Role of *recBC* Function in Formation of Chromosomal Rearrangements: A Two-Step Model for Recombination

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ABSTRACT

The role of *recBC* functions has been tested for three types of chromosomal recombination events: (1) recombination between direct repeats to generate a deletion, (2) recombination between a small circular fragment and the chromosome, and (3) recombination between inversely oriented repeats to form an inversion. Deletion formation by recombination between direct repeats, which does not require a fully reciprocal exchange, is independent of *recBC* function. Circle integration and inversion formation are both stimulated by the *recBC* function; these events require full reciprocality. The results suggest that half-reciprocal exchanges can occur without *recBC*, but *recBC* functions greatly stimulate completion of a fully reciprocal exchange. We propose that chromosomal recombination is a two-step process, and *recBC* functions are primarily required for the second step.

THE *recBC* gene product (*Exo*V nuclease) is a major enzyme involved in bacterial recombination (for review see CLARK 1973; STAHL 1979a, b; SMITH 1983, 1988). However, its precise role *in vivo* remains unclear. Results of *in vitro* experiments indicate that double-stranded ends are the major substrate for the RecBC enzyme (GOLDMARK and LINN 1972; KARU *et al.* 1973; TELANDER-MUSKAVITCH and LINN 1982; SMITH *et al.* 1984; PONTICELLI *et al.* 1985; TAYLOR *et al.* 1985). *In vivo* data also support the proposal that double-stranded ends are required for RecBC activity. Studies of recombination stimulated by the double strand ends of lambda (*cos*), and ends generated by *Eco*RI cutting, have led STAHL and coworkers to propose that double-stranded ends provide an entry site for a recombinase (KOBAYASHI *et al.* 1983, 1984; STAHL *et al.* 1983; KOBAYASHI, STAHL and STAHL 1984).

We are interested in examining the RecBC dependence of exchange events that occur between sequences within the circular bacterial chromosome, since these substrates, at least initially, have no double-stranded ends. If double-stranded ends are a required substrate for RecBC, then a RecBC dependence of chromosomal recombination would suggest that double-stranded ends are intermediates in the recombination events that rearrange the chromosome. We have tested the effect of *recBC* mutations on events that require half- and fully reciprocal exchanges. Fully reciprocal recombination involves the rejoining of both pairs of flanking markers from a single recombination event (Figure 1); *A* is joined to *b* and *a* is joined to *B*. Half-reciprocal recombination involves the rejoining of only one pair of flanking markers

from a single recombination event, *either A* is joined to *b or a* is joined to *B*; by this definition, a half-reciprocal exchange would leave double-strand breaks adjacent to the unjoined flanking sequences.

These tests have been made using a genetic assay for observing recombination events within the bacterial chromosome (MAHAN and ROTH 1988). In this assay, all substrates and recombination functions are exclusively provided by the bacterial chromosome. We previously used this assay to provide evidence for full reciprocality of intrachromosomal exchanges. Here we use the system to study the effects of *recBC* mutations on three types of recombination events: (1) recombination between direct repeats (deletion formation), (2) chromosomal integration of the circular by-product of an intrachromosomal deletion event, and (3) inversion rearrangement of the bacterial chromosome. A model is presented which proposes that the RecBC enzyme enters recombining molecules at double-stranded breaks that are generated as intermediates in recombination.

MATERIALS AND METHODS

Bacterial strains: All strains used in this study (Table 1) were derived from *Salmonella typhimurium* strain LT2. Strains with directed transpositions were constructed according to methods described previously (CHUMLEY and ROTH 1980; SCHMID and ROTH 1980). Isolation and characterization of *recB*::MudJ and *recC*::MudJ insertions is described elsewhere (MAHAN and ROTH 1989). *recB10* allele was obtained from A. EISENSTARK (EISENSTARK *et al.* 1969).

Isolation of His⁺ recombinants and Hol⁻ segregants: Strains from a frozen culture of the parent strain to be tested (*e.g.*, TT13870, see RESULTS) were streaked for single colonies on nutrient broth solid medium and grown 20–24 hr at 37°. One colony was resuspended in 1 ml of E medium. This suspension was split into 3 fractions; one fraction was used for each of the following determinations.

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TABLE 1

Bacterial strains

Strain[a]	Genotype
TT11077	DEL646[(cob21*MudJ*hisF9951)]
TT13870	ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13871	recB10, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13873	zgb-3669::MudJ, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13874	recB497::MudJ, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13875	recC498::MudJ, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13880	ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13881	recA1, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13882	recB10, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13883	recA1, recB10, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13884	pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13885	recB10, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13886	zgb-3669::MudJ, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13887	recB497::MudJ, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13888	recC498::MudJ, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13889	pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13890	recA1, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13891	recB10, ara-651::(Tn10-hisOGDC8691-Tn10), DUP885[(hisOGD646-C8691)*Tn10*(hisO-E⁺)], proAB47
TT13892	recB10, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13899	recC498::MudA, ara-651::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47
TT13900	recC498::MudA, pncB165::(Tn10-hisOGDC8691-Tn10), hisOGD646, proAB47

[a] All strains used in this study were derived from *Salmonella typhimurium* LT2; all strains were constructed in this laboratory.

Fully Reciprocal
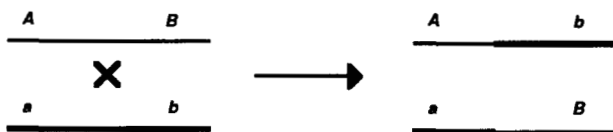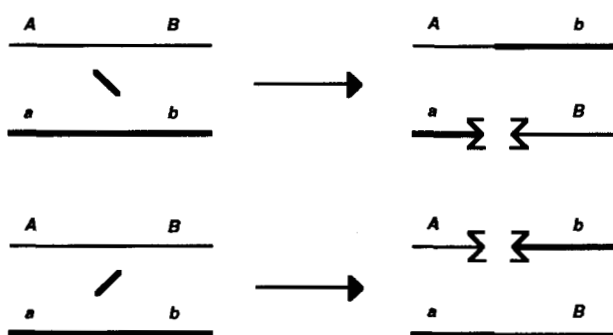

Half-Reciprocal

FIGURE 1.—Reciprocal recombination. Fully reciprocal recombination involves the rejoining of both pairs of flanking markers from a single recombination event; A is joined to b and a is joined to B. Half-reciprocal recombination involves the rejoining of only one pair of flanking markers from a single recombination event; *either* A is joined to b or a is joined to B.

