Genetic Recombination Through Double-Strand Break Repair: Shift From Two-Progeny Mode to One-Progeny Mode by Heterologous Inserts

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Manuscript received May 23, 1996
Accepted for publication December 21, 1996

ABSTRACT

Double-strand break repair models of genetic recombination propose that a double-strand break is introduced into an otherwise intact DNA and that the break is then repaired by copying a homologous DNA segment. Evidence for these models has been found among lambdoid phages and during yeast meiosis. In an earlier report, we demonstrated such repair of a preformed double-strand break by the *Escherichia coli* RecE pathway. Here, our experiments with plasmids demonstrate that such reciprocal or conservative recombination (two parental DNAs resulting in two progeny DNAs) is frequent at a double-strand break even when there exists the alternative route of nonreciprocal or nonconservative recombination (two parental DNAs resulting in only one progeny DNA). The presence of a long heterologous DNA at the double-strand break, however, resulted in a shift from the conservative (two-progeny) mode to the nonconservative (one-progeny) mode. The product is a DNA free from the heterologous insert containing recombinant flanking sequences. The potential ability of the homology-dependent double-strand break repair reaction to detect and eliminate heterologous inserts may have contributed to the evolution of homologous recombination, meiosis and sexual reproduction.

AN important and unresolved issue in modern biology is our failure to adequately explain and identify the forces underlying the evolution and maintenance of the mechanisms of genetic (homologous) recombination, meiotic recombination and sexual reproduction. One possibility is that these processes are required for the repair of damaged DNA or the elimination of deleterious mutations. Alternatively, it has been suggested that they could represent defense mechanisms against parasites (MICHOD and LEVIN 1988). Recent elucidation of the mechanisms of homologous recombination provides clues that may help to resolve these questions.

The double-strand break repair models of homologous recombination propose that recombination is initiated by introducing a double-strand break on one of the duplex DNAs (Figure 1B) (RESNICK 1976; SZOSTAK *et al.* 1983; KOBAYASHI 1992). The break is repaired by copying a homologous DNA (Figure 1, C and D), and this copying (gene conversion) is often accompanied by crossing over of the sequences flanking the break (Figure 1C). Accumulating evidence shows that meiotic recombination in yeast *Saccharomyces* is initiated by double-strand breakage as predicted by these models (LICHTEN and GOLDMAN 1995). One peculiar feature

of these models is that double-strand breakage, a destructive event for a gene, is introduced apparently "on purpose" into an otherwise intact DNA.

One explanation may be that this double-strand break repair mechanism represents a versatile repair system for various types of DNA damage (repair hypothesis) (BERNSTEIN *et al.* 1987). According to this hypothesis, the conversion of single-stranded damage to double-stranded damage triggers double-strand break repair. This repair capacity might be the driving force for the evolution of homologous recombination, meiosis and sexual reproduction. A central problem that has impeded the wide acceptance of the repair hypothesis is the observed association of flanking crossing-over events with double-strand break repair. If DNA repair is the "purpose" of recombination, there should be no need for flanking crossing-over events. Another problem of the repair hypothesis as applied to sexual reproduction is the occurrence of outcrossing. Recombination between chromosomes is not limited to identical sister chromosomes.

There exists strong evidence in support of the double-strand break repair model for homologous recombination in the prokaryote, *Escherichia coli* (KOBAYASHI *et al.* 1983; KOBAYASHI 1992). Repair of a preformed double-strand break as envisaged by the model (Figure 1, A through D) was demonstrated for two recombination systems in *E. coli* (KOBAYASHI and TAKAHASHI 1988; TAKAHASHI and KOBAYASHI 1990; YOKOCHI *et al.* 1995): the enzymatic machinery of both bacteriophage lambda

This article is dedicated to Mary M. Stahl.

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FIGURE 1.—Effect of heterologous insertion on homology-dependent double-strand break repair. (A–D) The double-strand break repair model. A double-strand break is introduced into an otherwise intact duplex DNA (A and B). The double-stranded ends are processed to yield single-stranded tails, which pair with a homologous duplex (B). The double-stranded break is repaired by copying this homologue. The products are two progeny duplex DNAs with crossing over (reciprocal recombination) of flanking sequences (C) or without crossing over (D). We define this type of homologous recombination that generates two duplex DNA segments out of two duplex DNA segments as conservative recombination or two-progeny recombination in this report. The recombination initiated at the double-strand break sometimes results in only one product (G). We define this type of homologous recombination that generates one duplex DNA segment out of two duplex DNA segments as nonconservative recombination or one-progeny recombination. The half-crossing-over shown here (G) is an example of nonconservative (one-progeny) recombination. It is defined as recombination that produces one recombinant progeny segment with recombinant flanking sequences, leaving one or two ends. When a heterologous insertion is present in the upper duplex DNA (E), a double-strand break at or near it (F) may lead to the configuration in which heterologous DNA is attached to one end of the double-strand break. The nonconservative (one-progeny) route is preferred over the conservative (two-progeny) route. The recombinant progeny (half-crossing-over type) (G) is free from the heterologous insertion.

[Red pathway of recombination (KOBAYASHI 1992)] and the cryptic prophage (provirus), *Rac*, present in some *E. coli* strains [RecE pathway of recombination (CAMPBELL 1994; KOLODNER *et al.* 1994)].

These repair events were frequently accompanied by flanking crossing over (Figure 1C) as predicted by the double-strand break repair models (KOBAYASHI and TAKAHASHI 1988; TAKAHASHI and KOBAYASHI 1990). We identified two enzymes (the *recJ* and *recQ* gene product)

that are specifically involved in the flanking crossing over associated with break repair (KUSANO *et al.* 1994a). Mutational inactivation of these enzymes did not decrease the level of double-strand break repair without crossing over (Figure 1D) in the plasmid transformation experiments. Correlation was found, in various genetic backgrounds, between the level of crossing-over events associated with the double-strand break repair after plasmid transformation and cell survival after treatments with DNA damaging agents (KUSANO *et al.* 1994a). These results suggested that double-strand break repair with crossing over (Figure 1C) is somehow more relevant to cellular recovery than repair without crossing over (Figure 1D). This is in contrast to the simple predictions of the above mentioned repair hypothesis.

The recombination events discussed so far generate two progeny DNAs from two parental DNAs (Figure 1, C and D). Such events are defined as “reciprocal,” “conservative” or “two-progeny” recombinations in this article. The recombination stimulated by a double-strand break might produce only one progeny DNA, as demonstrated in one mutant strain (*recBC sbcBC*) of *E. coli* (Figure 1G) (TAKAHASHI *et al.* 1992). (The homologous recombination in this genetic background is often referred to as RecF pathway of recombination.) These one-progeny events are defined as “nonreciprocal,” “nonconservative” or “one-progeny” recombinations. In the present work, we show that recombination is frequently conservative (two-progeny) even when there is an alternative opportunity for nonconservative (one-progeny) recombination. The presence of a heterologous insert at the double-strand break causes a shift from the conservative (two-progeny) mode to the nonconservative (one-progeny) mode. We discuss the implications of this capacity to remove heterologous inserts with respect to the possible roles of the double-strand break repair mechanism and present a specific hypothesis for the evolution of meiotic recombination and sexual reproduction.

MATERIALS AND METHODS

Bacterial strains: *E. coli* K-12 strains, JC8679 (= F^- *recB21 recC22 sbcA23 rac⁺ thr-1 ara-14 leuB6 Δ (gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 λ^-*) (GILLEN *et al.* 1981; KOBAYASHI and TAKAHASHI 1988), JC7623 (= F^- *racdel recB21 recC22 sbcB15 sbcC201 hisG4 thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 argE3 rfbD1 mgl-51 kdgK51 Δ (gpt-proA)62 rpsL31 tsx-33 supE44 λ^-*) (LLOYD and BUCKMAN 1985; TAKAHASHI *et al.* 1992), and JC8691 (= *recB21 recC22 rac⁺ sbcA23 recE159*) (GILLEN *et al.* 1981; TAKAHASHI *et al.* 1993) were used. The DH1 and DH5 strains have already been described (HANAHAN 1983). They are recombination-defective (*recA1*) strains and were used for propagation of plasmids.

