Orientation Dependence in Homologous Recombination

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ABSTRACT

Homologous recombination was investigated in *Escherichia coli* with two plasmids, each carrying the homologous region (two defective *neo* genes, one with an amino-end deletion and the other with a carboxyl-end deletion) in either direct or inverted orientation. Recombination efficiency was measured in *recBC sbcBC* and *recBC sbcA* strains in three ways. First, we measured the frequency of cells carrying *neo*$^+$ recombinant plasmids in stationary phase. Recombination between direct repeats was much more frequent than between inverted repeats in the *recBC sbcBC* strain but was equally frequent in the two substrates in the *recBC sbcA* strain. Second, the fluctuation test was used to exclude bias by a rate difference between the recombinant and parental plasmids and led to the same conclusion. Third, direct selection for recombinants just after transformation with or without substrate double-strand breaks yielded essentially the same results. Double-strand breaks elevated recombination in both the strains and in both substrates. These results are consistant with our previous findings that the major route of recombination in *recBC sbcBC* strains generates only one recombinant DNA from two DNAs and in *recBC sbcA* strains generates two recombinant DNAs from two DNAs.

THE difference of the recombination frequency between directly repeated sequences and between inversely repeated sequences (orientation-dependence) has been observed in various recombination systems. In site-specific recombination, the resolvases of the Tn3 family of transposons specifically recombine directly repeated recombination sites (GRINDLEY and REED 1985), and the Gin system of bacteriophage Mu is specific for recombination of inverted recombination sites (PLASTERK *et al.* 1983). Such orientation-dependence in several site-specific recombination systems can be explained by juxtaposition of two recombination sites and the recombination machinery under topological constraints (MIZUUCHI and CRAIGIE 1986).

Similar orientation-dependence in homologous recombination has been reported for bacterial chromosomes (MAHAN and ROTH 1988–1990), mammalian chromosomes (LISKAY and STACHELEK 1986) and mammalian extra-chromosomal elements (SEIDMAN 1987; KITAMURA *et al.* 1990). The model for the orientation-dependence in the site-specific recombination systems does not appear suitable for that in the homologous recombination systems because the size and the sequence of the recombining DNAs are variable. One possible explanation is that the recombination is nonreciprocal (or nonconservative, making one intact duplex DNA out of two duplex DNAs; see Figure 1A for definitions) so that it produces linear, inviable recombinant from inverted repeats on a circular substrate (see Figure 5. below).

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It has been controversial whether RecBCD-mediated recombination in bacteria is reciprocal (or conservative, making two intact duplex DNAs out of two duplex DNAs) or nonreciprocal. Earlier works with chromosomes and bacteriophage lambda suggested reciprocality (MAHAN and ROTH 1988; KOBAYASHI *et al.* 1984; for example). Further analysis, however, revealed that this view is too simple (MAHAN and ROTH 1990; STAHL *et al.* 1995). The reactions catalyzed by RecBCD enzyme are also against the hypothesis of simple reciprocality (KOWALCZYKOWSKI and EGGLESTON 1994).

We have demonstrated that the elementary recombination process is nonreciprocal or nonconservative in an *Escherichia coli recBC sbcBC* strain (TAKAHASHI *et al.* 1992). YOKOCHI *et al.* (1995) have shown that the elementary recombination process is often conservative in an *E. coli recBC sbcA* strain, at least, in the presence of a double-strand break. Homologous recombination appears often conservative in this "RecE pathway active" strain (TAKAHASHI *et al.* 1992).

This explains why apparent gene conversion is very rarely accompanied by crossing-over of the flanking sequences in this "RecF pathway" active strain. (YAMAMOTO *et al.* 1992).

In the present study, we report orientation-dependence in RecF pathway (*RecBC sbcBC*) and orientation-independence in RecE pathway (*recBC sbcA*), exactly as predicted by the nonreciprocality in the RecF pathway and the reciprocality in the RecE pathway.

MATERIALS AND METHOD

Bacterial strains: The isogenic strains JC7623 (*recB21 recC22 sbcB15 sbcC201*) (KUSHNER *et al.* 1971; LLOYD and BUCK-

