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$_2$SO$_4$ 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$_2$SO$_4$ 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).
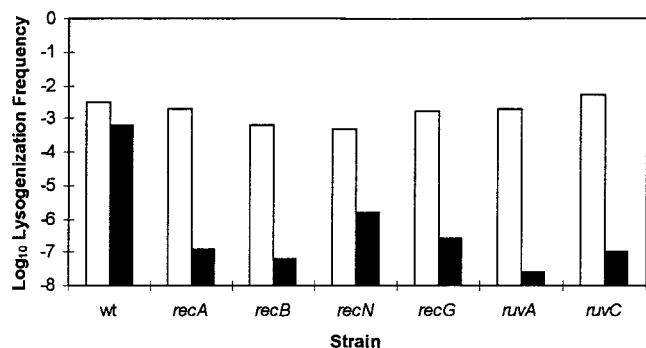
Figure 1.—Effects of *recA*, *recB*, *recN*, and Holliday junction resolution mutations (*recG*, *ruvA*, and *ruvC*) on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The strains used were AB1157, N2691, N2362, DL1106, DL1104, DL1100, 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
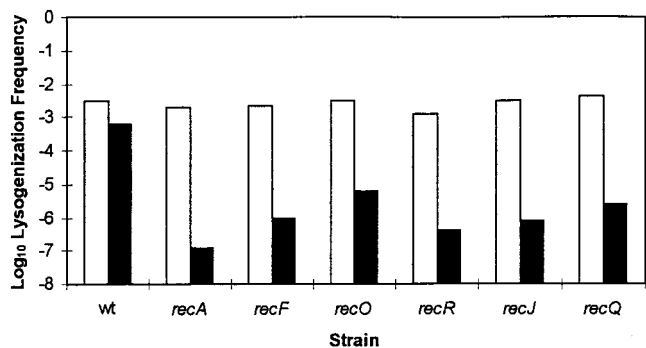

Figure 2.—Effects of RecF pathway mutations (*recF*, *recO*, *recR*, *recJ*, and *recQ*) on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The effect of *recA* is shown for comparison. The strains used were AB1157, N2691, DL1092, DL1108, 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.
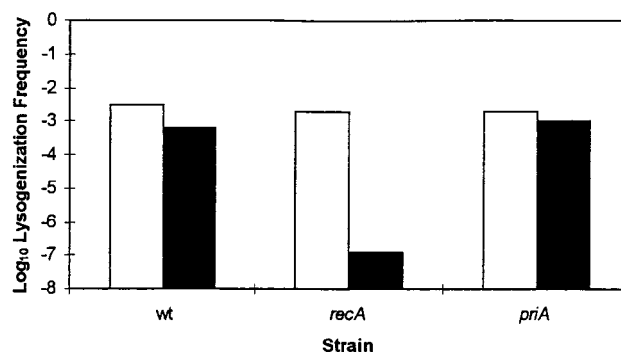

Figure 3.—Effects of a *priA* mutation on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The effect of *recA* is shown for comparison. The strains used were AB1157, N2691, and DL1133. The results are the geometric means of at least two 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
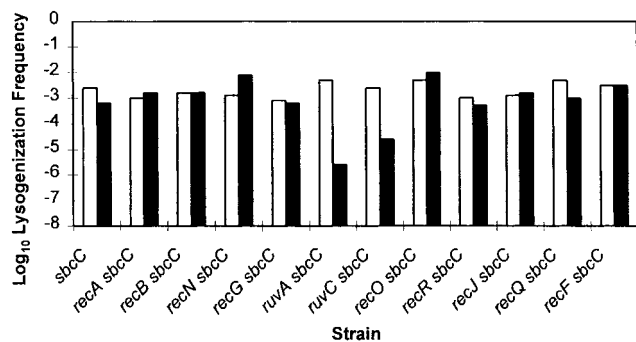
Figure 4.—Effects of recombination mutations on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars) in an *sbcC* background. The strains used were N2679, N2693, N2365, DL1107, DL1105, DL1101, DL1122, DL1093, DL1109, DL1111, DL1097, and DL1113. Lysogenization was carried out as described in materials and methods. The results are the geometric means of at least two independent experiments.
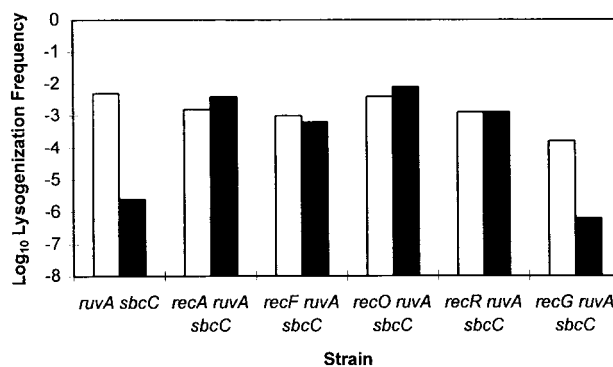