Plasmids: The plasmids and viral DNAs used are listed in Table 1. Construction of the 14,795-bp plasmid pIK43 (Figure 2) has been described previously (YAMAMOTO *et al.* 1988). The coordinates proceed clockwise starting from the upper *EcoRI*

site. The two homologous *neo* segments (coordinates 3–2074 and coordinates 10374–12472) are drawn as parallel lines. The top *neo* segment has a 283-bp-long deletion (deletion *a*) between two *NaeI* sites, which removed one end (the carboxyl end) of the *neo* gene. The *NaeI* site has been inactivated by insertion of an *XhoI* linker (5' CCTCGAGG). The bottom *neo* segment has a 248-bp-long deletion (deletion *b*) between two *NaeI* sites, which removed the other end (the amino end) of the *neo* gene. The two deletion mutations are separated by 506 bp of homology and are flanked by 1079 bp of homology to the left and 231 bp of homology to the right. The left part (2322 bp) of pIK43, including the *amp* (ampicillin resistance) gene and the ColE1 replication origin, is derived from pBR322. The right part (8298 bp) is derived from SV40 and bovine papillomavirus type 1. The restriction enzymes and their site coordinates are as follows: *XhoI* at 1083; *EcoRI* at 8025, 14794.

pKEN33 (Figure 4A) (TAKAHASHI *et al.* 1992) is derived from pIK43 and pACYC184 (CHANG and COHEN 1978; ROSE 1988) and is 9000-bp long. The upper *neo* gene, derived from pIK43 (Figure 2A), has the 283-bp-long deletion (deletion *a*) with an *XhoI* linker. The lower *neo* segment also from pIK43 (*SalI/EcoRV* fragment) carries the 248-bp-long deletion (deletion *b*). These two deletions are separated by a 506-bp-long region. There exists a 1079-bp homologous region downstream and a 231-bp-long homologous region upstream as in pIK43. The left unique (nonhomologous) part (2295 bp long), including the ampicillin-resistance gene (*amp*) and the ColE1 replication origin (white circle), is derived from pBR322 (*EcoRI/PvuII* fragment). The right unique (nonhomologous) part, including the chloramphenicol resistance gene (*cml*) and the p15A replication origin (black circle), is derived from the *SalI/HindIII* fragment of pACYC184, but a segment of 1094 bp was deleted during construction apparently by homologous recombination between sequence repeats in pACYC184 (ROSE 1988; TAKAHASHI *et al.* 1992). pKEN30 is identical with pKEN33 except that the 1094-bp-long segment near *cml* gene is intact.

Transformation: Plasmids for transformation were prepared in a recombination-defective *recA* strain (DH1 or DH5) and grown with a minimal number of generations to minimize the appearance of *neo*⁺ and other types of recombinants. They were purified in CsCl/EtBr gradients and by agarose gel electrophoresis. Transformation by the rubidium method was as follows (TAKAHASHI *et al.* 1993). A tube of competent cells (200 μ l) was incubated with DNA on ice for 15 min, then shifted to 42° for 90 sec, and returned to ice for a further 15 min. The cells were then mixed with 800 μ l of SOC (HANAHAN 1985) and incubated at 37° for 1 hr. The competent cells were in a volume of 100 μ l and SOC was 900 μ l in some sets of experiments. The cells were immediately spread on L agar plates (pH was adjusted to 7.5 with Tris-HCl) containing various combinations of antibiotics and were incubated overnight at 37°. For the selection of chloramphenicol resistance incubation was for two nights. We avoided use of *cml* as a selection marker whenever possible. The concentrations of antibiotics were as follows: kanamycin, 50 μ g/ml; ampicillin, 50 μ g/ml (used together with methicillin, 150 μ g/ml); chloramphenicol, 15 or 25 μ g/ml. The relative transformation efficiencies on various antibiotics plates has been described (TAKAHASHI *et al.* 1992). Other methods of analysis were performed as described previously (KOBAYASHI and TAKAHASHI 1988; YAMAMOTO *et al.* 1988).

RESULTS

Inhibition of conservative (two-progeny) double-strand break repair by a heterologous insert at the

break: Figure 2 illustrates the experimental design used to demonstrate the presence of a conservative (two-progeny) double-strand break repair mechanism in an *E. coli* strain (JC8679) expressing the RecE pathway of homologous recombination (genotype *recBC sbcA23*) (KOBAYASHI and TAKAHASHI 1988; YOKOCHI *et al.* 1995). Its *sbcA23* mutation allows expression of *recE* and *recT* genes that are necessary for the double-strand break repair (TAKAHASHI *et al.* 1993). The *recBC* mutations have been used to study other kinds of homologous recombination mediated by the RecE pathway, but the presence of these mutations did not affect very much the double-strand break repair assay as measured in the present system (TAKAHASHI *et al.* 1993).

Our substrate plasmid pIK43 (Figure 2A) carries the *amp* gene and two deletion heteroalleles of the *neo* gene in an inverted orientation. A wild-type *neo* gene confers resistance to kanamycin. Because an oligonucleotide containing a site for the restriction enzyme *XhoI* is inserted at one of these deletions (deletion *a*), cleavage of this plasmid with *XhoI* generates a double-stranded gap corresponding to deletion *a* (Figure 2B). We introduced such gapped plasmid into *E. coli* cells and counted the number of Amp^R transformants as well as Kan^R transformants. The lower, wild-type sequence is expected to be used in the cells as a template to repair the gap in a conservative (two-progeny) recombination process. The number of Kan^R transformants served as a measure of the cells that had generated *neo*⁺ plasmids by homologous recombination through the double-strand break repair mechanism. The two predicted types of repaired products corresponding to gene conversion without and with crossing over (Figure 2, C and D, respectively) were found in these Kan^R cells. The number of the Amp^R transformants served as a measure of changes in transformation efficiency resulting from the break and its repair.

Transformation experiments with plasmid pIK43 are shown in Figure 3A. The double-strand break decreased Amp^R transformants by 0.9×10^{-3} in a highly competent *recA* strain, DH5, that was used as a negative control (data not shown). The same break decreased Amp^R transformants only by 0.12 in JC8679 (= *recBC sbcA*) (Figure 3A, first and second columns). In the control strain DH5, the fraction of Kan^R transformants was $<10^{-5}$ (Kan^R transformants/Amp^R transformants) and was not increased by the double-strand break (data not shown). In JC8679, the Kan^R transformants were $\sim 10^{-3}$ or less of the Amp^R transformants when uncut plasmid was used (Figure 3A, first column). Introduction of an *XhoI*-generated double-strand break increased Kan^R transformants ~ 100 -fold (Figure 3A, second column).

The possibility that these recombination events could be bimolecular has been excluded in a reconstruction experiment employing two marked plasmids in a design detailed earlier (YOKOCHI *et al.* 1995). We repeated this reconstruction experiment under the condition used in

TABLE 1
Plasmids and DNA

Name	Figure	Parent	Description	Source/reference
pBluescript SK(+)			A cloning vector with the ColE1 origin, fl origin, truncated <i>lacZ</i> gene, <i>amp</i> gene and T3 promoter with known sequence	STRATAGENE
Adenovirus, type 2			ds DNA with known sequence	BRL/GINGERAS <i>et al.</i> (1982)
pIK43	Figure 2		ColE1 origin; <i>amp</i> gene; inverted repeats each with a deletion mutant <i>neo</i> gene	YAMAMOTO <i>et al.</i> (1988)
pIK518 (=pKEN51)			The same as pIK43 except that a single <i>EcoRV</i> site is changed into a <i>SacI</i> site	TAKAHASHI <i>et al.</i> (1992)
pIK443	Figure 2	pIK518	A 943-bp-long fragment from adenovirus (8244 <i>XhoI</i> -9185 <i>EcoRV</i>) and a 10-bp-long fragment from pBluescript (<i>EcoRV</i> through <i>Clal</i>) inserted to the left of <i>XhoI</i> site of pIK518	This work
pKEN30		pIK43 & pACYC184	Double origin (ColE1 ori and p15A ori); Amp ^R and Cml ^R ; two different deletion alleles of <i>neo</i> gene in direct orientation. 10094 bp long.	TAKAHASHI <i>et al.</i> (1992)
pKEN33	Figure 4	pIK43 & pACYC184	Double origin (ColE1 ori and p15A ori); Amp ^R and Cml ^R ; two different deletion alleles of the <i>neo</i> gene in direct orientation; differs from pKEN30 in carrying a small deletion near <i>cml</i> gene. 9000 bp long.	TAKAHASHI <i>et al.</i> (1992)
pKEN32	Figure 6	pKEN33	Double origin (ColE1 ori and p15A ori); Amp ^R and Cml ^R ; two different deletion alleles of the <i>neo</i> gene in direct orientation; relative orientation of the repeats opposite to that in pKEN33.	TAKAHASHI <i>et al.</i> (1992)
pKEN34	Figure 6	pKEN33	Double origin (ColE1 ori and p15A ori); Amp ^R and Cml ^R ; one deletion allele and one insertion allele of the <i>neo</i> gene in direct orientation. 9283 bp long.	TAKAHASHI <i>et al.</i> (1992)
pBNT48	Figure 4	pKEN33	Recombination product; Kan ^R ; Cml ^R ; p15 ori. 4875 bp long.	This work
pBNT47	Figure 4	pKEN33	Recombination product; Amp ^R ; ColE1 ori. 4408 bp long.	This work
pBNT50		pKEN33	Recombination product, formally through gene conversion at the <i>XhoI</i> site without flanking crossing-over; Double origin (ColE1 ori and p15 ori); Amp ^R ; Cml ^R ; Kan ^R . 9283 bp long.	This work

