





INHIBITION OF CELL DIVISION IN ESCHERICHIA  
COLI BY ULTRAVIOLET RADIATION

by Valerie Darby B.Sc. (London)  
Genetics Department,  
University of Leicester

A thesis submitted for the degree of Doctor of  
Philosophy at the University of Leicester

February 1981

UMI Number: U316516

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U316516

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



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



THESIS  
623089  
26 5 81

x75294-8325

### ACKNOWLEDGEMENTS

I am happy to thank Dr. Barry Holland for his encouragement, unfailing interest in the project, and most stimulating supervision. I am also indebted to my immediate colleagues in the laboratory, both past and present, for their friendly interest in this work. In particular I wish to thank Drs Julie Pratt, Peter Meacock and Brian Spratt for academic help, and Ms Julie Crisp for occasional technical support.

The project was carried out in a congenial atmosphere, for which I thank all the members of the department, particularly other members of the technical staff, for their willing cooperation and frequent expert advice.

I am very grateful to Sheila Mackley for her accurate and efficient typing of the manuscript.

Finally, my grateful thanks go to Tim Burland for his friendship (from afar) throughout this work, for his critical interest and for his practical help in the last stages of preparation of this volume.

## CONTENTS

	PAGE NO
<u>CHAPTER 1</u> <u>INTRODUCTION</u>	1
1.1      Coupling of division and DNA replication	2
1.2      Cell shape and the septation process	6
1.3      Genetic analysis of division control	8
1.4      RecA and SOS division inhibition	11
1.5      Induction of recA synthesis	13
1.6      Mutations affecting the division response to UV	22
1.7      Aims of the project	26
<u>CHAPTER 2</u> <u>MATERIALS AND METHODS</u>	27
2.1      Bacterial strains	27
2.2      Growth of bacterial cultures	27
2.3      UV irradiation	31
2.4      Measurement of bacterial cell number	31
2.5      Growth of bacteriophage $\lambda$	32
2.6      Radioactive labelling of protein and DNA <u>in vivo</u>	34
2.7      Preparation of whole cell lysates and envelopes for PAGE analysis	36
2.8      Polyacrylamide gel electrophoresis	39
2.9      Preparation of phage DNA	45
2.10      Preparation of plasmid DNA	47
2.11      Agarose gel electrophoresis	49
2.12      SDS lysates for rapid plasmid screening	50
2.13      Digestion of DNA by restriction endonucleases; ligation of DNA fragments	52
2.14      Transformation of bacteria by plasmid DNA	52

CHAPTER 2      CONTINUED

2.15	Transduction of chromosomal markers carried by $\lambda$	53
2.16	Preparative electrophoresis of DNA restriction fragments	54
2.17	Expression of genes carried by specialised $\lambda$ transducing phages in UV irradiated cells	55
2.18	Construction of $\lambda$ GH200 cI <sup>ts</sup> <u>ind</u> <sup>-</sup>	55
2.19	<u>In vitro</u> protein synthesis	56
2.20	Construction of a recombinant plasmid from DNA fragments with non-complementary ends	62
2.21	Sources of chemicals	66
2.22	Abbreviations	67

CHAPTER 3      THE PHYSIOLOGICAL EFFECTS OF UV IRRADIATION

		70
3.1	Introduction	70
3.2	Cell division after UV in <u>lon</u> <sup>+</sup> and <u>lon</u> <sup>-</sup> strains	70
3.3	RecA is essential for division switch-off	72
3.4	Effect of UV on DNA synthesis	72
3.5	Induction of recA protein	73
3.6	Stability of recA protein	75
3.7	The effect of Rifampicin on recA induction and division	77
3.8	Other effects of UV, and the <u>lon</u> gene product	78
3.9	Effect of UV on synthesis of envelope proteins	79

	PAGE NO
<u>CHAPTER 4</u> <u>THE ELUSIVE <u>SULB</u> GENE</u>	80
4.1      Introduction	80
4.2      The <u>sulB</u> mutation and models for <u>sulB</u> action	81
4.3      Genetics of <u>sulB</u> and cloning strategy	84
4.4      Attempts to isolate specialised transducing phage $\lambda$ <u>envA</u> <sup>+</sup>	87
4.5      Extension <u>in vivo</u> of $\lambda$ <u>env</u> <sup>+</sup> to $\lambda$ <u>env</u> <sup>+</sup> <u>fts</u> <sup>+</sup>	89
4.6      Tests for <u>sulB</u> <sup>+</sup> on $\lambda$ GH200 and $\lambda$ <u>fts</u>	90
 <u>CHAPTER 5</u> <u>CLONING OF <math>\lambda</math><u>fts</u> DNA FRAGMENTS INTO A PLASMID</u>	 93
<u>VECTOR</u>	
5.1      Introduction	93
5.2      Choice of a cloning vehicle	92
5.3      Construction and identification of recombinant plasmids	95
5.4      The effect of pLG510 on division after UV	97
5.5      Amplification and stability of pKN410 derivatives	99
 <u>CHAPTER 6</u> <u>EXPRESSION AND IDENTIFICATION OF CLONED GENES</u>	 104
6.1      Introduction	104
6.2      Radioactive labelling of plasmid-coded proteins <u>in vivo</u>	105
6.3      Radioactive labelling of phage coded proteins in UV-irradiated cells	107
6.4 <u>In vitro</u> synthesis of plasmid-coded and restriction fragment-coded polypeptides	110
6.5      Recloning the 2.0 Kb fragment	115

	PAGE NO
<u>CHAPTER 7</u>	
<u>INTERACTION BETWEEN RECA* AND THE DIVISION TARGET</u>	118
7.1 <u>In vivo</u> experiments	118
7.2 Experiments in heavily UV irradiated cells	118
7.3 The need for an <u>in vitro</u> system	119
7.4 <u>In vitro</u> experiments	120
7.5 Assay of tif activity in the S.30 extract	122
7.6 Future prospects for <u>in vitro</u> experiments	123
 <u>CHAPTER 8</u>	
<u>AN APPRAISAL OF THE PROJECT IN THE LIGHT OF RECENT DEVELOPMENTS</u>	125
8.1 Kinetics of induction and recovery from the division block	125
8.2 Location of <u>sulB</u>	128
8.3 The inducible inhibitor	129
8.4 RecA and DNA replication	131
8.5 Pathways of division control	133
 BIBLIOGRAPHY	138

## Chapter 1

### INTRODUCTION

In the study of bacterial cell division, three principle approaches have been used. First, an analysis of the relationships between different physiological parameters over the cell cycle has led to the formulation of a mathematical description of growth and division. In this approach it is assumed that division is controlled by a balance between mass increase, surface growth and DNA replication rather than by timed, discrete molecular events specific to septation. This assumption could be justified by the difficulties involved in elucidating such controlling events, difficulties which have only recently been overcome.

In a second approach, biochemical studies on the biosynthesis of the rigid peptidoglycan layer of the cell envelope (responsible for cell shape), while revealing in Streptococcus a pattern of surface growth that accounts for septum formation, have been unsuccessful in this respect when applied to other organisms.

Thirdly, a genetic analysis has been attempted by the isolation of mutants defective in the division process. The complexity of division control was indicated by the large number of mutants obtained, and the list may not yet be complete. Genetic analysis per se has not revealed any detailed mechanism of division control and this aspect is still poorly understood.

A critical discussion of all these studies is beyond the scope of this chapter and recent reviews have analysed the data in depth (Shockman et al.,

1974, Slater and Schaecter, 1974, Pritchard, 1974, Daneo-Moore and Shockman, 1977, Helmstetter et al., 1979). Therefore only those features from each aspect will be considered, which have contributed significantly to current ideas on division control, and the work of this project in particular. Recent investigations have uncovered another area of great significance in a study of bacterial cell division, linking the recA gene and functions under its control to division regulation. The role of this gene will also be dealt with in some detail.

### 1.1 Coupling of division and DNA replication

Maintenance of a population of physically and genetically identical bacteria requires division and DNA replication to be coordinated to ensure correct segregation of daughter chromosomes into daughter cells. In the simplest case, the chromosome must be completely duplicated before segregation, but control of the timing of these events in E.coli must allow for an increase in complexity observed at fast growth rates when the time between divisions becomes shorter than the time taken to replicate the chromosome (Yoshikawa et al., 1964). Under these conditions, initiation of one cycle of DNA replication occurs before the previous cycle has terminated, resulting in segregation of more than one genome equivalent at division.

1.1a The cell cycle of E.coli      The cell cycle of E.coli (growing with a generation time, " $\tau$ ", of 20-60 min) has been described as consisting primarily of a period "C", the time taken to replicate the chromosome, and a further period "D" between termination of DNA replication and division. The D period was observed by Cooper and Helmstetter (1968) when DNA

synthesis was measured in a population of cells fractionated according to age by the technique, now well-known, of membrane elution. In an analysis of division and DNA synthesis at different growth rates, these workers found that C and D were constant and independent of growth rate (within certain narrow limits). They were also able to demonstrate that at fast growth rates successive rounds of DNA replication overlap, and that under these conditions replication continues throughout the cell cycle.

Unfortunately, the membrane elution technique (and hence the application of the results obtained) is limited to B/r strains. Also the binding of parent cells to the filter may lead to artefacts (Boyd and Holland, 1977), but it has been used successfully in many studies of physiological changes over the cell cycle.

At slower growth rates in some strains a third period, G, may elapse between division and initiation of DNA replication (Helmstetter and Pierucci, 1976), and thus the G, C and D periods have been suggested to correspond with the  $G_1$ , S and  $G_2$  periods identified in the cycles of eukaryote cells (Cooper, 1979).

1.1b The requirements of models for division control      Observed relationships between mass increase, DNA synthesis and division have given rise to a mathematical description of the E.coli cell cycle (Donachie, 1968, Pritchard et al., 1969). A resulting model (reviewed by Pritchard, 1974) has been useful in the design of experiments concerned with a numerical analysis of division control, as well as a basis from which to investigate genetic and biochemical controls.

The mathematical studies focussed on initiation of DNA replication as the critical event in the bacterial cell cycle. Membrane elution

experiments had shown that since replication cycles can overlap, termination of one "round" is neither the trigger, nor even essential, for the initiation of the next round. Donachie (1968) suggested that initiation took place at a constant cell size (mass) and that new synthesis of protein(s) required for initiation was necessary in each cycle. It was also observed (Pierucci and Helmstetter, 1969) that a fixed amount of protein synthesis, taking place during DNA replication, was an essential requirement for division. These findings led to the conclusion that the cell must somehow measure its mass or size in order to regulate replication and ultimately division correctly. This could be achieved by strictly regulating the synthesis of initiation protein(s) so that these are kept in balance with the cell's growth rate. Alternatively this regulation could be carried out negatively by a repressor synthesised at the start of replication which is gradually diluted out by growth (Pritchard et al., 1969, Sompeyrac and Maaløe, 1973). This idea, though originally proposed for control of initiation of DNA replication, could also time division. An alternative means of timing division was proposed by Jones and Donachie (1973) who suggested that termination of replication triggered the synthesis of a protein which then promoted division, when the (independent) requirement for the period of protein synthesis concurrent with DNA replication was completed. Donachie et al. (1976) later proposed the concept of a minimum "unit cell" size from measurements of cell lengths at different growth rates, suggesting that division was triggered when cells reached a critical length, twice the length of the unit cell. Koch (1977) lent some support to this proposal by observing that since the distribution of cell size at initiation of DNA synthesis was much larger than at

division (and therefore initiation size is less well regulated than size at division), initiation cannot "time" division.

Experiments relating to these ideas have yielded data of great interest, but have yet to provide a universally acceptable model for division and its control in E.coli. The author's opinion, in agreement with that of Daneo-Moore and Shockman (1977), is that the object of studies on division is ultimately to "describe each event, and the mechanisms regulating it, on a biochemical and molecular level". While the theoretical approaches described above have provided a fundamental framework for the understanding of growth and division, other approaches will be required to take advantage of recent technical developments which allow a more fruitful investigation of genetic and biochemical aspects of division control.

A major criticism of the early experiments relating DNA synthesis and division is now apparent. In these studies it was tacitly assumed that effects on division resulting from perturbation of DNA replication were passive. Recently, investigations of DNA repair and associated activities in E.coli following DNA damage (including that shown to be inflicted by some of the treatments used in many early experiments) have demonstrated that, on the contrary, highly specific mechanisms operate in these circumstances, overriding the normal division controls. (Reviewed by Witkin, 1976; discussed here in 1.4 and 1.5). A possible further difficulty arose with the realisation that strain B/r, on which many of the experiments described in this chapter were performed, carries two mutations affecting division control, particularly that operating after DNA damage. These are lon and sul (see 1.6).

## 1.2 Cell shape and the septation process

In Streptococcus, the rigid polymer peptidoglycan is the chief component of the cell "wall" and is responsible for maintenance of cell shape (Daneo-Moore and Shockman, 1977). Biochemical studies on the synthesis and assembly of this polymer have provided a basis for a plausible model of the division process. This simple approach identified a system of growth zones in which new polymer is introduced into the peptidoglycan layer. The activity of these growth zones appears to provide successive cycles of wall and septum growth. Attempts have been made to identify such zones in E.coli (in which a rigid layer of peptidoglycan is also responsible for cell shape). Ryter et al. (1973 and 1975) and Begg and Donachie (1973, 1977), using autoradiographic techniques to identify newly synthesised peptidoglycan, and the location of new bacteriophage receptors in the E.coli surface, showed that peptidoglycan "growth" in this rod-shaped organism is more complex than in the spherical Streptococcus. These studies did not produce an acceptable model for surface growth and division for E.coli. However, that peptidoglycan is probably a critical surface component of E.coli with respect to division, is not disputed; clearly, structural alterations to the polymer are necessary for septum formation. While this may also be true for the inner and outer membrane of E.coli, these are relatively much more fluid in structure. Thus septum formation (perceived as a shape change) could be brought about by modifications to the peptidoglycan assembly mechanism resulting in a different crosslinking pattern, or a slightly different composition. Consequent shape changes might thereby form the basis of the division process. Such differences in chemical composition or structure would probably be very small and difficult to detect, explaining why no septum-

specific modifications have been clearly identified. Daneo-Moore and Shockman, reviewing data from several organisms, observed that, for example a reduction in teichuronic acid content of the peptidoglycan in some Bacillus and Micrococcus species, was often associated with defects in cell shape or division. Thus shape might be regulated by controlling the activity of the enzyme(s) responsible for assembly of teichuronic acid into the peptidoglycan. These enzyme(s) might be found closely associated with the peptidoglycan (for example as cytoplasmic membrane components) under the control of membrane or cytoplasmic factors (or both). Daneo-Moore and Shockman suggest that in E.coli, lipoprotein may be important for shape maintenance : although it can be removed from sacculi by trypsin digestion without loss of shape, these authors suggest that its incorporation in vivo in a particular sequence of synthetic processes might be critical in shape determination. Supporting this possibility, Sonntag et al. (1978) found that double mutants of E.coli lacking lipoprotein and an outer membrane protein (ompA) had lost shape control, and that the peptidoglycan and outer membrane in this mutant appeared to be dissociated.

In a search for envelope components specifically associated with division, the B-lactam antibiotics have been most useful. These compounds interfere with the synthesis and assembly of peptidoglycan by binding to the peptidoglycan transpeptidases (Spratt, 1978). Seven proteins in the E.coli envelope that bind penicillin (PBPs) have been identified (Spratt, 1975) and some of these have been genetically defined; PBP3 is essential for septation and PBP2 is necessary for maintenance of the rod shape of E.coli. Further genetic analysis and identification of the gene products is in progress and it is anticipated that these studies will do much to elucidate the mechanism of septation and elongation of the E.coli rod.

With the exception of the PBPs no other proteins involved in division have been clearly identified in the E.coli envelope, probably indicating that such proteins, if present, are in very low concentration. In Streptococcus the peptidoglycan-degrading enzyme autolysin, found tightly bound to the cell wall, appears to have a potentially critical role in controlling the shape of the peptidoglycan layer, particularly in facilitating the post-synthetic incorporation of teichuronic acid as well as in cell separation itself (Daneo-Moore and Shockman, 1977). The activity of this enzyme must be tightly regulated as its unlimited action is lethal; its activation by proteases has been demonstrated but nothing is known of any genetic controls. Similar autolytic activities have been demonstrated in E.coli (Van Heijenoort et al., 1975; Holtje et al., 1975), but the role of these enzymes in peptidoglycan assembly has not yet been determined.

### 1.3 Genetic analysis of division control

In attempts to identify genes controlling division, many mutants defective in septation have been isolated - a recent review lists 35 (Helmstetter et al., 1979). However, the physiological studies by which these mutants were mostly characterised have revealed surprisingly little information regarding control mechanisms. It is quite probable that some division control genes remain undiscovered.

Many of the mutants studied have their primary defect in an aspect of DNA synthesis or a DNA repair function (see next section) but of more specific interest, several appear to be conditional division mutants. Two of these map close together near 2 min on the E.coli chromosome in a large cluster of genes involved in surface growth and septum formation (see

Table 1.1Induction of SOS functions

1. Inducing treatments: UV,  $\gamma$  and X-irradiation.  
 Nalidixic acid, novobiocin, bleomycin.  
 Mitomycin C, NMG, aflatoxin and other mutagens.  
 Thymine starvation.  
 Introduction of irradiated (double-stranded) replicons.
2. SOS responses:
  - 1) Increased synthesis of recA protein.
  - 2) Inhibition of division.
  - 3) Induction of prophage  $\lambda$  in lysogens.
  - 4) Error-prone repair of chromosomal lesions.
  - 5) Increased survival of irradiated phage (Weigle reactivation).
  - 6) Error-prone repair of phage DNA lesions.
  - 7) Cessation of respiration <sup>1</sup>.
  - 8) Induction of colicins in col<sup>+</sup> strains.
  - 9) Alleviation of restriction <sup>2</sup>.
  - 10) "Stable" DNA replication.

<sup>1</sup> Swenson et al. (1978)

<sup>2</sup> Dharmalingham and Goldberg (1980)

Functions 4, 5 and 6 are reviewed by Witkin (1976)

All others are dealt with in the text

Chapter 4), and new investigations, making use of recently developed molecular techniques, have begun to yield significant data.

The gene coding PBP3 (see section 1.2), also known as sep or ftsI has been identified (Fletcher et al., 1978) using specialised transducing phages. DNA from one phage has been used as a specific probe for mRNA and the transcription of pbp3 through the cell cycle is under investigation (J. Walker, personal communication). The gene product has also been identified (Irwin and Walker, 1980) and it is expected that such experiments, in combination with physiological studies, will elucidate the mechanism of action of the PBP3 protein.

Transducing phages have also been used most effectively to investigate the ftsA gene and adjacent regions mapping close to pbp3. Mutations in ftsA, coding a 50K polypeptide (Lutkenhaus and Donachie, 1979) cause filamentation at non-permissive temperature. The isolation of amber mutations in ftsA (Donachie et al., 1979) allowed physiological studies to identify accurately a 10 min period in the cell cycle in which ftsA synthesis must take place if division is to follow.

Modifications to the ftsA transducing phages (e.g. by in vitro manipulation of the phage DNA) have enabled Luktenhaus et al. (1980) to identify a second division locus ftsZ adjacent to ftsA. Further isolation of amber mutants has facilitated the identification of two more genes in this cluster, ftsQ (Begg et al., 1980) and murG (Salmond et al., 1980). The new fts mutants have a conditional division defect (i.e. formed filaments at 42°) and the murG mutant lost control of cell shape at 42° and then lysed. Finally, a study of the proteins synthesised from transducing phages carrying parts of the fts - pbp3 region enabled these groups, also

Lutkenhaus and Wu (1980), to deduce details of the transcriptional organisation of this region.

These approaches represent a considerable breakthrough in the study of genetic control of division and show that the techniques are now available by which the specific molecular events controlling septation might be elucidated.

#### 1.4 RecA and SOS division inhibition

It is now known that treatments damaging DNA or disturbing DNA replication result in the induction (or switch-on) of a series of responses collectively termed "SOS" functions (Radman, 1975), summarised in table 1.1. These phenomena were suggested to constitute an "emergency" response to potentially lethal DNA lesions, perhaps primarily designed to allow DNA replication to proceed past lesions which would otherwise cause a permanent block to the replication apparatus. Lesions appear to be negotiated by a mechanism of error-prone repair. A second feature of the SOS response is the inhibition of division during the repair of DNA damage by the constitutive, error-free repair processes (excision and recombination). When E.coli is exposed to a low dose of UV, any damage is removed relatively rapidly and the SOS effects are transient. All the SOS functions are thought to be coordinately controlled since they are induced simultaneously and the expression of each is dependent on functional recA and lexA gene products (Witkin, 1976).

The primary effect of UV irradiation on DNA is the crosslinking of adjacent pyrimidine bases forming non-coding dimers that distort the double helix and block DNA replication presumably because non-pairing bases

inserted by polymerase III are immediately excised by its 3'-5' editing activity (see below 1.4b). The presence of these lesions somehow leads to greatly increased synthesis of a protein of M.W. 40K (Inouye and Pardee, 1970; Gudas and Pardee, 1976; Sedgwick, 1975), later identified as the product of the recA gene (Gudas and Mount, 1977; Little and Kleid, 1977; McEntee, 1977; Emmerson and West, 1977). Expression of other SOS functions is associated with this induction of recA synthesis. Cell division presumably comes under the control of recA in inducing conditions in order to prevent segregation of damaged genomes into daughter cells. This aspect of division control is triggered by a specific event (damage to DNA) and overrides the normal controls since cells in the D period may also be prevented from dividing (Chapter 3).

Witkin had suggested (1967) that filamentation in response to UV could be brought about by derepression of a gene coding an inhibitor of septum formation. Subsequently, George et al. (1975) proposed similarly that division was controlled by a radiation-induced inhibitor (RADI). An increasing understanding of SOS induction lent credence to these proposals. Also, the use of UV as an inducing treatment offered a unique opportunity to investigate this specific mechanism of division control with minimal disturbance to other physiological parameters. In contrast to other continuous SOS-inducing treatments (e.g. mitomycin or nalidixic acid treatment), low doses of UV proved to be effective for transient induction without significantly disturbing mass increase (Darby and Holland, 1979). Thus in formulating a project to investigate the nature and control of the block to division during SOS induction, UV treatment was chosen as the inducing stimulus.

The first requirement of such an investigation is an understanding of the mechanism by which *recA* is induced since this might also reveal the mechanism of control of other SOS functions. In the following pages this mechanism will be discussed particularly in relation to division control, though wider implications will also be included. The induction mechanism illustrates some important principles in control of gene expression, which later suggested ways of probing the control of division inhibition at the molecular level.

## 1.5 Induction of *recA* synthesis

1.5a Properties of *recA* protein      The *recA* product is a multifunctional molecule essential for genetic recombination as well as expression of SOS functions. *RecA*<sup>-</sup> mutants degrade their DNA after damage (e.g. by UV). The protective effect of *recA* against this degradation appears to reside in the ability of the molecules to bind to single stranded DNA (Gudas and Pardee, 1975; Satta *et al.*, 1979) and hence limit the action of the *recBC* coded exonuclease. The unwinding of duplex DNA by single-stranded DNA, and the subsequent rewinding of homologous strands was also shown to be promoted by *recA* binding to single stranded DNA (Cunningham *et al.*, 1979; McEntee *et al.*, 1979). Further, an endonucleolytic incision of the duplex DNA associated with the rewinding effect was shown to be *recA* dependent in vivo and in vitro (Howard-Flanders, 1978). These events are thought to be the basis of the recombination mechanism. Mutations in *recA* (see table 1.2) have separated recombination functions from control of SOS induction, so the molecule must have at least two functional domains.

Table 1.2Mutations in *recA* and *lexA*

<u><i>recA</i> allele</u>		<u>Recombination</u>	<u><i>recA</i> induced</u>	
			<u>by UV</u>	<u>in <i>recA</i> <i>tsl</i></u>
<u><i>recA1</i></u>		-	+	+
" <u>56</u>	missense	-	-	+
" <u>13</u>		-	-	+
" <u>200</u>		-	+	+
" <u>12</u>	small deletion	-	-	+
" <u>7</u>	total deletion	-	-	-
" <u>99</u>	amber	-	-	-
<u>tif</u>		+	+	+
<u>zab</u>		+	-	-
<u>lexB</u>		+/-	+/-	?
<u><i>lexA</i> allele</u>				
<u>lexA3</u>	(dominant)	+	-	
<u>spr</u>		+	Constitutive	+
<u>tsl</u>		+	t.s.	+

### 1.5b Mechanism of recA regulation

The identification of the recA protein was based on observations involving derepression of its synthesis in various recA mutants (table 1.2). These studies also showed that functional recA protein was required for derepression of its own synthesis (e.g. Emmerson and West, 1977) and that induction was constitutively expressed without any detectable DNA damage, and therefore presumably without any effector, in the tif mutant (mapped in recA by Morand et al., 1977). A dominant mutation at lexA was shown to block induction (Mount et al., 1972) while constitutive (and temperature sensitive) mutations in lexA (table 1.2) resulted in derepression of recA and SOS functions, suggesting that lexA is a repressor of the recA gene (Mount et al., 1975; Gudas and Pardee, 1975; Gudas and Mount, 1977). Many groups proposed models of recA regulation based on these observations : references include Bailone et al. (1979), Castellazzi et al. (1977), Satta and Pardee (1978) Sedgwick et al. (1978), as well as authors mentioned above.

Recent studies have confirmed the basic premises of these models at the molecular level. Induced synthesis of recA mRNA detected by hybridisation was blocked by lexA and certain recA mutations, and increased by mutations assumed to inactivate or reduce the efficiency of the lexA repressor (McPartland et al., 1980). Derepression of recA was shown to be accompanied by cleavage of the lexA gene product (Little et al., 1980), whose synthesis is also repressed by lexA itself (Brent and Ptashne, 1980). On the basis of these findings, and incorporating the basic ideas proposed in earlier models, Brent and Ptashne proposed the following mechanism to account for control of recA synthesis (illustrated in fig.1.1). In normally growing cells lexA represses both its own synthesis and that of

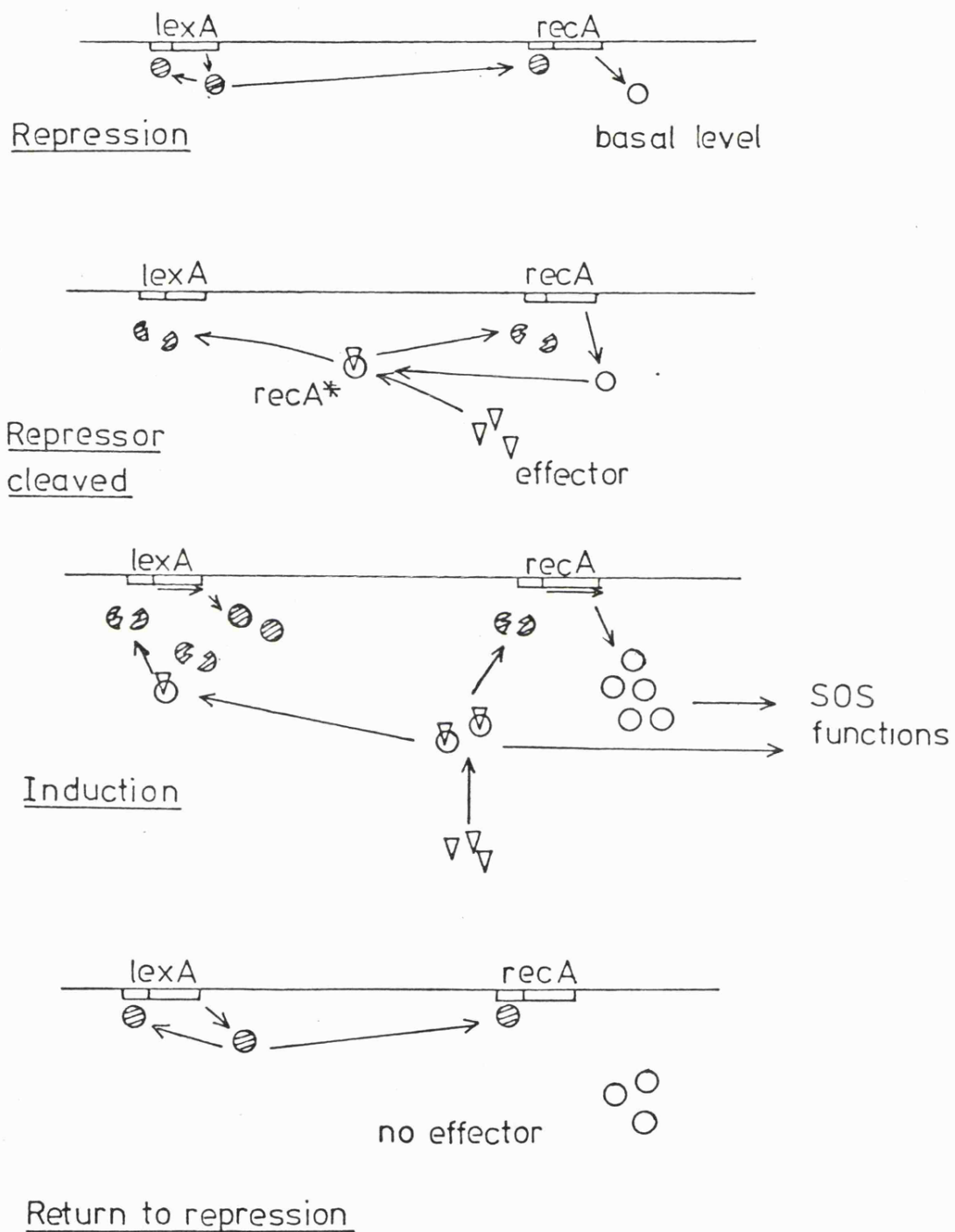


Figure 1.1 Model of *recA* regulation

recA. Damage to DNA produces an effector molecule (see below), presumed to interact with low (constitutive) levels of recA molecules to produce an allosteric change conferring a new protease activity on recA. This new form of recA (termed here recA\*) cleaves lexA and thus allows recA transcription to proceed at a high rate, producing large quantities of (proteolytically inactive) recA molecules. Until repair nears completion, the presence of the effector presumably ensures "activation" of a small number of recA molecules to recA\*, maintaining transcription of recA and expression of other SOS functions (see below). The massive production of recA (as opposed to recA\*) is assumed to provide protective binding to single stranded DNA formed during repair processes, and perhaps to aid the efficiency of recombination. As the DNA damage is repaired and the effector concentration falls, continuing transcription from the derepressed lexA gene produces new repressor molecules which gradually (on the vital assumption that recA\* activity is proportional to effector concentration) re-establishes repression.

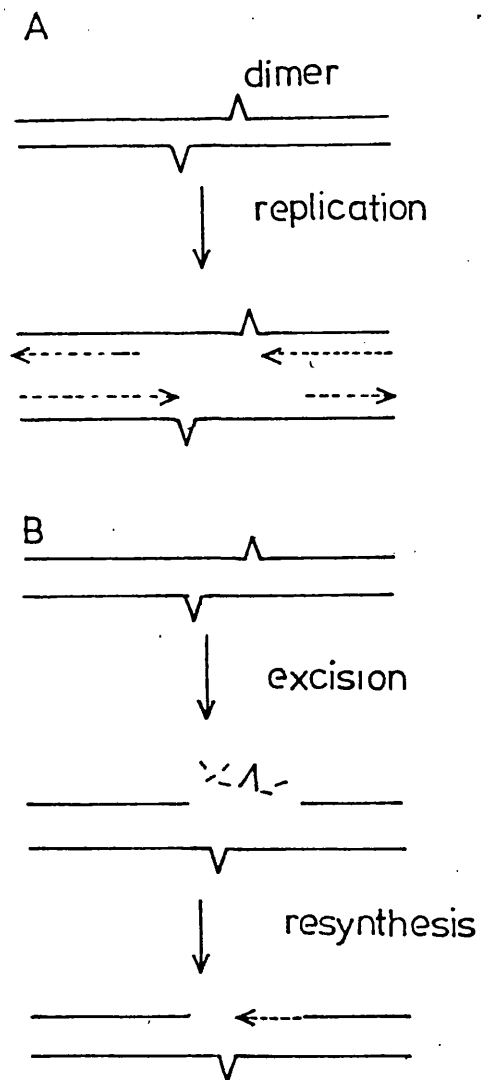
This model explains many of the properties of mutants mentioned above and in table 1.2. The tif mutant appears spontaneously to produce a protein at 42° similar to recA\* which promotes expression of SOS functions including extensive tif (recA) synthesis. The tif protein was shown to possess a slightly altered isoelectric point from that of the recA<sup>+</sup> product and is therefore probably the result of a point mutation, spontaneously changing to an active conformation at 42° (Emmerson and West, 1977, and others quoted). The dominant lexA3 mutation seems to result in an altered repressor, apparently not affecting the basal level of recA (Moody et al., 1973) but resistant to the protease activity of recA\*. Another class of

mutations in lexA (tsl, spr) appear to produce a repressor whose recognition of recA (and presumably lexA) operator sites is severely impaired. Other mutations identified in recA and lexA can be similarly accounted for by the model.

1.5c The inducing signal      There are several ways in which DNA damage could lead to derepression of recA. Perhaps the simplest "signal" would be that of an altered DNA conformation caused by interference with the supercoiled structure of the genome by bulky unpaired lesions and single strand incisions at these lesions, leading to alterations in the recA or lexA promoters. This latter possibility has been investigated : Herrero et al. (1981) showed that inactivation of the gyrB subunit of DNA gyrase, and therefore loss of supercoiling did not lead to recA induction, and further, that this loss of supercoiling did not interfere with UV induction of recA.

Studies by Radman et al. (1977) and Caillet-Fauquet et al. (1977) using irradiated, single stranded phage  $\phi$ X174, showed that a thymine dimer posed a complete block to the replication complex in vivo.

In vitro experiments showed that at a "stalled" replication fork there was repeated insertion of nucleotides opposite the lesion in an attempt to proceed, but that these were removed by the polymerase III editing function, effectively turning over dNTPs to dNMPs (Radman et al., 1977). Boiteux et al. (1978) suggested that these dNMPs could provide the inducing signal. Though plausible this increase in dNMP concentration resulting from the "idling" replication complex could not be the only means of effector production since infection of normal cells with UV irradiated  $\phi$ X174 does not induce SOS functions; it has also been shown that induction does not necessarily require an active replication fork (see below).



**Figure 1.2 "Unrepairable" overlapping lesions induced by UV**

(After Witkin, 1976)

Sedgwick (1976) suggested that closely spaced lesions cannot be substrates for the cell's well characterised constitutive repair systems. Lesions can be considered to overlap if their repair by excision or recombination would lead to double strand breakage or loss of template for repair synthesis after the removal of the lesion (see fig.1.2). Post replication gaps were suggested to be left by replication complexes blocked at lesions, apparently followed by reinitiation downstream, at least on the lagging strand (Hanawalt and Smith, 1978). Such gaps have been detected (Rupp and Howard Flanders, 1968) and recently visualised (Johnson and McNeill, 1978). If these gaps on sister strands overlap, no intact template is available for repair synthesis after recombination (fig.1.2). Sedgwick suggested that this impasse could lead to induction of error-prone repair activity, the single stranded gaps acting as the inducing signal. Again, however, induction in non-replicating cells and at low levels of damage is not accounted for. Baluch et al. (1980b) developed a similar argument, suggesting that activation of recA occurs when recA molecules bind cooperatively at the single-strand DNA at lesions, and is followed by derepression of recA synthesis. However, it is difficult to imagine how the structure of such lesions could lead to the effective activation of recA; although recA might well bind to single stranded DNA as suggested, and conceivably alter the properties of the blocked replication complex, activation of molecules bound at this site could not easily promote cleavage of lexA and hence derepression of SOS functions.

The most general assumption for the activation of recA has been that small oligonucleotides produced by repair activity (e.g. by the recBC coded exonuclease) form the effector. Support for this proposal came from

the observations that tif expression is modified by nucleotides (George et al., 1975) and that one SOS function, induction of  $\lambda$ , has been shown to require added polynucleotide in vitro (see 1.5d). In addition, Oishi and Smith (1978) showed that addition of oligonucleotides to permeabilised lysogens resulted in the induction of  $\lambda$ . However, there appear to be several ways of generating the effector. Thus while recBC is essential for induction in replicating bacteria by nalidixic acid treatment (Gudas and Pardee, 1976), this exonuclease is not necessary for induction by UV (Little and Hanawalt, 1977; Bockrath and Hanawalt, 1980). Genetic studies have failed to reveal a mechanism common to all inducing situations, and have, on the contrary, revealed some intriguing complications. Thus Rothman et al. (1979) identified alternative pathways of induction by UV involving recF and recL in strains deficient in excision repair (uvr<sup>-</sup>). Baluch et al. (1980a) also found that induction of SOS functions by UV was dependent on single-stranded DNA binding protein coded at the lexC/ssbA locus. These examples demonstrate that there may be several mechanisms of effector production, each of which can lead to expression of SOS functions including division inhibition. The relationship between these pathways is not clear.

1.5d Coordinate control of SOS functions      Although it is difficult to accept without qualification Radman's original suggestion that all of the SOS functions act to promote cell survival, it is clear that division inhibition does contribute to survival after DNA damage, by allowing time for DNA repair. This effect even operates at low levels of damage which, theoretically can easily be repaired. In recA and lexA mutants division continues after irradiation and many DNA-less cells are produced; even in

untreated cultures of these mutants some DNA-less cells are detected suggesting that recA and lexA may have a continuous role in coordinating DNA replication and division.

Coordinate control of division and SOS DNA repair functions was suggested by their dependence on recA and lexA, their coordinate induction after UV (see Chapter 3) and their coordinate expression in constitutive mutants at these loci (recA and lexA). Experiments by Meyn et al. (1977) showed that  $\lambda$  induction, mutagenic repair and filamentation were all blocked by protease inhibitors, indicating that the proteolytic activity of recA\* might have a broader significance. Witkin had suggested in 1976 that this principle of induction by (recA\* - mediated) cleavage of a repressor might be general for control of SOS functions. Thus the division inhibitor postulated earlier by Witkin and George et al. might be regulated by a repressor which is cleaved by recA\* after UV. Parallel with the development of these ideas, experiments on the mechanism of  $\lambda$  induction revealed precisely such a system. In 1975 Roberts and Roberts showed that the  $\lambda$  cI repressor was cleaved after UV; subsequently they demonstrated that this cleavage was due to a proteolytic activity which copurified with recA (Roberts et al., 1978). In vitro experiments gave further confirmation and also detected a requirement for ATP and added polynucleotide for the reaction to proceed (Craig and Roberts, 1980). Though some reservations persist concerning the details of these experiments, these findings are now generally accepted. A similar mechanism seems to operate in the induction of P22 phage (Craig and Roberts, 1980), and evidence is accumulating that the induction of colicin synthesis may proceed by a similar mechanism, (Tessman et al., 1978; Tessman and Peterson,

Table 1.3Mutations affecting SOS filamentation

Mutation	Map position	SOS filamentation	Inducing agent	Comment
<u>recA</u>	56	-	UV	
<u>tif</u>	"	+	42°	
<u>lexA</u>	90	-	UV	
<u>tsl</u>	"	+	42°	
<u>rnmA</u>	"	+	UV	
<u>tsl recA99</u>		+	42°	
<u>tsl recA1</u>		+	42°	
<u>recF</u>	82	-	UV	} in presence of <u>uvr</u> <sup>-</sup> only
<u>recL</u>	?	-	UV	
<u>ssbA</u>	91	+	42°	} Constitutive
<u>lexC</u>		+		
<u>lon</u>	10	+	UV	
<u>sul/sfiA</u>	22	-	UV	} Suppressors of <u>lon</u> , <u>tif</u>
<u>sul/sfiB</u>	2	-	UV	
<u>tsl infB</u> <sup>1</sup>	66-83	-	42°	Suppressors
<u>tif infB</u>		-	42°	of <u>tsl</u> , <u>tif</u>

<sup>1</sup> Huisman et al. (1980b)

1980). These results are clearly consistent with the presence of an inducible inhibitor of division in E.coli regulated by lexA or another repressor cleavable by recA\*. A recent experiment by Huisman (1980a) added further support to this idea; filamentation was induced in tif strains by incubation at 42° and continued after returning the cultures to 30°; this treatment also effectively induced  $\lambda$ . If however, chloramphenicol (an inhibitor of protein synthesis) was added during the 42° incubation and then removed,  $\lambda$  induction still took place, but filamentation was blocked. This experiment was interpreted as showing that new synthesis of tif protein at 42° was not essential for induction of  $\lambda$ , but filamentation did require new protein synthesis, indicating that activated tif alone was not sufficient for induction and that an induced inhibitor was also required.

This "inducible inhibitor" principle which formed the basis of a working hypothesis for this project was thus validated. At the time when the project was started, the distinction between recA and recA\* had not been convincingly established. The first experiments were therefore designed to test the possibility that the synthesis of large quantities of recA was itself responsible for division inhibition (i.e. recA is the inhibitor). None of the foregoing arguments precludes this possibility. These experiments are described in Chapter 3 and in the accompanying paper (Darby and Holland, 1979).

#### 1.6 Mutations affecting the division response to UV

Although the principle of coordinate control of division inhibition with other SOS functions is generally supported by the evidence available (see previous section), the effects of certain mutations (table 1.2) indicate that the simple model of control proposed above may not sufficiently account for all the data.

Mount (1977) reported that when a tsl (recA<sup>+</sup>) strain was incubated at 42°, filamentation and derepression of recA synthesis took place. Under these conditions DNA synthesis was not disturbed and thus the induction occurred in the absence of effector molecules. Analysis of lexA102 revertants by Clark and Volkert (1978) and Volkert et al. (1979) showed that secondary mutations (rnmA) in lexA suppressed part of the LexA<sup>-</sup> phenotype, restoring control of DNA breakdown and filamentation after UV, but no other SOS functions. Notably, there was no induction of recA protein. Thus mutations at lexA can affect division inhibition independently of recA indicating that lexA itself might have a direct role in filamentation control, perhaps even as the repressor of the division inhibitor. The recF mutation (defective in post-replication repair) described by Rothman et al. (1979) blocked expression of most SOS functions while induction of recA protein proceeded normally after UV. A study of this and other mutant repair functions led Clark and Volkert (1978) to suggest that several pathways are available for production of effector; these pathways are recF, recBC, uvr or ssb - dependent, and probably operate simultaneously. This separation of various SOS effects ("split" phenotypes) in multiple mutants could indicate that specific induction mechanisms (and perhaps effectors) are therefore associated with each SOS function, all subsequently acting through recA.

The lon gene is of particular interest since it specifically affects the division response. Lon<sup>-</sup> mutants filament persistently after UV even at low doses (Adler and Hardigree, 1965) and other SOS inducing treatments, and are unable to recover even though the DNA repair capacity of the mutants is normal, (Howard-Flanders et al., 1964; Smith, 1969). Bridges

et al. (1977) showed that the lon mutation renders the cell extremely sensitive to the DNA lesions assumed to initiate the SOS response; in a lon uvr mutant (i.e. when excision is prevented) filamentation was triggered by the presence of only one or two lesions in the genome.

In addition, the lon mutation is extremely pleiotropic. Mutants overproduce capsular polysaccharide (colanic acid) giving colonies their characteristic mucoid appearance. This phenotypic effect has been extensively investigated (reviewed by Markovitz, 1977). Mackie and Wilson (1972) obtained evidence that the lon product acted as a repressor of the gal operon, thus the synthesis of galactose (a colanic acid precursor) is derepressed in the lon mutant, leading to production of excess colanic acid (i.e. mucoidy).

Protease activity has been ascribed to the lon gene product. In mutants originally designated degT, nonsense polypeptide fragments were shown to be degraded very slowly compared with deg<sup>+</sup> strains; these strains were shown to be lon mutants (Shineberg and Zipser, 1973).

Mutations in the lon gene reduce the frequency of lysogeny of some bacteriophages.  $\lambda$  multiplies normally in lon mutants but lysogens form very poorly (Walker et al., 1973). The prophage state of  $\lambda$  is established and maintained by the cI repressor; a double mutant phage ( $\lambda$  cI<sup>857</sup> cro) overproduces cI repressor at 30°. Therefore infection results in a 100% lysogenic response, and no plaques were formed on wild type hosts. In lon hosts, however, plaques were produced at 30° (Walker et al., 1973). Further, direct measurements (Truitt et al., 1976) showed that in lon strains lysogenised by normal phage, the level of cI repressor was only half that in lon<sup>+</sup> hosts, suggesting that the lon product is necessary for normal production (and perhaps stability) of the cI repressor.

In fast growing cultures of lon strains a proportion of the cells from filaments spontaneously, perhaps reflecting an imbalance in the coordination of DNA synthesis, mass increase and division. This "complex medium killing" (Gayda and Markovitz, 1978) is observed as an increase in sensitivity of the culture to various treatments. Gayda and Markovitz proposed that the low frequency of lysogen formation may be partly accounted for if the filamentous cells in the population are more likely to be killed by e.g.  $\lambda$  infection, than are the normal cells. This complex medium killing effect can be suppressed by limiting the growth rate of the cultures with pantoyl lactone, which interferes with lipid metabolism (Kantor and Deering, 1966). The observation that "holding" irradiated lon bacteria in buffer before plating on agar enhances survival, may be related to this phenomenon.

Do these phenotypic effects of lon mutations bear any relation to SOS functions? With respect to the lysogeny defect, Walker et al. (1973) suggested that the lon product might modulate the affinity of phage repressors for their corresponding operators. Conceivably lon might also act in this way to modify transcription of recA and lexA. Alternatively the proposed inducible division inhibitor might be controlled directly (rather than via rec and lex) by the repressor activity, or the repressor-modulating activity of lon.

In 1976, shortly before this project was begun Witkin suggested that the protease activity (or control of such activity), assigned to lon could be an important factor in the regulation of SOS expression, since the altered stability (resulting from lon action) of protein(s) induced during the SOS response could greatly affect the expression of SOS phenotypes.

These possibilities for the role of lon in SOS expression therefore suggested that further investigation of this mutant would be most worthwhile.

George et al. (1975) observed that the effects of tif and lon in tif lon double mutants were additive - they were more sensitive to UV than lon mutants, and tif expression at 42° was enhanced. These workers suggested that filamentation is probably induced by the same mechanism in the two single mutants since single site revertants of the double mutants were isolated in which filamentation was suppressed. Moreover, separation of the lon and tif mutations showed that the suppressor was still effective in the presence of either. These suppressor mutations (sfi/sul) were shown to map in genes quite distinct from recA and lon (Johnson, 1976b) and appear to identify possible genetic targets for the action of recA and lon, as discussed in detail in Chapter 4. Thus, at the start of the project, it was anticipated that steps in the pathway by which SOS filamentation is expressed might be elucidated by an investigation of these (sul/sfi) mutants.

### 1.7 Aims of the project

The initial aim of this work was to investigate any kinetic relationships between recA induction, DNA synthesis and filamentation. The effect of the lon mutation on these relationships was also determined. The second part of the project was concerned with the cloning of one of the possible recA/lon "target" genes mentioned above, the identification of the gene product, and a preliminary investigation of factors controlling expression of this gene. The concurrent development of ideas relating to the possible mechanism of division inhibition is discussed in the introductory part of each chapter.

## Chapter 2

### MATERIALS AND METHODS

#### 2.1 Bacterial strains

The bacterial strains used in this work were all E.coli K12. Their genotypes are shown in table 2.1. Cultures were maintained on nutrient agar at 4°C when in current use, and freeze dried or deep frozen at -20°C (in nutrient broth containing 10% glycerol) for long term storage.

The lon mutant of KN126 was isolated by selection of spontaneous resistance to chlorpromazine (Molnar et al., 1977). The bacteria were grown in minimal medium and plated on to minimal agar containing 65µg/ml chlorpromazine, when lon mutants appeared as mucoid colonies after 2-3 days; these were also UV sensitive. The rough map position of the mutation conferring mucoidy was confirmed when F'254 was transferred in (covering the 10 min region). Transconjugants were non-mucoid and UV resistant, and thus the mutation is probably located in the lon gene.

#### 2.2 Growth of bacterial cultures

Bacterial liquid cultures in Luria broth or M9 minimal medium prepared as indicated in table 2.2, were shaken in a New Brunswick Gyrotary Shaker, at temperatures indicated in the text. Bacterial cell mass in growing cultures was monitored by measurement of absorbance at 450nm using a Gilford Microsample spectrophotometer 300N. Solid media used were nutrient agar (2.5% Difco nutrient broth, 1.45% agar) and minimal medium as described in table 2.2, solidified with 1.2% agar. Vitamin B1 (0.1µg/ml); thymine (5-80µg/ml) and casamino acids (0.05-0.1%),

Table 2.1Bacterial strains

<u>Name</u>	<u>Genotype</u>	<u>Source</u>
KN126	<u>tyram</u> <u>ilv</u> <u>tryEam</u> <u>lac</u> <u>sup126</u> $\lambda^R$ (lamB)	B.G. Spratt
KN126 <u>lon</u>	" " " " " " <u>lon</u>	From KN126 by chlorpromazine selection
C600	<u>thr</u> <u>leu</u> <u>thi</u> $\lambda^S$ <u>supE</u> <u>tonA</u>	Laboratory stocks
AB1157	<u>thr</u> <u>leu</u> <u>thi</u> <u>his</u> <u>arg</u> <u>met</u> <u>pro</u> <u>str</u> <u>lac</u> <u>tsx</u> <u>mtl</u> <u>xyl</u> <u>ara</u> <u>gal</u> <u>supE</u>	"
KL16	Hfr <u>thi</u>	"
KL16-99	Hfr <u>thi</u> <u>recA1</u>	"
PAM162	<u>thr</u> <u>leu</u> <u>his</u> <u>met</u> <u>thi</u> <u>lon</u> <u>sulB26</u>	B. Bachmann <sup>2</sup>
PAM161	" " " " " " <u>sulB25</u>	" 2
D21	<u>trp</u> <u>pro</u> <u>his</u> <u>rpsL</u>	A. Salem
D22	" " " " <u>envA</u>	"
TKF12	<u>thr</u> <u>leu</u> <u>thi</u> <u>pyrF</u> <u>thyA</u> <u>ilv</u> <u>ftsA12</u> <u>his</u> <u>arg</u> <u>lac</u> <u>tonA</u>	W. Donachie
JM12	<u>thr</u> <u>leu</u> <u>thi</u> <u>pro</u> <u>his</u> <u>arg</u> <u>tif-1</u> <u>lac</u> <u>str</u> <u>gal</u>	B.G. Spratt
GC895	Hfr <u>tif-1</u> <u>sfiB</u>	"
159	<u>uvrA</u> <u>gal</u> <u>str</u>	G. Plastow
159 $\lambda$ 21	<u>uvrA</u> <u>str</u> <u>gal</u> $\lambda$ <u>imm</u> <sup>21</sup> lysogen	This work
833	<u>his</u> <u>try</u> ( <u>gal-att</u> - <u>bio</u> ) <u>tonA</u>	B.G. Spratt
KLF1	F' 0 $\rightarrow$ 2 min; as AB1157, <u>recA</u>	B. Bachmann

Table 2.1 Continued

<u>Name</u>	<u>Genotype</u>	<u>Source</u>
4282	F'254; as AB1157, <u>recA</u> <u>try</u> <u>purE</u> <u>azi</u> <u>tonA</u> ; <u>gal</u> <sup>+</sup>	B. Bachmann
4251	F'104; as AB1157; <u>recA</u> ; <u>met</u> <sup>+</sup>	"
DS410	<u>minA</u> <u>minB</u>	G. Boulnois
BS254	HfrH ( <u>gal-uvrB</u> ) <u>thi</u> , $\lambda$ cI857 integrated into <u>leuB</u>	B.G. Spratt/ K. Shimada <sup>3</sup>
Ymel	<u>tonA</u> <u>suIII</u> <u>tyr</u>	G. Plastow
W3550	<u>sup</u> <sup>o</sup> <u>gal</u>	G. Plastow
CV512	F <sup>+</sup> <u>leuA371</u>	B.G. Spratt
MRE600	RNase <sup>-</sup>	Porton Research Centre

Footnote:

1. J. Molnar et al., 1977
2. B.F. Johnson, 1977
3. K. Shimada and R.A. Weisberg, 1972

Table 2.2Media and BuffersA. Luria broth 1% w/v Tryptone

0.5% w/v Yeast Extract pH7.4

0.5% w/v NaCl

B. M9 minimal mediumNaH<sub>2</sub>PO<sub>4</sub> (anhydrous) 6.0 g/litre of mediumKH<sub>2</sub>PO<sub>4</sub> " 3.0 "

NaCl 0.5 "

NH<sub>4</sub>Cl 1.0 "

Glucose 0.4% w/v autoclaved separately

CaCl<sub>2</sub> 1.0mM " "MgSO<sub>4</sub> 10mM

Amino acids as required 20µg/ml " "

C. Antibiotics Ampicillin 25µg/ml

Tetracycline 10µg/ml

Sterilised by filtration

D. Bacterial bufferKH<sub>2</sub>PO<sub>4</sub> 3g/litreNa<sub>2</sub>HPO<sub>4</sub> 7 "

NaCl 4 "

MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 "E. λ buffer

Tris HCl pH7.2 6mM

MgSO<sub>4</sub> 10mM

Gelatin 0.05% w/v

in sterile solution, were also added to minimal medium as required. Strains harbouring plasmid borne drug resistance were grown in media containing the appropriate antibiotic as shown in table 2.2. All media containing antibiotics were freshly prepared. The constituents of bacterial and  $\lambda$  buffers are also noted in table 2.2.