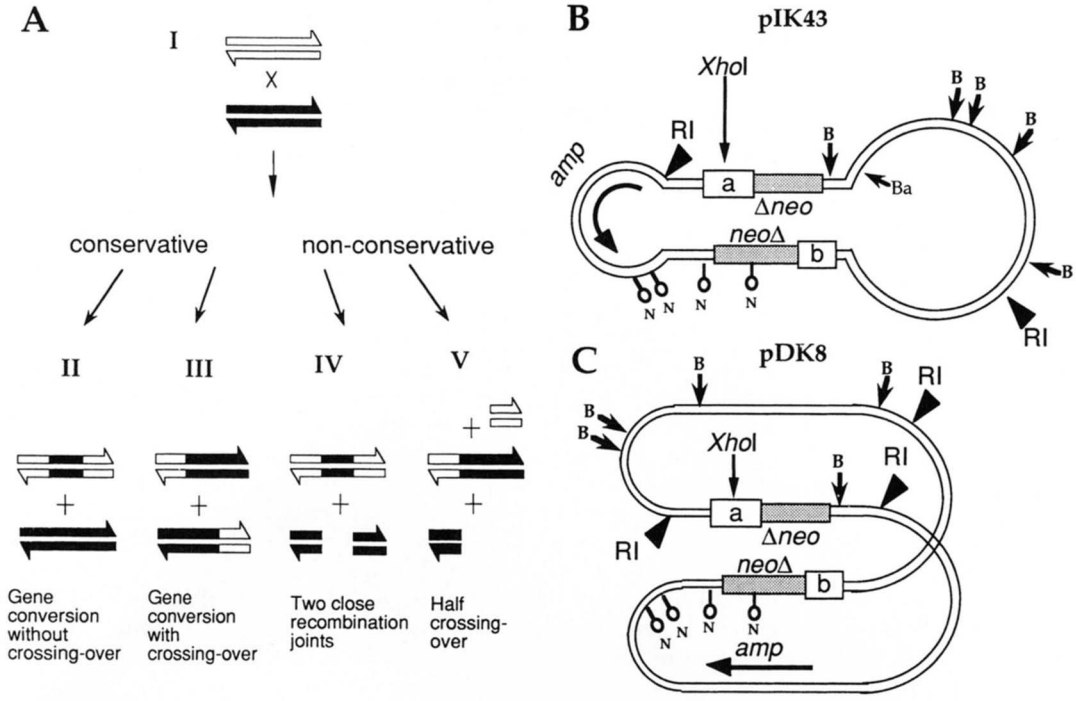

FIGURE 1.—Definitions and substrate. (A) Definitions about conservative and nonconservative recombinations. Homologous recombination generating two duplex DNA segments out of two duplex DNA segments is defined as conservative (or reciprocal) recombination. Homologous recombination generating one duplex DNA segments out of two duplex DNA segments is defined as nonconservative (or nonreciprocal) recombination. These definitions do not depend on the content of the progeny DNA segments. We define gene conversion as conservative recombination copying a sequence from one parental segment to the other parental segment (ii, iii). Half-crossing over is an example of nonconservative recombination. It is defined as recombination producing one recombinant progeny segment with recombination flanking sequences and leaves one or two ends (v). Nonconservative recombination might result in one recombinant molecule with parental flanking sequences (a molecule with two close recombination joints; iv). (B) Substrate plasmid pIK43 (inverted repeats). One of the two homologous duplex segments has a 283-bp deletion (deletion *a*) between *Nae*I sites, which removes one (C-terminal encoding) end of the *neo* gene. The *Nae*I site is inactivated by insertion of 8-bp long *Xho*I linker sequence. The bottom segment has a 248-bp deletion (deletion *b*), which removes the other (N terminal) end of *neo* gene. The two deletions are separated by 506-bp homology. The restriction enzymes are RI, *Eco*RI; XhoI, *Xho*I; B, *Bgl*II; N, *Nae*I; Ba, *Bam*HI. (C) Substrate plasmid pDK8 (direct repeats). *Bam*HI-*Eco*RI fragment of pIK43 was flipped through the *Bam*HI linker, which contains the *Eco*RI site. As a result, pDK8 contains three *Eco*RI sites.

MAN 1985) and JC8679 (*recB21 recC22 sbcA23*) (GILLEN *et al.* 1981) are from A. J. CLARK. A *recA1* strain, DH1 (HANAHAN 1983), is from B. HOHN.

Plasmid construction: Construction of pIK43 (Figure 1A) was described earlier (YAMAMOTO *et al.* 1988a). Its repeated sequence comes from pSV2neo (SOUTHERN and BERG 1982), which is derived from Tn5. The right unique part is from pML2d, a derivative of pBR322. The left unique part comes from SV40 replication origin and from bovine papilloma virus type 1. pDK8 is the same as pIK43 except that the orientation of the top *neo* segment is different and that the plasmid has three *Eco*RI site though pIK43 has two sites (Figure 1B). *Bam*HI-*Eco*RI fragment containing *Xho*I site of PIK43 was inserted into the *Bam*HI site of the pIK39 (SAKAGAMI *et al.* 1994) with the *Bam*HI linker which contains *Eco*RI site sequence.

TABLE 1

Recombinant frequency in stationary phase without selection

Bacterial strain	Plasmid[a]	Recombinant frequency[b]
recBC sbcA	pIK43 (inverted)	2.12 ± 0.12
recBC sbcA	pDK8 (direct)	7.06 ± 2.82
recBC sbcBC	pIK43 (inverted)	1.27 ± 0.65
recBC sbcBC	pDK8 (direct)	154 ± 87

Each of 10 ampicillin-resistant (Amp^R) colonies obtained by transformation with the parental plasmids (pDK8 or pIK43) was grown in L-broth with ampicillin selection to the stationary phase. The culture was diluted and plated on kanamycin agar plate and on ampicillin agar plate for overnight incubation. The recombinant frequency is shown together with the standard deviation among 10 clones examined. For all strains, $n = 10$.

[a] Orientation of repeats are indicated in parentheses.

[b] Kan^R colony former/Amp^R colony former × 10,000.

Detection of recombination and calculation of the recombinant frequency: Each of the ampicillin-resistant (Amp^R) colonies recovered after 13-hr incubation after transformation with the substrate plasmid (pIK43 or pDK8), was suspended and grown with ampicillin selection (100 μg/ml) for 5 hr in 5 ml of L-broth with aeration. The aliquots of the culture were spread on kanamycin (Kan) agar (50 μg/ml) plate and ampicillin (Amp) agar (100 μg/ml) plate. Recombinant frequencies were calculated as described (YAMAMOTO *et al.* 1988a,b).

Fluctuation test: Each of the Amp^R colonies obtained by transformation was grown to the exponential phase with ampicillin selection (100 μg/ml) in 10 ml. The culture was aliquoted into small sample tubes (500 μl) and stored at −80° with glycerol (final concentration: 15%). Content of one tube of the glycerol stock samples was diluted and cultured with