Determination of the number of cell generations: The number of cell generations was calculated. $N = N_0 2^x$, where x is the number of cell generations, N_0 is the number of cells at time zero, and N is the number of cells (viable and inviable) at time t; since the resuspended colony arose from a single cell, $N_0 = 1$. N was determined by counting the number of cell particles in suspension using a Coulter Counter (model

F, 30 μm orifice, Coulter Electronics). Populations tested further (see below) had undergone 24 ± 2 generations in forming the colony from the single plated parental cell.

Determination of Hol⁻ segregation frequency: Serial dilutions of fraction 2 were plated for single colonies (ca. 200/plate) nonselectively on solid nutrient broth medium and incubated overnight at 37°. The colonies were then replica printed to (1) minimal medium containing histidinol (Hol) and (2) minimal medium containing histidine in order to score the loss of the *hisD* chromosomal segment (Hol⁻ segregant). The frequency of Hol⁻ segregants was calculated as the number of Hol⁻ segregants per viable cell plated.

Determination of His⁺ recombinant frequency: His⁺ recombinants were selected on minimal medium. Since colonies continue to appear with extended incubation times, 43 hr of incubation at 37° was arbitrarily chosen for assaying colony number. The number of colonies arising on the plate was found to be dependent on the extent of growth on the selection medium and thus on the residual nutrients plated. To make this residual growth uniform, cells were plated on a lawn of a strain containing a *his* deletion (*hisO-E3050*) that consumes the plated nutrients. Under these conditions, the number of His⁺ recombinants detected at 43 hr of incubation showed a linear relation to the number of parental cells plated. The frequency of His⁺ recombinants throughout the paper is expressed as the number of His⁺ recombinant colonies scored per viable cell plated.

Detection of circle integration at the *his* region: The test strain (TT13870), described later in Figure 2, contains a chromosomal fragment at *ara* flanked by Tn10 elements in the same orientation. An intrachromosomal exchange event between direct repeats (Tn10 elements) at *ara* can result in the excision of *his* material as a circular fragment (Figure 3). The circular fragment can be captured by recombination at the *his* region of the parental strain; the *his* locus is deleted for the proximal portion of the *his* operon, but shares *hisD* and *hisC* gene sequences with the free circle. The His⁺ recombinant resulting from circle integration has a wild-type *his* operon and a duplication of *hisDC* material
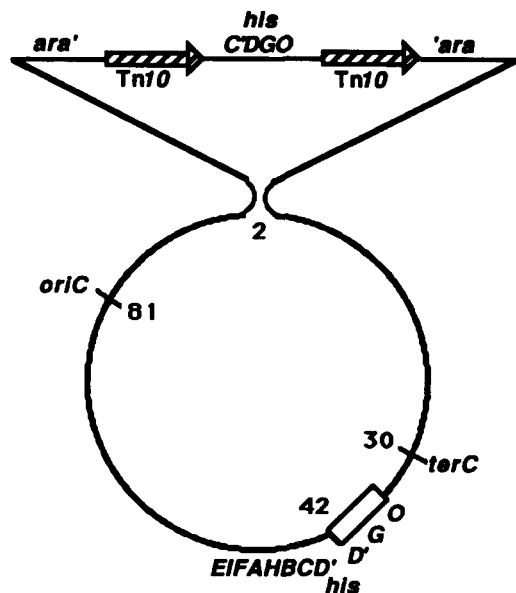
**Parental Strain
(TT13870)**

FIGURE 2.—Parental strain. Parent strain TT13870 contains a fragment of the *his* operon (*hisOGDC'*) at *ara* flanked by two Tn*10* elements in the same orientation (C' indicates that only part of the *hisC* gene is present here). It also contains a deletion in its standard *his* region (*hisOGD646*). The *his* sequences are in inverse order vis-à-vis the normal *his* operon.
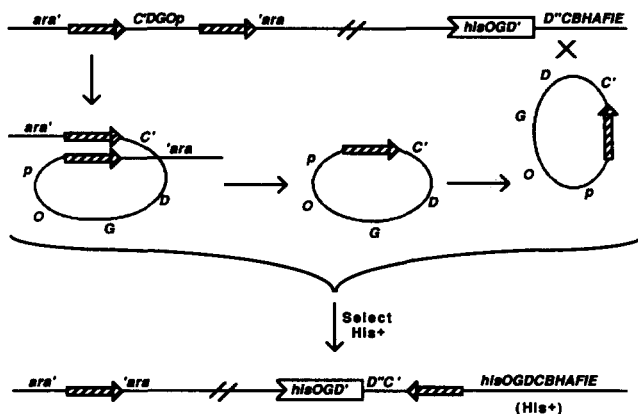

FIGURE 3.—Circle capture at *his*. Homologous recombination between direct order homologies (Tn*10* elements) within a single chromosome generates a free excised circle of DNA. Recapture of the excised circle at the *his* region occurs by a homologous recombination event between the shared *hisDC* homologies. Such exchange events give rise to prototrophic (His⁺) recombinants. Hatched arrows represent Tn*10* elements.

at *his*, which can be segregated at low frequency. If a linear fragment, rather than a circle, were generated from *ara*, it could not repair the *his* deletion, since the material present at *ara* is too short to include homology at the left side of the deleted region (*hisOGD646*). A more detailed description of the isolation and characterization of circle capture recombinants is given in MAHAN and ROTH (1988).

Detection of inversions: The *his* material at *ara* in parental strain TT13870 is in the opposite orientation vis-à-vis the normal *his* operon. When the shared *his* homologies recombine directly, a His⁺ recombinant is generated that carries an inversion of the chromosomal segment between

his and *ara* (Figure 4). Recombinants of this type can be detected because they show linkage disruption at both the *his* and *ara* regions. That is, both the *ara* and *his* regions have normal flanking material on only one side; transductional repair of either inversion join point with P22 phage grown on a wild-type donor would show a severe reduction in frequency.

Linkage disruption test: Strain TT11077 contains a large *his* deletion that is associated with a kanamycin resistance determinant (*cob21*MudJ*hisF9951*) (MAHAN and ROTH 1988). Linkage disruption at the *his* end point of the inversion was diagnosed as a reduction in the ability of His⁺ recombinants to inherit a kanamycin resistance marker introduced by P22 phage grown on TT11077.