TABLE 1

Continued

Name	Figure	Parent	Description	Source/reference
pNT141		pKEN33	80 bp (<i>Sall</i> - <i>SacI</i> fragment from pBSSK (+)) inserted to the left of the <i>XhoI</i> site.	This work
pNT101		pKEN33	80 bp (<i>Sall</i> - <i>SacI</i> fragment from pBS-SK (+)) inserted to the right of the <i>XhoI</i> site.	This work
pNT42		pKEN33	142 bp (9689–9831 from adenovirus) inserted to the left of the <i>XhoI</i> site.	This work
pNT47		pKEN33	142 bp (9689–9831 from adenovirus) inserted to the right of the <i>XhoI</i> site.	This work
pNT31		pKEN33	237 bp (9452–9689 from adenovirus) inserted to the left of the <i>XhoI</i> site.	This work
pNT317		pKEN33	237 bp (9452–9689 from adenovirus) inserted to the right of the <i>XhoI</i> site.	This work
pNT221 (=pNT22)		pKEN33	1208 bp (8244–9452 from adenovirus) inserted to the left of the <i>XhoI</i> site.	This work
pNT261 (=pNT26)		pKEN33	1208 bp (8244–9452 from adenovirus) inserted to the right of the <i>XhoI</i> site.	This work
pNT1-7		pKEN33	2587 bp (16744–19331 from adenovirus) inserted to the left of the <i>XhoI</i> site.	This work

the present work. We prepared pIK43 and its derivative (pIK500) that carries a single base-pair substitution mutation generating a unique *CpoI* site in the 283-bp-long region opposite the double-strand gap (Figure 2A). We transformed JC8679 with a mixture of 250 ng of pIK43 cut with *XhoI* and 250 ng of pIK500 cut with *XhoI*. Forty-two of the Kan^R transformants examined all turned out to carry a *XhoI*^R plasmid. The majority (37/42) of these plasmids inherited *CpoI* marker from only one of the parental plasmids (23 carried no *CpoI* site and 14 carried two *CpoI* sites). Only 5/42 carried markers from the two parents (one *CpoI* site). We concluded that the majority of the recombination events are unimolecular under these conditions. It is, therefore, highly unlikely that conservative (two-progeny) recombination is due to multiple rounds of nonconservative (one-progeny) recombination, although such a route was suggested by experiments done in a different genetic background (*recBC sbcBC*) (TAKAHASHI *et al.* 1992; YAMAMOTO *et al.* 1992).

To examine the effects of heterologous DNA on double-strand break repair, we constructed a plasmid (pIK443, Figure 2E) carrying a heterologous insert at the site of the double-strand break of pIK43. The insert is 954-bp-long, flanked by a unique *XhoI* site and a unique *EcoRV* site (see Table 1 for details). The insert

has a unique, internal *KpnI* site. Cleavage with *KpnI* generates the form shown in Figure 2F, with a 283-bp-long piece of DNA attached to the left of the double-strand break and a 671-bp-long piece attached to the right.

The plasmid pIK443 produced a very low fraction of Kan^R transformants when transferred into the *recA* control strain (DH5) (Kan^R/Amp^R < 1/80000). In this strain, *KpnI* cleavage decreased transformation efficiency (Amp^R) to 0.018, and no Kan^R transformants appeared (<10⁻⁴). In strain JC8679, uncut plasmid pIK443 showed a transformation efficiency comparable to that of pIK43 (Figure 3A, third column). *KpnI* cleavage increased the number of Kan^R transformants with pIK443, but the increase was not so large as that found with *XhoI* restriction of pIK43 (fourth column). This is one sign that heterology can reduce the efficiency of double-strand break repair. The decrease in the number of Amp^R transformants observed with *KpnI* cleavage of pIK443 was more severe than the decrease of Amp^R transformants observed with *XhoI* cleavage of pIK43 (Figure 3A, fourth column and 3B, second column). This is further evidence that heterology reduces the efficiency of double-strand break repair. We conclude that heterologous inserts attached to both sides of the

FIGURE 2.—Inhibition of the double-strand break repair reaction by a heterologous insertion attached to the double-stranded ends. (A) pIK43, a parental plasmid for the double-strand gap repair experiment. The two homologous *neo* segments are drawn as parallel lines. The restriction enzyme sites are as follows: *Xho* (*XhoI*) at 1083; *RI* (*EcoRI*) at 8025, 14794. (B–D). Double-strand gap repair during transformation. Restriction of pIK43 with *XhoI* results in a double strand gap in the upper segment (B). The double-stranded gap is repaired in *E. coli recBC sbcA* cells by gene conversion to copy the homologous sequence of the lower segment. This repair event restores a *neo*⁺ gene and makes the host cell resistant to kanamycin. The gap repair may take place without or with crossing over of the flanking sequences (C and D, respectively). The crossing over inverts the left part (D). (E) pIK443, a derivative of pIK43 with a 954-bp-long insertion at the *XhoI* site. The restriction enzyme sites are as follows: *Xho* (*XhoI*) at 1083; *Kpn* (*KpnI*) at 1366; *RV* (*EcoRV*) at 2026; *HindIII* at 3033 and others (not shown); *Sac* (*SacI*) at 11334. (F) A structure made by cutting pIK443 with *KpnI*. Heterologous DNAs are attached to both of the double-stranded ends at the double-strand break. These inhibit the double-strand break repair reaction leading to C and D.

double-strand break decrease conservative (two-progeny) double-strand break repair.

XhoI cleavage of pIK443 will leave the heterologous insert to the right of the double-strand break (Figure 2E), while *EcoRV* cleavage will leave the insert to the left (Figure 2E). Each of these enzyme treatments strongly decreased the number of Amp^R transformants (Figure 3B, third and fourth columns). When pIK443 is digested with both *EcoRV* and *XhoI*, most of the heterologous insert will be removed (Figure 2E) and the resulting form should be essentially the same as that of pIK43 digested with *XhoI* (except for 10 bp of heterologous DNA to the right of the break). In fact, pIK443 cut with *XhoI* and *EcoRV* gave signs of efficient double-strand break repair again. Relative to the uncut plas-

mid, there was only a slight decrease in the number of Amp^R transformants accompanied by a very large increase in the number of Kan^R transformants (Figure 3A, fifth column and 3B, fifth column). This resembles the data obtained with *XhoI*-digested pIK43. Thus, the inhibitory effect of a heterologous DNA on the double-strand break repair can also be detected when only one end at a double-strand break is blocked. In a control experiment, a short multiple cloning site derived from pBS-SK(+) was placed into pIK43 and transformation was examined after cleavage with various enzymes. We could not detect any difference between the efficiencies obtained with either a 5'-protruding end generated by *XhoI* cleavage, a 3'-protruding end generated by *KpnI* cleavage or a flush end generated by *EcoRV* cleavage.

