Restriction-Stimulated Homologous Recombination of Plasmids by the RecE Pathway of *Escherichia coli*

Anat Nussbaum, Moshe Shalit and Amikam Cohen

Department of Molecular Genetics, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

ABSTRACT

To test the double-strand break (DSB) repair model in recombination by the RecE pathway of *Escherichia coli*, we constructed chimeric phages that allow restriction-mediated release of linear plasmid substrates of the bioluminescence recombination assay in infected $EcoRI^+$ cells. Kinetics of DSB repair and expression of recombination products were followed by Southern hybridization and by the bioluminescence recombination assay, respectively. Plasmid recombinants were analyzed with restriction endonucleases. Our results indicate that a DSB can induce more than one type of RecE-mediated recombination. A DSB within the homology induced intermolecular recombination that followed the rules of the DSB repair model: (1) Recombination was enhanced by in vivo restriction. (2) Repair of the break depended on homologous sequences on the resident plasmid. (3) Break-repair was frequently associated with conversion of alleles that were *cis* to the break. (4) Conversion frequency decreased as the distance from the break increased. (5) Some clones contained a mixture of plasmid recombinants as expected by replication of a heteroduplex in the primary recombinant. The rules of the DSB repair model were not followed when recombination was induced by a DSB outside the homology. Both the cut and the uncut substrates were recipients in conversion events. Recombination events were associated with deletions that spanned the break site, but these deletions did not reach the homology. We propose that a break outside the homology may stimulate a RecE-mediated recombination pathway that does not involve direct participation of DNA ends in the homologous pairing reaction.

G ENETIC, physical and enzymatic analysis of homologous recombination in *Escherichia coli* suggests that DNA ends play a role in at least two distinct types of recombination pathways. In recombination by the RecBCD pathway, DNA ends are assumed to serve as entry-points for the RecBCD enzyme (KOBAYASHI *et al.* 1982; TAYLOR and SMITH 1985) that tracks the DNA processively (TAYLOR *et al.* 1985; STAHL *et al.* 1986), causing local denaturation at the regions of its presence (ROSAMOND, TELANDER and LIN 1979; TAYLOR and SMITH 1980). At specific DNA sequences (Chi sites), the RecBCD enzyme acts as a sequence-specific endonuclease (PONTICELLI *et al.* 1985; CHENG and SMITH 1987) that yields a putative substrate for the RecA-catalyzed homologous pairing reaction (for review see SMITH 1987, 1988). When recombination is mediated by the λ Red pathway, the closely related *E. coli* RecE pathway, and possibly the *E. coli* RecF pathway, DNA ends are proposed to be involved directly in the homologous pairing reaction. Several observations indicate a direct role for DNA ends in recombination by these pathways: the enhancing effect of DNA breaks on recombination (SYMINGTON, MORRISON and KOLODNER, 1985; STAHL, KOBAYASHI and STAHL, 1985; THALER, STAHL and STAHL, 1987a; THALER *et al.* 1989), the concentration

of crossover events at ends and breaks (STAHL *et al.* 1974; THALER, STAHL and STAHL 1987b), and the enzymatic activities of gene products that function in these pathways (JOSEPH and KOLODNER 1983; LOVETT and KOLODNER 1989; RADDING 1966).

By using intramolecular recombination substrates with double-strand breaks (DSB)[1] and analyzing plasmid recombinants, KOBAYASHI and TAKAHASHI (1988) have demonstrated that a DSB stimulates RecE-mediated recombination by a mechanism similar to that of the model put forth for DSB repair in yeast (RESNICK 1976; ORR-WEAVER and SZOSTAK 1983). According to the DSB-repair model, recombination is initiated at a DSB by exonucleolytic processing of the break to form a gap with overhanging 3′-single-stranded DNA ends. Invasion of a homologous duplex DNA by the 3′-single-stranded ends primes repair synthesis. This is followed by ligation and the formation of a pair of Holliday junctions that flank the repaired gap. Migration of the Holliday junctions elongates heteroduplex regions. Resolution of the two junctions in the same sense yields non-crossover products, and in the opposite sense, crossover products.

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[1] Abbreviations used: DSB, double-strand break; bp, base pair; moi, multiplicity of infection; $EcoRI^+$ or $EcoRI^-$ cells, cells that express or do not express EcoRI restriction-modification enzymes; IPTG, isopropyl-α-D-thiogalactopyranoside.

TABLE 1

E. coli strains

Strain[a]	Relevant genotype						Source or reference
	recA	recB	recC	sbcA	recE	Other	
AB1157	+	+	+	del	del		BACHMANN (1972)
JC8679	+	21	22	23	+		GILLEN et al. (1981)
JC8691	+	21	22	23	159		A. J. CLARK
JC9604	13	21	22	23	+		GILLEN et al. (1981)
DR100	del	+	+	del	del		LABAN and COHEN (1981)
AC165	+	21	22	23	+	(λ)	This work[b]
AC166	13	21	22	23	+	(λ)	This work[c]
AC173	+	21	22	23	159	(λ)	This work[d]
AC177	+	+	+	del	del	nhaA5::luxA⁺ luxB⁺ kan⁺	This work[e]

[a] All strains listed except DR100 encode: thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 txs-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1.
[b] λ(wt) lysogen of JC8679.
[c] λ(wt) lysogen of JC9604.
[d] λ(wt) lysogen of JC8691.
[e] Constructed by recombinational insertion of a linearized pBR322 derivative carrying the luxA luxB and kan genes and flanked by nhaA5 sequences into the homologous site of E. coli (JC7623) followed by P1 transduction to AB1157.

The RecE recombination pathway is functional in E. coli recB recC sbcA mutants (BARBOUR et al. 1970). sbcA mutations activate this pathway by promoting expression of the rac prophage recE gene that encodes exonuclease VIII (KUSHNER, NAGAISHI and CLARK 1974; GILLEN, WILLIS, and CLARK 1981). Exonuclease VIII may participate in recombination by a DSB-repair mechanism by digestion from the DSB, in a 5'→3' direction, to yield a recombinogenic 3'-single-stranded DNA end (JOSEPH and KOLODNER 1983).

To further investigate break-stimulated recombination in E. coli, we have constructed λ vector-based recombination substrates that facilitate kinetic studies and physical monitoring of break-stimulated recombination, as well as structural analysis of recombinants. These molecular constructs consist of plasmid substrates of the bioluminescence recombination assay (NUSSBAUM and COHEN 1988) ligated at or near EcoRI sites to λ phage arms. In vivo restriction of the chimera phage DNA releases linear plasmid recombination substrates in infected EcoRI⁺ cells, and in the appropriate genetic background, DSB-stimulated recombination is initiated. In this paper we report the application of this experimental system to physical and genetic analysis of DSB-stimulated recombination by the RecE pathway of E. coli.

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). All lysogens were lysogenic for λ wild-type. Cultures were grown in L-broth medium (LURIA and BURROUS 1957). Strains harboring plasmids were grown in media supplemented with the appropriate antibiotics (100 μg/ml ampicillin, 20 μg/ml kanamycin). Growth conditions of phage-infected cultures are described below.

