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Intermolecular Recombination between DNAs Introduced into Mouse L Cells Is Mediated by a Nonconservative Pathway That Leads to Crossover Products

FWU-LAI M. LIN,* KAREN SPERLE, AND NAT STERNBERG

E. I. du Pont de Nemours & Co., Inc., Central Research and Development Department, Experimental Station, P.O. Box 80328, Wilmington, Delaware 19880-0328

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We describe experiments designed to measure the efficiency of intermolecular recombination between mutant herpesvirus thymidine kinase (*tk*) genes introduced into mouse L cells. Recombinants were scored as stable transformants containing a functional *tk* gene. The two recombination substrates used were p*tk*B8, a pBR322-based plasmid containing a mutant *tk* gene, with a *Bam*HI linker in an *Sph*I restriction site that is centrally located within the gene, and mp10*tk*Δ3′Δ5′, an mp10 vector with a *tk* gene deleted at both the 3′ and 5′ ends. The only homology shared by the two DNAs is 885 base pairs within the *tk* gene. To determine whether the double-strand break repair model that has been used to explain recombination in yeast cells (J. W. Szostak, T. L. Orr-Weaver, R. J. Rothstein, and F. W. Stahl, Cell 33:25–35, 1983) can account for recombination during the introduction of these DNAs into mammalian cells, we transformed cells with *Bam*HI-linearized p*tk*B8 and supercoiled mp10*tk*Δ3′Δ5′ replicative-form DNA. These two DNAs should recombine efficiently according to that model and should generate gene conversion products. In this reaction, the supercoiled DNA acts as the donor of information to repair the cleaved *tk* gene. Our results indicated that the efficiency of this reaction was very low (<10 transformants were obtained per 0.1 μg of each DNA used in the reaction per 10^6 cells). In contrast, if *Bam*HI-cleaved p*tk*B8 DNA was cotransformed into cells along with a circular DNA molecule containing a *tk* gene deleted only at its 3′ end or only at its 5′ end (mp10*tk*Δ3′ or mp10*tk*Δ5′), then the efficiency of recombination could be more than 4 orders of magnitude higher than it was with circular mp10*tk*Δ3′Δ5′ DNA. Recombination frequencies were highest when the *tk*Δ3′ or *tk*Δ5′ DNA used was cleaved at the *tk* deletion junction. Southern analyses of DNA from TK^+ transformants generated with *Bam*HI-cleaved p*tk*B8 and *Bam*HI-cleaved mp10*tk*Δ3′ DNAs indicated that recombination was almost always associated with the reassortment of markers flanking the reconstructed *tk* DNA. Together, these results are more consistent with the nonconservative single-strand annealing model for recombination that we proposed several years ago (F.-L. Lin, K. Sperle, and N. Sternberg, Mol. Cell. Biol. 4:1020–1034, 1984) than they are with the double-strand break repair model.

To date, two working models have been proposed to explain results of homologous recombination for DNAs transfected into mammalian cells. One is the double-strand break repair (DSBR) model (2, 5, 6, 11, 13, 14, 29, 30, 37, 41), and the other is the single-strand annealing (SSA) model (1, 7, 9, 15, 16, 28, 40). Both models are based on the observation that double-strand breaks in DNA at certain locations stimulate recombination. In the DSBR model (34), recombination is initiated by a double-strand break in one of the DNA molecules (the recipient) that is enlarged to a double-strand gap. That gap is then repaired by using a second molecule (the donor) that shares homology with the region flanking the double-strand break in the first molecule. In this model, only the recipient molecule needs to contain a double-strand cut in the region of shared homology. In contrast, both substrates must be linearized to generate recombinants by the SSA model. According to that model, the DNA ends act as entry sites for a strand-specific exonuclease. Degradation of DNA by such a nuclease generates complementary single-stranded DNA for pairing and subsequent repair (15, 16). Alternatively, the single-stranded DNA can be generated by helicase unwinding of the linearized DNA duplex without extensive exonuclease degradation (40). The essential feature of the SSA model is that double-strand breaks made close to, but not necessarily in, the region of homology shared by each parental DNA molecule are required to initiate recombination.

