Recombination at the site of a long chromosomal palindrome in *Escherichia coli*

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Declaration

I declare that this thesis was composed by myself and the research presented is my own, except where otherwise stated.

Gareth Andrew Cromie
March 2000

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Abstract

Long palindromic sequences are targets of the SbcCD nuclease which cleaves hairpin DNA structures. The double-strand breaks generated by this activity require recombination functions for repair. In the absence of such repair the presence of a long chromosomal palindrome is lethal to the *E.coli* cell.

In this work a range of recombination mutants were screened for their ability to carry out successful recombinational repair of SbcCD-generated double-strand breaks at the site of a long chromosomal palindrome. The results obtained suggest that the components of both the RecB and RecF pathways are required for successful recombinational repair of SbcCD-generated breaks at the site of the palindrome. This breakage and repair process does not seem to involve replication fork breakage. In the absence of SbcCD recombination still occurs, apparently through the RecF gap-recombination pathway. Once again, this process appears to avoid replication fork collapse. In the absence of recombination, the RecQ helicase was found to be essential to the viability of *sbcC* cells possessing the palindrome. This suggests that RecQ is involved in a pathway allowing replicative bypass of secondary structure, probably through helicase unwinding of the secondary structure.

Using an *xerC* mutant deficient in the resolution of chromosome dimers, the relationship between recombination at the site of the palindrome and crossing-over was investigated. It was observed that double-strand break repair at the site of the palindrome is associated with crossing-over over whereas single-strand gap recombination is not. Using UV irradiation of cells deficient for excision repair it was demonstrated that the association of double-strand break repair, but not single-strand gap repair, with crossing-over is a general phenomenon.

The observation that P1 transduction leads to the formation of mainly crossover structures in the recipient cell supports the idea that there is a rule governing the resolution of Holliday junctions. The random resolution of Holliday junctions in *ruvABC* mutants suggests that this rule operates through the RuvABC Holliday junction resolution complex.

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Abbreviations

%	percent
A, C, G, T	adenine, cytosine, guanine, thymine
ADP, ATP	adenosine 5'-diphosphate, adenosine 5'-triphosphate
amp	ampicillin
bp	base pairs
Cm	chloramphenicol
Da	daltons
DNA	deoxyribonucleic acid
DSBR	double-strand break repair
E.coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid disodium salt
g	gram
Hfr	bacterial strain carrying integrated F plasmid
k	kilo (10^3)
kb	kilobases
-1	(fractions of a) litre
m	metre
μ	micro (10^{-6})
mol	moles
PAS	Primosome Assembly Site
pfu	plaque forming unit
pH	power of hydrogen ($-\log_{10}[H^+]$)
phage	bacteriophage
superscript "r" (^{r})	resistant
RNA	ribonucleic acid
rpm	revolutions per minute
superscript "s" (^{s})	sensitive
SSB	Single-Strand Binding Protein
SSGR	single-strand gap repair
Str	streptomycin
Tc	tetracycline
UV	ultra violet radiation
wt	wild-type
w/v	weight per volume

Chapter 1

Introduction

1.1 Propagation of palindromic sequences in *Escherichia coli*

A palindrome is a DNA sequence with rotational symmetry, i.e. 2 inverted repeats lacking a spacer region (Figure 1.1). The use of the term palindrome has also been extended to include inverted repeats with short spacer regions in the middle because of the similarity in behaviour between these 'interrupted palindromes' and perfect palindromes.

Early attempts to clone long palindromic DNA sequences in *wt E.coli* ran into difficulties. Either the replicon carrying the palindrome could not be replicated (inviability) or the palindrome suffered complete, or partial, deletion (instability) (Collins 1981; Lilley 1981; Mizuuchi *et al.*, 1982; Hagan and Warren, 1983; Leach and Lindsey, 1986). Inviability affects palindromes of lengths above 150-200 bp (Warren and Green, 1985; Yoshimura *et al.*, 1986). It was suggested that this effect was due to the ability of such long palindromes to form higher-order structures in DNA by forming intrastrand (as opposed to interstrand) pairing (Figure 1.1).

There are two kinds of structure that could be formed by palindromic sequences. In single-stranded DNA, palindromes could form hairpins, while in duplex DNA they could form cruciforms (Figure 1.1). Evidence exists for the formation of such structures both *in vivo* and *in vitro* (Mizuuchi *et al.*, 1982; Allers

and Leach, 1995; Dayn *et al.*, 1996). However several experiments have suggested that cruciform formation is in fact rare *in vivo* (Courey and Wang, 1983; Sinden *et al.*, 1983) and two lines of evidence have suggested that the problems associated with long palindromes *in vivo* are generally caused by hairpin formation, rather than cruciform extrusion. Firstly, there is a large kinetic barrier to cruciform extrusion (interstrand bonds must initially be broken) that would be expected to reduce its occurrence, even in situations (such as in negatively supercoiled DNA) where the final extruded state is energetically favourable (Courey and Wang, 1983; Gellert *et al.*, 1983; Sinden *et al.*, 1983). Secondly, studies have indicated that palindrome inviability is dependent on the replication of the palindromic sequence (Leach and Lindsey, 1986; Shurvinton *et al.*, 1987), which suggests that the DNA has to be single-stranded for the relevant event to occur. This is more consistent with a single-stranded process like hairpin formation rather than a double-stranded process like cruciform extrusion.

Further studies have suggested that hairpin formation occurs preferentially on the lagging strand, perhaps because of the extensive exposure of single-stranded DNA associated with lagging-strand replication. Supporting evidence comes from observations concerning the instability of long DNA palindromes. Such palindromes suffer deletions that tend to reduce their capacity to form secondary structure (shortening them and tending to introduce central asymmetries). Deletion stimulated by inverted repeat sequences seems to jump from small direct repeats causing deletion of all, or part of the inverted repeat sequence (Glickman and Ripley, 1984; Trinh and Sinden, 1991). Often a direct repeat inside the palindrome marks one end of the deletion and one outside the palindrome marks the other. It is believed that replication stalls inside the secondary structure formed by the palindrome and the strand being extended then 'slips' to pair with a direct repeat outside the hairpin structure (Weston-Hafer and Berg, 1989; Leach, 1994). This can be used to define the direction in which replication en-

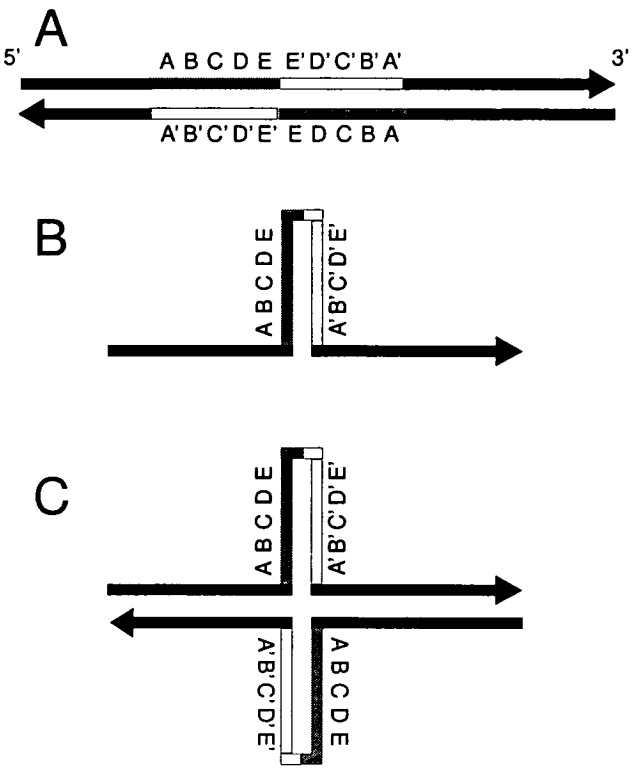

Figure 1.1: Palindromic sequences and their capacity to form secondary structure in single- and double-stranded DNA (adapted from Leach, 1994).
A) A palindrome is a closely spaced inverted repeat sequence with rotational symmetry in double-stranded DNA.
B) A palindrome can form a hairpin in single-stranded DNA by intrastrand base pairing.
C) A palindrome can form a cruciform in double-stranded DNA by intrastrand base pairing.

countered the hairpin in order to cause a particular deletion, i.e. the direction in which replication jumps from the repeat inside the structure to that outside, and not *vice versa*. As leading and lagging strand replication proceed in opposite directions this will define whether the event occurred on the leading or lagging strands. It is an analysis of the pattern of deletions on this basis that suggests that most deletion occurs on the lagging strand (Trinh and Sinden, 1991; Rosche *et al.*, 1995; Sharp and Leach, 1996; Pinder *et al.*, 1998). However, there is one contrary report suggesting that no strand preference exists (Weston-Hafer and Berg, 1991). The apparent preference for palindrome-stimulated deletion on the lagging strand suggests that structure formation is more prevalent on this strand.

Although long DNA palindromes cannot be propagated in *wt* cells, they can be propagated in cells carrying mutations in either of the genes *sbcC* or *sbcD* (Leach and Stahl, 1983; Chalker, 1988; Gibson et al, 1992).

1.2 The SbcCD nuclease

The *sbcC* and *sbcD* genes were identified by their ability (in combination with the *sbcB15* mutation) to suppress the recombination deficiency associated with mutations in the genes *recB* and *recC*. Initially it was believed that the *sbcB15* mutation by itself could suppress the severe recombination deficiency of these strains (Kushner *et al.*, 1971), but, although this mutation does indeed partially suppress this phenotype (Lloyd and Buckman, 1985), full suppression requires a further mutation in either *sbcC* or *sbcD* (Lloyd and Buckman, 1985; Gibson et al, 1992). This is why *recBC sbcB15* strains rapidly accumulate suppressors in *sbcC* and *sbcD* (Lloyd and Buckman, 1985; Gibson *et al.*, 1992). Single mutations in *sbcC* or *sbcD* do not restore recombination to any extent in a *recBC* strain or have any effect on recombination proficiency in *wt* cells (Lloyd and Buckman, 1985).

Both *sbcB* and *sbcCD* encode nucleases, and it is believed that mutations in these genes allow linear DNA to persist long enough that it is able to undergo

recombination using the genes of the RecF pathway (see Chapter 1.4.2). This restores the recombination proficiency normally associated with the function of RecBCD.

The *sbcC* and *sbcD* genes are encoded by a single operon and the last transcribed *sbcD* codon overlaps the first codon of *sbcC* so that it is believed that SbcC is (inefficiently) translated by the same ribosomes used by SbcD (Naom *et al.*, 1989). SbcC is a 118 kDa protein and SbcD a 45 kDa protein (Naom *et al.*, 1989). SbcC is a member of the SMC (Structural Maintenance of Chromosomes) family (Sharples and Leach, 1995) and SbcD is a phosphoesterase, believed to possess a nuclease function (Sharples and Leach, 1995). It is thought that 6 monomers of SbcC associate with 12 monomers of SbcD to form the functional nuclease (in the presence of Mn^{2+}) which has the structure of 2 globular 'head' domains linked by a coiled-coil 'rod' domain when examined by electron microscopy (Connelly *et al.*, 1997; Connelly *et al.*, 1998). This is the structure expected of SbcC so that it is not clear whether the complex seen using electron microscopy actually contains SbcD.

The SbcCD complex is an ATP-dependent double-strand exonuclease and an ATP-independent single-strand endonuclease (Connelly and Leach, 1996). The exonuclease activity appears to consist of a 3' to 5' nuclease activity operating from both ends of a linear DNA molecule (Connelly *et al.*, 1999). Significantly it also cleaves the closed ends of hairpin molecules, at the 5' side of the central loop (Connelly *et al.*, 1998; Connelly *et al.*, 1999).

The *in vitro* activity of SbcCD on hairpin molecules is consistent with a model which has been developed to explain its involvement in the viability of palindromes (Leach 1994; Leach *et al.*, 1997). If a palindrome forms a hairpin during replication then it could be cleaved by the SbcCD nuclease. This would be 'lethal' to the replicon involved unless this double-strand break was successfully repaired by recombination. Support for this model has come from studies using phage

λ carrying a long palindrome. It was observed that an imperfect palindrome of 246 bp present on phage λ could be propagated successfully in *sbcCD*$^+$ strains of *E.coli*, when the phage was present as a lysogen (Leach *et al.*, 1997). However, the viability of these cells was dependent on the recombination proteins RecBC and RecA, which are associated with the repair of double-strand breaks (Leach *et al.*, 1997) (see Chapter 1.4.1 and Chapter 1.5.1). Viability was not dependent on these proteins when the strain lysogenised was an *sbcC* mutant (Leach *et al.*, 1997). As with other palindromes, studies of deletion derivatives of this 246 bp sequence suggest it preferentially forms structures on the lagging strand during replication (Pinder *et al.*, 1998). These results are consistent with regular formation of a hairpin by the 246 bp palindrome on the lagging strand during replication, followed by cleavage of this structure by SbcCD (Figure 1.2). If this occurred, the resulting breaks could be successfully repaired by homologous recombination using the sister 'chromatid' as a template (Leach 1994; Leach *et al.*, 1997) (Figure 1.2). If a hairpin structure were then to form again this process could be repeated (Figure 1.2). Presumably the random occasions on which this sequence avoided hairpin formation would allow successful replication. This might only occur after several cycles of homologous recombination. In addition, if cleavage happened persistently then it might still be impossible to successfully replicate the palindrome sequence even with successful recombinational repair. In some cases (for example long palindromes in *wt* cells) the delaying effect of this process could lead to inviability without actual destruction of the parental DNA (Lindsey and Leach, 1989).

1.3 Recombination in *Escherichia coli*

Homologous recombination is a process essential to DNA repair and the generation of novel genotypes. Homologous recombination consists of exchanges between DNA molecules, or regions of the same molecule, of identical, or near identical

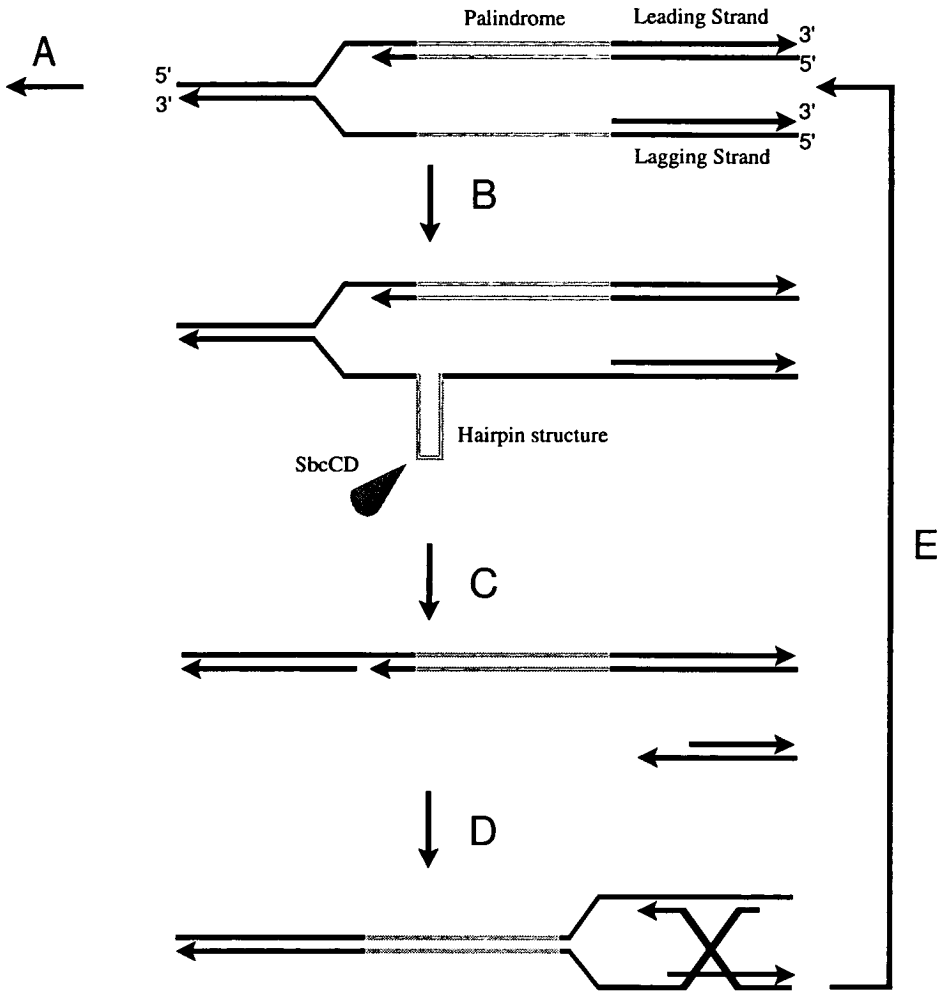

Figure 1.2: Model for SbcCD-generated double-strand breaks at palindromes, and the effect of this on replication (adapted from Leach *et al.*, 1997).
Initially replication uncovers the palindrome as a single-stranded sequence on the lagging strand template.
A) If the palindrome does not fold into a hairpin then replication proceeds normally past the problematic sequence.
B) If a hairpin does form it is cleaved by SbcCD.
C) This generates a double-strand break and a broken replication fork.
D) Recombination (using RecBCD and RecA) uses the broken end to invade the intact duplex and form a joint molecule.
E) Resolution of the Holliday junction in the joint molecule restores the replication fork and replication has another chance to progress through the palindomic sequence.

sequence. There are several proteins that can be involved in carrying out recombination in *E.coli*. The subset of recombination proteins that is involved in any given reaction depends on the nature of the DNA substrates undergoing recombination and the genetic background in which the reaction is taking place. However, in general terms, homologous recombination involves a three step process: presynapsis, synapsis and postsynapsis (Clark, 1971). In presynapsis single-stranded DNA is coated with the synapsis protein, which for the vast majority of *E.coli* recombination reactions is the RecA protein. During synapsis a joint molecule is formed by RecA-mediated strand invasion. During postsynapsis branch migration extends the heteroduplex region by catalysing the exchange of strands between the recombining DNA molecules. This converts the initial three strand intermediate into a four strand intermediate containing a four way Holliday junction. Resolution of this junction by appropriate strand cleavage is followed by replication and ligation to repair gapped or nicked strands. This completes postynapsis and the recombination reaction. The full process is illustrated for one kind of recombination ('ends-out' double-strand break repair (DSBR)) in Figure 1.3.

1.4 Presynapsis

Single stranded DNA is essential for RecA-loading and synapsis (Figure 1.3 (A). In some recombination reactions the initial DNA substrate undergoing recombination may possess a sufficiently large single-stranded DNA region to allow RecA to participate in efficient recombination. However, in other reactions single-stranded DNA has to be produced, or extended, by recombination enzymes. There are a large number of enzymes that can carry out these presynaptic steps and these have been grouped into three loose enzymatic 'pathways': the RecB pathway, the RecF pathway and the RecE pathway. This classification was initially made by analysis of the recombination proteins required in different genetic backgrounds, but has become less well defined over time. In this work 'pathways' have been de-

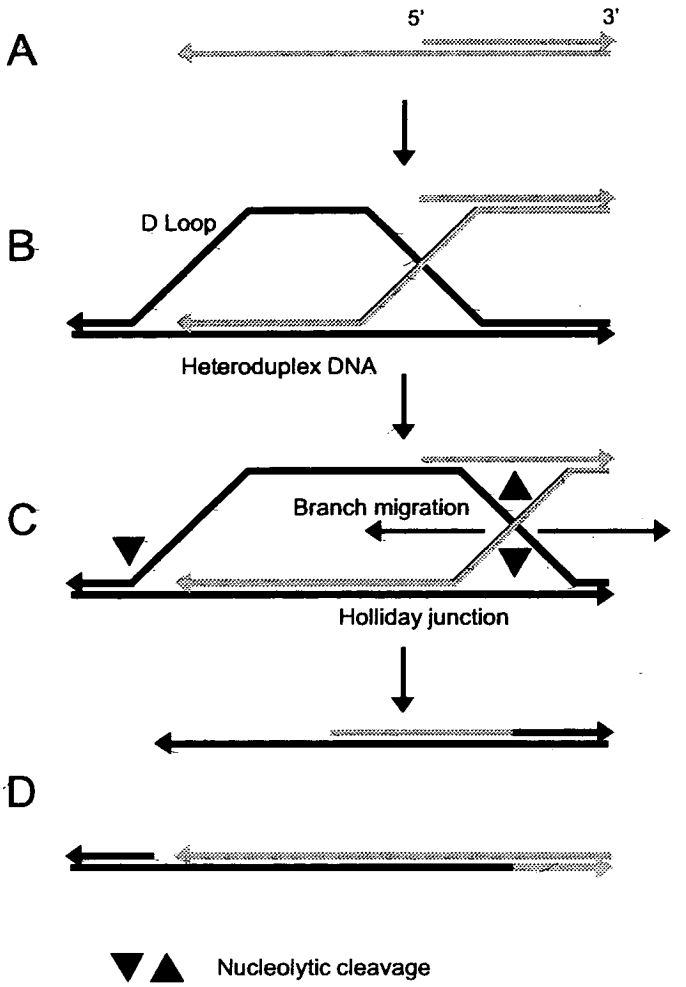

Figure 1.3: General scheme for recombination using a DNA double-strand end.
A) Presynapsis: A 3' single-stranded end is generated onto which RecA is loaded.
B) Synapsis: The invasive 3' single-stranded end coated with RecA invades a homologous duplex, generating a D loop.
C) Postsynapsis: The three strand junction is converted to a four strand junction. Branch migration moves the site of the Holliday junction and varies the extent of heteroduplex DNA. The Holliday junction is then resolved by strand cleavage (in this model the D loop must also be cleaved).
D) Recombinant DNA molecules are generated.

1985). It appears that overhanging ends may have to be processed by single-strand nucleases to produce a blunt end capable of being recognised by RecBCD (Thoms and Wackernagel, 1998). When RecBCD enters a DNA end it translocates along the DNA, unwinding and degrading both strands, with cleavage particularly directed at the 3'-ended strand (Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski 1993; Taylor and Smith, 1995 (2)) (Figure 1.4). It appears that the RecB subunit translocates along the strand 3' to 5' away from the DNA end while the RecC and RecD subunits translocate along the strand 5' to 3' away from the DNA end (Ganesan and Smith, 1993) (Figure 1.4). The ATPase activity of RecB seems to be important for cleavage of the 3'-ended strand while the ATPase activity of RecD is important for the degradation of the 5'-ended strand (Chen *et al.*, 1998).

Translocation and degradation of the 3'-ended strand continue until the enzyme comes upon a correctly orientated χ octamer sequence 5'-GCTGGTGG from the 3' side. It then makes a nick 4 to 6 bases 3' to the χ sequence (Taylor *et al.*, 1985; Ponticelli *et al.*, 1985) and its activity changes so that it no longer degrades the 3'-ended strand and cleavage on the 5'-ended strand is upregulated (Dixon and Kowalczykowski 1993; Taylor and Smith, 1995 (2); Anderson *et al.*, 1997 (2)) (Figure 1.4). This produces a 3' single-stranded overhang, onto which RecA protein loads, with the aid of the RecBCD complex (Anderson and Kowalczykowski, 1997 (1)) (Figure 1.4). The interaction between χ and RecBCD may involve the RecD subunit. Cells lacking RecD do not demonstrate χ-stimulated recombination but are still highly recombination proficient (Chaudhury and Smith, 1984 (1); Amundsen *et al.*, 1986; Dixon and Kowalczykowski, 1995). It is believed that interaction with χ causes a change to the structure of the enzyme (Taylor and Smith, 1992; Anderson *et al.*, 1997 (2); Yu *et al.*, 1998) rather than RecD ejection, as originally thought (Thaler *et al.*, 1989; Stahl *et al.*, 1990; Dixon *et al.*, 1994). The RecBCD enzyme appears to disassemble at some point after interaction with

χ, which perhaps is in order to ensure that only a single recombinational exchange occurs after this interaction (Taylor and Smith, 1999). Only about 25-40 % of RecBCD encounters with any given χ site are successful (Taylor and Smith, 1992; Dixon and Kowalczykowski, 1993). However, it appears that recombination via the RecB pathway (as measured by conjugational and transductional assays) is mainly through χ-mediated events (Dower and Stahl, 1981).

In recombination assays that involve DNA ends, such as conjugation or transduction, the RecB pathway predominates. In conjugation and transduction, mutations in *recB* or *recC* reduce the frequency of recombinant production by 100 to 1000 fold (Emmerson, 1968; Chaudhury and Smith, 1984 (2)). RecBCD is also important in the recombinational repair of UV damage. Mutations in *recB* or *recC* dramatically reduce the survival of UV irradiated cells (Ganesan and Smith, 1970; Wang and Smith, 1981; Wang and Smith, 1983). This is due to an inability to repair double-strand breaks by recombination (Wang and Smith, 1983; Wang and Smith, 1986). In contrast to the effect of mutations in *recB* or *recC*, mutations in *recD* are generally recombination-proficient, even hyper-recombinogenic (Chaudhury and Smith, 1984 (1) ; Biek and Cohen, 1986; Thaler *et al.*, 1989) and resistant to UV irradiation (Brcic-Kostic *et al.*, 1991). This reflects the functional helicase and constitutive RecA-loading activity of the RecBC protein (Churchill *et al.*, 1999).

1.4.2 The RecF pathway

The RecF pathway for the initiation of recombination was originally identified in *recBC* strains. The extreme deficiency of such strains in conjugation was found to be suppressed by mutations in either the *sbcC* or *sbcD* genes along with the *sbcB15* mutation (see Chapter 1.2). In such a background conjugational recombination was found to be highly dependent on the products of the *recF*, *recO*, *recR*, *recJ*, *recN*, *recQ* and *ruv* genes, whereas in *wt* cells none of these mutations have a

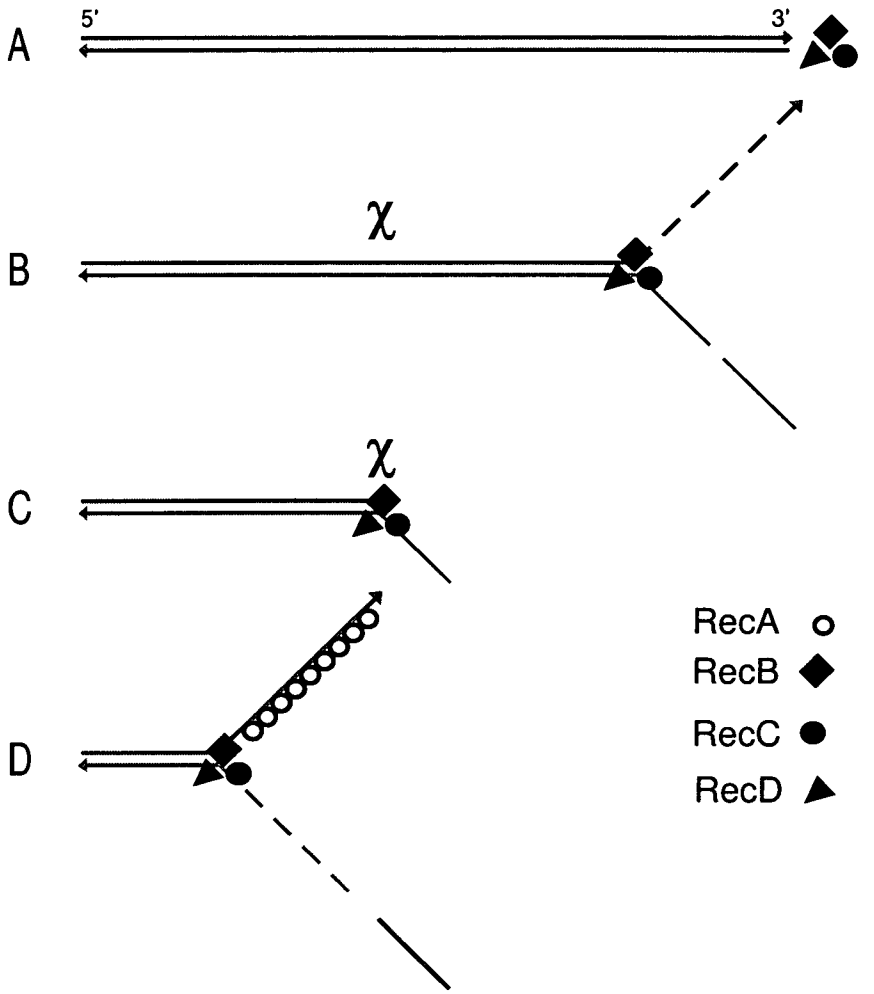

Figure 1.4: Action of RecBCD on duplex DNA.
A) RecBCD loads onto a DNA duplex at a blunt end.
B) RecB translocates 3' to 5' away from the DNA end, while RecC and RecD translocate 5' to 3' on the other strand. Degradation of both strands occurs with a more extensive activity on the 3'-ended strand.
C) RecBCD encounters a correctly orientated χ site and pauses, introducing a nick on the 3'-ended strand.
D) The enzyme continues translocating, but the nuclease activity is downregulated on the 3'-ended strand and upregulated on the 5'-ended strand. Meanwhile RecBCD loads RecA onto the 3'-ended strand.

dramatic effect on recombination (Horii and Clark, 1973; Lloyd *et al.*, 1983; Nakayama *et al.*, 1984; Lloyd *et al.*, 1984; Kolodner *et al.*, 1985; Mahdi and Lloyd, 1989).

The *ruv* genes encode components of a complex involved in carrying out post-synaptic branch migration and resolution and will be discussed later (see Chapter 1.6.1). The products of the other genes identified above appear to act presynaptically.

1.4.2.1 The RecFOR proteins

The RecF, RecO and RecR proteins appear to play a role together in the recruitment and localisation of RecA protein to single-stranded regions of DNA, particularly single-strand gaps.

Purified RecF protein can bind single-stranded and double-stranded DNA *in vitro*. Binding to double-stranded DNA is ATP-dependent, while binding to single-stranded DNA is not (Griffin and Kolodner, 1990; Madiraju and Clark, 1992). RecF has a weak ATP-hydrolytic capability, which leads to its dissociation from double-stranded DNA (Webb *et al.*, 1999). Purified RecO protein binds both single-stranded and double-stranded DNA and promotes the reannealing of complementary single-stranded DNA molecules (Luisi-DeLuca, 1994). No biochemical function has been shown for purified RecR protein on its own. The RecFOR proteins are also known to form complexes with one another. RecO and RecR associate strongly with one another, requiring no further cofactors and RecO also interacts with Single-Strand Binding Protein (SSB) (Umezu and Kolodner, 1993). RecF and RecR associate in the presence of double-stranded DNA and ATP (Webb *et al.*, 1995). Interactions have also been detected *in vitro* between RecF and RecR without the presence of DNA. Complexes of RecF, RecO and RecR have been detected in the same way (Hegde *et al.*, 1996).

The pre-incubation of single-stranded DNA with SSB hinders the formation of RecA filaments, and hence recombination, *in vitro* (Cox and Lehman, 1982,

Umezu *et al.*, 1993; Umezu and Kolodner, 1994). The presence of both RecO and RecR allows RecA to load onto regions of single-stranded DNA coated with SSB (apparently without displacement of the SSB) and to utilise this DNA just as efficiently as in a reaction where pre-incubation with SSB does not occur (Umezu *et al.*, 1993; Umezu and Kolodner, 1994). RecO and RecR also act together to stabilise RecA filaments on single-stranded DNA (Shan *et al.*, 1997). Although these reactions do not require RecF, the similar phenotypes of *recF*, *recO* and *recR* strains suggest that they form an epistasis group with their products acting together (Lloyd and Buckman, 1991). It has been suggested that the function of RecF may be to limit RecA loading specifically to single-stranded regions (Hegde *et al.*, 1996). Some support for this notion comes from the observation that, using small, partially single-stranded substrates, the RecF and RecR proteins act together to prevent the extension of RecA filaments beyond the single-stranded region (Webb *et al.*, 1997).

