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Control of Crossing Over

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Summary

The Holliday junction is a central intermediate in homologous recombination. It consists of a four-way structure that can be resolved by cleavage to give either the crossover or noncrossover products observed. We show here that the formation of these products is controlled by the *E. coli* resolvasome (RuvABC) in such way that double-strand break repair (DSBR) leads to crossing over and single-strand gap repair (SSGR) does not lead to crossing over. We argue that the positioning of the RuvABC complex and its consequent direction of junction-cleavage is not random. In fact, the action of the RuvABC complex avoids crossing over in the most commonly predicted situations where Holliday junctions are encountered in DNA replication and repair. Our observations suggest that the positioning of the resolvasome may provide a general biochemical mechanism by which cells can control crossing over in recombination.

Introduction

In 1964, Robin Holliday suggested a model for homologous genetic recombination that postulated a four-way junction intermediate that has subsequently been 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 "crossover" or "noncrossover" 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 (crossover or noncrossover) (Figure 1A).

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 interhomolog recombination, this falls to 10%–20% (Esposito, 1978; Haber and Hearn, 1985; Kupiec and Petes, 1988). The extent of crossing over in intersister 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 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 crossover 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 *E. coli*, the major Holliday junction resolution system involves the RuvABC proteins. RuvA and RuvB bind to junctions in the form of one or two tetramers of RuvA and two hexamer rings of RuvB. Together these proteins form a complex that carries out branch migration (reviewed in West, 1997). They also facilitate the action of the RuvC resolvase (van Gool et al., 1998; Zerbib et al., 1998) that cleaves two of the four strands that come together in the Holliday junction (Dunderdale et al., 1991; Iwasaki et al., 1991; Bennett et al., 1993). Cleavage occurs so that two strands of like polarity are cut, ensuring resolution of the four-strand junction into two daughter duplexes. Resolution of one pair of strands will lead to a crossover product, while resolution of the other pair will lead to a noncrossover product (Figure 1A). The polarity of cleavage is determined by the binding of the hexameric RuvB rings to the junction, which direct RuvC to cleave the strands exiting the rings in a 3′ direction toward the junction (van Gool et al., 1999). We realized that polar cleavage in relation to the RuvB rings implies that the positioning of the branch migration and resolution complex has the potential to control crossing over. Whether a crossover or noncrossover occurs depends on the identity of the cleaved strands with respect to the way in which they are connected to flanking chromosome arms. As this differs from one kind of substrate to another, some substrates could be associated with crossing over and others not.

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). Blunt DNA ends are processed by the RecBCD nuclease to form recombinogenic 3′ overhangs coated with RecA (Dixon and Kowalczykowski, 1991, 1993; Anderson and Kowalczykowski, 1997). These ends can then invade a duplex of homologous sequence, forming D loops and can either be oriented toward each other ("ends-in" DSBR) or away from each other ("ends-out" DSBR) (Figure 1B). Single-stranded gaps (Figure 1B) are processed by the RecFOR proteins, which are believed to load RecA specifically onto the gaps (Umezu and Kolodner, 1994; Webb et al., 1997). DNA ends with overhanging 3′ single-stranded ends may also be processed by the RecFOR proteins in cells lacking the nucleases RecBCD, SbcB, and SbcCD (Horii and Clark, 1973) and in wild-type cells under the particular circumstance where the 3′ overhang is generated from a gap (Cromie et al., 2000). After RecA has mediated strand invasion, both DSBR and SSGR pathways require joint molecule resolution. If resolution is random, it would be expected that both kinds of reaction could lead to crossover or noncrossover products with about 50% probability. However, if resolution is governed by the nature of the recombining substrates,

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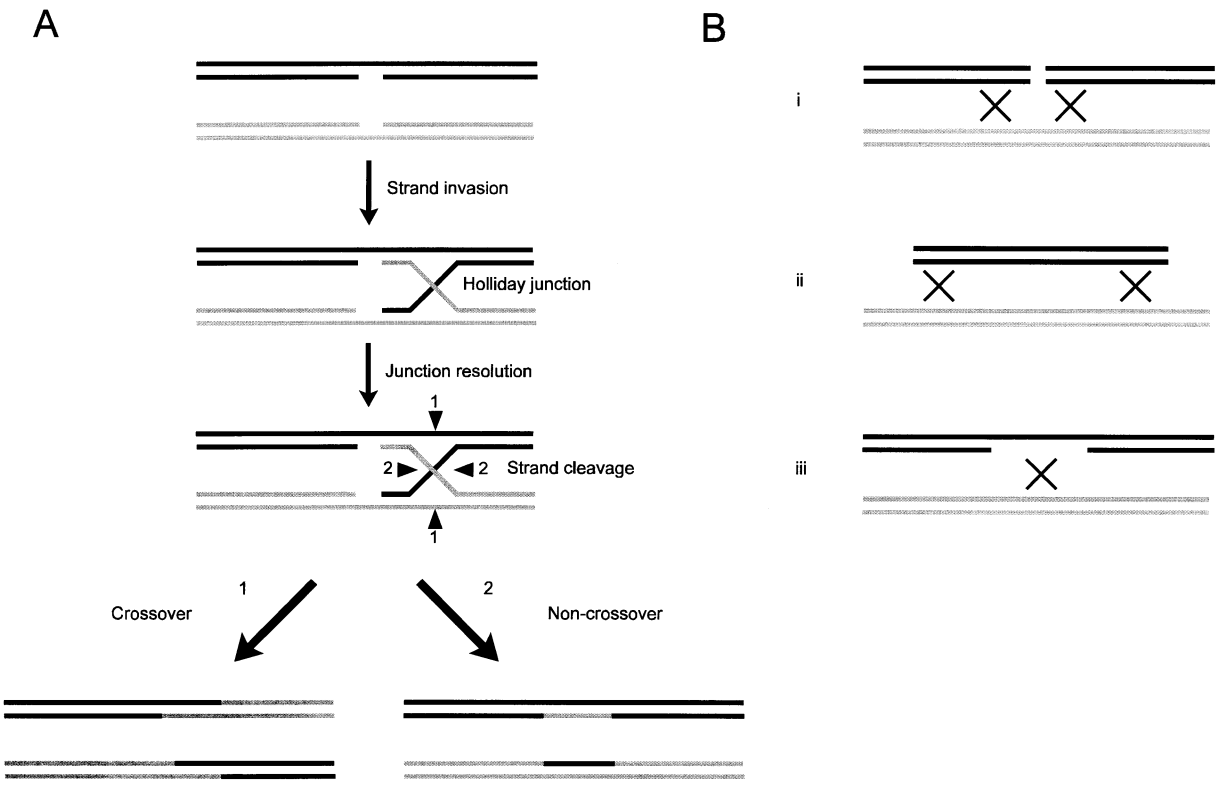

