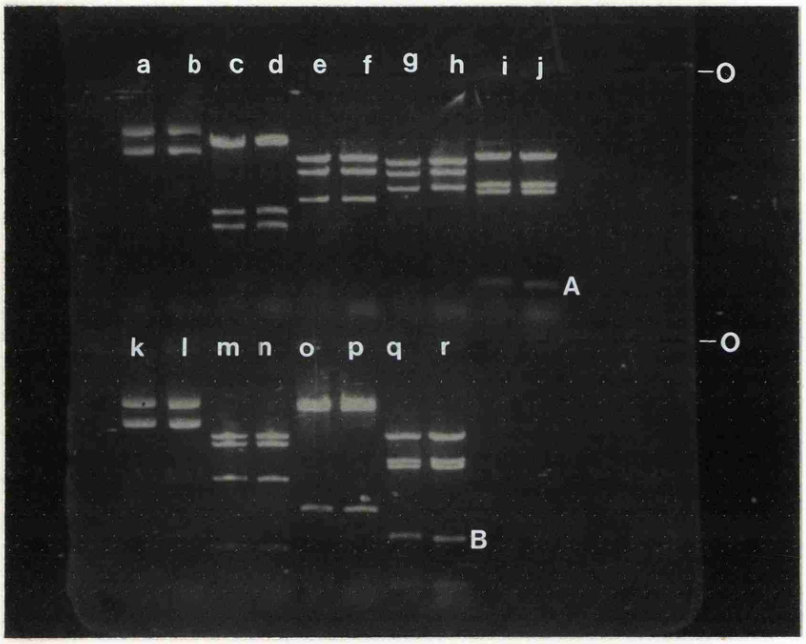

a b c d e f g h i j
O
A
k l m n o p q r
O
B

Fig. 4. 3.

Restriction Endonuclease Digestion of pOLIO 119:

Comparison with pOLIO SABIN.

Restriction fragments obtained following digestion of pOLIO SABIN with BglII, EcoRI and SstI are identical to those obtained following digestion of pOLIO 119 with the same enzymes. The 711bp KpnI B fragment of pOLIO SABIN corresponds to residues N2595 to N3306 of the cDNA. The C-T substitution at N2637 creates an additional KpnI site in the cDNA. As a result the KpnI B fragment of pOLIO 119 shows a small deletion compared to the KpnI B fragment of pOLIO SABIN. It also appears that the ScaI A fragment of pOLIO 119 is deleted compared with the ScaI A fragment of pOLIO SABIN. This fragment contains the PstI site at the 3' terminus of the cDNA. In this case the deletion may be the result of variation in the length of the poly(A) tract.

	pOLIO SABIN	pOLIO 119
uncut	a	b
X BglII	c	d
X EcoRI	e	f
X PstI	g	h
X ScaI	i	j
uncut	k	l
X SphI	m	n
X SstI	o	p
X KpnI	q	r

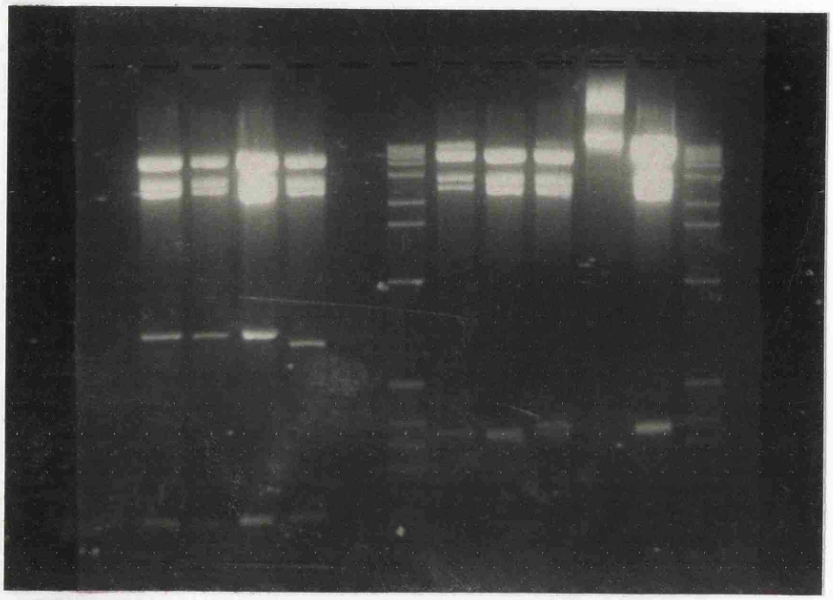


Fig 4. 4.

Restriction Endonuclease Digestion of S/119(1).

The mutation at N2634 in P3/WHO/119 generates an additional KpnI site in the cDNA. As a result, 45bp are deleted from the KpnI B fragment. The KpnI B fragment of S/119(1) comigrates with the KpnI B fragment of pOLIO SABIN, indicating that S/119(1) lacks the mutation at N2637. The ScaI A fragment of pOLIO 119 appears to carry a deletion compared to the ScaI A fragment of pOLIO SABIN, reflecting variation in the length of th poly(A) tract. The ScaI A fragment of S/119(1) comigrates with the ScaI a fragment of pOLIO 119, suggesting that S/119(1) carries the 3' end of the pOLIO 119 cDNA. These results are consistent with the predicted structure of S/119(1) (see text for details).

	X KpnI	X ScaI
pOLIO SABIN	a	h
pOLIO LEON	b	g
S/119(1)	c	f
pOLIO 119	d	e

size marker. One of these plasmids, designated S/119(1) was selected for further study.

S/119(1) was shown to contain a recombinant cDNA insert by the following procedure. Fig. 4.3. shows a comparison of the fragments obtained following restriction endonuclease digestions of pOLIO 119 and pOLIO SABIN. The 711bp KpnI fragment of pOLIO 119, spanning nucleotide residues N2595-N3305, appears to contain a small deletion when compared to the corresponding fragment of pOLIO SABIN. Examination of the nucleotide sequences of P3/Leon/12a$_1$b and P3/WHO/119 revealed that the base substitution mutation C-T at N2637 in P3/WHO/119 generates an additional KpnI site in the cDNA. Similarly, the 364bp ScaI fragment of pOLIO 119 appears to contain a small deletion when compared to the corresponding fragment of pOLIO SABIN. In this case the ScaI fragment spans the PstI site at the 3′ end of the cDNA. The deletion observed in the 364bp ScaI fragment of pOLIO 119 presumably reflects variation in the length of the poly(A) tail and/or the poly(G.C) tract. Digestion of S/119(1) with KpnI and ScaI revealed that the cDNA was Sabin-like at position N2637 but carried the 119 3′ end (see Fig. 4.4.).

4.3. The Construction of SABIN GDW.

The cDNA insert of S/119(1) differs in nucleotide sequence from P3/Leon/12a$_1$b at only three positions. S/119(1) carries two 119-specific mutations. These are C-T at N6034, a silent mutation in the region of the viral RNA encoding the protease, and G-A at N7431 in the 3′ non-coding region. In addition, reversion of a Sabin-specific mutation has occurred by direct back-mutation at N7432. SABIN GDW, a recombinant plasmid with a cDNA insert identical in nucleotide sequence to P3/Leon/12a$_1$b, was constructed by recombination between S/119(1) and pOLIO SABIN (see Fig. 4.6). This construction used the BglII site at N5064 in the poliovirus cDNA and the

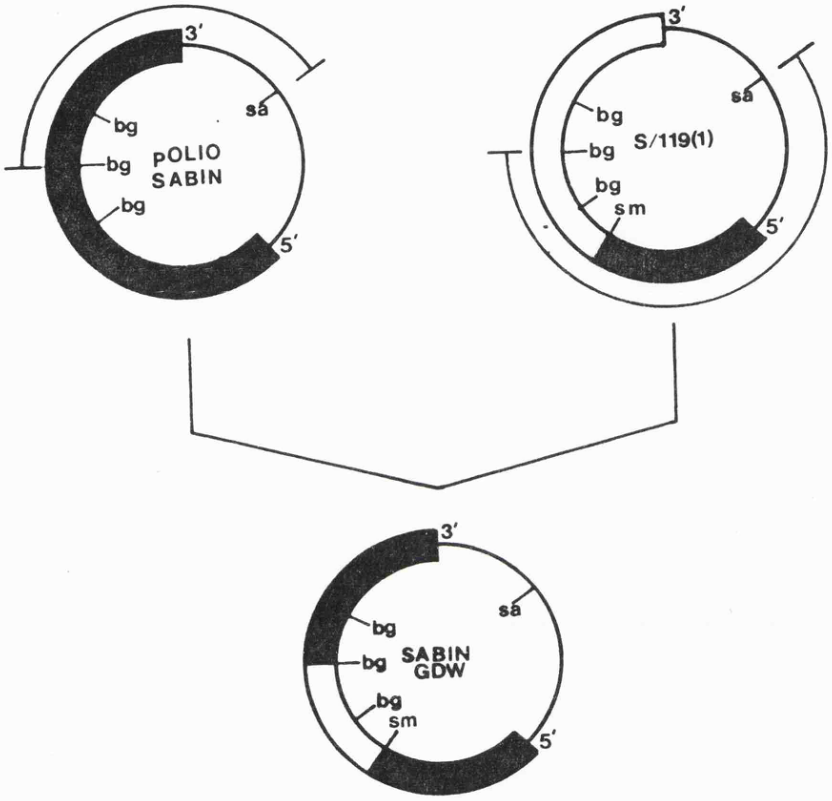

3'
sa
bg
bg
POLIO
SABIN
bg
5'
3'
sa
bg
bg
S/119(1)
bg
sm
5'
3'
sa
bg
bg
SABIN
GDW
bg
sm
5'

Fig. 4. 5.

The Construction of SABIN GDW.

SABIN GDW, an infectious clone containing a full-length cDNA of P3/Leon/12a$_1$b, was constructed from S/119(1) and pOLIO SABIN using the BglII site at N5064 in the cDNA and the SalI site in the vector, pAT 153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Filled double lines represent P3/Leon/12a$_1$b sequences and open double lines represent P3/WHO/119 sequences. Restriction fragments used to construct SABIN GDW are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

bg = BglII, Sm = SmaI, Sa = SalI.

SalI site at N651 in the vector. There are two additional BglII sites in the cDNA, at N3421 and N6308. pOLIO SABIN was digested to completion with SalI and the linearised plasmid was partially digested with BglII. The partial digestion products were resolved by electrophoresis on a 1% agarose gel and the 3.8kb fragment was electroeluted and purified. The second fragment used in the construction was obtained by SalI complete and BglII partial digestion of S/119(1).

SABIN GDW was formed by ligating the 3.8kb fragment of pOLIO SABIN to the 7.3kb fragment of S/119(1). Competent E.coli JA221 were transformed with 100ng of ligated DNA and plated out in the presence of tetracycline. A full length recombinant plasmid, isolated from a tet^r transformant, was designated SABIN GDW. SmaI and BglII digestion of SABIN GDW confirmed that the sites used in the construction had been recreated.

4.3. Transfection of Hep 2c monolayers with SABIN GDW.

Sub-confluent monolayers of Hep 2c were transfected with 20ug of SABIN GDW per 2×10^6 cells, using the calcium phosphate co-precipitation technique described in chapter 8. The transfected monolayers were incubated under liquid medium at 35°C. First signs of cytopathic effect were observed 5 to 6 days post transfection. Supernatent media were harvested by three cycles of freeze thawing at -20°C. The infectivities of the media were assayed in Hep 2c monolayers by plaque assay. Between 10^5 and 10^7 PFU/ml was obtained for each of four plates transfected. No infectivity was detected in media transfected with pOLIO SABIN, which was used as a negative control.

4.4. Characterisation of virus rescued from SABIN GDW by transfection.

The results of the following series of experiments provided strong evidence that virus rescued from SABIN GDW by transfection is not significantly different from reference preparations of P3/Leon/12a$_1$b.

(a) Antigenicity. Neutralisation tests were performed in Hep 2c monolayers using standard monospecific antisera. The results confirmed that virus rescued from SABIN GDW was a type 3 poliovirus (designated rP3/SABIN). 138 is a highly specific monoclonal antibody which neutralises P3/Leon/12a1b but not its progenitor P3/Leon/37 or other wild-type strains (Ferguson et al., 1984). rP3/SABIN was recognised by monoclonal antibody 138 in a neutralisation test, demonstrating that the cDNA derived virus was antigenically very similar to P3/Leon/12a$_1$b.

(b) Nucleotide sequence analysis of viral RNA. Partial nucleotide sequences were derived directly from purified viral RNA by primer extension. The presence of 5 of the 10 Sabin-specific mutations was confirmed (as shown in Table 4.1.). SABIN GDW was derived by recombination in vitro between pOLIO 119. In order to confirm that each step in the construction had been successful, nucleotide sequences were derived for regions of the viral RNA containing 119-specific mutations (see Table 4.2). The first step in the construction was verified by the observation that virus rescued from S/119(1) carried the 119-specific mutation at N6034 but was Sabin-like at positions N472 and N2637. Virus rescued from SABIN GDW was shown to be Sabin-like at position N6034. These results confirm that SABIN GDW is a complete cDNA copy of P3/Leon/12a$_1$b.

Table 4.1

Nucleotide sequence analysis of rP3/SABIN.

(a) Sabin-specific mutations.

Virus	Base at position 472	Base at position 2034	Base at position 3333	Base at position 3464	Base at position 7165
P3/Leon/37	C	C	A	A	G
rP3/SABIN	U	U	G	G	A
P3/Leon/12a$_1$b	U	U	G	G	A

Table 4.2

Nucleotide Sequence analysis of rP3/SABIN.

(b) 119-specific mutations.

Virus	Base at position 472	Base at position 1548	Base at position 1592	Base at position 2637	Base at position 6034
P3/WHO/119	C	A	A	U	U
rP3/S/119(1)	U	G	U	C	U
rP3/SABIN	U	G	U	C	C
P3/Leon/12a$_1$b	U	G	U	C	C

(c) Temperature sensitivity. The rct marker test is the most reliable biological test for vaccine standardisation in vitro. The test is based on the temperature sensitivity of the Sabin vaccine strains which replicate poorly at the supra-optimal temperature of 40°C. The reduction in infectivity titre, calculated as the ratio of the $\log_{10}$ titre at 35°C to the $\log_{10}$ titre at 40°C is considered to be a stably inherited character of any particular strain. The rct marker test was performed on rP3/SABIN as described in chapter 8. P3/Leon/37 and P3/WHO/119 were used as wild-type controls in each experiment. 71/303, a WHO reference preparation of P3/Leon/12a_1b, was included as a positive control. The results shown in Table 4.3 demonstrate that rP3/SABIN is indistinguishable from P3/Leon/12a_1b with respect to temperature sensitivity. The results also conform to National Control criteria which require that virus for use a a poliomyelitis vaccine should show a reduction in infectivity titre at 40°C of at least 5.00 $\log_{10}$ $TCID_{50}$ (WHO, 1983). Similar results were obtained for isolates of rP3/SABIN from three independent transfection experiments.

Table 4.3.

Temperature sensitivity of rP3/SABIN.

	Virus	Titre at 35°C	Titre at 40°C	Reduction in Infectivity Titre 35/40°C (log_{10})	
Experiment 1.	P3/Leon/37	7.25	6.00	1.25	rct/40$^+$
	71/303	6.75	0.75	6.00	rct/40$^-$
	P3/WHO/119	7.50	6.75	0.75	rct/40$^+$
	rP3/SABIN	8.25	1.75	6.50	rct/40$^-$
Experiment 2.	P3/Leon/37	7.00	6.00	1.00	rct/40$^+$
	71/303	6.75	0.75	6.00	rct/40$^-$
	P3/WHO/119	7.75	7.25	0.50	rct/40$^+$
	rP3/SABIN	7.75	1.50	6.25	rct/40$^-$

4.6 Neurovirulence of cDNA derived viruses.

Quantitative neurovirulence tests were performed in cynomolgus monkeys according to WHO guidlines (WHO, 1983). Briefly, each of four monkeys were inoculated with between 6.50 and 7.50 $\log_{10}$ $TCID_{50}$ of virus, by injection into the lumbar region of the spinal chord. Monkeys were observed over a period of 22 days for signs suggestive of poliomyelitis. Animals which became moribund or were severely paralysed were killed and autopsied. Surviving animals were killed and autopsied at the end of the observation period. For each monkey, representative sections of the brain and spinal chord were examined histologicaly for evidence of viral activity. A standard scoring system was employed to evaluate the extent of the virus specific damage. This system also takes into account the nature of the damage, whether immune infiltration or the destruction of neurons. A lesion score was calculated for each monkey and a mean lesion score was calcuted for each group of monkeys.

The neurovirulence of the cDNA derived viruses, rP3/LEON and rP3/SABIN are compared with results previously obtained for the respective parental strains, P3/Leon/37 and P3/Leon/12a_1b, in Table 4.4. rP3/LEON is indistinguishable from P3/Leon/37. Both viruses are highly neurovirulent, causing paralysis in all test monkeys during the first week of the test. In contrast, rP3/SABIN was shown to be highly attenuated. Each of the monkeys inoculated with rP3/SABIN survived the test with no indication of paralysis or clinical poliomyelitis. Virus specific damage in the CNS was minimal as indicated by the extremely low mean lesion score of 0.11. The lesion score is significantly lower than the range observed for reference preparations of P3/Leon/12a_1b in eight neurovirulence assays carried out over a period of two years at the N.I.B.S.C. (0.43-0.79) (D. Magrath, personal communication). On the basis of these results r P3/SABIN would be acceptable for use as a vaccine.

Table 4.4.

Neurovirulence of cDNA derived viruses.

Virus	Number of animals paralysed	Number of animals killed	Mean hisologic lesion score
P3/Leon/37	6/6	5/6	2.91
rP3/LEON	4/4	4/4	2.71
P3/Leon/12a_1b	0/18	0/18	0.72
rP3/SABIN	0/4	0/4	0.11

Scoring system for viral activity

1. Cellular infiltration only
2. Cellular infiltration with minimal neuronal damage
3. Cellular infiltration with extensive neuronal damage
4. Massive neuronal damage

4.7 Summary and conclusions.

This chapter reports the construction of SABIN GDW, a recombinant plasmid containing a full-length cDNA copy of P3/Leon/12a$_1$b. Virus rescued from SABIN GDW by transfection (rP3/SABIN) was shown to be indistinguishable from P3/Leon/12a$_1$b with respect to antigenicity, primary genome structure and temperature sensitivity. By the same criteria, virus recovered from pOLIO LEON (rP3/LEON) was shown to closely resemble the parental virus, P3/Leon/37.

The neurovirulence of these cDNA derived viruses was compared using the Standard WHO Neurovirulence Assay (WHO, 1983). The results were unequivocal. rP3/LEON was shown to be highly neurovirulent. Severe flaccid paralysis was induced in each of the test monkeys within one week of inoculation. Clinical disease was correlated in each case with massive neuronal damage throughout the CNS. rP3/SABIN, in contrast, was shown to be highly attenuated. All monkeys survived the test period without clinical symptoms and only a minimal degree of virus specific damage was observed in the CNS.

The observation that the cDNA derived viruses have the same biological characteristics of the respective parental viruses is of great significance. The complete nucleotide sequences of P3/Leon/37 and P3/Leon/12a$_1$b were derived from cloned cDNA (Stanway et al., 1984a,b). A major disadvantage with this approach is that individual cDNA clones may be unrepresentative of the total population of viral RNA. The observation that pOLIO SABIN is non-viable suggests that at least one of the sub-genomic cDNAs used in its construction contains an aberrant mutation (see chapter 3). An aberrant cDNA clone was more precisely characterised during nucleotide sequencing studies of P3/WHO/119 (Almond et al., 1984). A base substitution mutation was identified at N2570 which results in a glycine to arginine substitution mutation in the N-terminal region of VP1. Further DNA

sequencing revealed that alternative sub-genomic cDNAs of P3/WHO/119 do not possess this mutation. The results presented in this section provide direct evidence that the published sequences of P3/Leon/37 and P3/Leon/12a$_1$b accurately reflect the 'average sequence' of the parental virus populations. It also follows that the 10 base substitution mutations observed between P3/Leon/37 and P3/Leon/12a$_1$b must account for the marked differences in neurovirulence observed between the two strains.

The availability of infectious cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b allows a molecular genetic approach to the analysis of the determinants of attenuation. The results presented in Chapter 3 demonstrate that it is possible to segregate potential attenuating mutations from P3/Leon/12a$_1$b by constructing specific inter-strain recombinants <u>in-vitro</u> using conserved restriction endonuclease sites in the cDNA. Recombinant virus, rescued by transfection, could then be assayed for neurovirulence and in this way the significance of individual Sabin-specific mutations can be determined.

The construction of two specific inter-strain recombinants was reported in Chapter 3. Additional recombinants were required for a complete genetic analysis of the attenuation of P3/Leon/12a$_1$b. These were derived by the procedures described in Chapter 5.

CHAPTER FIVE

The Construction of inter-strain poliovirus recombinants.

5.1 Introduction.

Nucleotide sequence comparison revealed that only ten base substitution mutations distinguish the Sabin type 3 vaccine, P3/Leon/12a$_1$b, from its neurovirulent progenitor, P3/Leon/37 (Stanway et al., 1984). These mutations must account for the attenuated phenotype of the Sabin vaccine. Elucidation of the complete nucleotide sequence of P3/WHO/119, a neurovirulent revertant of the Sabin vaccine, allowed a re-evaluation of the likely significance of the individual Sabin-specific mutations (Cann et al., 1984).

Analysis of the data suggested that there were three possible genetic bases for attenuation. The first of these involved a C-U substitution at position 472 in the 5′ non-coding region. This mutation was implicated by evidence of direct back-mutation in the revertant. An A-G substitution at position 7432 in the 3′ non-coding region may also be significant. This mutation reverts by direct back-mutation in P3/WHO/119. However the revertant is not entirely wild-type in this region as an additional mutation was observed in the adjacent position (G-A at N7431).

The third possible mechanism is one of a functional change induced in a virus-specific protein by an amino acid substitution. Three of the mutations observed between the Sabin vaccine and its progenitor result in

amino acid changes. The A-U substitution at N2034 induces a serine-phenylalanine substitution in the structural protein VP3. The A-G substitution at N3333 causes the chemically more conservative, lysine-arginine change in the carboxy terminal region of VP1. Finally, the A-G substitution at N3464 induces a threonine-alanine change in the non structural protein, P2-3b. Any one or combination of these coding changes may contribute towards the attenuated phenotype of the Sabin vaccine. Here the mechanism of reversion would be suppression, as each of these mutations is conserved in P3/WHO/119.

The preceding chapters describe the construction of pOLIO LEON, a full-length cDNA clone of P3/Leon/37, and SABIN GDW, a full-length cDNA clone of P3/Leon/12a$_1$b. The infectious agents isolated from Hep 2c monolayers transfected with pOLIO LEON or SABIN GDW were shown to be identical to the respective parental viruses. Following these observations a direct approach to determining the effects of the mutations discussed above becomes possible. This involves the construction of recombinant genomes via infectious cDNA <u>in vitro</u> and rescue of recombinant virus by transfection.

The first experiments of this type were described in chapter 3. Two of the four recombinant cDNAs constructed between pOLIO LEON and pOLIO SABIN were shown to be infectious following transfection of Hep 2c monolayers. Virus rescued from the recombinant plasmid SLR 1 derives its 5′ terminal 2764 residues from the vaccine strain and the remainder of its genome from P3/Leon/37. rSLR 1 (for rescued SLR 1) therefore carries two of the mutations of interest, at positions 472 and 2034 (Ser-Phe in VP3). Virus rescued from S3′/L derives its 5′ terminal 5064 residues from P3/Leon/37 and the remainder of its genome from the Sabin vaccine. In this recombinant, therefore, the base substitution mutation at N7432 has been segregated from other potentially attenuating mutations.

This chapter describes the construction of additional inter-strain poliovirus recombinants. Each of the five potentially attenuating mutations were transferred independently to the parental virus genome. By subjecting these recombinants to neurovirulence assay it should be possible to identify the genetic determinants of attenuation.

5.2. The construction of recombinants between SLR 1 and pOLIO LEON.

5.2.1. The construction of SV3/L.

The plasmid SLR 1 contains a recombinant cDNA insert which derives its 5′ terminal 2764 residues from the pOLIO SABIN and the remainder of its genome from pOLIO LEON. The recombinant therefore carries four Sabin-specific mutations of which two were implicated as possible determinants of attenuation by nucleotide sequence comparisons (Stanway et al., 1984; Cann et al., 1984). These were the base substitution mutation at N2034 which results in an amino acid substitution Ser-Phe in VP3 and the C-U substitution at N472 in the 5′ non-coding region. The first construction was designed to segregate the VP3 mutation from SLR 1, using the NdeI site at N1580 in the cDNA (see Fig. 5.1.).

There are two additional NdeI sites in the cDNA, at N3374 and N6446. SLR 1 was digested to completion with NdeI and the 1.8kb fragment (corresponding to poliovirus nucleotides 1580-3374) was isolated and purified. The second fragment used in the construction was obtained by partial digestion of pOLIO LEON with NdeI. The partial digestion products were separated by electrophoresis on a 0.5% agarose gel. The required fragment of 9.3kb was only poorly resolved from partial digestion products of 11.1kb and 8.1kb. These three fragments were electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. The 11.1kb fragment and the 8.1kb fragment contained an internal SmaI fragment which

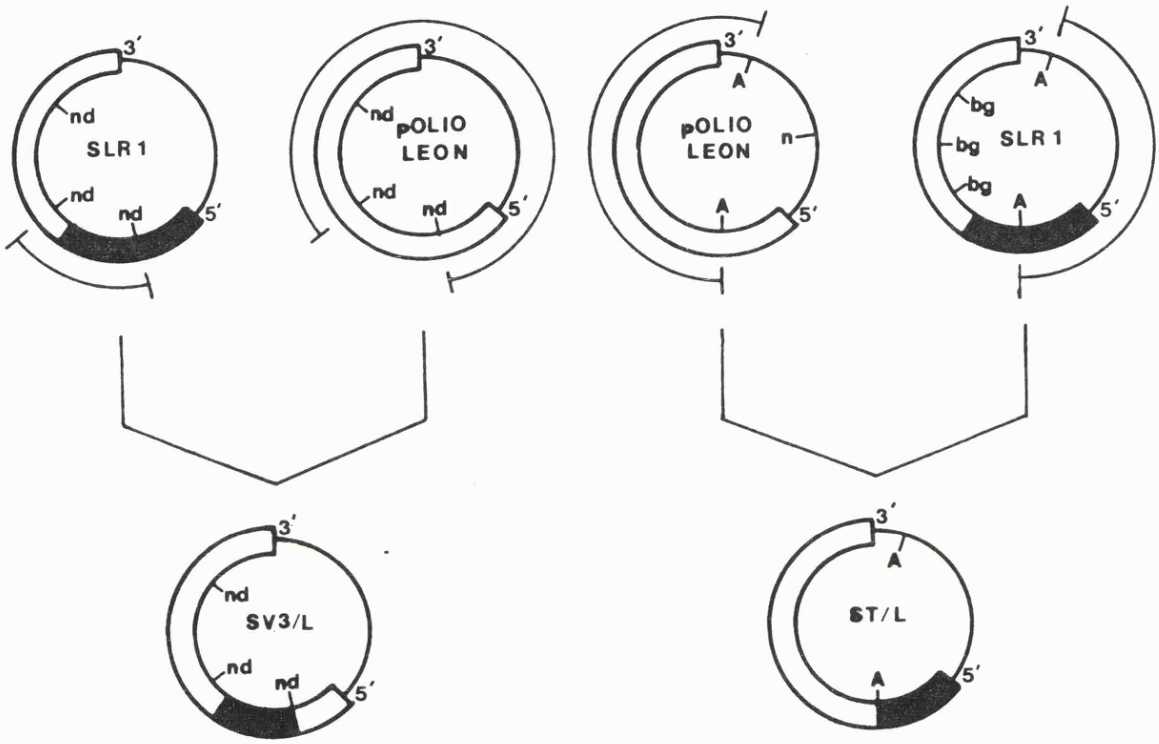

3′
nd
SLR 1
nd
nd
5′
3′
nd
pOLIO
LEON
nd
nd
5′
3′
A
pOLIO
LEON
n
A
5′
3′
A
bg
bg
SLR 1
bg
A
5′
3′
nd
SV3/L
nd
nd
5′
3′
A
ST/L
A
5′

Fig. 5. 1.