FIGURE 3.—Partial inhibition of the conservative double-strand break repair by heterologous insertions. A fixed amount of competent cells from an *E. coli recBC sbcA* strain (JC8679) was transformed by the rubidium method with 0.5 mg of plasmid pIK43 DNA or pIK443 DNA, either intact or after complete digestion with the indicated restriction enzymes. Aliquots of the transformation mixture were spread on ampicillin or kanamycin plates, which were then incubated overnight. The numbers of the ampicillin-resistant transformants and the kanamycin-resistant transformants per transformation reaction in duplicate are shown in the vertical axis. A and B represent different experiments. The kanamycin-resistant transformants were few in B and are not shown.

Analysis of the products confirmed the occurrence of the double-strand break repair reaction. Of the six Kan^R transformants examined following cleavage of pIK443 with both *XhoI* and *EcoRV*, three represented gene conversion with crossing over and three corresponded to gene conversion without crossing over. Of six Kan^R transformants obtained using *KpnI*-digested pIK443, five had gene conversion type with crossing over while one had gene conversion without crossing over.

Distinguishing conservative (two-progeny) and non-conservative (one-progeny) recombination with double-origin plasmids: The above experimental system (Figure 2) selected only conservative (two-progeny) recombination stimulated by the double-strand break. It seemed important to know how often recombination is nonconservative (producing only one progeny) when a double-strand break (with and without a heterologous insert) stimulates recombination.

The experimental design shown in Figure 4 was used

to address this question (TAKAHASHI *et al.* 1992). The “double-origin” plasmids (Figure 4A) carry two mutant alleles of the *neo* gene in direct orientation. They also carry two compatible replication origins, one from ColE1 and the other from p15A. Each of these origins is linked to a selectable marker: *amp*^R, for ampicillin resistance, or *cml* (also called *cat*), for chloramphenicol resistance. The plasmid was introduced into bacterial cells, and recombinants (*neo*⁺) were selected.

If this recombination is conservative, that is, if two products are generated, then two copies of the *neo* gene should be recovered. These two copies of the *neo* gene will be found either on two smaller separate plasmid molecules (if flanking sequences have been exchanged) or on one large plasmid molecule (if flanking sequences have not been exchanged) (Figure 4C, lower left). The number of transformants resistant to both kanamycin and ampicillin should be close to the number of transformants resistant to kanamycin.

If recombination is nonconservative, that is, if one product is generated, then only one plasmid molecule carrying a single *neo*⁺ gene without an *amp*^R gene should be recovered (Figure 4C, lower right). In this case, the number of transformants resistant to both kanamycin and ampicillin should be much smaller than the number of transformants resistant to kanamycin. In control transformation experiments, we found that the efficiency of colony formation is essentially independent of the method of antibiotic selection (MATERIALS AND METHODS). Note that if recombination is nonconservative (one-progeny) and without flanking exchange, the circular plasmid molecule will become linear and will not be able to replicate unless there is very efficient end-joining. Such high-efficiency end-joining was not detected within the transferred plasmid molecule under our experimental conditions as detailed in the next section.

The double-origin plasmids were prepared in the *recA* strain DH5. Cells were grown for a minimal number of generations to minimize *neo*⁺ recombinants. The plasmid preparations were then transferred back into the *recA* strain to check the background level of *neo*⁺ recombination. We found that the ratio of Kan^R (recombinant) transformants to the Amp^R transformants was always $<10^{-4}$ (Figure 5C).

When strain JC8679 (= *rac*⁺ *recBC sbcA*; RecE pathway active) and strain JC7623 (= *rac*^{del} *recBC sbcBC*) were transformed with pKEN30, contrasting results were obtained. The latter strain carries *recB recC sbcB sbcC* mutations and has been used as one of the standard strains in analysis of so-called “RecF pathway” of homologous recombination. In this strain (Figure 5A), the Kan^R Amp^R transformants were far fewer than the Kan^R transformants. This and other lines of evidence (TAKAHASHI *et al.* 1992) demonstrate that the recombination is non-conservative (producing only one progeny) in this *recBC sbcBC* strain.

FIGURE 4.—Shift from conservative (two-progeny) recombination to nonconservative (one-progeny) recombination as a result of a heterologous insert at the double-strand break. (A) pKEN33, our prototype double-origin plasmid, is derived from pIK43 and pACYC184. The upper *neo* gene (arrow) has deletion *a* (box) with an *XhoI* linker. The lower *neo* segment (arrow) also from pIK43 carries deletion *b*. There exists a 1079-bp homologous region downstream and a 231-bp homologous region upstream as in pIK43 (filled region; see Figure 2A). The left unique (non-homologous) part includes the ampicillin resistance gene (*amp*) and the ColE1 replication origin (○). The right unique (non-homologous) part includes the chloramphenicol resistance gene (*cml*) and the p15A replication origin (●). (B) pNT317, a derivative of pKEN33 carrying a 237-bp heterologous insertion at the double-strand break site (Table 1). (C) Distinguishing conservative (two-progeny) recombination and nonconservative (one-progeny) recombination by a double-origin plasmid. The arrows indicate *EcoRI* (RI) sites. (D) Effect of a heterologous insert at the double-strand break on the switch between conservative (two-progeny) recombination and nonconservative (one-progeny) recombination.

In strain JC8679 (using the RecE pathway) the number of Kan^R Amp^R transformants was comparable to that of Kan^R single-resistant transformants (Figure 5B). This was observed even at an input well below the apparent saturation level (Figure 5B). This result is expected if recombination is often conservative (producing two progeny). The following experiments provide further support for this interpretation.

Effect of a double-strand break: Because the double-origin plasmids carry an *XhoI* linker at one of the mutant *neo* alleles, treatment *in vitro* with *XhoI* generates a double-strand gap of ~280 bp. In recipient JC8679 (RecE pathway), this treatment before transformation increased the number of Kan^R transformants and Kan^R

Amp^R double-resistant transformants to a similar extent (Figure 5B and Figure 9A, left box). The number of Kan^R Amp^R transformants using the cut plasmid was comparable to the number of Kan^R transformants. This is the expected result if the break mostly stimulated conservative (two-progeny) double-strand break repair.

This is in contrast to the effect of the double-strand break in JC7623 (RecF pathway). A large increase in the number of Kan^R transformants was not accompanied by an increase in the number of Kan^R Amp^R transformants (Figure 5A). This and other lines of evidence demonstrate that double-strand-break stimulation of recombination in RecF pathway is nonconservative (producing only one progeny) (TAKAHASHI *et al.* 1992).

FIGURE 5.—Transformation of various *E. coli* strains with pKEN30, a double-origin plasmid. Competent cells were incubated with various amounts of pKEN30 DNA. After allowing time for expression, the cells were spread on agar plates supplemented with kanamycin, ampicillin or both and incubated overnight.

When pKEN30 digested with *XhoI* was used to transform the *recA*[−] recipient, DH5, there was an excess of Kan^R transformants over Kan^R Amp^R transformants. The number of Kan^R transformants was still quite low (6×10^{-4}) when compared to the bulk (Amp^R) transformation efficiency of this highly competent strain (Figure 5C). The digestion with *XhoI* decreased the number of Amp^R transformants considerably (4×10^{-3} -fold) in this *rec*[−] strain (Figure 5C). This indicates the upper limit of escape from the cutting and/or religation of cut plasmid molecules. In the RecE pathway strain (JC8679), the reduction in the number of Amp^R transformants due to the cut was very slight (Figure 5B) indicating efficient recombination to generate an *amp*⁺ (Amp^R) plasmid.

Analysis of the products: We analyzed plasmids from the Kan^R Amp^R transformants obtained after transformation of strain JC8679 (RecE pathway) with a double-origin plasmid (pKEN33, Figure 4A) cut with *XhoI*. The results were consistent with the occurrence of conservative (two-progeny) double-strand break repair as illustrated in Figure 4C (Figure 6A and Table 2). The *XhoI* site was absent in all the Kan^R Amp^R clones examined (Figure 6A). Furthermore, the two expected plasmids, a large *cml*⁺ *neo*⁺ plasmid (pBNT48) and a smaller *amp*⁺ *neo*[−] plasmid (pBNT47) (illustrated in Figure 4C) were observed in all these Kan^R Amp^R clones (Figure 7, lanes 3 and 4). One parental-sized recombinant plasmid that can be formally described as the product of gene conversion at the *XhoI* site without flanking crossing over (pBNT50) was detected in one of the Kan^R Amp^R clones (data not shown). These three types of plasmids (designated pBNT48, pBNT47 and pBNT50, respectively) were isolated by secondary transformation into a *recA* strain. Restriction analysis (*XhoI*, *EcoRI*, *HindIII*, *PvuI*,

SadI, *BglII*) confirmed their molecular structure (Figure 7, lanes 5–8).

