Molecular cloning and analysis of the *ruv* gene of *Escherichia coli* K12

by

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CONTENTS

CHAPTER 2 MATERIALS AND METHODS

Lloyd, R.G., Benson, F.E. and Shurvinton, C.E. (1984) Effect of ruv mutations on recombination and DNA repair in Escherichia coli. Mol. Gen. Genet. 194: 303-309.

Shurvinton, C.E., Lloyd, R.G., Benson, F.E. and Attfield, P.V. (1984) Genetic analysis and molecular cloning of the Escherichia coli ruv gene. Mol. Gen. Genet. 194: 322-329.

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Benson, F.E., Illing, G.T., Sharples, G.J. and Lloyd, R.G. (1988) Nucleotide sequencing of the ruv region of Escherichia coli K-12 reveals a LexA regulated operon encoding two genes. Nucl. Acids Res. 16: 1541-1549.

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Disclaimer

During the time that I was registered for a Ph.D. I was employed as a research assistant in Dr. R.G. Lloyd's group studying DNA repair and recombination in *E. coli*. As such the work presented in this thesis is to some extent the results of a team effort to investigate the organisation and function of the *ruv* gene of *E. coli*. I am indebted to Dr. R.G. Lloyd for his excellent supervision, particularly of the experiments that yielded the results presented in Tables 3.1 and 3.2, and in the isolation of the *ruv* transducing phages presented in section 4.2. I am also grateful to Dr. P.V. Attfield for allowing me to report the subcloning of the *ruv* gene into the plasmid pPVA101; and the isolation of pPVA105, and pPVA101::Tn*1000* derivatives. Dr. G.T. Illing and G. Sharples assisted in the nucleotide sequence determination.

ABBREVIATIONS

The abbreviations used in this thesis are those recommended for publications in the Proceedings of the National Academy of Sciences (USA), with the exception of the following:

Ap	Ampicillin
Cm	Chloramphenicol
ddATP	Dideoxyadenosine-5'-triphosphate
ddCTP	Dideoxycytidine-5'-triphosphate
ddGTP	Dideoxyguanosine-5'-triphosphate
ddTTP	Dideoxythymidine-5'-triphosphate
DS	Double-stranded
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
IPTG	Isopropyl B-D-thiogalactopyranoside
Km,Kan	Kanamycin
NaAc	Sodium acetate
Na cit	Sodium citrate
ONPG	O-nitrophenyl B-D-galactoside
PEG	Polyethylene glycol
R(superscript)	Resistant
S(superscript)	Sensitive
SDS	Sodium dodecyl sulphate
Sm,Str	Streptomycin
SS	Single-stranded
Tc,Tet	Chlortetracycline
TEMED	N,N,N',N',-Tetramethyl-ethylenediamine
X-GAL	5-Bromo-4-chloro-3-indolyl B-D-galactopyranoside

In addition, in figures, restriction enzyme cleavage sites are abbreviated as follows:- A, AvaI; B, BamHI; C, HincII; G, BglII; H, HindIII; K, KpnI; M, SmaI; P, PstI; R, EcoRI; S, SalI; V, EcoRV; and X, Sau3A.

LIST OF FIGURES

8.3 Identification of the cloned DNA in plasmid pFB3

8.4 Clones for sequencing the *ruv* gene *Kpn*I site 1 to the *Bgl*II site

8.5 Nucleotide sequence of the *ruv* region

8.6 Autoradiograph of DNA sequencing gel, showing proposed -10 region of promoter, sequence with homology to the Shine-Delgarno sequence and the proposed initiation codon of *ruvA*

8.7 Autoradiograph of restriction digested N2057 DNA probed with the 1kb fragment between *Eco*RV sites 2 and 3

LIST OF TABLES

ABSTRACT

Mutations in the *ruv* gene of *Escherichia coli* K-12 result in an increased sensitivity to agents that damage DNA. Studies presented in this thesis demonstrate that the *ruv* gene product is required for conjugational recombination in certain genetic backgrounds. From this it was inferred that the role of the *ruv* gene product was in the recombination repair of daughter strand gaps and double strand breaks in damaged DNA. In addition, the *ruv* gene product is shown to be required for the efficient recovery of F' transconjugants in certain genetic backgrounds, suggesting that recombination between transferred F' and the recipient chromosome may be an obligatory event in these strains.

Expression of *ruv* is regulated as part of the SOS response to DNA damage, by the *lexA* and *recA* gene products. The *ruv* gene product appears not to have any major role in its own regulation, however the basal level of expression of other SOS genes is increased in strains carrying *ruv* mutations.

The *ruv* gene has been cloned on a 10.4kb *Hind*III fragment into the low copy number vector pHSG415, to give plasmid pPVA101, which has been demonstrated to complement the UV sensitivity of strains carrying any of the 10 different *ruv* mutations tested. Analysis of the proteins synthesised by pPVA101, its deletion derivatives, and derivatives with Tn*1000* insertions inactivating the *ruv* gene, allowed the identification of the *ruv* coding region, and suggested that the *ruv* gene encoded a 41kd protein. In addition, regions of the cloned DNA coding for two further proteins of approximately 24kd and 33kd were identified.

The sites of insertion of Mu*d*(Ap)^{R}*lac* and Tn*10* elements in the *ruv* gene were mapped, which allowed the direction of transcription to be determined, and suggested that the 41kd protein may be cotranscribed with the 24kd protein from a promoter upstream of the

smaller protein. This was substantiated by the demonstration that two of the ruv mutations studied were chromosomal inversions, one of which had its end point within the coding region for the 24kd protein, and by the isolation of an SOS inducible promoter derived from the region upstream of the 24kd protein.

The nucleotide sequence of the ruv region revealed two open reading frames, designated ruvA and ruvB, with coding potential for proteins of 22087 daltons and 37177 daltons respectively, corresponding to the proteins with molecular weights estimated as 24kd and 41kd from SDS-polyacrylamide gels. A possible promoter, and two sequences with homology to the LexA binding site consensus sequence were identified upstream of the coding region of the 22kd protein. An amino acid sequence within the proposed RuvB protein was identified with homology to ATP binding sites of other proteins involved in DNA metabolism.

INTRODUCTION

1.1 Introduction

The maintenance of the integrity of the deoxyribonucleic acid (DNA) and its faithful replication is essential for the survival and propagation of any organism. Since the DNA molecule itself is highly reactive, capable of sustaining potentially harmful changes, both as a consequence of the reactions in which it partakes, such as replication and recombination, and as a consequence of the action of a variety of physical and chemical agents; a series of repair mechanisms have evolved to restore its integrity (Friedberg 1985). In eukaryotes, DNA damage has been clearly implicated in carcinogenesis (Heidelburger 1975), and the absence of components of the repair systems implicated in certain human disease syndromes (Cleaver *et al.* 1975). However, it is in prokaryotes, and in particular the bacterium *Escherichia coli* that repair mechanisms have been best characterised (Walker 1984); it is on these studies that I shall concentrate this introduction.

1.2 DNA damage

a) Spontaneous damage

The main source of DNA damage arising from the participation of DNA molecules in cellular processes is probably the mispaired base-pair, which may arise as a result of incorrect base incorporation during DNA replication (Wagner and Meselson 1976), or as a result of heteroduplex formation during genetic recombination (Holliday 1964). Although the base selection specificity and 3' - 5' exonuclease activity of DNA polymerase 1 reduce the incorporation of mispaired bases to approximately 10^{-7}, the observed spontaneous mutation rate

suggested the mispairing frequency was reduced further to approximately 10^{-10}, by the actions of a post-replicative mismatch correction system (Radman et al. 1981). Studies of mismatch repair have generally focussed on the repair of artificially constructed phage heteroduplex models introduced into E. coli (Nevers and Spatz 1975; Bauer et al. 1981).

b) DNA damage induced by external agents

A wide variety of chemical and physical agents can inflict damage on DNA (Friedberg 1985). However, although the precise effects of these agents may be different, essentially the resulting damage falls into four groups, namely (i) intrastrand links, (ii) strand breaks, (iii) interstrand cross links and (iv) base damage. Studies of repair of DNA damage have therefore concentrated on the study of repair of damage produced by a few well characterised agents, which have some biological relevance.

(i) Intrastrand link repair has focussed on the study of repair of the photoproducts formed in UV irradiated DNA, including the major lesion, the pyrimidine dimer (Setlow 1966) and the minor lesion the 6-4 photoproduct (Lippke et al. 1981); both formed between adjacent pyrimidine residues in the same DNA strand.

(ii) Strand break repair studies have focussed on the repair of DNA strand breaks, in particular double strand breaks which occur as major lesions in DNA exposed to ionising radiation (Ward 1975).

(iii) Repair of interstrand crosslinks has been studied by following the repair of rather poorly defined crosslinks produced by mitomycin C (Iyer and Szybalski 1963), and the rather better defined crosslinks produced by cis-diammine dichloro-platinum (II) (cis

platinum) between the O^6 and N^7 of guanine residues on different DNA strands (Roberts 1978).

(iv) Repair of base damage has centred on the study of repair of DNA alkylated resulting from treatment with monofunctional agents such as methylmethanesulphonate (MMS) and N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG), which alkylate the nitrogens and oxygens of the bases to differing relative extents, the N^7 position of guanine and the N^3 position of adenine being the most reactive (Roberts 1978).

1.3 Specific repair processes

In addition to the armoury of enzymes with rather general substrate specificity that the bacterium E. coli has at its disposal for repair of DNA damage (discussed in sections 1.6 - 1.10 of this introduction), it possesses a number of enzymes that recognise and initiate the repair of specific lesions, which may occur as a result of spontaneous DNA damage, or may be induced by external physical agents. These include:-

i) Uracil DNA glycosylase and hypoxanthine DNA glycosylase, which specifically remove uracil and hypoxanthine, the products of deamination of cytosine and adenine respectively, by cleaving the N-glyosylic bond between the base and the deoxyribose phosphate backbone, leaving apurinic and apyrimidinic sites which are subsequently excised by apurinic/apyrimidinic endonucleases (Lindahl et al. 1977, Lindahl 1979, Karran and Lindahl 1978, Lindahl 1982).

ii) The tag gene product, 3-methyl DNA adenine glycosylase I, which specifically removes 3-methyladenine by cleavage of the N-

glycosylic bond between the methylated base and the deoxyribose phosphate backbone (Lindahl 1976, Karran *et al.* 1980). A second 3-methyladenine DNA glycosylase (II) encoded by the *alkA* gene has a rather less specific substrate requirement and is induced as part of the adaptive response to alkylation damage (Evensen and Seeberg 1982) introduced in section 1.5.

iii) The 49kd *phr* gene product, DNA photolyase, which catalyses the monomerisation of thymine dimers, in a reaction dependent upon the absorption of near UV irradiation (wavelength 300 - 500nm), termed photoreactivation (Rupert and Sancar 1978, Sancar, Smith and Sancar 1983).

1.4 The SOS response to DNA damage

Damage to the DNA of *E. coli*, resulting from treatment with agents such as UV irradiation and mitomycin C, induces the expression of a group of coordinately regulated genes whose function is to aid cell survival in a response termed the SOS response. The existence of such a response was first suggested by Defais *et al.* (1971) and later elaborated on principally by Radman (1974, 1975). Several excellent reviews outline the details of the response (Walker 1984, Walker 1985, Little and Mount 1982).

The *lexA* gene encodes a repressor protein which represses a series of unlinked genes, including some of those required for excision repair, recombination repair and error prone repair of DNA damage (discussed in sections 1.7, 1.9, and 1.8 of this introduction respectively). In addition LexA protein represses its own synthesis, and that of the multifunctional protein RecA. In the presence of DNA damage, RecA protein is activated to a form which promotes cleavage of the LexA repressor, resulting in expression of the genes under its control. As DNA damage is repaired, the

inducing signal that results in activation of the RecA protein is removed, LexA protein accumulates and the repressed state is re-established (Figure 1.1). In addition to promoting cleavage of LexA protein, activated RecA protein also facilitates the cleavage of several phage repressors, including the CI and P22 repressors (Roberts and Roberts 1975, Sauer *et al.* 1982) resulting in induction of lytic growth as part of the SOS response to DNA damage.

Control of the SOS response has been characterised using strains carrying mutations in *recA* and *lexA* (Table 1.1), and more recently by molecular analysis of the genes induced as part of the SOS response.

Several genes have been demonstrated to be under SOS control by the isolation and characterisation of operon fusions, between chromosomal genes and the *lac* genes carried on a Mud(Ap)^{R}*lac* transducing phage (Casadaban and Cohen 1979). Fusions in SOS genes were identified by screening isolates containing random Mud(Ap)^{R}*lac* insertions for induction of B-galactosidase synthesis on treatment with DNA damaging agents, particularly mitomycin C. Kenyon and Walker (1980) identified five damage inducible din::Mud(Ap)^{R}*lac* insertions, in which expression of B-galactosidase was induced by treatment with DNA damaging agents, but was prevented in strains carrying *recA*(def) or *lexA*(ind^{-}) mutations (Kenyon and Walker 1980, Kenyon *et al.* 1982). Later studies demonstrated that one of the din::Mud(Ap)^{R}*lac* fusions was in *uvrA* (Kenyon and Walker 1981). Several more genes have since been demonstrated to be under SOS control by the isolation and characterisation of Mud(Ap)^{R}*lac* insertions, including *uvrB* (Fogliano and Schendel 1981, Schendel, Fogliano and Strausbaugh 1982), *uvrD* (Arthur and Eastlake 1983, Nakayama Irino and Nakayama 1983, Siegel 1983), *umuDC* (Bagg, Kenyon and Walker 1981), *sulA* (Quillardet *et al.* 1982), *himA* (Miller *et al.* 1981) and *recN* (Lloyd, Picksley and Prescott 1983).

The mechanism by which the *lexA* gene product is able to control

Key to figure 1.1

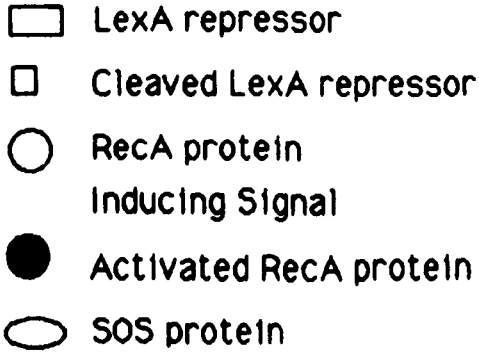
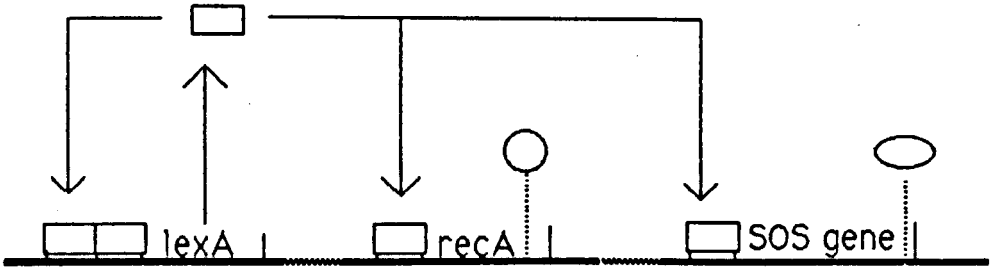
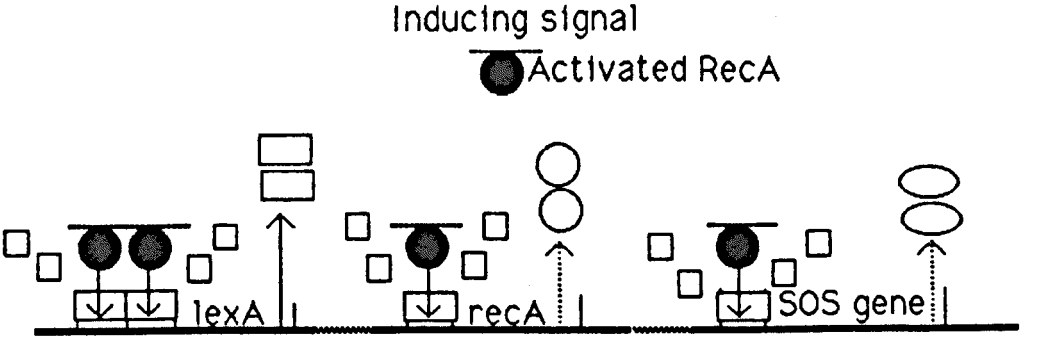


Figure 1.1 Model of SOS Regulation

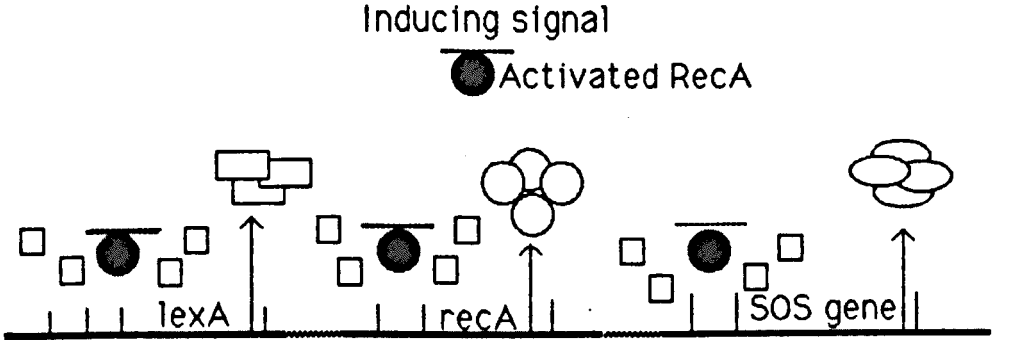
a) Repressed state



b) At induction



c) Induced state



d) Return to repressed state

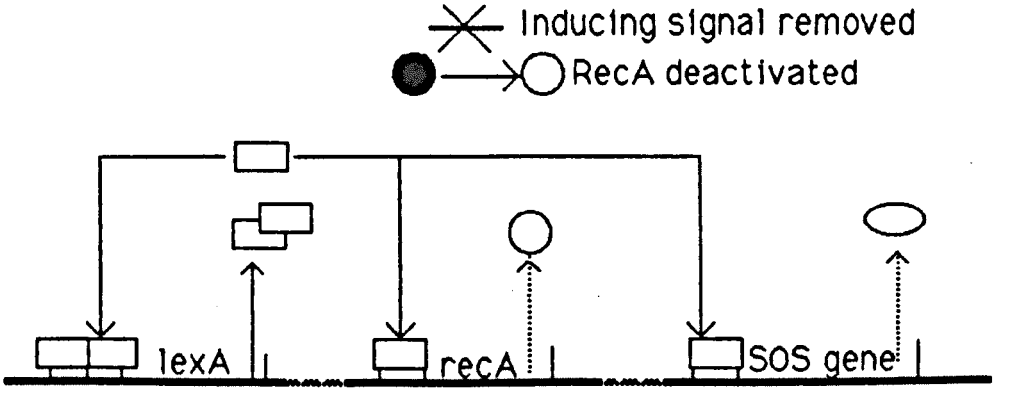


Table 1.1 Alleles of *recA* and *lexA* and their effect on expression of the SOS response

lexA/*recA* allele	SOS phenotype	Biochemical alteration of protein	References
lexA(ind$^-$)	Non-inducible SOS response	Proteolysis resistant LexA protein gly^{85} asp^{85}	Mount *et al*. (1972) Markham (1981)
lexA(ts)	Constitutive expression of SOS response at 42^{o}C	Degradation susceptible LexA protein gly^{85} asp^{85} and ala^{131} thr^{131}	Mount *et al*. (1973) Peterson and Mount (1987)
lexA(def)	Constitutive expression of SOS response	Defective repressor	Mount (1977)
recA(def)	Non-inducible SOS response	e.g. *recA1*, gly^{160} asn^{160} Defective DNA binding and ATPase activity of RecA protein	Clark (1973) Kawashima *et al*. (1984)
recA441(ts)	Constitutive expression SOS response at 42^{o}C (recombination proficient)	Constitutive protease at 42^{o}C glu^{38} lys^{38} (Prt^{c}) ile^{298} val^{298} (ts)	Kirby *et al*. (1967) Castellazzi *et al*. (1972) Knight *et al*. (1984) Wang and Tessman (1985)

expression of the SOS genes was revealed by *in vitro* studies on the interaction between purified LexA protein and the promoter regions of the SOS genes.

The nucleotide sequence of the *lexA* gene has been determined and the 22.7kd protein it encodes purified and characterised (Horii *et al.* 1981a,b, Little and Harper 1979, Brent and Ptashne 1981, Little *et al.* 1981). The site of LexA repressor cleavage has been identified as the peptide bond between ala^{84} and gly^{85} and the alteration in the *lexA*(ind$^-$) encoded protein identified as a change in the amino acid at position 85 from glycine to aspartate (Mount *et al.* 1983). Under certain *in vitro* conditions, purified LexA protein was demonstrated to effect autodigestion to the same 13.5kd and 9kd cleavage products obtained *in vivo* (Little 1984) which suggested the *in vivo* function of activated RecA protein may not itself be as a protease, but may be an effector of LexA protein autodigestion. A recent study by Slilaty and Little (1987) has suggested that the cleavage of LexA protein may occur by a mechanism similar to the cleavage of the serine proteases, activated RecA protein acting as a positive effector of the reaction.

Purified LexA protein has been shown to bind specifically to a 20bp sequence in front of the *recA* gene and to a 40bp sequence in front of the *lexA* gene itself, protecting those regions from DNAase I digestion, and repressing transcription of both genes (Little *et al.* 1981, Brent and Ptashne 1981). Analysis of the nucleotide sequence protected by LexA protein from DNAase I digestion revealed a perfect inverted repeat in front of the *recA* gene, and two similar inverted repeats in front of the *lexA* gene. These LexA binding sites were termed SOS boxes. Subsequent studies identified SOS boxes in front of several genes known to be under *lexA*/*recA* control (Table 1.2), a subset of these sequences have been clearly demonstrated to bind LexA protein. In addition the presence of SOS boxes in the control regions of the *recQ* and *dnaG* genes suggested

Table 1.2 SOS boxes identified in front of *lexA* regulated genes

Gene		SOS box sequence	Reference
Consensus		CTGtatatatataCAG	Wertman and Mount 1985
lexA	1	CTGTATATACTCACAG	(Horii *et al* 1981, Little *et al*. 1981
	2	CTGTATATACACCCAG	(Brent and Ptashne 1981
recA		CTGTATGAGCATACAG	Horii *et al*. 1981,Sancar *et al*. 1980
recN	1	CTGTATATAAAACCAG	Rostas *et al*. 1987
	2	CTGTACACAATAACAG	
recQ		CTGTTTTTATTT-CAG	Irino, Nakayama and Nakayama 1986
uvrA		CTGTATATTCATTCAG	Sancar *et al*. 1982
uvrB		CTGTTTTTTTATCCAG	Finch and Emmerson 1983
umuDC		CTGTATATAAAAACAG	Perry *et al*.1985,Kitagawa *et al*.1985
sulA		CTGTACATCCATACAG	Cole 1983
dnaG		CTGGCGTTGATGCCAG	Lupski, Ruiz and Godson 1984
mucAB		CTGTATAAATAAACAG	Perry *et al*. 1983
impAB		CTGTATATACATACAG	Strike and Lodwick 1987

that they may be regulated as part of the SOS response to DNA damage (Irino, Nakayama and Nakayama 1986, Lupski, Ruiz and Godson 1984). This was subsequently confirmed by analysis of operon fusions for the recQ gene (Irino et al. 1986).

The binding specificity of the LexA repressor was further investigated by examining the effects of mutations in the SOS box of the recA gene on the production of galactokinase from a recA::galK fusion (Wertman and Mount 1985). From these studies, which demonstrated that mutations at symmetrically equivalent sites had the same effect on LexA repression, it became apparent that the symmetrical structure of the SOS box was important for LexA binding and a consensus half site for optimal LexA binding was proposed with the sequence 5'CTGTATAT. Sedgwick and Yarranton (1981) suggested that much of the fine control of the SOS response could be achieved by deviation from this optimal sequence, or by the presence of more than one SOS box. Further control could be achieved by the presence of additional lexA independent promoters, as is the case with the uvrB gene (Sancar et al. 1982, Backendorf et al. 1983).

The two SOS boxes in front of the lexA gene itself are relatively poorly conserved and are therefore not subject to such tight repression of their expression as is, for example, the recA gene product. This ensures that sufficient LexA repressor can be produced to repress all the genes under SOS control before its expression is turned off (Brent 1983). The recA gene has a relatively well conserved SOS box, for which the LexA repressor has in vitro an approximately 40 x greater affinity than it has to the SOS boxes in front of its own gene (Brent and Ptashne 1981), presumably ensuring in vivo that RecA protein is not fully induced unless the DNA damage is severe, and therefore preventing the potentially lethal cleavage of prophage repressors, which requires greater levels of activated RecA protein than does LexA cleavage (Slilaty et al. 1986).

The recA gene has been cloned and sequenced, its protein purified and many of its reactions characterised in vitro (Ogawa et al. 1978, Sancar and Rupp, Sancar et al. 1980, Horii and Ogawa 1980, reviewed in Radding 1982, Cox and Lehmann 1987). In addition to the DNA binding and ATPase activities required for its role in recombination (discussed in section 1.10 of this introduction) RecA protein has been demonstrated to promote the cleavage of both the LexA and cI repressors (Little et al. 1980, Roberts, Roberts and Craig 1978).

The nature of the signal required to activate RecA has been the subject of much study (Little and Mount 1982). In vivo studies suggested that different pathways may exist to generate a common inducing signal from the initial DNA damage. Induction of the SOS response by nalidixic acid, an inhibitor of DNA gyrase which therefore blocks replication, required the RecBCD enzyme function retained in recD mutants (McPartland et al. 1980, Karu and Belk 1982, Chaudhury and Smith 1985), whilst induction by UV irradiation was dependent on the recF gene product (McPartland et al. 1980, Karu and Belk 1982, Salles and Paoletti 1983, Calsou and Salles 1985). In vitro studies suggested that the inducing signal produced from the initial DNA damage may be regions of single stranded DNA in the presence of a nucleoside triphosphate cofactor, particularly dATP (Craig and Roberts 1980, 1981, Phizicky and Roberts 1980, 1981). The presence of such regions of single-stranded DNA can clearly be envisaged in vivo as a result of DNA damage.

The current repertoire of chromosomal SOS genes includes three required for excision repair (uvrA, uvrB and uvrD), three required for error prone repair (umuDC and recA), and three required for recombination repair (recA, recN and recQ); these are all discussed in later sections of this introduction. In addition genes regulated as part of the SOS response include the sulA gene, required for inhibition of cell division (Huisman and D'Ari), and the himA gene,

required for site specific recombination (Miller *et al.* 1981). It seems likely that further SOS genes remain to be discovered since several inducible phenomena such as restriction alleviation (Thomas and Wackernagel 1984) and repair of oxidative damage (Imlay and Linn 1986) have yet to have specific gene products associated with them.

1.5 The adaptive response

The adaptive response to alkylating agents was first reported by Samson and Cairns (1977) who discovered that pretreatment of cells with low levels of MNNG increased their capacity for survival and decreased their susceptibility to mutagenesis when subsequently exposed to higher doses.

Induction of adaptation to alkylating agents has since been demonstrated to be dependent upon the *ada* gene product which acts as an activator of the genes under its control (Jeggo 1979, Sedgwick and Robins 1980, Sedgwick 1982).

Induction of the adaptive response results in increased expression of the cotranscribed *ada* and *alkB* genes and also the *alkA* and *aidB* genes (Lemotte and Walker 1985, Yamamoto *et al.* 1978, Karran *et al.* 1982, Kataoka and Sekiguchi 1985, Evensen and Seeberg 1982, Volkert and Nguyen 1984).

The *ada* gene has been cloned, sequenced, and the O^6 methylguanine DNA methyltransferase (O^6 me G methyltransferase) that it encodes purified (Sedgwick 1983, Demple *et al.* 1985, Nakabeppu *et al.* 1985). It is an unusual protein in that it is required in stochiometric amounts as an acceptor for the transfer of methyl groups from alkylated DNA, which result in its inactivation (Karran *et al.* 1979, Olsson and Lindahl 1980). The O^6 mc G DNA methyl transferase has, amongst its cysteine residues, two cysteines which specifically function as methyl acceptor sites; the cysteine at

position 321 accepts methyl groups from potentially mutagenic O^6 methylguanine (O^6 me G) and O^4 methyl thymine (O^4 me T) residues, whilst the cysteine at position 69 accepts methyl groups from Sp stereoisomers of methylphosphotriesters (Demple et al. 1985, McCarthy et al. 1983, McCarthy and Lindahl 1985, Hamblin and Potter 1985). These reactions appear to be irreversible, once the acceptor sites are occupied, no further methyl transfer can occur.

The demonstration that the ada gene encoded O^6 me G methyltransferase led to the proposal that methylation of the protein may be the signal required for induction of the adaptive response (Teo et al. 1984). This proposal was borne out by in vitro studies which demonstrated that methylation of the cysteine at position 69 - the methyl phosphotriester methyl acceptor site was required for activation of the Ada protein and induction of the adaptive response (Teo et al. 1986, Nakabeppu and Sekiguchi 1986). In addition these studies demonstrated that binding of the methylated Ada protein to similar nucleotide sequences with the consensus AAANNAAGCGCA, in the promoter regions of both the ada and alkA genes stimulated transcription of these genes, providing further evidence for the proposed regulatory role of the methylated Ada protein. A similar mechanism of induction is envisaged for the aidB gene.

The alkA gene has been cloned and the 3-methyladenine DNA glycosylase II (3 me A glycosylase II) it encodes purified and characterised (Nakabeppu et al. 1984a,b, McCarthy et al. 1984). Unlike the constitutively expressed tag gene product, 3 me A glycosylase I, which has a very precise substrate specificity, removing only N^3 methyladenine residues (Bjelland and Seeberg 1987, Sakumi et al. 1986), the inducible alkA gene product 3-me A glycosylase II has a wide substrate specificity, excising potentially lethal N^3-methylguanine (N^3-me G), O^2 methyl cytosine (O^2-me C) and O^2-methylthymine (O^2-me T) in addition to N^3-me A

residues (McCarthy et al. 1984).

Although the functions of the 27Kd alkB product (Kataoka and Sekiguchi 1985) and the aidB product have not been determined and other genes involved in adaptation to alkylating agents may still be identified, it is clear that the ada and alkA gene products are capable of repairing most of the potentially mutagenic or lethal lesions (McCarthy et al. 1984). A clear role for the aidB gene product in survival or reduced mutagenesis has not yet been established (Volkert and Nguyen 1984, Volkert et al. 1986).

However, the sensitivity to alkylation of strains carrying alkB mutations (Kataoka et al. 1983) and the increased sensitivity of strains carrying both alkB and alkA mutations suggests that the alkB gene product is required for repair of some form of alkylation damage, and furthermore that it functions either in a pathway distinct from the alkA gene product or on different lesions to the alkA gene product (Volkert 1988). The precise role of the alkB gene product in the repair of alkylation damage, and its interaction with the ada and alkA gene product remains to be determined.

1.6 Interaction between the SOS and Adaptive Responses

The demonstration that methylation of the cysteine residue at position 69 of the Ada protein was necessary for induction of the adaptive response (Takano, Nakabeppu and Sekiguchi 1988) clearly explains the lack of induction of the adaptive response by agents (such as UV and nalidixic acid) which induce expression of the SOS response (Jeggo et al. 1977). However, the SOS response can be induced by agents that induce the adaptive response, presumably since lesions such as 3-methyl adenine inhibit DNA replication (McCarthy et al. 1984).

The relative contributions of the enzymes, induced as part of these responses, to the repair of alkylation damage depends

primarily on the size of the alkylating groups involved. The efficiency with which alkylation damage is repaired by SOS controlled excision mechanisms increases with the size of the alkylating group (Todd and Schendel 1983), whereas the efficiency with which alkylation damage is repaired by the enzymes induced as part of the adaptive response decreases with the size of the alkylating group (Volkert 1988).

1.7 Excision repair of damaged DNA

The removal of pyrimidine dimers from the DNA of *E. coli* on incubation in the dark was first demonstrated by following the transfer of dimers from acid insoluble UV irradiated DNA, to the acid soluble fraction (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). In UV sensitive strains carrying *uvrA*, *uvrB* or *uvrC* mutations, thymine dimers remained in the acid insoluble fraction (Howard-Flanders *et al*. 1966). Since strains carrying mutations in more than one of the *uvrA*, *uvrB* or *uvrC* genes were no more sensitive to UV irradiation than strains carrying single mutations, and strains carrying mutations at any of the three loci were unable to excise dimers from their DNA, Howard-Flanders *et al*. (1966) proposed that the *uvrA*, *uvrB* and *uvrC* genes encoded an excision nuclease that excised dimers from DNA.

The incision process has been well characterised and shown to be dependent on the products of the *uvrA*, *uvrB* and *uvrC* genes, by assaying the nicking activity of cell extracts from different mutants, on UV irradiated plasmid DNA (Seeberg *et al*. 1976, Seeberg *et al*. 1980).

The *uvrA*, *uvrB* and *uvrC* genes have since been cloned, their products identified as polypeptides of 114kd, 84kd and 70kd respectively, purified, and the UvrABC enzyme reconstituted *in vitro* (Brandsma *et al*. 1981, Yoakum *et al*. 1982, Seeberg and Steinum 1982,

Kacinski et al. 1981, Sancar et al. 1981a, Pannekoek et al. 1978, Sancar et al. 1981b, Yoakum et al. 1981, Yoakum et al. 1980, Sancar et al. 1981c, Seeberg 1978, Seeberg and Steinum 1982).

The earliest model for excision repair suggested that the DNA strand containing the thymine dimer was incised and the dimer removed by repair synthesis in a manner analogous to a nick translation reaction (Pettijohn and Hanawalt 1964). This model was subsequently modified when it was discovered that the UvrABC enzyme introduced two incisions into the damaged DNA strand, one at the 8th phosphodiester bond 5' to the dimer and another at the 4th or 5th phosphodiester bond 3' to the dimer (Sancar and Rupp 1983, Yeung et al. 1983a and b) releasing an oligonucleotide of approximately 13 nucleotide residues (Figure 1.2). Later studies demonstrated that similar specific incisions were introduced by the UvrABC enzyme at positions either side of sites of damage caused by cis-platinum and N-acetylamino fluorine (Beck et al. 1985, Fuchs and Seeberg 1984).

In addition to the uvrA, uvrB and uvrC gene products, required for the incision of damaged DNA, products of the uvrD and polA genes were implicated as having roles in the post incision stage of excision repair. Strains carrying uvrD mutations (isolated independently as recL, uvrE and mutU mutations (Rothman 1978, Smirnov and Skavronskaya 1971, Siegel 1973) were moderately sensitive to UV irradiation and showed a reduced capacity to reactivate UV irradiated phage, whilst the length of the repair synthesis patch was altered in UV sensitive strains carrying polA mutations (Cooper 1982).

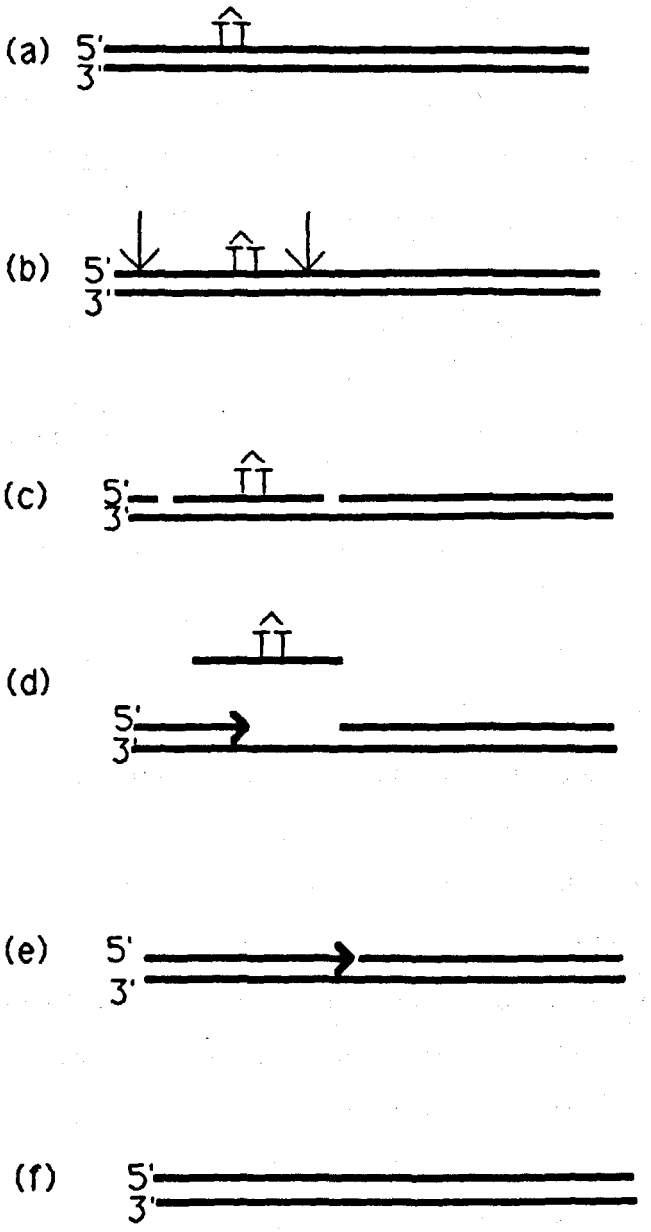
The elucidation of the role of the uvrD gene product in excision repair was facilitated by the cloning and subsequent identification of the uvrD gene product as DNA helicase II (Arthur et al. 1982, Kumura et al. 1983, Maples and Kushner 1982, Oeda et al. 1981, 1982). In vitro studies suggested that its role in excision repair may be in the release of the 12-13 nucleotide

Figure 1.2

Excision repair of thymine dimers

(a) Thymine dimer (T^T) present in DNA. (b) and (c) Nicks introduced at 8th phosphodiester bond 5' of lesion and at 4th or 5th phosphodiester bond 3' of lesion by UvrABC excision nuclease. (d) Initiation of repair synthesis by DNA polymerase I and action of *uvrD* product DNA helicase II results in release of 12-13mer oligonucleotide and release of UvrABC enzyme from it. (e) Repair synthesis and nick translation. (f) Nick sealed by the action of DNA ligase.

Figure 1.2 Excision repair of thymine dimers.



fragment excised by the UvrABC enzyme, and in promoting the removal of the UvrABC enzyme from the excised fragment (Caron *et al.* 1985, Husain *et al.* 1985, Kumura *et al.* 1985). The *polA* gene product, DNA Polymerase I, required *in vivo* for repair synthesis across the gap created by excision of the 12-13 nucleotide fragment, was also demonstrated *in vitro* to be required for the release of this fragment and for efficient turnover of the UvrABC enzyme suggesting that the release of the oligonucleotide, repair synthesis across the gap, and the release of the UvrABC enzyme from the excised oligonucleotide may be coupled *in vivo*.

The final stage of excision repair is thought to be the sealing of nick between newly synthesised and extant DNA, promoted by the *lig* gene product DNA ligase (Lehmann 1974).

1.8 Mismatch repair

Repair of mismatched base pairs was first demonstrated by analysis of progeny arising from the introduction of artificially constructed, genetically marked ØX174 and λ heteroduplexes containing mismatched base pairs, which clearly revealed that mismatches could be corrected to either of the parental strand genotypes prior to replication, and that closely spaced mismatches were generally repaired in favour of the same strand. These results were interpreted to suggest that mismatch repair occurred via an excision repair type mechanism with repair synthesis tracts of approximately 3Kb (Baas and Jans 1972, Wildenberg and Meselson 1975). Later studies demonstrated that mismatch repair was influenced by the presence and state of methylation of d(GATC) sequences (Lu *et al.* 1983, Pukkila *et al.* 1983, Laengle-Roualt *et al.* 1986), the substrate of the *dam* encoded DNA adenine methylase enzyme. The presence of d(GATC) sequences within DNA molecules containing mismatched base pairs was absolutely required for their

repair whilst the state of methylation of the sequences governed the direction of repair, mismatch correction occurring towards the methylated strand in hemimethylated duplexes (Kramer *et al.* 1984, Wagner *et al.* 1984). These results provided evidence for the proposal that the transient undermethylation of newly synthesised DNA may provide a basis for its discrimination from parental DNA and thus allow repair of mismatches arising as replication errors (Wagner and Meselson 1976).

Subsequent studies demonstrated that in addition to the *uvrD* gene product (Nevers and Spatz 1975), DNA helicase II (Arthur *et al.* 1982), which is also required for excision repair, the products of the *mutH*, *mutS* and *mutL* genes were required for methyl directed mismatch repair (Glickman and Radman 1980, Bauer *et al.* 1981). Strains carrying mutations in any of these genes have a mutator phenotype (Cox 1976, Glickman *et al.* 1980) presumably resulting from the inability, or the impaired ability, to perform methyl directed mismatch repair.

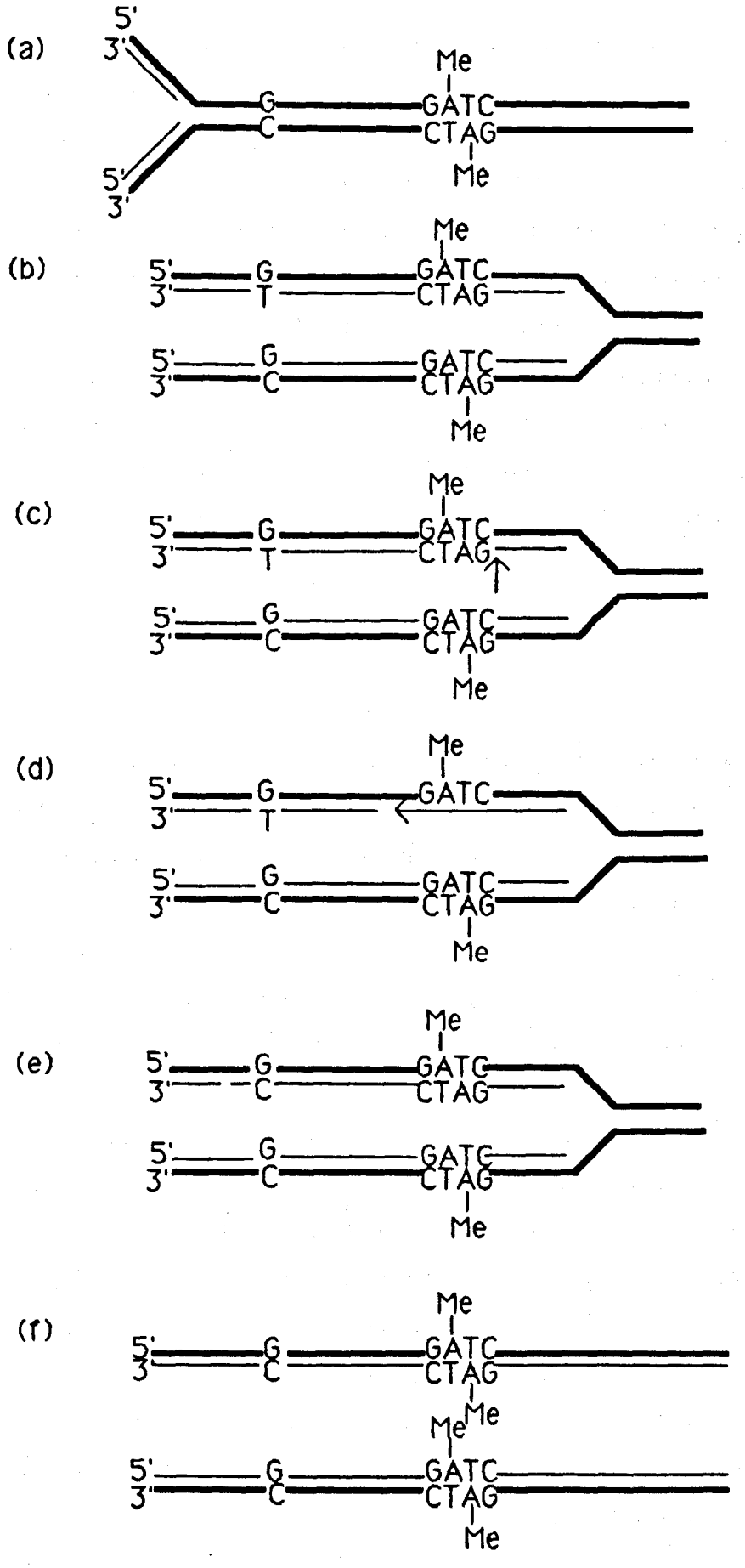
In the absence of methyl directed strand discrimination (in *dam* mutants), repair may be initiated on either strand, which according to the model of Glickman and Radman (1980) may lead to the generation of double strand breaks. This was substantiated by the observation that *dam recA*, *dam recB* and *dam lexA* double mutants were inviable (Marinus and Morris 1974), and that the inviability was probably due to an accumulation of double strand breaks (Wang and Smith 1986). The ability of mutations in *mutL* and *mutS* to suppress the inviability of such strains confirmed the involvement of the *mutL* and *mutS* gene products in a pathway involving the *dam* methylase (McGraw and Marinus 1980, Wang and Smith 1986). The products of the *mutH*, *mutS* and *mutL* genes have recently been isolated and purified from strains carrying the cloned genes using an *in vitro* mismatch repair complementation system (reviewed in Modrich 1987). The purified MutH protein has been shown to bind and introduce incisions

Figure 1.3

Mismatch repair

(a) Replicating DNA molecule, thick lines represent parental DNA, thin lines represent newly synthesised DNA. Both strands of the parental DNA are methylated at dGATC sites. (b) Mismatch incorporated in newly synthesised DNA (recognised by MutS protein). (c) Transient undermethylation of newly synthesised DNA allows strand discrimination. Nick introduced 5' of the G residue in the sequence dGATC on the unmethylated strand (by the MutH product). (d) The nick is translated along the DNA molecule in a 5' - 3' direction, in a reaction presumably dependent on the *uvrD* and *polA* products (in roles analogous to their roles in the excision of dimers). (e) The mismatched base is removed and replaced by the correct base. (f) The nick is sealed by the action of DNA ligase, and the daughter strand methylated.

Figure 1.3 Mismatch repair.



5' of dGATC sequences (Welsh *et al.* 1986), thus confirming the proposals that the *mutH* gene product was involved in strand discrimination (Kramer *et al.* 1984) and that mismatch correction may be initiated at d(GATC) sites (Lu *et al.* 1984, Laengle-Roualt *et al.* 1987). The *mutS* gene product is a 97Kd protein which has been demonstrated to bind mismatched base pairs with an affinity that reflects the ability of the correction system to repair different mismatches, suggesting that it is the *mutS* gene product that is responsible for recognition of mispaired bases (Su and Modrich 1986). A function for the *mutL* gene product has not yet been demonstrated. The current model for methyl directed mismatch repair is presented in Figure 1.3.

1.9 Error prone repair

The first observations of error prone repair were made by Weigle (1953) who noted that amongst the increased numbers of phage survivors obtained when host strains were UV irradiated prior to infection with UV irradiated phages, there were a significant number of mutants.

Later studies demonstrated a requirement for a UV inducible host product and the presence of pyrimidine dimers for error prone repair or UV mutagenesis (Witkin 1974).

Error prone repair has since been shown to be induced as part of the SOS response to DNA damage and to require the products of the *umuD* and *umuC* genes (Defais *et al.* 1971, Shinoura 1977, Steinborn 1978, Bagg *et al.* 1981), or their plasmid borne analogues such as *mucAB* (Perry and Walker 1982) or impAB (Glazebrook *et al.* 1986). The *umuDC* genes have been cloned, sequenced and shown to encode polypeptides of 16,000 and 45,000 daltons respectively, transcribed under SOS control from a promoter upstream of the coding region of the 16Kd *umuD* gene product (Elledge and Walker 1983, Kitagawa *et al.*

1985, Perry et al. 1985). Within the predicted amino acid sequence of the UmuD protein a region similar to the cleavage site of LexA protein was identified (Perry et al. 1985), which led to the suggestion that the UmuD protein may be subject to proteolytic cleavage.

In addition to the umuDC gene products, several studies suggested that RecA protein was required in its activated form for UV mutagenesis, in a role apparently distinct from its function in cleavage of LexA protein (Bagg et al. 1981, Calsou and Defais 1985, Ennis et al. 1985, Marsh and Walker 1987). Recent studies have demonstrated that one role for activated RecA protein is in mediating the cleavage of the bond between the cysteine and glycine residues at positions 24 and 25 of the UmuD protein, which activates the UmuD protein for its role in mutagenesis (Shinagawa et al. 1988, Nohmi et al. 1988, Burkhardt et al. 1988). A further role for activated RecA protein in UV mutagenesis has not been eliminated.

In addition to the products of the umuDC and recA genes, a role for DNA polymerase III in UV mutagenesis has been proposed (Bridges et al. 1976, Brotcorne-Lannoye et al. 1985, Bridges and Mottershead 1978).

Early proposals of the mechanisms of error-prone repair suggested that the UV inducible products of the umuDC and recA genes interacted with the replication complex resulting in a decreased fidelity of DNA synthesis allowing replication past lesions in DNA (Radman et al. 1971). Evidence for this proposal was subsequently obtained from studies on the replication of irradiated ØX174 DNA (Caillet-Fauquet et al. 1977).

A problem that arose from this proposed mechanism of error-prone repair was the question of whether the replication of template DNA was subject to a generally reduced fidelity, resulting in random misincorporation of bases, or whether decreased fidelity resulted in misincorporation of bases specifically opposite sites of damage in

DNA (Caillet-Fauquet et al. 1977).

Since replication of templates treated with different DNA damaging agents resulted in different spectra of mutations (Miller 1982, Coulondre and Miller 1977) it was concluded that most induced mutagenesis was targeted at sites of damage. However, studies by Caillet-Fauquet et al. (1984) suggested that mutagenesis may not be targeted, but that non-targeted errors are efficiently removed by the mismatch correction system.

Studies on targeting of UV induced mutations in the lacI gene demonstrated rather surprisingly that the 6-4 photoproduct, rather than the pyrimidine dimer was the major premutagenic lesion (Haseltine 1983a, b) in UV irradiated DNA and that the role of pyrimidine dimers in error prone repair was mainly confined to the induction of the SOS response (Brash and Haseltine 1985).

