Crossing Over Between Regions of Limited Homology in *Escherichia coli*: RecA-Dependent and RecA-Independent Pathways

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ABSTRACT

We have developed an assay for intermolecular crossing over between circular plasmids carrying variable amounts of homology. Screens of *Escherichia coli* mutants demonstrated that known recombination functions can only partially account for the observed recombination. Recombination rates increased three to four orders of magnitude as homology rose from 25 to 411 bp. Loss of recA blocked most recombination; however, RecA-independent crossing over predominated at 25 bp and could be detected at all homology lengths. Products of recA-independent recombination were reciprocal in nature. This suggests that RecA-independent recombination may involve a true break-and-join mechanism, but the genetic basis for this mechanism remains unknown. RecA-dependent crossing over occurred primarily by the RecF pathway but considerable recombination occurred independent of both RecF and RecBCD. In many respects, the genetic dependence of RecA-dependent crossing over resembled that reported for single-strand gap repair. Surprisingly, ruvC mutants, in both recA+ and recA mutant backgrounds, scored as hyperrecombinational. This may occur because RuvC preferentially resolves Holliday junction intermediates, critical to both RecA-dependent and RecA-independent mechanisms, to the noncrossover configuration. Levels of crossing over were increased by defects in DnaB helicase and by oxidative damage, showing that damaged DNA or stalled replication can initiate genetic recombination.

Recombination can occur between exogenous DNA introduced into bacteria by conjugation, phage transduction, or DNA transformation and the bacterial genome. Recombination plays a critical role in shaping bacterial chromosomes: DNA can be integrated or deleted from the chromosome by recombination, and duplicated gene segments can be substituted for one another. Recombinational interactions between sister chromosomes also repair DNA damage and restore the integrity of chromosomes that are broken during the process of DNA replication.

The steps of recombination: Models for genetic recombination have been proposed to explain the properties of genetic exchange during fungal meiosis (Holliday 1964; Meselson and Radding 1975; Szostak et al. 1983). The combined genetic and biochemical analysis of recombination functions from the bacterium *E. coli* has confirmed many elements of these recombinational models (reviewed in Clark 1991; West 1992; Kowalczykowski 2000; and summarized below).

Single-strand DNA (ssDNA) is the recombinogenic substrate. It may be produced by incomplete replication or by exonucleolytic degradation or unwinding of broken DNA. The RecA protein initiates recombination by binding to ssDNA and catalyzing strand pairing and strand transfer. Loading of RecA is facilitated by specific interactions with the RecBCD nuclease (Anderson and Kowalczykowski 1997; Churchill et al. 1999) or by action of the RecFOR complex (Umezu and Kolodner 1994; Shan et al. 1997; Webb et al. 1997). Because of RecBCD’s specificity for dsDNA ends, recombination involving double-strand breaks (DSBs) in DNA initiation employs RecBCD in the initiation step. In contrast, gap repair appears to be initiated by RecFOR proteins (Wang and Smith 1984), which enhance the efficiency of RecA loading onto ssDNA gaps (Umezu et al. 1993; Shan et al. 1997; Webb et al. 1997).

After strand transfer from either type of substrate, branched intermediates (Holliday junctions) are processed by one of several enzymes that can branch migrate such structures (RecA, RuvAB, or RecG) and can be cleaved by resolvases such as RuvC (West 1997). Mature recombinants are then formed by DNA ligase.

The genetics of recombination is assay specific: Genetic effects on the efficiency of recombination in bacteria depend on the type of recombination assay employed. This presumably reflects differences in the recombinational substrates, intermediates, or products involved in these reactions. Most early studies of recombination genes of *E. coli* employed assays of conjugational recom-

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bination (Clark 1973). Since that time, studies of genetic rearrangements in bacteria have revealed that there are differences between the genetic dependence of these processes as compared to conjugation. For example, although conjugation is mediated primarily by the RecBCD pathway and only in a minor way by the RecF pathway in E. coli (Horii and Clark 1973), recombination between direct repeats in Salmonella is catalyzed equally well by either the RecF or the RecBCD pathway (Galitski and Roth 1997).