Genetic assay revealing the state of *his* sequences at the *ara* region: P22 phage grown on TT11077, which contains a large *his* deletion associated with a Km determinant (*cob21*MudJ*hisF9951*), was used to transduce His⁺ recombinants to kanamycin resistance. Inheritance of the deletion removes the *his*⁺ material from the *his* region, thereby allowing the detection of *hisD* (Hol⁺) sequences at *ara* (or *pncB*). A more detailed description is given in MAHAN and ROTH (1988).

Media: The E medium of VOGEL and BONNER (1956) supplemented with 0.2% glucose was used as the defined minimal medium. Selection for growth on alternative carbon sources was done on NCE medium supplemented with 0.2% of the appropriate carbon source as described by BERKOWITZ *et al.* (1968). The complex medium was nutrient broth (8 g/liter, Difco Laboratories) with added NaCl (5 g/liter). Solid medium contained Difco agar at 1.5% final concentration. Auxotrophic requirements were included in media at final concentrations as described by DAVIS, BOTSTEIN and ROTH (1980). Final concentrations of antibiotics were as follows: tetracycline hydrochloride (Sigma Chemical Co., 16 μg/ml in rich medium, or 10 μg/ml in minimal medium); kanamycin sulfate (Sigma Chemical Co., 50 μg/ml in rich medium, or 100 μg/ml in minimal medium); ampicillin (Sigma Chemical Co., 40 μg/ml in rich medium, or 15 μg/ml in minimal medium); chloramphenicol (Sigma Chemical Co., 20 μg/ml in rich medium, or 5 μg/ml in minimal medium).

Transductional methods: The high frequency generalized transducing bacteriophage P22 mutant HT 105/1, *int*-201 (SCHMIEGER 1972) was used for all transductional crosses. Unless otherwise specified, 0.1 ml of an overnight culture grown in complex medium (*ca.* 2–4 × 10⁹ cfu/ml) was used as a recipient of 0.1 ml transducing phage (*ca.* 10⁸–10⁹ pfu/ml) and plated directly on selective plates. Transductional crosses involving the selection of kanamycin or chloramphenicol resistance were preincubated overnight on solid nonselective complex medium, then replica printed onto selective medium. Transductants were purified and phage-free isolates were obtained by streaking for single colonies on green indicator plates (CHAN *et al.* 1972). Phage-free colonies were tested for phage sensitivity by cross streaking with P22 H5 (a clear plaque mutant) phage.

Nomenclature: Nomenclature is generally as described in DEMEREC *et al.* (1966), CAMPBELL *et al.* (1977), and CHUMLEY, MENZEL and ROTH (1979). The nomenclature "*z*-::Tn*10*" refers to a Tn*10* insertion in a "silent" DNA region; the "*z*-" describes the map position of the insertion (SANDERSON and ROTH 1983). The nomenclature used for chromosomal rearrangements is described in CHUMLEY and ROTH (1980), SCHMID and ROTH (1983), and more recently in HUGHES and ROTH (1985).

RESULTS

The assay system: A single strain has been constructed that permits assay of three sorts of recombination events (MAHAN and ROTH 1988). By comparing the behavior of recBC⁺ and recBC⁻ derivatives of this strain, we can observe the effect of the recBC deficiency on all three events.

The parent strain TT13870 is diagrammed in Figure 2. The arabinose (ara) locus at minute 2 (SANDERSON and ROTH 1983) contains a chromosomal segment that carries a fragment of the his operon (hisOGDC') flanked by Tn10 elements in the same orientation (C' indicates only a fragment of the hisC gene is present). The his material at ara is in the opposite orientation vis-à-vis the normal his operon. At the normal his locus is a deletion mutation that removes the promoter. Strain TT13870 requires histidine, since it has no functional hisC gene, but it is able to grow on histidinol (Hol⁺) due to a functional hisD gene at ara (the hisD gene product catalyzes the conversion of the final intermediate histidinol to histidine). A second parent strain (TT13884) is identical to strain TT13870 except the hisOGDC' chromosomal fragment is placed in the pncB locus (minute 20); the his material in this strain is in the same orientation as the normal his operon.

Several genetic events can be scored using these strains. Methods for detection and classification of these recombinants are given in MATERIALS AND METHODS.

Deletion events: Homologous exchange between direct repeats (Tn10 elements) at ara (or pncB) results in the loss of his material (including hisD) between the direct order homologies. This can be scored by the loss of ability to use histidinol as a source of histidine.

Circle capture: An intrachromosomal recombination event between direct repeats (Tn10 elements) generates an excised circle that can be recaptured by recombination at the his operon (Figure 3). These events can be scored by identifying His⁺ recombinants that carry the functional His⁺ region at the standard his locus. These recombinants are distinguished from His⁺ recombinants arising by inversion as described in MATERIALS AND METHODS.

Inversion formation: Homologous exchange between separated his homologies in inverse order (strain TT13870) results in an inversion of the chromosomal material between the inverse order homologies. These recombinants are identified as His⁺ recombinants that have a disruption of genetic linkage at both the his and ara loci, due to the inversion. Criteria for scoring inversions is as described in MATERIALS AND METHODS.

In the experiments presented here, we distinguish between half- and fully reciprocal recombination (Figure 1). That is, a fully reciprocal exchange involves the joining of both pairs of flanking markers, resulting in the formation of two recombinant products from a single recombination event. Half-reciprocal exchange involves the joining of only one pair of flanking markers, resulting in the formation of only one recombinant product from a single recombination event.

Recombination between direct order homologies is RecBC independent: The parental strain TT13870, becomes Hol⁻ (unable to use histidinol as a histidine source) if an exchange event between the Tn10 elements at ara results in deletion of the hisD gene. This can occur by a single intrachromosomal exchange between the two flanking Tn10 elements, excising the his material as a free circular fragment (Figure 3). Hol⁻ clones can also arise by interchromosomal sister-strand exchanges, giving rise to a duplication in one sister chromosome and a deletion in the other. It should be noted that segregation of his material by either route does not require a fully reciprocal exchange. A half-reciprocal exchange, in which only one pair of strands is joined, is sufficient to generate the Hol⁻ recombinant that is scored.

