Heteroduplex Strand-Specificity in Restriction-Stimulated Recombination by the RecE Pathway of *Escherichia coli*

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ABSTRACT

The RecE recombination pathway is active in *Escherichia coli* *recB recC sbcA* mutants. To isolate and characterize products and intermediates of RecE-mediated, break-induced, intramolecular recombination, we infected *recB recC sbcA* mutants, expressing *EcoRI* endonuclease, with chimeric λ phages that allow *EcoRI*-mediated release of cloned linear recombination substrates. Substrates with direct terminal repeats recombined to yield a circular product with one copy of the repeated sequence. Some recombinants were heteroallelic for the recombining markers. Markers distant to the break were recovered in the circular product at a higher frequency than markers close to the break. To examine the heteroduplex structures that may have yielded the heteroallelic recombinants, nonreplicative substrates were employed. Some of the nonreplicative recombination products contained heteroduplexes, with a strong bias for paired strands ending 3' at the break. This strand bias in heteroduplex formation is consistent with recombination models that postulate homologous pairing of protruding 3' single-stranded ends.

The RecE recombination pathway of *Escherichia coli* is activated in *recB recC* mutants by *sbcA* mutants (BARBOUR *et al.* 1970). The *sbcA* mutations induce exonuclease VIII, a *recE* gene product that digests double-stranded DNA in a 5' to 3' direction to yield 3' single-stranded DNA overhangs (KUSHNER, NAGAISHI and CLARK 1974; JOSEPH and KOLODNER 1983). *recE* also encodes a protein that promotes homologous pairing of complementary single-stranded DNA (S. HALL, M. KANE and R. KOLODNER, submitted for publication). This pairing activity may explain *recA*-independent plasmid recombination in *recB recC sbcA* mutants (LABAN and COHEN 1981; FISHEL, JAMES and KOLODNER 1981). The homologous pairing activity of the *recE* product also suppresses RecA deficiency in plasmid recombination by the RecF recombination pathway (I. BERGER and A. COHEN, unpublished).

Recombination by the RecE, RecF and λ Red pathways is stimulated by double-strand breaks (DSB),¹ inflicted *in vitro* or *in vivo* by restriction endonucleases, or by λ terminase activity at λ *cos* sites (SYMINGTON, MORRISON and KOLODNER 1985; STAHL, KOBAYASHI and STAHL 1985; THALER, STAHL and STAHL 1987a; LUISI-DELUCA, LOVETT and KOLODNER 1989; NUSSBAUM, SHALIT and COHEN 1992). This observation, the high concentration of crossover events near

the break (STAHL *et al.* 1974; THALER, STAHL and STAHL 1987b), the inhibitory effect of proteins that protect DNA double-stranded ends (THALER, STAHL and STAHL 1987c) and substrate specificity of enzymes that are involved in these pathways (RADDING 1966; JOSEPH and KOLODNER 1983; LOVETT and KOLODNER 1989) suggest a direct role for DNA ends in the homologous pairing reaction.

The homologous pairing mechanism may be dictated by substrate configuration and the location of the break with respect to the homologous sequences. A break within one of the two recombining sequences stimulates a RecE-mediated recombination that follows the rules of the DSB-Repair model (KOBAYASHI and TAKAHASHI 1988; NUSSBAUM, SHALIT and COHEN 1992) as put forth for recombination in yeast (RESNICK 1976; ORR-WEAVER and SZOSTAK 1983). In linear intramolecular recombination substrates with direct terminal repeats, the break is located between the homologous sequences (see Figure 1). Therefore, either one or both sequences may be subjected to exonuclease processing before homologous pairing. The single-stranded DNA annealing (SSA) model proposes strand-specific digestion at both ends and annealing of the homologous overhangs (LIN, SPERLE and STERNBERG 1984). Recombination by a strand-specific pairing mechanism may also follow exonucleolytic digestion at one end. Such a mechanism could involve pairing of the homologous strands by strand-invasion and postsynapsis degradation of the displaced strand (SYMINGTON, MORRISON and KOLODNER 1985; MARYON and CARROLL 1991).

¹ Abbreviation used: DSB, double-strand break; SSA, single-strand annealing; IPTG, isopropyl- β -D-thiogalactopyranoside; *EcoRI*⁺ or *EcoRI*⁻ cells, cells that express or do not express *EcoRI* restriction-modification enzymes; nt, nucleotides; moi, multiplicity of infection. *HindIII*⁻ and *XmnI*⁻ designate *luxA* mutations at the *HindIII* and *XmnI* site, respectively.

TABLE 1
E. coli strains

Strain ^a	Relevant genotype				Other	Source or reference
	<i>recA</i>	<i>recB</i>	<i>recC</i>	<i>sbcA</i> ^b		
JC8679	+	21	22	23		GILLEN <i>et al.</i> 1981
AC224 ^c	+	21	22	23	[λ CI(Ind ⁻)]	This work
AC250 ^d	+	21	22	23	<i>mutS215::Tn10</i> [λ CI(Ind ⁻)]	This work
AC177	+	+	+	del	<i>nhaA::luxA⁺ luxB⁺ kan⁺</i>	NUSSBAUM and COHEN (1988)
AC217 ^e	$\Delta 306::Tn10$	+	+	del	<i>recD1009</i>	This work
DR100	$\Delta 306$	+	+	del		LABAN and COHEN (1981)
JC15502	+	+	+	del	<i>recD1009</i>	A. J. CLARK
JC15503	$\Delta 306::Tn10$	+	+	del		A. J. CLARK
MM294A <i>mutS::Tn10</i>	+	+	+	del	<i>mutS215::Tn10</i>	HABER <i>et al.</i> (1988)

^a All strains listed except DR100 and MM294A *mutS::Tn10* also carry *thr-1 ara-14 leuB6* Δ (*gpt-proA*)62 *lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 ml-1 argE3 thi-1*.