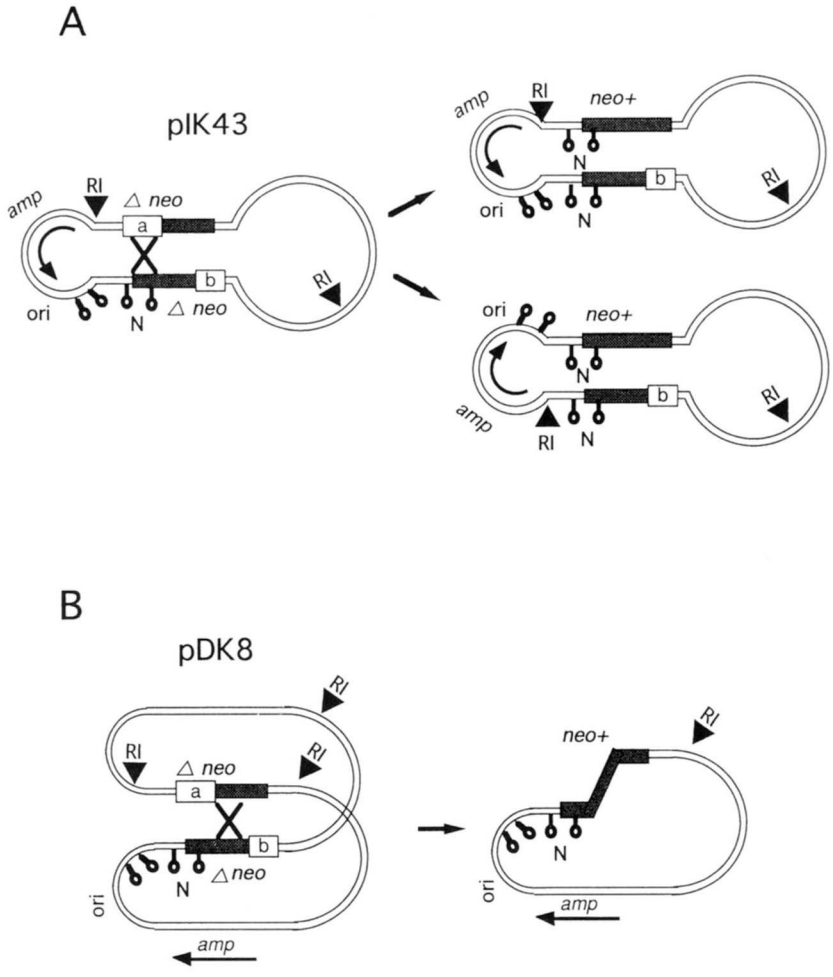

FIGURE 2.—Predicted recombination plasmids. (A) Gene conversion type products from pIK43. These two types of *neo*$^+$ plasmids have a structure that could be formed by intramolecular gene conversion at site *a*. Top right, without flanking crossing-over; bottom right, with flanking crossing-over. (B) Crossing-over type product from pDK8. This type of *neo*$^+$ plasmid has a structure that could be formed by intramolecular crossing-over or half crossing-over between site *a* and site *b* of two homologous sequences of pDK8.

ampicillin selection (100 μg/ml) in a plastic microtiter plate with 96 wells (12 row × eight column) so that each well receives 1–10 cells in 150 μl L-broth. At time $t = 0$, half (75 μl) of the content of each well of the first row of the 96-well plate was spread on ampicillin agar plates (100 μg/ml) to estimate the number of the plasmid-carrying cells in the well. Then, 75 μl of kanamycin-containing L-broth (100 μg/ml) was added to each of the wells of the first row. At time $t = t_1$ (30 or 60 min), half (75 μl) of the content of each well of the second row was treated as before. At time $t = t_n$ (30 × n

TABLE 2

Structural analysis of recombination products

Substrate	Type of recombinant plasmid	JC8679 (=*recBC sbcA*) Exp. 1	JC8679 (=*recBC sbcA*) Exp. 2	JC7623 (=*recBC sbcBC*) Exp. 3	JC7623 (=*recBC sbcBC*) Exp. 4
pIK43	Gene conversion at deletion *a* without crossing-over (Figure 2A, top; Figure 3, No. 1)	7	6	10	6
	Gene conversion at deletion *a* with crossing-over (Figure 2A, bottom; Figure 3, No. 2)	3	1	0	0
	Gene conversion at deletion *b* without crossing-over	0	1	2	0
	Gene conversion at deletion *b* with crossing-over	0	0	0	0
	Reciprocal crossing-over between *a* and *b*	1	0	0	0
	Others	0	0	0	0
		Exp. 5	Exp. 6	Exp. 7	Exp. 8
pDK8	Crossing-over or half crossing-over between direct repeat (Figure 2B; Figure 3, No. 3)	11	5	12	6
	Others	0	0	0	0

Kan^R clones generated during growth of cells carrying the parental plasmid (pDK8 or pIK43) were isolated as colonies. Plasmid DNA (monomer closed circles) from each of them was analyzed with restriction enzymes, *Nae*I *Eco*R I*Bgl*I I*Xho*I and classified (YAMAMOTO *et al.* 1988a).

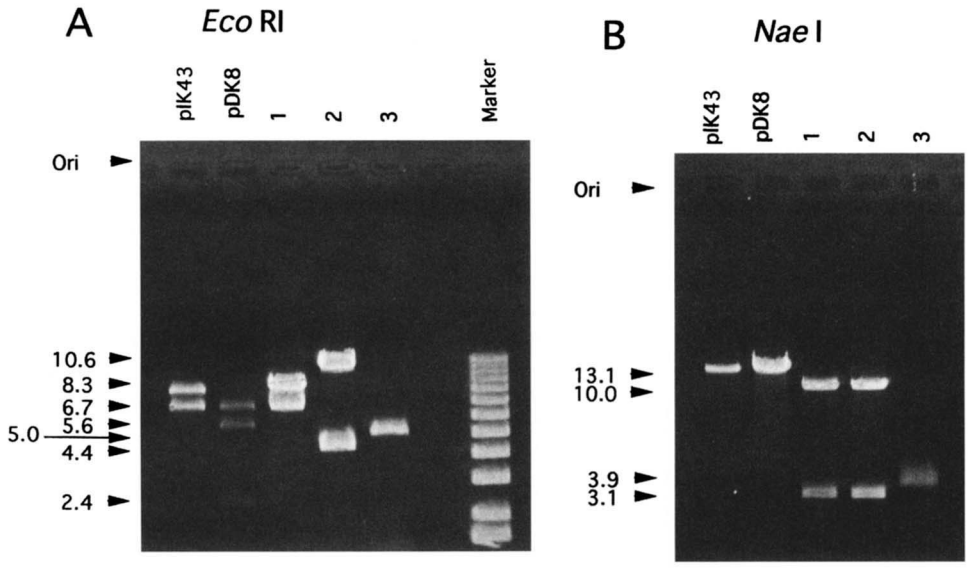

FIGURE 3.—Restriction enzyme analysis of the recombination products. Plasmid DNA was isolated from Kan^R colonies obtained by streaking of an Amp^R transformant of JC8679 with pIK43 or pDK8. Closed circle monomer DNA was purified by agarose gel electrophoresis. It was cut with restriction enzymes and electrophoresed through 0.7% agarose. With the pIK43 products, *Xho*I can detect apparent gene conversion at site *a* and *Eco*RI can detect apparent crossing-over of the flanking sequences (Figures 1 and 2). The numbers in the left indicate fragment lengths in kilobases. Ori, origin. Kan^R-1: gene conversion type at deletion *a* without flanking crossing-over (Figure 2A, top). Fragments (8 and 7 kb) can be found with *Eco*RI digestion (A, lane 1) and 10- and 5-kb fragments with *Nae*I digestion (B, lane 1). Kan^R-2: gene conversion type at deletion *a* with flanking crossing-over (Figure 2A, bottom). Fragments (10 and 5 kb) can be found with *Eco*RI digestion (A, lane 2) and 10- and 3-kb fragments with *Nae*I digestion (B, lane 2). Kan^R-3: crossing-over type product from pDK8 (Figure 2B); 5.5-kb fragments can be found with *Eco*RI digestion (A, lane 2) and 4-kb fragments with *Nae*I digestion (B, lane 3). By *Eco*RI digestion, we can find 8- and 7-kb fragments for the parent plasmid pIK43 and 7-, 6- and 2-kb fragments for the parent plasmid pDK8 (A, two leftmost lanes; see Figure 2). By *Nae*I digestion, we can find 13-kb fragments for both pIK43 and pDK8 (B, two leftmost lanes; see Figure 2).