The gene conversion that repairs the double-strand break may or may not be associated with flanking crossing over (Figure 1, C and D). Preparations of pBNT50 (gene-conversion without crossing-over) from a *recA* strain contained a small but significant amount of a smaller *amp* plasmid that was indistinguishable from pBNT47 in mobility and restriction pattern. After transformation into strain JC8679 (RecE pathway) and selection for ampicillin and kanamycin resistance, pBNT50

FIGURE 6.—Plasmids in the transformants. The indicated double-origin plasmids were cut with *XhoI* and used to transform a *recBC sbcA* strain (JC8679). Plasmids were isolated from the resulting Kan^R (K^R) or Amp^R Kan^R (A^R K^R) transformants, cut with the indicated restriction enzyme, and electrophoresed through agarose gels. Marker lanes contain a circle ladder (BRL). (A) pKEN33. (B) pNT317.

TABLE 2
Products from the double-origin plasmids

Plasmid	Selection	Experiment	With <i>Xho</i> I site	Without <i>Xho</i> I site		Total clones analyzed
				One plasmid ^a	Two plasmids ^b	
pKEN33	Kan ^R and Amp ^R	I	0	0	6	6
		II	0	0	2	2
pKEN32	Kan ^R and Cml ^R	III	0	0	6	6
		IV	0	0	6	6
pKEN34	Kan ^R and Amp ^R	V	0	0	6	6
		VI	0	0	6	6
pNT31	Kan ^R and Amp ^R	VII	0	0	4	4
		VIII	0	0	3	3
pNT31	Kan ^R	VII	0	5	1	6
		VIII	0	2	1	3
pNT317	Kan ^R and Amp ^R	VII	0	0	6	6
		VIII	0	0	3	3
pNT317	Kan ^R	VII	0	5	1	6
		VIII	0	2	1	3

Plasmids carrying two mutant alleles of the *neo* gene were cut with *Xho*I at one of the mutant sites and transformed into *E. coli* JC8679 (= *recBC sbcA*) cells. The cells where recombination had occurred between the *neo* alleles to generate a *neo*⁺ plasmid (Figure 4) were selected by spreading on agar plate containing the indicated antibiotics. Plasmids from the transformant clones were analyzed with restriction enzymes, *Xho*I, *Eco*RI (8999, 3592), *Pvu*II (8372), *Sad*I (2761), and *Bgl*II (1750). Here the numbers indicate coordinates in pKEN33.

^a A 4875-bp-long *neo*⁺ circle of the type of pBNT48 (Figure 4).

^b A 4875-bp-long *neo*⁺ circle of the type of pBNT48 (Figure 4) and a 4408-bp-long *amp*⁺ circle (Figure 4), except for pKEN32. A 6.5-kb-long *neo*⁺ *amp*⁺ plasmid and a 5.4-kb-long *cml*⁺ circle were obtained with pKEN32. Some clones also carried a varying amount of a parental-size plasmid of the type that can be formed either by gene conversion at the *Xho*I site without crossing over or by reciprocal recombination between the two smaller plasmids (pBNT50 except for pKEN32).

produced two smaller plasmids indistinguishable from pBNT47 and pBNT48 (data not shown). This suggests that the rate of homologous recombination that converts the “non-crossing-over” type to the “crossing-over” type is high in the RecE pathway strain. Therefore, we did not attempt to classify the conservative (two-progeny) double-strand break repair recombination into crossing-over and non-crossing-over types.

From all of the above results, we conclude that the RecE pathway performs conservative (two-progeny) double-strand break repair frequently, even when there exists an alternative route of nonconservative (one-progeny) recombination.

More double-origin plasmids: The same conclusion is supported by results obtained using two different double-origin plasmids (Figure 8).

Exchange of origins and markers: We inverted both of the *neo* alleles on one of the double-origin plasmids, pKEN33 (Figure 4C and 8B), to make plasmid pKEN32 (Figure 8B). This results in the association of the *neo*⁺ gene with the other replication origin (ColE1 instead of p15A) and the other selectable marker (*amp* instead of *cml*) after recombination. Results again indicated that the RecE pathway causes the frequent occurrence of conservative (two-progeny) double-strand break repair. That is, the number of Kan^R Cml^R transformants

obtained using *Xho*I-digested plasmid was close to that of Kan^R transformants (Figure 8A). We found the two expected recombination products (*neo*⁺ *amp*⁺ plasmid and *cml*⁺ plasmid) in all the examined Kan^R Cml^R transformants from pKEN32 (Table 2). These results eliminate the possibility that the observed similarity in transformant numbers solely resulted from artifacts arising from antibiotic selection.

Replacing the *neo* allele: In the above double-origin plasmid (pKEN33), both the *neo* alleles carried a deletion of >200 base pairs. One is a deletion between two *Nae*I sites, spanning the C terminus of the *neo* protein, marked with an 8-bp *Xho*I linker. We replaced this deletion allele with an insertion allele, a simple insertion of the 8-bp *Xho*I linker into the *Nae*I site within the *neo* coding region. The resulting plasmid, pKEN34 (Figure 8B), gave us essentially the same results (Figure 8A); the number of the Kan^R Amp^R transformants was close to that of the Kan^R colonies (Figure 8A). We found the two expected recombination products (*neo*⁺ *cml*⁺ plasmid and *amp*⁺ plasmid) in all the examined Kan^R Amp^R transformants from pKEN34 (Table 2).

Effect of heterologous insertions attached at the double-strand break on the double-origin plasmids: We examined the effect of heterologous DNA at the double-strand break on the preference for conservative (two-

FIGURE 7.—Recombination products. Lanes 1 and 2, the parental plasmid pKEN33 (Figure 4 and Table 1) prepared in DH5 with ampicillin selection. Lanes 3 and 4, the plasmid from a Amp^R Kan^R transformant of JC8679 (= *recBC sbcA*) from pKEN33. Lanes 5 and 6, pBNT47 (Figure 4 and Table 1) prepared in DH5 with ampicillin selection. Lanes 7 and 8, pBNT48 (Figure 4 and Table 1) prepared in DH5 with kanamycin selection. The plasmids were subjected to electrophoresis through 0.8% agarose. The numbers to the left indicate the size of two *Eco*RI fragments of pKEN33. The numbers to the right indicate the size of linearized pBNT47 and pBNT48, respectively.

progeny) recombination or nonconservative (one-progeny) recombination using the double-origin plasmid system (Figure 4, B and D). We constructed plasmids carrying heterologous inserts of varying lengths at the double-strand break site (*Xho*I site) of the prototype double-origin plasmid, pKEN33 (Table 1). Depending on the orientation of the insert, the *Xho*I site is regenerated at the left end of the insertion (Figure 4, B and D) or at the right end of the insertion. When these plasmids are cut with *Xho*I, the resulting form is the same as pKEN33 cut with *Xho*I (Figure 4C) except that the heterologous insertion is attached to the right or the left end of the double-strand break (Figure 4D).

One of these pKEN33-based plasmids carrying an insert (pNT221) produced Kan^R transformants at a level similar to that of the plasmid without the insert (pKEN33) (Figure 9A). The Kan^R Amp^R transformants and the Kan^R transformants were comparable in number. When the plasmid was cut (pNT221) with *Xho*I before transformation, which leaves the heterologous DNA to the left of the double-strand break, a large difference between the Kan^R transformants and the Kan^R Amp^R transformants was observed. In other words, the conservative (two-progeny) reaction as a fraction of total recombination events, including nonconservative (one-progeny) reactions, was decreased by the presence of the heterologous insertion. This effect also was observed when the heterologous DNA was present at the

B.

FIGURE 8.—Transformation of a *recBC sbcA* strain with the double-origin plasmids. (A) Transformation. The procedure is as described in Figure 5 legend, except that Kan^R Cml^R transformants (colonies on agar containing kanamycin and chloramphenicol) instead of Kan^R Amp^R transformants were scored for pKEN32. (B) Double-origin plasmids. See Table 1 for details.

right side of the double-strand break (Figure 9, plasmid pNT261 cut with *Xho*I).