^b del designates the absence of the *Rac* prophage from the indicated strains.

^c λ CI(Ind⁻) lysogen of JC8679.

^d AC250 was constructed by transduction of AC224 by P1.MM294A *mutS::Tn10* and selection for tetracycline resistance.

^e AC217 was constructed by transduction of JC15502 by P1.JC15503 and selection for tetracycline resistance.

The molecular structure of heteroduplexes, formed in the recombination process, may serve as a distinguishing feature for different classes of mechanisms (ROSENBERG 1987; HAGEMANN and ROSENBERG 1991; SIDDIQI, STAHL and STAHL 1991). If recombination involves strand-specific pairing, one heteroduplex type will be formed. Conversely, if heteroduplexes are produced by a mechanism that involves reciprocal strand exchange at duplex DNA regions, two chemically distinct heteroduplexes will be produced by branch migration (HOLLIDAY 1964). With the substrate depicted in Figure 1, one heteroduplex will be formed by pairing of the strands ending 3' at the break, and the other by pairing of the strands ending 5' at the break (see Figure 7).

To gain insight into the nature of the homologous pairing reaction in RecE-mediated intramolecular recombination, by linear substrates with direct terminal repeats, we determined the polarity of the strands incorporated into heteroduplex products. To isolate primary products of intramolecular recombination, we employed a λ -vector-based substrate delivery system that facilitates restriction-mediated release of linear substrates within *E. coli* cells (NUSSBAUM, SHALIT and COHEN 1992). We report here the formation of heteroduplexes by RecE-mediated recombination with a strong bias for paired strands ending 3' at the break.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Bacterial strains used in this study are listed in Table 1. All strains except for DR100 were isogenic derivatives of AB1157 (BACHMANN 1972).

Cultures were grown in L-broth medium (LURIA and BURROUS 1957). Strains harboring plasmids were grown in the presence of the appropriate antibiotics (100 μ g/ml am-

picillin, 20 μ g/ml kanamycin). Growth conditions of phage-infected cultures are described below.

Plasmids and phage: Plasmids and phage used in this study are listed in Table 2. λ MS805 (Figure 1) is a λ EMBL4 (FRISCHAUF *et al.* 1983) derivative, constructed by ligation of *Eco*RI cleaved pMS805 to *Eco*RI-generated arms of λ EMBL4. pMS805 is an intramolecular recombination substrate with a duplication of the *luxA luxB* genes of *Vibrio fischeri*, cloned downstream of the *Plac* promoter. Each copy of the *luxA* gene on pMS805 is mutated at a different restriction site. To construct pMS805 a *kan* gene was inserted into pAC602 (NUSSBAUM and COHEN 1988) by replacing a *Hind*III-*Sal*I fragment of pAC602 by the appropriate *Hind*III-*Sal*I fragment of pKC31 (THALER, STAHL and STAHL 1987a). The two *Eco*RI sites on pAC602 were destroyed by *Eco*RI-cleavage, conversion of 5' single-stranded ends to blunt ends by DNA polymerase I (Klenow fragment) and religation, to yield pMS804. The *luxA luxB* duplication in pMS805 was achieved by replacing an *Eco*RV-*Sal*I fragment of pMS804 by the appropriate *Bal*I-*Sal*I fragment of pAP601 (NUSSBAUM and COHEN 1988).

λ ZS820 is isogenic to λ MS805, except that the cloned recombination substrate in λ ZS820 lacks the pACYC184 (CHANG and COHEN 1978) replication origin. To construct the cloned substrate in λ ZS820, pMS805 and pBR322 (BOLLIVAR *et al.* 1977) were cleaved by *Eco*RI and fused by ligation (pZS819). pACYC184 origin was then deleted from the hybrid plasmid by digestion with *Sac*II and *Xba*I, conversion of the protruding 3' and 5' ends to blunt ends by T4 DNA polymerase-mediated synthesis and end-digestion (SAMBROOK, FRITSCH and MANIATIS 1989) and ligation (pZS820). The recombination substrate was separated from pBR322 by *Eco*RI digestion and ligated to *Eco*RI-generated λ EMBL4 arms (λ ZS820). To minimize recombination during phage stock preparation, λ MS805 and λ ZS820 were grown on a *recA recD* strain (AC217). *Eco*RI-modified phage was grown on an AC217 derivative harboring pMB4.

Analysis of recombinants: To select for Kan^R recombinants, samples taken 120 min following infection were plated on kanamycin-supplemented solid media. Kan^R cell-frequency was defined as the ratio of Kan^R cells to infected cells in the culture. Assuming a Poisson distribution of phage entering each unit of surface area, the calculated proportion of infected cells at moi of 0.2 was 19.5% and at moi of 2.0