The Construction of SV3/L and ST/L.

pOLIO LEON is a clone containing a full-length cDNA of P3/Leon/37. SLR 1 contains a recombinant cDNA insert in which the 5' terminal 2764 residues were derived from P3/Leon/12a$_1$b and the rest of the genome from P3/Leon/37. A pair of recombinants were constructed between SLR 1 and pOLIO LEON (see text for details). SV3/L and ST/L are genetic reciprocals (although the restriction sites used in their construction were not identical). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences (derived from pOLIO LEON) and filled double lines represent P3/Leon/12a$_1$b sequences derived from pOLIO SABIN). The restriction fragments used to construct SV3/L and SV1/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

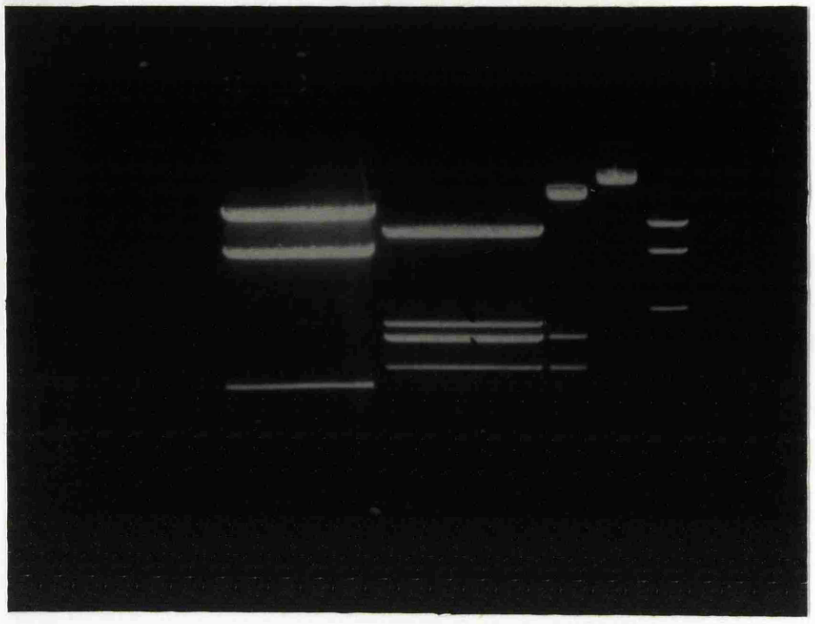
nd = NdeI, n = NruI, bg = BglII, A = AatI.

was absent from the 9.3kb fragment. The recovered DNA was therefore digested to completion with SmaI and treated with calf-intestinal phosphatase. The purpose of this treatment was to inactivate the contaminating fragments, both of which would form viable plasmids following self-ligation or ligation with the 1.8kb NdeI fragment of SLR 1.

The 1.8kb fragment of SLR 1 was ligated to the partially purified 9.3kb fragment of pOLIO LEON. Competent E.coli JA221 were transformed with approximately 100ng of ligated DNA and plated out in the presence of tetracycline. 12 out of 24 tetracycline resistant transformants appeared to contain full-length recombinant plasmids as judged by agarose gel electrophoresis using pOLIO LEON as a size marker. Several plasmids were digested with EcoRI and a plasmid in which the insert was in the correct orientation was selected. Recreation of the sites used in the construction was confirmed by digestion with NdeI. This clone, designated SV3/L, contained a recombinant cDNA insert which carried the Sabin specific VP3 mutation but was otherwise entirely Leon-like.

5.2.2. The construction of ST/L.

There are two AatII sites in pOLIO LEON, located at 1809 in the poliovirus cDNA and at N3585 in the vector. The N1809 site was used to segragate the three non-coding changes from SLR 1. AatII digests pOLIO LEON to generate fragments of 6.3kb and 4.8kb. Experience had shown that prolonged electrophoresis would be required to resolve these fragments. It was therefore decided to use additional enzymes to increase the resolution of the desired fragment and prevent cross-contamination. SLR 1 was digested with AatII and BglII to generate the 4.8kb AatII fragment and four smaller fragments produced by BglII digestion of the 6.3kb AatII fragment. Similarly, digestion of pOLIO LEON with NdeI in addition to AatII increased the resolution of the 6.3kb AatII fragment (see Fig. 5.2). The 4.8kb



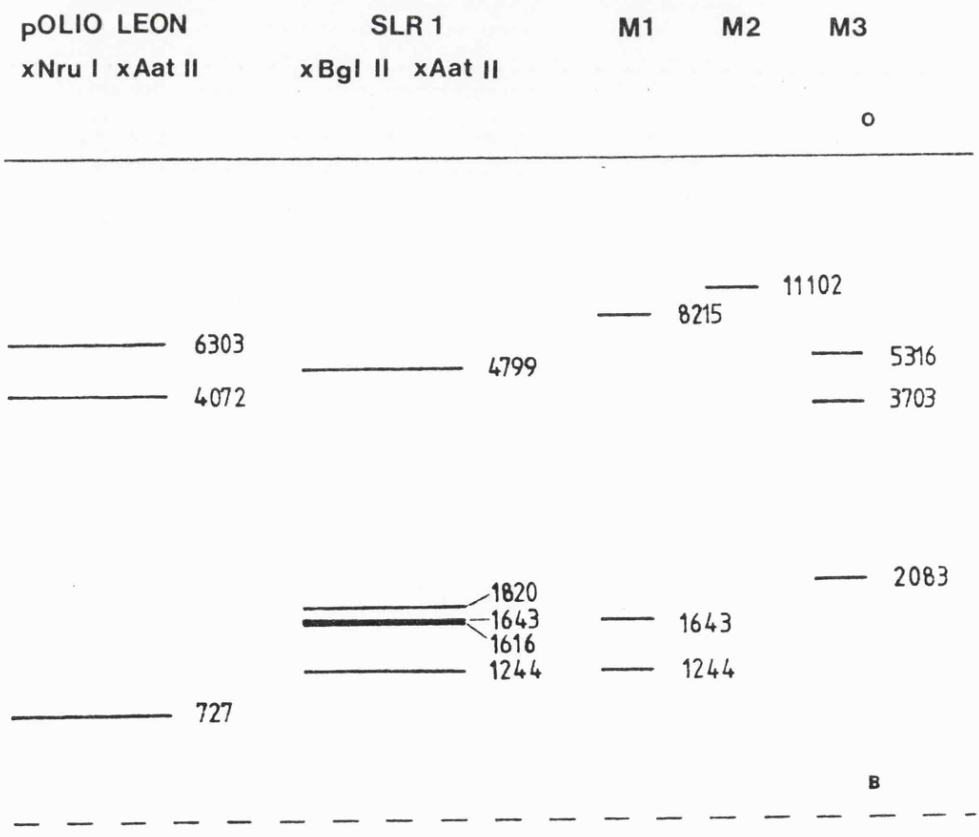
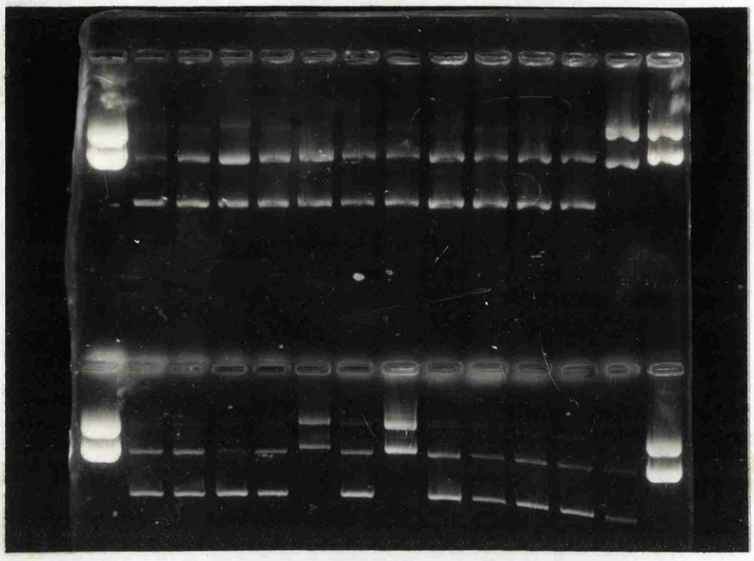

pOLIO LEON
xNru I xAat II
SLR 1
xBgl II xAat II
M1
M2
M3
O
11102
8215
6303
4799
5316
4072
3703
2083
1820
1643
1616
1643
1244
1244
727
B

Fig. 5. 2.

The Construction of ST/L: Isolation of the 6.3kb AatII Fragment of pOLIO LEON and the 4.8KB Fragment of SLR 1.

SLR 1 was digested to completion with AatII and BglII. Digestion products were separated by electrophoresis on a 0.8% agarose gel. BglII digestion increased the resolution of the required AatII fragment of 4.8kb by degrading the reciprocal AatII fragment of 6.3kb. Similarly, digestion with NruI increased the resolution of the 6.3kb AatII fragment of pOLIO LEON. The predicted size of each digestion product (bp) is shown in the diagram. pOLIO LEON digested with BglII (M1), SmaI (M2) and EcoRI (M3) were used as size markers.

O = origin, B = position of bromophenol blue.



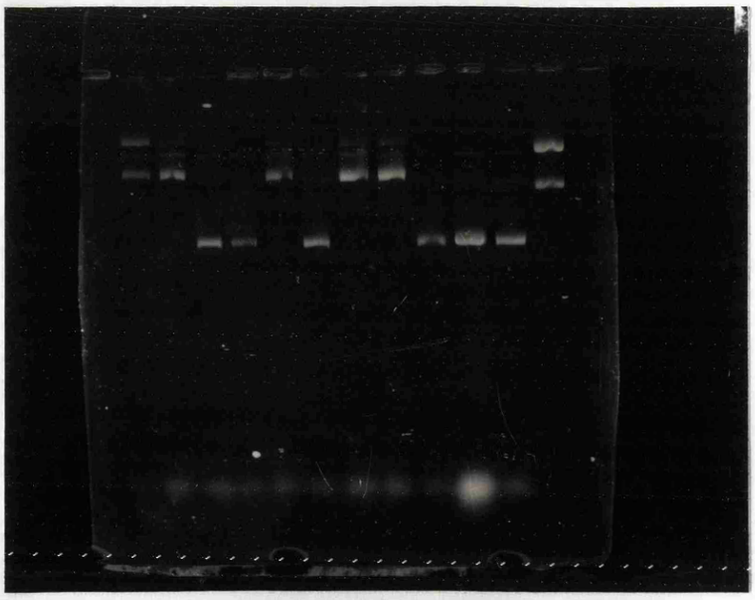
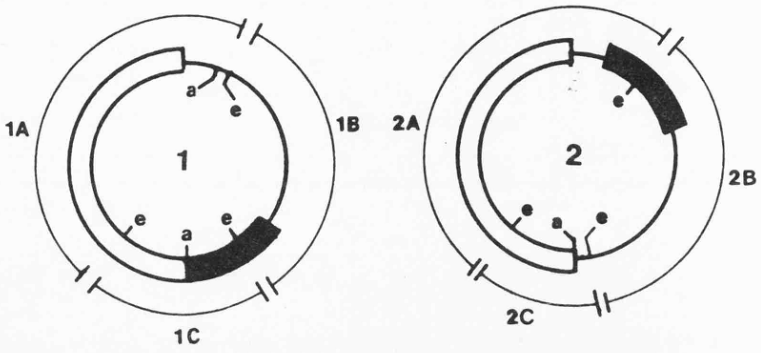


Fig. 5. 3.

The Construction of ST/L: Characterisation of Plasmid DNA Isolated From 36 Tetracycline Resistant Transformants.

Competent E.coli JA221 were transformed to tetracycline resistance with 100ng of a ligation mix containing the 6.3kb AatII fragment of pOLIO LEON (phosphatase treated) and the 4.8kb fragment of SLR 1. Plasmid DNA was isolated from 36 transformants and characterised by agarose gel electrophoresis using pOLIO LEON as a size marker. 7 of the isolates appeared to be full-length recombinant plasmids (12, 17, 19, 25, 28, 31 and 32). The remainder of the plasmids were probably formed by self-ligation of the 4.8kb AatII fragment which contains the pAT 153 origin of replication and a functional tetracycline resistance gene.

M = pOLIO LEON (1ug), 1 - 36 = plasmid isolates (1ug).



X EcoRI

1A —— 5.3
1B —— 3.7

1C —— 2.1

X EcoRI

2A —— 6.3
2B —— 3.7

2C —— 1.1

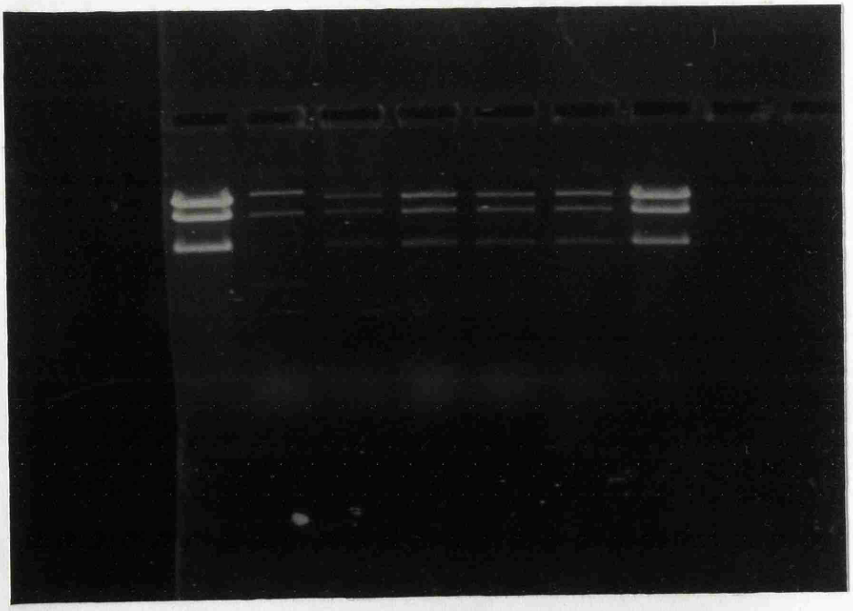


Fig. 5. 4.

Construction of ST/L: Orientation of Recombinant Plasmids by Restriction Endonuclease Digestion.

The reciprocal AatII fragments used in this construction may form viable plasmids by ligating in either of two orientations (see diagram). With the insert ligated in the correct orientation (plasmid 1), EcoRI digestion generates fragments of 5.3kb, 3.7kb and 2.1kb. With the insert ligated in the wrong orientation (plasmid 2), EcoRI fragments of 6.3kb, 3.7kb and 1.1kb are generated. Five full-length recombinant plasmids were digested with EcoRI. Digestion products were analysed by agarose gel electrophoresis. In four of the isolates (2 - 5), the insert was ligated in the desired orientation. Isolate 1 carries the insert in the wrong orientation.

M = pOLIO LEON x EcoRI, 1 - 5 = plasmid isolates x EcoRI.

fragment of SLR 1 contained the entire vector sequence and was therefore treated with calf-intestinal phosphatase to prevent self-ligation.

The 4.8kb fragment of SLR 1 was ligated to the 6.3kb fragment of pOLIO LEON. Approximately 100ng of cDNA was used to transform E.coli JA221. As shown in Fig. 5.3., seven of the plasmids isolated from thirty-six tetracycline resistant transformants appeared to be full-length recombinants on the basis of agarose gel electrophoresis. Five of these plasmids were digested with EcoRI. Fig. 5.4. shows that all five plasmids were full-length recombinants, although in one of the plasmids the insert was ligated in the wrong orientation.

One of these clones was selected for further study and designated ST/L. Recreation of the sites used in the construction was confirmed by digestion with AatII. ST/L therefore contains a recombinant cDNA insert which carries three Sabin-specific mutations. The C-U substitution at N472 in the 5′ non-coding region was implicated in attenuation by evidence of direct back mutation in a neurovirulent revertant of the vaccine (Cann et al., 1984). The two additional mutations, at N220 and N871, are conserved in the revertant and, therefore, must be silent with respect to attenuation. In ST/L, the mutation at N472 has been segragated from other potentially attenuating mutations.

5.3. The construction of recombinants between SABIN GDW and pOLIO LEON.

5.3.1. The construction of SCC/L.

The three Sabin-specific coding changes were segragated from SABIN GDW using the PvuII sites at N492 and N3750 in the poliovirus cDNA, as shown in Fig. 5.5. SABIN GDW was digested to completion with PvuII and the 3.3kb fragment (corresponding to residues N492 to N3750 of the cDNA) was isolated and purified. The reciprocal fragment of 7.8kb was obtained

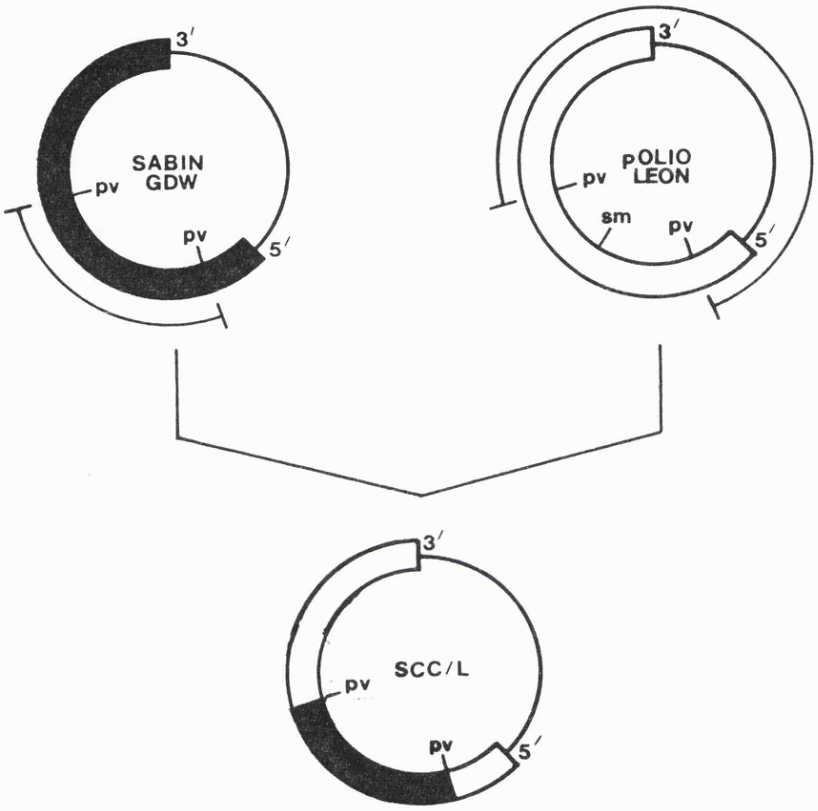

3′
SABIN
GDW
pv
pv
5′
3′
POLIO
LEON
pv
sm
pv
5′
3′
SCC/L
pv
pv
5′

Fig. 5. 5.

The Construction of SCC/L.

pOLIO LEON and SABIN GDW are infectious clones containing complete cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. A recombinant clone was constructed in which an internal PvuII fragment of the pOLIO LEON cDNA has been substituted with the corresponding region of SABIN GDW (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Filled double lines represent P3/Leon/12a$_1$b sequences (derived from SABIN GDW) and open double lines represent P3/Leon/37 sequences (derived from pOLIO LEON). The restriction fragments used to construct SCC/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

pv = PvuII, sm = SmaI.

from a PvuII digest of pOLIO LEON. Fragments of this size co-migrate with the supercoiled plasmid DNA. pOLIO LEON was therefore digested with SmaI prior to digestion with PvuII to reduce the possibility of contamination. The 7.8kb PvuII fragment of pOLIO LEON contains the entire vector sequence and was therefore treated with calf-intestinal phosphatase before ligating to the 3.3kb PvuII fragment of SABIN GDW. As shown in Table 5.1, phosphatase treatment failed to prevent self-ligation of the 7.8kb PvuII fragment.

Table 5.1

Transformation of E.coli JA221.

		Tet^r Transformants.
1) 1ng pAT 153		61
2) 100ng lig. mix 1.	pOLIO LEON 7.8kb*	91
	SABIN GDW 3.3kb	
3) 100ng lig. mix 2.	pOLIO LEON 7.8kb*	398
4) 100ng lig. mix 3.	SABIN GDW 3.3kb	-
5) 100ng pOLIO LEON 7.8kb*		-

* Treated with calf intestinal phosphatase.

In order to isolate full-length recombinant plasmids from the total population of tet^r transformants, a colony hybridisation experiment wsa performed using the method of Grunstein and Hogness. A radiolabelled probe was prepared by nick-translation of the 3.3kb PvuII fragment of SABIN GDW, as described in Chapter 8. Tet^r transformants were streaked onto nitrocellulose filters and grown overnight at 37°C. Colonies were

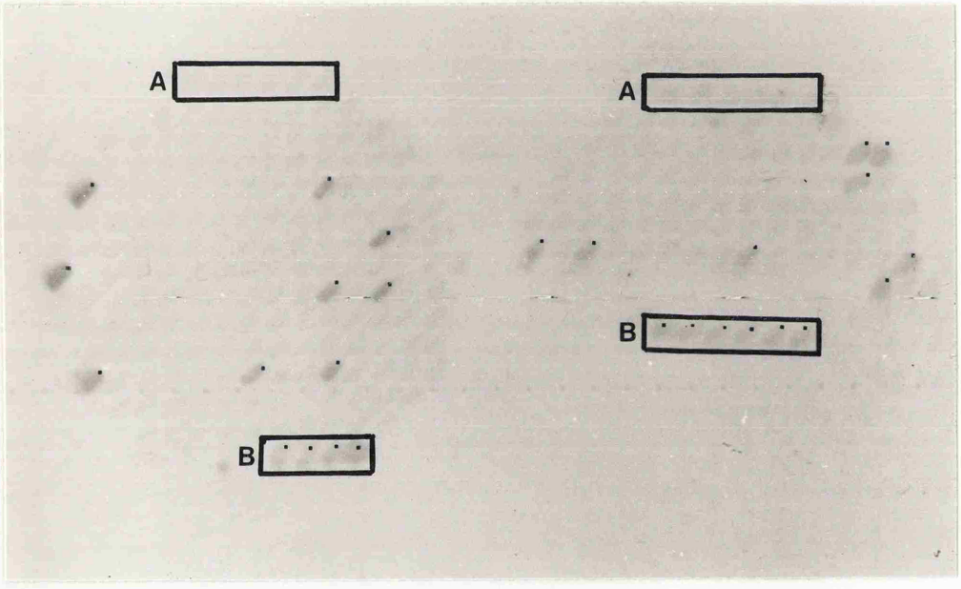

A
A
B
B

Fig. 5. 6.

The Construction of SCC/L: Identification of Full-length Recombinant Plasmids by Colony Hybridisation.

Competent E.coli JA221 were transformed with 100ng of a ligation mix containing the 7.8kb PvuII fragment of pOLIO LEON (phosphatase treated) and a threefold molar excess of the 3.3kb PvuII fragment of SABIN GDW (see text for details). Transformants were streaked onto nitrocellulose filters and grown overnight at 37°C. A colony hybridisation experiment was performed using a radiolabelled probe prepared by nick translation of the 3.3kb PvuII fragment of SABIN GDW (see text for details). An autoradiograph developed after 2 days exposure revealed 17 strongly hybridising colonies. Each of these colonies contained a full-length recombinant plasmid.

A = Negative control: colonies containing pAT 153.

B = Positive control: colonies containing SABIN GDW.

prepared for hybridisation by lysis and denaturation in 2M NaOH, as described in Chapter 8. The nick-translated probe was passed through a spun-column of Sephadex G100 to remove unincorporated label before denaturing by boiling for 10 minutes. The probe was added to 20ml of 5X denhardts solution, pre-warmed to 65°C, and poured over the nitrocellulose filters. Hybridisation was allowed to proceed for 24 hours at 65°C. Filters were then washed in 5X Denhardts solution before drying and subjecting to autoradiography.

Fig. 5.6. shows an autoradiograph developed after 2 days exposure. Plasmid DNA was prepared from the 17 positive colonies. Each of these plasmids contained a full-length cDNA insert, as judged by agarose gel electrophoresis using pOLIO LEON as a size marker. A recombinant plasmid in which the 3.3kb PvuII fragment was ligated in the correct orientation was identified by restriction endonuclease mapping and designated SCC/L.

5.3.2. The construction of SV3,V1/L and SP2/L.

Fig. 5.7. shows the construction of a pair of reciprocal recombinants between SCC/L an pOLIO LEON using the BglII site at N3421 in the poliovirus cDNA and the ClaI site at N23 in the vector, pAT 153. As in previous constructions an additional enzyme was used in each digest to increase the resolution of the desired fragments and to reduce the possibility of contamination with supercoiled plasmid DNA. pOLIO LEON was digested to completion with ClaI and SalI. The DNA was then partially digested with BglII. Fig. 5.8. shows the partial digestion products following electrophoresis on a 1% agarose gel for 2 hours. The 4.8kb fragment was electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. The second fragment used in the construction was obtained by complete digestion of SCC/L with ClaI, BglII and XhoI.

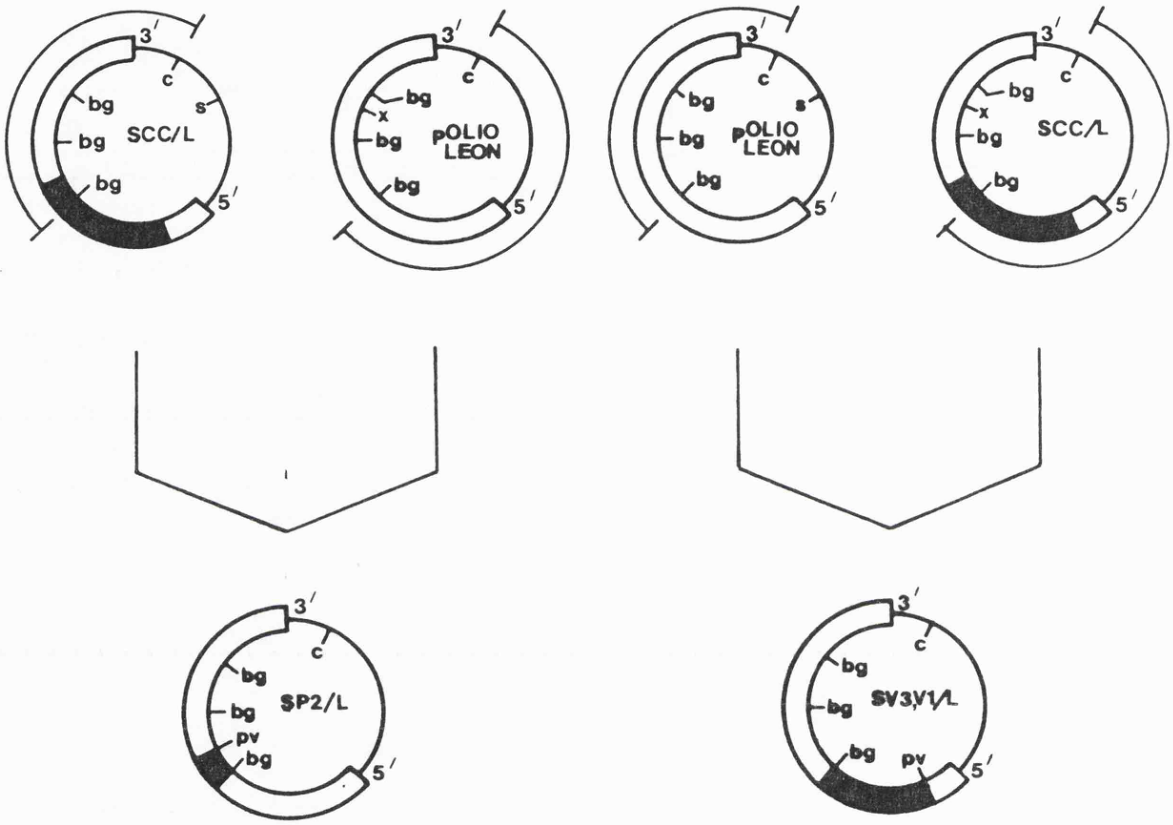
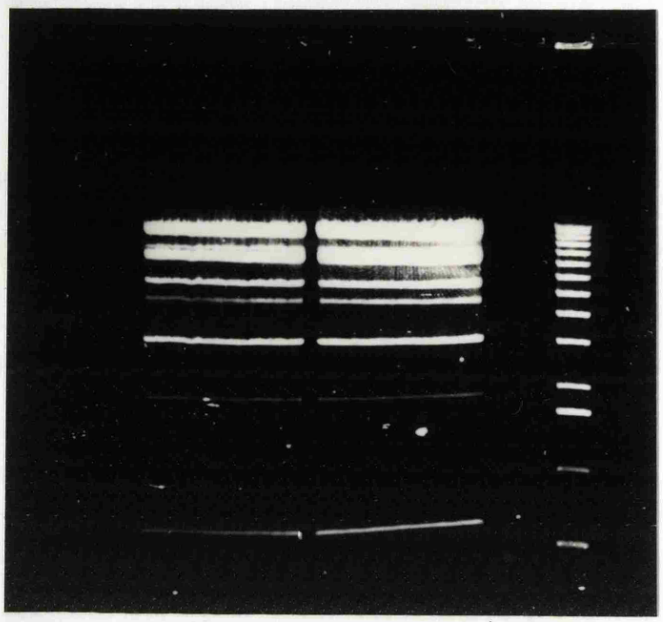

3′
c
bg
s
SCC/L
bg
bg
5′
3′
c
bg
x
bg
POLIO
LEON
bg
5′
3′
c
bg
s
bg
POLIO
LEON
bg
5′
3′
c
bg
x
bg
SCC/L
bg
5′
3′
c
bg
bg
SP2/L
pv
bg
5′
3′
c
bg
bg
SV3,V1/L
bg
pv
5′

Fig. 5. 7.