TABLE 3

Fluctuation analysis with a direct-repeat plasmid in the *recBC sbcA* strain

Experiment	Duration of incubation (min)	No. of Amp^R colony former/presence or absence of Kan^R colony former									Average Amp^R colony former[a]
1	0	4/−	6/−	7/−	4/−	4/−	5/−	3/−	6/−	4/−	5
	60	1/−	3/−	1/−	2/−	2/−	3/−	4/−	1/−	3/−	2
	120	6/−	2/−	4/−	3/−	4/−	2/−	4/−	6/−	2/−	4
	180	4/−	15/−	2/−	4/−	5/−	8/−	7/−	4/−	7/−	6
	240	21/−	32/−	5/−	45/−	52/+	19/−	62/−	25/−	38/+	33
	300	26/−	378/+	287/+	79/−	38/+	129/+	221/+	199/+	189/+	172
	360	547/+	705/+	156/+	721/+	853/+	487/+	421/+	689/+	312/+	543
2	0	2/−	2/−	5/−	6/−	15/−	1/−				5
	60	9/−	3/−	5/−	5/−	5/−	1/−				5
	120	4/−	3/−	6/−	7/−	2/−	5/+				5
	180	5/−	6/−	5/−	7/−	4/−	2/−				5
	240	55/+	20/−	27/−	23/−	39/−	27/−				32
	300	51/−	48/−	73/−	100/+	107/+	41/−				70
	360	483/+	271/+	314/+	197/+	58/−	593/+				319

An *Escherichia coli recBC sbcA* strain (JC8679) carrying pDK8 was cultured with ampicillin selection (100 μg/ml) in 96-well micrototer plate so that a well has 1–10 cell(s), on the avarage, in 150 μl L-broth. At time $t = 0$, 75 μl out of each well of the first row of the 96-well plate was spread on ampicillin agar (100 μg/ml) for estimation of the number of cells in the wells. Then, 75 μl of kanamycin-containing L-broth (100 μg/ml) was added to each of the wells of the first row. At time $t = t_1$ (30 or 60 min), 75 μl out of each well of the second row was treated in the same way as above. At time $t = t_n$, 75 μl of each well of the n+1-th row was treated. After overnight incubation, a clear well was judged to have contained no kanamycin-resistant (Kan^R) cell (−) and a turbid well to have contained at least one Kan^R cell (+), as indicated after the number of Kan^R colonies.

[a] Values are average of each respective row.

TABLE 4

Fluctuation analysis with an inverted-repeat plasmid in the *recBC sbcA* strain

Experiment	Duration of incubation (min)	No. of Amp^R colony former/presence or absence of Kan^R colony former									Average Amp^R colony former
1	0	1/−	2/−	2/−	3/−	3/−	4/−	3/−	5/−	4/−	3
	60	2/−	1/−	4/−	2/−	1/−	2/−	1/−	1/−	3/−	2
	120	2/−	1/−	4/−	3/−	2/−	3/−	4/−	2/−	2/−	3
	180	2/−	1/−	4/−	4/−	5/−	2/−	2/−	2/−	4/−	3
	240	2/−	8/−	19/−	6/−	18/−	1/−	12/−	8/−	3/−	9
	300	47/+	29/−	21/−	1/−	37/−	7/−	16/−	6/−	52/+	24
	360	98/−	89/−	201/+	37/−	25/−	139/+	98/−	75/+	52/+	90
	420	73/+	90/−	278/−	621/+	843/+	348/+	292/+	269/+	159/+	330
	0	10/−	5/−	5/−	9/−	7/−					7
	60	7/−	7/−	6/−	7/+	9/−					7
	120	6/−	9/−	9/−	9/−	5/−					8
	180	5/−	11/−	12/−	20/−	20/−					14
	240	9/−	20/−	23/−	43/−	24/−	3/−	22/−	23/−		21
	300	88/−	140/−	47/−	24/−	40/−	37/−	111/−	103/+		74
2	360	289/−	226/−	189/−	253/+	159/−	187/−	207/−	437/+		243
	420	500/+	152/+	190/−	870/+	730/+	850/+	1030/−	830/+		644

An *Escherichia coli recBC sbcA* strain (JC8679) carrying pIK43 was cultured with ampicillin selection (100 μg/ml) in 96-well plate. The procedure of each experiment is the same as that in Table 3. After overnight incubation, a clear well was judged to have contained no kanamycin-resistant (Kan^R) cell (−) and a turbid well to have contained at least one Kan^R cell (+), as indicated after the number of Kan^R colonies.

TABLE 5

Fluctuation analysis with a direct-repeat plasmid in the *recBC sbcBC* strain

Experiment	Duration of incubation (min)	No. of Amp^R colony former/presence or absence of Kan^R colony former									Average Amp^R colony former
1	0	6/−	8/−	5/−	4/−	4/−	2/−	1/−	2/−	4/−	6/−	4
	30	7/−	5/−	2/−	4/−	4/−	6/−	4/−	5/−	4/−	5/−	5
	60	3/−	3/−	5/+	6/−	3/−	5/−	3/−	4/−	3/−	4/−	4
	120	8/−	6/−	13/−	8/+	13/−	3/−	8/−	12/−	9/−	7/+	9
	180	5/−	46/−	17/−	0/−	7/−	14/−	3/−	16/+	16/−	18/−	16
	240	23/−	88/+	25/+	7/−	35/+	22/−	21/+	51/+	28/+	0/−	33
	300	90/+	37/−	78/+	29/+	27/−	31/+	51/+	59/+	68/+	59/+	53
	360											
	420	245/+	155/+	188/+	439/+	527/+	502/+	479/+	61/+	421/+	245/+	326
2	0	8/−	10/−	9/−	6/−	11/−	7/−	12/−	5/−			9
	60	11/−	5/−	10/−	7/−	11/−	10/−	9/−	11/−			9
	120											
	180											
	240	4/−	29/+	45/+	27/−	31/+	48/+	24/−	37/−			31
	300	50/+	73/−	133/+	79/+	22/−	13/−	54/−	71/+			62
	360	69/−	139/+	198/+	106/+	188/+	56/−	153/+	92/−			125
	420	88/+	436/+	240/+	59/+	320/+	187/+	163/+	172/+			208
	480											
	540											
	600											
	660											
	720											
	780											
	840	2100/+	9500/+	5110/+	3400/+	10300/+	3600/+	0/−	6800/+			5830

An *Escherichia coli recBC sbcBC* strain (JC7623) carrying pDK8. The procedure is the same as that in Table 3.