TABLE 2
Plasmids and phage

Plasmid or phage	Vector	Description	<i>luxA</i> mutation site ^a	Reference or source
pMB4		Amp ^R EcoRI ⁺	n.r. ^b	BETLACH <i>et al.</i> (1976)
pBR322	pMB1	Amp ^R Tet ^R	n.r.	BOLIVAR <i>et al.</i> (1977)
pKC31	λ dv	Kan ^R	n.r.	THALER, STAHL and STAHL (1987a)
pAP601	pBR322	Amp ^R LuxA ⁻ LuxB ⁺	HindIII	NUSSBAUM and COHEN (1988)
pAC602	pACYC184	Cam ^R LuxA ⁻ LuxB ⁺	XmnI	NUSSBAUM and COHEN (1988)
pMS804	pACYC184	Kan ^R LuxA ⁻ LuxB ⁺	XmnI	This work
pMS805	pACYC184	Kan ^R LuxA ⁻ LuxB ⁺	XmnI/HindIII ^c	This work
pZS819	pBR322/pACYC184	Kan ^R Amp ^R Tet ^R LuxA ⁻ LuxB ⁺	XmnI/HindIII	This work
pZS820	pBR322	Kan ^R Amp ^R Tet ^R LuxA ⁻ LuxB ⁺ Δ Ori _{pACYC184} ^d	XmnI/HindIII	This work
pMS807	pACYC184	Kan ^R LuxA ⁺ LuxB ⁺	+	This work
pMS808	pACYC184	Kan ^R LuxA ⁻ LuxB ⁺	XmnI	This work
pMS809	pACYC184	Kan ^R LuxA ⁻ LuxB ⁺	HindIII	This work
λ cl(Ind ⁻)		Ind ⁻	n.r.	A. OPPENHEIM
λ EMBL4		Red ⁺	n.r.	FRISCHAUF <i>et al.</i> (1983)
λ MS805	λ EMBL4	Kan ^R Red ⁻ LuxA ⁻ LuxB ⁺	XmnI/HindIII	This work
λ ZS820	λ EMBL4	Kan ^R Red ⁻ LuxA ⁻ LuxB ⁺ Δ Ori _{pACYC184}	XmnI/HindIII	This work

^a For the location of the indicated restriction sites, see Figure 1.

^b n.r. indicates not relevant.

^c Indicates a duplication of the *luxA luxB* genes. One copy has a *luxA* mutation at the HindIII site and the other at the XmnI site.

^d Indicates deletion of a fragment with pACYC184 replication origin from the cloned recombination substrate.

was 85.4%. For the determination of the Lux phenotype of Kan^R recombinants, individual colonies were grown overnight in 2 ml of L-broth supplemented with kanamycin, and bioluminescence activity of diluted samples was determined as described below. To determine *luxA* genotypes, plasmid preparations made from the overnight cultures by the method of HOLMES and QUIGLEY (1981) were subjected to restriction endonuclease analysis, using enzymes that cut at sites that had been destroyed (*HindIII*) or created (*BglII*) as genetic markers on pMS805.

The bioluminescence recombination assay: Bioluminescence activity of cultures infected by λ MS805 was determined essentially as described by NUSSBAUM, SHALIT and COHEN (1992). Phage were allowed to adsorb to cells suspended in TM (10 mM Tris, pH 7.4, 10 mM MgSO₄), by incubation for 30 min. at 0°. The suspension was then transferred to L-broth, prewarmed to 38° and supplemented with 1 mM IPTG. Incubation on a gyrotory shaker was at 37° for 5 min and then at 28°. Transfer to the prewarmed medium marked time 0 in all experiments. Bioluminescence was measured with a liquid scintillation spectrometer, set to read 10⁶ cpm for a suspension of 10⁷ AC177 cells grown on L-broth containing 1 mM IPTG.

Heteroduplex analysis: To prepare artificial mixtures of homoduplex and heteroduplex fragments with a single mismatch at the XmnI site (8 mispaired bases) of the *luxA* gene, pAC600 (*luxA*⁺) and pAC602 (*luxA*⁻) DNAs were mixed, cleaved by *XhoI*, heat-denatured (94° for 5 min) and annealed (65° for 5 min). Mixtures of homoduplexes and heteroduplexes with mismatches at the XmnI and HindIII (4 mispaired bases) sites were prepared by a similar method except that pAP601 [*luxA*⁻ (*HindIII*⁻)] was substituted for pAC600 in the denaturation-annealing reaction. The mixture of homoduplexes and heteroduplexes was digested with *PvuII* or with *PvuII* and *NdeI* and subjected to electrophoresis on 4% polyacrylamide gels in TBE buffer (NAGAMINE,

CHAN and LAU 1989). The separated fragments were analyzed by the SOUTHERN (1975) hybridization procedure using the appropriate ³²P-labeled *NdeI-PvuII* fragment of the *luxA* gene as a probe. To distinguish between the two complementary heteroduplexes, the separated fragments were hybridized to two complementary oligonucleotide probes, specific for the mutated *luxA* sequence. These probes consisted of a 6-nt sequence of the *BglII* linker inserted at the XmnI site and the adjacent 13 nt on the *luxA* gene. Sequences of the two probes homologous to the strands ending 5' and 3' at the break of *EcoRI*-restricted λ ZS820 were 5'GATCTGCATTCAACGTGAT3' and 5'ATCACGTTGAATGCAGATC3', respectively.

Hybridization: Total DNA (plasmid and chromosomal) of samples taken at the indicated times following infection was prepared as described elsewhere (NUSSBAUM, SHALIT and COHEN 1992) except that samples were poured into a KCN-EDTA solution to give a final concentration of 0.01 M KCN and 0.005 M EDTA before centrifugation. Hybridization of electrophoretically separated DNA fragments was according to the SMITH and SUMMERS (1980) (Figure 3) or CHURCH and GILBERT (1984) (Figures 5 and 6) modifications of the SOUTHERN (1975) procedure. When oligonucleotide probes were used, hybridization was at 42°. Blotting of DNA fragments from agarose or polyacrylamide gels to nitrocellulose filters (SCHLEICHER and SCHUELL BA-58S) was by capillary transfer. To prevent sticking of the polyacrylamide gel to the nitrocellulose filter, a slab of 2–3-mm thick 0.4% agarose gel (SeaKem LE) in TBE buffer was placed on top of the polyacrylamide gels before applying the nitrocellulose filters.