Although both the DSBR and SSA models emphasize the importance of generating single-stranded DNA for pairing steps in the reactions, the outcomes of recombination predicted by the two models are very different. In the SSA model, recombination is nonconservative (9, 28, 40) and produces exclusively crossover products, namely, recombinant DNA whose flanking sequences are reassorted. In the DSBR model, recombination is associated with crossover of the flanking markers only about 50% of the time, assuming that there is no bias in the resolution step of the reaction. The close association of recombination with crossover has been demonstrated to be the case for DNA-mediated transformation of yeast cells (23). In mammalian cells, the situation is not as clear.

In past studies with mammalian cells, investigators have used data on transformation efficiency, DNA substrate requirements, and DNA product analysis to support their views on the mechanisms of extrachromosomal recombination. Perhaps the most convincing experiments to date that deal with these issues are those from Seidman (28) and Chakrabarti and Seidman (9). These experiments, which directly measure the fate of DNA substrates transferred into African green monkey kidney cells before they become

* Corresponding author.

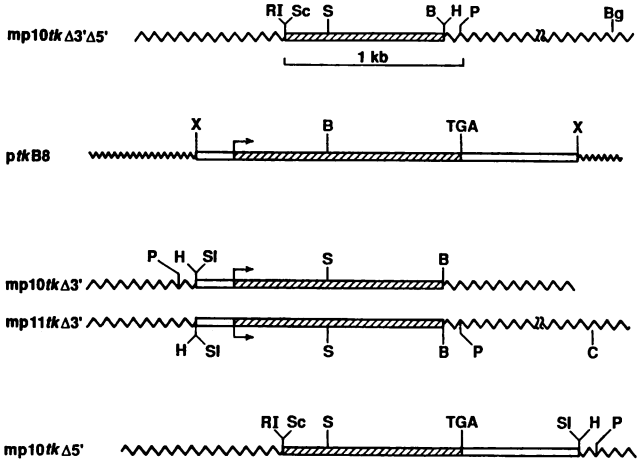

FIG. 1. Mutant *tk* genes used for studies of recombination. Symbols: □, herpesvirus DNA; ▨, herpesvirus *tk* DNA; ∿∿, pBR322 DNA; ∧∧, M13 DNA; ⌐→, *tk* transcription start site and direction; TGA, translation stop codon. Abbreviations for restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pvu*I; RI, *Eco*RI; S, *Sph*I; Sc, *Sac*I; Sl, *Sal*I; X, *Xho*I. Homology of the *tk* gene and its promoter sequence is drawn in vertical alignment. *Sph*I site in the *tk* gene is 493 bp downstream of the mRNA start site. It was converted to a *Bam*HI site by linker insertion in plasmid p*tk*B8. The unique *Pvu*I site is shown in all M13 *tk* clones to orient the M13 vector relative to the *tk* gene.

integrated into host chromosomes, indicate that recombination between DNAs containing repeated sequences occurs by a nonconservative pathway that destroys the starting substrate molecules. In this study, we have designed an alternate set of recombination substrates that can recombine by either the DSBR or SSA pathway. Recombination is assayed by the reconstruction of the herpesvirus thymidine kinase (*tk*) gene from two mutant genes in mouse L cells, and the criteria used to distinguish between the two pathways are the number of stable transformants produced and Southern analyses of markers flanking the recombinant DNA.

MATERIALS AND METHODS

Cell strains and media. The mouse cell line used was the LM*tk*$^-$ *aprt*$^-$ (L*tk*$^-$) line of Wigler et al. (42). It was grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 50 μg of gentamicin sulfate per ml in an atmosphere of 5% CO_2. Hypoxanthine-aminopterine-thymidine medium was used for selection of thymidine kinase-positive (TK$^+$) transformants (35). Bacterial strains HB101 and JM101 were used to prepare plasmid and M13 DNAs, respectively.