### 2.3 UV irradiation

Liquid cultures were irradiated without pretreatment at  $A_{450}$  0.15-0.2 ( $5 \times 10^7$  cells/ml). Up to 20ml of culture in a prewarmed 18cm glass petri dish (giving a liquid layer 2-3mm deep) was swirled beneath a Hanovia Bactericidal lamp. The dose rate was calibrated using a Latarjet dosimeter and the dose used was  $10 \text{ Jm}^{-2}$ , given in 20 secs. For plate tests, overnight broth cultures were diluted to about  $2 \times 10^7$  cells/ml and long streaks of these suspensions made across the surface of a nutrient agar plate. The plate was then irradiated in sections, so that graded doses were given along each streak. The plate was foil-wrapped for incubation.

### 2.4 Measurement of bacterial cell number

0.2ml samples were taken from cultures in minimal medium and fixed by dilution into 2.0ml 0.8% v/v formaldehyde in saline (0.8% w/v NaCl). Samples were further diluted in 25 volumes saline for counting using a Model B Coulter Counter fitted with a 30 $\mu$  orifice. Counting was normally performed on the same day as sampling. Occasionally samples were stored overnight at 4° with no reduction of particles counted. Instrument settings were as follows:- aperture current  $^{-1}=1$ ,

amplification  $-1 = \frac{1}{8}$ , lower threshold = 8, upper threshold =  $\infty$ . All saline was filtered through a 0.22 $\mu$  pore size Millipore filter to remove dust particles and then autoclaved before use.

## 2.5 Growth of bacteriophage $\lambda$

$\lambda$ 616 and derivatives were turbid plaque formers and were UV inducible. For preparation of stock suspensions, lysogens were grown in minimal medium containing 1% casamino acids to  $A_{450}$  0.6-0.7, and diluted 4-fold in  $\lambda$  buffer (see table 2.2) before irradiation (30Jm<sup>-2</sup>). The irradiated bacteria were then collected by centrifugation, resuspended in Luria broth to their original volume and shaken at 37° until lysis occurred (1-2 hours). Lysates were treated with chloroform and centrifuged to remove cell debris. Titres were usually  $> 10^{10}$  pfu/ml. Phage assays were performed by mixing 0.1ml of different phage dilutions with 0.2ml of an overnight culture of E.coli C600, standing at 37° for 15 min in the presence of 10mM Mg<sup>++</sup> to allow adsorption. Samples were then diluted with 3.5ml molten Trypticase soft agar, poured on to Trypticase agar plates containing 10mM MgSO<sub>4</sub>, and plates incubated at 37°.

Soft Trypticase agar:	Trypticase	1% w/v
-----------------------	------------	--------

	NaCl	0.5% w/v
--	------	----------

	Agar	0.7% w/v
--	------	----------

Trypticase agar for plates:	Trypticase	1% w/v
-----------------------------	------------	--------

	NaCl	0.5% w/v
--	------	----------

	Agar	1.5% w/v
--	------	----------

Preparation of  $\lambda$  cI<sup>ts</sup> (usually  $> 10^{10}$  pfu/ml) were made by heat

induction. Lysogens were grown in Luria broth at 30°C to  $A_{450}$  0.6-0.7,

then shifted to  $42^{\circ}$  and incubated until lysis occurred (1-2h). Lysates were processed and assayed as above. Specialised transducing phage were prepared by the method of Schrenk and Weisberg (1975). E.coli 833 was grown in Luria broth into stationary phase, the bacteria collected by centrifugation and resuspended in 0.4 volumes of  $\lambda$  buffer. 0.2ml of bacterial suspension were infected with 0.1ml  $\lambda$  NM627 ( $cI^{ts}$  Sam7) (MOI = 7)<sup>1</sup>. After 15 min at  $32^{\circ}$  for phage adsorption,  $10^{-1}$ - $10^{-4}$  dilutions of the mixture were spread on to Trypticase agar plates together with  $2 \times 10^9$   $\lambda$  c h82, to kill non-lysogenic cells thus selecting lysogens. After incubation at  $30^{\circ}$ , several plates were chosen bearing about 1000 colonies, and the bacteria washed off the agar surface with Luria broth. The optical density was adjusted to 0.3 at 450nm, and the culture (500ml) vigorously shaken at  $30^{\circ}$  until the  $A_{450}$  reached 0.8. It was then incubated at  $42^{\circ}$  for 30 min, followed by 2h at  $37^{\circ}$ . Cells were collected by centrifugation and resuspended in 10ml  $\lambda$  buffer and 0.5ml chloroform. Lysis occurred after 20 min incubation at  $37^{\circ}$  and cell debris was removed by centrifugation. The lysate was assayed on E.coli Ymel tonA suIII, on which  $\lambda$   $cI^{ts}$  s7 can grow (since  $\lambda$  h82 uses the tonA receptor) and on KN126 ( $\lambda^R$ ) on which only  $\lambda$  h82 can grow. The lysate contained  $5 \times 10^{10}$   $\lambda$   $cI^{ts}$  s7/ml and  $6 \times 10^6$   $\lambda$  h82/ml.

1 MOI = multiplicity of infection.

## 2.6 Radioactive labelling of protein and DNA *in vivo*

2.6a Pulse-labelling of proteins was achieved by incubating growing cells in minimal medium with  $[^{35}\text{S}]$ -methionine for short periods.

Typically, 1ml of exponentially growing culture at  $A_{450}$  0.2-0.5, was incubated for 5 min at  $37^\circ$ , with 5 $\mu\text{Ci}$  L- $[^{35}\text{S}]$ -methionine specific activity 50 $\mu\text{Ci}/\mu\text{g}$ . Incorporation was terminated by addition of ice-cold methionine to 4.0mg/ml and chloramphenicol to 600 $\mu\text{g}/\text{ml}$ . Pulses of 10 $\mu\text{Ci}$  for 7 min were used when cell envelope preparations were to be analysed since only 10% of incorporated counts are recovered in the envelope fraction.

2.6b Uniformly labelled bacteria: In most experiments, cells uniformly labelled with  $[^3\text{H}]$ -leucine were added to each sample as an internal standard. These were prepared by incubating an exponentially growing portion of the starting culture (at  $A_{450}$ =0.1-0.2), with 6 $\mu\text{Ci}/\text{ml}$   $[^3\text{H}]$ -leucine (specific activity 10 $\mu\text{Ci}/\mu\text{g}$ ), for 2.5h (i.e. 2-3 generations) at  $37^\circ\text{C}$ . Incorporation was terminated by addition of cold leucine to 4mg/ml and chilling on ice.

To monitor incorporation into proteins, 5 $\mu\text{l}$  portions of washed cells (see section 2.7) were solubilised in Aquasol scintillation fluid and counted in a Packard Tri-Carb 3255 liquid scintillation counter. This rapid estimation of incorporation allowed equal counts of all samples to be loaded onto the polyacrylamide gels. 1ml samples, pulse labelled as described, normally incorporated  $5-8 \times 10^5$  c.p.m. When  $[^3\text{H}]$  labelled cells were to be added, the ratio of  $[^3\text{H}]$  to  $[^{35}\text{S}]$  c.p.m. was adjusted to between 5:1 and 10:1, so that overspill of  $[^{35}\text{S}]$  counted in the  $[^3\text{H}]$  channel was less than 25% of the total c.p.m.  $[^3\text{H}]$  overspill

counting as  $[^{35}\text{S}]$  was negligible. 2-3ml of  $[^3\text{H}]$  labelled cells prepared as above, were sufficient for each sample.

2.6c Stability of labelled proteins. Stability of labelled proteins was measured in a pulse-chase experiment. 10ml of an exponential culture was pulse labelled for 10 min as described above, starting 15 min after irradiation. Unincorporated isotope was then removed by rapidly filtering the cells on to a 0.45 $\mu$  pore size Oxoid membrane filter; the cells were washed on the filter with warm medium without isotope, containing 300 $\mu\text{g}/\text{ml}$  methionine, and resuspended in the same medium. 1.5 ml samples, withdrawn during subsequent growth at 37 $^{\circ}$  were chilled, chloramphenicol added to 600 $\mu\text{g}/\text{ml}$  and 5 $\mu\text{l}$  counted in Aquasol prior to analysis by SDS-PAGE. Equal counts in each sample were loaded on to the gel, and thus loss of counts with time from individual bands was estimated (see 2.8c) .

2.6d DNA synthesis was measured by accumulation of  $[^{14}\text{C}]$ thymidine. Using thy<sup>+</sup> strains, cultures were grown in minimal medium containing 1 $\mu\text{Ci}/\text{ml}$   $[^{14}\text{C}]$  thymidine (specific activity 2 $\mu\text{Ci}/\mu\text{gm}$ ) and 1.5mM uridine, for about 5 generations before sampling began to ensure steady state growth and labelling conditions. For measurement of incorporation, 1ml samples from exponentially growing cultures were mixed with 2ml ice-cold 10% trichloroacetic acid (TCA). After 1h, precipitated nucleic acids were collected on Sartorius membrane filters (0.45 $\mu$  pore size), and washed with 2 volumes 5% TCA followed by at least 10 volumes of hot (90-95 $^{\circ}\text{C}$ ) water. Filters were dried and counted in non-aqueous scintillation fluid consisting of 0.5% diphenyloxazole (PPO) and 0.0033% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in toluene.

Rates of DNA synthesis were measured by pulse-labelling 0.5ml amounts of minimal medium cultures in exponential phase with 1 $\mu$ Ci [ $^3\text{H}$ ] thymidine (specific activity 85 $\mu$ Ci/mM), for 3 min at 37 $^{\circ}$ . Pulses were terminated by addition of 0.5ml ice-cold 10% TCA containing 400  $\mu$ g/ml unlabelled thymidine. Samples were processed for counting as described above.

## 2.7 Preparation of whole cell lysates and envelopes for PAGE analysis

2.7a Whole cell lysates for 1-dimension electrophoresis. Samples consisted of [ $^{35}\text{S}$ ]-methionine pulse-labelled cells and [ $^3\text{H}$ ]-uniformly labelled cells mixed as described in 2.6; to these were added  $10^{10}$  cells from a chilled but otherwise untreated portion of the same culture, as carrier cells. The mixed bacteria were then washed twice with 10mM sodium phosphate buffer pH7.4 by centrifugation at 4 $^{\circ}\text{C}$ , and the pellets resuspended in 50 or 100 $\mu$ l of the same buffer and transferred to small, capped plastic tubes. At this stage 5 $\mu$ l samples were withdrawn for counting (2.6). An equal volume of SDS Sample Buffer (table 2.3) was added to each tube and the samples boiled for 5 min to complete lysis and solubilisation of proteins. Vortexing to shear DNA in the preparations was often necessary. These samples were loaded on to polyacrylamide gels immediately or stored at -20 $^{\circ}$ . If stored, they were boiled again before analysis.

2.7b Envelopes for 1-dimension analysis. Cell envelopes were prepared by sonication and differential centrifugation. Samples of pulse-labelled cells (and uniformly labelled cells where indicated in the text) were mixed with  $5 \times 10^{10}$  unlabelled carrier cells in 25ml beakers and the volume

adjusted to 9.0ml with 10mM sodium phosphate buffer pH7.0. Sonication was then carried out in an ice-water bath, for 3 periods of 30 sec at an amplitude of 6 $\mu$ , with 30 sec cooling intervals, using an M.S.E. 100W Ultrasonic Disintegrator with a large probe. Unbroken cells were removed by centrifugation at 7,000 rpm at 4°C for 7 min, in the Sorvall SM24 rotor. The supernatants were then transferred to 10ml polycarbonate tubes and envelopes pelleted by 45 min centrifugation at 35,000 rpm in the Beckman 40 or 50Ti rotor at 4°C. The pellets were washed with sodium phosphate buffer by a similar centrifugation procedure and finally resuspended in 25 $\mu$ l of the same buffer. 25 $\mu$ l SDS sample buffer (table 2.3) was added and the samples boiled for 5 min before loading on to the gels.

2.7c Whole cell lysates for 2-dimension analysis. [<sup>35</sup>S]-methionine pulse labelled bacteria mixed with uniformly labelled and carrier cells as indicated in the text, were washed as described above and the final cell pellet was resuspended in 100 $\mu$ l I.F. sample buffer (table 2.4). The following additions were made to each sample (in a small, capped plastic tube): 1 $\mu$ l RNase (4.0mg/ml), 1 $\mu$ l egg white lysozyme (10mg/ml) and in some experiments 1 $\mu$ l of the protease inhibitor phenyl-methylsulphonyl fluoride (PMSF : 0.1M in 50% v/v ethanol). The bacteria were lysed by 5 cycles of freezing and thawing, 1 $\mu$ l DNase I (4mg/ml) added and the samples kept on ice for 1h. Each preparation was then brought to 8.5M Urea by addition of 50mg urea crystals, and then boiled for 5 min before loading on to isoelectric focussing gels, or storage at -20°C.

The procedure described here is based on those of O'Farrell (1975) and McEntee (1977).

Table 2.3Electrophoresis solutionsA. Separating gel buffer:-

0.75M Tris HCl pH8.8, 0.2% w/v SDS

B. Stacking gel buffer:-

0.25M Tris HCl, pH6.8, 0.2% w/v SDS

C. Acrylamide solution:-

I 44% w/v acrylamide, 0.8% w/v N,N'-methylene-bis-acrylamide (bis)

II 44% w/v acrylamide, 0.3% w/v bis

D. Electrophoresis buffer:-

0.125M Tris, 0.192M glycine, 0.1% w/v SDS (pH8.3 with no adjustment)

E. SDS sample buffer:-

0.125M Tris HCl pH6.8, 40% w/v glycerol, 4% w/v SDS, 10% mercapto-ethanol, 0.05% bromophenol blue

F. Gel compositionSeparating gel

	15%	11%
Buffer A	20ml	20ml
Acrylamide	13.6ml	9.4ml
Dist. water	5.4ml	8.4ml
	mixed and degassed	
Ammonium persulphate <sup>1</sup> (freshly prepared, 10mg/ml)	0.95ml	0.95ml
N,N,N',N',-tetramethyl <sup>1</sup> ethylenediamine (TEMED)	0.075ml	0.075ml

<sup>1</sup> Gels were formed without delay after addition of these polymerising agents

## 2.8 Polyacrylamide gel electrophoresis (PAGE)

2.8a Preparation of gels. For one-dimension analysis, gels were formed in the Biorad 220 slab gel apparatus without cooling using the system of Laemmli (1970) with minor modifications. Solutions used and the mixing procedure are listed in table 2.3. Gels consisted of a 10 cm separating gel of 11 or 15% acrylamide and a stacking gel in which the loading wells were formed, of 6.3% acrylamide, with 1 cm of effective stacking distance between the wells and the surface of the separating gel.

5-60 $\mu$ l samples containing bromophenol blue (table 2.3E) were loaded under electrophoresis buffer and 15 milliamps (m.a.)/gel applied from a Stogate power supply until the dye front was well into the stacking gel (about 1h). Then 25 m.a./gel was applied for 3-4h, when the dye front was close to the bottom of the gel. After removal from the apparatus, gels were stained in Coomassie blue (0.05% w/v in 10% v/v acetic acid, 25% v/v isopropanol) or fixed in acetic acid (10% v/v) and isopropanol (25% v/v) overnight; stained gels were then destained by diffusion in several changes of the same acetic acid - isopropanol mix over 24h. A [ $^{14}\text{C}$ ]-methylated protein mixture was applied to some gels as molecular weight markers. The mixture contained myosin (200,000), phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000; apparent M.W. 43,000), carbonic anhydrase (30,000) and lysozyme (14,300), mixed together at equal radioactive concentrations.

2.8b Autoradiography and fluorography. Gels containing fewer than  $10^5$  c.p.m. of [ $^{35}\text{S}$ ] per sample were then treated for fluorography by the method of Bonner and Lasky (1972) as follows: fixed or destained gels were soaked in 2 changes of dimethylsulphoxide (DMSO) for 30 min each,

then in 22% PPO in DMSO for 2h; the gels were then rehydrated by soaking in water for at least 1h.

Fixed, stained or fluorographed gels were then dried on to Whatman chromatography paper No.17, or Whatman No.1 filter paper (if bands were to be cut out) using a Biorad Model 224 slab gel drier connected to a KNF Laboport vacuum pump.

Fluorography or autoradiography was carried out by placing the dried gel in an X-ray cassette with a sheet of Kodak RP Royal X-ray film, and storing at room temperature for autoradiographs, or  $-80^{\circ}\text{C}$  for fluorographs, for times varying from 24h for samples containing  $> 10^6$  cpm, to 2 weeks for fluorographs of samples containing  $1 \times 10^4$  cpm. In experiments where both  $[^{35}\text{S}]$  and  $[^3\text{H}]$  were used, control samples containing only one isotope showed that blackening of the film was due to  $[^{35}\text{S}]$  only at these exposures.

When gels contained very low amounts of radioactivity, film was prefogged by limited exposure to a photographic flash, which increased the sensitivity of the film and reduced the exposure time.

Films were developed in darkness by immersion in Kodak DX-80 developer for 4 min, followed by an acid wash (in 1% acetic acid), and immersion for 4 min in Kodak FX-40 fixer .

2.8c Estimation of radioactivity in individual gel bands. The method of Ames (1974) was employed: bands were identified in stained, dried gels by comparison with their autoradiographs. After cutting from the gel, each slice, measuring approximately 1mm x 5mm, was placed in a plastic scintillation vial, without removing the paper backing strip. The slices were covered with 50 $\mu$ l distilled water and rehydrated for 10 min at room

temperature. 5ml NCS scintillation fluid (see below) was then added, well mixed, and the samples incubated at 37° overnight. After thorough mixing, the vials were chilled and counted in the Packard scintillation counter, using automatic [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ] settings. Analysis of the counts obtained, based on a method devised by A. Boyd (1979), allowed direct comparison of samples by reference to the [ $^3\text{H}$ ] internal standard. Thus for each sample

$$\frac{[\text{S}^{35}] \text{ cpm in band x}}{[\text{H}^3] \text{ cpm in band x}} \text{ estimates relative amount of x}$$

and by comparison of sample x and a reference band (y)

$$\frac{\text{relative amount of x}}{\text{" " of y}} \text{ estimates relative rate of synthesis of x}$$

In practice, the relative rate of synthesis of recA protein, which changed during the experiments, was calculated with respect to  $\beta\beta'$  RNA polymerase subunits (MW 160,000) or protein elongation factor EFTu (MW 44,000), as a reference protein having a constant rate of synthesis throughout the experiments, (described in Chapter 3).

NCS scintillation fluid:

77ml NCS solubiliser

3.75gm PPO

56mg dimethyl POPOP

Toluene to 1 litre.

2.8d Two dimension analysis. 2-dimension gel electrophoresis was performed essentially as described by O'Farrell (1975). Isoelectric focussing gels were prepared in glass tubes, 3mm internal diameter and 13 cm long, using solutions and mixing procedure listed in table 2.4. The gel tubes were sealed at one end with Nescofilm and supported

Table 2.4Isoelectric focussing solutionsA. Stock solutions

1. Acrylamide solution:-  
30% w/v acrylamide, 0.8% w/v bis
2. Nonidet NP40:- 10% w/v in distilled water
3. Ampholines : Biorad "Bio-lyte" carrier ampholytes,  
pH ranges 5-7 and 3-10

B. Buffers

1. I.F. sample buffer:-  
9.5M Urea, 2% w/v Nonidet, 2% v/v ampholines, 5% w/v mercaptoethanol.  
The ampholines comprised 1.7% v/v of pH5-7 and 0.3% v/v of pH3-10
2. Sample overlay buffer:-  
50% I.F. sample buffer
3. Anode solution:-  
0.02M sodium hydroxide (degassed by boiling)
4. Cathode solution:-  
0.01M phosphoric acid

Table 2.4 ContinuedC. Gel composition

5.5gm Urea

1.33ml acrylamide (solution A1)

2ml Nonidet (A2)

1.97ml distilled water

0.5 ml ampholines (0.4ml pH5-7

0.1ml pH3-10)

0.15ml ammonium persulphate, 10mg/ml, <sup>1</sup>

freshly prepared

10µl TEMED <sup>1</sup>

swirled to dissolve

urea, then

thoroughly degassed

<sup>1</sup> Gels were formed quickly after addition of these polymerisers.

vertically. Gel solution C (table 2.4) was introduced to the bottom of the tubes using a long steel cannula attached to a syringe, and the tubes were filled to 10 cm. After polymerisation, the tubes were fitted into a Canalco Disc Electrophoresis Apparatus Model 1200, and samples, containing  $1-2 \times 10^6$  cpm, in 10-50  $\mu$ l were loaded without prerunning the gels. The samples were overlayed and then filled to the top with anode buffer which also filled the upper reservoir. Electrofocussing was carried out by application of 400 volts at 2 watts from an LKB 2103 power supply, for 16h, followed by 800 volts for 1h. Gels were expelled from the tubes after electrofocussing by compressed air and collected in small plastic bags, in which they were immediately deep frozen ( $-20^{\circ}$ ) if not used. A control gel run without proteins was cut into 1cm pieces for estimation of the final pH gradient established during the run; each piece was then soaked overnight in 0.4ml 0.1M KCl, and the pH of the solution measured using a Russel CMW72 microelectrode and a Pye PW9418 pH meter.

For the second dimension, focussed gels were soaked in SDS sample buffer (table 2.3) for 1h and then applied to the top edge of a slab gel formed in the Biorad 220 apparatus as described for 1 dimension gels, but with only 3 loading wells at one side, in which control samples ( $10^5$  cpm/sample) were run as M.W. standards. The focussing gels were sealed in place with 0.5% agarose solution in 50% v/v stacking gel buffer, just above setting point. Electrophoresis and autoradiography were as described above (2.8a,b).

## 2.9 Preparation of phage DNA

For each preparation, a 100ml culture of C600 (in exponential phase) in Luria broth was grown at 37° to  $A_{450}$  0.6. The  $\lambda$  lysate was added to give a phage multiplicity of infection of 1, and  $MgSO_4$  was added to 20mM. The phage was adsorbed for 15 min without shaking, then the culture was incubated with vigorous shaking until lysis occurred. Phage titres obtained were often low ( $5 \times 10^9$  p.f.u./ml). The lysates were treated with chloroform and cell debris removed by centrifugation. Solid sodium chloride was added to 0.5M, then polyethylene glycol (PEG) 6000 to 10% w/v, which was stirred gently until dissolved. The mixture was stored at 4°C overnight to precipitate the phage which was then collected by centrifugation in the Sorvall SS34 rotor at 5000 rpm for 8 mins at 4°C. The pelleted phage were gently resuspended in 6ml  $\lambda$  buffer, and then purified by caesium chloride step gradient separation as follows. Stock solutions of different densities were prepared by mixing saturated caesium chloride solution with  $\lambda$  buffer to give refractive indices of 1.39, 1.38 and 1.36, corresponding to densities of 1.7, 1.5 and 1.3 g/cc respectively. Gradients were prepared by slowly pipetting layers of 1.5 ml of density 1.7, 3ml of 1.5 and 3ml of 1.3 into cellulose nitrate tubes, and finally the phage suspensions were carefully layered on to the top. Gradients were centrifuged in the Beckman SW40 rotor at 24,000 rpm for 3h at 8°C. Phage bands, usually very narrow but distinct, were seen towards the bottom of the 1.5 g/cc block, while PEG and protein remained in the 1.3 g/cc block. The side of the tube was punctured by a  $1\frac{1}{2}$  in. 21g syringe needle and the phage band withdrawn into a 5ml syringe in about 1.5ml. This was then diluted to 10ml with sterile TE buffer (see

Table 2.5Reagents used in the preparation of plasmid DNA

1. Tris-sucrose : 10% w/v sucrose, 50mM Tris HCl pH8.0
2. Lysozyme : 5mg/ml in 0.25M Tris HCl pH8.0
3. EDTA : 0.2M, adjusted to pH8.0 with NaOH
4. Sarkosyl : 2% w/v in distilled water
5. Phenol mix : 100g phenol dissolved in 100ml chloroform; 4ml isoamyl alcohol and 0.1g 8-hydroxyquinoline added. Stored under 10mM Tris - HCl pH7.5 at 4°C
6. TE buffer : 10mM Tris-HCl pH7.5, 1mM EDTA
7. Caesium chloride - isopropanol mix: 10ml saturated caesium chloride added to 250ml isopropanol; 10ml amounts of distilled water added and mixed well until an aqueous phase persisted, and all the CsCl was in solution

table 2.5) and transferred to a sterile siliconised Corex tube. The phage coats were then removed by 2 extractions with phenol mix (table 2.5), separating the phases by 30 min centrifugation at 10,000 rpm in the Sorvall HB4 rotor at 10°C. The aqueous phase was then transferred to a dialysis bag and dialysed overnight against 2 changes, each of 1 litre sterile TE buffer, and the DNA precipitated by ethanol at -20°C (see next section, 2.10). The dried pellet, usually containing 100-300µg phage DNA, was resuspended in 100µl sterile TE buffer, and stored at -20°C.

#### 2.10 Preparation of plasmid DNA

Cultures of strains containing pKN410 and its derivatives were grown at 30°C in Luria broth with 25µg/ml ampicillin. Strains containing pSC101 derivatives were grown at 37°C, in Luria broth with tetracycline at 10µg/ml. Overnight cultures of the 'runaway' plasmids were diluted into 100ml fresh Luria broth + ampicillin, to  $A_{450}$  0.2 and grown at 30°C to  $A_{450}$  0.5. These cultures were then diluted into 400ml fresh medium and incubated at 40°C for 2.5 hours to amplify the plasmids before harvesting. Derivatives of pSC101 are not amplifiable and 500ml overnight cultures ( $A_{450}$  1.2-1.5) were harvested without further treatment.

Bacteria were collected by centrifugation and resuspended in 25ml ice-cold Tris-sucrose (table 2.5). 2.15ml lysozyme (table 2.5) were added, followed by 8.3ml EDTA solution and after gentle mixing, the suspension was held on ice for 10 min. Then, after warming for 10 min at 37°C, 4.2ml Sarkosyl (table 2.5) were added with rapid but brief swirling, when the suspensions cleared instantly, lysing completely in a further 30 min at 37°C.

From this point, all glassware contacting the lysates was siliconised; throughout, glass, plastic ware, buffer and other aqueous reagents used were sterilised by autoclaving, and plastic gloves were worn for all manipulations. Lysates were 'cleared' of chromosomal DNA by centrifugation at 18,000 rpm for 20 min in the Sorvall SS34 rotor at  $10^{\circ}$ ; the upper 2/3 of the supernatant was taken into 30ml coated Corex tubes, and extracted twice with phenol mix (table 2.5), centrifuging for 30 min at  $10^4$  rpm at  $10^{\circ}$  in the Sorvall HB4 rotor to separate the phases. DNA in the clear aqueous phase was then precipitated by addition of 1/10 volume 3M sodium acetate and 2 volumes of absolute ethanol at  $-20^{\circ}\text{C}$ , and overnight storage at  $-20^{\circ}$ . DNA precipitates were collected by centrifugation at 10,000 rpm for 30 min at  $-10^{\circ}\text{C}$  in the Sorvall HB4 rotor and the supernatant discarded; pellets were dried under vacuum and re-suspended in 1-2ml TE buffer. These partly purified DNA suspensions contained some chromosomal DNA, protein and much RNA; the plasmid DNA was separated from the other components by isopycnic centrifugation in caesium chloride gradients consisting of 5.82ml saturated caesium chloride solution, 0.54ml ethidium bromide (EtBr; 5mg/ml) and the DNA sample to be purified, in 2ml TE. Since the pKN plasmids used in this work did not amplify greatly, all the cleared lysate from 500ml starting culture was normally loaded on to one gradient. The gradients were mixed in cellulose nitrate centrifuge tubes and the refractive index adjusted to 1.3910 with TE (1.0ml was usually added). The tubes were filled to the top with liquid paraffin, capped and centrifuged for 40-42h in the Beckman 40 or 50 Ti rotor, at 35,000 rpm, and  $15^{\circ}\text{C}$ . The plasmid band, about 2/3 from the top of the gradient, and below the chromosome band, was visualised

with long wave UV, when the ethidium bromide intercalating in the DNA was seen fluorescing. This allowed collection of the plasmid DNA by piercing the side of the tube.

The plasmid DNA was then transferred to a siliconised glass Sorvall tube. Ethidium bromide was extracted with isopropanol saturated with caesium chloride (see table 2.5); three such extractions were sufficient to remove all the pink colour from the aqueous phase. Caesium chloride was removed by dialysis : 3 changes of 500ml TE/DNA sample, followed by 1 change of 1/10 TE or water if the DNA sample was very dilute and required a significant concentration step to follow. This concentration was performed by repeated extractions with butan-2-ol, with 2 min centrifugation to separate the phases, when the volume of the aqueous phase, in which the DNA remained, decreased with each extraction. The yield was normally 100-300µg.

### 2.11 Agarose gel electrophoresis

Analysis of DNA was performed by electrophoretic separation in agarose slab gels. Gel solutions were prepared by boiling 0.5-1.2% w/v agarose in Tris-acetate buffer (13mM Tris, 0.33mM EDTA, adjusted to pH7.7 with glacial acetic acid). A wall of sticky tape was applied to the edges of a glass plate (19cm x 19cm or 10cm x 10cm) and the gel solution, cooled to 50°C was poured on to the plate to a depth of 3-5mm. Loading wells were formed 2cm from one end by a perspex comb held in place with plasticine. When highly purified DNA was analysed, 0.05µg/ml ethidium bromide was added to the gel solution immediately before pouring, and to the electrophoresis buffer, but when SDS-containing samples were

analysed, the gels were stained after electrophoresis. Samples were mixed with 1/5 volume DNA sample buffer (0.1% w/v bromophenol blue, 20% w/v glycerol) and heated to 65°C for 10 min prior to loading (up to 25µl/well). After setting, gels were placed in a horizontal electrophoresis tank submerged under Tris-acetate buffer, samples loaded and 50-200 volts applied using a Kingshill stabilised Power supply (Model 10A05C), for 2-3 hours. Gels were stained after electrophoresis by soaking in 5µg/ml ethidium bromide in Tris-acetate for 40-60 min. The rinsed gels were then photographed over a short wave UV Birchover Spectro-light Transilluminator using a Nikon camera fitted with an orange filter, exposing for 30-90s.

#### 2.12 SDS lysates for rapid plasmid screening

This method was developed by J. Broome-Smith (1980). 5ml cultures of strains under test were grown in Luria broth overnight and amplified when appropriate. Bacteria were collected by centrifugation in plastic tubes, and resuspended in 0.2ml TSE (25% w/v sucrose, 10mM Tris HCl pH8.0, 1mM EDTA). The cells were then lysed by addition of 10-20µl 10% SDS and standing at room temperature for 15 min. Lysates were then centrifuged in the Sorvall SM24 rotor at 18,000 rpm for 20 min at 10°C to sediment chromosomal DNA. 100µl supernatant was removed using Gilson or Finn pipetters, and transferred to small plastic tubes. RNA and protein were digested by a 20 min incubation at 37°C with 5µl ribonuclease I (4mg/ml) followed by a further 20 min with 5µl Pronase (4mg/ml). Both enzymes were pretreated for 10 min at 80°C to inactivate contaminating nucleases. After addition of 20µl DNA sample buffer (2.11), samples were analysed as described in 2.11, on 0.5% agarose gels.

Table 2.6Restriction and ligation buffers

<u>Enzyme</u>	<u>Buffer</u>				
	mM Tris-HCl pH7.4	mM NaCl	mM KCl	mM MgCl <sub>2</sub>	mM Dithiothreitol
EcoRI	500	250	-	50	2.5
HindIII	30	250	-	30	2.5
HpaI	50	-	250	50	2.5
BclI	30	500	-	30	2.5
XbaI	30	750	-	30	- *
BamHI	30	250	-	30	- *
Sst	70	450	-	30	- *
HincII	50 (pH7.9)	300	-	30	2.5
SauIIIA	30	250	-	30	2.5
SalI, PstI,	30	-	-	30	2.5
AvaI, PvuII,					
CauII					

\* 30mM mercaptoethanol instead of DTT

Ligation buffer:      15mg dithiothreitol

0.66ml 1M Tris HCl pH7.4	}	autoclaved
0.1 ml 1M MgCl <sub>2</sub>		
0.1 ml 5mg/ml gelatin		
0.1 ml 10mM ATP (freshly made)		

### 2.13 Digestion of DNA by restriction endonucleases; ligation of DNA fragments

For agarose gel analysis, restriction digests were prepared using 0.2-0.5µg DNA, in a total volume of 7.5µl. Buffers and incubation temperatures are listed in table 2.6. When larger quantities of DNA fragments were required (e.g. for ligation) the volume of the reaction mix was correspondingly increased. Digestions were terminated by heating at 65° for 10 min.

Recombinant plasmids were constructed by ligating together DNA fragments with complementary ends produced by restriction endonuclease digestion. 10µl of each DNA preparation (containing 1µg DNA in TE buffer) were mixed and held on ice for 60 min. 6µl ligation buffer (table 2.5) and one unit T4 ligase (a gift from Dr R. Wilson) were added, and the mixture incubated for 16-18h at 12°C, after which it was used directly to transform competent bacteria.

### 2.14 Transformation of bacteria by plasmid DNA

Cultures were subjected to treatment with CaCl<sub>2</sub> which facilitated efficient entry of circular DNA molecules, (M. Brown, 1980). Bacteria growing exponentially in 40ml Luria Broth were harvested at A<sub>450</sub> 0.3 by centrifugation, resuspended in 20ml ice-cold sterile CaCl<sub>2</sub> (100mM) and MgCl<sub>2</sub> (10mM), and held on ice for 40 min. One of the strains used (E.coli GC895) was treated with 70mM CaCl<sub>2</sub>, 10mM MgCl<sub>2</sub> for only 30 min since survival was severely reduced by the standard treatment. Cells were collected by centrifugation and resuspended in 0.5ml of the same Ca/Mg solution. To 100µl of this suspension in a plastic vial, 1µg plasmid

DNA was added, the volume made up to 200 $\mu$ l with TE buffer, and the mixture held on ice for 60 min. The transformed bacteria were then incubated at 42 $^{\circ}$  for 2 min followed by dilution into 5ml warm Luria broth, and grown for 2.5h at 30 or 37 $^{\circ}$ C (i.e. permissive temperature for normal plasmid replication). Bacteria were then harvested and resuspended in 0.3ml bacterial buffer and 10 $^0$ , 10 $^{-1}$  dilutions plated, using all of the suspension, on to agar containing antibiotics to select for appropriate plasmid bearing transformants. Further dilutions of 10 $^{-6}$  and 10 $^{-7}$  were spread on to nutrient agar to assay total viable cells. The yield was normally one transformant per 10 $^5$  - 5x10 $^6$  viable cells.

#### 2.15 Transduction of chromosomal markers carried by $\lambda$

The presence of leuA $^{+}$ , ftsA $^{+}$  or envA $^{+}$  on transducing phages was tested by the following methods: (1) ftsA (Lutkenhaus and Donachie, 1979). 0.1ml of an overnight culture of strain TKF12 (ftsA) was plated on to nutrient agar and phage dilutions spotted on to the surface. Plates were incubated at 42 $^{\circ}$  selecting fts $^{+}$ . (2) leuA. Strain CV512 (leuA) and phage dilutions were plated as in (1) but using minimal agar to select for leuA $^{+}$  colonies. (3) envA. Transductants of D22 (envA) required expression time before survivors could be detected on selective medium. Therefore 0.2ml of an overnight culture of D22 in Luria broth were mixed with phage at MOI = 5 in the presence of 10ml MgSO $_4$ , then diluted after 15 min, 4-fold in Luria broth and grown for 2h at 37 $^{\circ}$ . Dilutions were then plated on to nutrient agar containing 5 $\mu$ g/ml rifampicin, selecting env $^{+}$  (rif. resistant) transductants (Normark, 1970).

## 2.16 Preparative electrophoresis of DNA restriction fragments

This method was communicated by Dr. A. Jeffreys, (see also Smith, 1980). Several  $\mu\text{g}$  of DNA were digested with the appropriate restriction endonuclease as described above (2.13). Preparative agarose gels were of 0.5 or 0.8% agarose and  $0.05\mu\text{g/ml}$  EtBr, with loading wells of  $3\text{mm} \times 50\text{mm}$ . The digested DNA was diluted to  $0.2\text{ml}$  with TE and DNA sample buffers, and loaded on to the gel. Electrophoresis was at 50 volts for 3-5h until the band required was seen to be well separated from the other DNA species when viewed by overhead long wave UV. To recover the DNA fragment, a slice of gel containing the band was cut out of the gel under UV (with the current off) and placed in a dialysis bag, just covering the slice with electrophoresis buffer. This dialysis bag was then re-submerged in the electrophoresis tank, parallel with the electrodes such that when the power was re-applied, the DNA would migrate out of the gel by the shortest route. After several hours at 50 volts, the DNA could be seen by overhead UV to have collected on the inner surface of the dialysis membrane closest to the cathode. The current was then reversed for 2 min by exchanging the leads; the bag was removed from the electrophoresis tank and gently agitated to redistribute the DNA. The buffer from the bag, now containing the pure DNA fragment, was then collected taking care not to collect particles of agarose. Extraction with butanol (2.10) removed ethidium bromide and reduced the volume of the aqueous phase. The butanol was removed by ether extractions and the DNA then ethanol-precipitated as described in 2.10 to remove the (now) concentrated electrophoresis buffer solutes. Dried, pure fragment pellets were then dissolved in  $20\text{-}50\mu\text{l}$  TE or distilled water.

### 2.17 Expression of genes carried by specialised $\lambda$ transducing phages in UV irradiated cells

The method was based on that of Ptashne (1967). Strain 159 or 159  $\lambda_{imm}^{21}$  were grown in minimal medium containing 0.4% w/v glycerol and 0.2% w/v maltose at 37°. These carbon sources were used to minimise catabolite repression of gene expression, and maximise phage adsorption, respectively. At  $A_{450}=0.5$ , 20ml of culture were irradiated with 12,000 J.m<sup>-2</sup> UV to block protein synthesis programmed by the chromosome. Cells were then harvested and resuspended in 0.5ml of the same minimal medium, with MgCl<sub>2</sub> added to 10mM. 0.1ml amounts were infected with phage at MOI=6-7, for 15 min, then diluted with 4ml warm medium and incubated for a further 10 min. In order to label polypeptides coded by the phage, 40 $\mu$ Ci [<sup>35</sup>S]-methionine (50 $\mu$ Ci/ $\mu$ g) were added to each sample and incubation continued for 30 min at 37°. Labelling was terminated by addition of chloramphenicol and excess cold methionine, and whole lysates or envelopes prepared as described in 2.7, followed by SDS-PAGE (2.8).

### 2.18 Construction of $\lambda$ GH200 cI<sup>ts</sup> ind<sup>-</sup>

$\lambda$  h80 cI<sup>ts</sup> ind<sup>-</sup> (obtained from W. Brammar) was first confirmed as temperature-, but not UV-inducible, using the tonA<sup>+</sup> (i.e. coding the h80 receptor) host W3350. Titres of lysates from heat induced lysogens were at least 1000-fold higher than after UV. For the cross, 0.3ml W3550 at 10<sup>9</sup> cells/ml was infected with 0.2ml phage suspension containing equal numbers of  $\lambda$  h80 cI<sup>ts</sup> ind<sup>-</sup> and  $\lambda$  GH200 at a total multiplicity of infection of 10. After 15 min at 30° for adsorption of phage, 3ml Luria broth were added and the bacteria were pelleted by centrifugation. The pellet was

resuspended in 3ml Luria broth and grown at 30° for 2.5h when most of the cells had lysed. After chloroform treatment the lysate was assayed on C600 tonA lacking the h80 receptor, so that only phage with  $\lambda$  host range could grow. The plates were incubated at 40° so that recombinants carrying  $cI^{ts}$  were identified as clear plaques.

## 2.19 In vitro protein synthesis

The in vitro coupled transcription-translation system used was that of Zubay (1973) with minor modifications. The components of the system are listed in table 2.7. All glassware was oven-sterilised, plastic tubes treated with DEPC water (table 2.7), and gloves were worn for all manipulations after harvesting the culture.

2.19a Preparation of the S30 fraction. E.coli C600 was grown in 100ml medium (table 2.7) at 30° overnight. This starter culture was diluted into 4l of medium and grown overnight again. Before harvesting the culture was shifted to 42° for 2h to simulate conditions for induction of tif protein in strain GC895 (to be used later). Cultures were chilled on ice and bacteria harvested by centrifugation. The bacterial pellets were then washed 3 times with S30 buffer to which 0.5ml mercaptoethanol had been added per litre; 250ml were used to resuspend each 10g cells. The washed bacteria were then centrifuged for 20 min in the Sorvall GSA rotor at 10,000 rpm, and the cell paste obtained was stored overnight at -80°C.

After thawing slowly at 4°, the paste was slowly resuspended without vigorous agitation, in S30 buffer (100ml/10g) and mercaptoethanol (0.05ml/10g cells). The bacteria were again collected by centrifugation and the

Table 2.7

Preparation of the components of an *E.coli* transcription  
translation system

1. Growth medium

56g	$\text{KH}_2\text{PO}_4$	}	Dissolved in 10 L of $\text{H}_2\text{O}$ and autoclaved 45 min
289g	$\text{K}_2\text{HPO}_4$		
10g	Yeast extract		
10-15mg	Thiamine		
0.5g	20 amino acids		
Add:- 400ml	25% glucose		
100ml	100mM Mg Acetate		autoclaved separately 20 min

2. Preparation of water used for reagents and S30 buffer:-

1ml of diethylpyrocarbonate (DEPC) was slowly added to 1 L of distilled water, (with stirring). This was allowed to stir for 1 h, then autoclaved and stored at  $0^\circ\text{C}$ .

3. S30 buffer (Made in DEPC treated water)

10mM	Tris acetate pH8.2
14mM	Mg Acetate
60mM	K Acetate
1mM	DTT

1 L of a 10 x stock solution of the above was made without DTT and a stock of 100mM DTT prepared separately. The two were mixed when finally diluted just prior to use.

Table 2.7 Continued4. Stock solutions and chemicals

<u>Component</u>	<u>Manufacturer</u>	<u>Concentration</u>	<u>Volume</u>
Tris acetate	Sigma	2.2M pH8.2	200ml
Mg acetate	Fisons AR	3M	10ml
<u>Inorganic Mix</u>			
NH <sub>4</sub> acetate	BDH AR	1.1M	10ml
K acetate	Fisons SLR	2.2M	10ml
Ca acetate	Sigma	296mM	10ml
Polyethylene glycol 6000	Fisons	40%	10ml
20-L amino acids	Sigma (Kit LAA-21)	50mM each	5ml
DTT	Sigma	0.55M	1ml
ATP (Na)	Sigma	38mM pH7.0	20ml
Phosphoenol pyruvate (Na <sub>3</sub> )	Sigma	0.42M pH7.0	10ml
Cyclic AMP	Sigma	100mM pH7.0	1ml
Folinic acid	Sigma	2.7mg/ml	1ml
CTP (Na) (yeast)	Sigma	48.3mg	} 88mM each pH7.0
GTP (Na)	Sigma	47.6mg	
UTP (Na) (yeast)	Sigma	48.4mg	
<u>Other requirements</u>			
Diethylpyrocarbonate	Sigma		25ml
Pyruvate kinase	Sigma		
tRNA (E.coli)	Sigma (R-4251)		
[ <sup>35</sup> S] methionine	Amersham		

N.B. Solutions were brought to the correct pH by adding either Tris or acetic acid. The use of HCl was specifically avoided as chloride ions inhibit transcription.

Table 2.7 Continued5. Preincubation mix

Pyruvate kinase	(10mg/ml in $(\text{NH}_4)_2\text{SO}_4$ )	25 $\mu$ l
Tris acetate	pH8.2 (2.2M)	1.0ml
Mg acetate	3.0M	23 $\mu$ l
ATP	pH7.0 38mM	2.63ml
PEP	pH7.0 0.42M	1.5ml
DTT	0.55M	60 $\mu$ l
20 amino acid mix	50mM	6 $\mu$ l
H <sub>2</sub> O	to	7.5ml

Continued ...

Table 2.7 Continued6. Preparation of the low molecular weight mix (LMM)

The low molecular weight mix was prepared in advance and stored at  $-20^{\circ}\text{C}$ . The mix was prepared by adding the components listed below in the order shown.

	<u>Stock solution</u>	<u>Volume</u>
1.	Tris-acetate 2.2M pH8.2	40μl
2.	Dithiothreitol 0.55M	5μl
3.	ATP 38mM pH7.0	50μl
4.	CTP } GTP } each 88mM pH7.0 UTP }	15μl
5.	Phosphoenol pyruvate 0.42M pH7.0	100μl
6.	19 amino acids 55mM each (suspension) (no methionine)	10μl
7.	Polyethylene glycol-6000 40% in H <sub>2</sub> O	75μl
8.	Folinic acid 2.7mg/ml	20μl
9.	cAMP 50mM	20μl
10.	tRNA <u>E.coli</u> 17.4mg/ml	15μl
11.	Inorganic mix	
	Ammonium acetate 1.4M	} 40μl
	Potassium acetate 2.8M	
	Calcium acetate 0.38M	
12.	Water to total volume of 400μl	

pellet weighed (the yield was 15g cells), then gently resuspended under vacuum in 15ml of S30 buffer. Cells were disrupted by passing the suspension through a French Press (at 4°C) at 8,400 p.s.i., and 100µl 0.1M DTT was added immediately to the resulting viscous fluid. This was cleared by centrifugation at 15,500 rpm for 30 min at 4°C in the Sorvall SS34 rotor, after which the top 4/5 of the supernatant was recentrifuged. The upper 4/5 of the second cleared supernatant (7-8ml) was then removed to a foil covered flask and 2.25ml freshly prepared preincubation mix (table 2.7) added. After incubation for 80 min at 37°C to exhaust endogenous mRNA, the fluid was dialysed 4 times against 50 volumes S30 buffer, for 45 min at 4°C to remove amino acids. Finally, the preparation was dispensed in 0.5ml aliquots and stored in liquid nitrogen. Activity was maintained for many months.

When strain GC895 was used, the growth medium was modified by reducing the yeast extract concentration to 0.03%, and methionine was omitted from the amino acid mixture so that induction of the tif protein could be monitored by pulse-labelling with [<sup>35</sup>S]-methionine. Adenine was also added to the medium (final concentration 50µg/ml), to maximise tif expression (Elphrati-Elizur *et al.*, 1976). Two preparations of GC895 S30 extracts were made, one with an additional 2h of growth at 42°C before harvesting, to induce the tif protein. Small portions of the cultures were removed immediately before, and one hour after the shift to 42°C, and pulse labelled with [<sup>35</sup>S]-methionine. Whole cell lysates were prepared and analysed by SDS-PAGE to monitor induction of tif.

2.19b Protein synthesis. Before use, the S30 fractions were centrifuged for 3 min in the Eppendorf centrifuge to remove any whole cells. For each preparation, the magnesium concentration was optimised using 0.1M magnesium acetate. Synthesis mixtures contained 15 $\mu$ l L.M.M. (table 2.7), 10 $\mu$ l DNA (2-5 $\mu$ g), 1 $\mu$ l [ $^{35}$ S]-methionine (0.5 $\mu$ Ci; 50 $\mu$ Ci/ $\mu$ g) 10 $\mu$ l S30 fraction, 5 $\mu$ l 0.1M Mg acetate and 19 $\mu$ l distilled water. Incubation was for 30 min at 37 $^{\circ}$  and the reaction was terminated by addition of 5 $\mu$ l 44mg/ml methionine and a further 10 min incubation. Control experiments carried out by Dr. J. Pratt in this laboratory had established that incorporation was linear for at least 30 min under the conditions used and was absolutely dependent upon added DNA. Samples were then mixed with an equal volume of SDS sample buffer (table 2.3) and analysed by PAGE (2.8).

2.20 Construction of a recombinant plasmid from DNA fragments with non-complementary ends

Two synthetic reactions were employed to produce complementary ends on the plasmid vector pKN410 (cut with EcoRI) and the EcoRI-PvuII fragment 'A' from the envA region of the chromosome (Chapter 7).