The most recent model for error prone repair, proposed by Bridges and Woodgate (1985) suggested that UV-induced mutagenesis is a two step process, the first step involving the misincorporation of a nucleotide opposite a DNA lesion in a reaction facilitated by RecA protein, the second step involving the incorporation of the correct nucleotides 3' of the misincorporated base in a reaction mediated by the UmuC and UmuD proteins. This model has been extended on the basis of evidence that demonstrated that RecA protein bound to pyrimidine dimers in UV irradiated DNA (Lu et al. 1986), and inhibited the 3' - 5' exonuclease activity of DNA polymerase III holoenzyme (Hirsht and Knill-Jones 1983). Recent studies that demonstrate that the UmuD protein may be proteolytically activated in a reaction dependent on RecA protein are consistent with this model, the processed UmuD product facilitating replication beyond a misincorporated nucleotide (Burckhardt et al. 1988). Confirmation of this mechanism is likely only to be obtained by studies on the purified umuDC products. As a step towards this the UmuD protein has been purified from an overproducing plasmid harbouring strain

```
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```

(Burkhardt 1988). If the proposed mechanism turns out to be correct, it will be remarkable in that the RecA protein is involved in three distinct steps (i) cleavage of the LexA protein resulting in derepression of the umuDC gene products (Walker 1984), (ii) cleavage of the UmuD protein to an activated form (Shinagawa et al. 1988, Nohmi et al. 1988, Burkhardt et al. 1988), and (iii) binding to DNA lesions and reducing the proofreading activity of DNA polymerase III (Lu et al. 1986, Fersht and Knill-Jones 1983).

1.10 Recombination repair of damaged DNA

A role for recombination in the repair of damaged DNA was first proposed by Rupp and Howard-Flanders (1968), who suggested that gaps (arising due to the inability of the replication complex to replicate past thymine dimers, and subsequent reinitiation downstream) could be filled by recombination between sister duplexes in the model for daughter strand gap repair outlined in Figure 1.4.

Recombination between sister duplexes has also been proposed to have a role in the repair of double strand breaks occurring as a result of DNA damage (Kraisin and Hutchinson 1977), possibly by a mechanism similar to that put forward to account for repair of DNA douple strand breaks in yeast as outlined in Figure 1.5.

The mechanisms proposed for repair of daughter strand gaps, and double strand breaks, are based on recombination models, proposed by Holliday (1964), and later adapted by Meselson and Radding (1975) to account for the assymmetric transfer of markers. Features of both models include invasion of duplex DNA by a free 3'OH single strand DNA end, formation of heteroduplex DNA, strand transfer, and the eventual resolution of the Holliday junction.

Since the genes implicated in the process of genetic recombination all exhibit DNA repair defects, studies of genetic

Figure 1.4

Repair of daughter strand gaps

(a) Thymine dimer (T^T) in DNA, thick lines represent parental DNA strands. (b) Gap in the newly synthesised daughter DNA (represented by thin lines), arising from the inability of DNA polymerase to replicate past the dimer, and subsequent reinitiation downstream. The dimer containing duplex is referred to as duplex 1, the dimer free duplex as duplex 2. (c) Nick introduced in the parental strand of duplex 2, opposite the thymine dimer. (d) Parental DNA from duplex 2 is transferred into the gapped region of duplex 1. (e) Strand transfer of parental DNA from duplex 2 to duplex 1 displaces newly synthesised DNA of duplex 1, which then invades the single stranded region of duplex 2 in a reciprocal event and initiates DNA synthesis using the complementary newly synthesised DNA as template. (f) The crossover is resolved. (g) Nicks are sealed by the action of DNA ligase.

Figure 1.4 Repair of daughter strand gaps

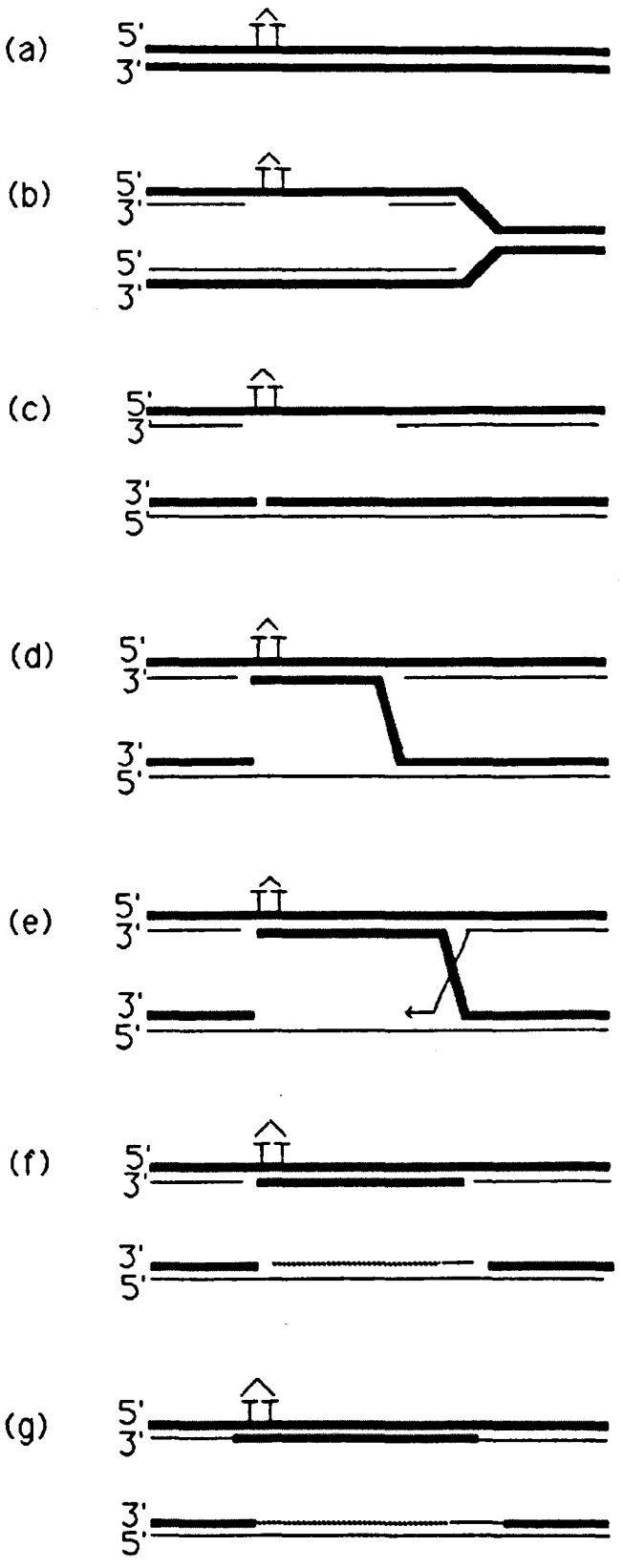
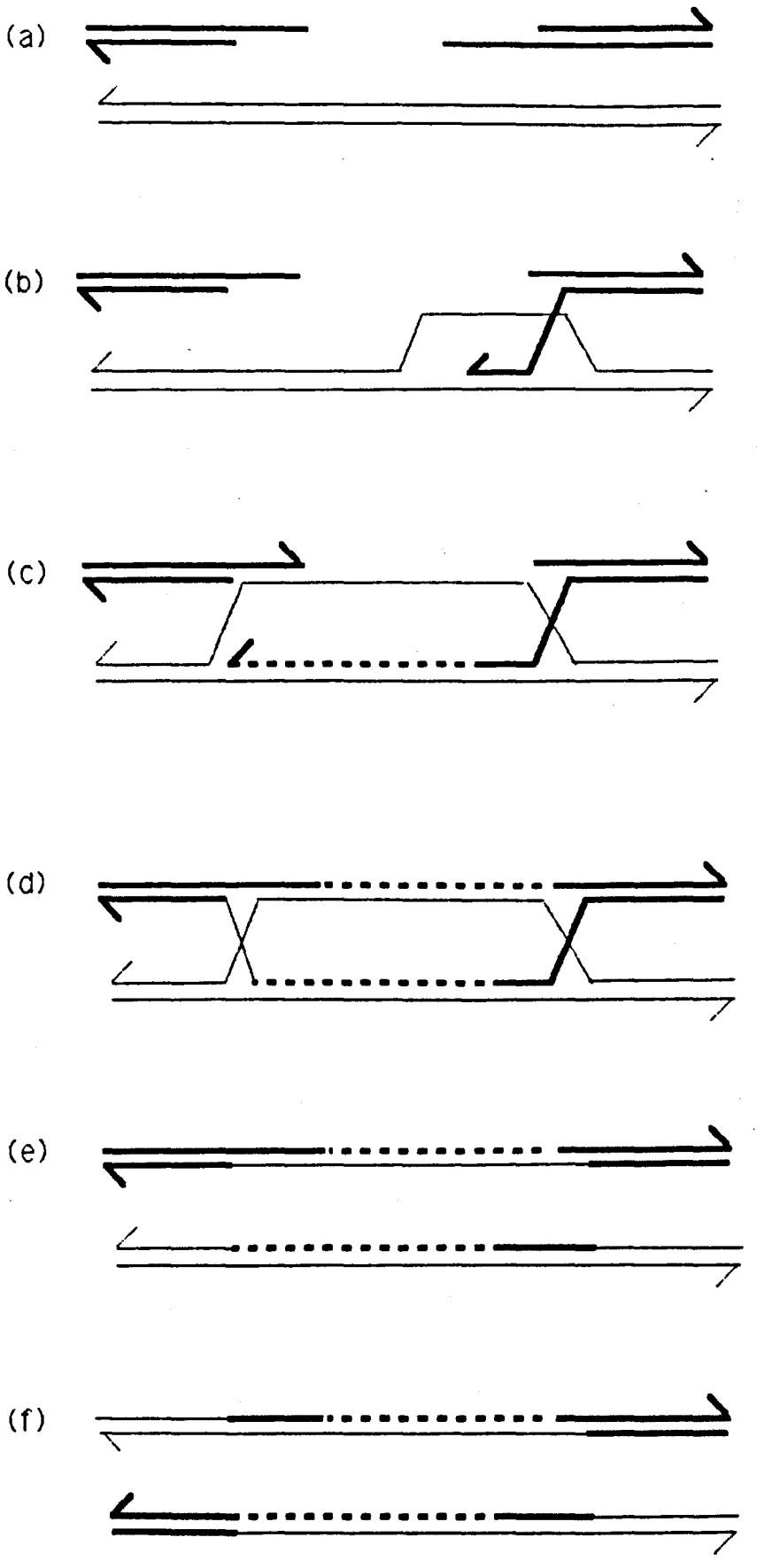


Figure 1.5

Double strand break repair model (Szostak *et al*. 1983)
(a) Duplex (thick lines), containing double strand break (or gap), and a homologous duplex (thin lines). (b) One 3' end of the broken duplex invades the homologous duplex, pairing with its complementary strand and displacing a D-loop. (c) The D-loop is enlarged by repair synthesis until the second 3' end can anneal to the complementary single stranded region of the D-loop. (d) A second round of repair synthesis is initiated across the gapped region and branch migration results in the formation of two Holliday junctions. (e) and (f) Holliday junctions are resolved to give 2 possible crossover and 2 possible non-crossover configurations. In the products illustrated, the right hand Holliday junction has been resolved in both cases by cleavage of the inner DNA strands.

Figure 1.5 Double strand break repair model



recombination and recombination repair have proceeded in parallel (Smith 1987). *In vivo* studies of recombination deficient mutants have so far implicated the products of nine genes in both genetic recombination and recombination repair, and have suggested that different gene products may be required for processing different recombination substrates and different DNA lesions. The current understanding of these genes and the role their products play in genetic recombination is presented in the following sections.

a) *recA*

The *recA* locus at 58' on the *E. coli* genetic map was identified by mutations which in recipient strains severely reduced the recovery of recombinants in crosses with Hfr donors (Clark and Margulies 1965, Willets, Clark and Low 1969). In addition *recA* mutants exhibited a variety of pleiotropic effects including sensitivity to UV and γ-irradiation, extensive post-UV degradation of DNA, deficiency in prophage induction and lack of mutability by UV light (Howard-Flanders and Theriot 1966, Clark *et al.* 1966, Brooks and Clark 1967, Witkin 1969). Subsequent studies led to the conclusion that in addition to its role in homologous recombination, the RecA protein was required for induction of the SOS response (Little *et al.* 1980) for recombination repair of daughter strand gaps (Smith & Meun 1970) and double strand breaks (Kraisin and Hutchinson 1977) and for error prone repair (Bagg *et al.* 1981, Calsou and Defais 1985, Ennis *et al.* 1985).

The *recA* gene has been cloned (Ogawa *et al.* 1978, Sancar and Rupp 1979), the DNA sequence determined (Sancar *et al.* 1980, Horii *et al.* 1980), the protein identified as a polypeptide of approximately 37.8kd (Sancar *et al.* 1980) and purified (Shibata *et al.* 1979, Roberts *et al.* 1978, Weinstock, McEtnee and Lehmann 1979). Extensive characterisation of the reactions mediated by the purified

protein *in vitro* has provided valuable insight into the possible role of the RecA protein *in vivo* (reviewed by Radding, 1982, Dressler and Potter, 1982, Cox and Lehmann, 1987). In a reaction stimulated by single-strand binding protein (SSB) (Cox *et al.*, 1983), RecA protein promotes the exchange of strands between a range of DNA substrates, of which the most extensively studied is strand exchange between circular single-stranded DNA molecules and homologous linear double stranded DNA molecules (Dasgupta *et al.* 1980, Flory *et al.* 1984), a reaction in some respects analogous to the formation of heteroduplex DNA proposed in widely accepted recombination models (Holliday 1964, Meselson and Radding 1975).

RecA protein mediates strand exchange *in vitro* in a reaction proposed to occur in three distinct phases each of which has been biochemically well-characterised.

i) RecA protein binds cooperatively to single stranded (SS) DNA in a reaction stimulated by SSB protein to form a RecA protein-SS DNA complex (Cox and Lehmann 1982, McEntee, Weinstock and Lehmann 1980, West *et al.* 1980).

ii) The RecA protein-SS DNA complex promotes a search of double-stranded (DS) DNA for sequences homologous to the complexed SS DNA, facilitated by a RecA protein-SS DNA complex stimulated unwinding of DS DNA (Cunningham *et al.* 1979) and the formation of large nucleoprotein networks (Chow and Radding 1985). Once a homologous sequence has been identified, RecA protein binds to duplex DNA resulting in an increased unwinding of DS DNA which is then aligned with the SS DNA of the RecA protein-SS DNA complex, in a structure termed a paranemic joint, (McEntee, Weinstock and Lehmann 1979, Cunningham *et al.* 1981, West, Cassuto and Howard-Flanders, 1981a, Bianchi, DasGupta and Radding 1983) which may be contained within a RecA spiral filament (Howard-Flanders, West and Stasiak 1984).

iii) RecA protein promotes the interwinding of single stranded and double-stranded DNA to form a plectonemic joint in a reaction that requires a free 3' end and promotes branch migration in a unique 5' - 3' direction relative to single-stranded DNA, and requires ATP hydrolysis (Cox and Lehman 1981, Kahn et al. 1981, West, Cassuto and Howard-Flanders 1981).

Although substrates used in the above experiment are not typical of those found in vivo, the activities promoted by RecA protein in vitro suggest that the central role for the RecA protein in homologous recombination is to promote pairing and strand exchange between homologous molecules. Subsequent studies have demonstrated that RecA protein can promote strand exchange between duplex molecules providing one molecule has a single stranded gap or tail and the other molecule has a 3' end homologous to the single stranded region of the first molecule (West, Cassuto and Howard-Flanders 1981b).

A role for RecA protein in recombination repair of daughter strand gaps and double strand breaks, similar to its role in homologous recombination, promoting pairing of homologous molecules and strand exchange to form heteroduplex DNA, was suggested by in vivo studies that demonstrated that strains with recA mutations were unable to regenerate high MW DNA from the low MW DNA synthesised after UV irradiation, or resulting from γ-irradiation (Smith and Meun 1970, Kraisin and Hutchinson 1977).

The proposed role in daughter strand gap repair was substantiated by an in vitro demonstration that RecA protein could bind to a small single stranded region within an otherwise double-stranded DNA molecule (similar to the proposed substrate for DSG repair) pair with, and initiate strand exchange from a homologous DS DNA molecule, providing the second molecule had a 3' end homologous to the single-stranded region of the first molecule (West et al. 1980, Cassuto et al. 1980, Cunningham et al. 1980, West, Cassuto

and Howard-Flanders (1981c) West, Cassuto and Howard-Flanders 1981). The observation that pyrimidine dimers had no effect on *in vitro* RecA mediated strand exchange provided further evidence for this role (Rupp *et al*. 1971).

Evidence for the proposed role for RecA protein in the repair of double strand breaks was obtained by an *in vitro* demonstration that RecA protein was able to promote strand exchange past a double strand break in DNA (West and Howard-Flanders 1984).

b) *recBCD*

The *recB* and *recC* loci were identified, and a role for the *recB* and *recC* gene products in homologous recombination proposed by the isolation of mutations which reduced the recovery of recombinant colonies in crosses with Hfr donors to 1% of that in isogenic *recB*$^+$ or *recC*$^+$ recipient strains. In addition, strains with mutations in *recB* or *recC* were sensitive to UV and γ-irradiation, exhibited a decreased capacity for post-UV DNA degradation and were poorly viable (Howard-Flanders and Theriot 1966, Emmerson and Howard-Flanders 1967, Capaldo-Kimball and Barbour 1971).

The *recB* and *recC* genes have since been demonstrated to encode subunits of the enzyme exonuclease V (Goldmark and Linn 1970, Buttin and Wright 1968, Oishi 1969, Barbour and Clark 1970).

Studies of the reactions mediated by exonuclease V, initially hampered by an inability to purify the enzyme in good yields due to its low cellular concentration ($10^{\text{molecules}}$/cell) (Taylor and Smith 1980) were facilitated by the cloning of the *recB* and *recC* genes, and the subsequent overproduction of their encoded proteins (Hickson and Emmerson 1981, Dykstra *et al*. 1984).

Analysis of exonuclease V purified from strains harbouring cloned *recB* and *recC* genes, suggested that in addition to the 135 Kd and 125 Kd subunits encoded by *recB* and *recC* respectively,

exonuclease V contained a third subunit of approximately 65 Kda (Lieberman and Oishi 1974, Dykstra, Palas and Kushner 1984). Although the origin of this subunit was as then unknown, it was recently demonstrated to be the product of a gene immediately downstream of recB, designated recD (Amundsen et al. 1986, Biek and Cohen 1986).

The nucleotide sequence of the entire region between thyA and argA containing the recC ptr recB and recD genes has now been determined and the protein sequences deduced (Finch et al. 1986a, b, c and d). Analysis of the DNA sequence and proteins synthesized in polar recB mutants suggests recB and recD may be coordinately transcribed (Finch et al. 1986d, Amundsen et al. 1986).

Exonuclease V or RecBCD enzyme has been demonstrated in vitro to have ATP-dependent DS and SS exonuclease activity, single strand endonuclease activity and DS DNA unwinding activity. However, in vivo under normal physiological conditions, the exonuclease activities of recBCD enzyme are likely to be unimportant (Telander-Muskavitch and Linn 1981, Cox and Lehmann 1987). Consequently, the RecBCD promoted unwinding of DS DNA and SS endonucleolytic cleavage reactions have been the most intensively studied, in order to attempt to elucidate the role of the RecBCD enzyme in homologous recombination. In vitro RecBCD enzyme binds to the ends of DS DNA molecules and then migrates along the DNA molecules unwinding and rewinding DNA in a reaction requiring ATP to produce single stranded DNA loops (Taylor and Smith 1985, Taylor and Smith 1980, Telander-Muskavitch and Linn 1982). During this unwinding RecBCD enzyme introduces nicks at specific sites called Chi on the single strand containing the sequence 5'GCTGGTGG3' only if it approaches the sequence from the right (Smith et al. 1981, Ponticelli et al. 1985). Since in vivo the presence of Chi sites stimulates phages recombination (reviewed by Smith 1983) the in vitro demonstration that RecBCD enzyme cuts at Chi led to the proposal that the role of

RecBCD enzyme in recombination was to cut at Chi and provide a 3' end for initiation of RecA mediated strand exchange (Taylor *et al.* 1985, Smith *et al.* 1984). Alternative models suggest that Chi dependent cleavage may resolve Holliday intermediates and therefore terminate RecA dependent strand exchange (Leach and Stahl 1983, Faulds *et al.* 1979).

Since *recD* mutants which lack the Chi specific endonuclease activity, but are assumed to retain the DNA unwinding activity of exonuclease V, are recombination proficient, it would seem that the unwinding activity ofRecBCD enzyme is the most important activity in recombination (Chaudhury and Smith 1984, Finch *et al.* 1986d). However, a comparison of recombinant progeny obtained in crosses between multiply marked Hfr donors and *recD*$^+$ or *recD* recipients, suggests that in wild type strains Chi dependent cleavage results in a specific polarity of recombination, which is abolished in recD mutants (Lloyd, Porton and Buckman 1988). From these results it was concluded that although chi specific endonuclease activity has a role in recombination in wild type strains, in its absence some other function is able to substitute efficiently in recombination. Recent studies suggest the product of *recJ* may perform this function (Lloyd *et al.* 1988). The sensitivity of *recB* and *recC* mutants to UV and irradiation led to the proposal that RecBCD enzyme was required for recombination repair of DNA damage (Clark 1973). Since *recD* mutants are resistant to UV irradiation, as in recombination, the unwinding activity of the RecBCD enzyme appears to be the important function in DNA repair (Chaudhury and Smith 1984).

Since the preferred substrate for RecBCD enzyme *in vitro* was double stranded DNA with flush, or nearly flush ends (Taylor and Smith 1985), the demonstration by Wang and Smith (1983) that *recBC* mutants were able to repair daughter strand gaps efficiently was not surprising. They propose that the role of RecBCD in repair of DNA damage is in repair of double strand breaks, arising from unrepaired

daughter strand gaps (Wang and Smith 1986), or resulting from irradiation (Sargentini and Smith 1986).

A further role for the RecBCD enzyme in the induction of the SOS response by nalidixic acid was reported in 1.4.

Mutations which suppress both the conjugational recombination deficiency and the DNA repair defects of *recBC* mutants have been isolated and mapped to *sbcA*, the regulatory gene for the *recE* encoded exonuclease VIII (Barbour *et al.* 1970, Kushner *et al.* 1974, Willis *et al.* 1985) *sbcB*, the structural gene for exonuclease I (Kushner *et al.* 1971, 1972) and *sbcC* (Lloyd and Buckman 1985). Mutations in *sbcA* result in synthesis of exonuclease VIII, an enzyme that degrades one strand of double stranded DNA in a 5' - 3' direction (Joseph and Kolodner 1983), and which presumably can substitute for the RecBCD enzyme in generating a free 3' single stranded end with which RecA can initiate recombination. Mutations in *sbcB* abolish synthesis of the enzyme exonuclease I, which degrades single stranded DNA in a 3' - 5' direction, and which, it has been proposed, normally removes the 3' leading end of transferred Hfr DNA (Lloyd and Thomas 1984). Presumably in the absence of exonuclease I the 3' leading end persists and is then a substrate for RecA mediated recombination. The product of the *sbcC* gene has not yet been characterised.

The observed suppression of the *recBC* mutant phenotype led Clark (1973) to propose the existence of alternative pathways for conjugational recombination. It was suggested that in wild type strains, recombination proceeded by the "recBCD pathway", dependent on the *recBCD* gene products, whilst in *recBC sbcA* strains, recombination proceeded by the "recE pathway" dependent on the *recE* gene product, and in *recBC sbcBC* strains recombination proceeded by the "recF pathway", dependent on the product of the *recF* gene. Conjugational recombination via the recE and recF pathways was proposed to play only a minor role in *recBC*$^+$ strains.

Mutations in the recA, recB and recC genes have a severe effect on conjugational recombination in otherwise wild type strains, the remaining genes implicated in the process of genetic recombination discussed below were identified on the basis of their effects on recombination in strains in which the recombination deficiency associated with recBC mutations was suppressed by either sbcA or sbcBC mutations.

c) recF

The recF gene, mapped to 82' on the E. coli chromosome, was the first gene characterised that encoded a product required for recombination in recBC sbcBC strains (Horii and Clark 1973, Clark 1973, Ream et al. 1980). In recBC sbcBC and recBC sbcA strains, recF mutation reduced the recovery of recombinant colonies in conjugational crosses with Hfr donors to less than 1% and 10% of the respective recF$^+$ parental strains, which led to the conclusion that the pathways originally called "recF" and "recE" were in fact branches of the same pathway which required the RecF protein and was therefore termed the "RecF pathway" (Horii and Clark 1973, Gillen, Willis and Clark 1981). In similar conjugational crosses, recF mutations had little effect on the recovery of recombinant colonies in recBC$^+$ recipients (Horri and Clark 1973).

In contrast to the effects of recF mutations on conjugational recombination, recF mutations decreased plasmid recombination in recBC$^+$ strains as well as in recBC sbcA and recBC sbcBC strains, suggesting a requirement for the recF gene product for recombination between certain substrates even in recBC$^+$ strains (Cohen and Laban 1983). In fact recBC mutations had no effect on plasmid recombination, suggesting that recombination between covalently closed circular molecules in wild type strains was independent of recBCD gene products, and provides evidence for a major role for

recF dependent recombination in wild type strains.

In addition to causing deficiencies in plasmidic recombination, and conjugational recombination (in *recBC sbcBC* and *recBC sbcA* genetic backgrounds), recF mutations confer a sensitivity to UV light on *recBC*$^+$, *recBC sbcBC* and *recBC sbcA* strains (Horii and Clark 1973, Gillen, Willis and Clark 1981) which led to the suggestion that the *recF* gene product was required for recombination repair of UV damaged DNA. This was substantiated by demonstration that *recF* mutants were unable to complete daughter strand gap repair efficiently (Rothman *et al.* 1975, Wang and Smith 1983). In addition *recF* mutants were demonstrated to be deficient in UV induction of the SOS response (McPartland, Green and Echols 1980) which led to the suggestion that interaction of the *recF*$^+$ gene product with daughter strand gaps may be required for SOS induction (Blanar *et al.* 1984).

In order to determine the precise role of the RecF protein in both homologous recombination and repair of DNA damage, the biochemistry of the reactions mediated by the *recF* gene product requires study. In order to facilitate such study the *recF* gene has been cloned, the product identified as a polypeptide of approximately 40kd, and the DNA sequence determined (Ream and Clark 1983, Blanar *et al.* 1984). To date, the purification and characterisation of the RecF protein has not been reported.

d) *recJ*

A mutation in the *recJ* gene, resulting in a deficiency in recombination in a *recBC sbcBC* strain was first isolated by Clarke (1973). Subsequently, a series of 6 Tn*10* insertions which resulted in a deficiency in conjugational recombination in *recBC sbcA* strains were isolated and mapped to *recJ* at 62' on the E. coli chromosome (Lovett and Clark 1984).

The effects of recJ mutations on recombination are similar to those of recF mutations, reducing the recovery of recombinants in conjugational crosses between Hfr donors and recBC sbcA and recBC sbcBC recipients to the level of recA mutants, whilst having little effect on conjugational recombination in $recBC^+$ strains, and reducing plasmid recombination to less than 0.1% of that measured in $recJ^+$ derivatives of $recBC^+$, recBC sbcA or recBC sbcBC strains (Kolodner, Fishel and Howard 1985).

However, although the effects of recJ mutations on recombination are similar to those of recF mutations, recJ mutations confer a significantly different DNA repair phenotype to recF mutations on wild type strains.

recJ single mutants are UV^R, whilst there appears to be two classes of recJ mutations with respect to UV sensitivity in recBC sbcB and recBC sbcA genetic backgrounds, one class resulting in a UV^R phenotype, one in a UV^S phenotype; both classes result in a severe recombination deficiency (Lovett and Clark 1984). These results suggest that recJ is only required for repair in the absence of the RecBCD enzyme, and that the recombination and repair activities promoted by recJ protein may be separate. In this respect, the deficiency in SOS induction reported by Lovett and Clark (1985) may contribute to the UV sensitivity conferred by recJ mutations in a recBC sbcBC mutant background.

Although neither recD single mutants nor recJ single mutants are sensitive to UV, the recD recJ double mutant is extremely UV sensitive, which led to the proposal that recD and recJ encoded products may be functionally similar (Lloyd et al. 1988).

Once again, the elucidation of the role of recJ in recombination and repair may be facilitated by studies of the reactions mediated by the recJ product in vitro. As a first step towards such studies, the recJ gene has been cloned and demonstrated to encode a polypeptide of approximately 53kd (Lovett and Clark

1985).

e) *recN*

The *recN* locus at 57.5' on the *E. coli* genetic map was identified by a damage inducible Mud(Ap)^{R}*lac* insertion that resulted in a deficiency in conjugational recombination in a *recBC sbcBC* genetic background. Subsequent studies demonstrated that expression of *recN* was regulated by the products of the *lexA* and *recA* genes as part of the SOS response to DNA damage (Lloyd, Picksley and Prescott 1983, Finch, Chambers and Emmerson 1985).

The effects of *recN* mutations on the recovery of recombinants in conjugational crosses, are similar to the effects of *recF* and *recJ* mutations, reducing recombinant recovery to 1% and 10% respectively of that in isogenic *recBC sbcBC recN*$^{+}$ and *recBC sbcA recN*$^{+}$ strains, whilst having no effect on recombinant recovery in *recBC*$^{+}$ strains (Picksley, 1985, Lloyd, Picksley and Prescott 1983, Lloyd, Buckman and Benson 1987). However, unlike *recF* and *recJ* mutations, *recN* mutations have no effect on plasmid recombination in *recBC*$^{+}$ strains (Kolodner, Fishel and Howard 1985).

Studies on the expression of the *recN* gene performed by following production of B-galactosidase from the *recN*::Mud(Ap)^{R}*lac* fusion in different genetic backgrounds, revealed significant variation in the basal levels of expression in *recBC*$^{+}$, *recBC*$^{-}$, and *recBC sbcBC* strains. This led to the suggestion that some of the recombination deficiency associated with *recBC* mutants could be due to an almost complete lack of *recN* expression, whilst the recombination proficiency of *recBC sbcB* mutants could be at least partly attributed to the high level of *recN* expression in these strains (Picksley, Lloyd and Buckman 1984).

recN mutants have a DNA repair phenotype distinct from any of the *rec* mutants so far described. *recN* single mutants are only

slightly sensitive to UV, survival only being significantly reduced by doses greater than 90 J/M^2. However, they are extremely sensitive to mitomycin C and γ-irradiation. recBC sbcBC strains carrying mutations in recN are sensitive to mitomycin C, and both UV and γ-irradiation.

Since recN mutants are resistant to low doses of UV and only show slight sensitivity to high doses, it was concluded that the recN gene product was probably not required for the repair of daughter strand gaps. However, the sensitivity to mitomycin C and γ-irradiation of the recN single mutants suggested that the recN gene product may have a role in double strand break repair. This was confirmed by studies that demonstrated that induction of the recN gene product was required for converting low MW DNA present in cells after γ-irradiation to normal high MW DNA (Picksley, Attfield and Lloyd 1984). The slight sensitivity of recN mutants to UV may be due to a requirement for recN in the repair of double strand breaks arising from unrepaired daughter strand gaps (Wang and Smith 1986) which it has been reported may be repaired by recBC dependent processes (Wang and Smith 1982). The increased UV sensitivity of recBC sbcBC recN mutants may reflect a greater requirement for the repair of UV induced double-strand breaks in the absence of recBCD enzyme. The relationship between recN-dependent repair of double-strand breaks and recBCD dependent repair of DS breaks is not yet clear and may await studies on the reactions promoted by the recN product. The recN gene has been cloned and the encoded protein identified as a polypeptide of approximately 60kd (Picksley, Morton and Lloyd 1985). The DNA sequence of the recN locus has been determined and the protein sequence deduced (Rostas et al. 1987). Identification of an ATP binding site, and a DNA binding fold similar to that identified in the RecA protein, may facilitate purification of the RecN protein, and allow its biochemical characterisation.

f) *recO*

The *recO* locus, at 55.4' on the *E. coli* genetic map, was identified by a Tn*5* insertion mutation which reduced conjugational recombination in a *recBC sbcBC* strain to 0.1% of the equivalent *recBC sbcBC recO*$^+$ strain (Kolodner, Fisher and Howard 1985). In addition *recO* mutations reduced the recovery of recombinants in conjugational crosses with *recBC sbcA* recipients to less than 1% of the isogenic *recO*$^+$ strain. In a manner similar to the effects of *recF* and *recJ* mutations, *recO* mutations have little effect on the recovery of recombinants in conjugational crosses in *recBC*$^+$ strains but reduce plasmid recombination to approximately 2% of *recO*$^+$ strains.

In addition to causing a deficiency in recombinant recovery in *recBC sbcBC* strains *recO* mutations reduce the recovery of F' transconjugants by a factor of 10, which was originally proposed to be due to a deficiency in DNA transfer, a proposal which may be discounted since efficient transfer of Hfr H (λind$^-$) into *recBC sbcBC recO* strains has since been demonstrated. This leaves the possibility that the deficiency in F' transconjugant recovery of *recBC sbcBC recO* strains is related to their recombination deficiency.

recO mutations result in yet another distinct DNA repair phenotype - causing sensitivity to UV light but resistance to mitomycin C in *recBC*$^+$ strains and an increased sensitivity to UV and extreme sensitivity to MC in *recBC sbcBC* strains.

The relationship between the role of *recO* in conjugational and plasmidic recombination and DNA repair is not yet clear. However, a role similar to that of *recF* in daughter strand gap repair could be postulated. Any role that *recO* may have in DS break repair may only be important in the absence of RecBCD enzyme.

To date, cloning of *recO* has not been reported.

g) recQ

The recQ locus, at approximately 85.5' on the E. coli genetic map, was identified by the isolation of a mutation that increased resistance to thymineless death in a recBC$^+$ strain (Nakayama et al. 1984). Although recQ$^+$ had no effect on conjugational recombination in this genetic background, it reduced the recovery of recombinants in conjugational crosses with recBC sbcBC recipients to 1-2% of the parent strain, suggesting that recQ was another gene required for recBCD independent recombination.

Although recBC sbcBC recQ strains are UV sensitive, recQ single mutants are resistant to UV, suggesting that the recQ product does not have a role in daughter strand gap repair. The effects of DNA damaging agents (such as mitomycin C and γ-irradiation) that cause strand breaks on the survival of recQ mutants has not been reported, precluding any suggestions of any role of the recQ product in DS break repair.

The recQ gene has been cloned, the DNA sequence determined, and shown to encode a 73kd protein (Irino, Nakayama and Nakayama 1986). Analysis of the DNA sequence of the recQ region revealed the presence of a putative LexA binding site, suggesting that recQ was under SOS control. This was subsequently demonstrated by analysis of B-galactosidase production from strains containing plasmids with recQ::lac fusions, and suggests the recQ product probably has a role in DNA repair (Irino, Nakayama and Nakayama 1986).

Thus, although several gene products have been implicated in recombination repair, with the exception of the RecA protein, the precise roles that each product plays remains far from clear. A more complete understanding of recombination repair awaits the purification and biochemical characterisation of all the products involved.

1.11 The ruv gene

The first mutations in the ruv gene designated ruvA4 and ruvB9 were identified by the isolation of mitomycin C sensitive derivatives of an MNNG mutagenised AB1157 (wild type) strain, and mapped close to the his gene at 41' on the E. coli chromosome (Otsuji, Iyehara and Hideshima 1974, Iyehara and Otsuji 1975, Bachmann 1983). In addition to their effect on mitomycin C sensitivity, strains carrying the ruvA4 or ruvB9 mutations showed increased sensitivity to UV and gamma irradiation. However a role for the ruv gene product in either excision or recombination repair of DNA damage was initially ruled out since ruv mutants were proficient at host cell reactivation of UV irradiated phages, and were proficient as recipients in conjugational crosses (Otsuji et al. 1974). The observation that strains carrying ruv mutations tended to form filaments, particularly when treated with DNA damaging agents, led Otsuji et al. (1974) to propose that ruv mutants had a cell division defect similar to that of lon mutants (Howard-Flanders, Simson and Theriot 1964). Subsequent studies, which demonstrated that strains carrying both ruv and lon mutations were no more sensitive to UV irradiation than strains carrying the single mutations, provided support for this proposal (Iyehara and Otsuji 1975). However this was later questioned when it was discovered that whereas sulA mutations suppressed both the filamentation and the UV irradiation sensitivity of lon strains, they suppressed only the filamentation of ruv strains, having no effect on the sensitivity to UV irradiation (Otsuji and Iyehara-Ogawa 1979, Shurvinton 1983).

The ruv gene was later demonstrated to be induced as part of the SOS response by the isolation of an ruv::Mud(Ap)^{R}lac fusion from which expression of B-galactosidase was induced by DNA damage in a manner dependent on the recA and lexA genes (Shurvinton and Lloyd 1982, Shurvinton 1983).

Early studies of the genetics of the ruv gene were initially hampered by the lack of selectable markers in its region and by the

high frequency of reversion of the eda marker with which it was initially reported to be 18-24% cotransduced (Iyehara and Otsuji 1975). This situation was improved by the isolation of a stable eda::Tn10 insertion which was cotransduced with the ruv gene with a frequency of approximately 40%, and which allowed ruv mutations to be moved between strains with much greater ease (Shurvinton 1983, Shurvinton et al. 1984). This also facilitated the isolation of strains carrying further ruv point mutations (ruv-52, ruv-53, ruv-54, ruv-55, ruv-56) and presumed ruv deletion mutations (ruv-57, ruv-58) (Shurvinton 1983, Lloyd, Shurvinton and Benson 1984), and confirmed that a mutation, later designated ruv-51, isolated by Stacey and Lloyd (1976) was in fact in the ruv gene. Two additional ruv mutations (ruv59::Tn10 and ruv60::Tn10) were isolated by Tn10 mutagenesis of the bacterial chromosome (Shurvington et al. 1984). Thus, prior to this study 14 different ruv mutations had been isolated (summarised in Table 1.3). However the function of the ruv gene product was still unknown.

The aims of this project were to attempt to elucidate the role of the ruv gene product, and to study its genetic organisation and expression.

The main mechanisms for repair of DNA damage in E. coli, and the gene products involved are summarised in Table 1.3b.

Table 1.3b Summary of genes involved in DNA repair in E. coli

Repair Mechanism	Gene	Product and in vitro activity	Control
Repair of alkylation damage	ada	06 me G methyl transferase	Adaptive response
	alkB	27kd protein (unknown function)	Adaptive response
	alkA	3 me A glycosylase II	Adaptive response
	aidB	?	Adaptive response
Excision repair	uvrA, uvrB, uvrC	UvrABC enzyme. Introduces single strand nicks 5' and 3' or damage.	SOS response
	uvrD	DNA helicase II. Promotes release of excised oligomer and turnover of UvrABC enzyme.	SOS response
	polA	DNA polymerase I. Promotes release of excised oligomer and synthesis of new DNA strand.	
Error prone repair	umuD	16kd protein (unknown function)	SOS response
	umuC	45kd protein (unknown function)	SOS response
	recA	RecA protein. Promotes cleavave of umuD protein to activated form, binds to irradiated DNA and inhibits 3'-5' exonuclease activity of DNA polymerase III.	SOS response
Recombination repair	recA	RecA protein. Promotes pairing and strand exchange between homologous molecules.	SOS response
	recB, recC, recD	RecBCD enzyme (exonuclease V) DS and SS exonuclease, DS DNA unwinding and SS endonuclease activities.	
	recF	40kd protein. Stimulates RecA promoted strand exchange.	
	recJ	53kd protein. SS exonuclease.	
	recN	60kd protein (unknown function)	SOS response
	recO	?	
	recQ	73kd protein (unknown function)	SOS response
Mismatch repair	dam	DNA adenine methylase. Methylates adenine in d(GATC) sequence.	
	mutH	MutH protein introduces incision 5' of d(GATC) sequence.	
	mutS	97kd protein. Binds mismatched base pairs.	
	mutL	?	

Table 1.3 Mutations in the *ruv* gene

ruv mutation	Nature of mutation and method of isolation
ruvA4	Probable point mutation, MNNG mutagenesis of AB1157
ruvB9	Probable point mutation, MNNG mutagenesis of AB1157
ruv-51	
ruv::Mud(Ap)^{R}*lac*$^{+}$	Mud(Ap)^{R}*lac* insertion in *ruv*, Lac^{+}
ruv::Mud(Ap)^{R}*lac*$^{-}$	Mud(Ap)^{R}*lac* insertion in *ruv*, Lac^{-}
ruv-52	Point mutations, MNNG mutagenesis of P1.CS40
ruv-53	Mutagenised P1.CS40 x AB1157 ⟶ Tc^{R}
ruv-54	Mitomycin C sensitive derivatives
ruv-55	Point mutations. Mutagenesised P1.CS40 x AB1157 ⟶ Tc^{R}
ruv-56	Temperature dependent mitomycin C sensitive derivatives. MC^{S} at 42°C
ruv-57	Mutations involving chromosomal rearrangements
ruv-58	Selected as TC^{S} derivatives of CS42 *eda*::Tn*10* *ruv*$^{+}$ Mitomycin C sensitive derivatives
ruv-59	*ruv*::Tn*10* insertions. Tn*10* mutagenesis
ruv-60	Mitomycin C sensitive derivatives

CHAPTER 2

MATERIALS AND METHODS

Materials and methods used throughout this thesis are presented in this chapter, those pertaining to specific sections of the work are detailed in the appropriate chapters.

2.1 Strains

Escherichia coli K12 bacteria, bacteriophages and plasmids used are detailed in Table 2.1.

2.2 Chemicals

Chemicals were obtained from Sigma, BDH, Fisons, or May and Baker unless otherwise stated. Restriction endonucleases, T4 DNA ligase, large fragment of DNA polymerase I (Klenow) and DNA polymerase I were obtained from BRL and used and stored as recommended.

2.3 Media

All media recipes are presented in terms of quantities necessary for the preparation of 1 litre of media, with distilled water.

(a) LB broth contained 10g Bactotryptone, 5g yeast extract, 0.5g NaCl and 0.08g NaOH. LB agar contained 15g of bactoagar per litre of LB broth.

(b) Mu broth contained 10g Bactotryptone, 5g yeast extract, 0.08g NaOH and 10g of NaCl. Mu plate agar (1%) and Mu overlay agar

Table 2.1 **Bacteria, bacteriophages and plasmids**

Strain	Genotype	Source or derivation
a)	Bacterial strains	
(i)	recBC$^+$ derivatives	
AB1157	F$^-$ thi-1 his-4 (gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfbD1 ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 supE44 rpsL31	Bachmann, 1972
W3110	F$^-$ IN(rrnD-rrnE)1	Laboratory strain
AB2463	a, recA13	Bachmann, 1972
N1372	F$^-$ thi-1 his-4 argE3 thrB1007 mtl-1 xyl-5 sup-37 nalA tsx	Laboratory strain
N1373	F$^-$ as N1372 but ruv-51	Laboratory strain
HI24	a, ruvA4	Otsuji et al.(1974)
HI36	a, ruvB9	Otsuji et al.(1974)
CS81A	a, eda-51::Tn10, b	Shurvinton 1983
CS40	a, eda-51 ruvA4	"
CS42	as N1372 but eda-51	"
CS81	a, eda-51 ruv-52	"
CS85	a, eda-51 ruv-53	"
CS86	a, eda-51 ruv-54	"
CS42	F$^-$ as N1372 but eda-51	"
CS114	F$^-$ as CS42 but Tet^S eda? ruv-57	"
CS115	F$^-$ as CS42 but Tet^S eda? ruv-58	"
CS123	F$^-$ as CS115 but λcI857	"
N2057	a, ruv-60::Tn10, b	R.G. Lloyd

Strain	Genotype	Source or derivation
N2058	a, *ruv-59*::Tn*10*, b	R.G. Lloyd
CSM7	F⁻ as N1372 but *his+ nalA+* (*lac-pro*)XIII *ruv*::Mu*d*(Ap)^{R}*lac*	Shurvinton 1983