Plasmid *tk* DNAs. pS*tk*-1 contains the 2.04-kilobase-pair (kb) *Pvu*II *tk* fragment (39) cloned between the unique *Aat*II and *Pvu*II sites of pBR322 DNA such that the direction of *tk* transcription is opposite that of the *bla* gene. Plasmid p*tk*B8 (Fig. 1) was produced by digesting pS*tk*-1 DNA at its unique *Sph*I restriction site within the *tk* gene, converting the resulting 3′ protruding ends to flush ends with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and finally linking the two blunt ends together with an 8-base-pair (bp) *Bam*HI linker (5′-CGGATCCG-3′; Collaborative Research, Inc.). In this manipulation, the unique *Sph*I site of the wild-type gene is replaced with a unique *Bam*HI site, resulting in a reading frameshift that inactivates the *tk* gene. The presumed structure of this construct was confirmed by DNA sequencing (26). Whereas pS*tk*-1 DNA produces 100 to 1,000 TK$^+$ transformants per ng of supercoiled plasmid DNA per 10^6 L*tk*$^-$ cells, p*tk*B8 DNA fails to produce transformants even when microgram quantities of DNA are used (see below).

M13 *tk* DNA. Construction of the 3′-end *tk* deletion mutants mp10*tk*Δ3′$_m$ and mp11*tk*Δ3′$_m$ and the 5′-end *tk* deletion mutant mp10*tk*Δ5′$_m$ has been previously described (16). We have not observed TK$^+$ transformants when L*tk*$^-$ cells are transformed with microgram quantities of any of these DNAs (see below). A 3′ and 5′ double-deletion mutant mp10*tk*Δ3′$_m$Δ5′$_m$ was constructed by using the individual single-deletion mutants. To simplify terminology, we no longer use the subscript "m" in referring to these mutants. All four M13 *tk* constructs are shown in Fig. 1. Replicative-form I supercoiled M13 *tk* DNA was isolated from bacteriophage-infected JM101 cells by the procedure of Holmes and Quigley (12). This DNA was purified through CsCl-ethidium bromide equilibrium gradients.

DNA-mediated cell transformation. We used a modified calcium phosphate procedure to introduce DNA into cells. Briefly, on day 1, L*tk*$^-$ cells were plated at a density of 5×10^5 cells per 100-mm-diameter tissue culture dish (Corning Glass Works) in 10 ml of growth medium. On day 2, 1 ml of a very fine DNA precipitate was added to each plate. That precipitate was generated by mixing two solutions. One solution contained, in 0.5 ml, the recombining DNAs in the amounts indicated in each experiment, 10 μg of carrier DNA from L*tk*$^-$ cells, 250 mM calcium chloride, 10 mM Tris hydrochloride (pH 7.4), and 1 mM EDTA. The other solution contained, also in 0.5 ml, 50 mM 1,4-piperazine diethanesulfonic acid (pH 6.95), 280 mM NaCl, and 1.5 mM sodium phosphate. On day 3, the medium on the plate containing the precipitated DNA was replaced with growth medium, and on day 4, drug selection was applied. On day 10, the medium in each plate was changed; on day 17, the medium was removed from the plates, and colonies were either cloned or stained. Enzyme-digested DNA was prepared for transformation either by phenol-chloroform extraction to remove proteins, followed by ethanol precipitation, or by passage through a NENSORB 20 column (Dupont, NEN Research Products). DNA concentrations were determined spectrofluorimetrically by Hoechst 33258 staining (8).

DNA hybridization. GeneScreen Plus (Dupont, NEN) or Zeta Probe (Bio-Rad Laboratories) nylon filter papers were used for blot hybridization analysis (32). *tk* probes were gel-purified DNA fragments labeled with [α-^{32}P]dCTP to a specific activity of 1×10^8 to 2×10^8 cpm/μg of DNA, using a nick translation kit purchased from Dupont, NEN. Protocols for hybridization and nick translation were used as specified by the vendors.