We have examined the effect of rec mutations on the frequency of Hol⁻ deletion recombinants. Table 2 shows no Hol⁻ clones in parental strains containing a recA mutation. Derivatives with either a recB or recC mutation show a nearly undiminished (<2-fold) frequency of Hol⁻ segregants. Note that the frequency of Hol⁻ clones is expressed as a fraction of viable cells present at the time of selection. This was done since cultures of both recA and recBC mutants accumulate appreciable numbers of inviable cells (CAPALDO-KIMBALL and BARBOUR 1971; MAHAN and ROTH 1989). Data in Table 2 show that formation of Hol⁻ clones (by loss of material between direct repeats) requires recA but does not depend on the presence of a functional recBC gene. The rec alleles used here have been extensively tested in transductional assays. The recA allele causes a >10⁴-fold reduction in transductant frequency, while the recB and recC alleles cause an 8- to 20-fold reduction in transductant frequency (per viable recipient cell plated).

RecBC enzyme stimulates the circle generation/capture process: In strains with outside his sequences in the same orientation as the his operon, His⁺ recombinants arise only by circle capture. This is shown in the top of Table 2; it can be seen that the presence of a recB or recC mutation reduces the frequency of His⁺ recombinants 14- to 20-fold. Since these recombinants require both the formation and the integration of a circle, the recBC function could contribute to either or both steps in the process. It should be noted that the circle capture is very efficient in rec⁺ strains. The frequency of His⁺ recombinants (in recBC⁺ cells) is typically only 20- to 100-fold less than the frequency of Hol⁻ segregants arising by exchange between 10-kb repeats of Tn10 elements that flank the outside his sequences. The efficiency of circle integration is striking when one considers that the circle shares only

TABLE 2

Effects of RecBC on deletion formation, inversion rearrangement and circle capture

Strain[a]	Site of *his* sequences	Rec mutation	Hol⁻ segregants per viable cell plated[b] (×10⁴)	His⁺ recombinants per viable cell plated[b,c] (×10⁶)
TT13884	*pncB*	*recA⁺B⁺C⁺*	61	36
TT13885	*pncB*	*recB10*	33	1.8
TT13886	*pncB*	*recA⁺B⁺C⁺*	47	287
TT13887	*pncB*	*recB497::MudJ*	30	20
TT13888	*pncB*	*recC498::MudJ*	24	20
TT13889	*pncB*	*recA⁺B⁺C⁺*	60	54
TT13890	*pncB*	*recA1*	<0.7	<0.2
TT13870	*ara*	*recA⁺B⁺C⁺*	45	105
TT13871	*ara*	*recB10*	28	5.5
TT13873	*ara*	*recA⁺B⁺C⁺*	37	631
TT13874	*ara*	*recB497::MudJ*	17	18
TT13875	*ara*	*recC498::MudJ*	33	13
TT13880	*ara*	*recA⁺B⁺C⁺*	31	95
TT13881	*ara*	*recA1*	<0.5	<0.1
TT13882	*ara*	*recB10*	21	2.7
TT13883	*ara*	*recA1 B10*	<0.5	<0.5

[a] The strains are grouped in isogenic sets. Strains in the top half contain *his* material at *pncB* in the same orientation *vis-à-vis* the *his* operon. Strains in the bottom half contain *his* material at *ara* in the opposite orientation *vis-à-vis* the *his* operon.

[b] The method for determining the frequency of Hol⁻ segregants and His⁺ recombinants is described in MATERIALS AND METHODS; the frequency was assayed after 24 generations of growth from a single cell.

[c] His⁺ recombinants were scored after 43 hr of incubation at 37°C on minimal medium. The fraction of His⁺ recombinants due to circle capture and inversion rearrangement is given in Table 3.

about 1 kb of homology with the *his* region, and that recombination between circular molecules is generally inefficient (LABAN and COHEN 1981). We have previously shown that the probability of circle capture can approach 1 if the extent of homology between the circle and the chromosome is increased only 3–4-fold (MAHAN and ROTH 1988). We will propose below that the majority of captured circles are formed by circularization of excised linear fragments that are "activated" by exposure to *recBC* function prior to circularization (see DISCUSSION).

The *recBC* function stimulates formation of inversions: In strains with recombining *his* sequences in inverse orientation, His⁺ recombinants can arise either by circle capture, or by direct recombination between inverse order homologies, which leads to formation of an inversion (Figure 4). Data for these strains are presented in the lower half of Table 2. In Table 2 it can be seen that the presence of a *recB* or *recC* mutation causes a 15–50-fold reduction in the number of His⁺ recombinants. To determine whether the effect of *recBC* deficiency bears on both recombinant types, we must classify the recombinant types arising with and without *recBC* function (see MATERIALS AND METHODS). These data are presented in Table 3; there it can be seen that the ratio of circle capture types and inversion types is affected very little by the presence of a *recB* or *recC* mutation. Thus, both types must be reduced in frequency by the re-

combination deficiency. Both of these recombinant types require a fully reciprocal exchange and both are stimulated by the presence of *recBC* function.

Residual recombination in the absence of RecBC appears fully reciprocal: If fully reciprocal recombination is reduced by the presence of a *recBC* mutation, while the half-reciprocal exchanges occur at normal frequency, one might expect the residual recombination in *recBC* mutant strains to be preferentially half-reciprocal. To test this, we used the same method applied previously to demonstrate reciprocality of intrachromosomal recombination (MAHAN and ROTH 1988). The rationale of this method is described briefly below.