CSM13	F⁻ as N1372 but *his+ nalA+* (*lac-pro*) XIII *ruv*::Mu*d*(Ap)^{R}*lac*	Shurvinton and Lloyd 1982
FB113	a, *ruvB9* λRL101	This study, c
FB114	a, *ruvB9* λRL102	"
FB115	a, *ruvB9* λRL104	"
FB116	a, *ruvB9* λRL103	"
FB117	a, *ruvB9* λRL105	"
FB118	a, *ruvA4* λRL101	"
FB119	a, *ruvA4* λRL102	"
FB120	a, *ruvA4* λRL104	"
FB121	a, *ruvA4* λRL105	"
FB122	F⁻ as N1373 but λRL102	"
FB123	F⁻ as N1373 but λRL103	"
FB124	F⁻ as N1373 but λRL105	"
FB129	a, *eda-51 ruv-52* λRL101	"
FB130	a, *eda-51 ruv-52* λRL102	"
FB131	a, *eda-51 ruv-52* λRL105	"
FB132	a, *eda-51 ruv-52* λRL103	"
FB133	a, *eda-51 ruv-52* λRL104	"
FB136	F⁻ as CS115 but λRL104	"
FB137	F⁻ as CS115 but λRL103	"
FB138	F⁻ as CS115 but λRL105	"
FB139	a, *eda-51 ruv-53* λRL102	"
FB140	a, *eda-51 ruv-53* λRL103	"
FB141	a, *eda-51 ruv-53* λRL105	"

Strain	Genotype	Source or derivation
pA1048	a, ruvA4, /pPVA101	P.V. Attfield
FB201	a, ruvA4, /pPVA101	This study, d
FB202	a, ruvB9, /pPVA101	"
FB203	F⁻ as N1373, /pPVA101	"
FB204	a, eda-51 ruv-52, /pPVA101	"
FB205	a, eda-51 ruv-53, /pPVA101	"
FB206	a, eda-51 ruv-54, /pPVA101	"
FB207	F⁻ as CS114 but /pPVA101	"
FB208	a, ruv-60, /pPVA101	"
FB210	F⁻ as CS115 but pPVA101	"
FB211	a, ruvA4, /pHSG415	"
FB212	a, ruvB9, /pHSG415	"
FB213	F⁻ as N1373 but pHSG415	"
FB214	a, eda-51 ruv-52, /pHSG415	"
FB215	a, eda-51 ruv-53, /pHSG415	"
FB216	a, eda-51 ruv-54, /pHSG415	"
FB217	F⁻ as CS114 but pHSG415	"
FB218	a, ruv-60, /pHSG415	"
FB219	a, ruv-59, /pHSG415	"
FB220	F⁻ as CS115 but pHSG415	"
FB272	a, ruvA4, /pPVA105	"
FB273	a, ruvB9, /pPVA105	"
FB274	a, ruv-60, /pPVA105	"
FB275	a, ruv-59 /pPVA105	"
FB276	a, eda-51 ruv-52 /pPVA105	"
FB277	a, eda-51 ruv-53 /pPVA105	"
FB278	a, eda-51 ruv-54 /pPVA105	"
FB292	a, ruvB9, /pFB500	"

Strain	Genotype	Source or derivation
FB293	F⁻ as N1373 but pFB500	This study, d
FB294	a, ruv-60, /pFB500	"
FB295	a, ruv-59, /pFB500	"
FB296	a, eda-51 ruv-52, /pFB500	"
FB297	a, eda-51 ruv-53, /pFB500	"
FB298	a, eda-51 ruv-54, /pFB500	"
FB299	F⁻ as CS114 but pFB500	"
FB300	F⁻ as CS115 but pFB500	"
FB330	a, ruvA4, /pFB501	"
FB331	a, ruvB9, /pFB501	"
FB332	F⁻ as N1373 but pFB501	"
FB333	a, ruv-60, /pFB501	"
FB334	a, ruv-59, /pFB501	"
FB335	a, eda-51 ruv-52, /pFB501	"
FB336	a, eda-51 ruv-53, /pFB501	"
FB337	a, ruv-54, /pFB501	"
FB338	F⁻ as CS114 but pFB501	"
FB339	F⁻ as CS115 but pFB501	"
FB345	a, ruvA4, /pFB502	"
FB346	a, ruvB9, /pFB502	"
FB348	a, eda-51 ruv-53, /pFB502	"
FB349	a, eda-51 ruv-54, /pFB502	"
FB350	F⁻ as CS115 but pFB502	"
FB241	a, ruvA4, /F'42 lac⁺	"
FB242	a, eda51 ruv-52, /F'42 lac⁺	"
AB2480	F⁻ as AB1157 but recA uvrA	Howard-Flanders and Theriot 1966
FB368	F⁻ as AB2480 but pBR322	This study, d

Strain	Genotype	Source or derivation
FB369	F^- as AB2480 but pACYC184	This study, d
FB370	F^- as AB2480 but pUC19	"
PA1120	F^- as AB2480 but pPVA101	"
PA1121	F^- as AB2480 but pPVA105	"
PA1122	F^- as AB2480 but pFB500	"
PA1123	F^- as AB2480 but pFB501	"
PA1124	F^- as AB2480 but pFB502	"
FB371	F^- as AB2480 but pFB503	"
FB372	F^- as AB2480 but pFB504	"
FB373	F^- as AB2480 but pFB505	"
FB374	F^- as AB2480 but pFB507	"
FB375	F^- as AB2480 but pFB509	"
FB394	F^- as AB2480 but pFB511	"
FB542	a, uvrA λcI857	"
C600	F^- thi$^-$ thr-1 leu-6 lacY1 tonA21 SupII Str^S	Laboratory strain
JM101	supE thi Δ(lac, proAB)/F', traD36, proAV $lacI^qZ$ M15	Yannisch-Perron,C. et al. (1985)
(ii)	**recBC sbcBC derivatives**	
JC7623	a, recB21 recC22 sbcB15 sbcC201	Kushner et al.(1971)
FB153	as JC7623 but eda51	P1.CS81 x JC7623[e]
FB154	as JC7623 but eda51 ruv-52	P1.CS81 x JC7623[f]
FB155	as JC7623 but eda51 ruv-54	P1.CS86 x JC7623[f]
FB156	as JC7623 but ruv59	P1.N2085 x JC7623[f]
FB165	as JC7623 but eda?(Tc^S)	Tc^S selection of FB153

Strain	Genotype	Source or derivation
FB166	as JC7623 but *eda*?(Tc^S)*ruv-52*	Tc^S selection of FB154
FB167	as JC7623 but *eda*?(Tc^S)*ruv-54*	Tc^S selection of FB155
FB168	as JC7623 but *ruv*?	Tc^S selection of FB156
FB221	as FB165 but pHSG415	d
FB222	as FB166 but pHSG415	"
FB223	as FB167 but pHSG415	"
FB224	as FB168 but pHSG415	"
FB225	as FB165 but pPVA101	"
FB226	as FB166 but pPVA101	"
FB227	as FB167 but pPVA101	"
FB228	as FB168 but pPVA101	"
FB235	as JC7623 but *eda51* *recA*::Tn*10*	P1.N3072 x FB165[g]
FB236	as JC7623 but *eda51* *ruv-52* *recA*::Tn*10*	P1.N3072 x FB166[g]
FB433	as JC7623 but *eda51* *ruv-54* *recA*::Tn*10*	P1.N3072 x FB167[g]
FB434	as JC7623 but *eda51* *recA*? (Tc^S)	Tc^S selection of FB235
FB435	as JC7623 but *eda51* *ruv-52* *recA*? (Tc^S)	Tc^S selection of FB236
FB437	as JC7623 but *eda51* *ruv-54* *recA*? (Tc^S)	Tc^S selection of FB433
N2282	as JC7623 but *recAo*[c] *recA200*	R.G. Lloyd
FB322	as JC7623 but *recAo*[c] *recA200* *eda51*	P1.CS81 x N2282[e]
FB323	as JC7623 but *recAo*[c] *recA200* *eda51* *ruv53*	P1.CS85 x N2282[f]

Strain	Genotype	Source or derivation
FB324	as JC7623 but recAo^{c} recA200 eda51 ruv54	P1.CS86 x N2282^{f}
FB325	as JC7623 but recAo^{c} recA200 eda51 ruv52	P1.CS81 x N2282^{f}
N2214	as JC7623 but recA200 srl::Tn10	R.G. Lloyd
N1434	a, lexA(ind^{-}) recAo^{c} srl^{+}	"
N1435	a. lexA(ind^{-}) recAo^{c} recA200 srl^{+}	"
FB271	as JC7623 but recA200	P1.W3110 x N2214 srl^{+}
FB415	as JC7623 but recA200 eda51	P1.CS81 x FB271^{e}
FB416	as JC7623 but recA200 eda51 ruv-52	P1.CS81 x FB271^{f}
FB417	as JC7623 but recA200 eda51 ruv-53	P1.CS85 x FB271^{f}
FB418	as JC7623 but recA200 eda51 ruv-54	P1.CS86 x FB271^{f}
(iii)	**recBC sbcA derivatives**	
JC8679	recB21 recC22 sbcA23	Gillen et al (1981)
N2241	as JC8679 but eda51	R.G. Lloyd
N2242	as JC8679 but eda51 ruv-53	"
N2243	as JC8679 but eda51 ruv-52	"
N2244	as JC8679 but eda51 ruv-54	"
FB282	as JC8679 but eda?(Tc^{s})	Tc^{s} selection of N2241
FB283	as JC8679 but eda?(Tc^{s})ruv-53	Tc^{s} selection of N2242
FB284	as JC8679 but eda?(Tc^{s})ruv-52	Tc^{s} selection of N2243

Strain	Genotype	Source or derivation
FB285	as JC8679 but eda?(Tc^S)ruv-54	Tc^S selection of N2244
N1627	a, srl::Tn10 recA200	
FB269	as JC8679 but srl::Tn10	P1.N1627 x JC8679[e]
FB270	as JC8679 but srl::Tn10 recA200	P1.N1627 x JC8679[e]
FB286	as JC8679 but $recAo^C$ recA200	P1.N1435 x FB269 - srl^+
FB289	as JC8679 but recA200	P1.N1434 x FB270 - srl^+
FB311	as JC8679 but $recAo^C$ recA200 eda51	P1.CS81 x FB286[e]
FB312	as JC8679 but $recAo^C$ recA200 eda51 ruv-52	P1.CS81 x FB286[f]
FB318	as JC8679 but recA200 eda51	P1.CS81 x FB289[e]
FB319	as JC8679 but recA200 eda51 ruv-53	P1.CS85 x FB289[f]
FB320	as JC8679 but recA200 eda51 ruv-52	P1.CS81 x FB289[f]
FB321	as JC8679 but recA200 eda51 ruv-54	P1.CS86 x FB289[f]
FB387	as JC8679 but eda51 recA::Tn10	P1.N3072 x FB282[g]
FB388	as JC8679 but eda51 ruv-52 recA::Tn10	P1.N3072 x FB284[g]
FB389	as JC8679 but eda51 ruv-54 recA::Tn10	P1.N3072 x FB285[g]

(iv) Strains used in expression studies

CS41	a, but thr^+ leu^+ (pro-lac) XIII MuC^+ruv::Mud$(Ap)^R$lac	Shurvinton 1983
FB244	as CS41 but pPVA101	This study, d

Strain	Genotype	Source or derivation
N2070	F⁻ *ruv*::Mud(Ap)^R*lac* Mu*c*$^+$ (*lac-pro*)XIII *malE*::Tn*5* *sfiA11* *argE3* *his-4* *rpsL31*	R.G. Lloyd
N2080	as N2070 but *recA*::Tn*10*	"
N2184	as CS41 but *malE*::Tn*10* *lexA*(ts)	Shurvinton & Lloyd (1983)
CSMCZ4	as N1372 but *his*$^+$ *nalA*$^+$ (*lac-pro*) XIII *sulA*::Mud(Ap)^R*lac*	Shurvinton 1983
FB160	as CSMCZ4 but *ruv-59*	P1.N2058 x CSMCZ4[f]
FB161	as CSMCZ4 but *eda51*	P1.CS81 x CSMCZ4[f]
FB162	as CSMCZ4 but *eda51* *ruv-52*	P1.CS81 x CSMCZ4[f]
FB163	as CSMCZ4 but *eda51* *ruv-54*	P1.CS86 x CSMCZ4[f]
CSMCZ7	as N1372 but *his*$^+$ *malA*$^+$ Δ(*lac-pro*) XIII *uvrA*::Mud(Ap^R*lac*	Shurvinton 1983
FB191	as CSMCZ7 but *eda51*	P1.CS81 x CSMCZ7[f]
FB193	as CSMCZ7 but *ruv-59*	P1.N2058 x CSMCZ7[f]
N1234	(JC12334) a, *tna-300*::Tn*10* *recF143*	Ream *et al*. 1980
N1563	(DM1411) a, (*tif-1*) *sulA11* (*lexA3*) *lexA*(Def) *recA*	D. Mount
N1564	DM49 *lexA*(ind⁻)	
N1182	AB2470 *recB21*	Bachmann 1972
N1193	JC5489 *recC22*	"
SP254	a, *recN262*	Picksley *et al*.
FB463	N1563/pFB4	This study, d
FB464	N1563/pFB7	"
FB445	N1563/pFB12	"
FB446	N1563/pFB14	"
FB455	N1204/pFB4	"

Strain	Genotype	Source or derivation
FB456	N1204/pFB7	This study, d
FB457	N1204/pFB12	"
FB458	N1204/pFB14	"
FB482	N1564/pFB14	"
FB501	N1182/pFB14	"
FB504	N1193/pFB14	"
FB507	N1234/pFB14	"
FB513	SP254/pFB14	"
FB516	JC8679/pFB14	"
FB461	a, /pFB1	"
FB462	a, /pFB3	"
FB463	a, /pFB4	"
FB464	a, /pFB7	"
FB465	a, p/FB12	"
FB466	a, /pFB14	"
FB467	a, /pFB17	"
FB468	a, /pFB22	"
(v)	**Hfr and plasmid donor strains**	
KL548	F' 128 *proAB*$^+$ *lacI3* *lacZ813*/ Δ(*pro-lac*)III *recA* *rpsE*	K.B. Low
KL226	HfrC (PO2A) *relA* *tonA22*	"
GY2200	Hfr (Hayes) (λ*ind*$^-$)$^+$ *thi-1* *mal*	R. Devoret
N1671	HfrC (PO2A) *relA* *tonA22* *car98*::Tn*10*	R.G. Lloyd
NH4104	F' lac$^+$/*uvrA6* *proA2* *leu8* *thi* *thr-4* *ara-14* *lac-1* *his*	K.B. Low

Strain	Genotype	Source or derivation
FB449	NH4104/pBR322	This study, d
FB451	NH4104/pPVA101	"
FB414	NH4104/pFB511	"
FB356	F' 101 Thr^+ Leu^+/C600	N1515(F'101) x C600 Thr^+ Leu^+
PA1011	R6K ($Ap^R Sm^R$)/*pro met*	P.V. Attfield
PA1012	R1 ($Ap^R Sp^R$)/*pro met*	"
N1722	R136 (Tc^R)/*pro met*	R.G. Lloyd
N2392	NK7380 as W3110 pOX::38::pTAC + mini-Kan	N. Kleckner
KL96	Hfr *thi-1 rel-1 lac-42* -	K.B. Low
KL99	Hfr *thi-1 rel-1 lac-42* -	"
N1674	as KL208 but *gal*::Tn*10*	R.G. Lloyd

b) *Bacteriophages*

λ^+	Wild-type	This laboratory
λ *vir*	as λ^+ but virulent	"
λ c(*int*) h80	as λ^+ but cI(int) h80	"
λcI857	as λ^+ but cI857	"
λ *vis*	*E. coli Sau3A* library in PE11	D. Bramhill
λRL101	λ*ruv*$^+$ phage isolated from λ *vis*	This study
λRL102	"	"
λRL103	"	"
λRL104	"	"

Strain	Genotype	Source or derivation
λRL105	λ *ruv*$^+$ phage isolated from λ *vis*	This study
λRL106	"	"
M13mp18	M13 derivatives	Yannisch-Perron, Viera and Messing (1985)
M13mp19	M13 derivative	

c) Plasmids

pHSG415	Ap^R, Cm^R, Km^R low copy number plasmid	Hashimoto-Gotoh *et al*. 1977
pBR322	Ap^{R}Tc^R multicopy number plasmid	Bolivar *et al*. 1977
pACYC184	Cm^{R}Tc^R multicopy number plasmid	Chang and Cohen 1978
pUC18	Ap^{R}Lac$^+$ multicopy number plasmid	Yannisch-Perron *et al*. 1985
pPVA101	*ruv*$^+$, Km^S recombinant of pHSG415	This study
pPVA105	*ruv*$^+$, Cm^S deletion derivative of pPVA101	"
pFB500	*ruv*$^+$, deletion derivative of pPVA105	"
pFB501	*ruv*$^+$, deletion derivative of pFB500	Laboratory strains
pFB502	*ruv*$^-$, deletion derivative of pPVA101	"
pFB503	*ruv*$^-$, pBR322 recombinant harbouring 1.9kb pPVA101 DNA	"
pFB304	*ruv*$^-$, pBR322 recombinant harbouring 1.7kb pPVA101 DNA	"

Strain	Genotype	Source or derivation
pFB505	*ruv*⁻, pBR322 recombinant harbouring 8.1kb pPVA101 DNA	Laboratory strains
pFB507	*ruv*⁻, pACYC184 recombinant harbouring 4.6kb pFB502 DNA	"
pFB511	*ruv*⁻, pBR322 recombinant harbouring 4.6kb pFB502 DNA	"
pFB512	*ruv*⁻, pUC18 recombinant harbouring 4.6kb pFB502 DNA	"
pFB509	*ruv*⁻, pUC18 recombinant harbouring 2.6kb pPVA101 DNA	"
pGS700	*ruv*⁻, pUC18 recombinant harbouring 1.2kb pPVA101 DNA	G. Sharples
pKK232-8	Ap^R promoter cloning vector	Brosuis 1984
pPAK316	pACYC184 derivative harbouring Tn*1000* *tnpR* gene	D. Sherratt
pMA21	pBR322 derivative containing directly repeated Tn*1000* *res* sites	"
pMA2631	pBR322 derivative containing inverted repeated Tn*1000* *res* sites	"
pFB1	Cm^R derivative of pKK232-8, containing SOS inducible promoter, derived from pFB512 DNA	This study
pFB3	"	"
pFB4	"	"
pFB7	"	"
pFB12	"	"
pFB14	"	"
pFB17	"	"

Strain	Genotype	Source or derivation
pFB22	Cm^R derivative of pKK232-8, containing SOS inducible promoter, derived from pFB512 DNA	This study

Table 2.1

a. Otherwise as AB1157.
b. Transposon insertions are designated by allele number only after the first listing.
c. λ lysogens were purified from the centres of patches of lysis. (Produced by spotting dilutions of λ lysates onto lawns of appropriate host strains), by streaking on plates seeded with λc(int)h80.
d. Plasmids were introduced into bacterial strains by the standard transformation procedure outlined in section 2.11, selecting for the appropriate antibiotic resistance.
e. Selection for Tc^R transductants, 0.5ug/ml mitomycin C resistant isolates at 32°C (0.2ug/ml mitomycin C sensitive at 42°C for recA200 isolates).
f. Selection for Tc^R transductants, 0.5ug/ml mitomycin C sensitive isolates (at 32°C for recA200 derivatives which are sensitive to 0.2ug/ml mitomycin C at 42°C).
g. Selection for Tc^R transductants, 0.2ug/ml mitomycin C sensitive derivatives.

(0.4% and 0.6%) were prepared by adding appropriate amounts of Bactoagar to Mu broth.

(c) TB broth contained 10g of Bactotryptone and 5g NaCl. TB agar was prepared by adding 10g or 4g of Bactoagar per litre of TB broth for plate and overlay agar respectively.

(d) Minimal 56/2 salts media (Willets et al. 1969) contained 2.64g of KH_2PO_4, 4.34g of Na_2HPO_4, 1ml of 10% $MgSO_4.7H_2O$, 10ml of 10% $(NH_4)_2SO_4$, 0.5ml of 1% $Ca(NO_3)_2$ and 0.5ml of 0.05% $FeSO_4$ $7H_2O$. 56/2 salts agar contained 15g of Bactoagar per litre of 56/2 salts. 56/2 salts medium was routinely used for diluting bacterial strains. It was supplemented with thiamine (1ug/ml), glucose (3.3mgs/ml) and required amino acids (50-80ug/ml) for growth of bacteria.

(e) Minimal M9 media contained 6g of Na_2HPO_4, 3g of KH_2PO_4, 0.5g of NaCl and 1g of NH_4Cl. 2ml of 0.5M $CaCl_2$ and 25ml of 0.4M $MgSO_4.7H_2O$ were added after autoclaving, and the media supplemented with glucose, thiamine and required amino acids as for 56/2 salts media.

(f) MacConkey agar contained 40g of MacConkey agar base (Difco). 50ml of 20% sugar solution was added after autoclaving.

(g) Agar for selection of tetracycline sensitive strains (TS agar) (Bochner et al. 1980) contained 5g of Bactotryptone, 5g of yeast extract, 10g of NaCl, 3ml of 2mg/ml tetracycline in 500ml, and 9g of $NaH_2PO_4.2H_2O$ and 5ml 20mM $ZnCl_2$ in 500ml, autoclaved separately and mixed 1:1 after autoclaving. Fusaric acid (6ml of 2mg/ml solution) was added immediately before pouring plates.

(h) YT broth contained 8g Bactotryptone, 5g yeast extract and 5g NaCl. YT agar contained 10g or 6g Bactoagar for plate and overlay agar respectively.

All media were routinely sterilised by autoclaving at 121°C for

15'.

Antibiotics were added to media after autoclaving as required:- Ampicillin (Ap) 20ug/ml, Chlortetracycline (Tc) 20ug/ml, Streptomycin sulphate (Sm) 100ug/ml, Kanamycin (Km) 40ug/ml and Chloramphenicol (Cm) 30ug/ml.

2.4 Phage buffers

(a) λ-buffer for bacteriophage λ contained 6ml 1M Tris-HCl pH 7.2, 2.4g of $MgSO_4$ $7H_2O$ and 0.05g gelatine.

(b) MC buffer (magnesium calcium) for P1 phage contained 2.46g of $MgSO_4.7H_2O$ and 0.735g of $CaCl_2.2H_2O$.

Buffers were sterilised by autoclaving at 121°C for 25'.

2.5 Growth and maintenance of bacterial strains

Bacterial strains were routinely grown to stationary phase in 5ml portions of LB broth in 'overnight' tubes on a rotator, or on LB agar plates, incubated at the appropriate temperature (usually 37°C).

Bacterial cultures were stored for periods of up to a month at 4°C - for long term storage, glycerol was added to fresh overnight cultures to a final concentration of 30% (V/V) and stored at -20°C. For experimental purposes, (e.g. transformation, transduction etc.) media were inoculated with one-fortieth volume of an overnight culture and incubated in a shaking water bath (Grants Instruments) with vigorous aeration. Cell growth was followed by measuring the OD_{650} of cultures (using a Spectronic 20 Bausch & Lamb), an OD_{650} of 0.4 being approximately equivalent to 2×10^8 cells/ml. Cells were routinely harvested by centrifugation - in a microcentaur microfuge for 2' at 15,000rpm for volumes of less than 1.5ml, in a Sorvall SS34 rotor for 5' at 5,000rpm for volumes of 1.5 - 30ml, and in a

Sorvall GSA rotor for 10' at 5,000rpm for volumes larger than 30ml.

2.6 Growth and maintenance of bacteriophages

Stocks of bacteriophage λ were prepared by the plate lysis method precisely as described by Silhavy et al. (1984).

Stocks of bacteriophage P1 were prepared in an analogous manner. Approximately 10^7 - 10^8 P1 Vir bacteriophages were mixed with 10^7 bacteria in 0.4% Mu overlay agar containing 5mM $CaCl_2$ and poured onto Mu agar plates containing 5mM $CaCl_2$ and 0.2% glucose. Plates were incubated right side up for 8-10 hours. The overlay agar was then scraped off the surface of the plate with a sterile spreader, vortexed vigorously with 0.5ml chloroform and centrifuged at 10,000rpm for 10' in a Sorvall SS34 rotor to remove agar and bacterial debris. Bacteriophages and P1 were stored at $4^{\circ}C$ over chloroform. Phages were titered by the spot titer method described by Silhavy et al. (1984).

Methods for growth of bacteriophage M13 were adapted from those of Yannisch-Perron et al. (1985). M13 phage stocks were prepared by incubating single plaques (containing 10^5 - 10^6pfu) with an initial innoculum of approximately 10^7 host SM101 cells in 1-2ml YT broth for 5 hours at $37^{\circ}C$. Intact cells and cell debris were removed by centrifugation. Phage supernatants containing 10^{11} - 10^{12}pfu/ml were stored at $4^{\circ}C$.

2.7 P1 transduction of antibiotic markers

P1 transductions were performed by a method adapted from those of Miller (1972) and Lloyd (1983). Bacteria to be transduced were grown to stationary phase in LB broth, harvested, and resuspended in one-fifth volume MC buffer. P1 vir stock was added to a multiplicity of infection of 1-10pfu/cell, the mixture incubated at

37°C for 20' to allow phage adsorption, and sodium citrate added to a final concentration of 0.2M to prevent reinfection of cells by P1 Vir. Excess LB broth was then added and incubation continued with aeration for a further 2 hours to allow expression of antibiotic resistance genes. Transduced cells were harvested, spread on appropriate selective plates and incubated for 24-48 hours prior to purification of transductants.

2.8 Conjugation

Donor and recipient cells were grown to OD_{650} = 0.4 (approximately 2 x 10^8 cells/ml) in Mu broth at 37°C either with very slow shaking in a water bath, or on a rotator. Cells were mixed in either a 1:10 or 1:4 ratio of donors to recipients and incubated for appropriate times, again with very slow shaking in a water bath. Hfr strains were routinely mated with recipients for 40', F' strains for 30', and R-plasmid strains for 30'. When necessary, mating mixtures were further incubated with vigorous aeration to allow expression of antibiotic resistance genes. At the end of the mating period, mixtures were vigorously vortexed and aliquots of suitable dilutions plated out on selective media in either 0.75% water agar or 0.6% Mu agar, containing 0.04% streptomycin. In some experiments Mu broth was added to the water agar overlay (2.5ml Mu per 150ml 0.75% water agar) to minimise the shift down effect on transferring cells from complex to minimal media (Riley & Pardee 1962). Plates were incubated for 2-5 days at appropriate temperatures prior to scoring transconjugants.

2.9 U.V. Survivals

UV irradiation survival was determined essentially as described by Lloyd & Barbour (1974). Bacteria were grown to approximately 2 x

10^8 cells/ml in LB broth. Serial dilutions of the bacteria down to a 10^{-5} dilution were made in 56/2 buffer and 10ul aliquots of each dilution spotted onto a series of LB plates. The spots were allowed to dry into the agar and the plates irradiated with known UV doses from a Hanovia bactericidal UV light. Irradiated plates were incubated along with an unirradiated control for 12-24 hours before scoring survivors.

2.10 γ-Survivals

Cells were grown, diluted and spotted onto LB plates as for UV survivals. Plates were exposed to known doses of γ-irradiation from a ^{60}Co source. Plates were incubated, along with an unirradiated control for 12-24 hours before scoring survivors.

2.11 Transformation

Bacteria were transformed by a method adapted from that of Mandel & Higa (1970). Cells were grown to mid-exponential phase in 8ml of LB broth, in a shaking water bath, harvested and resuspended in 1ml ice-cold 100mM $CaCl_2$. The $CaCl_2$-cell suspension was held on ice for 20'-30', the cells harvested again and resuspended in 0.2ml ice-cold 100mM $CaCl_2$. A minimum volume - usually approximately 5ul - of plasmid DNA solution was added and held on ice for a further 30'. The mixture was then heat shocked at 42°C for 2', plunged back into ice and 4ml of LB broth added. Transformed cells were incubated for a further 2 hours with vigorous aeration, to allow expression of antibiotic resistance genes, then plated out onto suitable antibiotic plates. Plates were incubated for 12-24 hours at suitable temperatures prior to purification or analysis of transformants.

Replicative form M13 DNA was transformed into cells made

competent by the $CaCl_2$ treatment described above, with the exception that aliquots of transformation mixtures were plated out immediately after heat shock (since expression of antibiotic resistance genes was not required), in 2.5ml of 0.7% YT agar containing 40ul of 20mg/ml X-GAL, 40ul of 20mg/ml IPTG and 0.1ml of a fresh overnight culture of host strain JM101, onto YT plates. Plates were incubated overnight at 37°C prior to purification and subsequent analysis of transformants.

2.12. Phage DNA Extraction

Phage λ DNA was prepared by a method adapted from that of Yamamoto et al. (1970). A 200ml liquid λ lysate was prepared by the method of Silhavy et al. (1984) and chloroform (0.2ml) and NaCl (5.8g) added. The lysate was clarified by centrifugation at 6,000rpm for 10', the supernatant retained and polyethylene glycol added to 10% (w/v) to precipitate the phages. The mixture was shaken thoroughly at room temperature to dissolve the PEG, and then held in iced water for 60'. Phages were harvested by centrifugation (6,000rpm, 10') and resuspended in 1-2ml of λ buffer. The PEG phage suspension was extracted with an equal volume of chloroform, and the phases separated by centrifugation. The aqueous phase was then carefully layered onto the top of a 5-40% glycerol step gradient in λ buffer prepared in a SW41 polyallomer tube. Phages were pelleted by centrifugation at 35K for 1 hour at 4°C in the SW41 rotor of a Kontron Centrikon T2055 ultracentrifuge. The supernatant was decanted off and phages were resuspended in 0.5ml of λ buffer. RNase A and DNase I were added to a final concentration of 10ug/ml and 1ug/ml respectively and the mixture incubated at 37°C for 30'. A one-fifth volume of STEP buffer (0.5% SDS, 50mM Tris-HCl pH 7.5, 0.4M Na_2 EDTA and 1mg/ml protease K added immediately before use) was added and the mixture incubated at 55°C for 15'. The mixture

was then sequentially extracted with equal volumes of (i) phenol (ii) 1:1 phenol:chloroform and (iii) chloroform - in each case the upper aqueous phase was retained. λ DNA was precipitated from the final aqueous phase by the addition of 2 volumnes of 95% ethanol, spooled and redissolved in a minimum volume of 10mM Tris-HCl pH 8.0 1mM Na_2 EDTA (TE buffer). DNA was stored at -20°C.

2.13 Plasmid and M13 replicative form DNA Extraction

Plasmid DNA was routinely prepared from 3ml 'overnight' cultures by a modification of the alkaline lysis method of Birnboim & Doly (1979). 2 x 1.5ml portions of cells were harvested by centrifugation, resuspended in 100ul 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM Na_2 EDTA and held on ice for 5'. 200ul of 0.2M NaOH 1% SDS was added and mixed by inverting at room temperature until lysis occurred. Chromosomal DNA and cellular protein were then precipitated by the addition of 150ul of ice cold 5M potassium acetate and pelleted by centrifugation. The supernatant was decanted off and extracted with an equal volume of phenol:chloroform 1:1, then twice with equal volumes of chloroform. Plasmid DNA was precipitated by the addition of two volumes of ice-cold absolute ethanol, pelleted by centrifugation and excess alcohol removed by freeze drying. DNA was redissolved in a minimum volume of 10mM Tris-HCl pH 8.0 1mM Na_2 EDTA and stored at -20°C. The preparation was frequently scaled up by a factor of 10 to prepare stocks of DNA - in this case lysozyme was added to the glucose-Tris-EDTA solution to a concentration of 5mg/ml to ensure good lysis.

Replicative form M13 DNA was prepared in an identical manner from cultures of JM101 infected in early log phase with M13 phage supernatant at a multiplicity of infection of 10pfu per bacterial cell, then incubated a further 12-18 hours at 37°C.

2.14 M13 single-stranded DNA extraction

M13 single-stranded DNA was prepared by an adaptation of the polyethylene glycol precipitation method described by Yamamoto et al. (1970). Phage supernatant (1ml) was added to 2.5M NaCl 20% PEG (200ul), the mixture briefly vortexed then left on ice for 30'. The PEG-phages precipitate was harvested by centrifugation, all traces of PEG-NaCl solution carefully removed, and the pellet resuspended in TE buffer (200ul). Phages were deproteinised by extraction with an equal volume of phenol, DNA was then further purified by two chloroform extractions, and finally precipitated by the addition of one-tenth volume of 3M N Ac, 2 volumes of ethanol at -20°C for 12-24 hours. DNA was harvested by centrifugation for 20' at 15K, freeze dried and resuspended in a minimum volume (5-6ul) of TE buffer. Sufficient DNA was obtained by this method to perform up to three sets of sequencing reactions.

2.15 E. coli chromosomal DNA extraction

Chromosomal DNA was extracted from E. coli by the method of Berman et al. (1981) 30ml 'overnight' cultures of cells were harvested and resuspended in 2ml 50mM Tris HCl pH 8.0 50mM Na_2 EDTA. Lysozyme was then added to a final concentration of 1mg/ml and the mixture held on ice for 30'. A one-fifth volume of STEP solution was added and the mixture incubated at 55°C for 60' with occasional gentle mixing. The bacterial debris was then removed by extracting with an equal volume of phenol, followed by a further extraction with an equal volume of phenol:chloroform 1:1. DNA and RNA were precipitated from the aqueous phase by the addition of 2 volumes of ice-cold 95% ethanol. Precipitated DNA and RNA were spooled, drained of excess ethanol, redissolved in 2ml 50mM Tris-HCl pH 7.5 1mM Na_2 EDTA 200ug/ml RNase A and incubated for 1 hour at 37°C. The

mixture was then re-extracted with phenol followed by two further chloroform extractions. Chromosomal DNA was precipitated from the final aqueous phase by the addition of two volumes of absolute ethanol, spooled, redissolved in a minimum volume (100-200ul) of TE buffer and stored at -20°C.

2.16 Restriction Endonuclease Digests

Restriction endonuclease digests were routinely set up in 20ul volumes of the appropriate buffer, containing 0.2-1ug DNA and 1 unit of restriction enzyme, and incubated for 2 hours at 37°C. Restriction enzymes were inactivated prior to electrophoresis of digested DNA by the addition of one quarter volume of 10% Ficoll (Pharmacia) 0.06% bromophenol blue, 0.5% SDS solution, followed by heating at 65°C for 10'.

DNA required for cloning purposes was purified from digests by sequential phenol, phenol:chloroform (1:1), chloroform, extractions, precipitated by the addition of one tenth volume of 3M NaAc and two volumes of ethanol, pelleted by centrifugation for 15' at 10K, freeze-dried, and finally resuspended in a minimum volume of 10mM Tris-HCl (pH 7.5) 10mM $MgCl_2$ 50mM NaCl.

2.17 Ligations

Ligation reactions containing 0.1ug vector DNA, 0.5-1ug insert DNA and 0.1 units of T4 DNA ligase in a minimum volume of 33mM Tris-HCl 6mM $MgCl_2$, 33mM NaCl, 30mM DTT and 1mM ATP were incubated overnight at 4°C for ligating 'sticky' ends or for 2 hours at 12°C for ligating blunt ends.

2.18 End Filling Reactions

DNA with a 5' overhang produced by restriction endonuclease digestion was blunt ended by filling in ends using Klenow polymerase. The reaction mixture, containing 0.2-1ug DNA, 5uM dATP, 5uM dGTP, 5uM dTTP, 5uM dCTP and 1 unit of Klenow polymerase in 1 X BRL CORE buffer was incubated at 20°C for 30'. The reaction was terminated by heating the mixture to 65°C for 10'.

2.19 Gel electrophoresis of DNA

DNA fragments of greater than 1Kb were generally separated on 0.4 - 2% agarose gels, those of less than 1Kb were separated on 5 - 10% polyacrylamide gels.

a) Agarose gels (0.4 - 2%) were prepared and run in either Tris-acetate electrophoresis buffer (5mM NaAc 1mM Na_2 EDTA 40mM Tris-HCl pH 7.9) or Tris-borate-EDTA electrophoresis buffer (TBE - 0.089M Tris-borate, pH 8.3, in a horizontal submarine type gel apparatus. Gels were routinely run at 50 - 150V for 1 - 15 hours.

b) Polyacrylamide gels (5 - 10%), containing a 20:1 ratio acrylamide:methylenebisacrylamide, polymerised by the addition of N,N,N^1,N^1-tetramethyl ethylenediamine (TEMED) in the presence of 0.25% ammonium persulphate, were prepared and run in TBE electrophoresis buffer in a vertical gel electrophoresis apparatus. Gels were routinely run at 100 - 150V for 2 - 4 hours.

Power for electrophoresis was supplied via a Shandon Vokan 500-500 powerpack.

After electrophoresis, gels were stained with ethidium bromide at 0.5ug/ml in electrophoresis buffer, visualised on a UV

transilluminator and photographed on Ilford or Polaroid film.

2.20 Isolation of DNA from agarose gels

A strip of gel (agarose or polyacrylamide) containing the required DNA placed in dialysis tubing containing one-tenth concentration electrophoresis buffer and the DNA electroeluted for 30' at 100V. The buffer was then removed from the tubing, reduced in volume by two extractions with an equal volume of isobutanol, further extracted with two volumes of chloroform and the DNA precipitated by the addition of two volumes of absolute ethanol, in the presence of 300mM sodium acetate.

2.21 Nick Translation of DNA

DNA was nick translated essentially according to the method of Rigby et al. (1977). 0.05-0.1ug of DNA was incubated at 15°C for 60' in a 25ul reaction volume containing 50mM Tris-HCl pH 7.8, 5mM $MgCl_2$, 10mM β-mercaptoethanol, 5uM dATP, 5uM dGTP, 5uM dTTP, 1.5ul (α-^{32}P)dCTP (10mCi/ml) 1ul 8ng/ml DNase I and 1ul DNA polymerase I. The reaction was stopped by adding 25ul of 10mM Tris-HCl, 10mM EDTA, 0.5% SDS, extracted with phenol:chloroform 1:1 and the labelled DNA separated from unincorporated ^{32}P dCTP by ethanol precipitation using herring sperm DNA as a carrier. The final precipitate was redissolved in 500ul TE buffer and stored at -20°C.

2.22 Transfer of DNA to nitrocellulose filters

DNA was transferred from agarose gels to nitrocellulose filters (Schleicher & Schuell) by the method of Southern (1975). DNA was partially depurinated by soaking the gel in 0.2M HCl for 15', denatured in 1.5M NaCl 0.5M NaOH for 15' and neutralised with 3M

NaCl 0.5M Tris-HCl pH 7.0 for 30'. The gel was placed on a filter paper soaked in 20 x SSC (20 x SSC contained 175.3g/l NaCl, 88.2g/l sodium citrate, brought to pH 7.0 with NaOH) and the DNA transferred overnight to a nitrocellulose filter soaked in 2 x SSC placed on top of the gel. The filter was washed for 20' in 2 x SSC and then baked *in vacuo* at 80°C for 2 hours. Filters were stored in sealed polythene bags at +4°C.

2.23 Filter hybridisation

Filters were prehybridised in 4 x SSC 0.1% SDS 5 x Denhardts solution (0.1% Polyvinylpyrrolidone, 0.1% Ficoll, 0.1% BSA) at 65°C in a sealed plastic bag for 4-6 hours prior to addition of probe. The nick translated probe was denatured by heating in a boiling water bath for 10', added to the bag containing filter and prehybridisation solution and the hybridisation reaction left at 65°C for 12-16 hours.

The hybridisation solution was then decanted off (occasionally it was reused) and the filter washed three times in 4 x SSC 0.1% SDS for 20' at 65°C. The filter was finally rinsed in 2 x SSC, and dried at room temperature.

2.24 Autoradiography

Hybridised filters were exposed to pre-flashed Kodak X-Omat film with an intensifying screen for periods ranging from one hour to several days. Film was developed in LX24 developer as recommended.

2.25 Nucleotide sequence determination

Nucleotide sequence was determined by the chain termination

method of Sanger et al. (1977) using DNA subcloned into M13mp18 and M13mp19 vectors (Yannish-Perron et al. 1985) as a source of template DNA.

a) Annealing of primer to template

Approximately 2-4ul of a 400ng/ml solution of commercially obtained sequencing primer (Amersham) was annealed to 1-4ug of template DNA in a total volume of 10ul in 10mM Tris-HCl pH 8.0 5mM $MgCl_2$ by boiling the mixture for 3' then incubating it at 55°C for $1\frac{1}{2}$ - 2 hours.

b) Sequencing reactions

DNA Polymerase I large fragment (1-5 units) and α-^{35}S dATP (10mCi.ml) 1-2ul were added to each annealed primer-template mix. The resulting mixture was then divided between four tubes labelled A, C, G and T each containing 2ul of the appropriate ddNTP/dNTPs sequencing reaction mix (Table 2.2), the tubes centrifuged briefly to mix the contents and then incubated at 37°C for 20'. A 'chase' of 2ul of a solution containing dATP, dCTP, dGTP and dTTP each at 0.5mM was then added and the mixtures incubated a further 15' at 37°C before terminating the reactions by the addition of 3ul STOP dye mix (0.3% xylene cyanol 0.3% bromophenol blue, 10mM Na_2 EDTA in deionised formamide).

Table 2.2 Composition of sequencing reaction mixtures

	A mix	C mix	G mix	T mix
dCTP	62.5uM	4 uM	80 uM	80 uM
dGTP	62.5uM	80 uM	4 uM	80 uM
dTTP	62.5uM	80 uM	80 uM	4 uM
ddATP	50 uM	-	-	-
ddCTP	-	50 uM	-	-
ddGTP	-	-	150 uM	-
ddTTP	-	-	-	250 uM
Tris-HCl (pH 7.0)	1.25uM	1.6uM	1.6uM	1.6uM
Na_2 EDTA	12.5uM	16 uM	16 uM	16 uM

c) Electrophoresis of sequencing reaction products

Products of sequencing reactions were separated by electrophoresis in adjacent lanes on 0.4mm thick 6% polyacrylamide gels containing 7.66M urea prepared either uniformly in 1 x TBE electrophoresis buffer or in a top to bottom buffer gradient of 1 x TBE to 5 x TBE, polymerised by the addition of TEMED to 0.1% in the presence of 0.025% ammonium persulphate.

Gels were run in a vertical electrophoresis apparatus in 1 x TBE buffer at 29mA (approximately 1400V) for 2 - 5 hours.

After electrophoresis, gels were fixed by soaking in 10% methanol 10% acetic acid for 20', transferred to Whatman 3MM paper and dried under vacuum at 80°C.

Dried gels were exposed directly to autoradiographic film for 24 - 48 hours prior to developing and direct reading of sequence from the film.

2.26 Preparation of maxicell extracts

The method used was based on that of Sancar et al. (1979). Plasmid harbouring derivatives of N1644 recA13 uvrA6 were grown to approximately 2 x 10^8 cells per ml in M9 media supplemented with required amino acids, irradiated with 10J/M^2 UV light, and incubated in the dark for one hour. Cycloserine was then added to a final concentration of 200ug/ml and incubation continued for a further 16 hours. Cells were harvested and washed twice in M9 media minus $MgSO_4$, resuspended in the same medium and incubated for a further 30' to deplete any remaining SO_4^{2-} in the media. ^{35}S-methionine (Amersham) was then added to 20uCi/ml and incubation continued for another hour. Cells were harvested, washed twice in M9 media and lysed with loading buffer (2% SDS, 20% glycerol, 5% β - mercaptoethanol, 0.001% Bromophenol blue, 0.125M Tris-HCl pH 6.8). Proteins were disaggregated by boiling for 3'.

The incorporation of ^{35}S-methionine was determined by scintillation counting of samples in 'Fisofluor' scintillant in a Packard Minaxi Tricarb 4000 series liquid scintillation counter. 10,000cpm were routinely loaded onto SDS polyacrylamide gels.

2.27 Preparation of phage λ encoded proteins

The method used was the UV-irradiated host system based on that of Ptashne (1966) adapted as described by Stoker et al. (1984).

The host strain was grown to mid exponential phase in M9 media, containing 0.2% maltose, at 32^{o}C.

Cells were heavily irradiated with a UV dose of 1200Jm^{-2}, with constant stirring to ensure all cells were irradiated. Aliquots of 500ul of bacterial suspension (for each phage) were harvested and resuspended in 100ul M9 media containing 0.2% maltose and 20mM $MgCl_2$ replacing $MgSO_4$. Extensively dialysed phage in λ buffer were

added to a multiplicity of infection of 5-10 pfu/cell and the mixture incubated at 32°C for 10' to allow phage adsorption. A further 400ul of the same media, prewarmed to 32°C was then added and incubation continued for 20' to deplete SO_4^{2-} prior to the addition of ^{35}S-methionine to a concentration of 20uCi/ml. The mixture was incubated for 20' and then a 'chase' of cold methionine added followed by a further incubation of 20'. Cells were harvested, washed in M9 buffer and lysed in 25ul of loading buffer (as for maxicells). Samples were counted as for maxicells. 10,000cpm were loaded onto SDS-polyacrylamide gels.

2.28 SDS polyacrylamide gel electrophoresis

Proteins were separated by electrophoresis in SDS-polyacrylamide stacking gels Hames and Rickwood (1981), and electrophoresed in a vertical gel electrophoresis apparatus.

Gels were sealed with 1-2% agarose in 0.375M Tris-HCl pH 8.8. Resolving gels contained 15% acrylamide 0.4% bisacrylamide in 0.375M Tris-HCl pH 8.8, 0.1% SDS, polymerised with TEMED in the presence of 0.075% Ammonium persulphate.

Stacking gels contained 3.75% acrylamide, 0.1% bisacrylamide in 0.125M Tris-HCl pH 6.8, 0.1% SDS, polymerised with TEMED in the presence of 0.075% ammonium persulphate.

Reservoir buffer was 0.025M Tris-HCl pH 8.3 0.192M glycine 0.1% SDS. Proteins were electrophoresed through the stacking gel with a current of 10mA and then through the resolving gel with a current of 30mA.

Molecular weight markers (Pharmacia) were visualised by staining the gel with 0.25% Coomassie Brilliant Blue G in 50% (w/v) trichloroacetic acid for 30' at 65°C, followed by destaining for 4-6 hours in 8% acetic acid at 65°C. ^{35}S-methionine labelled proteins were visualised by fluorography. Gels were soaked for 60' at room

temperature in 55% acetic acid, 15% absolute ethanol, 30% xylene, 0.5% 2, 5, diphenyloxazole (PPO), washed twice and left overnight in water.

The gel was then dehydrated on an 'ATTO' gel drier and exposed to autoradiographic film (as per nitrocellulose filters).

2.29 Assay of β-galactosidase

β-galactosidase was assayed by a method adapted from that of Miller (1972). Aliquots (0.5ml) of samples to be assayed were added to 2.5ml 56/2 buffer and lysed by adding 3 drops 0.1% SDS, 6 drops of chloroform and vortexing vigorously. Samples were held on ice until ready to assay and warmed to 28°C just before commencement of the assay. The assay was started by the addition of 1ml of 0.75M ONPG in 0.1M phosphate buffer pH 7.25 and incubation continued at 28°C until sufficient yellow colour to quantify by spectrophotometry was observed. The reaction was then terminated by the addition of 2ml 1M Na_2CO_3 8M urea solution and the assay time (t) noted. Bacterial debris was removed by centrifugation at 10K for 10' and the OD_{420} determined using a Cecil CE292 Digital UV spectrophotometer. Enzyme activity was calculated according to the following formula:-

$$Eu/ml = \frac{\text{Vol. assay mix} \times OD_{420}}{\text{Sample vol.} \times t \times 4.7 \times 10^{-3}}$$

2.30 Assay of chloramphenicol acetyl transferase

(i) Preparation of cell extracts

Cells were grown to mid-exponential phase in LB broth, harvested, resuspended in one-half volume of 50mM Tris-HCl pH 7.8 30uM DTT and sonicated on ice using an MSE ultrasonic sonicator. Cell debris was removed by centrifuging for 15' at 10K in an IEC

CENTRA 3RS refrigerated microfuge at 4°C. Extracts were aliquoted and stored at -20°C.

(ii) Assay

Chloramphenicol acetyl transferase was assayed by the spectrophotometric method described by Shaw (1975).