**What initiates recombination?** Many of the most common genetic recombination assays involve introduction of foreign DNA (by conjugation, transduction, or transformation) in which the DNA is broken. In these cases, recombination is initiated by the provision of a DNA end and so occurs at a conveniently high frequency. We know much less about what initiates recombination events between bacterial genes in situ. If recombination is initiated by strand breaks, the cellular processes that provide such breaks are unknown.

**Critical recombination intermediates:** After recombination has been initiated, branched DNA structures including Holliday junctions are formed. The branch migration of these structures can drive the recombination reaction forward or reverse it (West 1997). Instability of these initial joint molecules may act as a proofreading step for recombination, to prevent recombination between spurious regions of short homologies. Alternative resolution by cleavage of the Holliday junctions can produce either crossover or noncrossover products.

**Assay design:** We wished to design an assay with properties that would allow several unknown features of bacterial recombination to be explored. Our two-plasmid integration assay detects recombination between resident replicas; therefore, this assay should respond to factors that initiate recombination by production of strand breaks or other recombinogenic lesions. Our selection detects only a subset of recombination events—those involving reciprocal crossing over—and is insensitive to other types of recombination such as nonreciprocal gene conversions. Since gene conversions and crossing over are believed to be outcomes of differential processing of a common intermediate, the Holliday junction, genes that bias the resolution of recombination would also be revealed by our assay. Our system also includes recombination substrates with very short sequence homologies, amount of plasmid DNA in any of the mutants assayed was at revealed by our assay. Our system also includes recombination substrates with very short sequence homologies, amount of plasmid DNA in any of the mutants assayed was at

**MATERIALS AND METHODS**

**Strains:** The strains used in this study are listed in Table 1. Strains were constructed by P1virA transduction using the selections noted in Table 1 (Miller 1992). Growth in the presence of trimethoprim (Tp, 10 μg/ml) was used to select thyA mutant derivatives as described (Miller 1992; Viswanathan et al. 2000) and Thy+ transductants were subsequently selected on 56/2 minimal medium (Willett et al. 1969) supplemented with 0.4% glucose; 0.001% thiamine; and 0.01% arginine, proline, histidine, threonine, and leucine. All other experiments employed Luria-Bertani (LB) medium supplemented with the antibiotics ampicillin (Ap, 30 μg/ml), chloramphenicol (Cm, 20 μg/ml), or tetracycline (Tc, 15 μg/ml) as indicated. Growth was at 37°C unless otherwise noted.


**Assays:** Fluctuation assays for rate determination were performed by inoculation of 8–24 independent single colonies and aerobic growth for 2 hr in LB broth + Ap + Cm. Subsequent serial dilution and plating on LB + Ap + Cm and LB + Ap + Cm + Tc determined the number of plasmid-bearing cells and plasmid recombinants, respectively, in each culture. Using these values, recombination rates were calculated from either the method of the median or the maximum-likelihood method as described (Lea and Coulson 1949). The maximum-likelihood method was used in cases where 25% or more of the cultures contained zero Tc colonies. For hydrogen peroxide experiments, independent cultures