When these two plasmids (pNT221 and pNT261) are cut with the restriction enzyme *Kpn*I, which cuts in the middle of the heterologous insert, the heterologous sequence will be attached to both ends of the double-strand break (Figure 9B). The ratio of Kan^R Amp^R transformants to Kan^R transformants was less with these plasmid preparations than with the plasmid without the insertion (Figure 9A). The heterologies apparently interfered with conservative (two-progeny) recombination and caused more of the repair to be nonconservative.

We examined a series of plasmids carrying various lengths of heterologous insert after different modes of cutting (Figure 10, right diagram). The decrease in the fraction of conservative (two-progeny) recombination roughly paralleled the increase in the total length of the heterologous inserts (Figure 10, left). These would be the results expected if a shift had occurred from conservative (two-progeny) recombination to nonconservative (one-progeny) recombination.

FIGURE 9.—Transformation with double-origin plasmids with heterologous insertions at the double-strand break. The plasmids (0.5 μ g) shown in B were cut with the indicated restriction enzymes and used to transform a *recBC sbcA* strain. Transformants were selected with the indicated antibiotics. Their number per reaction is plotted in A. The straight lines indicate recombination to *neo*⁺ in (B).

We analyzed the products from two of these plasmids, pNT31 and pNT317. All the Kan^R Amp^R transformants examined contained plasmids without an *XhoI* site. They all contained two plasmids indistinguishable from pBNT48 and pBNT47 (Figure 4). This is what one would expect from conservative (two-progeny) double-strand break repair (Figure 6B and Table 2). In all the Kan^R transformants examined, the plasmid had lost the *XhoI* site (Figure 6B and Table 2). Most had only one

plasmid species, the *neo*⁺ *cml*⁺ plasmid, indistinguishable from pBNT48 (Figure 6B and Table 2).

***recE*-dependence of the nonconservative (one-progeny) recombination:** Is the nonconservative (one-progeny) recombination event observed in the presence of the DNA heterology mediated by the RecE pathway of homologous recombination or by some other mechanism? As shown in Table 3, we found that the level of total recombination (recombination to Kan^R transformants) is lowered by at least 10-fold by the *recE159* mutation that inactivates exonuclease VIII, an essential component of the RecE pathway (KOLODNER *et al.* 1994; KUSANO *et al.* 1994b). Therefore, these nonconservative (one-progeny) recombination events are mediated by the RecE pathway.

DISCUSSION

Two-progeny or one-progeny: Our results demonstrate that repair of a double-strand break by the RecE pathway frequently leads to conservative (two-progeny) recombination even when there exists an alternative route of nonconservative (one-progeny) recombination. This is consistent with the high efficiency (close to 100%) of conservative (two-progeny) double-strand break repair under several conditions (TAKAHASHI and KOBAYASHI 1990; KUSANO *et al.* 1994b; YOKOCHI *et al.* 1995). Heterologous DNAs attached to one or both ends at the double-strand break caused an apparent shift from the conservative (two-progeny) mode to nonconservative (one-progeny) mode.

The Red system of lambda is very similar to the RecE recombination pathway. Heterologous DNAs caused a shift from the two-progeny mode to one-progeny mode

FIGURE 10.—Effect of the length of heterologous DNA on the fraction of conservative (two-progeny) recombination events. The plasmids described in Table 1 were cut with various restriction enzymes to generate one of the forms shown to the right and used to transform a *recBC sbcA* strain. Kan^R Amp^R transformants and Kan^R transformants were counted. The ratio ($\{[Kan^R Amp^R \text{ transformants}] / [Kan^R \text{ transformants}]\}$ from cut DNA) / ($\{[Kan^R Amp^R \text{ transformants}] / [Kan^R \text{ transformants}]\}$ from intact DNA) was taken to be the fraction of conservative (two-progeny) recombination and was plotted against the total length of the heterologous DNA. The straight lines in the right diagram indicated recombination to *neo*⁺.

TABLE 3
Effect of a *recE* mutation on recombination

Plasmids	Cell genotype	Kan ^R transformants from <i>Xho</i> I-cut plasmid	Amp ^R transformants from intact plasmid	Ratio
pNT31	<i>recBC sbcA</i>	6.8×10^3	33×10^3	0.21
	<i>recBC sbcA recE</i>	0.093×10^3	42×10^3	0.0021
pNT317	<i>recBC sbcA</i>	0.84×10^3	21×10^3	0.040
	<i>recBC sbcA recE</i>	0.067×10^3	17×10^3	0.0039

Plasmids carrying two deletion *neo* genes were cut with *Xho*I at one of the deletion mutations and transformed into *E. coli* strains of the shown genotype. The cells where recombination between the *neo* genes generated *neo*⁺ plasmids (Figure 4) were selected by spreading on agar plate containing kanamycin. Bulk transformation efficiency was measured by counting Amp^R transformants from plasmids without the *Xho*I cut.

in Red pathway (N. TAKAHASHI and I. KOBAYASHI, unpublished results). Red-mediated homologous recombination between lambda and a plasmid is stimulated by a restriction cut *in vivo* in lambda sequences (THALER *et al.* 1987). This result is consistent with both the conservative (two-progeny) mode and nonconservative (one-progeny) mode. THALER *et al.* (1987) performed lambda crosses of the type {A B C D} × {a b c d} under *red*⁺ *gam*⁺ conditions. B/b and C/c are closely linked. A double-strand break was delivered at B or b by restriction *in vivo* and {a D} recombinants were selected. When the break was at B, there were more {a c D} than {a C D} and many heteroduplexes at C/c. When the break was at b, there were more {a C D} than {a c D} and only few heteroduplexes at C/c. They interpreted these results as evidence for the "nonreciprocity" of the recombination. However, such a bias is also entirely consistent with the conservative (two-progeny) double-strand break repair mechanism as illustrated in Figure 1, B and C, as well as the nonconservative (one-progeny) mechanism in Figure 1, B–G. In the upper product of Figure 1C and in the product of Figure 1G, the exchange reaction and resulting heteroduplex are likely to be to the right of the double-strand break. Recombination stimulation at *cos*, the site for double-strand breakage for lambda packaging, is apparently nonconservative (nonreciprocal) in a bacteriophage lambda cross (STAHL *et al.* 1985; STAHL and STAHL 1985). This nonreciprocity was explained by interference between the packaging machinery and recombination (FEISS *et al.* 1983; STAHL *et al.* 1985; STAHL and STAHL 1985).

Unequal recovery of two crossover-type products was observed in a study where the recombinants in a RecE pathway were physically monitored after *in vivo* restriction cuts of the infecting phagemid (SILBERSTEIN *et al.* 1995). The inequality was stronger when the double-strand break was located close to the end of the homologous region. The restriction cuts were not complete in these experiments. Asynchrony of the two restriction cuts *in vivo* would leave long heterologous DNA at the double-strand break under these experimental conditions and may have decreased the level of the two-prog-

eny reaction. Indeed, our present results demonstrate that heterologous inserts at a double-strand break suppress conservative (two-progeny) double-strand break repair relative to the nonconservative (one-progeny) recombination mechanism. Such a relative shift is consistent with the insensitivity of the ratio {(transformants from linearized tandem dimer)/(transformants from intact dimer)} to the attachment of heterologous DNA at the double-strand break in the RecE pathway as described in an earlier work (LUISI-DELUCA and KOLODNER 1992).

Taken together with reports from other laboratories, results from our group are consistent with the notion that the double-strand-break-stimulated recombination in RecE and Red pathways is reciprocal (conservative, two-progeny) but can be made nonreciprocal (nonconservative, one-progeny) under several conditions.