Figure 1. Classical View of the Formation of Crossover and Noncrossover Products and the Structure of the Major Classes of Recombination Substrate

(A) Classical view of the formation of crossover and noncrossover 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. Crossover and noncrossover 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).

(B) Classes of recombination reaction based on the structure of the recombining substrates. (i) "Ends-in" double-strand break repair (DSBR). Recombination occurs on both sides of a double strand DNA break. (ii) "Ends-out" double-strand break repair (DSBR). Recombination occurs at both ends of a linear double stranded DNA molecule. (iii) Single-strand gap recombination (SSGR). Recombination occurs at the site of a single-stranded gap.

then these two different kinds of substrate might show a different propensity to form crossover products.

Recently it has been proposed that certain recombination events in *E. coli* may be linked to extensive replication. The structure formed by a DNA end invading a recipient duplex is the same as that of a replication fork. It is believed that the PriA protein can convert such intermediates into actual replication forks (Kogoma et al., 1996). In the case of ends-out recombination, where a linear molecule recombines with a circular chromosome, this process can allow recombination without the additional strand cleavage needed for break-join recombination. First, both ends of the linear molecule recombine with the chromosome to form replication forks. This is followed by complete replication around the chromosome, leading to the production of two daughter chromosomes, one with a recombinant genotype and the other with the original recipient genotype (Smith, 1991; Kogoma et al., 1996). The observation that mutations in *priA* have a deleterious effect on recombinant frequencies, especially during transduction, illustrates the importance of this kind of recombination (Kogoma et al., 1996).

In contrast to replicative ends-out reactions, ends-in DSBR initiated at a site of cleavage of a long palindromic sequence does not require PriA (Cromie et al., 2000). This reaction involves the SbcCD nuclease that recognizes and cleaves hairpin DNA to generate a double-strand break (Leach et al., 1997). It is possible that ends-in DSBR does not require PriA since it is not necessary to reestablish lagging strand synthesis. This is in contrast to ends-in DSBR in yeast, where there is a requirement for lagging strand synthesis (Holmes and Haber, 1999). Palindrome-stimulated DSBR in the presence of SbcCD has provided a useful system to initiate recombination at a specific chromosomal location. Furthermore, it has been shown that in the absence of SbcCD, a long palindrome initiates SSGR (Cromie et al., 2000). This is understood to be due to the hairpin impeding lagging strand synthesis, resulting in the persistence of a single-strand gap. Palindrome-initiated recombination in the presence and absence of SbcCD provides a useful experimental system to switch between DSBR and SSGR at a specific chromosomal location.

In the context of a circular chromosome, resolution of recombination intermediates to form crossovers

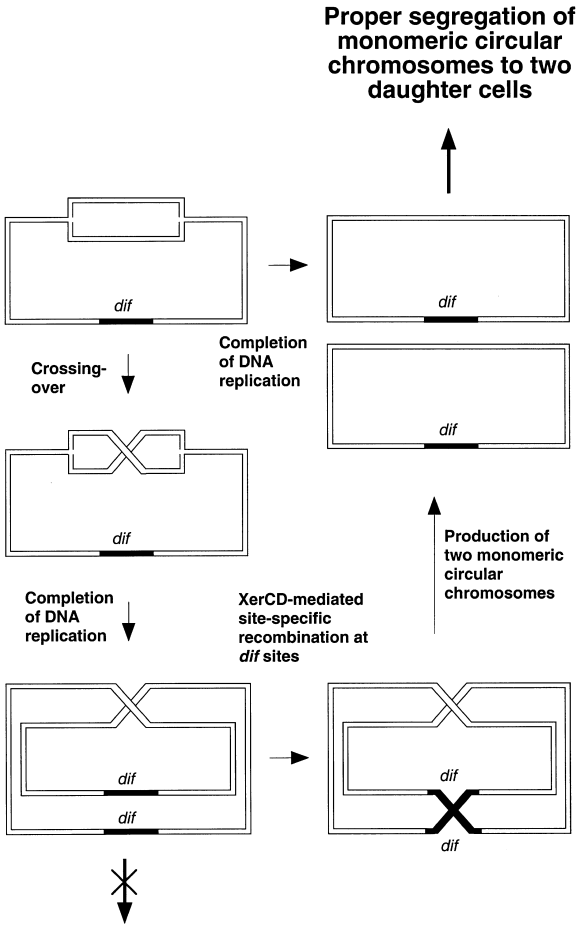

Figure 2. The Problem with Crossing Over in the Circular Chromosome of *E. coli* Is Solved by XerCD-Mediated Site-Specific Recombination