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TABLE 1
E. coli K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Derivation</th>
</tr>
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<tbody>
<tr>
<td>AB1157</td>
<td>rec&lt;sup&gt;c&lt;/sup&gt;</td>
<td>BACHMANN (1996)</td>
</tr>
<tr>
<td>CS140</td>
<td>ruvC53</td>
<td>SHURVINTON et al. (1984)</td>
</tr>
<tr>
<td>JC10287</td>
<td>(recA-srlΔ)304</td>
<td>CSONKA and CLARK (1979)</td>
</tr>
<tr>
<td>N2096</td>
<td>ruvAΔ63</td>
<td>R. Lloyd</td>
</tr>
<tr>
<td>N2731</td>
<td>recG258::miniTn10kan</td>
<td>R. Lloyd</td>
</tr>
<tr>
<td>STL230</td>
<td>recO1504::Tn5</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 RDK1504 (R. Kolodner) × AB1157</td>
</tr>
<tr>
<td>STL970</td>
<td>ruvC53 ruvG258::miniTn10kan</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 N2731 × CS140</td>
</tr>
<tr>
<td>STL1131</td>
<td>ruvC53 cysC59::Tn10</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; Cys&lt;sup&gt;-&lt;/sup&gt; transductant P1 CAG12173 (SINGER et al. 1989) × CS140</td>
</tr>
<tr>
<td>STL1143</td>
<td>ruvC53 (recA-srlΔ)304</td>
<td>Cys&lt;sup&gt;-&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 JC10827 × STL1131</td>
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<tr>
<td>STL1270</td>
<td>dnaB107(s) malE::Tn10kan</td>
<td>(SAVESON and LOVETT 1997)</td>
</tr>
<tr>
<td>STL3607</td>
<td>recF400::kan</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 JC18970 × AB1157</td>
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<tr>
<td>STL3919</td>
<td>recR252::miniTn10kan</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 AM207 (R. Lloyd) × AB1157</td>
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<td>Km&lt;sup&gt;+&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 STL3893 × AB1157</td>
</tr>
<tr>
<td>STL4955</td>
<td>recF400::kan thyA</td>
<td>T&lt;sup&gt;r&lt;/sup&gt; spontaneous mutant of STL3998</td>
</tr>
<tr>
<td>STL4959</td>
<td>recB2053::Tn10kan recF400::kan</td>
<td>Thy&lt;sup&gt;-&lt;/sup&gt; UV&lt;sup&gt;-&lt;/sup&gt; transductant P1 STL3893 × STL4955</td>
</tr>
</tbody>
</table>

All strains carry the additional markers F<sup>+</sup> lacI44 thi-1 hisG4 Δ(gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfhD1 ara-14 lacY1 galK2 mtl-5 mil-3 tss-30 supE44 rpsL31 (BACHMANN 1996).

were grown to an OD<sub>660</sub> of 0.3 and split into four 2-ml cultures. Two of the split cultures were treated with 5 mM H<sub>2</sub>O<sub>2</sub> and two remained untreated. After 20 min of growth, 50 µg/ml of catalase (Sigma, St. Louis) was added to the cultures to inactivate the H<sub>2</sub>O<sub>2</sub>. Each culture was diluted and plated on the appropriate media in duplicate to determine the number of recombinants and the total colony-forming units of surviving plasmid-containing cells. Average cell survival at this dose was 16%. Determinations were made for four independent cultures and the averages and range of values are presented.

**Product analysis:** Minipreparations of plasmid DNA were purified (QIAGEN) from independent tetracycline-resistant isolates. This DNA was subjected to agarose gel electrophoresis and sequenced to determine the homologies between the two plasmids, which vary from 25 to 411 bp among the constructs, restores a functional tet<sub>A</sub> gene and can be selected by tetracycline resistance. This assay, as designed, is specific to integrative recombinational events within a limited region and will not detect “gene-conversion” type recombination in which a patch of genetic information has been transferred from one replicon to the other. Strains of interest were transformed with pSTL330 and then one of the pSTL331–336 series plasmids, producing a set of six strains with two plasmids that share variable regions of sequence homology. Fluorescence analysis of fluorescently labeled probe hybridized to Southern blots of the DNA from the various strains was obtained.