Mechanism: Figure 11 illustrates one of the possible mechanisms underlying the shift to the one-progeny mode induced by heterologous inserts. These mechanisms are consistent with the known properties of RecE-mediated recombination (TAKAHASHI *et al.* 1993; KOLODNER *et al.* 1994; KUSANO *et al.* 1994a,b) and those of Red-mediated recombination (KMIEC and HOLLOMAN 1981; MUNIYAPPA and RADDING 1986; POTEETE 1993). Note that double-strand break repair is independent of the RecA function (TAKAHASHI *et al.* 1993). We propose that after annealing with a homologue, the 3' OH end of the invading single-strand primes DNA synthesis and copies the template when there is no heterologous DNA (A–F). This DNA synthesis will be necessary at both ends of the break for the completion of the conservative (two-progeny) double-strand break repair reaction. Heterologous DNA attached to the end may allow pairing but would inhibit priming (G–J). Priming of DNA synthesis would be relatively unnecessary for the nonconservative (one-progeny) recombination. The dependence on the length of the insert may be explained by removal of the inserts by exonucleases or endonucleases inside the cell (FISHMAN-LOBELL and HABER 1992).

Role: What do the present findings tell us about the roles, or the selective advantages, of homologous recombination in general and the Red- and RecE-medi-

FIGURE 11.—A possible mechanism underlying the shift from conservative (two-progeny) to nonconservative (one-progeny) recombination by a heterologous insertion. The action of RecE or Reda exonuclease at a double-strand break (A and B) exposes a recombinogenic single-strand tail (C and D). It pairs with a homologue aided by RecT protein or Redb protein (D) and primes DNA synthesis (E). Two products are generated (F). When a heterologous DNA is present (G), the single-stranded tail (H) can pair with a homologue (I) but cannot prime DNA synthesis. Only one product is produced (J).

ated double-strand break repair mechanism in particular? In the Introduction, we discussed the repair hypothesis, together with its two difficulties, the existence of flanking (half) crossing over and outcrossing.

Intraclonal recombination could help a bacteriophage clone survive an encounter with a host bacterial population carrying a particular restriction modification system. Cleavage of a recognition site in the phage genome would stimulate its recombination with an uncut site. This type of recombination could be conservative or nonconservative, although the conservative mode would be more advantageous for the phage genome. Nonconservative recombination between two chromosomes cut at different loci would allow reconstitution of one intact chromosome. This would explain why flanking crossing over (for conservative recombination) or half-crossing over (for nonconservative recombination) can be beneficial.

The effect of restriction-break-stimulated recombination on phage survival as a population would be larger if recombination takes place with a partner who is a member of another phage clone possessing, if possible, a diverged genome (outcrossing). When the partner

FIGURE 12.—An anti-restriction-modification hypothesis for the double-strand break repair mechanism of lambdoid phage recombination. (i) A phage chromosome. ABC and abc are different in sequence. (ii) A restriction enzyme recognizes and cuts at site B. The double-strand break stimulates homologous recombination of the cut chromosome and a homologous uncut chromosome without the recognition site (b). (iii) When there is no large heterology around the double-strand break, conservative (two-progeny) recombination regenerates two intact duplex DNAs. Because the two chromosomes differ at B/b, the site recognized by the restriction modification system (site B) is lost. (iv) When there is large heterology around the break, nonconservative (one-progeny) recombination generate one intact duplex DNA. The site recognized by the restriction modification system (site B) is lost. There may be a decrease of this restriction modification system and increase of restriction modification systems that would recognize site b through frequency-dependent selection. Alternatively, the phage population might encounter bacterial populations with a different combination of restriction modification systems. The attack of such a restriction enzyme at b would regenerate genotype B. This cycle of chase between the host (phage genome) and the parasites (restriction modification systems) would continue forever.

genome lacks the recognition site, recombination may result in a genome devoid of the particular restriction site (Figure 12). This should have short-term as well as long-term selective advantage in the presence of the restriction modification system. As a phage population encounters bacterial populations with various combinations of restriction modification systems, the fitness of a particular genotype will be changed forever. This hypothesis that double-strand-break-stimulated homologous recombination is advantageous is reminiscent of the anti-parasite hypothesis for sex (meiosis and outcrossing) (HAMILTON *et al.* 1990). Indeed, some type II restriction modification systems resemble parasites in that they force their maintenance on host cells by killing host cells that have failed to maintain them (KUSANO *et al.* 1995; NAITO *et al.* 1995; KOBAYASHI 1996; Y. NAKAYAMA, T. NAITO and I. KOBAYASHI, unpublished results).

The double-strand break repair mechanism might also help bacteriophages in their competition with transposons. Some transposons may leave a double-strand break after cut-and-paste transposition (*e.g.*, HAGEMANN and CRAIG 1993). The double-strand break repair mechanism will repair the double-strand break in the phage genome made by the transposon as suggested

for the *P* element in *Drosophila* (NASSIF *et al.* 1994). When the cleavages at the two sides of an inserted transposon are not coordinated (HANIFORD and KLECKNER 1994), the resulting genome carries double-strand breaks connected to a long heterologous insert when compared with a homologous genome without transposons (Figure 1F). Recombination between these genomes would favor formation of a progeny DNA free from the transposon as demonstrated in this work. The chromosomes would again benefit from outcrossing because the double-strand break can be repaired by copying a homologue without the transposon and possibly without its preferred insertion site.

Such competition with other “selfish gene” units such as restriction systems and transposons may have been important in the evolution and maintenance of the homologous recombination systems of bacteriophages, although there is not enough data to exclude DNA damages of a nonbiological origin as important factors. In fact, the shift from the conservative mode to the nonconservative mode should eliminate many types of DNA damages, just as it is able to eliminate heterologous DNA (Figure 1). It would repair damages not recognized by any repair machinery in the cell. This may explain why there is a correlation between damage repair and the association of flanking exchange with double-strand break repair (KUSANO *et al.* 1994a). Although recombinational repair with a prophage could explain the presence of outcrossing, the advantage would not be so large as in the case of anti-restriction-modification and anti-transposon, which prefer specific sequences.

What about cellular recombination as opposed to bacteriophage recombination? The mode of action of the RecBCD homologous recombination system of *E. coli* (KOWALCZYKOWSKI *et al.* 1994) may be different from the conservative double-strand break repair mechanism described above (ROSENBERG and HASTINGS 1991; TAKAHASHI *et al.* 1993; MYERS and STAHL 1994) but can still be understood as another kind of adaptation to the competition among selfish genes. This could occur through the following series of events. After double-strand cleavage by a restriction system, the RecBCD enzyme would enter duplex DNA and initiate exonucleolytic degradation. This would destroy infecting phage DNAs (SIMMON and LEDERBERG 1972). If the enzyme encounters a chi sequence, which would serve in this case as an identification marker for the *E. coli* chromosome, degradation would stop and promotion of recombinational repair with the sister chromosome would occur. These two reactions are uncoupled in the combination of one mutant RecBCD enzyme and one chi variant sequence (N. HANDA, S. OHASHI, K. KUSANO, and I. KOBAYASHI, unpublished results). Thus, the bacterial RecBCD system collaborates with the restriction-modification system to counteract infecting phage DNA but defends the bacterial chromosome from attack by the restriction modification systems. A double-strand

FIGURE 13.—Patrol model: an anti-insertion hypothesis for the double-strand break repair mechanism of meiotic recombination. (i) Two homologous chromosomes in a diploid cell. (ii) A transposon inserts at site B of the upper chromosome. (iii) A “patrol” endonuclease somehow senses the insertion and introduces a double-strand break there. (iv) This stimulates homologous recombination of the cut chromosome and the homologous chromosome. (v) When the insert is not large (when the insertion is not serious), conservative (two-progeny) recombination regenerates two intact duplex DNAs free from the insertion. When the two chromosomes are different at B, the site preferred by the transposon is lost. (vi) When the insert is large (when the insertion is serious), nonconservative (one-progeny) recombination generates one duplex DNA without the insertion. When the two chromosomes are different, the site preferred by the transposon (site B) is lost. The chromosomal region defined by the combination of A and C is replaced by the chromosomal region defined by the combination of a and C. Decrease of genotype ABC and increase of genotype abC would lead to decrease of this transposon and increase of transposons which favor genotype abC. Its attack would regenerate genotype ABC. This cycle of chase between the host (chromosomes) and the parasites (transposons) would continue forever.

break in chromosomal DNA by restriction modification systems or by transposons, may cause a cascade of non-conservative (one-progeny) recombination (Figure 5A) by the RecF pathway of recombination in *E. coli* (TAKAHASHI *et al.* 1992) and reconstitute one intact chromosome (YAMAMOTO *et al.* 1988, 1992, 1996).