It has long been recognized that "double-sized" circular chromosomes have segregation problems, and Barbara McClintock suggested that a single crossover between the sister "chromatids" in a circular chromosome could generate the problematic "double-sized" (dimeric) molecule (McClintock, 1938). In the circular chromosome of the bacterium *E. coli*, this problem is solved by the operation of a site-specific recombination system (see Sherratt et al., 1995 and references therein). In this system, the XerCD protein acts at the *dif* site to resolve dimeric chromosomes back to monomers. In *xerC* mutants, any chromosomal dimers that remain after crossing over and completion of replication give rise to a segregation problem.

leads to chromosome dimerization (Figure 2), whereas resolution that avoids crossing over does not. In this work, we have investigated whether crossing over is associated with ends-out DSBR, ends-in DSBR, and SSGR by investigating the association of these processes with chromosome dimerization. To do this, we used inactivation of the XerCD site-specific recombination system that is responsible for the resolution of *E. coli* chromosome dimers at the *dif* site (Sherratt et al., 1995). The effects of *xerC* and *dif* mutations were used to assess the frequency of crossover products versus noncrossover products using defined *E. coli* recombinational substrates. Using the palindrome-initiated assay for ends-in DSBR and SSGR, we observed an association between break recombination and crossing over. By contrast, there was no association between gap recombination and crossing over. These observations were generalized using gapped and broken substrates during the replication of UV-irradiated chromosomes. Once again, we observed that recombination of the broken substrates was linked to crossing over, while recombination 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.

Our results demonstrate that crossing over is determined by the nature of the substrate initiating recombination and its interaction with the RuvABC resolvasome. We propose that the positioning of RuvABC controls crossing over, and we extend our analysis to derive the consequences for recombination events restoring replication forks. In conclusion, we argue that directional cleavage of Holliday junctions by resolvasome complexes can provide a biochemical basis for the control of crossing over, and the RuvABC complex has evolved its particular directional cleavage in order to minimize crossing over in a circular genome.

Results

Ends-In DSBR Is Frequently Associated with Crossing Over

The existence of a mechanism controlling crossing over predicts that specific recombinational substrates should give rise either to crossover or noncrossover products, not both. This hypothesis was initially tested using a recombination system that uses either DNA double-strand breaks or single-strand gaps as substrates at a specific chromosomal location.

In vitro, the SbcCD nuclease has been shown to cleave DNA hairpins formed by palindromic sequences (Connelly et al., 1998). There is genetic evidence that when a 246 bp interrupted palindrome is present on the *E. coli* chromosome, it is the target of nucleolytic attack by the SbcCD nuclease (Leach et al., 1997). The resulting double-strand break requires the products of both the RecB and RecF pathways for repair (Cromie et al., 2000). In the absence of SbcCD, the palindromic sequence promotes RecF-mediated gap recombination (Cromie et al., 2000). This system offers a method of analyzing the association of break and gap recombination at a defined chromosomal location with crossing over by using mutations in *xerC* or *dif*. 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* or *dif* mutant strain of *E. coli*. To test this hypothesis, two isogenic phage were used to carry out lysogenization frequency tests. The first contained the 246 bp interrupted palin-

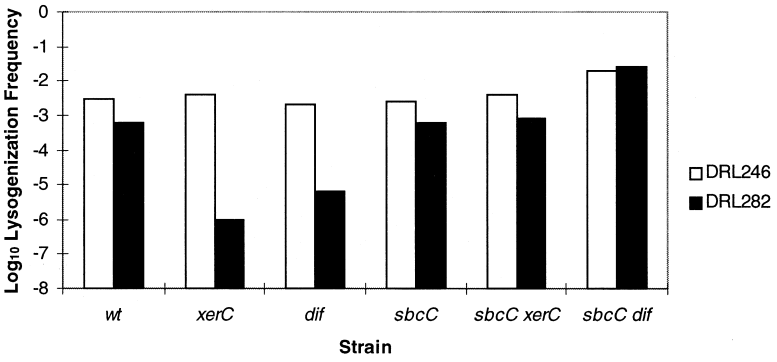

Figure 3. Effects of *xerC* Mutations on Lysogenization Frequency of Phages Undergoing DSBR and SSGR

The results show the lysogenizaton frequencies of phages DRL246 (palindrome-free, open bars) and DRL282 (246 bp palindrome, filled bars) in *xerC* and *dif* strains. The results are compared to *wt* and *sbcC* controls (Cromie et al., 2000). The strains used were AB1157, DL1132, DL1387, N2679, DL1122, and DL1400. Lysogenization was carried out as described in Experimental Procedures. The results are the geometric means of at least two independent experiments.

drome mentioned above (λDRL282), and the second was a palindrome-free control phage (λDRL246). The phage encode resistance to the antibiotic zeocin, allowing the selection of lysogens.

It was previously shown that the palindrome phage could lysogenize the wild-type strain at the same frequency as the control phage (Cromie et al., 2000) (Figure 3). This is because in the presence of the full complement of proteins of the RecB and RecF pathways, DSBR is carried out successfully at the site of the palindrome and there is no viability problem. However, the story was quite different in an *xerC* or *dif* mutant strain. In this case, the palindrome phage lysogenized at a much-reduced frequency compared to the control phage (Figure 3). This indicates that palindrome-induced DSBR frequently causes crossing over. The resulting dimerization leads to a viability problem in the *xerC* or *dif* mutant strain.