The Construction of SV3,V1/L and SP2/L.

pOLIO LEON and SABIN GDW are infectious clones containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. SCC/L is a recombinant clone in which an internal PvuII fragment of pOLIO LEON (residues N492 - N3750 of the cDNA) has been replaced with the corresponding region of SABIN GDW. A pair of reciprocal recombinants were constructed between SCC/L and pOLIO LEON using the BglII site at N3421 and in the cDNA and the ClaI site in the vector pAT 153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences and filled double lines represent P3/Leon/12a$_1$b sequences. The restriction fragments used to construct SV3,V1/L and SP2/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

bg = BglII, c = ClaI, pv = PvuII, S = SalI, x = XhoI.



pOLIO LEON x Cla I

M xSal I xBgl II p

o

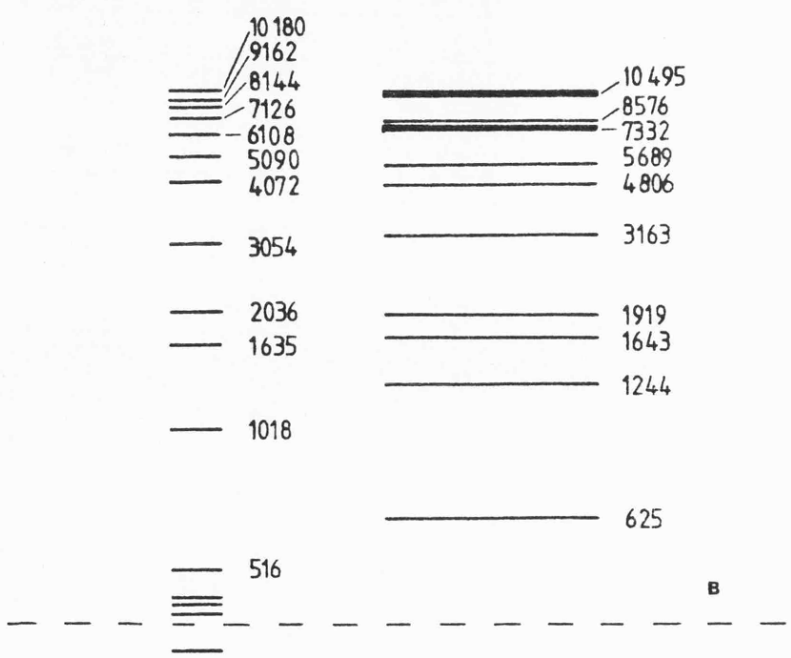


Fig. 5. 8.

The Construction of SV3,V1/L: Isolation of the 4.8kb BglII/ClaI Fragment of pOLIO LEON.

pOLIO LEON was digested to completion with SalI and ClaI and then partially digested with BglII. Partial digestion products were separated by electrophoresis on a 1% agarose gel for 2hrs at 100V. The 4.8kb fragment was excised from the gel, electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. BRL kb ladder was used as a size marker (M). The sizes of partial digestion products are shown in the diagram.

O = origin, B = position of bromophenol blue.

SV3,V1/L was constructed by ligating the 4.8kb ClaI/BglII fragment of pOLIO LEON to the 6.3kb ClaI/BglII fragment of SCC/L. In this recombinant an internal restriction endonuclease fragment of the pOLIO LEON cDNA has been replaced by the corresponding region of SABIN GDW. SV3,V1/L therefore carries three Sabin-specific mutations. G-A at N871 is a silent change in the region of the viral RNA encoding VP4. C-U at N2034 results in a serine-phenylalanine substitution in VP3. Finally, A-G at N3333 results in a lysine-arginine substitution in VP1. SP2/L was constructed as the genetic reciprocal of SV3,V1/L, as shown in Fig. 5.7. This recombinant carries a single Sabin specific mutation, A-G at N3464, which results in a threonine-alanine change in the non-structural protein P2-3b.

5.3.3. The construction of SV1/L.

The A-G substitution at N3333 results in a lysine-arginine change in the structural protein VP1. This mutation was transferred to P3/Leon/37 by constructing a recombinant between SV3,V1/L and pOLIO LEON using the SmaI site at N2769 in the poliovirus cDNA and the SalI site at N652 in the vector pAT 153 (see Fig. 5.9.). The products of SmaI and SalI digestion of pOLIO LEON are fragments of 5.0kb and 6.1kb. To increase the resolution of the desired fragment and to reduce the possibility of cross-contamination, a third enzyme was used in each digest. Fig. 5.10. shows the products of a SmaI, SalI and BglII digestion of pOLIO LEON, following electrophoresis on a 1% agarose gel for two hours. The 5.0kb SmaI/SalI fragment was electroeluted and purified by phenol/chloroform extraction and ethanol precipitation. The reciprocal SmaI/SalI fragment was obtained from a SmaI, SalI and SstI digest of SV3,V1/L, as shown in Fig. 5.11.

The 5.0kb SmaI/SalI fragment of pOLIO LEON was ligated to the 6.1kb SmaI/SalI fragment of SV3,V1/L. Competent E.coli JA221 were transformed with 100ng of ligated DNA and plated out in the presence of

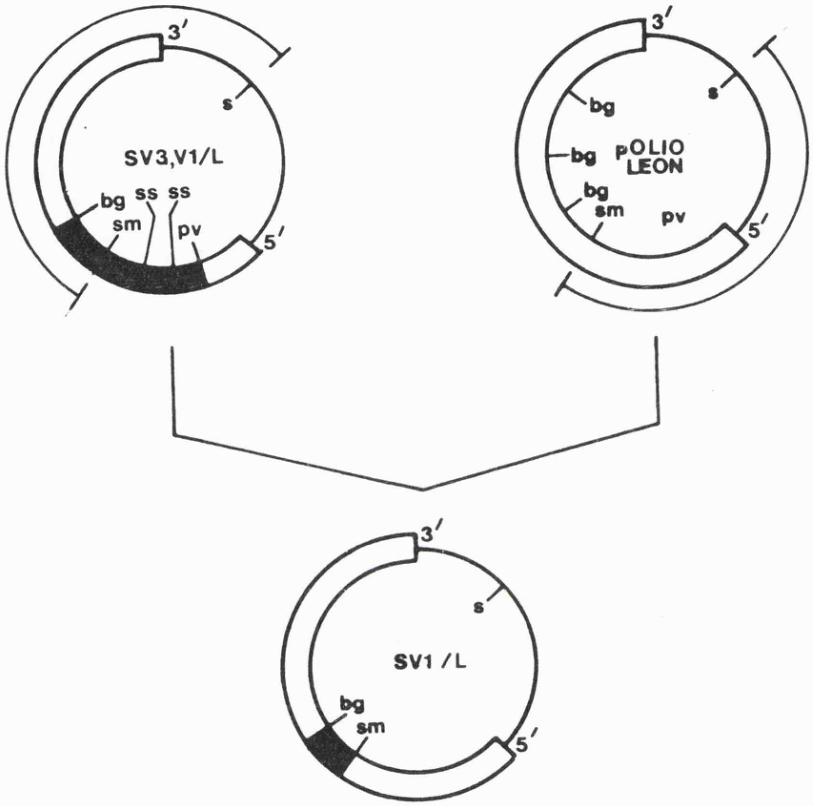
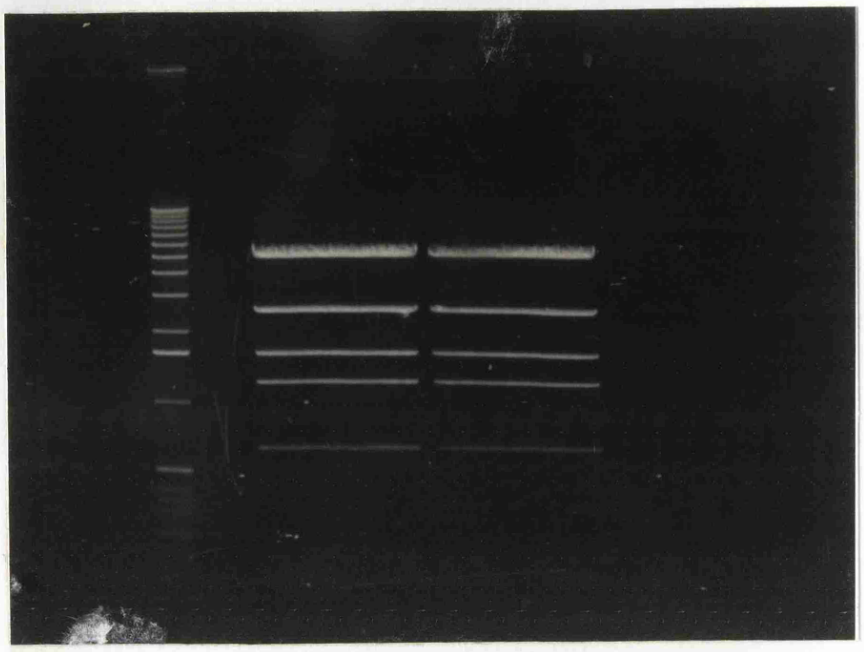

3′
s
SV3,V1/L
bg
ss
ss
sm
pv
5′
3′
bg
s
bg
POLIO
LEON
bg
sm
pv
5′
3′
s
SV1 /L
bg
sm
5′

Fig. 5. 9.

The Construction of SV1/L.

pOLIO LEON and SABIN GDW are infectious clones containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b. SV3,V1/L is a recombinant clone in which the 2.5kb PvuII/BglII fragment of pOLIO LEON (residues N492 to N3421 of the cDNA) has been replaced by the corresponding region of SABIN GDW. A recombinant plasmid was constructed between SV1,V3/L and pOLIO LEON using the SmaI site at N2769 in the cDNA and the SalI site in the vector, pAT 153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences and filled double lines represent P3/Leon/12a$_1$b sequences. The restriction fragments used to construct SV1/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

bg = BglII, s = SalI, sm = SmaI, ss = SstI.



M

pOLIO LEON xSma I

xSal I xBgl II

o

7126
6108
5090
4072
3054
2036
1635
1018
516

5037
2546
1643
1244
650

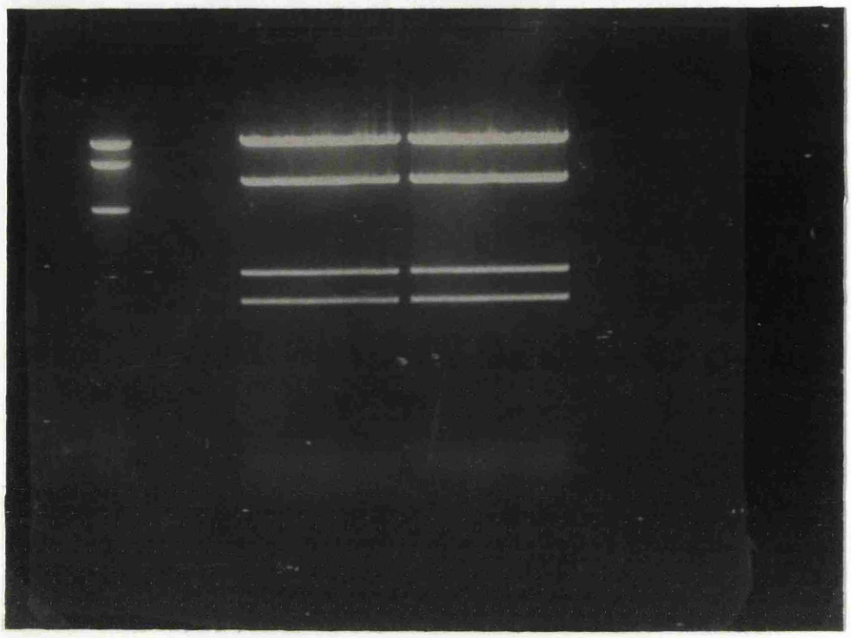
B

Fig. 5. 10.

The Construction of SV1/L: Isolation of the 5.0kb Fragment of pOLIO LEON.

pOLIO LEON was digested to completion with SmaI, SalI and BglII. BglII digestion increased the resolution of the required 5.0kb SalI/SmaI fragment by degrading the recipocal 6.1kb fragment, thereby precluding the possibility of cross-contamination during purification. Sizes of digestion products (bp) are shown in the diagram. BRL kb ladder was used as a size marker (M).

O = origin, B = position of bromophenol blue.



M

SV3,V1/L xSal I
xSst I xSma I

o

5320
3703
2083

6085
3020
1148
869

B

Fig. 5. 11.

The Construction of SV1/L: Isolation of th 6.1kb SmaI/SalI Fragment of SV3,V1/L.

SV3,V1/L was digested to completion with SmaI, SalI and SstI. Digestion with SstI increased the resolution of the required 6.1kb SalI/SmaI fragment by degrading the reciprocal fragment of 5.0kb, thereby precluding the possibility of cross-contamination during purification. The sizes of digestion products (bp) are shown in the diagram. pOLIO LEON digested with EcoRI was used as a size marker (M).

O = origin, B = position of bromophenol blue.

tetracycline. As expected each of the tet^r transformants characterized contained a full-length recombinant plasmid. One of these was selected for further study and designated SV1/L. In this recombinant an internal restriction endonuclease fragment of P3/Leon/37, spanning nucleotide residues N2769-N3421, has been replaced with the corresponding region of SABIN GDW. Therefore, SV1/L carries the A-G substitution at N3333, but is otherwise entirely Leon-like.

5.4. Nucleotide sequence analysis of recombinant polioviruses.

Fig. 5.12. shows the genome structure of the four recombinant polioviruses described above. Each was shown to be infectious following transfection of sub-confluent monolayers of Hep 2c by the calcium phosphate co-precipitation technique. The cDNA derived viruses were shown to be type three polioviruses in a virus neutralisation assay. Virus rescued from the recombinant plasmid SCC/L was designated rSCC/L (for rescued SCC/L). The same nomenclature was used for the other recombinants. Partial nucleotide sequences were obtained from purified viral RNA by adapting the di-deoxy chain termination method to an oligonucleotide primed reverse transcription reaction. The results presented in Table 5.2. are compatible with the genome structures of the recombinant cDNAs. For example, the recombinant plasmid SCC/L contains a cDNA insert in which an internal restriction endonuclease fragment of the pOLIO LEON, spanning nucleotide residues N492-N3750, has been replaced with the corresponding fragment of SABIN GDW. rSCC/L was shown to be Leon-like at N472. The recombinant virus carries the three Sabin-specific coding changes at N2034, N3333 and N3421, but is Leon-like at N7165.

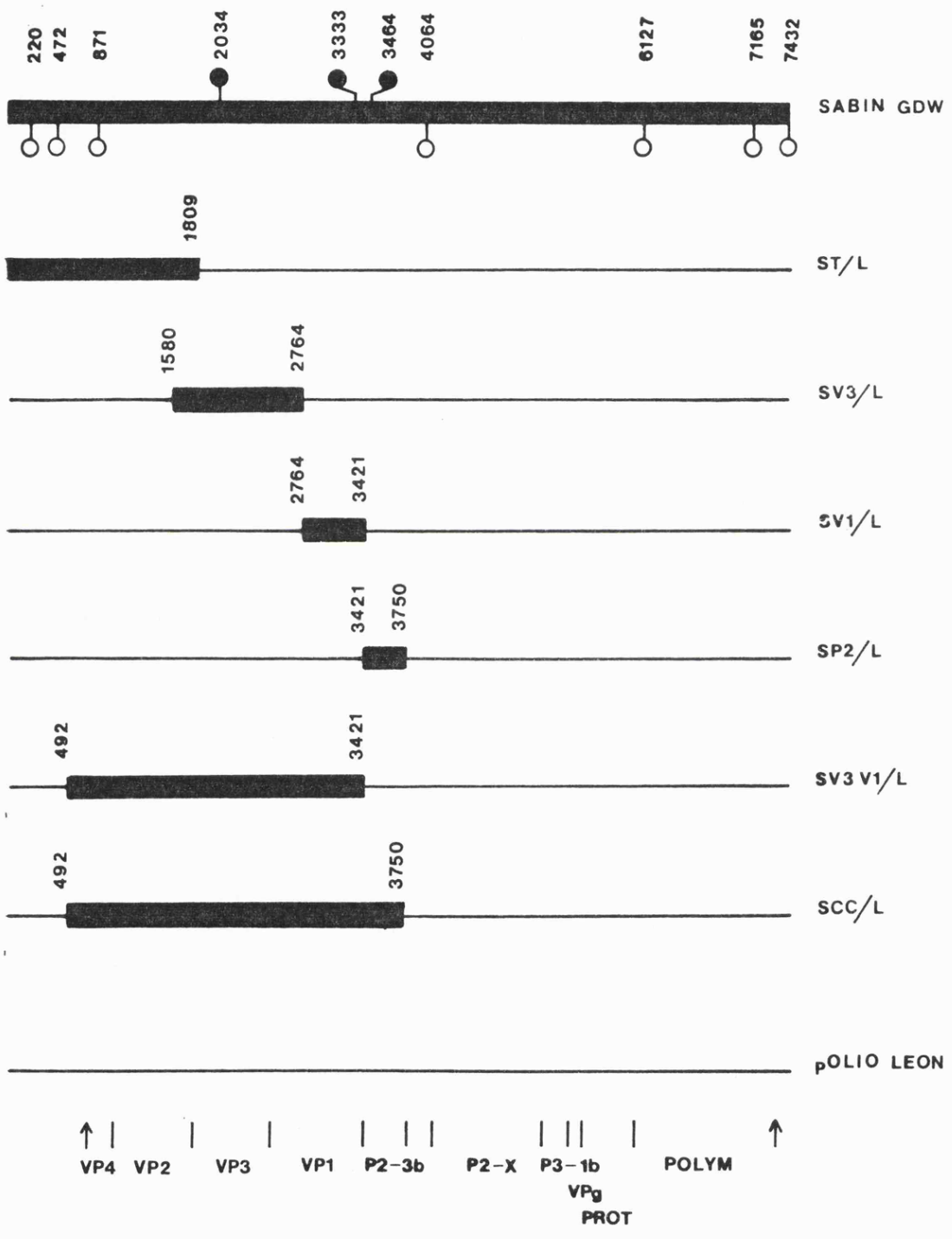

220
472
871
2034
3333
3464
4064
6127
7165
7432
SABIN GDW
1809
ST/L
1580
2764
SV3/L
2764
3421
SV1/L
3421
3750
SP2/L
492
3421
SV3 V1/L
492
3750
SCC/L
pOLIO LEON
VP4
VP2
VP3
VP1
P2-3b
P2-X
P3-1b
VPg
PROT
POLYM

Fig. 5. 12.

Structure of Recombinant Poliovirus cDNAs

pOLIO LEON and SABIN GDW are infectious clones containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. The cDNA inserts of pOLIO LEON and SABIN GDW are shown as a single line and a heavy line respectively. Open circles represent non-coding changes observed between P3/Leon/37 and P3/Leon/12a$_1$b. Filled circles correspond to mutations which result in amino acid substitutions. The structure of six recombinant cDNAs are shown with single lines representing P3/LEON/37 sequences, derived from pOLIO LEON, and heavy lines representing P3/Leon/12a$_1$b sequences, derived from SABIN GDW. Positions of the restriction endonuclease sites used to construct the recombinant cDNAs are shown in bp.

Table 5.2

Nucleotide sequence analysis of recombinant polioviruses.

Virus	Base at position 472	Base at position 2034	Base at position 3333	Base at position 3464	Base at position 7165
P3/Leon/37	C	C	A	A	G
rST/L	U	C	A	A	G
rSV3/L	C	U	A	A	G
rSV1/L	C	C	G	A	G
rSP2/L	C	C	A	G	G
rSV1,V3/L	C	U	G	A	G
rSCC/L	C	U	G	G	G
P3/Leon/12a$_1$b	U	U	G	G	A

In view of the high spontaneous mutation frequencies associated with replication of RNA genomes, the nucleotide sequencing gels were also examined for evidence of aberrant mutations. As a result, a deletion mutation was identified at N417 in the 5′ non-coding region of virus rescued from ST/L. This mutation was shown to be present in virus rescued from the other plasmids which derive their 5′ non-coding regions from pOLIO SABIN, i.e SLR 1 and SABIN GDW.

Examination of the sequence of pSAG 24, the 5′ terminal sub-genomic cDNA used in the construction of pOLIO SABIN, confirmed that the deletion at N417 was present in the cDNA. This mutation could not be detected when RNA purified from authentic P3/Leon/12a1b was sequenced by primer extension. This suggested that pSAG24 was unrepresentative of the population of RNA from which the cDNA was derived. The remainder of this chapter describes the construction of a recombinant designed to determine whether the deletion at N417 is biologically significant.

5.4 The construction of S5′/L.

The full-length cDNA clone SABIN GDW contains an aberrant deletion mutation at N417. Two of the poliovirus recombinants also carry this mutation: these are SLR 1 and ST/L. In order to determine whether this mutation is biologically significant, an additional poliovirus recombinant was constructed. It was not possible to segregate the mutation from SABIN GDW because of the absence of any convenient restriction endonuclease site. It was therefore decided to replace the aberrant region of ST/L with the corresponding region of pSAG 21, the sub-genomic cDNA used to derive the sequence of P3/Leon/12a$_1$b. S5′/L was constructed by recombination between ST/L and pSAG 21, as shown in Fig. 5.13. The significance of the deletion mutation at N417 could then be determined indirectly by comparing virus rescued from S5′/L and ST/L in temperature sensitivity and neurovirulence assays.

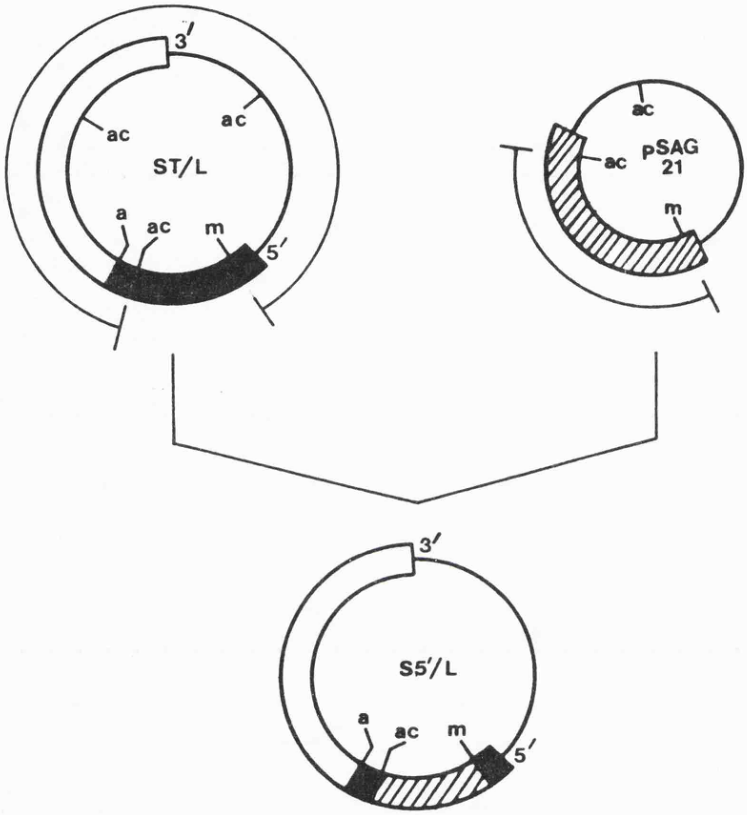

3′
ac
ac
ST/L
a
ac
m
5′
ac
ac
pSAG
21
m
3′
S5′/L
a
ac
m
5′

Fig. 5. 13.

The Construction of S5'/L.

S5'/L was constructed by substituting the 1.2kb MluI/AccI fragment of ST/L (residues N279 - N1522 of the cDNA) with the corresponding region of pSAG 21 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences, derived from pOLIO LEON, and filled double lines represent P3/Leon/12a$_1$b, sequences derived from pOLIO SABIN. Cross-hatched double lines represent P3/Leon/12a$_1$b sequences derived from pSAG 21. The restriction fragments used to construct S5'/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

ac = AccI, m = MluI.

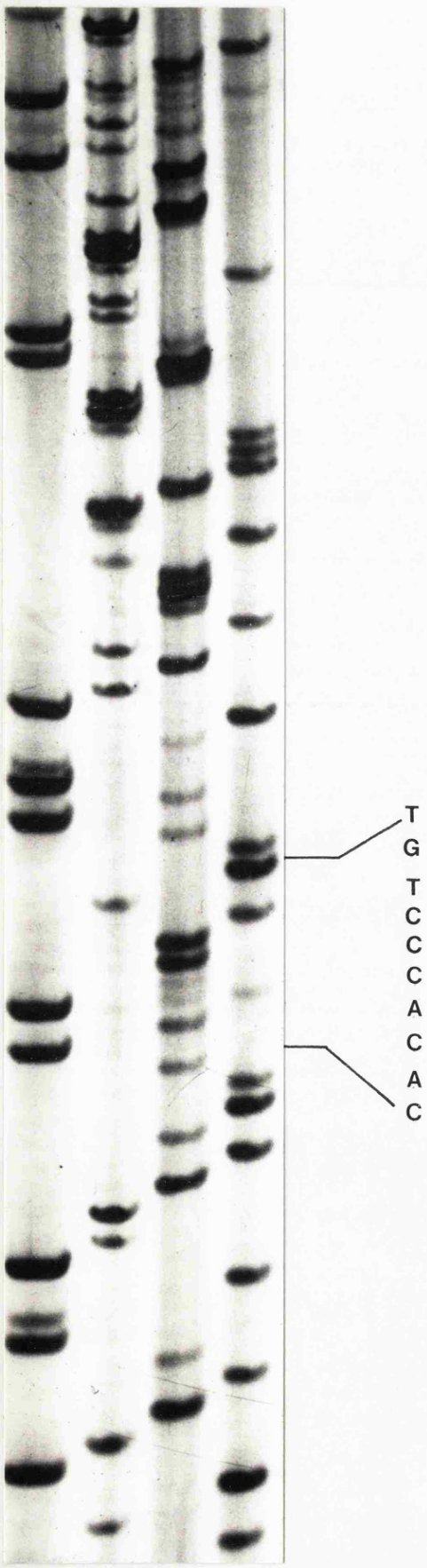

T
G
T
C
C
C
A
C
A
C

Fig. 5. 14.