The biochemical evidence pointing to a role for the RecFOR proteins in the recruitment of RecA to single-stranded DNA fits well with the phenotypes of *recF*, *recO* and *recR* mutants. The recombinational, and UV-repair defects of mutations in *recF*, *recO* and *recR* can be partially suppressed by certain mutations in *recA* (Volkert and Hartke, 1984; Wang and Smith, 1986; Wang *et al.*, 1993). The basis of this suppression appears to involve a greater ability of the product of the mutant *recA* gene to load onto single-stranded DNA, especially in the presence of SSB (Dri and Moreau, 1991; Madiraju *et al.*, 1992). This suggests that the RecFOR proteins are involved in promoting RecA binding to single-stranded DNA *in vivo*, a process that would otherwise be hindered by SSB. The similarity in phenotypes between *recF* mutants and stains overproducing SSB (Moreau, 1988) also supports this idea.

One of the functions of RecBCD is to load RecA onto single-stranded regions at DNA ends (Anderson and Kowalczykowski, 1997 (1)). In *recBC* mutants the

RecFOR proteins may be needed to carry out this function. This would explain the requirement for the RecFOR proteins in conjugation and transduction (DNA end-directed recombinational processes) in *recBC* cells. In *wt* cells RecFOR may be required to recruit RecA to single-stranded regions not produced by RecBCD, especially internal single-strand gap regions. Support for RecFOR action on these kinds of substrates comes from the observation that *recFOR* mutants display UV sensitivity in $recBC^+$ cells (Horii and Clark, 1973; Kolodner *et al.*, 1985; Mahdi and Lloyd, 1989). This deficiency appears to represent a problem in the recombinational (RecA-dependent) filling of single-strand gaps generated by excision repair (Smith and Sharma, 1987). Mutations in *recF*, *recO* and *recR* also show a deficiency in recombinational repair in excision repair mutants, which once again appears to involve a deficiency in the recombinational filling of single-strand gaps (Tseng *et al.*, 1994).

Although many of the phenotypes of *recF*, *recO* and *recR* mutants are very similar there are some phenotypes that distinguish the mutants. Double null mutants of *recF* (but not *recO* or *recR*) and *priA* are inviable and other double mutants (involving less severe mutations) demonstrate an impairment in SOS induction greater than either of the single mutants (Sandler, 1996). PriA is a protein involved in the reestablishment of broken replication forks (Chapter 1.7) and RecF may be required for an alternative pathway to carry out this function (Sandler, 1996). In fact even in $priA^+$ cells the RecFOR proteins are required for the resumption of replication after UV irradiation (Courcelle *et al.*, 1997; Courcelle *et al.*, 1999; Courcelle and Hanawalt, 1999). This suggests that instead of RecF being involved in a cryptic pathway only active in *priA* strains, both PriA and RecF have important, but somewhat overlapping, functions in the resumption of interrupted DNA replication.

In *wt* cells, although the RecFOR proteins are not important for conjugational or transductional recombination, they are important for plasmid recombination.

Mutations in the *recF*, *recO* or *recR* genes have a dramatic effect on plasmidic recombination in *wt* cells (James *et al.*, 1982; Laban and Cohen, 1981; Cohen and Laban, 1983; Kolodner *et al.*, 1985; Mahdi and Lloyd, 1989). However in *recBC* strains, although these gene products are still necessary for efficient interplasmidic recombination, they are no longer necessary for intraplasmidic events (Cohen and Laban, 1983).

1.4.2.2 The RecJ protein

The RecJ protein is a 5' to 3' single-strand exonuclease whose role in recombination seems to be to extend regions of single-stranded DNA, in order to provide a substrate for RecA. The activity of RecJ may also aid in the early stages of branch migration.

RecJ is a 60 kDa monomer protein which demonstrates a Mg^{2+}-dependent 5' to 3' single-strand exonuclease activity (Franklin and Lindahl, 1988; Lovett and Kolodner, 1989).

The *recJ* gene was initially identified through its importance in recombination in *recBC sbcB sbcC* cells. In this background both conjugational recombination and recovery from UV irradiation are both strongly dependent on the RecJ protein (Lovett and Clark, 1984). In a *wt* background *recJ* mutants show little deficiency in either of these processes (Lovett and Clark, 1984). In the *recBC sbcB sbcC* background it appears that other proteins are able to take over the various activities of the RecBCD enzyme. Together the RecFOR proteins could replace the ability of RecBCD to load RecA onto single-stranded DNA ends, and it appears that RecJ may replace the (χ-activated) 5' to 3' nuclease activity of RecBCD that produces these ends. In support of this idea in *recD* mutant strains of *E.coli* (which are deficient in RecBCD nuclease activity, but not helicase activity) conjugational recombination and UV resistance become dependent on RecJ (Lovett *et al.*, 1988; Lloyd *et al.*, 1988). In this case it would appear that the nuclease activity of RecJ is acting along with the helicase activity of the RecBC

enzyme to produce 3' overhanging DNA ends coated with RecA.

As well as an involvement in the production of recombinogenic 3' single-stranded DNA ends, RecJ may also play a role in the promoting branch migration leading to a productive recombination event. Studies *in vitro* show that RecJ stimulates the rate of strand transfer and greatly improves the ability of branch migration to traverse regions of non-homology (Corrette-Bennett and Lovett, 1995). It is believed that RecJ does this by degrading the 5'-ended strand that is displaced as its complementary 3'-ended strand invades another duplex during recombination. This activity would have the effect of making strand exchange unidirectional by removing a potential competitor for pairing with the invasive strand (Whitby and Lloyd, 1995; Corrette-Bennett and Lovett, 1995; Friedman-Ohana and Cohen, 1998).

It appears that the normal function of the RecFOR proteins may be to help initiate recombination at single stranded gaps. There is evidence to suggest that RecJ may also be involved in gap recombination. In a *recB uvrB* mutant, recombinational repair of UV damage occurs through the RecF gap-repair pathway. In this background mutations in *recJ* cause a severe deficiency in the repair of single-strand gaps (Wang and Smith, 1988). In conjugational recombination measured by the synthesis of β-galactosadase in crosses between *lacZ* mutants, RecF recombination is as significant as the RecB pathway. It is believed that the substrates of this activity are also single-strand gaps. In this case *recJ* mutations are as deleterious as *recF* and *recO* mutations and the products of these three genes appear to operate in the same pathway, a pathway distinct from that of RecB (Lloyd *et al.*, 1987). In general the contribution of RecJ to gap-repair pathways might be to extend the putative recombinogenic single-stranded region (in conjunction with a helicase).

Mutations in *recF*, *recO* and *recR* have deleterious effects on plasmid recombination, even in *wt* cells. This is also true for mutations in *recJ*. In fact these

mutants are exceptionally deficient in plasmid recombination (Kolodner et al, 1985).

For RecJ to convert duplex DNA into single-stranded DNA it needs to operate in conjunction with a helicase. In *recD* mutants this helicase can be RecBC but it has been suggested that another partner for RecJ could be the RecQ helicase (Lovett and Kolodner, 1989). There is some evidence to support the idea of these enzymes working together in *wt* cells, for example at gaps formed by stalled replication forks (Courcelle and Hanawalt, 1999)

1.4.2.3 The RecQ helicase

RecQ is a helicase with an ability to unwind duplex DNA in the 3' to 5' direction (relative to the strand it loads onto) (Umezu *et al.*, 1990; Umezu and Nakayama, 1993). It can act on blunt-ended DNA although it seems to have a higher activity on DNA with a 3' single-strand overhang (Umezu *et al.*, 1990). The helicase activity of RecQ is greatly stimulated *in vitro* by the presence of SSB. There is evidence that RecQ is an SOS protein (Irino *et al.*, 1986).

Mutations in *recQ* cause a defect in conjugational recombination and an increase in UV sensitivity in *recBC sbcB sbcC* cells but have little effect on these processes in *wt* cells (Nakayama *et al.*, 1984, Nakayama *et al.*, 1985). This differs from the effect of mutations in the *recF*, *recO* and *recR* genes, which do cause UV sensitivity in *wt*, but is similar to the effects of *recJ* mutations. If RecJ is able to replace the recombinogenic 5' to 3' single-strand nuclease activity of RecBCD in *recBC sbcB sbcC* cells then RecQ is a good candidate for replacing the helicase activity of the RecBCD enzyme. In support of this, it has been demonstrated *in vitro* that RecQ can initiate recombination using a blunt-ended duplex by providing single stranded DNA for RecA loading (Harmon and Kowalkzykowski, 1998). The helicase activity of RecQ can also disrupt recombination by unwinding joint molecules generated by RecA-mediated strand invasion (Harmon and Kowalkzykowski, 1998). The ability of RecQ to unwind joint molecules may ex-

plain its ability to suppress illegitimate recombination, which appears to depend upon short homologous regions undergoing illegitimate pairing (Hanada *et al.*, 1997).

A different activity of RecQ is manifested in its ability to allow *E.coli* Topoisomerase III to fully catenate double-stranded DNA circles (Harmon *et al.*, 1999; Wu *et al.*, 1999). This appears to involve an ability of RecQ to unwind covalently-closed double-stranded DNA. This generates a substrate for TopoIII, even though molecules with single-stranded regions are not in themselves a substrate for the activity of Topo III (Harmon *et al.*, 1999). It has been suggested that this activity could allow RecQ (along with Topo III) to control the levels of homologous and non-homologous recombination in *E.coli* (Harmon *et al.*, 1999).

Finally, it has also been suggested that RecQ (and its eukaryotic homologues) could play a role in defending genome integrity. This might include suppressing illegitimate recombination events and resolving abnormal DNA structures (Chakraverty and Hickson, 1999).

1.4.2.4 The RecN protein

As is the case with *recJ* and *recQ*, mutations in *recN* cause a deficiency in conjugational recombination and an increase in UV sensitivity in *recBC sbcB sbcC* strains, but have much milder effects in cells that are *wt* for these functions (Lloyd *et al.*, 1983). RecN is an SOS protein and appears to be present in very low quantities in uninduced cells (Finch *et al.*, 1985).

Mutations in *recN* cause a major defect in the ability of UV irradiated cells to repair double-strand breaks (Sargentini and Smith, 1986 (2); Wang and Smith, 1988). Similarly RecN is needed to repair double-strand breaks caused by X rays (Sargentini and Smith 1986 (1); Sargentini and Smith 1986 (3)). In these processes RecN appears to act in a pathway along with RecB and in general it appears that RecN plays a role in DNA end-directed recombinational pathways. This suggests that the grouping of RecN with RecFOR, RecQ and RecJ (because

of the roles they all play in *recBC sbcB sbcC* cells) is inappropriate and, instead, the activity of RecN should probably be grouped with RecBCD.

1.5 Synapsis

After the production of single-stranded DNA and the loading of RecA protein the next stage in recombination is the invasion of a homologous duplex by the RecA-coated recombinogenic single strand (Figure 1.3 (B)). This process of synapsis results in the formation of a joint molecule which can then be extended by branch migration.

1.5.1 The RecA protein

The *recA* gene was initially identified by the exceptionally deleterious effects of *recA* mutations on conjugational recombination (Clark and Marguilies, 1965). Mutations in *recA* have a similar deleterious effect on most other recombination processes, including transduction (Hertman and Luria, 1967) and plasmid recombination that does not utilise the RecE pathway (Hobom and Hogness, 1974; Laban and Cohen, 1981; James *et al.*, 1982).

Mutations affecting *recA* also cause defects in the recombinational repair of UV damage (Clark and Marguilies, 1965), including the repair of both double-strand breaks and single-strand gaps (Smith and Meun, 1970; Wang and Smith, 1983). The recombinational repair of breaks occurring in X ray and γ irradiation is also dependent on RecA (Howard-Flanders and Theriot, 1966; Kapp and Smith, 1970; Krasin and Hutchison, 1977; Sargentini and Smith 1986 (1); Sargentini and Smith 1986 (3)). Similarly, the recombinational repair of single-strand gaps generated by excision repair is also RecA-dependent (Youngs *et al.*, 1974; Smith and Sharma, 1987). This demonstrates the involvement of RecA in nearly all *E.coli* recombinational pathways, whether utilising gaps or breaks, the RecF-group of presynaptic proteins or the RecB pathway.

The RecA protein is a 38 kDa protein that binds both single-stranded and double-stranded DNA (Sancar *et al.*, 1980; McEntee *et al.*, 1981). The protein can form regular right-handed helical filaments on the DNA to which it is bound, with close to 3 base pairs of DNA per RecA monomer (Di Capua *et al.*, 1982; Egelman and Stasiak, 1986). The protein is a DNA-dependent ATPase (Ogawa *et al.*, 1978; Roberts *et al.*, 1978) and can generate joint molecules from DNA molecules of homologous sequence *in vitro*, in an ATP-dependent manner (McEntee *et al.*, 1979; Shibata *et al.*, 1979). However the hydrolysis of ATP is not required to provide energy for this reaction (Menetski *et al.*, 1990). Instead, it modulates the DNA-binding activity of RecA, from a high affinity ATP-bound form to a lower affinity ADP-bound form (Menetski and Kowalczykowski, 1985). In turn, this process promotes RecA recycling (Menetski *et al.*, 1990; Rosselini and Stasiak, 1990).

Although the presynaptic generation of single-stranded DNA and loading of RecA onto this substrate requires further enzymatic functions *in vivo*, *in vitro* it is possible to initiate recombination reactions directly, simply using the DNA substrate and RecA. RecA nucleates randomly onto the single-stranded DNA and then binds co-operatively in a 5' to 3' polar manner to form a filament so that 3' ends are more likely to be coated with RecA than 5' ends (Griffith *et al.*, 1984; Register and Griffith, 1985). This is likely to explain why 3' ends are more invasive than 5' ends (Konforti and Davis, 1990). Disassembly of the RecA filament occurs in the same direction as assembly (Lindsey and Cox, 1990). RecA binding has the effect of extending the single-stranded DNA to 1.5 times its normal length (Flory *et al.*, 1984; Staskiak *et al.*, 1984). The functional presynaptic filament contains RecA, single-stranded DNA and ATP. The presence of SSB in the reaction removes secondary structures and allows longer presynaptic filaments to form that would otherwise be blocked by such structures (Kowalczykowski and Krupp, 1987). Once it has bound to single-stranded DNA the RecA filament can

easily be extended into double-stranded DNA (West *et al.*, 1980), whereas in the absence of a single-stranded region nucleation onto double-stranded DNA is a slow process (Kowalczykowski *et al.*, 1987; Pugh and Cox, 1987).

The actual process of synapsis consists of a search for homologous sequences to undergo homologous pairing with the DNA coated by RecA, followed by strand invasion and the setting up of a joint, homologously paired, molecule. The initial step of this process, the search for homology, is not well understood, but appears to involve transient extension and unwinding of possible targets with a homology check through attempted pairing in DNA-protein coagregates (Gonda and Radding, 1986; Rould *et al.*, 1992).

Once successful homologous pairing has occurred, RecA protein catalyses the exchange of DNA strands, with the target molecule being denatured locally, followed by the exchange of the invading DNA strand. If the invading DNA strand includes a DNA end then a D loop is formed (see Figure 1.3) (McEntee *et al.*, 1979; Shibata *et al.*, 1979). In this case the joint molecule will possess a stable interwound plectonemic joint (reviewed in Kowalczykowski *et al.*, 1994). A plectonemic joint is also formed if the free DNA end is provided by the target strand (complementary to that invading) rather than the invading DNA strand. If a single-strand gap region is involved, or recombination occurs between two duplex regions, then a non-interwound and unstable paranemic joint is formed (DasGupta *et al.*, 1980; Conley and West, 1989). However, *in vitro*, a topoisomerase can convert this initial unstable joint molecule into a stable, catenated plectonemic joint (Cunningham *et al.*, 1981).

1.6 Postsynapsis

After the completion of synapsis a structure consisting of a joint molecule incorporating both interacting duplexes and containing a four-way Holliday junction exists. This structure contains heteroduplex regions where single strands from

the two duplexes have been exchanged. The final stage of recombination is the movement of the four-way junction (which has the effect of varying the size of the heteroduplex regions) and resolution to form two discrete (recombinant) duplexes (Figure 1.3 (B)). These steps are described as postsynaptic.

1.6.1 The RuvABC proteins

The major pathway for the postsynaptic movement and resolution of Holliday junctions is the RuvABC system which combines a (junction-moving) branch migration function with a (junction-cleavage) resolution function.

Mutations in *ruv* genes were initially identified by their sensitivity to mitomycin C (Otsuji *et al.*, 1974). They also exhibit high sensitivity to UV but only a moderate deficiency in conjugational recombination in *wt* cells. However, they display a severe defect in recombination and UV-repair in *recBC sbcB sbcCD* strains (Otsuji *et al.*, 1974; Lloyd *et al.*, 1984; Lloyd, 1991). The phenotypes of mutations in the three different *ruv* genes are very similar.

The *ruvA* and *ruvB* genes form a single operon that is regulated by LexA as part of the SOS response (Shurvinton and Lloyd, 1982; Benson *et al.*, 1988; Shinagawa *et al.*, 1988). RuvC is encoded separately and is not SOS-regulated (Sharples *et al.*, 1990; Takahagi *et al.*, 1991).

RuvA is a 22 kDa protein that exists as a tetramer (Benson *et al.*, 1988; Tsaneva *et al.*, 1992). The protein possesses a 'pin' structure which may be important in separating strands during branch migration (Rafferty *et al.*, 1996). RuvA binds DNA and has a particularly high affinity for Holliday junctions (Shiba *et al.*, 1991; Iwasaki *et al.*, 1992; Parsons *et al.*, 1992). RuvA facilitates the binding of RuvB to DNA suggesting that it may recruit RuvB to Holliday junctions (Muller *et al.*, 1993 (2); Parsons and West, 1993). The structure of the Holliday junction is also altered by RuvA binding, the effect of which is to keep it in an open planar conformation (Parsons *et al.*, 1995, Rafferty *et al.*, 1996).

RuvB is a 37 kDa protein which forms a hexamer in the presence of DNA (Benson *et al.*, 1988; Mitchell and West, 1994). RuvB is a DNA-dependent ATPase (Iwasaki *et al.*, 1989), although, on its own, RuvB displays a low affinity for DNA (Muller *et al.*, 1993 (2)). RuvB is a helicase in the presence of RuvA (see below).

Together RuvA and RuvB form a complex of one tetramer of RuvA bound to the Holliday junction and two hexamers of RuvB on diammetrically opossed duplex arms entering the junction (Figure 1.5B) (Parsons *et al.*, 1995). Although RuvB can carry out branch migration on its own, the RuvAB complex is better able to carry out this reaction (Iwasaki *et al.*, 1992; Tsaneva *et al.*, 1992; Muller *et al.*, 1993 (1)). RuvAB exhibits a 5' to 3' helicase activity contributed by RuvB (Tsaneva *et al.*, 1993). The mechanism of RuvAB-mediated branch migration involves movement of the DNA through RuvA before entering the RuvB rings (Figure 1.5 (A)) (Hiom and West, 1995; West 1996).

RuvC is a 19 kDa protein which exists as a dimer (Iwasaki *et al.*, 1991).This dimer binds specifically to Holliday junctions (Dunderdale *et al.*, 1991). RuvC is an endonuclease and resolves junctions by symmetrical cleavage of strands of the same polarity. This cleavage shows a sequence specificity (5'-A/TTTG/C-3') with cleavage 3' to the run of Ts (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991; Bennett *et al.*, 1993; Shah *et al.*, 1994). Cleavage occurs when the cleavage site is positioned at, or within one base pair of, the crossover point of the strands in the junction (Bennett and West, 1996; Shida *et al.*, 1996).

Upon binding of RuvC to a Holliday junction *in vitro*, the structure of the junction becomes altered to form a more open structure with 2-fold symmetry (Bennett and West, 1995(1); Bennett and West, 1995(2)). This structure possesses wide and narrow angles at the Holliday junction and it is the wide angles that are subject to RuvC cleavage (at the consensus sequence) (Bennett and West, 1995 (2)) (Figure 1.5 (B)). After cleavage, the nicks present in the discrete

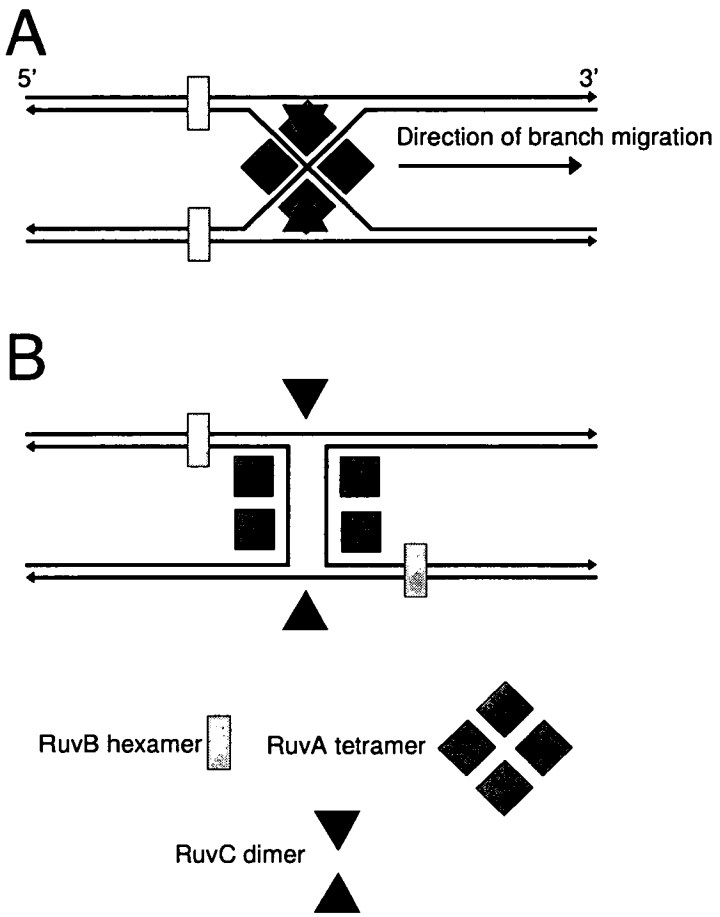

Figure 1.5: Action of RuvABC in branch migration and Holliday junction resolution.
A) A tetramer of RuvA binds to the Holliday junction and recruits two hexamer rings of RuvB. These are aligned so as to branch migrate the junction in one direction. A dimer of RuvC can also join this complex.
B) RuvC cleaves the strands passing 3' through RuvB and into the junction. Studies *in vitro* suggest that RuvC preferentially cleaves the wide angles of a junction with 2-fold symmetry. How the structures of junctions bound by RuvA and RuvC relate is unknown. RuvC cleaves at a consensus sequence (see text).

duplexes produced can be repaired by DNA ligase (Bennett *et al.*, 1993).

The genetic evidence demonstrating a similar phenotype for all 3 *ruv* single mutants suggests that they interact functionally. RuvA, RuvB and RuvC can form a complex together on Holliday junctions *in vitro* (Davies and West, 1998). In an *in vitro* recombination system carrying out branch migration and resolution in the presence of all 3 proteins, resolution of the junction was inhibited by antibodies raised against any one protein (Eggleston *et al.*, 1997). This suggests that all 3 proteins are present in an active complex. The sequence specificity of RuvC suggests that it would need to be coupled to a branch migration function in order to be able to cleave Holliday junctions, which might otherwise be located at unfavourable sequences. This idea is supported by the observation that RuvAB stimulates the junction-resolution activity of RuvC (Zerbib *et al.*, 1998). In the presence of RuvAB, RuvC specifically cleaves the strands which pass 3' through RuvB into the junction (van Gool *et al.*, 1999) perhaps through these strands being held in a conformation so that they form the more open angles of the junction.

Finally, although junction resolution *in vivo* appears to require a complex consisting of all 3 Ruv proteins, there are other reactions that only require RuvAB. These reactions involve the formation and resolution of four strand junctions from stalled replicaton forks (Seigneur *et al.*, 1998). This is consistent with the suggestion that active RuvAB complexes may exist alongside RuvABC complexes (see West, 1997).

1.6.2 The RecG protein

Although *ruv* mutants are extremely defective in the repair of UV damage they are only moderately defective in recombination, as measured by conjugation and transduction (see Chapter 1.6.1). This is surprising as the resolution of Holliday junctions would be expected to be an essential component of recombination. In

fact, it was discovered that this high residual level of recombination in all three *ruv* single mutants is dependent on the product of the *recG* gene (Lloyd, 1991). However, single mutations in *recG* confer only a moderate deficiency in recombination and DNA repair (Lloyd and Buckman, 1991). It therefore appears that there is a functional overlap between RecG and the RuvABC complex in the resolution of intermediates in the late stages of recombination.

RecG is an ATPase and possesses weak DNA 3' to 5' helicase activity in normal helicase assays (Whitby *et al.*, 1994). RecG binds specifically to branched DNA molecules and shows an enhanced ability to unwind such structures, in an manner dependent on ATP hydrolysis (Lloyd and Sharples, 1993; Whitby *et al.*, 1994; Whitby and Lloyd, 1998). RecG can bind to and branch migrate both three-strand and four-strand branched structures using its helicase activity although three-strand intermediates are better targets (McGlynn and Lloyd, 1999). A comparison of the much stronger unwinding activity of RecG compared to RuvAB on three-strand junctions has led to suggestions that RecG may convert three-strand intermediates into four-strand substrates for RuvABC (Whitby and Lloyd; 1995). RecG can also counter RecA-driven strand exchange (Whitby et al, 1993). It has been suggested that this process of 'reverse branch migration' could abort recombination using 5' single stranded ends, and enhance the use of 3' ends (Whitby and Lloyd, 1995).

The ability of RecG to bind to and unwind Holliday junctions is consistent with it being able to take over some of the functions of the RuvABC complex. However, RecG does not possess an endonucleolytic function that could replace the action of RuvC in cleaving Holliday junctions. Neither does RecG operate with RuvC, *recG ruvA* and *recG ruvB* double mutants are just as recombination deficient as *recG ruvC* mutants (Lloyd, 1991). This has led to suggestions that in *ruv* mutants recombination intermediates are resolved by a totally different kind of mechanism to that using RuvABC. In one model, branch migration by RecG

is combined with D-loop cleavage to resolve joint molecules possessing Holliday junctions (Kuzminov, 1996) (Figure 1.6).

RecG appears to interact with the protein PriA. PriA is involved in the establishment of replication forks at sites other than *oriC* (see Chapter 1.7), but it also possesses a helicase function which is not needed for establishing replication forks (Zavitz and Marians, 1992). Mutations affecting the helicase activity of PriA suppress the repair and recombination defects of *recG* mutants, but not the defects displayed by *recG* mutants in a *ruv* background (Al-Deib *et al.*, 1996). This suggests that the 'normal' role of RecG involves an interaction with PriA, and that its role in compensating for *ruv* mutations is a fundamentally different, cryptic one. PriA and RecG bind to similar structures (although there are differences) (McGlynn *et al.*, 1997) and both possess helicase activities. It has therefore been suggested that they compete for binding recombination intermediates, with the activity of the PriA helicase tending to abort recombination, and the activity of the RecG helicase tending to promote it or counter PriA in some other manner (McGlynn *et al.*, 1997).

1.7 Recombination and replication: the role of the PriA protein

It is now believed that recombination and replication are intimately related processes. Although replication forks initiated at *oriC* are highly processive it appears that they do not, in fact, usually complete replication of the *E.coli* chromosome in one uninterrupted process. Instead, it is believed that replication forks often stall, or collapse, and that recombination is necessary for them to be restarted (see Cox, 1998). This recombination is required both to repair double-strand breaks and to provide a substrate (the D loop) for the proteins carrying out the reinitiation of lagging strand synthesis. This latter process takes place in a manner different from that which occurs at *oriC* and is dependent on the PriA

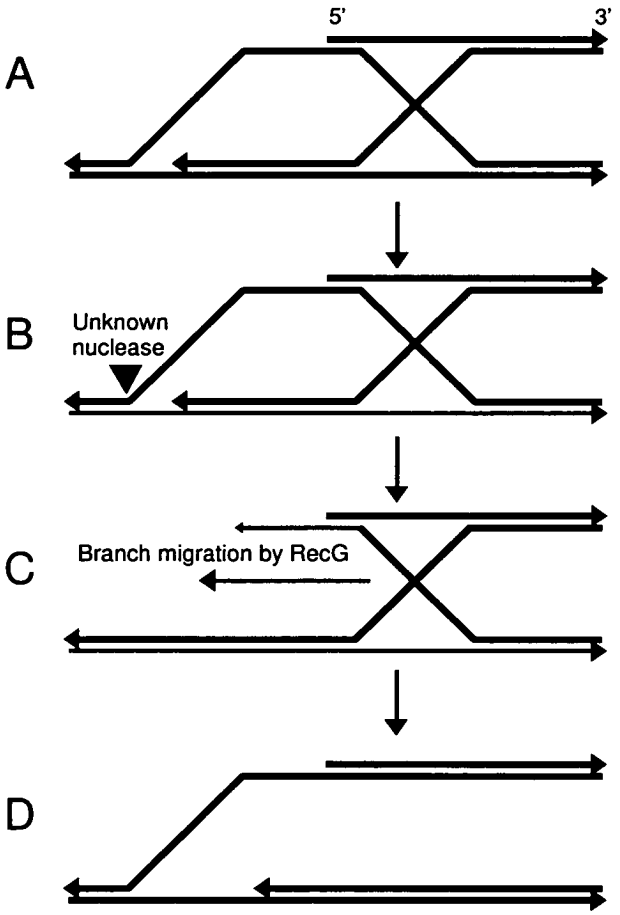

Figure 1.6: Model for RecG resolution of Holliday junctions without junction cleavage (adapted from Kuzminov, 1996).
A) Strand invasion sets up a D loop and a Holliday junction is formed.
B) A nuclease (unknown) is postulated to cleave the D loop. This activity is also needed to explain break-join recombination events and is not unique to the model presented here.
C). RecG 'reverse branch migrates' the Holliday junction until it encounters the double strand break.
D) The resulting structure is the same as that of a replication fork. Replication from this structure can produce a recombinant (see Chapter 1.7).

protein. In addition, if replication has been shown to require recombination it also appears that in reactions characterised as 'recombination' the generation of a replication fork is also important.

PriA is a 81.7 kDa protein with a 3' to 5' helicase activity (Lee and Marians, 1987; Lee *et al.*, 1990; Nurse *et al.*, 1990). It was discovered as one of 5 proteins required for the assembly of a primosome in ϕX174 replication. The other proteins are PriB, DnaT, DnaB, DnaC and DnaG (reviewed in Marians, 1992). The importance of a sixth protein, PriC, is less clear. In this reaction PriA recognises a specific sequence called PAS (Primosome Assembly Site) and intitiates assembly of the primosome (Sclomai and Kornberg, 1980), which in conjunction with DNA Polymerase III allows concurrent lagging and leading strand replication (reviewed in Marians, 1992). A primosome is a protein machine capable of movement along the lagging strand template and synthesis of RNA primers on that strand. The first role is carried out by the replicative helicase, DnaB, and the second by the primase, DnaG. The steps that occur in the PriA-dependent primosome assembly pathway are as follows (Ng and Marians, 1996): (i) PriA binds to the DNA, (ii) PriA recruits PriB, (iii) DnaT then joins the complex; (iv) DnaB is recruited from its complex with DnaC in solution, (v) finally DnaG is recruited (transiently) by DnaB. For primosomes that are assembled at *oriC* the proteins that are required are DnaA, DnaB, DnaC and DnaG (reviewed in Marians, 1992). Assembly of a primosome at *oriC* does not require PriA, PriB or DnaT (Kaguni and Kornberg, 1984).