In the circle capture process described above, if recombination is fully reciprocal, generation of a circle is accompanied by closing of the donor site, leaving a deletion (Figure 5). The circle generated can integrate into the same chromosome from which it was formed or into an uninvolved sister chromosome. If recombination is only half-reciprocal, circle formation leaves a break at the donor site, and circle capture can only be detected if it occurs in an intact uninvolved sister chromosome. To determine reciprocality, we recover His⁺ recombinants generated by circle integration and determine the state of the donor site. In our previous experiments (in *recBC⁺* strains), 60–65% of the His⁺ recombinants have *his* sequences at the donor site, suggesting that circle integration can occur
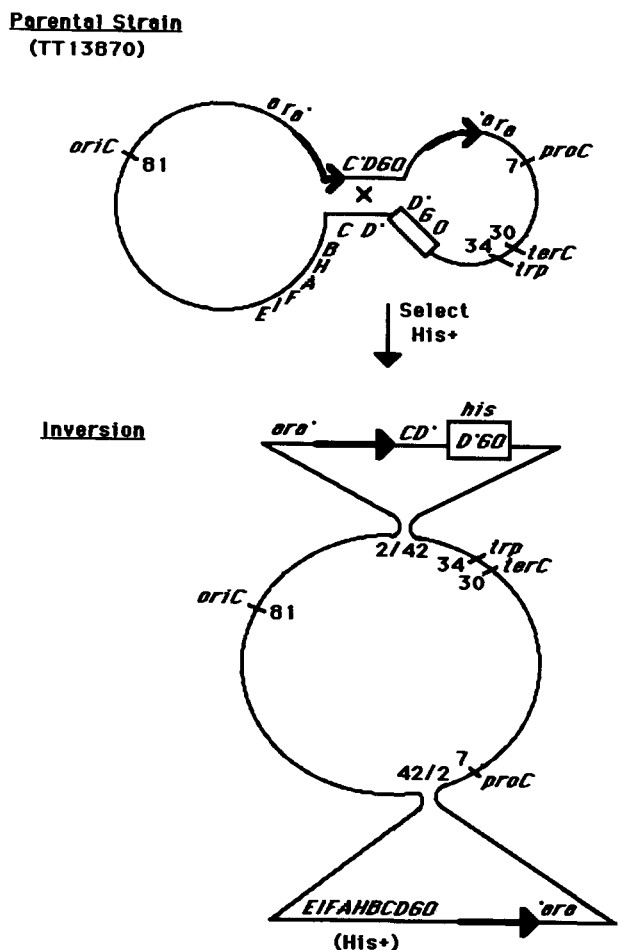
FIGURE 4.—Selection for spontaneous inversion rearrangement of the bacterial chromosome. Parent strain TT13870 contains a chromosomal fragment that contains the proximal portion of the *his* operon at *ara* flanked by Tn*10* elements in the same orientation. Selection for His⁺ yields recombinants, some of which have undergone an exchange event between the separated *hisDC* homologies. Such an exchange event results in inversion of the intervening material between the sites of exchange (*ara-his*). The dark arrows represent Tn*10* elements in the same orientation.

in a sister chromosome whose donor site was not involved in the recombination events. Approximately 35–40% of the His⁺ recombinants that captured a circle at the *his* locus have lost *his* sequences from the donor site (*ara* or *pncB*); that is, the donor site has been repaired leaving a deletion of *his* material. This indicates that capture of a circle at the *his* locus is frequently associated with the loss of those sequences from *ara* and recombination is frequently fully reciprocal.

Results of these experiments for *recB* and *recC* mutant strains are presented in Table 3. Although the frequency of circle capture was reduced in overall frequency (see above), the associated loss of *his* material from the donor site was at least as frequent as in *recBC*⁺ strains. This suggests that most residual recombination in *recBC* strains is fully reciprocal. This finding is contrary to our initial expectation and will be discussed below.
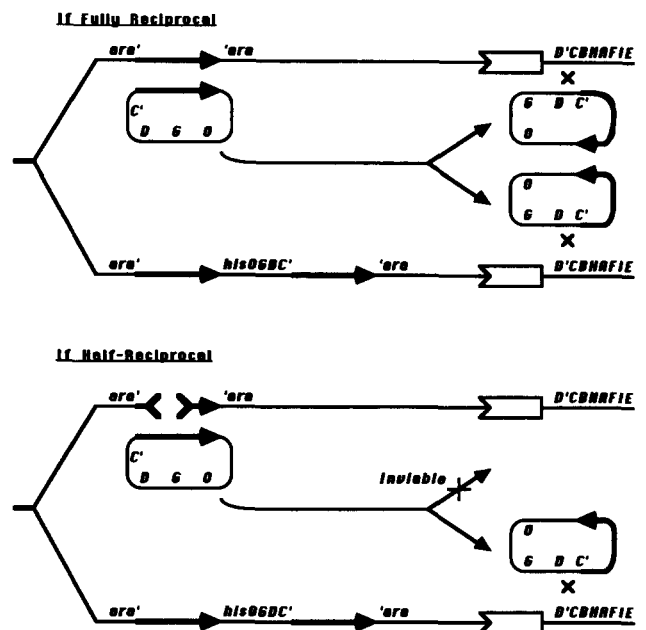

FIGURE 5.—Reciprocal recombination assay. Circles generated by fully reciprocal recombination between direct order sequences (represented by dark arrows) present in the same chromosome, may integrate into the *his* region of the same chromosome from which it was formed, or into an uninvolved sister chromosome. Such fully reciprocal exchanges result in His⁺ recombinants that are associated with *loss* of *his* sequences at the donor site (*ara*). Circles generated by half-reciprocal recombination must integrate into the *his* region of an uninvolved sister chromosome. Such half-reciprocal exchanges result in His⁺ recombinants that *retain his* sequences at the donor site (*ara*).

DISCUSSION

Data presented here suggest the following. (1) The frequency of recombination between direct repeats to remove an intervening segment from the chromosome is not affected by the *recBC* function. This result confirms an earlier observation of SCLAFANI and WECHSLER (1981) that the segregation of a duplication is independent of *recBC* function (2). The overall process of excising a circle and recapturing it at a distant site is stimulated by *recBC* function. (3) Inversion formation by recombination between inverse order homologies is stimulated by *recBC* function.

In interpreting these data, one should note that the RecBC independent event is one that does not require full reciprocality. Circle capture and inversion formation do require fully reciprocal exchanges and both are stimulated by *recBC* function. This suggests that *recBC* functions are involved in fully reciprocal exchanges and contribute to exchanges for which the initial substrates provide no double-stranded ends.

We would like to discuss these results in terms of a model for recombination. This model deals with the gross events involved in reciprocal exchanges, not the detailed molecular mechanism. The model proposes that reciprocality, under our conditions, is achieved by a two-step process in which one pair of flanking sequences is joined and a double-strand break is gen-

TABLE 3

RecBC dependent fully reciprocal recombination

			His+ recombinants		
Strain[a]	Site of *his* sequences	Rec mutation	Percent due to inversions[b]	Percent due to circle capture[b]	Percent of strains with captured circles that have lost *his* sequences from the donor site[c]
TT13870	*ara*	*recA+B+C+*	78 (39/50)	22 (11/50)	36 (4/11)
TT13871	*ara*	*recB10*	54 (61/114)	46 (53/114)	53 (28/53)
TT13899	*ara*	*recC498::*MudA	57 (57/100)	43 (43/100)	60 (26/43)
TT13884	*pncB*	*recA+B+C+*	NA[d]	100 (50/50)	42 (21/50)
TT13885	*pncB*	*recB10*	NA[d]	100 (80/80)	67 (54/80)
TT13900	*pncB*	*recC498::*MudA	NA[d]	100 (100/100)	66 (66/100)

[a] Strains in the top half contain *his* material at *ara* in the opposite orientation *vis-à-vis* the *his* operon. Strains in the bottom half contain *his* material at *pncB* in the same orientation *vis-à-vis* the *his* operon.