2.20a DNA polymerase I reaction. First, both purified DNA preparations were incubated with dATP, dTTP and DNA polymerase I (Klenow fragment)

so that the sequence AATT was added to the EcoRI ends ...G to  
...CTTAA

form a paired, blunt ended structure. Reaction conditions and reagents are shown in table 2.8. Vector DNA concentration was 13 picomole (p.M.) ends and that of fragment A (the EcoRI-PvuII fragment described in

Table 2.8Recombinant DNA techniquesa) DNA polymerase I fill-in reaction solutions volumes (μl)

	<u>Vector</u>	<u>Fragment A</u>
DNA	33μl	10μl
[ <sup>3</sup> H] dTTP <sup>1</sup> (1μCi/μl)	20	10
1mM dATP	2.8	2.5
1mM dTTP	2.8	2.5
10xHindIII salt	6.0	5
d. water	-	20
DNA polymerase I	5.0 (2.5 units)	5.0 (2.5 units)

<sup>1</sup> [<sup>3</sup>H] dTTP supplied in ethanol; vacuum dried and resuspended in distilled water for use

10xHindIII salts:	60mM Tris-HCl pH7.5	} autoclaved
	600mM NaCl	
	150mM MgCl <sub>2</sub>	
	60mM mercaptoethanol	

Table 2.8 Continuedb) Homopolymer tailing solution volumes (μl)

	<u>Vector</u>	<u>Fragment A</u>
DNA	20μl	20μl
<sup>32</sup> P dCTP	0.6μl (5μCi)	-
<sup>3</sup> H dGTP	-	10μl (5μCi)
Cacodylate buffer <sup>1</sup>	5.0	5.0
CoCl <sub>2</sub> (added last) <sup>3</sup>	5.0	5.0
1mg/ml BSA <sup>2</sup>	1.0	1.0
1mM dCTP	2.0	-
1mM dGTP	-	2.0
d. water	15	5
Terminal transferase	1μl (10 units)	1μl (10 units)

<sup>1</sup> Cacodylate buffer:- 0.1M potassium cacodylate p pH7.6

250mM Tris base

<sup>2</sup> Bovine serum albumin

<sup>3</sup> 10mM CoCl<sub>2</sub>

c) Reannealing buffer for poly-dC/poly-dG ends

0.1M NaCl

10mM Tris-HCl pH7.5 autoclaved

0.2mM EDTA

Chapter 7) was 5 p.M. ends. The reaction was monitored by incorporation of [ $^3\text{H}$ ]-dTTP into TCA precipitable material. Input counts were measured by taking 2 $\mu\text{l}$  of the mix directly onto a Whatman GF/C filter. A second sample of 5 $\mu\text{l}$  was taken into 0.5ml (50 $\mu\text{g}/\text{ml}$ ) salmon sperm DNA in 0.2M sodium pyrophosphate pH7.0, and 150 $\mu\text{l}$  50% TCA, on ice. The reaction was started by adding the polymerase. After 60 min incubation at 10 $^{\circ}\text{C}$ , a further 5 $\mu\text{l}$  sample was precipitated with TCA. Precipitates were collected on GF/C filters after 15 min on ice, and washed with 50ml 5% TCA followed by 10ml ethanol. Radioactivity on the dried filters was then counted in non-aqueous scintillation fluid (2.6) in the Packard scintillation counter. From these values, the specific activity of dTTP (input cpm/p.M.) and the total amount of dTTP incorporated in the reaction were calculated. The results indicated that about 65% of vector DNA molecules and 80% of fragment A molecules had been "filled in", and thus were blunt-ended. Each DNA sample was then extracted with an equal volume of phenol mix (table 2.5) and the separated phenol was re-extracted with an equal volume of water. Residual phenol in the combined aqueous phases was then removed by 3 ether extractions, and DNA in the samples was precipitated by addition of 10 $\mu\text{l}$  10mg/ml tRNA, 1/10 volume 3M sodium acetate and 2 volumes ethanol, and incubation in a methanol-solid  $\text{CO}_2$  bath for 5 min. After centrifugation, vacuum dried pellets were resuspended in 10 $\mu\text{l}$  distilled water.

2.20b Homopolymer tailing. Homopolymer sequences were attached to the blunt-ended molecules obtained by "filling-in", by terminal deoxynucleotidyltransferase (terminal transferase). Reaction conditions are shown in table 2.8. This enzyme can use the terminal 3' residue of a blunt ended DNA molecule as an efficient substrate (Nelson and Brutlag, 1979).

In order to regenerate the EcoRI sites on reannealing, (Chapter 7) dCTP was used to 'tail' vector molecules and dGTP for fragment A. The reaction was started by addition of the enzyme and incubation was continued for 60 min at 37°. Reactions were monitored by incorporation of  $\alpha$  [ $^{32}\text{P}$ ] dCTP and [ $^3\text{H}$ ] dGTP into TCA precipitable material as described in a) above. [ $^{32}\text{P}$ ] was estimated as Cerenkov counts using Packard [ $^3\text{H}$ ] settings. From the specific activity of each isotope, it was calculated that on average, each DNA molecule end in the reaction mixtures was extended by 4-5 residues. The preparations were then phenol extracted and ethanol precipitated as described above, resuspending the pellets in reannealing buffer (table 2.8).

2.20c Reannealing. The vector preparation was divided into two parts and the fragment A preparation mixed with one part. The volume of each was made up to 200 $\mu$ l with reannealing buffer. Samples were incubated at 65° for 10 min, then at 44°, and after 2h, the water bath was switched off allowing the preparations to cool slowly to room temperature. These samples were used directly to transform PAM162 (2.14).

## 2.21 Sources of chemicals and reagents

See table 2.9.

Table 2.9Sources of chemicals and reagents

1. Media	Oxoid or Difco
Agar	Davis
2. Common chemicals and solvents	Fisons
3. Biochemicals including	Sigma
Trizma base	
Pronase and RNase	
Antibiotics	
4. Acrylamide	Kodak
X-ray film and photographic chemicals	
5. SDS and other reagents used in	Biorad
acrylamide gels	
6. Radioactive compounds	The Radiochemical Centre, Amersham
7. Aquasol	New England Nuclear
NCS solubiliser	Amersham/Searle
8. PPO	Fisons
POPOP	Nuclear Enterprises Ltd
9. Agarose	Marine Colloids Inc.
10. Photographic chemicals and film for	Ilford
UV photography	
11. Restriction enzymes	Biolabs or
Terminal transferase	BRL
DNA polymerase I	

2.22 Abbreviations

Ap            Ampicillin

DNase        Deoxyribonuclease

dNMP        Deoxynucleotide monophosphate

dNTP        "        triphosphate

EDTA        Ethane diamine tetracetic acid

Genetic nomenclature : recA        =        gene

              recA        =        gene product

              RecA        =        phenotype

Kb           Kilobases

MOI        Multiplicity of infection

M.W.       Molecular weight (daltons)  
              (e.g. M.W. of 30K = 30,000 daltons)

PAGE       Polyacrylamide gel electrophoresis

Rif        Rifampicin

RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
Tc	Tetracycline
UV	Ultraviolet radiation,

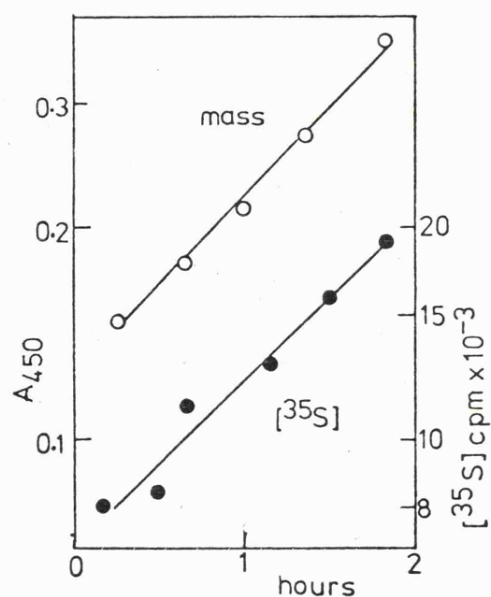


Figure 3.1 Incorporation of [<sup>35</sup>S]-methionine after 10 J.m<sup>-2</sup> UV  
 [<sup>35</sup>S]-methionine incorporated into whole cell lysates was determined by solubilising washed samples of labelled cells with SDS, then counting 5μl in Aquasol scintillation fluid.

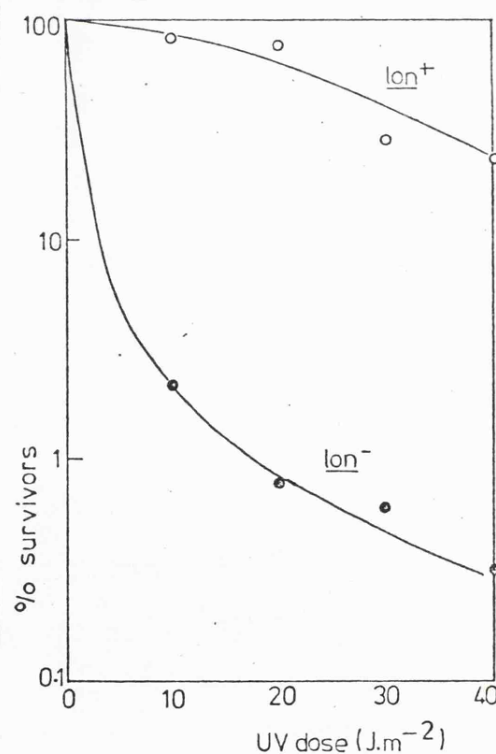


Figure 3.2 Survival of KN126lon<sup>+</sup> and lon<sup>-</sup> after UV

Cultures were grown in nutrient broth, diluted in buffer to A<sub>450</sub>=0.1 for irradiation, then diluted and plated onto nutrient agar immediately.

### Chapter 3

#### THE PHYSIOLOGICAL EFFECTS OF UV IRRADIATION

##### 3.1 Introduction

The first aim of this work was to investigate the relationship between UV induced *recA* synthesis and inhibition of cell division, to test the hypothesis put forward by several workers (see Chapter 1) that *recA* acts directly to inhibit division. Experiments designed to test this hypothesis are described in this chapter. In addition, similar experiments were performed on a *lon* mutant to ascertain whether the missing or mutant *lon* function had any effect on the induction process.

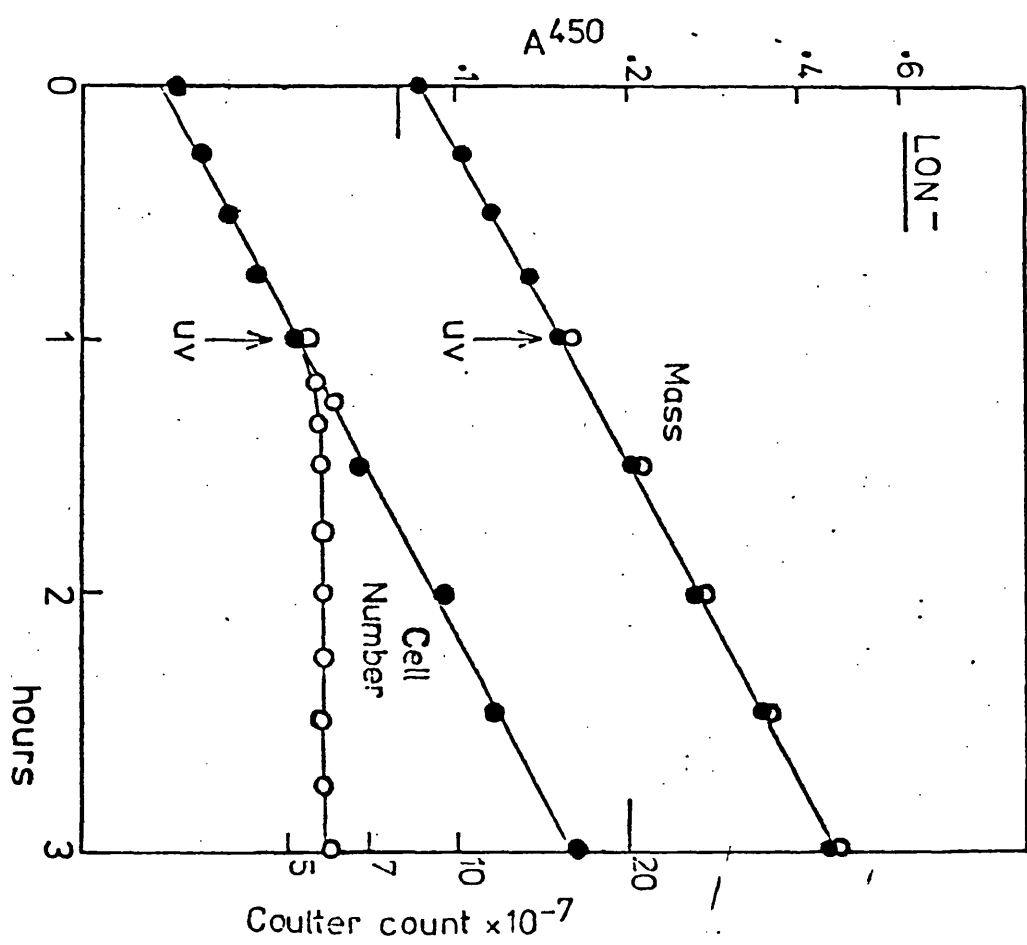
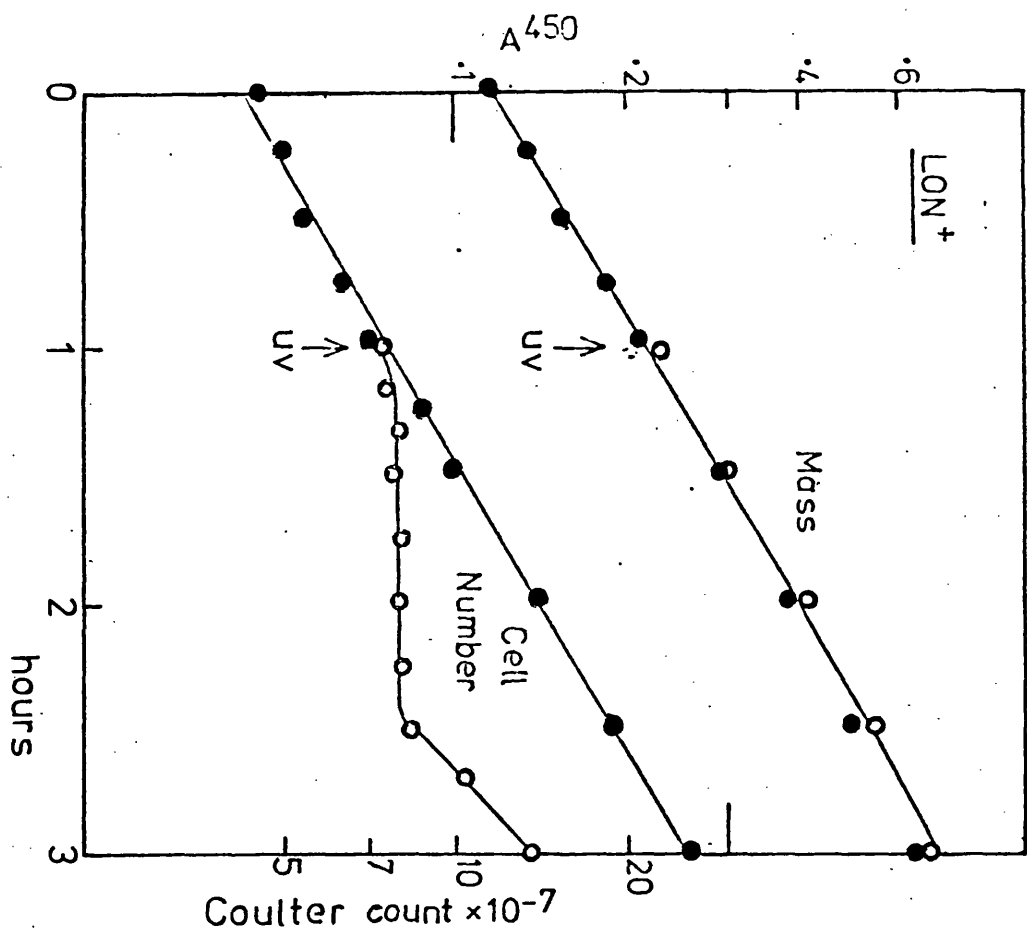
##### 3.2 Cell division after UV in *lon*<sup>+</sup> and *lon*<sup>-</sup> strains

Division was measured directly as change in cell number by counting instantaneous cell numbers in the Coulter counter. Survival (i.e. colony formation on agar) was measured separately. The UV dose used ( $10\text{Jm}^{-2}$ ) was relatively mild since mass increase, measured either as optical density or incorporation of [<sup>35</sup>S] methionine, was undisturbed (fig.3.1), and more than 90% of *lon*<sup>+</sup> bacteria survived to form colonies at this dose (fig.3.2) when plated on nutrient agar immediately after irradiation. Fig.3.3 shows that after irradiation, division ceased in both *E.coli* KN126 *lon*<sup>+</sup> and *lon*<sup>-</sup> cultures, within about 7 min of the treatment. After 90 min the *lon*<sup>+</sup> culture began to divide again but no recovery of division was seen in the *lon* mutant for at least 3 hours (i.e. 3 generations); microscopic examination of irradiated *lon* cultures 4-5 hours after treat-

Figure 3.3 Kinetics of cell division after UV

Portions of cultures of KN126lon<sup>+</sup> and lon<sup>-</sup> in minimal medium were irradiated (10 J.m<sup>-2</sup>) and sampled for Coulter counting as described in 2.3 and 2.4.

●——●     -UV  
○——○     +UV

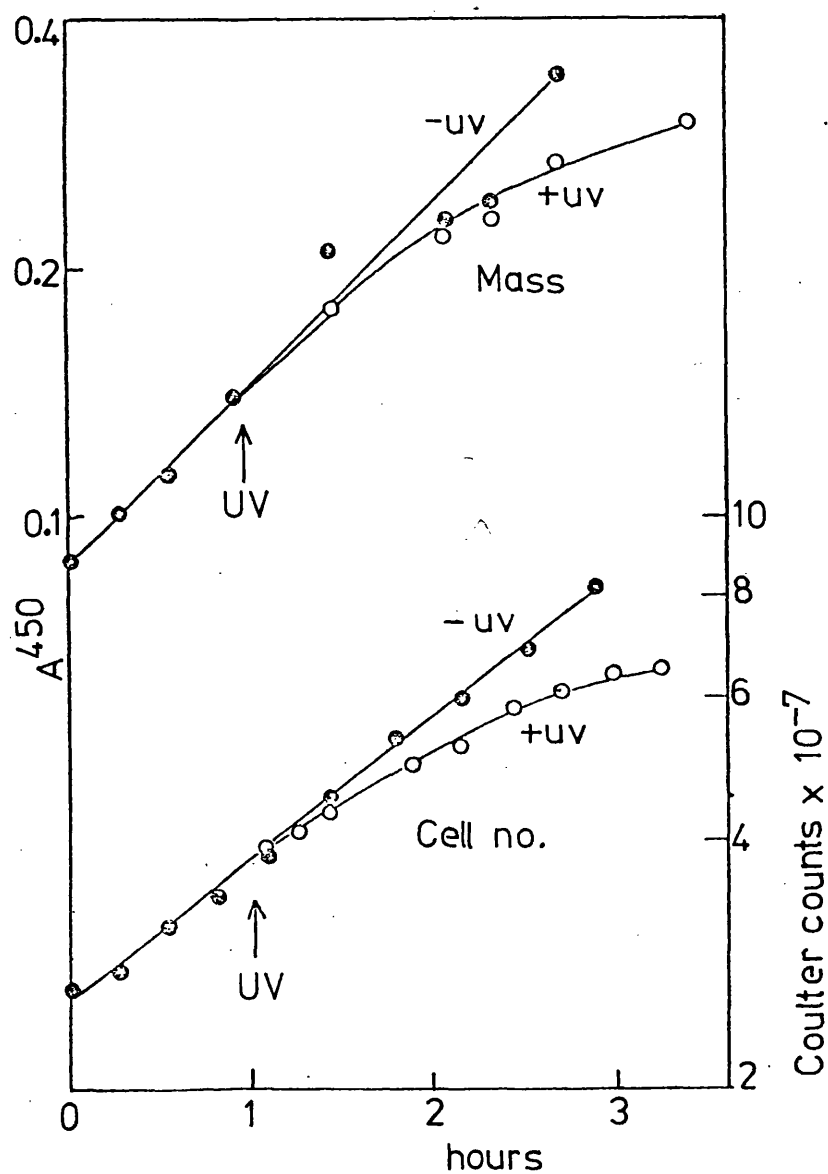


ment revealed long filaments 30-50 $\mu$  in length, and no normal cells whereas lon<sup>+</sup> bacteria were completely restored to normal length (2-2.5 $\mu$ ). In a control experiment to simulate the conditions during irradiation, bacteria were treated identically in a foil covered dish; no effect on division or mass increase was detected.

The timing of division recovery in lon<sup>+</sup> bacteria (at 90 min) was highly consistent in several independently derived wild-type strains under identical conditions. The fraction of cells which proceeded to divide after UV (12-15%) was also highly consistent in these strains. However, when thymine requiring derivatives of KN126 (lon<sup>+</sup> and lon<sup>-</sup>) were starved for thymine, a much larger division increment, of 25%, was obtained (data not shown). As discussed in Chapter 1, this increment theoretically measures the fraction of the bacterial population which are in the "D" period, i.e. which have completed chromosome replication prior to thymine starvation (Meacock, 1975) and thus cannot be prevented from dividing by the inability to terminate DNA replication. A value of 25% for cells in D is consistent with a C time of about 45 min when  $\tau^1$  is 65 min (Cooper and Helmstetter, 1968). This value for C was confirmed for KN126 when  $\Delta G^2$  was measured after rifampicin treatment (which prevents re-initiation of chromosome replication (Bremer and Churchward, 1977)). C was then determined by comparison of these data with those previously obtained by Pritchard and Zaritsky (1970), and found to be about 45 mins, indicating that the 25% increment is an accurate reflection of the proportion of cells in the D period. Since the number of cells dividing after UV is much smaller than the number dividing after thymine starvation, it is clear that some of the cells blocked by UV were in the

1  $\tau$  = doubling time

2  $\Delta G$  = residual DNA synthesis



**Figure 3.4** Kinetics of division after UV in KL16-99 (recA1)

A culture in minimal medium was irradiated ( $10 \text{ J.m}^{-2}$ ) and sampled for Coulter counting (see 2.3 and 2.4).

D period; the UV induced block to division was acting very late in the cell cycle and can operate independently of the requirement for termination of replication.

### 3.3 RecA is essential for division switch-off

The dependence of division inhibition after UV on normal recA function was confirmed when division after UV was determined in a recA1 mutant. Fig. 3.4 shows that mass increase was somewhat reduced after UV in this mutant, but division continued more or less undisturbed. George et al. (1975) reported that a lon recA double mutant also continued to divide after UV.

### 3.4 Effect of UV on DNA synthesis

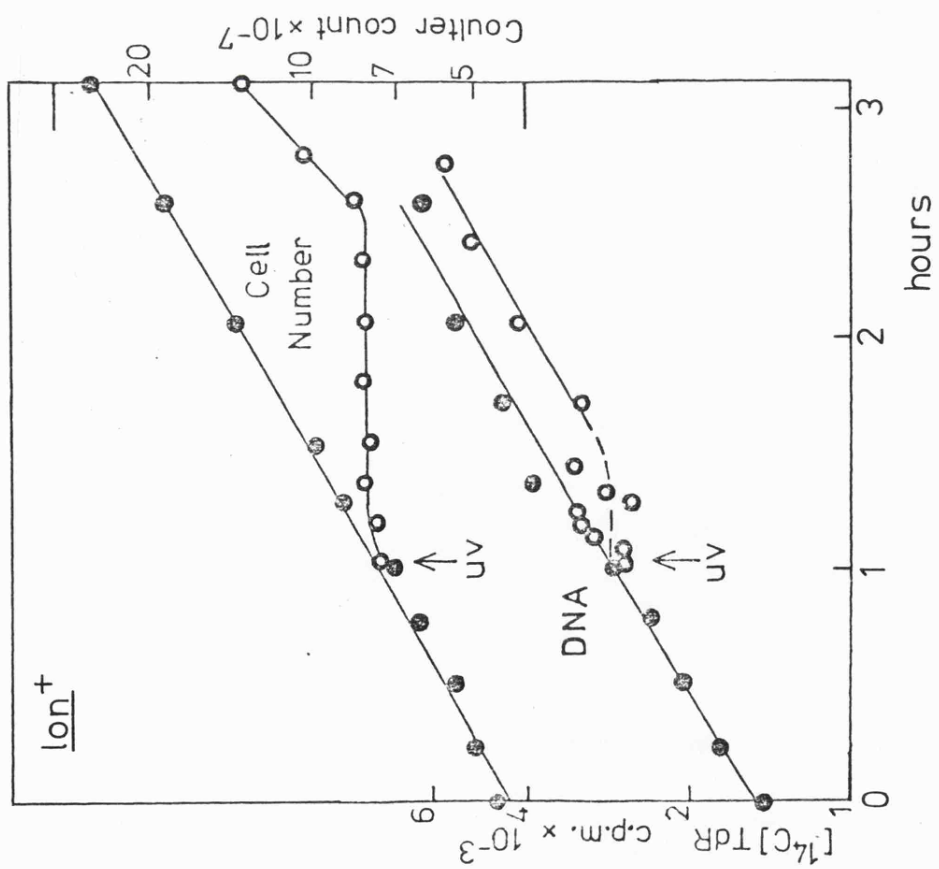
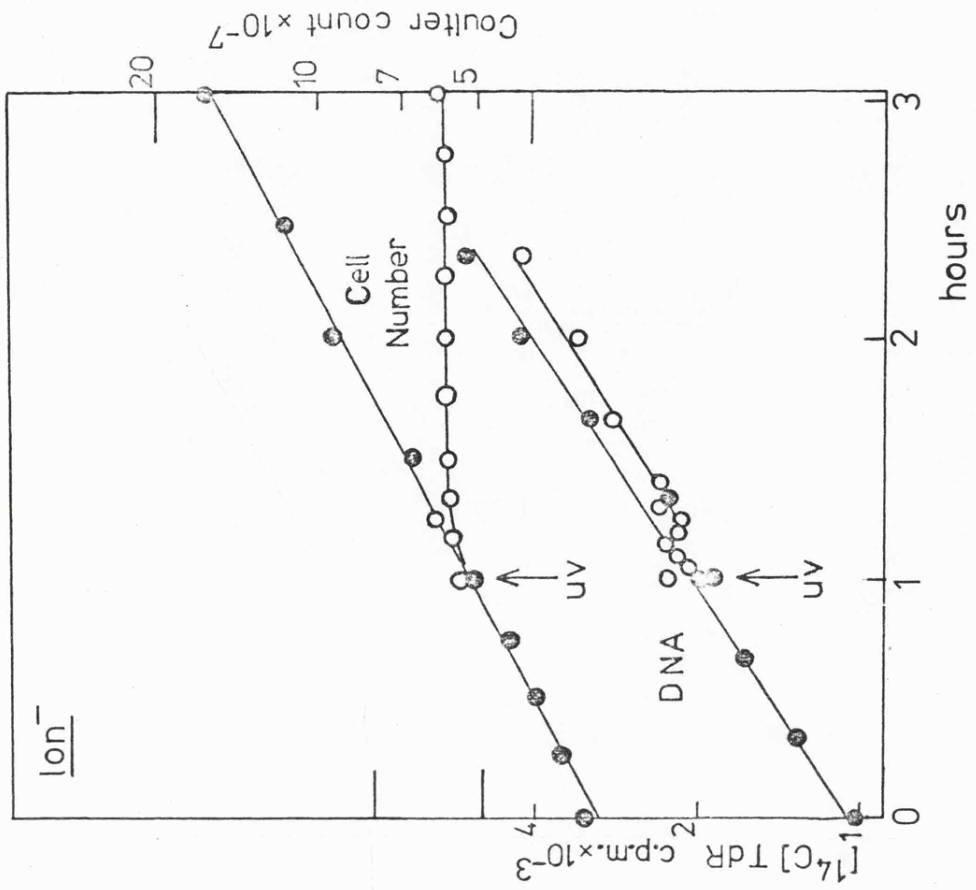
DNA synthesis was followed after irradiation by the incorporation of radioactive thymidine into strains KN126 (lon<sup>+</sup> and lon<sup>-</sup>), so that a comparison could be made between kinetics of DNA synthesis, division and recA induction. When accumulation of DNA was measured (fig. 3.5), incorporation of thymidine was inhibited for about 20 min after irradiation. During this period, incorporation was unusually irregular, presumably due to the combined effects of replication, repair synthesis and degradation of damaged DNA. Incorporation was restored to normal within 30-40 min and no differences were detected between lon<sup>+</sup> and lon<sup>-</sup> cultures. However, although the rate of accumulation returned to that found before irradiation, the DNA:mass ratio in both cultures was not fully restored in the period of the experiment. These data show no indication that the DNA deficit incurred (with respect to mass) during the

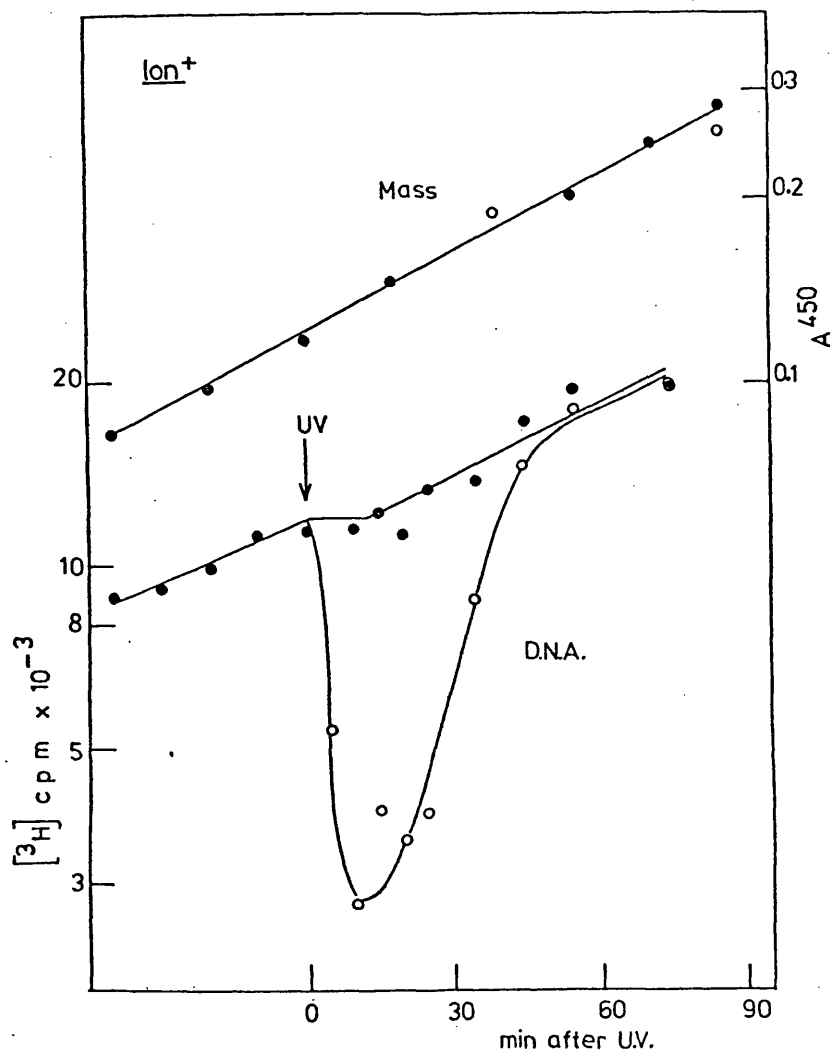
Figure 3.5 DNA synthesis after UV

Cultures of KN126 $\underline{\text{lon}}^+$  and  $\underline{\text{lon}}^-$  in minimal medium were continuously labelled with  $[^{14}\text{C}]$ -thymidine (see 2.6), and acid-precipitable counts and cell number (2.4) determined before and after irradiation ( $10 \text{ J.m}^{-2}$ ; see 2.3).

● — ● -UV

○ — ○ +UV





**Figure 3.6** Rate of DNA synthesis after UV

A culture of KN126 in minimal medium was pulse-labelled with  $[^3\text{H}]$ -thymidine and acid-precipitable counts were determined (2.6) before and after UV ( $10 \text{ J.m}^{-2}$ ; see 2.3).

● — ●    -UV  
○ — ○    +UV

20 min inhibition, is ever replaced. Interpretation of this observation is difficult since the DNA content of the culture, measured by thymidine incorporation, was not constant during inhibition but subject to breakdown and repair. It should also be noted here, that from the data in fig.3.3, it appears that the divisions omitted after UV were compensated only very slowly when division resumed, whereas the average DNA content per cell had almost doubled by this time, though DNA per unit mass was still lower than before UV. Further experiments would be required to clarify these confusing observations : it is clear that in terms of balanced physiology, the recovery process is extremely complex and may also be very slow.

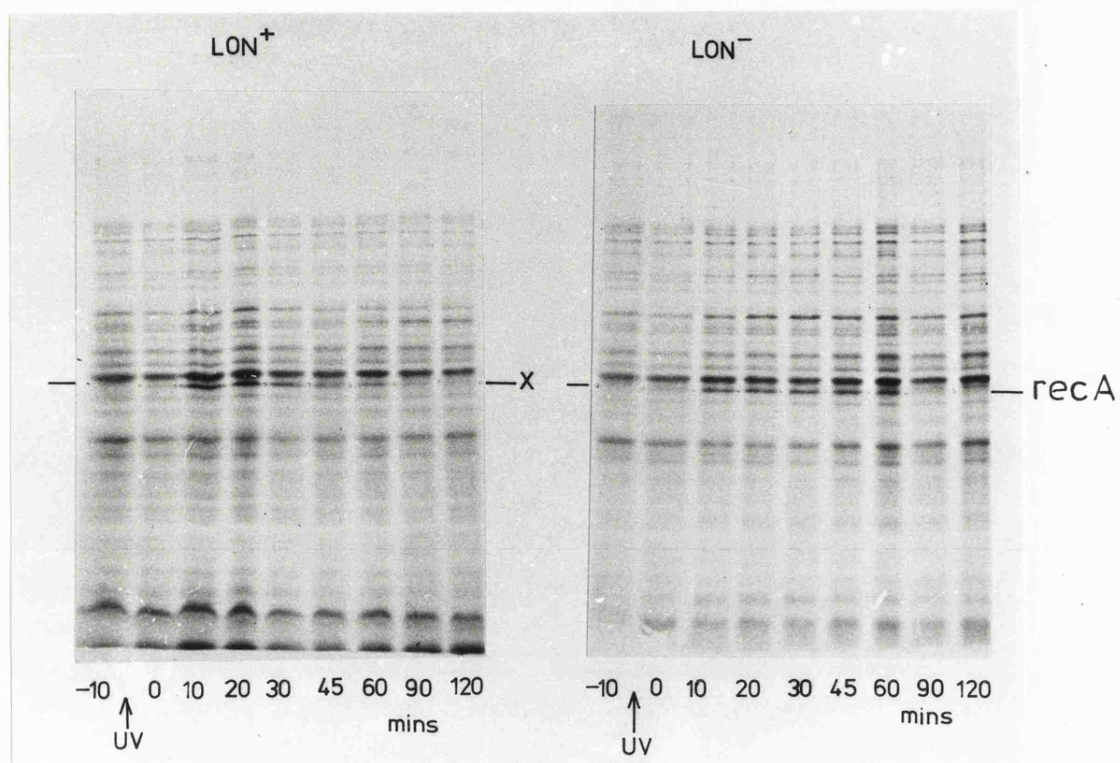
The rate of DNA synthesis after UV was measured by pulse-labelling with radioactive thymidine. After irradiation the rate of incorporation fell abruptly to 20-30% of normal in 5 mins, then gradually returned to the pre-treatment rate within 45 min (fig.3.6). Data for lon<sup>+</sup> and lon<sup>-</sup> cultures were essentially similar : the small variations found in individual experiments probably do not reflect real differences.

### 3.5 Induction of recA protein

Analysis of [<sup>35</sup>S] methionine labelled proteins in whole cell lysates by SDS-PAGE yielded autoradiographs (fig.3.7) which clearly showed induction of a polypeptide of M.W.40,000, corresponding with that of purified recA protein (Emmerson and West, 1977; Gudas and Mount, 1977; McEntee, 1977). Samples were pulse-labelled with [<sup>35</sup>S] methionine at intervals after UV treatment, and fig.3.7a shows that incorporation into the recA band was elevated for about 60 min, returning to the initial level at the end of this time.

Figure 3.7 Induction of recA protein after UV

Minimal medium cultures of KN126lon<sup>+</sup> and lon<sup>-</sup> were pulse-labelled with [<sup>35</sup>S]-methionine (2.6) before and after UV (10 J.m<sup>-2</sup>). To each sample, cells uniformly labelled with [<sup>3</sup>H]-leucine were added as an internal standard (2.6b). Whole cell lysates were prepared (2.7a) and analysed on 11% acrylamide gels (2.8). Autoradiographs (2.8b) of the gels are shown in Fig. 3.7a. The recA bands were then cut from the gels and the radioactivity of [<sup>35</sup>S] and [<sup>3</sup>H] in each was determined (2.8c). The relative rate of recA synthesis was calculated (2.8c) and is shown in Fig. 3.7b.



3.7a

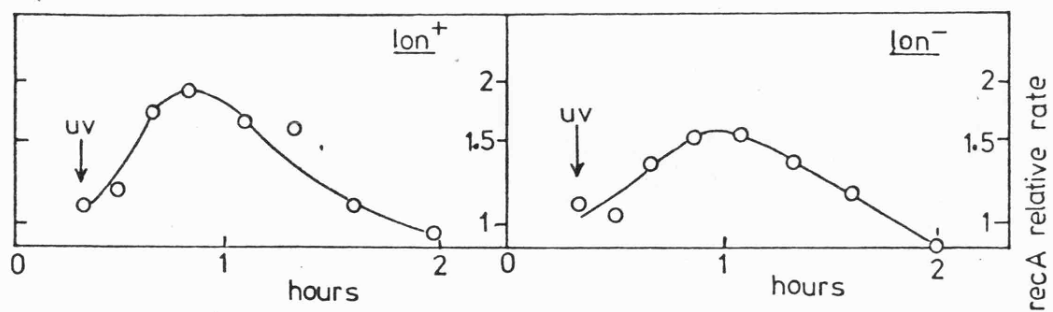
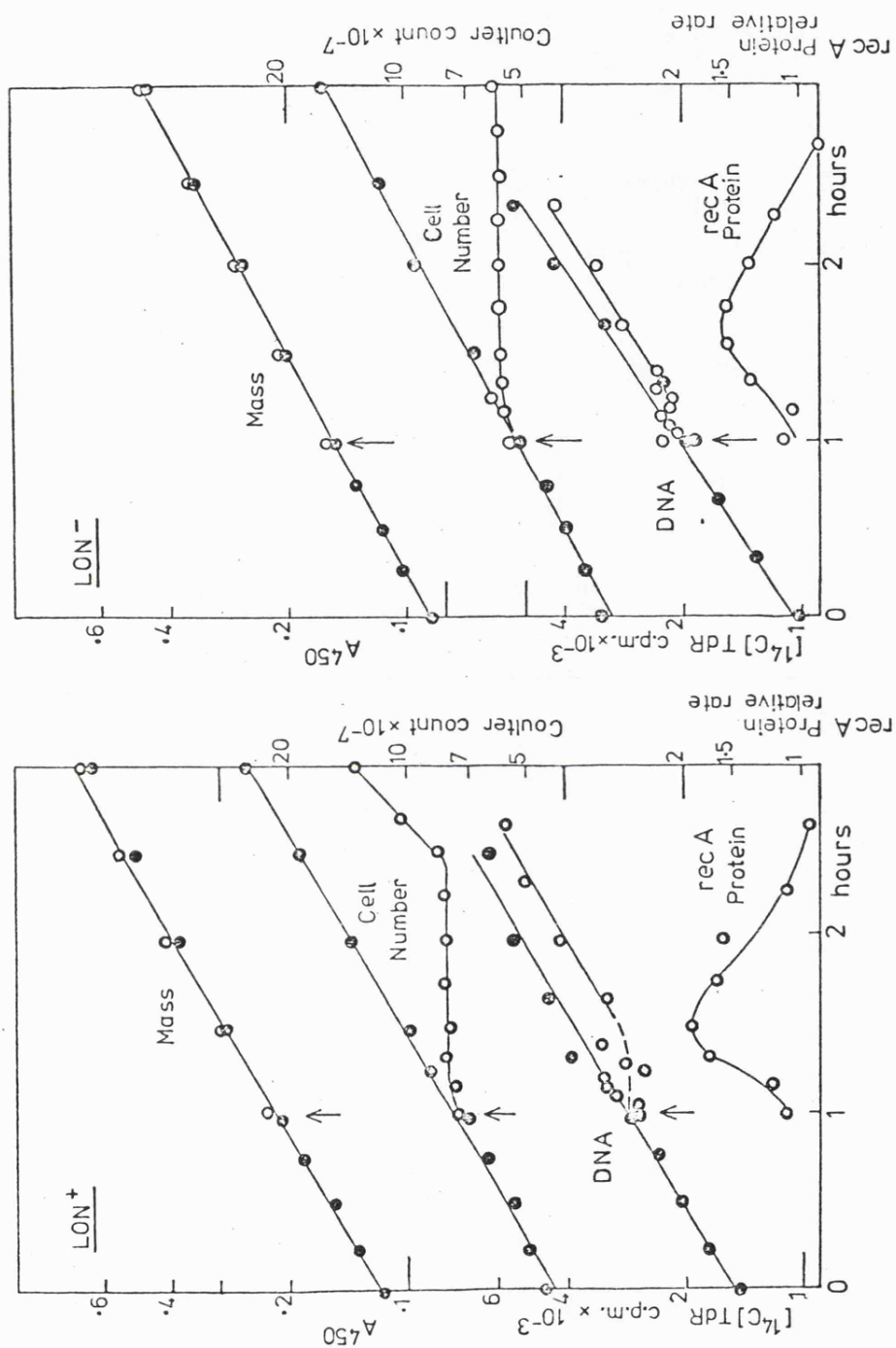


Fig 3.7b



**Figure 3.8** Composite of Figures 3.7, 3.5 and 3.3

To quantify this induction, cells uniformly labelled with [ $^3\text{H}$ ]-leucine were mixed with the pulse-labelled samples to provide an internal standard (see Chapter 2 for details) and lysates analysed by SDS-PAGE. The *recA* band was then cut from the gel after identification by comparison with the autoradiograph, and the ratio of [ $^3\text{H}$ ] to [ $^{35}\text{S}$ ] cpm in each sample was determined. A comparison of this ratio for *recA* and other, non-induced proteins then gave values for the relative rate of *recA* synthesis at each time. Fig.3.7b shows these data for  $\text{lon}^+$  and  $\text{lon}^-$  cultures of KN126 ; the relative rate of *recA* synthesis increased at least 2 fold and reached a maximum at 30 min. This was an underestimate since subsequent 2-dimension gel analysis showed at least 4 other proteins of MW 40,000 (fig.3.10), and from the autoradiographs, their combined rate of synthesis in pulse-labelling experiments was at least 5 times the basal rate of *recA* synthesis (i.e. before UV). When the total amount of *recA* protein induced was measured in the 2-dimension gels, (section 3.6) the relative amount of *recA* protein was found to increase at least 10 fold during induction.

Fig.3.7b shows that the induction kinetics were similar in  $\text{lon}^+$  and  $\text{lon}^-$  strains, and correspond well with data for other UV induced SOS functions, capacity for mutagenic repair, prophage  $\lambda$  induction and reactivation of irradiated  $\lambda$  (Defais *et al.*, 1976; Monk and Kinross, 1975), supporting the idea that these are coordinately controlled. However, the composite fig.3.8 clearly shows that induction kinetics of *recA* synthesis corresponded fairly well with inhibition of DNA synthesis, but not with division inhibition. Maximal division inhibition was reached more than 20 min before maximum *recA* synthesis, and continued after *recA* induction was

over. However, the 20 min delay in DNA synthesis, and presumably therefore, the DNA repair period, corresponded with the increasing phase of induction; when repair was complete *recA* synthesis no longer increased, and quickly returned to normal. These data do not rule out the possibility that division is inhibited by a high level of *recA* protein. However, this seems an unlikely mechanism since the rapid onset of division inhibition took place before much new *recA* protein had been made, and could not therefore have been triggered by high levels of *recA*. Further, this mechanism requires that *recA* is unstable or quickly degraded since 90 min growth would only dilute the new protein 2-3 fold.

### 3.6 Stability of the *recA* protein

The stability of the *recA* protein was estimated by analysis of whole lysate samples taken from a culture which had been pulse-labelled during the period of maximum *recA* protein synthesis and then chased. Autoradiographs of 1-dimension gels (fig. 3.9a) showed no decrease in intensity of the 40,000 MW polypeptide for at least 2 hours after labelling. This observation was confirmed in an experiment using [ $^3\text{H}$ ]-leucine labelled cells as an internal standard, when the relative amount of the 40K polypeptide was measured by comparison of [ $^{35}\text{S}$ ] : [ $^3\text{H}$ ] ratios in this gel band and that of  $\beta\beta'$  RNA polymerase (stable polypeptides). The results are shown in fig. 3.9b.

Further analysis by 2-dimension PAGE (fig. 3.10) also confirmed that *recA* was stable for at least 2 hours of chase. The stability of *recA* was similar in  $\text{lon}^+$  and  $\text{lon}^-$  cultures, and the isoelectric points of the 40K polypeptides synthesised in both cultures before and after UV were identical. This technique is capable of detecting single amino-acid

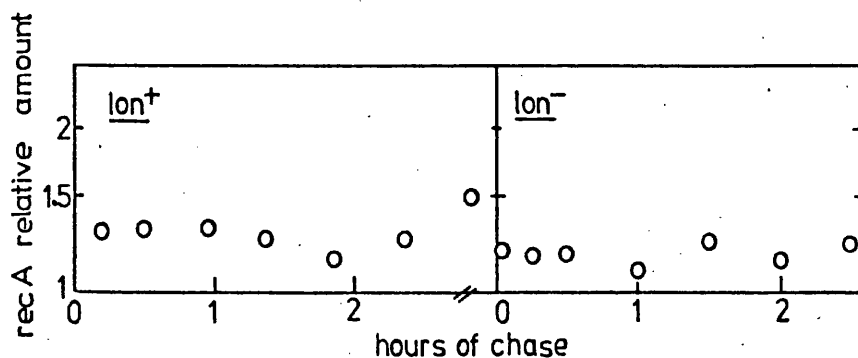


Figure 3.9b

Figure 3.9 Stability of recA protein

Cultures of KN126 $lon^+$  and  $lon^-$  in minimal medium were irradiated ( $10 \text{ J.m}^{-2}$ ; 2.3), pulse-labelled with  $[^{35}\text{S}]$ -methionine and chased (2.6c). Samples taken at intervals were analysed by SDS-PAGE (2.6c; 2.8). Autoradiographs of the gels are shown opposite in Fig. 3.9a. The recA bands were cut from the gel and the radioactivity in each was determined (2.8c), shown above in Fig. 3.9b.

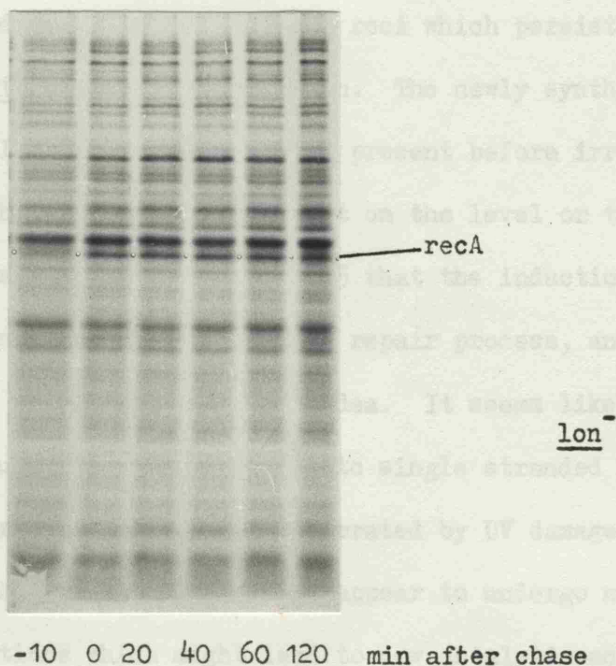
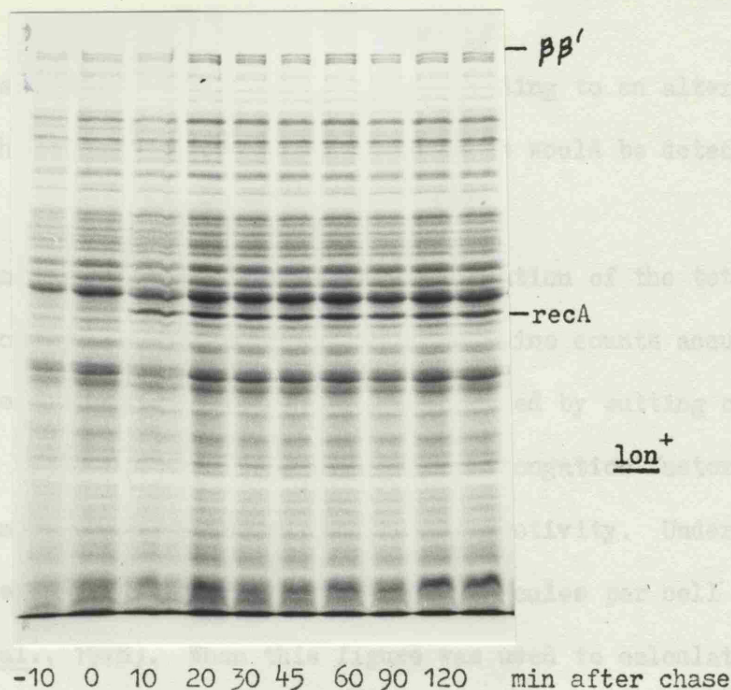


Figure 3.9a

changes or post-translational modifications leading to an alteration in net charge (though not all protein modifications would be detected in this system).

The 2-dimension analysis provided an estimation of the total amount of recA produced after induction.  $[^{35}\text{S}]$ -methionine counts accumulating during the whole period of induction were measured by cutting out the spots of recA and for comparison, the protein elongation factor EFTu from the 2-dimension gel and counting the radioactivity. Under the growth conditions used, EFTu was present at 58,000 molecules per cell (Pederson *et al.*, 1978). When this figure was used to calculate the value for recA, it was found that about 40,000 molecules of recA per cell were present after induction.

These experiments demonstrated that irradiated  $\text{lon}^+$  and  $\text{lon}^-$  cultures contained high levels of stable recA which persisted for at least one hour after recovery of division. The newly synthesised molecules were not detectably different from those present before irradiation, and the  $\text{lon}$  mutation had no detectable effect on the level or the stability of recA. It was suggested in section 3.5 that the induction of recA synthesis was closely related to the DNA repair process, and the results discussed in this section support this idea. It seems likely that newly synthesised molecules bind to single stranded DNA (as described by Weinstock *et al.*, 1979) generated by UV damage, in a stoichiometric fashion. These molecules appear to undergo no post-translational modifications which might lead to new catalytic activity associated with division inhibition. Even if the majority of newly synthesised recA is bound to DNA during repair, data in section 3.4

Figure 3.10 Stability of recA protein by 2-dimension gel analysis

Cultures of KN126 $\underline{\text{lon}}^+$  and  $\underline{\text{lon}}^-$  were grown in minimal medium and a portion of each was irradiated (2.3). After a further 20min, 10ml of culture was pulse-labelled with 50 $\mu$ Ci [ $^{35}\text{S}$ ]-methionine for 10min, and then chased (2.6c). Samples (1.5ml) were taken from the chased culture at intervals, cold carrier cells added (2.7a) and whole lysates prepared (2.7c). The lysates were analysed by 2-dimension gel electrophoresis, using SDS lysates (2.7a) as M.W. markers at the side of the second (SDS) separation; the gels were autoradiographed. Representative autoradiographs are shown (opposite and next page) for samples labelled before UV and immediately after the chase. Samples taken later after the chase gave identical autoradiographs.

When the total amount of recA protein was estimated, [ $^3\text{H}$ ] labelled cells were added to the samples before lysis as an internal standard.