A 1ml volume of reaction mixture containing 100mM Tris-HCl pH 7.8, 0.1mM Acetyl Coenzyme A, 0.4mg/ml DTNB and 10-50ul of cell extract was prewarmed in a 1ml cuvette with a 1cm light path at 37°C. The rate of increase in absorption at 412nm was followed for several minutes using a Unicam SP1800 recording UV spectrophotometer until the reaction mixture was equilibrated at 37°C A value, A(-Cm) was calulated from the slope of the line for the rate of increase in absorption before the addition of chloramphenicol. The reaction was then started by the addition of chloramphenicol to a final concentration of 0.1mM and the change in absorption followed. A value, A(+Cm) was calculated from the slope of the line for the rate of increase in absorption after chloramphenicol addition.

The units of enzyme present in the reaction mixture were calculated according to the following equation:

$$Eu = \frac{[A(+Cm)] - [A(-Cm)]}{13.6}$$

Values were standardised by relating Eu/ml of chloramphenicol acetyl transferase, to the total protein content of the extracts, determined by comparing the absorption at 205nm to known standards.

CHAPTER 3

The biological role of the *ruv* gene product

3.1 Introduction

Prior to this study, although several *ruv* mutants had been isolated (Otsuji *et al.* 1974, Stacey and Lloyd 1976, Shurvinton and Lloyd 1982, Shurvinton 1983) no known physiological role for the *ruv* gene product had been proposed and widely accepted. All *ruv* mutants isolated are sensitive to agents that damage DNA such as mitomycin C, UV irradiation and γ-irradiation. Additionally *ruv* mutants exhibit a cell-division defect, resulting in the formation of filaments, particularly after treatment with agents that damage DNA. This discovery led to the proposal that the *ruv* gene product might have a role similar to that of the *lon* gene product (Iyehara and Otsuji 1975), since *lon* mutants exhibit a similar phenotype (Howard-Flanders *et al.* 1964). This proposal was discounted when it was found that although *sulA* mutations suppressed both the filamentation and the UV sensitivity of *lon* mutants, only the filamentation of *ruv* mutants was suppressed, their sensitivity to UV irradiation was unchanged (Otsuji and Iyehara-Ogawa 1979, Lloyd *et al.* 1984). *lon* has since been shown to encode a heat inducible protease thought to be required for degradation of abnormal cellular proteins, including *sulA* induced as part of the SOS response (Mizusawa and Gottesman 1983).

The discovery that the *ruv* gene was a component of the SOS system regulated by *lexA* and *recA* gene products (Shurvinton and Lloyd 1982) led to the suggestion that the *ruv* gene product could play a role in one of the pathways for repair of UV-damaged DNA (Shurvinton 1983), since at least some of the gene products required for excision repair, error prone repair and recombinational repair

of damaged DNA had been demonstrated to be under SOS control (Walker 1984).

Since the UV sensitivity of an *ruv uvrA* double mutant was approximately the same as the sum of the sensitivities of the two single mutants, it was concluded that the *ruv* gene product did not play a role in excision repair (Iyehara and Otsuji 1975, Shurvinton 1983).

Since *ruv* mutants were as UV mutable as their parental *ruv*$^+$ strains, it was concluded that the *ruv* gene product was not required for error prone repair (Shurvinton 1983). Additionally, studies had demonstrated that the *ruv* gene product was not required for conjugational recombination in a strain otherwise wild type for recombination genes (Otsuji *et al*. 1974, Shurvinton 1983). This had led to the suggestion that the *ruv* gene product was not required for recombination repair. Thus, although *ruv* mutants had been subjected to considerable study, no proposed physiological role for the *ruv* gene product had been accepted.

Although clearly experimental evidence ruled out the possibility that the *ruv* gene product was involved in either excision repair, or error prone repair, studies on the effects of *ruv* mutation on recombination overlooked the existence of alternative mechanisms of recombination in *E. coli*.

From studies of mutants deficient in recombination, it was originally proposed that recombination in *E. coli* could initiate and proceed by two partially independent pathways, one dependent upon exonuclease V, the product of the *recB* and *recC* genes - the "recBC pathway", and the other dependent upon the products of the *recF* and other, as then unidentified genes - "the recF pathway", recombination by both pathways being absolutely dependent on the *recA* gene product (Clark 1973). Since this proposal was put forward, several more genes, the products of which are required for recombination, were identified. *recD* encodes the third subunit of

exonuclease V and is probably involved in recBC dependent recombination, (Amundsen et al. 1986, Biek and Cohen 1986). recJ (Lovett and Clark 1984), recN (Lloyd et al. 1983), recQ (Nakayama et al. 1984), recO (Kolodner et al. 1985) are required for "recF pathway" recombination.

It was originally suggested that the "recBC pathway" was the major pathway of conjugational recombination in wild type cells and that the "recF pathway" was only important in certain recBC strains having additional mutations in sbcB (Kushner et al. 1971), and sbcC (Lloyd and Buckman 1985) or sbcA (Barbour et al. 1970). More recent proposals suggest that the gene products required for recombination are dictated by the available substrate, particularly suggesting that recombination initiated at ends of double-stranded DNA is dependent upon the recBCD gene products, whilst recombination initiated at single-strand gaps is independent of recBCD and requires the products of some or all of the recFJQON genes (Lloyd and Thomas 1984).

In an analogous situation two different modes of post replication recombination repair have been described, they are recBCD dependent double strand break repair, and recF dependent daughter strand gap repair (Walker 1985); both types of repair are absolutely dependent on the recA gene product (Kraisin and Hutchinson 1977, Smith and Meun 1970). As in conjugational recombination, in those strains where the deficiency in recombination of recBCD strains is suppressed by mutation in sbcA or sbcBC, then some of the gene products such as recF required for recBC independent recombination are able to mediate repair of double-strand breaks (Wang and Smith 1985). However, the relationship between the recF dependent and the recBCD dependent modes of repair of double strand breaks remains unclear since the product of at least one gene (recN) identified as a "recF" pathway recombination gene, is required for double strand break repair,

Figure 3.1

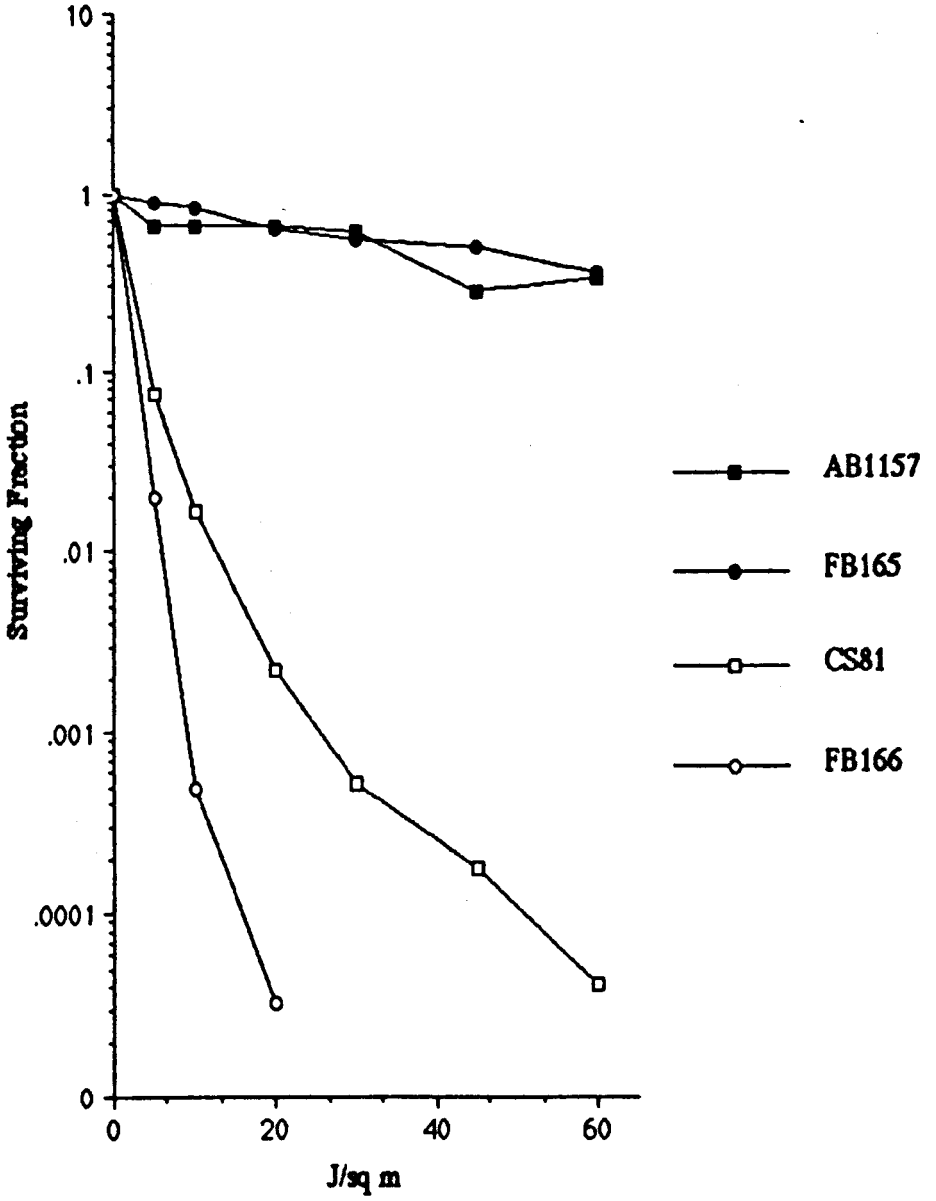


Fig. 3.1

UV radiation sensitivity of ruv and ruv recBC sbcBC strains. Strains used were AB1157 ruv^+ rec^+, FB165 recBC sbcBC, CS81 ruv-52 and FB166 recBC sbcBC ruv-52

Figure 3.2

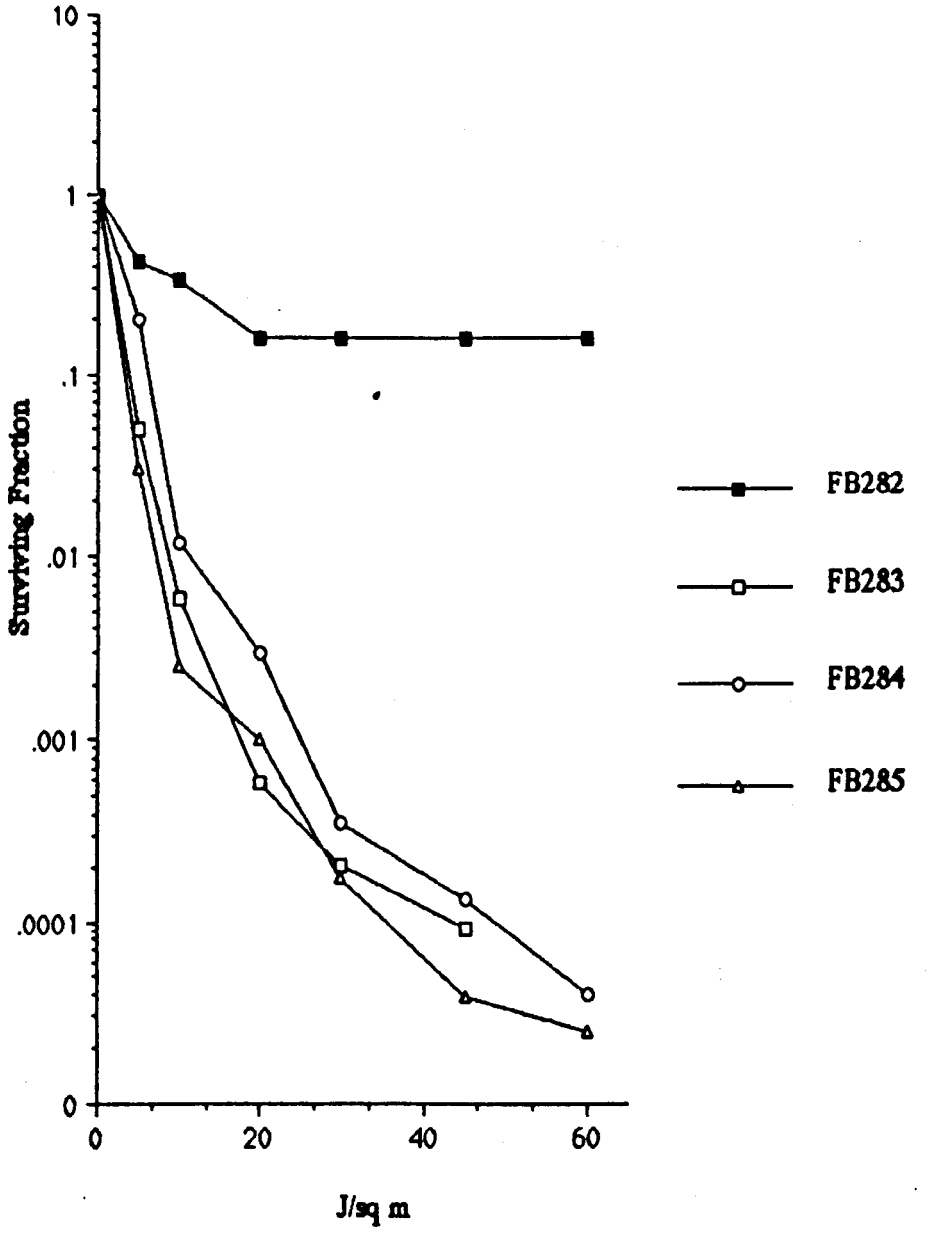


Fig. 3.2

UV radiation sensitivity of recBC sbcA ruv strains. Strains used were FB282 recBC sbcA, FB283 recBC sbcA ruv-53, FB284 recBC sbcA ruv-52 and FB285 recBC sbcA ruv-54

regardless of the availability of RecBCD enzyme (Picksley *et al.* 1984).

The observation that *recBC* independent recombination was inducible led to the proposal that its main function was in the repair of DNA damage (Lovett and Clark 1983, Armengod 1982).

In the light of this clarification of the roles of *recBCD* and *recFJOQN* gene products in DNA repair it was decided to re-examine the effects of *ruv* mutations on the phenotypes of both wild type strains, in which repair and recombination may be initiated either by *recBC* dependent or *recBC* independent mechanisms; and *recBC sbcBC* or *recBC sbcA* strains, in which recombination and repair can only proceed via *recBC* independent mechanisms, in order to evaluate any possible role of the *ruv* gene product in recombinational repair.

3.2 Sensitivity to U.V. irradiation

The UV survival curves of strains AB1157 *ruv*$^+$, CS81 *ruv*-52, FB165 *recBC sbcBC ruv*$^+$ and FB166 *recBC sbcB ruv*-52 are shown in Figure 3.1. The UV irradiation sensitivity of strain CS81 *ruv*-52, is similar to the irradiation sensitivity of otherwise wild type strains carrying any of the different *ruv* mutations. Typically, the survival of an *ruv* mutant is reduced to 0.01% of the unirradiated control by a dose of 60J/M^2 UV light.

Figure 3.1 clearly shows that the *ruv*-52 mutation has a much greater effect on the sensitivity to UV irradiation of a *recBC sbcBC* strain, in which repair can only proceed independently of RecBCD enzymes, (a dose of 20J/M^2 reducing the survival to less than 0.003% of the unirradiated control), than it does on the UV irradiation sensitivity of an otherwise wild type strain. Figure 3.2 shows that *ruv* mutations have a similar, although rather less severe effect on the UV irradiation sensitivity of *recBC sbcA* strains.

The sensitivity of *ruv* mutants to UV light even in an otherwise

wild type background suggested that the ruv gene product may be required for repair of daughter strand gaps, whilst the increased sensitivity conferred on recBC sbcBC strains may reflect a requirement for the ruv gene product (in the absence of RecBCD enzyme) in the repair of double strand breaks generated during the repair process,in addition to daughter strand gaps.

3.3 Sensitivity to ionising radiation

The ionising radiation survival curves of AB1157 ruv$^+$, CS81 ruv52, FB165 recBC sbcBC and FB166 recBC sbcBC ruv-52 are shown in Figure 3.3.

The ruv mutant CS81 shows a moderate sensitivity to ionising radiation at low doses, survival being reduced to 50% of the unirradiated control by a dose of 15Krads. In contrast, a dose of 15Krads reduces the survival of FB166 recBC sbcBC ruv-52 to approximately 0.01% of the unirradiated control, a similar survival to that of AB2463 recA$^-$ which is apparently completely deficient in double strand break repair (Kraisin and Hutchinson 1977). These results provide further evidence for a possible role of the ruv gene product in repair of double strand breaks in the absence of RecBCD enzyme.

The increasing sensitivity of CS81 ruv-52 to higher doses of ionising radiation may reflect the requirement for the ruv gene product in the repair of double strand breaks under conditions where insufficient RecBCD enzyme, reportedly present in cells at approximately 10 molecules/cell (Hickson et al. 1985) is available to efficiently repair double strand breaks.

3.4 Viability

Results presented in Table 3.1 show the effects of the ruv-52

Figure 3.3

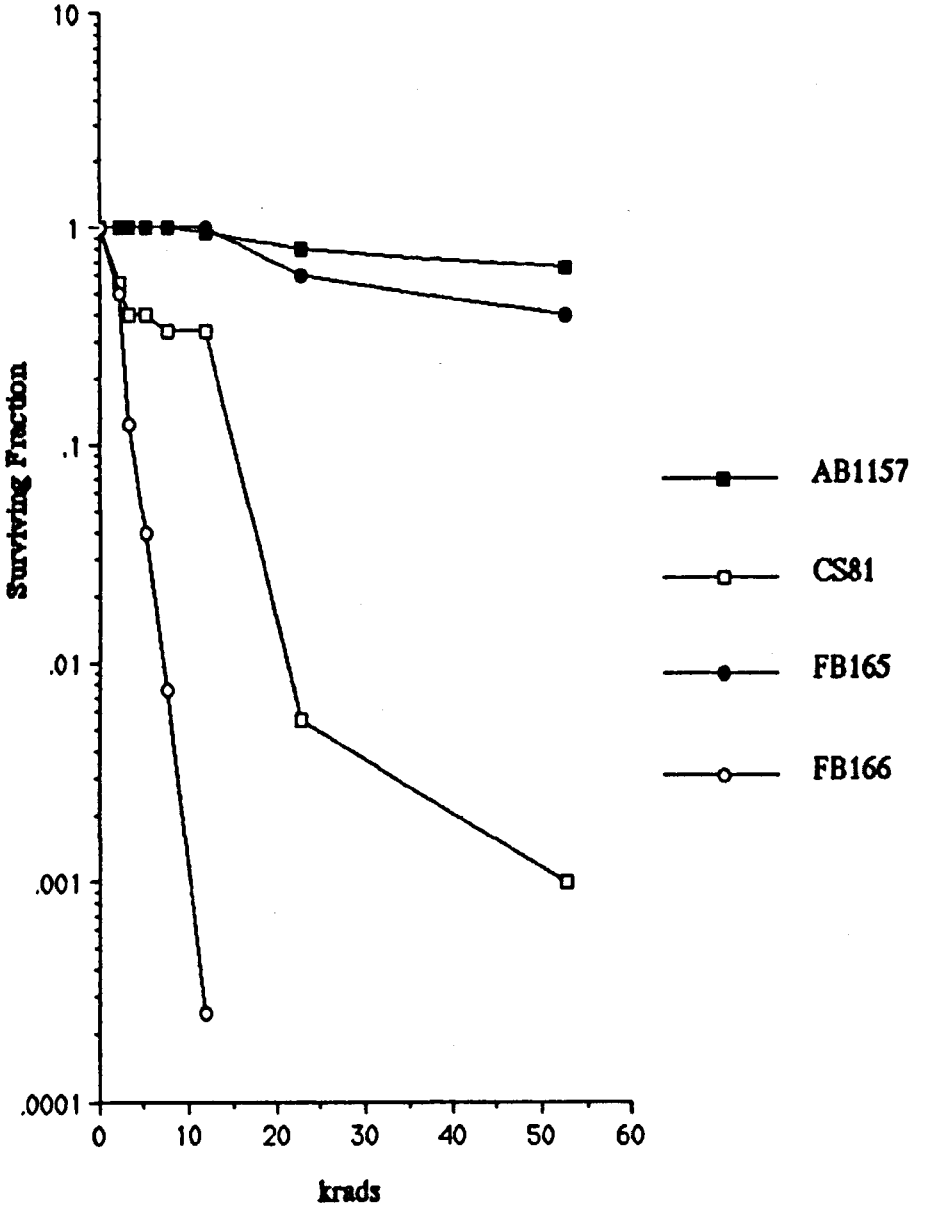


Fig. 3.3

Gamma radiation sensitivity of *ruv* *rec*$^+$ and *recBC* *sbcBC* *ruv* strains. Strains used were AB1157 *rec*$^+$ *ruv*$^+$, CS81 *ruv-52*, FB165 *recBC* *sbcBC* and FB166 *recBC* *sbcBC* *ruv-52*

mutation on the relative viabilities of *recBC*$^+$, *recBC sbcBC* and *recBC sbcA* strains. These show that while the *ruv*-52 mutation has some effect on the viability of an otherwise wild type strain, reducing it to 75% of the parental *ruv*$^+$ strain, it has a much greater effect on the viability of a *recBC sbcBC* strain reducing it to approximately 10% of the parental *ruv*$^+$ strain. Once again, the effect of the *ruv*-52 mutation on the viability of *recBC sbcA* strains is intermediate between the effects on *recBC*$^+$ and *recBC sbcBC* strains, reducing the viability to 44% of the parental *ruv*$^+$ strain.

Since the decreased viability of *recA* and *recBC* mutants has been proposed to be due to a deficiency in the repair of strand breaks that occur during normal DNA metabolism (Capaldo and Barbour 1975) it seems likely that the reduction in viability of strains harbouring the *ruv*-52 mutation may reflect the requirement for the *ruv* product in the repair of such strand breaks, particularly in the absence of *recBCD* gene products.

Table 3.1 The effect of *ruv* mutations on strain viability

Strain	Viable cells/ml(a)	Relative viability(b)
AB1157	2.0×10^8	1
CS81 (*ruv*-52)	1.5×10^8	0.75
FB165 (*recBC sbcBC*)	9.1×10^7	1
FB166 (*recBC sbcBC ruv*-52)	7.1×10^6	0.08
FB282 (*recBC sbcA*)	1.4×10^8	1
FB284 (*recBC sbcA ruv*-52)	6.1×10^7	0.44

Strains were grown to $O.D._{650}$ = 0.4 in LB broth, samples of appropriate dilutions plated out on LB agar plates and incubated overnight at 37°C. Colonies were counted and the number of viable cells per ml of the original culture calculated (a). The relative

viability of the ruv mutant compared with its parental strain is shown (b).

3.5 Recombination proficiency

Data presented in Table 3.2 show the effect of ruv mutations in wild type recipient strains on the recovery of F' transconjugants and recombinants in crosses with KL548 (F'128), Hfr Cavalli aand Hfr Hayes donor strains.

The data presented clearly show that the different ruv mutations have little effect on the recovery of transconjugants or recombinants in a recBC$^+$ strain in which recombination can proceed by mechanisms either dependent on, or independent of the recBCD gene products. Only in crosses with Hfr Hayes was the recovery of recombinants significantly reduced, and even then this was only to approximately 50% of the recovery attained in the ruv$^+$ control strain. In all cases the size of the recombinant colonies obtained were comparable in the ruv$^+$ and ruv$^-$ strains. These results confirm earlier observations of Otsuji et al. 1974 and Shurvinton (1983), which were interpreted to suggest the ruv gene product had no role in recombination repair.

Data presented in Table 3.3 show the effect of ruv mutation in recBC sbcBC recipient strains on the recovery of F' transconjugants and recombinants in crosses with KL548 (F'128), Hfr Cavalli and Hfr Hayes donor strains. This clearly suggests that the ruv gene product has a role in the recovery of recombinants in strains in which recombination can only proceed by mechanisms independent of the recBCD gene products.

The mutations ruv-52 and ruv-54 reduced the recovery of recombinants in recBC sbcBC recipients in crosses with Hfr Cavalli donors to approximately 30% of the ruv$^+$ parental strain and to approximately 1% in crosses with Hfr Hayes donors. It was also

Tables 3.2, 3.3 and 3.4

Experiments were performed as described in 2.8, using a 1:4 ratio of donors:recipients and plating in minimal water agar containing additional Mu broth for Pro^+ selection, and in Mu overlay agar on Mu plates containing Tc and Sm for Tc^R selection. λ plaque formation was assayed by plating out the mating mixture on Mu plates in Mu overlay agar, seeded with AB1157, and containing streptomycin to counterselect the donor. Figures in line (a) represent the actual numbers of selected colonies obtained per ml of mating mixture, in line (b) the recovery of selected colonies relative to the *ruv*$^+$ parent strain, and in line (c) the relative recovery of selected colonies corrected for the observed deficiency in viability of the *ruv* mutant strains compared with the parent *ruv*$^+$ strain.

Table 3.2 The effects of *ruv* mutations in recipient strains on the recovery of recombinants and F' transconjugants

Donor		xGY2200 (Hfr H)			xKL226 (Hfr C)	xKL548 (F'128)
Selection		**Viability**	**λ plaques**	**Thr$^+$ Leu$^+$**	**Pro$^+$**	**Pro$^+$**
Recipient						
CS81A	a	3.47×10^8	8.8×10^6	1.42×10^7	1.38×10^7	1.42×10^7
ruv$^+$	b	1	1	1	1	1
CS81	a	1.3×10^8	7.9×10^6	3.15×10^6	6.38×10^6	7.9×10^6
ruv-52	b	0.37	0.89	0.22	0.46	0.55
	c		>1	0.59	>1	>1
CS86	a	1.55×10^8	6.3×10^6	2.94×10^6	5.54×10^6	5.85×10^6
ruv-54	b	0.44	0.71	0.20	0.40	0.41
	c		>1	0.45	0.91	0.94
CS40	a	1.51×10^8	6.4×10^6	3.03×10^6	8.16×10^6	6.01×10^6
ruvA4	b	0.43	0.72	0.21	0.59	0.42
	c		>1	0.48	>1	0.98
N2058	a	1.54×10^8	7.7×10^6	3.37×10^6	7.13×10^6	1.31×10^7
ruv-59::Tn*10*	b	0.44	0.87	0.23	0.52	0.92
	c		>1	0.54	>1	>1

Table 3.3 The effects of *ruv* mutations in *recBC sbcBC* recipient strains on the recovery of recombinants and F' transconjugants

Donor		xGY2200 (Hfr H)		xKL226 (Hfr C)	xKL548 (F'128)
Selection	**Viability**	**λ plaques**	**Thr$^+$ Leu$^+$**	**Pro$^+$**	**Pro$^+$**
Recipient					
FB153	a 1.5×10^8	2.0×10^7	7.3×10^6	8.3×10^6	1.92×10^7
recBC sbcBC ruv$^+$	b 1	1	1	1	1
FB154	a 1.8×10^7	1.01×10^7	1.08×10^4	2.54×10^5	6.0×10^3
recBC sbcBC ruv-52	b 0.12	0.5	0.0015	0.03	0.00031
	c	>1	0.012	0.25	0.0026
FB155	a 1.67×10^7	1.02×10^7	1.18×10^4	2.65×10^5	2.0×10^3
recBC sbcBC ruv-54	b 0.11	0.51	0.0016	0.03	0.0001
	c	>1	0.014	0.29	0.0009

Table 3.4 The effects of *ruv* mutations in *recBC sbcA* recipient strains on the recovery of recombinants and F' transconjugants

Donor		xKL226 (HfrC)	xKL548 F'128	xN1671 (Hfr C *car*::Tn*10*)
Selection	**Viability**	**Pro$^+$**	**Pro$^+$**	**Tet^R**
Recipient				
FB282	a	1.35×10^8	1.08×10^6	6.2×10^6	7.5×10^6
recBC sbcA ruv$^+$	b	1	1	1	1
FB283	a	4.4×10^7	1.7×10^5	1.4×10^5	1.3×10^5
recBC sbcA ruv-53	b	0.32	0.15	0.023	0.017
	c		0.49	0.07	0.054
FB284	a	6.85×10^7	1.8×10^5	1.4×10^5	8.1×10^5
recBC sbcA ruv-52	b	0.51	0.16	0.022	0.108
	c		0.32	0.044	0.21
FB285	a	9.95×10^7	2.4×10^5	1.7×10^5	8.2×10^5
recBC sbcA ruv-54	b	0.73	0.22	0.027	0.109
	c		0.30	0.037	0.15

noted that most of the recombinant colonies obtained in these crosses were much smaller than those obtained in crosses with control *ruv*$^+$ strains, by a factor greater than could be accounted for simply by the viability difference between the strains.In addition *ruv* mutations dramatically decreased the recovery of F' Pro$^+$ transconjugants in *recBC sbcBC* recipients to approximately 0.1% of the *ruv*$^+$ level, an observation that initially led to the suggestion that *recBC sbcBC ruv* strains were deficient in the recovery of transferred DNA rather than in recombination (Shurvinton 1983). However, this was shown not to be the case since induction of *ind*$^-$ transferred by Hfr Hayes to a *recBC sbcBC* recipients was unaffected by an *ruv* mutation.

The results presented in Table 3.4 show that *ruv* mutations reduced the recovery of recombinants in *recBC sbcA* recipients, to between 30% and 50% of the equivalent *ruv*$^+$ control in crosses with HfrC selecting for Pro$^+$ recombinants and to between 5% and 20% in crosses with N1671 HfrC *car*::Tn*10* selecting for Tc^R recombinants. Again most of the recombinant colonies obtained in these crosses with *recBC sbcA ruv* recipients were significantly smaller than those obtained with the equivalent *ruv*$^+$ control strain.

In addition, *ruv* mutations reduced the recovery of F' Pro$^+$ transconjugants to less than 10% of the recovery in *ruv*$^+$ control strains.

These results suggest that *ruv* may be required for the efficient recovery of recombinants and F' transconjugants in strains in which recombination is mediated independently of *recBCD*. The appearance of small recombinant colonies in crosses with *recBC sbcA ruv* and *recBC sbcBC ruv* receipients could perhaps suggest that in the absence of the *ruv* gene product, recombination is initiated, albeit at a somewhat reduced level, but proceeds to completion much more slowly than in the presence of the *ruv* gene product. Thus, the Ruv product probably mediates a stage relatively late in

recombination. Since the role of the ruv gene product is likely to be similar in the recovery of both recombinants and transconjugants, the results in Tables 3.3 and 3.4 suggest that recovery of F' transconjugants in recBC sbcBC and recBC sbcA strains normally involves a recombinational event, which is prevented from completing by an additional ruv mutation. A further investigation of the effects of ruv mutations on transconjugant recovery is presented in a later chapter of this thesis (Chapter 7).

The above evidence that ruv is required for recovery of recombinants in conjugal crosses in strains deficient in recBCD gene products, lends further support to the proposal that ruv is required for recombination repair primarily of daughter strand gaps, and in addition for double strand break repair in strains in which the recBCD gene products are either absent or limited.

3.6 Discussion

A role for the ruv gene product in daughter strand gap repair and double strand break repair could explain the observed sensitivity of ruv mutants to DNA damaging agents. In addition the filamentation of ruv mutants observed during normal conditions and particularly after treatment with DNA damaging agents could be explained by assuming that the inability of ruv mutants to repair daughter strand gaps or double strand breaks leads to persistence of a low level of inducing signal resulting in a higher basal level of expression of other SOS genes, including sulA, resulting in filamentation. This possibility is further investigated in a later chapter of this thesis (Chapter 6).

The precise role of the ruv gene product remains to be determined. Since it was previously demonstrated using alkaline sucrose gradient sedimentation, that ruv mutants were proficient at converting low MW DNA (produced as a result of inability to

replicate past dimers) to high MW DNA (Otsuji *et al*. 1974) it must be concluded that any reaction mediated by the *ruv* gene product in daughter strand gap repair must occur at a late stage, perhaps in the resolution of recombination intermediates into viable products. The role of the *ruv* gene product in double strand break repair is similarly unclear. Experiments designed to demonstrate rejoining of double strand breaks were inconclusive. It is, however, likely that the *ruv* gene product has a similar role to that in daughter strand gap repair and may function late, resolving recombination intermediates into viable DNA products.

Thus although the precise role of the *ruv* gene product in recombination repair remains a mystery it seems clear that *ruv* is a member of the group of SOS inducible genes so far including *recA* *recN* and *recQ*, required for recombination repair, (Walker 1985, Lloyd *et al*. 1983, Picksley *et al*. 1984, Nakayama *et al*. 1984, Irino *et al*;. 1986).

CHAPTER 4

Cloning of the ruv locus and identification of encoded proteins

4.1 Introduction

Studies presented in the preceding chapter suggested that the ruv gene product may be required for recombination repair of daughter strand gaps and double strand breaks in damaged DNA. However, although genetic studies may provide further evidence for a role for the Ruv protein in repair and recombination it is envisaged that ultimately the precise role will be confirmed only by biochemical characterisation of the purified protein. As a first stage towards these studies, the cloning of the ruv region and identification of its encoded proteins is presented in this chapter.

4.2 Isolation and characterisation of λruv$^+$ transducing phages

λruv$^+$ phages were isolated from the library of D. Bramhill by their ability to complement the mitomycin C and UV light sensitivity or an ruv mutant strain.

The vector used for library construction was λPE11 (Hickson and Emmerson 1981), a cI857 derivative of λL47 (Loenen & Brammar 1980). The library consisted of a partial Sau3A digest of E. coli DNA ligated between the two BamHI sites of the vector, replacing the central portion of λ DNA. The size of the inserted DNA was limited to 4.7 - 19.3kb by the constraints on λ packaging (Maniatis 1982).

Strain CS123 ruv-58 λcI857 was infected with the λVI Sau3A library at a multiplicity of infection of 1pfu/cell. Cells and phages were incubated at 32°C for 60' to allow phage adsorption, plated out onto 0.15ug/ml mitomycin C plates, irradiated with 20J/m^2 UV and incubated for 24 hours at 32°C. Surviving colonies, presumed

to be CS123 ruv-58 λcI857 λruv$^+$ double lysogens were purified, gridded and replica plated to test for resistance to UV irradiation (and mitomycin C). Phages from the UV^{R}MC^R isolates were heat induced and the mixed λcI857/ λ"ruv$^+$" lysate tested for its ability to complement a range of strains carrying different ruv mutations by cross streaking on a 0.2ug/ml mitomycin C plate which was then irradiated with 40J/m^2 UV light. λ"ruv$^+$" phages were purified from those mixed lysates that complemented the UV and mitomycin C sensitivity of ruv mutants, by streaking mixed lysates to single plaques on a lawn of strain W3110 in TB overlay agar on TB plates. Six λ"ruv$^+$" phages designated λRL101-106 were identified on the basis of their smaller plaque size and their ability to complement the mitomycin C and UV irradiation sensitivity of ruv mutants on cross-streak plates. Since efficient complementation of several ruv mutants was observed with each phage, it was assumed that all phages carried the intact ruv$^+$ gene and that complementation did not require intragenic recombination between the chromosomal ruv mutation and λ"ruv$^+$". This could not be tested since the λ"ruv$^+$" phage do not possess the λ int gene required for integration at the chromosomal att site and therefore the RecA protein is required in order to integrate the λ"ruv$^+$" phage by homologous recombination in the first instance. In addition the extreme UV and mitomycin C sensitivity of recA mutants would make the detection of any complementation of ruv mutants by λ"ruv$^+$" phages impossible, even if it could occur.

Restriction endonuclease analysis of the six λ"ruv$^+$" transducing phages designated λ RL101-106 demonstrated that five had different restriction patterns containing some common fragments, when digested with HindIII, BamHI or EcoRI, the sixth, RL106 had the same restriction pattern as λRL102 (data not shown).

λ"ruv$^+$" lysogens of ruv mutants were constructed by spotting 10^6-10^8 phage in 10ul of λ buffer onto a lawn of the appropriate

host strain in TB overlay agar on TB plates, then incubating the plates at 32°C for 24-48 hours. Lysogens were purified from the centres of patches of lysis by streaking to single colonies on plates spread with approximately 10^9pfu λ c(int) h80. They were then further checked for resistance to λ c(int)Δ h80 superinfection and sensitivity to λ vir. In order to confirm the ability of the λ "ruv$^+$" phages to complement the UV sensitivity of ruv mutant strains, so far observed only on plate cross-streaks, the survival to UV irradiation of several strains carrying different ruv mutations lysogenised with each of the different λ"ruv$^+$" phages was determined and is shown in Fig. 4.1a-c. Clearly, lysogenisation with any of the λ"ruv$^+$" phages restores the sensitivity to UV irradiation of the ruv mutant strains to the level of resistance observed in ruv$^+$ strains. This provides further evidence to suggest that each of the λ"ruv$^+$" phages isolated carries the entire ruv region since if intragenic recombination between the λ ruv$^+$ phage and the mutant ruv allele were necessary to provide a functional ruv gene product, then an intermediate level of resistance to UV irradiation might be expected in some of the lysogenised mutant strains.

However, the further possibility that the λ"ruv$^+$" phages, isolated on the basis of complementation of the mitomycin C and UV irradiation sensitive phenotype of ruv mutants, carried not the ruv$^+$ gene itself but rather a supressor of the ruv mutant phenotype, could not be excluded.

This possibility was investigated by determining the approximate origin of the cloned DNA carried by the λ"ruv$^+$" phages by mapping their site of insertion in the chromosome in constructed lysogens. Since the λ ruv$^+$ phages do not encode the phage λ int gene product, integration cannot occur via int promoted site-specific recombination at the chromosomal att site, but must occur via recombination between the cloned DNA and its homologous region

Figure 4.1a

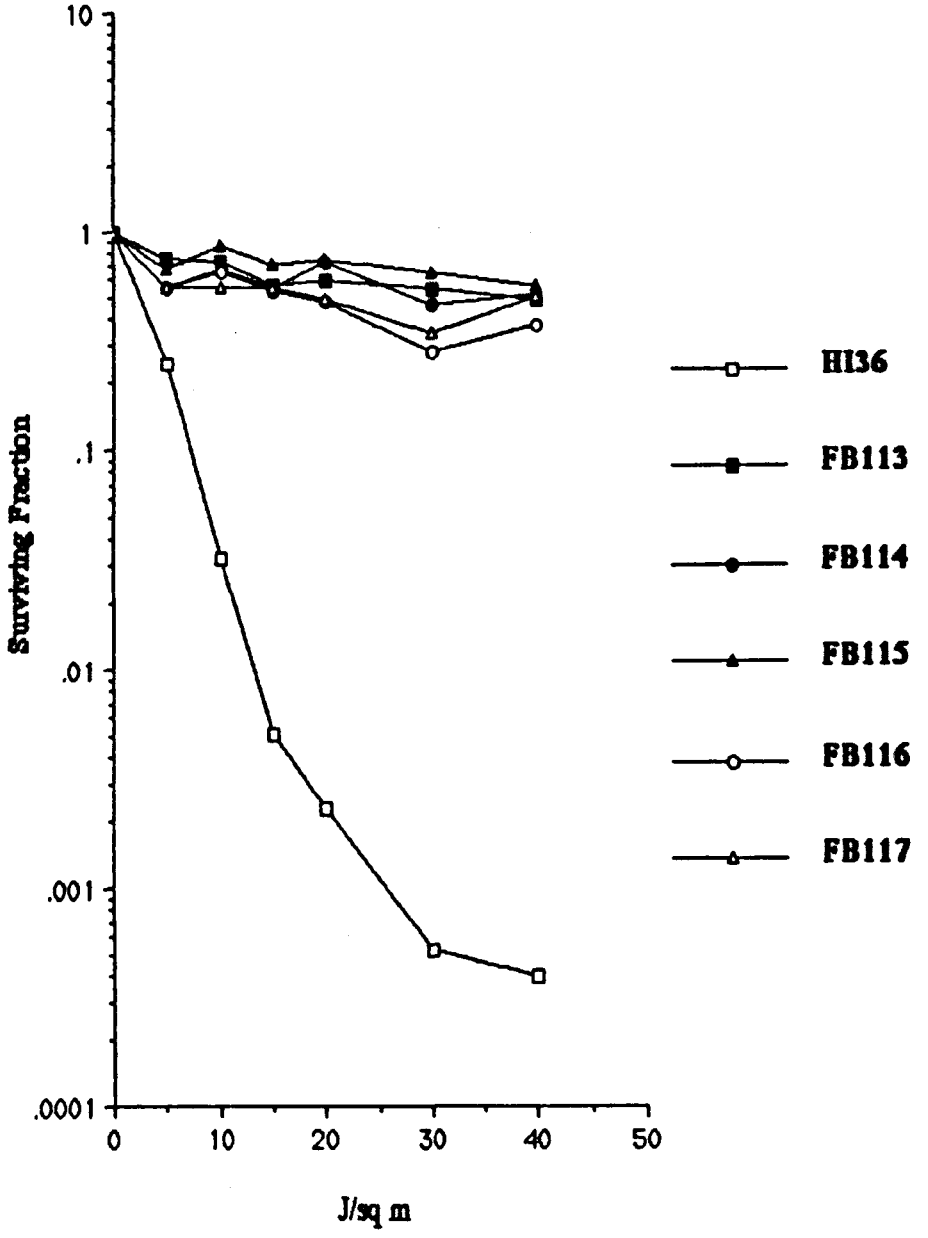


Fig. 4.1a

Complementation of UV sensitivity of an ruv mutant with ruv$^+$ phages. Strains used were HI36 ruvB9, FB113 ruvB9/ λRL101, FB114 ruvB9/ λRL102, FB115 ruvB9/ λRL104, FB116 ruvB9/ λRL103, and FB117 ruvB9/ λRL105

Figure 4.1b

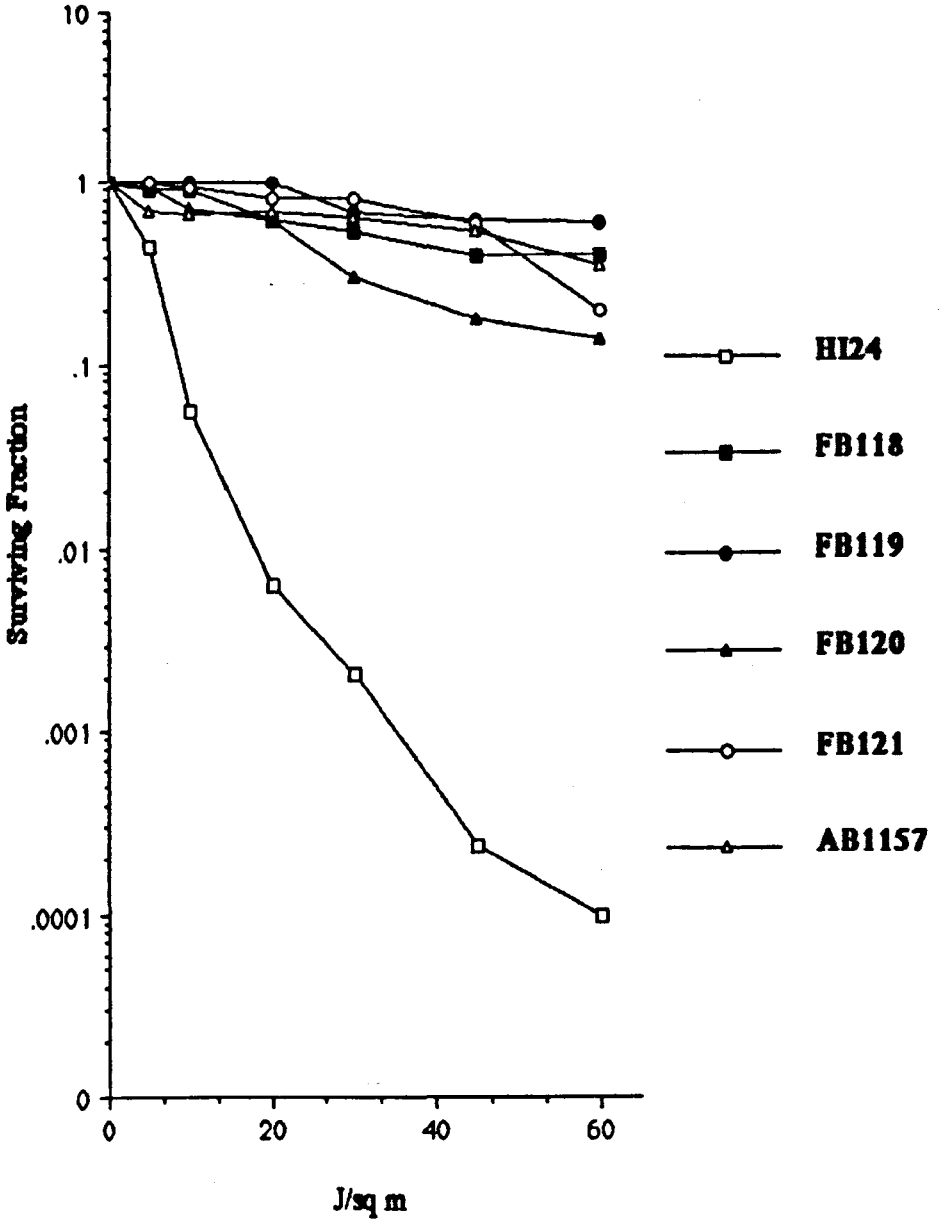


Fig. 4.1b

Complementation of UV sensitivity of an ruv mutant with ruv$^+$ phages. Strains used were HI24 ruvA4, FB118 ruvA4/ λRL101, FB119 ruvA4/ λRL102, FB120 ruvA4/ λRL104, FB121 ruvA4/ λRL105, and AB1157 ruv$^+$

Figure 4.1c

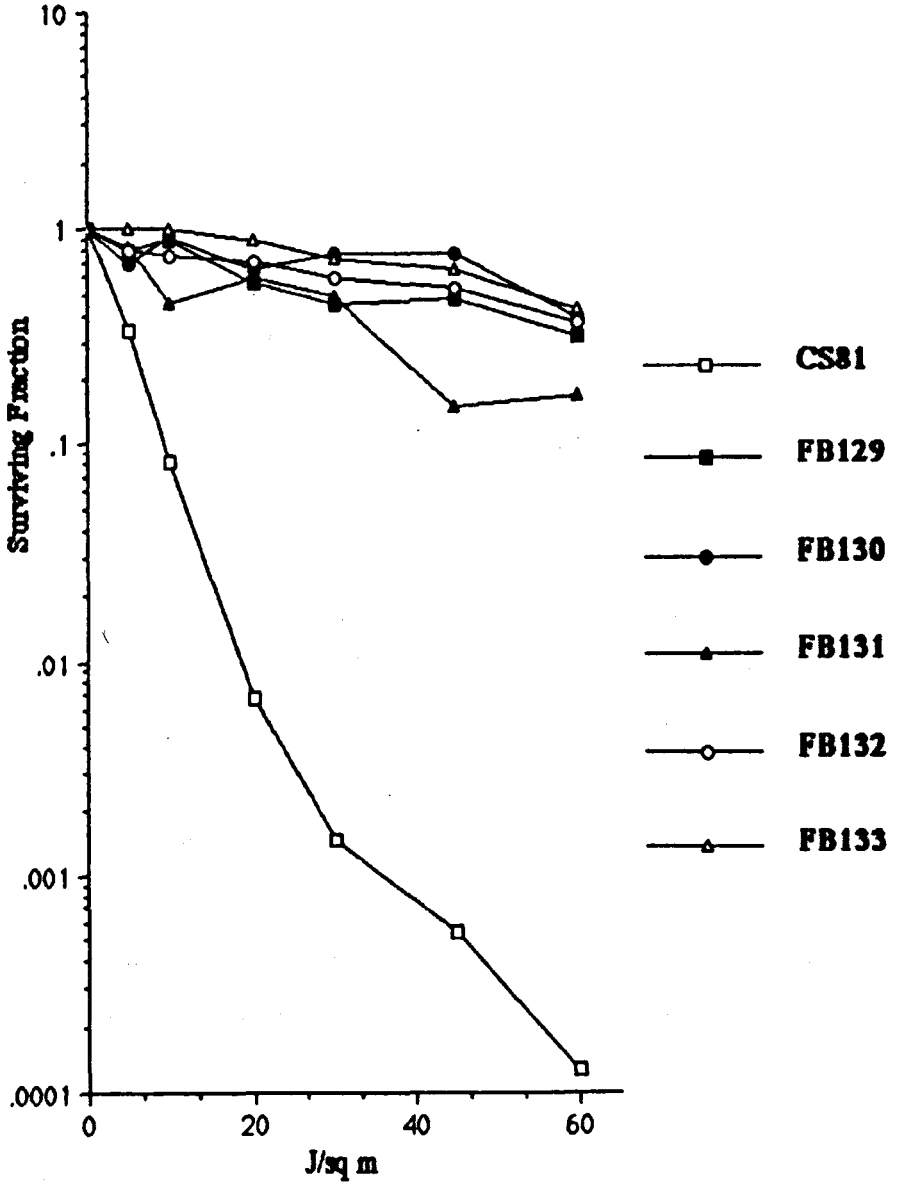


Fig. 4.1c

Complementation of UV sensitivity of an ruv mutant with ruv$^+$ phages. Strains used were CS81 ruv-52, FB129 ruv-52/ λRL101, FB130 ruv-52/ λRL102 FB131 ruv-52/ λRL104, FB132 ruv-52/ λRL103, and FB133 ruv-52/ λRL105

of the chromosome. Thus, determination of the site of insertion of the λ"*ruv*$^+$" prophages in constructed lysogens should give an indication of the map region from which the cloned DNA was derived.

The donor Hfr strains KL96 (which transfers DNA from an origin between 44' and 45' in an anticlockwise direction, and therefore transfers the *ruv* region as an early marker) and KL208 (which transfers DNA from an origin between 30' and 31' in an anticlockwise direction and therefore only transfers the *ruv* region as a late marker) were grown to approximately 2 x 10^8 cells/ml in Mu broth and mixed with λ*ruv*$^+$ lysogenic strains at the same cell density in a 1:4 ratio and allowed to mate for 30' at 37°C. Mating mixtures were then vortexed and plated out on minimal agar plates selecting for recombinants that inherited the donor *Pro*$^+$ marker. *Pro*$^+$ recombinants were then tested for the presence of λ*ruv*$^+$ by examination of growth at 42°C and of sensitivity to λ*c*(*int*)Δ*h80* at 32°C. In the absence of the *proA* marker in the recipient, KL99 (which transfers DNA from an origin between 22' and 23' in a clockwise direction and therefore transfers the *ruv* region relatively early) and N1674 (a derivative of KL208 with a *gal*::Tn*10* insertion providing a selectable marker at 17'), were used as donors, selecting for His$^+$ and Tc^R recombinants respectively. In all cases donor strains were counterselected by the presence of streptomycin at 0.10% in the selective plates.

Results presented in Table 4.1 clearly show that each of the 5 independently isolated λ*ruv*$^+$ phages preferentially integrates in the 30' to 44' interval as defined by the origins of transfer of the donor Hfr strains KL208 and KL96 or in the region extending approximately 20' clockwise from the origin of transfer of KL208 at 30'. Since λ*ruv*$^+$ phages can only integrate via homologous recombination, the observed preferred integration site between 30' and 44' which includes the *ruv* locus at 41' suggests that λRL101-106 do in fact carry the *ruv*$^+$ gene rather than a suppressor of the

Table 4.1 Mapping of insertion sites of λRL101-105 in *ruv*/ λ*ruv*$^+$ lysogenic strains

Recipient strain	Donor strain				Insertion site
	KL96	KL208	N1674	KL99	
FB129 *ruv-52* λRL101	35	0	ND	ND	30' - 44'
FB130 *ruv-52* λRL102	0	0	ND	ND	?
FB131 *ruv-52* λRL103	90	0	ND	ND	30' - 44'
FB132 *ruv-52* λRL104	80	0	ND	ND	30' - 44'
FB133 *ruv-52* λRL105	90	0	ND	ND	30' - 44'
FB139 *ruv-53* λRL102	30	0	ND	ND	30' - 44'
FB140 *ruv-53* λRL104	60	0	ND	ND	30' - 44'
FB141 *ruv-53* λRL105	65	0	ND	ND	30' - 44'
FB122 *ruv-51* λRL102	ND	ND	0	0	?
FB123 *ruv-51* λRL104	ND	ND	0	40	30' - 50'
FB136 *ruv-57* λRL103	ND	ND	0	90	30' - 50'
FB137 *ruv-57* λRL104	ND	ND	0	75	30' - 50'
FB138 *ruv-57* λRL105	ND	ND	0	75	30' - 50'

Crosses between donor and recipient strains, selection, and analysis of selected recombinants were performed as described in Section 4.2. Twenty recombinants from each cross were analysed. Results are presented as the percentage of selected recombinants sensitive to λ *c*(*int*)Δ h80 and resistant to growth at 42°C, i.e. those recombinants that have failed to inherit the λ*ruv*$^+$ prophage . The possible site of insertion of the λ *ruv*$^+$ prophage in each lysogenic strain was determined from the origins of transfer of the Hfr strains used.

ND = not determined

mitomycin C and UV irradiation sensitive phenotype of ruv mutants. Of course, these results cannot rule out the possibility that λ "ruv$^+$" transducing phages encode a closely linked suppressor.

The results presented in Table 4.1 also show that in two strains, FB130 and FB122, the site of insertion of the λruv$^+$ phage λRL102 is not within the ruv region. This was not entirely surprising since it had been reported that the VI E. coli library originally screened contained "jumbled" inserted DNA, i.e. DNA not conti guous in the chromosome ligated together and inserted between the vector arms (I. Hickson, pers. comm.). If λRL102 contained, in addition to the ruv region, DNA not contiguous with this region in the chromosome, then it could insert via homology with a second region in addition to the ruv region.

Further evidence that λRL101 - 106 carry the chromosomal ruv$^+$ region was provided by the observation that one phage, λRL103, was able to complement on a streak test the temperature sensitive growth phenotype of a second mutation tls-1 which is 40-50% cotransduced with ruv (Shurvinton, 1983).

Since the DNA carried by λRL101 - 105 is at least partly derived from the chromosomal region between 30' and 44' which includes the ruv locus at 41' and since each of the λRL101 - 105 phages complements efficiently the UV sensitivity of strains harbouring each of the different ruv mutations, it was concluded that λRL101 - 105 all carry the intact functional ruv$^+$ gene.

The proteins encoded by the five independently isolated λruv$^+$ phages were identified using the irradiated host system with FB542 a uvrA λcI857 host. The excision deficiency of a uvrA strain ensured that irradiated cells were unable to repair the massive damage to the chromosome inflicted by 12000J/m^2 UV light, thus reducing background labelling of chromosome encoded proteins to an acceptably low level. A λcI857 lysogen was used to provide intracellular cI repressor and thus prevent expression of the λ genes needed for

lytic growth of the adsorbed λ*ruv*$^+$ phage - the only λ encoded proteins expressed are the *c*I857 and *rex* gene products of 26,200 and 29,000 daltons respectively (Stoker *et al.* 1985). An autoradiogram of ^{35}S methionine labelled proteins encoded by λ*ruv*$^+$ phages RL101-105 separated by SDS-PAGE is shown in Figure 4.2.

The smallest λ*ruv*$^+$ phage, λ RL105 which has an insert of approximately 10kb encodes only two proteins - of approximately 25,000 and 41,000 daltons respectively, that are not encoded by the vector. Both these proteins are encoded by the other 4 λ*ruv*$^+$ transducing phages and are thus candidates for the *ruv* encoded protein.

4.3 Subcloning of ruv into the low copy number vector pHSG415

The *ruv* region was subcloned from λ*ruv*$^+$ phages into the plasmid vector pHSG415 by ligating a mixture of the 5 *Hin*dIII digested λ *ruv*$^+$ phages into the *Hin*dIII site of pHSG415 which lies within the gene encoding kanamycin resistance. The ligation mixture was transformed into HI24 *ruvA4* and Ap^{R}CM^R transformants selected. Transformants were tested for sensitivity to Kanamycin, UV light and mitomycin C. One Ap^R,Cm^R transformant, designated PA1048 was found to be sensitive to Kanamycin (confirming that a DNA fragment had been ligated into the *Hin*dIII site of pHSG415), and resistant to UV irradiation and mitomycin C, suggesting that the plasmid, designated pPVA101, present in the isolate PA1048 carried the intact *ruv* region (Dr. P.V. Attfield).

Plasmid DNA was extracted and used to transform a range of strains carrying different *ruv* mutations, selecting for Ap^{R}CM^R transformants. Data presented in Figure 4.3 show that pPVA101 complemented the sensitivity to UV irradiation of all the strains carrying single *ruv* mutations tested. A similar result was obtained for complementation of the mitomycin C sensitivity of these strains.

Figure 4.2

Fluorograph of λRL101-105 encoded proteins, labelled with ^{35}S methionine in the UV-irradiated host system, separated on a 15% polyacrylamide-SDS gel and visualised by fluorography. Lane a. λPE11. b. λRL101, c. λRL102, d. λRL103, e. λRL104, f. λRL105, (g.pPVA101). Lambda encoded proteins are arrowed. Molecular weight markers were phosphorylase b (94kd), bovine serum albumin (67kd), ovalbumin (43kd), carbonic anhydrase (30kd), soybean trypsin inhibitor (20.1kd) and α-lactalbumin (14.4kd).

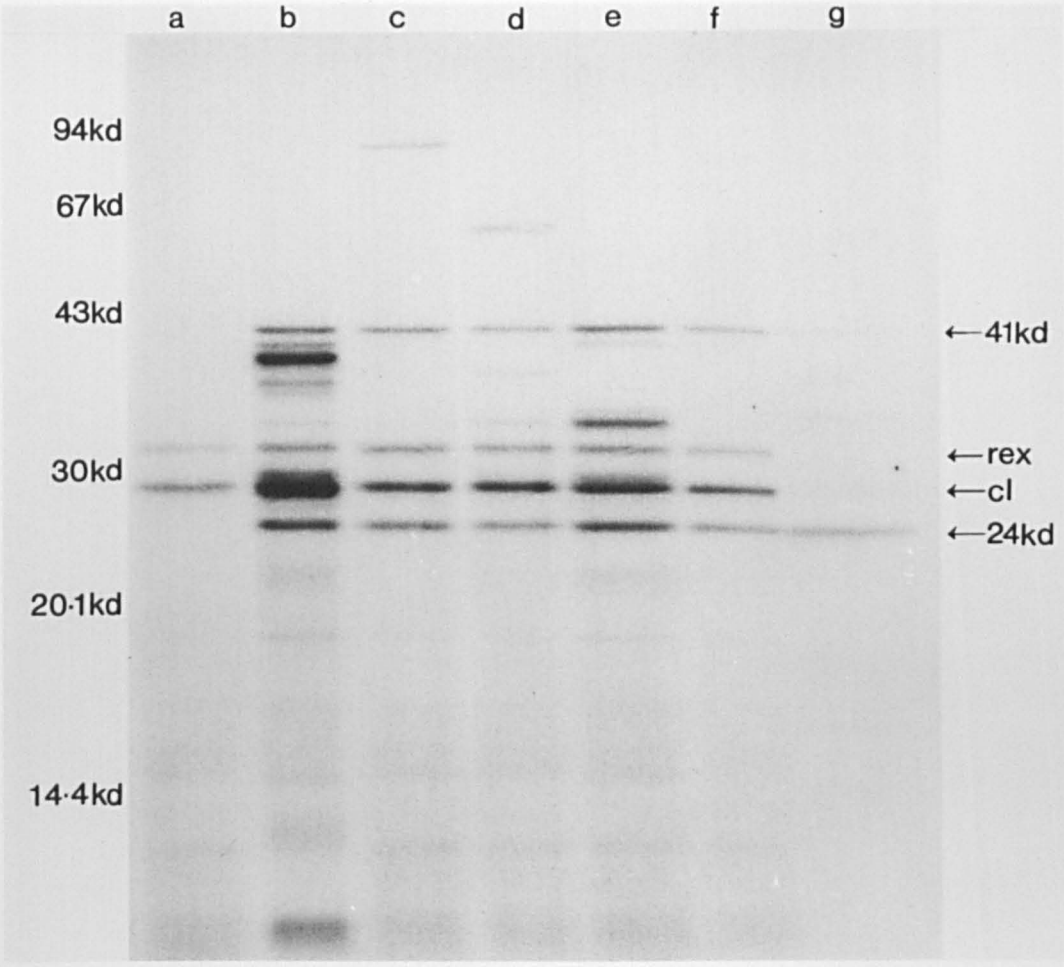

a
b
c
d
e
f
g
94kd
67kd
43kd
30kd
20·1kd
14·4kd
←41kd
←rex
←cI
←24kd

Figure 4.3a

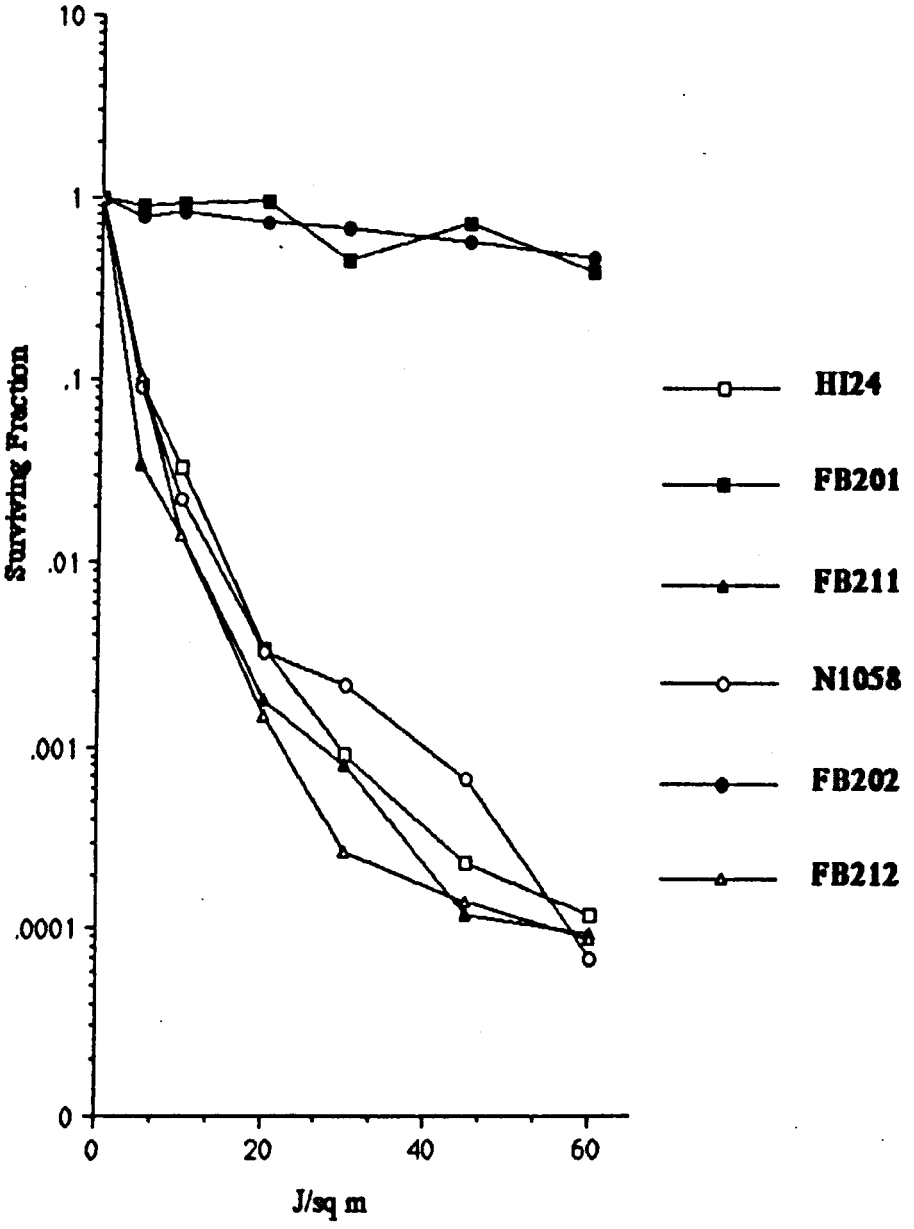


Fig. 4.3a

Complementation of UV sensitivity of ruv mutants with plasmid pPVA101. Strains used were HI24 ruvA4, FB201 ruvA4/pPVA101, FB211 ruvA4/pHSG415, HI36N1058 ruvB9, FB202 ruvB9/pPVA101, and FB212 ruvB9/pHSG415

Figure 4.3b

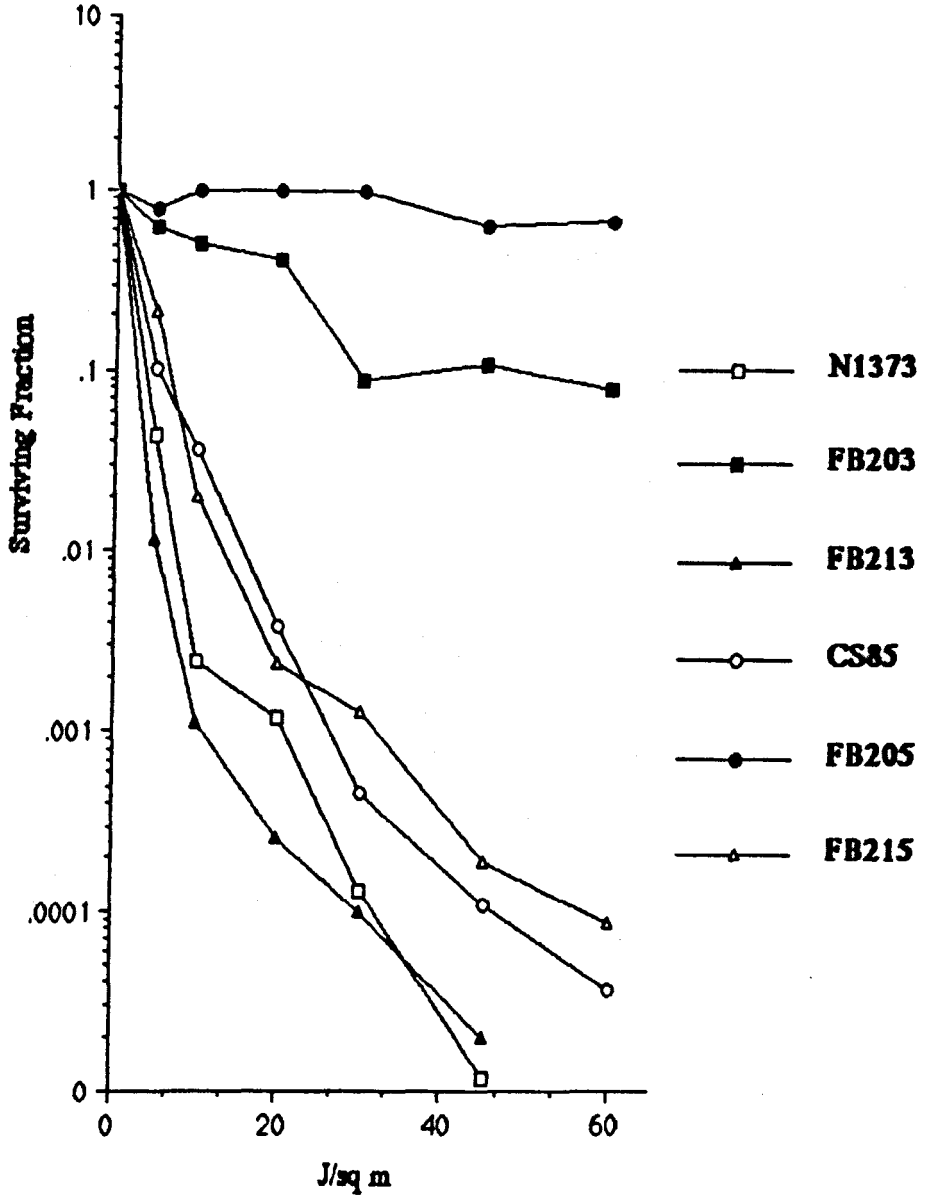


Fig. 4.3b

Complementation of UV sensitivity of ruv mutants with plasmid pPVA101. Strains used were N1373 ruv-51, FB203 ruv-51/pPVA101, FB213 ruv-51/pHSG415, CS85 ruv-53, FB205 ruv-53/pPVA101, and FB215 ruv-53/pHSG415

Figure 4.3c

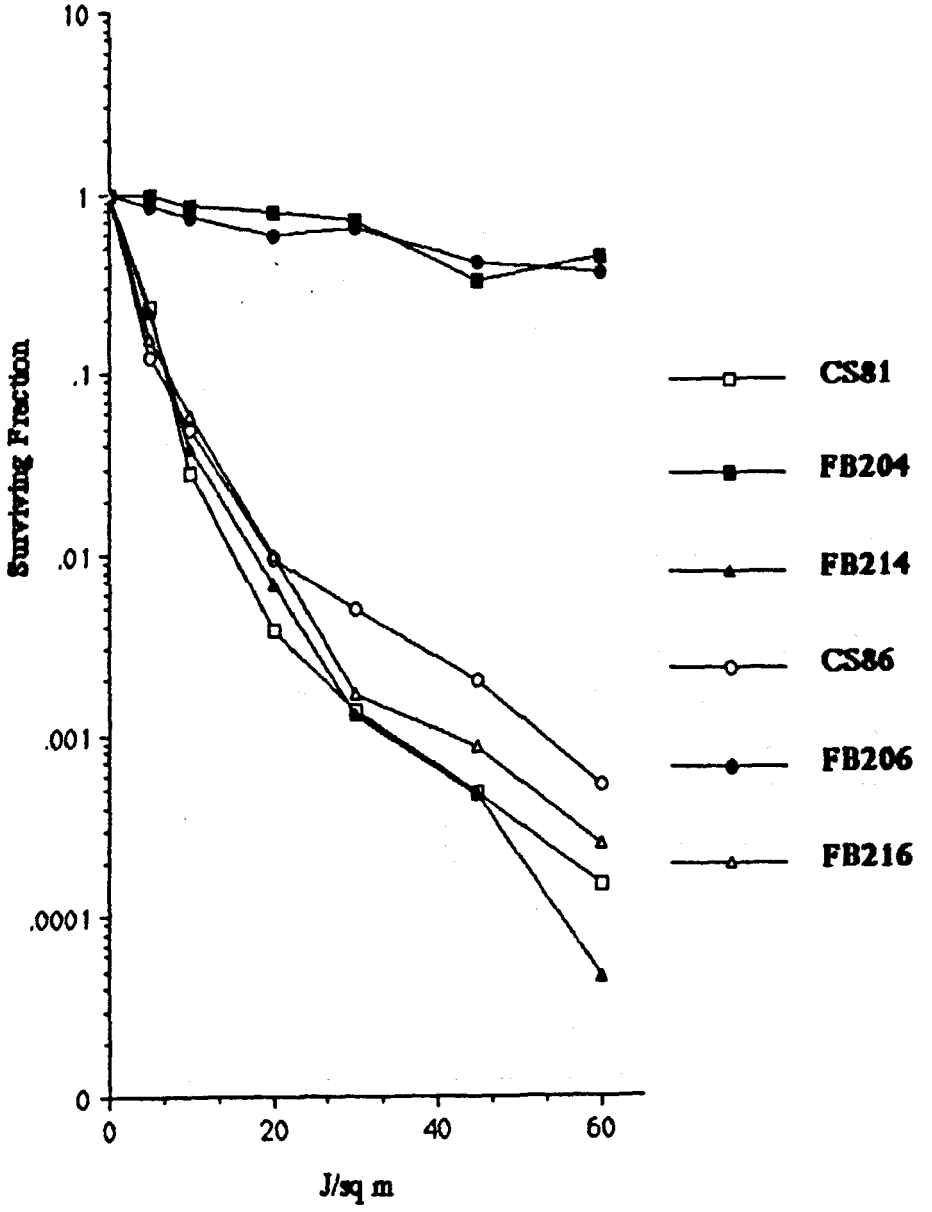


Fig. 4.3c

Complementation of the UV sensitivity of ruv mutants with plasmid pPVA101. Strains used were CS81 ruv-52, FB204 ruv-52/pPVA101, FB214 ruv-52/pHSG415, CS86 ruv-54, FB206 ruv-54/pPVA101, and FB216 ruv-54/pHSG415

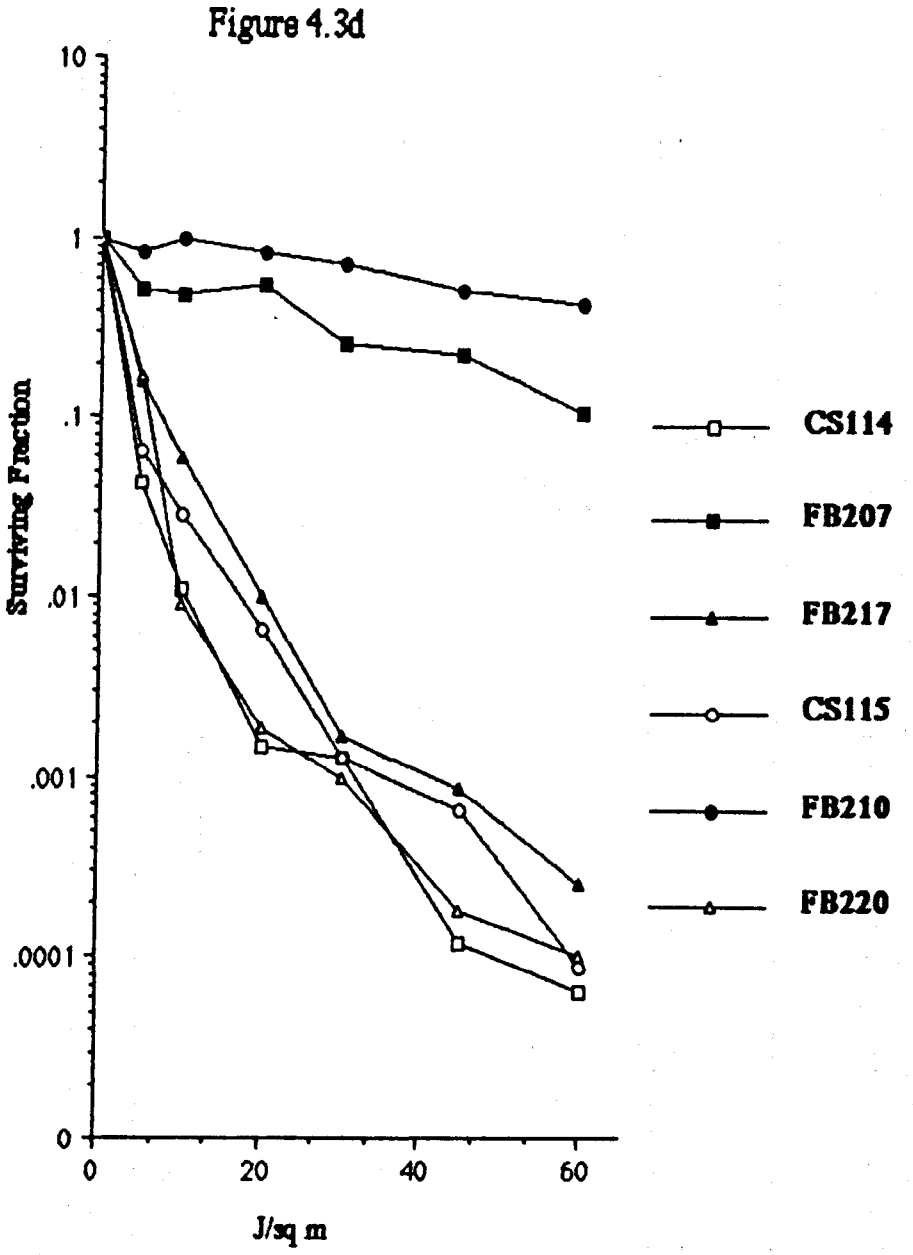


Fig. 4.3d

Complementation of the UV sensitivity of ruv mutants with plasmid pPVA101. Strains used were CS114 ruv-57, FB207 ruv-57/pPVA101, FB217 ruv-57/pHSG415, CS115 ruv-58, FB210 ruv-58/pPVA101, and FB220 ruv-58/pHSG415

TABLE 4.2 Restoration of recombination proficiency and F' transconjugant recovery in a *recBC sbcBC ruv* strain by pPVA101

Donor		x GY2200 (HfrH)		xKL548 (F'128)
Selection	**Viability**	**λ plaques**	**Thr⁺ Leu⁺**	**Pro⁺**
Recipient				
FB165	a. 1.6×10^8	1.5×10^6	4.9×10^6	1.23×10^7
recBC sbcBC	b. 1	1	1	1
FB166	a. 1.4×10^7	1.2×10^6	2.3×10^4	1.7×10^4
recBC sbcBC ruv-52	b. 0.087	0.8	0.0047	0.0013
	c.	> 1	0.05	0.015
FB225	a. 1.1×10^8	1.3×10^6	7.9×10^6	7.9×10^6
recBC sbcBC	b. 0.68	0.86	> 1	0.63
pPVA101	c.	> 1	> 1	0.93
FB226	a. 1.4×10^8	2.0×10^6	2.1×10^6	1.25×10^7
recBC sbcBC ruv-52	b. 0.87	> 1	0.42	1
pPVA101	c.	> 1	0.48	> 1

Experiments were performed as described in 2.8 using a 1:4 ratio of donors:receipients. Antibiotic selection (Ap) for pPVA101 was maintained throughout. Figures in line (a) represent the numbers of colonies (plaques) per ml of mating mixture, in line (b) the recovery of selected colonies (plaques) relative to the *ruv*⁺ parent strain and in (c) the recovery of selected colonies corrected for the observed viability deficiency.

In addition pPVA101 complemented fully the γ-sensitivity (Figure 4.4) the UV sensitivity (Figure 4.5) and the deficiency in recovery of recombinants and F' transconjugants (Table 4.2) of a recBC sbcBC ruv strain.

A preliminary restriction analysis demonstrated that pPVA101 had an insert of 10.4kb that could have originated from either λRL101 or λRL103 (Figure 4.6). Subsequent studies suggested that the 10.4kb HindIII bands observed in digests of λRL101 and λRL103 were not the same, and that the insert of pPVA101 was derived from λRL103 (data not shown).

Preliminary restriction analysis also revealed an EcoRI restriction site close to one end of the inserted DNA fragment, approximately 2.2kb from the EcoRI site present within the chloramphenicol resistance gene of the vector. This allowed the construction of the first in a series of deletion derivatives constructed to attempt to define the ruv coding region within the cloned DNA fragment.