TABLE 6

Fluctuation analysis with an inverted-repeat plasmid in the *recBC sbcBC* strain

Experiment	Duration of incubation (min)	No. of Amp^R colony former/presence or absence of Kan^R colony former										Average Amp^R colony former
1	0	6/−	11/−	7/−	11/−	1/−	7/−	10/−	7/−	5/−	6/−	7
	60	4/−	7/−	5/−	4/−	6/−	5/−	9/−	9/−	7/−	8/−	6
	120	15/−	11/−	16/−	8/−	18/−	17/−	17/−	12/−	15/−	1/−	15
	180	22/−	34/−	36/−	18/−	26/−	35/−	8/−	35/−	16/−	2/−	26
	240	52/−	16/−	53/−	55/−	65/−	77/−	56/−	36/−	62/−	4/−	52
	300	57/−	143/−	89/−	66/−	49/−	28/−	68/−	76/−	58/−	16/−	80
	360	259/−	137/−	178/−	329/−	227/+	231/−	51/−	157/−	168/−	259/−	200
	420	507/−	87/−	349/−	425/−	542/−	624/−	106/−	396/−	468/−	302/−	380
2	0	7/−	9/−	11/−	6/−	9/−	4/−	12/−	13/−			9
	60	12/−	5/−	7/−	11/−	11/−	9/−	8/−	6/−			9
	120											
	180											
	240	56/−	27/−	141/−	26/−	33/−	49/−	89/−	35/−			57
	300	105/−	133/−	73/−	79/−	203/−	125/−	72/−	56/−			106
	360	269/−	431/−	259/−	116/−	288/−	361/−	121/−	53/−			225
	420	650/−	263/−	201/−	409/−	320/−	787/−	98/−	172/−			363
	480											
	540											
	600											
	660											
	720											
	780											
	840	5500/+	2000/−	18300/+	23200/+	13100/+	20600/+	8800/+	7600/+			12388

An *Escherichia coli recBC sbcBC* strain (JC7623) carrying pIK43. The procedure of each experiment is the same as that in Table 3.

min or 60 × *n* min), 75 μl out of each well of the *n*+1th row was treated. After overnight incubation, a clear well was judged to have contained no kanamycin-resistant (Kan^R) cell at the time of addition of kanamycin and a turbid well to have contained at least one Kan^R cell. The calculating method for the estimated recombination frequency is described in the APPENDIX.

Transformation: Transformation by a calcium method is the same as described (YAMAMOTO *et al.* 1988a,b) except that the final cell concentration of the competent cells, for the *recBC sbcBC* strain, was 10^{10} in 200 μl to achieve a high efficiency.

Other methods: Substrate plasmid preparation, plasmid preparation by a boiling method, restriction enzyme analysis, electrophoresis and the other procedures were described (YAMAMOTO *et al.* 1988a,b).

RESULTS

Experimental design: Our substrate plasmid (Figure 1, B and C) carries two homologous *neo* segments in either inverted orientation (pIK43) or in direct orientation (pDK8). The upper segment has a deletion (deletion *a*) removing one end (C terminus) of the *neo* gene, and the lower segment has a deletion (deletion *b*) removing the other end (N terminus) (Figure 1, B and C).

These substrate plasmids were introduced into an *E. coli recBC sbcA* strain and a *recBC sbcBC* strain. The efficiency of recombination generating neo^+ plasmid (Kan^R cell) was measured by three different methods: measurements of Kan^R colony formation in a liquid

TABLE 7

Recombination frequency from fluctuation tests

Bacterial strain	Plasmid		Recombination frequency (×10,000)	95% confidence interval
recBC sbcA	pIK43 (inverted)	Exp. 1	58	29, 110
		Exp. 2	21	16, 44
recBC sbcA	pDK8 (direct)	Exp. 1	81	32, 160
		Exp. 2	120	58, 250
recBC sbcBC	pIK43 (inverted)	Exp. 1	(<20)	<20
		Exp. 2	2	0.7, 6.2
recBC sbcBC	pDK8 (direct)	Exp. 1	210	120, 350
		Exp. 2	200	120, 340

The recombination frequencies were calculated as described in MATERIALS AND METHODS. <20, less than 20 with 95% confidence.