**RESULTS**

**Assay design:** A set of moderate-copy-number plasmids was designed to detect crossing over between homologies of variable lengths (Figure 1). One plasmid, pSTL330, was derived from pBR322 (BOLIVAR et al. 1977), carrying a ColE1 origin of replication; this plasmid encodes ampicillin resistance and carries a mutant form of the tet<sub>A</sub> gene lacking promoter, ribosome-binding site, and initiation codon. Another set of plasmids, pSTL331–336, were derived from pACYC184 (CHANG and COHEN 1978), carrying the p15A origin of replication; these plasmids encode chloramphenicol resistance and carry various 3′ deletions of the tet<sub>A</sub> gene. These plasmids were designed to share between 25 and 411 bp of homology with the pSTL330 5′-deleted tet<sub>A</sub> allele. The ColE1 and p15A plasmids are not homologous in segments outside the tet<sub>A</sub> gene. Crossing over at the tet<sub>A</sub> homologies between pSTL330 and any one of the pSTL331–336 derivatives produces a dimeric integrant plasmid. This recombination event also restores a functional tet<sub>A</sub> gene and can be selected by tetracycline resistance. This assay, as designed, is specific to integrative recombinational events within a limited region and will not detect “gene-conversion” type recombination in which a patch of genetic information has been transferred from one replicon to the other. Strains of interest were transformed with pSTL330 and then one of the pSTL331–336 series plasmids, producing a set of six strains with two plasmids that share variable regions of sequence homology. Fluorescence analysis of fluorescently labeled probe hybridized to Southern blots of the DNA from the various strains was obtained.

**Figure 1.—Design of the two-plasmid crossing over assay.** One plasmid, pSTL330, confers ampicillin resistance and carries a 3′-truncated allele of tet<sub>A</sub>. The second plasmid in the series pSTL331–336 confers chloramphenicol resistance and carries a 3′-truncated allele of tet<sub>A</sub>. Crossing over in the region of homology shared between the two plasmids, which varies in length from 25 to 411 bp among the constructs, restores an intact tet<sub>A</sub> gene and can be selected by tetracycline resistance. The doubly deleted allele is also produced as a result of a reciprocal exchange and is not demanded by the selection.
independent recombination can occur between sister combination at all homology lengths (Figure 4A). Loss of H11003/tetA gene and the doubly deleted mutant (solid circles) strains.

Homology and RecA dependence: In a wild-type strain background, rates of recombination were quite low, $<10^{-4}$, at 25 bp and rose dramatically, over three orders of magnitude, with increasing length of homology (Figure 2). Rates appeared to approach a plateau at a rate of $2 \times 10^{-5}$ for homologies $>200$ bp. Restriction digestion of 6–10 independent tet+ recombinants at each homology length confirmed the reciprocal nature of the selected recombination event: All products were heteromultimeric and contained both an intact tetA gene and the doubly deleted tetA gene with both 3′- and 5′-deleted segments (Figure 1).

In a recA mutant strain, recombination rates remained at a low level of $\sim 10^{-8}$–$10^{-7}$/cell generation (Figure 2). There was a 10-fold elevation of recombination rates with increasing homology, suggesting that RecA-independent recombination is also more efficient with increasing homology, although not to the same extent as evident for RecA-dependent recombination. RecA-independent recombination was the major contributor to recombination between very limited homologies of 25 bp in length. Restriction analysis of 6–10 tet+ plasmid products formed independently of RecA, at each homology length, showed that they, like the RecA-dependent products, had experienced an apparent reciprocal exchange reaction. All products were plasmid heterodimers with one tetA+ and one doubly deleted tetA allele. (Although the tet+ allele is demanded by the selection, the reciprocal doubly deleted tet joint is not; therefore “illegitimate” joining at other sites could have occurred. Nevertheless, no such illegitimate joints were observed, even when only 25 bp of homology existed between the two plasmids.) Previous studies show that efficient RecA-independent recombination can occur between sister chromosomes (reviewed in Bzymek and Lovett 2001), probably during the process of DNA replication. The results reported here also suggest that RecA-independent crossing over between two separate replicons can indeed occur, albeit at low rates in the population. The genetic basis of this RecA-independent recombination remains unknown.