SSGR Is Not Frequently Associated with Crossing Over

These experiments were repeated using an *sbcC* mutant. Once again, the palindrome phage could lysogenize at the same frequency as the control phage (Cromie et al., 2000) (Figure 3). In this background, the palindrome phage is successfully undergoing SSGR in the presence of all of the proteins of the RecF pathway (Cromie et al., 2000). The ability of the two phage to lysogenize *sbcC xerC* or *sbcC dif* double mutants was then tested. Both the palindrome and nonpalindrome phage were able to lysogenize these strains equally well (Figure 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 what proportion of DSBR and SSGR events lead to dimerization, it is possible to say that this dimerization occurs frequently at the breaks and infrequently at the gaps.

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 is the case for palindrome-induced SSGR. However, these results refer only to recombination at a particular chromosomal location. To assess whether this difference between 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 postreplicational 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 a pathway that requires RecB, and the latter are repaired by a pathway that requires RecF (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*. In order to limit the number of preexisting dimers in the chromosomes undergoing repair, irradiation was carried out on cultures that had entered stationary phase. As described in previous studies (Ganesan and Smith, 1970; Horii and Clark, 1973; Wang and Smith, 1981, 1983), the addition of either *recF* or *recB* mutations to the *uvrB* strain greatly increased its sensitivity to UV irradiation (compare Figures 4A, 4B, and 4C). The effect of an *xerC* mutation on the *uvrB* strain was less dramatic but still significant (Figure 4A). 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, with no significant differences between the UV sensitivity of the *uvrB recB* and *uvrB recB xerC* strains (Figure 4B). 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 that is predicted to be associated with crossing over. This also turned out to be the case, with significant differences between the UV sensitivities of the *uvrB recB* and *uvrB recB xerC* strains (Figure 4C). Therefore, it can be seen that *xerC* mutations only cause sensitivity to UV in the presence of a functional RecB pathway (i.e.,

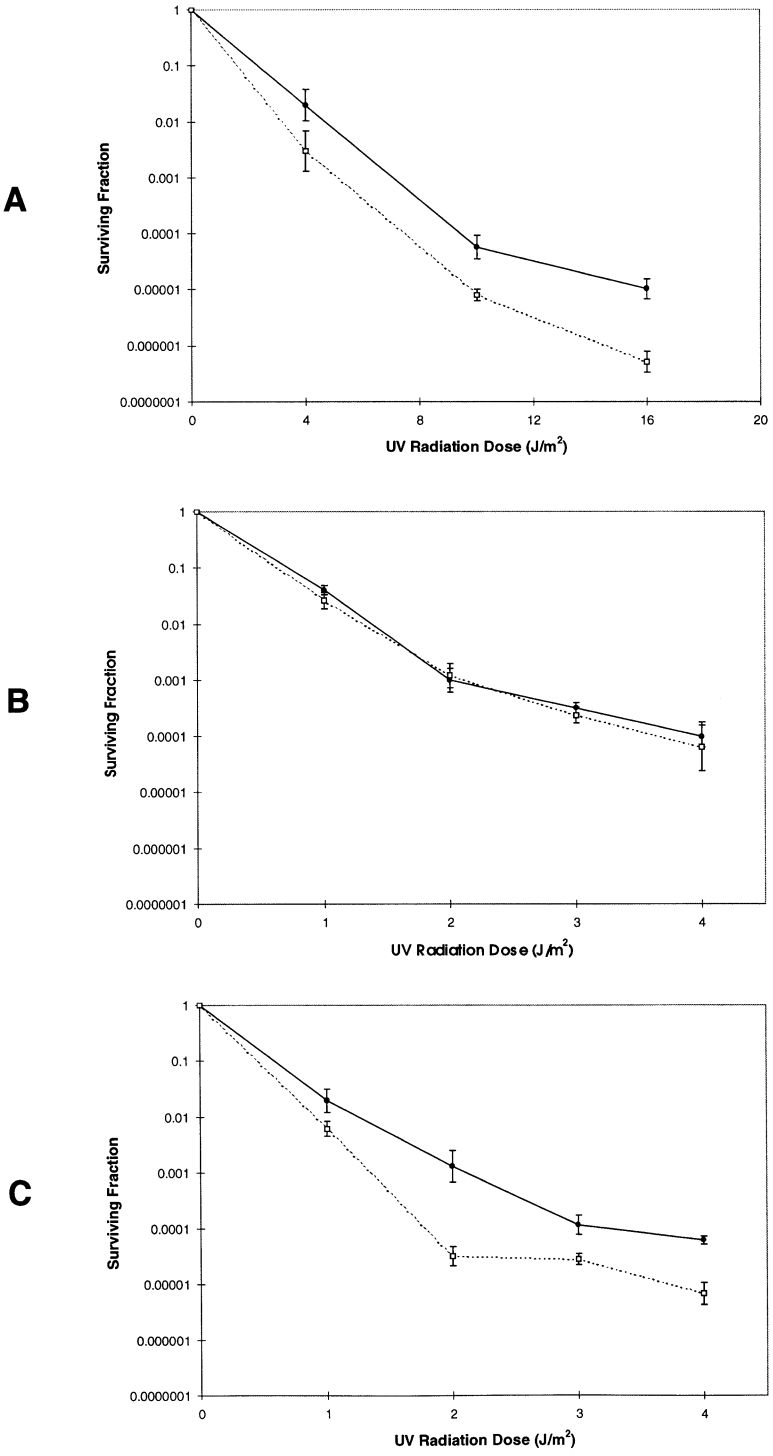

Figure 4. Sensitivity of *xerC* Mutants to UV Irradiation

Overnight, cultures of cells were exposed to UV irradiation as described in Experimental Procedures. Standard errors are shown for each of the data points. The data points are the geometric means of at least five independent measurements.