[b] Inversion rearrangements and circle capture were classified by linkage disruption (see RESULTS).

[c] The presence of *his* sequences at the donor site (*ara* or *pncB*) was determined by scoring Hol+ after inheritance of a large *his* deletion (see MATERIALS AND METHODS).

[d] Not applicable since *his* sequences are in direct order in the parent strain.
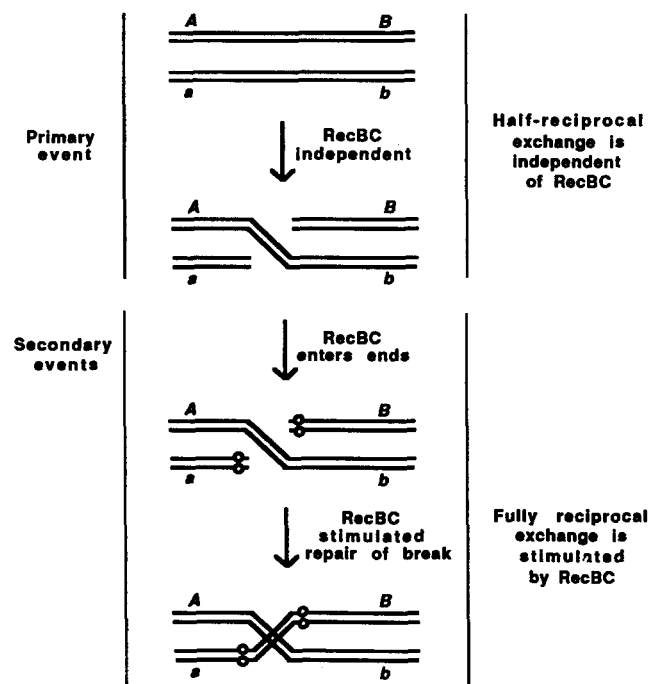

FIGURE 6.—Two step model for fully reciprocal recombination. The primary recombination event is a RecBC independent half-reciprocal exchange, generating one recombinant product (*A–b*) and two double-stranded ends at which RecBC may enter the bacterial chromosome. The RecBC-stimulated secondary exchange event joins the second pair of flanking markers (*a–B*), completing the fully reciprocal exchange.

erated as an intermediate. *According to this proposal, the two reciprocal products of recombination do not result from a concerted resolution of a single intermediate structure.*

We propose that, at least for intrachromosomal exchanges, the initial recombination event is half-reciprocal, generating one recombinant chromosome and leaving two double-strand ends (see primary event in Figure 6). We propose that the initial exchange has no double-stranded substrates and thus can not involve *recBC* function. However, this primary event

leaves a pair of unjoined flanking segments with double-stranded ends that permit RecBC to enter and play a role in subsequent degradation and resynthesis events required to join the second pair of flanking sequences (double-strand break/gap repair). The final result, after completion of double-strand break repair, is a fully reciprocal exchange. Since the *recBC* function acts only on the joining of the second pair of flanking sequences, it is not required for a half-reciprocal exchange, but it does stimulate the frequency of fully reciprocal recombination (secondary event, Figure 6).

We propose that the initial half-reciprocal event is sufficient to generate a deletion. The deletions detected are due primarily to those initial events that excise a linear fragment and do not disturb the continuity of the chromosome (*b* in Figure 7 below). The other half of the initial exchanges generate a circle and break the chromosome (*a* in Figure 7); such exchange events are lethal if the double-strand break remains unrepaired. If a *recBC* deficiency impairs such repair, one would expect *recBC* to cause at most a 2-fold decrease in the frequency of deletions. Our data (Table 2) show such a slight decrease in most experiments.

Inversion formation requires a fully reciprocal exchange, since both flanking sequences must be joined to restore the linear continuity of the chromosome. The frequency of this event is strongly reduced in RecBC deficient cells. In terms of the model for recombination, this reduction is due to impaired ability to perform the second step of the reciprocal exchange, repair of the intermediate double-strand break.

Circle capture also requires a reciprocal exchange. Thus, the fact that this process is less frequent in RecBC deficient strains is consistent with the model. However, we expected that, in RecBC deficient cells, we would find residual recombination to be less frequently fully reciprocal. We found no such reduction.
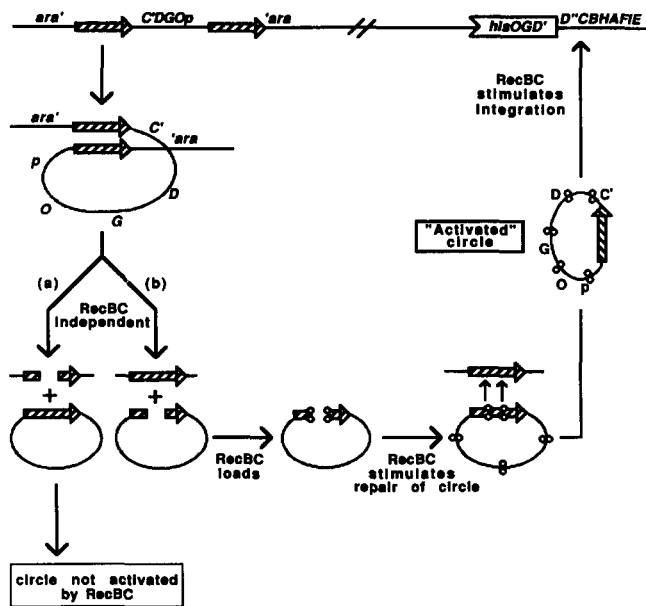
FIGURE 7.—RecBC dependent circle capture. A single intra-chromosomal exchange between direct repeats (Tn*10* elements) results in either: (a) a broken chromosome associated with an excised circle (that is not recombinationally activated); or (b) an intact chromosome associated with an excised linear molecule. The linear molecule is a source of double-stranded ends at which RecBC enzyme can enter and stimulate circularization. The rejoined circle, now recombinationally activated by RecBC, is integrated efficiently into the chromosome. Hatched arrows represent Tn*10* elements in the same orientation.

Upon close examination, this result is also consistent with the model.