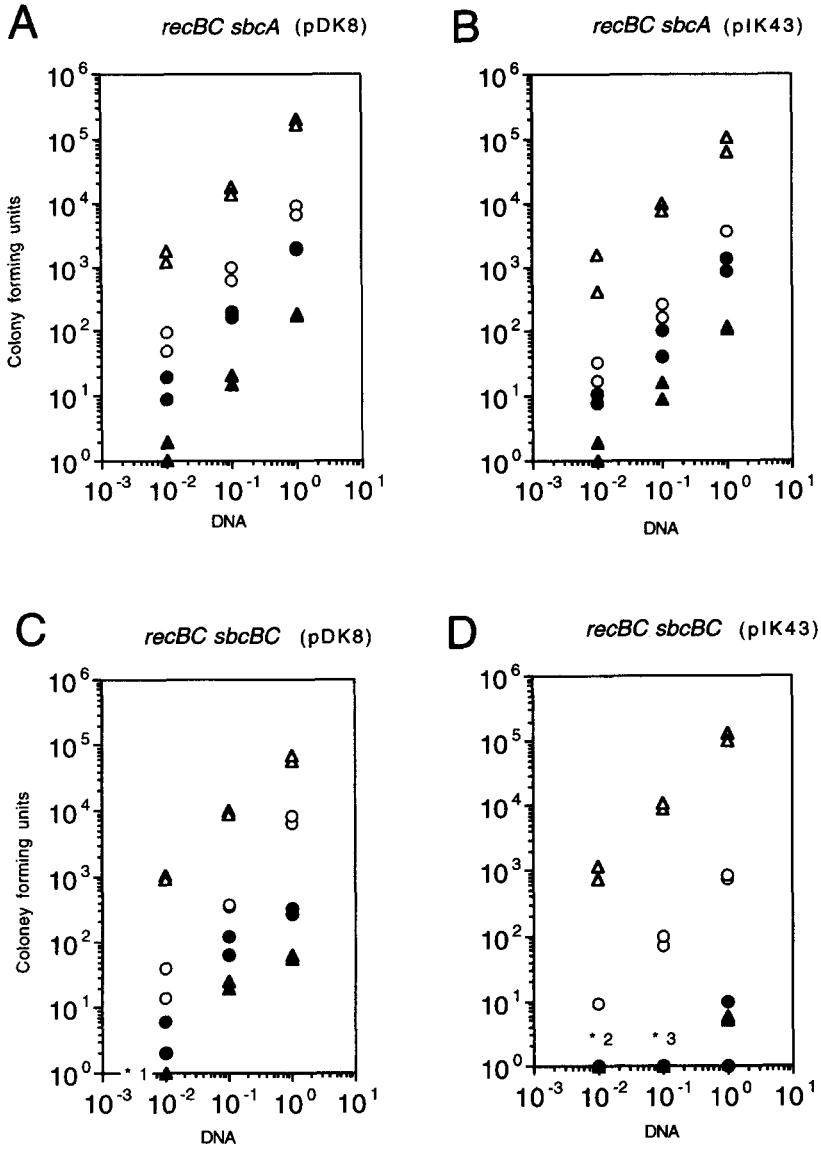

FIGURE 4.—Direct selection of recombination after transformation. △, uncut Amp^R; ○, cut Amp^R; ●, cut Kan^R; ▲, uncut Kan^R. The plasmids were propagated in DH1, purified in an ultracentrifuge with CsCl and cut with or without *Xho*I. Aliquots of the transformation mixture were spread on ampicillin (100 μg/ml) agar plates and kanamycin (50 μg/ml) agar plates, which were then incubated overnight. The numbers of ampicillin (Amp^R)- and kanamycin (Kan^R)-resistant transformants per transformation reaction are plotted in the vertical axis. *1 contains a double point of uncut Kan^R; *2 contains a double point of cut Kan^R, a duplicate point of uncut Kan^R and a single point of cut Amp^R; *3 contains a duplicate point of cut Kan^R and double point of uncut Kan^R.

culture of an Amp^R transformant, fluctuation tests and direct selection of Kan^R transformants after transformation.

Recombinant frequency: Each of the substrate plasmids (pIK43 and pDK8) was introduced into an *E. coli* strain with *recB recC sbcA* mutations (JC8679) or *recB recC sbcB sbcBC* mutations (JC7623) by ampicillin selection. Each of the resulting colonies was assayed for Kan^R colony formations and Amp^R colony formations. A large difference (two orders of magnitude) in the recombinant frequency was found between the two substrates with the *recBC sbcBC* strain, while such a large difference was not found with the *recBC sbcA* strain (Table 1).

Product analysis: The molecular forms of the recombinant plasmids in these Kan^R clones were analyzed with restriction enzymes, *Xho*I, *Eco*RI, *Nae*I and *Bgl*II (Figures 1 and 2). No heterogeneity in the plasmid structure was found within each of Kan^R clones unlike the case in a *rec*$^+$ strain (YAMAMOTO *et al.* 1988a). The plasmids were classified into expected types (Table 2) (YAMAMOTO *et al.* 1988b, 1992). In the case of the *recBC sbcA* strain, each of the clones contained the monomer-sized plasmid and its head-to-tail multimers. The *recBC sbcBC* cells contained the monomer form and its linear multimer forms. Figure 3 shows *Nae*I or *Eco*RI restriction patterns of each of three representative types of the *neo*$^+$ plasmids. The results were summarized in Table 2. With pIK43, we found both "with flanking crossing-over" type (5/19) and "without flanking crossing-over" type (14/19) in the *recBC sbcA* strain. We, however, found strong bias toward "without flanking crossing-over" type (0/18 *vs.* 18/18) in *recBC sbcBC* strain. This contrast was found in the previous works (YAMAMOTO *et al.* 1988b, 1992). With pDK8, all the *neo*$^+$ recombinants belonged to the type that would be obtained from crossing-over or half crossing-over between the direct repeats both in the *recBC sbcA* strain and in the *recBC sbcBC* strain.

Estimation of the recombination frequency by fluctuation tests: There was a possibility that the recombination frequency estimated above might have some bias caused