(A) DL698 (*uvrB*) (filled circle) and DL1155 (*uvrB xerC*) (open square).

(B) DL1159 (*uvrB recB*) (filled circle) and DL1161 (*uvrB recB xerC*) (open square).

(C) DL1156 (*uvrB recF*) (filled circle) and DL1157 (*uvrB recF xerC*) (open square).

in the *uvrB* and *uvrB recF* backgrounds). These results support the general conclusion that DSBR using RecB is frequently associated with crossing over, while SSGR using RecF is much less frequently (if at all) associated with crossing over.

Ends-Out DSBR Is Frequently Associated with Crossing Over

The existence of a mechanism that directs Holliday junction resolution predicts that recombination using a single defined substrate will generate a single kind of product (crossover or noncrossover). 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), it seems likely that recombination proceeds by the setting up of replication forks at the invading P1 ends. If this replication proceeds around the entire *E. coli* chromosome, then it should be possible to identify

Table 1. Relative Frequencies of P1 Transduction

Strain	Relevant Genotype	Relative Frequency[a] with P1.CAG18556	Relative Frequency[a] with P1.PLG292
AB1157	*wt*	1.00	1.00
DL1132	*xerCY17*::Mini-*cat thr1*	0.11	0.12
DL1387	*dif*Δ*6*::*kan*	[b]	0.067
DL1102	Δ *ruvAC65 eda51*::Tn*10*	0.39	0.33
DL1209	Δ *ruvAC65 eda51*::Tn*10* *xerCY17*::Mini-*cat*	0.15	0.15
DL1386	Δ *ruvAC65 eda51*::Tn*10 dif*Δ*6*::*kan*	[b]	0.14

[a] Relative to the frequencies with the *wt* parental strain AB1157. The values given are the means of at least ten measurements.
[b] These experiments were not carried out because the recipient strains were already resistant to kanamycin.

if a resolution bias is operating at the two Holliday junctions formed at each end of the recombining P1 fragment. In the absence of a resolution bias, it would be expected that upon completion of replication, 50% of the products would be monomers and 50% dimers. The operation of a resolution bias 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* or a *dif* mutant ran at approximately 10% of wild-type levels (Table 1). This corresponds to nine crossover events to one noncrossover event and compares to a ratio of 1:1 expected if junction resolution was random. Therefore, we conclude that resolution of the Holliday junctions is biased to crossing over. 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%.

In the Absence of RuvABC, Junction Resolution Is Approximately Random

The previous result suggests that the resolution of Holliday junctions is a nonrandom process and can be explained by the existence of a resolution bias. Resolution bias might be expected to operate through constraints on the activities of the RuvABC protein complex that normally carries out strand cleavage. We therefore investigated the effect of *xerC* or *dif* mutations on P1 transduction in a *ruvABC* deletion mutant. In this background, we observed that *xerC* or *dif* mutations only caused between a 2.2- to 2.6-fold reduction in transductant frequency. This is close to one crossover event for every noncrossover event (Table 1). If recombination continues to be highly linked to replication in these strains, then this means that the bias in crossover resolution has largely been removed. This in turn suggests that the bias for Holliday junction resolution operates through the RuvABC complex.

Discussion

Our results demonstrate three things, first, that the nature of the recombination substrate determines whether the product will be of a crossover or noncrossover kind; second, that a given substrate will produce primarily a single kind of product (crossover or noncrossover); and third, that the bias to crossover or noncrossover depends on the presence of the RuvABC complex. More specifically, all of our results can be explained if the structure of the recombination intermediate determines the productive positioning of the RuvABC resolvasome and this controls crossing over.

DSBR Is Frequently Associated with Crossing Over

We have 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. This argues that the processing of recombination intermediates to crossover or noncrossover products is not simply the consequence of random cleavage of Holliday junctions in the two possible modes depicted in Figure 1A. Instead, our results argue for biased resolution of junctions.

SSGR Is Not Frequently Associated with Crossing Over

We have 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 reinforces the conclusion that the resolution of Holliday junctions is not random.

These results may explain several confusing observations to be found in the literature. First, 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 conjugal 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

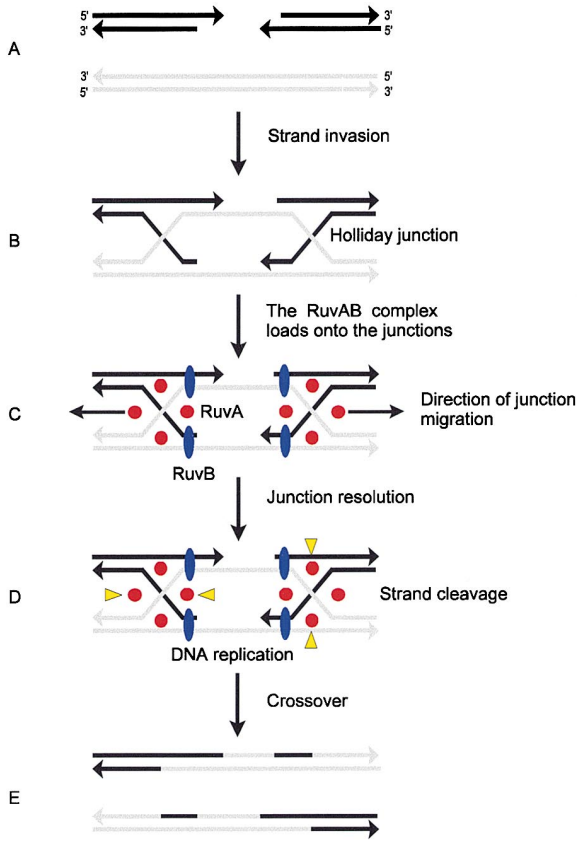

Figure 5. Scheme Showing the Biased Generation of Crossover Products from an Ends-In Double-Strand Break Substrate

(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 crossover product is formed.