pPVA101 DNA was digested with EcoRI, religated and transformed into AB2463 recA13. Restriction analysis of plasmid DNA prepared from an $Ap^{R}Cm^{S}$ transformant demonstrated that the plasmid, designated pPVA105 had lost the 2.2kb EcoRI fragment present in pPVA101 (Dr. P.V. Attfield). In order to determine whether the deleted plasmid derivative pPVA105 still carried the intact ruv region, it was transformed into a range of strains carrying different ruv mutations and the effect on survival after UV irradiation examined. Clearly the data presented in Figure 4.7 show that pPVA105 complements the UV sensitive phenotype of the ruv mutants, demonstrating that it still carries the intact ruv coding region.

Plasmid pPVA105 DNA was subjected to extensive restriction analysis using a combination of single, double and triple restriction endonuclease digests (Figure 4.8). Known sites were

Figure 4.4

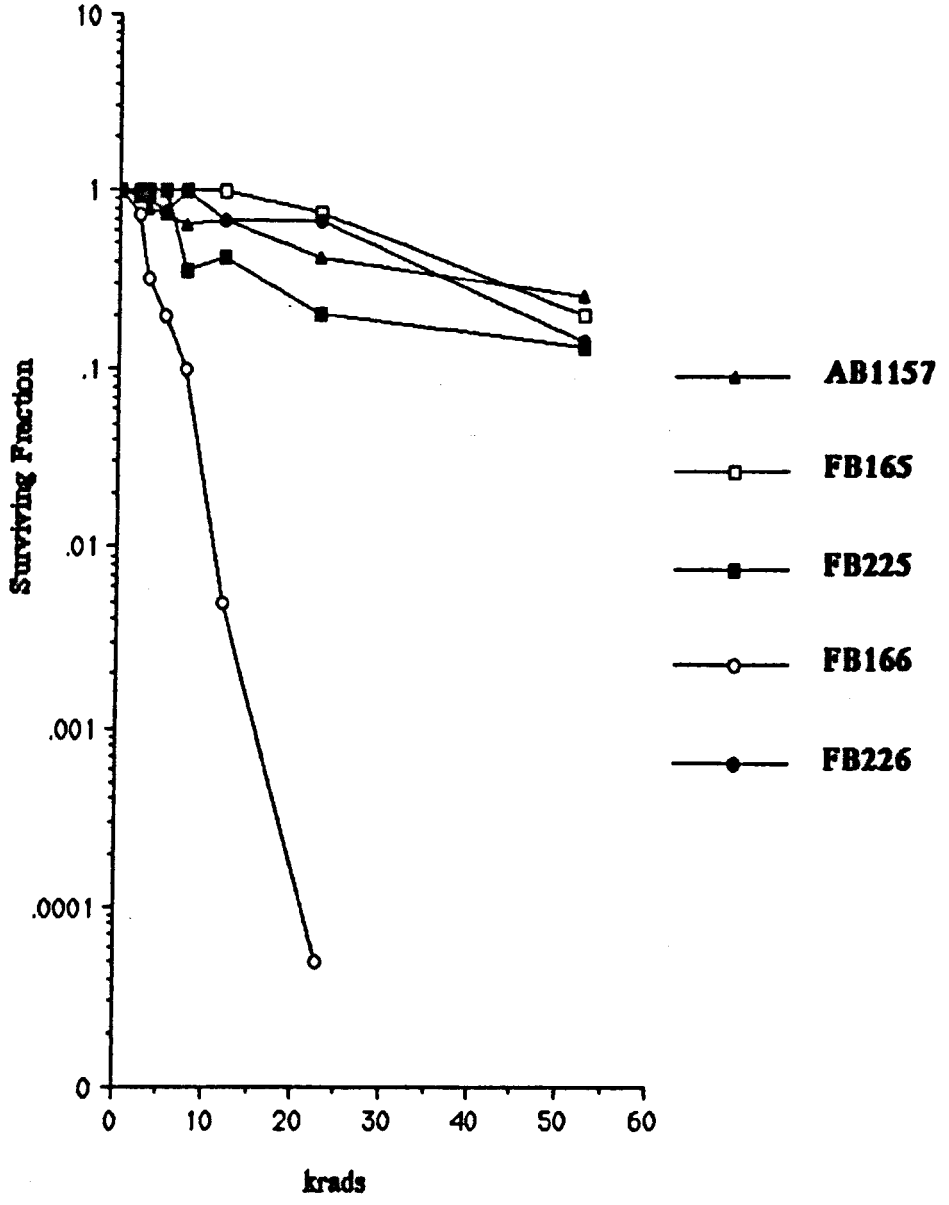


Fig. 4.4

Complementation of the gamma sensitivity of a recBC sbcBC ruv strain with pPVA101. Strains used were AB1157 ruv$^+$ rec$^+$, FB165 recBC sbcBC, FB225 recBC sbcBC pPVA101, FB166 recBC sbcBC ruv-52, and FB226 recBC sbcBC ruv-52 pPVA 101.

Figure 4.5

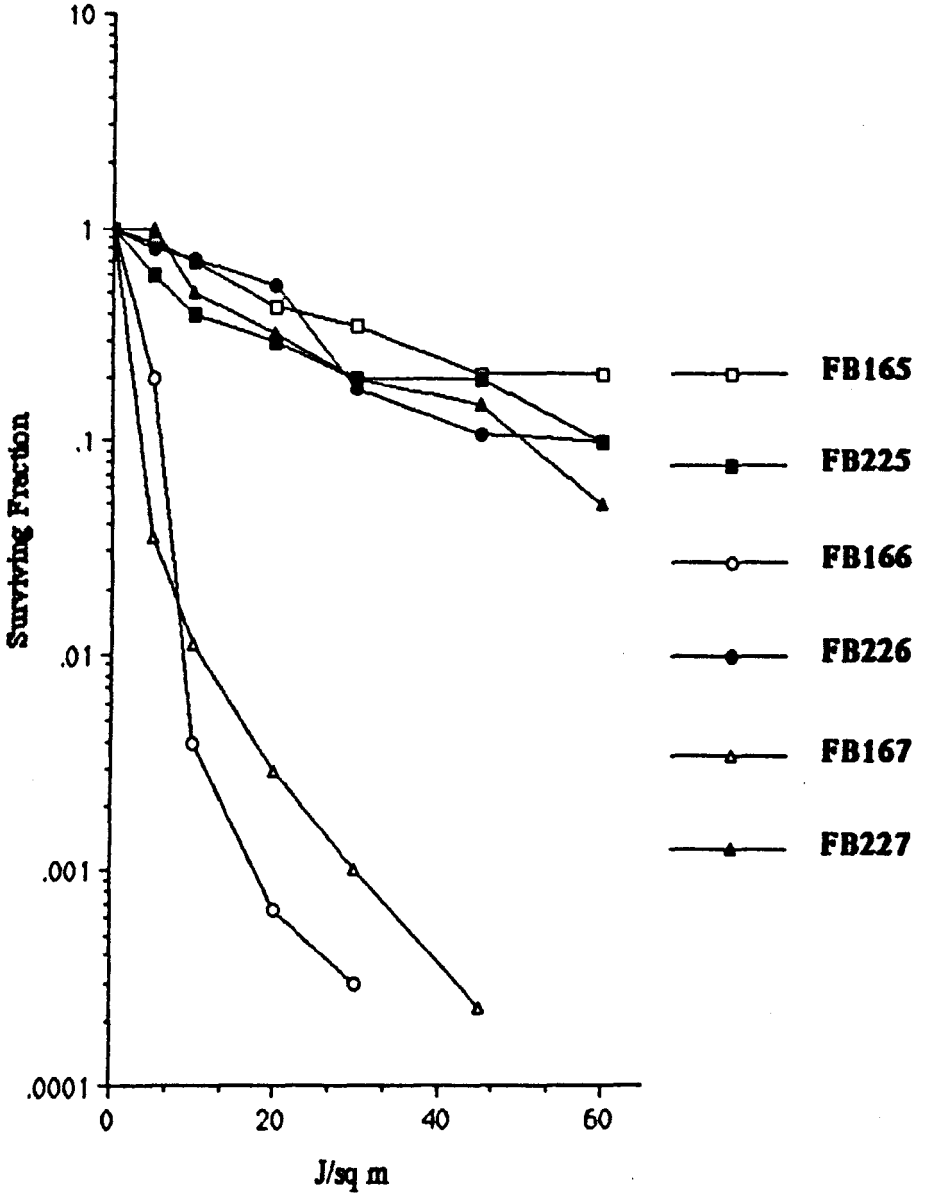


Fig. 4.5

Complementation of the UV irradiation sensitivity of recBC sbcBC ruv strains with plasmid pPVA101. Strains used were FB165 recBC sbcBC FB225 recBC sbcBC pPVA101, FB166 recBC sbcBC ruv-52, FB226 recBC sbcBC ruv-52 pPVA101, FB167 recBC sbcBC ruv-54, FB227 recBC sbcBC ruv-54 pPVA101

Figure 4.6

Restriction digests of λRL101-105 and pPVA101. Molecular weight markers (left) were the products of HindIII digestion of λ wild type, (λ w.t.).

A. HindIII digestion of λRL101-105 and pPVA101. The labelled bands are the products of HindIII digestion of the pHSG415 vector (which has a single HindIII site within the Km^R gene), and pPVA101 (which has cloned DNA inserted into the HindIII site of the vector).

B. EcoRI digestion of pPVA101 and pHSG415. Labelled bands are the products of EcoRI digestion of pHSG415 (which has a single EcoRI restriction site within the Cm^R gene) and pPVA101.

A
λRL101
λRL102
λRL104
λRL103
λRL105
λ wt.
pPVA101
pHSG415
10·4
7·1
9·46kb–
6·67kb–
4·26kb–
2·25kb–
1·96kb–
B
pPVA101
pHSG415
15
7·1
2·2

Figure 4.7a

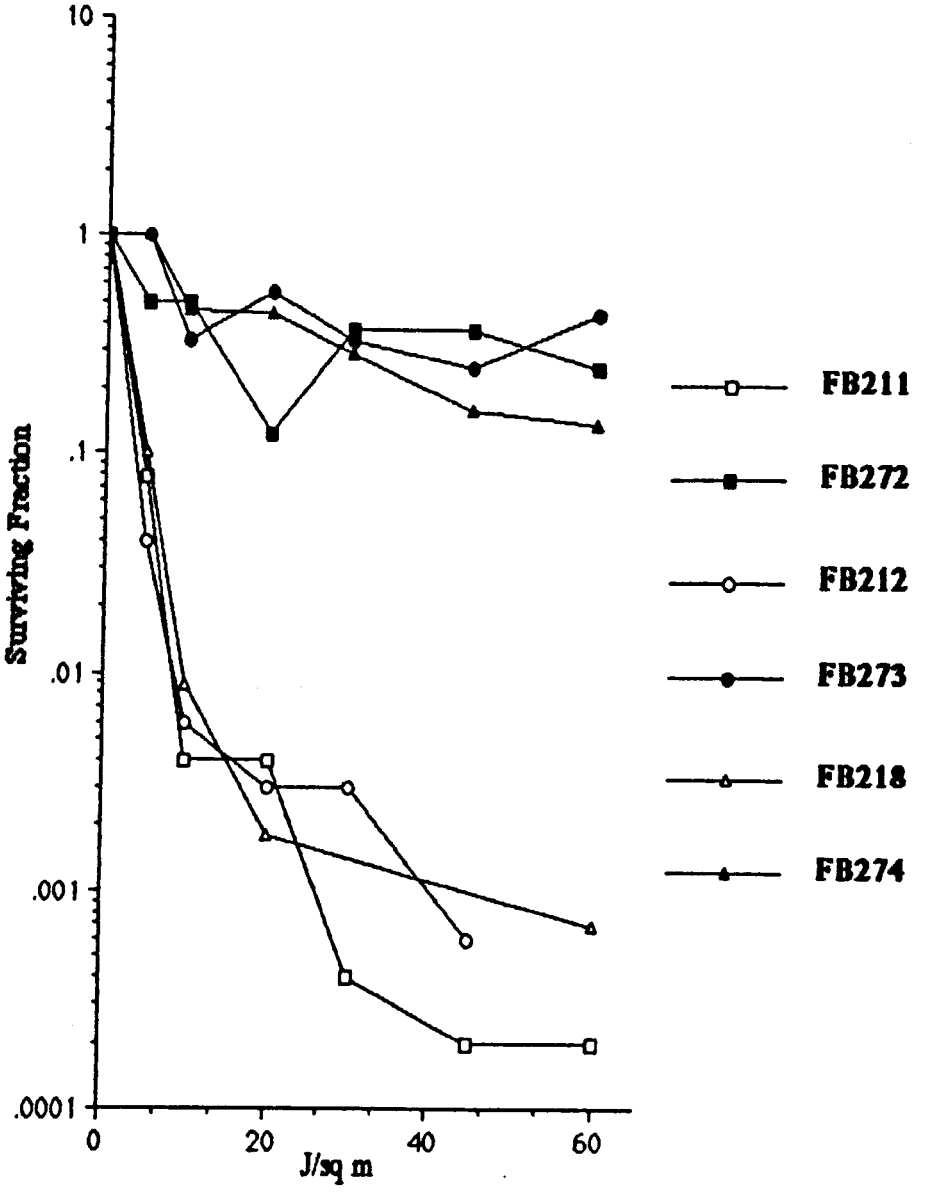


Fig. 4.7a

Complementation of the UV sensitivity of ruv strains with plasmid pPVA105. Strains used were FB211 ruvA4 pHSG415, FB272 ruvA4 pPVA105, FB212 ruvB9 pHSG415, FB273 ruvB9 pPVA105, FB218 ruv-60::Tn10 pHSG415 and FB274 ruv-60::Tn10 pPVA105

Figure 4.7b

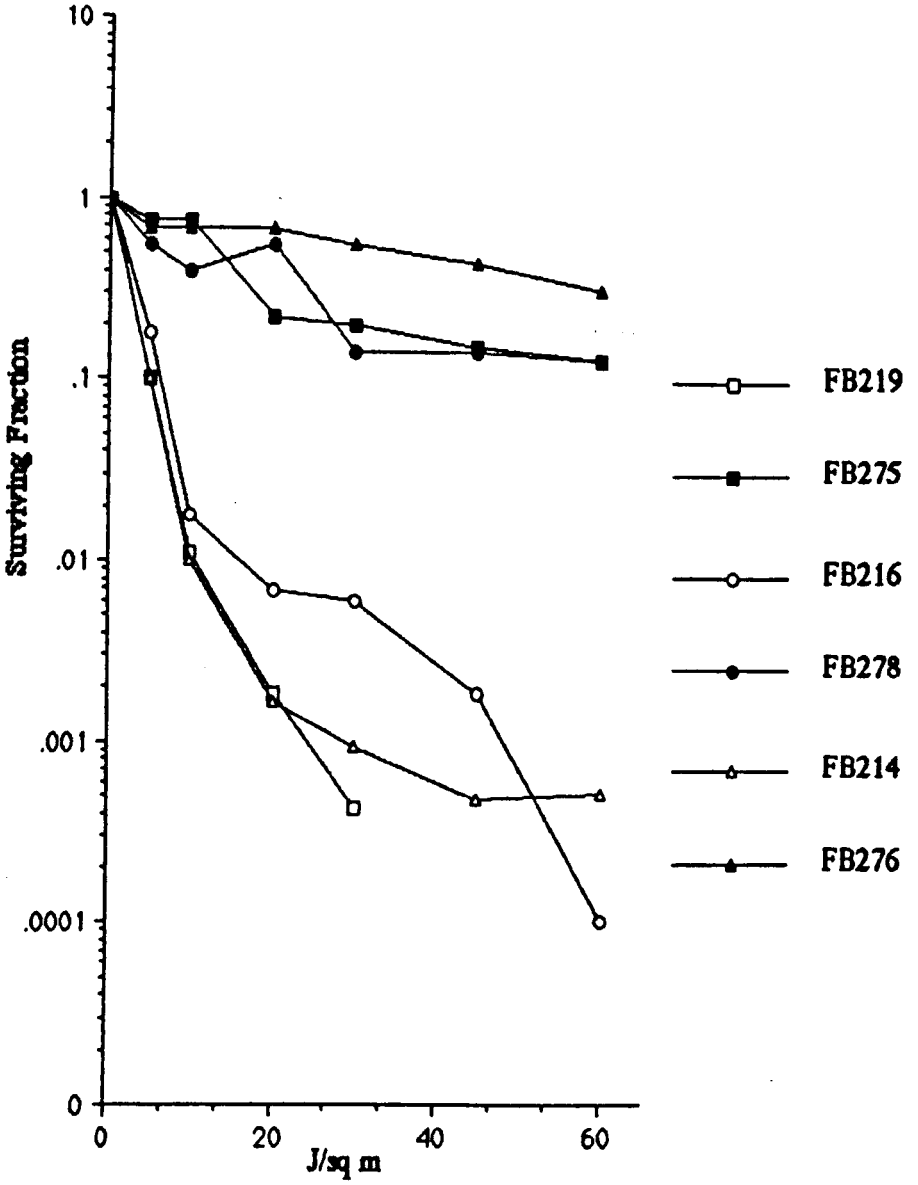


Fig. 4.7b

Complementation of the UV sensitivity of *ruv* strains with plasmid pPVA105. Strains used were FB219 *ruv59*::Tn*10* pHSG415, FB275 *ruv-59*::Tn*10* pPVA105, FB216 *ruv-54* pHSG415, FB278 *ruv-54* pPVA105, FB214 *ruv-52* pHSG415 and FB276 *ruv-52* pPVA105

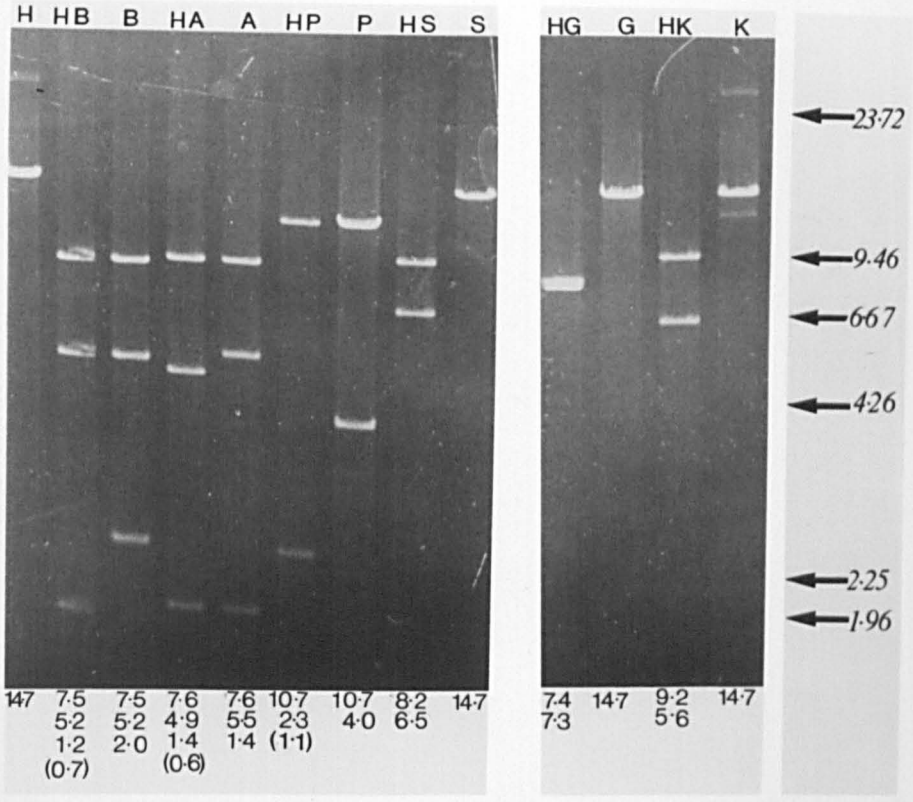
Figure 4.8 (a, b and c)

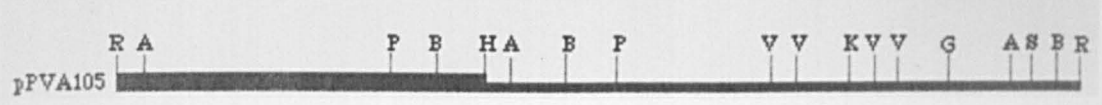
Restriction analysis of pPVA105 DNA.

pPVA105 DNA was digested with the enzymes BamHI(B), AvaI(A), PstI(P), SalI(S), BglII(G) and KpnI(K), in single digests, double digests with either EcoRI(R) or HindIII(H), and in triple digests with both EcoRI and HindIII. Fragments were separated on 1% agarose gels, stained with ethidium bromide, visualised on a transilluminator and photographed.

Figure 4.8a shows the results of digestion with the single enzymes, and also the results of the double digests with HindIII; the derived map has a scale of 0.85cm to 1 kilobase. Figure 4.8b shows the results of digestion with the single enzymes, and also the results of the double digests with EcoRI; the derived map has a scale of 1.05cm to 1 kilobase. Figure 4.8c shows the results of the triple digests with both EcoRI and HindIII, in addition to the 'test' enzyme; the derived map has a scale of 0.84cm to 1 kilobase. The products of HindIII digestion of wild type λ DNA, indicated at the right of the figures, were used as molecular weight standards. Sizes of the digestion products obtained, determined by comparison with the molecular weight standard are shown below each track on the gel. The bracketed figures on the products of digestion predicted from analysis of different enzyme combinations, which have electrophoresed to a point beyond the region photographed, or are stained with ethidium bromide to an intensity less than could be detected. The letter D indicates that the band observed is probably a doublet.

The accuracy with which the molecular weights were estimated was somewhat variable, particularly in the size range above approximately 6kb. Where possible restriction sites were positioned on the final map from analysis of small restriction fragments.


H HB B HA A HP P HS S
HG G HK K
23·72
9·46
6·67
4·26
2·25
1·96
14·7
7·5 5·2 1·2 (0·7)
7·5 5·2 2·0
7·6 4·9 1·4 (0·6)
7·6 5·5 1·4
10·7 2·3 (1·1)
10·7 4·0
8·2 6·5
14·7
7·4 7·3
14·7
9·2 5·6
14·7


R A P B H A B P V V K V V G A S B R
pPVA105

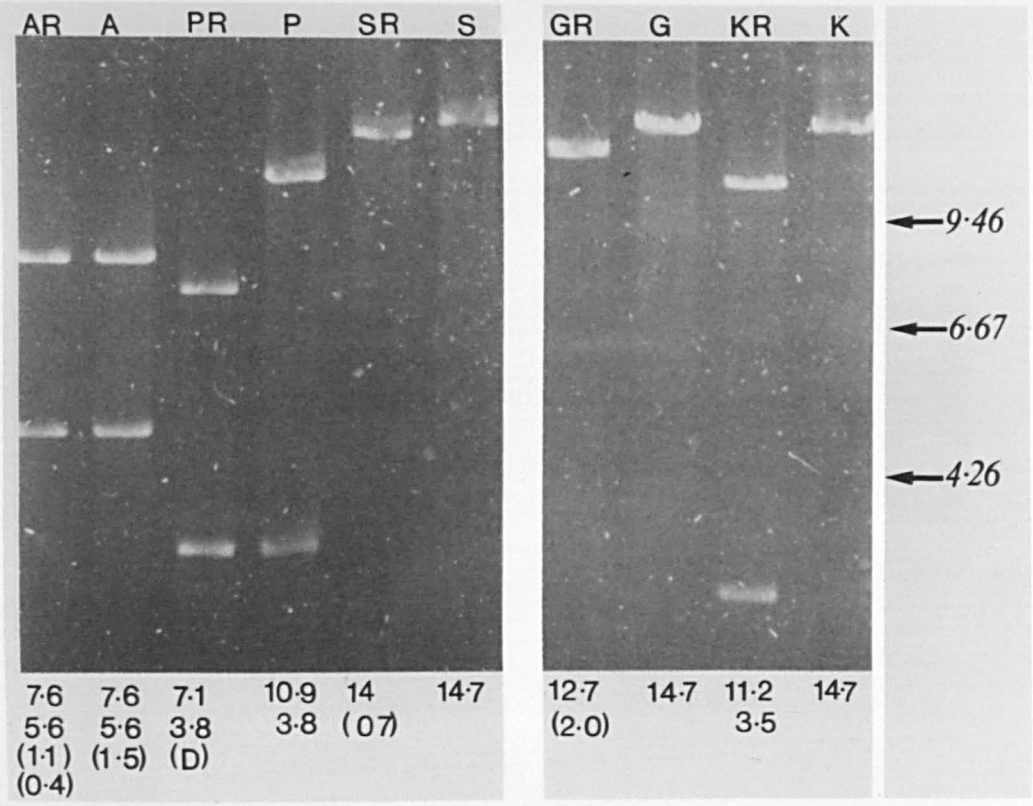
From these results it was clear that three enzymes: SalI, BglII and KpnI had single sites within the plasmid DNA. Since none of these enzymes had restriction sites within the pHSG415 vector, it was concluded that the inserted DNA possessed these sites, which were then positioned by analysis of the triple digest products. The SalI site to 0.8kb to the left of the EcoRI site, the BglII site to 2.0kb to the left of the EcoRI site, and the KpnI site to 3.5kb to the left of the EcoRI site.

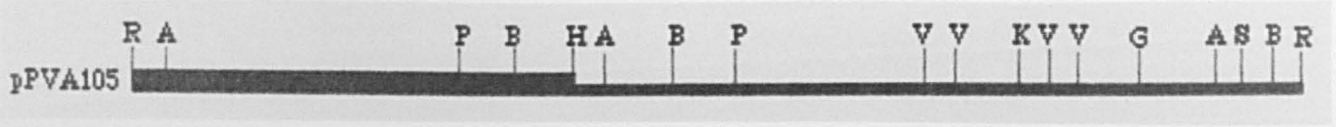
The enzyme PstI had two sites within the plasmid DNA. The position of one site was known from the published restriction map of pHSG415 (Hashimoto-Gotoh et al. 1981). The second was located to a position 2.3kb to the right of the HindIII site by analysis of the products of the single digest; the double digest in combination with HindIII; and the triple digest.

The enzyme BamHI had three restriction sites, one of which was within the pHSG415 vector DNA (Hashimoto-Gotoh et al. 1981). The remaining sites were positioned to 1.2kb to the right of the HindIII site, and to approximately 0.4kb to the left of the EcoRI site (the 0.4kb BamHI-EcoRI fragment was not detected on any of the mapping gels), from analysis of single, double and triple digests.

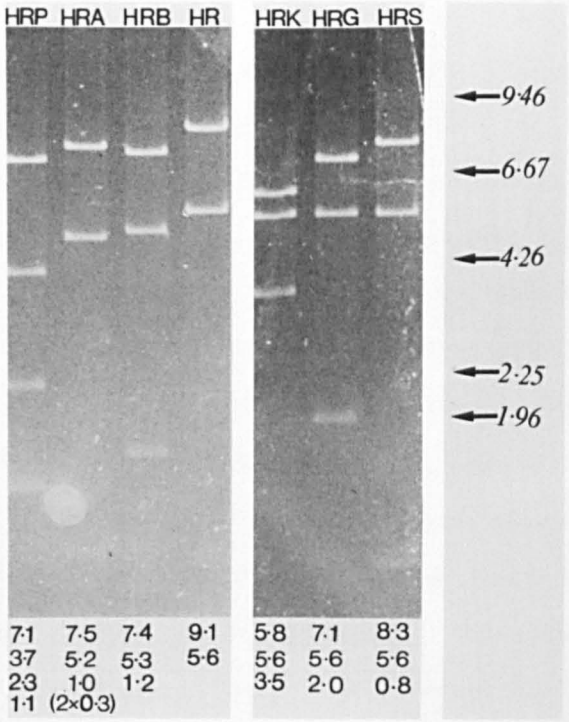
The enzyme AvaI had three restriction sites, one within the pHSG415 vector, the remaining two within the cloned DNA. These sites were mapped to positions 1.0kb to the left of the EcoRI site, and to approximately 0.6kb to the left of the HindIII site (although once again this HindIII-AvaI fragment was not detected on mapping gels), from analysis of single, double and triple restriction digests.

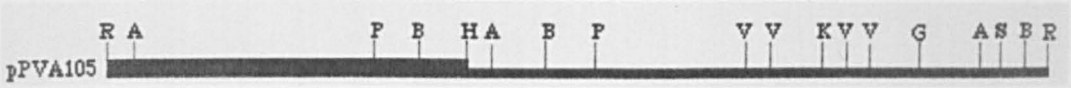
Sites for the enzyme EcoRV(V), detected at a later date, are indicated on the maps in each figure.


AR
A
PR
P
SR
S
GR
G
KR
K
9·46
6·67
4·26
7·6
5·6
(1·1)
(0·4)
7·6
5·6
(1·5)
7·1
3·8
(D)
10·9
3·8
14
(0·7)
14·7
12·7
(2·0)
14·7
11·2
3·5
14·7


R A
P B H A B P
V V K V V G A S B R
pPVA105

4·8b


HRP HRA HRB HR
HRK HRG HRS
9·46
6·67
4·26
2·25
1·96
7·1 7·5 7·4 9·1
3·7 5·2 5·3 5·6
2·3 1·0 1·2
1·1 (2×0·3)
5·8 7·1 8·3
5·6 5·6 5·6
3·5 2·0 0·8


R A P B H A B P V V K V V G A S B R
pPVA105

then used to further map pPVA101 (Figure 4.9).

In order to locate the *ruv*$^+$ coding region, further deletion derivatives of pPVA101 and pPVA105 were constructed.

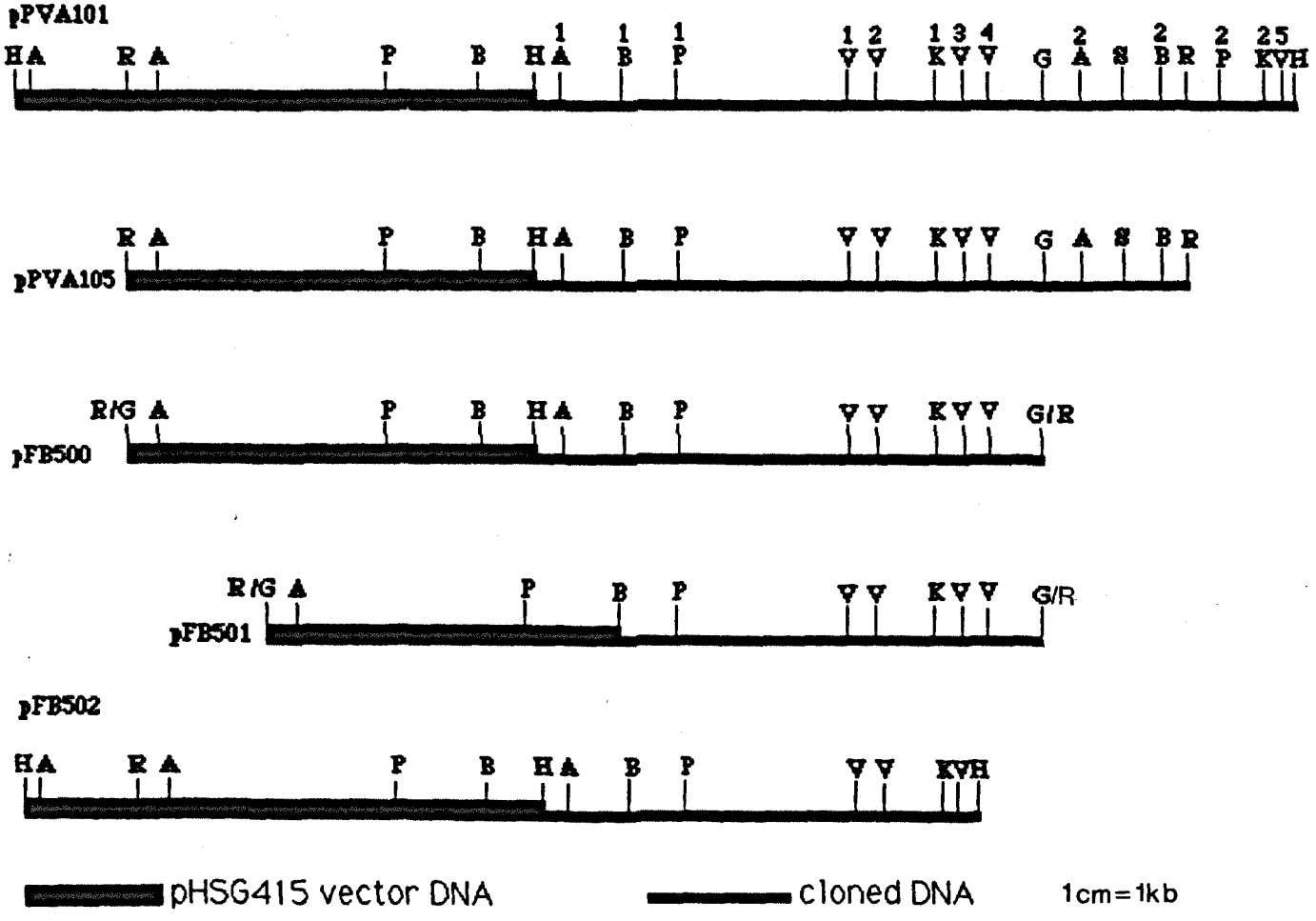
Plasmid pFB500 was obtained by digesting pPVA105 DNA with *Bgl*II and *Eco*RI, filling the ends using Klenow polymerase, blunt end ligating the resulting molecules and transforming into AB2463 *recA13*, selecting for Ap^R transformants. Plasmid DNA prepared from one such transformant was demonstrated by restriction analysis to have lost the *Eco*RI-*Bgl*II region of the parent plasmid pPVA105 (Figure 4.10). Data presented in Figure 4.11 show that the plasmid designated pFB500 retained the ability to restore resistance to UV irradiation to a range of strains carrying different *ruv* mutations, demonstrating that it still carries the intact *ruv* coding region.

Plasmid pFB501 was constructed by digesting pFB500 DNA with *Bam*HI to remove the 2.4kb *Bam*HI fragment, ligating the resulting molecules, and transforming into AB2463 *recA13*, selecting for Ap^R transformants. Plasmid DNA prepared from one such transformant was demonstrated by restriction analysis to have lost 2.4kb *Bam*HI fragment present in the parent pFB500 plasmid. The data presented in Figure 4.12 demonstrates that the plasmid deletion derivative designated pFB501 still complements the UV sensitivity of strains carrying different *ruv* mutations and must therefore still carry the *ruv* coding region.

Analysis of the restriction maps and complementation data obtained from the plasmids pPVA101, pPVA105, pFB500 and pFB501 clearly shows that the *ruv*$^+$ coding region must be contained within the 6kb *Bam*HI/*Bgl*II fragment in the centre of the insert in pPVA101.

A further deletion derivative, pFB502 was constructed by digesting pPVA101 DNA with *Kpn*I to remove the 4kb *Kpn*I fragment, ligating the resulting molecule, and transforming into AB2463 *recA13*, selecting for Ap^{R}Cm^R transformants. Plasmid DNA prepared from one such transformant carrying a plasmid designated pFB502 was

Figure 4.9 Restriction maps of pPVA101, pPVA105, pFB500, pFB501 and pFB502

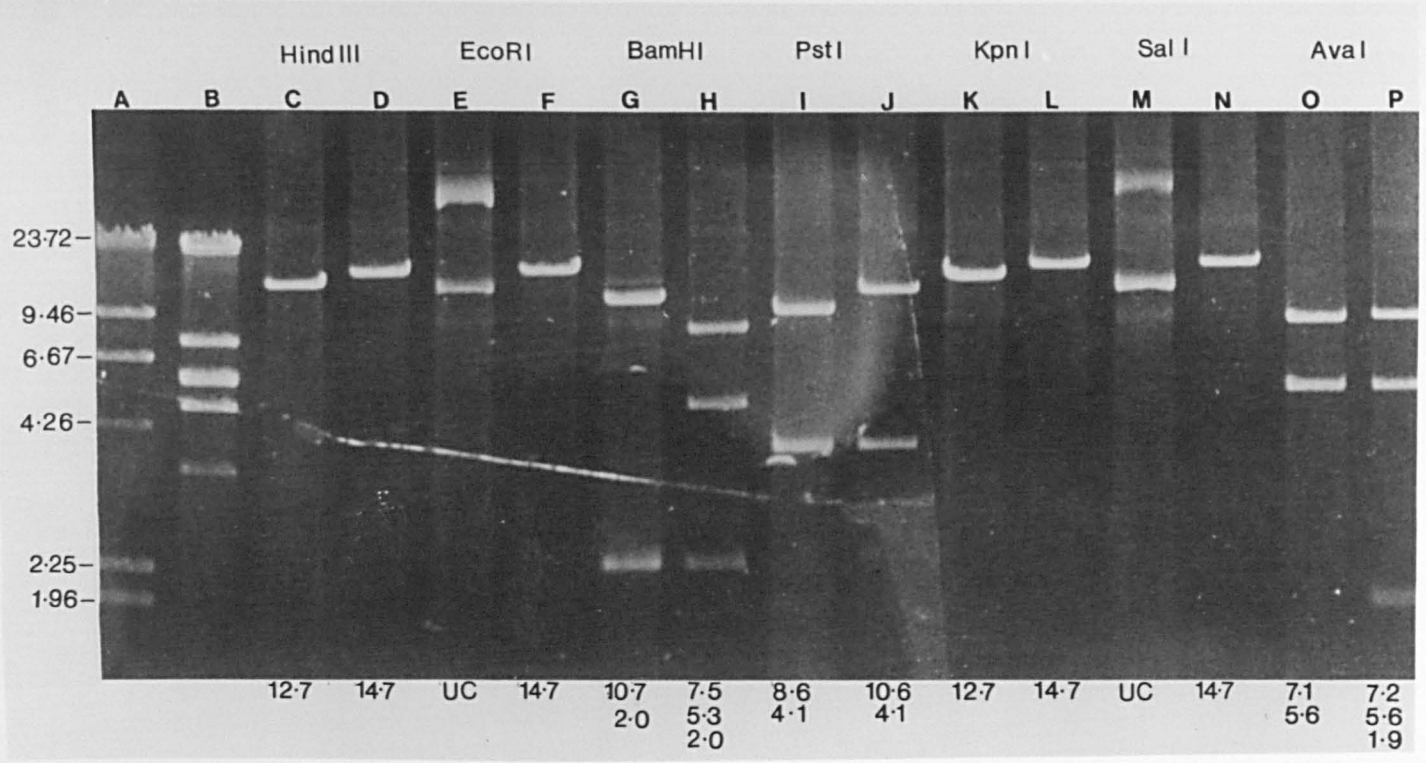


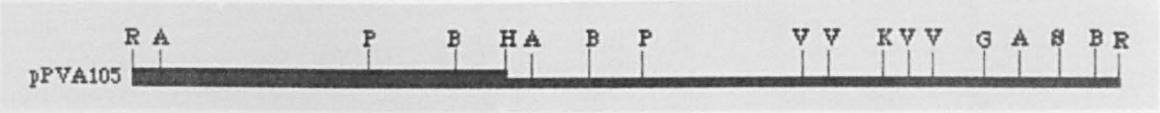
Restrictionsites are numbered from left to right in the inserted DNA of pPVA101as drawn.

Figure 4.10

Restriction analysis of pFB500 DNA.

Lanes A. and B. λDNA, A.*Hind*III digested to provide molecular weight markers, indicated at the left, B.*Eco*RI digested. Lanes C, E, G, I, K, M and O, contained pFB500 DNA. Lanes D, F, H, J, L, N and P contained pPVA105 DNA. The restriction enzymes used are indicated above the relevant lanes on the gel. The sizes of the digestion products, determined from a comparison with the molecular weight standards are shown below each lane. The results clearly show that the plasmid pFB500 is smaller than its parent pPVA105 by approximately 2kb, and has lost the *Bgl*II, *Ava*I, *Sal*I, *Bam*HI and *Eco*RI sites present in the parent plasmid.


Hind III
EcoRI
BamHI
Pst I
Kpn I
Sal I
Ava I
A B C D E F G H I J K L M N O P
23·72–
9·46–
6·67–
4·26–
2·25–
1·96–
12·7
14·7
UC
14·7
10·7
2·0
7·5
5·3
2·0
8·6
4·1
10·6
4·1
12·7
14·7
UC
14·7
7·1
5·6
7·2
5·6
1·9


pPVA105
R A P B H A B P V V K V V G A S B R

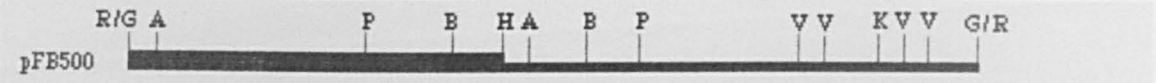

pFB500
R/G A P B H A B P V V K V V G/R

Figure 4.11a

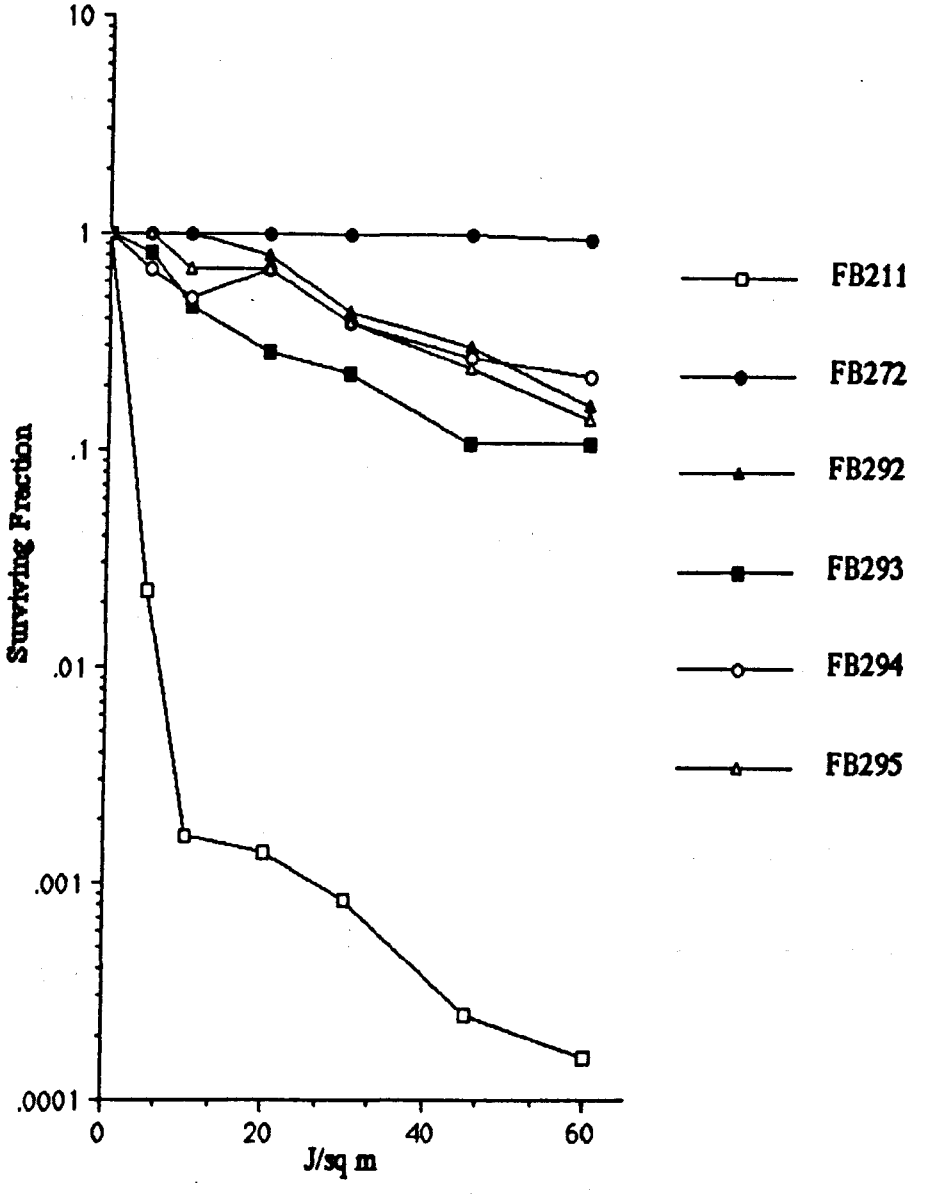


Fig. 4.11a

Complementation of the UV sensitivity of *ruv* strains with plasmid pFB500. Strains used were FB211 *ruvA4* pHSG415, FB272 *ruvA4* pPVA105, FB292 *ruvB9* pFB500, FB293 *ruv-51* pFB500, FB294 *ruv-60*::Tn*10* pFB500, FB295 *ruv-59*::Tn*10* pFB500

Figure 4.11b

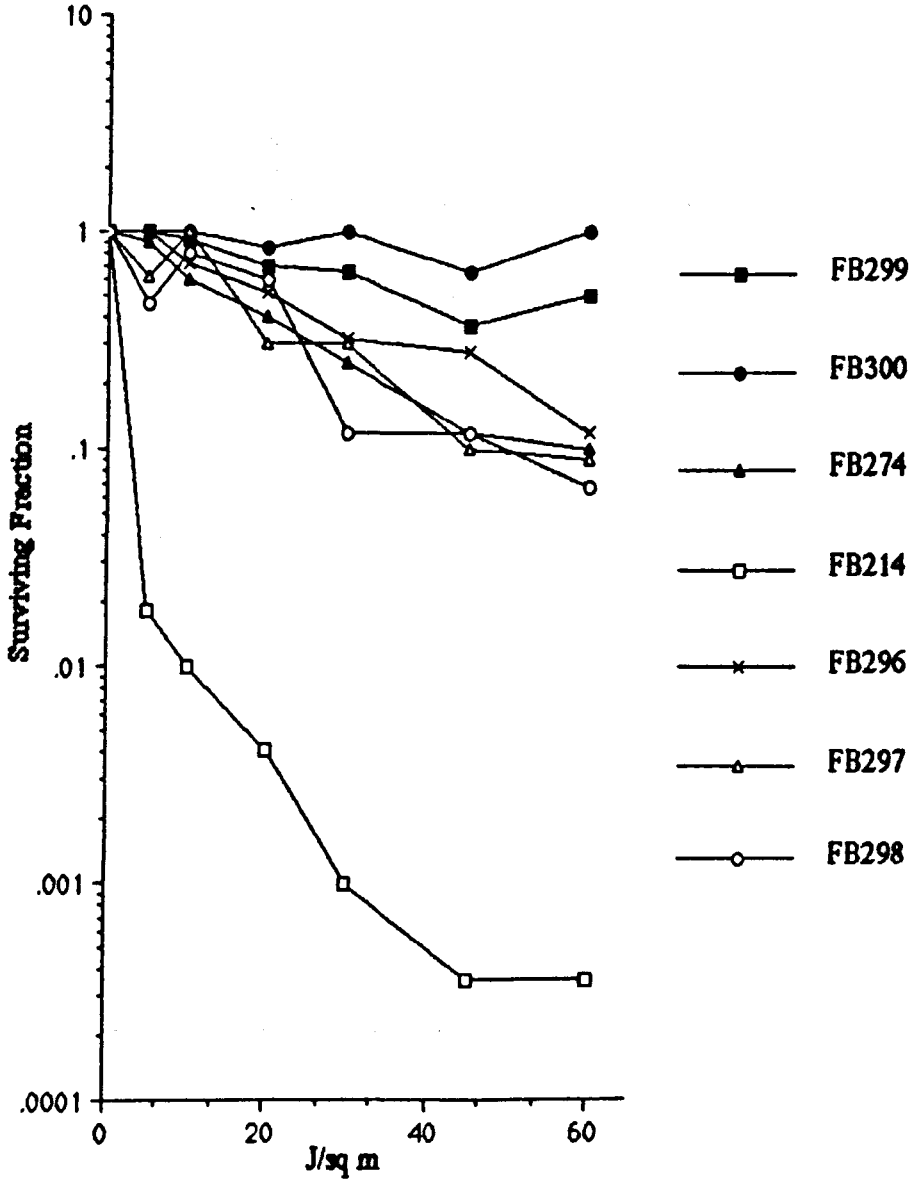


Fig. 4.11b

Complementation of the UV sensitivity of *ruv* strains with plasmid pFB500. Strains used were FB299 *ruv-57* pFB500, FB300 *ruv-58* pFB500, FB274 *ruv-60* pPVA105, FB214 *ruv-52* pHSG415, FB296 *ruv-52* pFB500, FB297 *ruv-53* pFB500 and FB298 *ruv-54* pFB500

Figure 4.12a

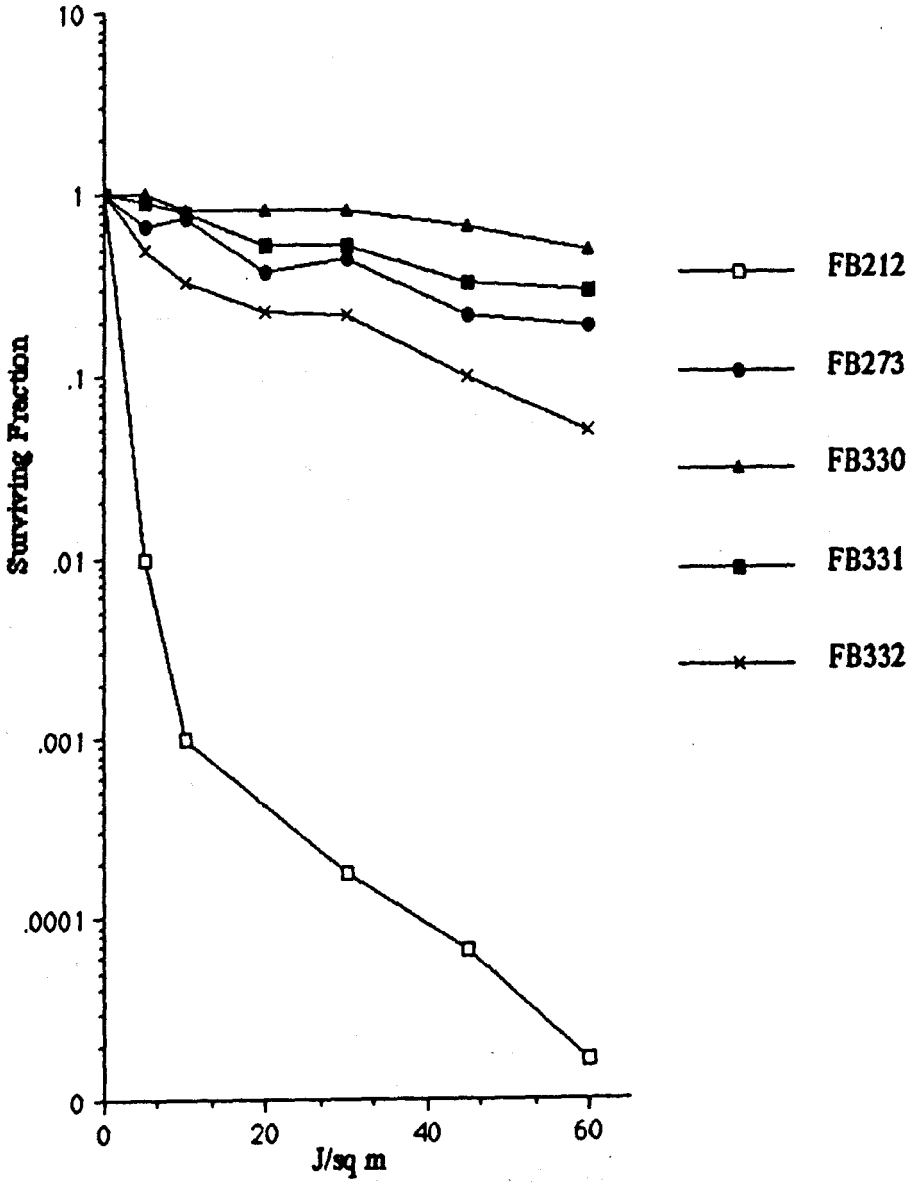


Fig. 4.12a

Complementation of the UV sensitivity of ruv strains with plasmid pFB501. Strains used were FB212 ruvB9 pHSG415, FB273 ruvB9 pPVA105, FB330 ruvA4 pFB501, FB331 ruvB9 pFB501, and FB332 ruv-51 pFB501

Figure 4.12b

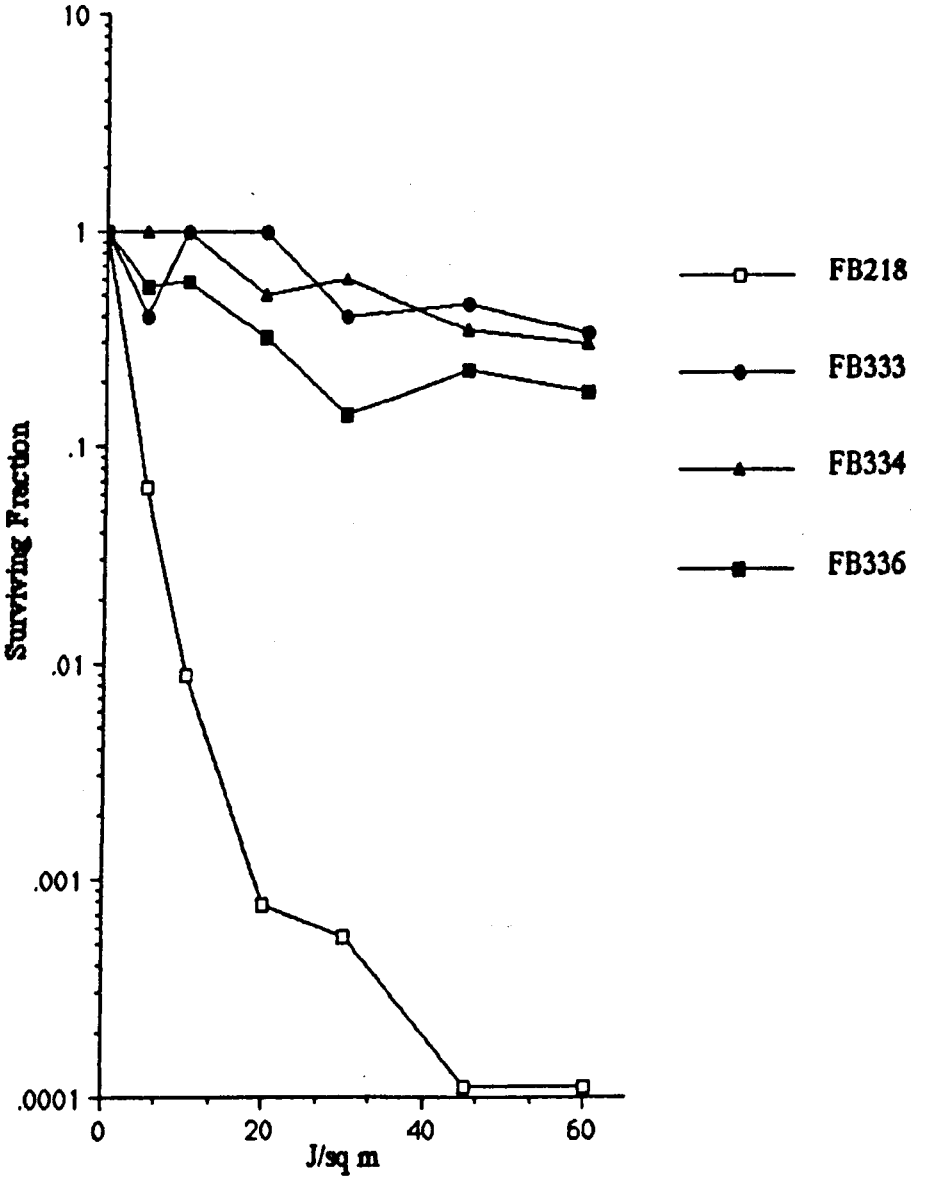


Fig. 4.12b

Complementation of the UV sensitivity of *ruv* strains with plasmid pFB501. Strains used were FB218 *ruv-60*::Tn*10* pHSG415, FB333 *ruv-60*::Tn*10* pFB501, FB334 *ruv-59*::Tn*10* pFB501, and FB336 *ruv-53* pFB501

Figure 4.12c

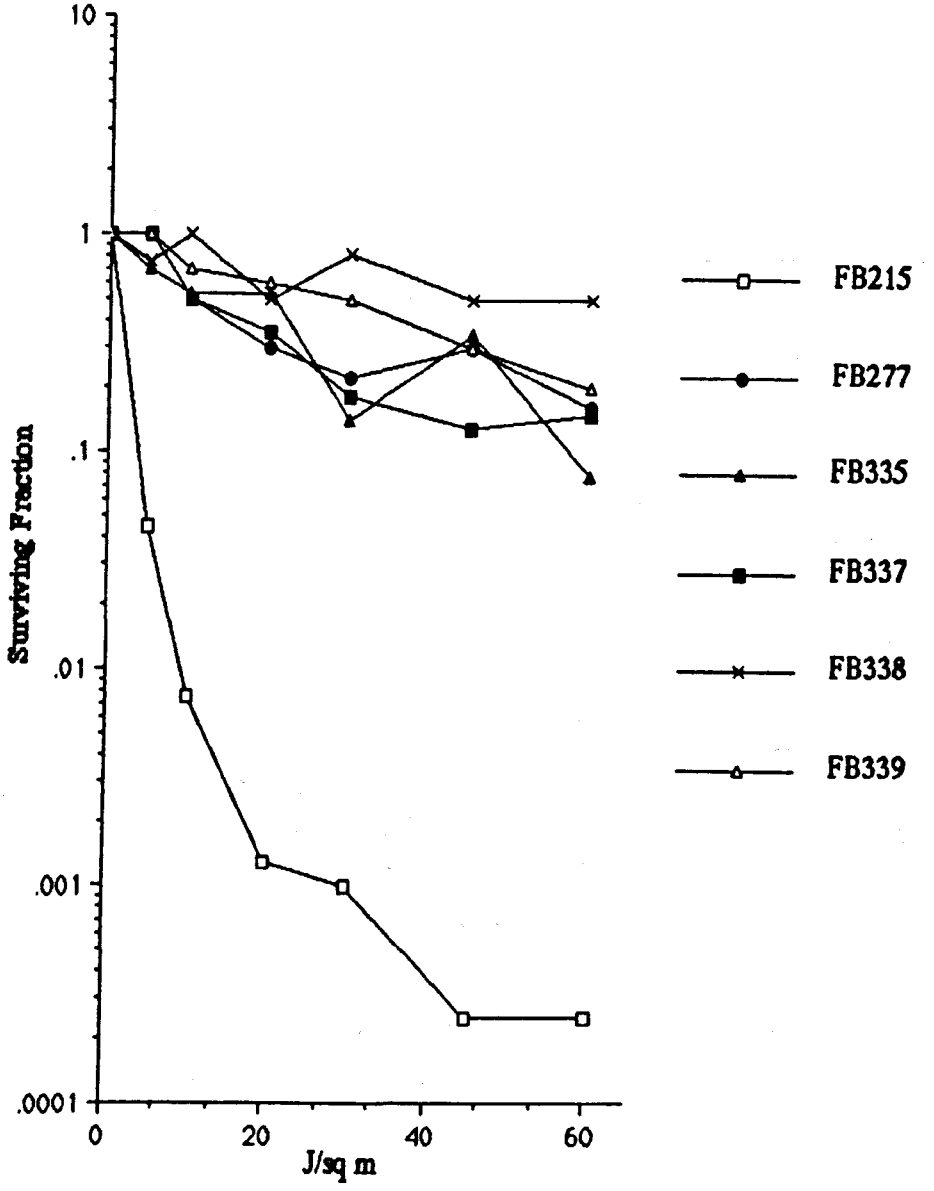


Fig. 4.12c

Complementation of the UV sensitivity of *ruv* strains with plasmid pFB501. Strains used were FB215 *ruv-53* pHSG415, FB277 *ruv-53* pPVA105, FB335 *ruv-52* pFB501, FB337 *ruv-54* pFB501, FB338 *ruv-58* pFB501, and FB339 *ruv-57* pFB501

Figure 4.13

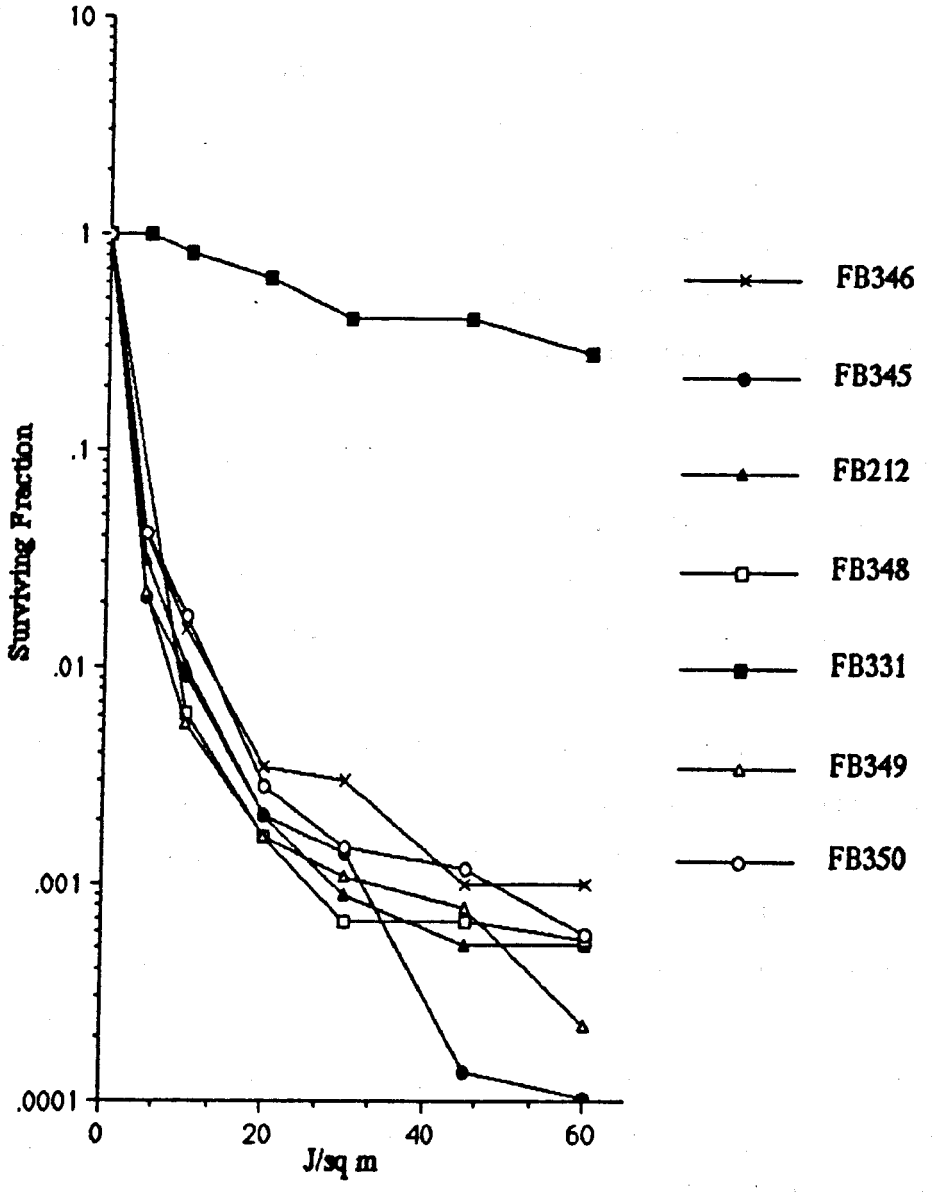


Fig. 4.13

Lack of complementation of the UV sensitivity of *ruv* strains by plasmid pFB502. Strains used were FB346 *ruvB9* pFB502, FB345 *ruvA4* pFB502, FB212 *ruvB9* pHSG415, FB348 *ruv-53* pFB502, FB331 *ruvB9* pFB501, FB349 *ruv-54* pFB502 and FB350 *ruv-57* pFB502

demonstrated by restriction analysis to have lost the 4kb KpnI fragment present in the pPVA101 parent plasmid. Data presented in Figure 4.13 show that pFB502 was unable to restore resistance to UV irradiation to a range of strains carrying ruv mutations, suggesting that pFB502 does not carry the intact ruv coding region.

This further limits the possible extent of the ruv coding region to the region overlapping KpnI site 1 extending to the BglII site.

In order to define more precisely the ruv$^+$ coding region, a series of pPVA101 derivatives with the Tn1000 insertion sequence ($\gamma\delta$) inserted in the ruv gene were obtained by the method of Guyer (1978).

Strain FB451 (F'lac$^+$) pPVA101 was used as the donor in a standard mating with an HI24 ruvA4 recipient. Transconjugants inheriting the Ap^R or Cm^R plasmid markers were selected and tested for the ability to complement the mitomycin sensitivity of the recipient ruv strain.

Despite screening approximately 2000 transconjugants, no isolates were detected (on the basis of mitomycin C sensitivity), with Tn1000 insertions in the ruv gene of the plasmids. Since the phenotype of ruv mutants varies in different genetic backgrounds it was possible that the presence of F'lac$^+$ masked the mitomycin sensitivity of ruv strains.

Results presented in Figure 4.14 suggested that this was probably not the case since strains carrying the ruv-52 or ruvA4 mutations were as sensitive to UV irradiation in the presence of F'lac$^+$ as in its absence.

Thus, this inability to recover ruv::Tn1000 insertions in an ruv recipient seemed to be genuine and suggested that the ruv$^+$ product may be necessary for the recovery of viable products from the transferred plasmid::Tn1000:: F-prime cointegrates.

The problem was circumvented by mating the FB451 donor with an

Figure 4.14

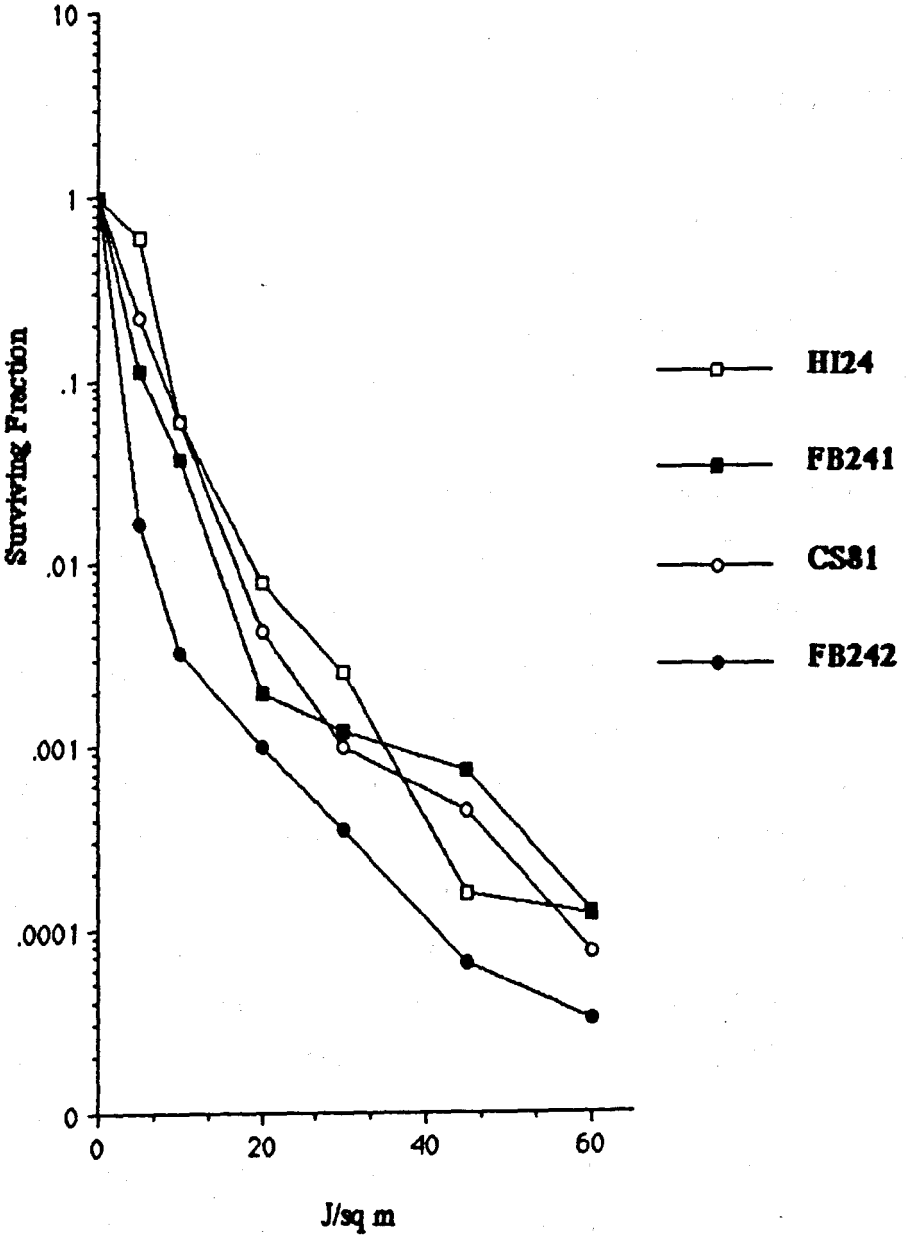


Fig. 4.14

UV sensitivity of ruv strains carrying F' lac$^+$. Strains used were HI24 ruvA4, FB241 ruvA4 F' lac$^+$, CS81 ruv-52 and FB242 ruv-52 F' lac$^+$

AB1157 *ruv*$^+$ recipient, selecting Ap^RCm^R transconjugants, preparing plasmid DNA from pooled transconjugants and transforming into strain HI24 *ruvA4* (Dr. P.V. Attfield). By this method, 12 different pPVA101 strain derivatives were obtained in which the insertion of the Tn*1000* element had abolished the ability of pPVA101 to restore UV resistance to *ruv* mutants. All 12 insertions were restriction mapped to a 2.2kb region spanning the *Kpn*I site 1 of pPVA101 and extending to the *Bgl*II site (Figure 4.15) (Dr. P.V. Attfield).

In order to identify the protein encoded by the *ruv* gene, the proteins encoded by pPVA101 and its deletion derivatives and pPVA101 *ruv*::Tn*1000* derivatives were selectively labelled using the maxicell system, separated on SDS-polyacrylamide gels which were then subjected to autoradiography.

The autoradiograph presented in Figure 4.16 shows that each of the plasmids pPVA101, pPVA105, pFB500 and pFB501 encode three proteins of approximately 41,000, 33,000 and 24,000 daltons respectively, in addition to the vector encoded Ap^R and CAT genes as appropriate. As expected, all three proteins are encoded by λ RL103. However, pFB502, which fails to complement the *ruv* defect, encoded only the 33,000 and 24,000 dalton proteins, suggesting that the 41,000 dalton protein was the product of the *ruv*$^+$ gene. This was substantiated by analysis of proteins encoded by pPVA101 *ruv*::Tn*1000* plasmids (Figure 4.17). In all cases, the 41,000 dalton protein was absent, confirming that this is the *ruv*$^+$ gene product. Unfortunately, no truncated proteins (which might have facilitated the determination of direction of transcription), were observed in the maxicell extracts of the pPVA101 *ruv*::Tn*1000* derivatives shown in Figure 4.17, or in the remaining 9 extracts analysed (detail not shown). Presumably any such products were unstable and rapidly degraded.

Figure4.15 Location of γδ insertions in pPVA101 that inactivate the ruv gene.

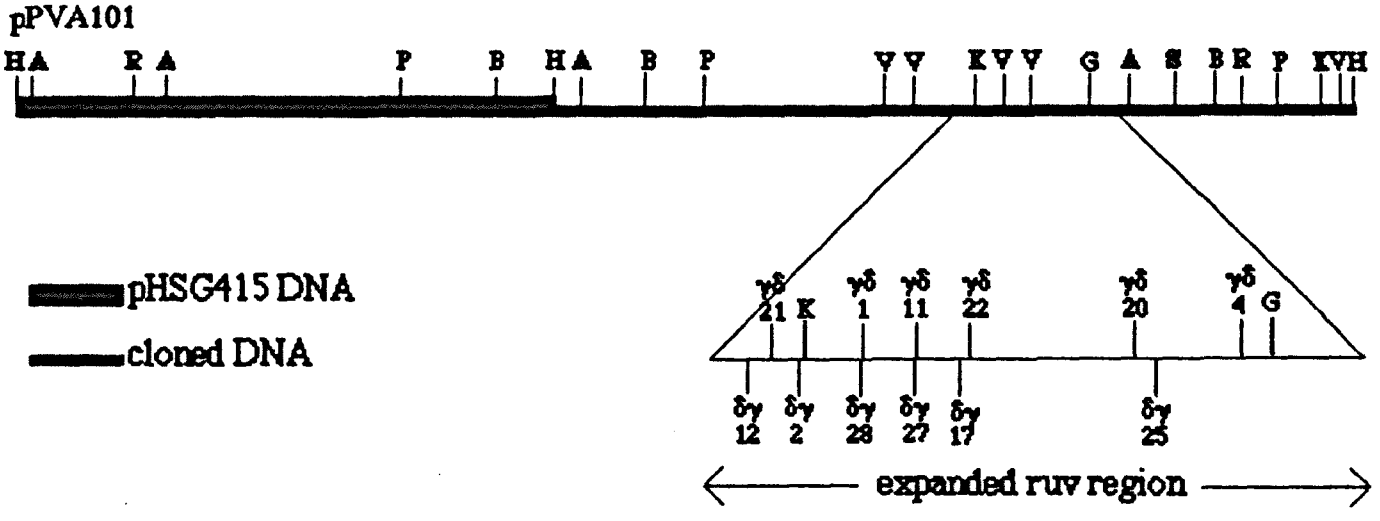


Figure 4.16

Fluorograph of plasmid encoded products of pPVA101 and its deletion derivatives, labelled in 'maxicells' with ^{35}S methionine, separated on a 15% polyacrylamide-SDS gel and visualised by fluorography. A.pHSG415, B.pPVA101, C.pPVA105, D.pFB500, E.pFB501, F.pFB502. Plasmid encoded proteins are arrowed, molecular weight markers were as in Fig. 4.2

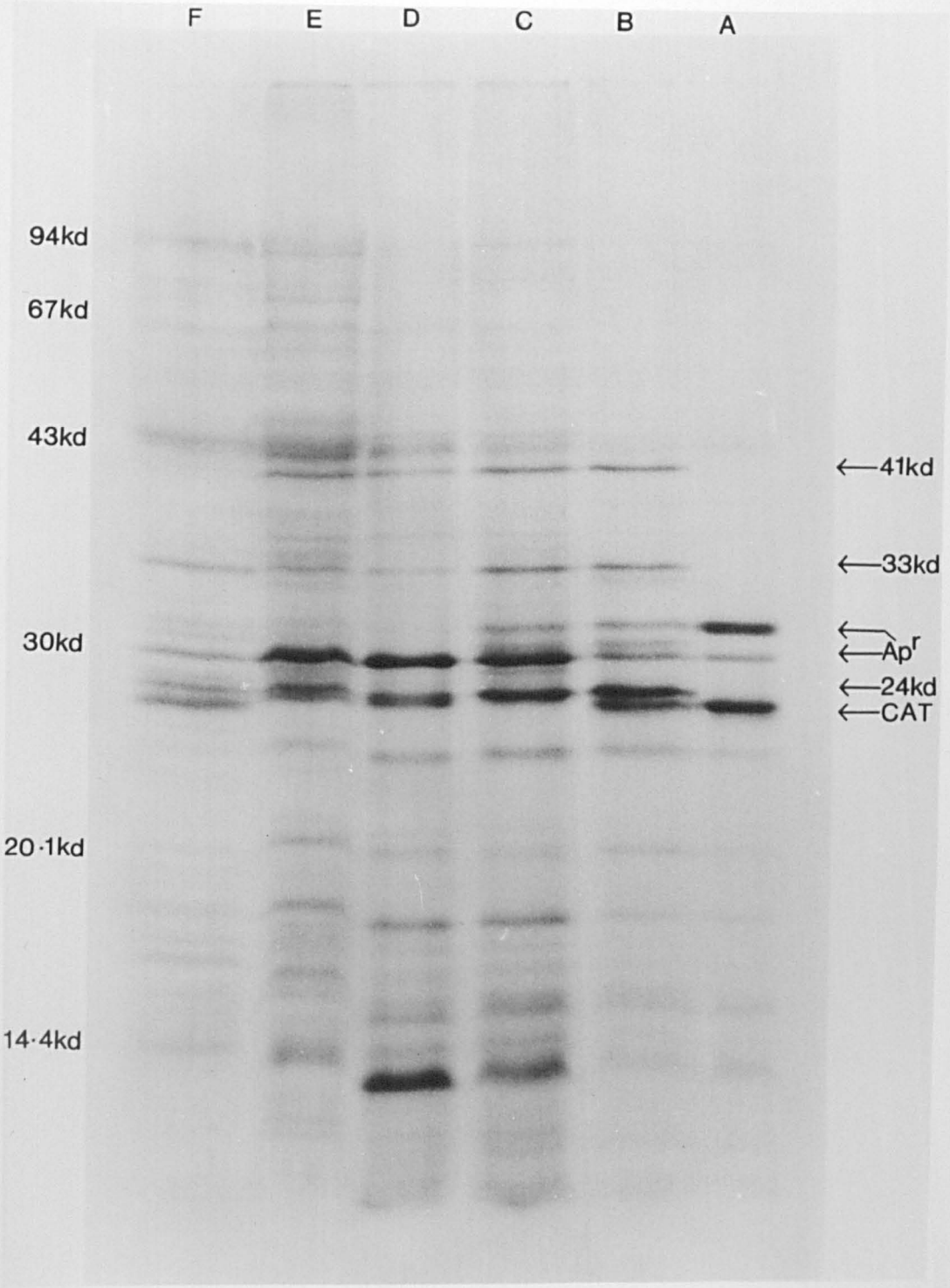
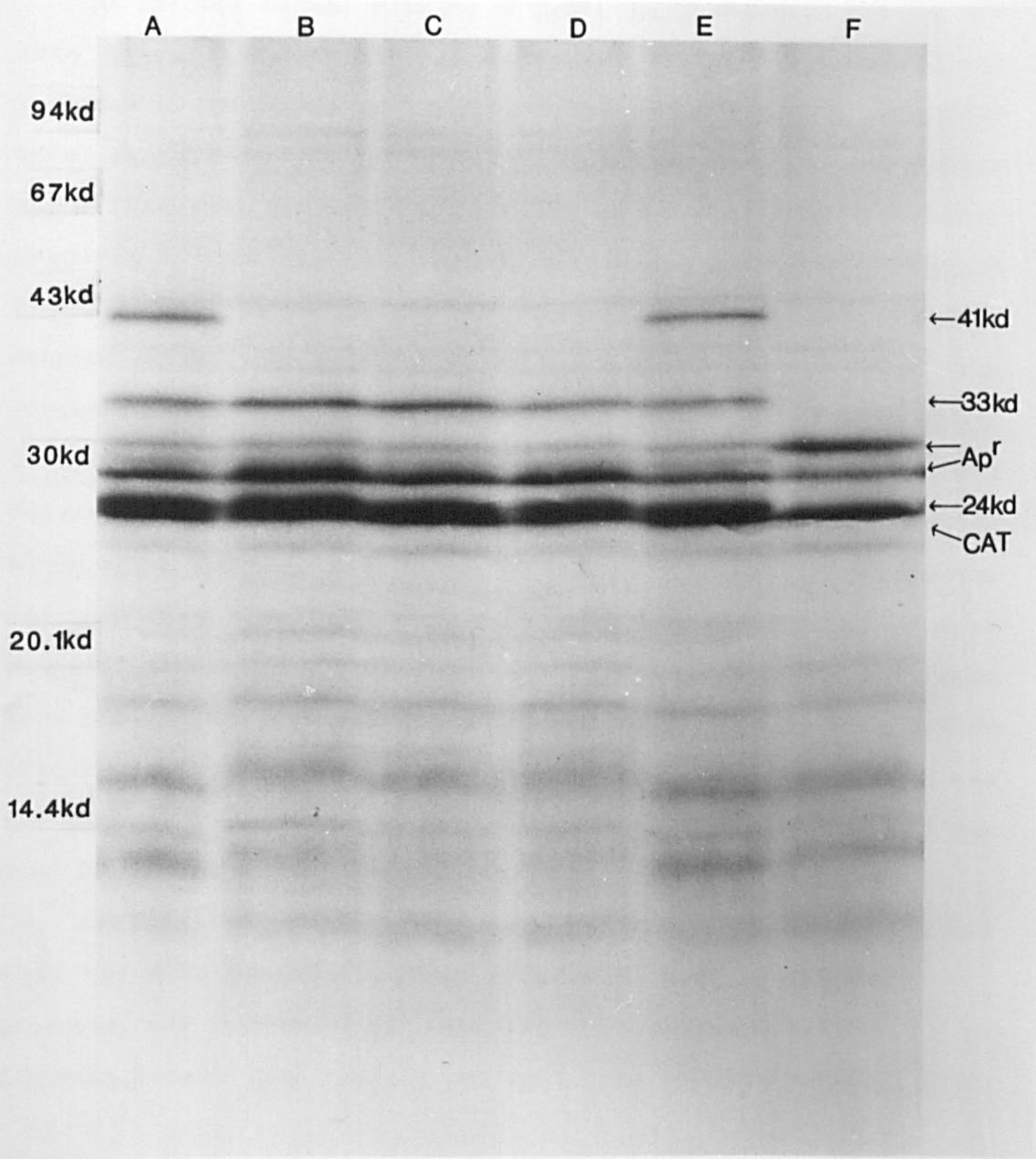

F
E
D
C
B
A
94kd
67kd
43kd
30kd
20·1kd
14·4kd
←41kd
←33kd
←Ap^r
←24kd
←CAT

Figure 4.17

Fluorograph of plasmid encoded products of pPVA101 *ruv*::Tn*1000* derivatives, labelled in maxicells with ^{35}S methionine, separated on a 15% polyacrylamide-SDS gel and visualised by fluorography. A.pPVA101, B.pPVA101::Tn*1000*.12, C.pPVA101::Tn*1000*.11, D.pPVA101::Tn*1000*.4, E.pPVA101, F.pHSG415. Plasmid encoded proteins are arrowed, molecular weight markers were as in Fig. 4.2


A
B
C
D
E
F
94kd
67kd
43kd
30kd
20.1kd
14.4kd
←41kd
←33kd
←Ap^r
←24kd
←CAT

4.4 Subcloning of the ruv region into multicopy plasmids

In order to facilitate further characterisation of the ruv nucleotide sequence and purification of the Ruv protein, an attempt to subclone the ruv^+ gene into high copy number plasmids was made.

An initial shotgun cloning of BamHI/BglII digested pPVA101 DNA into BamHI digested pBR322, selecting for Ap^R transformants, resulted in the production of the plasmids pFB503, pFB504 and pFB505 which contained the 2.4kb BamHI, the 1.7kb BamHI/BglII and the 8kb BamHI fragments respectively (Figure 4.18). The fourth plasmid, required to complete this series, containing the 6kb BamHI/BglII fragment which including the ruv^+ coding region, was not obtained, despite several attempts to isolate such a clone and evidence from Southern blots that such a ligation product was produced *in vitro*. This led to the conclusion that at least some of this region had a deleterious or lethal effect on transformed strains when present at high copy. Since the BamHI-HindIII fragment of pFB502 was successfully subcloned into both pACYC184 and pBR322 to give plasmids pFB507 and pFB511 respectively, and the KpnI-SalI fragment from pPVA101 was successfully subcloned into pUC18 to give pFB509 (Figure 4.18) it was concluded that the observed lethality of the 6kb BamHI-BglII fragment was probably due to overproduction of the 41Kd Ruv protein.

Analysis of proteins encoded by multicopy plasmids confirmed that the 41Kd protein was not encoded by any of the multicopy plasmids, and that the 2 Kd and 33Kd proteins were encoded by the fragment between BamHI site 1 and KpnI site 1(Figure 4.19).

4.5 Identification of the coding region for the 25Kd and 33Kd proteins

Coding regions for the 25Kd and 33Kd proteins were located by

Figure 4.18 Restriction maps of pPVA101 and its multicopy derivatives.

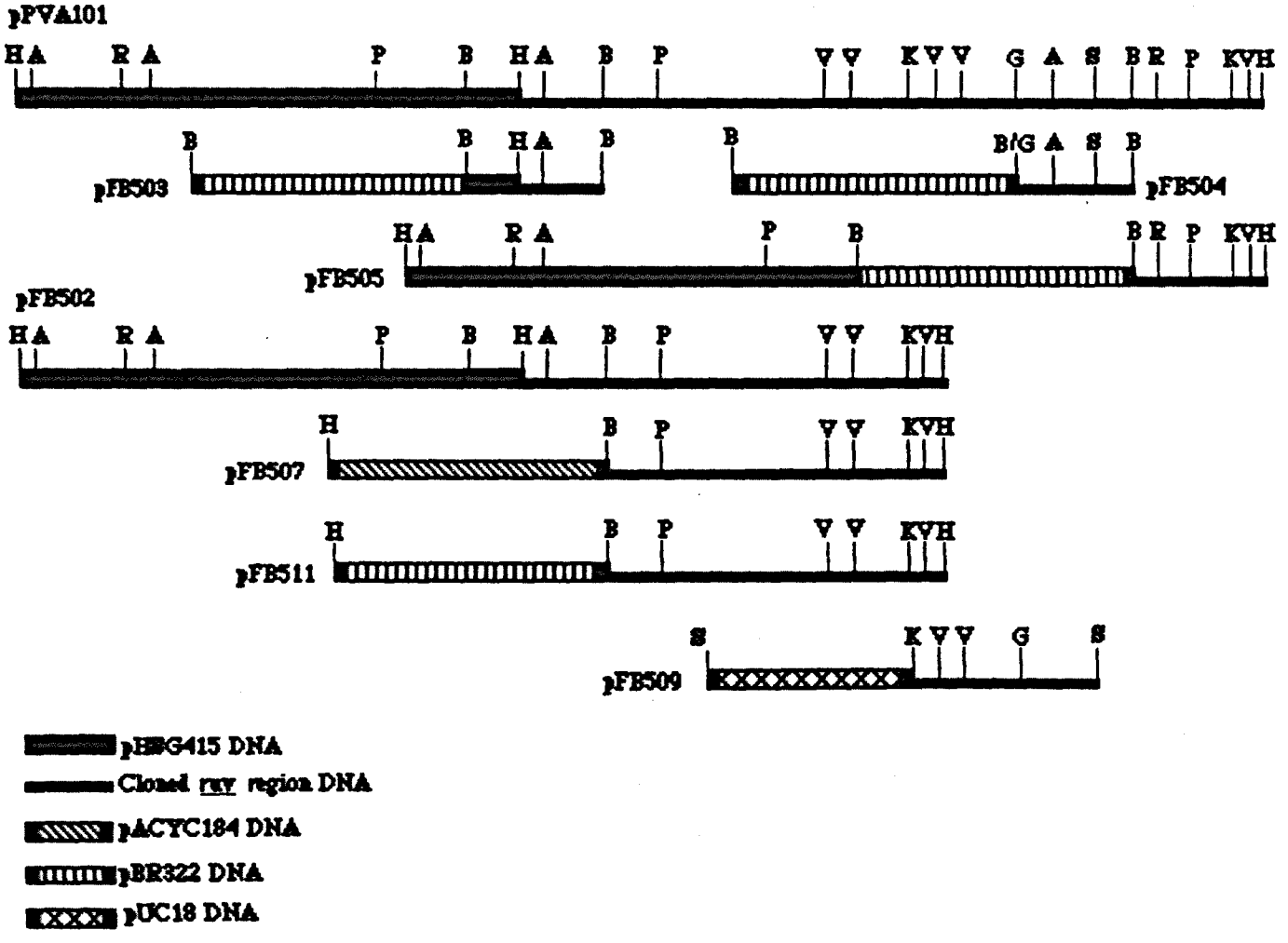
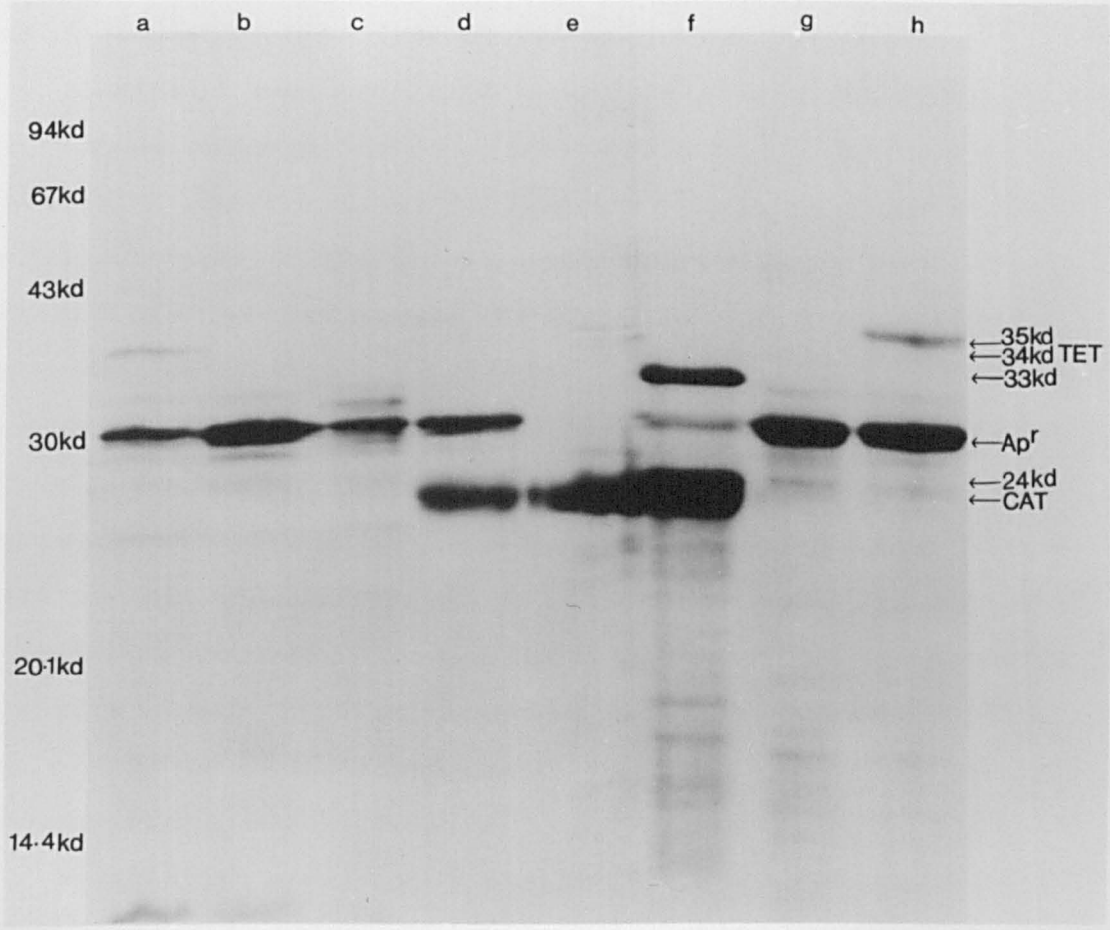


Figure 4.19

Fluorograph of multicopy plasmid encoded proteins, labelled in maxicells with ^{35}S methionine, separated on a 15% polyacrylamide-SDS gel, and visualised by fluorography. a.pBR322, b.pFB503, c.pFB504, d.pFB505, e.pACYC184, f.pFB507, g.pUC18, h.pFB509. Plasmid encoded proteins are arrowed, molecular weight markers were as in Fig. 4.2


a
b
c
d
e
f
g
h
94kd
67kd
43kd
30kd
20·1kd
14·4kd
←35kd
←34kd TET
←33kd
←Ap^r
←24kd
←CAT

Tn1000 insertion inactivation of the proteins (Guyer, 1978). Transconjugant colonies obtained from a mating between an FB414 F' lac$^+$ pFB507 donor and an AB1157 recipient, inheriting the plasmid encoded Cm^R gene were selected. Plasmid DNA was prepared from individual isolates and the site of any Tn1000 insertion mapped, using HindIII and BamHI double-digests, to either vector or insert DNA. Of 50 isolates screened, 23 had no Tn1000 insertions in either vector or insert DNA, 15 had Tn1000 insertions into the vector DNA and 11 had Tn1000 insertions in the insert DNA.

Maxicell extracts were prepared from AB2480 recA uvrA derivatives carrying those plasmids identified in which Tn1000 had integrated into the cloned DNA, and the labelled plasmid encoded proteins separated by SDS-PAGE. The autoradiograph presented in Figure 4.20 shows that amongst the pFB507::Tn1000 plasmids examined, only one, pFB507::Tn1000.26 had Tn1000 inserted in the coding region for the 33,000 dalton protein. It was impossible to identify any plasmids with Tn1000 inserted into the coding region for the 24,000 dalton protein because of the difficulty in distinguishing between this protein and the 25,000 dalton chloramphenicol acetyl transferase protein. To eliminate this problem, a series of plasmids with Tn1000 insertions were generated in plasmid pFB511 (which carries an identical insert to pFB507) selecting for Ap^R transconjugants in this case.

Restriction analysis of plasmid DNA from 30 such isolates revealed that 4 plasmids had no Tn1000 insertions, 15 had Tn1000 integrated in the insert DNA and 11 had Tn1000 inserted in the vector. The autoradiograph (presented in Figure 4.21) of labelled plasmid encoded proteins separated by SDS-PAGE revealed that two plasmids, pFB511::Tn1000.23 and pFB511::Tn1000.41 had Tn1000 inserted in the coding region for the 24,000 dalton protein and a third plasmid, pFB511::Tn1000.29 had a Tn1000 insertion in the coding region for the 33,000 dalton protein.

Figure 4.20

Fluorograph of plasmid encoded products of pFB507::Tn*1000* derivatives, labelled in maxicells with ^{35}S methionine, separated on a 15% polyacrylamide-SDS gel, and visualised by fluorography. A,B.pPVA101, C.pFB507, D.pFB507::Tn*1000*.25, E.pFB507::Tn*1000*.49, F.pFB507::Tn*1000*.56, G.pFB507::Tn*1000*.29, H.pFB507::Tn*1000*.77, I.pFB507::Tn*1000*.26, J.pFB507::Tn*1000*.25. Plasmid encoded proteins are arrowed, molecular weight markers were as in Fig. 4.2

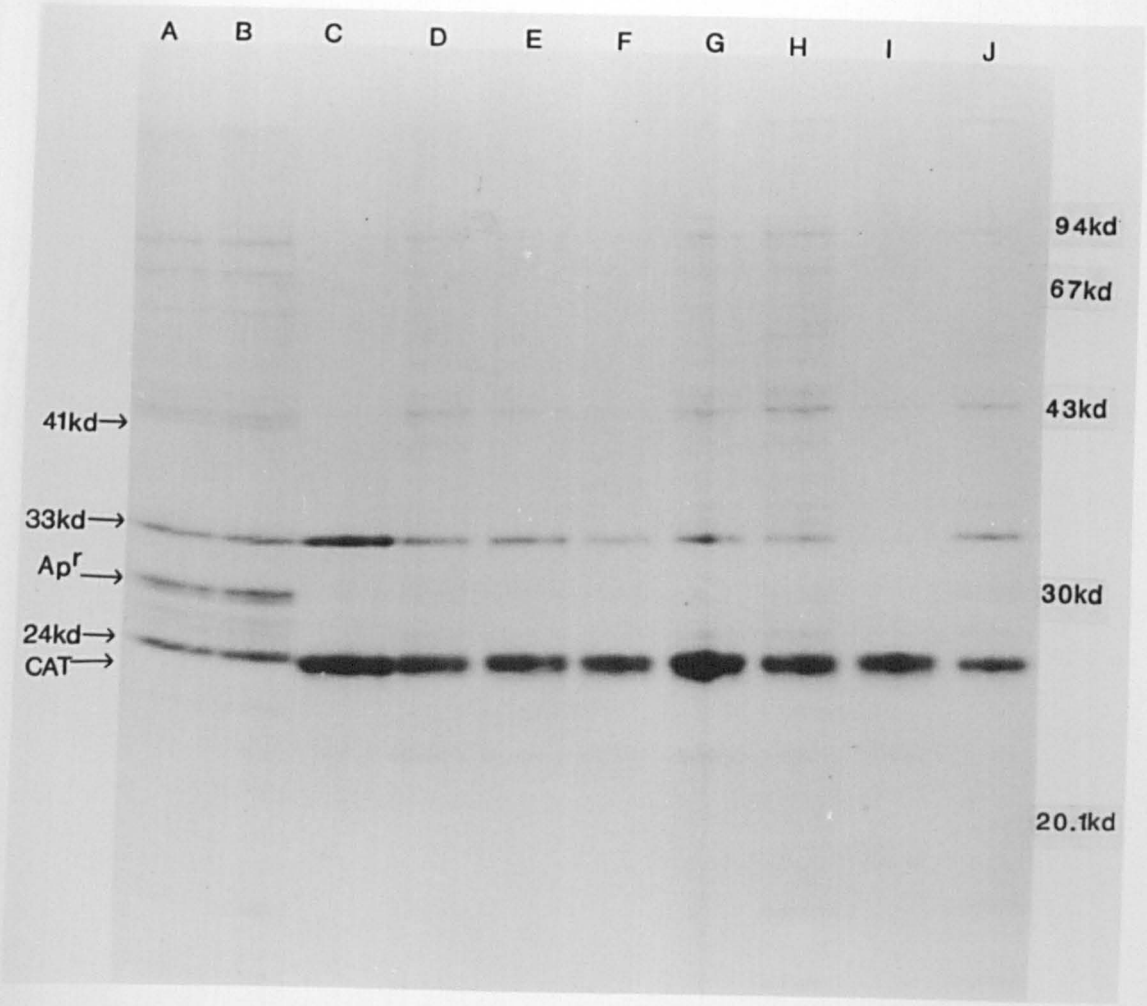
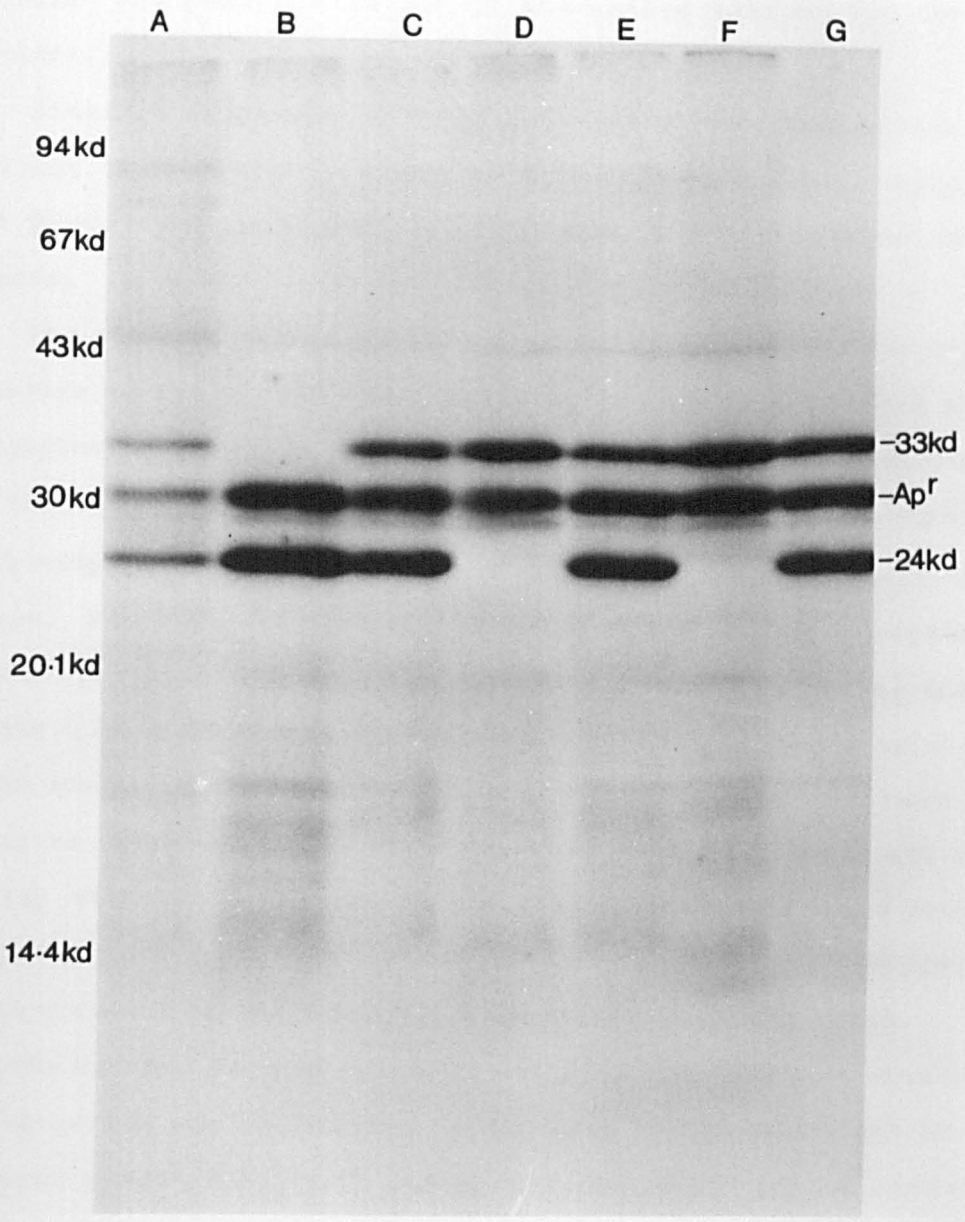

A
B
C
D
E
F
G
H
I
J
94kd
67kd
43kd
41kd→
33kd→
Ap^r→
30kd
24kd→
CAT→
20.1kd

Figure 4.21

Fluorograph of plasmid encoded products of pFB511::Tn1000 derivatives, labelled in maxicells with ^{35}S methionine, separated on a 15% polyacrylamide-SDS gel, and visualised by fluorography. A.pFB511, B.pFB511::Tn1000.29, C.pFB511::Tn1000.31, D.pFB511::Tn1000.41, E.pFB511::Tn1000.16, F.pFB511::Tn1000.23, G.pFB511::Tn1000.30. Plasmid encoded proteins are arrowed, molecular weight markers were as in Fig. 4.2.


A
B
C
D
E
F
G
94kd
67kd
43kd
30kd
20·1kd
14·4kd
–33kd
–Ap^r
–24kd

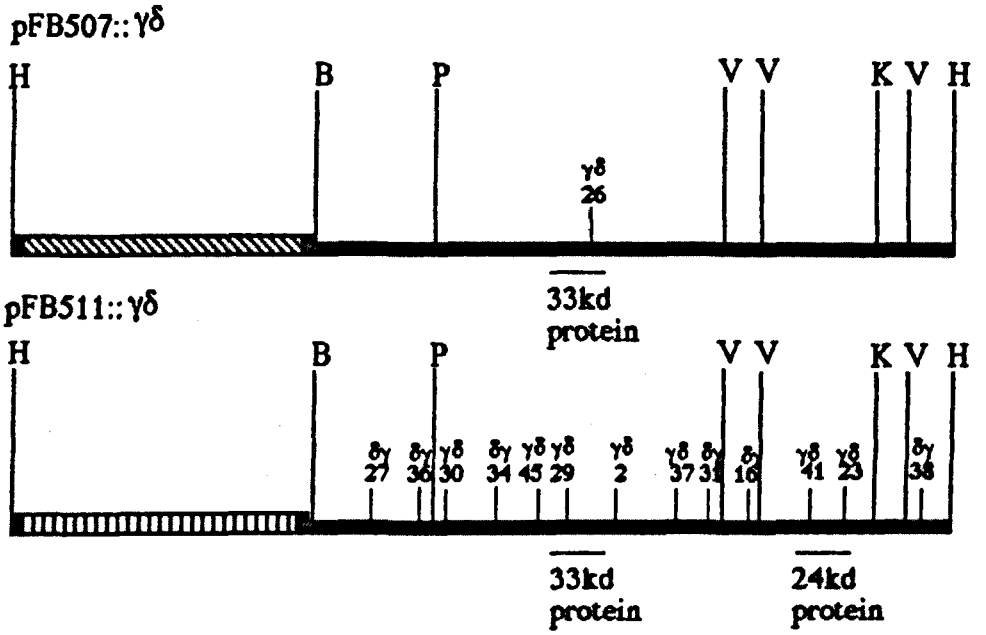
The coding regions for the 24kd and 33Kd proteins were approximately determined by mapping the sites of insertion of the Tn1000 elements in those plasmids in which Tn1000 insertion resulted in the loss of the plasmid encoded product. In addition, several insertion sites of Tn1000 within the insert DNA were mapped in order to limit the possible extent of the coding regions for the two proteins.

Sites of insertion of Tn1000 elements were determined from analysis of fragments generated by digestion of plasmid::Tn1000 DNA with HindIII and BamHI restriction enzymes in both single and double digests.

The sites of insertion and orientation determined for Tn1000 elements on the cloned DNA carried by pFB507 or pFB511 is shown schematically in Figure 4.22. These results place the coding region for the 33Kd protein in the centre of the cloned DNA approximately 2.5kb away from the extent of the region defined as the ruv coding region. However, the site of insertion of the Tn1000 elements in pFB511::Tn1000.23 and pFB511::Tn1000.41, places the region coding for the 2 Kd protein only 0.3kb from the ruv coding region which led to the suggestion that the two proteins could have related functions or may be cotranscribed. However, this possibility did not seem likely, since no insertions in the 24Kd protein were found amongst the pPVA101::Tn1000 derivatives selected on the basis of loss of complementation of the mitomycin C sensitivity of HI24 ruvA4.

The further possibility that the 33Kd and 24kd proteins were cotranscribed was eliminated on locating Tn1000 insertions in the plasmids pFB511::Tn1000.31 and pFB511::Tn1000.37 (which produced both proteins) to the region between the two identified coding regions.

Figure 4.22 Locations of γδ insertions in pFB507 and pFB511



The approximate locations of the coding regions for the 33kd and 24kd proteins are shown on the figure.

CHAPTER 5

Characterisation of the chromosomal *ruv* region

5.1 Introduction

In the previous chapter of this thesis, the cloning of the *ruv* gene, analysis of cloned DNA and identification of the encoded products were described. In order to investigate further the molecular organisation of the chromosomal *ruv* region, a restriction analysis of the chromosomal *ruv* region in *ruv*$^+$, *ruv*::Tn*10*, *ruv*::Mu*d*(Ap)^R*lac* and presumed *ruv* deletion strains was undertaken. More precisely, the aims of this investigation were:-

a) To determine the extent of contiguous DNA cloned in pPVA101 and map further restriction sites in the chromosomal *ruv* region outside the extents of the cloned fragment.

b) To map the sites and orientations of insertions in *ruv*::Tn*10* and *ruv*::Mu*d*(Ap)^R*lac* strains, to provide further confirmation that pPVA101 and its derivatives do in fact carry the *ruv* gene rather than a suppressor of the *ruv* mutant phenotype.

c) To determine the direction of transcription of the *ruv* gene on the plasmid pPVA101, by comparison with the orientation of the chromosomal *ruv*::Mu*d*(Ap)^R*lac* insertion in which β-galactosidase is expressed under control of the *ruv* promoter.

d) To determine the precise nature of the DNA rearrangements in the *ruv*-57 and *ruv*-58 mutants.

5.2 Restriction mapping of the chromosomal *ruv*$^+$region

Chromosomal DNA from the wild type strain W3110 was digested with appropriate restriction endonucleases, fragments separated by

electrophoresis on 1% agarose gels, and blotted onto nitrocellulose filters. Filters were then probed overnight with nick-translated ^{32}P labelled probes derived from the insert of pPVA101 and filters exposed to autoradiographic film.

The first observation made was that the 10.4kb HindIII insert of pPVA101 did not hybridise to a fragment of 10.4kb in HindIII digested W3110 DNA, but rather to a much larger fragment of approximately 15-20kb (Figure 5.1), suggesting that the HindIII restriction sites flanking the cloned fragment in pPVA101 were not both present in the chromosomal region, and therefore that the 10.4kb HindIII fragment cloned in pPVA101 was not present as a contiguous fragment in the chromosome.

In order to determine the extent of contiguity in the cloned fragment, further restriction digests of strain W3110 DNA were probed with the cloned 10.4kb HindIII fragment from pPVA101.

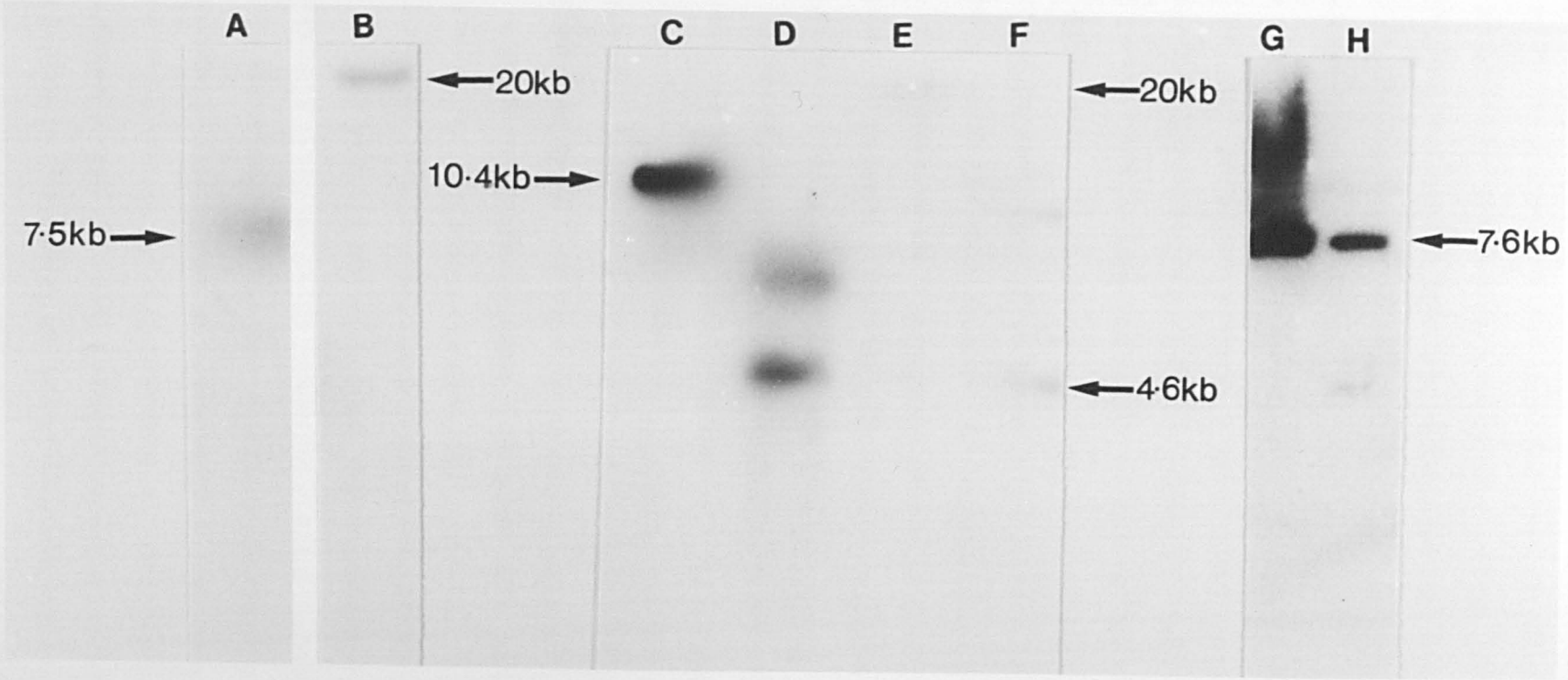
The autoradiograph presented in Figure 5.1 shows that the 10.4kb HindIII fragment hybridised to a band of approximately 20kb in BamHI digested W3110 DNA rather than to a band of 7.5kb which would be expected if the DNA between the BamHI sites in the clone was contiguous in the chromosome, and which can be seen in the BamHI digested plasmid DNA. Thus the 7.5kb BamHI fragment of pPVA101 is not contiguous in the chromosome.

The extent of the DNA cloned in pPVA101, contiguous with ruv in the chromosome was more precisely defined by analysis of hybridisation of the cloned ruv region to KpnI and PstI digested chromosomal DNA. The autoradiograph presented in Figure 5.1 shows that the 10.4Kb HindIII probe hybridised to a 4.6Kb fragment in KpnI digested DNA from strain W3110, corresponding to the fragment between KpnI sites 1 and 2 in pPVA101, and to a 7.6Kb fragment in PstI digested W3110 DNA, corresponding to the fragment between the PstI sites in the pPVA101 insert DNA.

From this it was concluded that the region of chromosomal

Figure 5.1

Autoradiograph of restriction digested W3110 DNA probed with the 10.4kb HindIII fragment of pPVA101. Lanes A, C, D and G, contained pPVA101 DNA; lanes B, E, F and H contained W3110 DNA. DNA in lanes A and B was digested with BamHI; in lanes C and E with HindIII; in lanes D and F with HindIII and KpnI; and in lanes G and H with PstI. Hybridisation bands discussed in the text are arrowed.


A
B
C
D
E
F
G
H
7·5kb
20kb
10·4kb
20kb
4·6kb
7·6kb

contiguity in the cloned DNA fragment extended 8.1kb from PstI site 1 to KpnI site 2 of the insert. The region of cloned DNA known to be contiguous in the chromosome was extended when it was found that the 10.4Kb HindIII fragment hybridised to a 3.5Kb fragment in HindIII/BglII double digested DNA from strain W3110, corresponding to the 3.5Kb fragment between the BglII site and HindIII site 2 of pPVA101. However, the origin of the DNA between HindIII site 1 and PstI site 1 in pPVA101 remained unknown. Since pPVA101 was obtained as an ruv$^+$ subclone from a λ ruv$^+$ phage generated by ligating a partial Sau3A digest of chromosomal DNA into the BamHI sites of the vector λPE11, the end point of the contiguous region must be at a Sau3A site. From the above observations, this must lie either somewhere between PstI site 1 and BamHI site 1, or even at the BamHI site 1, although clearly there is no BamHI site present at this position in chromsomal DNA. A BamHI site could have arisen at this position in the plasmid either by (i) ligating a Sau3A fragment into the BamHI sites of the vector such that a BamHI site was regenerated, in which case, the HindIII - BamHI region of pPVA101 DNA would be derived from the λPE11 vector, or (ii) ligating non-contiguous fragments of Sau3A digested E. coli chromosomal DNA, either to produce a BamHI site at the ligated junction or, at a Sau3A site between the BamHI and PstI sites of the plasmid, with no generation of a BamHI restriction site, the BamHI site and HindIII site 1 being present on the non-contiguous DNA fragment.

A comparison of the restriction fragments produced from digestion of λVI and pPVA101 DNA with AvaI, BamHI and HindIII ruled out the possibility that pPVA101 contained some λ DNA as was proposed in (i). Unfortunately, it was not possible to distinguish between the options in (ii) since no restriction sites between PstI and BamHI were detected in either plasmid or chromosomal DNA. However, in either case, it was perhaps surprising that DNA fragments hybridising to the region of the cloned DNA not contiguous

with ruv in the chromosome were not detected.

Further restriction sites present in the chromosome were approximately mapped by probing appropriately digested DNA with the 7.6Kb PstI fragment from pPVA101 which is known to be contiguous in the chromosome.

Results of these mapping studies are presented in Figure 5.2.

5.3 Mapping of ruv::Mud(Ap)^{R}lac insertions

Sites of insertion of Mud(Ap)^{R}lac in ruv in the strains CSM7, in which β-galactosidase is expressed under control of the ruv promoter, and CSM13, in which there is no β-galactosidase expression (Shurvinton & Lloyd 1982) were determined by probing PstI single and PstI/BglII double digested DNA from these strains with ^{32}P labelled 7.6kb PstI fragment from pPVA101. Restriction sites and relevant genetic markers of Mud(Ap)^{R}lac are shown in Fig. 5.4 (O'Connor and Malamy 1983).

The autoradiograph presented in Figure 5.3 shows the probe hybridised to two bands, of approximately 4.5kb and 13kb in PstI digested DNA from strain CSM7, and of 5.8kb and 12kb in PstI digested DNA from strain CSM13, indicating that the Mud(Ap)^{R}lac element had inserted within the 7.6kb PstI fragment present in wild type DNA in both cases. From the known restriction sites of Mud(Ap)^{R}lac, two possible sites of insertion can be postulated for each strain, one in each of the two possible orientations.

These possibilities were distinguished between by analysis of the probe hybridisation to PstI/BglII double digested DNA from each strain. The 7.6kb PstI probe hybridised to fragments of 2.2kb and (approximately) 12kb in PstI/BglII digested DNA from strain CSM7, in addition to the 2.1kb fragment also observed in PstI/BglII digested DNA from strain W3110, confirming the site of the Mud(Ap)^{R}lac insertion in strain CSM7 to be between KpnI site 1 and the BglII

Figure 5.2 Location of restriction sites in chromosomal ruv region outside cloned DNA.

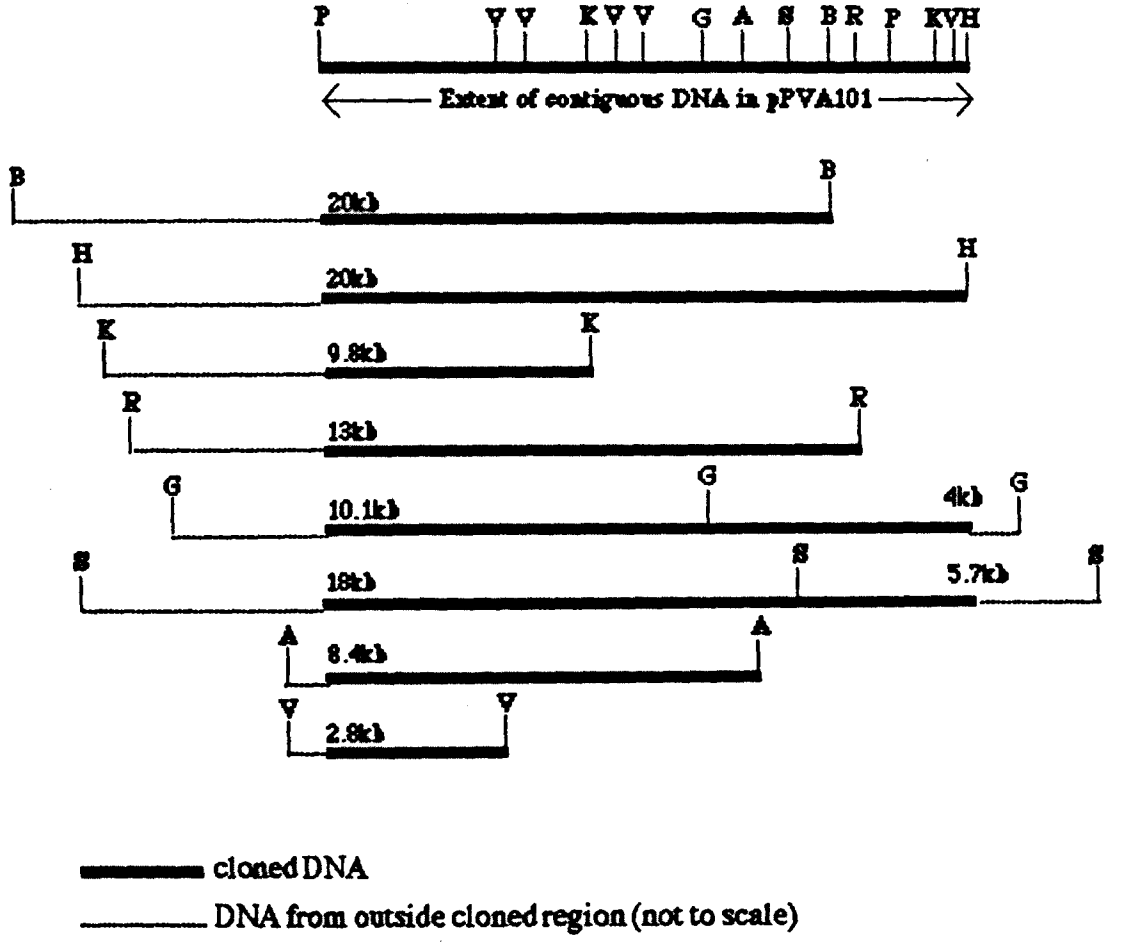
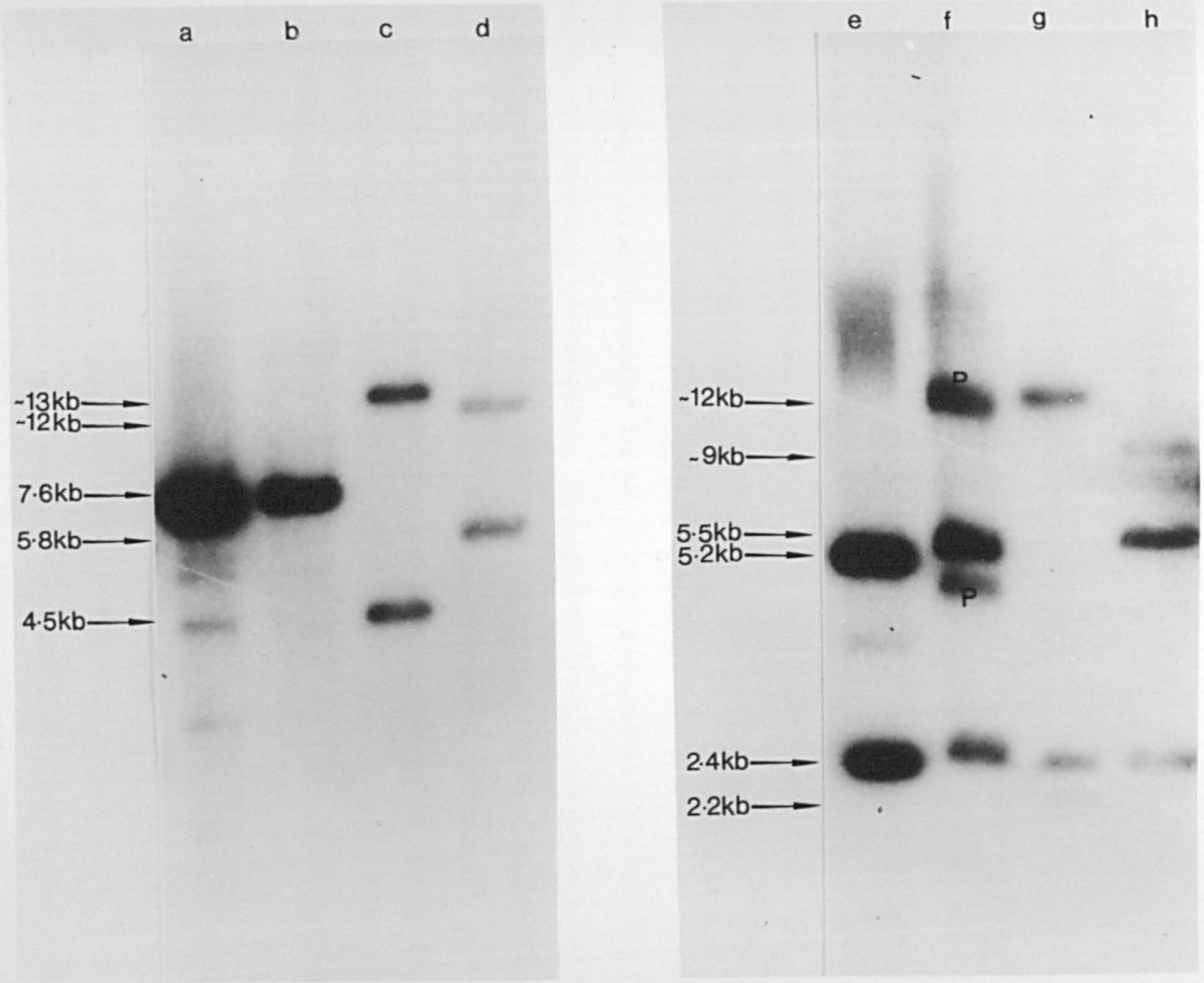


Figure 5.3

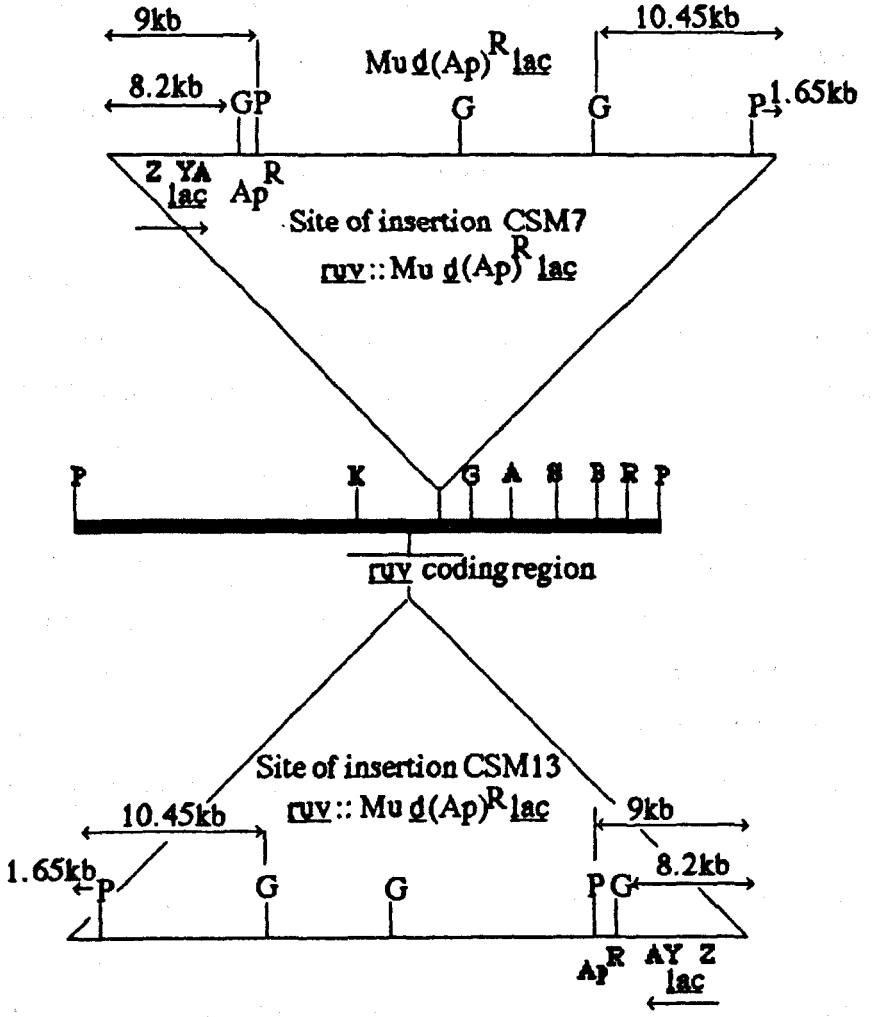
Autoradiograph of restriction digested DNA from strains CSM7 and CSM13 probed with the 7.6kb PstI fragment from pPVA101. Lanes a and e contained pPVA101 DNA; lanes b and f contained W3110 DNA; lanes c and g contained CSM7 DNA; and lanes d and h contained CSM13 DNA. DNA in lanes a-d was digested with PstI; and in lanes e-h was digested with PstI and BglII. Hybridisation bands discussed in the text are arrowed. Bands labelled P are presumed to result from partial digestion of the chromosomal DNA.


a
b
c
d
~13kb
~12kb
7·6kb
5·8kb
4·5kb
e
f
g
h
~12kb
~9kb
5·5kb
5·2kb
2·4kb
2·2kb
P
P

site, approximately 0.55kb from the BglII site (Figure 5.4). The observed hybridisation fragment of 2.2kb was derived from 1.65kb of Mud(Ap)^{R}lac DNA (between the end of the element and the first PstI site) plus approximately 0.55kb of DNA arising from the chromosomal region between KpnI site 1 and the BglII site immediately adjacent to the BglII site. The observed hybridisation fragment of approximately 12kb was derived from 8.2kb Mud(Ap)^{R}lac DNA (between the end of the element and the first BglII site) plus approximately 4kb of DNA arising from the chromosomal region between PstI site 1 and the BglII site, immediately adjacent to the PstI site. In this orientation transcription and translation are under the control of DNA sequences to the left of the insertion. Since β-galactosidase is expressed from the ruv promoter in strain CSM7, transcription of ruv must therefore be from left to right as drawn in the plasmid.

The probe hybridised to fragments of approximately 5.5 kb and 9kb in PstI/BglII digested DNA from strain CSM13 in addition to the 2.4kb fragment also observed in wild type DNA, thus confirming the site of Mud(Ap)^{R}lac insertion in strain CSM13 at a position 3.9kb to the right of PstI site 1 (Figure 5.4). The hybridisation fragment of approximately 5.5kb was derived from 1.65kb of Mud(Ap)^{R}lac DNA (between the end of the element and the first PstI site) plus approximately 3.9kb of DNA arising from the region between PstI site 1 and the BglII site, immediately adjacent to PstI site 1. The hybridisation fragment of approximately 9kb was derived from 8.2kb of Mud(Ap)^{R}lac DNA (between the end of the element and the first BglII site) plus approximately 0.8kb of DNA derived from the region between PstI site 1 and the BglII site, immediately adjacent to the BglII site. As expected, the Mud(Ap)^{R}lac insertion in strain CSM13 (in which the β-galactosidase is not expressed) is in the opposite orientation to that in strain CSM7. In both cases the Mud(Ap)^{R}lac insertions are within the DNA previously identified by Tn1000 insertion mutagenesis as the ruv coding region.

Figure 5.4 Restriction map, genetic organisation and sites of insertion of ruv :: Mu d(Ap)^R lac fusions.



——— Mu d(Ap)^R lac DNA (not to scale)

■■■■ cloned DNA

The ruv coding region, identified by mapping of γδ insertions is shown.
The Ap^R gene and the direction of transcription of the lac genes are also indicated.

5.4 Mapping of Tn10 insertions in ruv::Tn10 strains

The site of insertion of Tn10 in strains N2057 ruv60::Tn10 and N2058 ruv59::Tn10 was determined by probing appropriately digested DNA from these strains with the ^{32}P-labelled 7.6kb PstI fragment from pPVA101, previously demonstrated to be contiguous in wild type DNA. Relevant restriction sites in Tn10 DNA are diagrammed in Figure 5.6 The autoradiograph presented in the Figure 5.5 shows that the probe hybridised to a fragment of approximately 17kb in PstI digested N2057 DNA, corresponding to the 7.6kb PstI fragment plus 9.3kb of Tn10 DNA, which itself lacks PstI sites (Way et al. 1984). Identical results were obtained with N2058 DNA, confirming that Tn10 insertions in strains N2057 and N2058 were both within the 7.6kb PstI fragment present in wild type DNA.

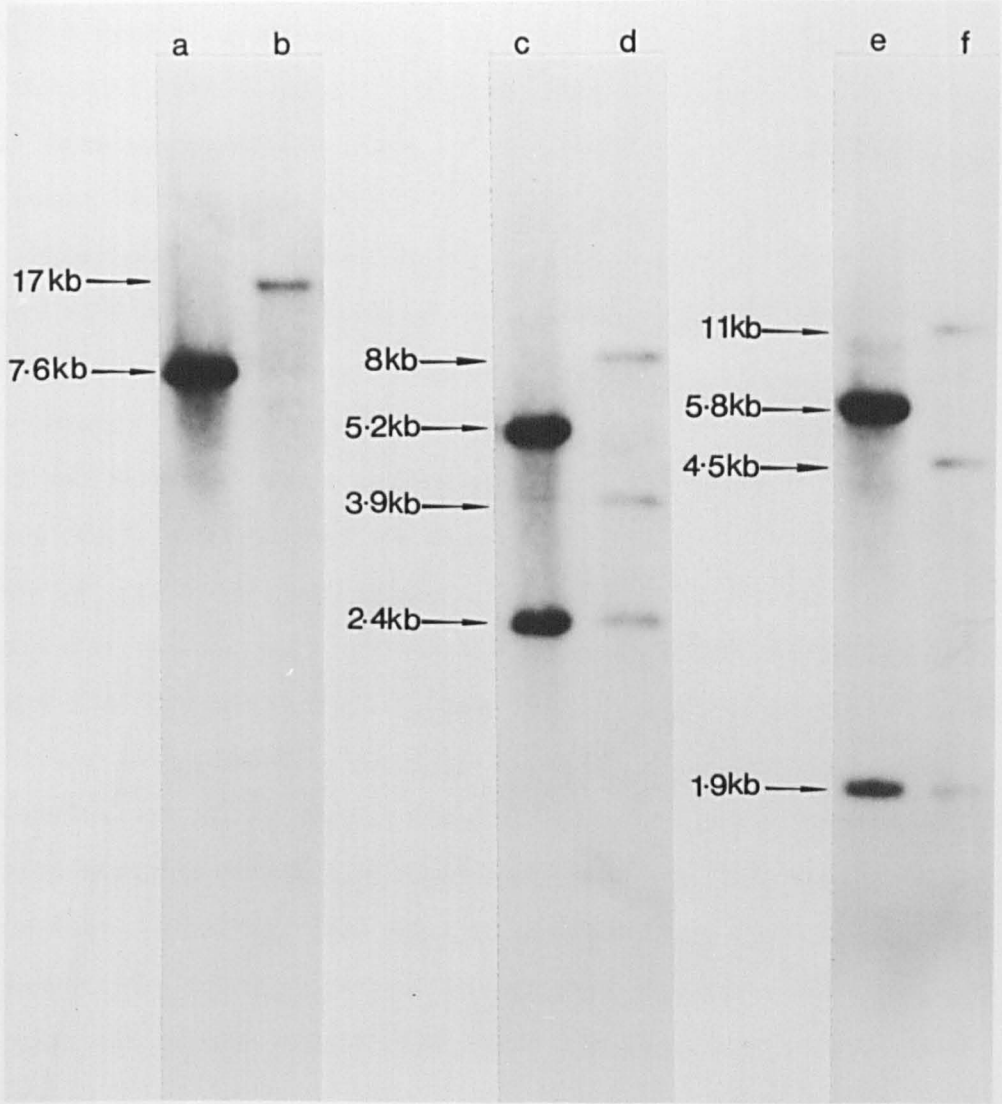
The probe hybridised to fragments of approximately 3.9kb and 8Kb in PstI/BglII digested DNA from strain N2057, in addition to the 2.4kb fragment also observed in PstI/BglII digested DNA from strain W3110, demonstrating that the Tn10 insertion in N2057 was not within the 2.4kb fragment between the BglII site and PstI site 2, but was located within the 5.2kb region between PstI site 1 and the BglII site. Identical results were obtained with strain N2058 DNA which suggested that the sites of Tn10 insertion in strains N2057 and N2058 may be identical.

From known restriction maps of Tn10 and the wild type ruv region, two possible sites of insertion could be postulated to account for the observed hybridisation fragments, one with each possible Tn10 orientation (Figure 5.6).

These possibilities were distinguished by analysis of the fragments obtained by hybridisation of the 7.6kb PstI probe to PstI/AvaI digested DNA from strains N2057 and N2058. In both cases the probe hybridised to bands of approximately 11kb and 4.5kb, (in addition to the 1.9kb band corresponding to the fragment between

Figure 5.5

Autoradiograph of restriction digested N2057 DNA probed with the 7.6kb *Pst*I fragment from pPVA101. Lanes, a, c and e contained pPVA101 DNA; lanes b, d and f contained strain N2057 DNA. DNA in lanes a and b was digested with *Pst*I; in lanes c an d with *Pst*I and *Bgl*II; and in lanes e and f with *Pst*I and *Ava*I. Hybridisation bands discussed in the text are arrowed.

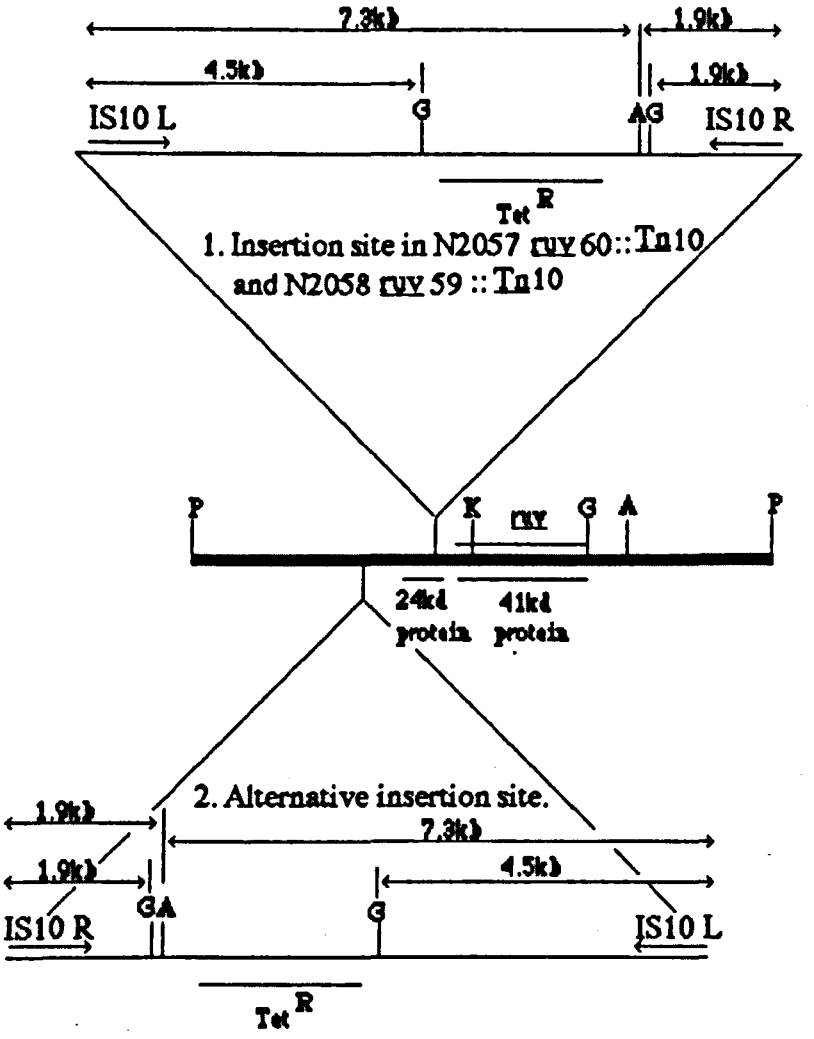

a
b
c
d
e
f
17kb
7·6kb
8kb
5·2kb
3·9kb
2·4kb
11kb
5·8kb
4·5kb
1·9kb

AvaI site 2 and PstI site 2, also seen in PstI/AvaI digested DNA from strain W3110, once again suggesting that the Tn10 insertions in these strains were identical. This also confirmed that the site of Tn10 insertion in both N2057 and N2058 was between the PstI site 1 and the BglII site at a position 2.3kb from the BglII site, the observed hybridisation fragment of 4.5kb being derived from 1.7Kb of Tn10 DNA (from the right end to the AvaI site) plus 3.4kb of DNA from the region immediately to the left of AvaI site 2, that of 11kb was derived from 7.5Kb of Tn10 DNA (from the left end to the AvaI site) plus approximately 3.5Kb of DNA from the region immediately to the right of PstI site 1.

Thus, the Tn10 insertions in both strains N2057 and N2058 were mapped to a position approximately 0.5Kb to the left of KpnI site (Figure 5.6). This was somewhat surprising, firstly since results from genetic analysis indicated that the Tn10 insertion in strain N2058 was upstream from the site of the Mud(Ap)^{R}lac insertion in strain CSM7, whilst that in strain N2057 was downstream from it (Lloyd et al. 1984), and secondly, the site of insertion in both strains was outside that previously identified as the coding region for the 41kd ruv gene product, and was in fact closer to the region identified as coding for the 25kd protein. Although limitations on the estimations of molecular weights from autoradiographs of probed Southern blots preclude the conclusion that the two Tn10 insertions are in fact identical, the mapping studies described unambiguously demonstrated that Tn10 insertions in both strains N2057 and N2058 were upstream of the Mud(Ap)^{R}lac insertion mapped in strain CSM7.