Figure 5.—Effects of RecF pathway (*recF*, *recO*, and *recR*), *recA* and *recG* mutations on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars). The strains used were DL1101, DL1140, DL1143, DL1146, DL1147, and DL1144. Lysogenization was 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-
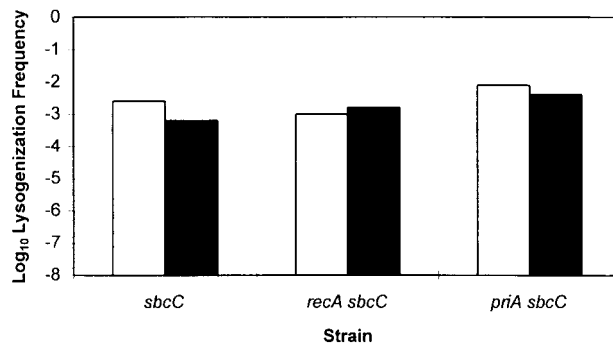

Figure 6.—Effect of *priA* mutation on lysogenization frequency of phages DRL246 (palindrome free, open bars) and DRL282 (246-bp palindrome, solid bars) in an *sbcC* background. The effect of *recA* is shown for comparison. The strains used were DL2679, N2693, and DL1134. Lysogenization was carried out 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.

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.

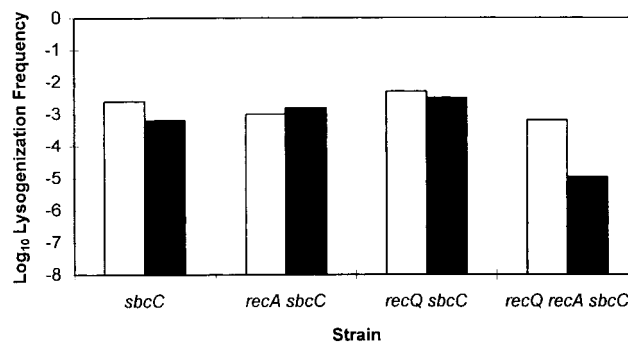In a wild-type *E. coli* cell the predominant fate of a long

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.

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 *shcB15* and *shcCD* 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 *shcB15 shcC* 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 *shcB15 shcC* 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-
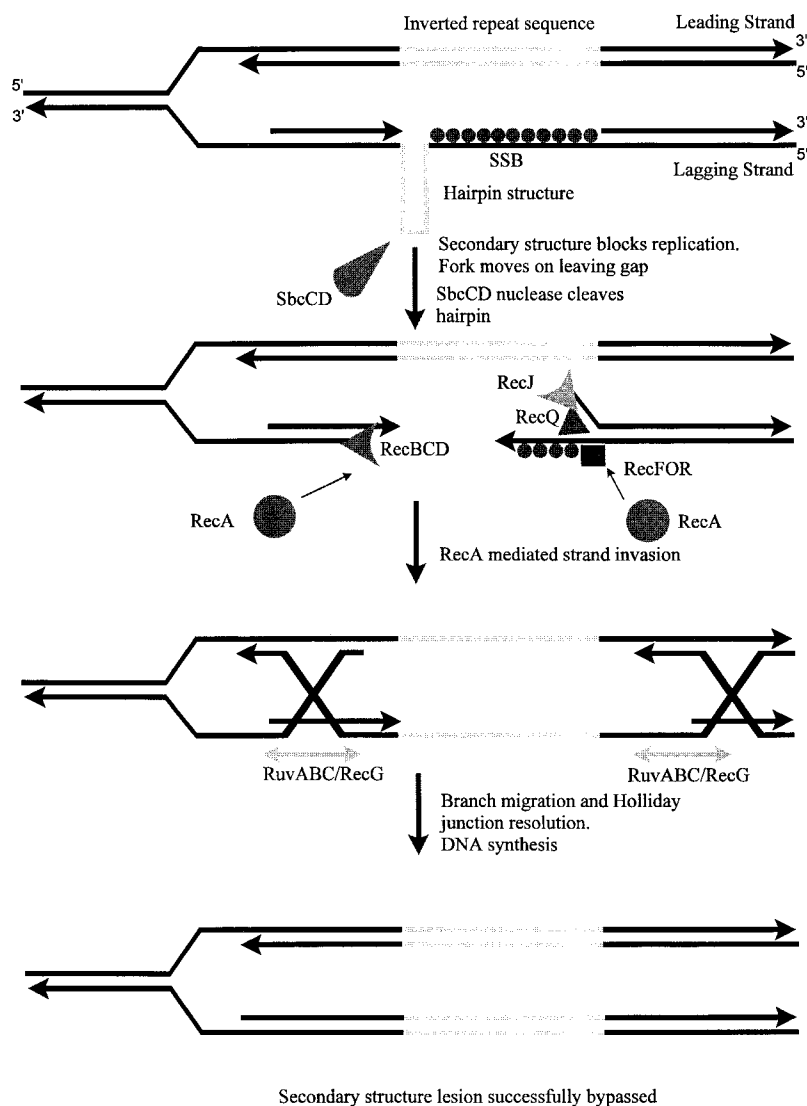
Figure 8.—Hypothetical scheme for recombinational repair of SbcCD-induced double-strand breaks utilizing both the RecB and RecF pathways.