Plasmids and phage: Plasmids used in this study are listed in Table 2. pAN603 is a pBR322 (BOLIVAR et al. 1977) derivative, with the luxA and luxB genes of Vibrio fischeri cloned downstream of the lac promoter. The luxA gene in this plasmid was mutated by insertion of a 8-bp BglII linker at the XmnI site. pAN603 was constructed by replacing a XhoI-SalI fragment of pAP601 (NUSSBAUM and COHEN 1988) with the homologous fragment of pAC602, carrying the XmnI mutation (NUSSBAUM and COHEN 1988). pAN601 and pAN602 (see Figure 1) were constructed by ligating a ClaI-SalI fragment of pMB4 (BETLACH et al. 1976; NEWMAN et al. 1981), that codes for EcoRI restriction-modification enzymes, to the ClaI-SalI fragments of pAP601 (NUSSBAUM and COHEN 1988) or pAN603, respectively. pAN606 and pAN607 are pACYC184 (CHANG and COHEN 1978) derivatives, with a kan gene of pKC31 (THALER, STAHL and STAHL 1987a) inserted between the HindIII and SalI sites, and luxA luxB genes, expressed from a lac promoter, inserted between the SalI and NruI sites of pACYC184. The XhoI site in the luxA gene of pAN606 was mutated by restriction, DNA polymerase I (Klenow fragment)-mediated filling of the recessed 3' termini, and blunt-end ligation (SAMBROOK, FRITSCH and MANIATIS 1990). The EcoRI site in the cat gene of pAN607 was mutated by a similar procedure. pAN611 is a heterodimer, constructed by ligating XhoI endonuclease-digested pAN607 and pAN602 and selecting for KanᴿAmpᴿ DR100 transformants. The phage precursor of the linear plasmid recombination substrate, λAN607 (see Figure 1), was constructed by ligating XhoI-digested pAN607 to λ phage arms generated by SalI digestion of λEMBL4 (FRISCHAUF et al. 1983) DNA. λAN606 was constructed by ligating λ phage arms generated by EcoRI endonuclease digestion of λEMBL4 to EcoRI-digested pAN606. bet, exo and gam are deleted in λAN606 and λAN607. Phage stocks were grown on AB1157 cells, EcoRI-modified phage was grown on an AB1157 derivative harboring pMB4.

The bioluminescence recombination assay: Infection of cells by the appropriate phage precursors of the bioluminescence assay substrates was essentially as described by BETTER and FREIFELDER (1983). Lysogenic E. coli cells, harboring plasmids that express EcoRI restriction-modification enzymes, were grown in 10 ml L-broth supplemented with 10 mM MgSO₄ and 0.2% maltose to a concentration of 2 × 10⁸ cells/ml. Cells were harvested by centrifugation,

TABLE 2

Plasmids and phage

Plasmid	Vector	Description	luxA mutation site[a]	Reference
pMB4		Amp^{R}EcoRI$^+$	n.r.[b]	BETLACH et al. (1976)
pAP601	pBR322	Amp^{R}LuxA$^-$LuxB$^+$	HindIII	NUSSBAUM and COHEN (1988)
pAC602	pACYC184	Cam^{R}LuxA$^-$LuxB$^+$	XmnI	NUSSBAUM and COHEN (1988)
pAN601	pBR322	Amp^{R}EcoRI$^+$LuxA$^-$LuxB$^+$	HindIII	This work
pAN602	pBR322	Amp^{R}EcoRI$^+$LuxA$^-$LuxB$^+$	XmnI	This work
pAN603	pBR322	Amp^{R}LuxA$^-$LuxB$^+$	XmnI	This work
pAN606^c	pACYC184	Kan^{R}Cam^{R}LuxA$^-$LuxB$^+$	XhoI	This work
pAN607	pACYC184	Kan^{R}LuxA$^+$LuxB$^+$	+	This work
pAN611^d	pACYC184/pBR322	Kan^{R}Amp^{R}LuxA$^+$LuxB$^+$	+/XmnI	This work
pAN613^e	pACYC184	Kan^{R}LuxA$^-$LuxB$^+$	XmnI	This work
pAN616^e	pBR322	Kan^{R}Amp^{R}LuxA$^+$LuxB$^+$	+/XhoIXmnI	This work
λEMBL4	λ	Red$^+$		FRISCHAUF et al. (1983)
λAN606	λEMBL4	Kan^{R}Red$^-$LuxA$^-$LuxB$^+$	XhoI	This work
λAN607	λEMBL4	Kan^{R}Red$^-$LuxA$^-$LuxB$^+$	XhoI	This work

[a] For the location of the restriction sites, see Figure 1.
[b] n.r. indicates not relevant.
[c] pAN606 has a single EcoRI site in the cat gene (see Figure 1).
[d] pAN611 is a heterodimer consisting of pAN607 and pAN602 monomers.
[e] pAN613 and pAN616 have deletions that flank the cut site. For a detailed map, see Figure 6.

suspended in 1 ml of TM buffer (10 mM Tris-HCl, pH 7.4, 10 mM MgSO$_4$) at 0° and phage was added at the indicated multiplicity. To allow phage adsorption, the suspension was incubated for 30 min at 0° and then transferred to L-broth, prewarmed to 37° and supplemented with 1 mM IPTG. The infected culture was incubated on a gyrotory shaker at 28°. One ml aliquots were taken at the indicated times and bioluminescence was measured in a liquid scintillation spectrophotometer as described before (NUSSBAUM and COHEN 1988). Transfer to prewarmed L-broth marked the time zero of all experiments. Sensitivity of the liquid scintillation spectrophotometer was set to read 10^6 cpm for a suspension of 10^7 AC177 cells carrying chromosomally cloned luxA luxB genes, expressed from the lac promoter, under induced conditions.

Hybridization procedure: To monitor recombination in infected cultures, 1.5-ml samples were taken at the indicated times following infection and total (plasmid and chromosomal) DNA was prepared as described (SILBERSTEIN et al. 1990). Hybridization of digested DNA preparations was by the SMITH and SUMMERS (1980) modification of the SOUTHERN (1975) procedure, with a ^{32}P-labeled ScaI-EcoRI fragment of pACYC184, homologous to the indicated fragment in λAN607 (see Figure 1).

Analysis of recombinants: For structural analysis of plasmid recombinants, samples were taken from infected cultures at 120 min following infection. Clones harboring kan^R plasmids were scored on kanamycin-supplemented solid medium. 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 2.0 was 85.4%. Individual colonies were inoculated into 2 ml L-broth, supplemented with 20 μg/ml kanamycin, cultures were grown overnight, and small scale plasmid extractions were prepared from 1.5 ml cultures by the method of HOLMES and QUIGLEY (1981). Plasmid lengths and structures were determined by restriction endonuclease analysis. Restriction endonucleases that cleave at sites that had been mutated in the recombination substrates (HindIII and XhoI endonucleases), or at sites created by linker insertion (BglII

endonuclease), were employed for structural analysis of plasmid recombinants. Where indicated, kan^R plasmids were subcloned by secondary transformation in recA mutants (DR100) before analysis.

RESULTS

Experimental design: To investigate DSB-stimulated recombination, chimeric phages were constructed that facilitate efficient and synchronous delivery of linear substrates into E. coli cells. Plasmid substrates of the bioluminescence recombination assay were cleaved by restriction endonucleases and ligated to λEMBL4 phage arms at or near EcoRI sites. The chimeric phages were used to infect E. coli cells, harboring plasmids that encode for EcoRI restriction-modification enzymes. In vivo restriction of the infecting phage DNA releases linear plasmid recombination substrates within the cells. Substrates designed to investigate DSB-stimulated intermolecular recombination are depicted in Figure 1.