Mapping of the Tn10 insertions to the region upstream of the coding region of the 41kd Ruv protein, probably within the coding region for the 25kd protein, suggested that either the absence of the 25kd protein resulted in a phenotype similar to that observed in ruv mutants independently of production of the 41kd protein, or that the 25kd and 41kd proteins were cotranscribed, the Tn10 insertion in

Figure 5.6 Location of Tn10 insertions in N2057 ruv60::Tn10 and N2058 ruv59::Tn10



cloned DNA

Tn10 DNA (not to scale)

The coding regions for the 24kd and 41kd proteins, identified by Tn1000 insertions are shown. The IS10 (L and R) sequences and Tc^R gene of Tn10 are alsoindicated.

the coding region of the 25kd protein exerting a polar effect on transcription of the 41kd protein. Since plasmids carrying only the 25kd protein fail to complement the MC sensitivity of either N2057 or N2058, the first possibility was discounted, leaving the possibility that the products of the 25kd and 41kd proteins are cotranscribed. Further evidence for the cotranscription of the 25kd and 41kd protein is presented later in this thesis (Chapters 6 and 8). On the basis of these results and in view of the fact that the genetic analysis may have been confounded by transposon mediated rearrangements the earlier map location of *ruv-60::Tn10* was corrected to the same location as *ruv59::Tn10*.

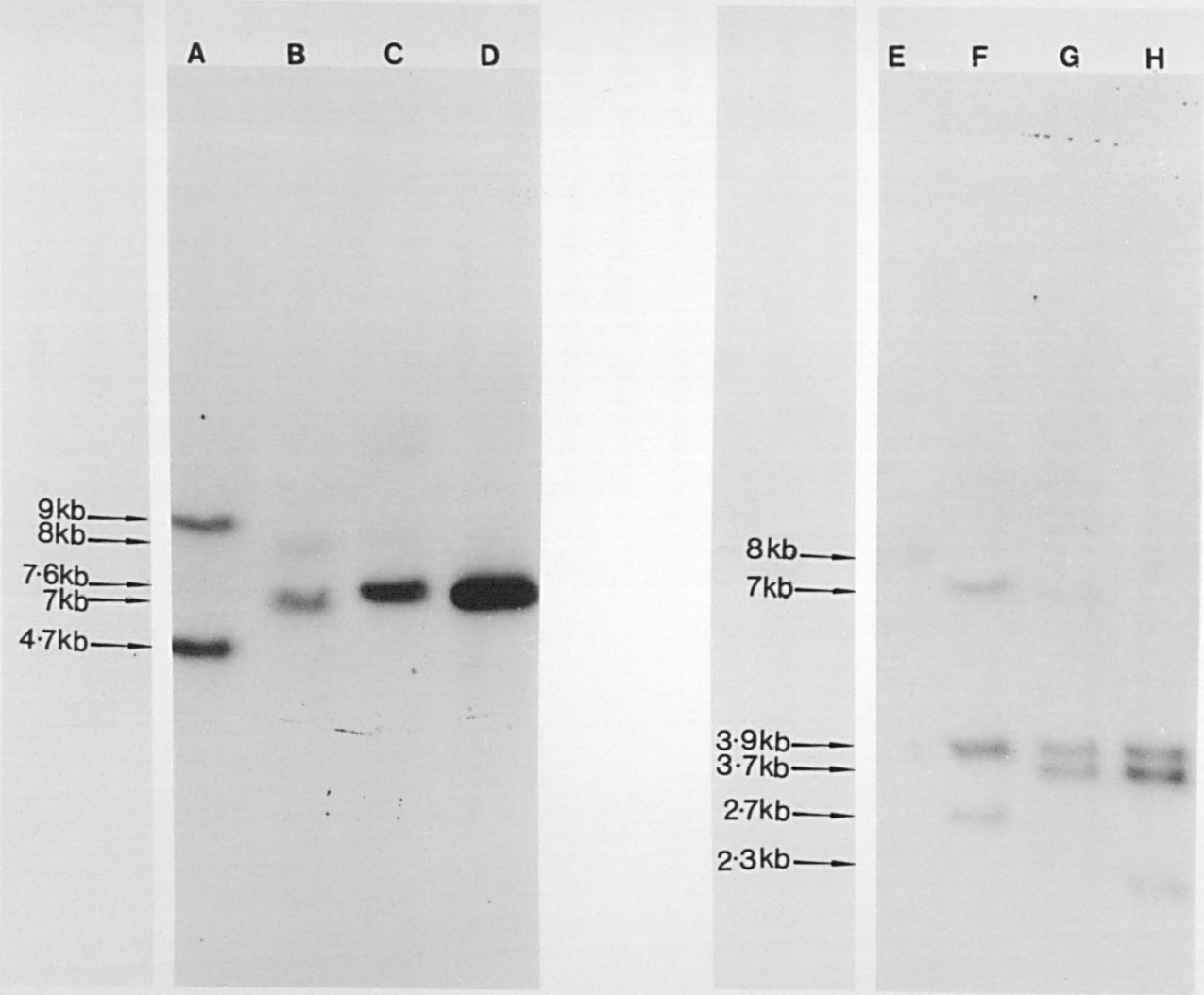
5.5 Mapping of chromosomal rearrangements in CS114 and CS115

Strains CS114 and CS115 were isolated as tetracycline sensitive derivatives of strain CS42 *ruv*$^{+}$*eda::Tn10*, which in addition to losing tetracycline resistance, had become sensitive to UV irradiation and mitomycin C. These strains harbour the only stable *ruv* mutations obtained from 2000 Tc^{S} colonies screened. Both strains were assumed to have simple deletions extending from *eda* into the *ruv* gene, although it had proved impossible to map the extent of the presumed deletions since in transductional crosses using P1 grown on strains carrying different *ruv* point mutations and strains CS114 and CS115 as recipients, *ruv*$^{+}$ recombinants were always obtained, suggesting that the deletions did not extend very far into *ruv*.

The nature of the chromosomal rearrangements was determined by probing Southern blots of restriction digested DNA from strains CS114 and CS115 with ^{32}P labelled fragments derived from pPVA101. The autoradiograph presented in Figure 5.7 shows that the 7.6kb *PstI* fragment hybridised to two bands - of approximately 7Kb and 8Kb in CS114 DNA and of approximately 4.7 and 9Kb in DNA from strain

Figure 5.7

Autoradiograph of restriction digested CS114 and CS115 DNA probed with the 7.6kb PstI fragment of pPVA101. Lanes A and E contained strain CS115 DNA; lanes B and F contained CS115 DNA; lanes C and G contained strain W3110 DNA and lanes D and H contained pPVA101 DNA. DNA in lanes A-D was digested with PstI; and DNA in lanes E-H was digested with PstI and KpnI. Hybridisation bands discussed in the text are arrowed.


A
B
C
D
9kb
8kb
7·6kb
7kb
4·7kb
E
F
G
H
8kb
7kb
3·9kb
3·7kb
2·7kb
2·3kb

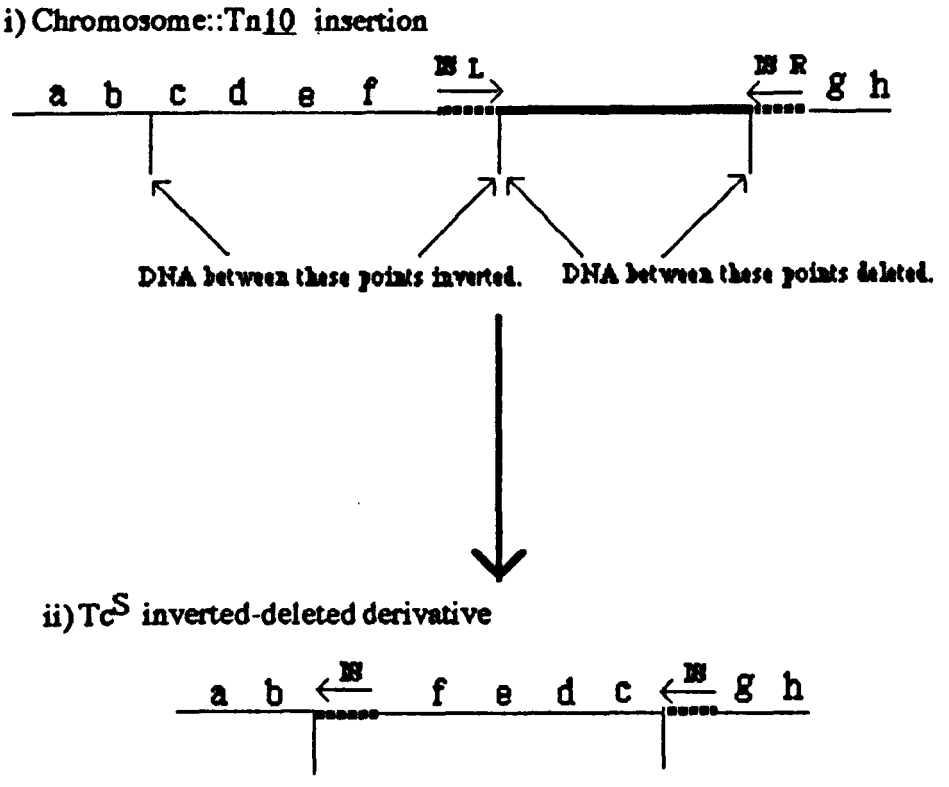
CS115 - rather than to the single 7.6kb fragment observed in *ruv*$^+$ strains. This suggested that a chromosomal rearrangement more complex than a simple deletion had occurred in both cases. This was perhaps not surprising since Tn*10* has been reported to mediate more complex rearrangements such as inversion/deletion of about the same frequency as simple deletions (Kleckner *et al.*, 1979; Kleckner and Ross, 1978). Hybridisation to two bands in *Pst*I digested CS114 and CS115 DNA is consistent with a Tn*10* promoted inversion of a chromosomal segment occurring in both strains, presumably of the general structure proposed by Raleigh and Kleckner, 1984 in which the tetracycline resistance genes of the Tn*10* are deleted and a region of the chromosome extending from the internal end point of one of the IS sequences, through the IS sequence into the chromosome, is inverted (Figure 5.8). Since the 7.6kb *Pst*I fragment present in *Pst*I digested DNA from strain W3110 is not intact in *Pst*I digested CS114 and CS115 DNA, the end point of the inverted segment must lie within the *Pst*I fragment.

The *Pst*I probe hybridised to a 3.9kb fragment, corresponding to the fragment between *Kpn*I site 1 and *Pst*I site 2 in *Pst*I/*Kpn*I digested DNA from both strains CS114 and CS115 confirming that the *ruv* mutations, *ruv-57* and *ruv-58* in strains CS114 and CS115 respectively are not simple deletions from *eda* (which lies downstream of *ruv* transcription - Shurvinton 1983; Lloyd *et al.* 1984).

In addition the *Pst*I probe hybridises to two further fragments in *Pst*I/*Kpn*I digested DNA of 2.7kb and 7kb in DNA from strain CS114 and of 2.3Kb and 8kb for CS115 demonstrating that the fragment between *Pst*I site 1 and *Kpn*I site 1 is not intact in strains CS114 and CS115 but is disrupted rather than deleted.

The autoradiograph presented in Figure 5.9 shows the 2.7kb *Pst*I/*Eco*RV probe hybridised to a fragment of 2.7kb in *Pst*I/*Eco*RV DNA digested from strain CS115, demonstrating that the region between

Figure 5.8 General structure of Tn10 promoted deletion - inversions.



IS10elements

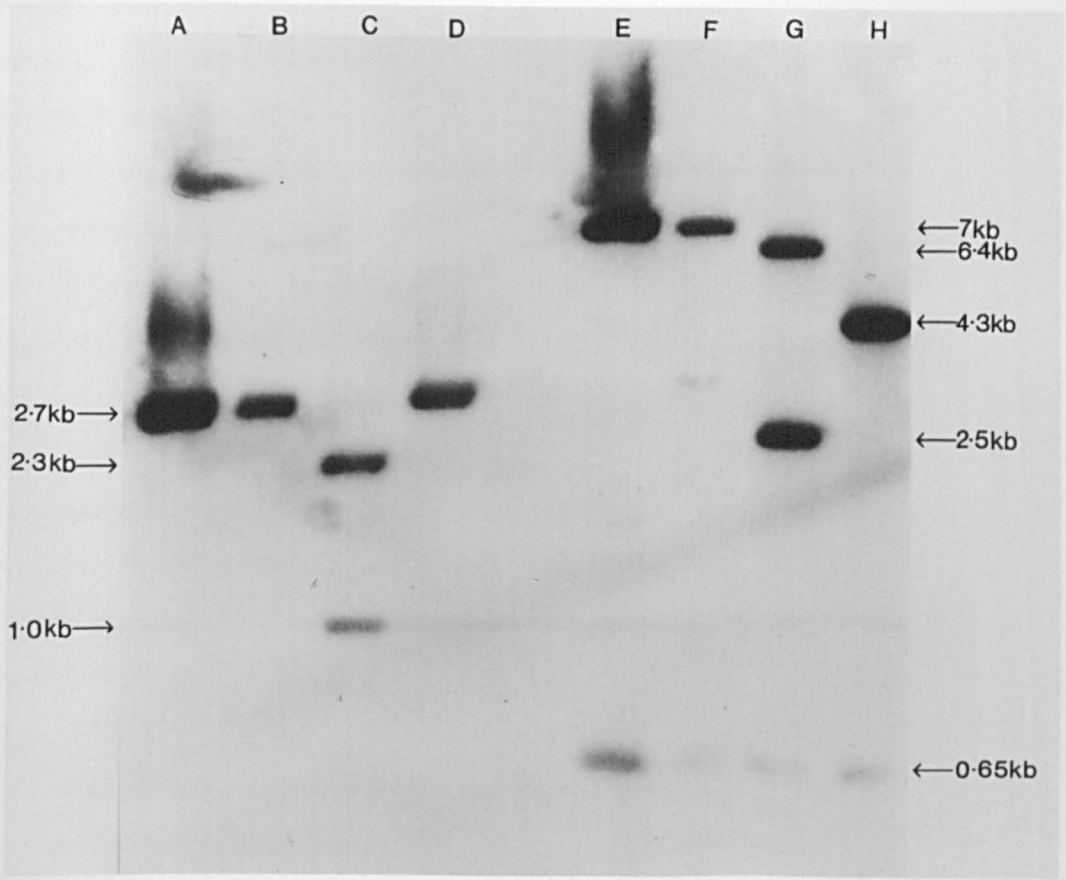
Central region of Tn10 DNA

Chromosomal DNA

a-h Hypothetical gene order

Figure 5.9

Autoradiograph of restriction digested CS114 and CS115 DNA probed with the 2.7kb PstI/EcoRV fragment of pPVA101. Lanes A and E contained pPVA101 DNA; lanes B and F contained W3110 DNA; lanes C and G contained CS114 DNA and lanes D and H contained CS115 DNA. Hybridisation bands discussed in the text are arrowed.


A
B
C
D
E
F
G
H
2·7kb⟶
2·3kb⟶
1·0kb⟶
⟵7kb
⟵6·4kb
⟵4·3kb
⟵2·5kb
⟵0·65kb

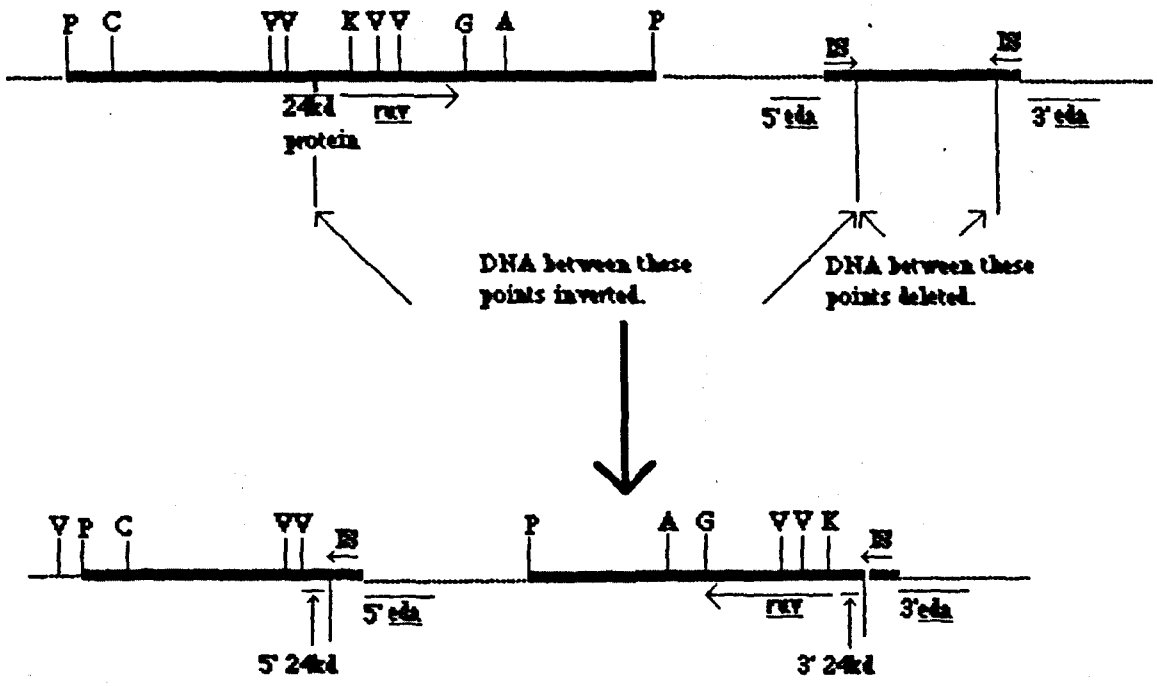
PstI site 1 and EcoRV site 1 is intact, which limits the inversion end-point to between the EcoRV site 1 and KpnI site 1 present in wild type DNA (Figure 5.10a).

The same probe hybridises to two fragments of 1.0kb and 2.3kb in PstI/EcoRV digested DNA from strain CS114 demonstrating that the 3.2kb PstI/EcoRV fragment present in wild type DNA is not intact in CS114. However, it hybridises to the same 0.65kb fragment in PstI/HincII digested DNA from strain CS114 as in the PstI/HincII digested DNA from strain W3110, demonstrating that at least the 0.65kb region between PstI site 1 and HincII site 1 is intact in CS114. The inversion 'end point' in strain CS114 must therefore be between HincII site 1 and EcoRV site 1, and on the basis of the 1.0kb hybridisation fragment observed in PstI/EcoRV digested DNA from strain CS114, must be within 1.0kb of EcoRV site 1.

Since cotransduction frequencies indicate that ruv and eda::Tn10 are separated by approximately 9-10kb, it is not surprising that only 2 stable ruv mutations were obtained out of 2000 tetracycline sensitive derivatives of CS42 screened, as Tn10 has previously been reported to mediate the formation of much smaller chromosomal deletions or inversions of up to 1kb in E. coli (Ross et al. 1979), although much larger inversions have been reported in S. typhimurium which may extend up to 20kb (Kleckner et al., 1979).

The discovery that the mutations in ruv-57 and ruv-58 are inversions rather than deletions and that in both cases the inversions extend through the region coding for the ruv protein and probably beyond that coding for the 25kd protein goes some way towards explaining the genetic mapping in which ruv$^+$ transductants were always obtained in crosses between strains CS114 and CS115 and donors carrying various ruv point mutations (Shurvinton et al., 1984).

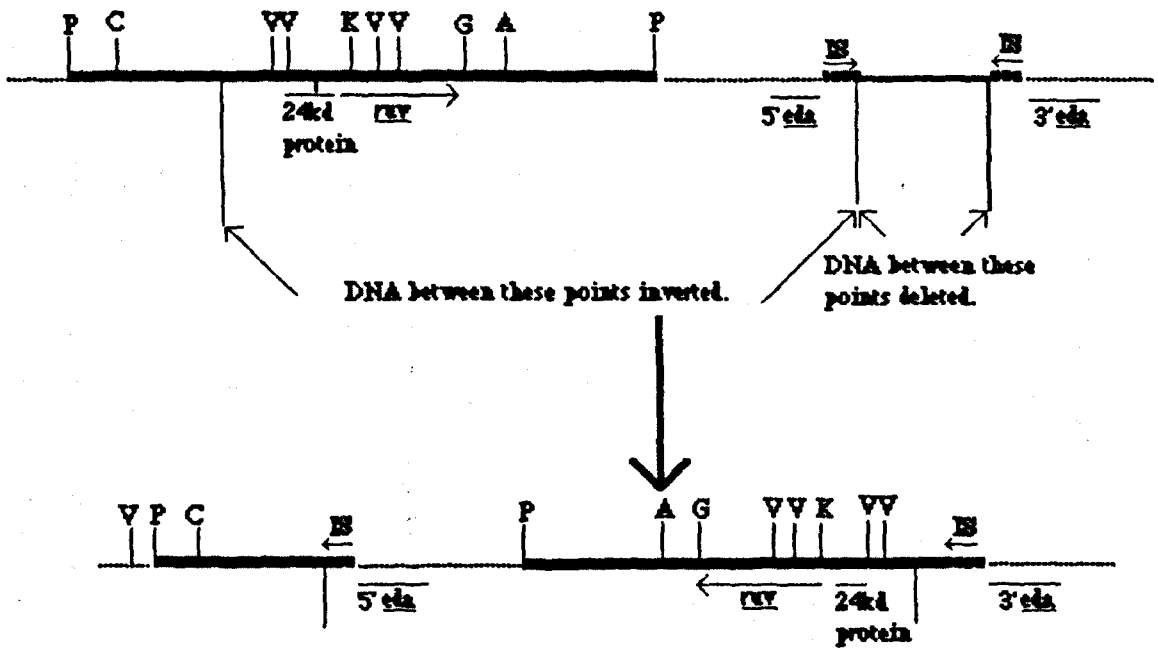
Figure 5.10a Structure of inversion - deletion in CS115 ruv-57.



DNA homologous to probe
IS10 sequence of Tn10 (not to scale)
Central region of Tn10 DNA (not to scale)
Chromosomal DNA (not to scale)

The coding regions for the 24kd protein, and the 41kd Ruv protein, identified by Tn 1000 insertions are shown. The approximate location of the eda ::Tn10 insertions are also shown.
The end points of the inversion/deletion event are indicated by the vertical lines.

Figure 5. 10b Structure of inversion - deletion in CS114 ruv -58



DNA homologous to probe
IS10 sequence of Tn 10 (not to scale)
Central region of Tn10 DNA (not to scale)
Chromosomal DNA (not to scale)

The coding regions for the 24kd protein, and the 41kd Ruv protein, identified by Tn 1000 insertions are shown. The approximate location of the eda ::Tn10 insertions are also shown.
The end points of the inversion/deletion event are indicated by the vertical lines.

CHAPTER 6

Expression of the *ruv* gene

6.1 Introduction

Observations presented in Chapter 3 of this thesis suggested that the *ruv* gene product may be required for recombination repair of daughter strand gaps and double strand breaks in damaged DNA. Previous studies of the expression of the *ruv* gene performed by following production of B-galactosidase expressed from a chromosomal *ruv*::Mud(Ap)^{R}*lac* fusion, had led to the conclusion that *ruv* was regulated by *lexA* and *recA* and induced as part of the SOS response to DNA damage (Shurvinton and Lloyd 1982, Shurvinton (1983). Thus, the *ruv* gene was identified as one of the group of genes, including *recA* *recN* and *recQ*, involved in SOS inducible recombination repair of DNA damage. In order to confirm that expression of the *ruv* gene was under SOS control, and to identify more precisely the nucleotide sequence involved in the SOS regulation, a further study of the *ruv* gene expression was undertaken.

6.2 Expression of the *ruv* gene in strains carrying *sulA* mutations

Earlier studies on the expression of the *ruv* gene were performed with strains carrying the wild type *sulA* gene (formerly *sfiA*) which results in cell filamentation which may be lethal on prolonged exposure to SOS inducing conditions (George *et al*. 1975, Mount 1977). Since the precise effect of filamentation on the expression of SOS inducible genes remains unclear, it was decided to re-examine the expression of the *ruv* gene in strains carrying a *sulA11* mutation.

Results presented in Figure 6.1 show the induction of B-

Figure 6.1

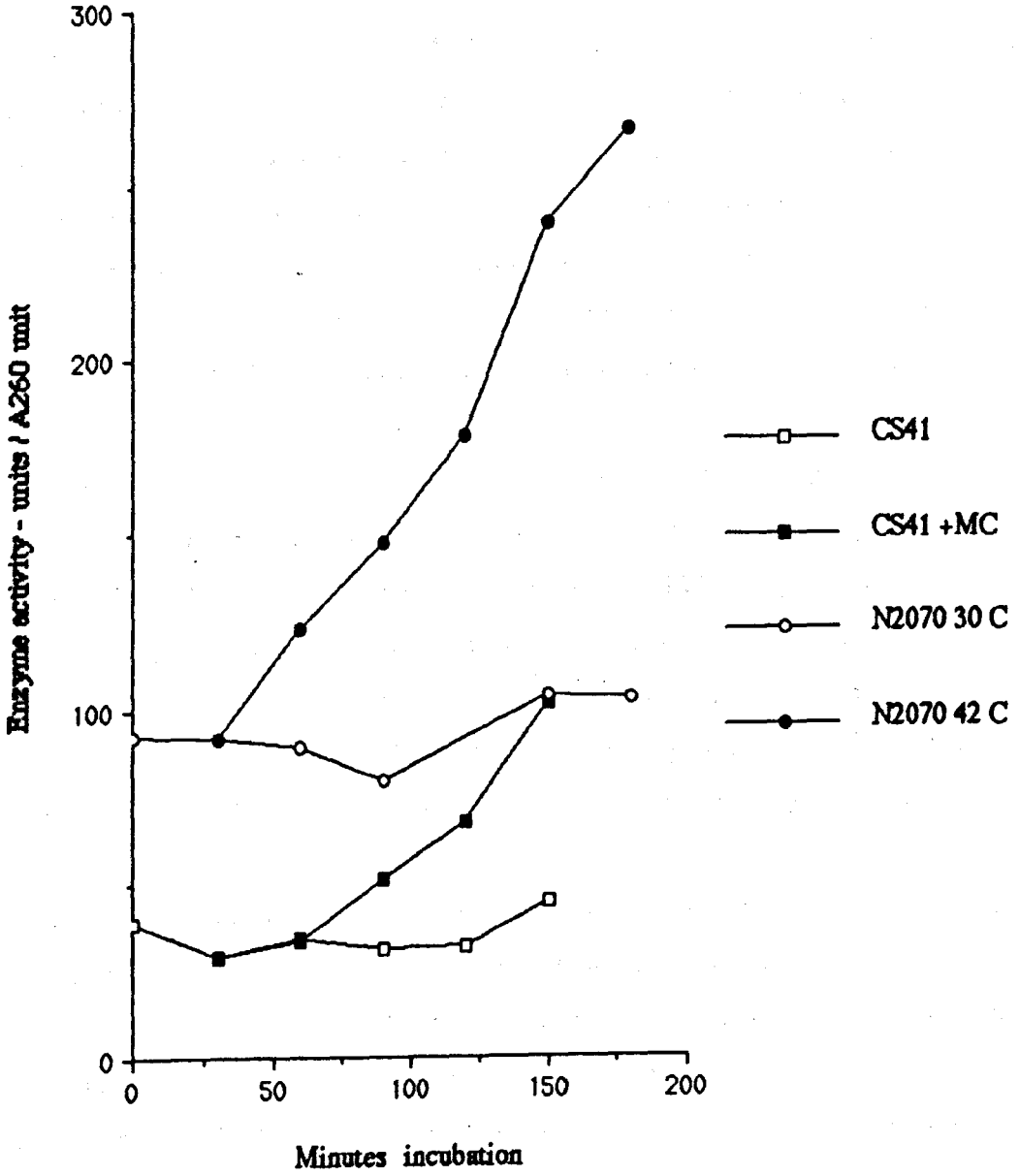


Fig. 6.1

Induction of B-galactosidase expression from an ruv::Mud(Ap)^{R}lac fusion, by mitomycin C (0.1ug/ml) treatment of CS41, ruv::Mud(Ap)^{R}lac, and by shifting the growth temperature of N2070 lexA(ts) ruv::Mud(Ap)^{R}lac from 30°C to 42°C.

galactosidase expression, produced by shifting the temperature of an exponentially growing culture of strain N2070 ruv::Mud(Ap)^{R}lac Muc$^+$ sulAll lexA(ts) from 32°C to 42°C, clearly paralleled the mitomycin C induction of B-galactosidase expression in strain CS41 ruv::Mud(Ap)^{R}lac as was used in earlier studies (Shurvinton 1983). In both cases the level of B-galactosidase was increased to approximately 3x the basal level after 5 hours incubation under inducing conditions, although both the basal and induced levels of ruv expression were considerably higher in strain N2070 than in strain CS41, presumably as a result of the decreased stability of the mutant LexA protein and its increased susceptibility to Lon protease mediated degradation, compared with the wild type LexA protein (Peterson and Mount, 1987).

However, since induction of B-galactosidase (by temperature shift) of strain N2070 ruv::Mud(Ap)^{R}lac Muc$^+$ sulAll lexA(ts) paralleled that observed in strain N2184 ruv::Mud(Ap)^{R}lac lexA(ts)Muc$^+$ (Shurvinton and Lloyd, 1982) and also by mitomycin C treatment of CS41 ruv::Mud(Ap)^{R}lac, it was concluded that induction of B-galactosidase in strains carrying the ruv::Mud(Ap)^{R}lac fusion by SOS inducing treatments was not an artifact of sulA dependent filamentation.

In order to confirm that expression of the ruv gene was controlled by LexA repressor (rather than another repressor subject to recA dependent cleavage), the effect of temperature shift on the expression of ruv in strains harbouring mutations in both recA and lexA was studied (again in strains carrying additional mutations in sulA).

Results presented in Figure 6.2 show that the induction of B-galactosidase expression produced by shifting the temperature of strain N2080 ruv::Mud(Ap)^{R}lac Muc$^+$ sulAll lexA(ts) recA::Tn10 from 32°C to 42°C again paralleled the induction of B-galactosidase expression produced by mitomycin C treatment of strain CS41

Figure 6.2

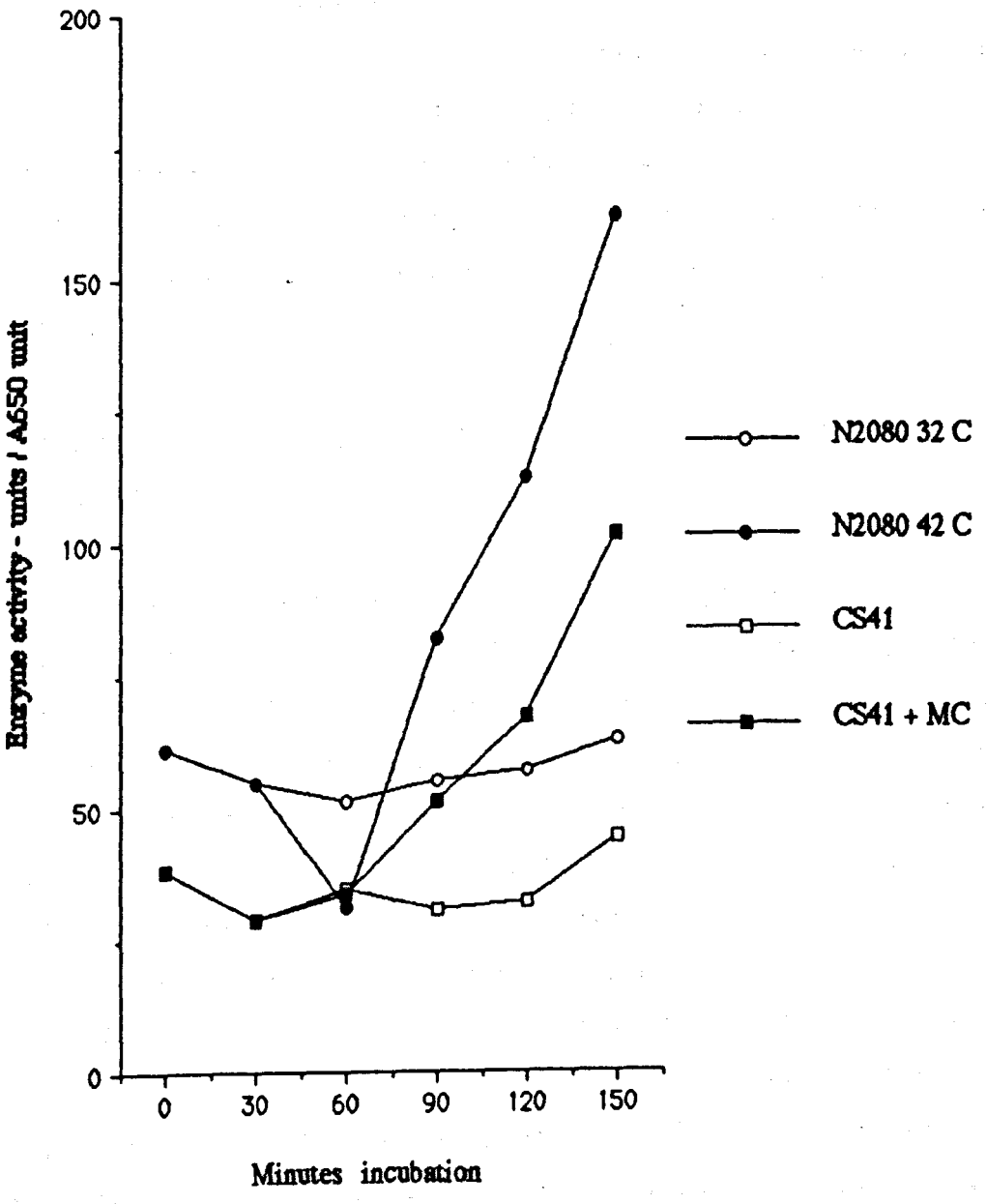


Fig. 6.2

Induction of B-galactosidase expression from an *ruv*::Mud(Ap)^{R}*lac* fusion, by mitomycin C (0.1ug/ml) treatment of CS41 *ruv*::Mud(Ap)^{R}*lac* and by shifting the growth temperature of N2080 *lexA*(ts) *recA*(Def) *ruv*::Mud(Ap)^{R}*lac* from 32°C to 42°C.

ruv::Mud(Ap)^{R}*lac* providing confirmation that the *ruv* gene is directly repressed by LexA protein.

Once again, however, both the basal and induced levels of *ruv* expression were higher in strain N2080 than in strain CS41, although they were not as high as in the parent *recA*$^{+}$ strain N2070, suggesting that the RecA protein may still have some role in modulating the SOS response in *lexA*(ts) strains. These results therefore provided further confirmation that the *ruv* gene promoter was directly repressed by the *lexA* protein and that induction of *ruv* gene expression by DNA damaging agents was not a *sulA* dependent artifact.

6.3 Investigation of the possible autoregulation of *ruv* gene expression

Previous studies of the expression of the *ruv* gene performed on strains carrying the *ruv*::Mud(Ap)^{R}*lac* fusion disregarded any effect the Ruv protein itself might have on *ruv* gene expression (Shurvinton and Lloyd 1982, Shurvinton 1983). In order to investigate whether expression of the *ruv* gene was subject to autoregulation, the induction of B-galactosidase by mitomycin C was examined in strains carrying the *ruv*::Mud(Ap)^{R}*lac* fusion and a plasmid borne *ruv*$^{+}$ gene (provided by pPVA101).

Results presented in Figure 6.3 show that both the basal level of expression and the induction of B-galactosidase produced by mitomycin C treatment of FB244 *ruv*::Mud(Ap)^{R}*lac* pPVA101 *ruv*$^{+}$ were similar to the results obtained in the plasmid free parent strain CS41 *ruv*::Mud(Ap)^{R}*lac*, suggesting that expression of the *ruv* gene was not subject to any autoregulation.

Figure 6.3

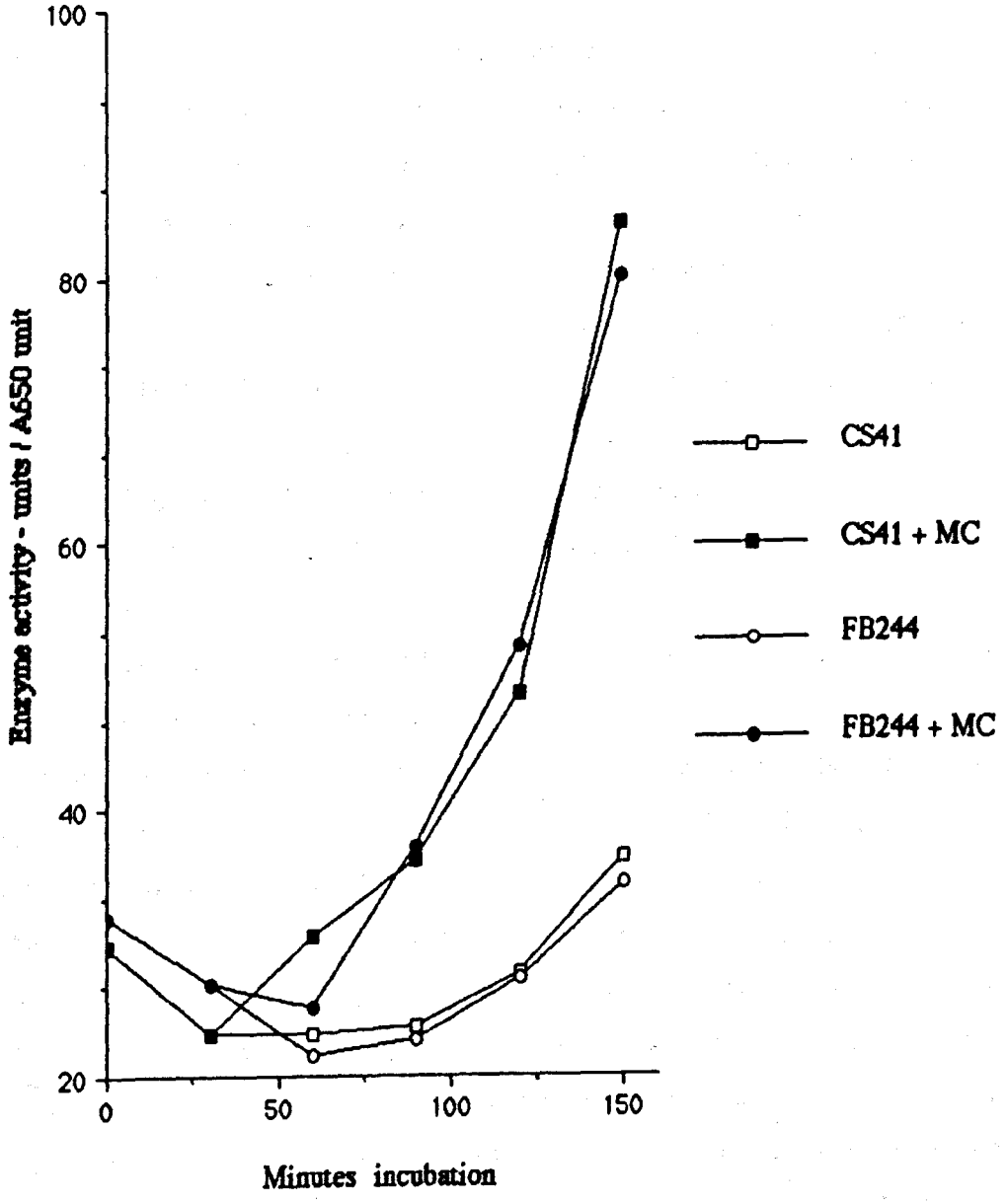


Fig. 6.3

Induction of B-galactosidase expression from an ruv::Mud(Ap)^{R}lac fusion, by mitomycin C (0.1ug/ml) treatment of CS41 ruv::Mud(Ap)^{R}lac and FB244 ruv::Mud(Ap)^{R}lac/pPVA101.

6.4 Induction of ruv gene expression by nalidixic acid

Studies by Shurvinton (1983) had shown that although expression of the ruv gene could be induced by treatment with either UV irradiation or mitomycin C, expression was not induced by treatment with nalidixic acid. This was somewhat surprising since nalidixic acid induced expression of other SOS genes in wild type strains, although induction was prevented in recBC strains (Gudas and Pardee 1975b, McPartland et al. 1980, Chaudhury and Smith 1984).

Three proposals were put forward to account for the lack of nalidixic acid induction of ruv gene expression (Shurvinton 1983):

i) Nalidixic acid directly inhibited ruv transcription (as was demonstrated for the lac and gal operons, Sanzey 1979).

ii) The ruv^+ gene product was required for nalidixic acid induction of the SOS response (as was demonstrated for the recBC gene products).

iii) The level of SOS induction produced by nalidixic acid was insufficient for induction of ruv gene expression.

Since nalidixic acid does not noticeably lower the basal level of ruv gene expression even when cells were subjected to prolonged exposure to it, the first of these proposals was eliminated.

In order to distinguish between the two remaining options, the effect of providing the ruv gene product, encoded by the plasmid pPVA101 on the induction of ruv gene expression by nalidixic acid, was investigated. If the ruv gene product was required for induction of the SOS response by nalidixic acid, then expression of B-galactosidase in strain FB244 ruv::Mud(Ap)^{R}lac pPVA101 ruv^+ should be inducible by nalidixic acid treatment.

Results presented in Figure 6.4a and 6.4b show the production of B-galactosidase in strains CS41 ruv::Mud(Ap)^{R}lac and FB244 ruv::Mud(Ap)^{R}lac pPVA101 ruv^+ treated with 10ug/ml and 40ug/ml nalidixic acid respectively. For comparison the B-galactosidase

Figure 6.4a

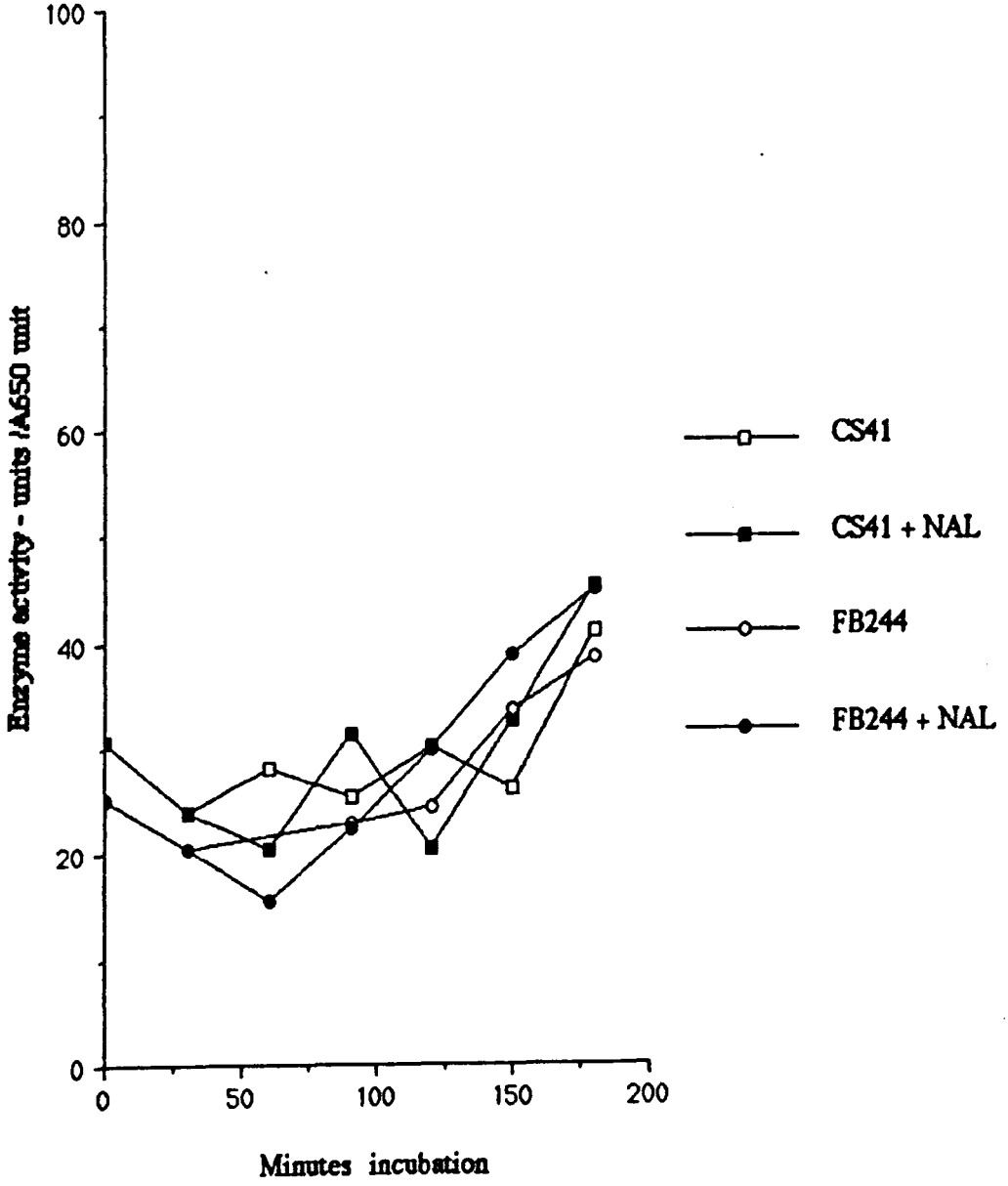


Fig. 6.4a

Expression of B-galactosidase from an ruv::Mud(Ap)^{R}lac fusion in strains treated with 10ug/ml nalidixic acid. Strains used were CS41 ruv::Mud(Ap)^{R}lac and FB244 ruv::Mud(Ap)^{R}lac/pPVA101.

Figure 6.4b

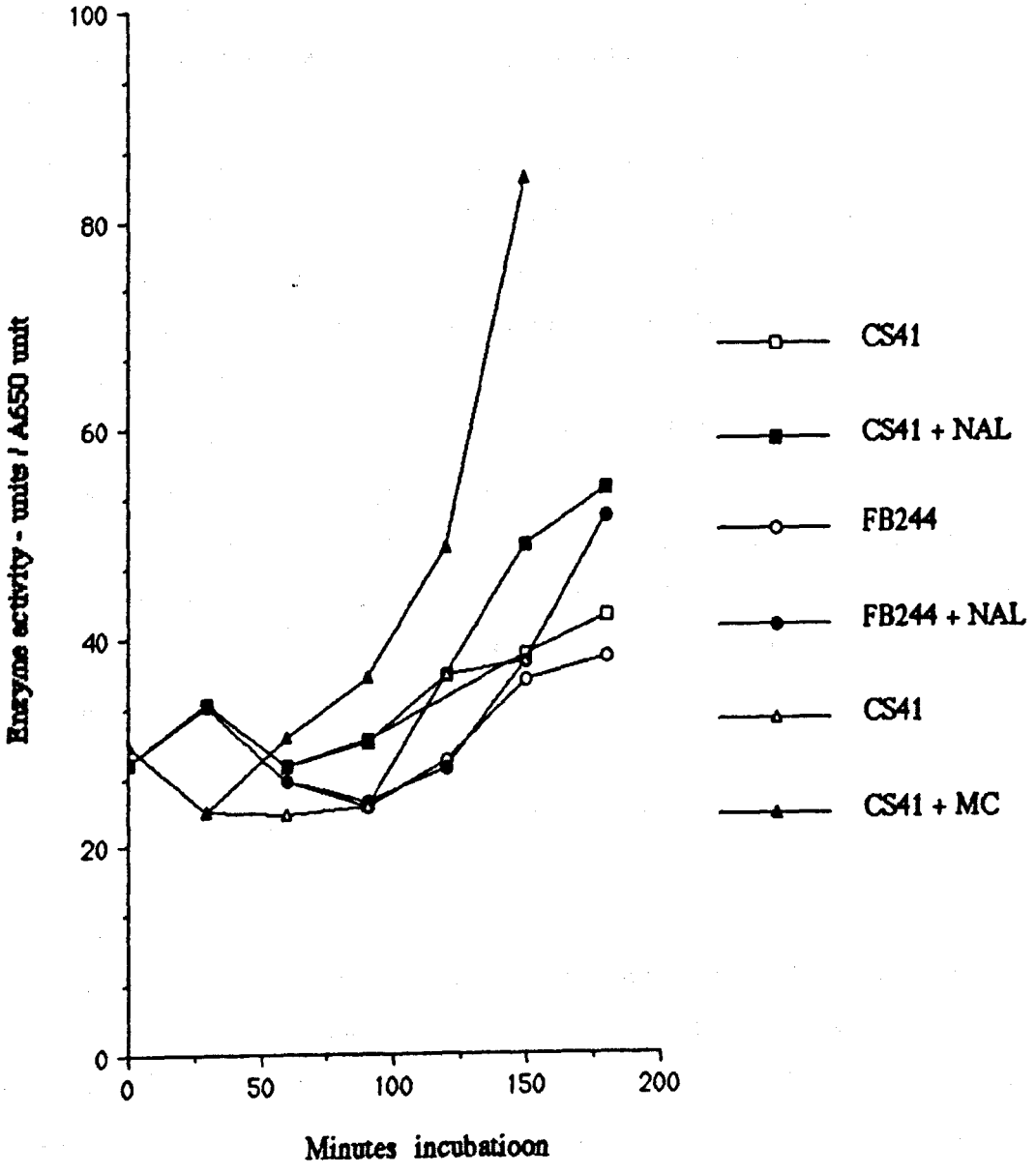


Fig. 6.4b

Induction of B-galactosidase expression from an ruv::Mud(Ap)^{R}lac fusion by mitomycin C (0.1ug/ml) and nalidixic acid (40ug/ml) in the presence of pPVA101 ruv^{+}. Strains used were CS41 ruv::Mud(Ap)^{R}lac and FB244 ruv::Mud(Ap)^{R}lac pPVA101

activity produced by mitomycin C treatment of CS41 *ruv*::Mud(Ap)^{R}*lac* is shown. Clearly, the presence of the *ruv* gene product has little effect on the failure of nalidixic acid to induce expression of the *ruv* gene. This suggested that a functional *ruv* gene product was not required for induction of the SOS response by nalidixic acid in a manner similar to the *recBC* gene products as was proposed in (ii) above.

This was confirmed by examining the effect of an *ruv* mutation on the nalidixic acid induction of *sulA* expression. Results presented in Figure 6.5 show that an *ruv* mutation did not prevent nalidixic acid induction of expression of B-galactosidase in strain FB160 *sulA*::Mud(Ap)^{R}*lac* *ruv*::Tn*10*.

Thus, the failure of nalidixic acid to induce expression of B-galactosidase from the *ruv*::Mud(Ap)^{R}*lac* fusion was not due to there being a general requirement for the *ruv* gene product for nalidixic acid induction of SOS genes, but was probably due to the level of SOS induction produced by nalidixic acid being insufficient for induction of *ruv* gene expression.

6.5 Expression of other SOS inducible genes in strains carrying *ruv* mutations

During the course of strain construction for the above experiments it was noticed that the basal level of expression of B-galactosidase in *sulA*::Mud(Ap)^{R}*lac* strains was consistently higher in strains with *ruv* mutations than in otherwise isogenic *ruv*$^{+}$ strains. Since increased expression of the *sulA* gene in *ruv* mutants could account for the filamentation associated with strains carrying *ruv* mutations, a further investigation of this phenomenon was undertaken.

Results presented in Figure 6.6 confirm the observations originally made on MacConkey lactose plates that B-galactosidase

Figure 6.5

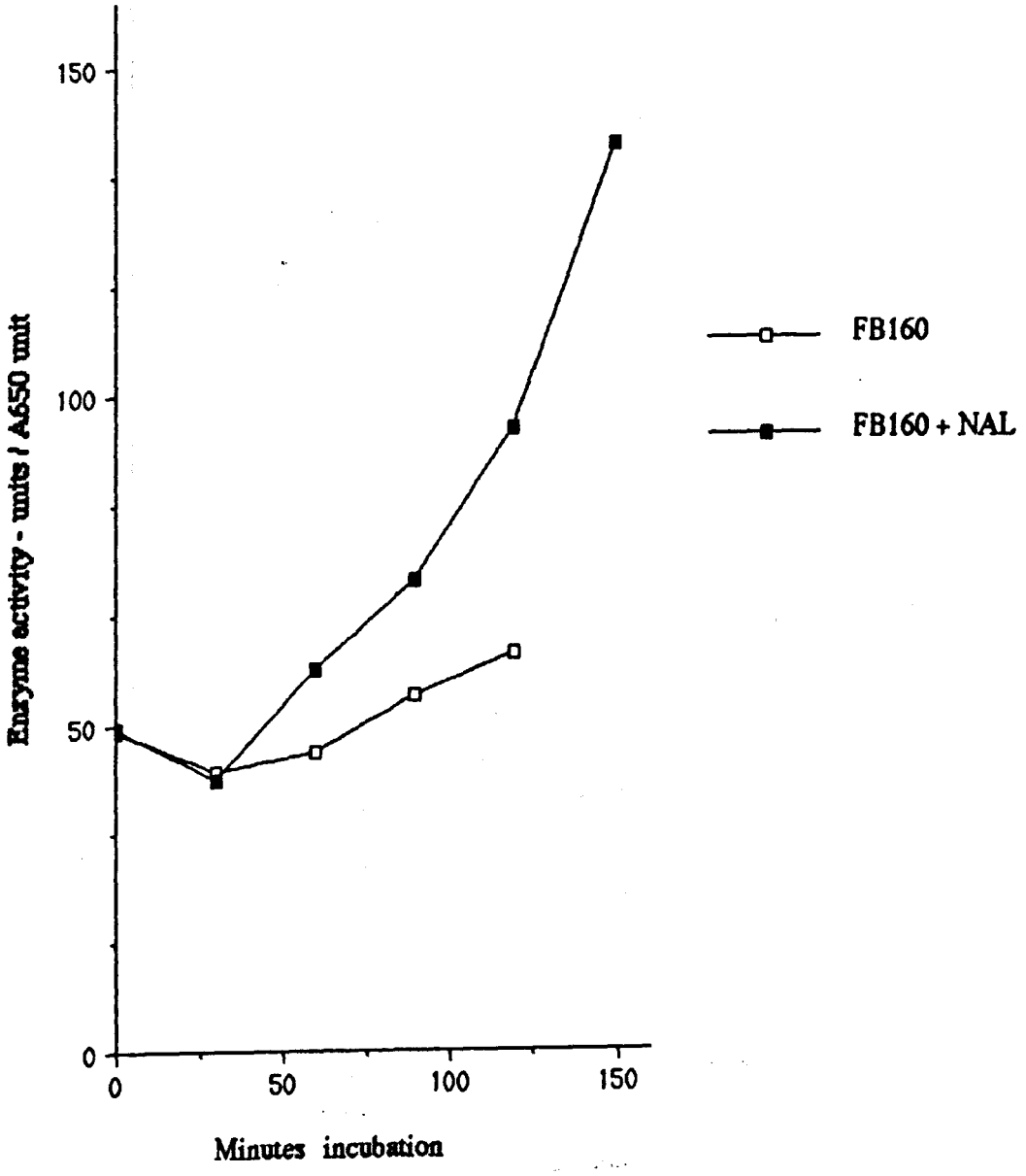


Fig. 6.5

Induction of B-galactosidase expression from a sulA::Mud(Ap)^{R}lac fusion by nalidixic acid (10ug/ml) in a strain carrying an additional ruv mutation. The strain used was FB160 sulA::Mud(Ap)^{R}lac, ruv-59::Tn10

Figure 6.6

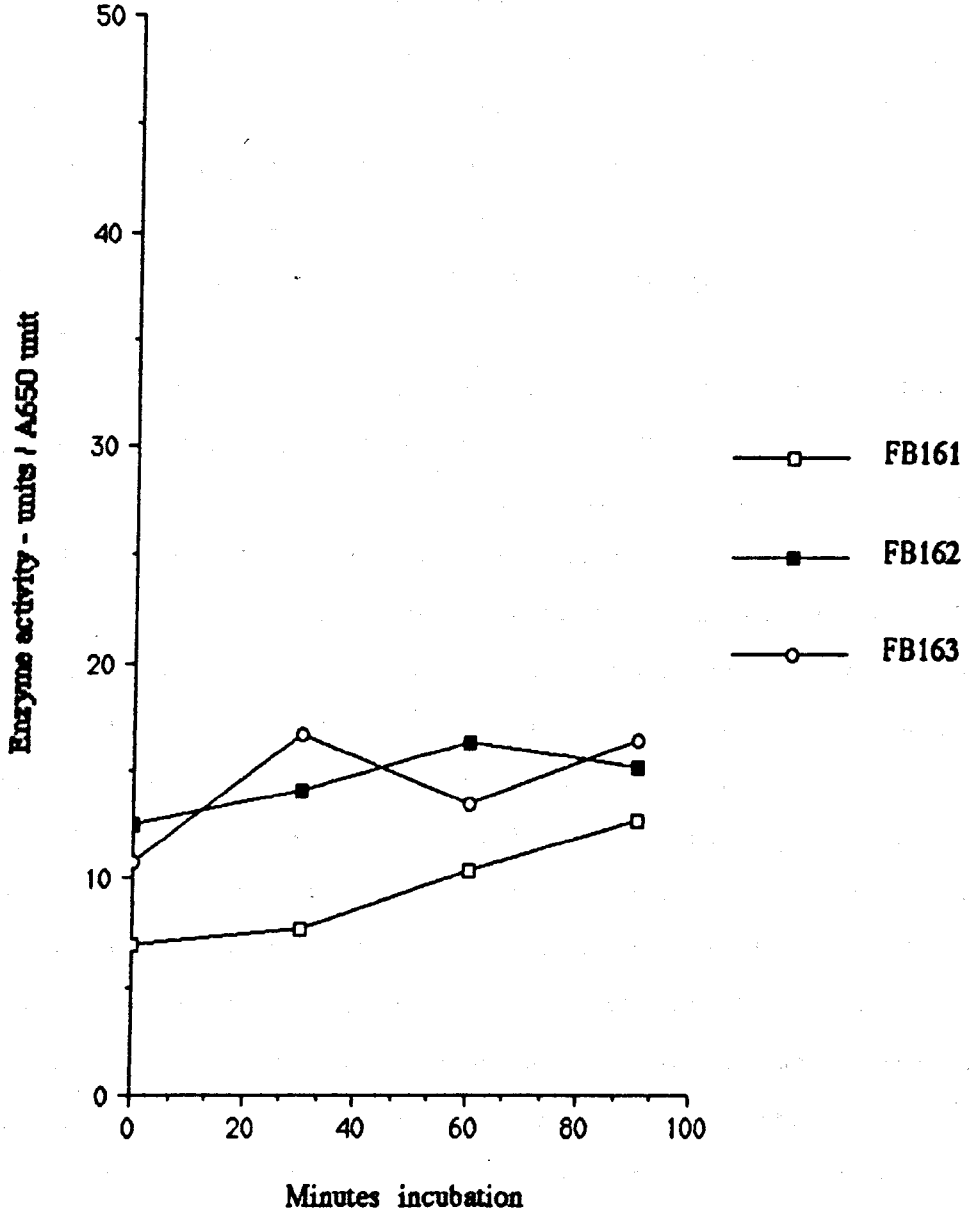


Fig. 6.6

The effects of *ruv* mutations on the expression of B-galactosidase from a *sulA*::Mud(Ap)^{R} *lac* fusion. Strains used were FB161 *sulA*::Mud(Ap)^{R} *lac* *ruv*$^{+}$ FB162 *sulA*::Mud(Ap)^{R} *lac* *ruv-52* and FB163 *sulA*::Mud(Ap)^{R} *lac* *ruv-54*

activity in sulA::Mud(Ap)^{R}lac strains carrying additional mutations in ruv was approximately twice the activity measured in otherwise isogenic ruv^{+} strains. This could clearly account for the filamentation of strains carrying ruv mutations observed during normal growth (Shurvinton, 1983).

In order to determine whether the effects of ruv mutations on expression were confined to increasing the expression of the sulA gene, or whether ruv mutations increased the expression of other SOS genes, the effects of ruv mutations on the production of B-galactosidase in strains with a uvrA::Mud(Ap)^{R}lac fusion was examined.

Results presented in Figure 6.7 show that the basal level of expression of B-galactosidase in strains with a uvrA::Mud(Ap)^{R}lac fusion was greater in strain FB193 ruv::Tn10 than in the otherwise isogenic strain FB191 ruv^{+}, approximately by a factor of 1.5, suggesting there may be a general increase in expression of SOS genes in strains with ruv mutations. In addition, these results demonstrated that mitomycin C induction of B-galactosidase from a uvrA::Mud(Ap)^{R}lac fusion was unaffected by ruv mutations.

The increased expression of both sulA and uvrA observed in strains with ruv mutations suggested that there may be a general increase in expression of SOS genes in these strains. Earlier studies, presented in Chapter 3 of this thesis, had suggested that the ruv gene product may be required for recombination repair of damage occurring as a consequence of treatment with DNA damaging agents such as UV irradiation, or arising in the course of DNA metabolism. In the absence of the ruv gene product promoting repair in ruv mutants, such damage might be expected to persist and function as a signal to partially induce the SOS response resulting in the higher basal level of expression of SOS genes such as sulA and uvrA reported above.

Figure 6.7

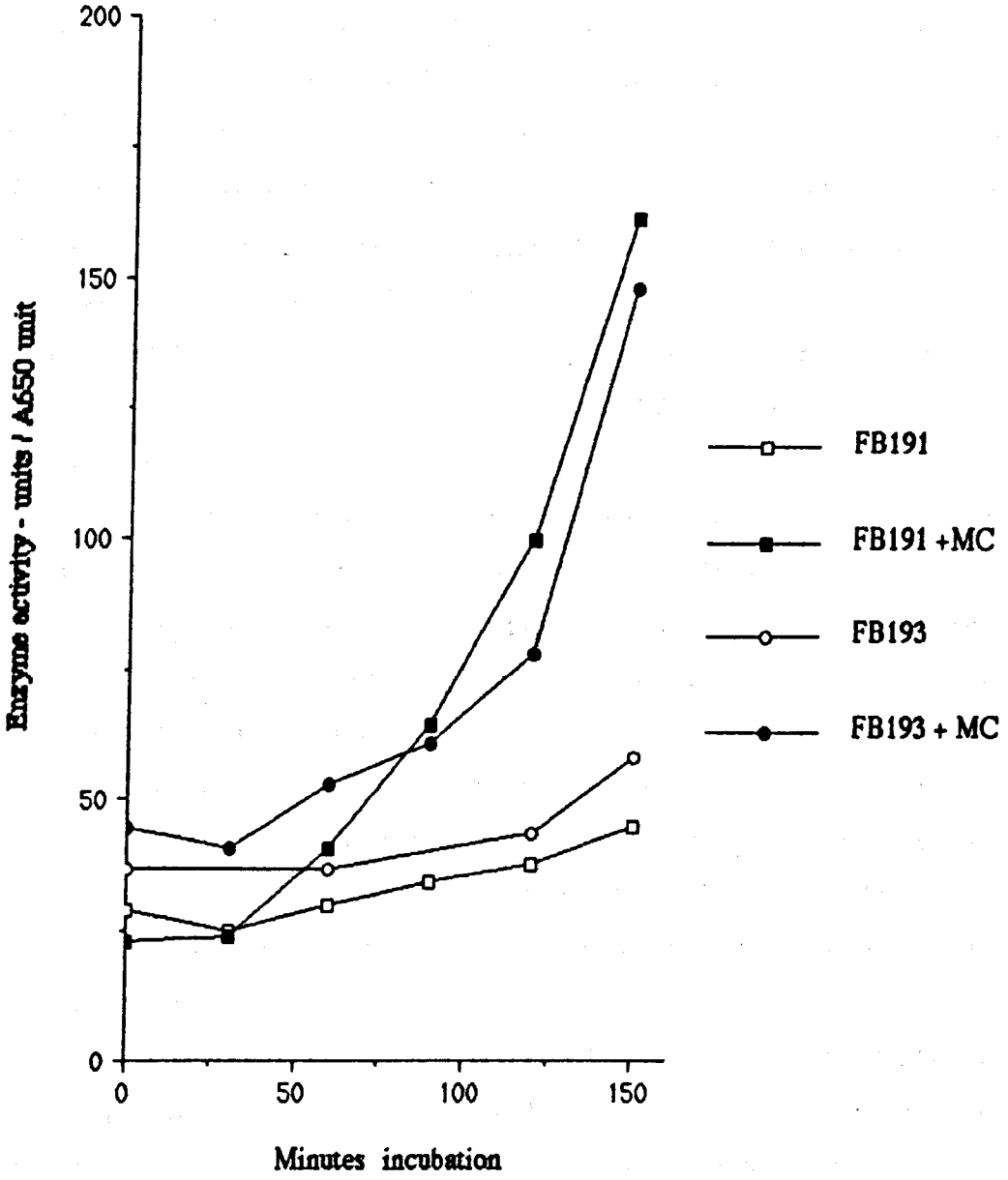


Fig. 6.7

The effect of an ruv mutation on the induction of B-galactosidase expression by mitomycin C (0.1ug/ml) treatment of strains carrying a uvrA::Mud(Ap)^{R}lac fusion. Strains used were FB191 uvrA::Mud(Ap)^{R}lac ruv^{+} and FB193 uvrA::Mud(Ap)^{R}lac ruv-60::Tn10

6.6 Cloning of the SOS inducible ruv promoter

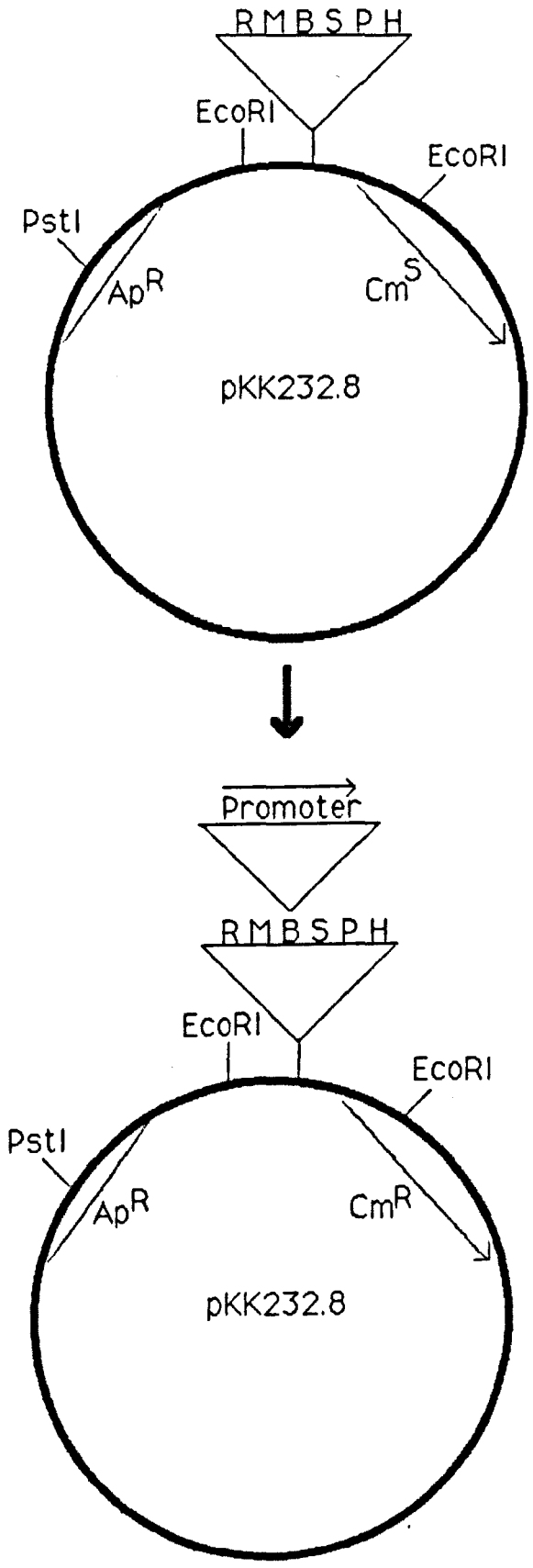
All studies on the expression of the ruv gene reported so far (Shurvinton and Lloyd 1982, Shurvinton 1983, and above) utilised the ruv::Mud(Ap)^{R}lac fusion isolated by Shurvinton (1983). In Chapter 5 of this thesis, the precise mapping of the Mud(Ap)^{R}lac insertion in the chromosomal ruv gene was described. This allowed the direction of transcription of the ruv gene to be determined as being from the KpnI site 1 towards the BglII site of plasmid pPVA101 and its derivatives and suggested that the ruv promoter was probably located at a position to the left of KpnI site (Figure 5.4).

In order to identify more precisely the promoter region of the ruv gene so that its nucleotide sequence could be determined, and to study the expression of ruv in a construct independent of the ruv::Mud(Ap)^{R}lac fusion, the ruv promoter was cloned into the promoter cloning vector pKK232.8 was used. pKK232.8 (Brosius 1984) is a pBR322 derivative with the promoter for the tetracycline resistance gene deleted and a multiple cloning site inserted in front of a promoterless chloramphenicol acetyl transferase (CAT) gene cassette. Cloning a fragment of DNA containing a promoter in the correct orientation into the multiple cloning site results in expression of the CAT gene under the control of the inserted promoter, and confers chloramphenicol resistance on transformed cells (Figure 6.8).

In order to clone the ruv gene promoter region, plasmid pFB512 DNA was digested with PstI and KpnI, fragments separated by agarose gel electrophoresis and the 3.7kb fragment isolated. This DNA was then digested with Sau3A and the resulting fragments ligated into BamHI digested pKK232.8.

The ligation mixture was transformed into strain N1563 lexA(Def) recA(Def) (in which the ruv promoter should be fully derepressed), and transformants selected on plates containing

Figure 6.8 Restriction map and genetic organisation of pKK232.8



20ug/ml ampicillin and 5ug/ml chloramphenicol.

Plasmid DNA was isolated from 24 such transformants and used to transform strain AB2463 recA13 (in which the ruv promoter should be repressed and uninducible), selecting for ampicillin resistant transformants. Plasmids were designated pFB1-24. Single colony isolates of N1563 pFB1-24 and AB2463 pFB1-24 derivatives were gridded onto ampicillin plates and after 8 hours incubation, replica plated onto plates containing 20ug/ml ampicillin and a range of chloramphenicol concentrations from 5ug/ml up to 100ug/ml, to determine whether CAT production was under SOS control in these plasmids and whether therefore they were likely to carry the ruv promoter. Plasmid DNA from those clones that conferred resistance to higher levels of chloramphenicol in strain N1563 lexA(Def) recA(Def) than in strain AB2463 recA(Def) was digested with EcoRI and Hind III, (which have restriction sites flanking the inserted DNA) and electrophoresed in order to identify cloned DNA fragments. The inserted DNA varied in size from approximately 0.6kb to 2.1kb and probably consisted of more than one Sau3A fragment ligated together, either due to partial digestion of the original PstI - KpnI fragment by Sau3A or by ligation of several Sau3A fragments together at the time of insertion into the vector.

Eight plasmid isolates, pFB1, pFB3, pFB4, pFB7, pFB12, pFB14, pFB17, and pFB22, were identified with different insert sizes and subjected to further study.

The plasmids were transformed into strain AB1157, selecting for ampicillin resistant transformants. Transformants were then gridded onto ampicillin plates and after 8 hours incubation at 37°C replica plated onto plates containing ampicillin or ampicillin and 0.1ug/ml mitomycin C, and a range of chloramphenicol concentrations from 5 to 80ug/ml.

Results presented in Table 6.1 show that of the 8 putative ruv promoter clones studied, all but one grew better at higher

Table 6.1 Growth of promoter clone harbouring derivatives of AB1157 on chloramphenicol plates +/- mitromycin C

	Chloramphenicol conc'n	Chloramphenicol conc'n (+ MC)
AB1157 pFB1	60ug/ml	80ug/ml
AB1157 pFB3	60ug/ml	80ug/ml
AB1157 pFB4	60ug/ml	80ug/ml
AB1157 pFB7	50ug/ml	60ug/ml
AB1157 pFB12	50ug/ml	60ug/ml
AB1157 pFB14	60ug/ml	80ug/ml
AB1157 pFB17	60ug/ml	80ug/ml
AB1157 pFB22	30ug/ml	30ug/ml

Transformants (10 of each strain) were gridded on to ampicillin plates, incubated for 8 hours then replica plated on to ampicillin and ampicillin and mitomycin C (0.1ug/ml) plates containing a range of chloramphenicol concentrations as described in 6.6. The highest chloramphenicol concentration on which growth of replica plated transformants was uninhibited is presented.

concentrations of chloramphenicol on the plates that contained mitomycin C at 0.1ug/ml than in the absence of mitomycin C, providing an indication that mitomycin C may be inducing expression of CAT from the cloned promoters, and therefore that the inserted promoter DNA may contain the ruv promoter which has been demonstrated to be induced by mitomycin C. The one plasmid pFB22, that did not confer greater chloramphenicol resistance on strain AB1157 in the presence of mitomycin C was not eliminated on the basis of this single plate test. In order to investigate more precisely the induction of expression of chloramphenicol acetyl transferase from the cloned promoters by mitomycin C, plasmid harbouring strains were grown in LB broth to an O.D.650 of 0.2, cultures divided into two, and mitomycin C added to a concentration of 1ug/ml to one portion. Incubation was continued for a further two hours then cells were harvested by centrifugation, resuspended in 50mM Tris HCl pH 7.8 30MM DTT and lysed by sonication on ice. Cellular debris was removed by centrifugation and the cellular extracts assayed for CAT activity as described in 2.30. The protein content of the cell extracts was estimated by a comparison of the absorbance at 205nm with the absorbance at 205nm of bovine serum albumin solutions of known concentrations. In order to directly compare the CAT activity in different strains either with or without mitomycin C treatment, the values obtained for CAT activity were normalised to give a figure relative to 1ug/ml total protein in the cell extract.

Results in Table 6.2 demonstrate that the expression of CAT is induced by mitomycin C in AB1157 derivatives carrying each of the 8 putative ruv promoter clones isolated, although the extent of induction varied considerably between derivatives carrying the different plasmids. The variation in expression was perhaps not surprising since the size of the DNA fragment inserted in the cloning site of pKK232.8 varied between 0.6kb and 2.1kb which would

Table 6.2 Induction of CAT expression by mitomycin C in promoter clone harbouring derivatives of AB1157

Bacterial Strain	Units CAT per lmg/ml total protein x 10^5 -MC	+MC	Induction ratio
FB463 pFB4	26	80.5	3.1
FB464 pFB7	22	36	1.66
FB465 pFB12	29	245	8.2
FB466 pFB14	13.7	192	14
FB467 pFB17	45	69	1.5
FB468	37	64	1.8

Experiments were performed as described in 6.6 and chloramphenicol acetyl transferase assayed as described in 2.30. Induction ratio was calculated by dividing the CAT activity in the presence of MC by the CAT activity in its absence.

be expected to affect CAT gene expression.

Enzyme assays on derivatives of strains AB2463 lexA(Def) recA(Def) and N1204 recA(Def) carrying the plasmids, pFB4, pFB7, pFB12 and pFB14, which showed greatest induction of CAT activity by mitomycin C in AB1157, were performed in order to confirm that mitomycin C induction of CAT production was under SOS control.

Results presented in Table 6.3 show that the expression of CAT was not induced by mitomycin C treatment of plasmid harbouring derivatives of strain AB2463 recA(Def), suggesting that the mitomycin C induction of CAT expression in strains carrying the putative ruv promoter clones was dependent on recA gene function and was therefore likely to be under SOS control.

This was confirmed by the results of assays of CAT activity in plasmid harbouring derivatives of strain N1563 lexA(Def), in which the SOS system is completely derepressed. These results, also presented in Table 6.3, demonstrate that the basal level of CAT expression in N1563 derivatives was approximately 3 x the basal level of expression in the equivalent AB2463 recA(Def) derivative, and that expression of CAT was not further induced by mitomycin C treatment.

It was therefore concluded that expression of CAT from the plasmids pFB4, pFB7, pFB12 and pFB14 was under SOS control, and that the DNA fragments inserted in the cloning site of pKK232.8 to generate these plasmids were likely to carry the ruv gene promoter.

Plasmid pFB14 was chosen to further study the regulation of CAT expression from the cloned putative ruv gene promoter. Plasmid pFB14 DNA was transformed into a series of strains with mutations in genes involved either in the SOS response or required for recombination, selecting for ampicillin resistant transformants. The CAT activity in plasmid harbouring strains with and without mitomycin C treatment was assayed as before.

Table 6.3 Expression of CAT from promoter clones in strains carrying *lexA*(Def) and *recA*(Def) mutations

	Units CAT per 1mg/ml total protein x 10^5 -MC	+MC	MC Induction Ratio	*lexA*(Def)/*recA*(Def) induction ratio (-MC)
FB455 *recA*(Def) pFB4	44	3.1	0.07	4.5
FB443 *lexA*(Def) pFB4	198	63	0.015	
FB456 *recA*(Def) pFB7	22	16	0.75	7.6
FB444 *lexA*(Def) pFB7	168	3.8	0.02	
FB457 *recA*(Def) pFB12	45	32	0.71	6.9
FB445 *lexA*(Def) pFB12	310	29	0.09	
FB458 *recA*(Def) pFB14	66	48	0.72	3.0
FB446 *lexA*(Def) pFB14	200	ND	ND	

Bacterial strains were grown, induced and lysed by sonication as described in 6.6. CAT activity was assayed by the method of Shaw (1975) described in 2.30. The MC induction ratio was calculated by dividing the CAT activity in the presence of mitomycin C by the CAT activity in its absence. The *lexA*(Def)/*recA*(Def) induction ratio was calculated by dividing the CAT activity in the *lexA*(Def) strain by that in the *recA*(Def) strain, in the absence of mitomycin C.

Results presented in Table 6.4 confirmed that the cloned promoter in pFB14 was under SOS control and was therefore likely to be the *ruv* promoter.

As was previously observed with *ruv*::Mud(Ap)^{R}*lac* fusion strains, expression from the *ruv* gene promoter was not inducible by mitomycin C treatment of derivatives of strains N1564 and AB2463 carrying *lexA*(ind^{-}) and *recA* mutations respectively, but was induced to approximately 3-4 x the basal level of expression by mitomycinC treatment of the AB1157 derivative. Both the basal level and mitomycin C induced expression of CAT from the *ruv* promoter in derivatives of strains CS81 *ruv*-52, N1182 *recB*, N1193 *recC*, N1234 *recF* and SP254 *recN* was found not to be significantly different from the expression in the otherwise isogenic wild type strain AB1157. Unfortunately, studies on the expression of CAT from the *ruv* promoter in a *recBC sbcBC* background, in which *ruv* mutations have their greatest effect on DNA repair and recombination, were prevented because of problems with plasmid stability in this genetic background. pFB14 could only be maintained in this genetic background with constant selection, which affected the assays.

In addition to the problems with plasmid stability, studies of gene expression using plasmid-borne promoter::CAT fusions, such as those obtained above by cloning DNA fragments into the multiple cloning site of pKK232.8 have several more inherent problems which have to be taken into account when analysing the results of these studies.

i) The precise effect of the presence of multiple copies of a promoter on the expression of CAT from that promoter was unknown.

ii) The efficiency of cell disruption by sonication was rather variable.

iii) The enzyme assay itself was not particularly sensitive for measuring such low enzyme levels.

Table 6.4 Expression of CAT from plasmid pFB14 in strains carrying mutations in genes required for (a) SOS control and (b) recombination

		Units CAT per 1mg/ml total protein x 10^5		Induction ratio
		-MC	+MC	
a)	FB466 (wt)	59	290	4.8
	FB446 lexA(def)	200	66	0.33
	FB482 lexA(ind$^-$)	31.3	28.3	0.9
	FB458 recA	54	25.5	0.47
b)	FB501 recB21	40.5	127	3.1
	FB504 recC22	36	220	6.1
	FB516 recBC sbcA	131	286	2.2
	FB513 recN262	61.2	310	5.1
	FB507 recF143	36	153	4.25
	CS81 ruv-52	60.5	346	5.7

Bacterial strains were grown, induced, and lysed by sonication as described in 6.6. CAT was assayed by the method of Shaw (1975) described in 2.30. MC induction ratios were calculated as described in Table 6.2.

iv) Total protein estimates based on the O.D.205 were rather rough and ready - better protein estimates could be obtained by an alternative method such as a Lowry determination.

v) As was the case with the B-galactosidase assays, the precise effect on the cells of prolonged exposure to mitomycin C was unknown particularly since all the strains used in these studies carried the *sulA*$^{+}$ gene.

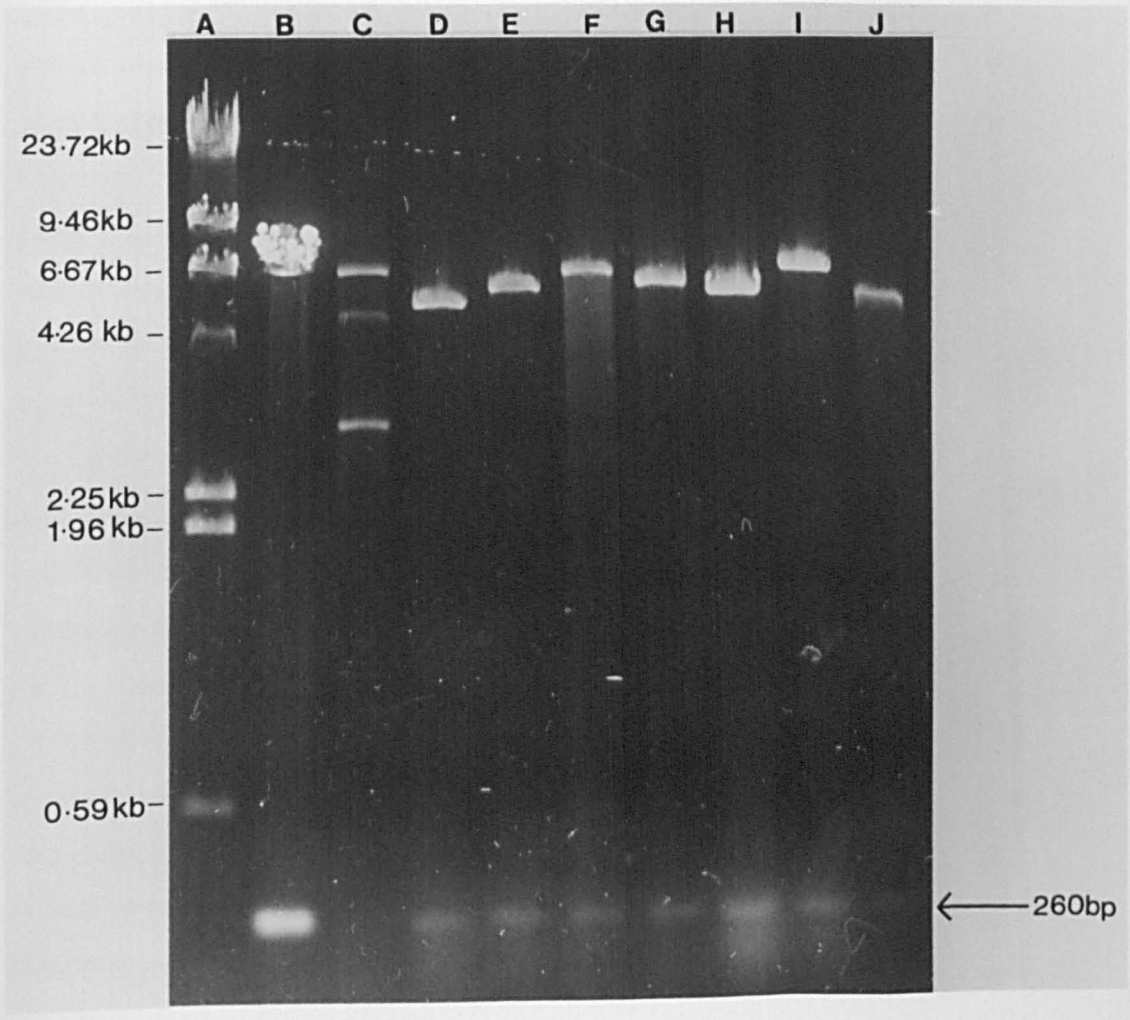
However, despite these problems, assays of CAT activity in strains harbouring the recombinant plasmids clearly suggested the cloned DNA fragment contained an SOS inducible promoter with similar induction properties to those previously characterised by means of the chromosomal *ruv*::Mu*d*(Ap)^{R}*lac* fusion. It seems reasonable to conclude that these are the same.

The main aim of subcloning the *ruv* promoter from the plasmid pPVA101 was to accurately locate it within the cloned DNA in pPVA101 in order to provide a starting point for nucleotide sequence determination. Since Tn*1000* insertions that inactivate the *ruv* gene had been mapped to a region spanning *Kpn*I site 1 and extending to the *Bgl*II site of pPVA101 (Chapter 4), and the direction of transcription of *ruv* had been shown to be from left to right in pPVA101 (Chapter 5), it was expected that the *ruv* promoter would be to the left of *Kpn*I site 1 of pPVA101. Since the only known restriction sites close to, and to the left of *Kpn*I site 1 of pPVA101 were a pair of *Eco*RV sites approximately 0.76kb and 1kb respectively to the left of the *Kpn*I site, plasmid DNA from the SOS inducible plasmid clones was digested with *Eco*RV, separated on an agarose gel, visualised and photographed.

The gel photograph presented in Figure 6.9 shows that all of the promoter clones carry the 240bp *Eco*RV fragment - which was subsequently shown to lack *Sau*3A restriction sites - suggesting that the *ruv* promoter was carried by the *Sau*3A fragment overlapping the two *Eco*RV sites. This placed the *ruv* gene promoter upstream of the

Figure 6.9

EcoRV restriction analysis of SOS inducible promoter clones. Lane A. HindIII digested wild type DNA. Lanes B-J EcoRV digested plasmid DNA. B.pFB512, C.pKK232.8, D.pFB1, E.pFB3, F.pFB4, G.pFB7, H.pFB12, I.pFB14, J.pFB17. Molecular weight markers are indicated at the left. The 260bp EcoRV fragment present in pFB512 and in the SOS inducible promoter clones, is arrowed.


A
B
C
D
E
F
G
H
I
J
23·72kb
9·46kb
6·67kb
4·26 kb
2·25kb
1·96 kb
0·59kb
260bp

position to which the region coding for the 24Kd protein had been mapped in pFB511 (Chapter 4), providing further evidence for the proposal that the expression of the 24Kd and the 41Kd proteins may both be regulated under SOS control from the same promoter.

6.7 Discussion

Studies of *ruv* gene expression using both the chromosomal *ruv*::Mud(Ap)^{R}*lac* fusion and the plasmid *ruv* promoter::CAT fusion presented in this chapter demonstrated that the *ruv* gene was regulated as part of the SOS response to DNA damage and confirmed its position in the group of genes so far including *recA*, *recN* and *recQ* required for inducible recombination repair of DNA damage. In addition, studies on expression of the *ruv* gene in chromosomal *ruv*::Mud(Ap)^{R}*lac* fusion strains suggested that like expression of *recN* (Picksley 1985), expression of *ruv* was not autoregulated.

Similar studies also demonstrated that the *ruv* gene product was not required for nalidixic acid induction of the SOS response, thus eliminating a possible role for the *ruv* gene product in the generation of SOS inducing signal in nalidixic acid treated cells, as had previously been demonstrated for the *recBC* gene products (Little and Mount 1982). The lack of induction of *ruv* gene expression by nalidixic acid was probably therefore due in part to the level of SOS induction produced by nalidixic acid being insufficient to induce expression of *ruv* and possibly partly due to the unknown effect on *ruv* transcription of decreased supercoiling in the promoter region brought about by nalidixic acid inhibition of the DNA gyrase A subunit (Gellert *et al.* 1977).

The increased expression of *sulA* and *uvrA* observed in strains carrying *ruv* mutations suggested that there may be a general increase in the basal level of expression of SOS controlled genes in *ruv* mutants, which may result from accumulation of unrepaired DNA

damage. In particular, the filamentation observed in strains carrying *ruv* mutations, which can be eliminated by a *sulA* mutation (Shurvinton 1983, Otsuji and Iyehara 1979) could be accounted for by the increased expression of *sulA*.