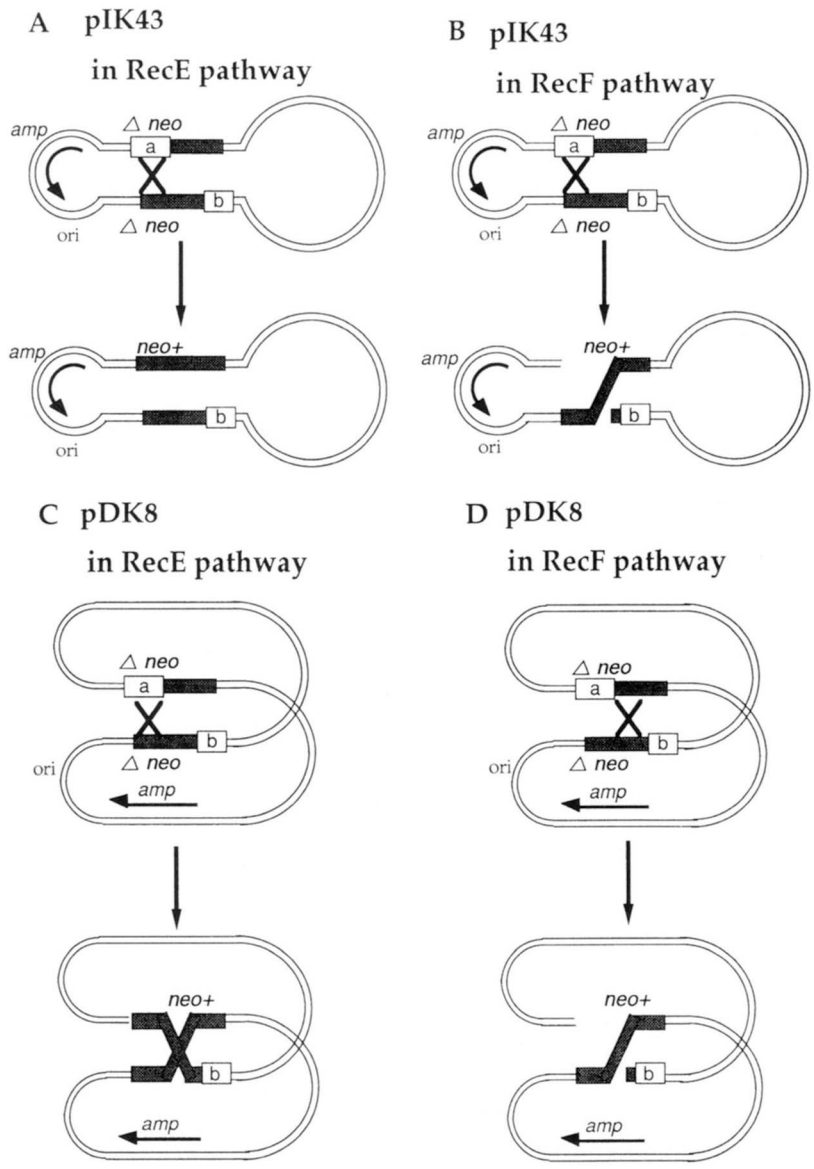

FIGURE 5.—A mechanism for the orientation-dependence model. (A) In RecE-pathway-active strain (*recBC sbcA*), conservative recombination between two duplexes leaves two DNA duplexes. Thus recombinant plasmid from pIK43 can replicate. (B) In recF-pathway-active strain (*recBC sbcBC*), half crossing-over (nonconservative) recombination between two duplex leaves only one DNA duplex. Thus recombinant plasmid from pIK43 cannot replicate. (C) In RecE-pathway-active strain (*recBC sbcA*), conservative recombination leaves two DNA duplexes. Thus recombinant plasmid from pDK8 can replicate. (D) In RecF-pathway-active strain (*recBC sbcBC*), nonconservative recombination leaves only one DNA duplex. The recombinant plasmid from pDK8 can replicate.

by different multiplication rates between the parental plasmid and the recombinants and among the various types of recombinants. Since some types of the *neo*+ recombinant plasmids are smaller than the parental plasmid, the cells carrying them may grow faster than those carrying the parent. Such advantage would lead to an estimation of the recombinant frequency larger than that expected from the recombination frequency. We tried a fluctuation test to estimate the recombination frequency to avoid such a bias. The overview of this experiment is as follows. First, we prepare small aliquots of bacterial culture that are likely to contain only recombinant-free cells. After some growth, we add kanamycin to them for the selection of cells containing a neo^+ plasmid. After their further incubation, the aliquots with at least one recombinant cell should become turbid because of the cell growth and those without any recombinant should remain clear because of the absence of cell growth. Recombination frequency can be estimated from the distribution of the turbid aliquots and the clear aliquots after various incubation times as explained in MATERIALS AND METHODS.

The primary data in these experiments are presented in Tables 3–6, and the estimated recombination frequencies are listed in Table 7. In the case of the recBC sbcA strain (JC8679), the estimated frequency between two substrates (direct *vs.* inverted) was not very different (Tables 3, 4 and 7). In the case of the *recBC sbcBC* strain (JC7623), on the other hand, there was a large difference in the recombination frequency (Tables 5, 6 and 7). Such large orientation-dependence is consistent with our results in Table 1.

Direct selection and the effect of the double-strand breaks: In the above experiments, we found significant orientation-dependence in recombination taking place during cell growth in RecF pathway but not in RecE pathway. To examine the recombination just after DNA transfer and possible effects of DNA double-strand breaks, we introduced the substrates with or without *Xho*I cut (Figure 1) into the cells and directly spread

them on ampicillin plate and kanamycin plate (Figure 4). Though no strong orientation-dependence was found in the case of the *recBC sbcA* strain (JC8679), we found the recombination rate of the direct repeats was higher than that of inverted repeats in the *recBC sbcBC* strain (JC7623). The recombination frequency was elevated by the double-strand break with both the direct-repeat and the inverted-repeat substrates in the *recBC sbcBC* strain (Figure 4, A and B). The recombination frequency was elevated by the break for the direct-repeat substrate (Figure 4C) but was not for the inverted-repeat plasmid (Figure 4D) in the *recBC sbcBC* strain.

DISCUSSION

We have shown here strong orientation-dependence in homologous recombination in *E. coli recBC sbcBC* strain (RecF pathway active) but not in *recBC sbcA* strain (RecE pathway active) with three different protocols: measuring the recombinant frequencies in the stationary-phase culture, fluctuation tests, and direct selection after transformation. Double-strand breakage of the substrates, increased the recombination in both the *recBC sbcBC* strain and the *recBC sbcA* strain.

We previously reported apparent gene conversion (nonreciprocal transfer of sequence information between homologous DNA sequences) in the *recBC sbcBC* strain (RecF pathway) (YAMAMOTO *et al.* 1992). This apparent gene conversion was rarely accompanied by apparent crossing-over. This feature was explained by the "successive half-crossing-over model". This model proposed that the elementary recombinational process is "half crossing-over" in the sense that it generates only one progeny DNA with flanking recombination out of two parental DNAs. We obtained direct evidence for such half crossing-over with a "double origin" plasmids that allow recovery of both progeny of recombination in the *recBC sbcBC* strain (TAKAHASHI *et al.* 1992).

In the *recBC sbcA* strain (RecE pathway), on the other hand, we have reported apparent gene conversion often accompanied by apparent crossing-over of the flanking sequences (YAMAMOTO *et al.* 1988b). Conservative recombination (two recombinant duplex DNA are generated from two parental duplex DNA) by double-strand break repair has been demonstrated (KOBAYASHI and TAKAHASHI 1988; YOKOCHI *et al.* 1995).

The orientation-dependence we found as above can be explained by these mechanisms as illustrated in Figure 5. That is, in RecE pathway, the elementary recombination is conservative by double-strand break repair system, which makes no difference for the recombination frequencies between the two substrates, pIK43 and pDK8 (Figure 5, A and B). While in RecF pathway, half crossing-over makes a viable recombinant with pDK8 but not with pIK43 (Figure 5, C and D). It is not necessary to invoke recognition of the orientation of the homologous sequences by the recombinational machinery.

KITAMURA *et al.* (1990) found similar orientation-dependence of homologous recombination with bovine papiloma vector, a mammalian plasmid, in mouse cells. The half crossing-over model can again explain this orientation-dependence. The nonconservative nature of homologous recombination in the mammalian cells has been suggested earlier (SEIDMAN 1987). If the relationship between half crossing-over and the orientation-dependence in *E. coli* RecF pathway is in parallel with that in the mammalian cells, it might have some meaning in their evolution.

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LITERATURE CITED

CHAKRABARTI, S., and M. M. SEIDMAN, 1986 Intramolecular recombination between transfected repeated sequences in mammalian cells is nonconservative. Mol. Cell. Biol. **6:** 2520–2526.

GILLEN, J. R., D. K. WILLIS and A. J. CLARK, 1981 Genetic analysis of the RecE pathway of genetic recombination in *Escherichia coli* K-12. J. Bacteriol. **145:** 521–532.

GRINDLEY, N. D. F., and R. R. REED, 1985 Transpositional recombination in prokaryotes. Annu. Rev. Biochem. **54:** 863–896.

HANAHAN, D., 1983 Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:** 557–580.