λAN607 is a phage precursor of a linear substrate for intermolecular recombination (Figure 1). It was constructed by ligation of XhoI-cleaved pAN607 to λ arms, generated by SalI digestion of λEMBL4. Restriction of λAN607 phage DNA by EcoRI nuclease yields a linear pAN607 flanked by the two 18bp SalI-EcoRI fragments of λEMBL4 multiple cloning sites. Recombinational repair of the break, with the mutated luxA sequence on the resident plasmid serving as a template, should yield a kan^R plasmid that may express the luxA gene.

The substrates depicted in Figure 1 enabled investigation of recombination at four levels. Expression of luxA$^+$ recombination products was followed by measuring bioluminescence activity of the infected cells.
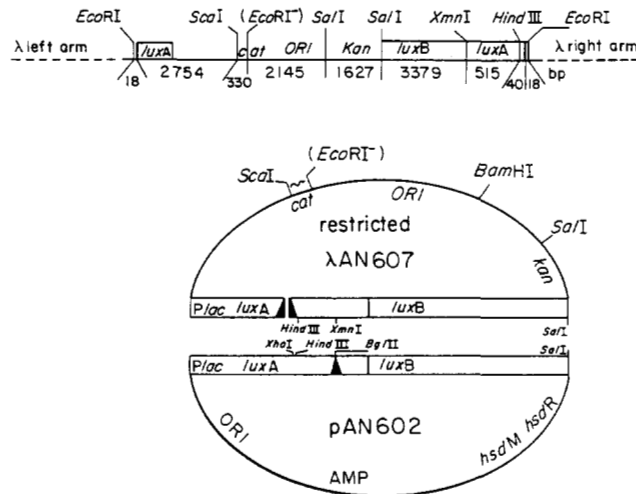
FIGURE 1.—Intermolecular substrates for DSB-enhanced recombination. The uncut substrate in the infected cells pAN602, encodes EcoRI restriction and modification enzymes and has a luxA mutation at the XmnI site. The cut substrate is an EcoRI-restricted λAN607. The lengths between restriction sites are marked on a linear map of λAN607. The locations of plasmid's replication origin (Ori), kan, luxA and luxB genes, EcoRI endonuclease, (hsdR) and EcoRI methylase (hsdM) genes, the relevant restriction sites, the mutated EcoRI site in the cat gene (EcoRI⁻), luxA mutations, (▲) and luxA gene boundary are indicated. Homology is designated by parallel boxes and λ arms of λAN607 (not to scale) by broken lines. The fragment of λAN607 that is homologous to the radioactive probe used in the hybridization experiment (Figure 4) is indicated by a wavy line.

Repair of the break was monitored by Southern hybridization of DNA samples taken at various times following infection, using plasmid-specific probes. Recombinant frequency was determined by scoring Kan^R cells in the infected cultures, and the structure of the kan^R plasmid recombinants was determined by restriction endonuclease analysis. To repress a lytic cycle by phage that had escaped EcoRI restriction, λ(wt) lysogens were used in all experiments.

DSB-induced recombination by the RecE pathway: Expression of the luxA⁺ product of DSB-induced recombination by the RecE pathway was monitored. A recB recC sbcA (λ) mutant strain (AC165), harboring pAN602, was infected with λAN607 (see Figure 1), and bioluminescence of samples taken at time intervals was determined. Bioluminescence was first detectable 60 min after infection and its level increased exponentially thereafter (Figure 2). We assume that the exponential increase in bioluminescence activity reflects replication of luxA⁺ plasmid recombinants. An increase in the total amount of pAN607 derivatives in the infected culture is also apparent from results of the hybridization experiment (see Figure 5).

At the time of recombinant frequency determination (120 min after infection), bioluminescence activity was about 10–20 fold lower than that expected on the basis of luxA⁺ recombinant frequency (Table 3). The reason for this apparent discrepancy is being investigated.
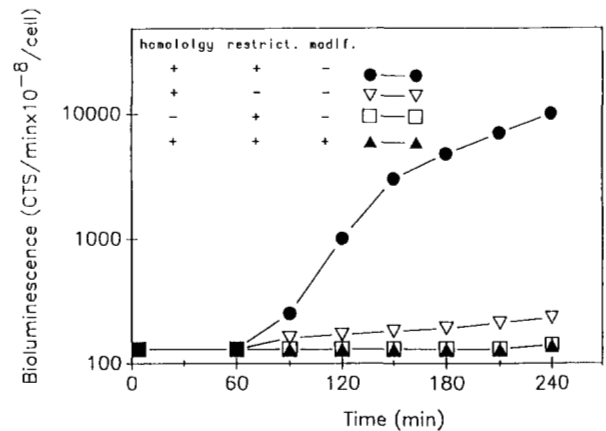

FIGURE 2.—The dependence of recombination in recB recC sbcA mutants on restriction. Cultures of AC165 [recB recC sbcA (λ)] cells, harboring pAN602 (luxA luxB⁺, EcoRI⁺), pAN603 (luxA luxB⁺) or pMB4 (EcoRI⁺) were infected by λAN607 or by λAN607 with modified EcoRI restriction sites. Bioluminescence activity of samples taken at the indicated times following infection was determined in a liquid scintillation spectrophotometer as described. Homology of the infecting phage with the resident plasmid expression of EcoRI endonuclease by the resident plasmid (restrict.) and methylation of EcoRI sites on the infecting phage (modif.) are indicated.

To determine the effect of in vivo restriction on luxA⁺ activity, bioluminescence kinetics of EcoRI⁺ cells, infected by λAN607, was compared to that of EcoRI⁻ cells (pAN603), infected by the same phage, or to that of EcoRI⁺ cells, infected by λAN607 with modified EcoRI restriction sites (Figure 2). In vivo restriction enhanced luxA⁺ activity. Bioluminescence of infected EcoRI⁺ cells was higher than that of infected EcoRI⁻ cells or of EcoRI⁺ cells infected by modified phage.

Dependence of luxA⁺ activity on homology was investigated by comparing bioluminescence activity of λAN607-infected cells harboring pAN602 (EcoRI⁺, luxA luxB⁺) to that of infected cells harboring pMB4 (EcoRI⁺). Bioluminescence was not detectable in infected cells that harbored pMB4 (Figure 2).