RESULTS

mp10*tk*Δ3′Δ5′ does not efficiently correct the *tk* insertion mutation in p*tk*B8. We cloned into pBR322 a 2.04-kb *Pvu*II DNA fragment that contains the functional *tk* gene of herpes simplex virus. From this clone, an insertion mutant, p*tk*B8, was generated by inserting an 8-bp *Bam*HI linker into the unique *Sph*I site at nucleotide 493 of the *tk* structural gene (20). The insertion causes a frameshift that inactivates the gene. We also produced a *tk* mutant that contains deletions of both ends of the *tk* gene, mp10*tk*Δ3′Δ5′ (see Materials and Methods). p*tk*B8 and mp10*tk*Δ3′Δ5′ share 885 bp of homology that is perfect except for the presence of the 8-bp *Bam*HI

TABLE 1. Recombination between p*tk*B8 and *tk*Δ3′Δ5′ DNAs[a]

Line	p*tk*B8 DNA		Donor DNA			No. of TK⁺ transformants
	Amt (ng)	Enzyme treatment	Source	Amt (ng)	Enzyme treatment	
1	100	None	mp10*tk*Δ3′Δ5′	100	None	1
2	100	None		100	*Sac*I	0
3	100	None		100	*Bam*HI	1
4	100	None		100	*Sac*I + *Bam*HI	1
5	100	*Bam*HI		100	None	0
6	100	*Bam*HI		100	*Sac*I	33
7	100	*Bam*HI		100	*Eco*RI	39
8	100	*Bam*HI		100	*Bam*HI	7
9	100	*Bam*HI		100	*Pvu*I	5
10	100	*Bam*HI		100	*Bgl*II	3
11	100	*Bam*HI		100	*Sph*I	0
12	100	*Bam*HI		100	*Sac*I + *Bam*HI	312
13	10	*Bam*HI	mp10*tk*Δ3′	10	*Bam*HI	280
14	10	*Bam*HI	mp11*tk*Δ3′	10	*Bam*HI	436
15	10	*Bam*HI		10	*Hin*dIII	0

[a] All data were collected from one experiment.

linker in p*tk*B8 DNA, which interrupts and destroys the one *Sph*I site in the wild-type gene in mp10*tk*Δ3′Δ5′ DNA (Fig. 1). The region of *tk* homology is divided into 236 bp upstream (the 5′ arm) and 649 bp downstream (the 3′ arm) of the *Bam*HI site and represents the only homologous region shared by the two mutant vectors.

In our initial experiments, we cotransformed Ltk⁻ cells either with the undigested p*tk*B8-mp10*tk*Δ3′Δ5′ pair of mutant *tk* DNAs or with that pair of DNAs predigested with various restriction enzymes. If the p*tk*B8 DNA was not linearized by restriction enzyme digestion, very few TK⁺ transformants were produced regardless of whether mp10*tk*Δ3′Δ5′ DNA was linearized (Table 1, lines 1 to 4). Few transformants also were produced if p*tk*B8 DNA was cleaved at its unique *Bam*HI site and mp10*tk*Δ3′Δ5′ DNA was not cleaved (Table 1, line 5). Since the *Bam*HI site in p*tk*B8 DNA is located in the region of homology shared by the two DNAs, the DSBR model predicts that these two DNAs should recombine efficiently. Clearly they did not. A double-strand break at the *Bam*HI site of p*tk*B8 stimulated recombination if the mp10*tk*Δ3′Δ5′ DNA was also cleaved at or close to one of the deletion junctions (Fig. 1 and Table 1, lines 6 to 9). Moreover, we consistently observed that cleavage of mp10*tk*Δ3′Δ5′ DNA near the 5′-end *tk* deletion junction (with *Eco*RI or *Sac*I) produced more recombinants than did cleavage of that DNA near the 3′-end *tk* deletion junction (with *Bam*HI or *Hin*dIII) (Table 1, compare lines 6 and 7 with line 8). We also observed that the cleavage of mp10*tk*Δ3′Δ5′ DNA at a *Bgl*II site located 680 bp away from the 3′ *tk* deletion junction in mp10 DNA generated transformants with an efficiency comparable to that observed with *Bam*HI-linearized mp10*tk*Δ3′Δ5′ DNA (Table 1, compare lines 8 and 10). If mp10*tk*Δ3′Δ5′ DNA was cleaved at the *Sph*I site, both of the cotransforming DNAs contained double-strand cuts at the same position within *tk*, and no detectable transformants were produced (Table 1, line 11). Surprisingly, when the doubly deleted *tk* fragment was excised from the mp*tk*Δ3′Δ5′ vector by digesting the vector with *Bam*HI and *Sac*I (Table 1, line 12), then recombination with *Bam*HI-cleaved p*tk*B8 DNA increased 10- to 100-fold compared with the level of singly digested mp10*tk*Δ3′Δ5′ DNA. The mechanism responsible for this stimulation is addressed in the accompanying paper (17).