It therefore appears that these protein groups define two separate primosomes, one generated at *oriC* by DnaA and one generated at PAS by PriA. There are no PAS sequences on the *E.coli* chromosome (see Sandler and Marians, 2000) but experiments still suggest that, although the PriA pathway is less essential than the DnaA/*oriC* pathway, it is relevant to normal chromosomal replication in an *E.coli* cell. Although cells carrying mutations affecting the primosome-assembly

function of PriA are viable, their viability is greatly diminished and they rapidly acquire suppressor mutations in *dnaC* (Lee and Kornberg, 1991; Sandler *et al.*, 1996) . They also display induction of the SOS response (leading to extensive filamentation), sensitivity to rich media and recombination and UV repair deficiencies (Lee and Kornberg, 1991; Nurse *et al.*, 1991; Masai *et al.*, 1994; Kogoma *et al.*, 1996). This led to the proposal that replication forks initiated at *oriC* often stall or break, perhaps by encountering a nick or other lesion and the PriA primosome pathway is necessary for the re-establishment of replication (Seufert and Messer, 1986; Zavitz and Marians, 1991; Cox, 1998). It was suggested that recombination could occur using the broken fork as a substrate and this could generate a D loop. PriA could then initiate primosome assembly by recognising such D loops, which have structural similarities to the hairpin believed to be formed by PAS (Masai and Kogoma, 1994; Kuzminov, 1995; Sandler *et al.*; 1996; Sandler and Marians, 2000).

In fact *in vitro* work has demonstrated that PriA does bind to D loops and that it can assemble a primosome there (Liu and Marians, 1999; McGlynn *et al.*, 1997; Nurse *et al.*; 1999). This in turn allows the initiation of replication from those D loops (Liu and Marians, 1999). Further evidence that the PriA pathway is responsible for replication reinitiated by recombination during normal growth comes from the observation that it is essential for iSDR (inducible Stable DNA Replication) in which replication is always initiated by recombination (Masai *et al.*, 1994).

If experiments with PriA have suggested that replication is often dependent on recombination they have also suggested that recombination is dependent on replication. As mentioned above, *priA* mutants are defective in conjugational and transductional recombination. The linear fragments introduced by transduction or conjugation are expected to set up D loops on the recipient chromosome using their invasive ends. It has been suggested that, instead of recombination occurring

through a conservative process involving breakage of these D loops, it could, instead, occur by initiating replication at the D loops (Figure 1.7) (Smith 1991; Kogoma *et al.*, 1996; Kogoma, 1996). This would be expected to involve the PriA-dependent primosome-assembly pathway and would explain the recombinational deficiency of *priA* mutants.

Although the role of the primosome-assembly function of PriA in normal cell growth is now becoming clearer the role of its 3' to 5' helicase activity is still a mystery. The deficiencies of *priA* null mutants can all be suppressed by complementation with *priA* allelles that have lost their helicase function (Zavitz and Marians, 1992). The helicase activity of PriA is evident on a number of branched substrates *in vitro* (McGlynn *et al.*, 1997). However, the only phenotpye associated with this activity *in vivo* is that it appears to be responsible for the mild recombination and DNA repair deficiencies observed in *recG* mutants (see Chapter 1.6.2.).

Finally, it appears that there are other pathways, involving RecF or PriB and PriC, that can achieve replication restart at the site of a stalled or broken replication fork without the action of PriA (Sandler, 1996; Sandler and Marians, 2000). The nature of these pathways and their relevance to the growth of *wt* cells is unclear.

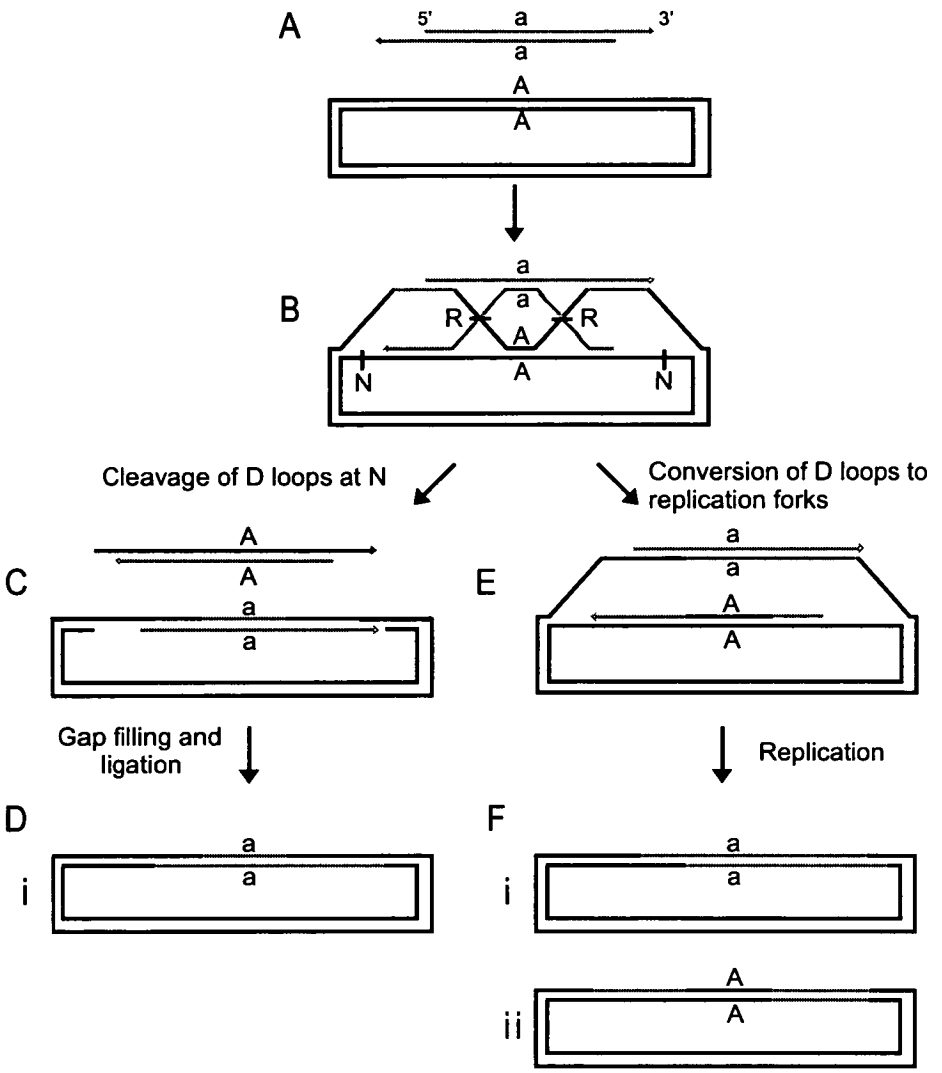

Figure 1.7: Model for production of recombinants with or without full chromosomal replication (adapted from Kogoma, *et al.*, 1996).
A) A linear fragment, introduced by transduction/conjugation etc., recombines with a chromosome. The linear DNA and chromosome possess different genetic markers ('A' and 'a') allowing parental and recombinant progeny to be distinguished.
B) The ends of the linear molecule invade the recipient chromosome. The joint molecule set up possesses Holliday junctions that are resolved by cleavage ('R'). For recombination without replication ('break-join') this is accompanied by cleavage of the D loops by an unknown nuclease (at 'N').
C). After cleavage at N a single chromosome is reconstituted along with an excised linear piece of the original chromosome.
D) Filling in of gaps and ligation produces a single recombinant chromosome ('a').
E) If cleavage at 'N' does not occur then the products of resolution are two replication fork structures.
F) Replication from these sites around the chromosome leads to the production of two chromosomes, one recombinant (i) and one parental (ii).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Stock solutions

Ampicillin (100 mg ml^{-1})

Ampicillin (Beecham Pharaceuticals) was made up in sterile (distilled water) and stored at -20 ^{o}C. It was used at a concentration of 100 μg ml^{-1}.

500 mM $CaCl_2$

Made up using sterile (distilled) water and autoclaved.

Chloramphenicol (20 mg ml^{-1})

Chloramphenicol (Sigma Chemical Company) was made up in 100 % ethanol and stored at -20 ^{o}C. It was used at a concentration of 50 μg ml^{-1}.

10 mM $FeCl_3$

Made up using sterile (distilled) water and autoclaved.

20 % (w/v) Glucose

Made up using sterile (distilled) water and filter sterilised.

Kanamycin (50 mg ml^{-1})

Kanamycin was made up in sterile (distilled) water and stored at -20 °C. It was used at a concentration of 50 μg ml^{-1}.

20 % (w/v) Maltose

Made up using sterile (distilled) water and filter sterilised.

1 M $MgSO_4$

Made up using sterile (distilled) water and autoclaved.

Phage Buffer

3 g KH_2PO_4, 7 g Na_2HPO_4, 5 g NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 1 % (w/v) gelatine per litre. Made up using sterile (distilled) water and autoclaved.

Spitzizen Salts

10 g $(NH_4)_2SO_4$, 70 g K_2HPO_4, 30 g KH_2PO_4, 5 g $Na_3C_6H_5O_7$ and 1 g $MgSO_4$ per litre. Made up using sterile (distilled) water and autoclaved.

Streptomycin (100 mg ml^{-1})

Streptomycin was made up in sterile (distilled) water and stored at -20 °C. It was used at a concentration of 100 μg ml^{-1}.

Tetracycline (50 mg ml^{-1})

Tetracycline was made up in a 1:1 ratio of ethanol and sterile (distilled) water and stored at -20 °C. It was used at a concentration of 50 μg ml^{-1}.

TM Buffer

10 mM Tris-HCl, 10 mM $MgSO_4$. Made up using sterile (distilled) water and autoclaved.

Kanamycin was made up in sterile (distilled) water and stored at -20 ^{o}C. It was used at a concentration of 50 μg ml^{-1}.

20 % (w/v) Maltose

Made up using sterile (distilled) water and filter sterilised.

1 M $MgSO_4$

Made up using sterile (distilled) water and autoclaved.

Phage Buffer

3 g KH_2PO_4, 7 g Na_2HPO_4, 5 g NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 1 % (w/v) gelatine per litre. Made up using sterile (distilled) water and autoclaved.

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Tetracycline (50 mg ml^{-1})

Tetracycline was made up in a 1:1 ratio of ethanol and sterile (distilled) water and stored at -20 ^{o}C. It was used at a concentration of 50 μg ml^{-1}.

TM Buffer

10 mM Tris-HCl, 10 mM $MgSO_4$. Made up using sterile (distilled) water and autoclaved.

Vitamin B1 (5mg ml^{-1})

Vitamin B1 (Sigma Chemical Company) was made up in sterile (distilled) water, filter sterilised and stored at 4 ^{o}C.

Zeocin (100 mg ml^{-1})

Provided at this concentration as a solution (Invitrogen Corporation). It was used at a concentration of 16 μg ml^{-1} in low salt media (this antibiotic is only effective in low salt conditions).

2.1.2 Media

LB Agar

10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl and 15 g Bacto-agar (Difco) per litre, adjusted to pH 7.2 with NaOH.

LB Agar (Low Salt)

As LB agar, but containing only 5 g NaCl per litre.

LC Agar

10 g Tryptone, 5 g yeast extract, 5 g Na Cl and 10 g Difco-agar per litre, adjusted to pH 7.2 with NaOH.

LC Top Agar

As LC agar, but containing only 7 g Difco-agar.

Minimal Agar

20 g Oxoid agar No. 3, per litre. Made up in distilled water. Spitzizen salts added on use to 25 % of volume.

L Broth

10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCL per litre, adjusted to pH 7.2 with NaOH.

Minimal Liquid Medium

Spitzizen Salts diluted to 25 % by volume in sterile (distilled) water and supplemented with 0.2% glucose 15 μg ml^{-1} threonine, 15 μg ml^{-1} histidine, 15 μg ml^{-1} arginine and 15 μg ml^{-1} leucine.

2.2 General methods

2.2.1 Overnight cultures

Overnight *E.coli* cultures were produced by streaking out the desired strain from the -70 ^{o}C stocks onto LB agar plates to obtain single colonies. A single colony was then used to inoculate 5 ml of L broth in a 1/2 oz bijou bottle, which was then shaken overnight at 37 ^{o}C.

2.2.2 Preparing a plating culture

An aliquot (0.5 ml) of fresh overnight culture was diluted 10-fold in L broth containing 2 % maltose and 5mM Mg_2SO_4 in a 1/2 oz bijou bottle and grown for 2.5 hours at 37 ^{o}C with shaking. This log phase culture was then diluted 2-fold with TM buffer and stored at 4 ^{o}C. The plating culture was used for up to 3 days after preparation.

2.2.3 Storage of bacteria

Stocks of *E.coli* were prepared by mixing 1 ml of an overnight culture of cells with 0.5 ml of sterile glycerol in a 1.5 ml Eppendorf tube. Stocks were prepared in duplicate and stored at -70 ^{o}C.

2.2.4 Storage of phage

Phage were stored at 4 °C.

2.2.5 Production of P1 plate lysates

An aliquot (500 μl) of an overnight culture of the *E.coli* strain carrying the desired marker (for P1 transduction work) was diluted 10-fold in fresh L broth containing $CaCl_2$ at a concentration of 2.5 mM. Growth was allowed to occur for 2 hours at 37 °C with shaking. After this time an aliquot (100 μl) of the culture was mixed with an equal volume of a range of 10 fold dilutions of a P1 lysate in 5 ml test tubes. Incubation then took place for 30 minutes at 37 °C followed by the addition of 2 ml of molten LC top agar supplemented with 5 mM $CaCl_2$ at a temperature of 46 °C to each test tube. The contents of each tube were then poured onto fresh LC plates supplemented with 5 mM $CaCl_2$. The plates were then incubated (lid upwards) at 37 °C for 6 to 8 hours. The plates giving best coverage of plaques were chosen and 5 ml of phage buffer was added to these plates. After maceration of the top agar with the buffer the top agar/buffer mixture was scrapped off into a sterile 30 ml glass bottle containing 100 μl of chloroform. The mixture was vortexed and left overnight at 4 °C. The following day the bottle was centrifuged at 5000 rpm and the supernatant transferred to a sterile 1/2 oz bijou bottle with 100 μl of chloroform.

2.2.6 Production of phage λ plate lysates

An aliquot (250 μl) of a plating culture of *E.coli* strain DL51 (which is permisive for the propagation of palindromes) was mixed with an equal volume of a range of 10-fold dilutions of a phage λ lysate in 5 ml test tubes. Incubation then took place for 10 minutes at room temperature followed by the addition of 2 ml of molten LC top agar (46 °C) to each test tube. The contents of each tube were then poured onto fresh LC plates supplemented with 3 % (w/v) maltose, 10 μg ml^{-1}

vitamin B1, 0.04 mM $FeCl_3$ and 8 mM$CaCl_2$. The plates were then incubated (lid upwards) at 37 °C for 6 to 10 hours. The plates giving best coverage of plaques (just touching) were chosen and 5 ml of TM buffer was added to these plates. After maceration of the top agar with the buffer the top agar/buffer mixture was scrapped off into a 30 ml glass bottle containing 100 μl of chloroform. The mixture was vortexed and left overnight at 4 °C. The following day the bottle was centrifuged at 5000 rpm and the supernatant transferred to a sterile 1/2 oz bijou bottle with 100 μl of chloroform.

2.2.7 Measuring the titre of phage λ stocks

The stock to be titred was diluted serially 10 fold in phage buffer and the dilutions covering the range 10^{-4} to 10^{-9} were used for titring. A 250 μl aliquot of a plating culture of DL51 was mixed with 2 ml of molten LC top agar at 46 °C, poured onto a fresh LC plate and allowed to set. Then 100 μl of the phage dilutions were spotted onto the plate and allowed to dry. The plate was then incubated, lid upwards at 37 °C for 6 to 10 hours. The spots where several distinct plaques were visible were chosen to determine the number of pfus (plaque forming units) per ml. The entire process was carried out in duplicate and the titre averaged.

2.3 Measuring Lysogenisation Frequency

A fresh plating culture of the strain to be lysogenised was prepared as described above, except that the overnight culture was subcultured to a specific optical density (A_{650}=0.9) corresponding to a cell density of 4 x 10^8 cells ml^{-1}, rather than for 2.5 hours. After dilution in TM buffer the final cell density was 2 x 10^8 pfu ml^{-1}. Phage λ lysates of strains λDRL246 and λDRL282 were diluted to 2 x 10^9 pfu ml^{-1}. An aliquot (0.15 ml) of phage was added to 0.15 ml of bacterial cells and allowed to adsorb for 60 min at 30°C. Infected cells were diluted in phage buffer and appropriate dilutions plated on LB agar (low salt) plates supplemented

with Zeocin to select for lysogens. Dilutions of the plating cultures were plated on LB agar to measure the initial number of cells. In each case colonies were counted after incubation at 37°C for 24 hours. Lysogenisation frequency was measured as the number of lysogens obtained per cell in the plating culture, i.e. by dividing the number of lysogens per ml by the initial number of viable cells per ml and multiplying by two (because of the dilution of the plating culture with the phage). When the *priA* strains DL1133 and DL1134 were used steps had to be taken to avoid the appearance of *dnaC* suppressor mutations. To this end the strains were grown on minimal liquid medium. Log phase cultures were then diluted with TM buffer and lysogenised in the same way as the other strains. The recombination efficiency of the strains was measured by P1 transduction frequency to check that suppressor mutations had not occurred.

2.4 P1 Transduction and measurement of P1 transduction frequencies

P1 transduction provides a mechanism for transferring a marker from one *E.coli* strain to another by homologous recombination between a fragment of chromosomal DNA packaged into a phage head when phage was grown on the first strain and the chromosome of cells of the second strain (see Masters, 1996). Overnight cultures of recipient strains were grown in LB broth containing 250 mM $CaCl_2$ to an approximate cell density of 1 x 10^9 cells ml^{-1}. An aliquot of 1 ml of this culture was spun down and resuspended in 100 μl of the 250 mM $CaCl_2$ L broth. This was mixed with an equal volume of the P1 lysate and incubated at 37°C for 20 minutes. Then 800 μl of L broth containing 500 mM $Na_3C_6H_5O_7$ was added and incubation at 37° continued for 60 minutes. Plating of 100 μl on selective media (plates lacking a particular amino acid or LB agar plates supplemented with an appropriate antibiotic) was then carried out. For novel strain construction this

sufficed, but when transduction frequency was to be measured the recipient culture (prior to infection with P1) was also plated on LB agar plates for counting initial cell density. Transduction frequency was deduced by dividing the number of selected transductants produced per ml of recipient culture by the number of cells per ml initially in that culture. Colonies were counted after incubation at 37°C for 24 hrs.

2.5 Conjugation

Conjugation using an Hfr donor strain provides a method for transferring a long linear (initially single-stranded) fragment of DNA from cells of one strain to another (see Firth *et al.*, 1996). Overnight cultures of the recipient and donor Hfr strains were prepared. Growth took place for at least 16 hours to allow entry into stationary phase. The 5 ml of overnight donor culture was then diluted into 100 ml of fresh L broth in a sterile 500 ml conical flask and grown for 2.5 hours at 37 °C with shaking. For conjugation with stationary phase recipient cells 1 ml of this donor culture was mixed with 1 ml of the recipient overnight culture (which would be in stationary phase) in a sterile 5 ml test tube. For conjugation using log phase recipient cells the recipient overnight culture was diluted in 100 ml of fresh L broth and grown for 2.5 hours as with the donor strain. A mixture of one ml of each of these cultures was then mixed in a sterile 5 ml test tube, as before. The mixed mating cultures were allowed to mate for the appropriate time and then plated on selective plates. For the experiments described in this work mating took place for 45 minutes and plating was done on minimal agar plates supplemented with streptomycin. After incubation of these plates for 48 hours at 37 °C 50 individual colonies from each mating were streaked out as small squares on a grid pattern onto minimal agar plates supplemented with streptomycin. These were also allowed to grow for 24 hours at 37 °C and then replica plated onto selective

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minimal plates (supplemented with zeocin, kanamycin or both). These plates were then allowed to grow for 24 hours at 37 °C before being scored for marker inheritance. This process was carried out in duplicate for each experimental mating (i.e. 100 recombinant colonies were characterised).

2.6 UV irradiation

Bacterial strains to be irradiated were grown overnight in LB broth for a minimum of 16 hours to ensure entry into stationary phase. For studies using stationary phase cells these cultures were resuspended in 10 mM Mg_2SO_4 and a 2 ml volume was irradiated in small (46 mm diameter) glass petri dishes (previously washed and autoclaved) for periods of 7.5s, 15s, 22.5s, 30s, 75s and 120s using light of wavelength 254 nm with vigorous shaking. This was carried out using a "Mineralight" (UVGL-58) (UVP Inc.) portable UV lamp placed at approximately 30 cm from the plates. For studies on log phase cells the overnight cultures were diluted 10 fold in L broth in 1/2 oz bijou bottles and allowed to grow for several hours at 37 °C with shaking until they were in log phase, they were then resuspended and irradiated in the same way as the stationary phase cells. Various dilutions of non-irradiated and irradiated cultures were plated on LB agar plates and grown overnight at 37 °C to allow calculation of the number of viable cells per ml at each dosage. Plating of irradiated cultures was carried out immediately after irradiation. All procedures were carried out in minimal light conditions. Survival at each dosage was calculated by dividing the number of cells per ml surviving the particular dosage in question by the number of viable cells per ml in the absence of irradiation. The dosages were calibrated by comparing the response of the wild-type, *recF* and *recB* strains to published data (Wang and Smith, 1983).

Strain	Genotype	Source or construction
CAG5054	*trpB39*::Tn*10*	Singer *et al.*, 1989
CAG18556	*purE3200*::Tn*10 kan*	Singer *et al.*, 1989
D7-8	*polA*^{ts}	William Donachie
DL1288	*polA*^{ts} *xerCY17*::Mini-*cat*	P1.DS984 x D7-8 (to Cm^r)
AB259	HfrH	
DL1312	HfrH *sbcC201 phoR*::Tn*10*	P1.476 x AB259 (to Tc^r)
N1411	*proB48 trpA9605 rpsL171*	Robert G. Lloyd
DL1306	*sbcC201 phoR*::Tn*10 rpsL171 proB48 trpA9605*	P1.476 x N1411 (to Tc^r)
DL1307	*sbcC201 phoR*::Tn*10 rpsL171 proB48 trpA9605 recF332*::Tn*3*	P1.JC13885 x DL1306 (to Ap^r)
AB1157	*his4 proA2 argE3 thr1 leuB6 ara14 galK2 lacY1 xyl5 mtl1 str*^{r}	
AB1157 derivatives:		
DL476	*sbcC201 phoR*::Tn*10*	Robert G. Lloyd
C876	*recB268*::Tn*10*	Franklin Stahl
DS984	*xerCY17*::Mini*cat*	David Sherratt
N2362	*recB21*	Lloyd and Buckman, 1985
N2365	*sbcC7623 recB21*	Lloyd and Buckman, 1985
N2679	*sbcC201*	Naom *et al.*, 1989
N2691	*recA269*::Tn*10*	Lloyd and Buckman, 1985
N2693	*sbcC201 recA269*::Tn*10*	Lloyd and Buckman, 1985
JC9387	*recB21 recC22 sbcB15*	Alvin J. Clark
JC13885	*recB21 recC22 recF*::Tn*3*	Alvin J. Clark
IN347	*recJ284*::Tn*10*	Robert G. Lloyd
N2057	*ruvA60*::Tn*10*	Shurvinton *et al.*, 1984
N3793	Δ*recG263*::*kan*	Al-Deib*et al.*, 1996
SP256	*recN262 tyrA16*::Tn*10*	Picksley*et al.*, 1984
N2445	*recO1504*::Tn*15*	Lloyd*et al.*, 1987
N2754	*recR252*::Tn*10 kan*	Robert G. Lloyd
N3343	*recQ1803*::Tn*3*	Lloyd and Buckman, 1991
N4155	*ruvAC65 eda51*::Tn*10*	Robert G. LLoyd
DB1318	*recA*::*cat*	Wertman*et al.*, 1986
CS85	*ruvC53 eda51*::Tn*10*	Shurvinton*et al.*, 1984
AG109	*priA2*::*kan*	Robert G. Lloyd
DL1092	*recF332*::Tn*3*	P1.JC13885 x AB1157 (to Ap^r)
DL1093	*sbcC201 recF332*::Tn*3*	P1.JC13885 x N2679 (to Ap^r)
DL1096	*recJ284*::Tn*10*	P1.IN347 x AB1157 (to Tc^r)
DL1097	*sbcC201 recJ284*::Tn*10*	P1.IN347 x N2679 (to ATc^r)
DL1100	*ruvA60*::Tn*10*	P1.N2057 x AB1157 (to Tc^r)

Strain	Relevant Genotype	Source or construction
DL1101	*sbcC201 ruvA60*::Tn*10*	P1.N2057 x N2679 (to Tc^r)
DL1102	*ruvAC65 eda51*::Tn*10*	P1.N4155 x AB1157 (to Tc^r)
DL1104	Δ*recG263*::*kan*	P1.N3793 x AB1157 (to Km^r)
DL1105	*sbcC201* Δ*recG263*::*kan*	P1.N3793 x N2679 (to Km^r)
DL1106	*recN262 tyrA16*::Tn*10*	P1.SP256 x AB1157 (to Tc^r)
DL1107	*sbcC201 recN262 tyrA16*::Tn*10*	P1.SP256 x N2679 (to Tc^r)
DL1108	*recO1504*::Tn*5*	P1.N2445 x AB1157 (to Km^r)
DL1109	*sbcC201 recO1504*::Tn*5*	P1.N2445 x N2679 (to Km^r)
DL1110	*recR252*::Tn*10 kan*	P1.N2754 x AB1157 (to Km^r)
DL1111	*sbcC201 recR252*::Tn*10 kan*	P1.N2754 x N2679 (to Km^r)
DL1112	*recQ1803*::Tn*3*	P1.N3343 x AB1157 (to Ap^r)
DL1113	*sbcC201 recQ1803*::Tn*3*	P1.N3343 x N2679 (to Ap^r)
DL1120	*sbcC201 xerCY17*::Mini*cat*	P1.DS984 x N2679 (to Cm^r)
DL1121	*ruvC53 eda51*::Tn*10*	P1.C585 x AB1157 (to Tc^r)
DL1122	*sbcC201 ruvC53 eda51*::Tn*10*	P1.C585 x N2679 (to Tc^r)
DL1132	*xerCY17*::Mini*cat*	P1.DS984 x AB1157 (to Cm^r)
DL1133	*priA2*::*kan*	P1.AG109 x AB1157 (to Km^r)
DL1134	*sbcC201 priA2*::*kan*	P1.AG109 x N2679 (to Km^r)
DL1140	*ruvA601504*::Tn*10 recA*::*cat*	P1.DB1318 x DL1101 (to Cm^r)
DL1143	*ruvA601504*::Tn*10* *recF332*::Tn*3*	P1.JC13885 x DL1101 (to Ap^r)
DL1144	*ruvA601504*::Tn*10* Δ*recG*::*kan*	P1.N3793 x DL1101 (to Km^r)
DL1146	*ruvA601504*::Tn*10* *recO1504*::Tn*5*	P1.N2445 x DL1101 (to Km^r)
DL1147	*ruvA601504*::Tn*10* *recR252*::Tn*10 kan*	P1.N2754 x DL1101 (to Km^r)
DL1152	*sbcC201 recA*::*cat* *recQ1803*::Tn*3*	P1.DB1318 x DL1113 (to Cm^r)
DL1209	*ruvAC65 eda51*::Tn*10* *xerCY17*::Mini*cat*	P1.DS984 x DL1102 (to Tc^r)
AB1885	*uvrB5*	
AB1885 derivatives:		
DL1155	*uvrB5 xerCY17*::Mini*cat*	P1.DS984 x AB1885 (to Cm^r)
DL1156	*uvrB5 recF332*::Tn*3*	P1.JC13885 x AB1885 (to Ap^r)
DL1157	*uvrB5 recF332*::Tn*3* *xerCY17*::Mini*cat*	P1.JC13885 x DL1155 (to Ap^r)
DL1159	*uvrB5 recB268*::Tn*10*	P1.C876 x AB1885 (to Tc^r)
DL1161	*uvrB5 recB268*::Tn*10* *xerCY17*::Mini*cat*	P1.C876 x DL1155 (to Ap^r)

Table 2.1: *Escherichia coli* strains used.

2.7 Strains

2.7.1 Bacterial strains

The *Escherichia coli* strains used are listed in Table 2.1

2.7.2 Bacteriophage λ strains

λSKK43 (*pal S'*, *red3*, *gam1342::kan*, *imm21*)(Kulkarni, 1990).

λDRL243 is a deletion derivative of λSKK43 which has lost the 246 bp palindrome (Pinder *et al.*, 1998).

λDRL246 was previously constructed (this laboratory) by cloning a Zeocin resistance marker in an *EcoR*I-*Bgl*I fragment from pZeoSV2(+) (Invitrogen Corporation) into the multicloning site of TXF97 (St. Pierre and Linn, 1996) using the *BamH*I and *EcoR*I sites. A 246 bp interrupted palindrome consisting of inverted repeats of 111 bp separated by a 24 bp spacer had previously been cloned from λSKK43 into pUC18 and was cloned into the multicloning site of λDRL246 as an *EcoR*I fragment to form DRL282 (Schmidt, 1999). Two internal mismatches were introduced into the palindrome during this process.

λDRL154 (*pal571*, Δ*spi6*, *cI857*, χ^+) contains a 571 bp palindromic sequence (this laboratory). λDRL152 is an isogenic phage lacking the palindrome sequence.

Chapter 3

A Palindrome can Stimulate Recombination via Double-Strand Break and Single-Strand Gap Pathways in *Escherichia coli*

3.1 Introduction

As discussed in Chapter 1, it appears that the SbcCD nuclease generates double-strand breaks at palindromic sequences during replication. This is believed to occur because of the ability of these sequences to form hairpin structures when transiently single-stranded on the lagging strand. These structures are then targeted and cleaved by SbcCD.

Cell viability requires that a double-strand break be repaired through recombination. The major pathway of end-directed recombination in *Escherichia coli* is the RecB pathway in which the RecBCD prepares 3'-ended single-stranded DNA onto which it loads RecA (see Chapter 1). A 246 bp interrupted palindrome confers inviability in *recB*, *recC* or *recA* backgrounds (Leach *et al.*, 1997). This inviability is dependent on the presence of functional SbcCD (see Chapter 1). This is consistent with the idea that the activity of SbcCD necessitates recombinational repair at a double-strand break generated at the site of the palindrome

This chapter describes experiments designed to further elucidate the mechanisms of recombinational repair of SbcCD-generated double-strand breaks at the site of palindromes. The viability of a long palindrome in a range of recombination-mutant backgrounds was investigated using a lysogenisation-frequency assay. These experiments were carried out in both *wt* and *sbcC* strains. Recombination initiated at the site of the palindrome was then demonstrated directly by allowing a chromosomal palindrome to initiate recombination against homologous DNA lacking the palindromic sequence. This homologous DNA was introduced by conjugation.

3.2 An assay allowing identification of the genetic requirements for palindrome-stimulated recombinational repair

In a previous study two isogenic phage, one containing a 246 bp interrupted palindrome and the other lacking this sequence, were used to identify the genetic requirements of recombinational repair at the site of the palindrome (Leach *et al.*, 1997). The palindrome-containing phage lysogenised a *wt* strain at approximately equal frequency to the palindrome-free control phage. However the lysogenisation frequency of the palindrome phage was several orders of magnitude lower than that of the control in *recA*, *recB* or *recC* backgrounds (Leach *et al.*, 1997). This reflected the inviability conferred by the palindrome when present on the chromosome in these mutant backgrounds. This in turn identified the products of these genes as essential for efficient recombinational repair at the site of the palindrome.

In this study a different strain of phage λ carrying the 246 bp palindrome was used to carry out similar lysogenisation frequency studies (λDRL282). An isogenic palindrome-free control phage was also used (λDRL246). These phage encode resistance to the antibiotic Zeocin, allowing selection of lysogens in a

wide variety of strains, including those resistant to the antibiotics tetracycline, ampicillin, kanamycin and chloramphenicol.