RecBCD vs. RecFOR pathways: Loss of recF (Figure 3A) produced a 6- to 40-fold reduction of recombination at every homology length except the smallest one. Recombination at 25 bp of homology was independent of both RecF and RecA; therefore, the lack of effect of RecF at this homology confirms that the reduction of recombination at higher homologies was not due to artifacts of plasmid maintenance or copy number problems. Mutants in recO and recR also exhibited reduction of recombination (five- and threefold, respectively) when assayed with the pSTL330 and STL336 plasmids sharing 411 bp of homology. The RecA-dependent crossing over measured in this assay, therefore, primarily involves the RecF pathway, as do other assays of recombination measured with plasmids (Cohen and Laban 1983; Kolodner et al. 1985; McFarlane and Saunders 1996). The plasmids employed in our assay for recombination naturally lack Chi sites, which attenuate the nuclease activity of RecBCD and promote its ability to activate recombination (Dabert et al. 1992; Dixon and Kowalczykowski 1993; Kuzminov et al. 1994). Therefore, it was not surprising that the recB mutant exhibited no reduction but, rather, especially high levels of recombination (Figure 3B). Broken plasmid DNA would be rapidly degraded by the RecBCD nuclease and therefore inactivation of this enzyme should stabilize broken DNA, permitting it to recombine with its homologous partner. This enhanced recombination in the recB mutant is again mostly due to the RecF-dependent pathway, since rates are reduced in the double recB recF mutant relative to the single recB mutant (Figure 3B). However, substantial RecF-independent recombination is evident. Recombination rates in recF are considerably higher than those in recA mutants; recombination rates in recB recF strains are similar to those of wild type. Since the RecBCD and RecFOR pathways differ in the initiation step of recombination, this may reflect an alternative way in which ssDNA is created or by which RecA is loaded on ssDNA. This could involve an unknown factor or, alternatively, a stochastic process in which a subset of substrates is generated that does not require RecFOR or other additional factors to support initiation.

Resolution factors: Since these reactions require crossing over, which may involve cleavage of a Holliday junction, it was of interest to determine the effect of functions that are known to either branch migrate or cleave Holliday junctions. A mutation in ruvC, which encodes the only known Holliday junction cleavage protein normally expressed in E. coli, promoted hyperrecombination at all homology lengths (Figure 4A). Loss
of ruvAB, encoding the Holliday junction helicase associated with ruvC resolvase, did not significantly affect recombination rates, except for a modest elevation at the highest homology, 411 bp (Figure 4A). A mutant in recG, which has been proposed to provide an alternative mode of Holliday junction processing (Whitby et al. 1993), showed hyperrecombination at the lowest homology (25 bp) and a reduction, two- to fivefold, or no effect at higher homologies (Figure 4B). A ruvC recG double mutant had a modest reduction, two- to fourfold in rates at all homology lengths, except the smallest and the largest, which showed no effect (Figure 4B). If Holliday junction resolution is required for these integrative reciprocal reactions, our experiments suggest that it is not mediated by RuvC. This may be because RuvABC resolution is biased to noncrossovers in these reactions, as has recently been proposed (Cromie and Leach 2000; Michel et al. 2000). A RecG-dependent pathway may contribute partially, but considerable crossing over remains after the inactivation of both RuvABC and RecG. This crossing over is probably not due to RuvA, a cryptic bacteriophage-encoded resolvase (Mandal et al. 1993; Sharples et al. 1994), since it is not normally expressed in E. coli K12. Furthermore, recombination rates of rus ruvC double mutants at 411 bp of homology were not lower than rates in ruvC mutants (data not shown).

At lowest length of homology, 25 bp, crossing over in wild-type strains is independent of RecA. We wished to know whether the hyperrecombination of ruvC mutants at 25 bp of homology was likewise independent of recA. With 25 bp of homology shared between plasmids STL330 and pSTL331, the rate of crossing over in the recA ruvC mutant was $3.9 \times 10^{-8}$ (16 assays) compared to the rate of $4.0 \times 10^{-8}$ (16 assays) seen with the ruvC single mutant. Therefore, the absence of RuvC stimulates RecA-independent recombination at short homologies.