Plasmid recombination in recB recC sbcA cells depends on recE activity (LABAN and COHEN 1981). The recA mutation enhances the frequency of intramolecular plasmid recombination (FISHEL, JAMES and KOLODNER 1981) but reduces the frequency of intermolecular plasmid recombination (LABAN and COHEN 1981). The effect of recE and recA mutations on restriction-induced intermolecular recombination in recB recC sbcA (λ) cells was determined (Figure 3). Bioluminescence was not detectable in infected recB recC recE sbcA (λ) cells (AC173) harboring pAN602. Bioluminescence activity was observed in infected recA recB recC sbcA (λ) (AC166) cells, harboring pAN602. This recA-independent activity was 5–10 times lower than that following infection of isogenic recA⁺ cells. Consistent with earlier reports is the observation that when intramolecular recombination substrates were used, bioluminescence activity in recA mutants was

TABLE 3

Kan^R clones of infected recB recC sbcA (λ) cells

Recombining[a] substrates	n[c]	Kan^R/infected cells[d] × 10^2	LuxA$^+$/Kan^R clones × 10^2	N[e]	(genotype 1)	(genotype 2)	(genotype 3)	(genotype 4)	(genotype 5)
λAN607 / pAN602	10	1.5 ± 0.50	61.4 ± 3	110	60 (55%)	50 (45%)	0	0	0
λAN607 / pAN60i	4	1.5 ± 0.56	17.7 ± 4.6	88	13 (15%)	75 (85%)	0	0	0
λAN607 / PMB4	4	0.025 ± 0.011	<2	15[f]	0	0	0	0	15 (100%)
λAN606 / pAN602	4	0.079 ± 0.03	10.4 ± 4.7	34[g]	1 (3%)	24 (71%)	7 (20%)	2 (6%)	0
λAN606 / PMB4	4	1.4 × 10^{-3} ± 6 × 10^{-4}	<2	15[f]	0	0	0	0	15 (100%)

[a] Diagrams depict the configuration of the break and the recombining markers on the restricted phage and resident plasmids. Homology is represented by parallel open boxes and mutations by triangles. The upper substrate in each diagram is the EcoRI-restricted DNA of the indicated phage. The lower substrate is the resident plasmid in the infected cells.

[b] Diagrams represent the configuration of markers on kan^R (Amp$^-$) plasmids. Detailed analysis of crossover products is depicted in Tables 4 and 6. Left marker is the XhoI site, mutated on the infecting phage, right marker is the luxA mutation on the resident plasmid. Broken line represents deletions.

[c] n represents the number of independent experiments, 40–100 colonies were scored in each experiment.

[d] Number of infected cells was calculated as described in text. In all experiments presented in this table moi was 2.0.

[e] N represents the number of kan^R (Amp$^-$) plasmids analyzed.

[f] All monomers had deletions that spanned the break site. The length of the deleted fragment varied and ranged from 1 to 6 kb.

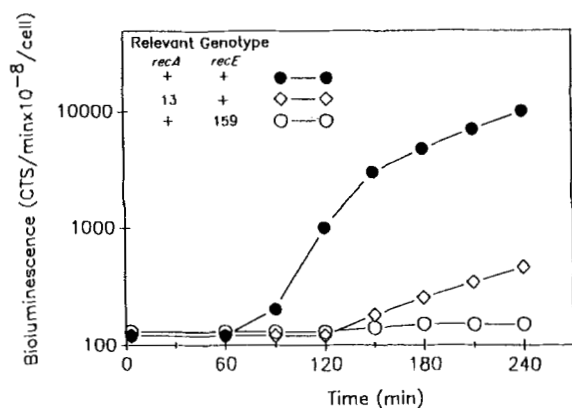[g] All monomers of this cross had deletions that spanned the break site. For a detailed map, see Figure 6.

FIGURE 3.—The dependence of intermolecular recombination on recA and recE activities. AC165(pAN602) and isogenic derivatives carrying the designated mutations were infected by λAN607 and bioluminescence was determined at the indicated times following infection.

higher than that in recA$^+$ cells (M. SHALIT and A. COHEN, unpublished results).

Physical monitoring of DSB repair. To monitor repair of the break on EcoRI-restricted λAN607 DNA, SalI-digested DNA preparations of cells in-

fected by λAN607 were hybridized to a ^{32}P-labeled DNA fragment, homologous to the mutated cat gene on λAN607. The mutated cat gene is located on a 5.2-kb SalI-EcoRI fragment of λAN607 (see Figure 1). Therefore, in vivo restriction of the infecting phage DNA should yield a hybridizable SalI fragment of this length. Repair of the EcoRI-induced break would yield a hybridizable SalI fragment of 9.2 kb (see Figure 1). Three hybridization bands were detectable in DNA preparations extracted immediately following infection. The two major bands corresponded in electrophoretic mobility to the expected SalI digestion products of linear λAN607 (25.0 kb) and SalI-EcoRI-restricted λAN607 (5.2 kb). The minor band corresponded to SalI-digested circular λAN607 (38.4 kb) (see Figure 1). The observation that the hybridizable 5.2-kb fragment was also detectable in infected cells that harbored pMB4, but not in infected cells that harbored pAN603, is consistent with the assumption that this band represents EcoRI-restricted λAN607.

A hybridizable fragment that corresponded in length to SalI-digested pAN607 (9.2 kb) was visible

in DNA preparations of cells harboring pAN602 that were incubated for 30 min or longer following λAN607-infection. The intensity of the 9.2-kb band increased with time. The appearance of this band indicated repair of the break on restricted λAN607 to yield pAN607 or its derivatives (see Tables 3, 4). Replication of pAN607 or its derivatives may contribute to the observed increase in intensity of the 9.2-kb band.

Recombination of restricted λAN607 and pAN602 is expected to yield monomeric noncrossover, as well as heterodimeric crossover products (see DISCUSSION). Since the SalI site on both recombination substrates is at the end of the homology (see Figure 1), the hybridizable SalI fragment of the expected heterodimer is of the same length as the hybridizable SalI fragment of pAN607. Therefore, the hybridization pattern in Figure 4 does not allow for a distinction between crossover and noncrossover repair products. Hybridizable bands that corresponded in electrophoretic mobility to SalI-digested circular λAN607 DNA were detectable in all preparations and their intensity increased in time. These bands may represent phage DNA that escaped restriction and then was replicated from the pACYC184 origin. An additional noteworthy feature of the data in Figure 4 is the reappearance of the hybridizable 5.2-kb fragment in preparations extracted at 120 min following infection. Replication of plasmids or phage in recB recC sbcA cells by a rolling circle type mechanism (COHEN and CLARK 1986), may yield undermethylated linear multimers (see PUKKILA et al. 1985) that are partially EcoRI endonuclease-sensitive.

The effect of substrate structure on bioluminescence kinetics: The effect of the location of the break and recombining markers on luxA⁺ activity was investigated. To investigate the effect of break location relative to the homology on recombination, we compared restricted λAN607, and restricted λAN606, as the cut recombination substrates. λAN606, like λAN607, was constructed by ligation of a linearized pACYC184 derivative, carrying the luxA luxB genes, to λEMBL4 arms. The two phages differ by the site of cleavage on the inserted plasmid. On λAN607 the phage-plasmid junction is at the XhoI site in the luxA gene (Figure 1), and on λAN606 the junction is at the EcoRI site in the cat gene (see Figure 1). The XhoI site of the luxA gene on λAN606 is mutated. EcoRI restriction of λAN607 would yield a linear substrate with a break within the homology. On the other hand, EcoRI restriction of λAN606 would yield a linear substrate with a break at a region that shares no homology with the resident plasmid pAN602.