The patrol model: meiotic recombination and sex as an anti-transposon strategy: Based on the above findings and considerations and also other experimental and theoretical work, we propose that the capacity of this double-strand break mechanism to eliminate selfish genes, more specifically, transposon insertions, underlies the evolution and maintenance of meiosis and sexual reproduction. We propose that meiotic recombination through the double-strand break repair mechanism allows the detection and the elimination of insertions (such as viral and nonviral transposons) from the genome as illustrated in Figure 13.

In meiosis with inbreeding (two identical homologous chromosomes), a diploid cell is formed by endo-

mitosis or by fusion of haploid cells of identical genotype. When a transposon inserts itself into one chromosome of a diploid cell (or has already been inserted into one chromosome before fusion), an endonuclease somehow senses the resulting changes in the chromatin structure and introduces a double-strand break at the transposon site. Recombination triggered by the break results in chromosomes free of the transposon.

The endonuclease and the recombination machinery are proposed to sense the nature of the transposon and/or the chromatin changes, and one of three possible modes of recombination are open. When the transposon is long or in some way represents a serious menace for the genome, nonconservative (nonreciprocal, generating only one-progeny DNA out of two parental DNAs) recombination with an intact homologue will reconstitute one intact chromosome free from the insertion (vi). The regions flanking the transposon/break are exchanged. One haploid cell and one dead aneuploid cell will result. When the insertion is not large or does not represent a serious menace, the double-strand break is repaired by an intact homologous chromosome through the conservative (generating two progeny DNAs out of two parental DNAs) double-strand break repair mechanism with flanking exchange. This results in elimination of the insertion (v). The cell may remain diploid (a prototype of meiosis) or may produce two haploid cells (one-step meiosis). The third mode of recombination is repair of the break by gene conversion unaccompanied by flanking exchange (not shown). Because the ability of the endonucleases to discriminate between "self" and "non-self" states in the chromatin structure is not perfect, there are "background" double-strand breaks even in the absence of insertion. These would lead to two-progeny recombination (either with or without flanking exchange). We do not know the features of the insert that result in differential recognition by the endonuclease. We hypothesize that these may be related to the chromatin structural changes that cause stronger double-strand breaks and stronger hot spots in *Saccharomyces* meiosis (LICHTEN and GOLDMAN 1995).

When the two chromosomes are not identical, the above mechanisms have the advantage of eliminating the genotype or local chromosome configuration that favored transposon insertion and should thus prevent further infection with copies of this transposon. Among the wide variety of transposons that have been identified, there exist unique patterns of preference with regard to their insertion sites (BERG and HOWE 1989). Many levels of chromosome organization and structure could be involved in the determination of the preferential insertion site. Particular nucleotide sequences might be favored (LICHTENSTEIN and BRENNER 1982). The term "regional specificity" with reference to some chromatin structure is appropriate for many transpo-

sons. The mechanism in Figure 12 would eliminate the site (B as opposed to b) chosen by the transposon along with the transposon itself. This would decrease the likelihood of further insertions by copies of this transposon in the same locus. An increased fitness of the genome would result if the transposon insertions had deleterious effects. The frequency of allele b would thus increase and the frequency of transposons preferring site B would decrease. The transposons preferring site b would insert at b. The same cycle of double-strand-initiated gene recombination would replace allele b with other alleles including B, which have now selective advantage. Accordingly, there would be cyclic changes in the frequency of the host alleles (B, b etc.) and "renewal" of transposons with alternative preferences.

This recombination mechanism would also replace the chromatin region defined by the combination of the flanking sequences (alleles) AC, which had been chosen by the transposon, by the chromatin region defined by other combinations such as aC and Ac. This should decrease the further likelihood of infection by this transposon. The appearance of the new host genotypes, aC and Ac, would be followed by an increase in the number of transposons favoring these genotypes. Accordingly, the old host genotype AC would increase in frequency. We suggest that such "arms-race" type coevolution of the host and the "molecular parasites" would continue and could eventually favor the evolution of recombination and outcrossing.

The evolutionary "arms race" between host organisms and their parasites was proposed to be responsible for the evolution of meiotic recombination and sex (anti-parasite hypothesis (HAMILTON *et al.* 1990)). A difficulty with this model is that meiotic recombination occurs more or less everywhere along the chromosomes, but not all the genes on the chromosome appear to be involved in defense against parasites. Our model proposes that the relevant parasites are transposons, a group of genomic parasites, whose potential target is the whole chromosome itself. The parasite model for sex works well when many loci are involved in the defense against parasites. There exist numerous transposons for a given host. Each of the numerous sites along the chromosomes of the host represent a potential site for transposon insertion. If there exist numerous alleles in a locus, sex will be favored. One locus (which can be defined as the region surrounding a gene) can take on many allelic states with respect to transposon preference. Our model should allow faster evolution of recombination than the parasite model because the very act of parasite infection triggers elimination of the susceptible genotype.

There are proposals that meiosis has evolved for anti-mutation (KONDRASHOV 1988), anti-damage (repair) (BERNSTEIN *et al.* 1987) and anti-meiotic drive (HAIG and GRAFEN 1991) among many others (MICHOD and LEVIN 1988). Our model, which will be detailed else-

where, tries to incorporate the respective merits and overcome the difficulties posed by these hypotheses. ROBIN HOLLIDAY proposed that the role of meiotic gene conversion might be the elimination of selfish DNA (HOLLIDAY 1982). MATTHEW MESELSON proposed that a principal advantage of sexual reproduction is the reduction in deleterious load that would otherwise be caused by mobile elements (ARKHIPOVA *et al.* 1995).

There are several known mechanisms for the inactivation of repeated genes that may depend on homologous interaction (KOBAYASHI *et al.* 1995). These include point mutagenesis (RIP, repeat-induced point mutation) (SELKER 1990), methylation (MIP, methylation induced premeiotically) (ROSSIGNOL and FAUGERON 1994), head-to-head fusion of free DNA ends (KUNES *et al.* 1990), illegitimate recombination (SAKAGAMI *et al.* 1994; FUJITA *et al.* 1995; K. KUSANO, K. SAKAGAMI, T. NAITO, Y. TOKINAGA, and I. KOBAYASHI, unpublished results), and repeat-induced gene silencing in plant cells (MATZKE *et al.* 1994). The efficiency of RIP is not 100%, but it still results in a genome free from active transposons (KIMSEY *et al.* 1994).

Meiosis and sex evolved in the protists, the first type of eukaryotes (MARGULIES and SAGAN 1986). A characteristic of eukaryotes has been the symbiosis of bacteria (MARGULIES 1993; GOLDING and GUPTA 1995), which have developed into the present day organelles. The chromosomes of the eukaryotic nuclei would assure the partition of genes to daughter cells at mitosis and represent a comfortable habitat for parasitic genes. It appears that many genes on the symbiotic bacterial chromosome moved into the evolving eucaryotic chromosome (*e.g.*, BROWN and DOOLITTLE 1995). We hypothesize that meiosis and sex evolved at this stage to fight against infection by these parasitic DNAs. The parasitic genes include homing endonucleases that are still present in the organelles of many organisms (BELFORT 1995). The "fighters" themselves may include homing endonucleases that may have moved in earlier during evolution.

This model was motivated by the finding that insertion of foreign DNA into *Saccharomyces* can create a hot spot for double-strand breakage that is at the same time a recombination hot spot (CAO *et al.* 1990; WU and LICHTEN 1994). The other features of meiotic recombination in yeast and of sexual reproduction in other organisms that can be explained by our hypothesis will be reviewed elsewhere. Part of the theoretical framework presented here has appeared before in the literature (KOBAYASHI 1994, 1996; KOBAYASHI *et al.* 1995). Quantitative treatments of the model will be presented elsewhere.

I.K. appreciates discussions with AKIRA SASAKI, YOH IWASA, TETSUKAZU YAHARA, ROBIN HOLLIDAY, MATTHEW MESELSON, JOHN MAYNARD SMITH. The work at IMS was supported by grants to I.K. from Ministry of Education, Science, Sports and Culture of the Japanese government (Recombination; Molecular Evolution; Gene Therapy; DNA Re-

pair) and Ministry of Health and Welfare (Gene Therapy) of the Japanese government.

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Communicating editor: S. JINKS-ROBERTSON