KANAAR, R., A. KLIPPEL, E. SHEKHTMAN, J. M. DUNGAN, R. KAHMANN *et al.*, 1990 Processive recombination by the phage Mu Gin system: implications for the mechanism of DNA strand exchange, DNA site alignment, and enhancer action. Cell **62:** 353–366.

KITAMURA, Y., H. YOSHIKURA and I. KOBAYASHI, 1990 Homologous recombination in a mammalian plasmid. Mol. Gen. Genet. **222:** 185–191.

KOBAYASHI, I., and N. TAKAHASHI, 1988 Double-stranded gap repair of DNA by gene conversion in *Escherichia coli.* Genetics **119:** 751–757.

KOBAYASHI, I., M. M. STAHL, F. R. FAIRFIELD and F. W. STAHL, 1984 Coupling with packaging explains apparent non-reciprocality of chi-stimulated recombination of bacteriophage lambda by RecA and RecBC functions. Genetics **108:** 773–794.

KOWALCZYKOWSKI, S. C., and A. K. EGGLESTON, 1994 Homologous pairing and DNA strand exchange proteins. Annu. Rev. Biochem. **63:** 991–1043.

KUSHNER, S. R., H. NAGAISHI, A. TEMPLIN and A. J. CLARK, 1971 Genetic recombination in *Escherichia coli.* The role of exonuclease I. Proc. Natl. Acad. Sci. USA **68:** 824–827.

LISKAY, R. M., and J. L. STACHELEK, 1986 Information transfer between duplicated chromosomal sequences in mammalian cells involves contiguous regions of DNA. Proc. Natl. Acad. Sci. USA **83:** 1802–1806.

LLOYD, R. G., and C. BUCKMAN, 1985 Identification and genetic analysis of *sbcC* mutations in commonly used *recBC sbcB* strains of *Escherichia coli* K-12. J. Bacteriol. **164:** 836–844.

MAHAN, M. M., and J. R. ROTH, 1988 Reciprocality of recombination events that rearrange the chromosome. Genetics **120:** 23–35.

MAHAN, M. M., and J. R. ROTH, 1989 Role of recBC function in formation of chromosomal rearrangements: a two-step model for recombination. Genetics **121:** 433–443.

MAHAN, M. M., and J. R. ROTH, 1990 Rearrangement of the bacterial chromosome: forbidden inversions, Science **241:** 314–1318.

MIZUUCHI, K., and R. CRAIGIE, 1986 Mechanism of bacteriophage Mu transposition. Annu. Rev. Genet. **20:** 385–429.

PLASTERK, R. H. A., T. A. M. ILMER and P. VAN DE PUTTE, 1983 Site specific recombination by Gin of bacteriophage Mu: inversions and deletions. Virology **127:** 24–36.

SAKAGAMI, K., Y. TOKINAGA, H. YOSHIKURA and I. KOBAYASHI, 1994 Homology-associated nonhomologous recombination in mammalian gene targeting. Proc. Natl. Acad. Sci. USA **91:** 8527–8531.

SEIDMAN, M. M., 1987 Intramolecular recombination between

transfected sequences in mammalian cells is primarily nonconservative. Mol. Cell. Biol. **7:** 3561–3565.

SOUTHERN, P. J., and P. BERG, 1982 Transformation of mammalian cells to antibiotic resistance with bacterial gene under control of SV40 early region promoter. J. Mol. Appl. Genet. **1:** 327–341.

STAHL, F. W., C. E. SHURVINTON, L. C. THOMASON, S. HILL and M. M. STAHL, 1995 On the clustered exchange of the RecBCD pathway operating on phage λ. Genetics **139:** 1107–1121.

TAKAHASHI, N., K. YAMAMOTO, K. KITAMURA, S.-Q. LUO, H. YOSHIKURA *et al.*, 1992 Nonconservative recombination in *Escherichia coli.* Proc. Natl. Acad. Sci. USA **89:** 5912–5916.

YAMAMOTO, K., H. YOSHIKURA, N. TAKAHASHI and I. KOBAYASHI, 1988a Apparent gene conversion in an *Escherichia coli rec*$^+$ strain is explained by multiple rounds of reciprocal crossing-over. Mol. Gen. Genet. **212:** 393–404.

YAMAMOTO, K., H. YOSHIKURA, N. TAKAHASHI and I. KOBAYASHI, 1988b Homologous recombination involving a large heterology in *Escherichia coli.* Genetics **119:** 759–769.

YAMAMOTO, K., K. KUSANO, N. TAKAHASHI, H. YOSHIKURA and I. KOBAYASHI, 1992 Gene conversion in the *Escherichia coli* RecF pathway: a successive half crossing-over model. Mol. Gen. Genet. **234:** 1–13.

YOKOCHI, T., K. KUSANO and I. KOBAYASHI, 1995 Evidence for conservative (two-progeny) DNA double-strand break repair. Genetics **139:** 5–17.

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APPENDIX

Suppose that an experiment gives k samples (wells) and that these samples can be divided into m groups. Here, the ith group ($i = 1 \cdots m$) has samples (wells) with the same numbers (n_i) of cells. We have $\Sigma_{i=1}^{m} k_i n_i$ cells in total and $k = \Sigma_{i=1}^{m} k_i$. Here, k_i is the number of samples in the ith group. For simplicity, we assume that any cell in the ith group has experienced the same times (g_i) of cell division. The probability that we observe l_i samples without Kan^R cells in the ith group is given by ${}_{k_i}C_{l_i}\, p^{g_i l_i}\, (1 - p^{g_i})^{(k_i - l_i)}$, where $p = 1 - q$ and q is recombination frequency per division. We determined the most likely q value by setting $g_i = 2n_i - 2$. Assuming that the result $\{k_1, \ldots, k_m\}$, $\{l_1, \ldots, l_m\}$ and $\{n_1, \ldots, n_m\}$ is observed in an experiment, we estimate the most likelihood recombination frequency value q, which maximizes

$$F(\{l_i\}, \{n_i\}, \{k_i\}) = \prod_{i=1}^{m} {}_{k_i}C_{l_i} p^{g_i l_i} (1 - p^{g_i})^{(k_i - l_i)}$$

In case $m = 1$ and $k_1 \gg 1$ with $k_1 l_1 q$ value fixed, F is reduced to the Poisson distribution.

We have to avoid the preexistence of the *neo*$^+$ recombinants completely. Under this condition, the k_i value is not large enough in our experimental system, which leads us to use the calculation method described as above instead of the Poisson distribution. We assumed that no *neo*$^+$ recombinants was newly generated after the kanamycin selection.