Bioluminescence activity was observed following λAN606 infection of recB recC sbcA(λ) mutants harboring pAN602. However, the activity of λAN606-
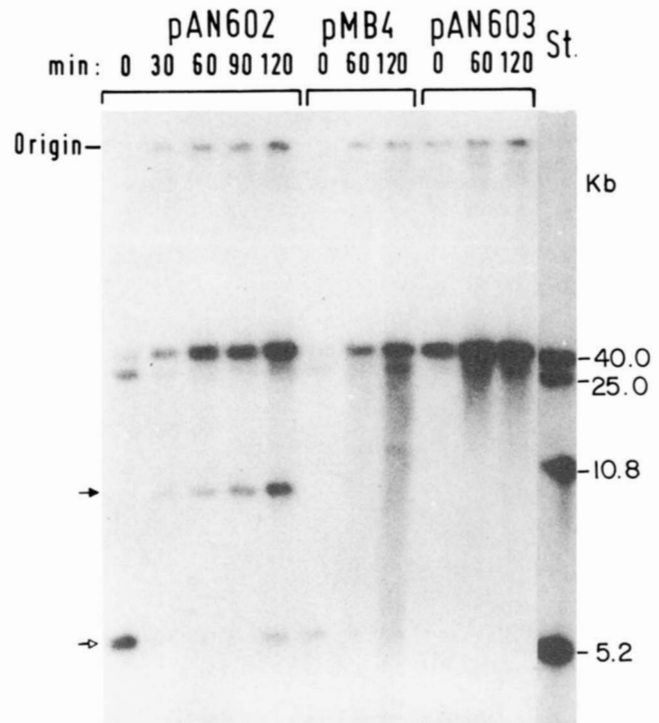

FIGURE 4.—Physical monitoring of intermolecular recombination. DNA preparations of samples, taken at the indicated times after λAN607 infection of recB recC sbcA (λ) cells harboring pAC602 (EcoRI⁺, luxA luxB⁺), pMB4 (EcoRI⁺), or pAN603 (luxA luxB⁺), were digested by SalI endonuclease and subjected to the SOUTHERN hybridization procedure. A ³²P-labeled DNA fragment, homologous to the mutated cat gene on λAN607 (see Figure 1) was used as a probe. Molecular length standards (St.) were pAN607 digested by XhoI (10.8 kb) or by XhoI and SalI (5.2 kb), λAN607 DNA (40 kb) and SalI endonuclease-digested λAN607 DNA (25.0 kb). The expected locations of SalI digestion products of EcoRI-restricted λAN607 (open arrow) and pAN607 recombination products (filled arrow) are indicated.

infected cells was about 10-fold lower than that following λAN607 infection (Figure 5). Consistent with these results is the observation that Kanᴿ recombinant frequency in λAN606-infected cultures was about 30 times lower than that in λAN607-infected cultures (Table 3). The effect of break location on the molecular structure of plasmid recombinants is discussed below.

Bioluminescence activity of λAN606-infected cells, like that of λAN607-infected cells, depended on EcoRI restriction of the infecting phage, the presence of luxA luxB⁺ genes on the resident plasmid and on recE activity. Bioluminescence was not detectable when the EcoRI sites on λAN606 DNA were modified, when pMB4 (EcoRI⁺) substituted for pAN602 (EcoRI⁺ luxA luxB⁺) as the resident plasmid in the infected cells, or when the infected cells carried a recE mutation (Figure 5).

Conversion of wild type luxA sequences on restricted λAN607 by their respective mutated alleles on the resident plasmids should have yielded non-bioluminescent Kanᴿ recombinants (see Table 4). The

TABLE 4

Crossover products induced by restriction within the homology

Recombination substrates N^a		pAN607 / pAN602 36		pAN607 / pAN601 16	
Description	Diagrams	Classification of products in Kan^R clones			
		Total		Total	
Conversion at the XhoI site A		8		0	
B		3	11 (30%)	0	
Independent conversion C		0		0	
Co-conversion D		18	18 (50%)	16	16 (100%)
Heteroallelic clones	A + C	1		0	
	A + D	5		0	
	B + D	1	7 (20%)	0	

Classification of crossover products isolated from λAN607-infected *recB recC sbcA* (λ) mutants. The configuration of the DSB and the markers on the recombining substrates are shown in the top diagrams. X, H and Xm designate *XhoI*, *HindIII* and *XmnI* sites, respectively. The left column shows diagrams of crossover products. The left marker is the *XhoI* site (mutated on the infecting phage), the right marker designates the site that is mutated on the resident plasmid (*HindIII* or *XmnI*). Triangles designate mutation.

^{a} N represents number of clones with heterodimers analyzed.

DSB-repair model predicts that conversion frequency of markers on the cut substrate decreases as the distance from the break increases. Therefore it is expected that the yield of LuxA$^+$ recombinants will increase with the distance between the *luxA* mutation on the non-cut substrate and the site allelic to the break. To test this prediction, cultures of *recB recC sbcA*(λ) mutants harboring either pAN601 or pAN602 were infected by λAN607 and bioluminescence kinetics and molecular structure of plasmid recombinants were determined. pAN601 and pAN602 differ from each other by the distance of their respective *luxA* mutations from the *XhoI* site that is allelic to the break on restricted λAN607 (see Figure 1). On pAN601 the *luxA* mutation is at the *HindIII* site and on pAN602 at the *XmnI* site. The corresponding nonmutated alleles on restricted λAN607 are at 58 and 573 bp from the break, respectively. Bioluminescence activity of infected cultures harboring pAN601 was about 10-fold lower than that of infected cultures harboring pAN602 (Figure 5). However, the frequency of Kan^R recombinants in the two cultures was similar (Table 3). These findings suggest that the difference in biol-
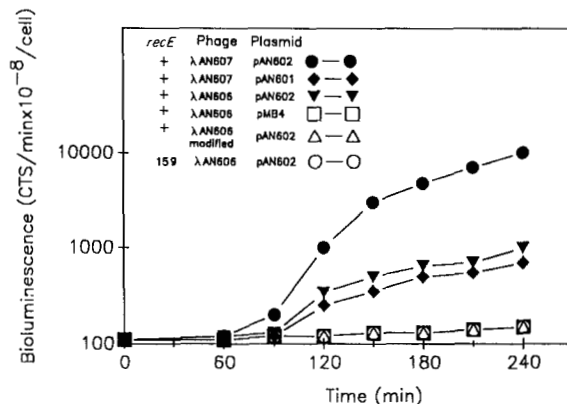

FIGURE 5.—The effect of DSB location and the location of the recombining markers on bioluminescence activity. AC165 cells harboring pAN601 (*HindIII$^-$*) or pAN602 (*XmnI$^-$*) were infected by λAN607 (see Figure 1) or λAN606. Bioluminescence was determined at times following infection. Dependence of recombination, induced by a DSB outside the homology (in λAN606-infected cells), on *recE* activity, homology, and restriction, was tested by infecting *recE* mutants, cells that harbor pMB4 (no homology), and using modified phage strains (open symbols).

uminescence kinetics is not due to a difference in the efficiency of recombination-mediated break-repair, but to a difference in the structure of plasmid recombinants. Determination of the LuxA phenotype of the Kan^R recombinants (Table 3), and the molecular structure of the respective *kan^R* plasmids (Tables 3, 4) confirm this hypothesis.