RESULTS

Restriction-stimulated intramolecular recombination: The plasmid intramolecular recombination

FIGURE 1.— λ MS805: A substrate for restriction endonuclease-induced intramolecular recombination. *In vivo* restriction of the infecting phage DNA by *EcoRI* endonuclease releases a linear plasmid recombination substrate with direct terminal repeats. The mutations at the recombining markers on the *luxA* gene are a 4 nucleotide insertion at the *HindIII* site and an 8 nucleotide *BglII* linker, inserted at the *XmnI* site. The location of the plasmid's origin of replication (*ori*), *kan*, *luxA* and *luxB* genes and the relevant restriction sites are indicated. Homology is designated by parallel boxes, mutations by triangles and λ arms (not to scale) by broken lines.

substrate, pMS805, has a direct duplication of the *V. fischeri luxA luxB* luciferase genes. Each repeat is mutated at a different restriction site in the *luxA* gene. pMS805 was linearized by *EcoRI* digestion and cloned between the *EcoRI* sites in the λ EMBL4 vector to yield λ MS805 (Figure 1). Infection of *EcoRI*⁺ cells by λ MS805 leads to a restriction-mediated release of linear recombination substrates with direct terminal repeats within the cell. With this type of substrate, recombination is conveniently monitored by measuring bioluminescence activity of *luxA*⁺ recombinants or by Southern hybridization analysis of DNA from infected cells (NUSSBAUM, SHALIT and COHEN 1992). To repress a lytic cycle by phage that had escaped *EcoRI* restriction, λ *cl*(Ind⁻) lysogens were used in all experiments.

To monitor RecE-mediated intramolecular recombination, *recB recC sbcA* [λ *cl*(Ind⁻)] cells (AC224), expressing *EcoRI* endonuclease from pMB4, were infected with λ MS805. Samples taken at time intervals after infection were analyzed for bioluminescence (Figure 2) and for the molecular structure of substrate derivatives in DNA preparations (Figure 3). The effect of *in vivo* restriction on recombination was ascertained by comparing bioluminescence kinetics and hybridization patterns of DNA preparations of infected *EcoRI*⁺ cells to that of infected *EcoRI*⁻ cells, or to that of *EcoRI*⁺ cells infected with phage that had been subjected to *EcoRI* modification.

Bioluminescence was first detectable 90 min following λ MS805 infection of *EcoRI*⁺ cells and its level increased thereafter (Figure 2). Bioluminescence activity of the infected cultures was restriction-dependent. At 180 min after infection, the bioluminescence

FIGURE 2.—The dependence of intramolecular recombination on *EcoRI* restriction. Cultures of AC224[*recB recC sbcA* (λ *cl*(Ind⁻))] cells that harbor or do not harbor pMB4(*EcoRI*⁺) were infected with λ MS805 or with λ MS805 bearing modified *EcoRI* restriction sites. Bioluminescence activity of samples taken at the indicated times following infection was determined as described.

activity of *EcoRI*⁺ cells infected with nonmodified phage, was 50–100-fold higher than that of *EcoRI*⁻ cells infected by the same phage or that of *EcoRI*⁺ cells infected with *EcoRI*-modified phage.

The genetic requirements for recombinant activity in *recB recC sbcA* mutants, as measured by the bioluminescence assay, were similar to the reported requirements for plasmid recombination (LABAN and COHEN 1981; FISHEL, JAMES and KOLODNER 1981; LUISI-DELUCA, LOVETT and KOLODNER 1989). Bioluminescence was not detectable in infected *recE recB recC sbcA* [λ *cl*(Ind⁻)] cells. In *recA recB recC sbcA* [λ *cl*(Ind⁻)] cells bioluminescence activity was about twofold higher than that in the isogenic *recA*⁺ cells (data not presented).

FIGURE 3.—Physical monitoring of intramolecular recombination. DNA preparations of samples taken at the indicated times after λMS805 infection of AC224 cells that harbor or do not harbor pMB4 were digested with *SalI* endonuclease and subjected to the Southern hybridization procedure using a 32 P-labeled *HindIII-SalI* fragment that carries the *kan* gene (see Figure 1) as a probe. The locations of molecular length standards are indicated. The expected locations of the *SalI* digestion products of *EcoRI*-restricted λMS805 (filled arrow) and plasmid recombination products (open arrow) are indicated.

To monitor restriction-mediated release of linear recombination substrates and formation of recombination products, total cellular DNA preparations of λMS805-infected cells were digested by *SalI* endonuclease and subjected to Southern hybridization analysis, using a probe homologous to the *kan* gene on pMS805 (Figure 3). The *SalI* product of *EcoRI*-restricted λMS805 was identified in DNA preparations of samples taken immediately following infection. Fragments that correspond in electrophoretic mobility to *SalI*-digested linear and circular λMS805 were also detectable in these preparations. The intensity of the band representing *EcoRI*-restricted λMS805 decreased with time following infection. A band that corresponded in electrophoretic mobility to a *SalI*-digested circular intramolecular recombination product was detectable in samples taken 30 min following infection. This product is isogenic to pMS805 but has a single copy of the *luxA luxB* genes. Replication of the plasmid recombination product probably contributed to the observed increase over time of the intensity of this band. As expected, restricted λMS805 was not detectable in DNA preparations of infected *EcoRI*⁻ cells. In these cells recombination products were not detectable before 180 minutes following infection. At this time, a faint band that comigrated with the *SalI*-digested recombination product was observed. The intensity of this band increased with time (not shown). The absence of a band that corresponds to the *SalI* product of circular pMS805 confirms an earlier indication that in *recB recC sbcA* mutants circularization of linear intramolecular recombination substrates is mainly by homologous recombination