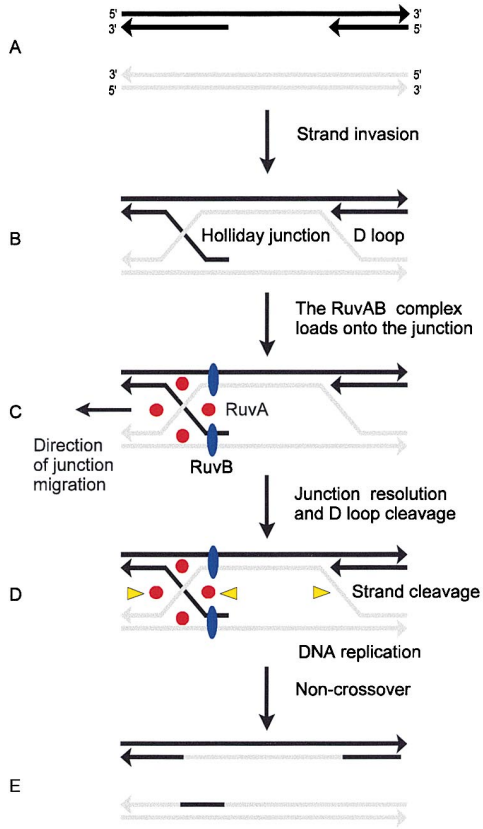

Figure 6. Scheme Showing the Biased Generation of Noncrossover Products from a Gapped Substrate

(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-crossover product is formed.

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.

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 crossover events that lead to dimerization are caused by a pathway that utilizes RecF (Steiner and Kuempel, 1998). It is important at this point to emphasize that our predictions about crossing over are based on the structure of the substrates involved, and not on which proteins carry out the early stages of recombination, RecBCD or RecFOR, for instance. In this work, we have looked at the relationship between defined substrates and crossing over in UV-repair, where RecF is implicated in SSGR and RecB is implicated in DSBR; in palindrome-induced recombination, where RecF is implicated in SSGR and both RecBCD and RecF are implicated in DSBR; and in P1 transduction, where RecBCD is implicated in DSBR. In a wild-type cell under normal conditions, it is not clear what is the major substrate acted upon by RecFOR. However, substantial evidence has pointed to a role for RecFOR-mediated recombination at stalled replication forks (Courcelle at al., 1997, 1999; Cox, 1998). As our predictions 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 our results. Interestingly, the application of our hypothesized bias for resolution to one substrate, suggested to be the RecF target at stalled forks (Cox, 1998), would lead to a crossover. See below