TABLE 2. Recombination between p*tk*B8 and either *tk*Δ3′ or *tk*Δ5′ DNA

Line	p*tk*B8 DNA		Donor DNA			No. of TK⁺ transformants	
	Amt (ng)	Enzyme treatment	Source	Amt (ng)	Enzyme treatment	Expt 1	Expt 2
1	50	None	mp10*tk*Δ3′	50	None	ND[a]	26
2	100	None		100	None	47	ND
3	50	None		50	*Bam*HI	ND	133
4	100	None		100	*Bam*HI	746	ND
5	10	*Bam*HI		10	None	6	6
6	100	*Bam*HI		100	None	486	404
7	10	*Bam*HI		10	*Bam*HI	185	485
8	100	*Bam*HI		100	*Bam*HI	2,410	>3,000
9	10	*Bam*HI		10	*Sal*I	2	ND
10	100	*Bam*HI		100	*Sal*I	191	ND
11	10	*Bam*HI	mp11*tk*Δ3′	10	None	10	9
12	10	*Bam*HI		10	*Bam*HI	268	1,250
13	10	*Bam*HI		10	*Cla*I	221	ND
14	10	*Bam*HI	mp10*tk*Δ5′	10	None	0	1
15	100	*Bam*HI		100	None	3	34
16	10	*Bam*HI		10	*Sac*I	7	102
17	100	*Bam*HI		100	*Sac*I	861	1,735
18	100	*Bam*HI	mp10*tk*Δ3′Δ5′	100	None	1	ND

[a] ND, Not done.

A mutant *tk* gene deleted at only one end can correct the lesion in the linker insertion mutant efficiently. The structures of singly deleted *tk* genes cloned into M13 vectors (mp10*tk*Δ3′, mp11*tk*Δ3′, and mp10*tk*Δ5′) are shown in Fig. 1, and the results of cotransformations with these DNAs and p*tk*B8 DNA are shown in Tables 1 and 2. Recombination between *Bam*HI-cleaved p*tk*B8 and either *Bam*HI-cleaved mp10*tk*Δ3′ or *Bam*HI-cleaved mp11*tk*Δ3′ DNA was much more efficient than comparable cotransformation with the *Bam*HI-cleaved mp10*tk*Δ3′Δ5′ DNA (Table 1, compare lines 13 and 14 with line 8). Indeed, the efficiencies of recombination with the *tk*Δ3′ substrates were so high (Table 2) that the amounts of DNA used in the reaction in Table 1 were reduced from 100 to 10 ng to ensure that DNA would be a limiting component in the reaction. When the colony count was less than 1,000 per plate, then the concentration dependence of colony formation was nearly second order; i.e., a 10-fold increase in amounts of both cotransfecting DNAs resulted in about a 100-fold increase in the number of recombinants (Table 2, compare lines 5 and 6, 9 and 10, and 16 and 17). Since plasmid with an intact *tk* gene generated 20 to 150 transformants per ng of linearized DNA (16), the

TABLE 3. Analyses of flanking markers in recombinants resulting from cotransformation of *Bam*HI-linearized p*tk*B8 DNA and uncut mp10*tk*Δ3′ DNA

Class	Type	No. of clones	No. with given DNA fragment size (kb)					
			*Dde*I			*Dde*I + *Sph*I		
			2.04	2.23	1.90	1.13	1.10	0.94
I	Crossover	5	+	−	+	−	+	+
		7	+	−	−	−	+	+
II	Crossover with 3′ rearrangement	1	−	−	−	−	−	+
III	Crossover and noncrossover	1	+	+	+	+	+	+

recombination observed with *Bam*HI-cleaved p*tk*B8 DNA and *Bam*HI-cleaved mp10*tk*Δ3′ DNA was about 10%.