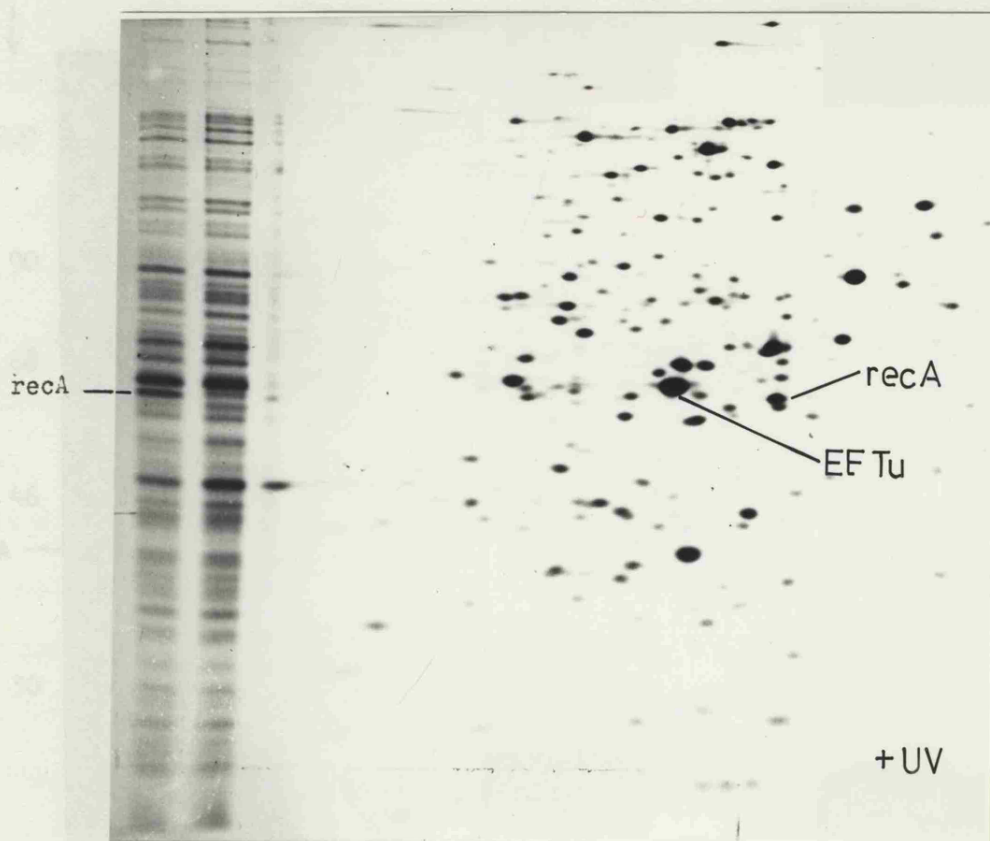


Figure 3.10 - KN126lon<sup>+</sup>

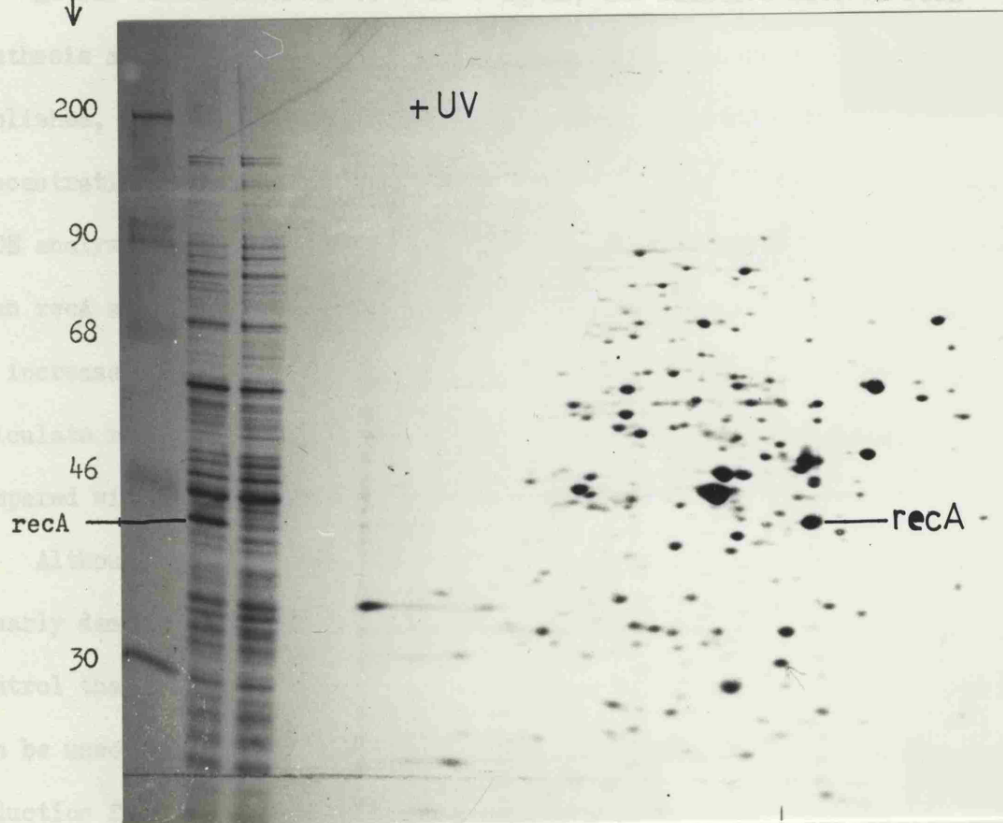
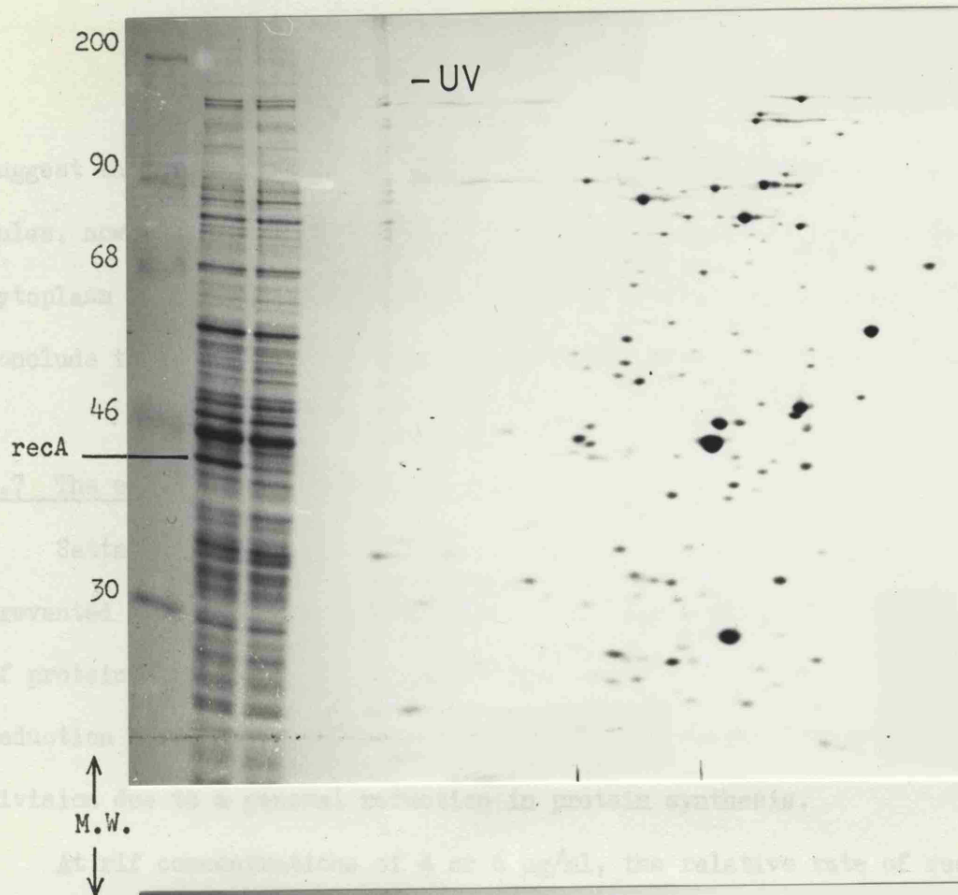


Figure 3.10 - KN126lon<sup>-</sup>

suggest that this process is completed in 30-40 mins and thus recA molecules, now released from the repaired DNA would again be free in the cytoplasm in high concentration at the time of division recovery. We conclude that newly synthesised recA probably does not inhibit division.

### 3.7 The effect of Rifampicin on recA induction and division

Satta and Pardee (1978) observed that low levels of Rifampicin (rif) prevented the synthesis of recA without gross effects on the overall level of protein synthesis. It was therefore possible to study the effect of a reduction in the level of recA after UV without direct disturbance to division due to a general reduction in protein synthesis.

At rif concentrations of 4 or 6 µg/ml, the relative rate of recA synthesis after UV was greatly reduced (fig.3.11), though not completely abolished, whereas division inhibition was not affected. At these rif concentrations protein synthesis was reduced by only 7-12% (fig.3.12) and PAGE analysis (fig.3.11) revealed no effects on individual proteins other than recA and the  $\beta\beta'$  subunits of RNA polymerase whose rate of synthesis is increased under these conditions (Blumenthal and Dennis, 1978). To calculate relative rates,  $[^{35}\text{S}] : [^3\text{H}]$  ratios for recA were therefore compared with those for EF<sub>Tu</sub> (M.W. 44,000).

Although recA synthesis was not entirely abolished, these experiments clearly demonstrate that the amount of recA present after UV does not control the division machinery in irradiated cells, and that recA synthesis can be uncoupled from the division block. Attempts to reduce recA induction further by increasing the concentration of rif, led to significant disturbance to overall protein synthesis and division.

Figure 3.11 Effect of rifampicin on division and recA induction

Part of a culture of KN126 in minimal medium was irradiated (2.3) and 4 $\mu$ g rif added immediately. Samples were removed for Coulter counting (2.4) and for pulse-labelling with [ $^{35}$ S]-methionine (2.6a).  $A_{450}$  was measured in the culture that was not treated with rif. Whole cell lysates were prepared from the [ $^{35}$ S] labelled samples and analysed by SDS-PAGE. The autoradiograph of the resulting gel is shown opposite. Rate of synthesis of recA protein was estimated as before by cutting out the gel bands, using EFTu (M.W. 44K) for comparison. A composite figure showing cell division kinetics and recA synthesis is shown overleaf.

Figure 3.12 Effect of rifampicin on incorporation of [ $^{35}$ S]-methionine

Samples of an exponential culture of KN126 in minimal medium were pulse-labelled (2.6a) with [ $^{35}$ S]-methionine and acid-precipitable counts determined.

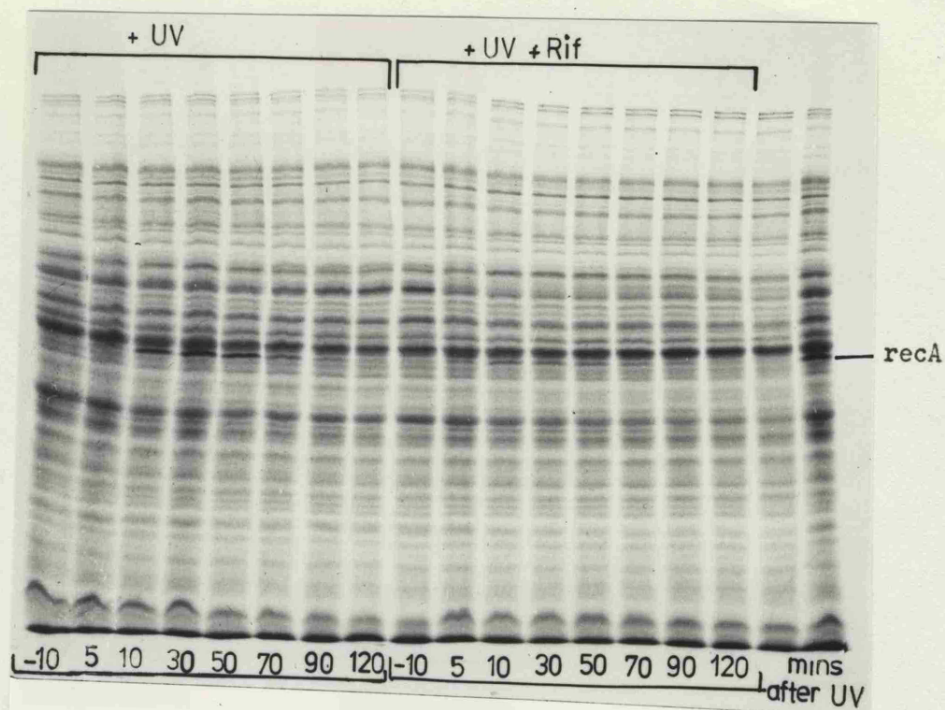


Figure 3.11

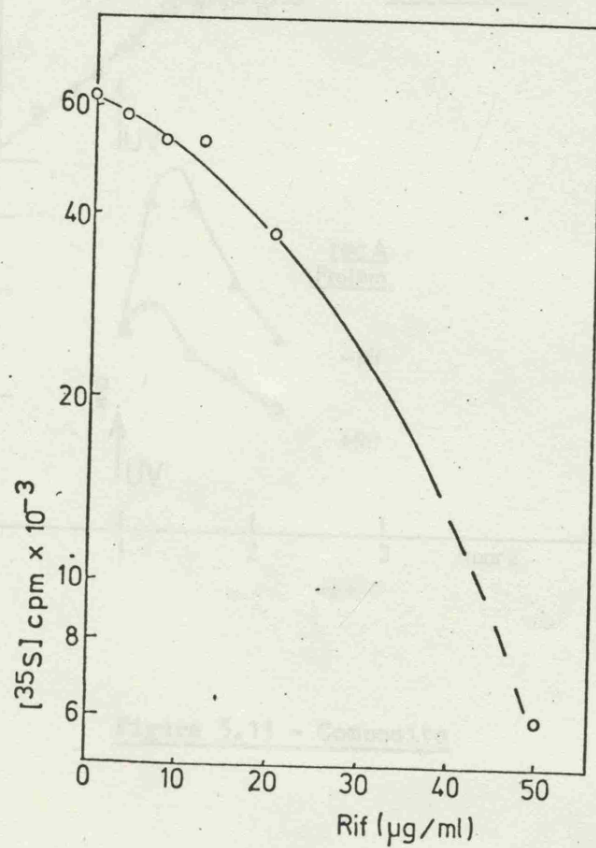


Figure 3.12

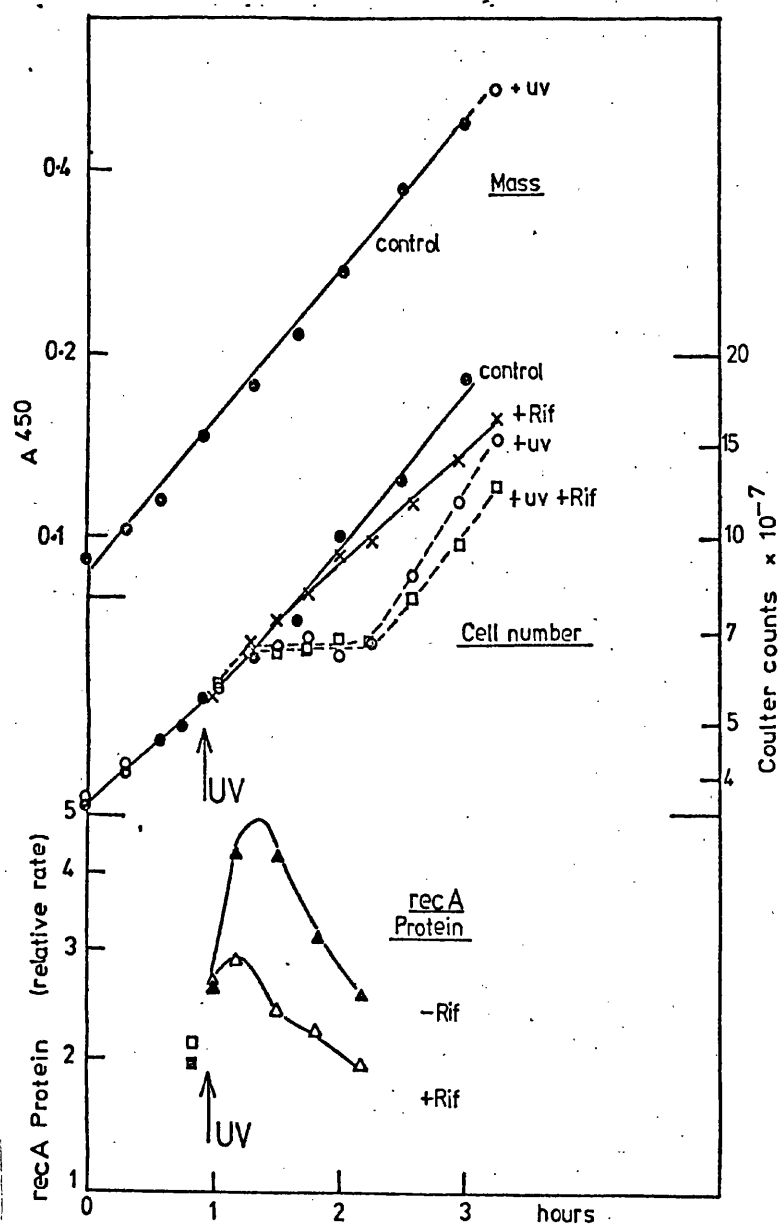
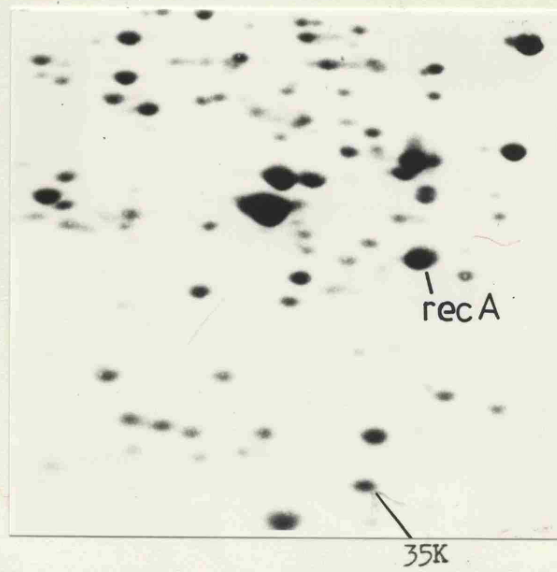


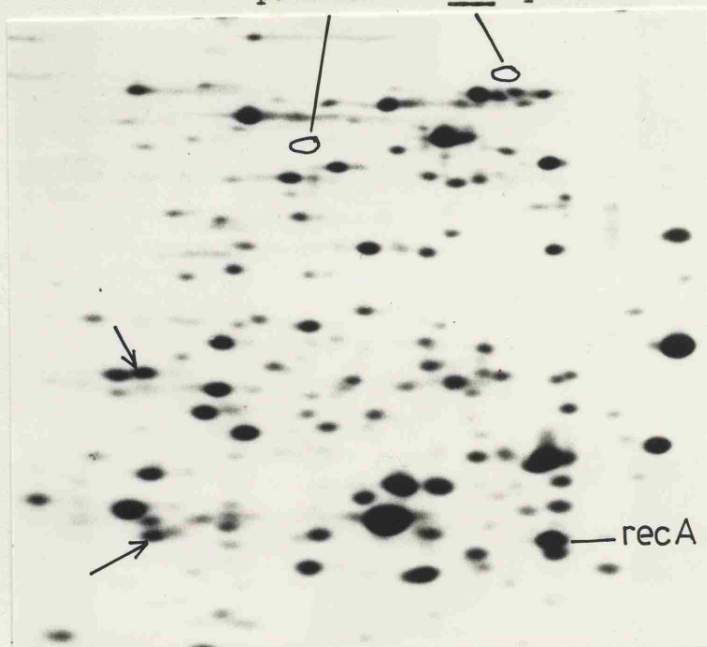
Figure 3.11 - Composite



lon<sup>-</sup>; +UV

positions of lon<sup>-</sup> proteins

arrows show  
proteins  
absent in  
lon<sup>-</sup> lysate



lon<sup>+</sup>; +UV

Figure 3.13 Enlarged areas of autoradiographs from Fig. 3.10

### 3.8 Other effects of UV, and the lon gene product.

Autoradiographs of the 2-dimension analyses described in the previous section (fig.3.10) were examined thoroughly for any other changes in whole cell lysates after UV. Though there was variation between experiments, pairs of gels run at the same time matched well and allowed comparison of many minor species before and after UV. A polypeptide of MW 33-35,000 appeared to be induced by UV in both lon<sup>+</sup> and lon<sup>-</sup> cultures pulse-labelled 30 min after irradiation, though less dramatically than recA. This protein was identified in at least 2 independent analyses for each strain, in gels where the resolution was good in the low MW region (fig.3.10). Other minor differences were found but these were not consistent over several experiments.

The lon gene product has recently been identified by Zehnbauer and Markovitz (1980) using a fragment of DNA from an F' carrying the lon region cloned into pSC101, which complemented UV sensitivity and mucoidy of a lon mutant. In this study a 94,000 polypeptide was identified (in maxicells) whose synthesis was greatly reduced by a recessive lon mutation in the recombinant plasmid. Furthermore this 94K protein was overproduced in minicells carrying a recombinant plasmid with a dominant lon mutation.

The 2-dimension autoradiographs described in section 3.7 were examined for a 94K polypeptide. Two possible candidates were observed in some autoradiographs (fig.3.13 for example), but these were present in lon<sup>-</sup> and not lon<sup>+</sup> lysates, whereas the data of Zehnbauer predicted that in the (recessive) mutant used in this work, synthesis of the lon polypeptide should be reduced. Two other polypeptides, about 57K and 45K, were absent from lon<sup>-</sup> compared with lon<sup>+</sup> lysates (fig.3.13). However, all of these

polypeptides were very minor species and therefore only identifiable in the best gels. In particular, the high molecular weight regions were often rather streaked and difficult to compare.

### 3.9 Effect of UV on synthesis of envelope proteins

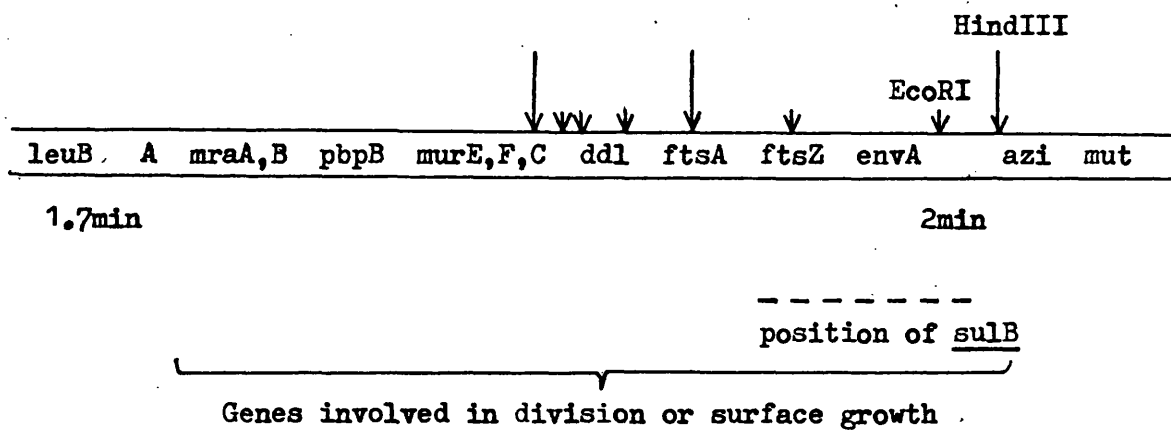
Earlier attempts to demonstrate the synthesis of specific polypeptides during septum formation in synchronous cultures have been unsuccessful (unpublished data from this laboratory; Lutkenhaus et al., 1979). To look more specifically at envelope proteins synthesised after UV, inner and outer membrane fractions were isolated from pulse-labelled irradiated cultures and analysed by 1-dimension PAGE. Autoradiography revealed (data not shown) that UV had no effect on the synthesis of any of the major envelope components. In contrast, other treatments which inhibited division resulted in gross disturbances to envelope protein synthesis (Herrero et al., in press). Thus the block to septum formation imposed by UV appears to be a specific process, perhaps acting through a septum protein which is a minor component of the cell envelope. This experiment also confirmed that *recA* protein was not detectable in either the inner or the outer membrane after UV, despite initial reports (Gudas and Pardee, 1975, Inouye and Pardee, 1970) that up to 10% of the induced *recA* protein was isolated with the envelope. These earlier findings were probably artefacts of the preparation procedures.

## Chapter 4

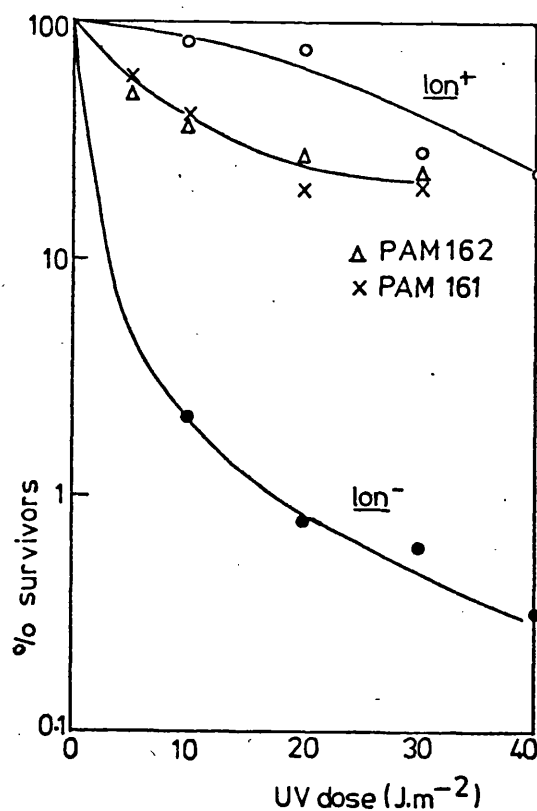
### THE ELUSIVE SULB GENE

#### 4.1 Introduction

Experiments described in Chapter 3 show that newly synthesised recA protein is not the inhibitor of division, yet the division block is recA dependent. These apparently conflicting observations may be reconciled by a consideration of the mechanism of UV induced derepression of recA synthesis (described in Chapter 1). A signal generated by DNA damage is thought to "activate" recA (probably by a conformational change) to recA\*, which then proteolytically cleaves the lexA repressor of the recA gene, allowing derepression of recA synthesis. In the case of UV induced division inhibition, it is presumably recA\* which (directly or indirectly) initiates the division block rather than the new recA molecules subsequently synthesised. The temperature sensitive tif (recA) mutant protein, at nonpermissive temperature, appears in some respects to be identical with recA\*, since division is permanently switched off due to the presence of the tif protein despite the apparent absence of DNA damage. In lon mutants after UV, division is also permanently inhibited, but this cannot be due to elevated levels or prolonged production of recA\* as this would lead to prolonged derepression of recA synthesis which is not the case. However, tif and lon mediated filamentation probably involve the same mechanism since extragenic suppressors of tif were found also to suppress lon, and vice versa (George et al., 1975). In addition, either type of suppressor was shown to prevent



**Figure 4.1** Part of the genetical and physical map of the 2min region of the *E. coli* chromosome



**Figure 4.2** Survival of *sulB* strains after UV

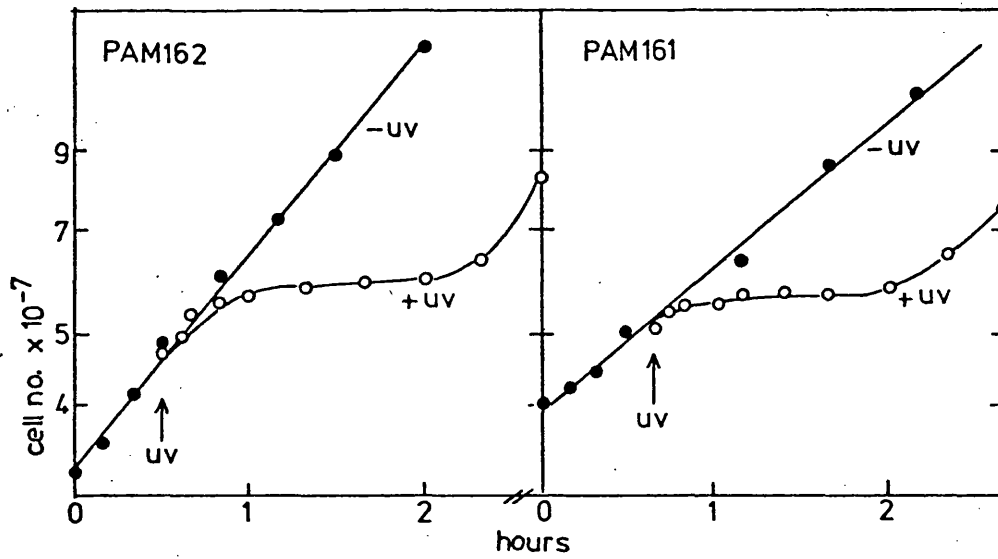
Exponential cultures at  $A_{450}=0.1$  were irradiated (2.3), diluted and plated immediately on nutrient agar.

filamentation in tif lon double mutants. Genetic analysis showed that these suppressors, known as sfi (tif) or sul (lon) could be divided into two distinct classes mapping at separate loci, sfiA/sulA at 22 min, and sfiB/sulB at 2 min on the E.coli chromosome (George et al., 1975; Gayda et al., 1976; Johnson, 1977); these loci are now widely assumed to represent single genes. Finally, sulB/sfiB maps in a cluster of genes involved in envelope synthesis and cell division (Bachmann and Low, 1980), shown in fig. 4.1, making this gene an obvious choice for further study.

How do these suppressors work? The lon phenotype (persistent filamentation after UV) might be reversed in two ways. First, division kinetics could be restored to those of lon<sup>+</sup> strains, that is a delay of 90 min (after a UV dose of 10J.m<sup>-2</sup>) followed by recovery. (Refer to fold-out appendix I). Alternatively, division might continue without delay after UV (as in recA mutants), that is, they would be insensitive to the division inhibitor or blocked in its production. These possibilities were investigated in experiments described below.

#### 4.2 The sulB mutation and models for sulB<sup>+</sup> action

Two lon sulB double mutants were obtained (Johnson, 1977) bearing different sulB alleles and their survival after UV (fig.4.2) and kinetics of division after UV were determined (fig.4.3). One of these, PAM161 (sulB25) responded exactly like a lon<sup>+</sup> sul<sup>+</sup> strain, with rapid onset of division inhibition, and an abrupt recovery at 90 min. The second mutant PAM162 (sulB26) showed intermediate kinetics, with a larger division increment (25% instead of 12-15%) and a more gradual resumption of division. This mutant may, for example, be only partially sensitive to



**Figure 4.3** Kinetics of division after UV in *lon sul* strains

Cultures of PAM161 (*lon sulB25*) and PAM162 (*lon sulB26*) were grown in minimal medium, and sampled for Coulter counting (2.4) before and after irradiation ( $10 \text{ J.m}^{-2}$ ; 2.3).



the inhibitor and thus behave as though the target for the division inhibitor is altered, or alternatively, less inhibitor is produced. We therefore considered that sulB could be a gene positively required for or negatively regulating division.

Interaction between sulB and an SOS induced factor involving recA\* and lexA proteins results in a block to septum formation. When DNA repair has been completed, the lon product is apparently required in sul<sup>+</sup> strains to reverse the effect of the SOS signal and to promote recovery of division; the mutation sulB25 overcomes the requirement for lon, and division can resume as soon as the SOS signal concentration falls, i.e. as repair is completed. Many models for these interactions may be devised, of which two examples are shown in fig. 4.4, and will now be discussed in detail.

A. Division negatively regulated by sulB. For example, lexA normally represses sulB synthesis. Formation of recA\* after UV treatment leads to cleavage of lexA and consequently to derepression of sulB synthesis and hence division inhibition. As DNA repair is completed, lex repression is re-established. Since the lon product is also essential for the recovery of division, it is therefore suggested that switch-off of sulB synthesis must be accompanied by degradation by the lon protease, of existing sulB molecules including those bound at the site of inhibition, before recovery of division can take place. A mutation in sulB might lead to an unstable form of the protein or molecules with reduced affinity for their "substrate", resulting in a low efficiency or even complete absence of division inhibition. Mutations preventing the binding of the lex repressor to the sulB operator would be lethal; other (cis-

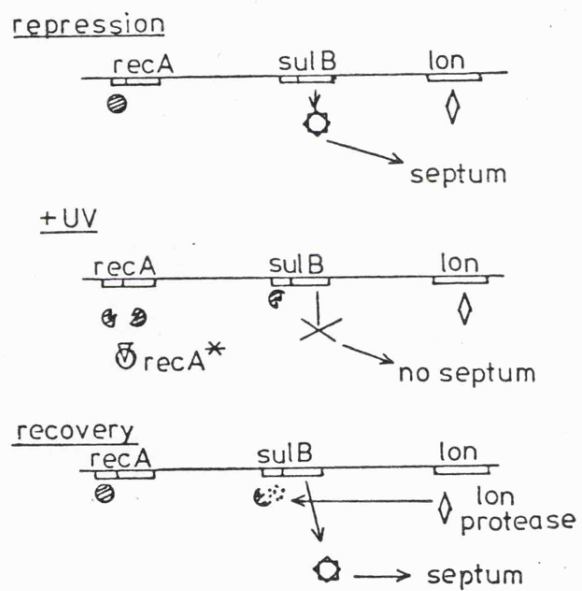


Figure 4.4 Model for *sulB* action

B. Positive control of division.

dominant) operator mutations could cause binding of *lex* so that the efficiency of cleavage by *recA\** was reduced, giving rise to the "mutant target" phenotype discussed above.

B. SulB positively required for division. In this model, *sulB* is envisaged as a septum component, and its synthesis is switched-off after UV, by direct or indirect action of the *recA\**-*lex* system. In this case *lon* may be required to reverse the SOS induced repression of the *sulB* gene, before division can recover. Alterations to the regulatory region of *sulB* rendering it less sensitive to the *recA\**-*lex* signal would also give rise to the "mutant target" phenotype.

C. A third hypothesis (not illustrated) assumes that the *sulB* gene product rather than the gene itself, is the division target, and is proteolytically cleaved after UV by *recA\**, leading to loss of an essential septum protein or alternatively, derepression of a division inhibitor.

These models were based on well established properties of *recA*, *lexA* and *lon*, and ascribe no new functions to any of them. Furthermore, known mutations in *recA* and *lexA* (*tif*, *tsl*, *spr*) have been considered in their design. The gene interactions predicted here are testable and therefore formed a useful basis for the planning of further experiments, beginning with the cloning of *sulB* and identification of its product. Several systems, both in vitro and semi- in vivo, are available in which gene products may be readily identified (discussed in Chapter 6) and it is reasonable to expect that certain interactions between gene products could be demonstrated in these systems. For example, proteolytic cleavage of the  $\lambda$ cI repressor by *recA* has been demonstrated both in vivo

and in vitro by Roberts et al. (1975, 1978). Similarly, control of transcription of one gene by the product of another should also be demonstrable. The possibilities of such an approach are discussed at length in Chapter 7.

In summary, a consideration of possible mechanisms of sulB action suggested that the identification of the gene product and a study of its synthesis and stability after UV would reveal essential information regarding its mode of action. If sulB was induced by UV, a negatively controlling protein (i.e. a division inhibitor) would be indicated; if synthesis was switched-off after UV, an essential septum component would be indicated. Post-translational cleavage of sulB in response to UV would be consistent with either possibility.

#### 4.3 Genetics of sulB and cloning strategy

As mentioned above, sulB mutations have been mapped to the envA-ftsA region near 2 min on the E.coli chromosome. Several workers have studied this region with respect to ftsA and envA (Wijsman and Koopman, 1976; Fletcher et al., 1978; Lutkenhaus et al., 1980); the order of the genes (excluding sulB) between leu and azi (fig.4.1) has been confirmed by complementation.

Studies by Johnson and in this laboratory have shown that sulB is distinct from both ftsA and envA, since sulB mutants, unlike ftsA mutants, continue to divide at 42° in minimal media, and unlike envA mutants, show no increase in sensitivity to a wide variety of detergents, dyes and antibiotics. In short, the effect of sulB mutations on filamentation in lon mutants after UV is the only detectable phenotype. In cloning experiments however, the use of UV as a selection procedure is un-

satisfactory for two reasons: 1) it is mutagenic, 2) at the physiologically low doses necessary to allow complete recovery of lon<sup>+</sup> cultures, significant fractions of lon<sup>-</sup> bacteria survive (2-10%). We therefore proposed to use the readily detectable outside markers ftsA and envA to identify the sulB - containing region of DNA. Regarding the source of DNA to be used for cloning, we considered that the number of genes implicated in division control and SOS functions is extremely large (probably 50-100) and several of these may affect the response of cells to UV (e.g. sulA). We therefore decided to use DNA specifically from the ftsA-envA region rather than genomic DNA, since the inadvertant isolation of suppressor gene(s) other than sulB could not be avoided in "shotgun" experiments. Lastly, we felt that multicopies of genes involved in regulating division might be lethal, and therefore decided to use bacteriophage  $\lambda$  as the initial vehicle.

As a preliminary to the cloning experiments, and to establish a method of screening clones for sul status, the dominance of sulB was investigated, expecting to find that if it were dominant, the UV resistance of lon sulB mutants would be lost on introduction of sul<sup>+</sup>.

Initially F' 104 (covering minutes 0-6 on the chromosome) was mated into PAM162 (arg leu pro thr his lon sulB) selecting leu<sup>+</sup> arg<sup>+</sup> transconjugants; these were obtained with a frequency of 0.1% of donors. These transconjugants were as sensitive as lon<sup>-</sup> sul<sup>+</sup> strains when irradiated on the surface of nutrient agar, and microscopic examination of 6 of these strains in liquid culture showed that they continued to filament for several hours after UV. The kinetics of division after UV of one transconjugant (which had also received pro<sup>+</sup> from the donor)

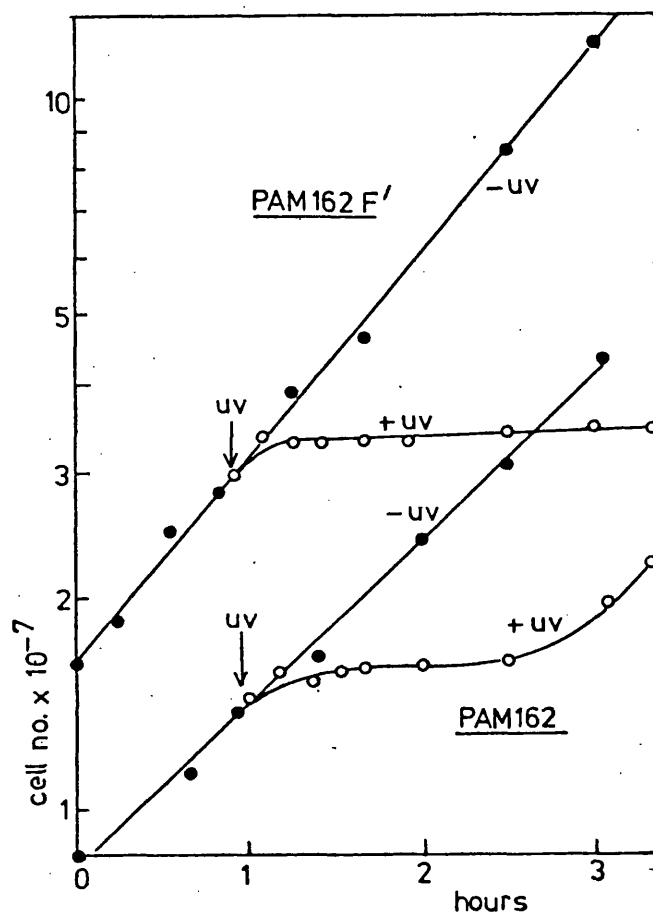


Figure 4.5 Kinetics of division after UV of PAM162F'

Cultures of PAM162, and the same strain carrying F'104 were grown in minimal medium and sampled for Coulter counting (2.6) before and after irradiation (2.3).

were determined (fig.4.5). The results confirmed that division was rapidly inhibited by UV and filamentation persisted for several hours, as in the original lon<sup>-</sup> sul<sup>+</sup> mutant. These results suggested that as expected, sul<sup>+</sup> on the F' was dominant. However, attempts to confirm partial diploidy by secondary transfer of met<sup>+</sup> from 5 of the transconjugants were unsuccessful, indicating that recombination between the chromosome and plasmid was taking place or that the donor was in fact an Hfr. In support of these explanations, only 50% of the transconjugants were also pro<sup>+</sup>, reflecting recombination with the loss of some F' DNA, or a gradient of transfer from an Hfr. It was not possible to transfer F'104 to a recA<sup>-</sup> recipient to test for dominance since the test for sulB involves UV irradiation.

To test whether the donor was in fact an Hfr, a mating was carried out with C600 (gal<sup>+</sup>) selecting for leu<sup>+</sup>; transconjugants were replica-plated on to eosin-methylene blue (EMB)-galactose agar and the results showed that none had received gal<sup>-</sup> from the donor. This marker is close to the end of F'104 but not actually carried by it, thus the result suggested that plasmid transfer had taken place rather than mobilisation from an Hfr donor. The fact that no secondary transfer was detected from these C600 (recA<sup>+</sup>) transconjugants either, showed that loss of transfer ability was not due to mutations specific to PAM162. On the other hand, we cannot explain the observation that the smaller F' KLF1, which also covers the thr leu region but does not extend to envA (our data), when transferred to PAM162 did show secondary transfer of thr<sup>+</sup> to C600. Although the initial experiments did show that the introduction of F'104 DNA into PAM162 resulted in the restoration of UV sensitivity to a lon<sup>-</sup>

sul<sup>-</sup> strain, the failure to demonstrate that the transconjugants were partial diploids prevented an unambiguous interpretation regarding the dominance of sul<sup>+</sup>. In view of the limitations imposed by recA on this approach to dominance tests, we decided to proceed with cloning experiments, hoping to obtain information on dominance by other methods. This question will be discussed with the relevant experiments in subsequent chapters.

#### 4.4 Attempts to isolate specialised transducing phage $\lambda$ envA<sup>+</sup>

Isolation of transducing phage  $\lambda$  is a well established technique and has recently been developed by Schrenk and Weisberg (1975) for the preparation of phage carrying DNA from many sites on the E.coli chromosome. Host strains deleted in the att $\lambda$  region can still be lysogenised, when the prophage may integrate at many secondary sites throughout the genome. Aberrant excisions (from these sites) then result in transducing phages carrying segments of DNA adjacent to the secondary sites. Phages carrying specific markers may then be identified by transduction and homogenous lysates prepared. Since sulB was mapped between leu and azi, it was hoped that transducing phage carrying the markers leu and envA should also contain sulB. Following the method of Schrenk and Weisberg,  $\lambda$  NM627 (ci<sup>857</sup> Sam7) was used to lysogenise strain 833  $\Delta$ (gal-att-bio) and a lysate was prepared which was found to transduce leuA or envA with low frequency (all methods are described in chapter 2). A second lysate was prepared from the secondary site lysogen BS254 in which  $\lambda$  ci<sup>857</sup> is integrated in leuB. Note that the gene order in this region is as follows:-

----- leuB-leuA ----- ftsA --- envA ---

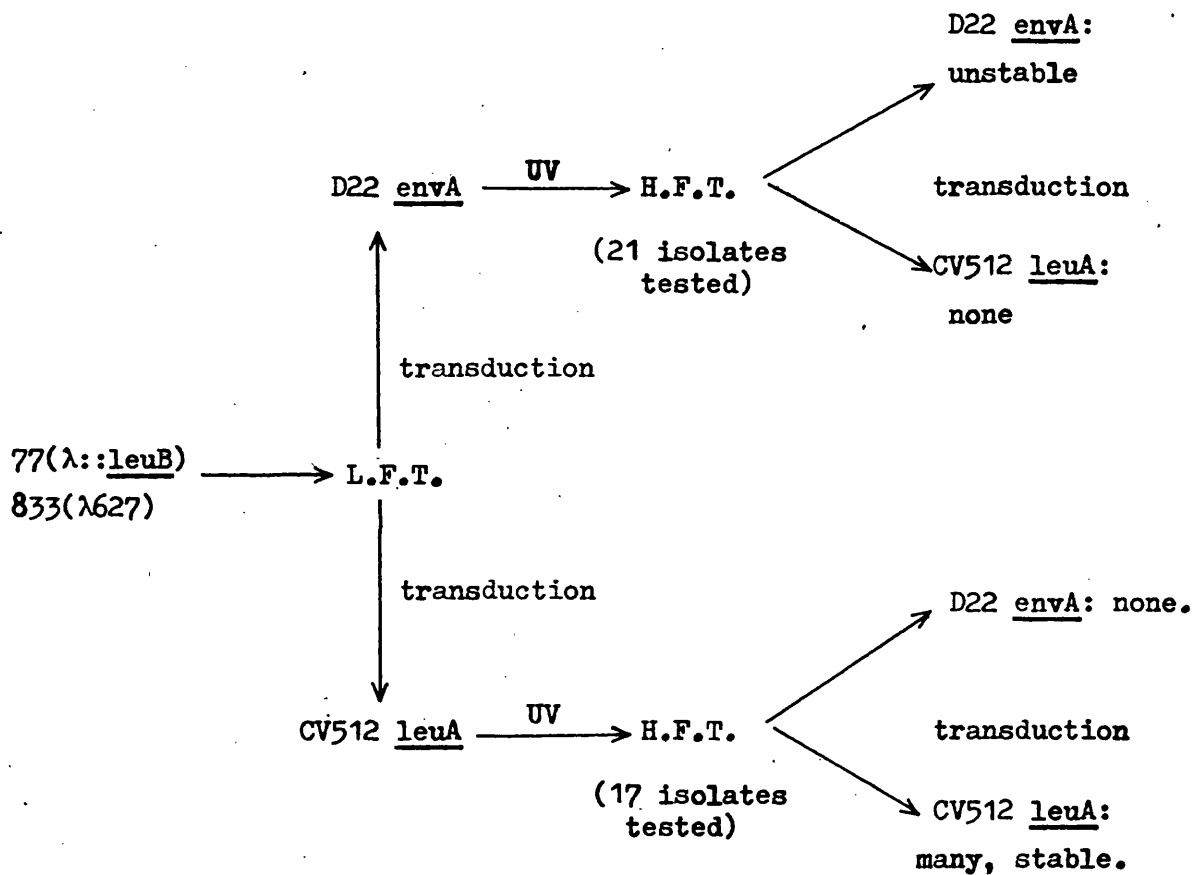
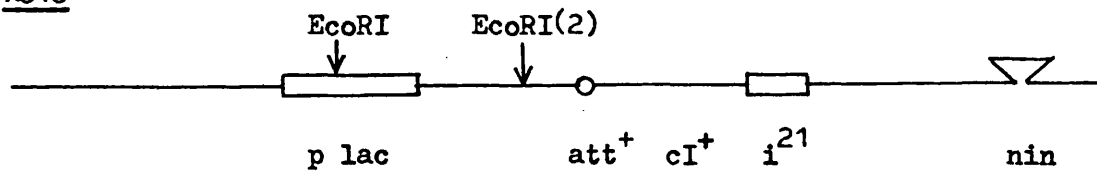


Figure 4.6    Transduction experiments performed in preparation of pure  
envA-transducing lysates

This lysate was found to transduce leuA and envA but both lysates showed a frequency of transduction of envA at least 10-fold lower than that of leuA, indicating that the site in leuB is probably the nearest  $\lambda$  secondary attachment site to envA.

In order to prepare pure envA transducing (HFT) lysates, E.coli D22 (envA) was transduced with both of the low frequency transducing (L F T) lysates and env<sup>+</sup> transductants collected. These were induced to give HFT lysates and tested for high frequency of transduction of leuA and envA. (These experiments are summarised in fig.4.6). All gave a high frequency of env<sup>+</sup> transductants when tested within 24 hours of preparation but failed to transduce either marker in subsequent tests. In contrast, HFT lysates prepared from leu<sup>+</sup> transductants of E.coli CV512 (leuA), using either LFT lysate, were quite stable but did not carry envA<sup>+</sup>. These results support the suggestion that the secondary attachment site in leuB is the nearest one to env, and that the phage carrying the whole of the leuA-envA region were unstable. Recent studies by Irwin et al. (1979) mapped leuA and envA 20Kb apart, and at least 8 other genes have been identified in the intervening DNA. Transducing phage carrying inserts of this size have been reported, but parent phage with large deletions must be used if the transducing DNA is to be packaged efficiently and stably. The phage used in this experiment was probably unable to package such a large insert. In addition both phage and hosts used here were recombination proficient, whereas a large insert of chromosomal DNA would have been more stably maintained in recombination deficient host and vector. However, use of a recA host was again precluded since the test for sulB involves irradiation.

λ616



(EcoRI sites 1,3,4 and 5 are abolished)

λGH200

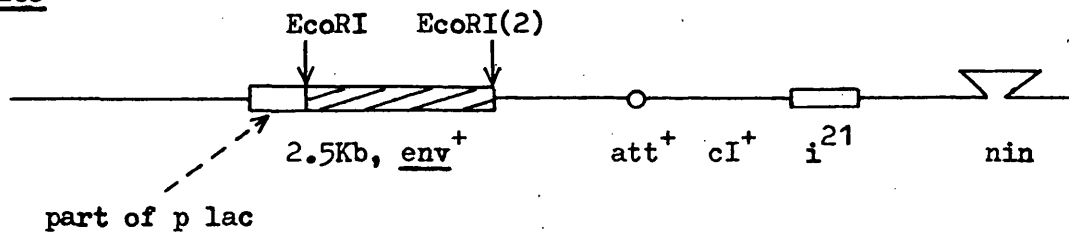
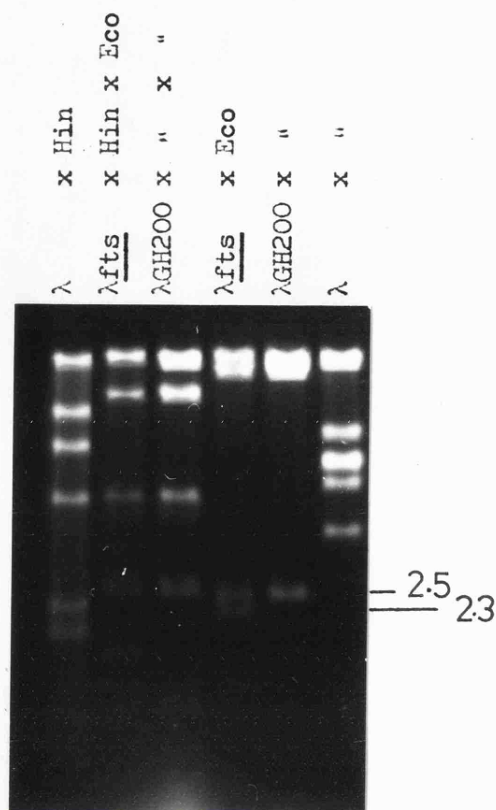


Figure 4.7    Structure of λ616 and λGH200

#### 4.5 Extension in vivo of $\lambda$ env<sup>+</sup> to $\lambda$ env<sup>+</sup> fts<sup>+</sup>

As an alternative source of "sulB<sup>+</sup> DNA",  $\lambda$  GH200 was obtained from G. Hatfull. This is an envA transducing phage constructed by cloning the 2.5Kb EcoRI fragment from transducing phage  $\lambda$  16-2 $\Delta$ E of Lutkenhaus et al. (1980) (carrying the envA region) into the replacement vector  $\lambda$  616. These phage genomes are shown diagrammatically in fig.4.7. The chromosomal DNA carried by  $\lambda$  GH200 does not extend to ftsA (confirmed by transduction experiments) and it was therefore anticipated that it might not contain the whole of the region provisionally identified as the sulB site. Consequently, the insert was extended to the ftsA gene using the procedure described by Lutkenhaus and Donachie (1979); E.coli 833 ( $\Delta$ att $\lambda$ ) was lysogenised and integration was assumed to take place by homologous recombination at envA. Subsequent excisions were expected to give rise to some phages which had packaged a larger insert containing ftsA. Such phages were obtained, and identified by transduction of E.coli TKF12 (ftsA) to fts<sup>+</sup> (i.e. able to grow at 42°), and were also shown by transduction to complement envA. This extended phage, called  $\lambda$  fts carries the region identified as the probable position of sulB.