The promoter containing DNA fragments cloned into pKK232.8 to generate plasmids pFB1, pFB2, pFB4, pFB7, pFB12, pFB14, pFB17 and pFB22 regulated CAT production in a manner similar to the regulation of B-galactosidase production in strains carrying the *ruv*::Mud(Ap)^{R}*lac* fusion. Expression of CAT was induced approximately 3-fold by mitomycin C treatment of plasmid harbouring derivatives of wild type strains, but was prevented in strains carrying *lexA*(ind^{-}) or *recA*(Def) mutations. It was therefore concluded that the promoter directing CAT production in the various plasmid derivatives was in fact the same as the promoter regulating the B-galactosidase production in *ruv*::Mud(Ap)^{R}*lac* fusion strains, and that the DNA cloned in the plasmid derivatives therefore contained the *ruv* gene promoter. Restriction analysis of the cloned DNA allowed the *ruv* gene promoter to be mapped to a region approximately 0.5kb to 1kb to the left of *Kpn*I site 1 of pPVA101, which provided further evidence for the proposal that the 24Kd and 41Kd proteins may be coregulated. This possibility is readdressed in the final two chapters of this thesis.

CHAPTER 7

The effect of *ruv* mutations on F' transconjugant recovery

7.1 Introduction

During the course of experiments designed to study the effect of *ruv* mutations in the recipient on the recovery of recombinants in crosses with Hfr donors, it was discovered that in addition to reducing the recovery of recombinants in *recBC sbcBC* and *recBC sbcA* recipients, *ruv* mutation had a severe effect on the recovery of F' Pro^+ transconjugants, reducing the viable F' Pro^+ transconjugants obtained to 0.1% of the *recBC sbcBC* parent strain and to 1% of the *recBC sbcA* parent strain (Chapter 3). Since the *ruv* gene product was required for recombination in *recBC sbcBC* and *recBC sbcA* strains it was postulated that the inability to recover F' Pro^+ transconjugants in these strains with additional *ruv* mutations was due to a block at in recombination at a stage normally mediated by the *ruv* gene product.

However, on the basis of these results alone several other possible explanations cannot be excluded. The aim of studies presented in this chapter is to ascertain the role of the *ruv* product in the recovery of F' transconjugants in *recBC sbcBC* and *recBC sbcA* recipients.

7.2 DNA transfer into *ruv* recipients

Table 7.1 shows the absolute number of transconjugants obtained in crosses between F' Pro^+ donors and FB165 *recBC sbcBC*, FB 166 *recBC sbcBC ruv*-52, FB282 *recBC sbcA* and FB284 *recBC sbcA ruv*-52 recipients.

Since mutation in *ruv* resulted in a reduction in viability

Table 7.1 Recovery of F' Pro$^+$ transconjugants in *recBC sbcBC ruv-52* and *recBC sbcA ruv-52* strains

Recipient strain	Viability[a]	Relative viability[b]	xKL548 F' Pro$^+$ transconjugants[c]	xKL548 Relative F' Pro$^+$[d]	F' Pro$^+$ recovery deficiency[e]
FB165 *recBC sbcBC*	9.25×10^7	1	1.71×10^7	1	1
FB166 *recBC sbcBC ruv-52*	3.9×10^6	0.042	2.3×10^3	0.000134	0.0032
FB282 *recBC sbcA*	1.6×10^8	1	8.95×10^6	1	1
FB284 *recBC sbcA ruv-52*	7.05×10^7	0.44	2.4×10^5	0.027	0.06

Experiments were performed with a 1:4 ratio of donor cells to recipient cells as described in 2.8.

a) Viable cells per ml of recipient strain (prior to mixing with donor) determined on supplemented minimal media.
b) Viability of *ruv* derivatives relative to *ruv*$^+$ parent strain.
c) Numbers of F' Pro$^+$ transconjugants per ml of mating mixture.
d) Yield of F' Pro$^+$ transconjugants relative to *ruv*$^+$ parent strain.
e) Relative F' Pro$^+$ transconsjugant yield corrected for observed viability deficiency of *ruv* strains.

compared with the ruv$^+$ parent strains, yields of transconjugants are shown relative to the parent strain and are then corrected for the observed viability deficiency. As was previously demonstrated in Chapter 3, the recovery of F' Pro$^+$ transconjugants was significantly reduced by mutation in ruv to approximately 0.3% and 6% of the recBC sbcBC and recBC sbcA parent strains respectively, even when allowing for the reduced viability of the ruv$^-$ derivatives. In addition the F' Pro$^+$ transconjugants obtained in crosses with both recBC sbcBC ruv and recBC sbcA ruv recipients were considerably slower growing than those obtained with the equivalent ruv$^+$ strain - more so than could be attributed simply to the viability difference between the two strains.

Since these results could imply that recBC sbcA ruv and recBC sbcB ruv recipients were defective for DNA transfer the efficiency of transfer of the R-plasmids R6K, R1 and R136, which encode the same conjugation system as F (Jacob et al. 1977), but which have no known homology with the E. coli chromosome, was examined. Plasmid-bearing transconjugants were selected on the basis of their encoded resistance to tetracycline for R136 and to ampicillin for R6K and R1, donors were counterselected by addition of streptomycin.

Data presented in Table 7.2 shows that the yields of R-plasmid transconjugants obtained in crosses between R6K, R1 and R136 harbouring donors and recBC sbcBC and recBC sbcA recipients were not greatly affected by additional mutations in ruv, the relative recovery of plasmid transconjugants in the strains carrying ruv mutations was between 28% and 100% of that obtained with the parent strains. In all cases growth of the transconjugant colonies was similar with ruv$^+$ and ruv$^-$ recipients.

Efficient recovery of R-plasmid transconjugants in these strains suggested that they were not defective as recipients for DNA transfer, and therefore that the greatly reduced recovery of F' Pro$^+$ transconjugants was unlikely to be due to a deficiency in the

Table 7.2 Recovery of R plasmid transconjugants in crosses with FB165 *recBC sbcBC* and FB166 *recBC sbcBC ruv-52* recipients

Donor			PA1011 (R6K)	PA1012 (R1)	N1722 (R136)
Selection		Viability	Ap^R	Ap^R	Tc^R
Recipient					
FB165 *recBC sbcBC*	a.	3.6×10^8	1.1×10^5	5.3×10^5	1.16×10^6
	b.	1	1	1	1
FB166 *recBC sbcBC ruv-52*	a.	6.05×10^7	2.7×10^4	7.17×10^4	5.4×10^4
	b.	0.16	0.24	0.13	0.046
	c.		> 1	0.85	0.28

Experiments were performed as described in 2.8 with a 1:4 ratio donor:recipient cells. At the end of the mating period the mixtures were vortexed then incubated for 1 hour with vigorous shaking to allow expression of antibiotic resistance genes.

Viable cells per ml of the recipient strain were determined on Mu plates.

Figures in lines (a) represent the numbers of viable cells or transconjugants obtained per ml of mating mixtures. Figures in line (b) represent the recovery of transconjugants in *ruv* derivatives relative to the ruv^+ parent strain and in line (c) the figure in line (b) corrected for the observed viability deficiency of the *ruv* strain.

recovery of transferred DNA in the recipient.

The difference in recovery of R-plasmid transconjugants and F' Pro$^+$ transconjugants in crosses with recBC sbcBC ruv recipients could reflect the different capacities of the transferred plasmid to undergo recombination in the recipient strain. R-plasmids have no known homology with the chromosome and are not therefore substrates for homologous recombination with the chromosome in the recipient, whereas F' Pro$^+$ has homology with the recipient chromosome and may therefore be a substrate for initiation of recombination in the recipient. Since ruv reduced the recovery of recombinants in recBC sbcBC recipients (Chapter 3) then it would perhaps be expected to reduce the number of transconjugants obtained if recombination were initiated between the transferred DNA and the recipient chromosome.

Although the above experiments clearly demonstrated that recBC sbcBC ruv recipients were proficient in recovery of transferred R6K and R1 DNA, the possibility remained that the inability to recover F' Pro$^+$ transconjugants was an effect specifically associated with the inability to recover transferred F plasmid DNA.

In order to eliminate this possibility, the transfer of a mini-F plasmid containing a Kanamycin resistance gene marker in to recBC sbcBC ruv and recBC sbcA ruv recipient strains was examined. The mini F-plasmid pOX38::mini Kan is a deletion derivative of F that lacks the natural insertion sequences of F and thus has no homology with the E. coli chromosome (Guyer et al. 1981). It has, however, a Kanamycin resistance gene marker, allowing it to be selected in recipient strains (Way et al. 1984).

Data presented in Table 7.3 (which unfortunately does not include a viability estimate) demonstrates that the mini F::Kan plasmid is transferred between 15% and 40% as efficiently to UV derivatives of recBC sbcBC and recBC sbcA strains as to the parent ruv$^+$ strains. Clearly if a correction for the reduced viability of the ruv derivatives were made, there would be no difference in

Table 7.3 Recovery of pOX38::mini F Km^R in *ruv* derivatives of *recBC sbcBC* and *recBC sbcA* strains

Recipient	xN2392 Km^R transconjugants	Relative yield Km^R transconjugants
FB165		
recBC sbcBC	4.1×10^6	1
FB166		
recBC sbcBC ruv-52	1.4×10^6	0.33
FB167		
recBC sbcBC ruv-54	5.1×10^5	0.12
FB168		
recBC sbcBC ruv	1.15×10^6	0.28
FB282		
recBC sbcA	1×10^7	1
FB283		
recBC sbcA ruv-53	3.1×10^6	0.31
FB284		
recBC sbcA ruv-52	4.85×10^6	0.485
FB285		
recBC sbcA ruv-54	4.35×10^6	0.435

Experiments were performed as described in 2.8 using a 1:4 ratio of donors to recipients. An expression time of 50' was allowed at the end of the mating period before plating in Mu overlay agar on Km Str plates. No viability control was included.

recovery of pOX38::mini Kan in the *ruv*$^+$ and *ruv*$^-$ strains respectively, thus confirming that the transfer of F DNA into these strains is unaffected by *ruv* mutation.

Since the inability to recover F' Pro$^+$ transconjugants does not appear to be due to a defect in DNA transfer into recipient *recBC sbcBC ruv* and *recBC sbcA ruv* strains, the question arose as to whether this effect was observed with F' plasmids bearing regions of the chromosome other than the *proAB* region or whether the effect was specific for F' 128 (Pro$^+$).

It has been reported that close to *proA* on the *E. coli* genetic map there exists a region of the chromosome designated *fre-1* (for frequent recombination exchanges) which increases the frequency of *recF* dependent recombination exchanges, relative to the rest of the map (Bresler, Krivonogov and Lanzov 1981). This presents the possibility that in crosses between F' Pro$^+$ donors and *recBC sbcBC* and *recBC sbcA* recipients, recombination is initiated at a higher frequency than with other F' donors, and that recovery of F' Pro$^+$ transconjugants may be prevented in *ruv* derivatives since the Ruv product is required for the recovery of recombinants in these strains.

In order to investigate this possibility, the recovery of F' transconjugants in crosses between donors carrying F' 101 (Thr$^+$ Leu$^+$) donors and *recBC sbcBC ruv* recipients was examined.

Data presented in Table 7.4 shows that recovery of F' Thr$^+$ Leu$^+$ transconjugants is reduced in *ruv* derivatives of *recBC sbcBC* strains to approximately the same extent as was previously shown for F' Pro$^+$ recovery. This demonstrated that the deficiency in recovery of F' transconjugants in *ruv* derivatives of *recBC sbcBC* strains was not simply a consequence of the transferred Pro$^+$ region initiating abortive recombination at high frequency in the recipient.

Thus, the inability to recover F' transconjugants in *recBC sbcA ruv* or *recBC sbcBC ruv* strains appeared not to be due to either a

Table 7.4 Recovery of F' Thr$^+$ Leu$^+$ transconjugants in FB165 *recBC sbcBC* and FB166 *recBC sbcBC ruv* recipient strains

Recipient	Viability[a]	Relative viability[b]	xFB356 [c] F' Thr$^+$ Leu$^+$	xFB356 [d] Relative F' Thr$^+$ Leu$^+$	F' Thr$^+$ Leu$^+$ [e] recovery deficiency
FB165 *ruv*$^+$	1.1×10^8	1	6.03×10^6	1	1
FB166 *ruv-52*	1.25×10^7	0.11	840	0.00014	0.0013

Experiments were performed with a 1:4 ratio of donor:recipient cells as described in 2.8.

a) Recipient viable cells per ml (prior to mixing with donor) were determined on minimal media.
b) Viability relative to *ruv*$^+$ parent strain.
c) Number of F' Thr$^+$ Leu$^+$ transconjugants obtained per ml of mating mixture.
d) Yield of F' Thr$^+$ Leu$^+$ transconjugants relative to *ruv*$^+$ parent strain.
e) Relative F' Thr$^+$ Leu$^+$ transconjugant yield corrected for observed viability deficiency of *ruv* strains.

defect in F' DNA transfer or to a specific effect associated with the transferred Pro^+ region of the chromosome.

The possibility therefore remained that the reduction in recovery of F' transconjugants in *ruv* derivatives of *recBC sbcA* strains to between 1% and 10% and of *recBC sbcBC* strains to between 0.1% and 1% of the parent strains respectively, reflected the relative contribution of the Ruv protein in the production of viable recombinants in these genetic backgrounds. This was supported by earlier observations (Chapter 3, Table 3.3 and 3.4) that show that an *ruv* mutation had an approximately 10 fold greater effect in reducing the recovery of recombinants in crosses with Hfr donors in *recBC sbcBC* recipients compared with *recBC sbcA* recipients.

7.3 F' transfer into recipients with altered capacities for recombination

Recombination between transferred F' plasmids and the recipient chromosome has been shown to occur at high frequency even in *recBC* strains (Hall and Howard-Flanders 1972). However, since mutation in *recA* had no effect on the recovery of F' transconjugants it was concluded that recombination was not required for F' recovery. However, although such a conclusion is valid in the case of *recA* mutants, this may not be so in wild type strains. The possibility that in $recA^+$ strains recombination is normally initiated between transferred DNA and the recipient chromosome and that completion of recombination is required for the recovery of viable F' transconjugants cannot be eliminated on the basis of these results.

In order to examine whether the Ruv product is required for recovery of transconjugants in those situations in which recombination is not initiated in the recipient, the transfer of F' Pro^+ into *recBC sbcA ruv* and *recBC sbcBC ruv* recipients carrying an additional *recA* mutation was studied.

Table 7.5 Recovery of F' Pro$^+$ transconjugants in *recA*::Tn*10* derivatives of *recBC sbcA ruv* recipients

Recipient[a]	Viability[b]	Relative Viability[c]	xKL548 Pro$^+$ [d]	xKL548 Relative Pro$^+$ [e]	F' Pro$^+$ recovery deficiency [f]
FB282 *ruv*$^+$	1.6×10^8	1	8.9×10^6	1	1
FB284 *ruv-52*	7.05×10^7	0.44	2.3×10^5	0.025	0.058
FB285 *ruv-54*	5.3×10^7	0.33	1.1×10^5	0.012	0.038
FB387 *ruv*$^+$ *recA*::Tn*10*	1.1×10^8	0.68	6×10^6	0.68	1
FB388 *ruv-52 recA*::Tn*10*	7.9×10^7	0.49	5.6×10^6	0.625	> 1
FB389 *ruv-54 recA*::Tn*10*	9.5×10^7	0.59	3.1×10^6	0.34	0.58

Results presented are means of two experiments performed as described in 2.8 using a 1:4 donor:recipient ratio.

a) All recipient strains are *recBC sbcA* derivatives.
b) Viability of recipient cells prior to mixing with donors was determined on minimal agar plates.
c) Viability relative to FB282 *recBC sbcA*.
d) Projected number of F' Pro$^+$ transconjugants obtained per ml of mating mixture.
e) Yield of F' Pro$^+$ transconjugants relative to yield in FB282 recipient.
f) Relative F' Pro$^+$ transconjugant yield corrected for measured viability deficiency compared with FB282.

Table 7.6 The effects of *recA* mutations in *recBC sbcBC ruv* recipients, on the recovery of F' Pro$^+$ transconjugants

Recipient[a]	Viability[b]	Relative Viability[c]	xKL548 Pro$^+$ [d]	xKL548 Relative Pro$^+$ [e]	F' Pro$^+$ recovery deficiency [f]
FB165 *ruv*$^+$	1.3×10^8	1	4×10^6	1	1
FB166 *ruv-52*	2.8×10^7	0.2	1.9×10^3	0.00047	0.0023
FB167 *ruv-54*	3.5×10^7	0.27	650	0.00016	0.00059
FB434 *ruv*$^+$ *recA*	9×10^6	0.07	3.5×10^5	0.0875	> 1
FB435 *ruv-52 recA*	2.4×10^7	0.18	5.5×10^5	0.1375	0.76
FB437 *ruv-54 recA*	4.6×10^7	0.35	1×10^6	0.25	0.71

Experiments were performed as described in 2.8 with a 1:4 donor:recipient ratio.

a) All strains are *recBC sbcBC* derivatives.
b) Viability of recipient cells was determined on minimal agar plates.
c) Viability relative to FB165 *recBC sbcBC*.
d) Number of F' Pro$^+$ transconjugants obtained per ml of mating mixture.
e) Yield of F' Pro$^+$ transconjugants relative to yield in FB165 recipient.
f) Relative F' Pro$^+$ yield corrected for observed viability deficiencies compared with FB165.

Data presented in Tables 7.5 and 7.6 show that the recovery of transconjugants obtained in crosses with recBC sbcA ruv and recBC sbcBC ruv recipient strains was restored to the level of the parental ruv$^+$ strains by an additional mutation in recA, which prevents recombination initiation.

These results suggest the Ruv product is only required for recovery of F' transconjugants in recipients in which recombination is initiated. In addition since the effect on recovery of F' transconjugants is so great (reducing it to 0.1% of the ruv$^+$ parent in the case of recBC sbcBC strains) these results imply that recombination between the transferred F' and the recipient chromosome is a very common event and may even be obligatory in recA$^+$ recipients.

In order to further study the recombination between transferred F' DNA and the recipient chromosome, the transfer of F' Pro$^+$ into a series of strains harbouring the recA200 allele was investigated. The recA200 allele encodes a temperature sensitive RecA protein that is able to promote recombination at low temperature (32°C) at approximately 50% of the level of the wild type RecA protein, but is almost completely defective at higher temperature (42°C) reducing the recovery of recombinants to less than 0.10% of equivalent recA$^+$ strains (Lloyd et al. 1974).

F' donor strains were grown to approximately 2 x 10^8 cells per ml in Mu broth at 37°C, mixed in a 1:4 ratio with recipient strains grown to the same cell density in Mu broth at 32°C, 40°C or 42°C and incubated with slow shaking at the appropriate temperature for 30'.

Mating mixtures were then vortexed vigorously and suitable dilutions (prepared in buffer held at the appropriate temperature) plated out in 0.75% water agar to which a trace of Mu broth had been added, on to prewarmed selective plates. Plates were incubated for 48-72 hours before scoring transconjugant colonies.

Results of initial experiments to investigate the effect of ruv

mutations on the transfer of F' Pro$^+$ into *recBC sbcBC recA200* strains at 32°C and 42°C are presented in Table 7.7. These results show that at 42°C the *ruv* mutation had no effect on the recovery of F' Pro$^+$ transconjugants; comparable yields of rapidly growing Pro$^+$ transconjugants were obtained irrespective of whether strains were *ruv*$^+$ or *ruv*$^-$, providing further evidence that the Ruv protein was only required for recovery of transconjugants when recombination was initiated between the transferred F' DNA and the recipient chromosome. At 32°C, although the yields of transconjugants in the *ruv* derivatives were reduced to approximately 25% of the recovery in the control *ruv*$^+$ strain and transconjugant colonies were smaller and weaker than could be accounted for simply by the differences in the relative viabilities of the *ruv*$^+$ and *ruv*$^-$ strains, it was clear that the reduction in transconjugant recovery was by no means as large as that previously observed in *recBC sbcBC ruv* recipient strains. This suggested that the RecA200 protein may be sufficiently deficient even at 32°C to prevent significant initiation of recombination between the transferred F' DNA and the recipient chromosome as had previously been reported (Lloyd and Low 1974).

In an attempt to eliminate this problem by overproducting the RecA200 protein, a *recAO*^{c} mutation was introduced into strains which already had the *recA200* allele; it was hoped that overproduction of the protein, slightly defective at 32°C may compensate for its deficiency and allow recombination to proceed between transferred plasmid and recipient chromosome as in normal *recA*$^+$ derivatives.

Table 7.8 shows the effect of the *ruv*-52 mutation on the recovery of F' transconjugants in *recBC sbcBC recAO*^{c} *recA200* recipients at 32°C. The results demonstrated that the Ruv product was required for transconjugant recovery at 32°C in strains in which the RecA200 protein was overproduced due to an additional *recAO*^{c} mutation. However, the effect of *ruv* mutations on the recovery of transconjugants at 42°C could not be examined since these strains

Table 7.7 Recovery of F' Pro$^+$ transconjugants in recBC sbcBC recA200 ruv recipients at 32°C and 42°C

Selection		Viability on[a] minimal media	Relative[b] viability	xKL548 F' Pro$^+$ [c] transconjugants	Relative F' Pro$^+$ [d] transconjugants	Deficiency in F' Pro$^+$ [e] transconjugant recovery
Recipient and temperature						
FB415 ruv$^+$	32°C	7.6×10^7	1	3.9×10^6	1	1
	42°C	2.5×10^7	0.33	7.9×10^5	0.20	0.61
FB416 ruv-52	32°C	2.8×10^7	0.36	3.5×10^5	0.089	0.24
	42°	2.5×10^7	0.33	5.8×10^5	0.15	0.45
FB417 ruv-53	32°C	1.11×10^7	0.144	1.15×10^5	0.029	0.20
	42°C	1.8×10^7	0.23	4.5×10^5	0.11	0.50
FB418 ruv-54	32°C	6.1×10^7	0.9	9×10^5	0.23	0.25
	42°C	3.2×10^7	0.42	8.66×10^5	0.222	0.52

Experiments were performed as described in 7.3. All recipient strains are recBC sbcBC recA200 derivatives.

a) Viability of recipient strains prior to mixing with donor was determined on prewarmed supplemented minimal 56/2 plates.
b) Viability relative to FB415 at 32°C.
c) Numbers of F' Pro$^+$ transconjugants per ml of mating mixtures.
d) Yield of F' Pro$^+$ transconjugants relative to yield of F' Pro$^+$ transconjugants on FB415 at 32°C.
e) Relative yield F' Pro$^+$ transconjugants, corrected for observed viability deficiency.

Table 7.8 Recovery of F' Pro$^+$ transconjugants in FB322 *recBC sbcBC recAo*^{C} *recA200* and FB325 *recBC sbcBC recAo*^{C} *recA200 ruv* at 32°C

Recipient		Viability [a]	Relative Viability [b]	xKL548 F' Pro$^+$ transconjugants [c]	Relative F' Pro$^+$ transconjugants [d]	Deficiency in F' Pro$^+$ transconjugant recovery [e]
FB322 *ruv*$^+$	32°C	1.42×10^8	1	7.2×10^6	1	1
FB325 *ruv-52*	32°C	9.4×10^6	0.066	100	0.000014	0.00021

Strains were *recBC sbcBC recAo*^{C} *recA200* derivatives. Experiments were performed as described in 7.3 using a 1:4 ratio donors:recipients.

a) Viability of recipient strains was determined prior to mixing with donor on supplemented minimal media plates.
b) Viability relative to FB322 at 32°C.
c) Numbers of F' Pro$^+$ transconjugants per ml of mating mixture.
d) Yield of F' Pro$^+$ transconjugants relative to yield with FB322 at 32°C.
e) Relative yield at F' Pro$^+$ transconjugants corrected for observed viability deficiency.

exhibit a severe viability problem at this temperature.

In an attempt to circumvent this problem, the recovery of F' transconjugants in these strains at 40°C was investigated. Results presented in Table 7.9 demonstrate that the Ruv gene product was required for recovery of F' transconjugants in recBC sbcBC recA0^{c} recA200 strains at 40°C. This was at first somewhat surprising, since it was expected that the mutant RecA200 protein would prevent the initiation of recombination between the transferred F' DNA and the recipient chromosome and therefore the Ruv product would not be required for the recovery of F' transconjugants. However, a comparison of the UV irradiation sensitivity of these strains demonstrated that they were no more sensitive to UV at 40°C than at 32°C suggesting they were not phenotypically RecA^{-} at this temperature. Once again it appeared that overproduction of the RecA200 protein by means of the recA0^{c} mutation was somehow compensating for the deficiency of the RecA200 protein in promoting recombination between the transferred F' DNA and the recipient chromosome, which therefore prevented the recovery of F' transconjugants in ruv derivatives.

Thus, the studies on the effects of ruv mutations on the recovery of F' transconjugants in recBC sbcBC strains carrying recA200 mutations gave rise to a rather complex set of results:- (i) At 32°C, recovery of F' transconjugants was almost as efficient in ruv^{-} strains as in ruv^{+} strains (Table 7.7), unless the strains carried an additional recA0^{c} mutation, in which case recovery of F' transconjugants was reduced to the levels previously observed in recBC sbcBC ruv strains (Table 7.8). (ii) At 42°C, recovery of F' transconjugants was as efficient in ruv^{-} strains as in ruv^{+} (Table 7.7), providing further evidence that the ruv gene product was only required for F' transconjugant recovery when recombination is initiated in the recipient. At 42°C recBC sbcBC recA0^{c} recA200 strains were inviable. At 40°C recBC sbcBC recA0^{c} recA200 strains

Table 7.9 Recovery of (i) F' Pro$^+$ and (ii) F' Thr$^+$ Leu$^+$ transconjugants in *ruv* derivatives of *recBC sbcBC recA0*^{c} *recA200* strains at 32°C and 40°C

Recipient and temperature		Viability [a]	Relative Viability [b]	xKL548 F' Pro$^+$ transconjugants [c]	Relative F' Pro$^+$ transconjugants [d]	Deficiency in F' Pro$^+$ transconjugant recovery [e]
(i)						
FB322	32°C	7.8×10^7	1	6.2×10^6	1	1
ruv$^+$	40°C	1.03×10^8	1.32	4.8×10^6	0.72	0.55
FB323	32°C	2.5×10^6	0.03	< 100	< 0.000016	< 0.0005
ruv-53	40°C	7.4×10^6	0.095	1.5×10^3	0.00024	0.0025
FB325	32°C	1.2×10^6	0.015	200	0.000032	0.0021
ruv-52	40°C	3×10^6	0.038	2.4×10^3	0.00039	0.0101
(ii)						
FB322	32°C	7.4×10^7	1	3.6×10^6	1	1
ruv$^+$	40°C	4.1×10^7	0.55	2.8×10^6	0.772	> 1
FB324	32°C	7×10^6	0.094	435	0.00012	0.0013
ruv-54	40°C	1.06×10^7	0.14	235	0.000065	0.00046

Experiments were performed as described in 2.8 and text 7.3. All recipient strains were *recBC sbcBC recA0*^{c} *recA200* derivatives.

a) Viability of recipient strains prior to mixing with donors was determined on prewarmed supplemented 56/2 plates.
b) Viability relative to FB322 at 32°C in appropriate sets of results.
c) Numbers of (i) F' Pro$^+$ (ii) F' Thr$^+$ Leu$^+$ transconjugants per ml of mating mixture.
d) Yield of (i) F' Pro$^+$ (ii) F' Thr$^+$ Leu$^+$ transconjugants relative to yield of (i) F' Pro$^+$ (ii) F' Thr$^+$ Leu$^+$ transconjugants with FB322 at 32°C.
e) Relative yield of (i) F' Pro$^+$ (ii) F' Thr$^+$ Leu$^+$ transconjugants corrected for observed viability deficiency.

were not phenotypically RecA$^-$ as had been expected. Recovery of F' transconjugants in these strains was severely reduced by an ruv mutation (Table 7.9).

Results presented in Table 7.10 show that the recA200 allele alone had a similar effect on F' Pro$^+$ recovery in recBC sbcA ruv strains at 32^{o}C and 42^{o}C as had been demonstrated in recBC sbcBC ruv strains (Table 7.7). At 32^{o}C, although the yield of F' transconjugants was reduced in recBC sbcA recA200 ruv strains to between 30% and 60% of the equivalent ruv$^+$ strain, this reduction was not as great as had previously been observed in recBC sbcA ruv strains (Table 7.1) once again suggesting that the RecA200 protein may be deficient at promoting recombinants between transferred F' DNA and the recipient chromosome, even at this temperature. At 42^{o}C the yield of F' transconjugants was not affected by an ruv mutation, again suggesting the ruv gene product was only required for F' transconjugant recovery in those recipients undergoing recombination between transferred F' DNA and the recipient chromosome.

Data presented in Table 7.11 demonstrate that, as with derivatives of recBC sbcBC recA200 strains, the introduction of the recAO^o mutation into recBC sbcA recA200 strains (resulting in overproduction of the RecA200 protein), appeared to compensate for the deficiency of the RecA200 protein in promoting recombination between F' DNA and the recipient chromosome at 32^{o}C, resulting in a reduction in the recovery of F' Pro$^+$ transconjugants in ruv derivatives to less than 2% of the recovery in equivalent ruv$^+$ strains, similar to the reduction in F' Pro$^+$ transconjugant yield previously observed in ruv derivatives of recBC sbcA strains.

In contrast to recBC sbcBC recAO^c recA200 strains recBC sbcA recAO^c recA200 strains were pefectly viable at 42^{o}C, and in addition were phenotypically RecA$^-$. Data presented in Table 7.10 show that at 42^{o}C, F' transconjugant recovery was at least as good as in ruv$^+$ strains at 32^{o}C and was independent of an ruv mutation, once again

Table 7.10 Recovery of F' Pro$^+$ transconjugants in *recBC sbcA recA200 ruv* strains at 32°C and 42°C

Recipient and temperature		Viability [a]	Relative Viability [b]	xKL548 F' Pro$^+$ transconjugants [c]	Relative F' Pro$^+$ transconjugants [d]	Deficiency in F' Pro$^+$ transconjugant recovery [e]
FB318	32°C	1.6×10^8	1	6.1×10^5	1	1
ruv$^+$	43°C	2.9×10^7	0.18	1.6×10^5	0.26	> 1
FB320	32°C	1.4×10^7	0.0875	3×10^4	0.049	0.56
ruv-52	42°C	4.2×10^7	0.26	5.9×10^5	0.96	> 1
FB321	32°C	1.1×10^8	0.68	1.4×10^5	0.23	0.33
ruv-54	42°C	6.6×10^7	0.41	4.1×10^5	0.67	> 1

Experiment was performed only once as described in 2.8 and text 7.3. All recipient strains were *recBC sbcA recA200* derivatives.

a) Viability of recipient strains prior to mixing with donors was determined on prewarmed supplemented 56/2 plates.
b) Viability relative to FB318 at 32°C.
c) Number of F' Pro$^+$ transconjugants per ml of mating mixture.
d) Yield of F' Pro$^+$ transconjugants relative to yield of F' Pro$^+$ transconjugants with FB318 at 32°C.
e) Relative yield of F' Pro$^+$ transconjugants corrected for observed viability deficiency.

Table 7.11 Recovery of F' Pro$^+$ transconjugants in *recBC sbcA recA0*^{C} *recA200 ruv* strains at 32°C and 42°C

Recipient and temperature		Viability[a]	Relative viability[b]	xKL548 F' Pro$^+$ transconjugants[c]	Relative F' Pro^{+d} yield	F' Pro$^+$ recovery[e] deficiency
FB311 *ruv*$^+$	32°C	1.1×10^8	1	4.1×10^6	1	1
	42°C	5.1×10^7	0.45	5.9×10^6	>1	>1
FB312 *ruv-52*	32°C	8.2×10^7	0.74	2.6×10^4	0.0063	0.0099
	42°C	6.5×10^7	0.59	5.4×10^6	>1	>1

Experiments were performed as described in 2.8 and text 7.3 Both strains were *recBC sbcA recA0*^{C} *recA200* derivatives.

a) Viable recipient cells per ml prior to mixing with donor was determined on prewarmed supplemented 56/2 plates.

b) Viability relative to FB311 at 32°C.

c) Numbers of F' Pro$^+$ transconjugants per ml of mating mixture.

d) Yield of F' Pro$^+$ transconjugants relative to FB311 at 32°C.

e) Relative yield of F' Pro$^+$ transconjugants corrected for observed viability deficiency.

suggesting the ruv gene product was required for F' Pro$^+$ recovery only when recombination was initiated in the recipient.

Thus, the above studies on F' transfer into strains carrying the recA200 allele provide further confirmation that ruv is required for F' recovery in recBC sbcBC and recBC sbcA strains only when recombination is initiated in the recipient. If the large deficiency in recovery of F' transconjugants in these ruv recipients is due to their inability to produce viable products from recombination intermediates due to an absence of the Ruv protein, then it follows that recombination must be initiated at high frequency between the transferred F' DNA and the recipient chromosome.

In order to demonstrate conclusively the transfer of F' DNA into recipients in which the recovery of F' transconjugant colonies was prevented by mutation in ruv, and to study the kinetics of recombination between transferred F' and recipient chromosome, the effect of mating F' donors with recBC sbcA recAO^c recA200 ruv$^{+/-}$ recipients at 32^{o}C and plating out at 42^{o}C and vice versa, on the recovery of transconjugant colonies was investigated.

Results presented in Table 7.12 show that an FB312 recBC sbcA recAO^c recA200 ruv-52 recipient mated with KL548 F' Pro$^+$ at 32^{o}C and plated out at 42^{o}C was able to form transconjugant colonies with the same efficiency as if the strain had been both mated and plated at 42^{o}C, whilst the same mixture plated out at 32^{o}C yielded F' transconjugants with a greatly reduced efficiency to approximately 0.1% of the equivalent ruv$^+$ strain. Conversely a mating between the same donor and recipient at 42^{o}C yielded transconjugants efficiently only when the mixture was plated at 42^{o}C, the yield was reduced by plating the mixture at 32^{o}C to approximately the same level as was obtained in the matings both conducted and plated at 32^{o}C. These results then conclusively demonstrated that DNA was transferred into strain FB312 recBC sbcA recAO^c recA200 ruv at 32^{o}C, since incubation

Table 7.12 Recovery of F' Pro$^+$ transconjugants in *recBC sbcA recAo^c recA200 ruv* strains mated and plated at 32°C and 42°C

Recipient and mating temperature		Plating temperature		Viability	Relative viability	xKL548 F'Pro$^+$ transconjugants	Relative yield F' Pro$^+$	F' Pro$^+$ recovery deficiency
FB311 *ruv$^+$*	32°C	(i)	32°C	8.5×10^7	1	4.6×10^6	1	1
		(ii)	42°C			6.1×10^6	1.3	1.3
FB311 *ruv$^+$*	42°C	(i)	32°C			3.9×10^6	0.84	2
		(ii)	42°C	3.5×10^7	0.41	3.5×10^7	0.76	1.8
FB312 *ruv-52*	32°C	(i)	32°C	7.9×10^7	0.92	4.6×10^3	0.001	0.0011
		(ii)	42°C			2.1×10^6	0.45	0.49
FB312 *ruv-52*	42°C	(i)	32°C			4.3×10^3	0.0009	0.0013
		(ii)	42°C	6.1×10^7	0.71	3.2×10^6	0.69	0.97

Experiments were performed as described in 2.8 and text 7.3. Both strains were *recBC sbcA recAo^c recA200* derivatives.

a) Viable cells/ml of recipient strains was determined, prior to mixing with donor on supplemented 56/2 plates prewarmed to the mating temperature.
b) Viability of strains relative to viability of FB311 at 32°C.
c) F' Pro$^+$ transconjugant yield per ml mating mixture plated at appropriate temperature.
d) Yield of F' Pro$^+$ transconjugants relative to yield with FB311 at 32°C.
e) Relative yield F' Pro$^+$ transconjugants corrected for observed viability deficiency.

at higher temperature resulted in the efficient recovery of transconjugant colonies. In addition these results suggested that recombination between transferred F' and recipient chromosome was not initiated immediately after DNA transfer, but was a slow process, which may not even have been initiated until mating aggregates were disrupted.

The above studies utilising strains with recA13, recA200 and recAO^{c} recA200 mutations were designed to investigate whether eliminating recombination in the recipient allowed recovery of F' transconjugants in recBC sbcA ruv and recBC sbcB ruv strains. The above evidence clearly suggests that this is the case. However, an alternative explanation is that it is the effect of recA mutation on preventing the expression of SOS genes in the recipient, that somehow allows recovery of F' transconjugants in ruv derivatives. Unfortunately, no lexA(ind^{-}) derivatives of recipients were available to investigate this possibility.

7.4 Plasmid mobilisation into ruv strains

A phenomenon which may be related to the deficiency of F' transconjugant recovery was reported earlier in this thesis (Chapter 4). Initial attempts to isolate Tn1000 insertions in the ruv gene by mobilisation of the plasmid pPVA101 into an N1057 ruvA4 recipient failed. Subsequently ruv::Tn1000 insertions were isolated in pPVA101 by mobilising the plasmid into an ruv^{+} recipient, preparing plasmid DNA from pooled pPVA101 transconjugants and transforming DNA into an ruvA4 strain. This led to the proposal that ruv function was required for the resolution of the transferred cointegrate to viable products.

To test this proposal, the F' mediated mobilisation of pBR322 into AB1157 ruv^{+}, CS 81 ruv-52 and N1057 was examined. Donor strain N1103 pBR322 grown to approximately 2 x 10^{8} cells/ml was mixed in a

1:4 ratio with the appropriate recipient strain and allowed to mate for 1 hour at 37°C. Mating aggregates were then disrupted by vortexing vigorously, excess Mu broth added and the mixtures incubated with vigorous shaking for a further 2 hours prior to plating out appropriate dilutions onto selective plates containing tetracycline and streptomycin.

The results presented in Table 7.13 show that the recovery of pBR322 transconjugants in strains CS81 *ruv*-52 and N1057 *ruvA4* was reduced to less than 1% of the recovery obtained with an AB1157 *ruv*$^+$ recipient, suggesting that the *ruv* gene product plays some role in the recovery of plasmid transconjugants in recipient cells. The data presented in Table 7.13 also demonstrate that the deficiency in recovery of pBR322 transconjugants in strains carrying *ruv* mutations was suppressed by an additional mutation in *recA*, but was not affected by a *lexA*(ind$^-$) mutation, suggesting that it was the block in recombination rather than the block in SOS induction produced by the *recA* mutation that resulted in suppression of the deficiency in recovery of plasmid transconjugants in *ruv* mutants.

The inability to recover plasmid transconjugants in *ruv* mutants could be due to either of two main possibilities: (i) the *ruv* gene product is required as a host factor to promote intramolecular recombination at the *res* sites of the Tn*1000* present in direct repeat within the transferred F'::Tn*1000*::pBR322 cointegrate, (ii) the *ruv* gene product is required to produce viable products from recombination intermediates produced by the initiation of homologous recombination between the transferred F'::Tn*1000*::pBR322 cointegrate and the recipient chromosome.

Although earlier studies had demonstrated that the *in vitro* resolution of synthetic cointegrates containing directly repeated Tn*1000* elements was dependent only on the presence of the Tn*1000* *tnpR* gene product and did not require any additional host factors (Reed 1981), the *in vivo* resolution of synthetic cointegrates in

Table 7.13 Mobilisation of pBR322 into strains carrying *ruv* mutations

Recipient strain	Viability[a]	Relative viability[b]	xN1103 pBR322 Tc^R transconjugants[c]	Relative recovery Tc^R transconjugants[d]	pBR322 transconjugant recovery deficiency[e]
AB1157 *ruv*$^+$	2.2×10^8	1	1.9×10^4	1	1
HI24 *ruvA4*	7.3×10^7	0.33	1.8×10^1	0.00095	0.003
CS81 *ruv-52*	9×10^7	0.41	1.8×10^1	0.00095	0.002
FB540 *recA*$^-$	1.22×10^8	0.55	1.1×10^4	0.57	1
FB525 *ruv-52 recA*$^-$	1.5×10^8	0.68	8.8×10^3	0.46	0.67
N2154 *ruvA4*	1.2×10^8	0.54	$<1 \times 10^1$	<0.0005	<0.001
N2155 *ruvA4 lexA*(ind$^-$)	1.1×10^8	0.5	$<1 \times 10^1$	<0.0005	<0.001

Experiments were performed as described in 7.4

a) Viable cells/ml of recipient strains were determined prior to mixing with donor on Mu plates.
b) Viability relative to AB1157 *ruv*$^+$.
c) Numbers of pBR322 transconjugants per ml of mating mixture.
d) Yield of Tc^R pBR322 transconjugants relative to AB1157.
e) Relative pBR322 transconjugant yield corrected for observed viability deficiency.

strains carrying ruv mutations was examined, to test the first possibility.

Two synthetic cointegrates were used (Figure 7.1) pMA21 is a pBR322 derivative, containing two res sites from Tn1000 in direct repeat, flanking the Tet^R gene. pMA2631 is a similar construct, however the res sites of Tn1000 are in the opposite orientation. The tnpR gene product, Tn1000 resolvase (Reed 1981), was provided by means of a compatible plasmid pPAK316 carrying the cloned tnpR gene. Resolution of cointegrate molecules was detected by loss of plasmid borne tetracycline resistance. Data presented in Table 7.14 show that cointegrate resolution was dependent upon the tnpR gene product and on the res sites being in direct repeat. Resolution was unaffected by mutations in either ruv or recA.