TABLE 3
luxA genotype of plasmids in *kan*^R recombinants

<i>kan</i> ⁺ plasmid	<i>luxA</i> genotype		Frequency (%)	
	<i>HindIII</i>	<i>XmnI</i>	<i>mutS</i> ⁺ , <i>N</i> ^a = 145	<i>mut-</i> <i>S215::Tn10</i> , <i>N</i> = 180
pMS807	+	+	6.2	5
pMS807/pMS808 ^b	+	+	5.5	6.1
pMS807/pMS809	+	+	<0.7	<0.6
pMS808	+	—	68.3	66.7
pMS809	—	+	11.7	15
	—	—	<0.7	<0.6
pMS808/pMS809	+	—	8.3	7.2
	—	+		

The *luxA* genotype of Kan^R clones, isolated from λMS805-infected cultures, was determined by analyzing plasmids with the appropriate restriction endonucleases (see MATERIALS AND METHODS). The *mutS* genotype of the infected cultures is indicated.

^a *N* indicates the number of recombinants analyzed.

^b Designates a heteroallelic clone harboring the indicated plasmids.

^c Designates a heteroallelic clone harboring plasmids with the indicated genotype.

and not by end-ligation (SYMINGTON, MORRISON and KOLODNER 1985).

Plasmid recombination products: To score and select for clones harboring plasmid derivatives of the linear *kan*⁺ recombination substrate, samples of λMS805-infected cultures were plated on kanamycin-supplemented solid medium. When infection was at a phage multiplicity (moi) of 0.2, 10% of the infected cells yielded Kan^R clones and 12% of these clones were bioluminescent (Table 3). Similar frequencies of Kan^R LuxA⁻ and Kan^R LuxA⁺ clones were measured at a moi of 2.0 (data not shown).

To determine the molecular structure of recombination products, plasmid preparations of the isolated Kan^R clones were analyzed by restriction enzymes that cleave at sites that had been destroyed (*HindIII*) or created (*BglII*) in the construction of pMS805 (see Figure 1). The *luxA* genotypes of plasmids in Kan^R clones are presented in Table 3. All Kan^R clones harbored, in addition to pMB4, derivatives of pMS805 with a single copy of the *luxA luxB* genes. As expected, all Lux⁺ clones harbored *luxA*⁺ (*HindIII*⁺*XmnI*⁺) plasmids (pMS807). However, the electrophoretic pattern of *BglII*-digested plasmid DNA preparations of about half of the LuxA⁺ clones indicated the presence of an additional *kan*^R plasmid, with a *BglII* linker inserted at the *XmnI* site in the *luxA* gene (pMS808) (Figure 4). To test the heteroallelic nature of these bioluminescent clones, plasmid preparations were used to transform a *recA* mutant (DR100), and the Kan^R transformants were tested for bioluminescence and *luxA* genotype. Transformation with plasmid preparations of the putative heteroallelic

FIGURE 4.—*Bgl*III-digestion products of plasmids from Kan^R recombinants. Plasmid DNA preparations of a non-bioluminescent Kan^R clone (lane 1) and two bioluminescent Kan^R clones (lanes 2 and 3) were digested by *Bgl*III endonuclease and subjected to electrophoresis on 0.5% agarose gels. The locations of *Bgl*III fragments of pMB4 (11.5 kb), pMS807 (*luxA*⁺, 9.8 kb) and pMS808 (*luxA*[−], 5.7 and 4.1 kb) are indicated.

clones yielded a mixed population of Lux⁺ and Lux[−] transformants. Five LuxA⁺ and 5 LuxA[−] transformants were tested for the *luxA* genotype of the *kan*^R plasmids. All Lux[−] transformants harbored pMS808 and all Lux⁺ transformants harbored pMS807. Lux⁺ clones heteroallelic for the markers at the *Hind*III site were not observed.

Most Lux[−] clones harbored a *kan*⁺ plasmid with a single *luxA* mutation at the *Xmn*I site (pMS808). Some (12%) harbored a *kan*⁺ plasmid with a *luxA* mutation at the *Hind*III site (pMS809), and some (9%) harbored a mixed population of pMS808 (*Hind*III⁺ *Xmn*I[−]) and pMS809 (*Hind*III[−] *Xmn*I⁺). Single colony isolates of these heteroallelic clones harbored either one of the two *luxA*[−] plasmids or a mixture of both.

We have considered the possibility that mismatch repair activity may affect marker distribution in Kan^R clones. To test this possibility, we compared plasmid *luxA* genotypes in Kan^R recombinants of infected *mutS*⁺ and *mutS215* derivatives of *Eco*RI⁺ *recB recC sbcA* [λ cI(Ind[−])] cells (Table 3). The *mutS* mutation had little or no effect on *kan*^R plasmid genotypes or on the frequency of heteroallelic Kan^R clones. These results are consistent with the observation that heteroduplexes with heterologies longer than 3 nt are poorly corrected by a *mutS*-dependent mismatch-repair activity (PARKER and MARINUS 1992).