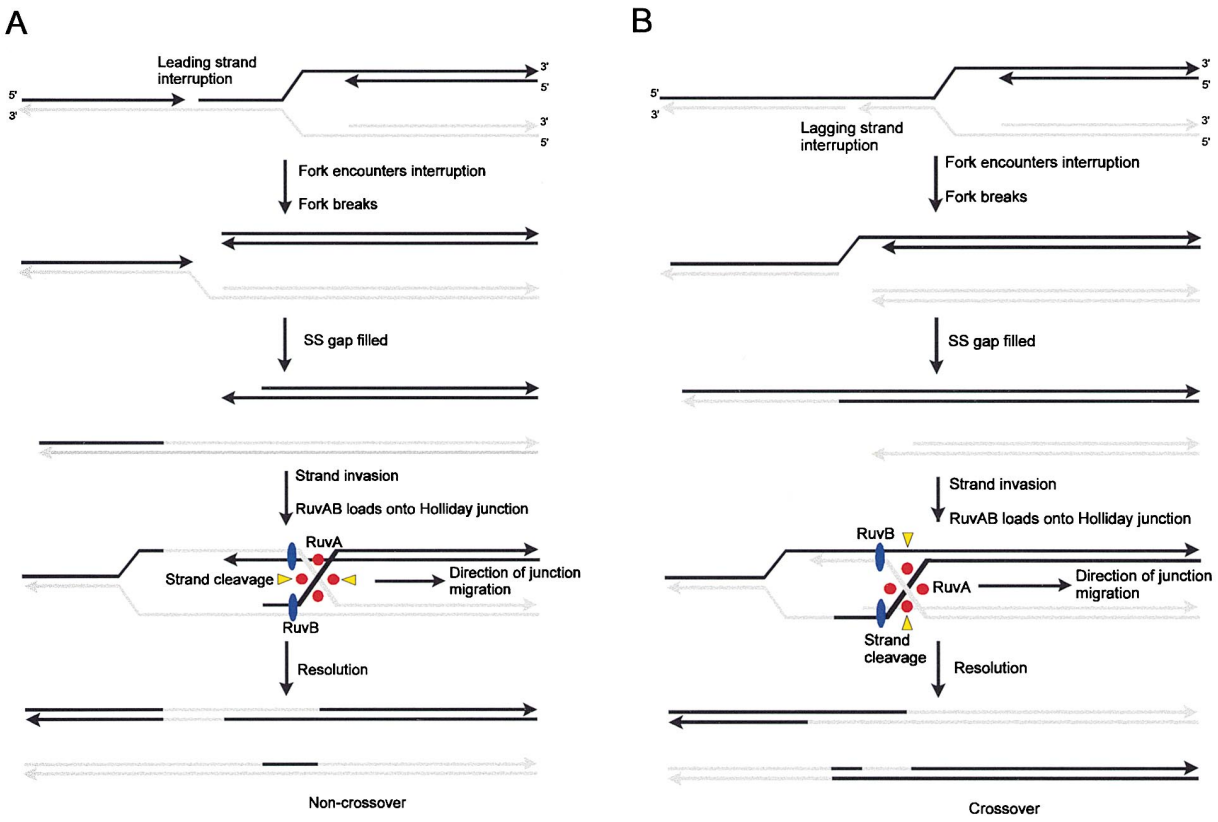

Figure 7. Scheme Showing the Biased Generation of Noncrossover Products from Forks Broken on the Leading Strand and of Crossover Products from Forks Broken on the Lagging Strand

The replication fork encounters a strand interruption on either the leading strand (A) or lagging strand (B). This causes fork breakdown. The resulting SS gap is filled, and the broken end is 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 by RuvC is directed by RuvB, and a replication fork is reassembled. In the case of a leading-strand break, a noncrossover product is generated (A). In the case of a lagging-strand break, a crossover product is generated (B).

for a discussion of how different substrates formed at broken replication forks are predicted to influence crossing over.

The Productive Positioning of RuvABC Can Explain the Observed Biases in Crossing Over

The bias toward crossing over in DSBR 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. 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). 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 that pass through the RuvB rings 3′ toward 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 5B and 6B, then "productive" placing of the RuvABC complex can generate the resolution bias 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 5C and 6C). 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) toward the junction along with cleavage of the D loop in the case of gap recombination would then occur (Figures 5D and 6D). This would lead to a crossover product in the case of DSBR and a noncrossover product in the case of SSGR (Figures 5E and 6E).

Crossing Over and the Repair of Broken Replication Forks

The constraints that this work places on recombination models have interesting implications for the repair of broken replication forks. Such broken forks may arise

Table 2. *E.coli* Strains Used in the Study

Strain	Relevant Genotype	Source, Reference, or Construction
AB1157 derivatives		
AB1157		Bachmann, 1996
N2679	*sbcC201*	Naom et al., 1989
DL1122	*sbcC201 xerCY17*::Mini-*cat*	P1.DS984 × N2679→Cm^r
DL1132	*xerCY17*::Mini-*cat*	P1.DS984 × AB1157→Cm^r
DL1102	Δ *ruvAC65 eda51*::Tn*10*	P1.N4155 × AB1157→Tc^r
DL1209	Δ *ruvAC65 eda51*::Tn*10 xerCY17*::Mini-*cat*	P1.DS984 × DL1102→Tc^r
DL1387	*difΔ6::kan*	P1.GR47 × AB1157→Km^r
DL1400	*sbcC201 difΔ6::kan*	P1.GR47 × N2679→Km^r
DL1386	Δ *ruvAC65 eda51*::Tn*10 difΔ6::kan*	P1.GR47 × DL1102→Km^r
AB1885 derivatives		
AB1885	*uvrB5*	Bachmann, 1996
DL1155	*uvrB5 xerCY17*::Mini-*cat*	P1.DS984 × AB1885→Cm^r
DL1156	*uvrB5 recF332*::Tn*3*	P1.JC13885 × AB1885→Ap^r
DL1157	*uvrB5 recF332*::Tn*3 xerCY17*::Mini-*cat*	P1.JC13885 × DL1155→Ap^r
DL1159	*uvrB5 recB268*::*Tn*10	P1.C876 × AB1885→Tc^r
DL1161	*uvrB5 recB268*::*Tn*10 *xerCY17*::Mini-*cat*	P1.C876 × DL1155→Tc^r
Other strains		
CAG18556	*purE32000*::Tn*10*	Singer et al., 1989
D7-8	*polA^{ts}*	William Donachie
DL1288	*polA^{ts} xerCY17*::Mini-*cat*	P1.DS984 × D7-8→CM^r
PLG292	*rif^R*	Noreen Murray
GR47	*difΔ6::kan*	Recchia et al., 1999
DS984	*xerCY17*::Mini-*cat*	David Sherratt
JC13885	*recB21 recC22 recF*::Tn*3*	Alvin J. Clark
C876	*recB268*::Tn*10*	Gerald R. Smith
N4155	Δ *ruvAC65 eda51*::Tn*10*	Robert G. Lloyd

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 the resolution bias caused by the productive positioning of the RuvABC complex is applied (Figure 7). It can be seen that the consequence of resolution bias is that leading-strand breaks will be accompanied by little crossing over, whereas lagging-strand breaks will be associated with substantial crossing over (Figure 7).