Recombinogenic factors: A number of cellular events may trigger recombination between intact DNA molecules. First, difficulties in replication may stall forks and elicit subsequent recombinational repair. Second, repair of DNA damage such as oxidative or other lesions may produce recombinogenic ssDNA that provokes crossing over between replicons. We investigated two situations in which recombination may be elevated: in a dnaBts mutant with defects in replication and with treatment of hydrogen peroxide to induce oxidative lesions. At its permissive temperature for growth, 30°C, introduction of a temperature-sensitive mutation in dnaB replicative helicase elevated recombination rates substantially relative to wild-type rates at 30°C. This effect was pronounced at all but the largest homology (Figure 5). At shorter homology lengths recombination rates

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**Figure 3.—Effects of recombination initiation pathways on recombination rates at different length homologies. (A) Wild-type (open squares, dashed line) and recF mutant (solid circles). (B) Mutants in recB (solid squares) or recB recF (solid diamonds).**

**Figure 4.—Effects of Holliday junction processing enzymes on crossing over between variable lengths of homology. (A) Recombination rates at different homology lengths for mutants in ruvC (solid triangles) or ruvAB (solid circles) compared to wild type (open squares, dashed line). (B) Recombination rates for mutants in recG (solid diamonds) or recG ruvC (solid squares).**
may be limited by the probability of an initiating lesion occurring in the region of homology shared between the two replicons. At the largest homology, other factors may become rate limiting. Difficulties in replication can trigger RecA-dependent recombinational repair and lead to genetic rearrangements, as supported by other analyses (Horiuchi and Fujimura 1995; Saveson and Lovett 1997; Michel 2000). Hydrogen peroxide stimulated recombination at all homologies tested, approximately two- to fivefold (Table 2). As with the stimulation conferred by dnaBts, stimulation of crossing over by H$_2$O$_2$ was relatively greater at the lower homology lengths.

**DISCUSSION**

**Advantages and constraints of our assay for crossing over:** We have developed a new assay to investigate crossing over between independent replicons that share limited regions of homology. Unlike many other assays for recombination used in bacteria, this one does not provide substrates with broken ends. The selected events can be produced only by crossing over and not by gene conversion-type reactions. Because of these features, such an assay may be useful to define the factors that initiate recombination in situ and those that affect the stability or processing of critical recombination intermediates. Indeed, our analysis shows that, depending on the length of sequence homology between recombining partners, factors affect recombination differentially. Recombination measured by our assay shows that the recombination genes identified by defects in repair or conjugation at present can only partially define the molecular events we observe.

No single recombination assay can reflect the diversity of genetic exchanges that occur in vivo. The genes that influence recombination can be assay specific, depending on the constraints of the particular assays. For example, our system may be insensitive to double-strand-break-initiated recombination because the recombining partners lack Chi sites that promote RecBCD-mediated exchange and attenuate the destruction of linear DNA by RecBCD. In addition, because our assay demands crossing over between independent replicons, factors that encourage recombination strictly between sister chromosomes would not lead to recombination scored by our assay and might, rather, inhibit interreplicon crossing over.

**Homology dependence of RecA-dependent and RecA-independent crossing over:** The length of the interacting homologies was a strong determinant of recombination rates. Between 50 and 400 bases, integrative recombination was dependent on RecA and strongly sensitive to the homology length. In this sense our results agree with previous studies of integrative recombination between bacteriophage λ and plasmid DNA (King and Richardson 1986; Shen and Huang 1986), although the absolute frequencies of recombination vary in the three analyses. At 25 bp of homology, recombination was independent of RecA, although it occurred at a low rate in the population. RecA-independent crossing over was weakly dependent on the length of sequence homology in contrast to RecA-dependent recombination, which showed a strong homology dependence. The apparent homology threshold for maximally efficient crossing over also was lower for RecA-independent than for RecA-dependent recombination: $\sim$50 vs. 150 bp, respectively.