Kan^R clones with a mixed plasmid population may be produced by heteroduplex replication or by two independent recombination events, following multiple phage infection. The second possibility seems unlikely, in view of the observation that similar frequen-

FIGURE 5.—Formation of heteroduplex structures in λ ZS820-infected cells. Total DNA preparations of AC224 cells harboring pMB4 and infected by λ ZS820 were digested with *Nde*I and *Pvu*II (see Figure 1) and subjected to polyacrylamide gel electrophoresis and Southern hybridization, with the appropriate ³²P-labeled *Pvu*II-*Nde*I fragment as a probe. Samples were taken at time 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4) and 120 min (lane 5) following infection. Hybridization of an artificial heteroduplex-homoduplex mixture, digested by *Pvu*II and *Nde*I, to the same probe (lanes 6 and 8) or to two complementary oligonucleotide probes homologous to the mutated *luxA* gene (lanes 9 and 10) is presented. The hybridizing oligonucleotide probes in lanes 9 and 10 are complementary to the strands ending 5' and 3' at the break of *Eco*RI restricted λ ZS820, respectively. This hybridization pattern distinguishes between the heteroduplex made by pairing of the strands ending 3' at the break (3' het) and the heteroduplex made by pairing of the strands ending 5' at the break (5' het). The mixed DNA preparation in lane 7 is similar to that in lane 6, except that the two heteroallelic fragments were denatured and annealed separately before mixing. The probe used for hybridization in lane 8 was the same as in lane 6.

cies of heteroallelic clones were measured in cultures infected at a moi of 0.2 and 2.0 (data not shown).

Strand-specific heteroduplexes: Two chemically distinct heteroduplex types may be formed by pairing of complementary strands of restricted λ MS805, one by annealing the strands ending 3' at the break and the other by annealing the strands ending 5' at the break. Since the mismatch at the *Xmn*I site is an 8-nt insertion, the two types may be resolved from each other, and from their respective homoduplexes, by polyacrylamide gel electrophoresis (NAGAMINE, CHAN and LAU 1989). The *Xmn*I site is located on a 528-nt *Pvu*II-*Nde*I fragment. Electrophoretic resolution of an artificial mixture of *luxA*⁺ and *luxA*[−] (*Xmn*I[−]) *Pvu*II-*Nde*I fragments and the respective *Xmn*I⁺/*Xmn*I[−] heteroduplexes (see MATERIALS AND METHODS) is depicted in Figure 5. Two heteroduplex bands with an electrophoretic mobility lower than the corresponding homoduplex bands were detected by hybridization to the appropriate radioactive *Pvu*II-*Nde*I fragment (lane 6). These two bands were absent from *luxA*⁺ and *luxA*[−] homoduplex preparations, treated separately by the same denaturation-reannealing procedure that yielded the homoduplex-heteroduplex mixture (see Figure 5, lane 7). As expected, the two heteroduplexes resisted digestion by *Xmn*I or *Bgl*II restriction endonucleases (not shown). Each of the two bands hybridized to one of two complementary 19-mer probes that

specifically hybridize to the *XmnI*⁻(*Bgl*II⁺) allele but not to the *XmnI*⁺ allele of the *luxA* gene (Figure 5, lanes 9 and 10). The pattern of hybridization to the strand-specific oligonucleotide probes allowed the identification of the upper and lower heteroduplex bands as the annealing products of the strands ending 3' and 5' at the break, respectively.

Heteroduplex production was investigated in infected *EcoRI*⁺ *recB recC sbcA* [λ CI(Ind⁻)] cells. To block heteroduplex replication, λ ZS820 was used as the infecting phage. λ ZS820 is isogenic to λ MS805 except that a 592 nt *Sac*II-*Xba*I fragment, which includes the pACYC184 origin of replication (see Figure 1), was deleted from the cloned linear recombination substrate in λ ZS820. To monitor heteroduplex production, total cellular DNA preparations of samples taken at time intervals following λ ZS820 infection were digested with *Nde*I and *Pvu*II and subjected to Southern hybridization, with the appropriate ³²P-labeled *Pvu*II-*Nde*I fragment as a probe (Figure 5). A hybridizable fragment of an electrophoretic mobility similar to that of the heteroduplex formed by pairing of the strands ending 3' at the break was detectable in samples taken 15 min following infection (Figure 5, lane 2). This band was not detectable in samples taken immediately after infection (lane 1) or when the infecting phage harbored a recombination substrate with pACYC184 replication origin (λ MS805) (not shown). The intensity of the heteroduplex band did not increase appreciably following 15 min after infection (lanes 3–5). A band that corresponded in electrophoretic mobility to the heteroduplex made by pairing of strands ending 5' at the break was not detectable at any time after infection. The stability of the heteroduplex band for a period of 120 min after infection is consistent with the notion that the 8-nt heterology at the *Xmn*I site is poorly corrected by mismatch repair activity.

To test for the presence of the heteroduplex structure in closed circular products, clear lysate preparations (CLEWELL and HELINSKI 1969) of samples taken 60 min after λ ZS820 infection were fractionated by cesium chloride-ethidium bromide density gradient centrifugation, and the plasmid fractions were tested for the presence of heteroduplex structures as described (MATERIALS AND METHODS). Hybridization of *Pvu*II-*Nde*I fragments of plasmid preparations from λ ZS820-infected cells revealed the presence of a heteroduplex formed by pairing of the strands ending 3' at the break (Figure 6, lane 1).