Nucleotide Sequence Analysis of S5'/L.

pOLIO SABIN and pOLIO LEON are clones containing full-length cDNAs of P3/Leon/12a$_1$b and P3/Leon/37, respectively. ST/L contains a recombinant cDNA insert in which the 5' terminal 1809 residues were derived from pOLIO SABIN and the remainder of the cDNA was derived from pOLIO LEON. Primer extension sequencing of virus rescued from ST/L revealed a deletion mutation at N417 which was not present in authentic P3/Leon/12a$_1$b. The origin of this mutation was shown to be pSAG 24, a sub-genomic cDNA used in the construction of pOLIO SABIN. pSAG 21, an alternative sub-genomic cDNA of P3/Leon/12a$_1$b, lacked the mutation at N417.

P3/Leon/12a$_1$b	ACAGGGTGTG
pSAG 24	ACAGGG-GTG
ST/L	ACAGGG-GTG
pSAG 21	ACAGGGTGTG
	410 420

pSAG 21 was used to repair the aberrant region of ST/L, as shown in Fig 5. 13. The resulting clone, designated S5'/L, was characterised by nucleotide sequencing in the vicinity of N417. A KpnI/EcoRI fragment of S5'/L (residues N71 - N785 of the cDNA) was sub-cloned into M13mp19. Nucleotide sequence was derived by the dideoxy chain-termination method. The sequence obtained is complementary to the genomic sequence.

ST/L	CAC-CCCTGT
S5'/L	CACACCCTGT
	420 410

The construction used the AccI site at N1522 and the MluI site at N279 in the poliovirus cDNA. There are two additional AccI sites in the full-length recombinant plasmid, at N6315 in the poliovirus cDNA and N653 in the vector, pAT 153. pSAG 21 was digested to completion with AccI and MluI and the 1.2kb fragment (corresponding to nucleotide residues N279-N1522 of the cDNA) was isolated and purified. The reciprocal fragment of 9.9kb was obtained from an MluI complete and AccI partial digest of ST/L.

S5′/L was constructed by ligating the 1.2kb MluI/AccI fragment of pSAG 21 to the 9.9kb MluI/AccI fragment of ST/L. An internal KpnI/EcoRI fragment of ST/L (corresponding to nucleotide residues N71-N785 of the cDNA) was sub-cloned into M13mp19. The insert was in the correct orientation for packaging of the strand corresponding to the positive sense poliovirus RNA. Single-stranded template, prepared as described in Chapter 9, could then be sequenced using the synthetic oligonucleotide primers synthesised for RNA sequencing. The nucleotide sequence of the region containing N417 was derived by the dideoxy chain-termination method. The results shown in Fig. 5.14. demonstrates that the construction of S5′/L was successful.

5.5 The construction of S5′3′/L.

The final recombinant constructed for this study is the genetic reciprocal of SCC/L. S5′3′/L was constructed by recombination between S5′/L and SABIN GDW using the unique XhoI site at N6051 in the poliovirus cDNA and the unique SalI site at N651 in the vector, pAT 153. This recombinant carries the C-U base substitution mutation at N472 in the 5′ non-coding region and the G-A base substitution mutation at N7432 in the 3′ non-coding region but is Leon-like in the region of the three Sabin-specific coding changes.

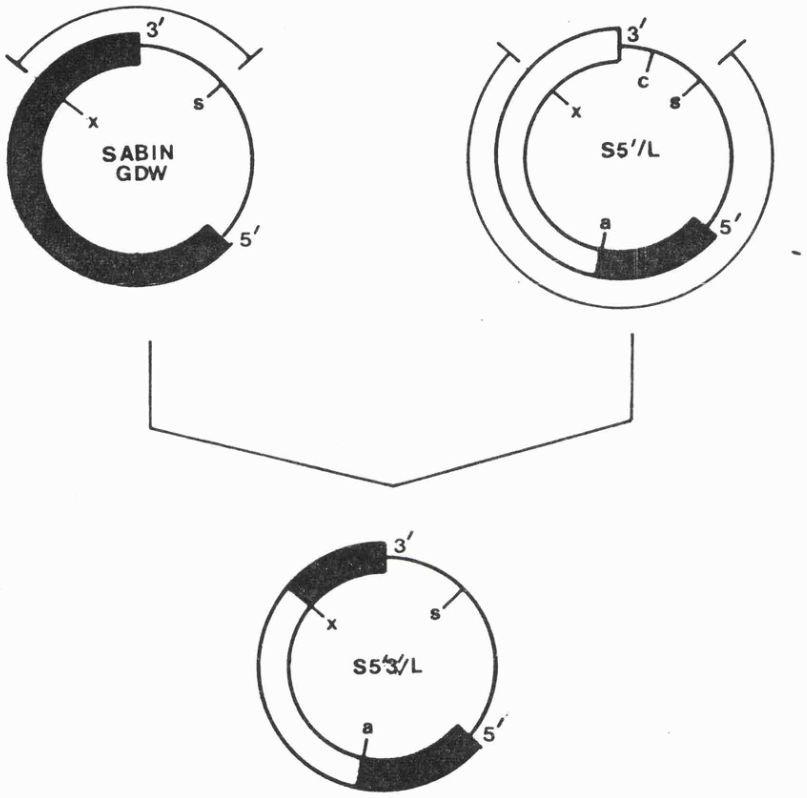

3′
x
s
SABIN
GDW
5′
3′
c
x
s
S5′/L
a
5′
3′
x
s
S5′3′/L
a
5′

Fig. 5. 15.

The Construction of S5'3'/L.

pOLIO LEON and SABIN GDW are infectious clones containing full-length cDNAs of P3/Leon/37 and P3/Leon/12a$_1$b, respectively. S5'/L is a recombinant cDNA clone in which the 5' terminal 1809 residues correspond to P3/Leon/12a$_1$b sequences and the rest of the cDNA from P3/Leon/37 sequences (see Fig. 5. 13). A recombinant plasmid was constructed between S5'/L and SABIN GDW using the unique XhoI site at N6051 in the poliovirus cDNA and the SalI site in the vector, pAT 153 (see text for details). Vector sequences are shown as single lines and poliovirus cDNA as double lines. Open double lines represent P3/Leon/37 sequences and filled double lines represent P3/Leon/12a$_1$b sequences. The restriction fragments used to construct S5'3'/L are shown as barred lines. 5' and 3' correspond to the termini of the poliovirus genome.

c = ClaI, s = SalI, x = XhoI.

SABIN GDW was digested to completion with SalI and XhoI and the 2.8kb fragment was isolated and purified. The reciprocal fragment was obtained from a SalI, XhoI and ClaI digest of S5′/L. S5′/L was constructed by ligating the 2.8kb SalI/XhoI fragment of SABIN GDW to the 8.3kb SalI/XhoI fragment of S5′/L, as shown in Fig. 5.15.

5.6 Nucleotide sequence analysis of recombinant polioviruses.

Fig. 5.16 shows the genome structure of the recombinant poliovirus cDNAs S5′/L and S5′3′/L. Both recombinants were infectious following transfection of sub-confluent monolayers of Hep 2c by the calcium phosphate co-precipitation technique. Virus neutralisation assay confirmed that the cDNA derived viruses were type 3 polioviruses.

Partial nucleotide sequences were derived from purified viral RNA by primer extension. The results shown in Table 5.3 demonstrate that the cDNA derived viruses are bone-fide recombinants. Nucleotide sequencing also confirmed that both viruses lack the deletion mutation at N417.

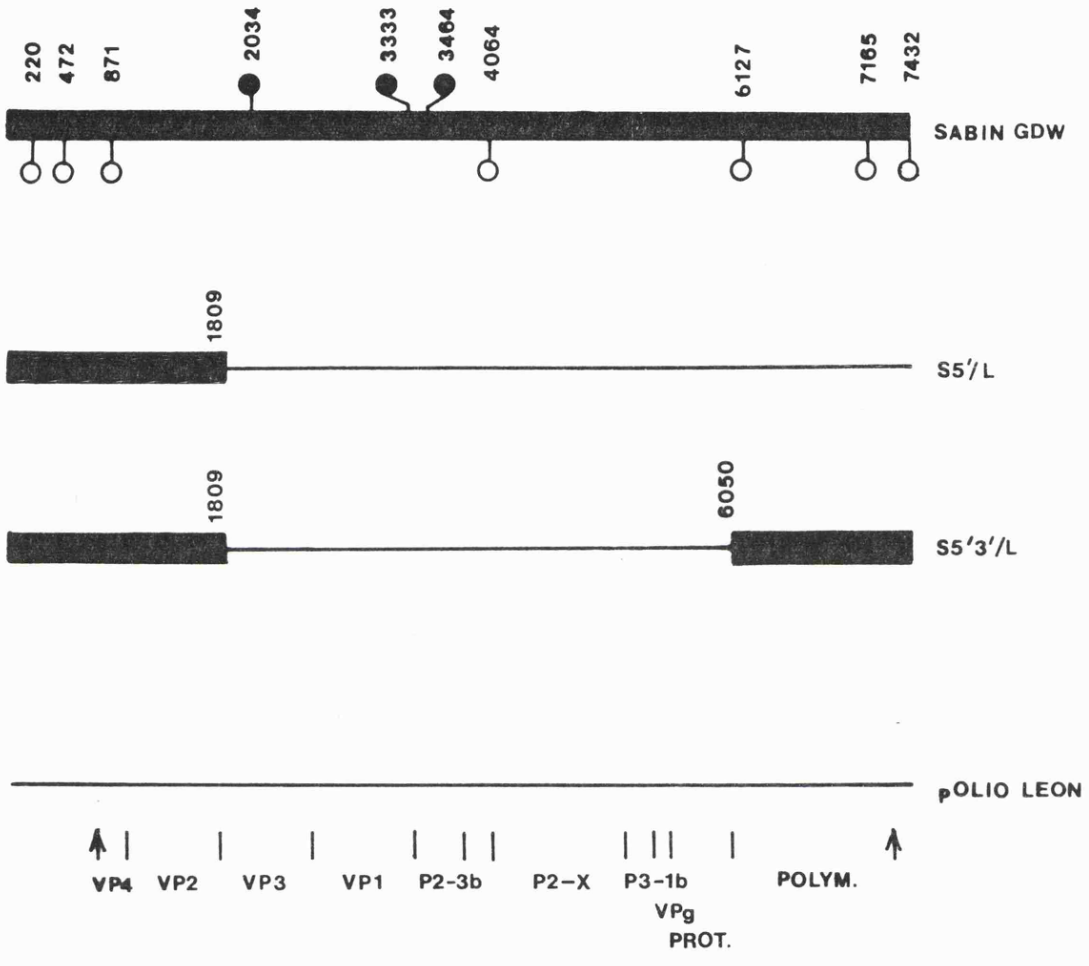

220
472
871
2034
3333
3464
4064
6127
7165
7432
SABIN GDW
1809
S5′/L
1809
6050
S5′3′/L
pOLIO LEON
VP4
VP2
VP3
VP1
P2-3b
P2-X
P3-1b
VPg
PROT.
POLYM.

Fig. 5. 16

Structure of Recombinant Poliovirus cDNAs.

Sequences derived from P3/Leon/37 are shown as single lines and sequences derived from P3/Leon/12a$_1$b are shown as heavy lines. Open circles represent non-coding changes observed between P3/Leon/37 and P3/Leon/12a$_1$b. Mutations which result in amino acid substitutions are shown as filled circles. The positions of restriction endonuclease sits used to construct the recombinant cDNAs are shown in bp.

Table 5.3

Nucleotide sequence analysis of recombinant polioviruses

Virus	Base at position 417	Base at position 472	Base at position 2034	Base at position 7165
P3/Leon/37	U	C	C	G
rS5′/L	U	U	C	G
rS5′3′/L	U	U	C	A
rP3/SABIN	G	U	U	A
P3/Leon/12a$_1$b	U	U	U	A

5.7 Summary and Conclusions.

This chapter reports the construction of a series of inter-strain poliovirus recombinants via cDNA in-vitro. These will be used, together with the recombinants described in Chapter 2, to determine the genetic bases for the temperature sensitive and attenuated phenotypes of the currently used poliovirus type 3 vaccine strain, P3/Leon/12a$_1$b. Each of the five potential attenuating mutations, identified by nucleotide sequence comparisons, has been segregated from P3/Leon/12a$_1$b.

Nucleotide sequencing studies revealed that SABIN GDW, a full-length cDNA clone of P3/Leon/12a$_1$b, contains an aberrant deletion mutation at N417 in the 5′ non-coding region. An additional recombinant was therefore constructed to determine whether this mutation was biologically significant.

CHAPTER SIX

Genetic Basis for Attenuation of the Sabin Type 3 Vaccine and Correlation with in-vitro Markers.

6.1 Introduction.

The attenuated Sabin type 3 vaccine was derived from the virulent `Leon´ strain by multiple passage through primary cultures of cynomolgus kidney cells (Sabin et al., 1954; Sabin and Boulger, 1975). In addition to their differences in the potential for causing disease, these two strains of virus differ in a number of biological characteristics, including antigenicity and temperature sensitivity.

Antigenic differences between the Sabin type 3 vaccine and `wild´ strains of poliovirus type 3 have been demonstrated using specific absorbed sera (van Wezel and Hazendonk, 1979). More recently, Ferguson et al. (1982) were able to differentiate between the Sabin type 3 vaccine, P3/Leon/12a1b, and its virulent progenitor, P3/Leon/37, on the basis of their reaction with a panel of strain specific monoclonal antibodies. One of these monoclonal antibodies, designated 138, neutralised P3/Leon/12a$_1$b but failed to react with P3/Leon/37 in neutralisation or D antigen blocking assays.

The Sabin type 3 vaccine replicates poorly at the supraoptimal temperature of 40°C whereas growth of the progenitor strain, P3/Leon/37, is only slightly inhibited. The type 1 and type 2 vaccine strains are also temperature sensitive mutants. The temperature sensitive mutation of the Sabin type 1 vaccine has been shown to inhibit the formation of 14S

particles during morphogenesis (Fezman et al., 1972). Furthermore, reversion of the temperature sensitive phenotype of the Sabin type 1 vaccine, by serial passage at successively higher temperatures, correlates with an increase in thalamic virulence for monkeys suggesting that the temperature sensitive mutation(s) may be responsible for or at least contribute towards the attenuated phenotype (Sabin and Lwoff, 1959).

Sabin-specific antigenicity and temperature sensitivity have been used as in-vitro markers for vaccine standardisation. The McBride (1959) and Wecker (1960) tests are designed to detect antigenic differences within serotypes using immune antisera for specific strains. These tests are based on the observation that homologous strains are more rapidly neutralised than heterologous strains. The standard test for temperature sensitivity is the rct marker test (reproductive capacity at different temperatures) in which the virus titre determined at 35°C is compared with the titre determined at 40°C. These tests are useful in that they allow a comparison to be made between vaccine preparations and the seed virus, revealing any changes that may occur during the in-vitro passages of the production cycle. However, neither test can substitute for neurovirulence assay because the relationship between the in-vitro markers and the attenuated phenotype has not been unequivocally determined.

The ten base substitution mutations observed between the Sabin type 3 vaccine, P3/Leon/12a1b, and its neurovirulent progenitor, P3/Leon/37, must account for the different biological characteristics of these strains (Stanway et al., 1984). Evaluation of the likely contribution of each mutation in determining the attenuated phenotype of the vaccine was made possible following the elucidation of the complete nucleotide sequence of P3/WHO/119, a neurovirulent revertant of the vaccine (Cann et al., 1984). As discussed in chapter 5, mutations at N472 in the 5′ non-coding region and at N7432 in the 3′ non-coding region are implicated in attenuation by evidence of direct back mutation in the revertant. Coding

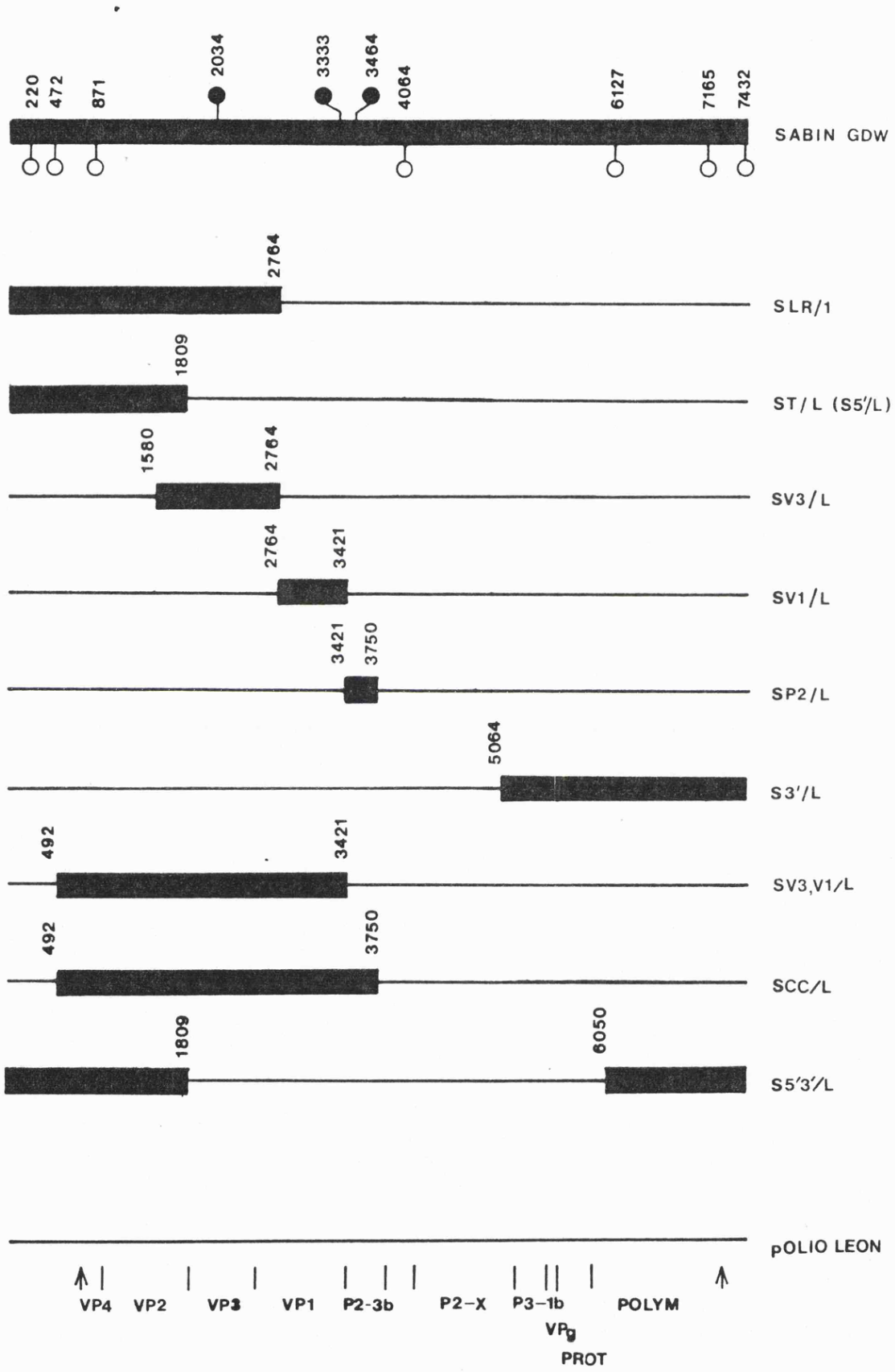

220
472
871
2034
3333
3464
4064
6127
7165
7432
SABIN GDW
2764
SLR/1
1809
ST/L (S5′/L)
1580
2764
SV3/L
2764
3421
SV1/L
3421
3750
SP2/L
5064
S3′/L
492
3421
SV3,V1/L
492
3750
SCC/L
1809
6050
S5′3′/L
pOLIO LEON
VP4
VP2
VP3
VP1
P2-3b
P2-X
P3-1b
POLYM
VPg
PROT

Fig. 6. 1.

Structure of Recombinant Poliovirus cDNAs

Sequences derived from P3/Leon/37 are shown as single lines and sequences derived from P3/Leon/12a$_1$b are shown as heavy lines. Open circles represent non-coding changes observed between P3/Leon/37 and P3/Leon/12a$_1$b. Mutations which result in amino acid substitutions are shown as filled circles. The positions of restriction endonuclease sites used to construct the recombinants are shown in bp

changes within the region of the genome encoding the virus capsid proteins may also be significant. These changes are located at N2034 (Ser-Phe in VP3) and N3333 (Lys-Arg in VP1).

To define precisely the contribution of each of the above mutations, a series of recombinants were constructed between the parent and the vaccine via cDNA in-vitro. Fig. 6.1 shows the the genome structures of the ten poliovirus recombinants described in chapter 2 and chapter 5. This chapter reports the results obtained when the panel of recombinants were subjected to the WHO monkey neurovirulence assay. The recombinants were also used to identify the mutations responsible for the differences in antigenicity and temperature sensitivity observed between the vaccine and its progenitor.

6.2. Antigenicity

Monoclonal antibody 138 neutralises the Sabin type 3 vaccine, P3/Leon/12a$_1$b, but fails to react with the progenitor strain, P3/Leon/37, or other 'wild' type 3 strains (Ferguson et al., 1982). Two of the mutations observed between the progenitor and the vaccine result in amino acid substitutions in the structural portion of the genome. These are C-U at N2034, which results in a serine to phenylalanine substitution in VP3, and A-G at N3333, which results in a lysine to arginine substitution in VP1. Either of these changes could be involved in recognition of the Sabin vaccine by monoclonal antibody 138.

Six of the recombinants constructed between the vaccine strain and its progenitor were characterised for their reaction with monoclonal antibody 138 in a virus neutralisation assay. As shown in table 6.1., neutralisation by 138 correlates with the presence of the Sabin-specific mutation at N3333. The results clearly demonstrate that the lysine to arginine substitution in VP1 is responsible for the Sabin-specific antigenicity detected by monoclonal antibody 138.

Table 6.1.

Reaction of Poliovirus Recombinants with Monoclonal Antibody 138 in a Virus Neutralisation Assay.

Virus	N2034 VP3	N3333 VP1	Reaction with 138*
rP3/SABIN	Phe	Arg	+
rSLR 1	Phe	Lys	-
rSCC/L	Phe	Arg	+
rSV3,V1/L	Phe	Arg	+
rSV3/L	Phe	Lys	-
rSV1/L	Ser	Arg	+
rSP2/L	Ser	Lys	-
rP3/LEON	Ser	Lys	-

* + titre >8

\- titre <2

Independent evidence in support of this conclusion was obtained by the isolation and characterization of mutants of the Sabin vaccine resistant to monoclonal antibody 138 (Minor et al., 1985). The Sabin-specific mutation at N3333 occurs in codon 285 from the 5′ end of the region coding for VP1. Nucleotide sequencing studies revealed that mutants resistant to 138 carry a base substitution mutation in the adjacent codon of VP1 which results in an asparagine to aspartate change. In addition, a series of isolates were obtained from a vaccinee with hypogammaglobulinaemia who excreted vaccine-derived type 3 poliovirus for a prolonged period after administration of the a monovalent type 3 vaccine. Six of the isolates failed to react with monoclonal antibody 138 and each of these carried a base substitution mutation which caused an asparagine to aspartate substitution 287 amino acid residues from the N-terminus of VP1.

On the basis of the results obtained with the poliovirus recombinants, mutant virus and excreted strains, it was concluded that monoclonal antibody 138 recognises a strain-specific antigenic site encompassing amino acid residues 285 to 287 from the N-terminus of VP1 (Minor et al., 1985). This site has been mapped onto the X-ray crystallographic structure of poliovirus (Hogle et al., 1985) where it is prominently exposed on the surface of the virion.

Of twenty-six neutralising monoclonal antibodies raised against several strains of poliovirus type 3, 138 was the only antibody which recognised the strain-specific site. The remaining antibodies were directed against an independent antigenic site of VP1, including amino acid residues 89 to 100 from the N-terminus. The latter site, designated site 1, is therefore considered to be the immunodominant site of poliovirus type 3 (Minor et al., 1985).

It is tempting to speculate that the lysine to arginine substitution in VP1 contributes to the attenuated phenotype of the Sabin vaccine by reducing the affinity of the virus for its cellular receptor.

The mutation is clearly significant with respect to specific protein-protein interactions, as shown by the different reactions of the progenitor and the vaccine with monoclonal antibody 138. It is also noteworthy that antigenic site 2, the site recognised by 138, is located in the vicinity of a structural motif of picornaviral capsids implicated in receptor recognition (Rossman et al., 1985). Finally, recent evidence implies that site 2 is a composite site composed of elements of VP3, including residues 58,59,77 and 79, and elements of VP1, including residues 285-290 (P. D. Minor, personal communication). This site could therefore be disrupted by the changes in conformation which accompany the shift from D to C antigenicity (Rueckert, 1976). If site 2 is involved in receptor recognition, an attractive model for the failure of C antigen to adsorb to cells can be produced. The inter-strain poliovirus recombinant, rSV1/L allows direct evaluation of the contribution of the lysine to arginine substitution towards attenuation. This recombinant carries the Sabin-specific mutation at N3333 but is otherwise Leon-like. The result obtained when rSV1/L was assayed for neurovirulence in cynomolgus monkeys is reported in section 6.4.

6.3 Temperature Sensitivity.

The rct marker test is based on the temperature sensitivity of the Sabin vaccine strains which replicate poorly at the supraoptimal temperature of 40°C. This test is considered to be the most reliable in-vitro assay for the evaluation of the quality of vaccine preparations (WHO,1983). The titre of the virus is determined at 35°C and at 40°C using a microtitre-plate $TCID_{50}$ assay and the rct/40 value is calculated as the ratio of the $\log_{10}$ titre at 35°C and the $\log_{10}$ titre at 40°C. The standard interpretation of the test is that virus with a reduction in titre at 40°C of less than 2.00 (+/- 0.50) $\log_{10}$ $TCID_{50}$ is regarded as wild-type or

Table 6.2.

Temperature Sensitivity of Recombinant Polioviruses.

Virus	Reduction in Infectivity Titre 35/40°C Log_{10} Expt. 1	Expt. 2	Expt. 3	Mean	rct/40
rST/L	1.50	0.75	1.25	1.17	$rct/40^+$
rS5′/L	1.75	2.25	2.00	2.00	$rct/40^+$
rS3′/L	2.00	2.00	2.25	2.08	$rct/40^+$
rS5′3′/L	2.00	2.25	1.75	2.00	$rct/40^+$
rSLR 1	4.75	4.50	5.00	4.75	$rct/40^-$
rSCC/L	5.50	4.50	5.00	5.00	$rct/40^-$
rSV3,V1/L	5.00	6.00	5.50	5.50	$rct/40^-$
rSV3/L	4.50	4.50	4.75	4.58	$rct/40^-$
rSV1/L	1.25	1.00	0.50	1.00	$rct/40^+$
rSP2/L	2.25	2.00	2.00	2.08	$rct/40^+$
rP3/LEON	2.00	1.75	1.50	1.75	$rct/40^+$
rP3/SABIN	5.75	7.00	4.75	5.83	$rct/40^-$
P3/Leon/37	2.00	1.75	1.00	1.58	$rct/40^+$
P3/30	0.00	0.00	0.00	0.00	$rct/40^+$
71/303	6.50	6.75	4.75	6.00	$rct/40^-$
411	5.25	6.50	4.25	5.33	$rct/40^-$

rct40$^+$. If the reduction in titre is greater than 5.00 (+/- 0.50) Log_{10} $TCID_{50}$, the virus is said to be rct/40$^-$ (Sabin, 1961). Control viruses are used in each experiment and the result is only considered valid if these viruses give the expected results.