Circle capture in the assay system is a very efficient process. As can be seen in Table 2, the frequency of circle formation and capture is typically only 20–100-fold less frequent than formation of a deletion by recombination between the 10 kb of closely placed Tn*10* elements that flank the outlying *his* sequences. The frequency of circle capture is surprising when one considers that only 1 kb of homology is shared by the small circle and the *his* locus into which it integrates. In our previous experiments, we found that the capture frequency can approach 100% if more extensive homology between the circle and the chromosome is provided (MAHAN and ROTH 1988). The high frequency of capture is especially surprising in view of the fact that recombination between closed plasmid circles is generally a low frequency event (LABAN and COHEN 1981). We propose that the method by which some circles are generated in our experiments explains the "activation" of circle capture that we see.

In our experiments, some circles form by the initial step of the recombination process, generating a circle with no intervening double-strand breaks (*a* in Figure 7). We propose that these circles, like plasmids, are "unactivated"; their involvement in circle-circle exchanges is inefficient and independent of *recBC* function (LABAN and COHEN 1981; JAMES, MORRISON and

KOLODNER 1982), since they never had a site for entry of RecBC enzyme. We suggest that these circles do not contribute significantly to the circle integration process we observe. Other circles are formed in the second step of recombination by circularization of linear molecules excised from the donor site. We propose that these linear molecules can be entered by the RecBC enzyme, which promotes circularization, and leaves an "activated" circle that has associated RecBC enzyme and is extremely efficient at recombination with the chromosome. This suggestion is diagrammed in Figure 7.

According to this picture, *recBC* function stimulates both the formation and the integration of "activated circles." In the absence of *recBC* function, no "activated" circles are formed. Under these conditions two sequences of events can be imagined. (1) The donor site can be healed by the primary event, excising a linear fragment that circularizes and integrates by an inefficient (RecBC independent) pathway. (2) Alternatively, the primary event may generate an unactivated circle, leaving broken ends at the donor site that are healed by an inefficient (RecBC independent) pathway. Since our experiments show that RecBC independent circle capture is associated with a normal frequency of donor site joining, we propose that the unactivated circles generated by alternative (2) above do not contribute to the events we observe, and alternative (1) above accounts for the events seen in *recBC* mutant strains. Strictly speaking, two activities of RecBC are proposed in Figure 7. First, RecBC can enter a duplex at a double-strand break and stimulate its repair; second, if RecBC is present within a double-stranded molecule lacking active ends, RecBC can stimulate the initial interaction of this molecule with another duplex molecule. While these activities are formally distinct, they could prove to be biochemically related.

The model fits well with other work on recombination. BIRGE and LOW (1974) and LLOYD and THOMAS (1984) described conjugation experiments focused on determining whether RecBC is involved early or late in the recombination process. These studies involve conjugation crosses between strains with differently mutant *lacZ* genes. Recombination intermediates, indicative of early events in recombination, are measured by monitoring appearance of β-galactosidase activity in the mating population. The frequency of Lac$^+$ recombinants is a measure of completed recombination. In these studies, a *recBC* defect causes only a 2-fold reduction in β-galactosidase appearance but a 100-fold reduction in the frequency of Lac$^+$ recombinants. These results are consistent with RecBC acting at a late step in recombination and fit well with results presented here. The initial (half-reciprocal, RecBC independent) event in Figure 7 would generate the functional *lacZ* gene and the later

(RecBC dependent) exchange would complete the final recombinant. (The importance of the second event is discussed later.)

More recent work (LLOYD, EVANS and BUCKMAN 1987) suggested an additional early role for RecBC. In strains lacking both RecBC and RecF, J or O activities, a reduction was seen in β-galactosidase as well as in recombinant formation. Neither defect was seen in strains lacking only RecF, J or O. This suggests that RecBC can also contribute to early events, but its early role is not apparent in the presence of RecF, J or O. The role we propose here for RecBC in activating circle integration may be the same as the early activity of RecBC proposed by LLOYD, EVANS and BUCKMAN (1987).

Conjugational and transductional recombination typically show a 10- to 100-fold reduction in frequency in recBC strains (CLARK 1973). In principle, neither process appears to require a fully reciprocal exchange (inheritance of both products of a single recombination event); recombinants could form by two unrelated half-reciprocal (RecBC independent) exchanges flanking the integrated material. We propose that the parameters that govern circle capture and inversion formation also govern transduction and conjugation. That is, RecBC acts primarily at a double-strand break in a recombinational intermediate and serves to stimulate the secondary rejoining of the bacterial chromosome. This is discussed below for transduction.

It seems likely that the ends of transduced fragments are protected in some way from the degradative activity of RecBC. Transformation is stimulated in exonuclease-deficient (recBC) cells (WINANS et al. 1985), providing evidence that DNA molecules with exposed ends are quickly degraded. Since transduced fragments do not show this sensitivity but recombine in a RecBC dependent way, it seems likely that fragment ends are protected from RecBC in some way. Studies of the ends of transduced fragments (SANDRI and BERGER 1980) have provided some evidence for the existence of protective devices that could limit access of RecBC to the ends of a transduced fragment. Therefore we propose that the initial event in transductional recombination (like the initial event in intrachromosomal recombination) is a half-reciprocal internal exchange, resulting in joining one pair of flanking sequences, A–B–c (Figure 8), and causing a double-strand break in the chromosome at the recombination site. The new double-stranded ends allow efficient entry of RecBC, which stimulates the second exchange or double-strand break/gap repair required to rejoin the chromosome and integrate the donor material. That is, *activation of the new double-strand ends by RecBC makes the secondary event much more likely than an independently initiated half-reciprocal exchange.*