Two types of heteroallelic Kan^R clones were observed in λ MS805-infected cultures: LuxA⁺ clones, heteroallelic for the *Xmn*I site, and LuxA⁻ clones, heteroallelic for both the *Xmn*I and the *Hind*III sites (Table 3). We have attempted to resolve the corresponding heteroduplex structures, with a mismatch at

FIGURE 6.—Circular heteroduplex recombination products. Plasmid preparations of AC224 cells harboring pMB4 and infected by λ ZS820 were digested by *Pvu*II and *Nde*I (lane 1) or *Pvu*II and *Xho*I (lane 3) and analyzed by polyacrylamide gel electrophoresis and Southern hybridization, with the appropriate ³²P-labeled *Pvu*II-*Nde*I fragment as a probe. Molecular markers are artificial heteroduplex-homoduplex mixtures digested with *Pvu*II and *Nde*I (lane 2) or *Pvu*II and *Xho*I (lanes 4–7), carrying a mismatch at the *Xmn*I site (lanes 2, 5, 7) or mismatches at both the *Xmn*I and *Hind*III sites (lanes 4 and 6). Hybridization of the heteroduplex-homoduplex preparations was to the ³²P-labeled *Pvu*II-*Nde*I fragment (lanes 2, 6 and 7) or to the ³²P labeled oligonucleotide probe, complementary to the strand ending 3' at the break (lanes 4 and 5). The mixed DNA preparation in lane 8 is similar to that in lane 6, except that the two heteroallelic fragments were denatured and reannealed separately before mixing. The probe used for hybridization in lane 8 was the same as in lane 6. Arrows indicate the position of 3' heteroduplexes. Electrophoresis at 300 V was for 1 hr (lanes 1 and 2) or 3 hr (lanes 3–8).

the *Xmn*I site or mismatches at both the *Xmn*I and *Hind*III sites, in plasmid preparations of λ ZS820-infected cultures. The *Hind*III and *Xmn*I sites are located on a 912 nt *Pvu*II-*Xho*I DNA fragment (Figure 1). Artificial mixtures of homoduplexes and heteroduplexes with a mismatch at the *Xmn*I site or mismatches at both the *Xmn*I and *Hind*III sites were prepared, digested by *Pvu*II and *Xho*I and resolved by polyacrylamide gel electrophoresis; the electrophoretic mobility of heteroduplexes with mismatches at the *Xmn*I and *Hind*III sites (lanes 4 and 6) is slightly lower than that of the corresponding heteroduplexes with a mismatch at the *Xmn*I site (lanes 5 and 7). To analyze heteroduplexes in plasmid preparations of λ ZS820-infected cells, *Pvu*II-*Xho*I fragments were subjected to polyacrylamide gel electrophoresis and hybridization to a radioactive *Nde*I-*Pvu*II fragment (Figure 6, lane 3). In addition to the homoduplex band, a double band that migrated with the artificial heteroduplexes, formed by annealing of the strands ending 3' at the break of restricted λ ZS820, was observed. Of this double band, the lower one corresponded in electrophoretic mobility to the *Pvu*II-*Xho*I heteroduplex fragment with a mismatch at the *Xmn*I site, and the upper one to the heteroduplex fragment with mismatches at both the *Xmn*I and *Hind*III sites.

The density of the upper band was higher than that of the lower band.

DISCUSSION

Intramolecular recombination of linear substrates in *recB recC sbcA* cells is *recA*-independent (SYMINGTON, MORRISON and KOLODNER 1985). It does depend on the activity of *recE* gene, which encodes a DSB-specific 5' to 3' exonuclease (KUSHNER, NAGAISHI and CLARK 1974; JOSEPH and KOLODNER 1983) and a single-strand annealing activity (S. HALL, M. KANE and R. KOLODNER, submitted for publication). The dependence on activities that may generate and anneal 3' single-stranded DNA ends is consistent with the SSA recombination model. This model predicts the formation of heteroduplex structures by pairing the strands ending 3' at the break (LIN, SPEARLE and STERNBERG 1984). Here we have tested this prediction and verified it. When replication of recombination products was blocked by deletion of the plasmid replication origin in the linear substrate, heteroduplexes were observed. Two possible types of heteroduplex structures may be formed by pairing of restricted λ 820 complementary strands, but only those formed by pairing the strands ending 3' at the break were detected. While the genetic requirements favor the SSA model, the results presented here do not discriminate against models that postulate strand-specific pairing following 3' end invasion (SYMINGTON, MORRISON and KOLODNER 1985; MARYON and CARROLL 1991).

We note that, with some constraints, the observed strand bias in heteroduplex formation may also be explained by models that postulate reciprocal exchange between homologous duplex regions, leading to the formation of Holliday junctions (SYMINGTON, MORRISON and KOLODNER 1985). By this mechanism, branch migration that follows the initial strand exchange yields two chemically distinct heteroduplexes (HOLLIDAY 1964). Intramolecular recombination by the linear substrate, depicted in Figure 1, would incorporate one of the two heteroduplexes into a circular crossover product (Figure 7). The structure of the heteroduplex in the circular product would be determined by the polarity of the strands that are initially exchanged and by the site of the reciprocal exchange (Figure 7). Therefore, to accommodate the observed strand bias by models that postulate heteroduplex formation by reciprocal exchange and branch migration, one must assume that the initial exchange is strand-specific and that it occurs on one side of the recombining markers. An alternative explanation for the observed strand bias is instability of 5' heteroduplexes due to repair synthesis, primed from the 3' ends into the heteroduplex region.