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 $3' \rightarrow 5'$ 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).

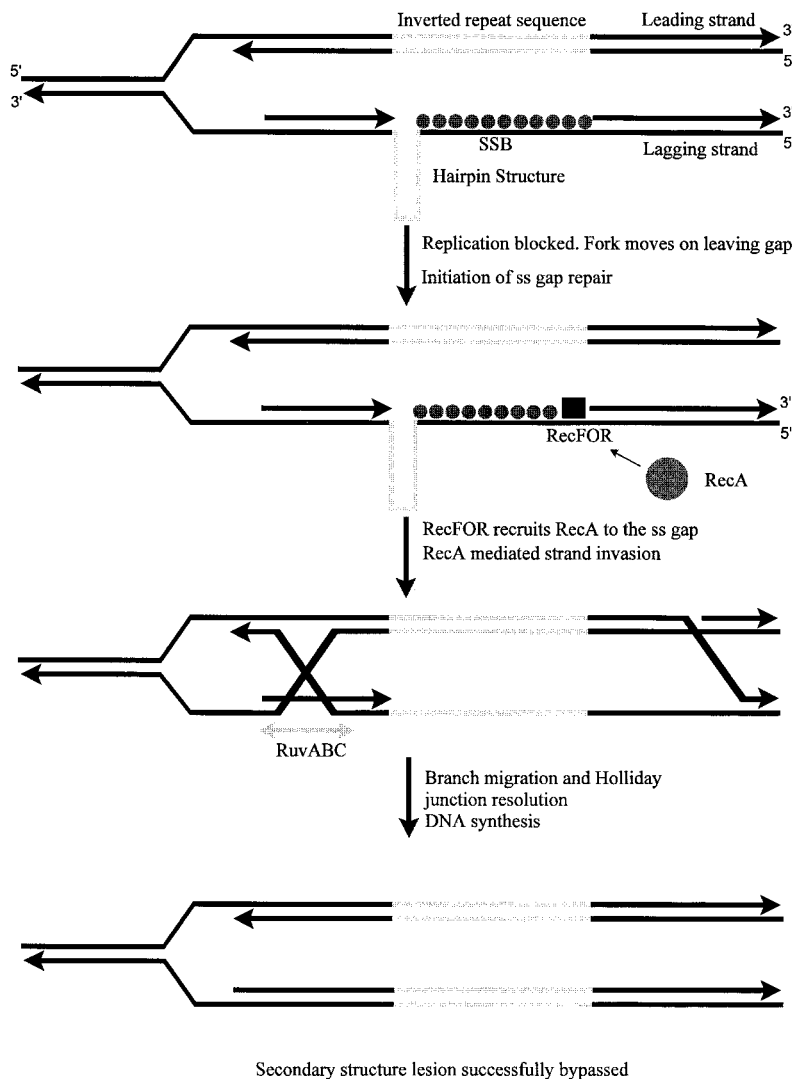*E. coli* does not possess long perfect palindromic se-

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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