In order to identify the mutations responsible for the temperature sensitive phenotype of the Sabin type 3 vaccine, the panel of ten inter-strain recombinants were characterized for their behaviour in the rct assay. P3/Leon/37, the progenitor of the vaccine and P3/30, an unrelated wild-type strain, were used as rct/40$^+$ controls. 71/303, a WHO reference preparation of the Sabin type 3 vaccine, and P3/411, a plaque-purified isolate of the vaccine, were used as temperature sensitive controls. Table 6.2. shows the results obtained in three independent experiments.

Replication of P3/Leon/37 was slightly inhibited at the supraoptimal temperature as shown by a reduction in titre of between 1.00 and 2.00 log_{10} $TCID_{50}$. This inhibition may be caused by the effect of elavated temperatures on the host cell. Lysosomal enzymes, which degrade the viral RNA, are released at temperatures above 38.5°C (Fiszman et al., 1970). An average reduction in titre of between 5.33 and 6.00 log_{10} $TCID_{50}$ was obtained for the temperature sensitive controls. However, the rct/40 values calculated for individual experiments varied between 4.25 and 6.75 for 71/303, and between 4.75 and 6.75 for 71/303. It should be noted that rct/40 values ranging from 4.83 to 6.75 were obtained for 71/303 in eight independent experiments conducted at the N.I.B.S.C. (D. Magrath, personal communication).

Four of the inter-strain recombinants were shown to be vaccine-like with respect to thermo-sensitivity. These were rSCC/L, rSV3/L, rSV3,V1/L and rSLR 1. For these recombinants the reduction in titre obtained at 40°C in each experiment fell within the range observed for the temperature sensitive controls. The remaining recombinants could not be differentiated from the progenitor strain, P3/Leon/37. As shown in Table

Table 6.3.

Correlation of Temperature Sensitivity with Sabin-specific Mutations.

Virus	NC 472	VP3 2034	VP1 3333	P23b 3464	NC 7432	Phenotype
rP3/SABIN	U	Phe	Arg	Ala	G	ts
rST/L	U	Ser	Lys	Thr	A	ts^+
rS5′/L	U	Ser	Lys	Thr	A	ts^+
rS3′/L	C	Ser	Lys	Thr	G	ts^+
rS5′3′/L	U	Ser	Lys	Thr	G	ts^+
rSCC/L	C	Phe	Arg	Ala	A	ts
rSV3,V1/L	C	Phe	Arg	Thr	A	ts
rSV3/L	C	Phe	Lys	Thr	A	ts
rSLR 1	U	Phe	Lys	Thr	A	ts
rSV1/L	C	Ser	Arg	Thr	A	ts^+
rSP2/L	C	Ser	Lys	Ala	A	ts^+
rP3/LEON	C	Ser	Lys	Thr	A	ts^+

6.3., temperature sensitivity correlates with the presence of the Sabin-specific mutation at N2034 (Ser-Phe in VP3). This appears to be the only temperature sensitive mutation in the Sabin vaccine.

Most national control criteria for the rct marker are based on WHO international recommendations . The 1983 recomendations include the use of two elevated temperatures for a more precise determination of the marker (WHO, 1983). The recombinant SV3/L carries the Sabin-specific mutation at N2034 but is otherwise Leon-like. Virus titres were determined at 39°C, 39°C and 40°C. As shown in Table 6.4., rSV3/L is identical to 71/303, indicating that the mutation at N2034 can account for the full temperature sensitive phenotype of the Sabin type 3 vaccine.

Table 6.4.

Temperature Sensitivity of rSV3/L.

				Reduction in Infectivity	
	$TCID_{50}$ Titre (Log_{10})			Titre (Log_{10})	
Virus	35°C	39°C	40°C	39/40°C	35/40°C
rSV3/L	8.13	5.34	3.50	2.79	4.63
71/303	7.00	3.84	2.17	3.16	4.83
P3/Leon/37	8.68	7.84	7.00	0.84	1.68

The results presented above were verified by investigating the rct/40 characteristics of independent isolates of each recombinant. The results of these experiments are shown in Tables 6.5. and 6.6. It was concluded that the temperature sensitive phenotype of the Sabin type 3 vaccine is the result of a single point mutation, C-U at N2034, which leads to a serine-phenylalanine substitution in VP3.

Table 6.5.

Temperature Sensitivity of Recombinant Polioviruses.

	$TCID_{50}$ Titre Log_{10}		Reduction in Infectivity Titre (Log_{10})	
	35°C	40°C	35/40°C	rct/40
rST/L (a)	7.00	5.25	1.75	$rct/40^+$
rST/L (b)	6.50	4.50	2.00	$rct/40^+$
rS5′/L (a)	7.00	4.75	2.25	$rct/40^+$
rS5′/L (b)	4.00	3.25	0.75	$rct/40^+$
rS3′/L (a)	8.25	6.25	2.00	$rct/40^+$
rS3′/L (b)	8.00	6.00	2.00	$rct/40^+$
rS3′/L (c)	8.25	6.00	2.25	$rct/40^+$
rS5′3′/L (a)	7.50	6.75	0.75	$rct/40^+$
rS5′3′/L (b)	8.00	6.50	1.50	$rct/40^+$
rS3′5′/L (c)	7.75	6.75	1.00	$rct/40^+$
P3/30	8.75	8.75	0.00	$rct/40^+$
P3/Leon/37	9.50	7.50	2.00	$rct/40^+$
71/303	7.25	0.75	6.50	$rct/40^-$
P3/411	7.50	2.25	5.25	$rct/40^-$

Table 6.6.

Temperature Sensitivity of Recombinant poloviruses.

	$TCID_{50}$ Titre Log_{10}		Reduction in Infectivity Titre (Log_{10})	
	35°C	40°C	35/40°C	rct/40
rSCC/L (a)	7.75	2.75	5.00	$rct/40^-$
rSCC/L (b)	7.25	2.50	4.75	$rct/40^-$
rSV3/L (a)	7.00	2.00	5.00	$rct/40^-$
rSV3/L (b)	7.25	2.25	5.50	$rct/40^-$
rSV3/L (c)	6.25	1.00	5.25	$rct/40^-$
rSV1/L (a)	7.50	5.25	2.25	$rct/40^+$
rSV1/L (b)	7.25	5.00	2.25	$rct/40^+$
rSV1/L (c)	6.75	4.75	2.00	$rct/40^+$
rSP2/L (a)	6.50	4.50	2.00	$rct/40^+$
rSP2/L (b)	6.50	4.75	1.75	$rct/40^+$
P3/30	8.25	8.25	0.00	$rct/40^+$
P3/Leon/37	8.25	7.25	1.00	$rct/40^+$
71/303	7.50	2.25	5.25	$rct/40^-$
P3/411	8.00	3.25	4.75	$rct/40^-$

This mutation is chemically the most drastic of the amino acid substitutions observed between the vaccine and its progenitor. When mapped onto the X-ray crystallographic structure of poliovirus type 1 (Hogle et al., 1985), it was shown that the mutation occurs within a region of structural importance. The serine-phenylalanine change is located 91 amino acid residues from the N-terminus of VP3. This corresponds to a region of α-helix, located on the bottom surface of the wedge shaped β-barrel.

6.4. The WHO Neurovirulence Assay.

Neurovirulence can be measured quantitatively in monkeys by correlating the incidence of paralysis or the development of neuronal lesions within the CNS with the concentration of virus determined in-vitro using a $TCID_{50}$ assay. In the past it was considered important to measure neurotropic activity of a virus by means of the intrathalamic and the intraspinal route of inoculation. However the intrathalamic test suffers from the relative insusceptibility of monkeys to the virus when it is injected by this route (Sabin, 1956; WHO, 1982). Very few animals show any evidence of virus activity following intrathalamic inoculation and, of these, the majority show only mild lesions limited to the inoculation trauma (Winter and Boulger, 1963). The intrathalamic test is capable of revealing only gross differences in neurovirulence and is no longer included as a mandatory test for vaccine standardisation (WHO, 1938).

In the original intraspinal test, the incidence of paralysis was observed following inoculation of monkeys with graded doses of virus. The test was designed to measure a 50% end point of virus activity and invariably involved a large number of negative monkeys. The revised WHO intraspinal neurovirulence assay, used throughout this study is based on a smaller number of monkeys. Greater emphasis is now placed on the character and the distribution of the lesions within the CNS.

Cynomolgus monkeys are now routinely used for neurovirulence assay. For each test, the concentration of virus was determined in-vitro using a $TCID_{50}$ assay. The virus preparation was diluted to give a final Concentration of between 5.5 and 6.5 $\log_{10}$ $TCID_{50}$ in 0.1ml. Monkeys, routinely four per virus, were inoculated with 0.1ml of the virus suspension by injection into the lumbar region of the spinal cord. All monkeys were observed for a period of 22 days for symptoms suggestive of poliomyelitis. Monkeys were sacraficed either at the onset of prostrating paralysis or at the end of the observation period. All animals were autopsied and representative sections of the brain and spinal cord were prepared for histological examination.

A standard scoring system was used to evaluate virus activity in the hemisections of the spinal cord and brain. This scoring system takes into account the nature of the virus specific damage which is considered to be important. A value 0,1,2,3 or 4 was assigned to each hemisection as follows:

1 : Cellular infiltration only.

2 : Cellular infiltration with minimal neuronal damage.

3 : Cellular infiltration with extensive neuronal damage.

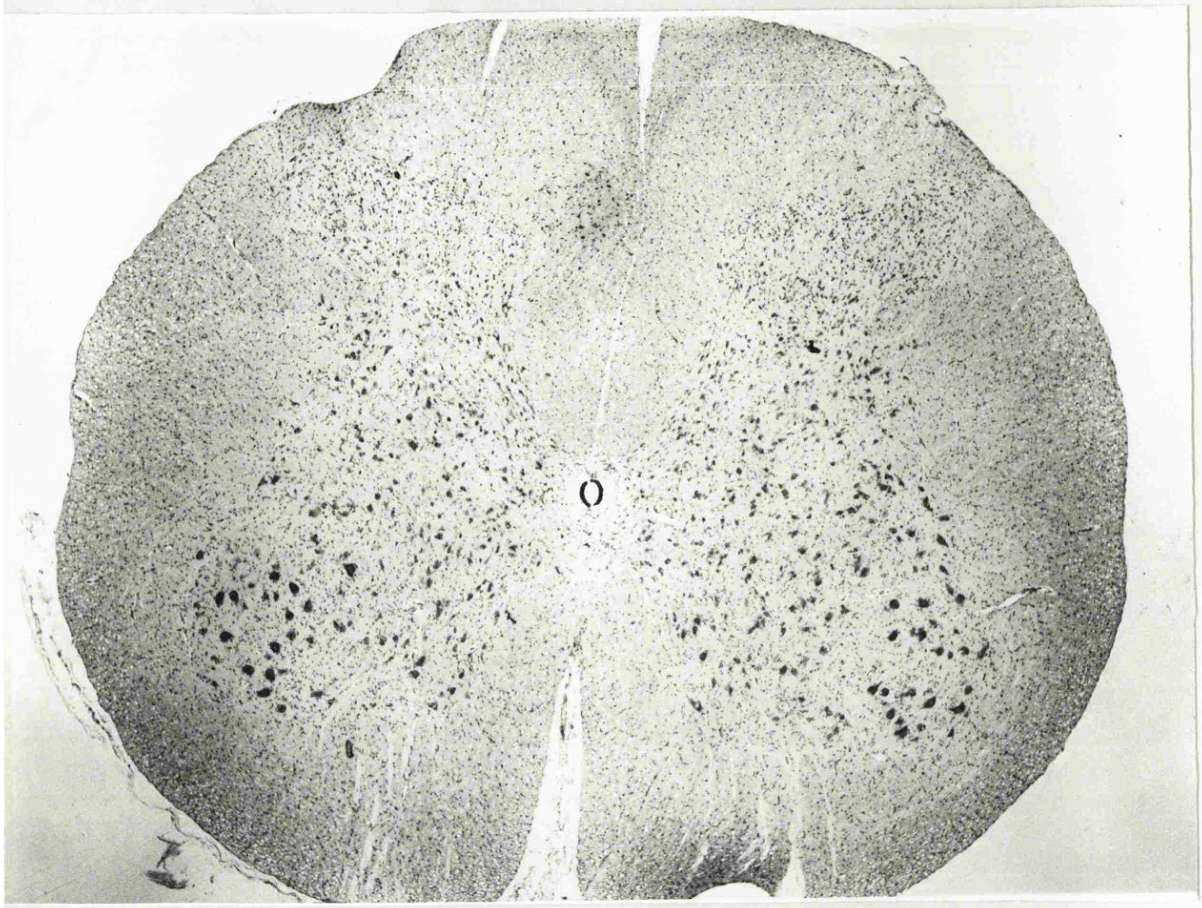
4 : Massive neuronal damage with or without cellular infiltration.

A lesion score, based on the hemisection readings of the lumbar (L), cervical cord (C) and brain (B) histological sections, was calculated as follows:

$$LS = \text{Mean L Score} + \text{Mean C Score} + \text{Mean Brain Score} \div 3$$

A mean lesion score was then calculated for each group of animals.

With experience it is possible to differentiate between the character of lesions induced by an attenuated strain of poliovirus and the character of lesions induced by a virulent strain. Fig. 6.2. shows a section of the lumbar region of the spinal cord of an uninfected animal at X30 and X125 multiplication. The lower motor neurons of the anterior horn of the spinal cord are clearly visible. Fig. 6.3. shows the corresponding region of an animal inoculated with 5.5 to 6.5 log10 TCID50 of a reference preparation of the Sabin type 3 vaccine. The most striking feature is the heavy immune infiltration concentrated in the region of blood vessels. This feature, termed "perivascular cuffing", is charactaristic of attenuated-type lesions (S. Marsden, personal communication). Neuronal damage is minimal, with the majority of lower motor neurones unnafected. Finally, Fig 6.4. shows a section of the corresponding region of an animal innoculated with P3/Leon/37. Immune infiltration is clearly more diffuse. The most striking feature of this section is the complete destruction of the lower motor neurons of the anterior horn. This section is typical of a virulent-type lesion.



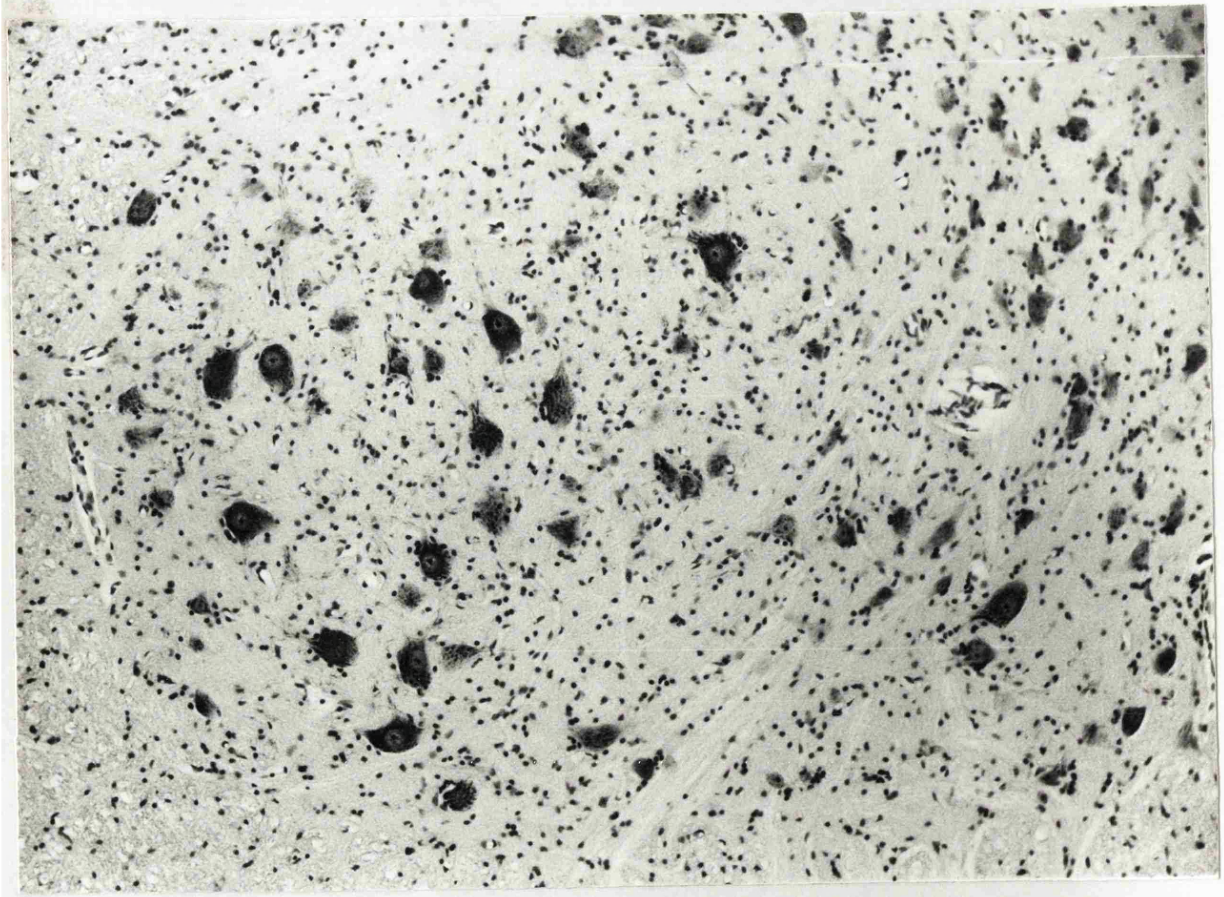
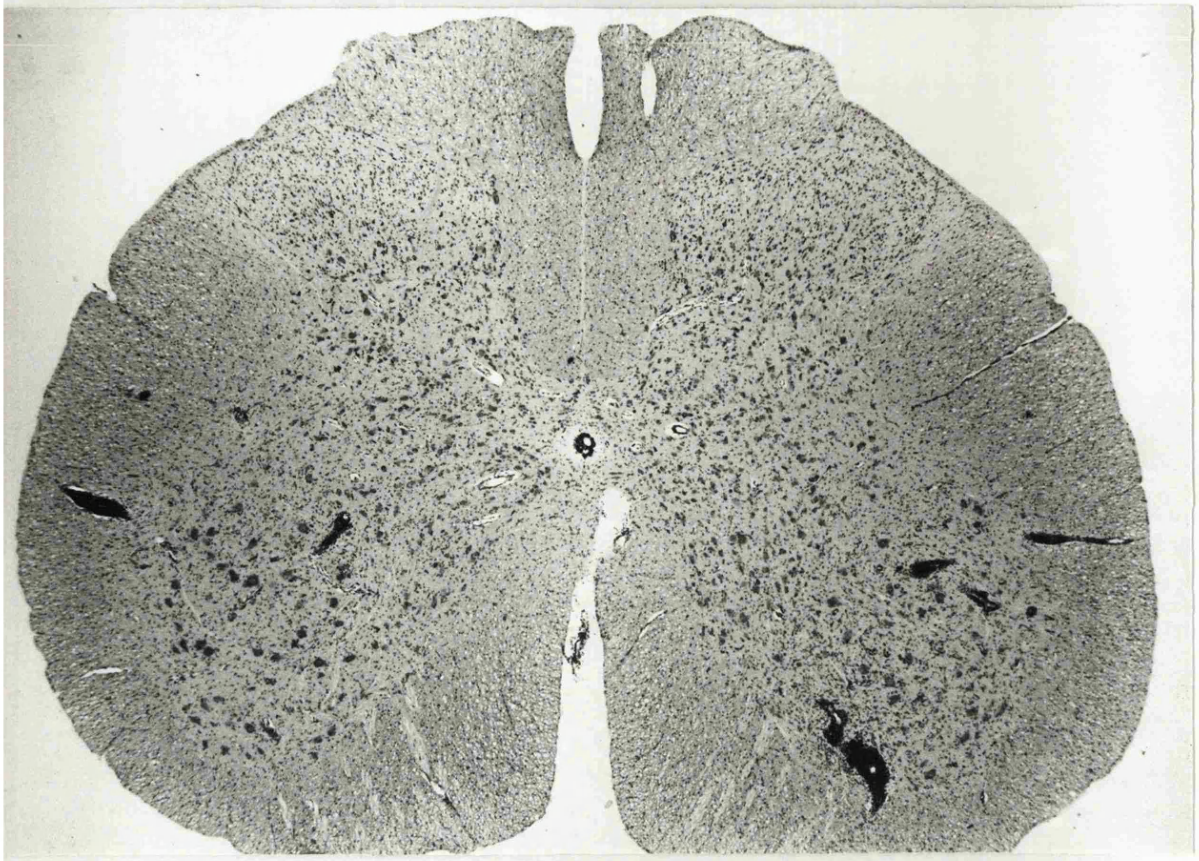


Fig. 6. 2.

Histological Section of Uninfected Spinal Cord

This figure shows a section from the lumbar region of the spinal cord of an uninfected animal at X30 and X125 magnification. The lower motor neurons of the anterior horn are clearly visible at the higher magnification.



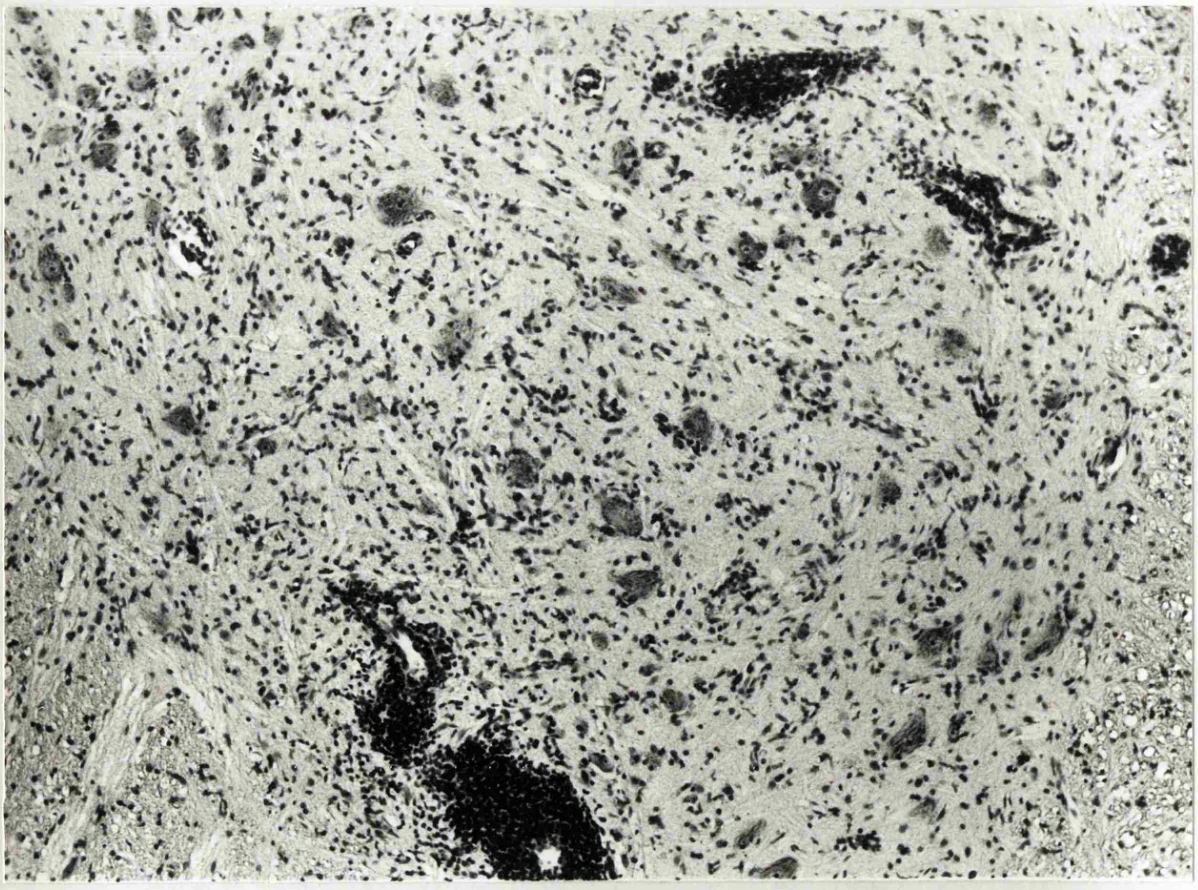
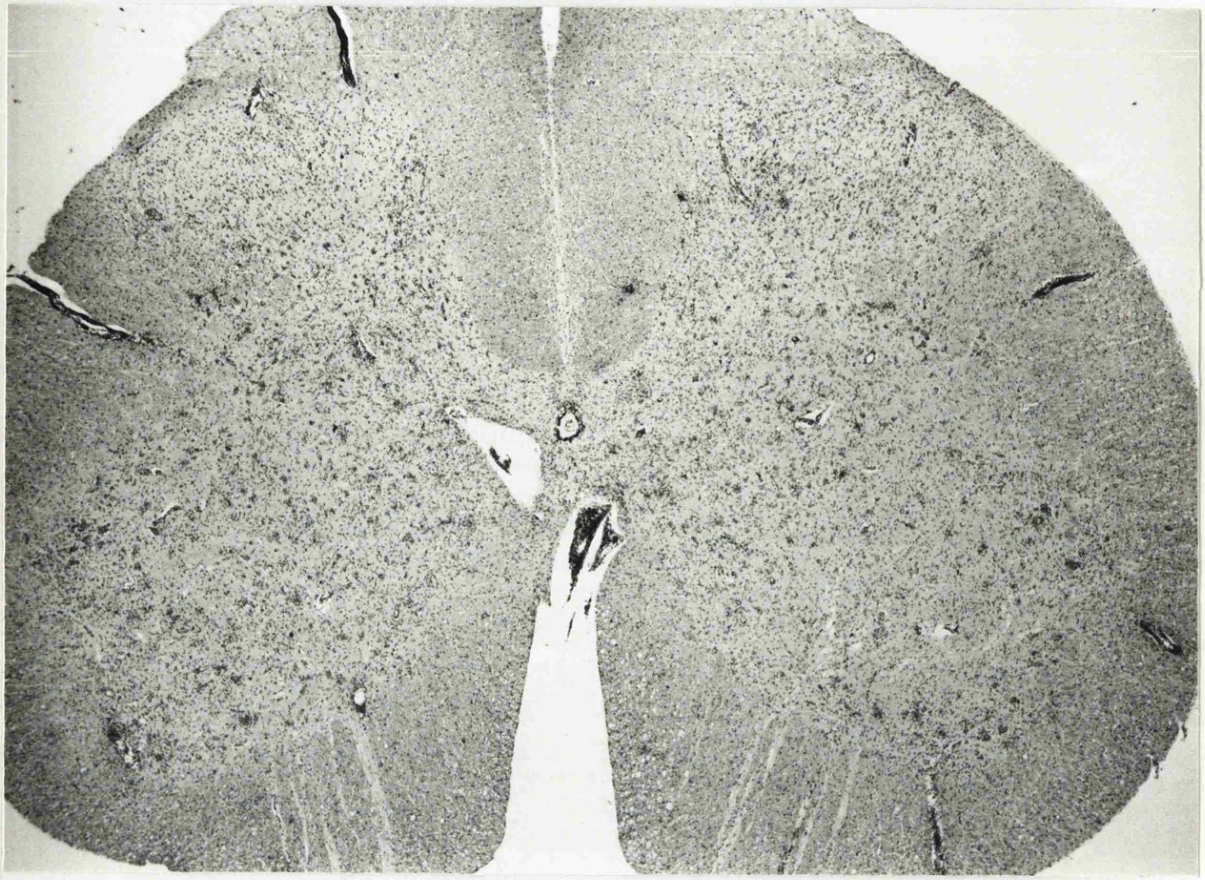


Fig. 6. 3.

Histological Section of an Infected Spinal Cord.