**RecA-dependent crossing over occurs primarily via the RecFOR pathway and may be initiated by gap repair:** RecA-dependent recombination contributes to most crossing over we observed with homologies $>$50 bp in length. This recombination appeared to be initiated via the RecFOR pathway and not via the RecBCD pathway. This is probably because our substrate plasmids lack Chi sites that potentiate the recombinogenic activity and diminish the degradative activity of RecBCD. The hyper-recombinational phenotype of mutants defective in

**TABLE 2**

<table>
<thead>
<tr>
<th>Homology (bp)</th>
<th>Fold elevation in recombinant frequency by 5 mM H$_2$O$_2$ (range)</th>
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</thead>
<tbody>
<tr>
<td>25</td>
<td>5.1 (1.8–9.0)</td>
</tr>
<tr>
<td>51</td>
<td>3.0 (2.2–4.1)</td>
</tr>
<tr>
<td>104</td>
<td>1.9 (1.5–2.3)</td>
</tr>
<tr>
<td>158</td>
<td>1.9 (1.1–3.6)</td>
</tr>
<tr>
<td>211</td>
<td>1.9 (0.8–2.8)</td>
</tr>
<tr>
<td>411</td>
<td>1.6 (1.5–1.8)</td>
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</table>

Four independent cultures in exponential growth phase were split and treated with H$_2$O$_2$ or not and recombinant frequency was determined in the cultures. All dilutions and plating were performed in duplicate. The average of the treated cultures is expressed relative to the untreated cultures. The range of values from lowest to highest is also given in parentheses.
RecBCD suggests that linear DNA is generated in vivo but restrained from recombinational interactions by degradation by RecBCD if the molecules lack Chi sites. The RecFOR pathway is presumed to be a mechanism for recombination initiated at ssDNA gaps. The RecFOR pathway contributes to approximately one-half of the crossing over detected between E. coli sister chromosomes (Steiner and Kuempel 1998) and to about one-half of the recombination between direct repeats in Salmonella (Galitski and Roth 1997). In the latter case, dependence on recF was diminished with the introduction of a DSB (Galitski and Roth 1997), suggesting that the RecFOR pathway recombination was primarily occurring via a gap-repair mechanism. In our two-plasmid crossing over assay, ~10% of RecA-dependent recombination was independent of the RecFOR pathway, as well as the alternate RecBCD pathway. With respect to its dependence on RecF, our two-plasmid crossing over assay resembles chromosomal gap repair observed by physical analysis after UV-irradiation: likewise, its major component is RecF dependent, RecBCD independent and its minor component is both RecF and RecBCD independent (Wang and Smith 1984). Whether the RecF- and RecBCD-independent recombination utilizes an unknown function or merely represents the proportion of initiating DNA molecules that do not require further processing is unknown.

**Holliday junction resolution and crossing over:** Molecular models of recombination (Holliday 1964; Meselson and Radding 1975; Szostak et al. 1983) presume that appropriate endonucleolytic resolution of Holliday junctions generates reciprocal exchanges, seen as genetic crossovers between chromosomes (Figure 6A). Alternatively, apparent reciprocal exchanges can be generated by sequential nonreciprocal exchanges. Although the selection imposed by our system demands a crossover and does involve reciprocal exchange between two DNA molecules, the only Holliday junction resolving activity expressed normally in E. coli, RuvC, was not required. In fact, ruvC mutants were hyperrecombinogenic. The hyperrecombinational phenotype of ruvC mutants suggests that RuvC normally cleaves critical intermediates, removing their potential to give rise to recombinants scored by our assay. The simplest explanation is that RuvC biases resolution of Holliday junctions toward the noncrossover configuration in certain recombination reactions (Cromie and Leach 2000; Michel et al. 2000; as in Figure 6B). Another possibility is that stalled replication initiates crossing over by a gap-filling mechanism; however, a competing reaction, which does not yield recombinants, could be retrograde fork movement to produce a Holliday-junction-like structure. The cleavage of this structure by RuvC, as demonstrated by Michel and collaborators (reviewed in Michel 2000), and the destruction of the resultant linear DNA by RecBCD could preclude recombination in our assay. Both RecA-dependent and RecA-independent recombination were found to be stimulated by inactivation of RuvC, suggesting that both modes of recombination share a critical branched intermediate.