Products induced by restriction within the homology: Repair of the break on an *EcoRI*-restricted λAN607 DNA would yield a *kan^R* plasmid derivative of pAN607. If a crossover event were associated with the recombinational-repair reaction, the expected product would be a *kan^R amp^R* heterodimer consisting of derivatives of the resident plasmid and pAN607 (see Figure 7). To score Kan^R recombinants, samples taken at 120 min following λAN607 infection of cells harboring pAN601 or pAN602 were plated on kanamycin-supplemented medium (Table 3). Kan^R colonies were tested for bioluminescence and the molecular structure of their *kan^R* plasmids was determined (Tables 3, 4). Of λAN607-infected cells, 1–5% yielded Kan^R clones. The ratio of Kan^R cells to infected cells did not change as the moi was lowered from 2 to 0.02 (not shown). The location of the *luxA* mutation on the resident plasmid did not affect Kan^R recombinant frequency. It did, however, affect the proportion of bioluminescent colonies among these recombinants. This proportion was 15% when the *luxA* mutation on the resident plasmid was at the *HindIII* site (pAN601), and 60% when the resident plasmid was mutated at the *XmnI* site (pAN602) (Table 3). In control experiments, where pMB4 substituted for the *luxA luxB$^+$* plasmids in the infected cells, the frequency of Kan^R cells was lowered by 100-fold. With pMB4 as the resident plasmid, none of the

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TABLE 5

Stability of crossover and noncrossover products in AC165(pAN602) cells

Transforming plasmid	Molecular structure	n^a	kan^R plasmids in cultures		
			pAN607	pAN611	pAN607 + pAN611
pAN607	Monomer	24	24	0	0
pAN611	Heterodimer	20	9	4	7

AC165(pAN602) cells were transformed by pAN607 or pAN611 (Table 2). Transformants were selected on kanamycin plates, inoculated to 1.5 ml kanamycin-supplemented L-broth and grown overnight. Plasmid preparations were analyzed by BamHI digestion.

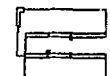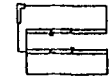^{a} n designates the number of cultures of independent transformants tested.
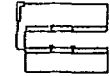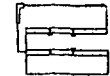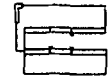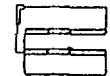
Kan^R colonies were bioluminescent and all analyzed plasmids suffered deletions that spanned the break site. The length of the deleted fragments varied from 1 to 6 kb (not shown).

The molecular structure of kan^R plasmids in Kan^R clones, derived from λAN607-infected cultures, was determined. Most clones (70–90%) harbored, in addition to the resident pAN601 or pAN602, monomeric derivatives of pAN607. These monomers may be primary noncrossover products, or products of secondary recombination events that affect heterodimeric crossover products (see below). The XhoI site that was cleaved and mutated in the process of λAN607 construction (see Figure 1) was converted 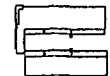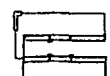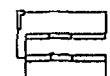to its wild type allele in all monomers tested. All kan^R luxA mutants had acquired the luxA mutation of the resident plasmid (pAN601 or pAN602).

To isolate crossover products following infection of cells harboring pAN601 or pAN602, recA mutants (DR100) were transformed by plasmid preparations of Kan^R clones, and Kan^R transformants were tested for Ampicillin resistance (20–30 transformants of each Kan^R clone). To distinguish between Kan^{R}Amp^R transformants harboring heterodimers from those that were cotransformed by kan^R and amp^R monomers, plasmid preparations were analyzed by BamHI endonuclease. pAN607 has a single BamHI site and pAN601 or pAN602 have none. Thirty percent of the Kan^R clones harbored heterodimers. However, most clones that harbored heterodimers (74%) also harbored kan^R monomers. This proportion was not lowered when infection multiplicity decreased from 2 to 0.02 (data not shown). Therefore, it seems unlikely that clones with a mixed kan^R plasmid population resulted from multiple independent primary recombination events following multiple λAN607 infection. To assess the possibility that secondary recombination events may have affected the structure of primary plasmid recombinants, AC165 cells harboring pAN602 were transformed by an enzymatically synthesized heterodimer (pAN611), or by pAN607 mon-

TABLE 6

Crossover products induced by restriction outside the homology

Recombination substrates		pAN606 ―――――― x pAN602	$n = 41$
Description	Diagrams	Products	Total
Reciprocal crossover		6	
		13	
		4	
		1	24 (58%)
Conversion of alleles on restricted substrate		1	
		1	
		3	
(Co-conversion)		6	11 (27%)
Conversion of alleles on non-cut substrate		2	
		1	
(Co-conversion)		3	6 (15%)

Classification of crossover products isolated from recB recC sbcA (λ) cells, harboring pAN602, following λAN606 infection. Configuration of the break and markers on the recombining substrates is shown in the top diagram. Diagrams illustrate crossover products that have been found, X and Xm designate XhoI and XmnI sites. Triangles designate mutations, open box designates deletions, marker designations are as in Table 4.

omer, and the plasmid content of cultures of the Kan^R transformants was examined (Table 5). Heterodimers were found to be unstable in the transformed cells. Most cultures derived from cells transformed by pAN611 harbored a mixture of pAN611 and its monomeric components, and some had lost the transforming heterodimer. On the other hand, pAN607 and pAN602 were stably comaintained in the Kan^R transformants. Heterodimers were not detectable in cul-

tures harboring the two plasmid monomers. This observation and the finding that most clones that harbored heterodimers also harbored kan^R monomers, suggest that in most clones with a mixed kan^R plasmid population, the kan^R monomers were products of secondary recombination events that affect the primary heterodimer recombinants. Heterodimers did not accumulate to detectable amounts in cells transformed by their monomeric components; This observation argues against the possibility that the heterodimers that were isolated from the infected culture originated by a secondary recombination event of kan^R monomers and the resident amp^R plasmids.

The molecular structures of heterodimers in Kan^R clones of pAN601 ($Hind$III$^-$) or pAN602 (XmnI$^-$)-harboring cultures, infected by λAN607, are shown in Table 4. The mutated XhoI site of the cut substrate was converted to its wild type allele in all heterodimers tested. The frequency of coconversion of markers on the cut substrate with the mutated XhoI site was affected by their distance from the break. The wild type allele at the $Hind$III site (58 bp from the break) coconverted with the mutated XhoI site in all 16 heterodimers isolated following infection of cultures harboring pAN601. On the other hand, when the $luxA$ mutation on the resident plasmid was at the XmnI site (573 bp from the break), coconversion of its wild-type allele on the cut substrate with the mutated XhoI site was observed in only 24 heterodimers of the 43 tested. Independent conversion at XhoI and XmnI sites was observed in only one heterodimer. The observation that 17% of the Kan^R clones from infected cells that harbor pAN601 ($Hind$III) were bioluminescent (Table 3), indicates that some recombination repair events did not involve conversion at the $Hind$III site of the cut substrate.

Seven out of the 24 clones that harbored heterodimers with a converted XmnI site harbored an additional heterodimer, with no conversion at this site. Since five of the seven clones with mixed heterodimer populations were detected in cultures infected at a multiplicity of 0.02, it is unlikely that the mixed heterodimer population was a result of two independent primary recombination events. We therefore propose that the simplest interpretation of this observation is the replication of a primary crossover product with a heteroduplex region that includes the XmnI site.

Products induced by restriction outside the homology: The break on restricted λAN606 is at the EcoRI site of the cat gene (see Figure 1), in a region that shares no homology with pAN602. Bioluminescence activity of cells harboring pAN602 was about 10-fold lower following λAN606 infection than following λAN607 infection (Figure 5). In both cases, bioluminescence depended on restriction of the in-

fecting plasmid and on $recE$ activity (Figure 5).