(1) P3/Leon/12a_1b

This figure shows a section from the lumbar region of the spinal cord of an animal infected with a reference preparation of the Sabin type 3 vaccine, P3/Leon/12a_1b. Neuronal damage is moderate and confined to the left anterior horn. The most striking feature of this section is the heavy immune infiltration concentrated in the vicinity of blood vessels. This feature, termed perivascular cuffing, is characteristic of an `attenuated type' lesion.



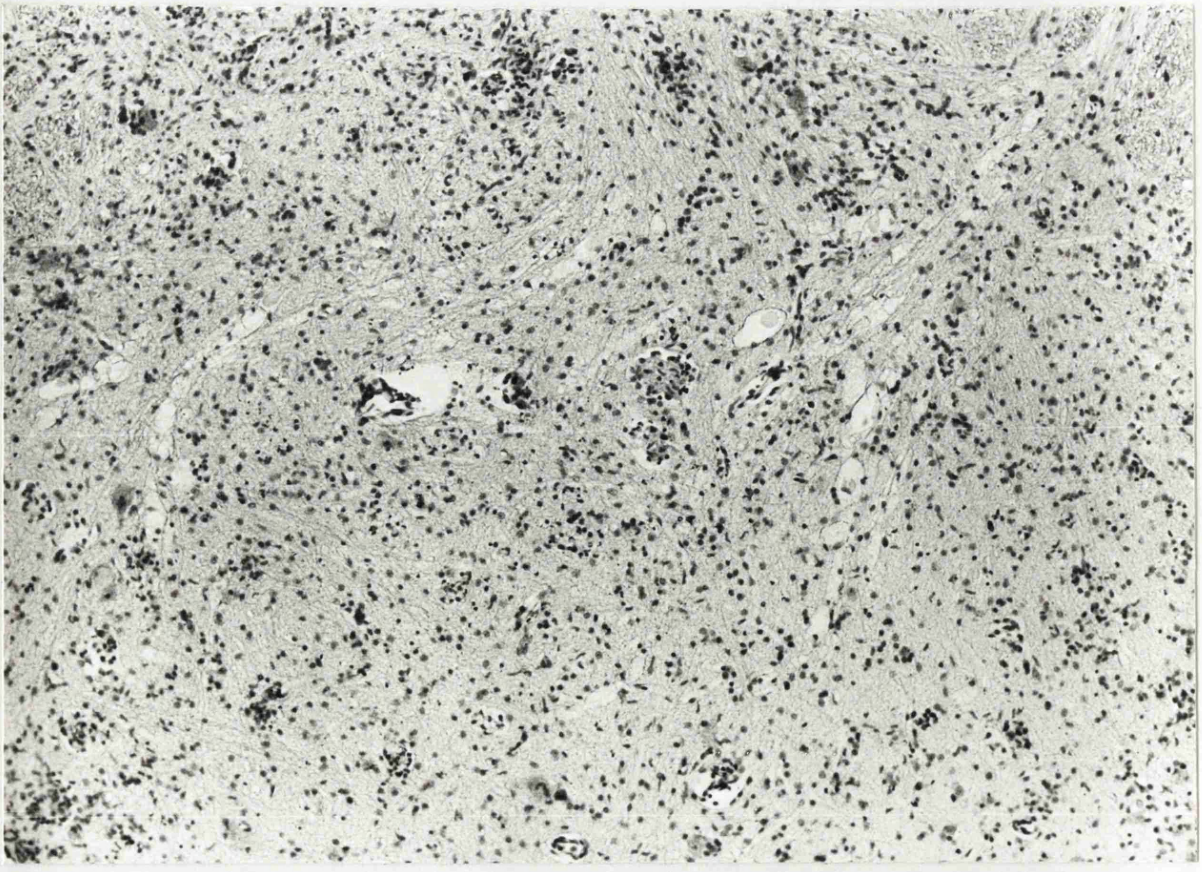


Fig. 6. 4.

Histological Section of an Infected Spinal Cord.

(2) P3/Leon/37

This figurs shows a section from the lumbar region of the spinal cord of an animal infected with P3/Leo/37. Immune infiltration is more diffuse than observed for an attenuated strain. The most striking feature of this section is the complete destruction of the lower motor neurons of the anterior horn.

Table 6.7.1.

Neurovirulence of rP3/LEON.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
D83	+	+	4.00	2.85	1.56	2.80
D84	+	+	4.00	2.45	1.06	2.50
D85	+	+	3.57	2.32	1.75	2.55
D86	+	+	4.00	3.45	1.56	3.00

Mean Histological Lesion Score : 2.71

Table 6.7.2.

Neurovirulence of rSV1/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
100	+	+	3.93	2.20	1.38	2.50
101	+	-	3.30	2.20	2.25	2.59
102	+	+	4.00	3.10	2.00	3.03
103	+	+	4.00	2.70	1.13	2.61

Mean Histological Lesion Score : 2.68

Table 6.7.3.

Neurovirulence of rSP2/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
144	+	+	4.00	2.60	1.69	2.76
145	+	+	3.22	1.20	1.14	1.99
146	+	+	3.83	2.35	1.38	2.52
147	+	+	3.67	3.05	1.63	2.78

Mean Histological Lesion Score : 2.51

Table 6.7.4.

Neurovirulence of rSV3/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
164	+	-	3.63	2.25	2.13	2.67
165	+	-	1.96	0.10	0.13	0.73
166	-	-	0.58	0.00	0.00	0.19
167	+	-	2.00	1.75	1.94	1.90

Mean Histological Lesion Score : 1.37

Table 6.7.5.

Neurovirulence of SV3.V1/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
148	-	-	0.85	0.10	0.31	0.42
149	-	-	1.33	0.35	0.56	0.75
150	-	-	3.47	2.00	1.81	2.01
151	-	-	2.32	2.00	1.93	2.09

Mean Histological Lesion Score : 1.32

Table 6.7.6.

Neurovirulence of rSCC/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
168	+	+	3.31	2.35	1.63	2.43
169	-	-	1.15	1.40	0.63	1.06
170	+	+	3.26	2.90	2.13	2.76
171	-	-	2.18	2.00	1.88	2.02

Mean Histological Lesion Score : 2.06

Table 6.7.7.

Neurovirulence of rST/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
D260	+	-	1.96	0.15	0.31	0.81
D261	+	+	3.20	2.21	1.06	2.16
D264	-	-	1.18	0.00	0.15	0.44

Mean Histological Lesion Score : 1.14

Table 6.7.8.

Neurovirulence of S5´/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
180	-	-	0.74	0.20	0.06	0.33
181	-	-	0.34	0.00	0.25	0.20
182	-	-	1.15	0.15	0.31	0.54
183	-	-	1.00	0.45	0.50	0.65

Mean Histological Lesion Score : 0.43

Table 6.7.9.

Neurovirulence of rS5′3′/L.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
152	-	-	2.07	1.45	1.56	1.69
153	-	-	0.96	0.05	0.69	0.57
154	-	-	2.82	1.95	1.69	2.15
155	+	-	2.40	1.50	1.81	1.90

Mean Histological Lesion Score : 1.58

Table 6.7.10

Neurovirulence of S3′/L

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
176	+	+	3.11	2.05	1.56	2.24
177	+	+	4.00	3.85	1.85	3.39
178	+	-	2.89	1.80	1.20	1.71
179	+	+	3.65	1.95	0.95	2.26

Mean Histological Lesion Score : 2.40

Table 6.7.11.
Neurovirulence of rSLR 1.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
C261	-	-	1.74	1.55	1.56	1.62
C262	-	-	0.43	0.00	0.06	0.16
C263	+	-	2.42	1.55	1.31	1.76
C264	-	-	0.61	0.10	0.06	0.26
C266	-	-	2.54	1.00	1.25	1.60
C267	-	-	2.92	1.65	1.44	2.01
C268	-	-	2.14	0.75	0.63	1.17

Mean Histological Lesion Score : 1.23

Table 6.7.12.

Neurovirulence of rP3/SABIN.

	Clinical Polio	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score
E82	+	-	0.57	0.00	0.00	0.20
E83	-	-	0.39	0.00	0.19	0.19
E84	-	-	0.07	0.00	0.00	0.02
E85	-	-	0.14	0.00	0.00	0.05

Mean Histological Lesion Score : 0.11

Table 6.8.

Neurovirulence of Interstrain Poliovirus Recombinants.

Virus	Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score	Range
rP3/Leon	4/4	3.89	2.76	1.48	2.71	(2.50-3.03)
rSV1/L	3/4	3.80	2.55	1.69	2.68	(2.50-3.03)
rSP2/L	4/4	3.68	2.30	1.46	2.51	(1.99-2.78)
rS3′/L	3/4	3.41	2.41	1.39	2.40	(1.71-3.39)
rSV3/L	0/4	2.04	1.03	1.05	1.37	(0.19-2.67)
rSV3,V1/L	0/4	1.99	1.11	1.15	1.32	(0.42-2.09)
rSCC/L	2/4	2.48	2.16	1.57	2.06	(1.06-2.76)
rST/L	1/4	2.11	0.79	0.50	1.14	(0.46-2.16)
rS5′/L	0/4	0.81	0.20	0.28	0.43	(0.20-0.65)
rS5′3′/L	0/4	2.06	1.24	1.44	1.58	(0.96-1.79)
rP3/SABIN	0/4	0.29	0.00	0.05	0.11	(0.05-0.20)

6.5. Neurovirulence of Inter-strain Poliovirus Recombinants.

This section reports the results obtained when the ten interstrain poliovirus recombinants were characterized for their behaviour in the WHO intraspinal neurovirulence assay. The results of individual experiments are shown in Tables 6.7.1. to 6.7.12. These are summarized in Table 6.8.

6.5.1. The Sabin-specific coding changes.

Virus rescued from the recombinant plasmid SV1/L (designated rSV1/L) carries the Sabin-specific coding change at N3333 (Lys-Arg in VP1), but is otherwise Leon-like. On testing in cynomolgus monkeys, rSV1/L was found to be indistinguishable from the parental strain, P3/Leon/37. 3/4 animals developed severe flaccid paralysis within three days of inoculation. All animals showed massive neuronal damage throughout the lumbar region of the spinal cord, with clear evidence of viral replication at higher levels in the CNS. The mean lesion score of 2.68 is identical to that previously obtained for animals inoculated with rP3/LEON (2.71). This results demonstrates that the mutation at N3333 is not attenuating.

rSP2/L carries the Sabin-specific coding change at N3464 (Thr-Ala in P2-3b) but is otherwise Leon-like. Each of four animals inoculated with 5.5 to 6.5 $\log_{10}$ $TCID_{50}$ of virus developed full paralysis during the first week of the test. Clinical disease was correlated in each case with a level of neuronal damage consistent with a virulent phenotype. The mean lesion score of 2.51 is very similar to that obtained for rP3/LEON. The mutation at N3464 is clearly not significant to the attenuated phenotype. This result was expected because N3464 is conserved in a neurovirulent revertant of the vaccine, P3/WHO/119. The three potential suppressor mutations observed in the revertant are located in the structural region of the genome, precluding a role in the suppression of a mutation in the non structural protein P2-3b (Cann et al., 1984).

The mutation at N2034 (Ser-Phe in VP3) has been segregated from other Sabin-specific mutations in rSV3/L. This recombinant failed to induce clinical paralysis. However, the mean lesion score calculated for the group was 1.37, which is intermediate between an attenuated and a virulent phenotype. Analysis of the data available for individual animals indicated that the situation was more complex (see Table 6.7.4.). In contrast to the results obtained for the virulent recombinants, considerable variation in the level of viral activity was observed between animals. In animals 165 and 166, viral replication was confined to the lumbar cord where neuronal damage was minimal and the lesions induced were attenuated in character. However the level of virus-specific damage induced in animal 168 was similar to that observed for rP3/LEON (see Table 6.7.1.). In veiw of the extensive neuronal damage observed throughout the lumbar cord, it is surprising that this animal showed no clear signs of paralysis.

rSV3,V1/L carries the coding change at N3333 (Lys-Arg in VP1) in addition to the change at N2034 (Ser-Phe in VP3). Each of four animals survived the 22 day observation period without developing clinical paralysis. The mean lesion score of 1.32 is identical to that obtained for rSV3/L. A similar spectrum of viral activity was observed, ranging from attenuated (animals 148 and 149 in Table 6.7.5.), through `intermediate' (animal 151) to almost full virulence (animal 150).

rSCC/L carries the three Sabin-specific coding changes but lacks the non-coding changes at N472 and N7432. 2/4 animals inoculated with rSCC/L developed clinical paralysis during the first week of the test, suggesting that this recombinant may be more neurotropic than either rSV3/L or rSV3,V1/L. However, it should be noted that, in terms of the level of neuronal damage, the animals showing clinical paralysis could not be differentiated from animal 164 in the rSV3/L assay or animal 150 in the rSV3,V1/L assay (see Table 6.8.).

Table 6.8.

Neurovirulence of Recombinant Polioviruses.

Virus		Paralysis	Mean Lumbar Score	Mean Cervical Score	Mean Brain Score	Overall Lesion Score	
rSV3/L	164	-	3.63	2.25	2.13	2.67	V
rSV1,V3/L	150	-	3.47	2.00	1.81	2.01	V
rSCC/L	168	+	3.31	2.35	1.63	2.43	V
rSCC/L	170	+	3.26	2.90	2.13	2.76	V

V : Lesions induced in the CNS are virulent in character

Wide variation in the level of virus activity was observed between animals (see Table 6.7.6.). In this respect rSCC/L is clearly distinct from rP3/LEON and resembles rSV3/L and SV3,V1/L. Animals 168 and 170 showed a virulent type response correlated with clinical paralysis, animal 171 was `intermediate' and the level of virus specific damage induced in animal 169 is more consistent with an attenuated phenotype.

Fig. 7.5. compares the pattern of virus-specific damage induced by the recombinants rSV3/L, rSV3,V1/L and rSCC/L with that observed for rP3/LEON. These recombinants appear to have a similar phenotype. In approximately half the animals inoculated with 5.5 to 6.5 $\log_{10}$ $TCID_{50}$ of virus, extensive neuronal damage was observed in the lumbar cord with spread of the infection to higher levels of the CNS. The lesions were virulent in character and the level of neuronal damage approached the threshold required to induce flaccid paralysis. In the remaining animals, the virus failed to establish a clinically significant infection. Viral replication was largely confined to the lumbar cord where the neuronal damage was minimal and the lesions induced were attenuated in character.

This variable response may represent the 50% end point of viral activity. It would therefore be interesting to compare the effect of graded doses of P3/Leon/37 and rSV3/L on cynomolgus monkeys. It was concluded that the mutation at N2034 (Ser-Phe in VP3) contributes towards attenuation. However, this mutation does not account for the full attenuated phenotype of the vaccine.

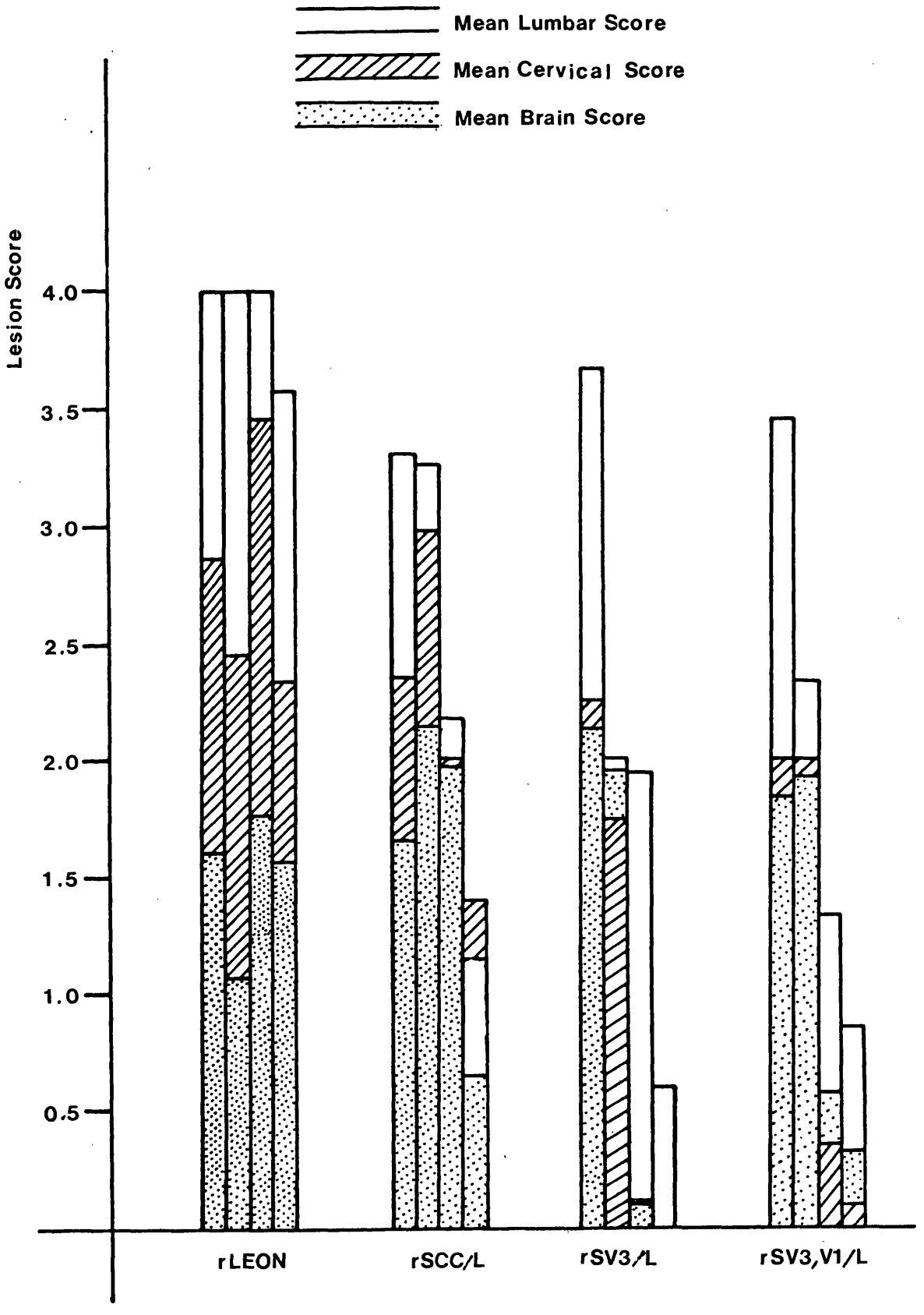

Mean Lumbar Score
Mean Cervical Score
Mean Brain Score
Lesion Score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
rLEON
rSCC/L
rSV3/L
rSV3,V1/L

Fig. 6. 5.

Pattern of Virus Specific Damage Induced by Poliovirus Recombinants.

Mean lesion scores were calculated for the lumbar and cervical regions of the spinal cord and the brain. This figure shows the data plotted in the form of a histogram. For animals inoculated with poliovirus recombinants carrying the Ser-Phe mutation in VP3 (i.e. SV3/L, SV3,V1/L and SV1/L), considerable variation was observed both in the extent and the distribution of virus specific damage. In contrast, animals innoculated with the parental strain, P3/Leon/37, showed similar patterns of virus specific damage (see text for explanation).

6.5.2. The Non-coding Changes.

Virus rescued from S3′/L carries three of the Sabin-specific mutations. These are the silent mutations at N6127 and N7165 together with the A-G substitution at N7432 in the 3′ non-coding region. 3/4 animals developed clinical paralysis within three days of inoculation. The mean lesion score of 2.40 is similar to that obtained for rP3/LEON. It was concluded that the mutation at N7432 is not attenuating. In support of this conclusion, sequence variation has been observed at the homologous position of different preparations of the virulent type 1 strain, P3/Mahoney, without any reported effects on neurovirulence (Kitamura et al., 1981; Racaniello and Baltimore, 1981a).

Virus rescued from the recombinant plasmid ST/L derives its 5′ terminal 1809 nucleotides from the Sabin vaccine and the remainder of its genome from P3/Leon/37. rST/L therefore carries the mutation at N472, which has been implicated in attenuation by nucleotide sequence comparisons, together with the presumed silent changes at N220 and N871. Nucleotide sequence analysis of purified viral RNA revealed that the recombinant carried an additional mutation, a deletion at N417 in the 5′ non coding region.

The results of the neurovirulence assay on rST/L were similar to those obtained for recombinants carrying the Ser-Phe mutation in VP3. 1/3 animals developed full flaccid paralysis on day four of the test which was correlated with a level of virus specific damage similar to that observed for rP3/LEON. In the remaining animals, replication of the virus was clearly confined to the lumbar cord, inducing only minimal neuronal damage. The mean lesion score of 1.14 suggested an intermediate level of virulence.

The mutations at N220 and N871 are conserved in the revertant of the vaccine, P3/WHO/119 (Cann et al., 1984). It should also be noted that virus rescued from a full-length cDNA of P3/WHO/119 was shown to be highly

neurotropic (M. Skinner, personal communication). It can therefore be concluded that the mutations at N220 and N871 are not significant. However, the possibility that the deletion at N417 contributes towards the partially attenuated phenotype of rST/L cannot be ruled out. An additional recombinant was therefore constructed.

rS5′/L carries the Sabin-specific mutations at N472, N220 and N871 but lacks the deletion at N417. On testing in cynomolgus monkeys, the recombinant appeared to have a completely attenuated phenotype. The virus failed to induce clinical paralysis. The mean lesion score of 0.43 falls within the range observed for the reference preparations of the vaccine (D. Magrath, personal communication). In view of the observation that S5′/L is at least as attenuated as ST/L, it is clear that the mutation at N472 can account for the attenuated phenotypes of both recombinants. The results also imply that the deletion at N417 is not significant.

The results obtained for the final recombinant suggest that the low mean lesion score for S5′/L is an artifact of a test involving only four animals. rS5′,3′/L carries the mutation at N472 in the 5′ non-coding region and the mutation at N7432 in the 3′ non-coding region. This recombinant failed to induce clinical paralysis. However the mean lesion score of 1.58 suggests an intermediate level of virulence and therefore supports the result obtained for rST/L. It was concluded that the mutation at N472 contributes towards attenuation, but cannot account for the full attenuated phenotype of the vaccine.

6.6. Summary and Conclusions.

6.6.2. The Genetic Basis for Attenuation.

The attenuated phenotype of the Sabin type 3 vaccine strain results from the concerted effect of two independent point mutations. The first is a C-U substitution at N472 in the 5′ non-coding region. The second is a C-U substitution at N2034 which induces a serine to phenylalanine change in VP3. The data presented in support of this conclusion is undeniably complex. Nevertheless, each of the above mutations has been shown to reduce the potential of P3/Leon/37 for causing a clinically significant infection of cynomolgus monkeys following intraspinal administration of high doses of virus. Similar or identical results were obtained for three idependently derived recombinants carrying the mutation at N472, i.e. rST/L, rS5′/L and rS5′3′/L and three independent recombinants carrying the mutation at N2034, i.e. rSV3/L, rSCC/L and rSV1,V3/L.

The variable response obtained for recombinants carrying N472 or N2034 suggested that these viruses had a 50% end point of neurovirulence near to the concentration used as an inoculum. If this interpretation is correct, these recombinants have a phenotype which is intermediate between attenuated and virulent. It follows that neither mutation alone can account for the full attenuated phenotype of the Sabin type 3 vaccine. However, an alternative explanation for the observed variation is possible.

rST/L carries a single attenuating mutation, C-U at N472 in the 5′ non-coding region. It is reasonable to assume that reversion to neurovirulence must occur by direct back mutation. Estimates of the frequency of RNA genome mutation have been obtained using monoclonal antibodies to select for antigenic variants. Minor and co-workers (1984) calculated that the average frequency with which resistant mutants appear under monoclonal selection is about 10^{-4} or 1 PFU per 10^{4} wild-type PFU.

Escape from neutralisation frequently involved mutation at a single nucleotide position (Evans et al., 1984). The mutation at N472 may therefore revert with a similar high frequency.

The results obtained when rST/L was assayed for neurovirulence are shown in Table 6.7.7. In two of the animals, the infection was confined to the lumbar region of the spinal cord, which is consistent with a full attenuated phenotype. However it is clear that replication of the virus has occurred. Reversion of the mutation at N472 may occur during the course of viral replication. Once formed, such revertants would be subject to strong selection pressure. Animal D261 developed full flaccid paralysis within 3 days of innoculation. Clinical disease was correlated with a level of neuronal damage approaching that observed for P3/Leon/37. This result could be the consequence of in-vivo reversion of the mutation at N472. Reversion of the mutation at N2034 may occur with a slightly higher frequency because in this case reversion could be achieved by direct back-mutation at N2034 or suppression by mutation at a distal site.

The experiments described in section 6.5. have identified the mutations involved in attenuation. However, it is not possible to draw any firm conclusions on the relative importance of these mutations. A more valid quantitative comparison of the level of virulence of rSV3/L, rST/L and rP3/SABIN could be achieved by investigating the effect of graded doses of virus on cynomolgus monkeys. It would also be of interest to characterize virus isolated from infected animals to investigate the frequency of in-vivo reversion.

Finally, Almond et al. (1984) reported that virus rescued from the recombinant plasmid rSLR 1, which carries the mutations at N472 and N2034, was not fully attenuated. It should be noted that this refers to a pool of virus rescued at 37°C. Passage of the Sabin vaccine at this temperature may lead to reversion of the temperature sensitive phenotype. The result is therefore not directly comparable to the results obtained for the remaining recombinants which were rescued at the permissive temperature of 35°C.

6.6.2. Correlation of Attenuation with in-vitro markers.

rSV1/L carries the mutation at N3333 (Lys-Arg in VP1) but is otherwise Leon-like. The recombinant is neutralised by monoclonal antibody 138, demonstrating that the lysine to arginine substitution is responsible for the antigenic differences observed between the Sabin vaccine and its progenitor. In view of the conservative nature of the substitution, together with its location in the C-terminal extension of VP1 (Hogle et al., 1985), the mutation is unlikely to induce distant effects on the conformation of the protein. It was therefore not suprising to find that rSV1/L was wild-type with respect to temperature sensitivity. It is also clear that the mutation does not contribute to the attenuated phenotype of the vaccine. rSV1/L is highly neurotropic and cannot be differentiated from the progenitor P3/Leon/37.

The temperature sensitive phenotype of the Sabin vaccine was shown to be the result of the C-U substitution at N2034, which induces a serine to phenylalanine change in VP3. In rSV3/L, the mutation at N2034 has been transfered to the genome of P3/Leon/37 idependently of the other Sabin-specific mutations. rSV3/L was shown to be less neurotropic than P3/Leon/37, providing clear evidence of a correlation between temperature sensitivity and attenuation.

CHAPTER SEVEN

Discussion

7.1. Introduction

Although developed countries have experienced a dramatic reduction in the incidence of poliomyelitis since vaccination was introduced, those countries using the live attenuated vaccines developed by Sabin have noted a residual level of approximately 0.02 to 0.20 cases/ million population/ year (Melnick, 1982). Evidence has accumulated that this persistant low level of disease may be caused by the vaccines themselves. It is now clear that the type 3 vaccine (and most probably the type 2 vaccine) can revert to neurovirulence during replication in recipients of the trivalent vaccine or their contacts, although this rarely if ever seems to be the case for type 1 (Cossart et al., 1977; Nottay et al., 1981; Minor, 1980; Cann et al., 1984). Prospects for improving the Sabin vaccines would be enhanced by a clearer understanding of the molecular basis for attenuation and reversion.

Poliovirus type 3 is the the serotype most frequently implicated in vaccine associated poliomyelitis. Complete nucleotide sequences have been determined for the neurovirulent progenitior of the Sabin type 3 vaccine, P3/Leon/37, the type 3 vaccine itself, P3/Leon/12a$_1$b, and a virulent revertant strain isolated from a fatal case of vaccine associated poliomyelitis, P3/WHO/119. Analysis of the data suggested that the following mutations were involved in attenuation and reversion: (1) a single base substitution mutation (C-U) at position 472 in the 5'

non-coding region of the genome; (2) nucleotide substitutions resulting in amino acid substitutions in the capsid proteins (Ser-Phe in VP3 and Lys-Arg in VP1); and (3) a single base substitution mutation (A-G) at the end of the 3′ non-coding region.