The possibility remains that an unknown activity in E. coli resolves Holliday junctions and gives rise to recombinants in our assay. If so, this activity is at least partially dependent on RecG branch migration helicase since mutations in recG produce a modest reduction in crossing over both in ruvC+ and ruvC mutant backgrounds. With respect to its peculiar response to ruv, recG, or ruv recG mutations as well as a dependence on recF, our two-plasmid crossing over assay shares a striking resemblance to the recombinational repair pathway reported for etheno-adducts in DNA (Pandya et al. 2000).

To explain our observations we favor a gap-filling mechanism for crossing over similar to that shown in Figure 7 that generates a Holliday junction intermediate concomitant with a template switch. Since one DNA strand of this structure is already recombinant, replication of the intermediate without resolution could generate both crossover and noncrossover products. Or an unknown resolvase may generate these products by the appropriate strand cleavage.

An alternative model is that seemingly reciprocal products detected by our assay may be generated by exchanges that do not involve formation of Holliday junctions. For example, as shown in Figure 8, linear DNA generated by cleavage or by rolling circle replication could invade the second partner, producing the first recombinant joint. Generation of a second end by
Figure 7.—Initiation of crossing over by gap-filling recombination. The genetic dependence of crossing over as detected in our assay is reminiscent of gap-filling repair. (A) The initial gapped substrate. (B) Strand invasion between the gapped DNA and homologies present on the second plasmid. (C) A template switch allows the gapped material to be synthesized from the other partner and the displaced strand is cleaved. (D) This forms a Holliday junction intermediate. (E) Replication of this intermediate without resolution can produce both crossover and noncrossover products. Alternatively, Holliday junction resolution produces either product.

cleavage or by fork collapse could produce a second linear end that invades or anneals at homology to form the circular crossover product. We think this type of mechanism is less likely to explain our observed recombination since it invokes two linear intermediates that should be unstable to RecBCD degradation. However, such a mechanism may explain the very high levels of crossovers observed in recB mutants.

Initiation of recombination: Defects in replication or oxidative damage elevate crossing over detected with our two-plasmid assay. A temperature-sensitive mutation of the DnaB helicase, at its permissive temperature of growth, and hydrogen peroxide treatment promoted exchange that was particularly evident at the lower homologies tested. This result emphasizes the importance of examining recombination between substrates of limited homology, since at larger homologies initiation may not be rate limiting. Mutations in dnaB have been found to promote both RecA-dependent and RecA-independent genetic rearrangements (Saveson and Lovett 1997, 1999) and can result in chromosome breakage (Michel et al. 1997). Presumably, in this mutant replication frequently arrests and replication gaps initiate crossing over between replicons. It has been noted previously that oxidative damage stimulates recombination within bacteria (Chen and Bernstein 1987; DeRosa and Claycamp 1991; Steiner and Kuempel 1998; Onda et al. 1999), because of the DNA strand breaks produced directly by reactive oxygen species (Imlay et al. 1988) or by subsequent enzymatic processing of oxidative lesions in DNA (Onda et al. 1999). Whether or not intrinsic replication arrest or oxidative damage contributes substantially to spontaneous recombination rates remains to be investigated. Mutants or treatments that produce lower spontaneous crossing over rates may reveal which processes initiate recombination during normal cell growth.

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LITERATURE CITED


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