To investigate recombination events that were stimulated by a break outside the homology, AC165 cells harboring pAN602 were infected by λAN606, Kan^R recombinants were scored, their Lux phenotype determined, and their kan^R plasmids were analyzed. Kan^R recombinant frequencies in cultures infected by λAN606 were twenty fold lower than that in cultures infected by λAN607 (Table 3). This difference and the difference in the proportion of LuxA$^+$ recombinants among the Kan^R cells (Table 3), is consistent with the observed differences in bioluminescence kinetics between the cultures infected by the two phages (Figure 5).

About half of the Kan^R recombinants in cultures infected by λAN606 harbored $kan^R amp^R$ plasmids (not shown). Unlike the crossover products in λAN607-infected cultures, the $kan^R amp^R$ plasmids of λAN606-infected cultures were stably maintained in cells grown in the presence of kanamycin and were not resolved to yield kan^R and amp^R monomers. The reason for this stability is apparent from the molecular structure of these plasmids. Crossover products in cultures infected by λAN606 suffered deletions that spanned the cut site and included the pAN606 replication origin (Figure 6). A secondary, intramolecular crossover reaction would have resolved these primary products to yield a replicative amp^R plasmid and a non-replicative circle that carries the kan gene. Noncrossover products also suffered a deletion that spanned the cut site (Figure 6). However, the deletions in these plasmids did not include the pAN606 replication origin. Restriction endonuclease analysis of several kan^R and $kan^R amp^R$ products indicates that most deletions in each one of the two classes ended at the same, or almost the same, site. The deletion did not reach the region of homology with pAN602 in any of the products tested. The length of the deleted fragment was 1.7 kb in all kan^R plasmids tested. In most $kan^R amp^R$ plasmids (35 of the 41 tested), the length of the deletion was 2.8 kb, and in some (6 plasmids), 2.5 kb. The distance from the end of the deletion to the homology was 100 bp in the kan^R plasmids and 500 bp or 200 bp in the $kan^R amp^R$ plasmids (Figure 6).

The molecular structures of crossover products induced by restriction outside the homology are depicted in Table 6. Conversion events were apparent in about 40% of the tested plasmid recombinants. However, the conversion products differed from those induced by restriction within the homology. When conversion was induced by restriction within the homology, the cut substrate was almost exclusively the recipient molecule (Table 4). On the other hand, when conversion was induced by restriction outside the homology, either one of the two substrates could serve as a recipient (Table 6). With the break outside
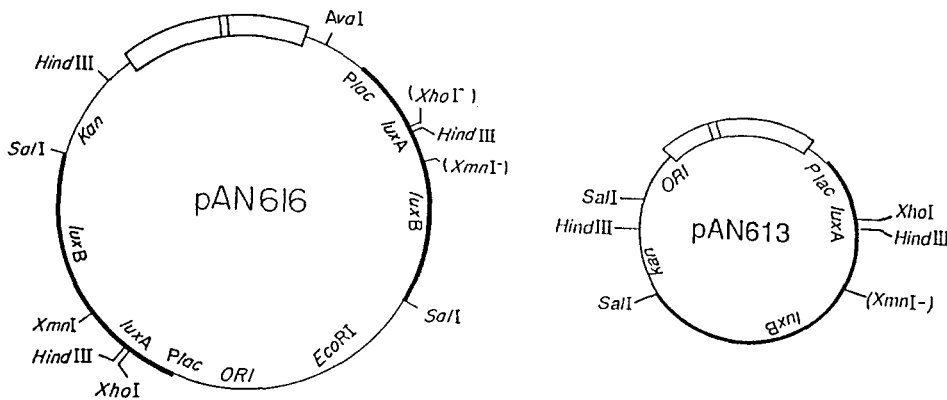
FIGURE 6.—Products of recombination induced by a DSB outside the homology. A luxA/luxA⁺ crossover product (pAN616) and a non-crossover luxA product (pAN613) are presented. The recombining substrates were restricted λAN606 and pAN602 (see Table 2). Restriction sites and relevant genes are designated as in Figure 1. The deletions that span the DSB of the cut substrate are designated by an open box. The DSB site is indicated within the box. The region of homology in the recombining substrates is indicated by heavy lines and the luxA mutations at the XmnI site by (XmnI⁻).

the homology, coconversion of markers at the XhoI and XmnI sites was observed in about half of the analyzed conversion events (Table 6).

DISCUSSION

An experimental system for the efficient and synchronous delivery of linear recombination substrates into E. coli cells was applied to the investigation of DSB-stimulated plasmid recombination by the RecE pathway. Results indicate that in recB recC sbcA mutants, a DSB can induce more than one type of a recE-dependent recombination pathway, and that the nature of the pathway depends on the location of the break with respect to the homology. A break within the homology induces a recombination pathway that is consistent with predictions of the DSB-repair model (RESNICK 1976; ORR-WEAVER and SZOSTAK 1983). On the other hand, a break outside the homology induces a pathway that does not involve direct participation of DNA ends in the homologous pairing reaction. In this pathway, both the cut and noncut substrates can serve as recipients in conversion events. A similar effect of the configuration of the DSB and the recombining markers on DSB-stimulated recombination has been recently observed in yeast cells (NICKOLOFF et al. 1989).

Recombination induced by a break within the homology: The following observations support an earlier proposal that a break within the homology induces RecE-mediated recombination by a DSB-repair mechanism (KOBAYASHI and TAKAHASHI 1988). (1) Recombination was greatly enhanced by in vivo restriction. (2) Repair of the break on the cut substrate depended on the presence of a sequence, homologous to the break region, on the non-cut substrate. (3) Break-repair was associated with replacement of alleles that were cis to the break by alleles of the noncut homolog. (4) Alleles that were close to the break were replaced at higher frequencies than alleles that were further from the break. (5) Some of the KanR clones harbored plasmid recombinants that were mixed for

the luxA character, consistent with the primary recombinant having a heteroduplex structure.

The results presented above are interpretable in the context of the DSB-repair recombination model. The dependence of recombination on in vivo restriction at the region of homology indicates a direct role for a DSB in the recombination reaction. The dependence on recE activity is explained by the role of exonuclease VIII in processing the DSB to form the recombinogenic 3′-single-stranded DNA ends (SYMINGTON, MORRISON and KOLODNER 1985; KOBAYASHI and TAKAHASHI 1988). According to the DSB-repair model, recombinational repair of the break is associated with enlargement of the break to a gap, and creation of a conversion region by gap repair, with the non-cut homolog serving as a template (ORR-WEAVER and SZOSTAK 1983). The frequent replacement of alleles, cis to the break, by alleles of the noncut homolog is consistent with this feature of the model. The size distribution of the repaired gap may be estimated by determining the effect of the allele's distance from the break on the frequency of its replacement. The mutated XhoI site at 18 bp from the break on restricted λAN607 was replaced by its wild-type allele in all kanR plasmids tested. The nonmutated HindIII and XmnI sites are located at a distance of 58 and 573 bp from the break, respectively. Coconversion of these markers with the mutated XhoI allele would have yielded nonbioluminescent recombinants. With a luxA mutation at the HindIII site on the resident plasmid, 85% of the KanR recombinants were nonbioluminescent, and with a luxA mutation at the XmnI site only 40% were nonbioluminescent (Table 3). The effect of the distance from the break on conversion frequency is also apparent from restriction analysis of crossover products (Table 4). Conversion at the HindIII site occurred at higher frequencies than at the XmnI site (Table 4). This difference is also reflected in the difference in bioluminescence activity between infected cultures of cells harboring plasmids with a mutated HindIII site (pAN601) or a mutated XmnI site (pAN602) (Figure 5).