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 reestablish these replication forks. Interestingly, *ruv* mutations also cannot be combined with *polA* mutations (Ishioka et al., 1998), even though *ruv* mutants display only a small defect in DNA end-directed recombination as measured by conjugation and transduction assays (see Kowalczykowski et al., 1994 and references therein). If recombination, caused by fork breakdown in a *polA* mutant, is mostly of the leading-strand break variety, then we would predict that RuvABC would direct cleavage to avoid crossing over and dimerization (Figure 7A). This avoidance of dimerization could help to explain the inviability of *polA ruv* double mutants and predicts that *polA xerC* double mutants should be viable. In fact, we were able to construct *polA xerC* double mutants by P1 transduction (data not shown), and a *polA^{ts} xerC* mutant grew at both restrictive (42°C) and nonrestrictive (30°C) temperatures. This suggests that recombination occurring at leading-strand breaks mainly avoids crossing over; the prediction made on the basis of the resolution bias in Figure 7. Similar results have been obtained by Kuempel et al. (1991), who have shown that a *polA^{ts}* dif strain can form colonies at both 42°C and 28°C. Although viable, this strain showed reduced plating efficiency and poor growth in LB medium at the restrictive temperature, arguing that some of the damage caused by *polA* deficiency results in crossing over. This may either be because some damage results in lagging-strand breaks (e.g., is derived from interruptions of the newly synthesized leading strand), or because the bias to avoid crossing over from leading-strand breaks is not 100%.

Another potential source of breaks may be Holliday junction cleavage following the reversal of replication forks, a reaction shown to be catalyzed 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.

General Consequences of the Control of Crossing Over

The combination of our observations 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) place constraints on models of homologous recombination. Together, they argue that if RuvAB is positioned so as to give productive branch migration, then in SSGR, as in DSBR, Holliday junctions are formed adjacent to recombinogenic single strands that run 5′ to 3′ away from the junction (Figures 5C and 6C).

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. It may not be surprising that an organism with a circular chromosome has evolved a mechanism that favors the resolution of Holliday junctions to minimize the formation of dimers. We have shown that SSGR and leading-strand breaks tend to avoid crossing over. Leading-strand breaks might be expected to be more common than lagging-strand ones because the newly synthesized lagging strand (that is potentially interrupted due to its discontinuous mode of synthesis) becomes the leading-strand template in the next round of replication. Furthermore, cleaved fork-reversal products are also equivalent to leading-strand breaks.

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 crossover and noncrossover products is no longer tenable for *E. coli* recombination. Instead, the resolvasome is placed specifically to generate crossover or noncrossover outcomes in relation to the nature of the recombining substrates. In an organism with a circular genome such as *E. coli*, it makes sense to evolve a mechanism of resolution of Holliday junctions that favors noncrossover outcomes in the most commonly encountered situations involving Holliday junctions. In this way, the generation of potentially lethal dimers is minimized. Our observation that crossing over in *E. coli* is determined by the interaction between the nature of the recombination intermediate and the resolvasome opens up new ways of thinking about the control of crossing over in other organisms. The existence of rules for the resolution of Holliday junctions in yeast are implied by the patterns of recombination observed (e.g., see Gilbertson and Stahl, 1996). It is possible that crossing over in eukaryotes is also regulated by positioning of the branch migration apparatus, though there may be additional levels of control that determine the positioning of that apparatus.

Experimental Procedures

Bacterial and Phage Strains

The *E. coli* strains used are listed in Table 2.

λDRL246 was constructed by cloning an EcoRI-BglII fragment containing a Zeocin resistance marker from pZeoSV2(+) (Invitrogen corporation) into the multicloning site of λTX97 (St. Pierre and Linn, 1996) using the BamHI and EcoRI sites. A 246 bp interrupted palindrome encoding 111 bp inverted repeats separated by a 24 bp spacer had previously been cloned from SKK43 (Kulkarni, 1990) into pUC18 and was inserted into the multicloning site of λDRL246 in an EcoRI fragment to form λDRL282 (this laboratory). During this process, two internal mismatches were introduced into the palindromic sequence.

Lysogenization Frequencies

Bacterial strains undergoing lysogenization were grown overnight and then diluted 10-fold in broth containing 2% maltose and 5 mM Mg_2SO_4. They were then grown to a cell density of 4×10^8 cells ml^{-1} ($A_{650} = 0.9$). These cultures were then diluted 2-fold in 10 mM Tris, 10 mM Mg_2SO_4 (pH 8) buffer, giving a final cell density of 2×10^8 cells ml^{-1}. λ phage lysates were diluted to 2×10^9 pfu ml^{-1}. Equal volumes of phage and cell cultures (0.15 ml of each) were mixed and incubated at 30°C for 60 min to allow phage adsorption. Infected cells were diluted in phosphate buffer and a range of dilutions plated on L AGAR plates and low salt (85 mM NaCl) L AGAR plates supplemented with Zeocin (Invitrogen Corporation) at a concentration of 16 μg ml^{-1}.

UV Irradiation

Bacterial strains to be irradiated were grown overnight for a minimum of 16 hr to ensure entry into stationary phase. The cultures were resuspended in 10 mM Mg_2SO_4 and irradiated for periods of 15 s, 30 s, and 75 s using light of wavelength 254 nm. The dosages were calibrated by comparing the response of the wild-type, *recF*, and *recB* strains to published data (Wang and Smith, 1983). Various dilutions of nonirradiated and irradiated cultures were plated on L AGAR plates. Plating of irradiated cultures was carried out immediately after irradiation. All procedures were carried out in minimal light conditions.

Determination of P1 Transduction Frequencies

Overnight cultures of recipient strains were grown in L broth containing 250 mM $CaCl_2$ to an approximate cell density of 1×10^9 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 min. Then 800 μl of L broth containing 500 mM Na citrate was added and incubation at 37°C continued for 60 min. Plating of 100 μl on selective media was then carried out. The selective plates used were L AGAR supplemented with either 50 μg ml^{-1} rifampicin or 50 μg ml^{-1} kanamycin. The recipient culture (prior to infection with P1) was also plated on L AGAR plates for counting initial cell density. Plates were counted after incubation at 37°C for 48 hr.

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