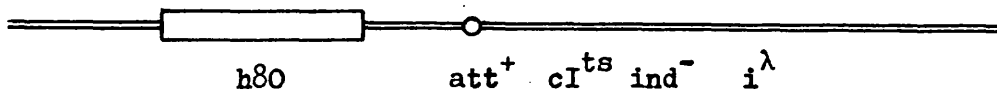
DNA was prepared from  $\lambda$  GH200 and  $\lambda$  fts as described in chapter 2, and digestion by restriction enzyme EcoRI followed by agarose gel electrophoresis, showed that  $\lambda$  GH200 contained a 2.5Kb insert (fig.4.8).  $\lambda$  fts contained fragments of 2.5Kb and 2.3Kb, consistent with the physical map of envA and ftsA - transducing DNA first suggested by Irwin et al. (1979) and later confirmed by Lutkenhaus et al. (1980). This map identifies envA on a 2.5Kb EcoRI fragment and ftsA on an adjacent 2.3Kb EcoRI fragment (fig.4.1).



**Figure 4.8** Restriction digests of  $\lambda$ GH200 and  $\lambda$ fts

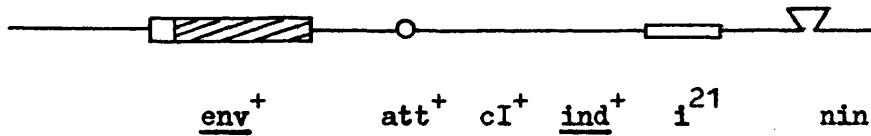
Phage DNA was prepared (2.9) and restricted (2.13) with EcoRI. Samples were loaded onto a gel of 0.7% agarose (2.11) and run for 2.5h at 100V. The gel was stained with ethidium bromide and photographed under UV (2.11). Samples of  $\lambda$ CI<sup>857</sup> DNA digested with EcoRI and HindIII were used as M.W. markers and the fragment sizes in the transducing phage DNA digests were calculated from these.

$\lambda$ h80cI<sup>ts</sup>ind<sup>-</sup>



X

$\lambda$ GH200



recombinant phage

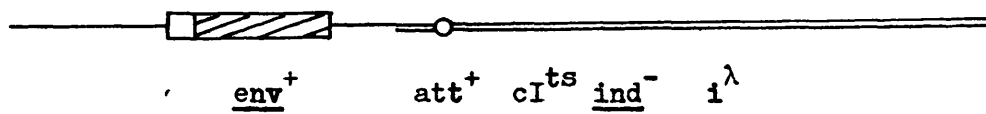


Figure 4.9     Construction of recombinant phage  $\lambda$ cI<sup>ts</sup>ind<sup>-</sup>env<sup>+</sup>

#### 4.6 Tests for sulB<sup>+</sup> on $\lambda$ GH200 and $\lambda$ fts

Although these phage strains were UV inducible, the level of UV sensitivity of various lysogens was estimated as an indication of the presence of sulB. Diluted overnight cultures were streaked on to nutrient agar and parts of the plate exposed to different UV doses. In these streak tests, PAM162 (lon sul) lysogenised by either  $\lambda$  GH200 or  $\lambda$  fts appeared more sensitive to UV for doses between 1 and 20 Jm<sup>-2</sup>, than the non-lysogen or a lysogen of the parent phage  $\lambda$  616. However, these tests gave somewhat variable results and were not considered to be a reliable method for the unambiguous detection of sulB.

Division kinetics after UV could not be studied in these lysogens since cell lysis due to phage induction was observed in less than 60 min after irradiation. In order to avoid this problem, a UV-non-inducible derivative of  $\lambda$  GH200 was constructed by crossing  $cI^{ts}$  ind<sup>-</sup> into it (see Chapter 2 section 18). When this experiment was performed, evidence had already been obtained from other experiments (Chapter 5) that the 2.5Kb EcoRI fragment contained the region of interest, hence the use of  $\lambda$  GH200 and not  $\lambda$  fts for this cross; recombinant phage were formed from the left arm of  $\lambda$  GH200 (carrying the cloned chromosomal DNA) and the right arm of  $\lambda$  cI<sup>ts</sup> ind<sup>-</sup> (shown diagrammatically in fig.4.9). When the progeny of this cross were screened at 40°, about 1 in 200 plaques formed on C600 had clear centres indicating the presence of  $\lambda$  cI<sup>ts</sup>. These phages were collected and purified by replating to single plaques on C600. Lysates of 34 recombinant phages were prepared and all were shown by transduction to carry env<sup>+</sup>. In order to test for the presence (and dominance) of sulB<sup>+</sup>, PAM162 (lon sulB26) was lysogenised with the

recombinant phage identified by formation of clear plaques at 40°. Unfortunately lon mutants are only poorly lysogenised by  $\lambda$  (Walker et al., 1973); consequently lysogens of only 9 of the recombinant phages were obtained, and these with a frequency of about 10% of that in a lon<sup>+</sup> host. A second possible reason for this poor lysogeny concerns the phage itself. The right arms of the two parent phage are unequal in size (fig.4.9), since  $\lambda$  GH200 has a 6% nin deletion, and its immunity region (imm<sup>21</sup>) is 5% smaller than that of imm $\lambda$ . Lack of homology between h<sup>80</sup> and the  $\lambda$  GH200 left arm, and selection for cI<sup>ts</sup> forced the recombination event to take place in the region between the left arm and the cI region of each phage, i.e. in the att region. Recombinant phages would therefore be 11% larger than the parent phage  $\lambda$  GH200, possibly leading to difficulties in phage packaging. Thus, phages may have been selected with new deletions in the region of recombination to allow packaging, and may therefore have lost integration function(s).

The PAM162 lysogens obtained were rechecked for induction by heat and not UV, and for env<sup>+</sup>. In two of these, cell division after UV was measured directly. Neither lysogen showed division kinetics substantially different from those of the non-lysogen, suggesting that sulB<sup>+</sup> was either absent from these phages, including the possibility that it had been lost during construction of the ind<sup>-</sup> derivatives, or it was recessive to sulB26 on the chromosome.

As mentioned above, an alternative approach was under way at the time of the phage cross experiment; the 2.5Kb EcoRI fragment had been recloned into a plasmid vector (described in the next chapter) and had already produced evidence that a gene carried by the fragment was involved

in UV-induced division inhibition. Therefore inspite of the failure of experiments described in this chapter to resolve the question of dominance, or even demonstrate clearly the presence of sulB in the cloned DNA, it was considered that further experiments using the recombinant plasmid were justified.

In addition, although most sulB alleles which have been studied by other workers were found to be recessive (Huisman et al., 1980b), at least one dominant allele has been reported (J.R. Walker, personal communication), though this was not the allele used here. It was therefore reasoned that since dominance of sulB26 was a significant possibility, further studies were justified.

Chapter 5CLONING OF  $\lambda$ fts DNA FRAGMENTS INTO A PLASMID VECTOR5.1 Introduction

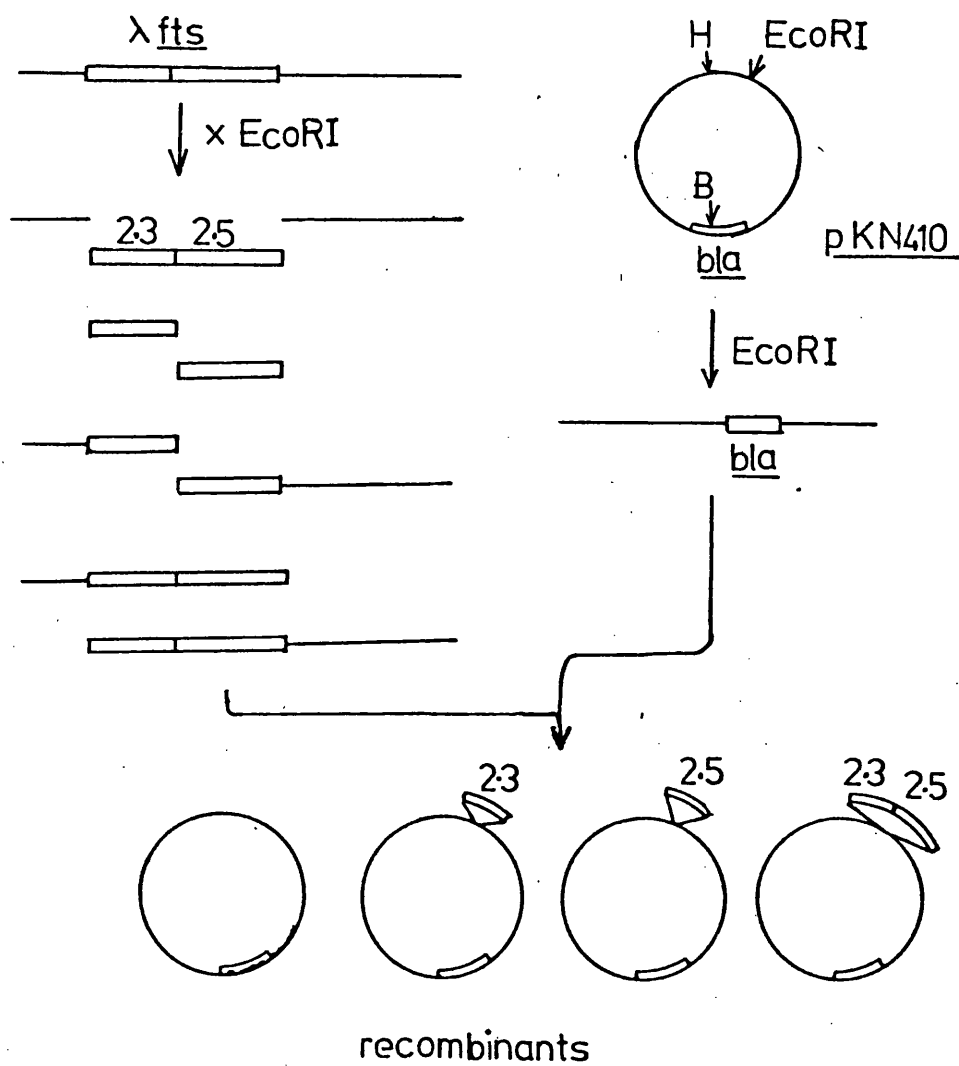
The requirement for sulB<sup>+</sup> transducing phages to be made ind<sup>-</sup> posed certain problems concerning the use of these phages. Although the ind<sup>-</sup> mutation was, with some difficulty, crossed into  $\lambda$ GH200, (Chapter 4.6), the actual site of the recombination event could not be determined without extensive mapping experiments, and thus the precise structure of the central region of the phage was uncertain. Interpretation of the restriction patterns of such phages might be ambiguous since the recombination event could have generated one or more new restriction sites. Similarly, in gene expression experiments (Chapter 6) assignment of gene products to phage or cloned DNA might be extremely difficult: not only might new fusion polypeptides be formed by the recombination event, but also it would be nearly impossible to construct the identical parent phage for comparison. Therefore, although the ind<sup>-</sup> phage was important for testing the presence of sulB in the cloned ftsA - envA region, it was clearly essential to develop concurrently an alternative approach, particularly for the identification of gene products coded by cloned DNA, and the investigation of their role (if any) in the UV response. To this end, EcoRI fragments from  $\lambda$ fts were recloned into a plasmid vector by in vitro techniques, so that precisely characterised DNA was available for further experiments.

## 5.2 Choice of a cloning vehicle

The recent isolation of plasmids containing mutations in copy number control or replication control genes theoretically permits simple experiments to be devised in which the copy number of (recombinant) plasmids can be increased, sometimes several hundred times. This amplification appears to take place without grossly affecting the host cell's physiology for at least 1-2 generations under some conditions. Thus the R1 derivatives isolated by K. Nordstrom's group (Uhlin et al., 1979) carry a temperature sensitive mutation (cop<sup>-</sup>) which leads to "runaway" DNA replication at nonpermissive temperature. The copy number of these plasmids was reported to increase from 5 at 30° to several hundreds after 2-3 generations at 40° while mass increase was not affected.

In this investigation, the ability to follow physiological parameters under different conditions is essential since we are concerned with the kinetics of division after UV. A copy mutant plasmid was therefore an ideal choice of vector since division kinetics after UV could be followed at different levels of plasmid amplification (i.e. at different temperatures), and thus the effect of increasing the level of gene products coded by the recombinant plasmid could be investigated under controlled conditions. Moreover, it was anticipated that this amplification might facilitate the identification of the products of cloned genes.

A preparation of pKN410 (Uhlin et al., 1979) DNA was kindly provided by Dr. B.G. Spratt. This plasmid is a 15Kb temperature sensitive copy mutant of R1d<sub>rd</sub>-19 carrying resistance to ampicillin (Ap) mediated by  $\beta$ -lactamase (bla<sup>+</sup>). The plasmid was reported to have several specific



**Figure 5.1** Cloning of  $\lambda$  *fts* fragments into pKN410

H = HindIII site; B = BamHI site

restriction sites into which DNA fragments could be cloned (fig.5.1), including an EcoRI site outside the bla gene allowing Ap resistance to be used in the selection of plasmid transformants.

### 5.3 Construction and identification of recombinant plasmids

To generate fragments of DNA for cloning,  $\lambda$  fts DNA was partially digested with EcoRI; serial dilutions of the enzyme were used and the products analysed by agarose gel electrophoresis to establish an optimum enzyme concentration. A partial digest was obtained, containing at least 9 different sized DNA fragments, including 2.3, 2.5 and 4.8 Kb (2.3 and 2.5 still joined - see fig.5.1), identical with the expected chromosomal fragments from the ftsA - envA insert. The plasmid vector was opened up with EcoRI, and the 2 digests mixed and ligated, as illustrated in fig.5.1. The mixture was then used to transform strains PAM162 (lon sulB) and D22 (envA) made competent by  $\text{Ca}^{++}$  treatment, and transformants were selected by plating on to nutrient agar containing 50 $\mu\text{g}/\text{ml}$  Ap. The plates were incubated at 30 $^{\circ}$ .

At least 4 different classes of bla<sup>+</sup> recombinant plasmids were expected, (illustrated in fig.5.1) containing (i) recircularised vector, (ii) vector + 2.5Kb fragment, (iii) vector + 2.3Kb fragment, and (iv) vector + 4.8Kb fragment. Larger combinations of plasmid and  $\lambda$  DNA were also possible.

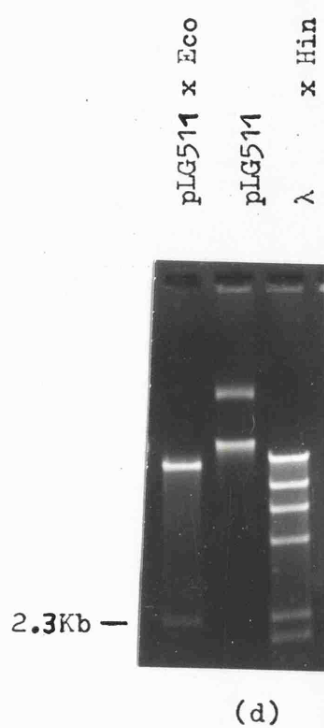
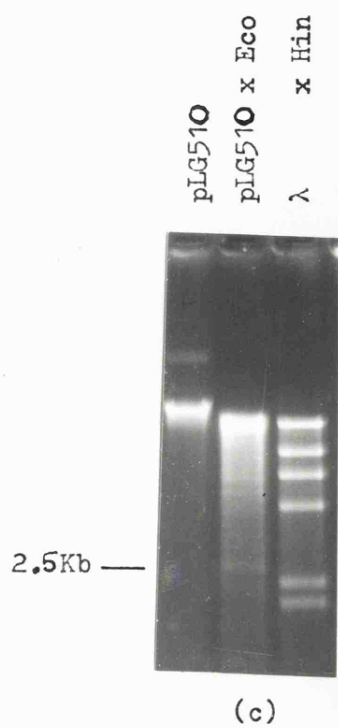
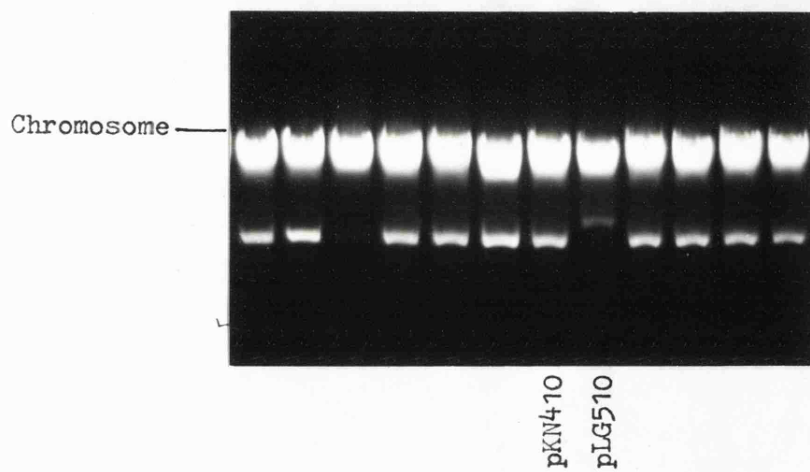
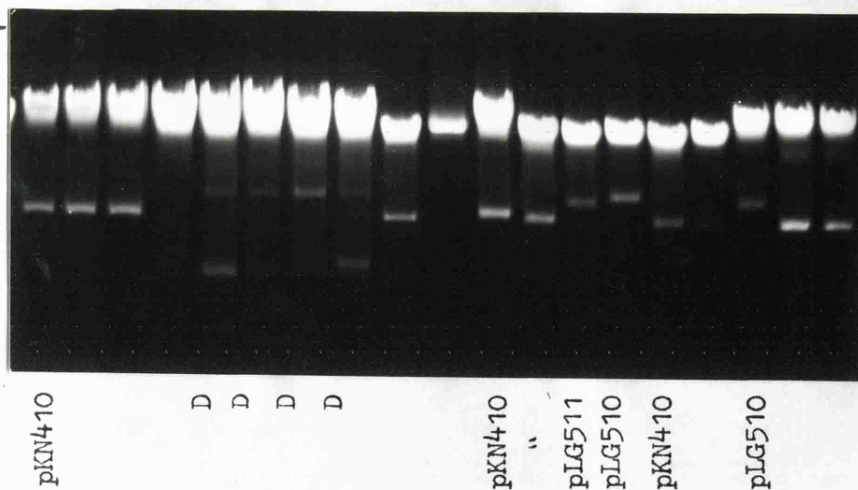
Transformants were obtained with frequencies of 1 per  $2 \times 10^5$  survivors (PAM162) and 1 per  $3 \times 10^6$  (D22). These were purified on fresh Ap agar plates before testing further. 450 PAM162 transformants were then patched onto nutrient agar in duplicate and one of each pair of

plates was irradiated with  $20\text{Jm}^{-2}$  UV. At this UV dose, the plasmidless host (lon sulB) was not affected, but a lon sul<sup>+</sup> strain was killed, thus bacteria containing sul<sup>+</sup> plasmids were expected, assuming sulB26 to be recessive, to be UV sensitive. None of the 450 clones tested showed this highly UV sensitive phenotype, but 47 which appeared to be slightly more sensitive than the others were tested further. Diluted overnight cultures were streaked across nutrient agar plates, UV irradiated in sections, incubated at  $30^{\circ}$  and then examined for growth. In addition exponential cultures grown in Luria broth to  $A^{450}=0.1$  were irradiated ( $10\text{Jm}^{-2}$ ) and examined microscopically for filaments after several hours of post-UV incubation at  $30^{\circ}$ . These tests did not reveal any truly UV sensitive transformants and therefore suggested that none contained sul<sup>+</sup> recombinant plasmids. The D22 transformants obtained did, however, confirm that some recombinant plasmids had been formed, since 35 out of 105 tested were resistant to  $5\mu\text{g/ml}$  rifampicin (i.e. were now env<sup>+</sup>) when tested on nutrient agar containing the drug (Normark, 1970).

To ascertain whether the results of the UV tests really reflected the structure of the plasmids present, SDS cleared lysates of 31 PAM162 transformants were prepared, after 2h. growth at  $40^{\circ}$  to amplify the plasmids. The lysates were analysed on agarose gels, and the results (fig.5.3) showed that, despite the lack of genetic evidence, some of the PAM162 clones tested did contain plasmids of increased size suggesting that chromosomal fragments had been cloned. The two plasmids indicated in fig.5.3 are different in size and both are larger than the vector pKN410. Preparations of purified plasmid DNA were made from these two strains and restriction with EcoRI showed that each contained only one species of

Figure 5.3 Plasmid screening of PAM162 and D22 transformants

SDS lysates of transformants were prepared (2.12), loaded onto 0.7% agarose gels (2.11) and run for 2.5h at 100V. Gels were stained with ethidium bromide and photographed over UV (2.11). Examples of these screening gels are shown (a and b). Lysates marked "D" were prepared from D22 and show "breakdown" plasmids (see section 5.5). Figures 5.3c and d show 1% agarose gel analyses of EcoRI digests of pLG510 and pLG511 DNA respectively, prepared as in 2.10.



plasmid. The restriction digests also revealed that each of the two plasmids, termed pLG510 and pLG511, consisted of 2 EcoRI fragments, one of 15Kb (i.e. vector-sized) and a smaller fragment of 2.5Kb (pLG510) or 2.3Kb (pLG511). The physical structure of these new plasmids therefore confirms that they are indeed the expected recombinants as illustrated in fig.5.1. From the fragment sizes, pLG510 contained the envA DNA and pLG511 the ftsA DNA from the transducing phage  $\lambda$ fts. When pLG510 was used to transform D22 (envA), rif resistance (i.e. env<sup>+</sup>) was found to be co-transformed with Ap resistance, but only in 18% of the transformants, which were obtained at an unusually low frequency (20% of normal). The reasons for this are discussed later (5.5).

Despite the continued lack of genetic evidence for the presence of sulB, the new plasmids appeared to contain the required DNA fragments covering the supposed sulB region, and these were further investigated. We had hoped to obtain a recombinant plasmid containing the two cloned fragments still joined, but later results reduced the need to test such a plasmid.

#### 5.4 The effect of pLG510 on division after UV

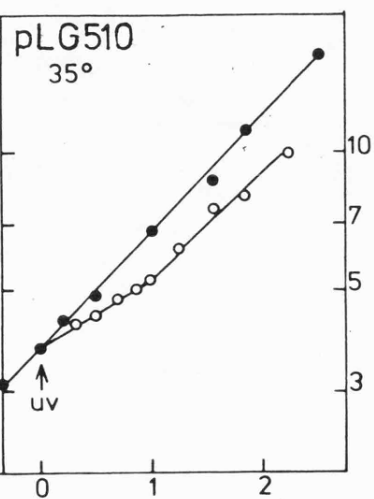
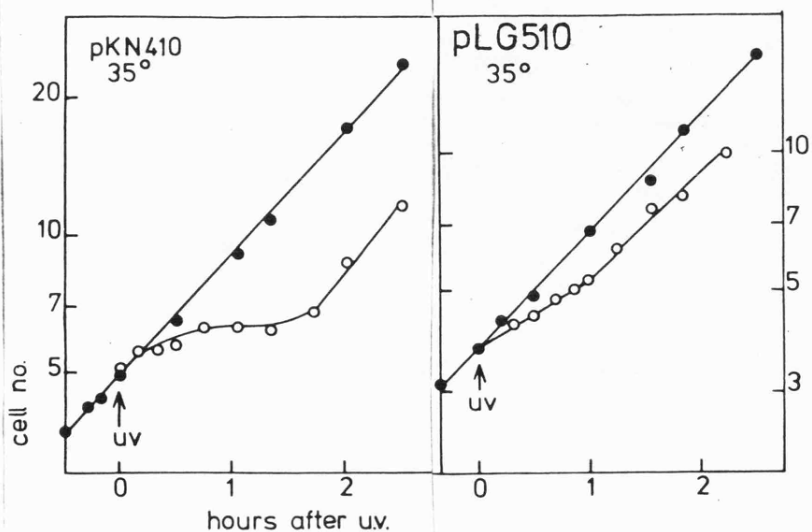
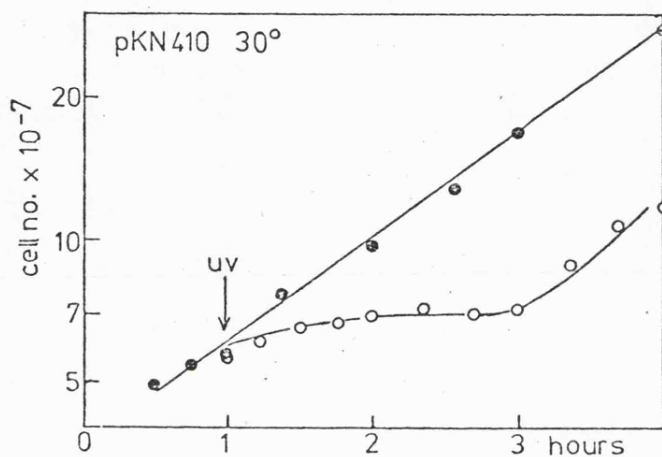
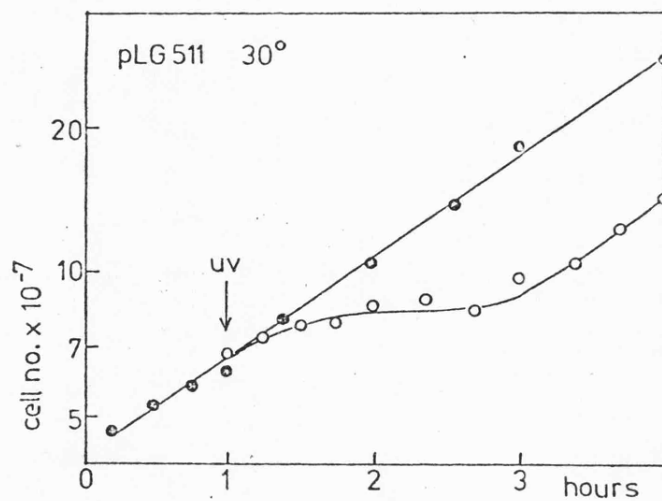
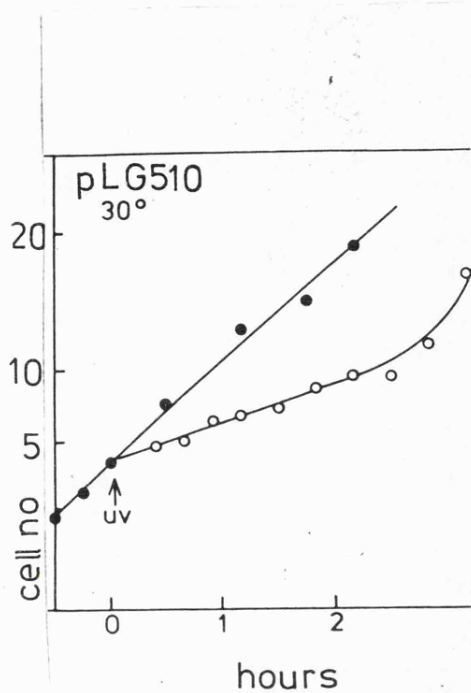
The kinetics of division after UV were determined for PAM162 containing pKN410, pLG510 or pLG511. (The reader is referred to fold-out Appendices I and II for continuous reference to (I) a summary of division kinetics experiments and (II) plasmid structure). As shown in fig.5.4a, when the strains were growing at 30°, a clear difference was seen between PAM162 bearing pLG510 and all other strains after UV. The

Figure 5.4 Kinetics of division after UV in PAM162 carrying pKN plasmids

Cultures of PAM162 containing pKN410, pLG510 or pLG511 were grown in minimal medium containing 0.25%w/v Casaminoacids at 30° or 35° as indicated. Irradiation ( $10 \text{ J.m}^{-2}$ ) and cell number determination were carried out as in 2.3 and 2.4.

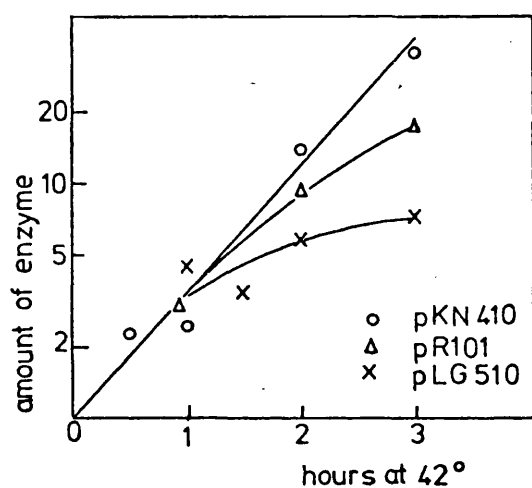
●—● -UV

○—○ +UV



presence of pLG510 considerably reduced the amount of inhibition of division after UV, whereas neither pKN410 nor pLG511 affected the division response, showing that the increased resistance to UV was a specific effect of the 2.5Kb cloned fragment in pLG510. At 30°, the copy number of these plasmids should be about 5 (Uhlin *et al.*, 1979). At 35 and 38°, when the plasmid copy number should be considerably increased (see 5.5 below), the effect of pLG510 was more striking (fig.5.4b); the block to division was increasingly reduced so that at 38°, division continued almost normally after UV. Under the same conditions pLG511 had no effect. These data suggest that the 2.5Kb insert codes for a gene which in high multiplicity interferes with the expression of the SOS-induced division block, consistent with the proposed function of sulB as the target of the recA\* controlled division inhibitor. Multicopies of sulB, then, (or some other cloned gene), appeared to titrate out the recA\* dependent inhibitor (or perhaps recA\* itself), leaving unaffected sulB (either genes or products) allowing division to continue despite the SOS-inducing DNA damage. The implication of this type of mechanism, where an excess of sulB suppresses division inhibition, is that the recA\* - dependent inhibitor switches off the transcription of a factor required for division (i.e. sulB) after UV. An increase in the copy number of a gene coding a factor which negatively controls division would not be predicted to alter division kinetics after UV in the manner observed. On the contrary, this should lead to a more rapid onset of division inhibition and more persistent filamentation.

In summary, although sulB has not been genetically identified, the results nevertheless demonstrated the presence of a gene on the 2.5Kb



**Figure 5.5** Production of  $\beta$ -lactamase by pKN plasmids

(Data of G.S. Plastow)

Extracellular  $\beta$ -lactamase was assayed in whole nutrient broth cultures of strain C600 carrying pKN410 or pR101 (tonB) and PAM162 pLG510 after shifting from 30° to 42°. "Amount" of  $\beta$ -lactamase is expressed as  $A_{450}$  units /  $A_{450}$  units culture.

fragment, which is involved in the UV response and is apparently the target for the *recA*\*-dependent inhibitor, inviting experiments to investigate the interaction between this gene and the *recA*\* to be carried out.

### 5.5 Amplification and stability of pKN410 derivatives

The successful preparation of SDS lysates described above (5.3), containing large amounts of plasmid DNA (relative to chromosomal DNA) indicated that the plasmids were considerably amplified by 2h. incubation of the host strain at 40°.

When transformants containing pLG510 and 511 were first isolated, it was noticed that patch growth on agar at 40° was nearly normal compared with growth of host strains carrying pKN410, suggesting that these recombinant plasmids did not amplify much at 40°. To investigate amplification, G. Plastow working in this laboratory made direct measurements of  $\beta$ -lactamase production by plasmid bearing strains after temperature shift. Fig.5.5 shows that in a culture of *E.coli* C600 containing pKN410, levels of extracellular  $\beta$ -lactamase increased 10-fold during the first 2h. after shift from 30° to 40°. These data are quantitatively similar to those described by Uhlin *et al.* (1979) for the original isolate of pKN410. Our experiment showed that in a recombinant pKN410 plasmid carrying the *E.coli tonB* region,  $\beta$ -lactamase production also increased 10-fold after shift to 40°, but PAM162 pLG510 showed a smaller and somewhat variable increase in  $\beta$ -lactamase production, on average only 2-4-fold after 2h. at 40°. Thus it appears that pKN410 recombinant plasmids did not invariably amplify to several hundred copies but reached about 20 copies even when amplification was poor.

Table 5.1Stability of pLG510 in various strains

<u>Strain</u>	<u>Relevant</u> <u>Genotype</u>	<u>Purpose of transformation</u> <u>with pLG510</u>	<u>Stability of plasmid</u> <u>physical structure</u>
PAM162	<u>lon</u> <u>sulB</u>	Isolation of pLG510	stable
GC895	<u>tif</u> <u>sfiB</u>	Investigation of UV- and tif-induced division kinetics	plasmid breakdown
JM12	<u>tif</u>		"
AB1157	<u>tif</u> <sup>+</sup> <u>sfi</u> <sup>+</sup>		"
D22	<u>envA</u>	Test for <u>env</u> <sup>+</sup> in pLG510	"
C600	W.T.	Control strain	"
KN126	<u>lon</u>	UV-division kinetics	"
KN126	W.T.	Control strain	"
DS410	<u>minA</u> <u>minB</u>	Identification of gene products in minicells	"

The selection for transformants was Ap resistance

During this work there were several indications that the pKN410 derivatives were not stably maintained in the host strains over many generations of growth. It was found necessary to maintain Ap selection at all times to avoid loss of plasmids from up to 50% of the cell population which took place in 2-3 generations of growth in the absence of Ap (measured by plating for viable colonies on agar with or without the drug). Particular care was necessary when overnight cultures were diluted, since a reduction in the Ap concentration during overnight growth of bla<sup>+</sup> bacteria resulted in a significant amount of plasmid loss, and thus a reduced viability of the culture when diluted into fresh Ap medium.

The loss of structural integrity of individual recombinant pKN410 plasmids (not seen with the vector plasmid) was a more serious problem in many backgrounds. This second type of instability resulted in actual breakdown of plasmids in vivo, so that after transformation with pLG510, several strains were found to contain only smaller plasmids which had lost the cloned insert and some non-essential plasmid DNA.

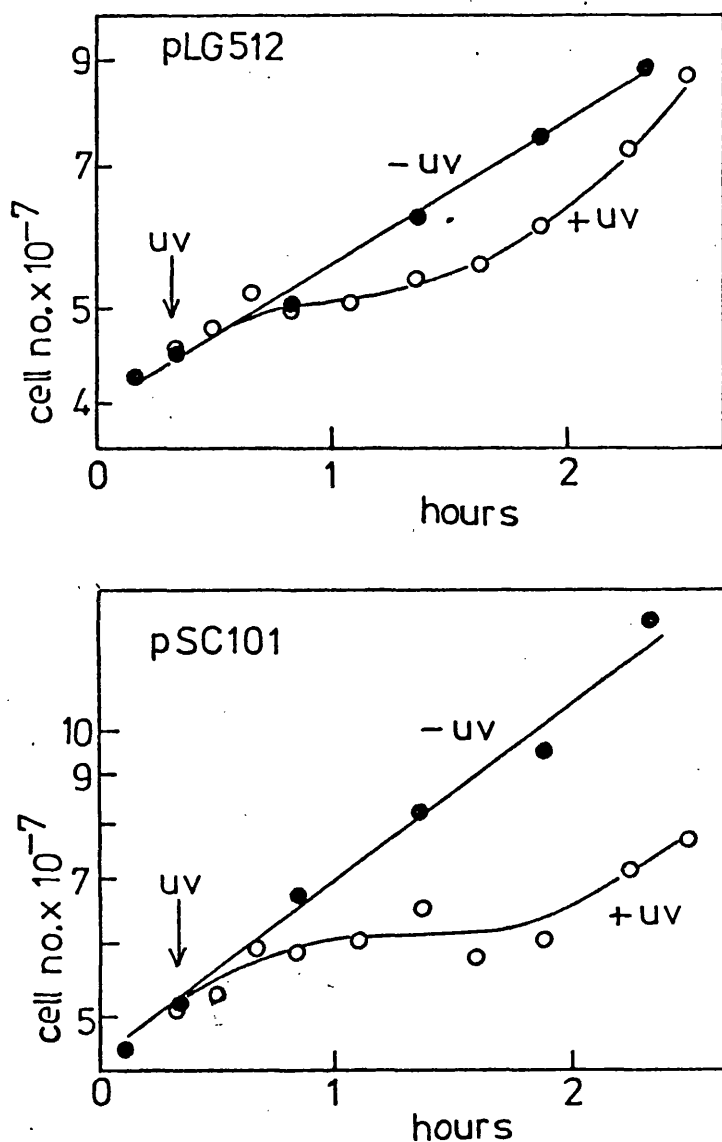
pLG510 was only able to maintain its structural integrity in the host strain PAM162. In 8 other host strains (table 5.1), only smaller plasmids were found in SDS lysates after transformation; these plasmids were bla<sup>+</sup> and assumed to be "breakdown products" of pLG510. The genetic backgrounds of the host strains were varied, but no common genetic feature could easily account for the plasmid breakdown.

Interestingly, most isolates contained a similar sized smaller plasmid which appeared to be a "preferred" product. In further studies, this plasmid, termed pV3, was no longer cut by EcoRI. It was stably

maintained by Ap selection and showed no loss of structural integrity in hosts C600 and D22 (envA) in which the parent plasmid had broken down. When transformed into D22, no complementation of envA was observed, and in the host PAM162, no effect was detected on the kinetics of division after UV. These results suggested that the original 2.5Kb insert was not present in the new plasmid, and this was confirmed by a later experiment discussed in chapter 6.

This unpredictable property of pLG510 is difficult to explain since pKN410 did not breakdown in C600. Although copy mutant plasmids are known frequently to generate "mini" plasmids (indeed this fact has been utilised in isolation of copy mutants), it is unclear why the effect should be dependent on the genetic background of the host strain. It should be remembered, however, that the host strains were all rec<sup>+</sup> so that if homologous recombination played any part in the breakdown process, the inadvertant selection of new, more stable forms of plasmid could not be avoided.

A further possibility was that multicopies of a gene contained in the 2.5Kb cloned DNA fragment could not be tolerated in certain host strains. However, strong evidence that this was not the case came at the end of this study when the 2.5Kb fragment was recloned into the plasmid vector pSC101. After ligation of the insert and EcoRI-digested pSC101 DNA, the mixture was used to transform strains D22 (envA) and C600. Selection of D22 transformants on agar containing both tetracycline (selecting pSC101) and rif (selecting env<sup>+</sup>) yielded bacteria harbouring a new recombinant plasmid pLG512 containing the 2.5Kb (env<sup>+</sup>) fragment from pLG510 inserted into the EcoRI site of pSC101 (see



**Figure 5.6** Kinetics of division after UV in PAM162 pLG512

Cultures of PAM162 pLG512 and PAM162 pSC101 were grown in minimal medium at 37°, irradiated (10 J.m<sup>-2</sup>; 2.3) and sampled for Coulter counting (2.4).

Appendix II). This was confirmed by a restriction digest of reisolated plasmid DNA. The new recombinant plasmid was quite stable in D22, PAM162, and C600, and since the copy number of pSC101 has been estimated to be  $\geq 4$  in exponential growth (Hashimoto-Gotoh and Sekiguchi, 1977), it seems that at least this number of the cloned genes is therefore well tolerated in this vector, whereas pLG510 is very unstable in these strains.

The kinetics of division after UV were determined for PAM162 pLG512 at 37° (fig.5.6) and were similar to those of PAM162 pLG510 at 35°, consistent with the presence of a gene able to titrate out the SOS division inhibitor as suggested for pLG510. Plasmids derived from pSC101 are not amplifiable and so this experiment was performed at 37° only. Although evidence had now been obtained that the 2.5Kb fragment indeed contained a gene involved in SOS division inhibition, the opportunities to investigate the interaction of this gene with other genes involved in SOS functions were severely impaired by the plasmid's instability in most strains. It had been hoped that a study of tif and lon strains in particular, when transformed by pLG510, would reveal informative patterns of division after SOS induction. The alternative approach to the study of these molecular interactions, involving in vitro techniques, required first the identification of gene product(s) coded by the 2.5Kb fragment. The next chapter deals with the demonstration of one such polypeptide and attempts to establish its genetic identity.

## Chapter 6

### EXPRESSION AND IDENTIFICATION OF CLONED GENES

#### 6.1 Introduction

This chapter describes experiments in which polypeptide synthesis programmed by the 2.5Kb fragment was analysed. Several methods were used, each having certain limitations, but yielding complementary results. Thus, while genes cloned with incomplete control regions may not be transcribed in most systems, if such genes are cloned, for example, in the correct orientation downstream of  $P_L$  of phage  $\lambda$ , polypeptides may be produced under the control of this promoter regardless of the promoter defect in the cloned gene. In the opposite orientation, however, even with an intact control region, synthesis may be blanketed by strong promoter activity from  $P_L$  acting against the direction of transcription of the cloned gene.

In both the phage clones  $\lambda$  GH200 and  $\lambda$  fts, the orientation of the inserts was unknown, with respect both to gene order and direction of transcription, and it was not therefore possible to predict whether the expression of the cloned genes would be affected by transcription from  $P_L$ . Further, digestion of phage DNAs with HindIII (results not shown) indicated that during the construction of  $\lambda$  GH200, an extra piece of DNA, identified by the presence of an additional HindIII site, had been recloned with the 2.5Kb EcoRI fragment from  $\lambda$  16.2 $\Delta$  E (see Chapter 5). The origin, size and position of this fragment were unknown, which might further complicate interpretation of gene expression data. Phage clones

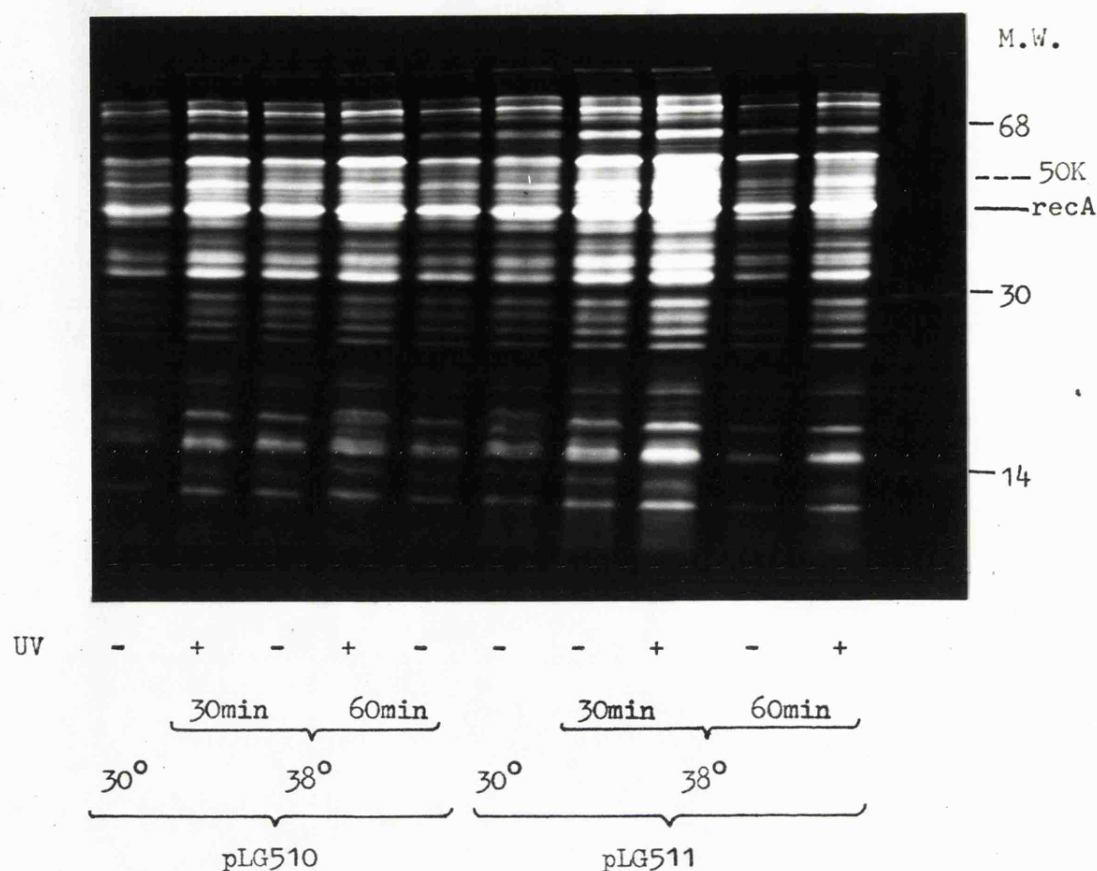


Figure 6.1 Radioactive labelling of proteins synthesised in PAM162 carrying pLG510 or pLG511

Proteins synthesised in exponential cultures of the two strains growing in minimal medium at 30° were pulse-labelled with [<sup>35</sup>S]-methionine (2.6). At A<sub>450</sub>=0.2, the cultures were shifted to 40°, and 20min later, a portion of each was irradiated (10 J.m<sup>-2</sup>; 2.3). Samples from both irradiated and unirradiated cultures were then pulse-labelled with [<sup>35</sup>S]-methionine as before, 30 and 60min after UV. Whole lysates and envelopes were prepared from the labelled samples and analysed by SDS-PAGE followed by fluorography. For technical reasons, only the whole cell lysates are shown.

could not therefore be relied upon as the sole system for identification of the sulB and envA products. For these reasons the plasmid clone pLG510 was also used to programme polypeptide synthesis in vivo.

While these experiments were in progress, an in vitro protein synthesising system became available in this laboratory developed by Dr. J.M. Pratt (Pratt et al., 1981). This system was to yield the most unambiguous data on expression of polypeptide(s) coded by the 2.5Kb fragment, and further, was to offer a system in which the molecular interactions which were discussed in Chapter 5 could best be studied. The gene expression experiments are described below; preliminary experiments exploring the ability to detect interactions in vitro between cloned genes and recA are described in Chapter 7.

## 6.2 Radioactive labelling of plasmid-coded proteins in vivo

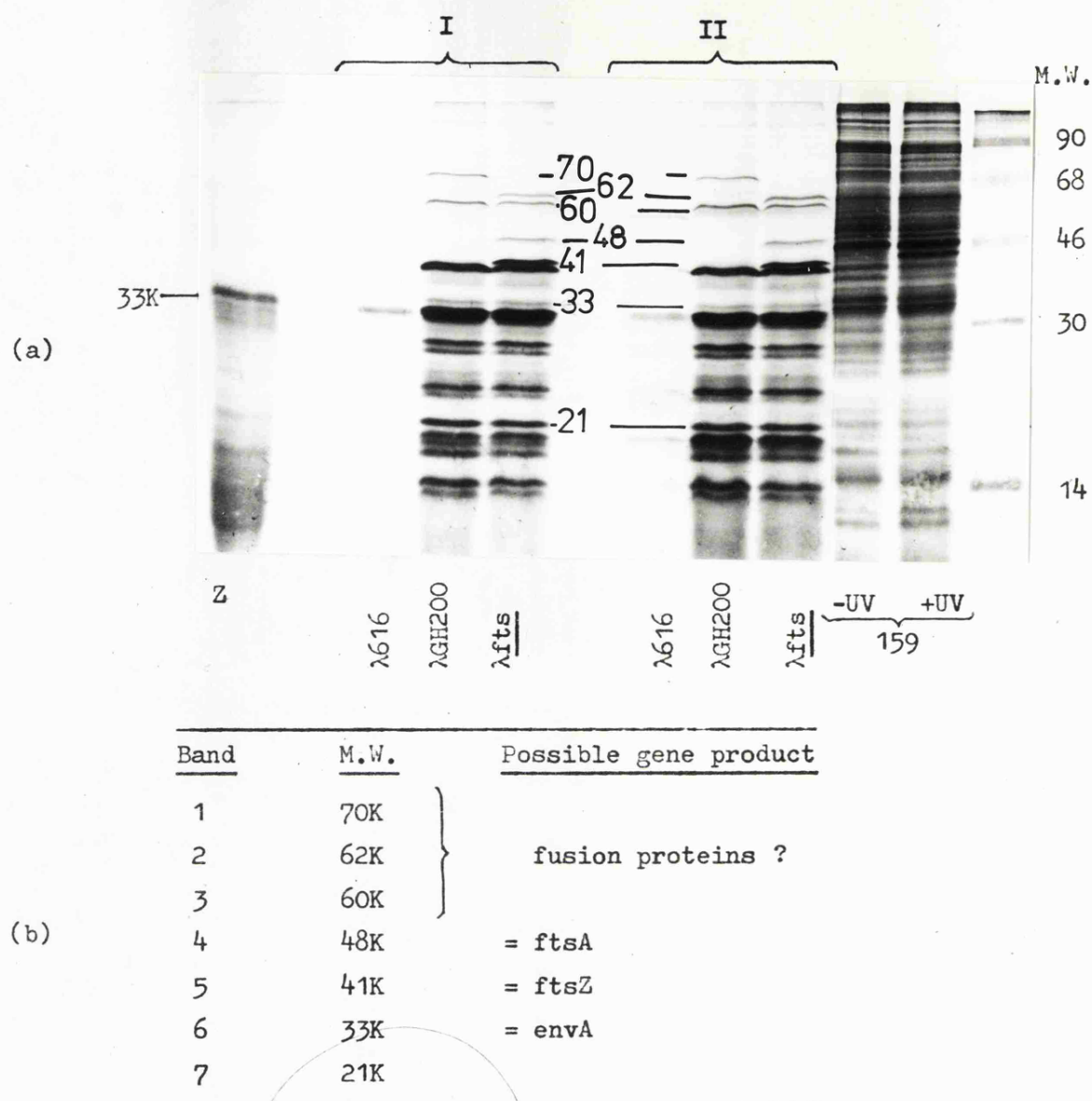
PAM162 (lon sulB) containing pLG510 or pLG511 was pulse-labelled with [ $^{35}\text{S}$ ]-methionine during growth at 30°, then at 38°, when the plasmid copy number increased several fold (see 5.5). Under these conditions, plasmid coded proteins should be amplified (Uhlir et al., 1979), and thus may theoretically be detected by SDS-PAGE even in whole cell lysates. In order to test the effect of UV on transcription of plasmid coded gene products, part of the culture was irradiated ( $10\text{J.m}^{-2}$ ) shortly after the temperature shift (for other details see legend to figure 6.1). Whole lysates and envelopes were prepared from the labelled samples and analysed by SDS-PAGE. The results, shown in figure 6.1, demonstrated that in whole lysates, although recA protein (MW 40,000) was synthesised

in large quantities 30 min after UV, the temperature shift alone did not produce any detectable proteins coded by the amplified plasmid. Induction of *recA* was not affected by the increase in temperature.

In the envelope preparations, two possible new proteins were seen (in small amounts) after the shift to 38°. These had a MW of 30-33 and 45-47K and were found in the presence of both pLG410 and pLG511 and were therefore probably coded by the vector DNA rather than the cloned inserts. It was interesting to note that neither of these proteins were detectable in envelopes prepared from irradiated cells, indicating that perhaps UV had a generalised inhibitory effect on transcription of plasmid-coded genes. Direct UV damage to the transcription template would not be expected to interfere with gene expression since most plasmids would not sustain lesions at the dose used. Perhaps a generalised effect on transcription, mediated by the "SOS" system, was responsible; the implication being that even if the *sulB* product were identified by this method, it might not be possible to demonstrate a specific "SOS" effect on *sulB* transcription if all plasmid transcription (or translation) is depressed after UV.

Recently Lutkenhaus and Donachie (1979) identified the *ftsA* gene product, a 50K protein, and showed that it is coded by the 2.3Kb EcoRI fragment adjacent to that coding *envA* on the chromosome (Lutkenhaus *et al.*, 1980). This fragment forms the clone pLG511. The autoradiographs in fig. 6.1b were inspected for this protein but it was not detected.