Another feature of the investigated recombination

FIGURE 7.—The expected effect of recombination mechanism on heteroduplex structure. One heteroduplex type is expected when linear substrates with direct terminal repeats (parallel lines) recombine by a SSA mechanism (right pathway). Exonuclease processing in a 5' to 3' direction allows the formation of a heteroduplex structure by annealing the homologous strand ending 3' at the break. Two heteroduplex types would be formed by migration of a Holliday junction that follows reciprocal strand exchange in a duplex DNA region (left pathway). The structure of the heteroduplex incorporated into the circular crossover product is determined by the polarity of the strands initially exchanged, and by site of the reciprocal exchange.

reaction is the frequent loss of the mutated *HindIII* and the non-mutated *XmnI* sites from the plasmid recombination products (Table 3). These two markers are located at a distance of 776 and 1,291 nt from one end of the linear substrate, respectively. The frequently incorporated markers—the mutated *XmnI* site and the nonmutated *HindIII* site are located at a distance of 2,403 and 2,918 nt from the other end, respectively (see Figure 1). Loss of markers that are close to the break is also apparent from heteroduplex structures. All the recombinant heteroduplex for the *HindIII* marker are heteroduplex for the *XmnI* marker as well. Conversely, among the recombinants heteroduplex for the *XmnI* marker about half have lost the *HindIII* marker that is close to the break. If recombination involves pairing of 3' single-stranded tails, the frequent loss of markers that are close to the end may be explained by their relative susceptibility to end-degradation. If pairing is by 3' end-invasion, degradation must be presynaptic. On the other hand, recombination by the SSA mechanism allows either pre- or postsynaptic end-degradation. Postsynaptic degradation of the 3' end may occur if annealing along the entire length of the homology is prevented by limited degradation of the strands ending 5' at the break. In such an event, sequences that are close to the end may not pair, and circles with single-stranded tails may be produced (Figure 7). Postsynaptic trim-

FIGURE 8.—A model to account for the distribution of genetic markers in recombination by restricted λ MS805. X and H designate *Xmn*I and *Hind*III sites on the *luxA* gene. (▼) designates a mutation and arrows 3' ends. Exonucleolytic processing of the break yields a gap, bounded by complementary 3' single-stranded DNA tails. Both ends may be processed to the same degree (center and right lanes), or one end may be processed to a higher degree than the other (left lane). Enlargement of the gap beyond the mutated *Hind*III site (center), or beyond the nonmutated *Xmn*I site (right) (B), complementary strand-annealing (C), and repair synthesis (D) yield recombinants with a heteroduplex structure at the *Xmn*I site and a non-mutated *Hind*III site (center) or with a mutated *Xmn*I site and non-mutated *Hind*III site (right). If 3' end-processing does not reach the *Hind*III site, a heteroduplex with a double mismatch, at the *Hind*III and *Xmn*I sites, will be formed (not shown). Preferential degradation of the substrate arm with the nonmutated *Hind*III site (left) would yield a recombinant with a *luxA* mutation at the *Hind*III site.

ming of the unpaired single-stranded tails may lead to the loss of markers that are close to the end.

The model in Figure 8 attempts to explain the distribution of *luxA* genotypes in recombination products by the rules of the SSA model and the assumption that end-degradation occurs, as postulated for the DSB-repair model (RESNICK 1976; ORR-WEAVER and SZOSTAK 1983), before homologous pairing. A *recE* product (exonuclease VIII) processes a DSB to yield 3' single-stranded overhangs (KUSHNER, NAGAISHI and CLARK 1974; JOSEPH and KOLODNER 1983) that may serve as substrates in the homologous pairing reaction. If one 3' single-stranded overhang is degraded to a point between the mutated *Hind*III site and the *Xmn*I site (central pathway), strand annealing would yield a circle with a single-stranded gap at the *Hind*III site and a mismatch at the *Xmn*I site. Gap-repair and replication would generate clones heter-

oallelic for the *Xmn*I site, as observed in about half of the *Lux*⁺ recombinants (Table 3). If end-degradation does not reach the *Hind*III site (not shown), a heteroduplex with mismatches at the *Hind*III and *Xmn*I sites would yield the observed *Lux*⁻ heteroallelic clones. The difference in intensity between the two heteroduplex bands (Figure 6, lane 3) suggests that end degradation stops more frequently in the 776-nt interval between the break and the mutated *Hind*III site (interval III) than in the 515-nt interval between the mutated *Hind*III and *Xmn*I sites (interval II). If end-degradation proceeds to a point beyond the *Xmn*I site (right pathway), annealing of the single-stranded overhangs and repair of the single-stranded gap would yield the abundant pMS808. To generate pMS809 (*Hind*III⁻*Xmn*I⁺) by a similar mechanism (left pathway), the end that is distal to the mutated *Hind*III site must be degraded to a larger extent than the end that is closer to this mutation. Such degradation would allow repair of the single-stranded gap with the strand carrying the mutated *Hind*III site serving as a template. Loss of one of the two incompatible plasmids during propagation of heteroallelic recombinants may have yielded some of the clones with a single, *luxA*⁺ or *luxA*⁻, plasmid population.

The observed marker distribution in circular recombination products may also be explained by a mechanism that involves crossover events. The ratio of the *Hind*III⁺*Xmn*I⁻:*Hind*III⁺*Xmn*I⁺:*Hind*III⁻*Xmn*I⁺ recombination products genotypes is 6.5:1:1.7 (Table 3). These plasmid genotypes would have been generated by crossover events in intervals I, II, and III (Figure 1), respectively. The length ratio of these recombination intervals (4.1:1:1.5) is roughly proportional to the ratio of the corresponding genotypes.

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