3.2.1 The components of both the RecB and RecF pathways are required for palindrome viability in the presence of SbcCD

Initially the previous lysogenisation results for the *wt*, *recA* and *recB* backgrounds were replicated (Figure 3.1). As previously observed (Leach *et al.*, 1997), there was a reduction in the lysogenisation frequency of the palindrome phage (λDRL282) compared to the palindrome-free phage (λDRL246) in the *recA* and *recB* backgrounds. This means that recombination involving the RecA and RecBCD proteins is required for the viability of cells containing the palindrome.

This analysis was then extended to a range of other recombination mutants. Given that recombination was known to be occurring, it was expected that there would be a requirement for the post-synaptic recombination proteins RecG, RuvA and RuvC (see Chapter 1.6.1 and 1.6.2) and indeed the lysogenisation frequency of λDRL282 was severely reduced in *recG*, *ruvA* and *ruvC* backgrounds compared to the *wt* (Figure 3.1). λDRL282 lysogenisation was also impaired in the *recN* background (Figure 3.1). This is consistent with the role of RecN in other DNA end-based recombination assays (see Chapter 1.4.2.4). Lysogenisation of λDRL246 (palindrome-free) was unaffected by these mutations (Figure 3.1).

The effect of mutations in genes of the RecF pathway was a more open question. In fact mutations in all of the RecF pathway genes studied (*recF*, *recO*, *recR*, *recQ* and *recJ*) caused palindrome-mediated inviability and a specific reduction in the efficiency of λDRL282 lysogenisation (Figure 3.2).

These results indicate that efficient repair of the SbcCD-generated double-strand break requires a wide range of recombination functions, including the components of both the RecB and RecF pathways.

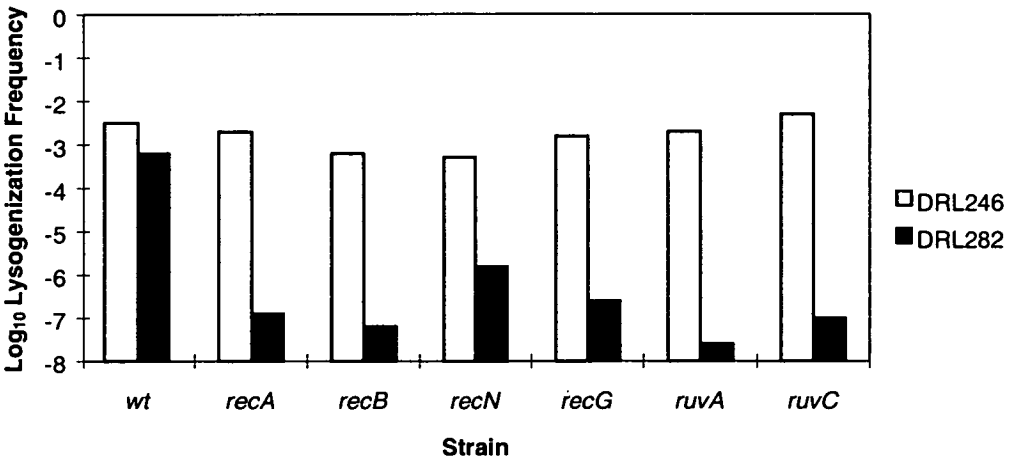

Figure 3.1: Effects of *recA*, *recB*, *recN* and Holliday junction resolution mutations (*recG*, *ruvA* and *ruvC*) on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars). The strains used were AB1157, N2691, N2362, DL1106, DL1104, DL1100 and DL1121. The results are the geometric means of at least two independent experiments. No λDRL282 lysogens were isolated in the *ruvA* or *recB* backgounds, so that the values given are maximum estimates.

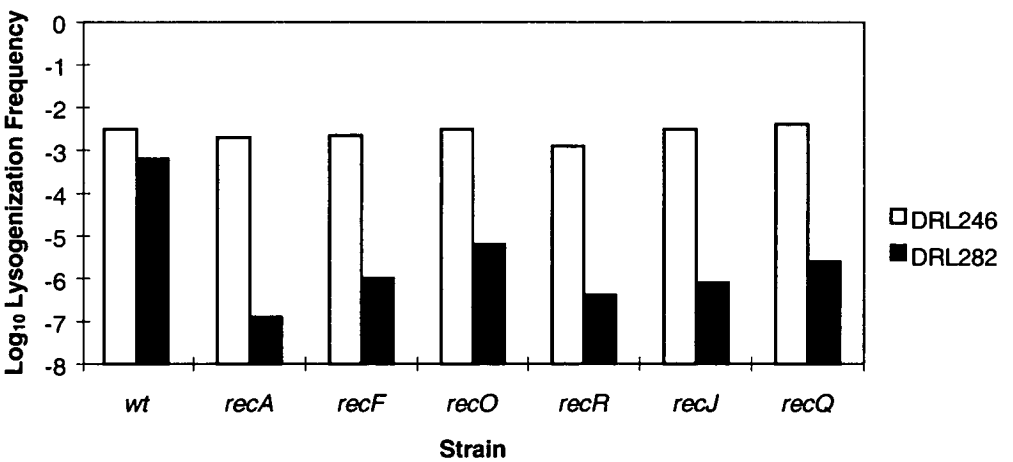

Figure 3.2: Effects of RecF pathway mutations (*recF*, *recO*, *recR*, *recJ* and *recQ*) on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars). The strains used were AB1157, N2691, DL1092, DL1108, DL110, DL1096 and DL1112. The results are the geometric means of at least two independent experiments.

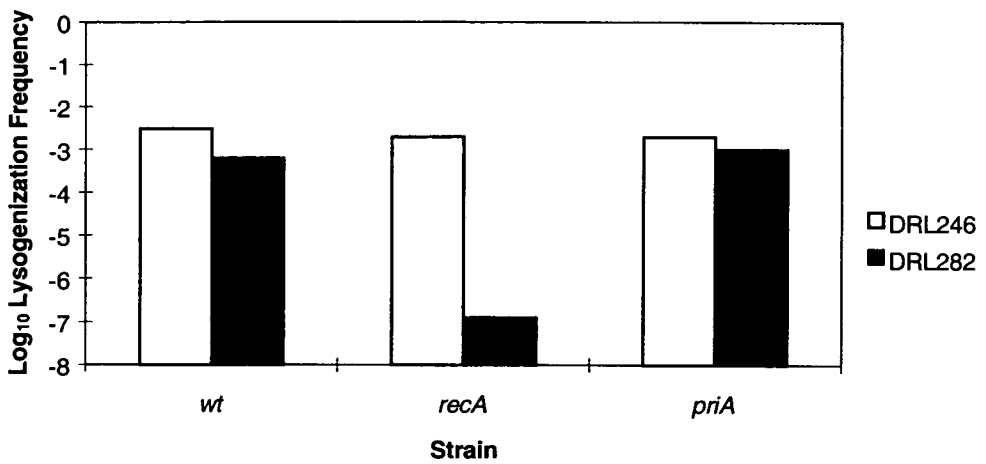

Figure 3.3: Effects of a *priA* mutation on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars). The strains used were AB1157, N2691 and DL1133. The results are the geometric means of at least two independent experiments.

3.2.2 SbcCD-induced double-strand breaks are not associated with PriA-dependent replication fork repair

Double-strand breaks, caused by replication encountering a nick or lesion, are believed to lead to replication fork collapse and probable disassembly of the replication protein complex (Cox, 1998 and references therein). For fork progression to resume it is believed that strand invasion sets up a D loop which the PriA protein then binds to, initiating the reassembly of a primosome and the reestablishment of a full replication fork (see Chapter 1.7). To assess whether SbcCD cleavage at the 246 bp imperfect palindrome leads to replication fork collapse the ability of the palindrome and palindrome-free control phage to lysogenise a *priA* mutant was tested. Both the palindrome and control phage were able to lysogenise the mutant at a high frequency (Figure 3.3). This indicates that replication fork collapse is not occurring, despite genetic evidence suggesting the formation of a double-strand break during replication. The alternative would be that fork collapse does occur, but is repaired via a PriA-independent mechanism.

3.2.3 In the absence of SbcCD the palindrome stimulates recombination via the RecF pathway

The lysogenisation assay was repeated using the same range of recombination mutants as above, but with each carrying an additional mutation in *sbcC*. Previous analysis of *recA*, *recB* and *recC* mutants in an *sbcC* background had demonstrated lysogenisation of the palindrome phage at an equally high frequency to that of the palindrome-free control (Leach *et al.*, 1997). This suggested that the formation of double-strand breaks at the site of a palindrome requires SbcCD. Without SbcCD there are no double-strand breaks and therefore no apparent need for functional recombinational repair.

The results of this study generally support these findings. λDRL282 could lysogenise most of the recombination mutants carrying the additional *sbcC* mutation at the same high frequency as λDRL246 (Figures 3.4 and 3.5). However there were two exceptions: the palindrome conferred inviability in the *ruvA sbcC* and *ruvC sbcC* double mutants (Figure 3.4). The RuvA and RuvC proteins are late components of recombination, comprising a component of the RuvAB branch migration complex and a Holliday junction resolvase enzyme, respectively (see Chapter 1.6.1). It seems likely that the inability to propagate palindromes in these backgrounds represents the presence of lethal unresolved Holliday junctions, which cause chromosome partitioning problems (Ishioka *et al.*, 1998). This lethality indicates that recombination is occurring at high frequency even though recombination also appears to be unnecessary for viability (for instance the palindrome is viable in an *sbcC recA* double mutant (Figure 3.4)).

The substrate involved in this reaction is unlikely to be a DNA break as this would represent a lethal event in the absence of recombination. This suggests, instead, that a single-strand gap is the substrate. This would also allow another mechanism to fill the gap in the absence of recombination. If this interpretation is correct then it should be possible to suppress the lethality of the unresolved

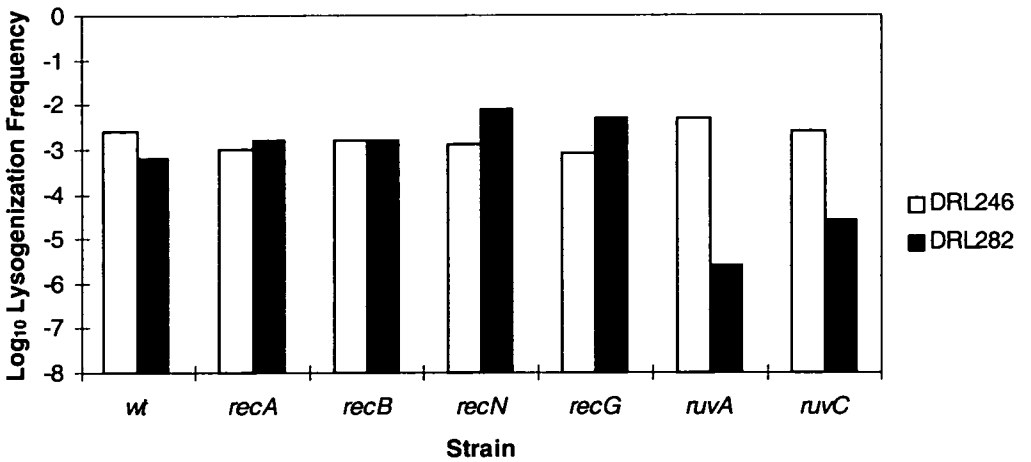

Figure 3.4: Effects of *recA*, *recB*, *recN* and Holliday juction resolution mutations (*recG*, *ruvA* and *ruvC*) on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars) in an *sbcC* background. The strains used were N2679, N2693, N2365, DL1107, DL1105, DL1101 and DL1122. The results are the geometric means of at least two independent experiments.

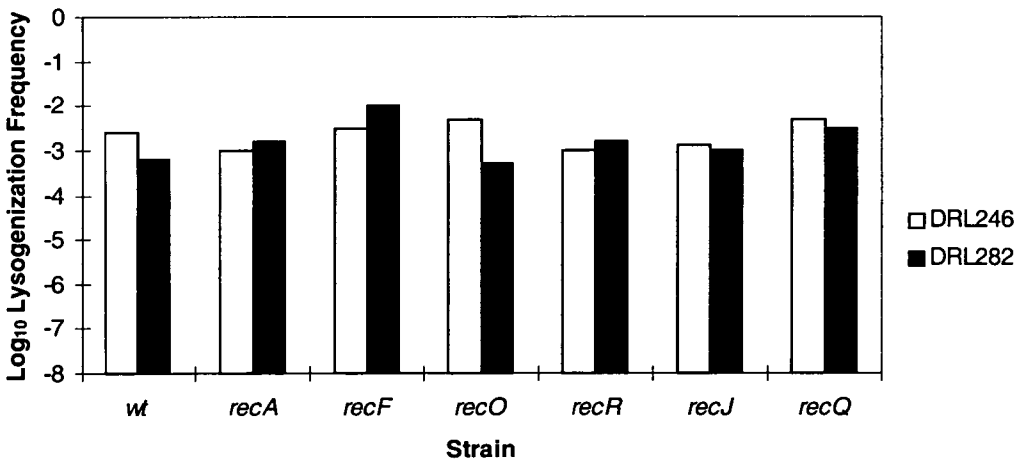

Figure 3.5: Effects of RecF pathway mutations (*recF*, *recO*, *recR*, *recJ* and *recQ*) on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars) in an *sbcC* background. The strains used were N2679, N2693, DL1093, DL1109, Dl111, DL1097 and DL1113. The results are the geometric means of at least two independent experiments.

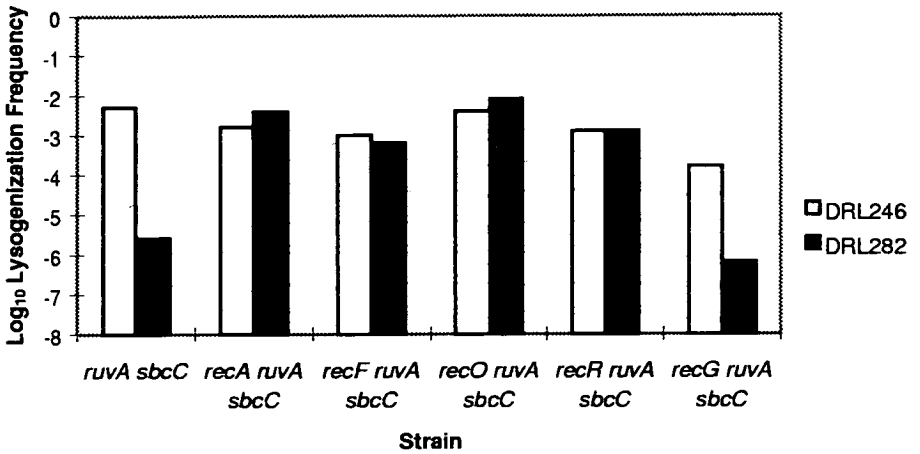

Figure 3.6: Effect of RecF pathway (*recF*, *recO* and *recR*), *recA* and *recG* mutations on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars) in an *sbcC ruvA* background. The strains used were DL1101, DL1140, DL1143, DL1146, DL1147 and DL1144. The results are the geometric means of at least two independent experiments. No λDRL282 lysogens were isolated in the *recG ruvA sbcC* background, so that the value given is a maximum estimate.

Holliday junctions by preventing their formation. To test this, a range of triple mutants was constructed carrying mutations in *ruvA*, *sbcC* and in a third recombination gene. As expected, mutations in genes encoding proteins involved in the early stages of gap recombination (*recA*, *recF*, *recO*, *recR*) restored the ability of the palindrome phage to lysogenise a *ruvA* background (Figure 3.6). A mutation in *recG*, a gene encoding a late acting junction resolving protein(see Chapter 1.6.2), did not restore viability (Figure 3.6). This means that RecG is not acting prior to the Ruv proteins. The lack of any palindrome-mediated viability problem in the *sbcC recG* background (Figure 3.4) suggests that RecG is not itself acting at a late stage when unresolved intermediates would be lethal. This implies that RecG may have little role to play in gap recombination.

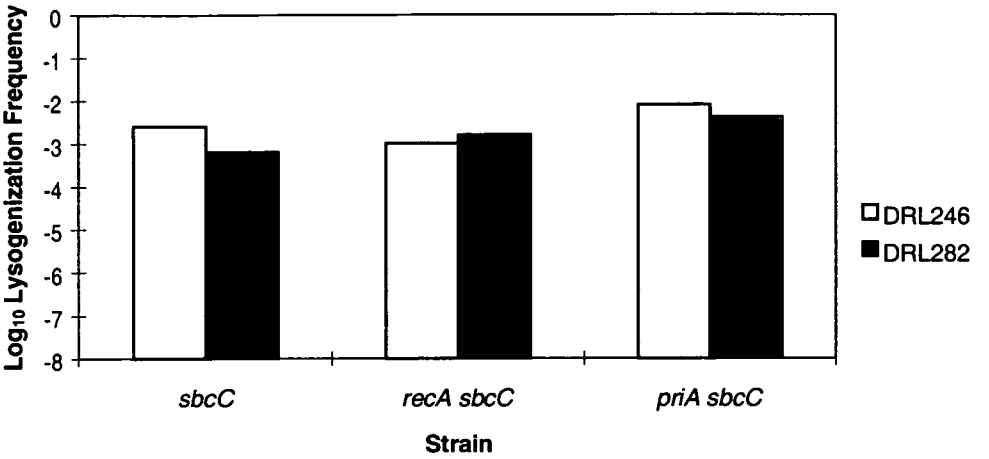

Figure 3.7: Effects of a *priA* mutation on lysogenisation frequency of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars) in an *sbcC* background. The strains used were AB1157, N2691 and DL1133. The results are the geometric means of at least two independent experiments.

3.2.4 Gap recombination at the site of the palindrome is not associated with PriA-dependent replication fork repair

In an *sbcC* background both the palindrome and control phage were able to lysogenize a *priA* mutant at high frequency (Figure 3.7) indicating either that palindrome-induced replication fork collapse does not occur in *sbcC* cells or that it is repaired in a PriA-independent manner.

3.2.5 In the absence of recombination the viability of cells carrying the uncleaved palindrome requires the presence of the RecQ helicase

The 246 bp imperfect palindrome used in this study appears to stimulate the formation of recombinogenic single-strand gaps during replication in an *sbcC* mutant background. This indicates that replication has difficulty progressing through the secondary structure formed by this sequence. Presumably recombination using RecFOR fills in this gap. However this gap can also be filled in the absence of recombination as is demonstrated by the ability of λDRL282 to lysogenise *sbcC recA* cells (Figure 3.4). One possible method of filling the gap without recombi-

nation would be to use a helicase to unwind the secondary structure and so allow replication. There was an indication that the RecQ helicase might fulfil such a function. It was observed that λDRL154 (containing a 571 bp palindrome) could not form plaques on an *sbcC recQ* background. Normally this phage will form plaques on *sbcC*, but not *wt* cells. The phage produced plaques on all of the *sbcC* strains used in this study, with the exception of the *recQ sbcC* double mutant (results not shown). A palindrome-free control, λDRL152, was able to form plaques on all backgrounds. It is possible that the difference between the ability of λDRL282 to form lysogens in *sbcC recQ* cells and the inability of λDRL154 to form plaques on the same background represents differences in the lengths of the palindromes or differences between chromosomal and lambda lytic DNA replication. The result of such differences might be to make λDRL154 lytic replication more reliant on a recombination-independent replication bypass mechanism than λDRL282 replication as a lysogen. The plating behaviour of λDRL154 suggested that RecQ could have a role in such a recombination-independent system especially as the effect was not observed for mutations in the other RecF pathway genes (*recF*, *recO*, *recR*, *recJ*) or *recA* in an *sbcC* background.

The role of RecQ in palindrome viability in an *sbcC* background in the absence of recombination was then addressed directly by lysogenising an *sbcC recA recQ* triple mutant. In this background the palindrome conferred inviability (Figure 3.8) indicating that RecQ is required to process palindromes in the absence of recombination in an *sbcC* mutant.

3.3 Conjugational recombination can be used to investigate recombination stimulated by a chromosomal palindrome sequence

In a replicating *E.coli* chromosome possessing a palindromic sequence recombinational repair at the site of the palindrome appears to occur with or without

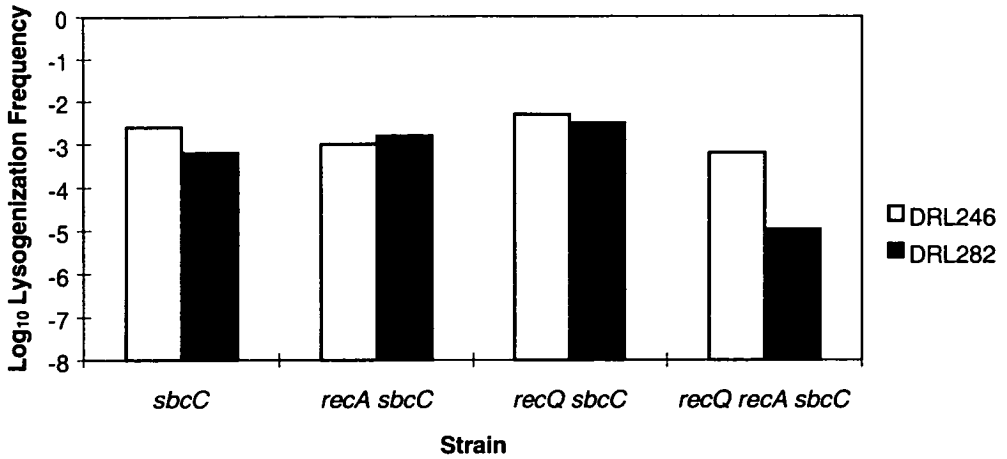

Figure 3.8: Effect of a *recA recQ* double mutation on lysogenisation frequency of phages λDRL246 (palindrome-free, opn bars) and λDRL282 (246bp palindrome, filled bars) in an *sbcC* background. The strains used were N2679, N2693, DL1113 and DL1152. The results are the geometric means of at least two independent experiments.

the activity of the SbcCD nuclease. Normally this recombination occurs against the intact sister duplex which is used as a template to repair the double-strand break or single-strand gap. However there is no reason to suppose that if another molecule possessing a sequence homologous to that around the palindrome was present that recombination could not take place against this molecule. One method of introducing such a molecule is through conjugation. By the use of different selectable markers on the recipient chromosome and the second DNA molecule introduced by conjugation it should be possible to directly identify if recombination between these two molecules is actually occurring. This kind of assay could provide insights into the mechanisms and positions of recombinational events in a way impossible for recombination against the sister chromosome, the sequence of which is identical to the DNA initiating recombination.

3.3.1 The presence of a palindrome on a replicating chromosome leads to the recombinational loss of the DNA region possessing the palindrome

Conjugation was carried out using a recipient strain (N1411 or a derivtive of that strain) that was a lysogen for either λDRL282 (palindrome) or λDRL246 (no palindrome). Both of these phage carry a zeocin-resistance gene. In λDRL282 the palindrome sequence is adjacent to this marker (Figure 3.9). The recipient strains were pro^- and trp^-, but str^r (Figure 3.9). The donor strain used was a λDRL243 lysogen of strain AB259. λDRL243 uses the same immunity system as λDRL246 and λDRL282 but possesses a kanamycin-resistance gene and a large region that is non-homologous to λDRL246/λDRL282 (Figure 3.9). This region includes the zeocin-resistance gene and the site of the palindrome. The donor strain was pro^+, trp^+ and str^s. The conjugation was carried out using log phase recipient and donor cells for 45 minutes to allow transfer of both *pro* and *trp*. Selection for transconjugants was done using minimal plates, requiring colonies to be pro^+ and trp^+ for growth. The plates were supplemented with streptomycin to counterselect against the donor strain. The result of this selection was to isolate recipient cells that had acquired pro^+ and trp^+, this ensured that the region of the chromosome containing the λ lysogen was transferred and was available for recombination against the chromosomal lambda sequence. The cross is shown in Figure 3.9.

The results of these crosses are shown in Table 3.1. When no palindrome was present it can be seen that the donor lambda markers (kan^r zeo^s) were inherited 35% of the time while the recipient markers (kan^s zeo^r) were inherited 31% of the time. Markers indicative of recombination between the *kan* and *zeo* loci (kan^r zeo^r and kan^s zeo^s) or mixed colonies (consisting of two different populations, one kan^r zeo^s and one kan^s zeo^r) made up the rest of the transconjugants. The fact that the donor and recipient genotypes were inherited at about the same frequency

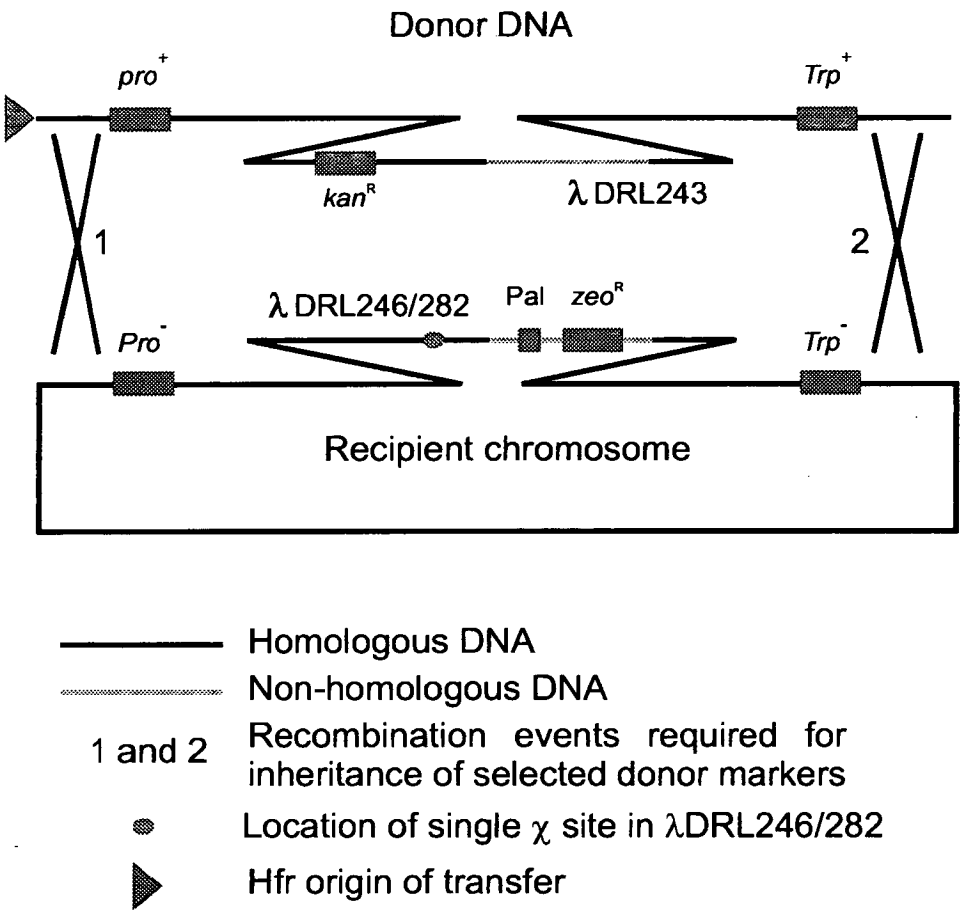

Figure 3.9: Illustration of Hfr crosses and markers present on the donor and recipient chromosomes.

was unexpected, as selection for donor markers on the flanking sequences on both sides was expected to bias inheritance towards the λ sequences present on the donor DNA. Inheritance of the donor λ sequences only requires the two crossover events occurring 'before' *pro* and 'after' *trp* (Figure 3.9) whereas inheritance of the recipient λ sequences requires two extra crossover events. Why this should occur with such a high frequency is unclear, but not in itself important in analysing the effect of the presence of a palindrome on the recipient chromosome.

Recipient lysogen	***kan*^{r} *zeo*^{r} + mixed colonies**	***kan*^{r} *zeo*^{s}**	***kan*^{s} *zeo*^{r}**	***kan*^{s} *zeo*^{s}**
λDRL246	26%	35%	31%	8%
λDRL282	0%	59%	2%	39%

Table 3.1: Effect of a recipient palindrome on selectable marker inheritance, using log phase N1411 recipient cells. The donor strain was a λDRL243 lysogen of AB259.

When the same cross was carried out using a λDRL282 (palindrome) N1411 lysogen as the recipient the results were different (Table 3.1). Inheritance of the *zeo*^{r} marker dropped dramatically (57% to 2%), but the inheritance of the *kan*^{r} marker was unchanged (61% to 59%). Looking at the haplotypes of the individual colonies, it appears that the result of the presence of the palindrome is to convert the classes possessing *zeo*^{r} into the equivalent classes lacking the *zeo*^{r} marker (*kan*^{r} *zeo*^{r} converted to kan^r *zeo*^{s} and *kan*^{s} *zeo*^{r} converted to *kan*^{s} *zeo*^{s}) (Table 3.1). This suggests that recombination stimulated by the palindrome against the donor DNA (so that the palindrome and its associated *zeo*^{r} marker are lost) is extremely common. In addition these results are consistent with the formation of a double-strand break at the site of the palindrome because the sequence containing the palindrome is lost. A double-strand break at the site of the palindrome could only be repaired against the DNA introduced by conjugation by a recombination event on either side of the break, in the homologous sequences

beyond the non-homologous region containing the palindrome (Figure 3.9). This would have the result of replacing the *zeo*^{r} classes of exconjugants with *zeo*^{s} ones, exactly as is observed. Finally it appears the 'leftward' recombination event is occurring almost entirely between the *kan* marker and the beginning of the non-homologous region (Figure 3.9). This is interesting as there is only one χ site in this region. RecBCD only recognises any given χ about 20-40% of the time (Taylor and Smith, 1992; Dixon and Kowalcvzykowski, 1993), but recombination using RecBCD seems to occur almost entirely at χ sites (Dower and Stahl, 1981). If the palindrome-stimulated recombination was RecBCD-dependent then it might be expected that a large proportion of recombination events would occur at the χ sites to the left of the *kan* marker. The fact that this is not observed suggests that recombination in this region may not be occurring through the action of RecBCD. Interestingly RecF end-directed recombination may be more likely to be focused at the extreme ends of a DNA molecule as the main activity chewing back DNA ends in *E.coli* is that of RecBCD. Recombination using RecFOR might be expected to use the homologous sequences immediately adjacent to the non-homologous region (i.e. to the 'right' of the *kan* marker), as is observed.

In the model for recombinational repair of SbcCD-initiated double-strand breaks (see Chapter 1.2) it was envisaged that the palindromic sequence would form a hairpin and then be recognised by SbcCD. In order to fold back on itself to form this kind of secondary structure the palindrome would have to be single-stranded. The model envisaged that this would occur when the lagging strand is transiently single-stranded during replication. Therefore recombination initiated by a double-strand break at the site of the palindrome should be dependent on replication of the recipient chromosome. To test this the conjugational experiments above were repeated using recipient cells in stationary phase. The results of these experiments are shown in Table 3.2. As can be seen, the effect of having the recipient cells in stationary phase is to abolish the effect of the palindrome.

The pattern of markers inherited when using N1411 recipients that were lysogens of λDRL282 was the same as that seen when recipients that were lysogens of λDRL246 were used. Not only does this indicate that palindrome-induced recombination is dependent on replication through the palindrome sequence, but it also indicates that the effect of the palindrome on the pattern of inherited markers is not due to a viability problem specifically affecting the palindrome-containing transconjugants. If this were the case then the same effect would be expected to be observed with recipient cells in stationary phase as is observed for those in log phase. If the effect of the palindrome on marker inheritance is not a selective one then this, in turn, strongly suggests that the effect of the palindrome actually occurs through recombination.

Recipient lysogen	***kan*^{r} *zeo*^{r} + mixed colonies**	***kan*^{r} *zeo*^{s}**	***kan*^{s} *zeo*^{r}**	***kan*^{s} *zeo*^{s}**
λDRL246	11%	45%	38%	6%
λDRL282	11%	42%	32%	15%

Table 3.2: Effect of a recipient palindrome on selectable marker inheritance, using stationary phase N1411 recipient cells. The donor strain was a λDRL243 lysogen of AB259.