The major objective of the work presented for this thesis was to define precisely the contribution of the above mutations to the attenuated phenotype of the Sabin type 3 vaccine. To achieve this, full-length cDNA copies of the RNA genomes were constructed, each within the prokaryote vector pAT 153. Transfection of Hep2c monolayers with the full-length cDNAs generated virus which was indistinguishable from the respective parental strains, confirming and extending the earlier observation of Racaniello and Baltimore (1981). Recombinant genomes were then constructed in-vitro at the level of cDNA using conserved restriction endonuclease sites. Transfection of Hep 2c monolayers with these recombinant cDNAs yielded virus with defined sets of mutations. These were characterised for their reaction in the WHO monkey neurovirulence assay.

7.2. The Genetic Bases for Attenuation of the Sabin type 3 Vaccine.

The results presented in chapter 6 provide strong evidence that the attenuated phenotype of the Sabin type 3 vaccine is the concerted effect of two independent mutations: C-U at N472 in the 5′ non-coding region and C-U at N2034 which results in a serine to phenylalanine change in VP3. The remaining Sabin-specific mutations have no effect on the neurovirulence of P3/Leon/37. Independent evidence in support of this conclusion has been obtained by extensive nucleotide sequence analysis of 13 strains of poliovirus type 3 which, in addition to P3/WHO/119, had been implicated in vaccine associated paralysis (Minor; 1980, Kew et al., 1981; Minor, 1982). Partial nucleotide sequences were derived directly from the viral RNA by primer extension. The results (Evans et al., personal communication) are summarised in Table 7.1.

Table 7.1.

Comparison of Poliovirus type 3 Strains at Critical Regions in the Genome.

Virus	Neurovirulence	NC 472	VP2 1548	VP2 1592	VP3 2034	VP1 2637	VP1 3333
P3/Leon/37	+	C	Arg	Leu	Ser	Ala	Lys
P3/Leon/12a_1b	-	U	Arg	Leu	Phe	Ala	Arg
P3/WHO/119	+	C	Lys	Met	Phe	Val	Arg
Revertant							
P3/106	+	C	Arg	Leu	Phe	Ala	Arg
P3/115	+	C	Arg	Leu	Phe	Ala	Arg
P3/116	+	C	Arg	Leu	Ser	Thr	Arg
P3/122	+	C	Arg	Leu	Phe	Ala	Arg
P3/131	+	C	Arg	Leu	Phe	Val	Arg
P3/132	+	C	Arg	Leu	Phe	Ala	Arg
P3/146	+	C	Arg	Met	Phe	Ala	Arg
P3/156	+	C	Arg	Leu	Ser	Thr	Lys
P3/158	+	C	Arg	Met	Phe	Val	Arg
P3/161	+	C	Arg	Leu	Ser	Thr	Arg
P3/263	+	C	Arg	Leu	Phe	Ala	Arg
P3/382	+	C	Arg	Leu	Phe	Thr	Arg
P3/414	+	C	Arg	Leu	Phe	Ala	Arg

In all 13 revertants the nucleotide at N472 was cytidine, as in P3/Leon/37. In contrast the nucleotide at this position in two batches of the vaccine was uridine, confirming the previous findings of Stanway et al. (1984) and Toyoda et al. (1984). The consistency with which back mutation at N472 was detected amongst the vaccine derived revertant viruses suggested that this nucleotide was of significance to attenuation and reversion to neurovirulence. Further information on the importance of position N472 came from studies on viruses isolated after human passage (Evans et al., 1985).

Poliovirus type 3 was isolated from the faeces of a six month old infant following routine administration of the trivalent live attenuated vaccine. Virus pools obtained from faecal samples taken various times post- vaccination were sequenced in the region surrounding N472. As shown in Table 7.2., virus shed in the first two positive samples contained U at N472. In contrast, faecal samples taken at 47 hours and later predominantly contained virus with C at N472. Virus prepared from the 35 hour sample appeared to consist of a mixed population, since U and C bands of equal intensity were detected on the sequencing gel. This result implied that growth in the human gut provided a strong selection pressure for viruses containing C at N472. To determine whether this change also influenced neurovirulence, viruses DM2 (U at N472) and DM4 (C at N472) were tested in monkeys (Table 7.2.).

DM4 was found to be significantly more neurovirulent than the vaccine strain with respect to both histological lesions in the CNS and the paralysis induced, although it was not as neurovirulent as the revertant, P3/WHO/119, or the vaccine progenitor P3/Leon/37. The conclusion was complicated slightly by the observation that DM2 had higher lesion score than the Sabin vaccine strain. One interpretation of this finding is that DM2 contains a small proportion of virus with C at N472 which was undetectable by the sequencing methods used.

Table 7.2

Neurovirulence of Sequential Isolates of Poliovirus Type 3.

Isolate Number	Time (hr)	Base at N472	Paralysis	Mean Lesion Score
DM1	24	U	ND	ND
DM2	31	U	1/5	1.46
DM3	35	U/C	ND	ND
DM4	47	C	4/10	2.48
DM5	51	C	ND	ND
DM6	55	C	ND	ND

The mean lesion score obtained for the revertant P3/WHO/119 was 3.34

DM4 was shown to be temperature sensitive or rct/40$^-$, as is the Sabin vaccine. In contrast, the revertant P3/WHO/119 and each of the other revertants tested were wild-type or rct/40$^+$ (WHO Collaborative Study, 1981). Therefore, reversion at N472 alone does not account for the neurovirulent and rct/40$^+$ phenotypes of revertants of the Sabin vaccine.

Nucleotide sequence analysis was subsequently extended to the region of the viral RNA encoding the capsid proteins (D.M.A. Evans, personal communication). Three of the revertants (P3/116, P3/156 and P3/161 in Table 7.1.) showed back-mutation to serine in VP3 at N2034, suggesting that this may be the basis of their revertant phenotype and, therefore, that the mutation to phenylalanine at this position in the vaccine is important for attenuation. It also seems likely that the mutations in P3/WHO/119 responsible for suppression of the attenuated phenotype (in addition to N472) are those at position 1592 and/or position 2637. Three of the revertants (P3/146, P3/131 and P3/158 in Table 7.1.) shared common mutations with P3/WHO/119 at one or both positions 1592 and 2637. Four additional revertants (P3/116, P3/156, P3/161 and P3/382) mutated to Thr at position 2637.

The data as a whole suggests that at least two independent mutations are required for reversion to neurovirulence. The first involves direct back mutation at N472 in the 5′ non-coding region. The second mutation is required for reversion of the serine to phenylalanine change in VP3. This may occur by direct back mutation at N2034 or by a suppressor mutation involving one of the sites implicated in P3/WHO/119 or an additional uncharacterised site(s).

The results obtained for the revertant strains and the sequential isolates are consistent with those obtained for the recombinant polioviruses. It should be noted that the neurovirulence of DM4 was very similar to that observed for the poliovirus recombinants which carried the Ser-Phe mutation in VP3 but lacked the mutation at N472. The lesion score

calculated for DM4 (2.48) was slightly higher than the range observed for the recombinants rSV3/L, rSV3,V1/L and rSCC/L (1.32-2.06). However, in an independent experiment, DM4 failed to induce clinical paralysis and gave a lesion score of 1.58 (D.M.A. Evans, personal communication). DM4, in common with all recombinants carrying the VP3 mutation, could not be distinguished from the Sabin vaccine with respect to temperature sensitivity.

In an independent study, Agol and co-workers (1984) isolated a series of inter-typic recombinants from doubley infected cells. Guanidine resistance and type-specific antigenicity were used as the selectable markers. The crossover point for each recombinant should therefore map within the centre of the genome, between the major antigenic site for poliovirus type 3 (residues 89-100 of VP1) and the locus for guanidine resistance (non-capsid protein P2-X). One of these recombinants derived the 5′ half of its genome from the type 3 vaccine and the 3′ half of its genome from the virulent Mahoney strain of poliovirus type 1. On testing in cynomolgus monkeys, using the intra thalamic route of inoculation, the recombinant was shown to be attenuated. This result is again consistent with the conclusion that N472 and N2034 are the attenuating mutations of the Sabin type 3 vaccine.

These two mutations undoubtedly influence virulence by separate and distinct mechanisms. The degree of sequence homology between the serotypes of in the 5′ non-coding region is high (about 74%) (Toyoda et al., 1984). This strong conservation suggests that the region has functional significance. Possible functions include replicase binding on the negative strand, packaging of RNA during morphogenesis, uncoating and the initiation of translation. Such functions could depend on RNA secondary structure. It may therefore be significant that the C-U substitution at N472 has a drastic effect on the predicted secondary structure of P3/Leon/37 (Evans et al., 1985).

Recent work suggests that the mutation at N472 reduces the efficiency of the poliovirus message (Svitkin et al., 1985). The template activities of RNAs purified form P3/Leon/37, the Sabin type 3 vaccine and P3/WHO/119 were compared in-vitro in Krebs-2 cell extracts. RNA from the type 3 vaccine (U at N472) showed a diminished translation efficiency compared to RNA from the virulent strains (C at N472). The defective step appeared to be the initiation of translation, as shown by the relatively low yield of P1-1a in lysates programmed with the Sabin type 3 RNA. The authors speculated that the mutation at N472 alters the affinity of the viral RNA for initiation factor(s), which may be limiting in the in-vitro system.

7.3. Prospects for Alternative Attenuated Vaccines.

The high frequency of mutation that occurs during poliovirus genome replication (Reanny at al., 1984) makes it difficult to maintain the attenuated phenotype of the live poliovirus vaccines. This is a particular problem with the type 3 vaccine which shows a higher tendency to revert to neurovirulence on passage in tissue culture than the type 1 and type 2 strains (Boulger et al., 1979; Marsden et al., 1980). The majority of type 3 vaccines in use at the present time are prepared from an SO +2 working seed and are therefore at the SO +3 level of passage (i.e three in-vitro passages removed from the Sabin Original vaccine). Preparations of virus at higher levels of passage may be unsuitable for use as a vaccine (Boulger et al., 1979). Global control of poliomyelitis may eventually be hampered by the limiting amount of SO +2 vaccine stock.

The construction of SABIN GDW, an infectious cDNA clone of the Sabin type 3 vaccine, was described in chapter four. Virus rescued from SABIN GDW by transfection of Hep 2c monolayers (rP3/SABIN) was shown to be indistinguishable from the Sabin vaccine with respect to antigenicity and

temperature sensitivity. On testing in cynomolgus monkeys, rP3/SABIN was found to be highly attenuated. In fact, the mean lesion score of 0.11 was unusually low, suggesting that the cDNA derived virus may induce a milder infection than reference preparations of the Sabin vaccine. One explanation for this observation is that transfection with cDNA may be equivalent to a stringent plaque-purification, in that a population of genomes are derived from a single molecule (in this case a cDNA clone).

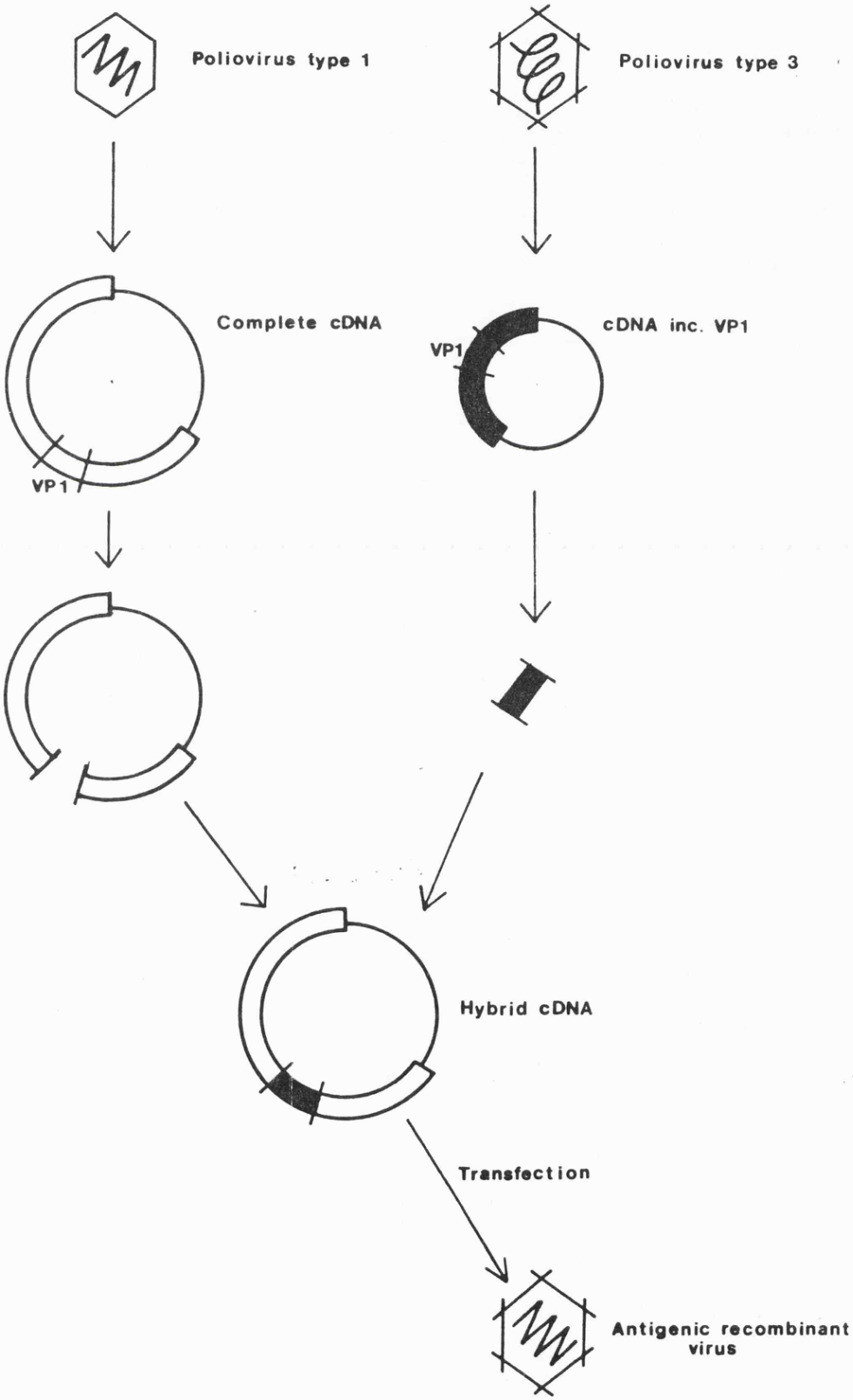
The results demonstrate that the genetic information of the Sabin type 3 vaccine can be stored as cDNA and propagated in E.Coli. The possibilty of using virus rescued from SABIN GDW as an alternative seed for vaccine production is currently under investigation at Lederle Laboratories (M. Sulzinski, personal communication).

Ultimately, however, the most satisfactory solution to the problems encountered with the Sabin type 3 strain is to develop a more stably attenuated vaccine. The molecular definition of attenuation represents a significant step towards this goal. Of the two attenuating lesions, the base substitution at N472 in the 5′ non-coding region is of particular interest. Using the techniques of site-directed mutagenesis, it may be possible to introduce additional mutations into this region of the genome via cDNA, thereby reducing the probabilty of reversion.

It is generally accepted that the type 1 vaccine is more stably attenuated than the type 2 or type 3 strains. Indeed comparison of the Sabin type 1 vaccine with its neurovirulent progenitor revealed 55 nucleotide substitutions, distributed over the entire length of the genome. These result in 21 amino acid substitutions within the viral polyprotein. Using an approach analgous to that described for this thesis, a series of inter-strain recombinants have been constructed between Sabin type 1 and its neurovirulent progenitor (Omata et al., 1985). These authors failed to assign the attenuated phenotype to any specific mutations, or even to specific viral proteins. However, the results were at least consistent with

Fig. 7. 1.

The Construction of Poliovirus Inter-typic Recombinants Via cDNA in vitro.



the conclusion that the type 1 vaccine owes its stable phenotype to multiple attenuating mutations.

This suggests a further interesting possibility for vaccine design. That is to use the stable type 1 vaccine as a basal strain, but to modify its antigenicity so that it immunises against poliovirus type 3. The first experiment of this type involved the construction of a a recombinant plasmid, pOT 5, using the scheme outlined in Fig. 7.1. A 220 base pair SphI-HinfI fragment of the Sabin type 1 cDNA has been replaced by the equivalent fragment of the Sabin type 3 cDNA. This region incorporates the coding region for the 12 amino acids corresponding to the major antigenic site of poliovirus type 3. Unfortunately, this initial construct proved to be non-viable (G. Stanway, Journal of Virology, in press). In addition to eight amino acid substitutions within the major antigenic site, Sabin 3 differs from Sabin 1 at seven additional amino acid positions within the coding region of the 220 base pair fragment. It is possible that one or more of these mutations are lethal for Sabin type 1. pOT5 was designed before the high-resolution structure of poliovirus was determined (Hogle et al., 1985). This information together with our improved understanding of antigenicity could be used to design a viable type 1/type 3 hybrid vaccine.

CHAPTER EIGHT

Materials and Methods

10.1 Materials.

10.1.1 Reagents.

Specialised reagents were obtained from the following sources:

Amersham International, Amersham, UK.

BDH - British Drug Houses Ltd., Poole, UK.

Boehringer-Mannheim, Lewis, UK.

BRL - Bethesda Research Laboratories, Cambridge, UK.

Calbiochem, La Jolla, California, USA.

CBL - Cambridge Biotechnology Laboratories, Cambridge, UK.

Cambrian Chemicals, Croyden, UK.

Difco Laboratories, Detroit, Michigan, USA.

Fisons, Loughborough, UK.

Gibco Europe Ltd., Uxbridge, UK.

NEN - New England Nuclear, Boston, Massachusetts, USA.

Pharmacia Fine Chemicals, Uppsala, Sweden.

Serva Feinbiochemica, Heidelberg, GDR.

Sigma Chemical Company, Poole, UK.

Uniscience, Cambridge, UK.

Acrylamide (2X crystalised, Analytical Grade)	Uniscience.
Agar (Difco Bacto Agar)	Difco.
Ammonium persulphate (AnalR)	BDH.
Antibiotics	Sigma.
(ampicillin, tetracycline, actinomycin D)	
DL-dithiothreitol (DTT)	Sigma.
Ficoll (Type 400 - Mr 400,000)	Sigma.
Formamide (AnalR)	BDH.
HEPES	Sigma.
8-hydroxyquinoline	Sigma.
N,N´-methylene bis acrylamide	Uniscience.
Phenol	Fisons.
Polyethylene Glycol (PEG) (Mr 8000)	Sigma.
Sodium dodecyl sulphate (SDS) (Specially pure)	BDH.
Tris-(hydroxymethyl) aminomethane acetate	Sigma.
(Tris acetate)	
Tris-(hydroxymethyl) methylamine (Tris)	Fisons.
Urea	Fisons.

Radioisotopes

All radioisotopes were obtained from Amersham International:-

[α - ^{32}P]dATP (2000-3000 Ci/mmol)

[α - ^{35}S]dATP (at least 400 Ci/mmol)

[5 - ^{3}H]dUTP (18-30 Ci/mmol)

Carrier-free ^{32}P

Enzymes

T4 DNA Ligase	NEN.
E.coli DNA polymerase I	Boerhinger, BRL.
Large fragment E.coli DNA polymerase I	Boerhinger, BRL,
(Klenow fragment)	CBL.
Proteinase K	Sigma.
Restriction endonucleases (various)	BRL.
RNase T1	Calbiochem.

Reverse transcriptase was a gift from DR J.W. Beard, NIH, Bethesda, USA

Tissue Culture Reagents

All tissue culture reagents were obtained from Gibco Europe Ltd. :-

Newborn Calf Serum (NCS)

Foetal Calf Serum (FCS)

Penicillin-Streptomycin Solution (10,000 units penicillin and 10,000 mcg Streptomycin/ml)

Fungizone (Amphorotericin B, 250mcg/ml)

MEM - Minimal essential medium (Eagle) 10x

10.1.2 Solutions and Buffers

40% Acrylamide	380g	Acrylamide
	20g	Methylbisacrylamide
	Make up to 1 litre with distilled water.	
10X TBE Buffer	432g	Tris
pH 8.3	220g	Boric Acid
	37.2g	EDTA
	Make up to 4 litres with distilled water.	
20% Acrylamide	100g	Urea
	20ml	10X TBE
	100ml	40% Acrylamide
	Make up to 200ml with distilled water.	
8% Acrylamide	82g	Urea
Gel Mix	20ml	10X TBE
	40ml	40% Acrylamide
	Make up to 200ml with distilled water.	
0.5M TBE Gel	460g	Urea
Mix	50ml	10X TBE
	150ml	40% Acrylamide
	Make up to 1 litre with distilled water.	
5M TBE Gel	460g	Urea
Mix	250ml	20X TBE
	5g	Sucrose
	50mg	Bromophemol Blue
	Make up to 1 litre with distilled water.	

Formamide Dye	100ml De-ionised Formamide 100mg Xylene Cyanol FF 100mg Bromophenol Blue 2ml 500mM EDTA
5 x Denhardt's solution	0.1% (w/v) BSA, 0.1% (w/v) ficoll, 0.1% (w/v) poly-vinylpyrrolidone in 6 x SSC. Filter sterilised and kept at 4°C.
EDTA	0.5M EDTA in distilled water, adjusted to pH 8.0 with NaOH.
Ethidium bromide (EtdBr)	5 mg/ml in TE.
Saline citrate (SSC)	0.15M NaCl, 0.15M sodium citrate in distilled water.
Tris-EDTA (TE)	10mM Tris-HCl pH 7.4, 1mM EDTA in distilled deionised water.
Elfo buffer	40mM Tris, 1mM EDTA in distilled water, adjusted to pH 7.7 with glacial acetic acid.
Elfo sample Buffer	100mg agarose, 5g glycerol, 50ml 10mM Tris-acetate, 2ml 0.5M EDTA, 0.01% (w/v) bromophenol blue. Autoclaved 15min at 15lb/in^3 cooled and expelled from syringe to form agarose `beads'.

10.1.3 Bacterial Growth Media.

Luria broth — 10g/l Difco Bacto Peptone, 5g/l Difco yeast extract, 5g/l NaCl in distilled water.

L-tet — Luria broth containing 10ug/ml tetracycline.

L-amp — Luria broth containing 100ug/ml ampicillin.

All agar plates contained 1.5% (w/v) Difco Bacto agar. Soft top agars contained 0.7% (w/v) Difco Bacto agar.

Minimal agar — Agar - 3% (w/v) Difco Bacto agar in distilled water.
Salt solution - 10.5g/l K_2HPO_4, 4.5g/l KH_2PO_4, 1g/l $(NH_4)_2SO_4$, 0.5g/l sodium citrate, 0.2g/l $MgSO_4$ in distilled water.

Solutions autoclaved seperately, then 100ml of the salt solution is mixed with 100ml of agar. When cool, 2ml of glucose solution (200g/l) and 1ul of thiamine HCl (1mg/ml) were added before pouring.

10.1.4 Tissue Culture Buffers, Solutions and Media

Phosphate Buffered Saline (PBS) — 8.0g/l NaCl, 0.2g/l KCl, 1.15g/l $Na_2HPO_4.7H_2O$ 0.2g/l KH_2PO_4.

Puck's Saline — 8.0g/l NaCl, 0.4g/l KCl, 0.045g/l $Na_2HPO_4.7H_2O$, 0.03g/l KH_2PO_4.

Glutamine — 200mM Glutamine in sterile distilled water. Filter sterilise and store at -20°C.

$NaHCO_3$ — 7.5% (w/v) $NaHCO_3$ in sterile distilled water. Filter sterilise and store at 4°C.

MEM + 5% NCS

50ml	10X Minimal Essential Medium (Eagle)
5ml	Penecillin/Streptomycin
5ml	Fungizone
5ml	200mM Glutamine
25ml	Newborn Calf Serum
14ml	7.5% $NaHCO_3$

Make up to 500ml with sterile distilled water.

MEM + 2% FCS

50ml	10X Minimal Essential Medium (Eagle)
5ml	Penicillin-Streptomycin
5ml	Fungizone
5ml	200mM Glutamine
10ml	Foetal Calf Serum
14ml	7.5% $NaHCO_3$

Make up to 500ml with sterile distilled water.

All tissue culture media contained antibiotics and glutamine. FCS and $NaHCO_3$ were varied as indicated in text.

10.2 Methods

10.2.1 Recombinant DNA Methods

Restriction Endonuclease Digestions.

Restriction digests were carried out under the following conditions:-

AccI -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 1mM DTT.

AatII -10mM Tris-HCl pH 7.5, 50mM KCl, 7mM $MgCl_2$, 7mM B-mercaptoethanol.

BamHI -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT.

BglII -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

ClaI -10mM Tris-HCl pH 8.0, 10mM $MgCl_2$.

EcoRI -50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

HincII -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

HindIII -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

KpnI -6mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 100mM NaCl, 1mM B-mercaptoethanol

MluI -10mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 100mM NaCl, 1mM B-mercaptoethanol

PstI -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

PvuII -6mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 60mM NaCl, 6mM B-mercaptoethanol.

SalI -50mM Tris-HCl pH 8.0, 10mM $Mgcl_2$, 100mM NaCl, 1mM DTT.

SmaI -15mM Tris-HCl pH 8.0, 6mM $MgCl_2$, 15mM KCl.

SphI -50mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 50mM NaCl, 6mM B-mercaptoethanol.

XhoI -50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT.

Restriction endonuclease buffers were prepared as 10X solutions and stored at -20°C. One unit of enzyme was required for every µg of DNA to be digested. For complete digests, reactions were incubated at 37°C for 90 minutes (except for digests with SmaI which were incubated at 30°C. For partial digests, samples were taken at timed intervals and the reactions

terminated by addition of EDTA to a final concentration of 20mM. Aliquots were stored on ice before analysis of the size of the partial digestion products by electrophoresis on a 1% agarose gel. In this way optimum conditions for a single, long digest could be determined.

Agarose Gel Electrophoresis.

Agarose gels were prepared by boiling agarose (routinely 1% v/v) in ELFO buffer (40mM Tris pH 7.7, 1mM EDTA). The solution was cooled to 50°C before adding ethidium bromide (5mg/ml in TE) to a final concentration of 0.5 µg/ml. Gels were poured onto 11.2 x 12.2cm plates to a depth of 0.5cm. Agarose gels were placed into electrophoresis tanks and submerged in ELFO buffer containing 0.5µg/ml EtdBr. DNA samples were mixed with agarose sample buffer and loaded into preformed wells. 1 to 2µg of DNA was added to each well, depending on the size of the fragments to be separated. Electrophoresis was generally carried out at 100V and 100mA.

Isolation of DNA Fragments from Agarose Gels

Following the resolution of DNA fragments by agarose gel electrophoresis, the desired fragment was excised from the gel with a sterile scalpel blade and placed in a dialysis bag containing ELFO buffer. After removing most of the buffer, the dialysis bag was sealed, taking care to exclude air bubbles. Electrophoresis was carried out (as described above) until the DNA had eluted from the gel to form a discrete band at the edge of the dialysis bag (visualised by long-wave UV illumination). The polarity of the current was reversed for 30 seconds to release the DNA from the dialysis membrane. The contents of the dialysis bag was removed and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA solution was concentrated by 3-4 cycles of extraction with equal volumes of isobutanol before ethanol precipitation. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 50µl of sterile distilled water to give a DNA concentration of 20-100ng/µl.

Ethanol Precipitation of DNA

To precipitate DNA from solution, 1/20th volume of 2M Sodium Acetate followed by 2.5 volumes of ethanol were added. After mixing the solution was frozen in a dry-ice/ethanol bath. The DNA was pelleted by centrifugation at 10K for 10 minutes. The supernatent was removed and the pellet washed once in 70% ethanol. The pellet was then dried under vacuum for at least 1 hour and resuspended in sterile distilled water.