Recombination between uncut p*tk*B8 and mp10*tk*Δ3′ DNAs during transformation was relatively inefficient (Table 2, lines 1 and 2), but was much higher than that observed with the double-deletion mutant (compare Tables 1 and 2). Cutting mp10*tk*Δ3′ at the *Bam*HI site stimulated recombination with uncut p*tk*B8 about 5-fold when the two pairs of DNAs are compared at 50 ng of DNA per plate (Table 2, experiment 2, lines 1 and 3) and 16-fold when the two pairs of DNAs are compared at 100 ng of DNA per plate (Table 2, experiment 1, lines 2 and 4). If p*tk*B8 DNA was cut at the *Bam*HI site and then used with either uncut mp10*tk*Δ3′ DNA or *Bam*HI-cut mp10*tk*Δ3′ DNA to cotransform cells, there were 30 to 80 times more recombinants with the *Bam*HI-cleaved *tk*Δ3′ DNA than with the uncleaved DNA (Table 2, lines 5 and 7). In contrast, cleavage of mp10*tk*Δ3′ DNA at the *Sal*I site not only failed to stimulate recombination but, in fact, decreased the number of recombinants detected relative to that seen with mp10*tk*Δ3′ DNA (Table 2, compare lines 5 and 9). The effect of the location of the cleavage site on recombination is reminiscent of what we observed in studies of recombination between a *tk*Δ3′ and *tk*Δ5′ gene (15, 16); namely, cleavage at or near the *tk* deletion junction greatly stimulated recombination, but cleavage at the other end of the *tk* DNA did not. High levels of recombination also were observed when *Bam*HI-cleaved p*tk*B8 DNA was cotransformed into cells along with appropriately cut mp11*tk*Δ3′ and mp10*tk*Δ5′ DNAs (Table 1, lines 14 and 15; Table 2, lines 11 to 17). The number of transformants obtained with the pair p*tk*B8-mp10*tk*Δ3′ was always higher than that obtained with the pair p*tk*B8-mp10*tk*Δ5′ (Table 2, compare lines 7 and 8 with lines 16 to 17). We also found that it was not necessary to cut mp11*tk*Δ3′ DNA at precisely the deletion junction to see the stimulatory effect. Cleavage at the *Cla*I site in M13 DNA more than 600 bp downstream of the deletion junction had a stimulatory effect comparable to that seen with cleavage at the *Bam*HI site (Fig. 1 and Table 2, lines 12 and 13). In order to see a sizable stimulation of TK^+ transformation, it was necessary to carry out transformation at a limiting amount of the two DNAs. At higher DNA concentrations, the number of tranformants increased disproportionately to the increase in the amount of DNA used, suggesting that the number of transformable cells may be limiting. For example, *Bam*HI-linearized p*tk*B8 DNA was 30 times more active than uncut p*tk*B8 DNA at 10 ng per plate but only 5 times more active at 100 ng per plate (Table 2, experiment 1, compare lines 5 and 7 and lines 6 and 8)

Most of the TK^+ recombinants generated by cotransforming cells with *Bam*HI-digested p*tk*B8 DNA and *Bam*HI-digested mp10*tk*Δ3′ DNA have their flanking markers exchanged. The DSBR and SSA models make different predictions about the disposition of flanking markers among the TK^+ recombinants generated by the above-described set of DNAs. Assuming that there is no bias in the resolution of Holliday structure intermediates, the DSBR model predicts that about 50% of the recombinants will have flanking markers exchanged. The SSA model predicts that all of the recombinants will be of the crossover type. We cloned 30 TK^+ transformants produced by cotransformation at low DNA concentrations. Such conditions ensure that most of the transformants will have few copies of the introduced DNA. Genomic DNA of the individual transformants was digested either with restriction enzyme *Dde*I alone or with *Dde*I and *Sph*I. If there is no crossover of the flanking markers, one should see a 2.23-kb *Dde*I fragment. If exchange of the *tk* arms occurs, one should see a 2.04-kb *Dde*I band. Double digestion with *Dde*I and *Sph*I permits one to determine whether the wild-type sequence has been restored at the mutation site (Fig. 2A). A functional *tk* gene will have either a 2.23-kb *Dde*I fragment or a 2.04-kb *Dde*I fragment that is cleaved by *Sph*I to generate two smaller fragments. In contrast, if the 2.23-kb *Dde*I fragment is produced by the in