3.3.2 In the absence of SbcCD, replication-dependent recombination still occurs at the site of the palindrome, using RecF

From the analysis of lysogenisation frequencies in the absence of SbcCD it appeared that recombination still occurs at the site of the palindrome, even without SbcCD-dependent double-strand break formation. The genetic data suggested that this recombination is an example of the RecF pathway acting on gaps.

To test these hypotheses the conjugation experiments described above were repeated using *sbcC* mutants. The effect of a *recF* mutation in this background

was also examined. The results of the crosses carried out using log phase recipient (and donor) *sbcC* cells are shown in Table 3.3. When no palindrome was present it can be seen that the donor lambda markers (kan^r zeo^s) were inherited 23% of the time while the recipient markers (kan^s zeo^r) were inherited 57% of the time. Markers indicative of recombination between the *kan* and *zeo* loci (kan^r zeo^r and kan^s zeo^s) or mixed colonies (consisting of two different populations, one kan^r zeo^s and one kan^s zeo^r) made up the rest of the recombinants. The reason for the substantially higher inheritence of the kan^s zeo^r haplotype in the *sbcC* background compared to the wild type is unclear.

Recipient lysogen	**kan^r zeo^r + mixed colonies**	**kan^r zeo^s**	**kan^s zeo^r**	**kan^s zeo^s**
DRL246	17%	23%	57%	3%
DRL282	16%	62%	21%	1%

Table 3.3: Effect of recipient palindrome on selectable marker inheritance in an *sbcC* background, using log phase DL1306 recipient cells. The donor strain was a λDRL243 lysogen of DL1312.

Once again when the same cross was carried out using a λDRL282 (palindrome) DL1306 lysogen as the recipient the results were different (Table 3.4). Inheritance of the zeo^r marker decreased markedly (57% to 21%), and the inheritance of the kan^r marker increased by a similar amount (23% to 62%). Looking at the haplotypes of the individual colonies, it appears that the result of the presence of the palindrome is to convert approximately half of the kan^s zeo^r class into kan^r zeo^s. This is consistent with gap recombination occurring at the site of the palindrome. This would result in the production of a heteroduplex at the site of the gap so that the recipient sequence on one of the two recipient DNA strands would be replaced with sequence from the donor. This in turn would lead to a 50% reduction in the inheritance of the recipient sequence, consistent with what

is observed. Why the *kan*^{r} *zeo*^{r} and mixed classes do not show a similar 50% conversion to their *zeo*^{s} equivalents is not apparent. The pattern of conversion of the *kan*^{s} *zeo*^{r} class to the *kan*^{r} *zeo*^{s} class suggests that recombination is occurring to the 'left' of the *kan* marker. This in turn would suggest that the single-strand gap is extensively extended in this direction. Interestingly this is exactly the direction in which RecQ and RecJ could act together to extend single-stranded DNA (see Chapter 1).

It would be expected that replication of the recipient chromosome would be necessary to generate a single-stranded gap at the site of the palindrome, just as replication is necessary for the production of a double-strand break. To test this the conjugation experiments carried out above in *sbcC* strains in log phase were repeated using stationary phase recipient cells. The results of these experiments are shown in Table 3.4. It can be seen that in these experiments there is little effect of the presence of the palindrome on the pattern of marker inheritance.

The results of the lysogenisation experiments suggest that the gap recombination carried out at the site of a palindrome in an *sbcC* strain can be suppressed by mutations in the RecF pathway. To test if this is the case conjugation experiments using log phase DL1307 recipient cells that were *sbcC recF* and donor cells that were *sbcC* were carried out. It was unnecessary to use donor cells carrying the *recF* mutation as the *recF* locus is at 83 minutes on the standard *E.coli* genetic map and so is not transferred during these experiments. The results of these experiments are shown in Table 3.5. From these results it can be seen that in this background there was no effect of the palindrome on the inheritance of the *zeo* (and *kan*) markers, despite the fact that the recipient cells were in log phase. This supports the idea that in the absence of SbcC the recombination that occurs at the site of the palindrome is a gap recombination process dependent on RecF.

Recipient lysogen	*kan*^{r} *zeo*^{r} + mixed colonies	*kan*^{r} *zeo*^{s}	*kan*^{s} *zeo*^{r}	*kan*^{s} *zeo*^{s}
λDRL246	15%	32%	49%	4%
λDRL282	7%	41%	51%	1%

Table 3.4: Effect of a recipient palindrome on selectable marker inheritance in an *sbcC* background, using stationary phase DL1306 recipient cells. The donor strain was a λDRL243 lysogen of DL1312.

Recipient lysogen	*kan*^{r} *zeo*^{r} + mixed colonies	*kan*^{r} *zeo*^{s}	*kan*^{s} *zeo*^{r}	*kan*^{s} *zeo*^{s}
λDRL246	13%	50%	35%	2%
λDRL282	13%	56%	30%	1%

Table 3.5: Effect of a recipient palindrome on selectable marker inheritance in an *sbcC recF* background, using log phase DL1307 recipient cells. The donor strain was a λDRL243 lysogen of DL1312.

3.4 Discussion

The resolution of a 246 bp imperfect palindrome in *E.coli* appears to be a complex affair, where the palindromic substrate can be the target of double-strand break repair, single-strand gap repair or replicative bypass, depending on the genetic background. In the presence of SbcCD the components of both the RecB and RecF pathways are required for the viability of palindrome-containing cells. This palindrome-stimulated recombination was demonstrated directly by its effect on the pattern of inheritance of markers during conjugation.

In the absence of SbcCD, palindrome-stimulated recombination still appears to occur at a high frequency as *ruvA* mutations that trap late recombination intermediates are lethal. This recombination, occurring in the absence of SbcCD, was also demonstrated directly using the conjugational assay. The recombination was shown to require RecF. Despite the frequency with which it occurs this recombination is not necessary for the viability of palindrome-containing cells, as demonstrated by the viability of *recA* mutants possessing the palindrome. In the absence of both SbcCD and recombination the RecQ helicase is required for cell viability in the presence of a chromosomal palindrome.

3.4.1 Recombinational repair of double-strand breaks at the site of a palindrome

In a *wt E.coli* cell the predominant fate of a long palindrome appears to be to stimulate the formation of a double-strand break on the lagging strand. In contrast to what is believed to happen with double-strand breaks derived from fork interactions with a nick or other lesion (Kuzminov, 1995; Cox, 1998) this does not seem to lead to the breakdown of the replication fork and its associated protein replication apparatus. This was inferred from the observation that reinitiation of lagging strand synthesis by PriA is not frequently associated with the SbcCD-initiated double-strand break. This might be explained if nick or lesion-induced

double-strand breaks occur at or 'in front' of the fork whereas in the case of a palindrome the break occurs 'behind' the fork. Double-strand breaks initiated by radiation or other DNA damage could occur by the replicative helicase uncovering a nick, or by breakage of a stalled fork. The palindrome, however, has to be processed into a double-strand break, there is no physical interruption to the DNA when it is initially uncovered by the replicative helicase. What might be envisaged to occur is unimpeded helicase progression followed by the formation of a hairpin structure on the lagging strand template. This could then impede lagging strand DNA synthesis, but only with the result that the hairpin would be left in a gapped region with replication progressing 'past' this lesion in a manner similar to that suggested for UV damage-induced gaps (Rupp and Howard-Flanders, 1968; Kuzminov, 1995). The hairpin structure left behind in this gap could then be cleaved by the SbcCD nuclease to form a double-strand break, but this break would be physically removed from the replication fork and would avoid causing it to collapse. It is also possible that the 246 bp palindrome causes fork breakdown but that this is repaired in a PriA-independent manner. This seems unlikely however, given that *priA* mutants are highly deficient in the repair of other kinds of double-strand breaks (Kogoma *et al.*, 1996). In fact the general effects of *priA* mutations on cell viability suggest that in the absence of PriA cells are very deficient in replication fork repair (see Chapter 1.7). However, as replication fork repair is a poorly understood phenomenon, the existence of a PriA-independent fork repair mechanism involved in replication past secondary structures such as hairpins, cannot be ruled out. There is some evidence for the existence of PriA-independent replication fork repair pathways (see Chapter 1.7).

Repair of the double-strand break was expected to involve the products of the RecB pathway which appears to be the dominant end-directed recombination system in *wt E.coli*. It was already known that the RecA and RecB proteins are essential for repair of the palindrome-initiated double-strand break (Leach

et al., 1997) and this work demonstrates that the RecN protein is also involved. Although the RecN protein is poorly understood it has been implicated in end recombination involving the RecB pathway as well as RecF recombination which is directed to DNA ends (Chapter 1.4.2.4). In addition, the post-synaptic proteins that are common to both the RecF and RecB pathways, RuvA and RuvC, are essential for successful recombinational repair of palindrome-induced double-strand breaks. This is also true for the post-synaptic protein RecG.

More surprising was the discovery that the proteins of the RecF pathway, RecF, RecO, RecR, RecJ and RecQ, are also essential for viability in the presence of a chromosomal palindrome and SbcCD. In the absence of the RecB pathway, RecF recombination can substitute for its function at DNA ends, but only in *sbcB15* and *sbcCD* mutant strains. In these cases recombinogenic 3' DNA ends are being protected by the mutations affecting the two nucleases, and this is needed for efficient recombination. In *wt* cells the RecF pathway appears to act at DNA ends very infrequently (approximately 1 time in 100 (Howard-Flanders and Theriot, 1966)) with the RecB pathway predominating. In this work, however, both the RecB and RecF pathways have been shown to operate frequently on DNA ends. One possibility is that both sets of proteins cooperate to process the same substrates. However, while it is possible to envisage the RecFOR proteins helping to load RecA onto a 3' end produced by the action of the RecBCD nuclease, it is more difficult to imagine how the RecQ and RecJ proteins could cooperate with RecBCD when they appear to substitute for one another as helicases linked to 5' to 3' nucleases. Another possibility is that both the RecF and RecB pathways are used by the DNA ends at approximately equal frequency (so that mutants in either pathway have a lethal phenotype). The third possibility is that the two ends produced by the SbcCD cleavage event have different recombinational requirements, with one utilising the RecF pathway and the other the RecB pathway. RecBCD can only utilise DNA ends that are blunt or nearly blunt (Taylor

and Smith, 1985), so that if one of the DNA ends had a long overhang it could not be used as a substrate by RecBCD. This is similar to the suggestion that UV-induced single-strand gaps could be broken to produce DNA ends and that the RecF pathway could act on these if they possessed long single-stranded overhangs (Wang and Smith, 1985). A model describing this scheme is diagrammed in Figure 3.10. It would seem necessary for this putative substrate to be protected, both to allow RecF recombination and to prevent processing to a RecB end. One possibility is that the RecF pathway loads RecA onto the single-stranded DNA of the putative hairpin-containing gap region and that when this is converted to a double-strand break the RecA protects the end it now finds itself on.

Recombination against homologous DNA stimulated by the presence of the palindrome was demonstrated directly using a conjugational assay. The results of these experiments support the model described above. Recombination was shown to be dependent on replication, just as would be expected in order to allow single-stranded DNA to form secondary structure on the lagging strand. This recombination caused the complete loss of a marker absolutely linked to the site of the palindrome, as would be expected for double-strand break repair. Finally the recombination at the side of the break distal to the replication fork was shown to occur at a position that suggested it was not χ-dependent. This supports the idea that this recombination at this DNA end is carried out by the RecF pathway, not the RecB pathway with its strong dependence on χ sequences.

3.4.2 Recombinational repair of single-strand gaps at the site of a palindrome

In the absence of the SbcCD nuclease, palindromic sequences still stimulate recombination at high frequency. As recombination is unnecessary for viability in these circumstances, and there is no hairpin nuclease, it seems unlikely that the substrate in this situation is a double-strand break. The alternative would be recombination stimulated by a single-strand gap (Figure 3.11) and the involvement

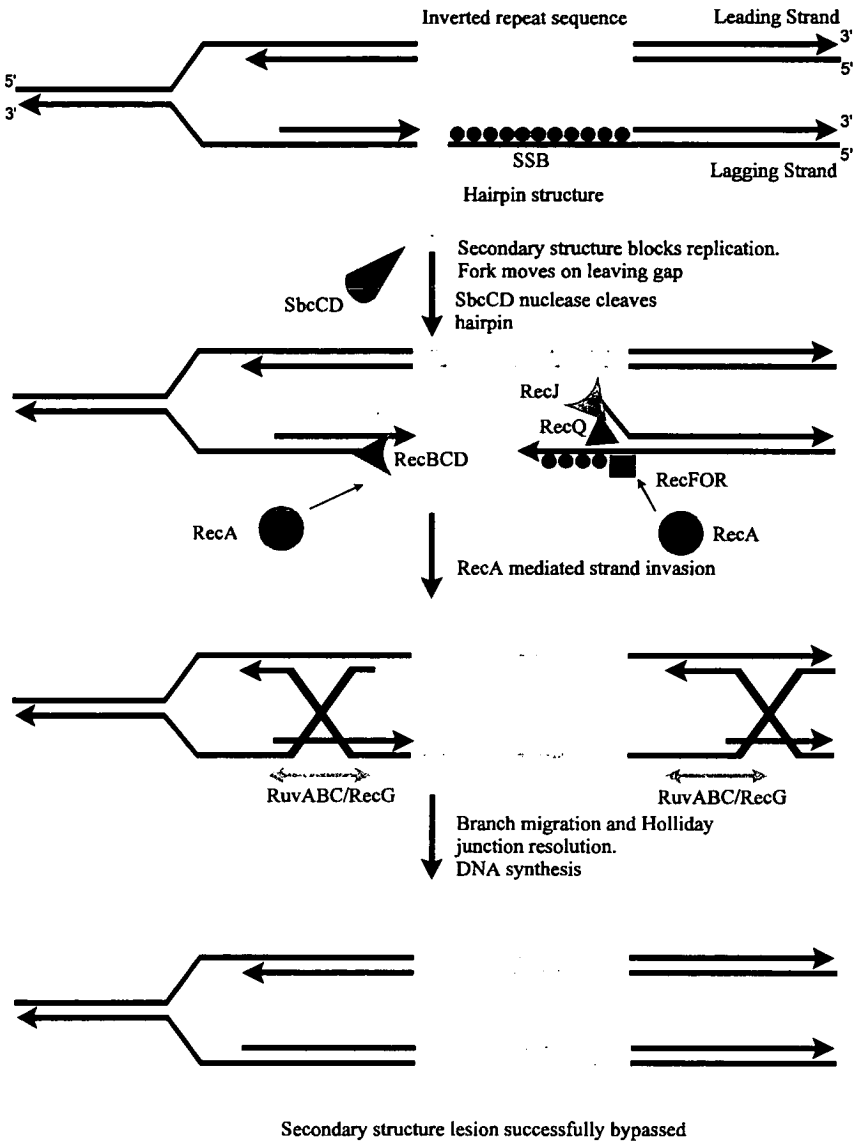

Figure 3.10: Hypothetical scheme for recombinational repair of SbcCD-induced double-strand breaks.

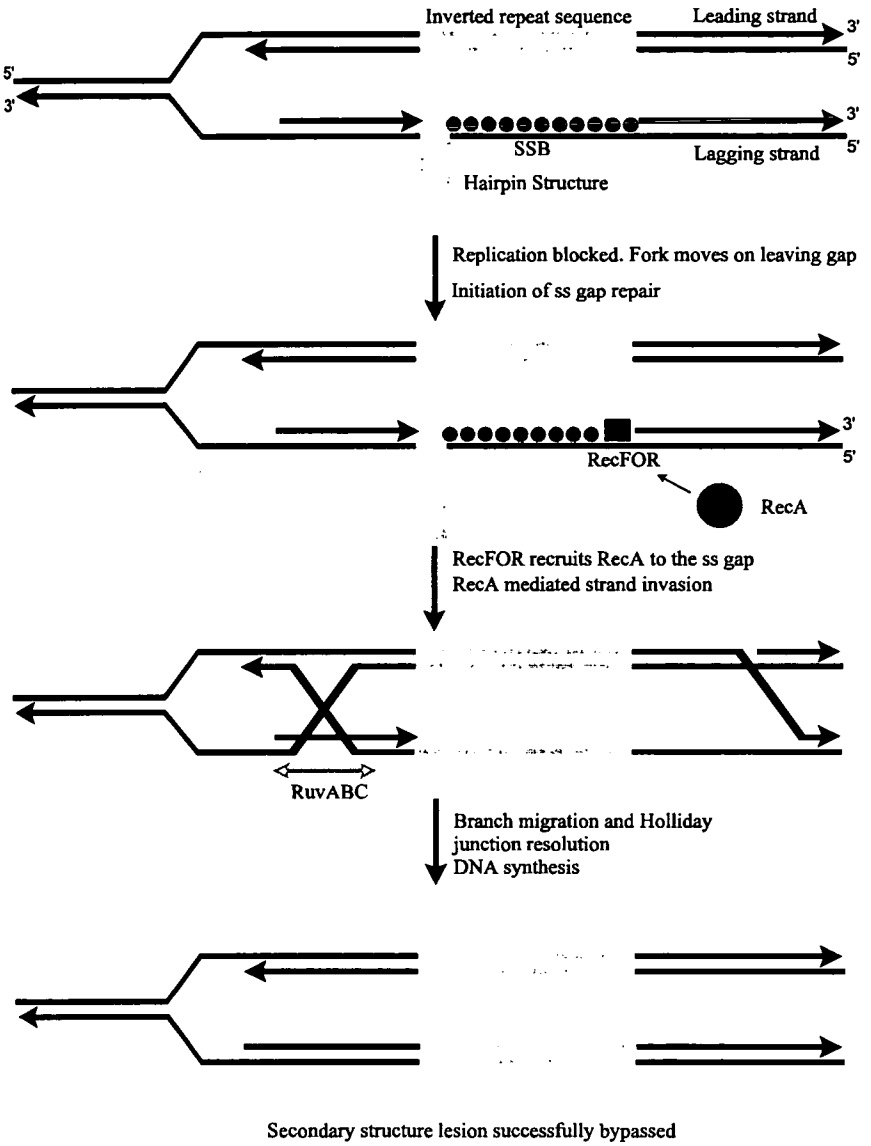

Figure 3.11: Hypothetical scheme for recombinational repair of single-strand gaps caused by hairpin blockage of lagging strand DNA synthesis.

of the RecF pathway proteins RecF, RecO, RecR suggests that this is in fact the case. Although the RecF pathway proteins can efficiently stimulate recombination at ends in the absence of RecB in an *sbcB15 sbcC* mutant background, they have an independent role in gap-based recombination (see Chapter 1)

Once again it was possible to directly demonstrate palindrome-stimulated recombination in the absence of SbcCD using the conjugational assay. The results of these experiments support the idea that it is gap recombination that occurrs at the site of the palindrome in these circumstances. This is because instead of

a total loss of the marker absolutely associated with the palindrome the loss of only 50% of this marker was observed. This is consistent with recombination involving a single-strand gap where only one of the two recipient strands is replaced with donor sequence. The recombination stimulated by the palindrome was shown to be dependent on RecF as had been deduced from the lysogenisation data. Once again, consistent with the model, this process required replication of the palindrome-containing DNA.

It appears that in both the presence and absence of SbcCD the existence of a long palindromic sequence leads to the formation of a single-strand gap containing the palindrome (probably at one side). The lysogenisation results with *priA* strains show that this process of fork progression with a gap left behind does not involve fork collapse. In this sense whether or not the single-strand gap is then converted to a double-strand break by SbcCD is irrelevant, the progression of the fork will not be affected in either event.

3.4.3 Replicative bypass of palindrome-generated structures using the RecQ helicase

It is surprising that the gap recombination which appears to occur frequently in the absence of SbcCD is not necessary for palindrome viability. Replication is unable to process the secondary structure and leaves a gap which would lead to a viability problem if left unfilled. Recombination must be able to unwind the secondary structure and allow the gap to be filled by replication using the other sister as a template. The exact mechanism by which this unwinding occurs is unclear, but it could occur during strand exchange or branch migration. The question then arises as to how the single-strand gap is filled in the absence of recombination. It appears that the RecQ helicase is central to this process. The RecQ protein is a 3' to 5' DNA helicase that acts on duplex DNA or duplex DNA with single-stranded overhangs (see Chapter 1.4.2.3). Its role in the recombination-independent resolution of secondary structure could be explained

by RecQ acting along with a repair polymerase (perhaps DNA Polymerase I), with the helicase unwinding the secondary structure and allowing replication to pass through the palindromic sequence. This is consistent with the proposal that RecQ (and its eukaryotic homologues) could defend genome integrity by targeting secondary structures (Chakraverty and Hickson, 1999). Interestingly the eukaryotic RecQ homologues BLM and Sgs1p have been shown to unwind G4 tetraplex structures and guanine-guanine paired DNA (Sun *et al.*, 1998; Sun *et al.*, 1999). Similarly, in hyperthermophilic archaebacteria it has been suggested that reverse gyrase, which has a helicase as well as a topoisomerase activity, may be involved in eliminating various kinds of abnormal DNA structures (Kikuchi and Asai, 1984).

Chapter 4

Control of Crossing-Over in *Escherichia coli*

4.1 Introduction

In 1964 Robin Holliday suggested a model for homologous genetic recombination that postulated a four-way junction intermediate that has subsequently become known as the 'Holliday junction' (Holliday, 1964). He also proposed that there were two alternative modes of resolution of this junction by endonucleolytic cleavage. These two modes of resolution would result in the 'cross-over' or 'non cross-over' types of recombinant that are both associated with gene conversion. It has generally been assumed that any Holliday junction can be resolved by the cleavage of either of these two possible pairs of strands. This means that a single intermediate can give rise to two kinds of products (cross-over or non cross-over) (Figure 4.1).

However, it is not clear that resolution in both directions is equally probable. In yeast meiotic recombination, on average 35% of conversions are associated with crossing-over (Fogel *et al.*, 1981) while in mitotic inter-homologue recombination this falls to 10% to 20% (Esposito, 1978; Haber and Hearn, 1985; Kupiec and Petes, 1988). The extent of crossing-over in inter-sister mitotic recombination is not known. What is clear is that the proportion of recombination events that involve crossing-over is different in different recombinational situations. This

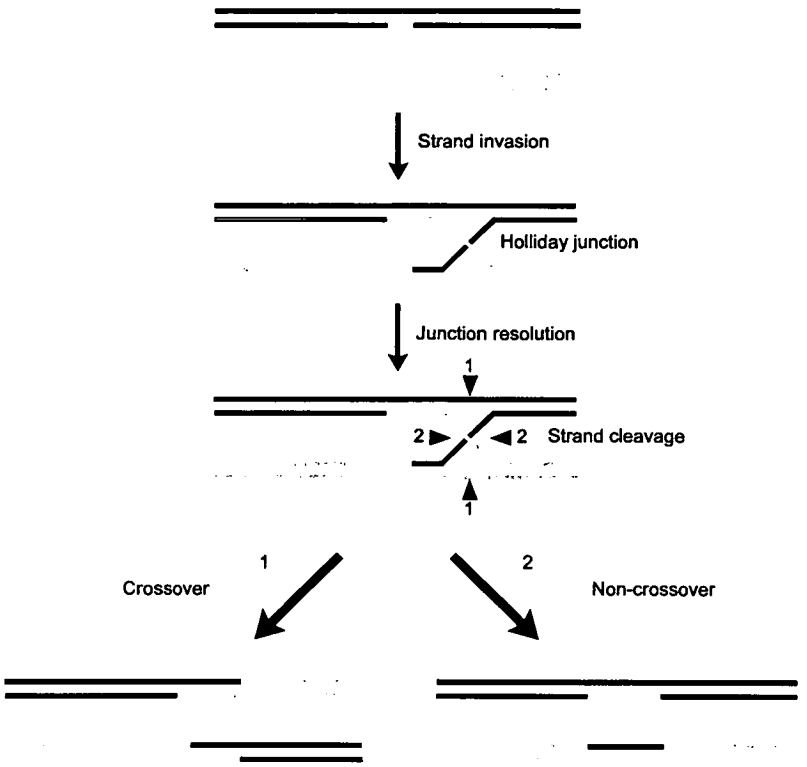

Figure 4.1: Classical view of the formation of cross-over and non cross-over products from a single substrate by recombination (adapted from Holliday, 1964).Two nicked strands undergo reciprocal strand invasion. The resulting joint molecule contains a Holliday junction. Cross-over and non cross-over products can be formed from this single intermediate by cleaving one or the other pair of permissable strands in the Holliday junction. The same result holds true for cleavage of Holliday junctions in more complex recombinational models (Meselson and Radding, 1975; Szostak *et al.*, 1983).

implies that recombination can be regulated to control the level of crossing-over. In meiosis, it is normally desirable to ensure that at least one cross-over occurs per chromosome in order to facilitate proper chromosome disjunction. By contrast, in many organisms it may be desirable to limit crossing-over in recombinational DNA-repair reactions. In a bacterium with a circular chromosome, crossing-over has the unfortunate consequence of generating a dimeric chromosome (Figure 4.2). Control of crossing-over is likely to be determined at several different levels and one important level is that of Holliday junction cleavage, which is addressed in this chapter.

Logically the outcome of a resolution reaction must depend on the identity of the cleaved strands with respect to the way in which they are connected to flanking chromosome arms. Therefore if cleavage is regulated to control crossing-over then it would be expected that this might operate through a rule determined by the structure of the recombining substrates. There are two broad types of recombinational substrate in *E.coli.* These are DNA ends (implicated in double-strand break repair, DSBR) and DNA gaps (implicated in single-strand gap repair, SSGR). The early stages of recombination using these substrates proceed via different enzymatic systems (see Chapter 1). If resolution is random it would be expected that both kinds of reaction could lead to cross-over or non cross-over products with about 50 % probability. However if resolution is governed by a rule based on the nature of the recombining substrates then these two different kinds of substrate might show a different propensity to form cross-over products.

If recombination occurs between daughter duplexes in a replicating circular molecule so as to cause a cross-over (or any odd number of cross-overs) then, upon completion of replication, a dimeric molecule is formed (Figure 4.2). This situation is obviously relevant to the circular *E.coli* chromosome and *E.coli* possesses an enzymatic system to resolve the problem. This is the XerCD site specific recombination system.

The XerC and XerD proteins form a site-specific recombination system that resolves dimeric chromosomes back to monomers by a conservative-break join reaction (Sherratt *et al.*, 1995; Cox, 1998). This reaction takes place at a specific chromosomal locus *dif* located near the terminus region of the chromosome. Similar sequences (*cer*, *psi* and *clf*) allow XerCD to resolve plasmid dimers (see Sherratt *et al.*, 1995 and references therein).

This chapter describes work carried out to determine if recombination at the site of a palindrome causes crossing over, and hence dimerisation. The presence of SbcCD determines the nature of the recombinogenic substrate generated at the site of the palindrome (break or gap). Any difference in the frequency of crossing-over between these two situations would be indicative that crossing-over is a regulated process in *E.coli*, determined by the nature of the recombining substrates.

To carry out this study we used inactivation of the *xerCD* site-specific recombination system. The effect of an *xerC* mutation was used to assess the frequency of cross-over products versus non cross-over products using the phage lysogenisation assay introduced in Chapter 3. It was observed that palindrome-directed break recombination (in the presence of SbcCD) was associated with frequent crossing-over, whereas palindrome-directed gap recombination (in the absence of SbcCD) was not associated with frequent crossing-over. Palindrome-directed recombination in the presence of SbcCD is an example of 'ends-in' double-strand break repair (DSBR) whereas recombination in the absence of SbcCD is a form of single-strand gap repair (SSGR).We investigated whether the results obtained for the two kinds of palindrome recombination also held true more generally for DSBR and SSGR. This was done by investigating the association of crossing-over with the use of gapped and broken substrates for recombination during the replication of UV-irradiated chromosomes. Once again we observed that recombination of the broken substrates was linked to crossing-over, while recombination

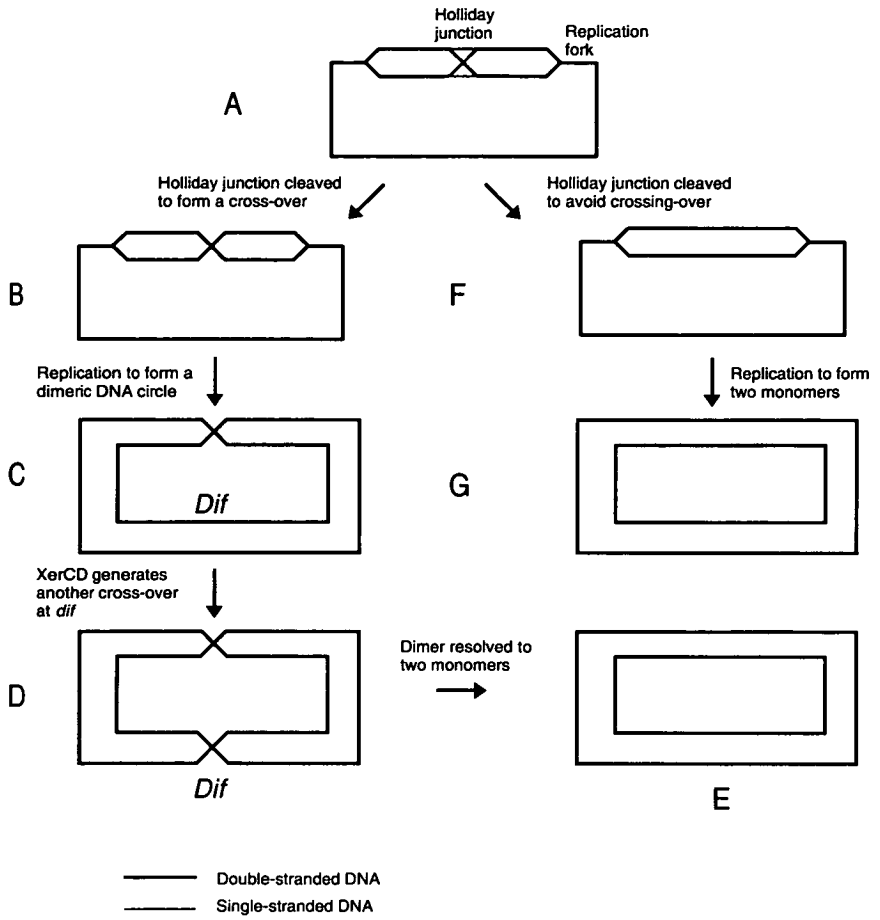

Figure 4.2: Formation of dimeric molecules by recombination between daughter duplexes in a replicating DNA molecule and resolution of the dimers by XerCD.
A) Recombination occurs between the daughter duplexes of a replicating circular DNA molecule forming a Holliday junction.
B) The Holliday junction is resolved to form a cross-over.
C) Replication is completed and produces a single dimeric molecule.
D) Site-specific recombination using XerCD at (for instance) *dif* creates an additional crossover.
E) The dimer is resolved into two monomer daughter molecules.
F) The Holliday junction is resolved to avoid a cross-over
G) Replication is completed and produces two monomer daughter molecules.

involving the gapped substrates avoided crossing-over. Finally we observed that using a single defined substrate, a linear double-strand DNA fragment undergoing 'ends-out' replicative recombination after P1 transduction, mainly a single type of product was produced: a chromosome dimer. This bias for crossing-over in DSBR was removed in the absence of the RuvABC complex. These results are consistent with the operation of a rule for crossing-over determined by directional branch migration leading to biased Holliday junction resolution. A prediction of this kind of rule would be that replication fork breakage occurring on the lagging strand would lead to crossing over whereas breakage on the leading strand would avoid it. It was shown using a *polA* mutant that leading strand fork breakdown does indeed largely avoid crossing over.