The model described fits well with the observation that the cis-acting recombinator site, Chi, when
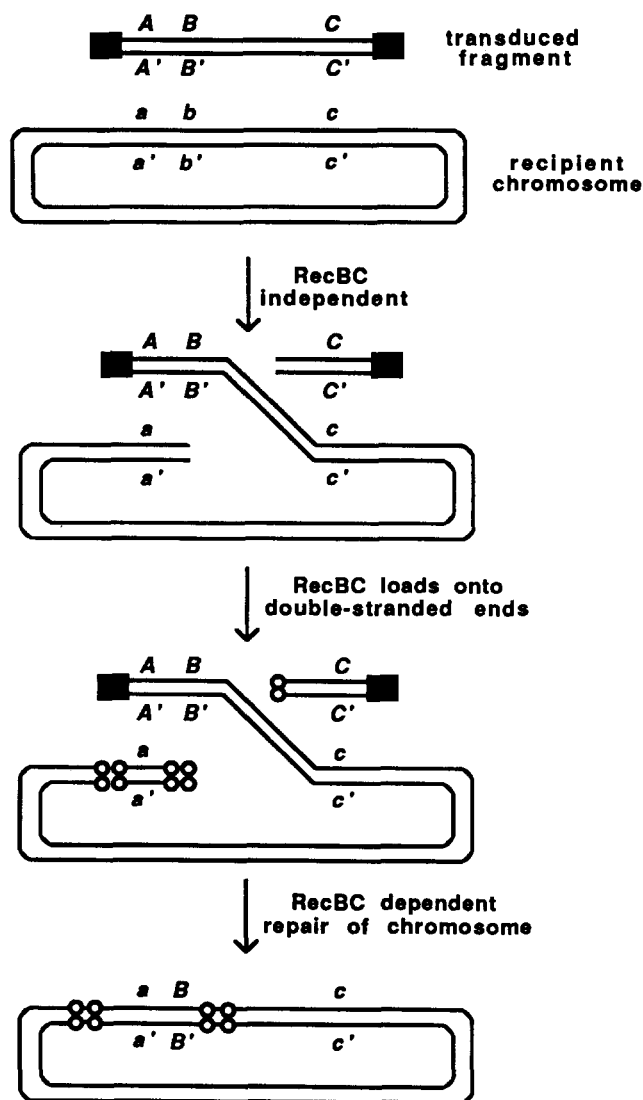

FIGURE 8.—RecBC dependent transductional recombination. The ends of a transduced fragment are capped (represented by dark squares), providing protection from the degradative activity of the RecBC enzyme. The initial recombination event is half-reciprocal; whereby one arm of the transducing fragment (A–B) is joined to the chromosome (c). This exchange event is associated with a double strand break at the donor site, providing a mechanism for RecBC to enter and stimulate the second event that rejoins the chromosome and integrates the donor material (a–B–c).

present in the recipient chromosome, stimulates recombination frequencies in a transduction cross (DOWER and STAHL 1981). That is, an initial half-reciprocal exchange would generate a double-strand break at which RecBC can enter the chromosome, be activated at a Chi site, and stimulate secondary events that rejoin the chromosome ends (see Figure 8).

One problem with the transduction model, as presented in Figure 8, is that it does not account for the fact that transductional recombination is very frequent compared to the rates of duplication segregation and plasmid integration. If the proposed capping of the ends of the transduced fragment completely prevented RecBC entry, one would expect the initial

exchange to occur at a low frequency, comparable to that of plasmid integration. The observed high frequency of transductional recombination might be due to a short period of RecBC access to the fragment ends before capping. Entry of RecBC at the end of a fragment would "activate" that fragment and allow RecBC to stimulate the initial exchange; the double-strand ends generated by this first exchange would be fully accessible by RecBC and provide the most frequent means of completing the event. Transduced fragments that are capped before RecBC entry could be the long-lived, infrequently recombining fragments responsible for "abortive" transduction (STOCKER, ZINDER and LEDERBERG 1953; reviewed in HARTMAN and GOODGAL 1959). The two activities proposed for RecBC in transduction would be identical to the two activities proposed (in Figure 7) to explain the high frequency of circle formation and integration and to the late and early activities inferred by LLOYD, EVANS and BUCKMAN (1987). The above considerations also fit with conjugational recombination if access of RecBC to the initial substrates is limited (e.g., by distance from a double-strand end or by capping).

In summary, we have observed the effects of recBC mutations on three different types of recombination events: deletion formation, circle integration and inversion rearrangement. Our results indicate that deletion formation, requiring only one half-reciprocal exchange event, is independent of recBC function. Circle integration and inversion formation, both requiring a fully reciprocal exchange event, are stimulated by recBC function. We propose that fully reciprocal recombination is a two-step process. The initial event is a half-reciprocal exchange joining one pair of flanking sequences and producing a double-strand break. In intrachromosomal recombination this initial event is independent of RecBC, since the enzyme does not have access to the substrates. However, if RecBC access is provided, the initial event can be stimulated by RecBC. The double-strand breaks generated by the initial event permit RecBC efficiently to enter and participate in the double-strand break/gap repair that completes the reciprocal exchange. The model is consistent with data obtained from yeast tetrad analysis [for a review see SZOSTAK et al. (1983) and ORR-WEAVER AND SZOSTAK (1985)]. Such data include the observation that gene conversion is frequently associated with exchange of flanking markers. This would occur if the initial exchange were half-reciprocal, joining one pair of flanking sequences and generating a double-strand break at the recombination site. If this site is near a central marker, repair of the double-strand break/gap joins the second pair of flanking sequences and sometimes results in conversion of the central marker. Thus the model seems consistent with observations made on fungal recombination.

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APPENDIX

His⁺ recombinant formation in *recBC* mutant strains does not require a suppressor of *recBC*: Circle integration (see text) can generate His⁺ recombinants with reduced frequency in a *recBC⁻* strain. We wanted to determine whether the His⁺ recombinants carried suppressors of the *recBC* defect. One test is based on the expectation that if His⁺ derivatives arose only in cells that had acquired a suppressor of *recBC* capable of catalyzing circle integration, a second test of circle integration in the same genetic background should not show the 20-fold reduction shown in the original His⁺ reversion test.

Starting with a His⁺ (circle capture) recombinant formed in a *recBC⁻* strain that retained *his* material at *ara* (TT13891), a Hol⁺ His⁻ derivative was isolated as a segregant that had lost the *his* material (by excising the material between the repeats of *hisDC* sequence) integrated at the *his* region. This strain (TT13892) is virtually identical to the parent strain TT13871 used in the His⁺ recombinant assay. It is Hol⁺ due to a functional *hisD* gene at *ara*; it is phenotypically His⁻ due to lack of expression of a functional *hisC* gene.

To test if His⁺ recombinant formation requires a suppressor of *recBC*, His⁺ recombinants were again selected on minimal medium. Again, a 20-fold reduction in recombination is observed when compared with the isogenic *recBC⁺* control strains.

Further, standard tests of transductional recombination, cell viability, and resistance to irradiation by ultraviolet light have demonstrated that *recBC* strains have the same defects before and after being subjected to the His⁺ selection. Thus, we conclude that the His⁺ derivatives isolated in a *recBC* genetic background did not arise in strains carrying a suppressor of *recBC*, but arose despite the *recBC* defect.