As discussed in Chapter 5, pLG510 probably amplifies poorly and this almost certainly was one reason why no plasmid-coded proteins were clearly identified in the previous experiment. In order to study



**Figure 6.2** Proteins coded by  $\lambda$ GH200 and  $\lambda$ fts

Proteins synthesised from the transducing phages were labelled with  $[^{35}\text{S}]$ -methionine after infection of irradiated strain 159 (see 2.17). Whole lysates were prepared (2.7) and analysed by SDS-PAGE (2.8) followed by autoradiography (samples I). A second series of samples (II) were similarly labelled and prepared using a culture of 159 which had received a low dose ( $10 \text{ J.m}^{-2}$ ) of UV 30min prior to the high dose; this experiment is described in Chapter 7. (The sample marked "Z" is an in vitro translation of The 2.5Kb fragment (see section 6.4)).

synthesis of plasmid products in the absence of host protein synthesis, pLG510 was used to transform the minicell-producing strain DS410, since in this strain plasmids segregate into the minicells without segregation of chromosomal DNA. After separation from the normal cells, protein synthesis continuing in minicells from plasmid templates can be radioactively labelled for analysis (Frazer and Curtiss, 1975). Unfortunately pLG510 was unstable in this strain, and the bla<sup>+</sup> transformants obtained were shown to contain smaller plasmids, some resembling pV3 (see Chapter 5) when lysates were analysed on agarose gels, and therefore this method could not be used to detect plasmid-coded polypeptides.

### 6.3 Radioactive labelling of phage coded proteins in UV-irradiated cells

After heavy irradiation of cells by UV, bacterial chromosomes cannot serve as efficient templates for transcription. Such cells, when infected with unirradiated phage, are still able to synthesise polypeptides coded by the phage DNA (Ptashne, 1967).

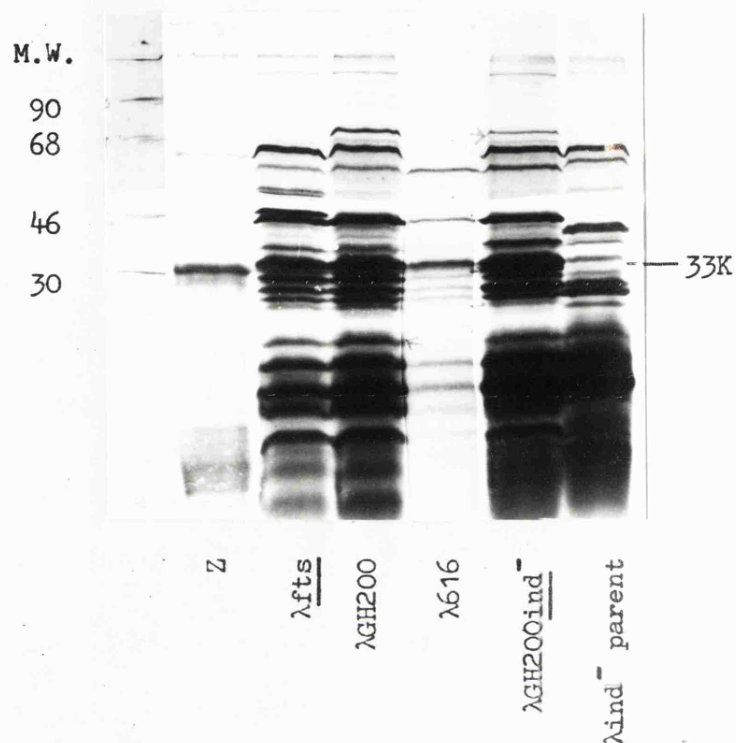
6.3a Proteins coded by  $\lambda$  fts and  $\lambda$  GH200. Suspensions of the phages  $\lambda$  fts,  $\lambda$  GH200 and  $\lambda$  616 (parent phage) were used to infect UV irradiated strain 159 (gal uvrA, non-lysogenic) and polypeptides were labelled with [<sup>35</sup>S]-methionine (see Chapter 2). Lysates were prepared and analysed by SDS-PAGE; an autoradiograph from such an experiment is shown in fig. 6.2. Compared with the parent phage  $\lambda$  616,  $\lambda$  fts and  $\lambda$  GH200 programmed the synthesis of several polypeptides. Some of these, polypeptides of M.W. 60, 32-33, 21 and 18.5K, were common to both phages. In addition,  $\lambda$  GH200 coded a unique 70K protein and  $\lambda$  fts, unique 62K, 46-48K and 41-43K proteins. Other smaller polypeptides were noticed but caution should be

used in the interpretation of the low M.W. region of the gels, as partial (incomplete) products may be present.

Comparison with the data of Lutkenhaus and Donachie (1979) and Lutkenhaus *et al.* (1980) suggested that the 46-48K and 41-43K polypeptides of  $\lambda$  fts seen in this experiment, correspond with their 50K and 45K proteins identified as ftsA and ftsZ respectively. Of the proteins common to  $\lambda$  fts and  $\lambda$ GH200, that of M.W. 33K probably corresponds with the 31K polypeptide very recently identified by Lutkenhaus and Wu (1980) as envA. The larger proteins (60, 62 and 70K) may be hybrid (fusion) proteins formed by transcription across the junction between cloned and vector DNA. These details are summarised in fig. 6.2b.

6.3b Interpretation of phage gene-expression data. While it was encouraging to find that some of the gene products produced in this experiment were comparable with those found by other workers, it was not possible to assign unambiguously a protein product to the sulB gene, and indeed this was not expected. At the time these results were obtained, in the absence of published data suggesting the 31K protein of Lutkenhaus was in fact envA, it was assumed that either the 33K or the 21K polypeptide could be sulB.

In order to minimise the synthesis of proteins under the control of phage promoters, strain 159 was lysogenised with  $\lambda$ imm<sup>21</sup> and heavily irradiated; under these conditions cloned genes with their own promoters should then produce the majority of the newly synthesised products. Unfortunately this modification did not clarify the earlier results due to very low levels of incorporation of [<sup>35</sup>S]-methionine into the infected cells. The reason for this was unknown.



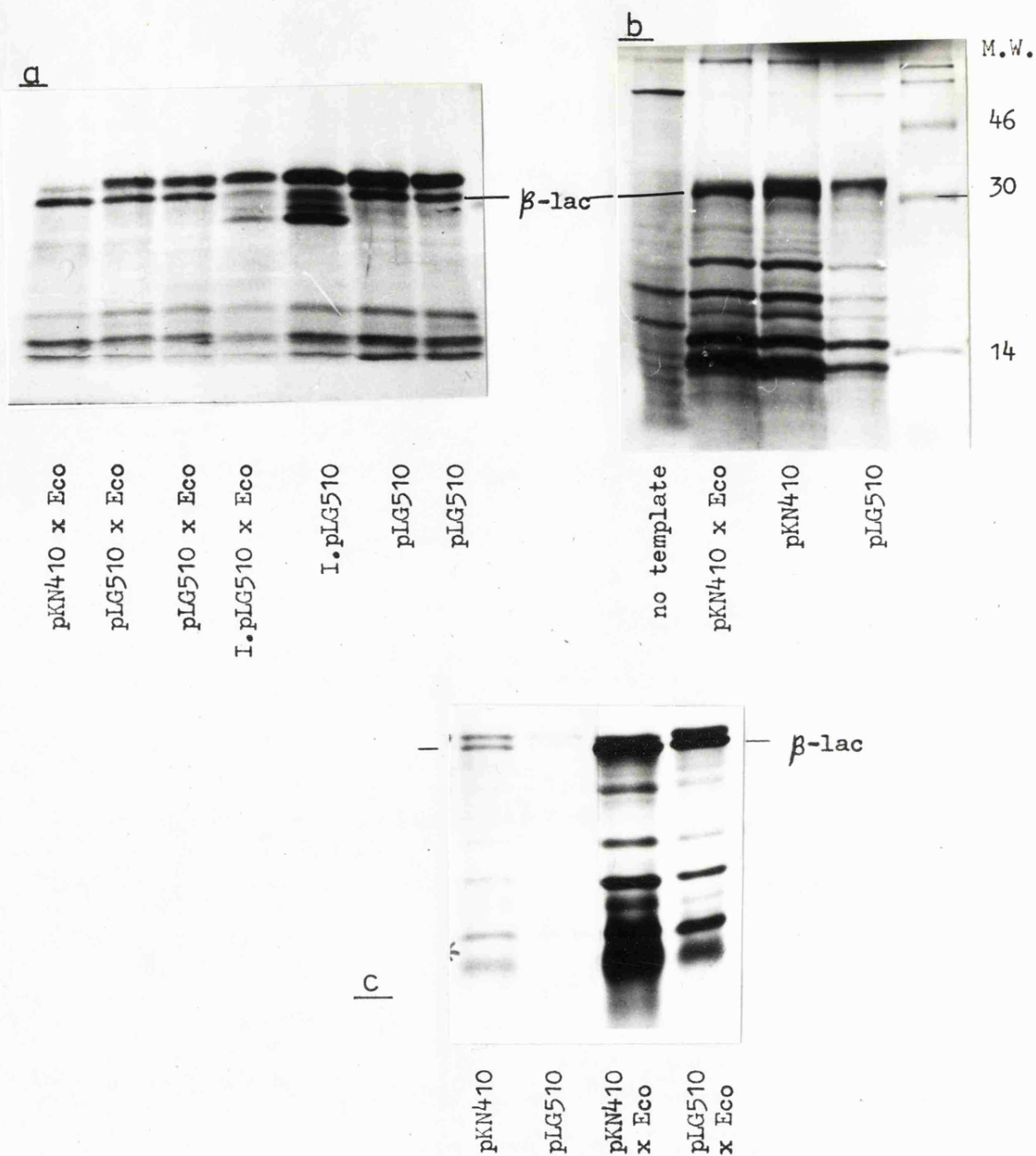
**Figure 6.3** Polypeptides coded by  $\lambda\text{GH200ind}^-$

Proteins synthesised in UV-inactivated strain 159 after infection with  $\lambda\text{GH200ind}^-$  were labelled with  $[^{35}\text{S}]$ -methionine (2.17). Whole cell lysates were prepared (2.7) and analysed by SDS-PAGE (2.8) on 15% gels, followed by autoradiography. The track marked "Z" on the autoradiograph above shows the 33K polypeptide synthesised in vitro (2.19) from the isolated 2.5Kb DNA fragment cloned from  $\lambda\text{fts}$  (see Chapter 6).

EDTA treatment was used to isolate phage with deletions (Parkinson and Huskey, 1971) so that loss of genetic markers could be correlated with the ability to synthesise certain polypeptides in the irradiated cells. Phages which have spontaneously deleted a (non-essential) part of their genome form, in the presence of EDTA, much larger plaques than the starting phage, and may therefore be selected at the appropriate EDTA concentration. In this case it would have been useful to check whether loss of the ability to complement envA corresponded with loss of the 33K or 21K proteins from  $\lambda$ GH200. A direct correlation for sulB could not have been tested in this way without first crossing in ind<sup>-</sup>, and the screening procedure would then depend on the sulB26 allele being recessive as discussed in Chapter 4.

In practice, deletions in  $\lambda$ GH200 could not be isolated by the EDTA method, though some deletions in  $\lambda$ fts were obtained. These were characterised genetically with respect to ftsA and envA, but analysis of polypeptides synthesised from these mutants had not been attempted when the new information regarding the envA product was published, rendering further analysis of these deletions redundant.

6.3c Polypeptides coded by  $\lambda$ GH200 ind<sup>-</sup>. The construction of  $\lambda$ GH200 ind<sup>-</sup> was described in Chapter 4; this phage showed no complementation of sulB but was envA<sup>+</sup>. When used to programme protein synthesis in irradiated cells (fig. 6.3) it was clear that compared with the ind<sup>-</sup> parent phage, the 33K protein coded by the original  $\lambda$ GH200 was still produced. The 21K region was obscured by a phage protein derived from the ind<sup>-</sup> parent phage and therefore no conclusions could be drawn concerning the 21K protein. Results are consistent with the 33K protein being envA<sup>+</sup> but add nothing to previous data.



**Figure 6.4** Proteins synthesised in vitro from pLG510 and pKN410

Plasmid DNA was used as a template for in vitro protein synthesis (2.19), with or without previous digestion with EcoRI (2.13). Samples marked "I" were treated with  $\beta$ -lactamase antibody (see p.110) and any resulting precipitate was removed by centrifugation before analysis. Samples were analysed by SDS-PAGE (2.8) on 15% gels; the autoradiographs are shown above.

#### 6.4 In vitro synthesis of plasmid-coded and restriction fragment-coded polypeptides

The coupled transcription - translation system based on the method of Zubay (1973) is described in Chapter 2. I am indebted to Dr. J.M. Pratt for her enthusiastic collaboration in these experiments.

6.4a The 33K polypeptide and  $\beta$ -lactamase. Initially, pLG510 and pKN410 were used as templates for transcription in vitro. Polypeptides synthesised were labelled with [ $^{35}$ S]-methionine and analysed by SDS-PAGE as described in Chapter 2. Fig. 6.4 shows that both plasmids programmed synthesis of several polypeptides of low M.W, including two major species for each plasmid at 30 and 33K, which appeared at first to be identical in the two plasmids. In both cases the lower band (30K polypeptide) was readily identified as  $\beta$ -lactamase by immunoprecipitation with antibody raised in rabbit, precipitated with S.aureus protein A, (Kessler, 1975) (fig. 6.4a). The upper band (33K polypeptide) proved to be a different molecule in the two plasmids, since synthesis of that from pKN410 was very much reduced by previous digestion of the plasmid DNA by EcoRI, whereas this treatment had no effect on the synthesis of the similar polypeptide from pLG510. This result suggested that the EcoRI-sensitive polypeptide was coded across the EcoRI site in the vector plasmid. In pLG510, this DNA was interrupted by the 2.5Kb fragment which also coded for a 33K polypeptide, but without an internal EcoRI site (fig. 6.4b).

It is notable that in this system, although  $\beta$ -lactamase is always detected among the translation products from pLG510, the amount produced is often much less than that of the 33K protein running just above it in the gels. After digestion with EcoRI, however, when the vector and insert

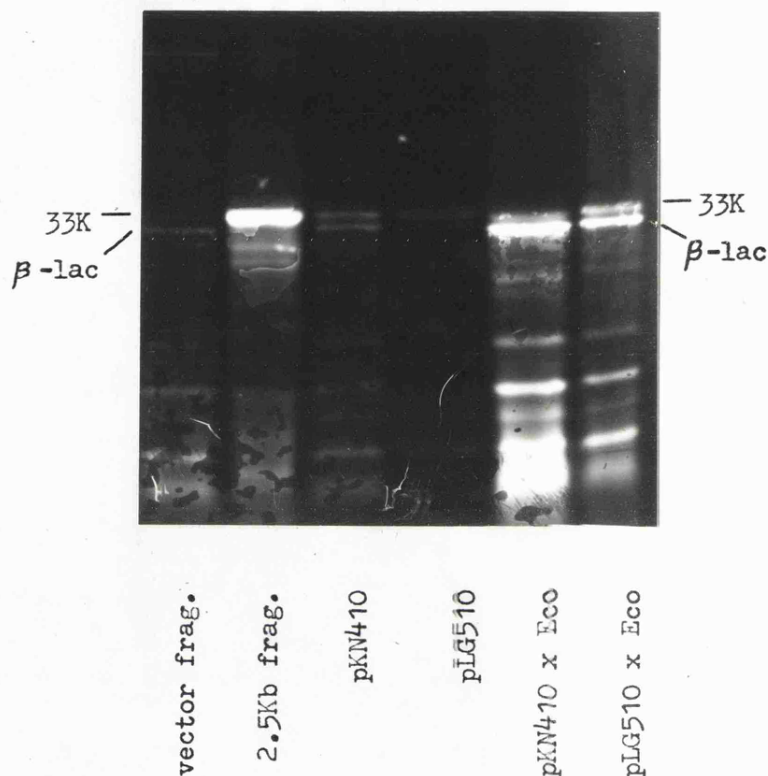


Figure 6.5 In vitro synthesis of polypeptides coded by isolated  
DNA restriction fragments

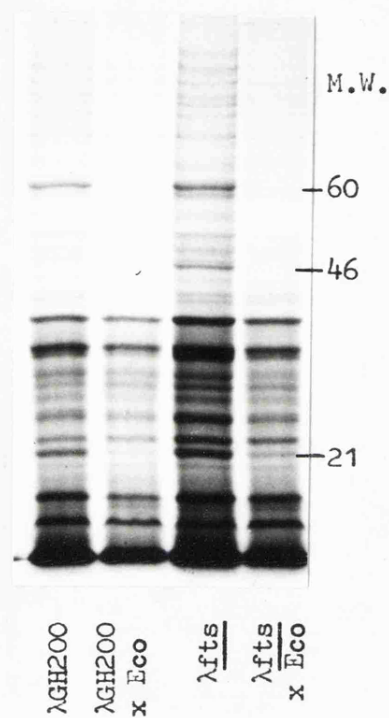
A preparation of pLG510 DNA was digested with EcoRI (2.13), the fragments were separated by agarose gel electrophoresis (0.5% agarose) and the two fragments (vector and insert) purified separately from the gel (2.16). These fragments were then used to programme in vitro protein synthesis (2.19); whole plasmids (pLG510 and pKN410) or unseparated digests were also used. Proteins synthesised in vitro were analysed by SDS-PAGE (15% acrylamide) and the resulting autoradiograph is shown above.

fragments become separated, the proportion of  $\beta$ -lactamase in the translation products increased, as if some cis-acting control leading to preferential transcription of the 33K protein, was abolished by EcoRI digestion (fig. 6.4c).

Translation products from both plasmids included several smaller polypeptides which were assumed to be vector-coded since they were identical from pKN410 and pLG510. As in the phage expression system, data concerning low M.W. polypeptides was interpreted with caution, allowing for the presence of partial translation products.

The results so far attributed genes coding  $\beta$ -lactamase and the smaller polypeptides to the vector DNA, and the gene coding the 33K protein to the 2.5Kb insert. The vector and insert portions of pLG510 were released by EcoRI digestion and collected separately by preparative agarose gel electrophoresis. The pure DNA fragments were then used to programme in vitro synthesis and the resulting polypeptides analysed (fig. 6.5) with the result that the assignments deduced above were confirmed. Since the DNAs were used in this experiment in equimolar amounts, the very large amount of 33K protein obtained, relative to the amount of  $\beta$ -lactamase, indicated that transcription was proceeding from a very strong promoter under the conditions used.

6.4b Coding capacity of the 2.5Kb fragment. In section 6.3 it was mentioned that a 21K polypeptide was found among the labelled proteins from transducing phages  $\lambda$  fts and  $\lambda$  GH200. Autoradiographs of translation products of pLG510 were examined for this polypeptide, but it was not detected. In particular, the 2.5Kb fragment when used alone did not programme a 21K protein. This does not rule out the possibility that the

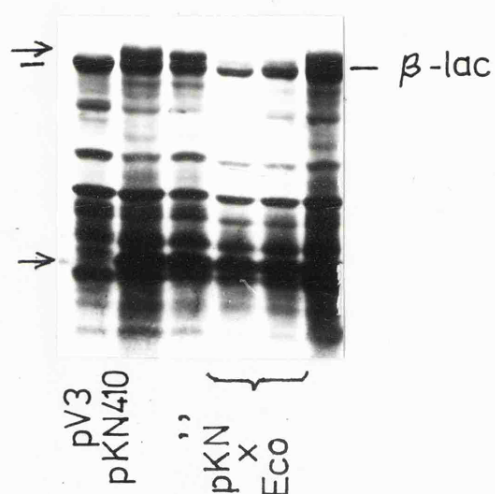


**Figure 6.6** In vitro synthesis of proteins from phage DNA

DNA was prepared from  $\lambda$ GH200 and  $\lambda$ fts (2.9), and samples were digested with EcoRI (2.13). Digested and undigested phage DNA was then used as template for in vitro protein synthesis (2.19), and samples were analysed by SDS-PAGE (15% acrylamide; 2.8). The resulting autoradiograph is shown above.

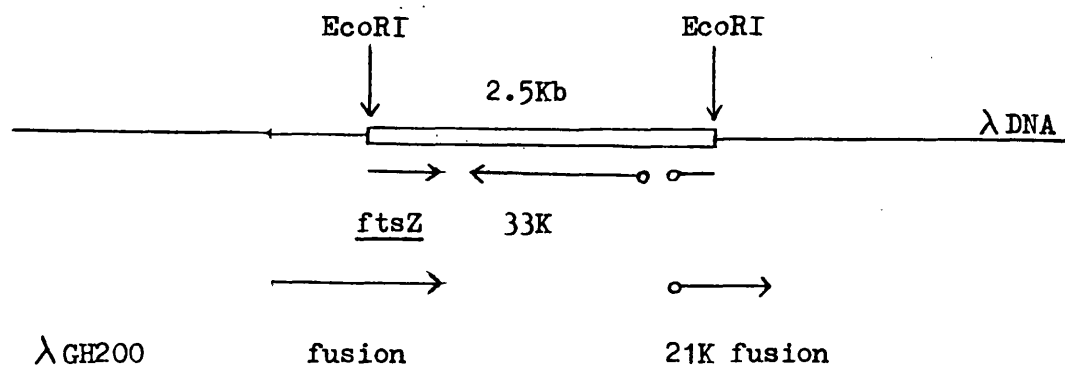
gene coding this protein, or part of it, exists on the 2.5Kb fragment, but for some unknown reason was not expressed in vitro.

The coding capacity of the 2.5Kb fragment is in fact sufficient to accommodate at least part of a third gene (coding up to 20K of protein) in addition to those coding the 33K protein and the major part of the C-terminus of the 45K, ftsZ gene product (see fig. 8.1 in Chapter 8). The latter has been assigned to this fragment by the recent work of Lutkenhaus and Wu (1980). The operator region for the ftsZ gene was shown to be in the adjacent (2.3Kb) EcoRI fragment by these workers, and therefore expression of this gene from the 2.5Kb fragment would not be expected either in vivo or in vitro. However, transcription through the C-terminus portion of the ftsZ gene from a phage promoter in  $\lambda$ GH200 could give rise to a fusion product, and such a product was identified in vivo (Chapter 6.3). When  $\lambda$ GH200 was used as a template for transcription in vitro, synthesis of two translation products was greatly reduced by prior digestion of the phage DNA with EcoRI (fig. 6.6). This result indicated that these two polypeptides were fusion products since the insert DNA was cloned into the single EcoRI site of the vector phage. Similar fusion products were identified from  $\lambda$  fts and one of them appeared to be the same 21K protein observed in irradiated cells after infection. This protein was coded by both phages and was therefore assumed to be coded across the right-hand boundary of the cloned insert and transcribed from a promoter within the 2.5Kb insert as shown below.



**Figure 6.7** Polypeptides coded by pV3

A DNA preparation (2.10) of the "breakdown" plasmid pV3 was used to programme in vitro protein synthesis (2.19), and the resulting polypeptides were analysed by SDS-PAGE (15% acrylamide; 2.8). The autoradiograph is shown above.



Because a fusion product at the ftsZ end of the 2.5Kb fragment must be initiated from a  $\lambda$  promoter, the second fusion product must be transcribed from a promoter within the fragment.

Fusion products were not detected among translation products from the plasmidic clone in vitro. It was assumed that the position of the insert in the phage clone fortuitously allowed fusion products to be formed, whereas the sequence of the neighbouring DNA in the plasmid clone prevented synthesis across the boundaries of the cloned insert.

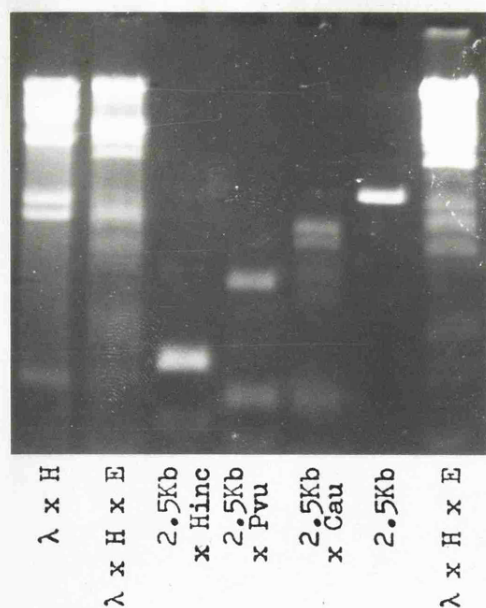
6.4c Products coded by pV3. The "breakdown product" plasmid resulting from the structural instability of pLG510 (see Chapter 5) was also used as a transcription template for in vitro protein synthesis, to ascertain whether the gene coding the 33K protein was still present. Fig. 6.7 shows that the 33K protein was not made from this plasmid, and that a vector gene had also been lost from pV3 (positions of the missing proteins are indicated).).

6.4d Is the 33K protein envA or sulB? When the experiments described had established that the 33K protein was coded by the 2.5Kb fragment, before the publication of Lutkenhaus and Wu's experiments, it was then essential to discover whether the gene coding this product was envA or sulB. Use

Table 6.1

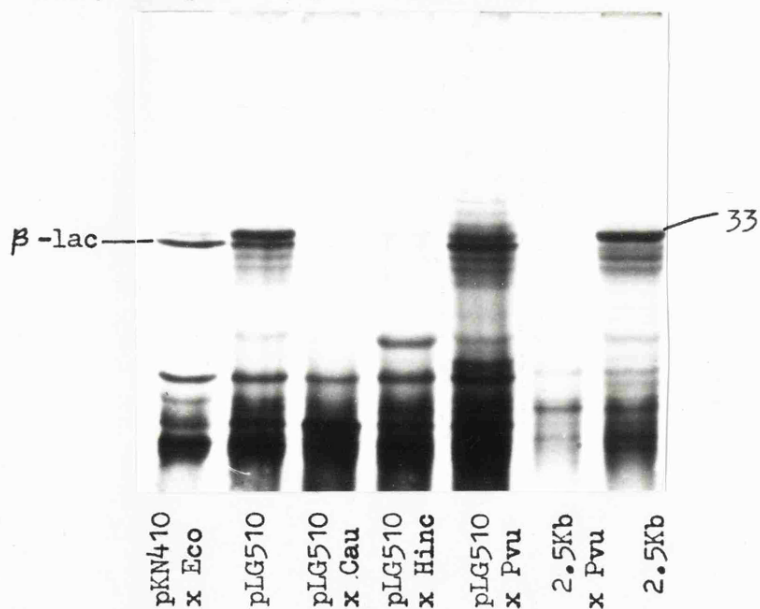
Digestion of the 2.5Kb DNA fragment by restriction enzymes

<u>Enzyme used</u>	<u>Sites in vector</u>	<u>Sites in fragment</u>
HpaI	Many	4
SalI	0	0
CauII	2	2
PstI	Many	0
BclI	0	0
XbaI	3	0
PvuII	2	1
BamHI	0	0
Sst	1	0
HincII	4	1
SauIIIA	Many	Many



**Figure 6.8** Restriction digests of the 2.5Kb fragment

Purified fragment DNA (2.16) was digested with PvuII, HincII or CauII (2.13) and analysed on a 1% agarose gel (2.11). M.W. standards used were EcoRI and HindII digests of  $\lambda$ DNA. The gel, shown above, was photographed under UV.



**Figure 6.9** Proteins synthesised from the digested 2.5Kb fragment

Purified 2.5Kb fragment, digested as in Fig. 6.8, was used to programme polypeptide synthesis in vitro (2.19). The proteins were analysed by SDS-PAGE (15% acrylamide; 2.8) and the resulting autoradiograph is shown above.

of the in vitro system allowed a search to be made for a restriction enzyme which cut the 2.5Kb fragment in the 33K gene. If such a (single) restriction site could be found, the two parts of the fragment resulting from digestion might be recloned separately, and tested for both complementation of envA, and the division inhibition suppressing effect discussed in Chapter 5.

First, the isolated 2.5Kb fragment was digested with a number of restriction enzymes, mostly with 5-6 base recognition sequences, and the digests analysed by agarose gel electrophoresis. The results are listed in table 6.1. 5 enzymes were found to cut the fragment; HpaI and SauIIIA at many sites, CauII at 2 sites, PvuII and HincII at one site only (fig. 6.8).

To determine whether the sites for PvuII, HincII and CauII were in the 33K gene, pLG510 was digested with these enzymes and then used to programme protein synthesis in vitro. The whole plasmid rather than the 2.5Kb fragment was used because preparation of the purified fragment in the quantity needed for this experiment was laborious and variable in yield. Fig. 6.9 shows that PvuII and CauII digestion prevented synthesis of the 33K protein and thus had cutting sites within the gene. The site for PvuII was only 20% from one end of the fragment, generating a new 2.0Kb piece containing most of the original DNA but unable to direct synthesis of the 33K polypeptide. If this new fragment could be recloned into pKN410 and tested for ability to complement envA, the genetic identity of the 33K protein might be unambiguously established.

### 6.5 Recloning the 2.0Kb fragment

Digestion with PvuII produces blunt ends at the cutting site, thus the 2.0Kb fragment had one staggered (EcoRI) end and one blunt end, and in order to reclone, the ends of the fragment and vector molecules had to be made complementary to allow covalent joining in vivo. Two methods were considered : in one, S1 endonuclease may be used to remove sequentially, unpaired bases from the protruding single stranded part of the EcoRI end. Blunt ended molecules may then be ligated directly with T4 ligase, or after "tailing" with complementary 3'-homopolymers using terminal transferase (see below). The homopolymer tails then allow re-annealing of vector and insert to form new recombinant plasmids which can be used to transform a suitable host.

However, the degradative action of S1 endonuclease is difficult to control and preparations of this enzyme may sometimes nick and degrade double stranded DNA; therefore an alternative method was preferred, involving first a synthetic reaction to fill in unpaired EcoRI ends, and then tailing to regenerate complementary ends. The reactions are illustrated in fig. 6.10. DNA polymerase I (Klenow large fragment) was used to insert dATP and dTTP opposite the unpaired single stranded EcoRI ends; this enzyme retains the "faithful" polymerisation activity but lacks the 5'-3' exonuclease of the full polymerase complex. The reaction was monitored by incorporation of [ $^3\text{H}$ ] dTTP into acid-precipitable material. To produce blunt ended 2.0Kb molecules, a whole PvuII digest of the 2.5Kb fragment was used, (i.e. the 0.5Kb piece was also present), because of the difficulty of obtaining large quantities of the purified fragments. It was felt that the two possible resulting recombinant plasmids containing either the 2.0 or the 0.5Kb insert would be easily

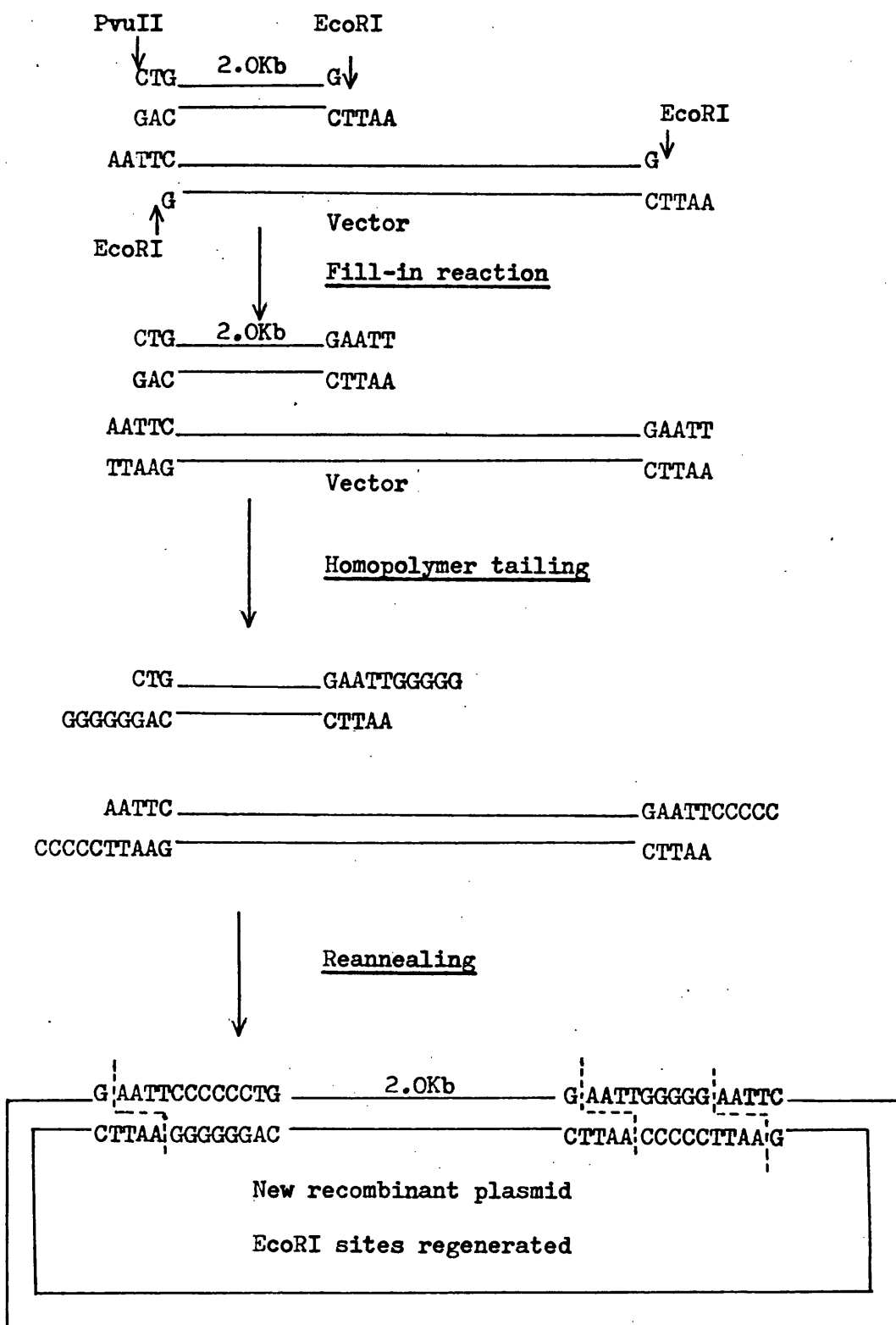


Figure 6.10 Recloning the 2.0Kb fragment

distinguishable in size by analysis of SDS lysates, as well as by restriction analysis.

To the blunt-ended molecules thus obtained, homopolymer tails were now added using terminal transferase to add poly-d-C to the vector and poly-d-G to the insert. Since this enzyme links bases to a 3' terminus as substrate, use of dC and dG in this way should regenerate the EcoRI sites at the junction between vector and insert when the fragment and vector reanneal (fig. 6.10).

The reannealed mixture of filled and tailed molecules (plus a control, of reannealed vector alone) was used to transform PAM162. In one experiment, the frequency of transformants obtained by vector alone was rather high, and when SDS lysates were screened on agarose gels, 200 potential recombinants proved to be all vector-sized molecules with no insert. This could have been due to the presence of vector molecules which had escaped the initial EcoRI digestion. In a second experiment, linear (filled and tailed) vector molecules were purified by agarose gel electrophoresis before reannealing and the high "background" level of transformation was eliminated. However, no recombinant plasmids were obtained. Since both the filling-in and tailing reactions were monitored and shown to have worked reasonably well, it seems likely that the reason for the failure of the experiment was complex. Terminal transferase can very occasionally produce short polymers without a "substrate" (primer) and may also sometimes produce single stranded breaks, when the new 3' end released is used as a primer, producing branched molecules (Roychoudhury and Wu, 1980). Further, although the 3' termini of blunt ended molecules are a more efficient substrate, homopolymers may also be added to a recessed 3'

end (i.e. an unfilled EcoRI end) and thus some incorporation may have been attributable to additions to unfilled ends. All these factors could contribute to an artificially high estimation of tailing efficiency. It is possible that direct ligation of the blunt ended molecules might have been more successful. Though this process is less efficient than ligation of complementary ends, the use of terminal transferase would be avoided. Unfortunately, circumstances did not permit further experiments, and the timely publication of Lutkenhaus and Wu (1980), suggesting that their 31K (this 33K) protein was indeed envA, has probably answered the question already.

## Chapter 7

### INTERACTION BETWEEN RECA\* AND THE DIVISION TARGET

#### 7.1 In vivo experiments

Results discussed in earlier chapters indicated that division inhibition after UV is mediated by *recA\** acting directly or indirectly on a target involved in the division mechanism. We have interpreted this to be a controlling effect of *recA\** on the transcription or stability of *sulB*, probably by switching-off synthesis of (or degrading) the *sulB* product. This chapter discusses some further investigations into how such gene interactions might be demonstrated.

Some experiments described in Chapter 6 indicated that this demonstration was not possible in vivo by UV-irradiation of bacteria containing pLG510, because the cloned gene products were not clearly detected due to poor amplification of the plasmid. It is possible that if a different copy mutant vector were used, with efficient amplification, the cloned gene products might be more easily identified and the effect of UV estimated. The effect of *tif*-induced SOS functions and the involvement of the *lon* gene product could not be investigated in vivo either, since pLG510 did not maintain its structure in *tif* and *lon* hosts.

#### 7.2 Experiments in heavily UV irradiated cells

A modification of the technique described in Chapter 6.3 was used to detect any effect of *recA\** on transcription of cloned genes in phage vectors. The host cells (*E.coli* 159) were "primed" to produce *recA\** by a

low ( $10\text{J.m}^{-2}$ ) dose of UV 30 min before the high UV dose used to inactivate the chromosome prior to infection.  $\lambda$  fts and  $\lambda$  GH200 therefore entered cells presumably containing *recA\**. The translation products were compared with those obtained without the "priming" dose of UV (fig. 6.2).

No effect of this low dose of UV was detectable on any of the cloned gene products coded by the transducing phages (identified in Chapter 6.3), although the induction of *recA* synthesis by the priming treatment was confirmed by pulse-labelling a portion of the culture with  $[^{35}\text{S}]$ -methionine, and analysis by SDS-PAGE (fig. 6.2). The success of this approach, however, depends on the assumption that *recA\** molecules, once produced, then persist in the cell for at least 60 min to be still active during transcription of DNA introduced upon infection. This may not be the case. To overcome this problem, the use of a conditional (tif) or constitutive (tif spr) mutant as the host strain in this experiment should allow heavily irradiated cells to be used in which SOS functions were turned on continuously (e.g. by temperature shift) prior to irradiation, rather than transiently as with the priming dose of UV.

### 7.3 The need for an *in vitro* system

All the systems described above have one major disadvantage concerning the complexity of division control. We know already that many factors contribute to the control of division (possibly with several levels of control operating) and new evidence (see Chapter 8) now suggests that the SOS pathways may be even more complex than was previously thought. In the light of these facts it should be admitted that experiments in vivo on division control will inevitably be difficult to

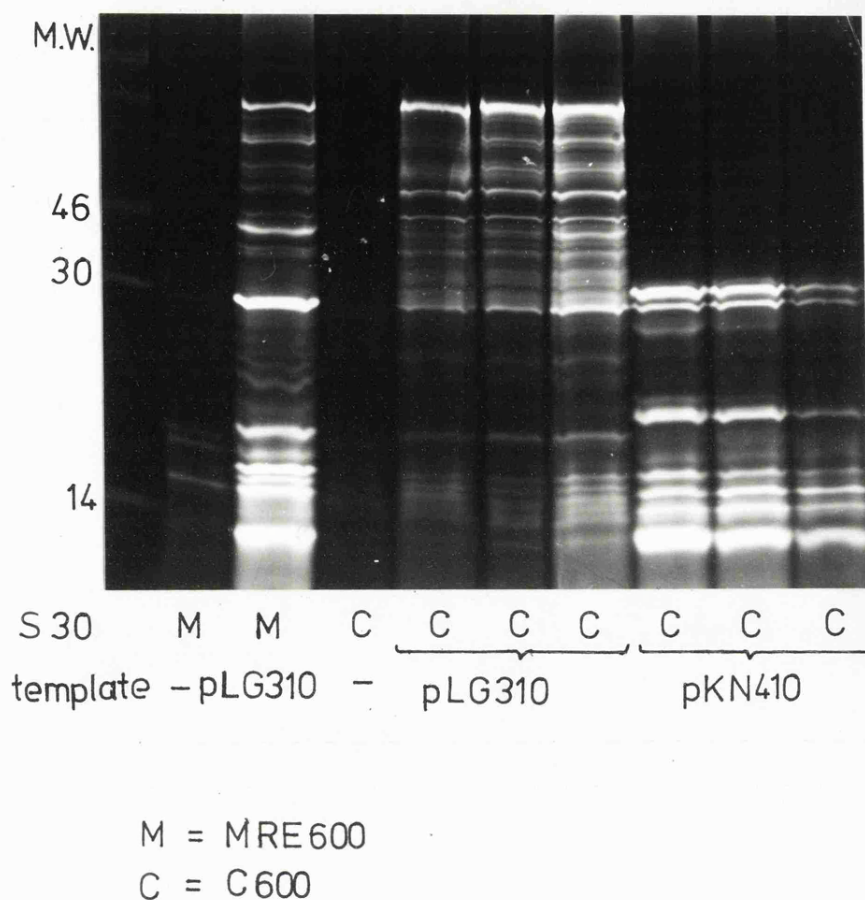
interpret because of possible interactions of the system under test with other levels of control. Thus we may ask : is the inhibition of division after UV due solely to the induction of a specific (UV-associated) "inhibitor", or is it also partly a consequence of the brief inability of the irradiated cells to terminate chromosome replication? (further discussion in Chapter 8).

These considerations suggest that the use of the in vitro transcription-translation system will be of paramount importance in the study of division control, since in this system the effect of a single gene product can be isolated from other interacting controls. The relationships of genetically well characterised factors can thus be studied in detail. In vitro studies have contributed significantly to the elucidation of many aspects of protein and DNA synthesis, together with biochemical and genetic approaches, and there is every reason to suppose that a similar contribution can be made by in vitro studies to the understanding of division control.

#### 7.4 In vitro experiments

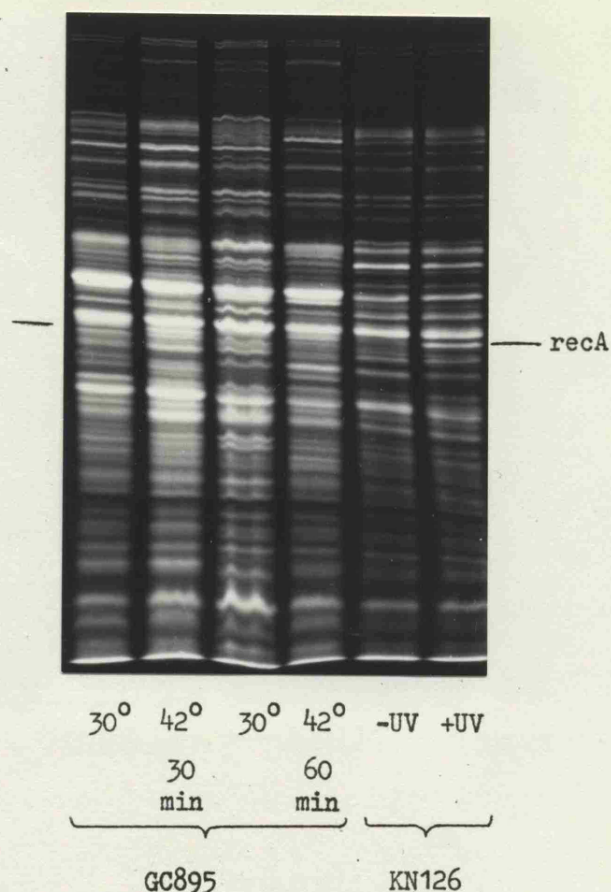
The identification of a 33K gene product coded by the 2.5Kb cloned DNA fragment was described in Chapter 6. At this time, in the absence of any evidence to identify this polypeptide as sulB or envA, some preliminary experiments were designed with the object of testing the effect of the tif (recA\*) protein on synthesis of the 33K protein in vitro. Although the stability of translation products was not tested specifically, the period of labelling of these products was 30 min (see Chapter 2) and this in itself gave an indication of stability.

Hitherto all S30 extracts (see Chapter 2.19) for coupled trans-



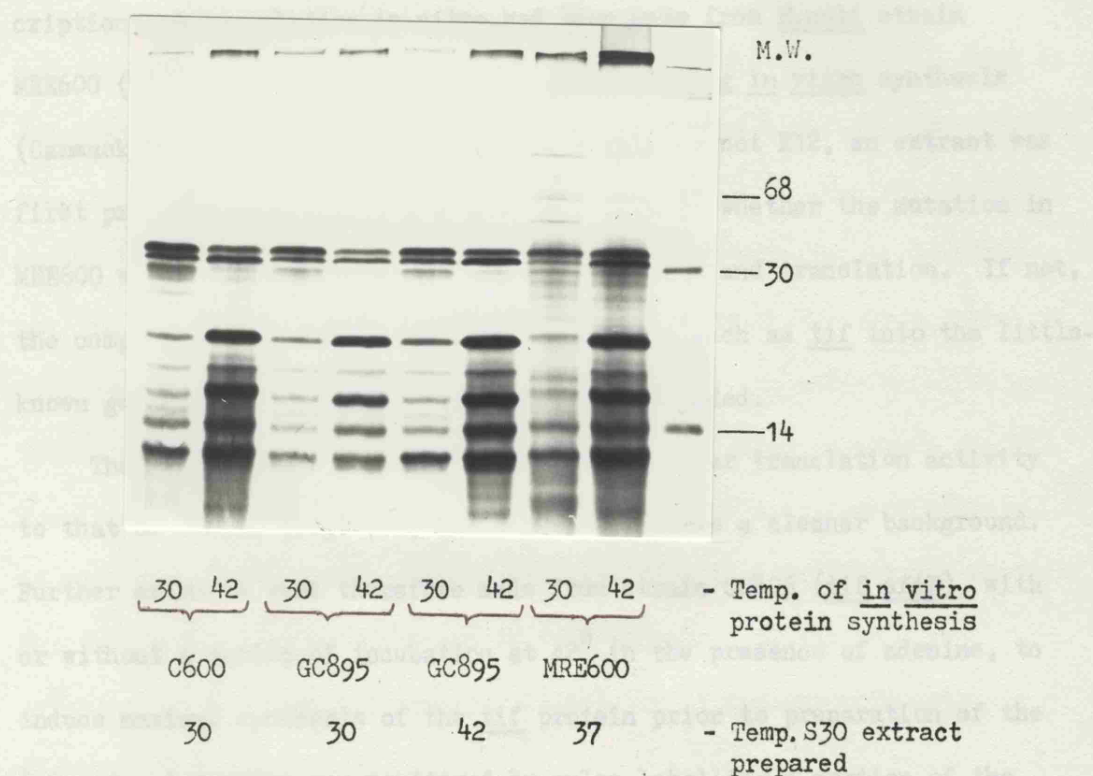
**Figure 7.2** In vitro protein synthesis using an E.coli K12 extract

An S30 extract for in vitro protein synthesis (2.19) was prepared from E.coli C600; plasmid DNA templates were used to programme protein synthesis, and the  $Mg^{++}$  concentration in the synthesis mix was varied to optimise the reactions. Proteins synthesised were analysed by SDS-PAGE (15% acrylamide; 2.8) and the resulting autoradiograph is shown above.



**Figure 7.3** Induction of tif protein in strain GC895

A culture of GC895 was grown to prepare an S30 extract (2.19), and shifted to 42° to induce the tif protein. Samples were removed before, and 30 or 60min after the shift, and proteins pulse-labelled with [<sup>35</sup>S]-methionine (2.6). Whole cell lysates were prepared (2.7) and analysed by SDS-PAGE (11% acrylamide; 2.8). As a control to show recA induction, pulse-labelled samples of KN126 were prepared before and after UV (as in Chapter 3) and also analysed. The resulting autoradiograph is shown above.



**Figure 7.4** Polypeptides synthesised from pLG510 in the presence of tif protein

Preparations of pLG510 (2.10) were digested with EcoRI (2.13) and used as templates for transcription *in vitro* (2.19) in S30 extracts from C600 and GC895 (prepared at 30° & after induction of tif at 42°). The *in vitro* reactions were carried out at 30° or 42° as indicated to ensure "activation" of tif protein. As a control, protein synthesis was also carried out using an MRE600 S30 extract. Proteins were analysed by SDS-PAGE (2.8); the autoradiograph is shown above.

cription and translation in vitro had been made from E.coli strain MRE600 (RNase<sup>-</sup>; this mutation protects mRNA during in vitro synthesis (Cammack and Wade, 1965)). Since this strain is not K12, an extract was first prepared from E.coli K12 C600 to determine whether the mutation in MRE600 was essential for efficient transcription and translation. If not, the complex genetics of transferring mutations such as tif into the little-known genetic background of MRE600 could be avoided.

The C600 extract showed qualitatively similar translation activity to that of MRE600 (fig. 7.2) and in addition gave a cleaner background. Further extracts were therefore made from strain GC895 (tif sfiB), with or without a period of incubation at 42° in the presence of adenine, to induce maximal synthesis of the tif protein prior to preparation of the extract. Induction was monitored by pulse-labelling a portion of the culture with [<sup>35</sup>S]-methionine, and analysis of lysates carried out by SDS-PAGE, showed that a moderate induction of tif took place (fig. 7.3). The resulting S30 extracts were active in transcription and translation.

When pLG510, previously digested with EcoRI to release the 2.5Kb fragment, was used as a template in the S30 tif extracts, the 33K polypeptide was synthesised at both 30° and 42° (fig. 7.4). Though the amount of the 33K protein made at 42° was lower relative to the amounts of the other newly synthesised proteins, this effect was also observed when the C600 (tif<sup>+</sup>) extract was used, and could not therefore be attributed to the presence of the tif protein.

### 7.5 Assay of tif activity in the S30 extract

Since the level of induction of the tif protein in the cells used to prepare the S30 extract was fairly low, it was considered possible that the final extract may not have contained sufficient tif protein, relative to the amounts of transcription and translation complexes and template DNA, to exert a detectable effect on transcription of the 33K protein, even assuming that such an effect occurs in vivo. In addition, although there is no reason to suspect that the S30 preparation procedure should result in differential loss or inactivation of any cytoplasmic protein, it was necessary to attempt a direct assay of tif activity in the extract. For this experiment it was hoped to take advantage of the well established fact that tif protein cleaves the  $\lambda$  repressor in vitro (Roberts et al., 1978).

As a source of labelled cI repressor, a heavily irradiated culture of 159  $\lambda$  ind<sup>-</sup> was infected with  $\lambda$ <sup>+</sup>, when few  $\lambda$  proteins other than cI are synthesised. Lysates of infected cells were prepared by freeze-thawing, and samples, containing labelled cI, were incubated with varying amounts of tif S30 extract in the presence of ATP and then analysed by SDS-PAGE (results not shown). No cleavage of the cI repressor was detected, either by loss of radioactivity from the 27K band (cI) or by the appearance of the 14K cleavage products (Roberts and Roberts, 1975). In a control experiment, a low dose of UV was used to prime the host cells with recA\* prior to infection in an attempt to demonstrate cleavage of the cI repressor in vivo. Although induction of recA synthesis was confirmed as in 7.2, again no cleavage of cI was detected by SDS-PAGE.

This experiment was also performed using a non-lysogenic host for the production of  $\lambda$  proteins after infection, in case the non-cleavable ind<sup>-</sup> repressor, abundant in the lysogenic host strain 159, bound induced recA\* in competition with newly synthesised cI of the superinfecting phage. The results were the same (i.e. no cleavage of cI). Conditions in which the  $\lambda$  -infection lysates were incubated with tif S30 extracts were comparable with those used by Craig and Roberts (1980) to demonstrate cleavage of cI in vitro, so it must be concluded that the tif S30 extract did not contain an easily detectable level of tif activity.