4.2 Association of crossing-over with DSBR but not SSGR at a specific site

4.2.1 'Ends-in' DSBR is frequently associated with crossing-over

In the presence of SbcCD a 246 bp palindrome stimulates DSBR whereas in the absence of SbcCD the palindromic sequence promotes RecF-mediated SSGR (see Chapter 3). This system offers a method of analysing the association of break and gap recombination at a defined chromosomal location with crossing-over by using mutations in *xerC*. If either the DSBR or SSGR initiated by the 246 bp palindrome is strongly associated with crossing-over this will cause a palindrome-induced viability problem in an *xerC* mutant strain of *E.coli*. To test this hypothesis two isogenic phage were used to carry out lysogenisation frequency tests as in Chapter 3. The first contained the 246 bp interrupted palindrome mentioned above (λDRL282) and the second was a palindrome-free control phage (λDRL246).

As previously shown, the palindrome-phage could lysogenise the wild-type

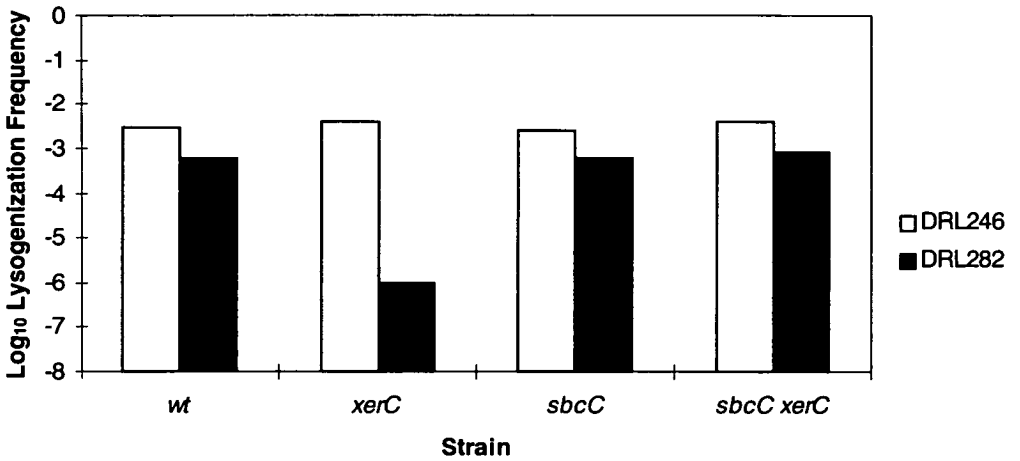

Figure 4.3: Effect of an *xerC* mutation on lysogenisation frequency of phages undergoing DSBR and SSGR. The results show the lysogenisaton frequencies of phages λDRL246 (palindrome-free, open bars) and λDRL282 (246 bp palindrome, filled bars). The strains used were AB1157, DL1132, DL515 and DL1122. Lysogenisation was carried out as described in Materials and Methods. The results are the geometric means of at least two independent experiments.

strain at the same frequency as the control phage (Figure 4.3). This is because, in the presence of the full complement of proteins of the RecB and RecF pathways, DSBR can be carried out successfully at the site of the palindrome and there is no viability problem. However the story was quite different in an *xerC* mutant strain. In this case the palindrome phage lysogenised at a much reduced frequency compared to the control phage (Figure 4.3). This indicates that palindrome-induced DSBR frequently causes crossing-over. The resulting dimerisation leads to a viability problem in the *xerC* mutant strain.

4.2.2 SSGR is not frequently associated with crossing-over

These experiments were repeated using an *sbcC* mutant strain. As observed previously the palindrome-phage could lysogenise at the same frequency as the control phage (Figure 4.3). In this background the palindrome phage can successfully undergo SSGR in the presence of all of the proteins of the RecF pathway. The ability of the two phage to lysogenise an *sbcC xerC* double mutant was then tested. Both the palindrome and non-palindrome phage were able to lysogenise

this strain equally well (Figure 4.3). This indicates that crossing-over is rarely associated with SSGR at the site of the palindrome.

It therefore appears that there is a difference in the relationship to crossing-over of break and gap recombination at a defined chromosomal location. Although it is not possible to say exactly what proportion of DSBR and SSGR events lead to dimerisation it is possible to say that this dimerisation occurs frequently at the breaks and infrequently at the gaps.

4.3 The association of crossing-over with DSBR but not with SSGR is a general phenomenon

In the previous experiments palindrome-induced DSBR was shown to be more strongly associated with crossing-over than was the case for palindrome-induced SSGR. However these results referred only to recombination at a particular chromosomal location. To assess whether this difference between the products of break and gap recombination is more generally true, a different system was needed. Such a system would have to involve breaks and gaps stimulating chromosomal recombination at many different sites. This is precisely the situation that occurs during the post-replicational repair by recombination of UV-induced damage.

In *E.coli* there are four systems for coping with UV-induced damage: photoreactivation, excision repair, recombinational repair and the UmuDC translesion-bypass system (see Friedberg *et al.*, 1995). In excision repair mutants (e.g. *uvrB*), where irradiation and recovery are carried out in low light conditions, recombinational repair is essential for recovery from UV irradiation. UV damage coupled with replication leads to double-strand breaks and single-strand gaps (Wang and Smith, 1983). The former are repaired by the RecB pathway and the latter by the RecF pathway (Horii and Clark, 1973; Wang and Smith, 1983). In the absence of RecB, repair is highly dependent on RecF and vice-versa. This allows RecF-dependent recombination at distributed gaps and RecB-dependent recombination

at distributed breaks to be isolated from one another.

The effect of these two pathways on crossing-over was compared by introducing further mutations in *xerC*. Initially experiments were carried out using cells in log phase. As described in previous studies (Ganesan and Smith, 1970; Horii and Clark, 1973; Wang and Smith, 1981; Wang and Smith, 1983) the addition of either *recF* or *recB* mutations to the *uvrB* strain greatly increased its sensitivity to UV irradiation (compare Figures 4.4, 4.5 and 4.6). The effect of an *xerC* mutation on the *uvrB* strain, however, was small (Figure 4.4). It was predicted that if SSGR avoids crossing-over generally, then an *xerC* mutation should have no effect on the UV sensitivity of a *uvrB recB* strain, which is dependent on RecF gap recombination. This indeed turned out to be the case (Figure 4.5). It was also predicted that *xerC* could have a negative effect on the viability of a *uvrB recF* strain. Such a strain would be dependent on RecB-dependent DSBR which is predicted to be associated with crossing-over. In fact, however, the addition of a *xerC* mutation to a *uvrB recF* strain greatly increased the resistance of the strain to UV irradiation (Figure 4.6). This result was entirely unexpected. It is difficult to imagine how a cellular deficiency in the resolution of the products of recombination could make cells more resistant to UV irradiation. Instead it seemed more likely that the chromosome dimers that already existed, or were on the way to being formed, in the *uvrB recF xerC* strain were more resistant to UV irradiation than the monomers present in the *uvrB recF* strain. In other words the RecB pathway may be better able to deal with UV lesions when operating on a chromosome dimer.

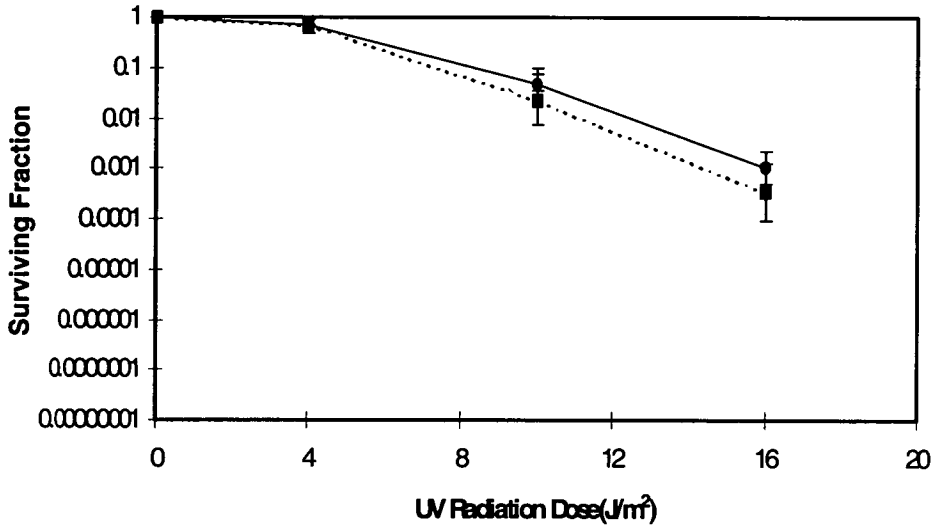

Figure 4.4: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB* background with growth in log phase. Cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least two independent measurements. The strains used were: DL698 (*uvrB*) (closed circles) and DL1155 (*uvrB xerC*) (closed boxes).

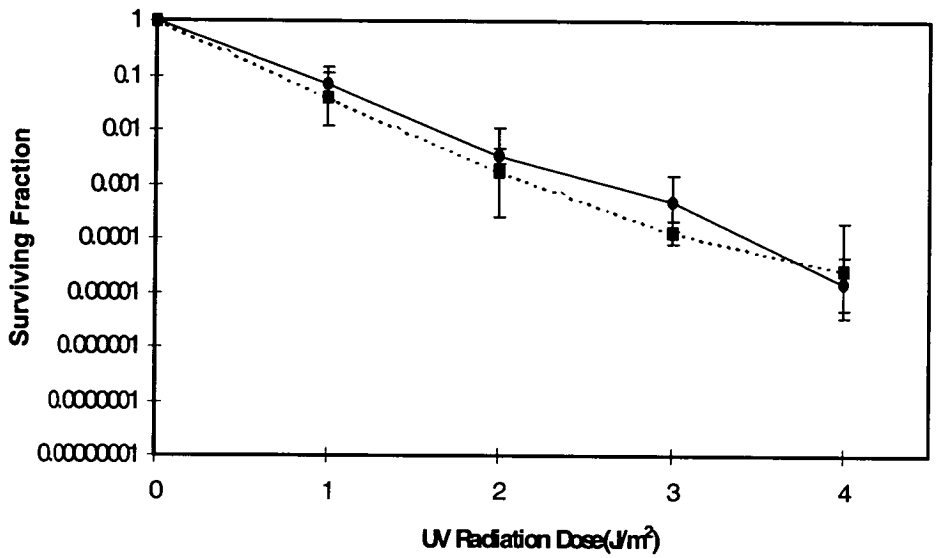

Figure 4.5: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB recB* background with growth in log phase. Cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least two independent measurements. The strains used were: DL1159 (*uvrB recB*) (closed circles) and DL1161 (*uvrB recB xerC*) (closed boxes)

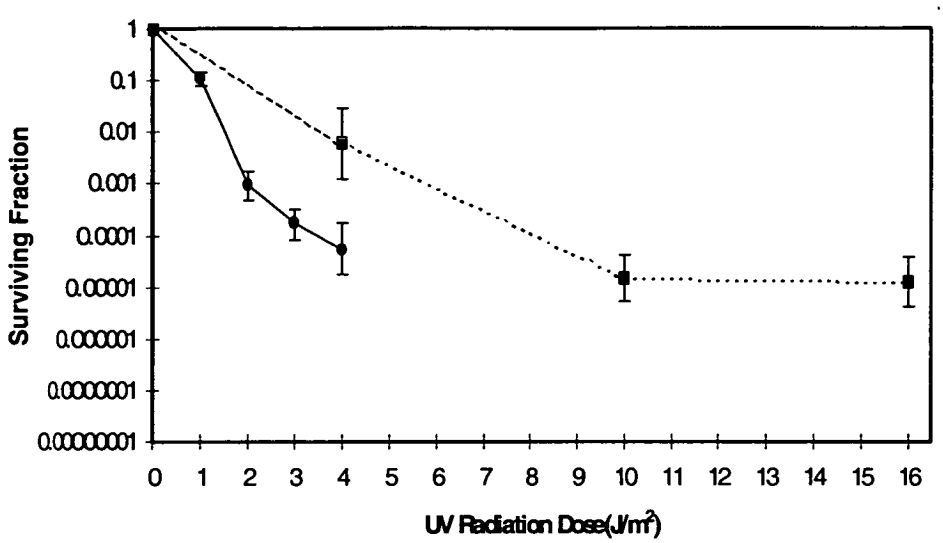

Figure 4.6: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB recF* background with growth in log phase. Cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least two independent measurements. The strains used were: DL1159 (*uvrB recF*) (closed circles) and DL1161 (*uvrB recF xerC*) (closed boxes).

With this in mind irradiation was carried out on cultures that had entered stationary phase. This was done in order to limit the number of pre-existing dimers in the chromosomes undergoing repair as partitioning problems should have led to the loss of cells containing such dimers. Once again, the addition of either *recF* or *recB* mutations to the *uvrB* strain greatly increased its sensitivity to UV irradiation (compare Figures 4.7, 4.8 and 4.9). The UV-sensitising effect of an *xerC* mutation on the *uvrB* strain was smaller but still significant ($p<0.05$ (student's T-test, 2-tailed)) at all but the lowest UV dose (Figure 4.7). Again, as predicted, an *xerC* mutation had no effect on the UV resistance of a *uvrB recB* strain, which is dependent on RecF gap recombination (Figure 4.8), any small differences between the UV sensitivities of the two strains were not significant ($p>0.05$ for all doses). However, in contrast to the results with log phase cells, *xerC* had the negative effect on the viability of a *uvrB recF* strain that was initially predicted (Figure 4.9). The *uvrB recF xerC* strain was significantly ($p<0.05$) more sensitive at all but the lowest UV dose. Therefore a mutation in *xerC* only

confers sensitivity to UV when the RecB presynaptic pathway is functional. This supports the general conclusion that DSBR using the RecB pathway is frequently associated with crossing over while SSGR using the RecF pathway is much less frequently (if at all) associated with crossing-over.

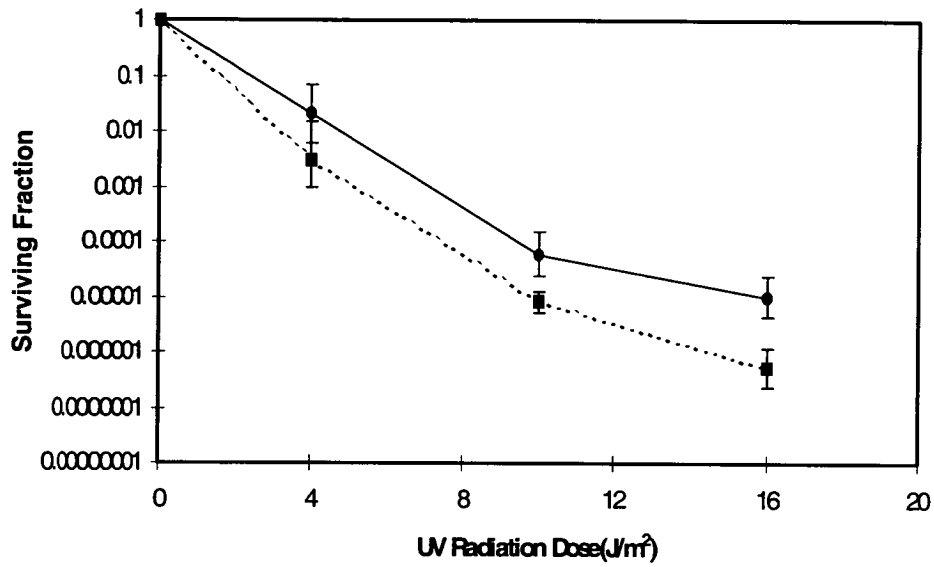

Figure 4.7: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB* background with growth in stationary phase. Overnight cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least five independent measurements. The strains used were: DL698 (*uvrB*) (closed circles) and DL1155 (*uvrB xerC*) (closed boxes).

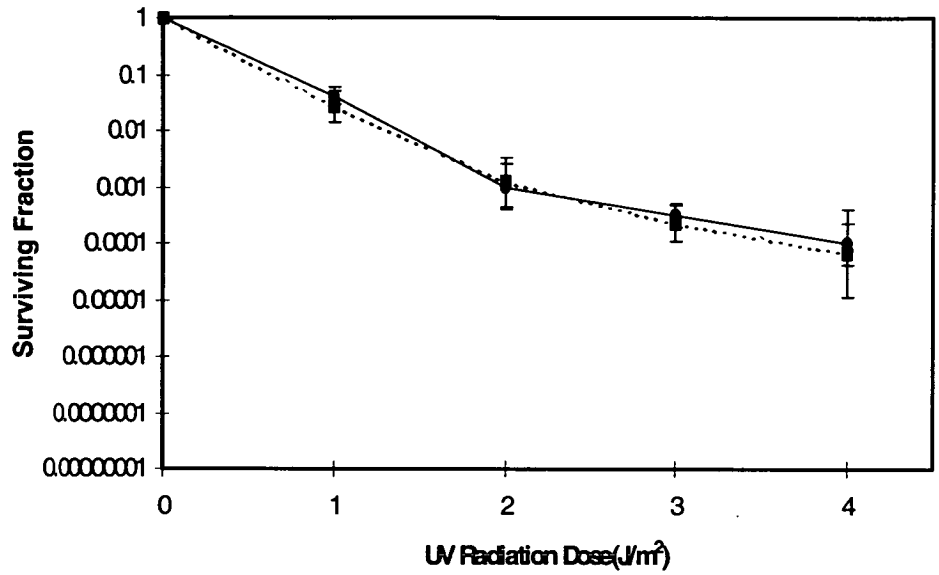

Figure 4.8: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB recB* background with growth in stationary phase. Overnight cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least five independent measurements. The strains used were: DL1159 (*uvrB recB*) (closed circles) and DL1161 (*uvrB recB xerC*) (closed boxes).

4.4 Evidence of a RuvABC-directed rule for the resolution of Holliday junctions

4.4.1 A defined recombinational substrate generates a single kind of cross-over product

The existence of a rule for Holliday junction resolution would predict that recombination using a single defined substrate would generate a single kind of product (cross-over or non cross-over). This hypothesis was tested using P1 transduction. This is an 'ends-out' DSBR event involving recombination via the RecBCD pathway at both ends of a linear double-stranded DNA fragment. As this process is strongly dependent on PriA (Kogoma *et al.*, 1996) (see Chapter 1.7), it seems likely that recombination proceeds by the setting up of replication forks at the invading P1 ends (Figure 1.7). If this replication proceeds around the entire *E.coli* chromosome then it should be possible to identify if a resolution rule is operating at the two Holliday junctions formed at each end of the recombining P1 fragment.

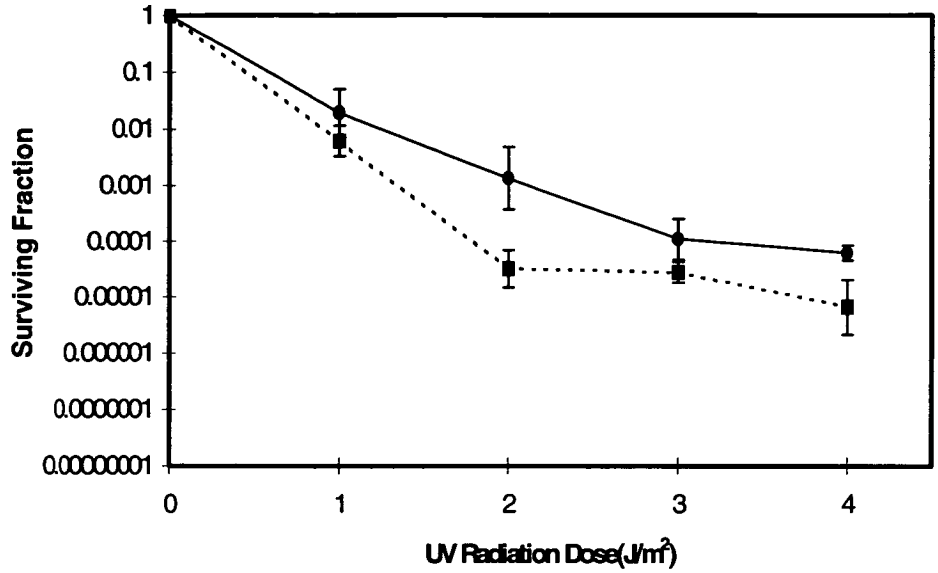

Figure 4.9: Sensitivity of *xerC* mutants to UV irradiation in a *uvrB recF* background with growth in stationary phase. Overnight cultures of cells were exposed to UV irradiation as described in Materials and Methods. 95% confidence intervals are shown for each of the data points. The data points are the geometric means of at least five independent measurements. The strains used were: DL1159 (*uvrB recF*) (closed circles) and DL1161 (*uvrB recF xerC*) (closed boxes).

In the absence of a resolution rule it would be expected that, upon completion of replication, 50% of the products would be monomers and 50% dimers. The operation of a resolution rule should lead to all of the resulting chromosomes becoming either monomers or dimers. It was possible to test this by looking at transduction frequencies in wild-type and *xerC* strains.

When this was done it was observed that transduction frequencies in an *xerC* mutant ran at approximately 10% of wild-type levels (Table 4.1). This compares to a value of 50% expected if junction resolution is random. Therefore resolution of the Holliday junctions seems to be non-random. In fact a significant percentage of replication forks are believed to break before completing replication and have to be reformed by recombination (see Cox, 1998 and references therein). This process could convert what would have been a chromosome dimer to a monomer and therefore the bias in resolution may actually approach 100%.

Strain	Relevant Genotype	Relative transduction frequency with P1.CAG18556	Relative transduction frequency with P1.CAG5054
AB1157	*wt*	1.00	1.00
DL1132	*xerCY17*::Mini-*cat*	0.11	0.078
DL1102	*ruvAC65*::Tn*10*	0.39	
DL1209	*ruvAC65*::Tn*10* *xerCY17*::Mini-*cat*	0.15	

Table 4.1: Effect of an *xerC* mutation on transduction frequency in a *wt* and *ruv* background. The relative transduction frequencies were measured with respect to the transduction frequencies using the *wt* parental strain AB1157. The standard error of the mean in these experiments is less than 25%. The values given are the means of at least 10 measurements. The transductions using P1.CAG5054 were not carried out with DL1102 or DL1209 as these strains were already resistant to tetracycline.

4.4.2 In the absence of RuvABC, junction resolution appears to be random

The previous result suggested that the resolution of Holliday junctions is a non-random process and could be explained by the existence of a resolution rule. Resolution bias might be expected to operate through constraints on the activities of the RuvABC protein complex which normally carries out strand cleavage. We therefore investigated the effect of an *xerC* mutation on P1 transduction in a *ruvABC* deletion mutant. In this background we observed that an *xerC* mutation only caused between a 2- and 3-fold reduction in transductant frequency (from 39% of wild-type levels to 15%) (Table 4.1). If recombination continues to be highly linked to replication in these strains then this means that the bias in cross-over resolution has largely been removed. This in turn suggests that the rule for Holliday junction resolution operates through the RuvABC complex.

4.5 Crossing-over and the collapse of replication forks

The constraints that this work place on recombination models have interesting implications for the repair of broken replication forks. Such broken forks may arise though the chance encounter of a nick or gap in either of the template strands (see Kuzminov, 1995 and references therein). If the discontinuity is in the template of the lagging-strand, the consequence is a 'lagging-strand break' whereas if the nick is in the template of the leading-strand, the consequence is a 'leading-strand break'. These two kinds of substrate lead to different kinds of product if a resolution rule based on the positioning of the RuvABC complex is applied (Figures 4.10 and 4.11). The rule illustrated is that the strand passing through the RuvB ring 3' towards the junction is cleaved (see discussion). It can be seen that the consequence of this rule is that leading-strand breaks are predicted to be accompanied by little crossing-over whereas lagging-strand breaks are predicted to be associated with substantial crossing-over (Figures 4.10 and 4.11).

It was not possible to test this prediction with respect to lagging-strand breaks, however leading-strand breaks are believed to predominate in *polA* mutants. In *polA* mutants nicks are left on the lagging strand after replication and during the next round of replication these nicks are converted specifically to leading-strand breaks (Cao and Kogoma, 1995). Mutations in *polA* cannot be combined with mutations in *recA* or *recB* (Gross *et al.*, 1971; Monk and Kinross, 1972), suggesting that leading-strand breaks frequently cause replication fork breakdown and recombinational repair is needed to re-establish these replication forks. Interestingly a *ruv* mutant also cannot be combined with a *polA* mutant (Ishioka *et al.*, 1998) even through *ruv* mutants display only a small defect in DNA end-directed recombination as measured by conjugation and transduction assays (see Chapter 1.6.1). This suggests that if even a moderate number of individual recombination

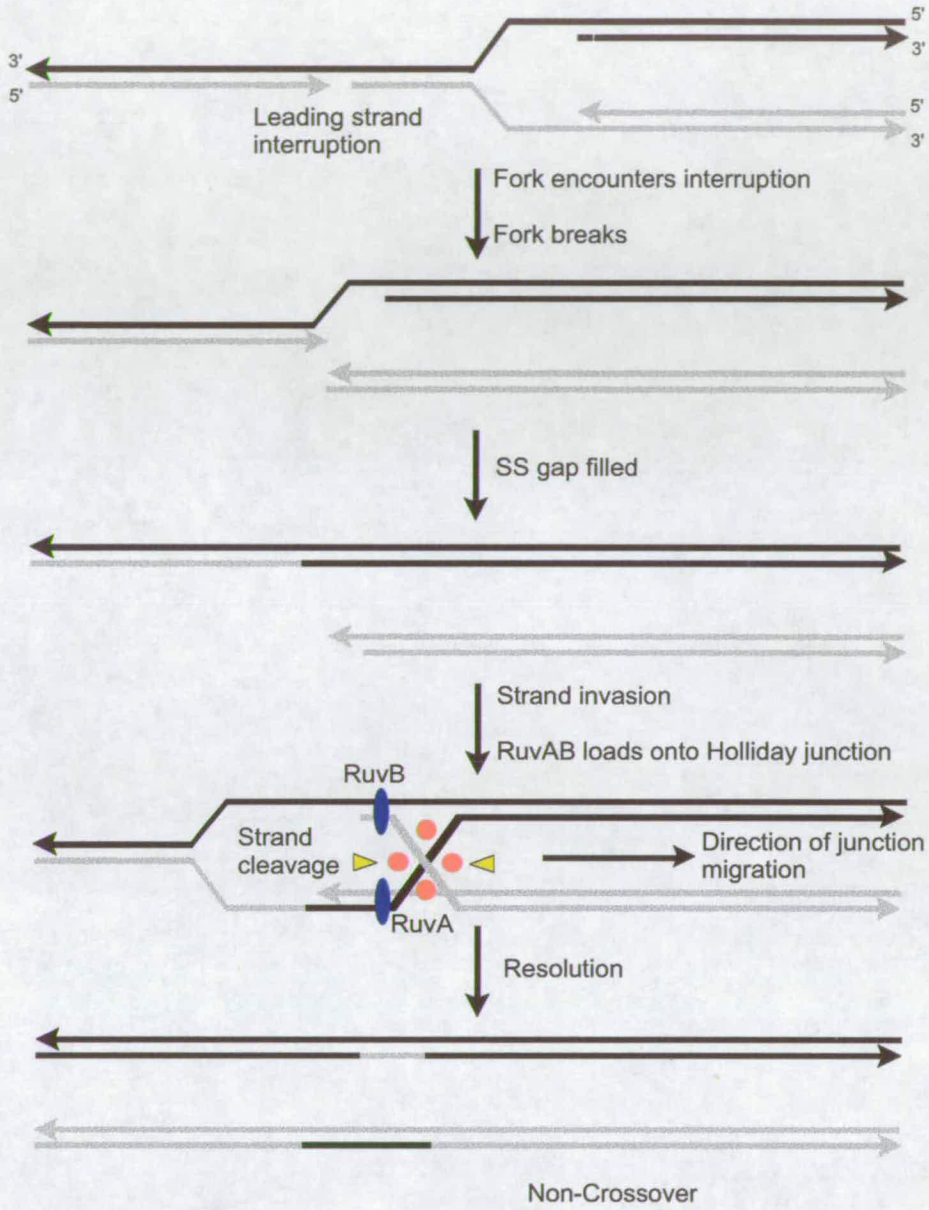

Figure 4.10: Scheme showing the avoidance of cross-overs when forks are broken on the leading strand. The replication fork encounters a strand interruption on the leading strand. This causes fork breakdown. The resulting SS gap is filled and the broken end processed by RecBCD to form a 3' overhang coated with RecA. RecA promotes strand invasion generating a joint molecule possessing a Holliday junction. The RuvAB complex loads onto this junction so as to branch migrate it 'productively' away from the DNA end. Strand cleavage is directed by RuvB to the strand passing 3' through RuvB toward the Holliday junction. A non cross-over product is generated.

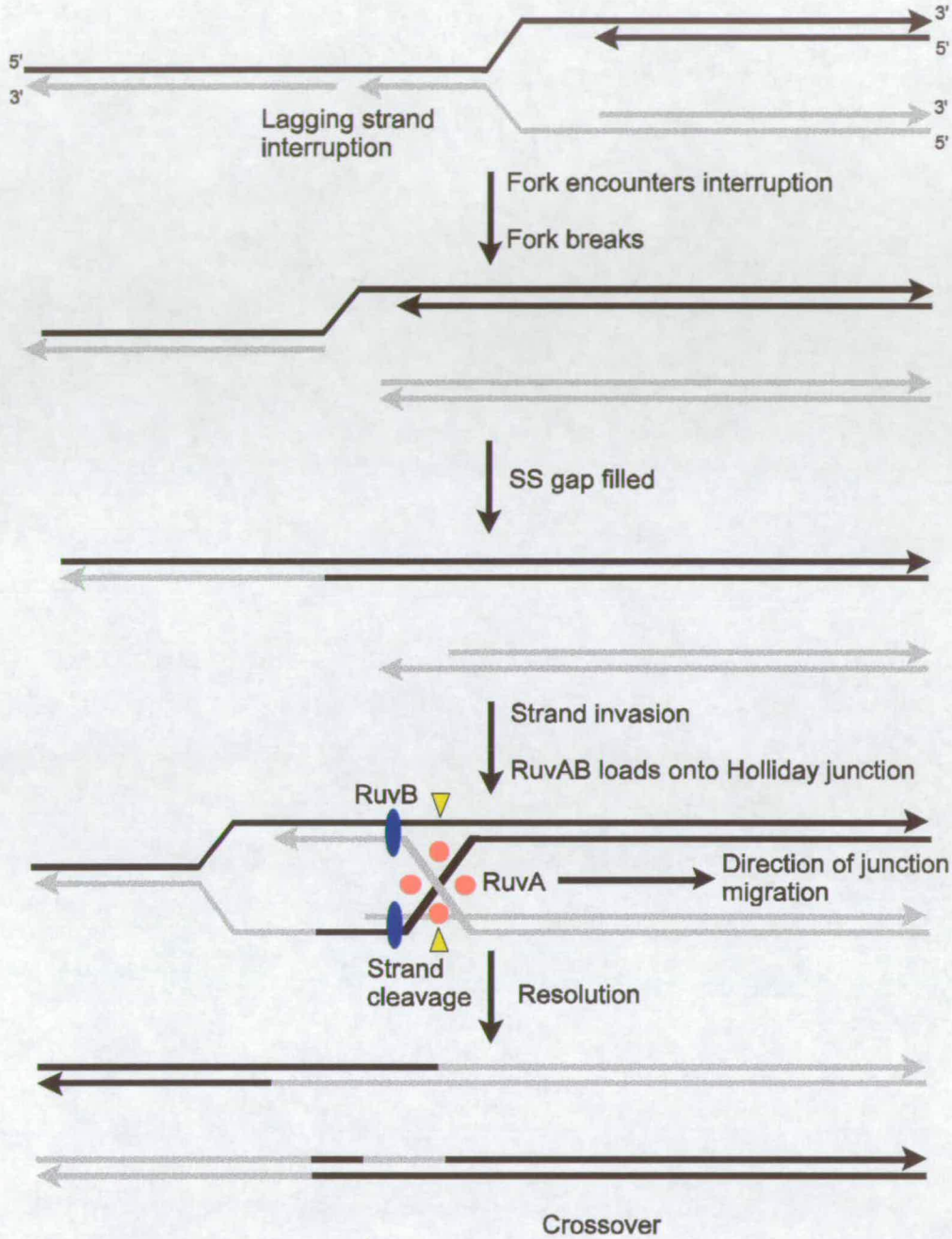

Figure 4.11: Scheme showing the formation of cross-overs when forks are broken on the lagging strand. The replication fork encounters a strand interruption on the lagging strand. This causes fork breakdown. The resulting SS gap is filled and the broken end processed by RecBCD to form a 3' overhang coated with RecA. RecA promotes strand invasion generating a joint molecule possessing a Holliday junction. The RuvAB complex loads onto this junction so as to branch migrate it 'productively' away from the DNA end. Strand cleavage is directed by RuvB to the strand passing 3' through RuvB toward the Holliday junction. A cross-over product is generated.

events fail in a *polA* background then the strain will be inviable.