Ligation

DNA fragments to be ligated were prepared in sterile distilled, deionised water. For sub-cloning into pAT 153 or for constructions in which one of the fragments contained the entire vector sequence, 200ng of vector (phosphatase treated if necessary) was mixed with a three fold molar excess of insert DNA. For constructions in which neither fragment could re-circularise to form a viable plasmid, equimolar amounts of fragments were ligated. The mixture was made up to 1mM rATP and 1X ligation buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT) using 10X stock solutions. 1 unit of T4 DNA ligase was added and the ligation mix was incubated at +14°C for 17 hours for ligation of fragments bearing cohesive ends and for 24 hours for blunt end ligations.

Preparation of competent E.coli

E.coli strain JA221 (hsdM$^-$, hsdR$^-$, LacY$^-$, leuB6$^-$, trpES, recA1/F) was used in all transformations involving pAT 153 vectors. Cells were made competent for transformation using the following method. Luria broth (routinely 40mls) was inoculated with 0.05 volumes of an overnight culture and grown with vigerous aeration at 37°C to an OD_{650} of 0.4-0.5. After chilling the culture on ice for 10 minutes, cells were harvested by centrifugation at 2K

(10 mins) and resuspended in 0.5 volumes of ice-cold 100mM $CaCl_2$. After 40 minutes incubation on ice, the cells were pelleted once more and gently resuspended in 0.05 volumes of cold $CaCl_2$. Competent cells were held on ice for at least one hour before use.

Transformation of E.coli

Ligated DNA was made up to a volume of 100µl in 1X SSC and incubated on ice with 200µl of competent cells. After 40 minutes, cells were heat shocked at 42°C for 2 minutes and then returned to ice for a further 20 minutes. 1ml of pre-warmed Luria broth was then added to each suspension and the cells were incubated at 37°C for 45 minutes to allow transformants to express tetracycline resistance. Cells were then pelleted by centrifugation at 2K and resuspended in 100µl of Luria broth before plating out on L-tet plates.

Plasmid Isolation Techniques

(1) Rapid isolation of plasmid DNA This technique was routinely used to screen transformants. Cell pellets from 10ml cultures, grown overnight in L-tet, were resuspended in 730µl of 50mM Tris-HCl pH 8.0, 15% (w/v) sucrose and transfered to sterile microfuge tubes. 70ul of lysozyme (10mg/ml freshly prepared) was added to each tube to give a working strength of 1mg/ml. After 10 minutes incubation at room temperature, 30ul of 10% (w/v) SDS was added followed by 75µl of 4M Pottasium Acetate (pH 7.5). The suspensions were incubated on ice for 30 minutes and then centrifuged for 15 minutes in a microfuge. The pellets were discarded and 2µl of RNaseA/T1 (2mg/ml) was added to each of the supernatents. After 15 minutes at room temperature the supernatents were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA was precipitated from the aqueous phase by addition of 750µl of cold ethanol. Following centrifugation for 15 minutes the pellet was resuspended in 300µl of 0.1M Sodium Acetate. DNA was re-precipitated by addition of 750µl of ethanol, followed by incubation in

an ethanol/dry-ice bath for 15 minutes. After two additional cycles of DNA precipitation, the DNA pellets were washed with cold 70% ethanol, dried under a vacuum for 1 hour and finally resuspended in 100µl of sterile destilled water. 10ul of the DNA solution was sufficient for size estimation by agarose gel electrophoresis or for characterization by digestion with restriction endonucleases.

(2) Large scale isolation of plasmid DNA. Recombinant plasmids containing full-length cDNA inserts were isolated in a highly purified form using the following `cleared lysate' method. A 1 litre culture in L-tet broth was grown overnight at 37°C to late stationary phase. The cells were harvested by centrifugation, washed once in TE buffer and resuspended in 24ml of 25% (w/v) sucrose, 50mM Tris-HCl pH 8.0. The cells were held on ice and 6ml of 10mg/ml lysozyme and 6ml of 0.5M EDTA was added whilst stirring. After 10 minutes on ice, this was followed by 40ml of Triton mixture (50mM Tris-HCl pH 8.0, 50mM EDTA, 1% (w/v) Triton X 100). When lysis had occured (indicated by the increased viscosity of the suspension), the cell debris was pelleted by centrifugation for 45 minutes at 20K. 8ml of 10% (w/v) SDS was added to the clear supernatent which was then extracted with 20ml phenol/chloroform (1v/1v). the nucleic acids were precipitated from the aqueous phase with ethanol and re-dissolved in 10ml of 10mM Tris-HCl pH 8.0. 50µl of a 20mg/ml solution of RNaseA/T1 was added. The mixture was incubated for 30 minutes at 37°C and then extracted with 10ml of phenol/chloroform. The DNA was ethanol precipitated from the aqueous phase and re-disolved in a total weight of 20g of TE buffer. 4ml of a 5mg/ml solution of Ethidium Bromide and 23.76g of CsCl were added to bring the density of the solution to 1.393-1.394g/cm^3. Gradients were formed by in 36ml polyallomer tubes by centrifugation in a Sorvall TV850 rotor at 40K for 18 hours. The lower, plasmid DNA band was collected with a 10ml syringe by side puncture of the tubes. Ethidium Bromide was removed by repeated

extractions of the DNA solution with equal volumes of CsCl/water saturated isopropanol, followed by two extractions with an equal volume of diethyl ether. The DNA was dialysed against 500ml of sterile distilled water with several changes over a period of 3-4 hours. The DNA was then ethanol precipitated and re-dissolved in sterile distilled water at a concentration of 1mg/ml.

Colony Screening

For most constructions, transformants containing recombinant plasmids could be identified by isolating plasmid DNA from 24 tetracycline resistant transformants. Recombinant plasmids were then identified on the basis of size, as determined by agarose gel electrophoresis, using appropriate size markers. Occasionaly, re-circularisation of the vector proved to be more efficient than recombination with the insert. In such cases, transformants containing recombinant plasmids could be identified by colony hybridisation using a radiolabeled probe prepared by nick-translation of the insert fragment (Grunstein and Hogness, 1975; Rigby et al., 1977).

(1) Colony hybridisation. tet^r transformants were streaked onto nitrocellulose filters and grown overnight at 37°C. Colonies were prepared for hybridisation by lysis and denaturation in 0.5M NaOH (5 mins), and neutralisation in 0.5M Tris-HCl pH 7.4 (4 mins) and 0.5M Tris-Hcl pH 7.4, 1.5M Nacl (4 mins). After drying the filters were baked in-vacuo at 80°C for 2 hours in 5X Denhardt's solution. Hybridisations were performed in 9cm pteri dishes in sealed plastic boxes. Up to 4 filters were placed in a single dish containing 10-20ml 5X Denhardt's solution with the radiolabeled hybridisation probe. Approximately 1 x 10^6 cpm were used per filter. Hybridisation was allowed to proceed at 65°C for 18-24 hours. Filters were then washed at 65°C for a period of about 3 hours in three 10-15ml changes of 3X SSC, 0.1% SDS (w/v). After drying, filters were covered with Seran-wrap and subjected to autoradiography.

(2) Preparation of radiolabelled probe. The fragment used as the insert in the construction was diluted in deionised water to a concentration of 20-100µg/ml. The DNA was boiled for 1 minute to denature it and quenched on ice prior to to the addition of the reaction components. This step increased the incorporation of radiolabel in the reaction by 50%. Nick-translation was carried out in a reaction containing 100ng DNA, Hin buffer (20mM Tris-HCl pH 7.4, 7mM $MgCl_2$, 60mM NaCl), 0.5mM dCTP, dGTP and dTTP, 0.1 mM dATP, 200µci/ml [α-^{35}S]dATP, 10ng/ml DNase I and 20u/ml E.coli DNA polymerase I. After incubation for 30 minutes at room temperature, the reaction was stopped by phenol extraction. The hybridisation probe was passed through a Sephadex G100 column before use to remove unincorporated label and very low molecular weight material, which would otherwise result in non specific hybridisation. The appropriate column fractions were boiled for 10 minutes to denature the probe before being added directly to the hybridisation dishes (pre-warmed to 65°C).

10.2.2 Tissue Culture Methods.

Cell Lines and Culture Conditions.

The human epithelial cell line, Hep 2c, was obtained from Dr. P. Minor, Division of Viral Products, National Institute of Biological Sandards and Control, Hampstead, London. The cell line was maintained by continuous passage in MEM (Eagle), supplemented with 5% NCS an 0.22% $NaHCO_3$. Cells were routinely grown in 175cm^3 dispossable flat-bottomed flasks. To passage cells, confluent monolayers were washed twice in calcium and magnesium free PBS. 10ml of trypsin suspension (0.25% in Puck's saline) was added to each flask. After 2 minutes adsorption at room temperature, the trypsin was removed and the monolayers were incubated at 37°C until 80-90% of the cells had rounded up, as judged by phase contrast microscopy. 20ml of medium was added to each flask and the cells were dislodged by shaking. Large

aggregates of cells were broken up by passing through a 10ml pipette. 5ml aliquots of trypsidised cell suspension were dispensed into 175cm^3 flasks and approximately 70ml of warm medium was added to each flask. The monolayers were grown at 37°C in a humid, 5% CO_2 atmosphere. Trypsidised cells plated out within 2 hours at 37°C and monolayers grew to full confluence within 3-4 days. Monolayers were split as soon as they reached confluence or else the growth medium was replaced with a maintenance medium of MEM + 2% FCS to preserve the monolayers for up to 7 days.

Cryogenic Preservation of Cells

Medium for freezing cells consisted of 1X MEM (Eagle), 25mM HEPES pH 12.5, 20% (v/v) glycerol and 20% (v/v) FCS. Trypsidised cells from a single 10cm petri-dish were resuspended in 4ml of the above medium. 1ml aliquots were transfered to cryogenic vials and frozen overnight in an insulated container at -70°C. The frozen cells were transferred to Liquid Nitrogen and stored for up to 12 months. To initiate a culture from frozen cells, a single vial was defrosted rapidly by submerging in a 37°C water bath. The vial was sterilised by flaming in IMS before pouring the contents onto a 10cm petri dish containing MEM + 5% NCS. The medium was changed as soon as the cells had plated out.

DNA Transfection.

(1) Buffers and solutions. HEPES-buffered saline was prepared at 2X strength and stored at 4°C in polyethylene tubes until ready for use. 2X HEPES buffered saline consisted of 1% (w/v) HEPES and 1.6% (w/v) NaCl, adjusted to pH 7.1 with 5M NaOH. 100X phosphate was prepared by mixing equal volumes of 70mM NaH_2PO_4 and 70mM Na_2HPO_4. 2M $CaCl_2$ was stored at -20°C in polyethylene tubes for up to 1 month. All solutions were prepared in Nanopure distilled water and filter sterilised.

(2) Preparation of monolayers. Monolayers were seeded the day before transfection. A single $175cm^3$ flask was split into eight 10cm petri dishes to give monolayers of between 70% and 90% confluence after 12 hours incubation at 37°C. The cells were grown in MEM + 2% FCS.

(3) Calcium phosphate/DNA co-precipitate. Transfection solutions were equilibrated to room temperature. Solution A and solution B were prepared in 10ml test-tubes as follows: solution A, 50µl of 100X phosphate and 2.5ml of 2X HEPES buffered saline; solution B, 300µl of 2M $CaCl_2$ and 200µl of supercioled plasmid DNA (1µg/µl), made up to a total volume of 2.5ml with sterile distilled water. Each solution was blended in a vortex mixer. Solution B was then added to solution A, dropwise and very slowly, under conditions of continuos aggitation achieved by blowing into solution A via a pasteur pipette/spit-tube. The resulting solution was blended in a vortex mixer for 30 seconds at maximal speed. The transfection cocktail was allowed to stand for 30 to 40 minutes at room temperature, with periodic aggitation. A fine white precipitate, with no visible aggregates, is formed during this period.

(4) Transfection. The medium was removed from each of four sub-confluent monolayers. 1ml of calcium phosphate/DNA co-precipitate was added to each monolayer (i.e. 40µg DNA per plate or approximately 10-20µg DNA per 2 x 10^6 cells). After 20 minutes adsorption at 37°C, 9ml of MEM + 10% FCS was added to each plate. The monolayers were then incubated for a further 4 hours at 37°C.

(5) Glycerol shock. The precipitate was removed from the each plate and the monolayers were washed with MEM + 2% FCS. 3ml of glycerol solution (20% (v/v) in HEPES-buffered saline) was added to each plate. After 3 minutes incubation at 37°C, the glycerol solution was removed and the monolayers were washed three times in MEM + 2% FCS. Finally, 10ml of MEM + 2% FCS was added to each plate and the transfected monolayers were incubated for 5-7 days at 35°C.

Poliovirus Plaque Assay and Plaque Purification.

Virus was harvested from transfected monolayers by three cycles of freeze-thawing. This treatment releases intra-cellular virus. The supernatent media was centrifuged for 10 minutes at 2K to remove cell-debris. The cleared supernatent was aliquoted into 2ml viles and stored at -20°C until use. Monolayers were prepared for plaque assay by splitting a single $175cm^3$ flask into eight 6-well multiwell dishes (each well 3cm). Cells grew to full confluence following three days incubation at 37°C. The virus suspension was titrated in 10-fold steps to 10^{-7} in MEM +2% FCS. 0.1 ml of each dilution was applied to separate wells. After 30 minutes adsorption at room temperature, 3ml of agar overlay was added. The overlay consisted of MEM + 2% FCS + 1% Noble agar and was prepared by mixing equal volumes of 2X MEM + 2% FCS and 2% Noble agar at 45°C. Monolayers were incubated at 35°C in an inverted position. Plaques could be observed after 3-4 days by examining the monolayers in oblique light. To count plaques the agar overlay was removed and the monolayers were fixed and stained with carbol fuschin. For plaque-purification, well separated plaques were picked with a pasteur pipette and transfered to glass vials containing 1ml of MEM + 2% FCS. Virus was eluted from the agar plugs by incubating overnight at 4°C. 0.1ml of the virus suspension was used to seed a confluent monolayer of cells grown on a 10cm plate in MEM + 2% FCS. 10ml of MEM was added following adsorption at room temperature and the monolayers were incubated at 35°C. CPE was observed following 3-4 days. Virus was then harvested as described above and stored in 1ml aliquots at -20°C.

Poliovirus Neutralisation Assay.

The poliovirus neutralisation assay was carried out in 96-well microtitre plates. Standard poliovirus antisera used were as follows:-

Type I	(Guinea pig)	Titre = 1:32,000
Type II	(Monkey)	Titre = 1:4,000
Type III	(Monkey)	Titer = 1:1,000

The assay was designed to serotype the poliovirus isolate and, at the same time, to screen for the presence of extraneous viruses. The virus was titrated to 10^3 $TCID_{50}$/ml in MEM (Eagle) supplemented with 4% FCS and 0.12% NaHCO3. 0.1ml of virus (i.e 100 $TCID_{50}$) was mixed with 0.1 ml of the following antisera mixes:-

(1) Type I and Type II

(2) Type II and Type III

(3) Type III and Type I

(4) Type I, Type II and Type III

(5) Virus titration medium.

Each of the components of the antisera mixes were diluted 1:200 in the virus tiration medium. The virus/antisera (routinely 4 wells/virus) were incubated for 3 hours at 35°C. A single 175cm^3 flask of Hep 2c cells was trypsidised and resuspended in 150ml of the virus tiration medium. 0.1ml of the cell supension was added to each well and the plates were sealed with pressure-sensitive film. The plates were scored for CPE following 7 days incubation at 35°C.

Poliovirus type 3 rct Marker Test

The rct marker test (reproductive capacity at different temperatures) is routinely used as an in-vitro test for vaccine standardisation. The test is based on the temperature sensitivity of the Sabin attenuated vaccine strains which replicate poorly at the non-permissive temperature of 40°C. The titre of the virus is determined at 35°C and 40°C using a $TCID_{50}$ assay and the rct value is calculated as the ratio of the Log_{10} titre at 35°C and the log_{10} titre at 40°C. The standard interpretation of the test is that virus with a reduction in titre at 40°C of < 2.0 log_{10} $TCID_{50}$ is regarded as wild-type or $rct/40^{+}$. If the reduction in titre is > 5.0 log_{10} $TCID_{50}$ the virus is said to be $rct/40^{-}$. Control viruses are included in each test and the result is considered to be valid only if these viruses give the expected results. The test is carried out in 96-well microtitre plates using the following procedure. A single 175 cm^3 flask of Hep 2c was trypsidised and the cells were resuspended in 200ml of MEM + 5% NCS. 0.1ml of the cell suspension was added to each well and the plates were incubated at 37°C. Monolayers grew to full confluence in 2 days. On the day of the test, the growth medium was replaced with 0.1 ml per well of maintenance medium which consisted of MEM (Eagle) supplemented with 1% FCS and 0.88% $NaHCO_3$. The virus was titrated in ten-fold steps to 10^{-9} in maintenance medium. Monolayers were inoculated with 0.1ml of virus per well at 4 wells per dilution as follows:-

	Plates incubated at 35°C	Plates incubated at 40°C
Wild type 3 control	10^{-9} - 10^{-5}	10^{-9} - 10^{-5}
Sabin type 3 contol	10^{-9} - 10^{-5}	10^{-8} - 10^{-1}
Unknown viruses	10^{-9} - 10^{-5}	10^{-8} - 10^{-1}

The plates were sealed with pressure sensitive film and submerged in water baths equilabrated to the appropriate temperature. The water bath temperatures should not vary more than 0.1°C. Monolayers were examined for CPE following 7 days incubation. The $TCID_{50}$ titre was calculated as the dilution which induces CPE in 50% of the moonolayers inoculated. At 4 wells per dilution each well is equivalent to 0.25 $\log_{10}$ $TCID_{50}$. For example 2 out of 4 wells positive at 10^{-4} = 4.00 $TCID_{50}$/0.1ml. Similarly, 3 out of 4 wells positive at 10^{-4} = 4.25 $TCID_{50}$/0.1ml.

10.2.3. Nucleotide Sequencing of Viral RNA

Partial nucleotide sequences were derived directly from purified viral RNA by adapting the di-deoxy chain termination method to an oligonucleotide primed reverse transcriptase reaction.

Purification of Synthetic Oligonucleotides.

Synthetic oligonucleotides, complementary to viral RNA sequences downstream from the sites of known Sabin type 3 specific mutations, were synthesised by J. Kyte, Department of Biochemistry, University of Leicester. The crude oligonucleotide mixture was disolved in 10µl of TBE dye and boiled for 2 minutes before loading onto a 20% polyacrylamide/urea gel. Electrophoresis was carried out at 45W in TBE buffer until the bromophenol blue had migrated to the bottom of the gel. The gel was transfered to a sheet of cling-film and layered over a fluorescent plate. The DNA was visualised by short wavelength UV light. The dark band positioned at the top of the ladder was excised with a sterile scalpel blade. The gel-slice was dissolved in 100ul of sterile distilled water by incubation at room temperature for 2 hours. The DNA was desalted by ethanol precipitation and resuspended in 100ul of sterile distilled water to give a concentration of approximately 20ng/µl.

Purification of Viral RNA.

For each virus 6 glass tubes (15cm by 1.3cm internal diameter) were each seeded with 10 Hep 2c cells in 1ml of MEM + 2% FCS. The tubes were incubated in a roller drum for 16 hours at 35°C. The medium was decanted and 50ul of virus suspension (10 PFU/ml) was added to each tube. After adsorption for 1 hour at 35°C, 1ml of fresh medium containing 25µci of [5-^{3}H] Uriidine was added to each tube. Rolling was continued for another day at 35°C. The cells were then lysed by freeze-thawing at -20°C. The supernatents were pooled and the cell-debris removed by centrifugation at 2K. Nonidet P-40 detergeant was added to 1% and the virus suspension was layered onto a 35ml gradient of 15 to 45% RNase-free sucrose, 10mM Tris-Hcl pH 7.4 and 50mM NaCl. The gradient was centrifuged at 24K for 4 hours at 4°C and fractions of approximately 1.5ml were collected by bottom puncture. Fractions containing labeled virus were pooled and virus was pelleted by centrifugation at 30K for 16 hours. The sucrose buffer was decanted and the pellet was resuspended in 500µl of 150mM sodium acetate. After transfering to a sterile microfuge tube, 2µl of 50mM B-mercaptoethanol was added. The suspension was extracted three times with equal volumes of phenol/chloroform/isoamyl alcohol (25/24/1) and once with H_2O saturated ether. RNA was precipitated from the aqueous phase by addition of 5ul of 5M NaCl and 2.5 volumes of etanol, followed by incubation in a dry-ice bath for 20 minutes. The pellet was dried under vacuum for 30 minutes and resuspended in 5µl of sterile distilled water to give a concentration of approximately 1µg/ml.

Nucleotide Sequencing of Viral RNA by Primer Extension.

(1) Sequencing reagents. dNTP and ddNTPs were prepared as 20mM master stocks and stored at -20°C. The dNTP mix was prepared as follows:-

dNTP Mix	20mM dATP	1µl
	20mM dGTP	20µl
	20mM dCTP	20µl
	20mM dTTP	20µl

dNTPs were diluted to 6.4mM before diluting for use as follows:-

ddNTPs	6.4mM ddATP	1µl + 24µl H_2O
	6.4mM ddGTP	1µl + 3µl H_2O
	6.4mM ddCTP	1µl + 7µl H_2O
	6.4mM ddTTP	1µl + 3µl H_2O

ddNTP/dNTP mixes were prepared for sequencing as follows:-

dd/dNTP mix	A	G	C	T	
dNTP Mix	2.5	2.5	2.5	2.5	µl
ddNTP	2.5	2.5	2.5	2.5	µl
H20	5	5	5	5	µl

(2) Sequencing protocol. 3µl of Liquid Parrafin was added to each of four labelled microfuge tubes. The RNA/primer hybrid mix was then prepared as follows:-

Hybrid mix	1µl RNA (1µg)
	1µl Primer (20ng)
	1µl 8X RT buffer
	1µl [α-^{32}P]dATP

The 8X RT buffer (reverse transcriptase buffer) consisted of 800mM Tris-HCl pH 8.3, 1.12M KCl, 80mM $MgCl_2$. dd/dNTP/RTase mixes were prepared as follows:-

dd/dNTP/RTase	5µl dd/dNTP mix (relevant base)
	4µl H2O
	1µl Reverse Transcriptase (11u/µl)

1µl of hybrid mix was dispensed into each of four parrafin'ed tubes. 1µl of the relavent dd/dNTP/RTase was added to the side of the tubes. the reagents were mixed by a short spin in the microfuge. The reaction was allowed to proceed for 15 minutes at 45°C and then terminated by addition of 8ul of 10mM EDTA. The parrafin was extracted by adding 150µl of ether which was then removed using a pasteur pipette and water-pump. 2.5µl of each reaction was mixed with 1µl of formamide dye. The solution was boiled for 3 minutes and chilled briefly in a dry-ice bath, before loading on an 8% polyacrylamide gel. Electrophoresis was carried out in TBE buffer at 16mA per gel.

10.2.4. Nucleotide Sequencing of Poliovirus cDNA.

M13 Sub-cloning of DNA Fragments.

Restriction endonuclease fragments were ligated into appropriately digested M13 vectors using the following procedure. Digested M13 RF DNA (1ug/ml) and the purified restriction endonuclease fragment (0.1-1 ug/ml or a 1:3 fold molar ratio of insert:vector) were incubated at 14°C for 18-24 h in the presence of 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP.

Transfection of E.coli.

M13 ligation mixtures (see above) were used to transform competent JM103 cells (see, "Preparation of Competent cells") using the following method. 0.1ml of competent cells were added to each 10ul ligation mixture and incubated on ice for 40 min, then heat shocked at 42°C for 2 min. The cells were plated out onto well-dried minimal agar plates in 3ml of Luria soft-top agar containing 6ug/ml BCIG, 5ug/ml IPTG and 0.15ml of a stationary phase culture of JM103. After allowing the soft top to solidify, the plates were incubated in an inverted position at 37°C for 18 h. Recombinant phage containing cDNA inserts formed turbid plaques ("whites") on incubation, easily distinguishable from the blue plaques formed by phage without inserts.

Preparation of single-stranded Recombinant M13 DNA.

Single stranded M13 DNA to be used as templates for nucleotide sequencing was prepared as follows. Recombinant plaques were harvested using sterile toothpicks and used to infect 1 ml cultures in Luria broth, seeded with a 1:100 dilution of a stationary phase culture of JM103. These cultures were grown at 37°C with vigerous aeration for 6 h. The cells were then pelleted by centrifugation in 1.5ml Eppendorf tubes at 15,000 x g for 5 mins in an Eppendorf microfuge. The cell pellets were discarded and the phage precipitated from the supernatents by the addition of 0.2ml of a solution of 10% (w/v) PEG, 2.5 M NaCl. After incubation for 30-60 min at room temperature, the phage were harvested by centrifugation for 15 min in an Eppendorf microfuge. The supernatent liquid was removed with a drawn out capillary. Tubes without phage pellets were discarded at this stage. The remaining pellets were resuspended in 0.1ml of 1.1M sodium acetate pH 7.0. The phage suspension was vortexed with 50ul of aqueous phenol. 50ul of chloroform/isoamyl alcohol (50v/1v) was added and the tubes were vortexed again. They were then centrifuged for 1 min in an Epindorf microfuge. The

aqueous phase was re-extracted with 0.1ml of chloroform/isoamyl alcohol and the DNA precipitated by the addition of 0.25ml of ethanol and chilling the tubes in a alcohol - dry-ice bath. Following centrifugation for 10 min in a microfuge, the supernatents were removed with a drawn out capillary. The pellets were dried under vacuum and redisolved in 20µl of 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.

DNA Sequencing.

Nucleotide sequencing of recombinant M13 phage bearing restriction endonuclease fragments of poliovirus cDNA was carried out using the method of Sanger et al., (1977). 2µl of template DNA was placed in a 0.4ml Eppendorf tube with 2µl of priming mixture containing 1ng primer (synthetic oligonucleotide complementary to poliovirus cDNA), 10mM Tris-HCl pH 8.5, 5mM $MgCl_2$. The tube was sealed, placed in a boiling water bath for 5 min, then allowed to cool to room temperature for 15 min. After priming, 4µl of 3mM DTT, 3uCi [a-35S]dATP and 0.4u E.coli DNA polymerase I (Klenow fragment) were added. To start the reaction, 2ul aliquots of the primed mixture were added to open 1.5ul Eppendorf tubes containing 1µl of A/G/C/T nucleotide mixture (see Table 8.1.). After 15 min at room temperature, 0.5µl of 0.5mM dATP was added. After a further 15 minutes, 2µl of formamide-dye mixture (containing 95% (v/v) formamide, 20mM EDTA, 0.1% w/v bromophenol blue, 0.1% (w/v) xylene cyanol) was added and the tubes were placed in a boiling water bath for 3 min. The samples were then loaded onto 6% (w/v) acrylamide TBE gradient gels (50cm x 20cm x 0.25cm), containing 8M urea. Electrophoresis was carried out at a constant 40W (aproximately 20mA, 2000V) with 0.5 x TBE in the upper chamber and 1 x TBE in the lower. Electrophoresis was terminated when the bromophenol blue marker had migrated to the bottom of the gel. The gel was fixed in 10% (v/v) acetic acid, transfered to Whatman 3MM paper and dried on a Bio Rad 1125B gel drier (Bio Rad Laboratories Ltd, Watford, UK). The gels were then subjected to autoradiogrephy for 12 h - 2 days.

Table 8.1.

Composition of Nucleotide Sequencing Reagents.

	A µl	G µl	C µl	T µl
0.5mM dGTP	250	12.5	250	250
0.5mM dCTP	250	250	12.5	250
0.5mM dTTP	250	250	250	12.5
5.0mM dATP	1	-	-	-
10mM ddGTP	-	8	-	-
10mM ddCTP	-	-	4	-
10mM ddTTP	-	-	-	25
TE*	250	500	500	500

*10mM Tris-HCl pH 8.0, 1mM EDTA

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