#### 7.6 Future prospects for in vitro experiments

The use of a plasmid carrying the cloned cI gene should provide a more sensitive and direct assay for tif activity in S30 extracts. This plasmid DNA could be used to programme the synthesis of cI protein in vitro, which might then be cleaved by endogenous tif protein. If the reaction conditions are sufficiently close to those in vivo to allow efficient transcription and translation, they should surely allow tif activity to be expressed. Also, a more active tif extract could probably be made from a constitutive tif-producing mutant (tif-spr) thus avoiding reliance on the rather variable induction of the tif protein.

Other methods are available for investigation of possible recA\* -sulB interactions in vitro. Instead of using an S30 extract from a tif mutant, purified tif (or recA) protein could be added directly to the translation mixtures containing a sulB template. Alternatively, both genes could be introduced into the translation mix as cloned DNA templates. Translation of a cloned tif gene carried on a plasmid vector would provide

a high concentration of the active product which might then be expected to interact efficiently with the cloned target gene or its product in an easily detectable manner. This technique could be used to detect "cross reactions" between any pairs of genes and/or their products, and a preliminary experiment in this laboratory has shown that such co-translation can be achieved. The lexA product, suggested by Witkin (1976) to be a repressor of several SOS controlled genes, has recently been cloned (Brent and Ptashne, 1980). Plasmid DNA containing this gene was used as an in vitro template and synthesis of the lexA product was obtained. When pLG510 was also added, products were obtained from both templates but as yet no effect on the synthesis of gene products coded in pLG510 has been demonstrated.

In conclusion, the experiments described in this chapter were somewhat preliminary in nature and should not be taken to indicate that, for example, S30 extracts containing active tif protein cannot be prepared. It is hoped that this system might quickly yield results if some of the modifications suggested here were introduced.

Recent evidence (see Chapter 6) that the 33K protein is in fact envA implies that the structural gene for sulB is not present on the cloned 2.5Kb fragment. Further experiments (discussed in Chapter 8) would therefore be necessary to locate and clone the structural gene before the transcriptional tests described in this chapter could be carried out in relation to this gene.

## Chapter 8

### AN APPRAISAL OF THE PROJECT IN THE LIGHT OF RECENT DEVELOPMENTS

#### 8.1 Kinetics of induction and recovery from the division block

An important feature of the division kinetics after UV was that a substantial proportion of cells in the D period were prevented from dividing (Chapter 3). This result indicated that the UV induced division block can veto the division of cells whose replication and protein synthesis requirements are fulfilled. In contrast, during thymine starvation (Chapter 3) none of the cells in D appeared to be prevented from dividing. Further, Gudas and Pardee (1976) reported that after cessation of DNA replication in a dnaA mutant at 42°, nalidixic acid did not induce synthesis of recA protein. These observations indicate that neither nalidixic acid (which acts at the replication fork) nor thymine starvation can generate the inducing signal in non-replicating cells. Moreover, the signal induced by UV in cells in D cannot be generated at, or by the replication complex, i.e. the inducing signal in these circumstances is not post-replication gaps, or dNMPs, or DNA breakdown from the replication fork by the recBC enzyme or any other consequence of a blocked replication complex.

Since the onset of division inhibition after UV is rapid, the signal must be produced during early activity of a constitutive repair process, e.g. degradation of DNA at small excision gaps. At the dose used in the experiments in this project, it may be calculated (Rupp and Howard-Flanders, 1968) that about 500 dimers per genome are produced, and it seems reasonable to suppose that excision repair could easily cope with most of these. It is unlikely that "unrepairable" overlapping lesions could be an inducing

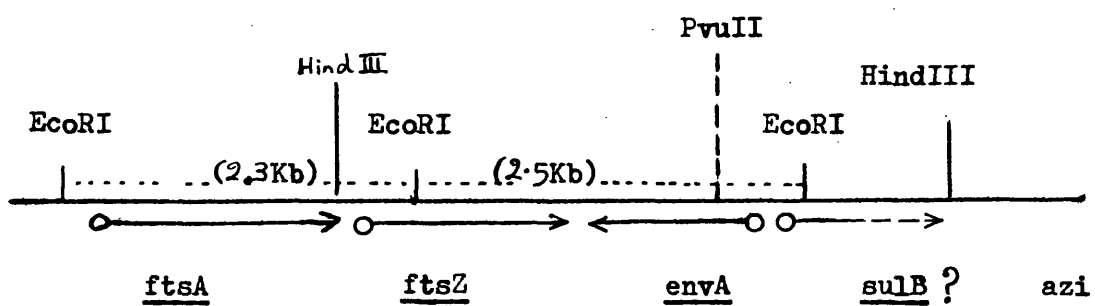
signal in this case since even if 10% of all the lesions were repaired by postreplication, long-patch mechanisms, less than 1% of the total genome would be subject to repair activity. Thus induction of SOS functions occurs at relatively low levels of damage, and is not restricted to an extreme situation, with a particular kind of lesion as the inducing signal. These considerations are consistent with speculations made in Chapter 1 that the signal may be generated by several different mechanisms depending on 1) the repair capacities of the cell, 2) the inducing stimulus and 3) the stage in the cell cycle. These predictions would be confirmed if it could be demonstrated that UV irradiation of synchronous cultures of cells in D results in immediate induction of any SOS function before the next round of replication begins. It would also be interesting to determine whether a single lesion could lead to induction in excision-deficient cells in D.

Two other interesting features of the kinetic experiments were noted. First, that the reduction in the DNA:mass ratio after UV was not corrected. Secondly, division kinetics during the recovery of lon<sup>+</sup> cells after UV revealed that although in many experiments there was a slight increase in the rate of division for a short period, there was no indication that the lost divisions were ever fully recovered. At the start of the recovery period after a UV dose of  $10\text{Jm}^{-2}$ , the cell number in irradiated lon<sup>+</sup> cultures was 55% lower than that of the unirradiated control; thus every cell had missed one division and many cells had missed 2 divisions before the block was lifted. It seems unlikely that either the division or the DNA deficits could be accounted for by killing of a significant proportion of cells since no effect on mass increase was detected. Thus, recovering

filamentous cells must either divide centrally to give a uniform population of larger cells, or more likely, asymmetrically, giving rise to one normal daughter cell and one larger cell (with decreased DNA/mass) in which the previously blocked division site is never used. However, it is difficult to attribute the alteration in DNA/mass wholly to the presence of oversized cells since these should rapidly become a very small proportion of the culture causing the DNA/mass defect to be quickly diluted out, contrary to the results obtained. Thus the precise explanation for these observations remains unclear.

It is possible that a more detailed study of the recovery period might reveal interesting features of the normal coupling between DNA replication and division as the SOS block is lifted. In this respect it is anticipated that the use of a low dose of UV as a transient inducing agent would minimise general disturbance to the cell, since even  $1\text{Jm}^{-2}$  produces an easily detectable effect on division (data not shown). UV has the advantage (over drugs like mitomycin or nalidixic acid) of producing repairable damage and can therefore be thought of as a normal physiological trigger for the SOS system. It is also likely that UV has been a major selective influence in the evolution of the SOS response. Several authors have suggested that a better understanding of the (error-prone repair) response to UV may contribute to an understanding of carcinogenesis (Hanawalt et al., 1978; Hiatt et al., 1977), since error-prone repair appears to occur in mammalian cells and to be implicated in carcinogenesis (e.g. in Xeroderma pigmentosa patients).

Recent experiments (P. Burton, personal communication) showed that the delay in division after UV was proportional to the dose (within the range



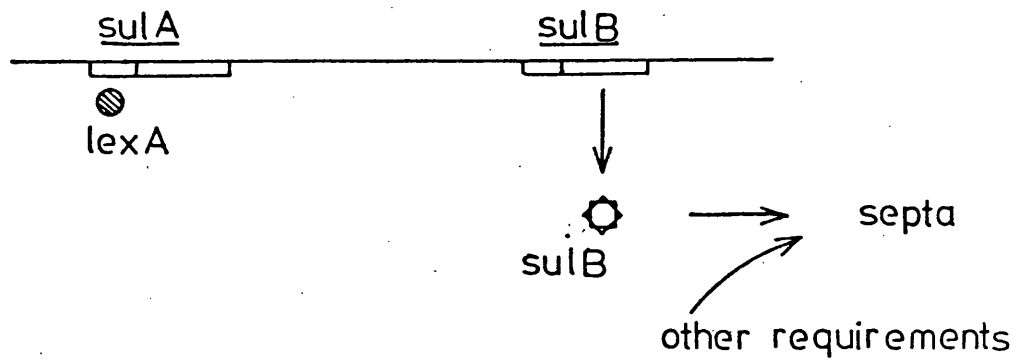
**Figure 8.1.** Proposed structure of the *ftsZ* - *sulB* region of the *E. coli* chromosome

$1-30\text{Jm}^{-2}$ ) suggesting that the amount of damage to the chromosome is an important factor in determining the duration of the division block in lon<sup>+</sup> cells. It would be interesting to know whether the kinetics of DNA synthesis and of recA synthesis (and therefore presumably recA\* activity) also vary with UV dose, as this would indicate whether recA and the proposed inducible division inhibitor have a common or similar induction mechanism.

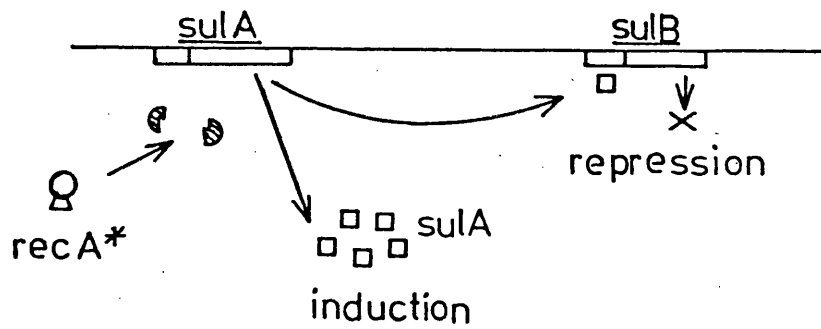
## 8.2 Location of sulB

A tsl, sfiB114 mutant, in which the sfiB(sulB) mutation was known to be recessive, was recently used to test for complementation by sulB<sup>+</sup> in  $\lambda\text{GH200}$  (R. D'Ari, personal communication after this project was completed). When the strain was lysogenised, sulB<sup>+</sup>, if present on the phage, should have restored temperature sensitive filamentation due to the tsl mutation, but failed to do so, indicating the absence of sulB<sup>+</sup> on  $\lambda\text{GH200}$ . This result also confirmed that sulB and envA are distinctly separate genes. These data bear on the interpretation of the effect of the multicopy plasmid (carrying the cloned fragment from this phage), in suppressing filamentation after UV. The best interpretation of the data is that the promoter region of the sulB gene is present on the 2.5Kb fragment (accounting for the titrating out of the division inhibitor in the multicopy situation) but that any coding sequence present is not sufficient to direct synthesis of an active or detectable polypeptide. The structural part of the sulB gene must therefore continue to the right of the 2.5Kb EcoRI fragment (fig.8.1). This region could now readily be isolated by lysogenisation with  $\lambda\text{GH200}$ , of a host deleted for att <sup>$\lambda$</sup>  as described for the extension of this phage to cover ftsA (Chapter 5), but using azi (azide

NORMAL



+UV



RECOVERY

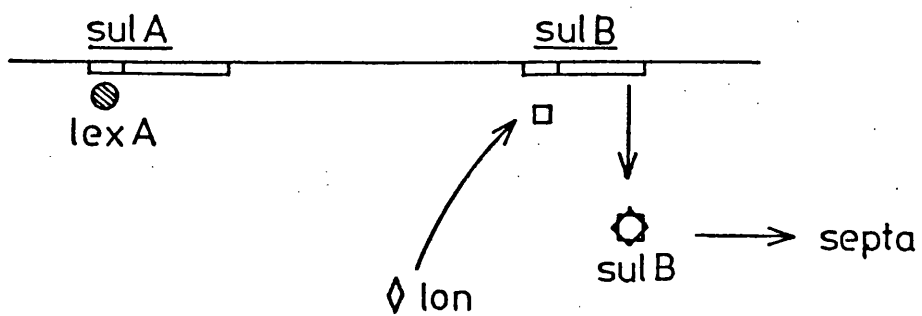


Figure 8.2 Model for *sulB* action

resistance) as a selective marker. The possible use of this marker is currently under investigation in this laboratory.

### 8.3 The inducible inhibitor

Using the technique of gene fusion (Casadaban and Cohen, 1979) D'Ari and coworkers have recently constructed strains in which the lacZ gene was fused to the sulA promoter region (R. D'Ari, personal communication). These workers found that several different SOS inducing treatments (UV, thymine starvation, tif or tsl, and several drugs) led to increased synthesis of  $\beta$ -galactosidase in the fusion mutant, confirming their earlier suggestion that sulA is inducible by the SOS signal.

Taking these data together with the multicopy effect of the proposed sulB promoter, models for the interaction of sulA and sulB can now be proposed. In one example (fig.8.2), conversion of recA to proteolytically active recA\* by the SOS effector leads to derepression of sulA by cleavage of a repressor (probably lexA). Newly synthesised sulA product then specifically inhibits division by repressing sulB, which codes a septum component (or enzyme or a cofactor essential for septum construction). The sulB product must normally be present in very low quantity, or turned over very rapidly for this mechanism to be effective, consistent with its activity being involved in a short, discrete event in the cell cycle (i.e. septation). The fact that mutations in sulB are isolated only rarely (Huisman *et al.*, 1980b) also suggests this type of vital or even essential role for sulB.

In this model, the supply of sulB is tightly (inversely) coupled to recA\* production after DNA damage. The model assumes that the sfiA,B system is responsible for the duration of the division delay (this point is

discussed further in 8.5), and that *recA\** is only active during the detectable period of *recA* induction (i.e. less than 60 min - Chapter 3).

Can this model account for the timing of division recovery, which takes place 30 min after *recA* induction is over after a UV dose of  $10\text{Jm}^{-2}$ ?

The *lon* gene product may have a role in this respect, the simplest proposal being that its protease activity may control the stability of some component in the system, possibly *sulA*. It is clear that the mechanism involved in transient division inhibition after UV involves *lon* in some way but it is difficult to see how these gene and gene product interactions could be further investigated without the use of operon fusions or *in vitro* studies with cloned genes. Gene fusion has already been used to identify several mitomycin C- and UV-inducible genes in *E.coli* (Kenyon and Walker, 1980).

Witkin (1976) discussed the way in which a single gene product (*recA*) can exert control over a diverse range of SOS functions. She suggested that the source of the diversity might reside in either a series of different repressors, all cleavable by one protease activity (*recA\**), or in a series of different inducible proteases. The evidence to date supports the first postulate, but further suggests that the (*lexA*) repressor might also be multifunctional, with different active sites enabling it to control several inducible genes. This would perhaps be surprising in such a small molecule (24K) but would nevertheless help to account for the split phenotypes of various *lexA* mutants described in Chapter 1.

#### 8.4 RecA and DNA replication

Some recent observations have suggested that *recA* may be involved in the DNA replication complex. D'Ari et al. (1979) noted that a *dnaB252* mutation, causing a conditional defect in initiation of DNA replication, suppressed expression of *tif*-mediated SOS functions. Since the *dnaB* product is an essential component of the replication complex (Kornberg, 1980), D'Ari et al. concluded that *tif* expression operated through the replication complex; since no effector molecule is necessary for *tif* expression, then *tif* (*recA*) itself must be part of the replisome.

One of the SOS functions listed in table 1.1 is "stable DNA replication" (Kogoma and Lark, 1970). Although DNA replication in progress is blocked by SOS inducing treatments, after a time new initiations at the origin of replication are observed, and replication follows which, e.g. after induction by thymine starvation, is semiconservative and appears to be normal in all other respects (Lark and Lark, 1979). However, the replication complexes assembled soon after an SOS inducing treatment which carry out this new synthesis, unlike normal replisomes, can continue to complete and to re-initiate new rounds of replication for many generations in the absence of protein synthesis (Kogoma and Lark, 1970). The formation of these stable replisomes is *recA* and *lexA* dependent and is also induced at non-permissive temperature in certain *dna*<sup>ts</sup> mutants (Kogoma et al., 1979; Lark and Lark, 1979). The stability of the "SOS" complexes was suggested to be due to *recA* acting in the complex, preventing the release or degradation of a replisome component, which would normally occur at termination of replication. Dominant conditional lethal mutations (*dnaT*) have been isolated in which the induction of stable replication is blocked, perhaps defining a protein

in the replication complex with which *recA* and *dnaB* might interact (Lark and Lark, 1979). These workers also showed that the *dnaT* mutation prevented error-prone repair, since in these mutants reversion of an auxotrophic defect by MMS was no longer enhanced. Evidence was also obtained that error-prone stable replication activity required continued protein synthesis after the SOS replisome had been assembled, indicating a requirement for a second inducible protein factor in addition to *recA*. Lark and Lark (1979) suggested a model in which *recA* (or presumably *recA\**) replaces *dnaT* in the replisome (removing it by proteolytic cleavage), producing a complex which is stable, and capable of error-prone repair. In this model it must be assumed that any essential function of *dnaT* during replication is served by *recA* (*recA\**). The transient nature of error-prone repair would be controlled by the second inducible factor and not directly by the stable replisome.

It is tempting to speculate that stable replication reflects a continuous involvement of *recA* in DNA replication, and perhaps also tempting to look for mechanisms by which this involvement could serve to couple DNA synthesis to division in normally growing cells. Such an involvement would explain the early observations (Chapter 1) that *recA* appeared to be the division inhibitor whatever stimulus is applied. However it should be remembered that *recA* cannot be an essential part of the replication complex since *recA<sub>am</sub>* and deletion mutants are viable and show normal DNA replication (McEntee, 1977; P. Oliver, personal communication).

## 8.5 Pathways of division control

This project focussed on what was thought to be a single pathway of division control elicited in response to DNA damage. However, while this work was in progress there have been indications that division inhibition after UV may involve more than one recA dependent pathway. Experiments in this laboratory by P. Burton have shown that at very low doses of UV ( $1-1.2\text{Jm}^{-2}$ ) the period of division inhibition observed in W.T. strains is abolished by mutations in sulA or sulB, and that filamentation induced by temperature shift in a tsl mutant is abolished by  $4\mu\text{g/ml}$  rif. In contrast, experiments described in Chapter 4 showed that rif had no effect on division inhibition after UV in wild type strains, and that sulA and B suppression of lon filamentation did not lead to abolition of the division block but allowed recovery after a period of filamentation. Taken together, these results show that at least two pathways must operate in irradiated cells. One of these pathways functions very rapidly after damage even at very low levels, is recA and sul dependent, rif sensitive and can be thought of as an "early" or "immediate" response. In sulA and sulB mutants, the division inhibition after a UV dose of  $10\text{Jm}^{-2}$  does not involve this early "component" and the division increment before the block is imposed is larger than in wild type strains (Chapter 4 and Appendix I). The concept of 2 independent pathways for division inhibition is consistent with certain observations which have been hitherto rather confusing; for example, Salta and Pardee (1978) had already reported that temperature-induced filamentation in tsl mutants was abolished by rif treatment. This can now be interpreted as further evidence that lexA itself has a direct role in the early response. Huisman et al., (1980c) observed that the onset of filamentation during

thymine starvation was delayed by sfiA and sfiB mutations. They also noted that in these mutants a persistent fraction (30%) of cells did not filament, and suggested that these are DNA-less cells which would be prevented from forming in sfi<sup>+</sup> cultures by the early, sfi dependent block.

Do these pathways reflect separate functions of division inhibition? It seems likely that the sfi/sul-dependent early system is the immediate "SOS" component - an emergency measure, triggered by the presence of DNA damage, to prevent segregation of damaged genomes or formation of DNA-less cells. The signal can be generated independently of replication and cells in D are subject to the block. The delayed onset of second-phase inhibition suggests that the production of the corresponding signal may depend in this case specifically on replication. Speculatively, this could reflect two possible mechanisms. The first is blockage of the replication complex at a non-coding lesion. These lesions, which somehow provide the trigger for the induction of error-prone replication capacity (i.e. error-prone "repair"), could also lead to a reinforcement of the division block by a separate recA dependent, rif resistant mechanism allowing more extensive post-replication and error-prone repair before division is allowed to resume. The other possibility is that the second-phase division block simply reflects delayed termination of replication. This would lead to the delayed production of a positive division control factor, normally synthesised at termination or released from the dissociating replication complex at termination, and probably involving recA or recA\* (as suggested in the account of stable replication). All the data are consistent with both division-inhibition pathways being recA dependent, and this factor has presented, and continues to present practical difficulties in distinguishing them.

All of these studies have still failed to reveal the role of lon. Evidence is accumulating (Huisman et al., (1980c), and experiments in progress in this laboratory) that the irreversible filamentation of lon strains after UV is triggered by the early pathway, suggesting that the inability to recover reflects the essential role of lon in turning off this pathway, as proposed above in a model of sulB action. If this suggestion is correct, the timing of the release from the 'early' division block should be controlled by lon-sul interaction. However, since this pathway cannot be isolated from the second-phase division block, it is not possible to determine which pathway finally fixes the timing of recovery.

The simple model described in 8.3, though useful, fails to account for all the data when the suppression of lon by sulB is considered. In a lon mutant the division block is permanently switched on; i.e. in terms of the model, sulA repression of sulB is irreversible. Mutations in sulA could lead to a reduction in the efficiency of repression of sulB and sulB transcription could therefore "escape" to promote division recovery. Taking into account that 1) nearly all sulB mutants studied so far are recessive, and 2) mutations in sulB which affect the binding of sulA (and hence the efficiency of repression) should be dominant, how can (recessive) mutations in sulB lead to escape of sulB synthesis?

This conceptual difficulty suggests that the proposed model of sulA and sulB and lon interaction is incomplete at best, and a rather more complex role for lon is required than has been hitherto suggested, since the ability of a recessive mutation in sulB to suppress the lon defect indicates a direct interaction between lon and sulB itself rather than sulA as suggested. However, many attempts to draw up an alternative, or improved model without substantially changing the role of sulB as a target for sulA

have not resolved this difficulty. Neither positive nor negative modulation of sulB transcription by lon, even acting independently of sulA, would allow for recessive suppressor mutations in sulB. Similarly, ascribing autoregulatory properties to sulB leads to the same impasse. If interactions at the level of transcription are precluded, could interactions of the gene products themselves provide the mechanism of suppression? Again recessiveness presents an insurmountable problem.

An alternative model in which sulA and sulB act together as the division inhibitor allows for recessive mutations in sulB to suppress the lon defect. In this case mutations in either sulA or sulB might reduce the effectiveness of the proposed sulA-sulB inhibitor complex, and such mutations could be recessive. When the sulB operator is present on a multicopy plasmid in a lon sulB background (Chapter 5), the SOS signal might still be titrated out if both sulA and sulB are repressed by lexA. In this situation, although cleavage of lexA may result in "derepression" of some genes, no sulB product can be made, and therefore no division block resulting from the sulA-sulB complex is possible. Inhibition of division due to the "second-phase" system, observed in the lon sulB strain was largely abolished in the presence of the multicopy plasmid. RecA\* molecules (on which the second pathway also depends) may have been titrated out by the extra copies of the sulB operator, leaving less recA\* activity available to induce the second-phase block.

It is perhaps important to remember that the multicopy effect of the proposed sulB operator region was observed in a lon<sup>-</sup> sulB<sup>-</sup> strain, i.e. the normal activity of lon was absent in this experiment, and thus the "titration" effect could have reflected an abnormal mechanism for promotion

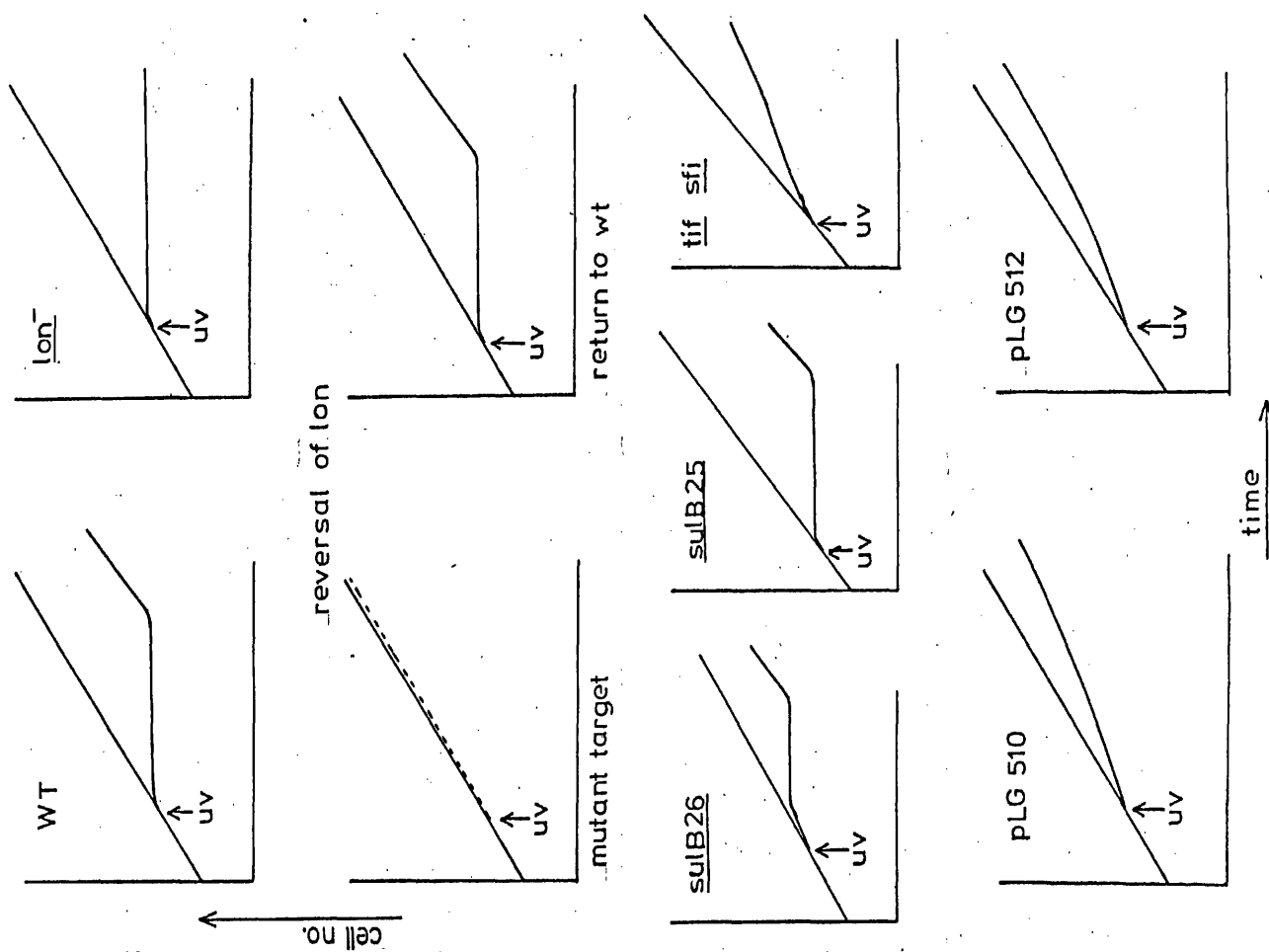
of division after UV. Since further physiological studies would be complicated by the inability to eliminate the "second phase" division block in order to study the sul pathway in isolation, it may well prove essential to perform in vitro studies on the (cloned) genes involved to elucidate sulA, sulB and lon interactions further.

Finally, any interpretation of these data should allow for the fact that new information regarding genetic control of division inhibition pathways is continually being obtained, (e.g. the infB mutation, see table 1.3). Though many of the new mutations have proved to map at well known loci, the probability still exists that important features of division control remain unrecognised.

In summary, this study has elucidated some aspects of division control after UV, characterising the contributions of some of the genetic elements involved. Investigation of the interaction of these elements has been made possible by the cloning of part of the sulB gene and much of the preparatory work has been done to enable the molecular mechanism of the division block to be demonstrated.

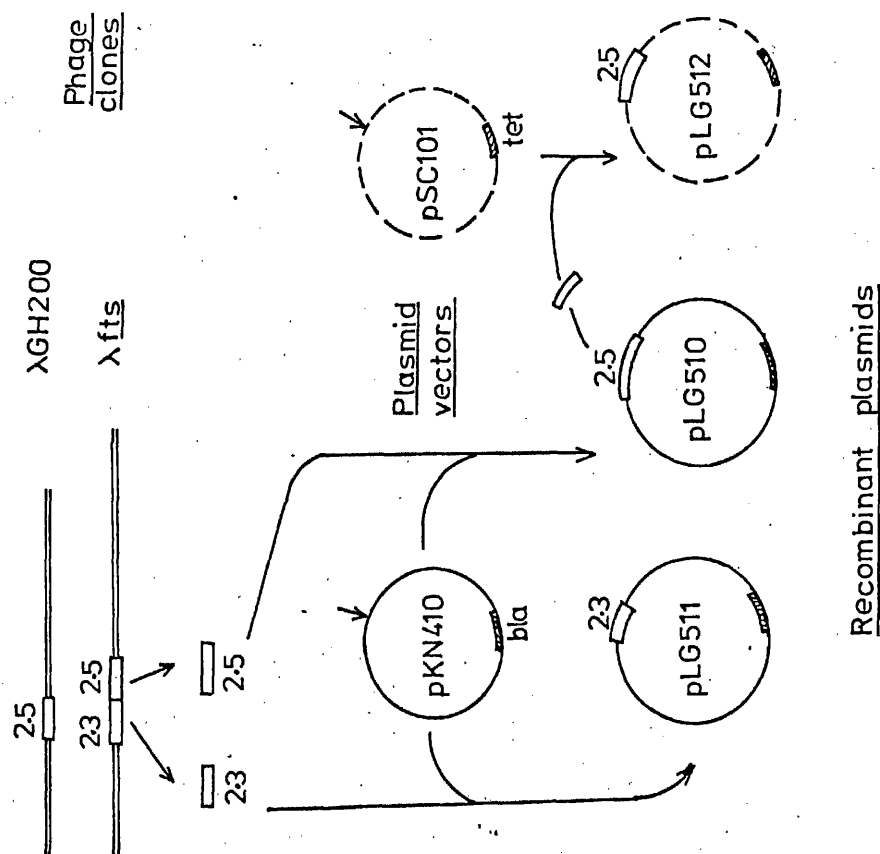
# Appendix I

Diagrammatic summary of division kinetics after UV for various strains, and theoretical kinetics for two mechanisms of reversal of the Lon phenotype. Data redrawn from figures in the text for comparative purposes.



## Appendix II

Summary of cloning experiments showing the relationship between recombinant plasmids constructed during the project.



Bibliography

- ADLER, H.I. and HARDIGREE, A.A. (1965)  
Growth and division of filamentous forms of Escherichia coli.  
J. Bacteriol. 90, 223-226.
- AMES, G.F. (1974).  
Resolution of bacterial proteins by polyacrylamide electrophoresis  
on slabs. J. Biol. Chem. 249, 634-644.
- BACHMANN, B. and LOW, K.B. (1980)  
Linkage map of Escherichia coli K-12, edition 6. Microbiol. Rev.  
44, 1-56.
- BAILONE, A., LEVINE, A. and DEVORET, R. (1979)  
Inactivation of prophage  $\lambda$  repressor in vivo. J. Mol. Biol.  
131, 553-572.
- BALUCH, J., CHASE, J.W. and SUSSMAN, R. (1980a)  
Synthesis of recA protein and induction of bacteriophage lambda in  
single-strand deoxyribonucleic acid-binding protein mutants of  
Escherichia coli. J. Bacteriol. 144, 489-498.
- BALUCH, J., SUSSMAN, R. and RESNICK, J. (1980b)  
Induction of prophage  $\lambda$  without amplification of recA protein.  
Molec. gen. Genet. 178, 317-323.
- BEGG, K.J. and DONACHIE, W.D. (1973)  
Topography of outer membrane growth in E.coli. Nature New Biol.  
245, 38-39.
- BEGG, K.J. and DONACHIE, W.D. (1977)  
Growth of the Escherichia coli cell surface. J. Bacteriol. 129,  
1524-1536.
- BEGG, K.J., HATFULL, G.F. and DONACHIE, W.D. (1980)  
Identification of new genes in a cell envelope - cell division gene  
cluster of Escherichia coli : cell division gene ftsQ. J. Bacteriol.  
144, 435-437.
- BLUMENTHAL, R.M. and DENNIS, P.P. (1978)  
Gene expression in E.coli during partial Rifampicin-mediated  
restrictions of transcription initiation. Molec. gen. Genet. 165,  
79-86.
- BOCKRATH, R.C. and HANAWALT, P.C. (1980)  
Ultraviolet light induction of recA protein in a recB uvrB mutant of  
Escherichia coli. J. Bacteriol. 143, 1025-1028.

- BOITEUX, S., VILLANI, G., SPADARI, S., ZAMBRANO, F. and RADMAN, M. (1978)  
In DNA repair mechanisms (ICN-UCLA symposia on molecular and cellular biology IX), eds. Hanawalt, P.C., Friedberg, E.C. and Fox, C.F. (Academic Press Inc.) pp.73-84.
- BONNER, W.M. and LASKY, R.A. (1974)  
A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46, 83-88.
- BOYD, A. (1979)  
Aspects of outer membrane protein synthesis in Escherichia coli B/r. Ph.D Thesis, University of Leicester.
- BOYD, A. and HOLLAND, I.B. (1977)  
Protein d, an iron-transport protein induced by filtration of cultures of Escherichia coli. *FEBS Letters.* 76, 20-24.
- BREMER, H. and CHURCHWARD, G. (1977)  
Inhibition of initiation of rounds of replication in E.coli B/r. *J. Bacteriol.* 130, 692-697.
- BRENT, R. and PTASHNE, M. (1980)  
The lexA gene product represses its own promoter. *Proc. Natl. Acad. Sci. USA.* 77, 1932-1936.
- BRIDGES, B.A., MOTTERSHEAD, R.P. and GREEN, M.H.L. (1977)  
Cell division in Escherichia coli B<sub>g</sub>-12 is hypersensitive to deoxy-ribonucleic acid damage by ultraviolet light. *J. Bacteriol.* 130, 724-728.
- BROOME-SMITH, J. (1980)  
Relaxation complexes and mobilisation properties of colE1 and related plasmids. Ph.D Thesis, University of Leicester.
- BROWN, M. (1980)  
Transformation of Escherichia coli by plasmid DNA. Ph.D Thesis, University of Liverpool.
- CAILLET-FAUQUET, P., DEFAIS, M. and RADMAN, M. (1977)  
Molecular mechanisms of induced mutagenesis. Replication in vivo of bacteriophage  $\phi$ X174 single-stranded, ultraviolet light - irradiated DNA in intact and irradiated host cells. *J. Mol. Biol.* 117, 95-112.
- CAMMACK, K.A. and WADE, H.E. (1965)  
The sedimentation behaviour of ribonuclease-active and -inactive ribosomes from bacteria. *Biochem. J.* 96, 671-680.
- CASADABAN, M.J. and COHEN, S.N. (1979)  
Lactose genes fused to exogenous promoters in one step using Mu-lac bacteriophage : In vivo probe for transcriptional control sequences. *Proc. Natl. Acad. Sci. USA.* 76, 4530-4533.

- CASTELLAZZI, M., MORAND, P., GEORGE, J. and BUTTIN, G. (1977)  
 Prophage induction and cell division in E.coli. V. Dominance and complementation analysis in partial diploids with pleiotropic mutations (tif, recA, zab and lexB) at the recA locus. Molec. gen. Genet. 153, 297-310.
- CLARK, A.J. and VOLKERT, M.R. (1978)  
 In DNA repair mechanisms (ICN-UCLA symposia on molecular and cellular biology IX), eds. Hanawalt, P.C., Friedberg, E.C. and Fox, C.F. (Academic Press Inc.) pp.57-72.
- COOPER, S. (1979)  
 A unifying model for the G1 period in prokaryotes and eukaryotes. Nature. 280, 17-19.
- COOPER, S. and HELMSTETTER, C.E. (1968)  
 Chromosome replication and the division cycle of Escherichia coli. J. Mol. Biol. 31, 519-540.
- CRAIG, N.L. and ROBERTS, J.W. (1980)  
E.coli recA protein - directed cleavage of phage  $\lambda$  repressor requires polynucleotide. Nature. 283, 26-30.
- CUNNINGHAM, R.P., SHIBATA, T., DASGUPTA, C. and RADDING, C.M. (1979)  
 Single strands induce recA protein to unwind duplex DNA for homologous pairing. Nature. 281, 191-195.
- DANEO-MOORE, L. and SHOCKMAN, D.G. (1977)  
 The bacterial cell surface in growth and division. Cell Surface Reviews. 4, 597-715.
- DARBY, V. and HOLLAND, I.B. (1979)  
 A kinetic analysis of cell division, and induction and stability of recA protein in U.V. irradiated lon<sup>+</sup> and lon<sup>-</sup> strains of Escherichia coli K12. Molec. gen. Genet. 176, 121-128.
- D'ARI, R., GEORGE, J. and HUISMAN, O. (1979)  
 Suppression of tif-mediated induction of SOS functions in Escherichia coli by an altered dnaB protein. J. Bacteriol. 140, 381-387.
- DEFAIS, M., CAILLET-FAUQUET, P., FOX, M.S. and RADMAN, M. (1976)  
 Induction kinetics of mutagenic DNA repair activity in Escherichia coli following ultraviolet irradiation. Molec. gen. Genet. 148, 125-130.
- DEFAIS, M., CAILLET-FAUQUET, P., RADMAN, M. and ERRERA, N. (1971)  
 Ultraviolet reactivation and ultraviolet mutagenesis of  $\lambda$  in different genetic systems. Virology. 43, 495-503.
- DHARMALINGAM, K. and GOLDBERG, E.B. (1980)  
 Restriction in vivo V. Induction of SOS functions in Escherichia coli by restricted T4 phage DNA, and alleviation of restriction by SOS functions. Molec. gen. Genet. 178, 51-58.

- DONACHIE, W.D. (1968)  
Relationship between cell size and time of initiation of DNA replication. *Nature*. 219, 1077-1079.
- DONACHIE, W.D. and BEGG, K.J. (1970)  
Growth of the bacterial cell. *Nature*. 227, 1220-1224.
- DONACHIE, W.D., BEGG, K.J., LUTKENHAUS, J.F., SALMOND, G.P.C., MARTINEZ-SALAS, E. and VINCENTE, M. (1979)  
Role of the ftsA gene product in control of Escherichia coli cell division. *J. Bacteriol.* 140, 388-394.
- DONACHIE, W.D., BEGG, K.J. and VICENTE, M. (1976)  
Cell length, cell growth and cell division. *Nature*. 264, 328-333.
- EMMERSON, P.T. and WEST, S.C. (1977)  
Identification of protein X of E.coli as the recA<sup>+</sup>/tif<sup>+</sup> gene product. *Molec. gen. Genet.* 155, 77-85.
- EPHRATI-ELIZUR, E. and LUTHER-DAVIES, S. (1977)  
Phenotypic instability in a tif-1 mutant of Escherichia coli. *Molec. gen. Genet.* 155, 163-169.
- FLETCHER, G., IRWIN, C.A., HENSON, J.M., FILLINGIM, C., MALONE, M.M. and WALKER, J.R. (1978)  
Identification of the Escherichia coli gene sep and organisation of the cell division - cell envelope genes in the sep-mur-ftsA-envA cluster as determined with specialized lambda bacteriophage. *J. Bacteriol.* 133, 91-100.
- FRAZER, A.C. and CURTISS, R. III. (1975)  
Production, properties and utility of bacterial minicells. *Curr. Top. Microbiol. Immunol.* 69, 1-84.
- GAYDA, R.C. and MARKOVITZ, A. (1978)  
Altered bacteriophage lambda expression in cell division mutants capR(lon) of Escherichia coli K-12. *Molec. gen. Genet.* 159, 1-11.
- GAYDA, R.C., YAMAMOTO, L.T. and MARKOVITZ, A. (1976)  
Second site mutations in capR(lon) strains of E.coli K12 that prevent radiation sensitivity and allow bacteriophage  $\lambda$  to lysogenise. *J. Bacteriol.* 127, 1208-1216.
- GEORGE, J., CASTELLAZZI, M. and BUTTIN, G. (1975)  
Prophage induction and cell division in E.coli. III Mutations sfiaA and sfiaB restore division in tif and lon strains and permit the expression of mutator properties of tif. *Molec. gen. Genet.* 140, 309-332.
- GUDAS, L.J. (1976)  
The induction of protein X in DNA repair and cell division mutants of Escherichia coli. *J. Mol. Biol.* 104, 567-587.

- GUDAS, L.J. and MOUNT, D.W. (1977)  
Identification of the recA (tif) gene product of Escherichia coli.  
Proc. Natl. Acad. Sci., USA. 74, 5280-5284.
- GUDAS, L.J. and PARDEE, A.B. (1975)  
Model for regulation of Escherichia coli DNA repair functions.  
Proc. Natl. Acad. Sci., USA. 72, 2330-2334.
- GUDAS, L.J. and PARDEE, A.B. (1976)  
DNA synthesis inhibition and the induction of protein X in Escherichia coli. J. Mol. Biol. 101, 459-477.
- HANAWALT, P.C., FRIEDBERG, E.C. and FOX, C.F. (Eds). (1978)  
ICN-UCLA symposia on molecular and cellular biology IX. DNA repair mechanisms. (Academic Press Inc.)
- HANAWALT, P.C. and SMITH, C.A. (1978)  
In : DNA repair mechanisms (ICN-UCLA symposia on molecular and cellular biology IX), eds. Hanawalt, P.C, Friedberg, E.C. and Fox, C.F. (Academic Press Inc.) pp.47-56.
- HASHIMOTO-GOTOH, T. and SEKIGUCHI, M. (1977)  
Mutations to temperature sensitivity in R-plasmid pSC101. J. Bact. 131, 404-412.
- HELMSTETTER, C.E. and PIERUCCI, O. (1976)  
DNA synthesis during the division cycle of three substrains of Escherichia coli B/r. J. Mol. Biol. 102, 477-486.
- HELMSTETTER, C.E., PIERUCCI, O., WEINBERGER, M., HOLMES, M. and TANG, M-S. (1979)  
In: The bacteria, vol. VII; Mechanisms of adaptation, eds. Sokatch, J.R. and Ornston, L.N. (Academic Press Inc.) pp.517-579.
- HERRERO, E., ORR, E. and HOLLAND, I.B. (1981)  
Induction of recA protein : effect of inactivation of DNA gyrase. FEMS. Lett. in press.
- HIATT, H.H., WATSON, J.D. and WINSTEN, J.A. (1977)  
Cold Spring Harbour conferences on cell proliferation, 4. Origins of human cancer. (Cold Spring Harbour Laboratory).
- HOLTJE, J.V., MIRELMAN, D., SHARON, N. and SCHWARZ, U. (1975)  
Novel type of murein transglycosylase in Escherichia coli. J. Bacteriol. 124, 1067-1076.
- HOWARD-FLANDERS, P. (1978)  
In DNA repair mechanisms (ICN-UCLA symposia on molecular and cellular biology IX) eds. Hanawalt, P.C., Friedberg, E.C. and Fox, C.F. (Academic Press Inc.) pp.105-111.

- HOWARD-FLANDERS, P., SIMSON, E. and THERIOT, L. (1964)  
A locus that controls filament formation and sensitivity to radiation in Escherichia coli K12. Genetics. 49, 237-246.
- HUISMAN, O., D'ARI, R. and GEORGE, J. (1980a)  
Dissociation of tsl-tif-induced filamentation and recA protein synthesis in Escherichia coli K-12. J. Bacteriol. 142, 819-828.
- HUISMAN, O., D'ARI, R. and GEORGE, J. (1980b)  
Further characterisation of sfiA and sfiB mutations in Escherichia coli. J. Bacteriol. 144, 185-191.
- HUISMAN, O., D'ARI, R. and GEORGE, J. (1980c)  
Inducible sfi dependent division inhibition in Escherichia coli. Molec. gen. Genet. 177, 629-636.
- INOUE, M. (1971)  
Pleiotropic effects of the recA gene of Escherichia coli : uncoupling of cell division from DNA replication. J. Bacteriol. 106, 539-542.
- INOUE, M. and PARDEE, A.B. (1970)  
Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of Escherichia coli. J. Biol. Chem. 245, 5813-5819.
- IRWIN, C.A., FLETCHER, G., SILLS, C.L. and WALKER, J.R. (1979)  
Expression of the E.coli cell division gene sep cloned in a  $\lambda$  Charon phage. Science. 206, 220-222.
- IRWIN, C.A. and WALKER, J.R. (1980)  
Cloning and expression of the Escherichia coli cell division gene sep. (Abstract) EMBO Workshop: Duplication of Bacteria, Noordwijkerhout, Netherlands.
- JOHNSON, B.F. (1977a)  
Genetic mapping of the lexC-113 mutation. Molec. gen. Genet. 157, 91-97.
- JOHNSON, B.F. (1977b)  
Fine structure mapping and properties of mutations suppressing the lon mutation in Escherichia coli K-12 and B strains. Genet. Res. Camb. 30, 273-286.
- JOHNSON, R.C. and McNEILL, W.F. (1978)  
In DNA repair mechanisms (ICN-UCLA symposia on molecular and cellular biology IX), eds. Hanawalt, P.C., Friedberg, E.C. and Fox, C.F. (Academic Press Inc.) pp.95-99.
- JONES, N.C. and DONACHIE, W. (1973)  
Chromosome replication, transcription and control of cell division in Escherichia coli. Nature. 247, 100-103.

- KANTOR, G.C. and DEERING, R.A. (1966)  
Ultraviolet radiation studies on filamentous Escherichia coli.  
J. Bacteriol. 92, 1062-1069.
- KENYON, C.J. and WALKER, G.C. (1980)  
DNA-damaging agents stimulate gene expression at specific loci in  
Escherichia coli. Proc. Nat. Acad. Sci. USA. 77, 2819-2823.
- KESSLER, S.W. (1975)  
Rapid isolation of antigens from cells with a Staphylococcal protein  
A - antibody adsorbent : parameters of the interaction of antibody-  
antigen complexes with protein A. J. Immunol. 115, 1617-1624.
- KOCH, A.L. (1977)  
Does the initiation of chromosome replication regulate cell division?  
Adv. Micro. Physiol. 16, 49-98.
- KOGOMA, T. and LARK, K.G. (1970)  
DNA replication in Escherichia coli : Replication in absence of protein  
synthesis after replication inhibition. J. Mol. Biol. 52, 143-164.
- KOGOMA, T., TORREY, T.A. and CONNAUGHTON, M.J. (1979)  
Induction of UV resistant DNA replication in Escherichia coli :  
Induced stable DNA replication as an SOS function. Molec. gen.  
Genet. 176, 1-9.
- KORNBERG, A. (1980)  
DNA replication. (San Francisco : W.H. Freeman).
- LAEMMLI, U.K. (1970)  
Cleavage of structural proteins during the assembly of the head of  
bacteriophage T4. Nature. 227, 680-685.
- LARK, K.G. and LARK, C. (1979)  
RecA dependent DNA replication in the absence of protein synthesis :  
characteristics of a dominant lethal replication mutation dnaT and  
requirement for recA<sup>+</sup> function. Cold Spring Harbour Symp. Quant.  
Biol. 43, 537-549.
- LITTLE, J.W., EDMISTON, S.H., PACELLI, L.Z. and MOUNT, D.W. (1980)  
Cleavage of the Escherichia coli lexA protein by the recA protease.  
Proc. Natl. Acad. Sci. USA. 77, 3225-3229.
- LITTLE, J.W. and HANAWALT, P.C. (1977)  
Induction of protein X in Escherichia coli. Molec. gen. Genet.  
150, 237-248.
- LITTLE, J.W. and KLEID, D.G. (1977)  
Escherichia coli protein X is the recA gene product. J. Biol. Chem.  
252, 6251-6252.

- LUTKENHAUS, J.F. and DONACHIE, W. (1979)  
Identification of the ftsA gene product. J. Bacteriol. 137, 1088-1094.
- LUTKENHAUS, J., MOORE, B.A., MASTERS, M. and DONACHIE, W. (1979)  
Individual proteins are synthesised continuously throughout the Escherichia coli cell cycle. J. Bacteriol. 138, 352-360.
- LUTKENHAUS, J.F., WOLF-WATZ, H. and DONACHIE, W. (1980)  
Organisation of genes in the ftsA-envA region of the Escherichia coli genetic map and identification of a new fts locus (ftsZ). J. Bacteriol. 142, 615-620.
- LUTKENHAUS, J.F. and WU, H.C. (1980)  
Determination of transcriptional units and gene products from the ftsA region of Escherichia coli. J. Bacteriol. 143, 1281-1288.
- MACKIE, G. and WILSON, D.B. (1972)  
Regulation of the gal operon of Escherichia coli by the capR gene. J. Biol. Chem. 247, 2973-2978.
- MARKOVITZ, A. (1977)  
In: Surface carbohydrates of the prokaryote cell. ed. Sutherland, I. Academic Press. pp.415-462.
- McENTEE, K. (1977)  
Protein X is the product of the recA gene of Escherichia coli. Proc. Natl. Acad. Sci. USA. 74, 5275-5279.
- McENTEE, K., WEINSTOCK, G.M. and LEHMAN, I.R. (1979)  
Initiation of general recombination catalysed in vitro by the recA protein of Escherichia coli. Proc. Nat. Acad. Sci. USA. 76, 2615-2619.
- McPARTLAND, A., GREEN, L. and ECHOLS, H. (1980)  
Control of the recA gene in E.coli : Regulatory and signal genes. Cell. 20, 731-737.
- MEACOCK, P.A. (1975)  
Chromosome replication and cell division in Escherichia coli. Ph.D. Thesis, University of Leicester.
- MEYN, S., ROSSMAN, T. and TROLL, W. (1977)  
A protease inhibitor blocks SOS functions in Escherichia coli. Antipain prevents  $\lambda$  repressor inactivation, ultraviolet mutagenesis and filamentous growth. Proc. Natl. Acad. Sci. USA. 74, 1152-1156.
- MOLNÁR, J., HOLLAND, I.B. and MÁNDI, Y. (1977)  
Selection of lon mutants in E.coli by treatment with phenothiazines. Genet. Res. Camb. 30, 13-21.

- MONK, M. and KINROSS, J. (1975)  
Kinetics of derepression of prophage  $\lambda$  following UV irradiation of lysogenic cells. *Molec. gen. Genet.* 137, 263-268.
- MOODY, E.E.M., LOW, K.B. and MOUNT, D.W. (1973)  
Properties of strains of *Escherichia coli* K12 carrying mutant lex and rec alleles. *Molec. gen. Genet.* 121, 197-205.
- MORAND, P., GOZE, A. and DEVORET, R. (1977)  
Complementation pattern of lexB and recA mutations in *Escherichia coli* K12; mapping of tif-1, lexB and recA mutations. *Molec. gen. Genet.* 157, 69-82.
- MOREAU, P.L., FANICA, M. and DEVORET, R. (1980)  
Induction of prophage  $\lambda$  does not require full induction of RecA protein synthesis. *Biochimie.* 62, 687-694.
- MOUNT, D.W. (1977)  
A mutant of *Escherichia coli* showing constitutive expression of the lysogenic induction and error-prone DNA repair pathways. *Proc. Natl. Acad. Sci.* 74, 300-304.
- MOUNT, D.W., LOW, K.B. and EDMISTON, S.J. (1972)  
Dominant mutations (lex) in *Escherichia coli* K12 which affect radiation sensitivity and frequency of ultraviolet light-induced mutations. *J. Bacteriol.* 112, 886-893.
- MOUNT, D.W., WALKER, A.C. and KOSEL, C. (1975)  
In: *Molecular mechanisms for repair of DNA*, eds. Hanawalt, P.C. and Setlow, R.B. (Plenum Publ. Corp., New York), Part A, pp.383-388.
- NELSON, T. and BRUTLAG, D. (1979)  
Addition of homopolymers to the 3'-ends of duplex DNA with terminal transferase. *Methods in Enzymology.* 68, 41-50.
- NORMARK, S. (1970)  
Genetics of a chain forming mutant of *E.coli* : Transduction and dominance of the envA gene mediating increased penetration to some bacterial agents. *Genet. Res.* 16, 63-78.
- O'FARRELL, P.H. (1975)  
High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021.
- OISHI, M. and SMITH, C.L. (1978)  
Inactivation of phage repressor in a permeable cell system : role of recBC DNase in induction. *Proc. Natl. Acad. Sci. USA.* 75, 3569-3573.
- PARDEE, A.B. (1974)  
The biochemical requirements for bacterial division. *Eur. J. Biochem.* 43, 209-213.