If recombination in a *polA* mutant is mostly of the leading-strand break variety then we would predict that this recombination would generally avoid crossing over and dimerisation (Figure 5A). Therefore a *polA xerC* mutant should be viable. In fact we were able to construct *polA xerC* double mutants by P1 transduction (data not shown), and similarly a $polA^{ts}$ *xerC* mutant grew at both restrictive (42°) and non restrictive (30°) temperatures (a similar result has been obtained by Bènèdicte Michel (personal communication)). This contrasts with the inviability of even moderately recombination-deficient mutations such as *ruv* in a *polA* background and suggests that recombination occurring at leading-strand breaks mainly avoids crossing over. This in turn supports the prediction made on the basis of the resolution rule in Figure 4.10.

4.6 Discussion

These results demonstrate two things: firstly that the nature of the recombination substrate determines whether the product will be of a cross-over or non cross-over kind and secondly that a given substrate will produce primarily a single kind of product (cross-over or non cross-over). The results are consistent with the existence of a rule for the resolution of Holliday junctions based on the structure of the recombination intermediates and their interaction with the RuvABC complex.

4.6.1 DSBR is frequently associated with crossing-over

It has been shown using three different assays ('ends-in' DSBR at a site of palindrome cleavage, DSBR after UV damage in a *recF uvrB* mutant and 'ends-out' DSBR following transduction) that recombination initiated at breaks is frequently associated with crossing-over.

4.6.2 SSGR is not frequently associated with crossing-over

It has been shown using two assays (SSGR associated with a palindromic sequence and SSGR after UV damage in a *recB uvrB* mutant) that recombination initiated at gaps is frequently not associated with crossing-over. This is in contrast to what is observed with DSBR and argues that the processing of recombination intermediates to cross-over or non cross-over products is not simply the consequence of random cleavage of Holliday junctions in the two possible modes depicted in Figure 4.1. Instead these results argue for a rule governing the resolution of junctions.

These results may explain several confusing observations to be found in the literature. Firstly it is strange that RecF can play such a significant role in the recombinational repair of UV irradiation (Horii and Clark, 1973) but seems to have very little effect on either the frequency or the pattern of inheritance of selectable markers in otherwise wild-type cells during conjugation (Horii and Clark, 1973; Lloyd and Buckman, 1995). In the case of UV irradiation, RecF recombination at internal gaps is measured through cell viability; the assay does not assess crossing-over. This contrasts with conjugational assays where RecF recombination would have to cause crossovers to alter the frequency or the pattern of inheritance of selectable markers. Our work would suggest that RecF-mediated SSGR may happen frequently during conjugation, but as it does not often cause crossing-over of flanking markers it is invisible to most conjugation assays. This idea is supported by a further observation from the literature. When recombination within a gene is assessed by the appearance of functional protein and not by inheritance then the RecF pathway is at least as important as the RecB pathway (Birge and Low, 1974; Lloyd *et al.*, 1987). Consistent with our observations, this can be explained by RecF-mediated SSGR occurring without crossing-over of flanking markers. This would generate a functional allele on the incoming DNA

that could not be inherited via this RecF pathway.

Similarly the observation by the Lloyd group that the production of unresolved recombination intermediates during conjugation in *ruv recG* mutants can be greatly reduced by a further mutation in *recF* (Ryder *et al.*, 1994) supports the conclusions reached in this work. This observation is consistent with frequent recombination using single-strand gaps as substrates in conjugation. The fact that mutations in *recF* do not seem to affect crossing-over during conjugation therefore supports the contention that recombination using single-stranded gaps avoids crossing-over.

Further examination of data from UV-irradiation studies also supports this conclusion. If recombination is associated with crossing-over on a random (1:1) basis then it would be expected that recombinational repair of UV lesions would be associated with the distribution of such lesions from parental to daughter strands on a equal basis. In fact, this is not what is observed. Instead UV lesions remain preferentially in the parental strands, although some distribution to daughter strands does occur (Rupp *et al.*, 1971; Ganesan, 1974). In UV irradiation both DSBR and SSGR are believed to occur, our work would suggest that only DSBR could lead to crossing-over and the distribution of UV lesions to daughter strands. It would be expected that all SSGR events and also some kinds of DSBR events (depending on the exact substrate used) would lead to the avoidance of crossing-over. This is consistent with the bias against crossing-over that is observed in the distribution of UV-induced lesions.

Although our work shows that RecF recombination acting at an internal gap largely avoids crossing-over it is not true that RecF recombination always avoids crossovers. In wild-type cells 50% of the cross-over events that lead to dimerisation are caused by a RecF pathway (Steiner and Kuempel, 1998). It is important at this point to emphasise that the predictions about crossing-over are based on the structure of the substrates involved, not on which proteins carry out the

early stages of recombination, RecBCD or RecFOR for instance. This work has looked at the relationship between defined substrates and crossing-over in situations where the RecF pathway was carrying out one kind of repair (SSGR) while the RecB pathway was carrying out another (DSBR). In a wild type cell under normal conditions it is not entirely clear what the major substrate acted upon by the RecFOR pathway is. However, substantial evidence has pointed to a role for RecFOR-mediated recombination at stalled replication forks (Courcelle *et al.*, 1997; Courcelle *et al.*, 1999; Cox, 1998). As the predictions in this work are based on the nature of the substrate, and in the case of normal growth the RecF substrate is not defined, the observed contribution of RecF-mediated events to crossing-over in normal populations of cells is compatible with the results presented in this study. Interestingly the application of the hypothesised rule for resolution to one substrate suggested to be the RecF target at stalled forks (Cox, 1998) would lead to a cross-over. See Figures 4.10 and 4.11 for an illustration of how different substrates formed at broken replication forks are predicted to influence crossing-over.

4.6.3 The productive positioning of the RuvABC complex can provide a rule that explains the observed biases in crossing-over

The rule for Holliday junction resolution demonstrated in this work is abolished in the absence of the RuvABC complex. This suggests that RuvABC may be involved mechanistically in the control of the direction of junction cleavage and, in turn, this provides a possible molecular mechanism for the resolution rule. The physical mechanisms by which Holliday junctions are cleaved in wild-type cells are now quite well understood. There is evidence in vitro that the RuvC resolvase acts on preferred sequences (Shah *et al*, 1994; Shida *et al.*, 1996) and that RuvC operates on an asymmetric junction structure so that only one of the two possible pairs of strands can be cleaved (Bennett and West, 1995 (2)) (see Chapter

1.6.1). Most significantly it has been shown in vitro that the orientation of the RuvABC complex (defined by which pair of arms RuvB is loaded on) determines which pair of strands are cleaved (van Gool *et al.*, 1999). Cleavage is directed to the strands which pass through the RuvB rings 3' towards the Holliday junction (van Gool *et al.*, 1999). The biochemistry alone does not clarify whether resolution will lead to crossing-over or not since potentially the RuvB rings can be positioned on either of two pairs of arms of a Holliday junction. However, if the structures of the recombination intermediates formed in DSBR and SSGR are as shown in Figures 4.12(B) and 4.13(B) then 'productive' placing of the RuvABC complex can generate the resolution rule observed in this work. The important feature is that the RuvABC complex be placed so as to extend the region of heteroduplex DNA (i.e. move the junction away from the initiating single-stranded region) (Figures 4.12(C) and 4.13(C)). Positioning in the other orientation, even if it does physically occur, would tend to abort recombination and so would not be expected to lead to the production of recombinants. Cleavage of the strands passing 3' through the RuvB rings (van Gool *et al.*, 1999) towards the junction, along with cleavage of the D loop in the case of gap recombination would then occur (Figures 4.12(D) and 4.13(D)). This would lead to a cross-over product in the case of the DSBR and a non cross-over product in the case of SSGR (Figures 4.12(E) and 4.13(E)).

4.6.4 Consequences of the rule governing crossing-over

The combination of the observations presented here concerning the preferred directions of Holliday junction resolution in DSBR and SSGR with the biochemical polarity of strand cleavage by RuvC in the presence of RuvAB (van Gool *et al.*, 1999) places constraints on models of homologous recombination. Together they argue that if RuvAB is positioned so as to give productive branch migration then

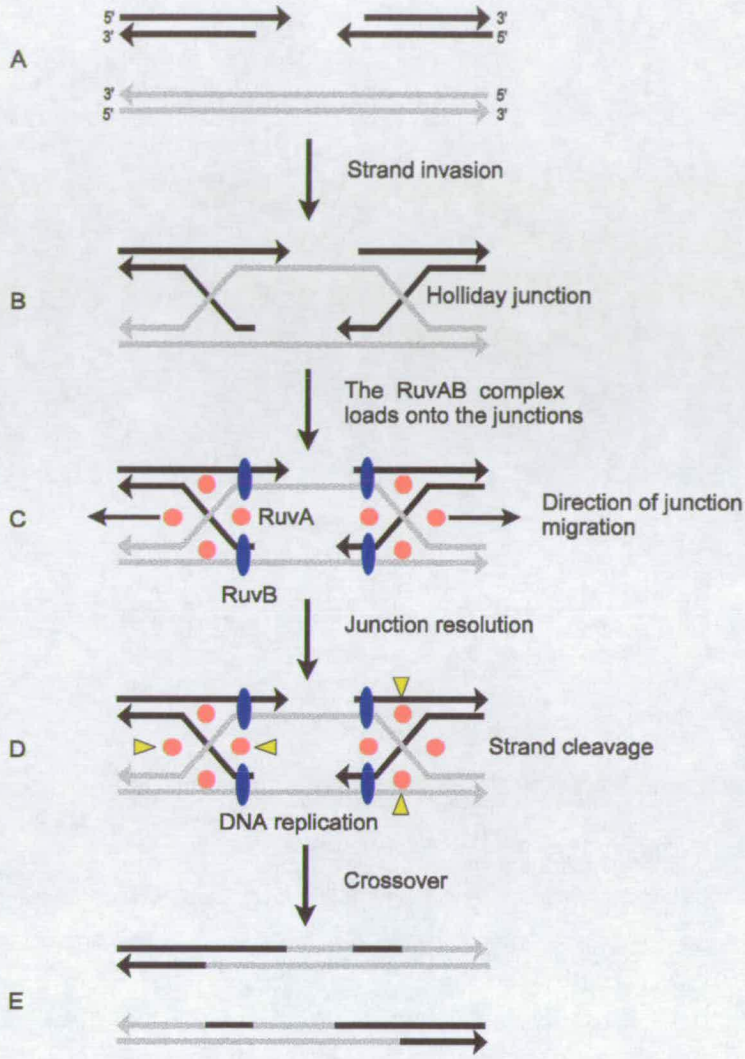

Figure 4.12: Scheme showing the biased generation of cross-over products from an 'ends-in' double strand break substrate.
(A) RecBCD proteins recruit RecA to a 5' recessed 'ends-in' double strand break substrate.
(B) RecA mediates strand invasion generating a joint molecule with two Holliday junctions.
(C) The RuvAB complex assembles on the Holliday junctions with RuvB positioned so as to branch migrate the junctions 'productively' away from the DNA ends.
(D) Strand cleavage by RuvC is directed by RuvB.
(E) DNA replication and ligation of the nicked strands completes the reaction. A cross-over product is formed.

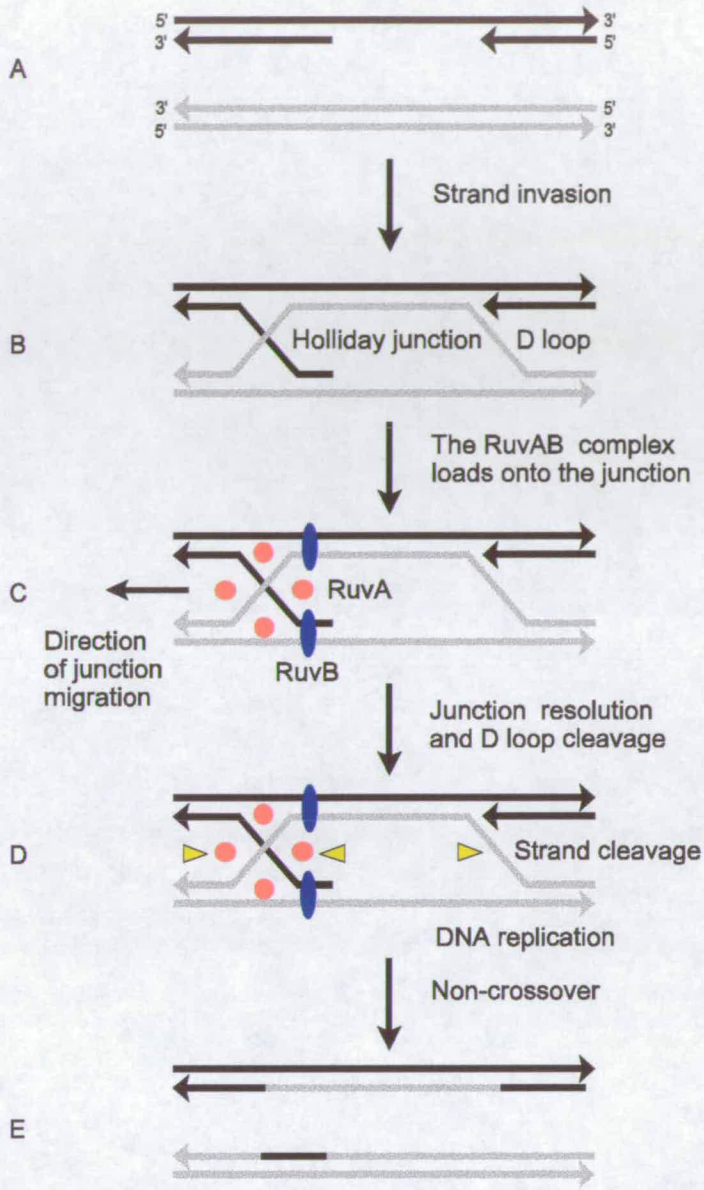

Figure 4.13: Scheme showing the biased generation of non cross-over products from a gapped substrate.
(A)RecFOR proteins recruit RecA to a single strand gap substrate.
(B) RecA mediates strand invasion generating a joint molecule with a Holliday junction (in the same orientation as with a 3' invasive end).
(C) The RuvAB complex assembles on the Holliday junction with RuvB positioned so as to branch migrate the junction 'productively' away from the DNA end.
(D) Strand cleavage by RuvC is directed by RuvB and the D loop is cut to complete resolution.
(E) DNA replication (from the 3' end generated by D loop cleavage) and ligation of the nicked strands completes the reaction. A non cross-over product is formed.

in SSGR, as in DSBR, Holliday junctions are formed at the 5' end of the recombinogenic single-strand (Figures 4.12(C) and 4.13(C)). The existence of rules for the resolution of Holliday junctions in yeast are implied by the patterns of recombination observed (Gilbertson and Stahl, 1996). It is possible that crossing-over in yeast (and in higher eukaryotes) also relates to the appropriate positioning of the branch migration apparatus, though there may be additional levels of control that determine the positioning of that apparatus.

The consequence of a rule for junction resolution for the repair of broken replication forks is that leading-strand breaks are expected to avoid crossing over while lagging strand breaks should lead to crossing over. This prediction is supported by the viability of *polA xerC* double mutants. It may not be surprising that an organism with a circular chromosome has evolved a rule for the resolution of Holliday junctions that minimises the formation of dimers. This study has shown that SSGR tends to avoid crossing-over and would predict that leading-strand breaks should also do so. Leading-strand breaks might be expected to be more common than lagging-strand ones as they would occur both through interaction of the replication fork with nicks and as a consequence of a second round of replication in the presence of unrepaired single-stand gaps on the lagging-strand. Another potential source of breaks may be Holliday junction cleavage following the reversal of replication forks, a reaction shown to be catalysed by RuvABC in *rep recBC* mutants (Seigneur *et al.*, 1998). It is interesting to note that the positioning of the RuvABC complex to promote fork reversal would orient it to generate leading-strand breaks and therefore no crossing-over when repaired.

It is clear that the simple proposal that a Holliday junction is free to be resolved at random in either of the two possible modes to generate cross-over and non cross-over products is no longer tenable for *E.coli* recombination. Instead the branch migration and resolution machinery is placed specifically to generate cross-over or non cross-over outcomes by a rule related to the nature of the recombining

substrates.

Chapter 5

Concluding Remarks

The results presented in this study demonstrate several things. The main conclusion must be that that the nature of the substrate of recombination is critically important in determining the course of the reaction. This is true both for determining the proteins that carry out the reaction as well as the structural nature of the DNA product. The results presented here show that gap and break recombination carried out at the site of a long palindrome use a different complement of proteins. This is in agreement with the generally accepted idea that double-strand breaks and single-strand gaps are the targets of different recombination proteins. However it was also observed in this study that recombination initiated by SbcCD generating a double-strand break at the site of a palindrome uses both the RecB and RecF 'pathways' for carrying out presynapsis. This contrasts with other studies in which a double-strand break is an important substrate for RecBCD, with little role for RecFOR etc.. This suggests that the idea of a simple division of the targets of recombination into 'breaks' and 'gaps' is untenable and that differences in the precise structure of breaks can lead to considerable variation in how different breaks are processed. It may also be true that different 'unbroken' structures (i.e. substrates possessing recombinogenic single-stranded regions) could be resolved by different groups of enzymes.

As well as determining which proteins are used for recombination the precise nature of the DNA also determines the outcome of recombination with respect to

crossing-over. It was demonstrated in this work that only DSBR and not SSGR can be associated with frequent crossing-over. Once again further observations showed that different kinds of double-strand break can be associated with more, or less, recombination depending on their exact structure. These differences can be explained by the resolution bias of the RuvABC complex if it is presumed that RuvABC operates in a 'productive' direction in any given recombination reaction.

Finally this work has shown that the resolution of secondary structure in *E.coli* is an 'enzymatic nexus' where the multi-protein systems carrying out replication, recombination and endonucleolytic cleavage come together.

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Palindromes as Substrates for Multiple Pathways of Recombination in *Escherichia coli*

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ABSTRACT

A 246-bp imperfect palindrome has the potential to form hairpin structures in single-stranded DNA during replication. Genetic evidence suggests that these structures are converted to double-strand breaks by the SbcCD nuclease and that the double-strand breaks are repaired by recombination. We investigated the role of a range of recombination mutations on the viability of cells containing this palindrome. The palindrome was introduced into the *Escherichia coli* chromosome by phage λ lysogenization. This was done in both *wt* and *sbcC* backgrounds. Repair of the SbcCD-induced double-strand breaks requires a large number of proteins, including the components of both the RecB and RecF pathways. Repair does not involve PriA-dependent replication fork restart, which suggests that the double-strand break occurs after the replication fork has passed the palindrome. In the absence of SbcCD, recombination still occurs, probably using a gap substrate. This process is also PriA independent, suggesting that there is no collapse of the replication fork. In the absence of RecA, the RecQ helicase is required for palindrome viability in a *sbcC* mutant, suggesting that a helicase-dependent pathway exists to allow replicative bypass of secondary structures.

LONG DNA palindromes and inverted repeat sequences separated by little intervening sequence confer inviability in *Escherichia coli* (see LEACH 1994 and references therein). This effect is dependent upon active replication of the palindrome-containing DNA (LEACH and LINDSEY 1986; SHURVINTON *et al.* 1987). It has been suggested that this effect is due to the formation of hairpin and hairpin-loop secondary structures on the template DNA when it is transiently single stranded at the replication fork (SHURVINTON *et al.* 1987; LEACH 1994). Analysis of the pattern of deletion occurring at palindromes, a process presumably promoted by secondary structure formation, suggests that such secondary structures may occur preferentially on the lagging strand template (TRINH and SINDEN 1991; ROSCHE *et al.* 1995; PINDER *et al.* 1998).

Palindrome-mediated inviability can be significantly suppressed by mutations in the *sbcC* or *sbcD* genes (CHALKER *et al.* 1988; GIBSON *et al.* 1992). *SbcC* mutants (along with the *sbcB15* mutation) were initially isolated as cosuppressors of recombination deficiency in *recB* strains of *E. coli* (LLOYD and BUCKMAN 1985). SbcC and SbcD together form a nuclease with an ATP-dependent double-strand exonuclease and an ATP-independent single-strand endonuclease activity (CONNELLY and LEACH 1996; CONNELLY *et al.* 1997). It has been postulated that suppression of the *recB* phenotype is due to the persistence of recombinogenic single-stranded DNA ends and that such ends cannot exist in the presence of the wild-type SbcB and SbcCD nucleases (HORII and CLARK 1973; LLOYD and THOMAS 1984).

SbcCD also acts as a hairpin endonuclease, cleaving hairpin loops near the 5′ junction with the duplex stem of the secondary structure (CONNELLY *et al.* 1998). The effect of SbcCD on palindrome viability could then be due to the SbcCD-dependent formation of double-strand breaks at palindromic sequences (LEACH 1994). Genetic evidence suggests that even a 246-bp imperfect palindrome that is short enough to be viable in *wt* cells is frequently cut by SbcCD. Recombination involving RecA and RecBC is then required to repair the resulting double-strand breaks (LEACH *et al.* 1997).

Recombination proteins in *E. coli* have been divided into two major systems: the RecB and RecF pathways. A third system, the RecE pathway, involves proteins encoded by a prophage present in only a subset of *E. coli* strains and is not discussed here. The RecB and RecF pathways involve different sets of proteins acting at the early, presynaptic, stages of recombination.

In the RecB pathway the RecBCD protein complex acts on blunt or near-blunt DNA ends (TAYLOR and SMITH 1985). The protein possesses a helicase and nuclease activity. It moves along the DNA duplex, unwinding the strands and preferentially degrading the 3′ strand (DIXON and KOWALCZYKOWSKI 1991, 1993). When it comes to an eight-base χ sequence its activity is altered, apparently through χ interaction with the RecD subunit (DIXON *et al.* 1994 and references therein). RecBCD continues to unwind the duplex but its nuclease activity becomes switched to the 5′ strand

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(DIXON and KOWALCZYKOWSKI 1991, 1993). This produces a 3′ single-stranded overhang on which RecA can polymerize and that can then initiate recombination. In some kinds of RecB recombination (*e.g.*, UV repair) the RecN protein can also be important (PICKSLEY *et al.* 1985; WANG and SMITH 1988).

The RecF pathway was identified as an alternative recombination system restoring high levels of recombination in *recB* strains (KOWALCZYKOWSKI *et al.* 1994 and references therein). In *recB* mutants recombination is restored by mutations in the *sbcB* and *sbcC* (or *sbcD*) genes. This recombination is dependent on the *recF*, *recO*, *recR*, *recJ*, *recN*, and *recQ* genes (KOWALCZYKOWSKI *et al.* 1994 and references therein). It is proposed that the RecJ 5′ → 3′ single-strand exonuclease and the RecQ helicase convert DNA ends into long 3′ overhangs of single-stranded DNA (LOVETT and KOLODNER 1989; UMEZU *et al.* 1990; KOWALCZYKOWSKI *et al.* 1994) onto which RecA can polymerize. RecFOR may aid RecA in loading, in a localized fashion, onto this single-stranded DNA (UMEZU and KOLODNER 1994; WEBB *et al.* 1997). It appears that, in the absence of functional RecBCD, the RecF proteins can substitute for its functions, but only when the SbcB and SbcCD nucleases are inactive. The RecF pathway may more normally be directed at single-strand gaps (SMITH 1988; CLARK 1991), where RecBCD cannot enter to initiate recombination; instead, RecFOR is able to load RecA onto the single-stranded gap to initiate recombination. An example of this kind of process is RecF-dependent UV repair. In this type of RecF-recombination RecN is unimportant (WANG and SMITH 1988).

The RecF and RecB pathways appear to act on different substrates and involve different presynaptic proteins. However, both feed into the same RecA-mediated pathway of strand exchange. This leads to the formation of Holliday junctions that are branch migrated by the RecG and RuvAB proteins and resolved by the RuvC nuclease (KOWALCZYKOWSKI *et al.* 1994 and references therein).

In this study the relationship between SbcCD and recombination at palindromic DNA sites was investigated further using a 246-bp imperfect palindrome and a range of recombination mutant backgrounds. In the presence of SbcCD the palindromic sequence was found to stimulate recombination using a large number of proteins, including the components of both the RecF and RecB pathways. In the absence of SbcCD the palindrome stimulates RecF-gap recombination at high frequency. In the absence of RecA, propagation of the palindrome requires the RecQ helicase.

MATERIALS AND METHODS

Bacteriophage λ strains: λDRL246 was constructed by cloning a Zeocin resistance marker in an *Eco*RI-*Bgl*II fragment from pZeoSV2(+) (Invitrogen Corp., San Diego) into the multicloning site of TXF97 (ST. PIERRE and LINN 1996) using the *Bam*HI and *Eco*RI sites. A 246-bp interrupted palindrome consisting of inverted repeats of 111 bp separated by a 24-bp spacer had been cloned previously from SKK43 (KULKARNI 1990) into pUC18 and was cloned into the multicloning site of λDRL246 as an *Eco*RI fragment to form λDRL282 (this laboratory). Two internal mismatches were introduced into the palindrome sequence during this process.

λDRL154 (*pal571*, Δ*spi6*, cI857, χ^+) contains a 571-bp palindromic sequence (this laboratory). λDRL 152 is an isogenic phage lacking the palindrome sequence.

Lysogenization: Overnight cultures of bacterial strains to be lysogenized were diluted 10-fold in L broth containing 2% maltose and 5 mM Mg_2SO_4 and grown to a cell density of 4×10^8 cells ml^{-1} ($A_{650} = 0.9$). Cultures were diluted with an equal volume of 10 mM Tris, 10 mM Mg_2SO_4 pH 8 buffer (TM buffer) to give a final cell density of 2×10^8 pfu ml^{-1}. Bacteriophage lysates were diluted to 2×10^9 pfu ml^{-1}. An aliquot (0.15 ml) of phage was added to 0.15 ml of bacterial cells and allowed to adsorb for 60 min at 30°. Infected cells were diluted in phosphate buffer and appropriate dilutions plated on low-salt (85 mM NaCl) L AGAR plates supplemented with Zeocin (Invitrogen Corp.) at a concentration of 16 μg ml^{-1} or on L AGAR plates. To prevent the appearance of *dnaC* suppressor mutations the *priA* strains DL1133 and DL1134 (Table 1) were grown on minimal liquid medium (Spitzizen Salts, supplemented with 0.2% glucose, 15 μg ml^{-1} threonine, 15 μg ml^{-1} histidine, 15 μg ml^{-1} arginine, 15 μg ml^{-1} leucine). Log phase cultures were then diluted with TM buffer and lysogenized in the same way as the other strains. The recombination efficiency of the *priA* strains was measured by P1 transduction frequency to check that suppressor mutations had not occurred.

RESULTS

The components of both the RecB and RecF pathways are required for palindrome viability in the presence of SbcCD: A 246-bp interrupted palindromic sequence consisting of inverted repeats of 111 bp separated by a 24-bp spacer is known to confer inviability on its host replicon in the presence of the SbcCD nuclease when the products of the *recA*, *recB*, or *recC* genes are absent (LEACH *et al.* 1997). These results were explained using a model in which SbcCD cleaves a hairpin structure formed by the palindromic sequence on the lagging strand during replication (LEACH 1994). Recombination involving RecA and RecBCD is then required to repair the resulting double-strand break.

Two isogenic phage, one containing a 246-bp interrupted palindrome and the other lacking this sequence, were used to identify these recombinational requirements of the 246-bp palindrome. The palindrome-containing phage lysogenized the *wt* (wild type) strain at approximately equal frequency to the palindrome-free control phage. However, the lysogenization frequency of the palindrome phage was several orders of magnitude lower than that of the control in *recA*, *recB*, or *recC* strains (LEACH *et al.* 1997). This reflected the lysogen inviability that was conferred by the palindrome in these backgrounds.

In this study a different strain of phage λ carrying the 246-bp palindrome was used to carry out similar lysogenization frequency studies (λDRL282). An isogenic palindrome-free phage was also used (λDRL246).

TABLE 1

***E. coli* strains used in the study**

Strain	Relevant genotype	Source, reference, or construction
AB1157		
AB1157 derivatives		
N2362	*recB21*	LLOYD and BUCKMAN (1985)
N2365	*sbcC7623 recB21*	LLOYD and BUCKMAN (1985)
N2679	*sbcC201*	NAOM *et al.* (1989)
N2691	*recA269*::Tn*10*	LLOYD and BUCKMAN (1985)
N2693	*sbcC201 recA269*::Tn*10*	LLOYD and BUCKMAN (1985)
JC13885	*recB21 recC22 recF*::Tn*3*	Alvin J. Clark
IN347	*recJ284*::Tn*10*	Robert G. Lloyd
N2057	*ruvA60*::Tn*10*	SHURVINTON *et al.* (1984)
N3793	Δ*recG263::kan*	AL-DEIB *et al.* (1996)
SP256	*recN262 tyrA16*::Tn*10*	PICKSLEY *et al.* (1984)
N2445	*recO1504*::Tn*5*	LLOYD *et al.* (1987)
N2754	*recR252*::Tn*10kan*	Robert G. Lloyd
N3343	*recQ1803*::Tn*3*	LLOYD and BUCKMAN (1991)
DB1318	*recA::cat*	WERTMAN *et al.* (1986)
CS85	*ruvC53 eda51*::Tn*10*	SHURVINTON *et al.* (1984)
AG109	*priA2::kan*	Robert G. Lloyd
DL1092	*recF332*::Tn*3*	P1.JC13885 × AB1157 → Ap^r
DL1093	*sbcC201 recF332*::Tn*3*	P1.JC13885 × N2679 → Ap^r
DL1096	*recJ284*::Tn*10*	P1.IN347 × AB1157 → Tc^r
DL1097	*sbcC201 recJ284*::Tn*10*	P1.IN347 × N2679 → Tc^r
DL1100	*ruvA60*::Tn*10*	P1.N2057 × AB1157 → Tc^r
DL1101	*sbcC201 ruvA60*::Tn*10*	P1.N2057 × N2679 → Tc^r
DL1104	Δ*recG263::kan*	P1.N3793 × AB1157 → Km^r
DL1105	*sbcC201* Δ*recG::kan*	P1.N3793 × N2679 → Km^r
DL1106	*recN262 tyrA16*::Tn*10*	P1.SP256 × AB1157 → Tc^r
DL1107	*sbcC201 recN262 tyrA16*::Tn*10*	P1.SP256 × N2679 → Tc^r
DL1108	*recO1504*::Tn*5*	P1.N2445 × AB1157 → Km^r
DL1109	*sbcC201 recO1504*::Tn*5*	P1.N2445 × N2679 → Km^r
DL1110	*recR252*::Tn*10kan*	P1.N2754 × AB1157 → Km^r
DL1111	*sbcC201 recR252*::Tn*10kan*	P1.N2754 × N2679 → Km^r
DL1112	*recQ1803*::Tn*3*	P1.N3343 × AB1157 → Ap^r
DL1113	*sbcC201 recQ1803*::Tn*3*	P1.N3343 × N2679 → Ap^r
DL1121	*ruvC53 eda51*::Tn*10*	P1.C585 × AB1157 → Tc^r
DL1122	*sbcC201 ruvC53 eda51*::Tn*10*	P1.C585 × N2679 → Tc^r
DL1133	*priA2::kan*	P1.AG109 × AB1157 → Km^r
DL1134	*sbcC201 priA2::kan*	P1.AG109 × N2679 → Km^r
DL1140	*ruvA60*::Tn*10 recA::cat*	P1.DB1318 × DL1101 → Cm^r
DL1143	*ruvA60*::Tn*10 recF332*::Tn*3*	P1.JC13885 × DL1101 → Ap^r
DL1144	*ruvA60*::Tn*10*Δ*recG::cat*	P1.N3793 × DL1101 → Km^r
DL1146	*ruvA60*::Tn*10 recO1504*::Tn*5*	P1.N2445 × DL1101 → Km^r
DL1147	*ruvA60*::Tn*10 recR252*::Tn*10kan*	P1.N2754 × DL1101 → Km^r
DL1152	*sbcC201 recA::cat recQ1803*::Tn*3*	P1.DB1318 × DL1113 → Cm^r

These phage encode resistance to the antibiotic Zeocin, allowing selection of lysogens in a wide variety of strains, including those resistant to the antibiotics tetracycline, ampicillin, kanamycin, and chloramphenicol.