Recombination induced by a break outside the homology: A break outside the homology induces recombination by a mechanism that does not involve conversion by gap repair. The observation that all recombinants suffered deletions that spanned the break site suggests that these substrates, like substrates with a break within the homology, were subjected to exonuclease digestion that enlarged the break to a gap. However, with no homology to serve as template, the gap was resealed rather than repaired. The deletions in the non-crossover products stopped short of the pAN606 replication origin. In the crossover products the pAN606 origin was deleted in the process of enlarging the break to a gap. The observation that the same, or nearly the same, fragment was deleted in all recombinants of each class suggests that the corresponding gaps were sealed at preferred sites. Sequence analysis of the deletion product should reveal these sites and may contribute to the understanding of the mechanism of gap sealing.

When considering a mechanism for recombination stimulated by a break outside the homology, the following observations must be taken into account. (1) The ends of the deletions share no homology with the uncut substrate (Figure 6). Therefore, the ends may not participate directly in the homologous pairing reaction. (2) Recombination depended on *recE* activity (Figure 5). (3) Conversion was not limited to markers on the cut substrate. Markers on the uncut substrates were converted as well (Table 6). A model that attempts to explain the observed effect of break location, relative to the homology, on *recE*-dependent recombination is presented in Figure 7. RecE enzyme (exonuclease *VIII*) digests double-stranded DNA from a DSB (A) in a 5'→3' direction to yield 3'-single-stranded DNA overhangs. Digestion of the overhangs by another nuclease may enlarge the break to a gap (B). If homology is available (left column) the overhangs may participate in a homologous pairing reaction (C) and prime a gap repair synthesis that yields a region of conversion, flanked by two Holliday junctions (D). In the absence of homology to the 3'-single-stranded DNA ends (right column), RecE-mediated digestion in the 5' to 3' direction will reach the homology (B), creating a single-stranded DNA region that may participate in a homologous pairing reaction, without direct participation of the nonhomologous ends (C). This reaction would yield a heteroduplex region on the uncut homolog. Mismatch correction of the heteroduplex may lead to conversion. To yield inheritable recombinants the gap must be sealed. The mechanism for gap-sealing is not yet understood. One possibility is that annealing of short homologies on the 3' overhangs primes synthesis of strands complementary to the 3' overhangs. Such synthesis would create a region of conversion on the cut homolog (D).
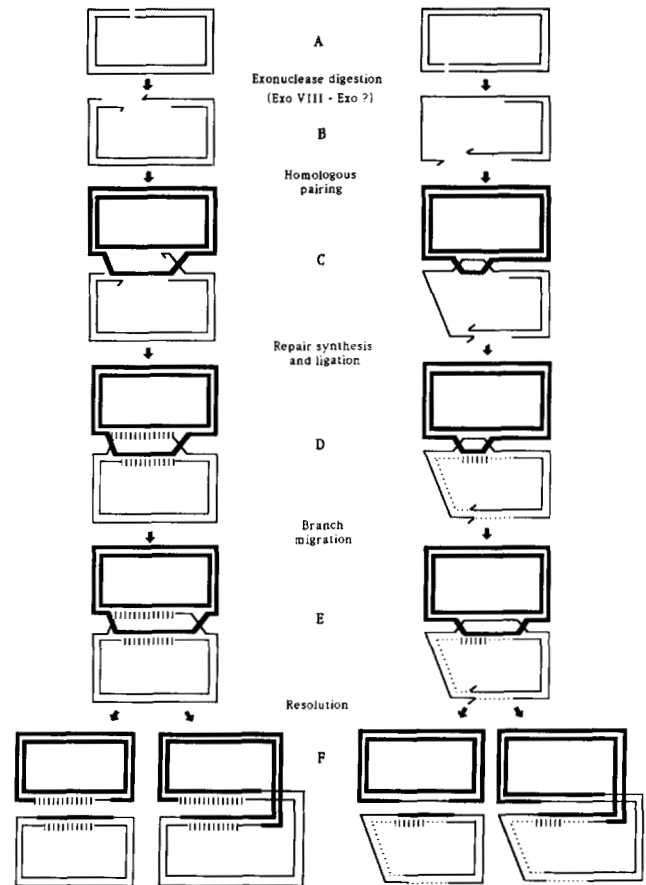

FIGURE 7.—Alternative *recE*-dependent, DSB-induced, recombination pathways. Cut substrates are shown in thin lines and noncut substrates in heavy lines. Homologous regions are located at the upper part of the rectangles that designate cut substrates and at the lower part of the rectangles that designate noncut substrates. Newly synthesized DNA is shown as discontinuous lines. Exonuclease digestion by the RecE enzyme (exonuclease VIII) and other nucleases enlarge the DSB on the cut substrates (A) to yield a gap with 3'-single-stranded DNA overhangs (B). If homology to the overhangs is available (left pathway), a homologous pairing reaction (C) can primes repair synthesis and ligation to create a region of conversion, that is flanked by a pair of Holliday junctions (D). If the break is outside the homology (right pathway), extension of the single stranded region by the RecE enzyme may reach the homology, yielding a homologous single-stranded region with non-homologous ends (B). Pairing of the single-stranded DNA with the noncut homolog would create a heteroduplex region on the noncut homolog. Annealing of short regions of homology on the 3' overhangs flanking the gap (C), may prime synthesis of complementary strands to create an intermediate with a deletion that spans the break site and a region of conversion on the cut homolog (D). In both pathways, migration of the Holliday junctions that flank the region of homologous pairing would extend the heteroduplex region (E). Endonuclease-mediated resolution of the junctions in the same, or different sense, would yield noncrossover and crossover products, respectively (F).

The observed deletions of sequences that flank the DSB may be a consequence of exonuclease digestion of the 3'-single-stranded tails. This exonuclease activity may take place before or after annealing of the short homologies. A mechanism of intramolecular recyclization that involves exonuclease processing of

exposed termini that generate substrates for short sequence annealing and deletion has been proposed by CONLEY et al. (1986). Intermediates of both pathways may be subjected to branch migration that extends the heteroduplex region (E) and endonucleolytic resolution that yields crossover or non-crossover products (F).

The observation that the frequency of Kan^R cells in λAN606-infected cultures that harbor pAN602 is higher than that in cells harboring pMB4, suggests a relationship between the gap-sealing reaction and recombination in the homologous region. Consistent with this suggestion is the finding that most kan^R plasmids in λAN606-infected cells that harbor pAN602 are either crossover products or noncrossover products that have acquired the luxA genotype of pAN602 (Table 6). While the model presented in Figure 7 does not account for this apparent relationship, it is possible that the reaction at the homology stabilizes the cut plasmid and allows sealing of the gap.

The proposed role for a DSB in RecE-mediated recombination, induced by a DSB outside the homology, is similar to that in RecBCD-mediated recombination (see THALER, STAHL and STAHL 1987b). In both cases the ends serve as entry points for recombination enzymes that travel along the DNA, creating single-stranded regions that serve as substrates in the homologous pairing reaction. The RecBCD enzyme catalyzes single-stranded DNA formation by helicase activity and the RecE enzyme by strand-specific exonuclease activity.

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