From these studies it was concluded that the role of the ruv gene product in the recovery of plasmid pBR322 transconjugants in $recBC^+$ strains may be similar to its role in the recovery of F' Pro^+ transconjugants in recBC sbcA and recBC sbcBC strains, and may function in both cases to produce viable products from recombination intermediates generated by the initiation of recombination between the transferred plasmid and the recipient chromosome. In addition it must be noted that whilst $recBC^+$ strains carrying ruv mutations are defective in recovery of plasmid transconjugants, they are proficient recipients for F' transfer, normal numbers of F' transconjugants being obtained. This suggests a critical difference between transferred F prime DNA and transferred F prime::plasmid cointegrate DNA which affects either (i) their capacity to participate in recombination in $recBC^+$ recipients, or (ii) the requirement for the Ruv protein in the recovery of viable products once recombination has been initiated in $recBC^+$ strains. Since essentially the only difference between the two types of transferred DNA molecule is the presence of an extra sequence flanking the plasmid DNA of the F'::plasmid cointegrate, it was concluded that

Figure 7.1 Restriction maps and organisation of plasmids pMA21 and pMA2631

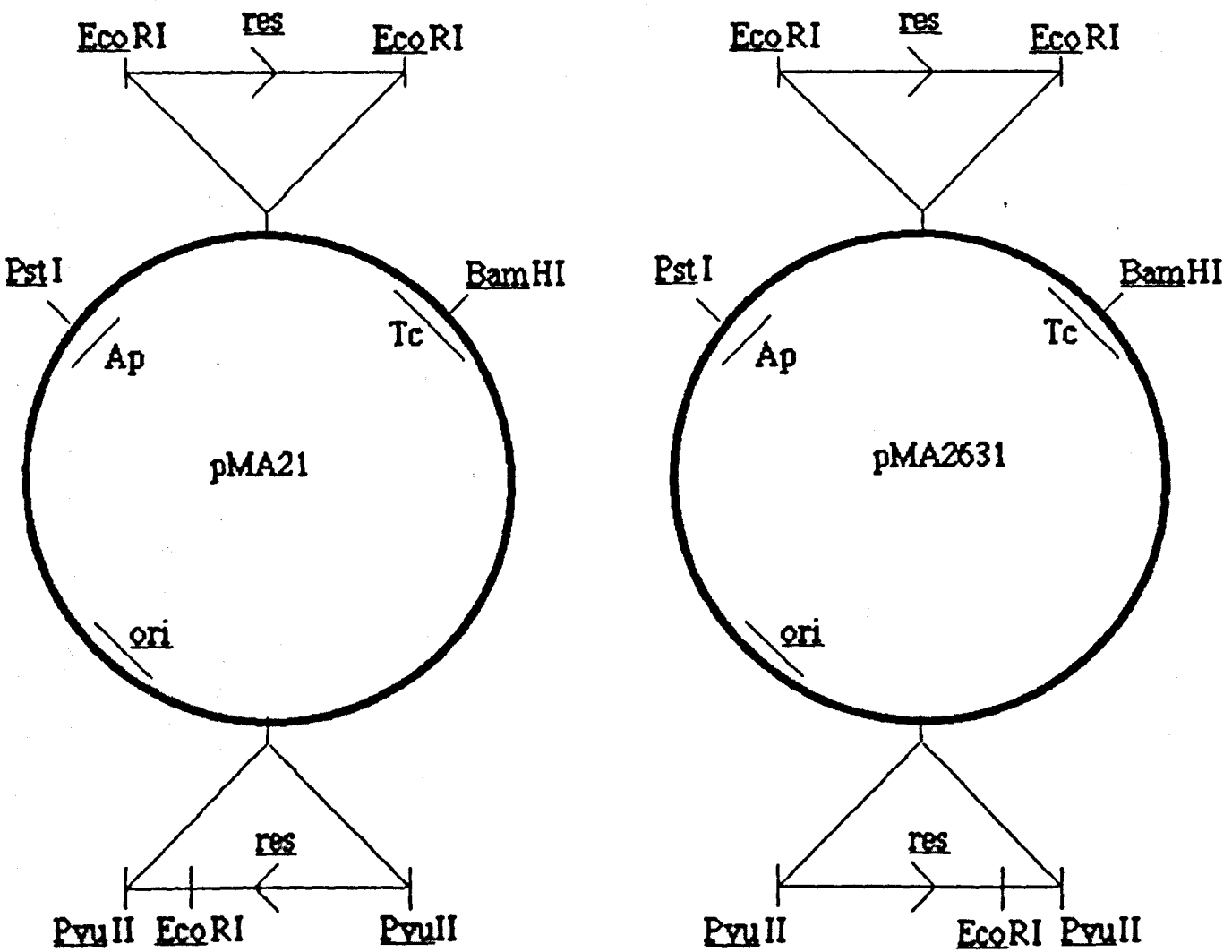


Table 7.14 Resolution of model Tn1000 cointegrates in strains carrying mutations in *recA* and *ruv* in the presence and absence of Tn1000 resolvase

Cointegrate	pMA21		pMA2631	
	Ap^RTc^R	Ap^RTc^S	Ap^RTc^R	Ap^RTc^S
Test Strain				
FB540 *recA*	99.2%	0.8%	100%	0%
FB524 *recA ruv-52*	100%	0%	100%	0%
FB540/pPAK316 *recA*	0%	100%	100%	0%
FB524/pPAK316 *recA ruv-52*	0%	100%	100%	0%

Plasmids were transformed into test strains selecting for Ap^R. Transformants were then gridded and printed on tetracycline plates. Chloramphenicol selection for plasmid pPAK316 was maintained throughout and 125 and 25 transformants of each strain were tested for resoltuion of pMA21 and pMA2631 respectively. Resolution of cointegrates was inferred by the inability of transformants to grow on tetracycline plates.

the difference in the recovery of the transferred DNA molecules in recBC$^+$ and recBC sbcBC strains was somehow related to the presence of homologous regions ($\gamma\delta$) within the transferred DNA, that may facilitate intramolecular recombination. It is possible that intramolecular recombination between the sequences, which may occur either by recA dependent processes, or by tnpR dependent processes may somehow prevent access of RecBCD enzyme at the ends of the transferred DNA molecule, so that recombination between the transferred F' and the recipient chromosome can only proceed via recF dependent processes, and for which the ruv gene product is required to produce viable products from recombination intermediates. In ruv mutants, this is prevented, with a resulting decrease in the recovery of plasmid transconjugants.

CHAPTER 8

The nucleotide sequence of the ruv region

8.1 Introduction

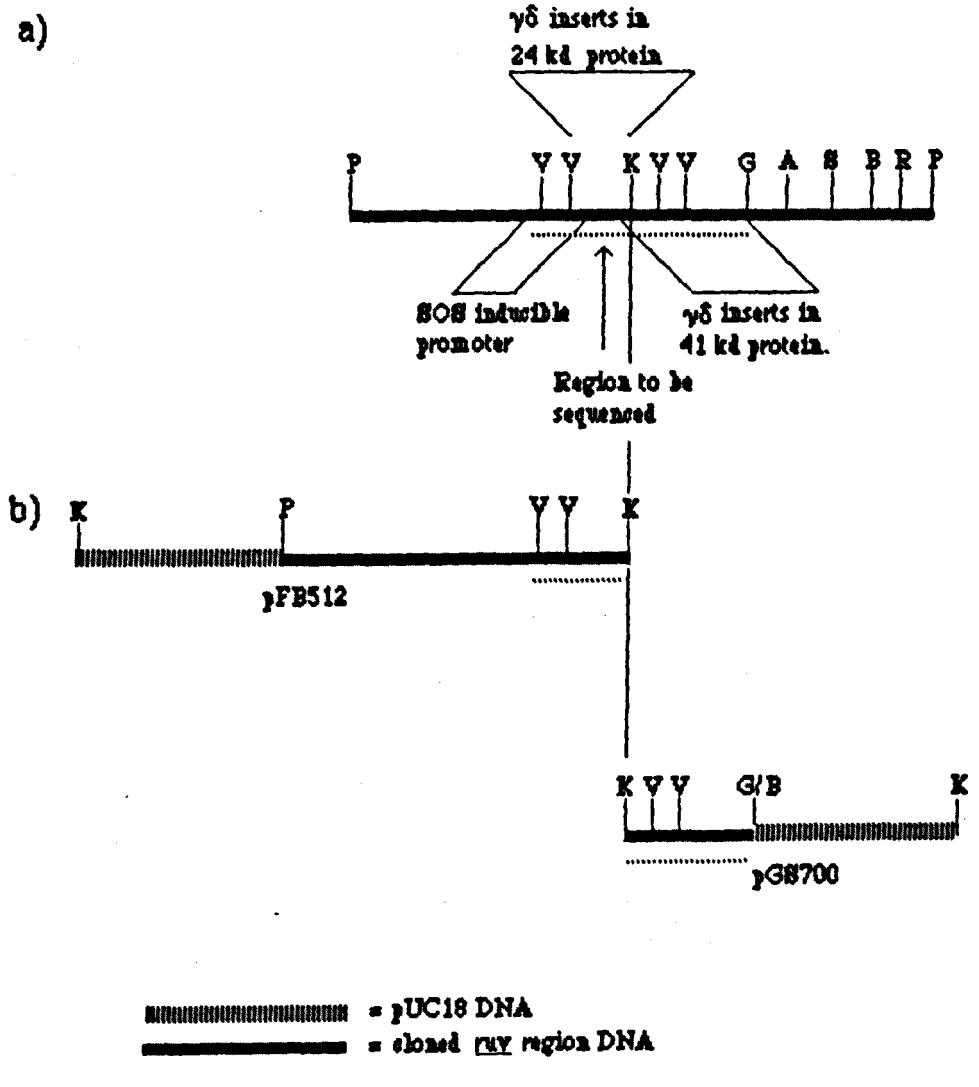
Results so far presented in this thesis suggest that the ruv gene encodes a 41Kd protein, required for DNA repair and recombination and induced as part of the SOS response. In addition studies of the SOS inducible promoter suggested a second polypeptide of approximately 24Kd may be transcribed in an operon with the 41Kd protein.

In order to resolve this problem the nucleotide sequence of the ruv region was determined and is presented in this chapter. As was reported earlier (Chapter 4), ruv was originally cloned on a 10.4kb HindIII restriction fragment, ligated into the HindIII site of pHSG415. Since Tn1000 insertion inactivation studies had identified the region from 0.2kb to the left of the KpnI site to the BglII site as the coding region for the 41Kd Ruv protein (Figure 8.1), and further studies had indicated the presence of an SOS inducible promoter within the Sau3A fragment overlapping the EcoRV sites 1 and 2 (Chapter 6), it was initially decided to determine the nucleotide sequence of the approximately 2.5kb region extending from EcoRV site 1 to the BglII site (Figure 8.1).

8.2 Nucleotide sequence assembly

The first requirement for sequencing the ruv region was a good source of the DNA to be sequenced, so that suitable subclones could be easily obtained. Since pPVA101 is a low copy number plasmid it could be obtained only with relatively poor yields. As was reported earlier (Chapter 4), attempts to clone this region intact into

Figure 8.1 a) Identification of region for nucleotide sequence determination and b) multicopy plasmid sources for M13 cloning



multicopy plasmids had proved fruitless, due, it was thought, to a lethal effect associated with overproduction of the 41Kd protein.

However, the region was successfully cloned in two parts into the multicopy plasmid pUC18, using the KpnI site within the 41Kd protein coding region, to give plasmids pFB509 and pFB512, harbouring DNA to the right and the left of the KpnI site respectively (Figure 4.14 and Figure 8.1). Sequencing of the ruv region was similarly divided into two parts; pFB512 provided an excellent source of DNA to the left of the KpnI site, whilst a further plasmid, pGS700, a pUC18 derivative harbouring the KpnI-BglII fragment (G. Sharples) provided an excellent source of DNA to the right of the KpnI site (Figure 8.1).

DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al. 1977) using M13 derivatives mp18 and mp19 (Yannisch-Perron et al. 1985) as cloning vectors to provide suitable template DNA. Methods used for the growth of M13 phages, the preparation of single-stranded and double-stranded phage DNA and methods used for dideoxy sequencing were detailed in Chapter 2 of this thesis.

Since analysis of standard sequencing reactions using mp18 and mp19 derivatives as templates, in conjunction with the universal sequencing primer, allows the accurate determination of the sequence of a maximum of approximately 250 nucleotides per clone, the first step towards obtaining the complete nucleotide sequence was to generate sufficient M13 clones with useful insert sizes.

Since the direction of transcription of the ruv gene had previously been demonstrated to be from left to right, and promoter analysis had suggested the presence of an SOS inducible promoter to the left of the KpnI site, sequencing the ruv region was started with the sequence to the left of the KpnI site.

The fragment between EcoRV sites 1 and 2 with an estimated size of 0.26kb, was of a suitable size to allow the nucleotide sequence

to be determined from end to end. In order to obtain suitable M13 clones, pFB512 DNA was digested with EcoRV, fragments separated on a 2% agarose gel, and the 0.26kb band cut out, electroeluted and ligated into HincII digested mp18 and mp19 vectors. (In theory, the EcoRV fragment could be inserted in either orientation into each vector. However, in practice, it was found that inserts were preferentially ligated in one orientation into one vector and in the opposite orientation into the other vector. As both orientations of insert were required to sequence both strands, inserts were routinely ligated into both vectors).

To obtain further clones of a suitable size for nucleotide sequence determination, the fragment of approximately 0.7kb between EcoRV site 2 and the KpnI site of pFB512 was isolated from an agarose gel and subjected to further digestion by three enzymes that restrict DNA at 4 base pair recognition sites - Sau3A, AluI and HpaII. No sites for AluI were identified, whilst HpaII appeared to generate 4 fragments ranging between 100bp and 400bp. Sau3A appeared to generate 4 restriction fragments ranging between 100bp and 250bp. Since the largest HpaII fragment generated was too large to allow accurate end to end nucleotide sequence determination, and since the Sau3A fragments generated were of a suitable size to allow end to end sequence determination, it was decided to attempt to obtain Sau3A clones of this region. Sau3A digested EcoRV-KpnI fragment was ligated into BamHI digested mp18 and mp19. In addition, the entire EcoRV-KpnI fragment was isolated and ligated (without further restriction) into HincII/KpnI digested mp18 and mp19. (Although this fragment was far too large to enable the nucleotide sequence to be determined from end to end, the sequence of the first 250 nucleotides from each end could be accurately determined and the next 100 approximately determined to allow positioning of nucleotide sequence obtained from Sau3A clones).

Ligation mixtures were transformed into strain JM101 and

transformation mixtures plated out in a lawn of the host JM101 in 2.5ml 0.6% YT agar containing 40ul 20mgs/ml X-GAL and 40ul 22.4mgs/ml IPTG, on a YT plate. Since occasionally DNA prepared from phages purified from colourless plaques was found not to contain an insert, in all cases, control uncut, cut, and cut and ligated, vector was transformed into JM101 along with the 'test' ligation mixtures, so that the probability of colourless plaques containing phages with insert DNA could be assessed.

After incubation for 12-24 hours, colourless plaques were purified and phage supernatants prepared. Samples of phage supernatants were spotted onto nitrocellulose filters, baked for 2 hours and probed overnight with nick translated pFB512 insert DNA in 4 x SSC 0.1% SDS 5 x Denhardts solution. Filters were subjected to autoradiography. (This step was omitted from those transformations which predicted a high insert to non-insert ratio amongst white plaques). Single stranded DNA was prepared from those phage supernatants that hybridised strongly to the probe, or were likely to contain inserts predicted from a comparison of the transformations obtained with cut and ligated vector control, and the cut vector ligated with insert.

Single stranded DNA to be sequenced was annealed to universal primer, α-^{35}S dATP and Klenow Polymerase added and four sequencing reactions per clone set up.

Products of sequencing reactions were separated by electrophoresis on denaturing acrylamide gels as described in 2.25c, then fixed, dried and exposed to autoradiographic film.

Sequences were read directly from developed film from the bottom of the gel upwards. Ideally sequences from within the mp18 or mp19 polylinker were read from buffer gradient gels enabling an accurate determination of the sequence from the beginning of the insert.

Analysis of sequence read from clones obtained from the above

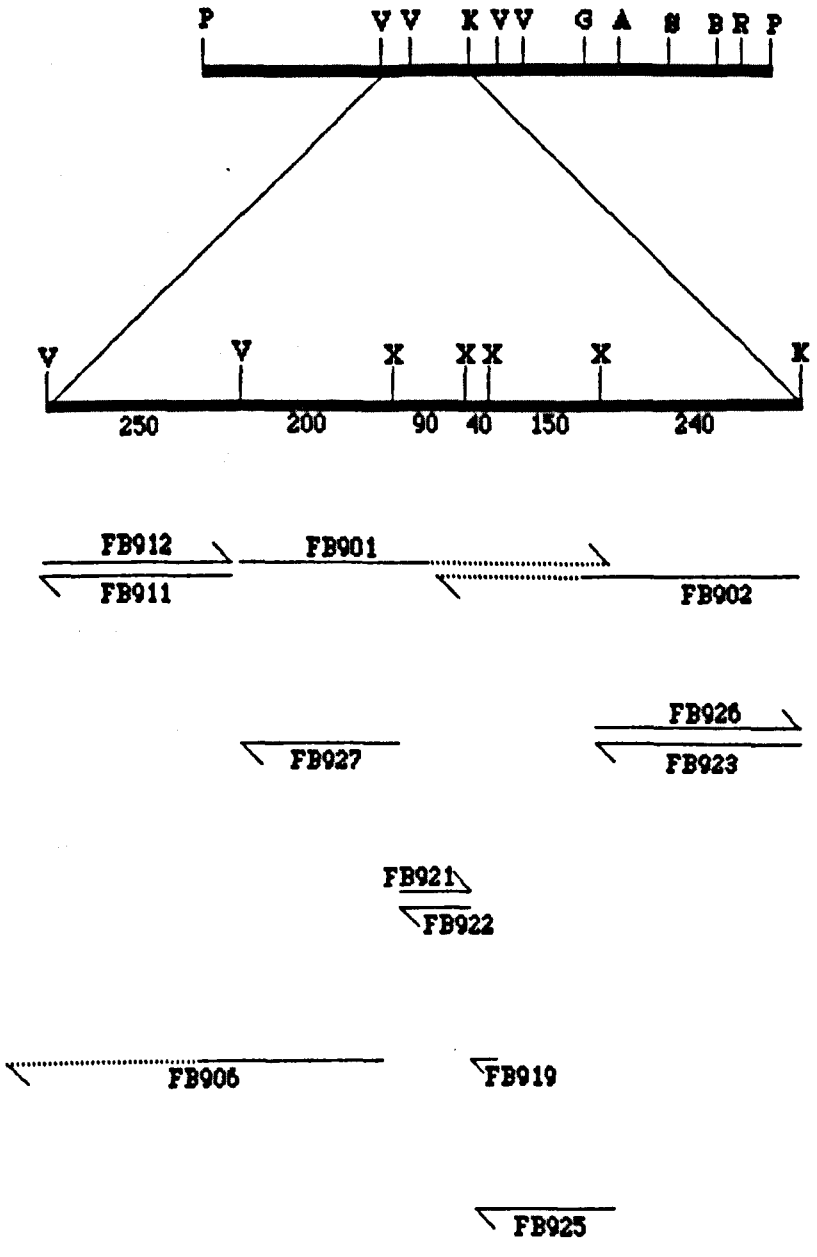
transformations revealed that all of the DNA between EcoRV site 1 and the KpnI site had been cloned (Figure 8.2) such that nucleotide sequence could be read at least on one strand for the entire region. In order to determine the complementary nucleotide sequence of the region covered by the insert of the mp18 derivative FB925, which had only been determined on one strand, double stranded DNA of FB925 was prepared, digested with HindIII and EcoRI, the insert isolated from an acrylamide gel and ligated into HindIII/EcoRI digested mp19. Determination of the nucleotide sequence of single-stranded DNA prepared from recombinant phages confirmed that these clones had the same insert as FB925, but in the opposite orientation, thus allowing determination of the complementary strand nucleotide sequence.

This completed the collection of clones required to determine the nucleotide sequence to the left of the KpnI site on both strands.

Since the nucleotide sequence of the 260bp fragment between EcoRV sites 1 and 2 was obtained independently of that between EcoRV site 2 and the KpnI site, its orientation with respect to the larger fragment was unknown. This was found by determining the nucleotide sequence of the insert of a clone FB906 obtained by subcloning a HindIII-EcoRI fragment from the promoter clone derivative pFB3 (Figure 8.3), which had previously been shown to carry an SOS inducible promoter (Chapter 6), into HindIII/EcoRI digested mp18. This confirmed the orientation of the EcoRV fragment was as shown in Figure 8.2, and that the SOS inducible promoter characterised in pFB3 and similar derivatives was in the correct orientation to direct synthesis of the Ruv product.

During the course of determining the nucleotide sequence of this region, several domains, particularly of local G-C richness, were encountered in which reading nucleotide sequence was difficult due to comigration of bands in different tracks. Such artifacts are apparently due to formation of secondary structures in the DNA

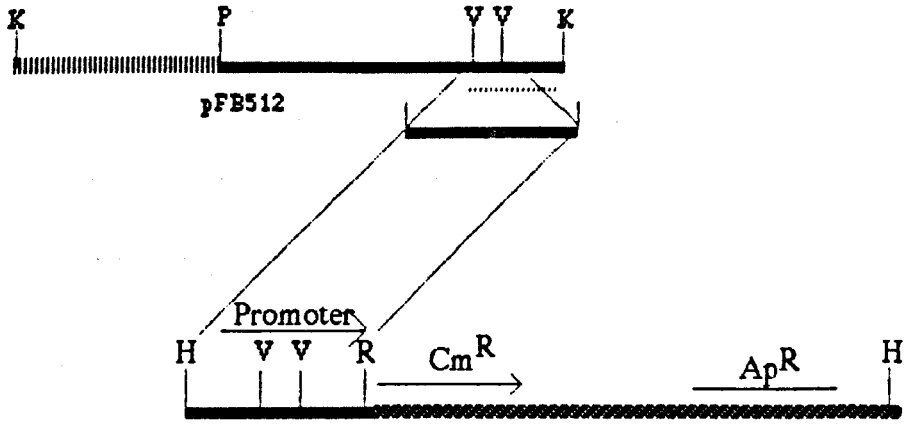
Figure 8.2 Clones for sequencing the ruv gene, EcoRV site 1 to KpnI site 1.



within the gel and were eliminated by replacing dGTP in nucleotide sequencing mixtures with dITP at 2mM and reducing the concentration of ddGTP in the 'G' mixture to 0.1 of that normally used (Mills and Kramer 1979).

Much of the nucleotdie sequence to the right of the KpnI site was determined by G.T. Illing and G.J. Sharples under my supervision. The plasmid pGS700 provided a good source of DNA for the preparation of suitable subclones for nucleotide sequence determination. Early restriction mapping of pPVA101 had suggested that there was a single EcoRV site between the KpnI and BglII sites. Further mapping of the insert of pGS700 led to the conclusion that there was not one but two EcoRV sites in this region as shown in Figure 8.4 (G.J. Sharples, pers. comm.). mp18 and mp19 clones with suitable insert sizes for nucleotide sequence determination were obtained in a similar manner to that previously described for the region to the left of the KpnI site:- KpnI-EcoRV$_3$, EcoRV$_3$-EcoRV$_4$, and EcoRV$_4$-HindIII fragments were purified from suitably digested pGS700 DNA, fractionated on agarose gels and ligated into KpnI/HincII, HincII and HincII/HindIII digested mp18 and mp19 respectively. In addition shotgun clones from Sau3A, HpaII and TaqI singly digested KpnI-HindIII fragment were obtained by ligating into BamHI, (for Sau3A digests) and AccI (for HpaII and TaqI digests) digested mp18 and mp19. Much of the nucleotide sequence, at least on one strand, was obtained by analysis of clones generated as described above (Figure 8.4). As with FB925 it was necessary to turn around some of the characterised clones in order to obtain nucleotide sequence of both strands. Curiously, no clones with useful insert sizes were obtained overlapping EcoRV site 4. Confirmation that the nucleotide sequence to the left of EcoRV site 4 determined from a clone harboring the fragment between EcoRV sites 3 and 4, was in fact contiguous with the nucleotide sequence to the right of EcoRV site 4, determined from a clone harbouring

Figure 8.3 Identification of the DNA region cloned in plasmid pFB3

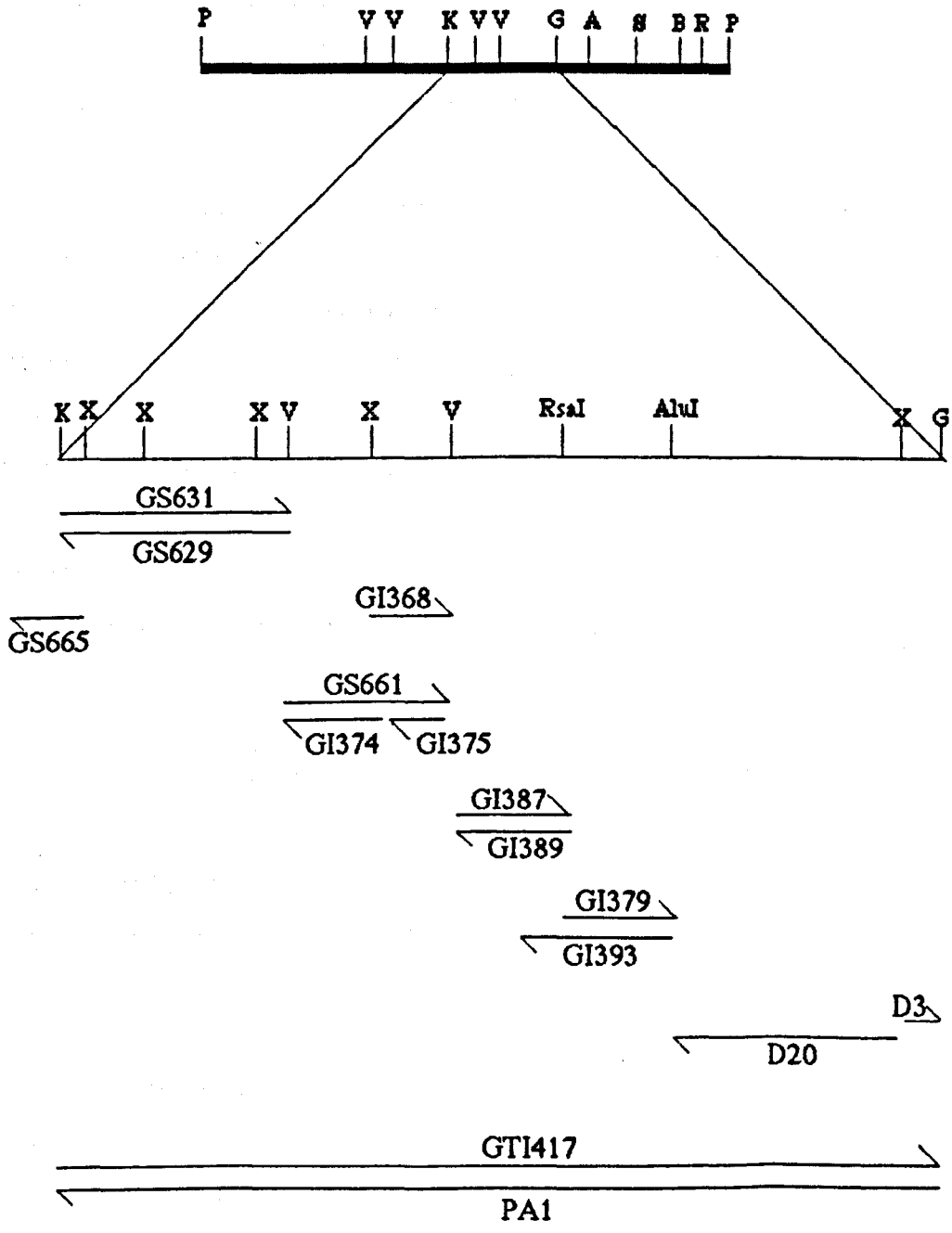


Cloned DNA

pKK232.8 derived DNA

pUC18 DNA

Figure 8.4 M13 clones for sequencing the KpnI to BglII region



the fragment between EcoRV site 4 and the BglII site, was provided by determining the nucleotide sequence from an oligonucleotide primer synthesised to be complementary to the nucleotide sequence to the left of EcoRV site 4 in the single-stranded DNA produced from clone GTI417, an mp19 derivative harbouring the entire KpnI-BglII fragment originally subcloned from pPVA101. The principal clones used to determine sequence to the right of the KpnI site are diagrammed in Figure 8.4.

In order to link the nucleotide sequences of the two parts of the ruv region, the approximately 1kb fragment between EcoRV sites 2 and 3, spanning the KpnI site, was cloned first into pUC18 to give pGS701 and then further subcloned into mp18. Nucleotide sequence determined from this clone FB930 confirmed the nucleotide sequences of the regions determined separately to the right and left of the KpnI site were contiguous in pPVA101.

Nucleotide sequence, totalling 2505 base pairs, determined on each strand at least 3 times is numbered from EcoRV site 1, and is presented in Figure 8.5.

8.3 Analysis of the nucleotide sequence

Basic analysis of the nucleotide sequence was performed using the DNA Inspector II package for the Apple Macintosh computer, further analysis was performed using the DNAstar programs for an IBM PC.

A search for possible LexA binding sites revealed the presence of 2 nucleotide sequences beginning at bp197 and bp250 respectively that resembled the nucleotide sequence of the LexA consensus binding site $CTG(N)_{10}$·CAG. These have been designated SOS box 1 and SOS box 2. A comparison of these sequences with other known LexA binding sites is presented in Table 8.1.

A visual search for potential promoter sequences revealed the

Figure 8.5

Nucleotide sequence of the *ruv* region. The sequence is numbered from *Eco*RV site 1. The location of the putative SOS boxes are indicated by asterisks above the nucleotide sequence. The proposed -10 and -35 regions of the promoter are underlined, and the sequences with homology to the Shine-Dalgarno sequence overlined and labelled S-D. The predicted protein sequences are presented under the nucleotide sequence, using the standard 1 letter amino acid code, positioned under the first nucleotide of the codon used. The sequence identified that may function as a transcriptional terminator is indicated by a string of crosses above the nucleotide sequence. Restriction sites mentioned in the text are labelled.

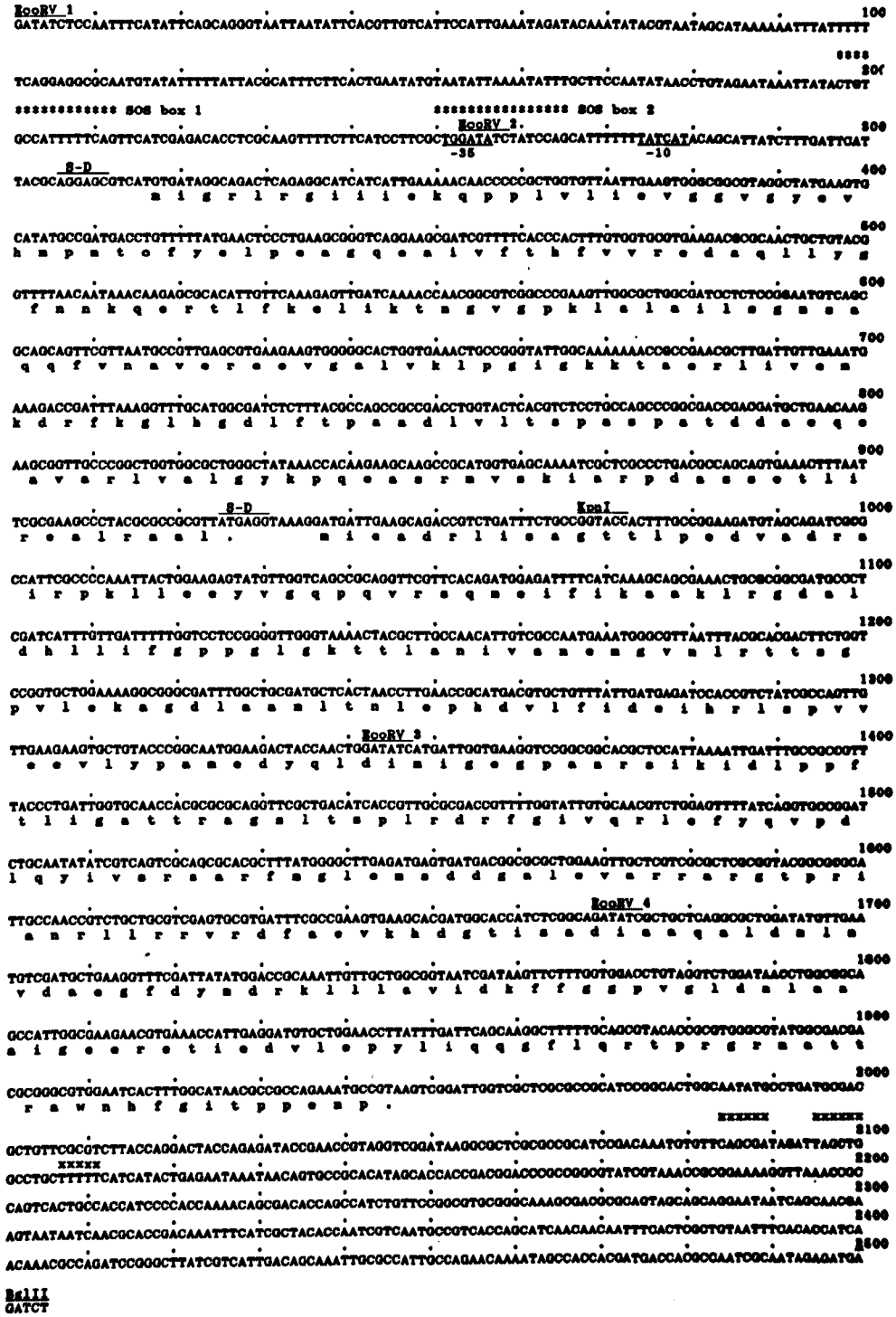

EcoRV 1
SOS box 1
SOS box 2
EcoRV 2
-35
-10
S-D
S-D
KpnI
EcoRV 3
EcoRV 4
BglII
GATCT

Table 8.1 Alignment of the two putative SOS boxes identified in front of the *ruv* gene with those of other *lexA* regulated genes

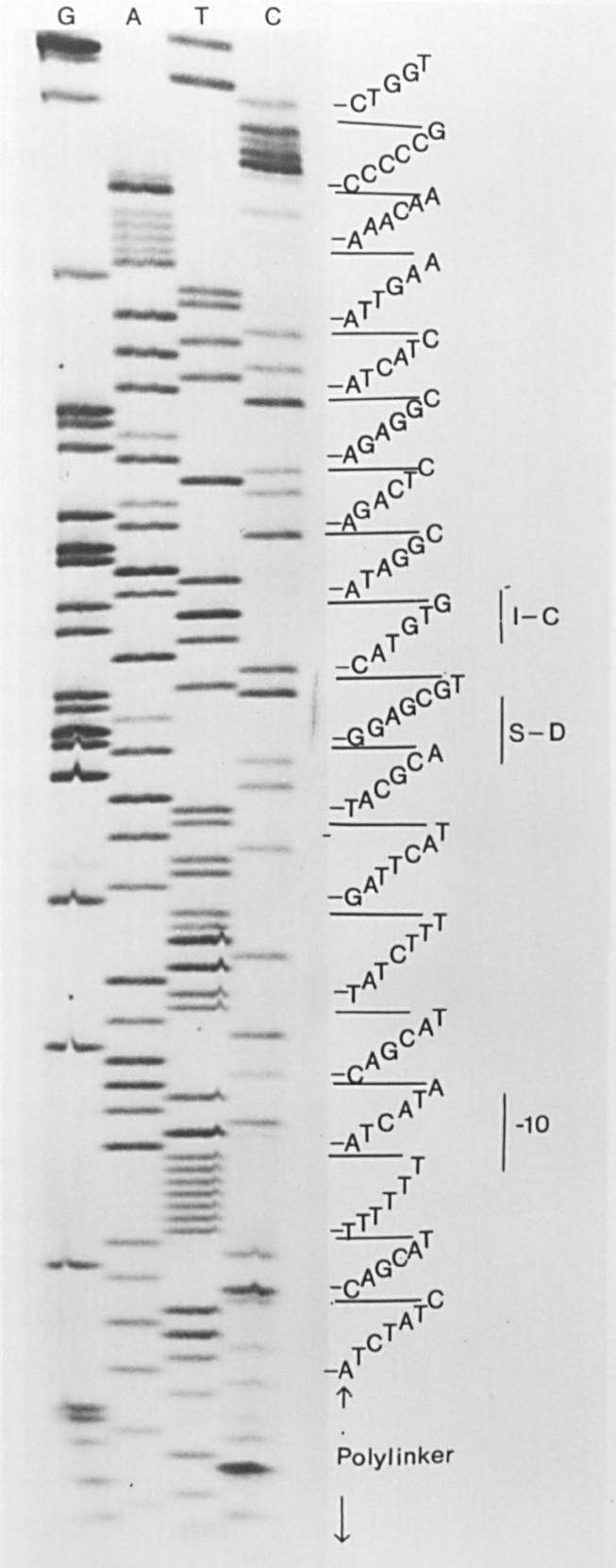
Gene		SOS box sequence	Reference
Consensus		CTGtatatatataCAG	Wertman and Mount 1985
ruv	1	CTGTGCCATTTTTCAG	
	2	CTGGATATCTATCCAG	
lexA	1	CTGTATATACTCACAG	(Horii *et al* 1981, Little *et al*. 1981
	2	CTGTATATACACCCAG	(Brent and Ptashne 1981
recA		CTGTATGAGCATACAG	Horii *et al*. 1981,Sancar *et al*. 1980
recN	1	CTGTATATAAAACCAG	Rostas *et al*. 1987
	2	CTGTACACAATAACAG	
recQ		CTGTTTTTATTT-CAG	Irino, Nakayama and Nakayama 1986
uvrA		CTGTATATTCATTCAG	Sancar A. *et al*. 1982
uvrB		CTGTTTTTTTATCCAG	Sancar G.B. *et al*. 1982
uvrD		CTGTATATATACCCAG	Finch and Emmerson 1983
umuDC		CTGTATATAAAAACAG	Perry *et al*.1985,Kitagawa *et al*.1985
mucAB		CTGTATAAATAAACAG	Perry *et al*. 1985
impAB		CTGTATATACATACAG	Strike and Lodwick 1987
sulA		CTGTACATCCATACAG	Cole 1983
dnaG		CTGGCGTTGATGCCAG	Lupski, Ruiz and Godson 1984

presence of a possible promoter overlapping SOS box 2. The nucleotide sequence (TGGATAT) beginning at bp251 has a reasonable match to the relatively poorly conserved -35 region TTGACAT consensus nucleotide sequence of E. coli promoters. The nucleotide sequence TATCAT beginning at bp274 has a good match to the E. coli promoter -10 consensus nucleotide sequence TATAAT, in particular to the highly conserved TA---T (Harley and Reynolds 1987). A potential transcription initiation site CAT is present beginning at 277bp. No further nucleotide sequences that could possibly function as promoters were identified in the region of the SOS boxes.

Computer analysis of open reading frames revealed two open reading frames, which, if translation were started from ATG initiation codons at bp404 and bp937 respectively could code for polypeptides of 19097 and 37177 daltons respectively. Since the proposed initiation codon at bp404 was 120 nucleotides downstream of the promoter identified and was not preceded by any putative Shine-Dalgarno sequence, a visual search for alternative initiation codons in this same reading frame was performed. This revealed a possible start site with a GTG initiation codon at bp317, preceded by a sequence AGGAGC at bp306 similar to the Shine-Dalgarno consensus AGGAGG (Shine and Dalgarno 1974, Stormo et al. 1982, Gren 1984). A section of an autoradiograph of a sequencing gel showing the proposed -10 region of the promoter, the possible transcription start site, the sequence similar to the Shine-Dalgarno consensus ribosome binding site, and the proposed initiation codon is shown in Figure 8.6. A comparison of this region with known E. coli translational initiation sites suggests that translation initiation could occur at the GTG resulting in a polypeptide of 22,087 daltons, closer to the estimate from SDS-PAGE of 24Kd. Termination of translation of this reading frame would occur at a TGA codon at bp926. Initiation of translation of the second open reading frame is expected to occur with an ATG codon at bp937, 11bp downstream of

Figure 8.6

Autoradiograph of a section of a sequencing gel, showing part of the sequence obtained from clone FB901. The nucleotide sequence of the cloned DNA begins with ATC, the half site for *Eco*RV digestion, and continues 5' - 3' through the proposed promoter region into the *ruvA* gene. The four tracks are labelled G, A, T and C from left to right, and the sequence is read from the bottom of the gel upwards. The sequence shown includes the proposed -10 region of the promoter, the sequence homologous to the Shine-Dalgarno consensus (S-D), and the proposed GTG initiation codon (I-C) for the *ruvA* gene.


G
A
T
C
I – C
S – D
-10
Polylinker

termination of the previous reading frame, and to terminate with a TAA codon at bp1943, resulting in a polypeptide of 37,177 daltons which is in reasonable agreement with the estimate from PAGE-SDS of 41Kd for the protein encoded by this region previously identified as the Ruv protein. The initiation codon is preceded by a sequence ATGAGG at bp925, overlapping by 1bp the coding region for the first polypeptide, that has a good match to the Shine-Dalgarno consensus nucleotide sequence. Analysis of nucleotide sequence upstream of the proposed translation initiation site revealed the presence of two regions that could possibly serve as additional promoters for transcription of the nucleotide sequence coding for the 37177 protein. The first of these has a putative -35 region beginning at bp807 of TTGCCGC which at least has a good match to the highly conserved TTG of the E. coli -35 consensus nucleotide sequence TTGACAT, and a possible -10 region at bp830 of TATAAA which although it has a reasonable match to the consensus -10 nucleotide sequence TATAAT, differs in the sixth position in which the T has sometimes been described as invariant - it is present in >90% of all E. coli promoter sequences so far analysed (Harley and Reynolds 1987). Several possible transcription initiation sites downstream of this putative promoter can be identified particularly within the nucleotide sequence AAGAAG beginning at bp840. The second possible promoter in this region begins with a nucleotide sequence TCGCCC at bp871 which has a relatively poor match to the -35 consensus nucleotide sequence TTGACA and is followed by a putative -10 region TTTAAT beginning at bp895, which although it has a good match to the E. coli consensus -10 region of TATAAT, it differs at the highly conserved second position which is reported to be an A in 90% of the E. coli promoter analysed. Several possible sites of initiation of transcription could be proposed particularly within the sequence GAAG at bp905. The relative contributions of the three possible promoter regions to transcription of the message for the 37177

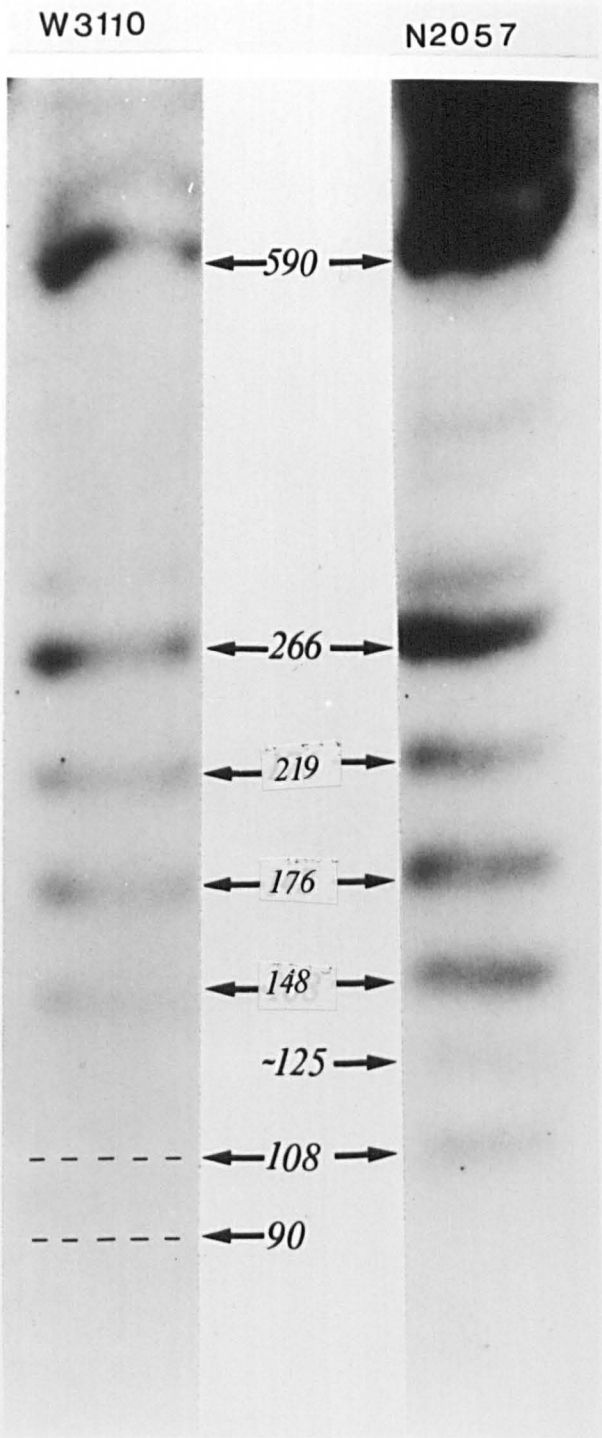
dalton protein remains to be investigated. However, it is unlikely that either of these putative promoters has a major role in transcription of the 37Kd protein coding region since *in vivo* studies on the expression of *ruv*, utilising the chromosomal *ruv*::Mu*d*(Ap)^{R}*lac* fusion (in which the Mu*d*(Ap)^{R}*lac* fusion has been mapped to a site within the 37Kd protein coding region [Chapter 5]) and the plasmid-borne promoter - CAT fusion (which carries DNA from upstream of the 22Kd protein coding region) show very similar induction ratios when exposed to SOS inducing treatments (Chapter 6). In addition, the Tn*10* insertion in strain N2057 *ruv*-60, mapped to a position between *Eco*RV site 2 and *Kpn*I site 1 in Chapter 5 (Figure 5.6) has been more accurately mapped to within the 90bp *Sau*3A fragment between bp451 and bp541 (Figure 8.7) of the nucleotide sequence. This clearly places the insertion within the coding region for the 22Kd protein. Since plasmid pFB502, which carries the intact 22Kd protein coding region failed to complement the UV sensitivity of N2057 *ruv*-60, it was inferred that the Tn*10* insertion had a polar effect on transcription of the 37Kd protein. It therefore seems likely that the 22Kd and 37Kd proteins are transcribed together in an operon from an SOS inducible promoter upstream of the 22Kd protein. The two open reading frames identified within the determined nucleotide sequence have therefore been designated *ruvA* (for the 22Kd protein coding region) and *ruvB* (for the 37Kd protein coding region).

A nucleotide sequence beginning at bp2084 has the characteristics of a transcriptional terminator, consisting of a region capable of forming a stem-loop structure, followed by a short run of T residues (Brendel *et al*. 1986).

Analysis of the nucleotide sequence of the region 3' of that coding for the 22Kd and 37Kd proteins failed to reveal any further significant open reading frames with suitable translation initiation signals.

Figure 8.7

Autoradiograph of restriction digested N2057 DNA probed with the 1kb fragment from between EcoRV sites 1 and 2. DNA was digested with Sau3A, fractionated on a 6% polyacrylamide gel and transferred to a nylon membrane. The hybridisation bands (sizes indicated in base pairs) in the Sau3A digested W3110 DNA are those predicted from the nucleotide sequence. Bands which were detected on the autoradiograph, but which have not photographed well are indicated by dashed lines. The hybridisation bands in the Sau3A digested N2057 DNA are those predicted from the nucleotide sequence of the wild type region, with the exception of the extra band at approximately 125bp, and the absence of a band at 90bp, confirming the site of insertion of Tn10 in N2057 at a position within the 90bp Sau3A fragment, between positions 451 and 541 of the nucleotide sequence.


W3110
N2057
590
266
219
176
148
125
108
90

8.4 Codon usage and amino acid composition of ruvA and ruvB

Initiation of translation of ruvA with the proposed GTG codon at bp317 suggests that the level of expression of ruvA may partly be controlled at a translational level. GTG codons occur as initiation codons in 8% of the translational initiation sites so far characterised in E. coli (Gren 1984). Recent studies demonstrate that they are approximately 66% as efficient in initiating translation as the more common ATG initiation codons (Reddy et al. 1985). Initiation of translation of ruvB with an ATG codon, which may occur more efficiently than initiation of translation of ruvA, could provide a mechanism by which the RuvB protein, transcribed coordinately with the RuvA protein can be maintained at a higher basal level. Interestingly, in ruvA the initiation codon is immediately followed by the rare codon ATA encoding isoleucine which on the basis of 288 translational start sites characterised has been predicted to occur only 3 times as a second codon per 1000 genes, but which is one of the most efficient second codons in terms of its effects on translation initiation (Looman et al. 1987). Further translational control has been suggested to depend on the relative use of rare codons in the translated and nontranslated reading frames respectively (Konigsberg and Godson 1983). The rare codons ATA(Ile), TCG(Ser), CCT(Pro), CCC(Pro), ACG(Thr), CAA(Gln), AAT(Asn), AGG(Arg) occur with a frequency of 7.4% within the ruvA coding frame and 8.8% and 13% in the two non-coding frames. This is consistent with moderately efficient translation of both genes, expression being primarily controlled at a transcriptional level and induced by DNA damage.

Table 8.2 shows the amino acid sequence of the RuvB protein contains a region between residues 55 and 77 which is homologous to a sequence conserved amongst adenine nucleotide binding proteins, such as ATPases (Walker et al. 1982), and which probably defines the

Table 8.2 Alignment of the putative ATP binding sequence of the RuvB protein with ATP binding sites of other *E. coli* enzymes involved in DNA metabolism

Protein	Residues	Sequence								Reference
UvrA	24- 45	DKLIV	V	TGLS	G	S	GKS	SLAFDT	L	Husain *et al.* 1986
	633-654	GLFTC	I	TGVS	G	S	GKS	TLINDT	L	
UvrB	32- 53	LAHQT	L	LGVT	G	S	GKT	FTIANV	I	Arikan *et al.* 1986
UvrD	22- 43	RSNLL	V	LAGA	G	S	GKT	RVLVHR	I	Finch and Emmerson 1984
RecB	16- 37	QGERL	I	EASA	G	T	GKT	FTIAAL	Y	Finch *et al.* 1986c
DnaB	223-244	SDLII	V	AARP	S	M	GKT	TFAMNL	V	Nakayama *et al.* 1984b
Rho	172-192	GQRGL	I	VAPP	K	A	GKT	MLLQNI	A	Pinkham and Platt 1983
RecN	22- 48	SGMTV	I	TGET	G	A	GKS	IAIDAL	G	Rostas *et al.* 1987
RecD	164-185	RRISV	I	SGGP	G	T	GKT	TTVAKL	L	Finch *et al.* 1986d
RecA	59- 80	GRIVE	I	YGPE	S	S	GKT	TLTLQV	I	Walker *et al.* 1982
			*		*	*	***		*	
RuvB	55- 77	LDHLL	I	FGPP	G	L	GKT	TLANIV	A	

ATP-binding site of those proteins (Fry, Kuby and Mildvan 1980). This suggests that the RuvB protein may require ATP as a cofactor for its function in repair and recombination.

The predicted secondary structure of the RuvA and RuvB proteins from Chou-Fasman analysis of the amino acids suggests both proteins have conformations typical of globular proteins with regions of alpha helix interspersed with short regions of extended beta sheet. The RuvA protein has 24.5% charged amino acids, 26 positively charged and 24 negatively charged, resulting in a net charge at pH6 of +2, consistent with the predicted isoelectric point of the 204 amino acid RuvA protein pI = 6.19. The RuvB protein has 25.8% charged amino acids, consisting of 39 positively charged and 48 negatively charged amino acids, resulting in a net charge at pH 6 of -13, consistent with the predicted isoelectric point of the 336 amino acid RuvB protein PI = 4.93.

CHAPTER 9

Discussion

9.1 The role of the ruv gene in the recovery of recombinants and F' transconjugants

Data presented in Chapters 3 and 7 of this thesis demonstrated that the ruv gene product was required for the recovery of recombinants and F' transconjugants in recBC sbcBC and recBC sbcA recipients, and for the recovery of plasmid transconjugants in recBC$^+$ strains. However, the extent to which the recovery of recombinants and transconjugants was affected by ruv mutations varied by a factor of 10 between recBC sbcBC and recBC sbcA strains. Presumably this difference reflects the requirement for the ruv gene product in recombination of the different substrates available in these genetic backgrounds (Lloyd et al. 1987). In recBC$^+$ strains, recombination can be initiated from the ends of DS DNA molecules by the action of the RecBCD enzyme, Exonuclease V, which can generate single stranded DNA tails from DS DNA, by its unwinding and nicking activities (Taylor et al. 1985). In recBC sbcBC strains, single-stranded DNA cannot be generated by exonuclease V action, however the single stranded region at the 3' terminus of the transferred DNA, which in wild type strains is presumably converted to a flush duplex substrate for the RecBCD enzyme, by the action of exonuclease I (Kushner et al. 1971), is allowed to persist, and is presumably a recombinogenic substrate, for recBC independent recombination. In recBC sbcA strains, the synthesis of a new enzyme, exonuclease VIII, which is able to generate single stranded regions from duplex DNA by a 5' - 3' exonucleolytic digestion of one strand of duplex DNA (Barbour et al. 1970, Joseph and Kolodner 1983), compensates for the loss of the RecBCD enzyme in generating single stranded DNA from

duplex DNA. The increased requirement for the ruv gene product for the recovery of recombinants in crosses with Hfr donors in recBC sbcBC recipients compared with recBC sbcA recipients, suggests that the ruv gene product may interact with the 3' single stranded end of transferred Hfr DNA, perhaps protecting it from nuclease digestion. The requirement for the ruv gene product in the recovery of F' transconjugants, only in recipient strains in which recombination can be initiated, clearly suggests however that the ruv gene product acts at some stage later than in the protection of the 3' transferred end, which is presumed to recircularise in the recipient fairly rapidly. It is difficult to reconcile the differences observed in the recovery of F' transconjugants in recBC sbcBC and recBC sbcA strains respectively, with the proposed role of the ruv gene product at a later stage, unless it is assumed that the activity of the enzyme exonuclease VIII in generating single-stranded DNA can influence the outcome of recombination events after they have been initiated. It is expected that the precise role of the ruv gene product in the recovery of recombinants in crosses with Hfr and F' donors will only be elucidated by the biochemical characterisation of the purified protein.

9.2 The role of the ruv gene product in recovery from DNA damage

The demonstration that the ruv gene product was required for recombination and recovery of F' transconjugants in recBC sbcBC and recBC sbcA strains led to the suggestion that the role of the ruv gene product in the repair of damaged DNA, was in the recombinational repair of daughter strand gaps and double strand breaks. This is consistent with suggestions that the main role for recombination in wild type bacteria in natural environments is not in the generation of diversity, as in sexually reproducing organisms, but is in the repair of damage occurring during DNA

metabolism or produced by external agents (Maynard-Smith 1986). Since early studies had demonstrated that ruv mutants were competent at rejoining low molecular weight DNA synthesised after UV irradiation, it was proposed that the ruv gene product acts at a late stage in recombination repair of both daughter strand gaps, and in situations where the RecBCD enzyme is limited, of double strand breaks. The observation that strains carrying mutations in both ruv and the gene for DNA adenine methylase (dam) were inviable (Peterson et al. 1985), provided support for the proposed role in double strand break repair. The inviability of dam recA and dam recB strains has been demonstrated, by the use of temperature sensitive mutations, to be correlated with the persistence of double strand breaks in the DNA of the inviable bacteria (Wang and Smith 1986b), and can be suppressed by mutations in mutL or mutS (McGraw and Marinus 1980). It will be interesting to determine whether the inviability of dam ruv strains can be similarly correlated with the presence of double strand breaks.

9.3 Genetic organisation of the ruv region

The ruv region has been cloned on a 10.4kb HindIII fragment into the low copy number vector pHSG415. Analysis of the proteins synthesised by pPVA101 deletion derivatives and derivatives carrying Tn1000 insertions that inactivated the ruv gene suggested that the ruv gene encoded a 41kd protein. In addition to the 41kd protein, the cloned DNA was demonstrated to encode two further proteins, of approximately 24kd and 33kd; one of which, the 24kd protein, was encoded by the DNA immediately adjacent to that coding for the 41kd protein, which first led to the suggestion that the proteins may have related functions or be contranscribed. This suggestion was supported by the mapping of a Tn10 element, (identified as being within the ruv gene on the basis of its effect on the sensitivity to

UV irradiation), to within the region coding for the smaller 24kd protein, and the observation that pPVA101 derivatives with Tn*1000* insertions in the 41kd protein failed to complement the UV sensitivity of the *ruv*::Tn*10* mutation (Dr. Paul V. Attfield).

The direction of transcription of the *ruv* gene on the plasmid was determined by mapping the site of insertion and orientation of a chromosomal Mu*d*(Ap)^{R}*lac* element in the *ruv* gene, from which B-galactosidase was expressed under the control of the *recA* and *lexA* genes and induced as part of the SOS response to DNA damage. An SOS inducible promoter that demonstrated similar induction properties to the *ruv*::Mu*d*(Ap)^{R}*lac* element was isolated from the DNA upstream of the coding region for the 24kd protein and cloned in front of the CAT gene of plasmid pKK232.8, again providing evidence that the 24kd and 41kd protein were cotranscribed.

Analysis of the nucleotide sequence of the *ruv* region revealed two open reading frames with coding potential for proteins of 22087 daltons and 37177 daltons respectively, which correspond to the proteins of 24kd and 41kd within the limits of molecular weight estimation from SDS-polyacrylamide gels. A possible promoter was identified upstream of the 24kd protein, which was overlapped by one of two sequences with homology to the *lexA* consensus binding site. The second putative SOS box was located approximately 40bp upstream of the proposed promoter. Purified LexA protein has been demonstrated to bind to both SOS boxes (G.T. Illing, pers. comm.). Two further possible promoters were identified upstream of the 37kd protein, however their role in the transcription of this protein was assumed to be relatively minor, since the induction response of the plasmid borne promoter::CAT fusion, and the chromosomal *ruv*::Mu*d*(Ap)^{R}*lac* were similar. However the possibility remains that the 37kd protein could be transcribed from one of these promoters at a low basal level in the absence of DNA damage.

From the above studies it was concluded that the 22kd and 37kd proteins were probably cotranscribed, however the relative contributions of the SOS controlled promoter and the two promoters in front of the 37kd protein remains to be determined. Clearly, the demonstration that the Tn*10* insertion in the *ruv-60* mutation mapped within the 22kd protein region suggested either that the two genes were cotranscribed, or alternatively that mutations in the 22kd protein also resulted in a UV and gamma irradiation sensitive and mitomycin C sensitive phenotype, which again suggested the functions of the two proteins may be related. Unfortunately, there are no known mutations in the 22kd proteins coding region that can be expected not to exert a polar effect on transcription of the 37kd protein.

9.4 Future directions

The determination of the nucleotide sequence and identification of the reading frames for the proteins encoded by the *ruv* region has opened the door for many different studies.

Firstly, analysis of *in vitro* and *in vivo* transcripts produced from the cloned DNA (by S1 nuclease mapping) should allow the relative contributions of the three promoters to be assessed, and the transcription initiation sites determined.

Secondly, analysis of the nucleotide sequence revealed further restriction sites which can be used to subclone the coding regions for the two proteins (both separately and together) into vectors in which their production can be regulated. The failure to clone the intact *ruv* region into multicopy plasmids (Chapter 4) had suggested that the production of the 37kd protein from multicopy plasmids was lethal to cells. However an alternative explanation is that it was over-production of both the 22kd and 37kd proteins that was lethal. The ability to separately subclone the coding regions for the two

proteins into plasmids in which their expression can be regulated should allow this alternative to be investigated.

Thirdly, the coding regions for the proteins can be subcloned into vectors such as those containing the λPL promoter in which expression of the cloned DNA can be tightly repressed by the presence of the λC1857 repressor and expression turned on simply by shifting the growth temperature of the plasmid harbouring strain. This should allow the production of sufficiently large quantities of both proteins such that purification can be initiated.

The purification and subsequent biochemical characterisation of the purified proteins should clarify the role of the ruv gene products in DNA repair and recombination.

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