Initially, the previous lysogenization results for the *wt*, *recA*, and *recB* backgrounds were replicated in this study (Figure 1). As previously observed (LEACH *et al.* 1997), there was a reduction in the lysogenization frequency of the palindrome phage (λDRL282) compared to the palindrome-free phage (λDRL246). This reflected the requirement for recombination involving the RecA and RecBCD proteins for the viability of the cell with the palindrome. This analysis was then extended to a range of other recombination mutants.

Given that recombination was known to be occurring, it was expected that there would be a requirement for the late-acting recombination proteins RecG, RuvA, and RuvC and indeed the lysogenization frequency of λDRL282 was severely reduced in *recG*, *ruvA*, and *ruvC* mutant backgrounds compared to the *wt* (Figure 1). λDRL282 lysogenization was also impaired in the *recN* background (Figure 1). This is consistent with the role of RecN in other DNA end-based recombination assays (KOWALCZYKOWSKI *et al.* 1994 and references therein).

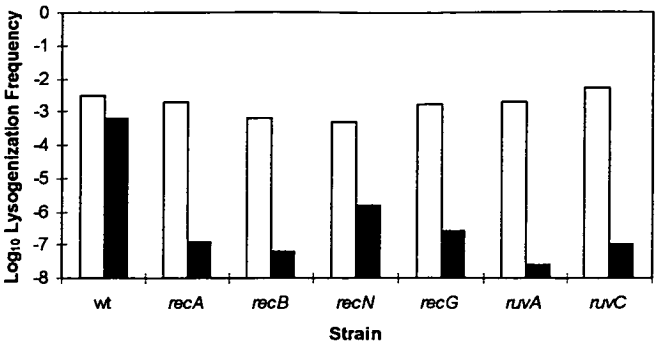

FIGURE 1.—Effects of *recA*, *recB*, *recN*, and Holliday junction resolution mutations (*recG*, *ruvA*, and *ruvC*) on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The strains used were AB1157, N2691, N2362, DL1106, DL1104, DL1100, and DL1121. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments. No DRL282 lysogens were isolated in the *ruvA* or *recB* backgrounds, so that the values given are maximum estimates.

Lysogenization of λDRL246 was unaffected by these mutations (Figure 1).

The effect of mutations in genes of the RecF pathway was a more open question. In fact mutations in all of the RecF pathway genes studied (*recF*, *recO*, *recR*, *recQ*, and *recJ*) caused palindrome-mediated inviability and a specific reduction in the efficiency of λDRL282 lysogenization (Figure 2).

These results indicate that efficient repair of the SbcCD-generated double-strand break requires a wide range of recombination functions, including the components of both the RecB and RecF pathways.

SbcCD-induced double-strand breaks are not associated with PriA-dependent replication fork repair: Double-strand breaks, caused by replication encountering

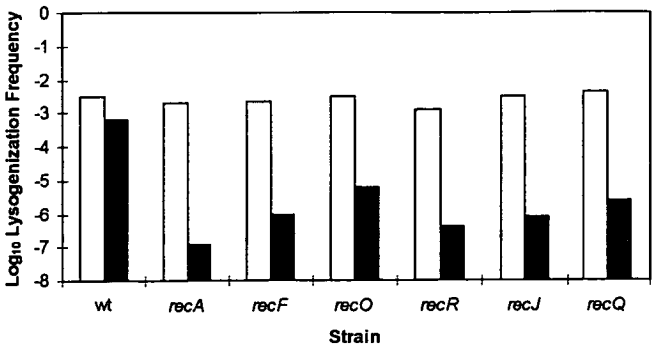

FIGURE 2.—Effects of RecF pathway mutations (*recF*, *recO*, *recR*, *recJ*, and *recQ*) on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The effect of *recA* is shown for comparison. The strains used were AB1157, N2691, DL1092, DL1108, DL1110, DL1096, and DL1112. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments.

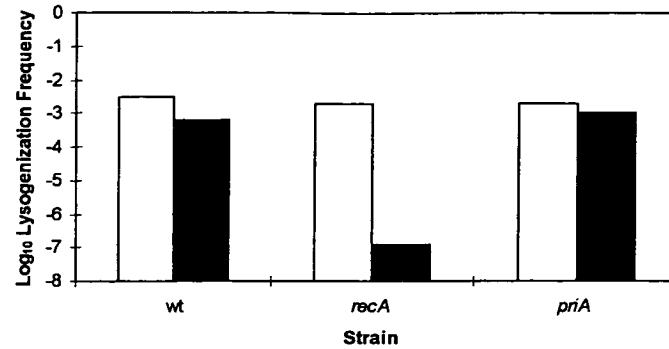

FIGURE 3.—Effects of a *priA* mutation on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The effect of *recA* is shown for comparison. The strains used were AB1157, N2691, and DL1133. The results are the geometric means of at least two independent experiments.

a nick or lesion, are believed to lead to replication fork collapse and probable disassembly of the replication protein complex (Cox 1998 and references therein). It is believed that for fork progression to resume strand invasion sets up a D loop, which the PriA protein then binds to, initiating the reassembly of a primosome and the reestablishment of lagging strand synthesis (McGLYNN *et al.* 1997). To assess whether SbcCD cleavage at the 246-bp imperfect palindrome leads to replication fork collapse, the ability of the palindrome and palindrome-free control phage to lysogenize a *priA* mutant was tested. Both the palindrome and control phage were able to lysogenize the mutant at a high frequency (Figure 3). This indicates either that replication fork collapse is not occurring, despite genetic evidence suggesting the formation of a double-strand break during replication, or that fork collapse is being repaired through a PriA-independent mechanism.

In the absence of double-strand breaks the palindrome stimulates recombination via the RecF pathway: The lysogenization assay was repeated using the same range of recombination mutants as above, but with each carrying an additional mutation in *sbcC*. Previous analysis of *recA*, *recB*, and *recC* mutants in an *sbcC* background had demonstrated lysogenization of the palindrome phage at an equally high frequency to that of the palindrome-free control (LEACH *et al.* 1997). This suggested that the formation of double-strand breaks at the site of palindromes requires SbcCD. Without SbcCD there are no double-strand breaks and therefore no apparent need for functional recombinational repair.

The results of this study generally support these findings. λDRL282 could lysogenize most of the recombination mutants carrying the additional *sbcC* mutation at the same high frequency as λDRL246 (Figure 4). However, there were two exceptions: the palindrome conferred inviability in the *ruvA sbcC* and *ruvC sbcC* double mutants (Figure 4). The RuvA and RuvC proteins are

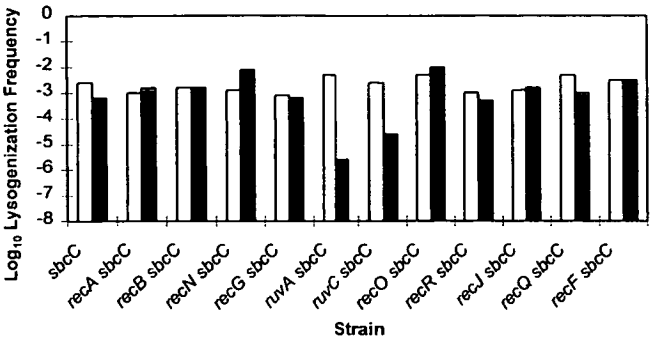

FIGURE 4.—Effects of recombination mutations on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars) in an *sbcC* background. The strains used were N2679, N2693, N2365, DL1107, DL1105, DL1101, DL1122, DL1093, DL1109, DL1111, DL1097, and DL1113. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments.

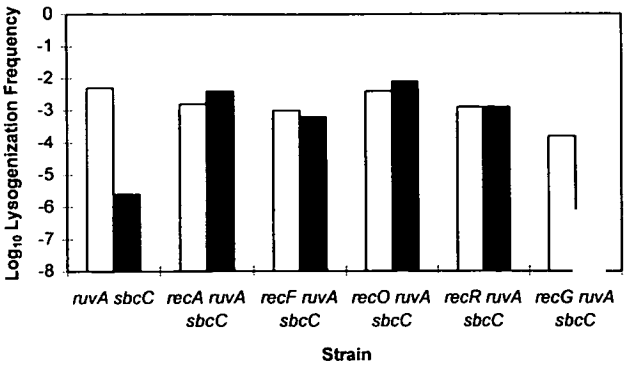

FIGURE 5.—Effects of RecF pathway (*recF*, *recO*, and *recR*), *recA* and *recG* mutations on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The strains used were DL1101, DL1140, DL1143, DL1146, DL1147, and DL1144. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments. No DRL282 lysogens were isolated in the *recG ruvA sbcC* background, so that the value given is a maximum estimate.

late components of recombination, comprising a component of the RuvAB branch migration complex and a Holliday junction resolvase enzyme, respectively (KOWALCZYKOWSKI *et al.* 1994 and references therein). It seems likely that the inability to propagate palindromes in these backgrounds represents the presence of lethal unresolved Holliday junctions, which cause chromosome partitioning problems (ISHIOKA *et al.* 1998). This lethality indicates that recombination is occurring at high frequency even though recombination also appears to be unnecessary for viability [for instance, the palindrome is viable in an *sbcC recA* double mutant (Figure 4)].

The recombination substrate involved is unlikely to be a DNA break as this would represent a lethal event in the absence of recombination. This suggests, instead, that a single-stranded gap is the substrate. This would also allow another mechanism to fill the gap in the absence of recombination. If this interpretation is correct, then it should be possible to repress the lethality of the unresolved Holliday junctions by preventing their formation. To test this, a range of triple mutants was constructed carrying mutations in *ruvA*, *sbcC*, and in a third recombination gene. As expected, mutations in genes encoding proteins involved in the early stages of gap recombination (*recA*, *recF*, *recO*, and *recR*) restored the lysogenization ability of the palindrome phage in a *ruvA* background (Figure 5). A mutation in *recG*, a gene encoding a late-acting junction resolving protein, did not restore viability (Figure 5). This means that RecG is not acting prior to the Ruv proteins, and the lack of any palindrome-mediated viability problem in the *sbcC recG* background (Figure 4) suggests it is not itself acting at a late stage when unresolved intermediates would be lethal. This implies that RecG may have little role to play in gap recombination.

Gap recombination at the site of the palindrome is not associated with PriA-dependent replication fork repair: In an *sbcC* background both the palindrome and control phage were able to lysogenize a *priA* mutant at high frequency (Figure 6), indicating either that palindrome-induced replication fork collapse is not occurring in *sbcC* cells or that it is being repaired in a PriA-independent manner.

In the absence of recombination the viability of cells carrying the uncleaved palindrome requires the presence of the RecQ helicase: The 246-bp imperfect palindrome used in this study appears to stimulate the formation of recombinogenic single-stranded gaps during replication in an *sbcC* mutant background. This indicates that replication has difficulty progressing through the secondary structure formed by this sequence. Pre-

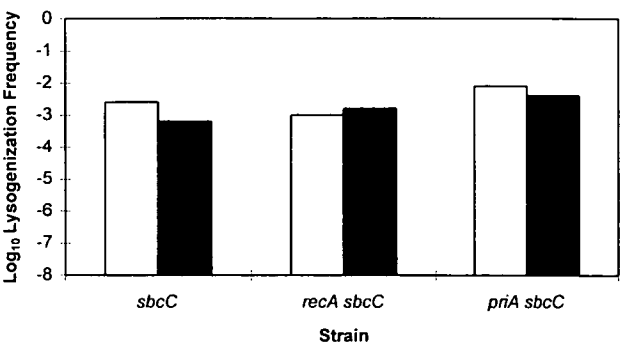

FIGURE 6.—Effect of *priA* mutation on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars) in an *sbcC* background. The effect of *recA* is shown for comparison. The strains used were DL2679, N2693, and DL1134. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments.

sumably recombination using RecFOR fills in this gap. However, this gap can also be filled in the absence of recombination as is demonstrated by the ability of λDRL282 to lysogenize *sbcC recA* cells (Figure 4). One possible method of filling the gap without recombination would be to use a helicase to unwind the secondary structure and so allow replication.

There was an indication that the RecQ helicase might fulfill such a function. It was observed that λDRL154 (containing a 571-bp palindrome) could not form plaques on an *sbcC recQ* background. Normally this phage will form plaques on *sbcC*, but not *wt*. The phage produced plaques on all of the *sbcC* strains used in this study, with the exception of the *recQ sbcC* double mutant (results not shown). A palindrome-free control, λDRL152, was able to form plaques on all backgrounds. It is possible that the difference between the ability of λDRL282 to form lysogens in *sbcC recQ* cells and the inability of λDRL154 to form plaques on the same background represents differences in the lengths of the palindromes or between chromosomal and λ lytic DNA replication. The result of such differences might be to make λDRL154 lytic replication more reliant on a recombination-independent replication bypass mechanism than λDRL282 when present on the *E. coli* chromosome. The plating behavior of λDRL154 suggested that RecQ could have a role in such a recombination-independent system, especially as the effect was not observed for mutations in the other recF pathway genes (*recF*, *recO*, *recR*, and *recJ*) or *recA* in an *sbcC* background.

The role of RecQ in palindrome viability in an *sbcC* background in the absence of recombination was then addressed directly by lysogenizing an *sbcC recA recQ* triple mutant. In this background the palindrome was inviable (Figure 7), indicating that RecQ is required to process palindromes in the absence of recombination in an *sbcC* mutant.

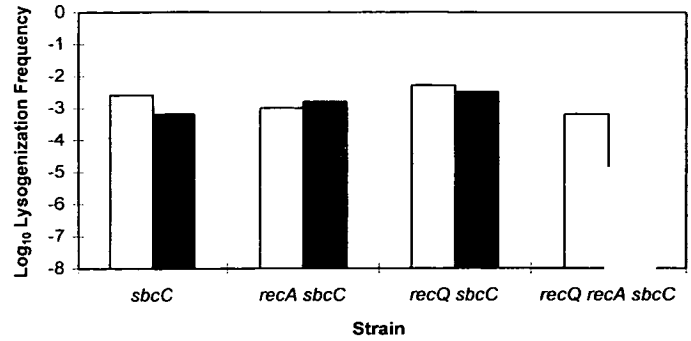

FIGURE 7.—Effect of a *recA recQ* double mutation on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars) in an *sbcC* background. The effects of single mutations in *recA* and *recQ* are shown for comparison. The strains used were N2679, N2693, DL1113, and DL1152. Lysogenization was carried out as described in MATERIALS AND METHODS. The results are the geometric means of at least two independent experiments.

DISCUSSION

The resolution of a 246-bp imperfect palindrome in *E. coli* appears to be a complex affair, where the palindromic substrate can be the target of double-strand break repair, single-strand gap repair, or replicative bypass, depending on the genetic background. In the presence of SbcCD the components of both the RecB and RecF pathways are required for the viability of the palindrome-containing cells. In the absence of SbcCD, recombination still appears to be occurring at a high frequency because *ruvA* mutations that trap late recombination intermediates are lethal. However, this recombination is not necessary for the viability of the palindrome-containing cells, as demonstrated by the viability of *recA* mutants. In the absence of both SbcCD and recombination the RecQ helicase is required for cell viability.

In a wild-type *E. coli* cell the predominant fate of a long palindrome appears to be to stimulate the formation of a double-strand break on the lagging strand. In contrast to what is believed to happen with double-strand breaks derived from fork interactions with a nick or other lesion (Cox 1998 and references therein), this does not seem to lead to the breakdown of the replication fork and its associated protein replication apparatus. This was inferred from the observation that reinitiation of lagging strand synthesis by PriA does not appear to be a frequent event associated with the SbcCD-initiated double-strand break. This might be explained if nick or lesion-induced double-strand breaks occur at or "in front" of the fork whereas in the case of a palindrome the break occurs "behind" the fork. Double-strand breaks initiated by radiation or other DNA damage could occur by the replicative helicase uncovering a nick or by breakage of a stalled fork. The palindrome, however, has to be processed into a double-strand break; there is no physical interruption to the DNA when it is initially uncovered by the replicative helicase. What might be envisaged to occur is unimpeded helicase progession followed by the formation of a hairpin structure on the lagging strand template. This could then impede lagging strand DNA synthesis, but only with the result that the hairpin would be left in a gapped region with replication progressing "past" this lesion in a manner similar to that suggested for UV-damage-induced gaps (RUPP and HOWARD-FLANDERS 1968; KUZMINOV 1995). The hairpin structure left behind in this gap could then be cleaved by the SbcCD nuclease to form a double-strand break, but this break would be physically removed from the replication fork and would avoid causing it to collapse. It is also possible that the 246-bp palindrome is causing fork breakdown but that this is repaired in a PriA-independent manner. This seems unlikely, however, given that *priA* mutants are highly deficient in the repair of other kinds of double-strand breaks (KOGOMA

et al. 1996). In fact, the general effects of *priA* mutations on cell viability suggest that in the absence of PriA cells are very deficient in replication fork repair (KOGOMA *et al.* 1996 and references therein). However, as replication fork repair is a poorly understood phenomenon, the existence of a PriA-independent fork repair mechanism involved in replication past secondary structures such as hairpins cannot be ruled out.

Repair of the double-strand break was expected to involve the products of the RecB pathway that appears to be the dominant end-directed recombination system in wild-type *E. coli*. It was already known that the RecA and RecB proteins are essential for repair of the palindrome-initiated double-strand break (LEACH *et al.* 1997) and this work demonstrates that the RecN protein is also involved. Although the RecN protein is poorly understood it has been implicated in end recombination involving the RecB pathway as well as RecF recombination that is directed to DNA ends (KOWALCZYKOWSKI *et al.* 1994 and references therein). In addition the postsynaptic proteins that are common to both the RecF and RecB pathways, RuvA and RuvC, are essential for successful recombinational repair of palindrome-induced double-strand breaks. This is also true for the postsynaptic protein RecG.

More surprising was the discovery that the proteins of the RecF pathway, RecF, RecO, RecR, RecJ, and RecQ, are also essential for viability in the presence of a chromosomal palindrome and SbcCD. In the absence of the RecB pathway, RecF recombination can substitute for its function at DNA ends, but only in *sbcB15* and *sbcCD* mutant strains. In these cases recombinogenic 3′ DNA ends are being protected by the mutations affecting the two nucleases, and this is needed for efficient recombination. In wild-type cells the RecF pathway appears to act at DNA ends very infrequently [~1 time in 100 (HOWARD-FLANDERS and THERIOT 1966)] with the RecB pathway predominating. In this work, however, both the RecB and RecF pathways are operating frequently together on DNA ends. One possibility is that both sets of proteins are cooperating to process the same substrates; however, while it is possible to envisage the RecFOR proteins helping to load RecA onto a 3′ end produced by the action of the RecBCD nuclease, it is more difficult to imagine how the RecQ and RecJ proteins could cooperate with RecBCD when they appear to substitute for one another as helicases linked to 5′ → 3′ nucleases. Another possibility is that both the RecF and RecB pathways are used by the DNA ends at approximately equal frequency (so that mutants in either pathway have a lethal phenotype). The third possibility is that the two ends produced by the SbcCD cleavage event have different recombinational requirements, with one utilizing the RecF pathway and the other the RecB pathway. RecBCD cannot load onto DNA ends that are not blunt or nearly blunt, so that if one of the DNA ends had a long overhang it could not be used as a substrate by RecBCD. This is similar to the suggestion that UV-induced single-strand gaps could be broken to produce DNA ends and that the RecF pathway could act on these if they possessed long single-stranded overhangs (WANG and SMITH 1985). A model describing this scheme is diagrammed in Figure 8. It would seem necessary for this putative substrate to be protected, both to allow RecF recombination and to prevent processing to a RecB end. One possibility is that the RecF pathway loads RecA onto the single-stranded DNA of the putative hairpin-containing gap region and that when this is converted to a double-strand break the RecA protects the end it now finds itself on. Experiments to address the recombinational requirements of the two DNA ends are underway.

In the absence of the SbcCD nuclease, palindromic sequences still stimulate recombination at high frequency. As recombination is unnecessary for viability in this system, and there is no hairpin nuclease, it seems unlikely that the substrate in this situation is a double-strand break. The alternative would be recombination stimulated by a single-strand gap (Figure 9), and the involvement of the RecF pathway proteins RecF, RecO, and RecR suggests that this is in fact the case. Although the RecF pathway proteins can efficiently stimulate recombination at ends in the absence of RecB in an *sbcB15 sbcC* mutant background, they have an independent role in gap-based recombination in plasmids and recovery from UV radiation (KOWALCZYKOWSKI *et al.* 1994 and references therein), as well as at sites of mismatch repair (FENG and HAYS 1995). This may be the normal substrate for RecF recombination, with the *sbcB15 sbcC* mutant background simply allowing RecF proteins to replace the activity of RecBCD. RecFOR appears to be involved in localizing and loading RecA onto single-stranded regions (UMEZU and KOLODNER 1994; WEBB *et al.* 1997) while together the RecQ helicase and RecJ 5′ → 3′ single-strand exonuclease could produce or extend such regions. The helicase and post-chi nuclease activities of RecBCD (DIXON and KOWALCZYKOWSKI 1991, 1993; DIXON *et al.* 1994) are equivalent to the joint activities of RecJ and RecQ while it appears that RecBCD may aid RecA loading onto the single-stranded DNA it produces after interaction with χ, an activity similar to that of RecFOR (ANDERSON and KOWALCZYKOWSKI 1997). It appears that together RecFOR, RecJ, and RecQ may have abilities similar to RecBCD in the production and extension of single-stranded DNA and in the loading of RecA. The difference may be that RecBCD acts on blunt-ended DNA and cannot act on gaps, while the RecF proteins can act on gaps but cannot act on DNA ends without nuclease mutations to stabilize the 3′ single-stranded ends.

It appears that in both the presence and absence of SbcCD the existence of a long palindromic sequence leads to the formation of a single-strand gap containing the palindrome (probably at one side). The lysogeniza-

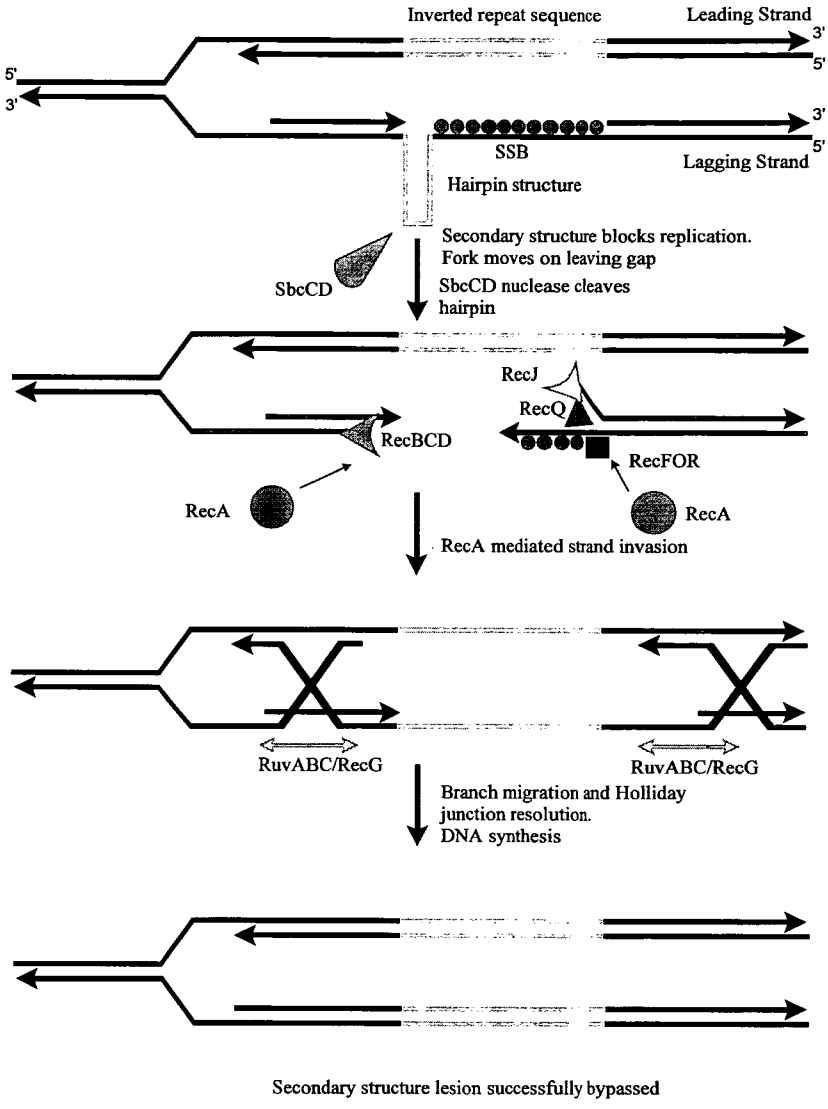

FIGURE 8.—Hypothetical scheme for recombinational repair of SbcCD-induced double-strand breaks utilizing both the RecB and RecF pathways.

tion results with *priA* strains show that this process of fork progression with a gap left behind does not involve fork collapse. In this sense whether or not the single-strand gap is then converted to a double-strand break by SbcCD is irrelevant; the progression of the fork will not be affected in either event.

It is surprising that the gap recombination that appears to occur frequently in the absence of SbcCD is not necessary for palindrome viability. Replication is unable to process the secondary structure and leaves a gap that would lead to a viability problem if left unfilled. Recombination must be able to unwind the secondary structure and allow the gap to be filled by replication using the other sister as a template. The exact mechanism by which this unwinding occurs is unclear, but it could occur during strand exchange or branch migration. The question then arises as to how the single-strand gap is filled in the absence of recombination. It appears that the RecQ helicase is central to this process. The RecQ protein is a 3′ → 5′ DNA helicase that acts on duplex DNA or duplex DNA with single-stranded overhangs (UMEZU *et al.* 1990). It appears to be the only helicase that can substantially replace the helicase activity of RecBC in recombination, in that it is required for recombination in *recBC sbcB15 sbcC* mutant strains (KOWALCZYKOWSKI *et al.* 1994 and references therein). It also has the ability *in vitro* to initiate and disrupt DNA recombination (HARMON and KOWALCZYKOWSKI 1998) and can suppress illegitimate recombination (HANADA *et al.* 1997). Its role in the recombination-independent resolution of secondary structure could be explained by RecQ acting along with a repair polymerase (perhaps DNA polymerase I), with the helicase unwinding the secondary structure and allowing replication to pass through the palindromic sequence. Interestingly, the eukaryotic RecQ homologs BLM and Sgs1p have been shown to unwind G4 tetraplex structures and guanine-guanine paired DNA (SUN *et al.* 1998, 1999). Similarly, in hyperthermophilic archaebacteria it has been suggested that reverse gyrase, which has a helicase as well as a topoisomerase activity, may be involved in eliminating various kinds of abnormal DNA structures (KIKUCHI and ASAI 1984).

E. coli does not possess long perfect palindromic se-

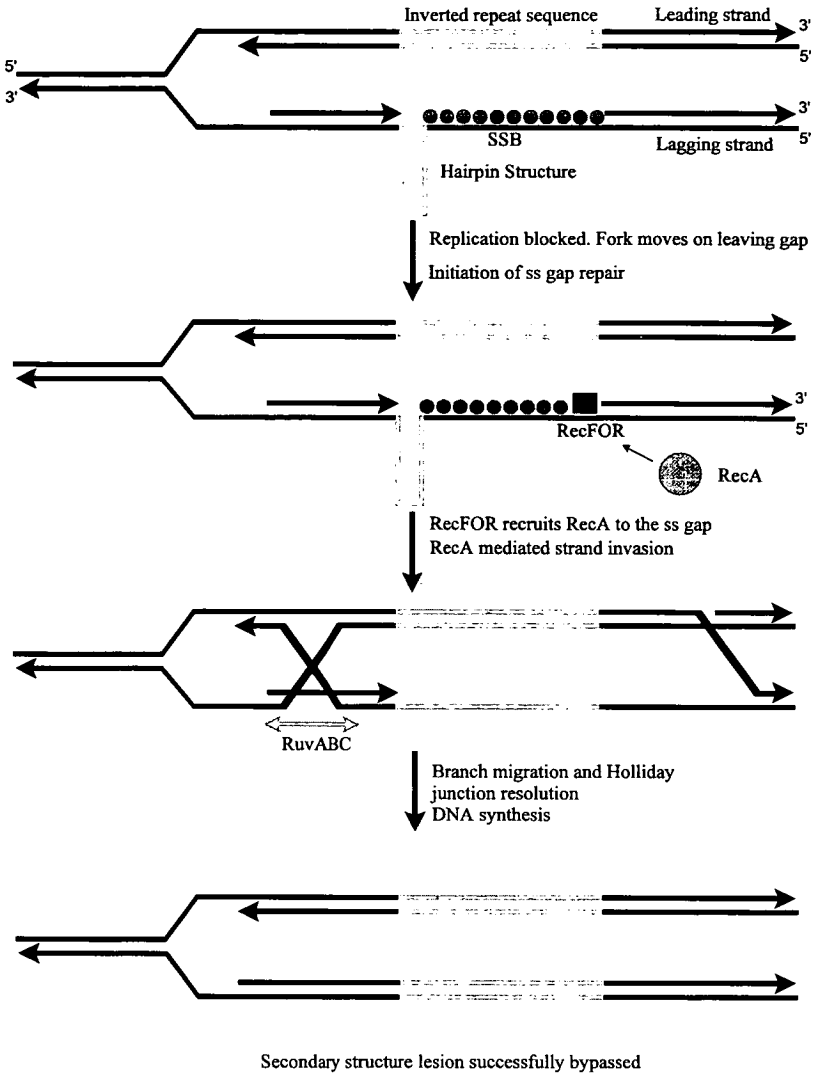

FIGURE 9.—Hypothetical scheme for recombinational repair of single-strand gaps caused by hairpin blockages of lagging strand DNA synthesis.

quences, although short or imperfect palindromes do exist. This is particularly true for the regions of the chromosome that encode rRNA and tRNA sequences. These sequences have the capacity to fold into complicated secondary structures. Even random single-stranded DNA sequences are capable of forming secondary structure of low stability, which may mean that infrequent formation of secondary structure is a normal consequence of DNA being single stranded. The SbcCD nuclease could act to prevent mutagenesis at any of these secondary structures by removing them (LEACH *et al.* 1997). The formation of the secondary structure also appears to block replication. This replication blockage is overcome in the replication associated with recombination. Recombination may therefore simply be a method of allowing replication of these difficult sequences, and the recombination stimulated by SbcCD-induced double-strand breaks may be the most robust in this respect. However, perhaps of more general interest is the ability of long palindrome sequences to promote recombination by multiple pathways at high frequency in a manner controlled by SbcCD. This may make long palindromes of interest to those seeking to elucidate recombinational mechanisms using entirely chromosomal substrates.

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