- PARKINSON, J.S. and HUSKEY, R.J. (1971)  
Deletion mutants of bacteriophage lambda. *J. Mol. Biol.* 56, 369-384.
- PEDERSON, S., BLOCH, P.L., REEH, S. and NEIDHARDT, F.C. (1978)  
Patterns of protein synthesis in *E.coli* : a catalogue of the amount of 140 individual proteins at different growth rates. *Cell.* 14, 179-190.
- PIERUCCI, O. and HELMSTETTER, C.E. (1969)  
Chromosome replication, protein synthesis and cell division in *Escherichia coli*. *Fed. Proc.* 28, 1755-1760.
- PRATT, J.M., HOLLAND, I.B. and SPRATT, B.G. (1981)  
*In vitro* synthesis and processing of precursor forms of penicillin binding proteins 5 and 6 of the *E.coli* cytoplasmic membrane. Submitted for publication.
- PRITCHARD, R.H. (1968)  
Control of DNA synthesis in bacteria. *Heredity.* 23, 472.
- PRITCHARD, R.H. (1974)  
On the growth and form of the bacterial cell. *Philos. Trans. Roy. Soc.* 267, 303-336.
- PRITCHARD, R.H., BARTH, P.T. and COLLINS, J. (1969)  
Control of DNA synthesis in bacteria. XIX Microbial growth, Symp. Soc. Gen. Microbiol. 263-297.
- PRITCHARD, R.H. and ZARITSKY, A. (1970)  
Effect of thymine concentration on the replication velocity of DNA in a thymineless mutant of *E.coli*. *Nature.* 226, 126-131.
- PTASHNE, M. (1967)  
Isolation of the  $\lambda$  phage repressor. *Proc. Natl. Acad. Sci. USA.* 57, 306-313.
- RADMAN, M. (1975)  
In: Molecular mechanisms for repair of DNA, eds. Hanawalt, P.C. and Setlow, R.B. (Plenum Publ. Corp. New York), part A, 355-369.
- RADMAN, M., VILLANI, G., BOITEAUX, S., DEFAIS, M., CAILLET-FAUQUET, P. and SPADARI, S. (1977)  
In: Origins of human cancer, eds. Hiatt, H.H., Watson, J.D. and Winstin, J.A. (Cold Spring Harbor). Book B. 903-922.
- ROBERTS, J.W. and ROBERTS, C.W. (1975)  
Proteolytic cleavage of bacteriophage lambda repression in induction. *Proc. Natl. Acad. Sci. USA.* 72, 147-151.
- ROBERTS, J.W., ROBERTS, C.W. and CRAIG, N.L. (1978)  
*Escherichia coli* *recA* gene product inactivates phage  $\lambda$  repressor. *Proc. Natl. Acad. Sci. USA.* 75, 4714-4718.

- ROTHMAN, R.H., MARGOSSIAN, L.J. and CLARK, A.J. (1979)  
W-reactivation of phage lambda in recF recL uvrA and uvrB mutants of E.coli K-12. *Molec. gen. Genet.* 169, 279-287.
- ROYCHOUDHURY, R. and WU, R. (1980)  
Terminal-transferase-catalysed addition of nucleotides to the 3' termini of DNA. *Methods in Enzymology.* 65, 43-62.
- RUPP, W.D. and HOWARD-FLANDERS, P. (1968)  
Discontinuities in the DNA synthesised in an excision-defective strain of Escherichia coli following ultraviolet irradiation. *J. Mol. Biol.* 31, 291-304.
- RYTER, A., HIROTA, Y. and SCHWARZ, U. (1973)  
Process of cellular division in Escherichia coli. Growth pattern of E.coli murein. *J. Mol. Biol.* 78, 185-195.
- RYTER, A., SHUMAN, H. and SCHWARTZ, M. (1975)  
Integration of the receptor for bacteriophage lambda in the outer membrane of Escherichia coli : coupling with cell division. *J. Bacteriol.* 122, 295-301.
- SALMOND, G.P.C., LUTKENHAUS, J.F. and DONACHIE, W.D. (1980)  
Identification of new genes in a cell envelope - cell division gene cluster of Escherichia coli : cell envelope gene murG. *J. Bacteriol.* 144, 438-440.
- SATTA, G., GUDAS, L.J. and PARDEE, A.B. (1979)  
Degradation of Escherichia coli DNA : evidence for limitation in vivo by protein X. *Molec. gen. Genet.* 168, 69-80.
- SATTA, G. and PARDEE, A.B. (1978)  
Inhibition of Escherichia coli division by protein X. *J. Bacteriol.* 133, 1492-1500.
- SCHRENK, W.J. and WEISBERG, R.A. (1975)  
A simple method for making new transducing lines of coliphage  $\lambda$ . *Molec. gen. Genet.* 137, 101-107.
- SEDGWICK, S.G. (1975)  
Ultraviolet inducible protein associated with error-prone repair in E.coli B. *Nature.* 255, 349-350.
- SEDGWICK, S.G. (1976)  
Misrepair of overlapping daughter strand gaps as a possible mechanism for UV induced mutagenesis in uvr strains of Escherichia coli : a general model for induced mutagenesis by misrepair (SOS repair) of closely spaced DNA lesions. *Mutation Res.* 41, 185-200.

- SEDGWICK, S.G., LEVINE, A. and BAILONE, A. (1978)  
Induction of recA<sup>+</sup>-protein synthesis in Escherichia coli. Molec. gen. Genet. 160, 267-276.
- SHIMADA, K., WEISBERG, R.A. and GOTTESMAN, M.E. (1972)  
Prophage lambda at unusual chromosomal locations. I Location of secondary attachment sites and the properties of lysogens. J. Mol. Biol. 63, 483-503.
- SHINAGAWA, K. and ITOH, T. (1973)  
Inactivation of DNA binding activity of repressor in extracts of lambda lysogens treated with mitomycin C. Mol. gen. Genet. 126, 103-110.
- SHINEBERG, B. and ZIPSER, D. (1973)  
The lon gene and degradation of B-galactosidase nonsense fragments. J. Bacteriol. 116, 1469-1471.
- SHOCKMAN, D.G., DANE0-MOORE, L. and HIGGINS, M.L. (1974)  
Problems of cell wall and membrane growth, enlargement and division. Ann. N.Y. Acad. Sci. 235, 161-197.
- SLATER, M. and SCHAECHTER, M. (1974)  
Control of cell division in bacteria. Bacteriol. Rev. 38, 199-221.
- SMITH, K.C. (1969)  
DNA synthesis in sensitive and resistant mutants of Escherichia coli B after ultraviolet irradiation. Mutation Res. 8, 481-495.
- SOMPEYRAC, L. and MAALØE, O. (1973)  
Autorepressor model for control of DNA replication. Nature. New Biol. 241, 133-135.
- SONNTAG, I., SCHWARZ, H., HIROTA, Y. and HENNING, U. (1978)  
Cell envelope and shape of Escherichia coli : multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. 136, 280-285.
- SPRATT, B.G. (1975)  
Distinct penicillin-binding proteins involved in the division, elongation and shape of Escherichia coli K12. Proc. Natl. Acad. Sci. USA. 72, 2999-3003.
- SPRATT, B.G. (1978)  
The mechanism of action of penicillin. Sci. Prog. Oxf. 65, 101-128.
- SWENSON, P.A., JOSHI, J.G. and SCHENLEY, R.L. (1978)  
Regulation of cessation of respiration and killing by cyclic 3',5'-adenosine monophosphate and its receptor protein after far-ultraviolet irradiation of Escherichia coli. Molec. gen. Genet. 159, 125-130.
- TESSMAN, E.S., GRITZMACHER, C.A. and PETERSON, P.K. (1978)  
Derepression of colicin E1 synthesis in the constitutive tif mutant strain (spr tif sfi) and in a tif sfi mutant strain of Escherichia coli K-12. J. Bacteriol. 135, 29-38.

- TESSMAN, E.S. and PETERSON, P.K. (1980)  
tif-dependent induction of colicin E1, prophage lambda, and filamentation in Escherichia coli K-12. J. Bacteriol. 143, 1307-1317.
- TRUITT, C.L., HALDENWANG, W.G. and WALKER, J.R. (1976)  
 Interaction of host and viral regulatory mechanisms : effect of the lon cell division defect on regulation of repression by bacteriophage lambda. J. Mol. Biol. 105, 231-244.
- UHLIN, B.E., MOLIN, S., GUSTAFSSON, P. and NORDSTROM, K. (1979)  
 Plasmids with temperature-dependent copy number for amplification of cloned genes and their products. Gene. 6, 91-106.
- VAN HEIJENOORT, J., PARQUET, C., FLOURET, B. and VAN HEIJENOORT, Y. (1975)  
 Envelope-bound N-acetylmuramyl-L-alanine amidase of Escherichia coli K12. Eur. J. Biochem. 58, 611-619.
- VOLKERT, M.R., SPENCER, D.F. and CLARK, A.J. (1979)  
 Indirect and intragenic suppression of the lexA102 mutation in E.coli B/r. Molec. gen. Genet. 177, 129-137.
- WALKER, J.R., USSERY, C.L. and SMITH, J.S. (1973)  
 Bacterial cell division regulation : Lysogenisation of conditional cell division lon<sup>-</sup> mutants of Escherichia coli by bacteriophage lambda. J. Bacteriol. 113, 1326-1332.
- WEINSTOCK, G.M., McENTEE, K. and LEHMAN, I.R. (1979)  
 ATP-dependent renaturation of DNA catalysed by the recA protein of Escherichia coli. Proc. Natl. Acad. Sci. USA. 76, 126-130.
- WIJSMAN, H.J.W. and KOOPMAN, C.R.M. (1976)  
 The relation of the genes envA and ftsA in Escherichia coli. Molec. gen. Genet. 147, 99-102.
- WITKIN, E. (1967)  
 The radiation sensitivity of Escherichia coli B : a hypothesis relating filament formation and prophage induction. Proc. Natl. Acad. Sci. USA. 57, 1275-1279.
- WITKIN, E. (1976)  
 Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol. Rev. 40, 869-907.
- YOSHIKAWA, H., O'SULLIVAN, A. and SUEOKA, N. (1964)  
 Sequential replication of the Bacillus subtilis chromosome, III. Regulation of initiation. Proc. Natl. Acad. Sci. USA. 52, 973-980.
- ZEHNBAUER, B.A. and MARKOVITZ, A. (1980)  
 Cloning of gene lon (capR) of Escherichia coli K12 and identification of polypeptides specified by the cloned deoxyribonucleic acid fragment. J. Bacteriol. 143, 852-863.
- ZUBAY, G. (1973)  
In vitro synthesis of protein in microbial systems. Ann. Rev. Genet. 7, 267-287.

### Addendum

Although it was suggested on page 71 that the increment of dividing cells during thymine starvation theoretically measures cells in the D period, it has not been proved that this is true experimentally. I therefore wish to state that the conclusion drawn in chapter 1, that cells in D are inhibited from division by UV, cannot be deduced with certainty from the experiments described here (although recent data not discussed in this thesis do support this proposal).

Chapter 3 describes experiments which show that the large amounts of recA protein which are induced after UV are not directly responsible for division inhibition. It is suggested that a modified form of the protein, recA<sup>\*</sup> is directly involved. Subsequently, in Chapter 4 and elsewhere, when it is stated that "newly synthesised" recA is not the division inhibitor, this phrase refers to unmodified recA molecules and does not include recA<sup>\*</sup>.

The reader's attention is drawn to pp. 98-99, 103, 124, where it is stated that the experiments discussed here have not provided conclusive evidence that sulB has been cloned. Where the text assumes that sulB may have been cloned, this was for the purpose of the discussion only, and should not be interpreted as a claim that sulB has been successfully cloned.

# Inhibition of cell division in *Escherichia coli* by ultraviolet radiation

by Valerie Darby

## Abstract

The inhibition of cell division after UV in wild-type bacteria is considered to be an "SOS" function under the control of recA; this control was the subject of the investigation.

A kinetic study of division inhibition and recA synthesis after a low dose of UV indicated that recA did not act directly to inhibit division. Further, recA synthesis was uncoupled from the division block in the presence of low levels of rifampicin. Nevertheless, recA is essential for division inhibition after UV.

A lon mutant, which unlike lon<sup>+</sup> strains did not recover from the division block, showed no alteration in the kinetics of recA induction. A mutation in a second gene, sulB, suppressed the lethal effect of UV on lon mutants by promoting recovery after a period of division inhibition; possible mechanisms of suppression are considered.

To investigate the interaction between sulB, recA and lon at the molecular level, a  $\lambda$  transducing phage was obtained carrying the proposed sulB region of the chromosome. The transducing DNA was recombined into a plasmid vector and a strain containing the recombinant plasmid showed an altered division response after UV, suggesting that a division control gene was present in the cloned DNA. Gene products coded by the transducing phage and the recombinant plasmids were demonstrated by in vitro and semi-in vivo techniques and the in vitro systems was developed further with the object of detecting possible transcriptional control of cloned genes by recA.

The results are considered in the light of some recent developments having important implications for the project.

## A Kinetic Analysis of Cell Division, and Induction and Stability of *recA* Protein in U.V. Irradiated *lon*<sup>+</sup> and *lon*<sup>-</sup> Strains of *Escherichia coli* K12

Valerie Darby and I.B. Holland

Department of Genetics, University of Leicester, Leicester, LE1 7RH, England

**Summary.** Kinetic analysis of induction of *recA* protein synthesis after U.V. irradiation does not show correspondence with the kinetics of division inhibition in *lon*<sup>+</sup> and *lon*<sup>-</sup> strains, but there is correlation between induction and DNA repair activity. Protein X is stable and identical in both *lon*<sup>+</sup> and *lon*<sup>-</sup> strains. When the induction of *recA* protein after U.V. is drastically reduced by rifampicin treatment, no effect on the kinetics of division inhibition is observed.

### Introduction

The *lon* mutation is a conditional division lesion in *E. coli* conferring U.V. sensitivity due to a permanent failure to divide after irradiation (Adler and Hardigree, 1965). This mutation does not impair excision and post-replication repair of U.V. induced DNA damage (Howard-Flanders et al., 1964; Smith, 1969) and it is therefore unlikely that permanent loss of division capability is accounted for, at least in excision proficient strains, by the continued presence of U.V. photo-products.

It is now well established that U.V. irradiation of wild-type bacteria initiates SOS or error-prone repair, a *recA* dependent system possibly involved in correcting damage to DNA which is inaccessible to the major repair enzymes. Evidence has been presented which indicates that SOS repair is an inducible system and therefore dependent upon protein synthesis (see Witkin, 1976). However, Bridges and Mottershead (1978) have obtained data which indicates that error-prone repair is also a constitutive function whose expression is perhaps enhanced by concomitant protein synthesis after irradiation. The SOS repair system is induced by most treatments which cause

damage to DNA or inhibit replication (Radman, 1975; Witkin, 1976) and includes a wide variety of effects: mutagenic or error-prone repair of DNA damage, induction of  $\lambda$  lysogens, inhibition of cell division, increased ability to repair damaged infecting phages (giving higher phage survival and mutation rates), and synthesis of large quantities of a new protein, termed "X" (Inouye and Pardee, 1970; Gudas and Pardee, 1975). Recent studies have established that protein X is the *recA* gene product (Emmerson and West, 1977; McEntee, 1977), a protein which binds to single stranded DNA causing "re-winding" of homologous strands (Weinstock et al., 1979) and has an associated proteolytic activity responsible for cleavage of the *cI* repressor of phage  $\lambda$  (Roberts et al., 1978). In addition, the properties of a number of *recA* mutants suggest (Emmerson and West, 1977; Gudas and Mount, 1977) that the *recA* protein is required for its own synthesis. This concept of self-regulation is consistent with the proposal that the variety of responses associated with SOS induction are all co-ordinately controlled by this protein, either directly or by regulating the expression of other genes, in the same way as it affects its own synthesis. The most commonly accepted hypothesis of the mechanism by which *recA* protein might carry out this control is that an effector molecule, widely assumed to be a DNA degradation product, interacts with *recA* protein to produce a change in the molecule. This change allows protein X to inactivate the *lexA*-coded repressor of the *recA* gene, leading to derepression, and expression of the SOS functions.

This general model assumes that U.V. induced division inhibition is an SOS function under *recA* control and there have been some specific suggestions how this might be brought about. Thus Satta and Pardee (1978), noting the affinity of *recA* protein for single stranded DNA, proposed that this protein might interfere directly with septum formation by

For offprints contact: V. Darby

binding to single-stranded DNA at the presumed chromosomal-envelope attachment site near the cell septum. George et al. (1975) suggested that a "repair-associated division inhibitor" might block septation in response to DNA damage, predicting the involvement of a molecule with properties similar to those of *recA* protein. Further, George et al. (1975) suggested that the *lon* gene product might affect the stability of such a division inhibitor, drawing on the observation that Deg (*lon*) mutants show increased stability of nonsense polypeptide fragments of  $\beta$ -galactosidase over that in wild type bacteria. The fact that *recA*, *lon* double mutants continue to divide almost normally after irradiation confirms the dependence of Lon expression on the *recA* gene (George et al., 1975).

The hypothesis that *recA* protein is directly responsible for filamentation cannot be adequately tested by simply observing synthesis of *recA* protein in the qualitative systems which have been widely used. The experiments described below were designed to measure the precise kinetics of *recA* synthesis in irradiated bacteria and thus to quantify the induction process; in addition a direct method of measuring instantaneous cell number provided accurate kinetic data for U.V. induced filamentation. Thus we were able to compare the induction kinetics of *recA* synthesis (and the stability of the induced protein) with the kinetics of the disturbance to division and DNA synthesis in irradiated cultures. The role of *recA* protein in DNA repair and division inhibition in both *lon*<sup>+</sup> and *lon*<sup>-</sup> cultures was assessed in this way. Our results show that *recA* protein is very stable and that its stability is not affected by the *lon* mutation. The induction kinetics do not support the idea that large amounts of newly synthesised *recA* protein directly inhibit septation but do support a direct involvement of the protein in the DNA repair process.

## Materials and Methods

### a) Strains

The strain used for this study was *E. coli* K12 KN126, *ilv*, *tyr*<sub>am</sub>, *tryE*<sub>am</sub>, *sup126*<sup>8</sup>, obtained originally from T. Nagata by B.G. Spratt. A *lon* derivative of this strain was isolated in this laboratory by B. Sneath, by selecting for spontaneous resistance to chlorpromazine (Molnar et al., 1977). Genetic analysis confirmed that the mutation carried by this strain was located close to the *lon* locus at min 10 on the *E. coli* map. The growth rate was not altered by the *lon* mutation. Other strains used were KL16 *thi*, and its *recA1* derivative KL16.99; C600 *thr leu thi lacY* (from laboratory stocks) and MX213 *thyA thi leu ilv* (from M. Chandler).

### b) Growth Conditions and U.V. Irradiation

Liquid cultures were grown at 37° in M9 medium supplemented with 0.4% w/v glucose and required amino acids to 20 µg/ml. Irradiation of exponential cultures ( $A^{450}$  0.1–0.2) was carried out

in 1–2 mm layers in glass petri dishes with swirling. Cells were not washed before irradiation. The U.V. dose, monitored by a Laterjet dosimeter, was 10 J·m<sup>-2</sup>, giving more than 90% survival of wild type cells when plated on nutrient agar immediately after irradiation. Under these conditions only 2% of irradiated *lon* bacteria survived.

### c) Measurement of Protein X Synthesis

1 ml aliquots of exponential cultures were mixed with 1 ml prewarmed M9 medium containing 5 µCi [<sup>35</sup>S]-methionine (specific activity 50 µCi/µgm), and incubated 5 min at 37°. Incorporation was terminated by addition of ice cold chloramphenicol (600 µgm/ml) and cold methionine (4 mg/ml). To each sample, 10<sup>10</sup> unlabelled carrier cells were added from a chilled but otherwise untreated portion of the same culture, together with 5 × 10<sup>8</sup> cells grown separately from the same inoculum for several generations in medium containing [<sup>3</sup>H] leucine (specific activity 10 µCi/µgm), as internal standard. The bacteria were then washed twice by centrifugation and lysed in 0.1 ml 10% w/v sodium dodecyl sulphate (SDS). Whole cell lysates were then analysed by SDS-polyacrylamide gel electrophoresis (PAGE), using the basic method of Laemmli (1970) but with an acrylamide monomer: dimer ratio of 44:0.3. The final concentration of acrylamide in the gels was 11%. Pure proteins of known molecular weight were applied to each gel as standards. Gels were stained with Coomassie blue and then dried on to Whatman No.1 filter paper, followed by autoradiography using Kodak XRP5 X-ray film. Gel bands were identified by comparison of the dried, stained gel with the corresponding autoradiograph. Bands were cut out and the radioactivity measured by counting in a scintillation fluid containing 'NCS' tissue solubiliser. The method for subsequent analysis of the data was developed by A. Boyd using the [<sup>3</sup>H] labelled internal standard, and measuring rate relative to another protein (e.g. RNA polymerase) which control experiments showed was not induced, to correct for experimental errors (Boyd, 1979).

### d) Measurement of Stability of Protein X

15 min after irradiation, an aliquot of culture was pulse labelled for 10 min, as described above. Unincorporated isotope was removed by filtering and washing the bacteria at 37° with unlabelled medium containing 300 µgm/ml methionine. The washed bacteria were resuspended in the same medium and incubated at 37°. Samples were removed at intervals and prepared for SDS-PAGE as above.

### e) Two-Dimension Gel Analysis

Exponential cultures were pulse labelled as above and the bacteria washed and lysed by freezing and thawing as described by O'Farrell (1975). These lysates were initially fractionated by isoelectric focussing, followed by SDS-PAGE in the second dimension at right angles to the focussing gel, essentially following the method of O'Farrell (1975). The ampholyte concentration was 2% v/v, comprising 1.6% pH range 5–7, 0.4% pH range 3–10, giving a linear gradient between pH5 and pH7 in the gels. Autoradiographs were prepared as described above. The amount of protein X induced was measured by cutting out the spot identified from the autoradiograph and counting the radioactivity in it as described above. When these counts were compared with a similar measurement of counts in the EFTu spot, and assuming that EFTu is present at 58,000 molecules/cell (Pederson et al., 1978), the amount of protein X could be calculated. These estimations were confirmed

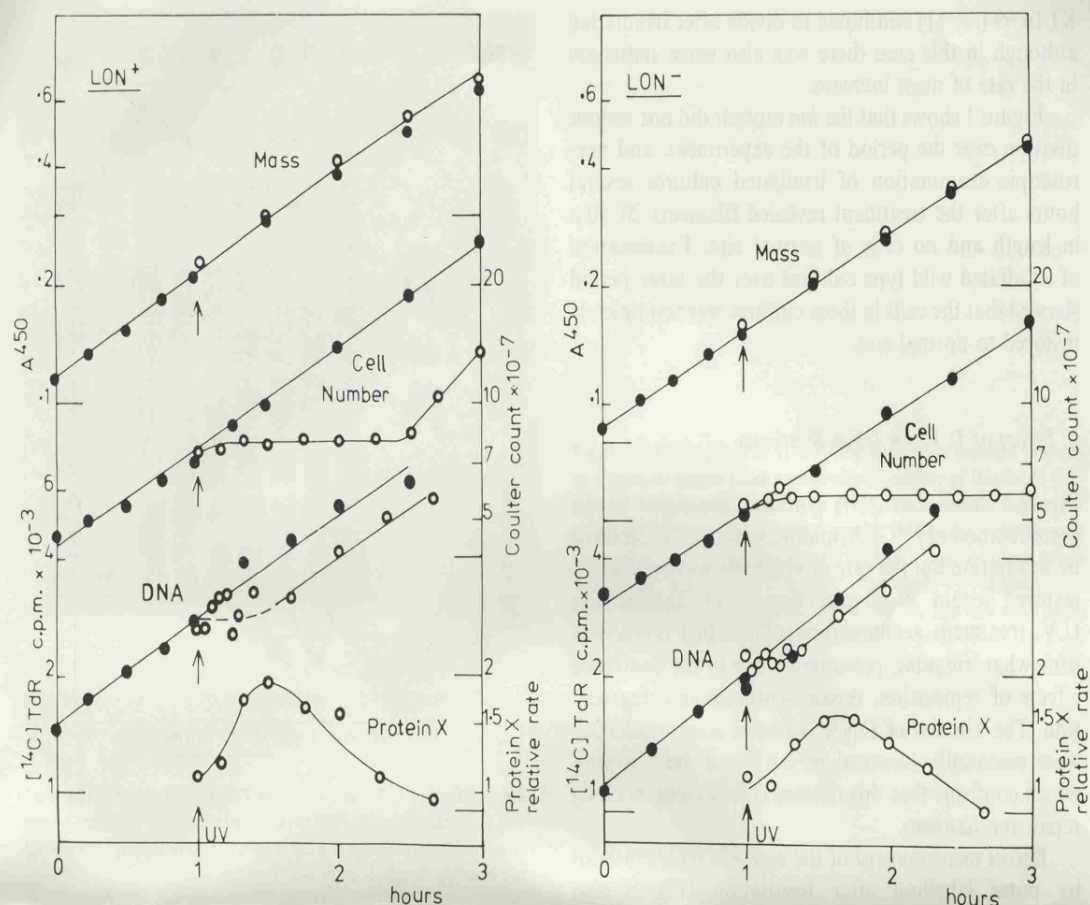


Fig. 1. Effect of U.V. on DNA synthesis, cell division and protein X synthesis: mass, cell number and TCA precipitated radioactivity were followed in both unirradiated and irradiated cultures of mutant (*lon*<sup>-</sup>) and wild type strains. ●—●, unirradiated; ○—○, irradiated. DNA synthesis is measured by continuous labelling in this experiment

by measuring directly the specific activity (c.p.m./μg. protein) in the whole cell lysate proteins, when, from the radioactivity in the spot, the specific activity of protein X was deduced.

#### f) Measurement of DNA Synthesis

Bacteria were labelled by diluting an overnight culture to about 10<sup>6</sup> cells/ml in medium containing [<sup>14</sup>C] thymidine (1 μCi/ml; 2 μCi/μgm) and 1.5 mM uridine and incubating for about 5 generations at 37°, before irradiation. Accumulation of labelled DNA before and after irradiation was determined after precipitation with 10% w/v trichloroacetic acid (TCA).

The rate of DNA synthesis was measured by pulse labelling 0.5 ml aliquots of exponential cultures with [<sup>3</sup>H] thymidine (1 μCi, specific activity 85 Ci/mM). Pulses were terminated by addition of ice cold 10% TCA and the precipitated counts measured.

#### g) Bacterial Cell Number

0.2 ml samples were treated with 2.0 ml 0.8% v/v formalin in saline and counted in a model B Coulter counter.

#### h) Chemicals

Acrylamide and bis-acrylamide were obtained from Eastman Kodak, Rochester, N.Y., U.S.A. All other chemicals used for PAGE were obtained from BioRad, Richmond, Calif., U.S.A. Radioac-

tively labelled compounds were from the Radiochemical Centre, Amersham, Bucks., England and 'NCS' Tissue Solubiliser from Amersham/Searle, Arlington Heights, Ill., U.S.A.

## Results

### 1. Effect of U.V. on Cell Division

The effect of the U.V. dose used in these experiments (10 J·m<sup>-2</sup>) was relatively mild since less than 10% of wild type bacteria were killed, and mass (optical density at A<sup>450</sup>) in both wild type and *lon* cultures continued to increase undisturbed (Fig. 1). In both cultures, division (detected as change in cell number measured by the Coulter counter) was completely inhibited within 10 min of irradiation (Fig. 1). After about 90 min, division abruptly resumed in the *lon*<sup>+</sup> but not in the *lon*<sup>-</sup> culture, and the timing of this recovery in *lon*<sup>+</sup> cultures was consistent over many experiments. This was also true for three other independently derived K12 strains. In contrast, a similar experiment (data not shown) showed that strain

KL16.99 (*recA1*) continued to divide after irradiation although in this case there was also some reduction in the rate of mass increase.

Figure 1 shows that the *lon* mutant did not resume division over the period of the experiment, and microscopic examination of irradiated cultures several hours after the treatment revealed filaments 20–50  $\mu$  in length and no cells of normal size. Examination of irradiated wild type cultures over the same period showed that the cells in these cultures were completely restored to normal size.

## 2. Effect of U.V. on DNA Synthesis

Figure 1 shows that DNA synthesis, measured by the accumulation of [ $^{14}$ C] thymidine, was briefly inhibited by irradiation but the rate of synthesis was apparently restored within 30–40 min. For about 20 min after U.V. treatment accumulation of labelled DNA was somewhat irregular, presumably due to the combined effects of replication, repair synthesis and degradation. The kinetics of DNA synthesis after irradiation were essentially identical in *lon*<sup>+</sup> and *lon*<sup>-</sup> strains, which confirms that this mutant is proficient in DNA repair mechanisms.

Direct measurement of the rate of DNA synthesis by pulse labelling after irradiation (Fig. 2) also confirmed the similarity of the responses of mutant and wild type cultures. The rate of incorporation of [ $^3$ H] thymidine fell abruptly after irradiation and gradually increased to approach the pre-treatment rate in about 40 min. Finally, a control experiment in which a portion of the wild type culture was swirled in a foil-covered dish to duplicate exactly the treatment conditions but prevent irradiation, showed only a brief (5 min) pause in the otherwise steadily increasing rate of DNA synthesis (Fig. 2).

## 3. Induction of Protein X

Analysis of whole cell lysates from pulse labelled irradiated cultures by SDS-PAGE yielded autoradiographs which clearly showed induction of a protein with a molecular weight of 40,000. This corresponds exactly with that reported for purified protein X (Emmerson and West, 1977; Gudas and Mount, 1977; McEntee, 1977). The band was cut from the original gel and its radioactive content at various times after UV treatment was determined, and as shown in Fig. 1, the induced synthesis of protein X continued for about 1 h. The data presented in Fig. 1 are based upon the relative rate of synthesis of protein X by comparison with the rate of synthesis of a second,

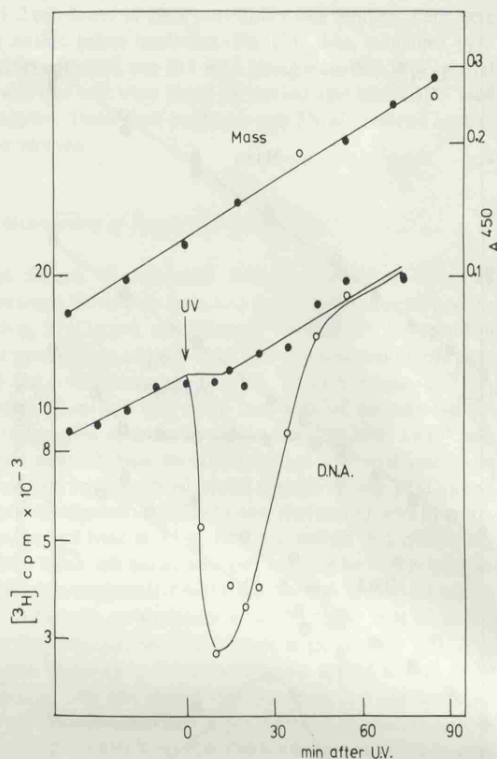


Fig. 2. Effect of U.V. on the rate of DNA synthesis. KN126 (*lon*<sup>+</sup>) was pulse labelled with [ $^3$ H] thymidine before and after irradiation. In a similar experiment the *lon* derivative gave closely corresponding kinetics. Symbols as in Fig. 1

uninduced protein ( $\beta\beta'$  subunits of RNA polymerase). The relative rate thus measured increased at least 2-fold within 30 min of irradiation and then gradually declined over the next 30 min to a level approaching that observed before irradiation. This measurement of a 2-fold increase in rate is an underestimate since 2-dimension gel analysis (Fig. 4) reveals the presence of several other uninduced proteins of molecular weight 40,000. Measurement of radioactivity in the protein X spot indicated at least a 10-fold increase after irradiation and calculation (see Methods) showed that about 40,000 molecules per cell were synthesised during the 60 min following UV treatment.

The kinetics of protein X induction were not significantly different in *lon*<sup>+</sup> and *lon*<sup>-</sup> cultures over several experiments. Inspection of Fig. 1 reveals that the timing of protein X induction did not coincide with inhibition of division, which was maximal within 10 min of irradiation, while the rate of protein X synthesis was maximal at about 30 min after irradiation. The period of increasing protein X synthesis corresponds closely with the period of maximum inhibition of DNA synthesis (Fig. 2) and therefore presumably with DNA repair.

The observation that division in the irradiated

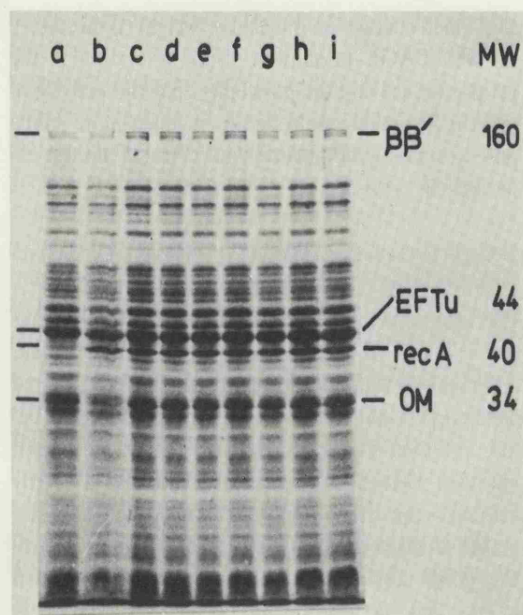


Fig. 3. Autoradiograph of SDS-PAGE analysis of labelled proteins in whole cell lysates from a pulse-chased culture of irradiated wild type bacteria showing stability of protein X. A similar experiment using KN126 *lon* gave identical results. Molecular weights were calculated from those of standard proteins. Lane *a* pulsed before irradiation. *b* pulsed 30 min after irradiation. *c-i* samples from pulse-chased culture taken 0, 15, 30, 60, 90, 120 min after chase

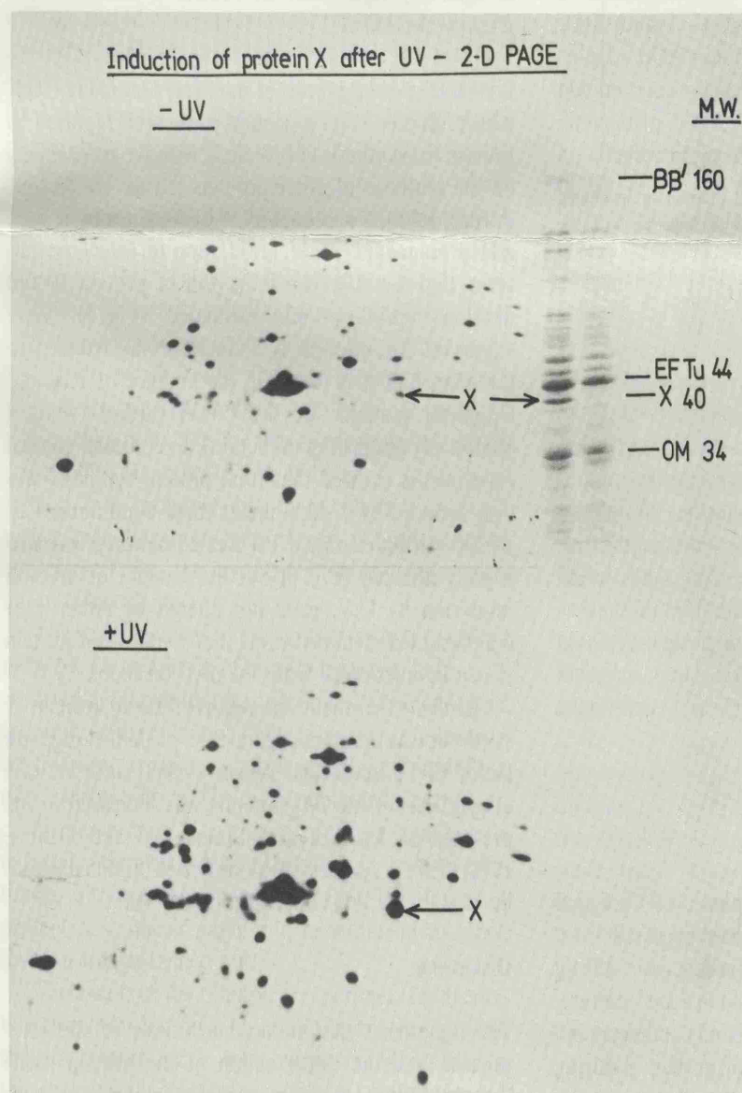


Fig. 4. Autoradiographs of 2-dimension gel analysis of whole cell lysates from pulse labelled irradiated and unirradiated wild type KN126. The isoelectric focussing separation was from left to right. One gel carries a one-dimension SDS-PAGE separation of the same sample as molecular weight markers and one sample (on left) showing the UV-induced protein X band

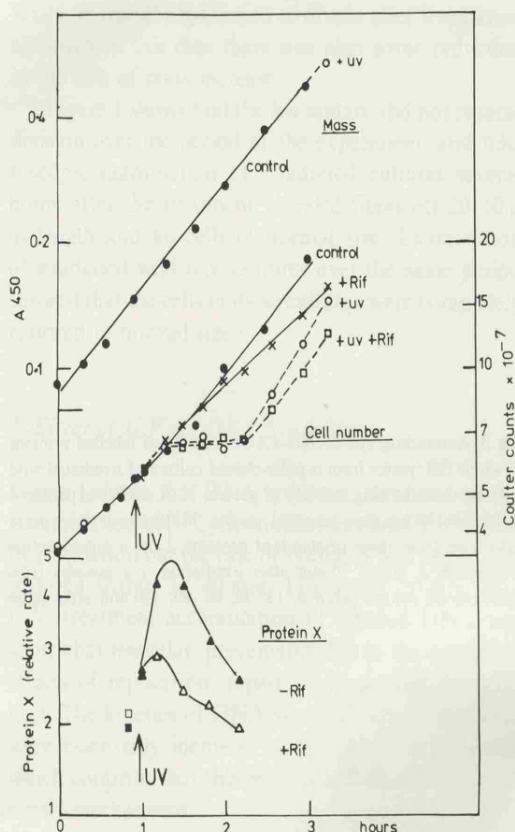


Fig. 5. The effect of Rif (4  $\mu$ g/ml) on cell division in irradiated and unirradiated wild type cultures is shown, together with the effect of U.V. alone. Mass increase ( $A^{450}$ ) was followed in cultures which did not receive Rif treatment. Protein X induction was measured in the same experiment. The square symbols in the lower figure indicate the level of synthesis in the 40K region before U.V.

*lon*<sup>+</sup> strain resumed shortly after protein X induction was complete could indicate that division was inhibited by the presence of large quantities of protein X. However, this argument requires the demonstration that active protein X is short-lived, and turned over within the filamentation period. The alternative, that an inhibitory concentration of protein X could be effectively diluted out during the latter stages of the filamentation period, although not specifically ruled out, seems to us unlikely.

#### 4. Stability of Protein X

It is clear from the autoradiograph shown in Fig. 3 that protein X in whole cell lysates prepared from a pulse-chased culture was not turned over during the period in which division resumed in a *lon*<sup>+</sup> culture. Quantitative analysis of the gel bands indicated no significant change in the amount of protein X during at least 2 h of chase, and that protein X was equally stable in *lon*<sup>-</sup> cultures (data not shown). However,

a distinct change in molecular weight is necessary for SDS-PAGE to show a visible alteration in the gel profile, leaving the possibility that the activity of protein X may be controlled by a much more subtle post-irradiation modification not detected in these experiments.

#### 5. 2-Dimension Gel Analysis of Whole Cell Lysates

This technique is capable of detecting any small terminal deletions of amino acids or post translational modification which results in proteins with an altered net charge. Cultures were therefore irradiated as before and lysates analysed by 2-dimension gel electrophoresis. Induction of protein X was again clearly demonstrated (Fig. 4) and comparison of autoradiographs failed to detect any difference in size or isoelectric point between the protein X molecules found in *lon*<sup>+</sup> and *lon*<sup>-</sup> cultures up to 3 h after irradiation.

#### 6. Effect of Rifampicin on Protein X Induction and Division Inhibition

Low levels (4–8  $\mu$ g/ml) of the drug rifampicin (Rif), which inhibits RNA synthesis, have been reported to prevent the synthesis of protein X without gross effects on the synthesis of other proteins (Satta, Gudas and Pardee, 1979). Consequently, 4 or 6  $\mu$ g/ml Rif were added immediately after irradiation to wild type cultures, and the effect on induction of protein X and division inhibition was estimated (Fig. 5). With 4  $\mu$ g/ml Rif, protein X induction was reduced to less than half that found in the absence of Rif, and 6  $\mu$ g/ml reduced the level even further (without completely abolishing induction). In contrast, control experiments showed that total protein synthesis was only reduced by 7–12% under these conditions. Cell division in unirradiated Rif treated cultures was also slightly affected (Fig. 5) but the kinetics of division inhibition by U.V. were not altered by either 4 or 6  $\mu$ g/ml Rif, demonstrating that synthesis of protein X can be uncoupled from the division block.

In this experiment the relative rate of protein X synthesis was measured against the protein elongation factor EFTu (molecular weight 44,000) since the rate of synthesis of the  $\beta\beta'$  subunits was increased in the presence of Rif (see also Blumenthal and Dennis, 1978). EFTu synthesis was not itself affected either by U.V. or by low concentrations of Rif.

#### Discussion

The hypothesis that induced synthesis of the *recA* protein (and the direct action of the protein itself) is responsible for division inhibition in irradiated bacteria was not supported by the results of the experi-

ments described above. The onset of division inhibition was very rapid, with only 10–12% of irradiated bacteria completing division and no further divisions were detected after 10 min of post-irradiation growth, that is 20 min before the maximum rate of *recA* protein synthesis was reached. Further, the fall in rate of protein X synthesis did not coincide with the recovery of division; this resumed at near normal rate 90 min after U.V. despite the continued presence of high levels of *recA* protein since this was shown to be stable for at least 60 min after division resumed.

The kinetics of protein X induction described here are very similar to the kinetics of induction of other SOS functions reported previously: the capacities for mutagenic repair, prophage  $\lambda$  induction and reactivation of irradiated  $\lambda$  (Defais et al., 1971; Defais et al., 1974). This result therefore further supports the hypothesis of co-ordinate control of SOS functions. In so far as the period of major DNA repair was indicated by the disturbance to DNA synthesis measured in irradiated cells, this also coincided very closely with the period of derepression of protein X synthesis, consistent with the suggestion that the chief function of newly synthesised *recA* protein is to bind to single stranded regions of DNA generated during repair (Satta et al., 1978).

Two-dimension gel analysis of lysates from induced cultures failed to detect any alteration in *recA* protein during and after division recovery, supporting the notion that protein X per se is not the division inhibitor. However, it should be recognised that there may be modifications to the protein during the recovery period which could alter its biological activity without affecting the isoelectric point of the molecule (e.g. methylation). Despite this reservation, the results discussed so far indicate that while newly synthesised protein X is probably directly involved with DNA repair, including mutagenic repair of both bacterial and phage DNA, the involvement with division inhibition is less clear.

The specific effects of low concentrations of rifampicin on the synthesis of certain proteins in *E. coli*, reported by Blumenthal and Dennis (1978), facilitated the study of the effects of reduced protein X induction on division, avoiding a more generalised inhibition of protein synthesis. The results obtained clearly indicated that protein X synthesis could be significantly reduced without any detectable effect on division inhibition, although the possibility that low levels of protein X synthesis might still be sufficient to block division was not ruled out.

The results of the rifampicin experiments indicated that the control of division inhibition by *recA* protein did not require its *de novo* synthesis. What then is the most likely explanation for the role of *recA* protein in U.V.-induced filamentation? One answer

may be derived from the proposed models of autoregulation of *recA* protein involving interaction with a 'signal' or effector molecule. Thus altered, *recA*\* protein might affect transcription of division control gene(s), the presence of *lon* protein being essential for reversing this process. Alternatively, the modified *recA*\* protein acting as a multifunctional molecule might cleave a septum-associated gene product, requiring expression of the *lon* gene to restore the intracellular level of this product. On either model we envisage that most newly synthesised molecules of *recA* protein are probably rapidly bound to single stranded DNA generated during damage and repair. As repair is completed and the effector concentration falls, protein X will be displaced from DNA to accumulate in the cytoplasm where it should compete with the activated complex to re-establish repression.

Any model of *recA* control of SOS functions must accommodate the phenotypes associated with two mutations, *tsl* (mapping at *lexA*) and *tif* (mapping at *recA*) (Mount et al., 1973; Castellazzi et al., 1972). Both mutations cause temperature sensitive expression of SOS functions including filamentation and derepression of the *recA* gene, presumably without damage to DNA, thus initiating induction without an effector molecule. In the case of *tif*, the product of the *recA* gene has been shown to be a mutant form of the protein, which is apparently able to promote derepression without DNA damage. The altered *lexA* repressor made by *tsl* cultures at 42° also allows derepression of protein X and division inhibition (our unpublished results) and we would argue that this altered *lexA* product affects the activity of protein X (perhaps in combination with it), to induce other SOS functions.

Our results are not consistent with the role suggested for the *lon* product by George et al. (1975); no effect of the *lon* mutation on the synthesis of *recA* protein or on the molecule itself were detected. However, since the *lon* gene product does appear to control gene expression (e.g. of the *gal* operon) and may indeed bind to DNA (Zehnbauer et al., 1979), we suggest an alternative hypothesis. In irradiated bacteria, *recA*\* and *lon* proteins might compete for an operator site at a third target gene, which regulates division. A likely candidate for this target gene is the *sfi/sul* locus near 2 min on the *E. coli* chromosome, where suppressors of filamentation have been mapped (Johnson, 1977). Further studies, aimed at demonstrating such interactions, should greatly clarify the roles of these genes in controlling cell division.

## References

- Adler, H.I., Hardigree, A.A.: Growth and division of filamentous forms of *Escherichia coli*. *J. Bacteriol.* **90**, 223–226 (1965)
- Blumenthal, R.M., Dennis, P.P.: Gene expression in *Escherichia*

- coli* during partial Rifampicin-mediated restrictions of transcription initiation. *Mol. Gen. Genet.* **165**, 79–86 (1978)
- Boyd, A.: Aspects of outer membrane protein synthesis in *Escherichia coli* B/r. Ph.D. Thesis, University of Leicester, England (1979)
- Castellazzi, M., George J., Buttin, G.: Prophage induction and cell division in *E. coli*. I. Further characterisation of a thermo-sensitive mutation *tif1* whose expression mimics the effect of U.V. irradiation. *Mol. Gen. Genet.* **119**, 139–152 (1972)
- Defais, M., Caillet-Fauquet, P., Fox, M.S., Radman, M.: Induction kinetics of mutagenic DNA repair activity in *E. coli* following irradiation. *Mol. Gen. Genet.* **148**, 125–130 (1976)
- Defais, M., Caillet-Fauquet, P., Radman, M., Errera, N.: U.V. reactivation and U.V. mutagenesis of  $\lambda$  in different genetic systems. *Virology* **43**, 495–503 (1971)
- Emmerson, P.T., West, S.C.: Identification of protein X of *Escherichia coli* as the *recA*<sup>+</sup>/*tif*<sup>+</sup> gene product. *Mol. Gen. Genet.* **155**, 77–85 (1977)
- George J., Castellazzi, M., Buttin, G.: Prophage induction and cell division of *E. coli*. III. Mutations *sfiA* and *sfiB* restore division in *tif* and *lon* strains and permit the expression of mutator properties of *tif*. *Mol. Gen. Genet.* **140**, 309–332 (1975)
- Gudas, L.J., Mount, D.W.: Identification of the *recA* (*tif*) gene product of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5280–5284 (1977)
- Gudas, L.J., Pardee, A.B.: A model for the regulation of *Escherichia coli* DNA repair functions. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2330–2334 (1975)
- Howard-Flanders, P., Simson, E., Theriot, L.: A locus that controls filament formation and sensitivity to radiation in *Escherichia coli*. *Genetics* **49**, 237–246 (1964)
- Inouye, M., Pardee, A.B.: Changes of membrane proteins and their relation to DNA synthesis and cell division of *E. coli*. *J. Biol. Chem.* **245**, 5813–5819 (1970)
- Johnson, B.F.: Fine structure mapping and properties of mutations suppressing the *lon* mutation in *Escherichia coli* K12 and B strains. *Genet. Res.* **30**, 273–286 (1977)
- Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970)
- McEntee, K.: Protein X is the product of the *recA* gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5275–5279 (1977)
- Molnár, J., Holland, I.B., Mándi, Y.: Selection of *lon* mutants in *Escherichia coli* by treatment with phenothiazines. *Genet. Res.* **30**, 13–21 (1977)
- O'Farrell, P.H.: High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021 (1975)
- Pederson, S., Bloch, P.L., Reeh, S., Neidhardt, F.C.: Patterns of protein synthesis in *E. coli*: a catalog of the amount of 140 individual proteins at different growth rates. *Cell* **14**, 179–190 (1978)
- Radman, M.: In: Molecular mechanisms for repair of DNA. (P.C. Hanawalt, R.B. Setlow, eds.). *Basic Life Sci.* **5**, 355–369 (1975)
- Roberts, J.W., Roberts, C.W., Craig, N.L.: *Escherichia coli recA* gene product inactivates phage  $\lambda$  repressor. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4714–4718 (1978)
- Satta, G., Gudas, L.J., Pardee, A.B.: Degradation of *Escherichia coli* DNA: Evidence for limitation *in vivo* by protein X, the *recA* gene product. *Mol. Gen. Genet.* **168**, 69–80 (1979)
- Satta, G., Pardee, A.B.: Inhibition of *Escherichia coli* division by protein X. *J. Bacteriol.* **133**, 1492–1500 (1978)
- Smith, K.C.: DNA synthesis in sensitive and resistant mutants of *Escherichia coli* B after ultraviolet irradiation. *Mutat. Res.* **8**, 481–495 (1969)
- Weinstock, G.M., McEntee, K., Lehman, I.R.: ATP dependent renaturation of DNA catalysed by the *recA* protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 126–130 (1979)
- Witkin, E.M.: Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriol. Rev.* **40**, 869–907 (1976)
- Zehnbauer, B., Foley, E.C., Markovitz, A.: DNA binding protein and the *lon* (*capR*) gene of *E. coli* K12. *Fed. Proc.* **38**, 623 (1979)

Communicated by B.A. Bridges

Received May 28, 1979

#### Note Added in Proof

In a recent paper, A. Bailone, A. Levine and R. Devoret (*J. Mol. Biol.* **131**, 553–572, 1979) presented evidence that UV irradiated *E. coli* contain only small amounts of an active form of *recA* protein which acts as the SOS inducer and they proposed, as suggested in this study, that the bulk of the newly synthesised *recA* protein is unmodified and acts primarily to facilitate repair of damaged DNA.