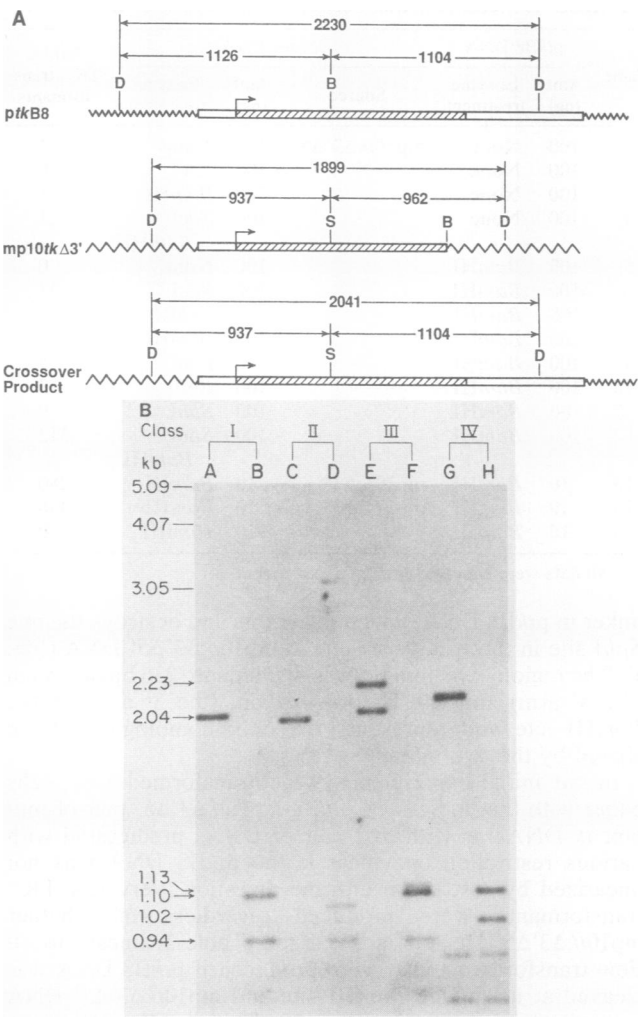

FIG. 2. (A) *Dde*I and *Dde*I-*Sph*I *tk* fragments of p*tk*B8 DNA, mp10*tk*Δ3′ DNA, and crossover recombination product DNA. Lengths are indicated in base pairs. All symbols and abbreviations are as for Fig. 1. Additional abbreviation: D, restriction enzyme *Dde*I. (B) Southern blot analyses of the four classes of TK^+ recombinants resulting from cotransformation of *Bam*HI-linearized p*tk*B8 and *Bam*HI-linearized mp10*tk*Δ3′ DNAs. Lanes: A, C, E, and G, DNAs digested with *Dde*I only; B, D, F, and H, DNAs digested with *Dde*I and *Sph*I. Class I recombinants have the crossover product. Class II recombinants are crossover products with 3′ DNA rearranged. Class III recombinants have both the crossover and noncrossover products in the same cell. The class IV recombinant cannot be assigned to crossover or noncrossover configuration. For the class III recombinant, the 2.23- and 2.04-kb *Dde*I fragments are further cleavable with *Sph*I to generate DNA fragments of 1.13 and 1.10 kb for the former and 1.10 and 0.94 kb for the latter. We expected the intensity of the 1.10-kb fragment to be about twice that at 1.13 kb, and that was the case (lane F). The probe used in these experiments was the 1.16-kb *Bgl*II-*Sma*I fragment of the *tk* DNA (39).

vivo religation of *Bam*HI-digested p*tk*B8 DNA, it will not be cleaved by *Sph*I.

DNAs from the 30 TK$^+$ transformants had very simple hybridization patterns (Fig. 2B). In no case could we detect a 1.9-kb *Dde*I fragment characteristic of the mp10*tk*Δ3′ substrate. Twenty-seven of the transformants had *Sph*I-cleavable 2.04-kb *Dde*I fragments characteristic of crossover products (classes I and III; Fig. 2B, lanes A, B, E, and F). One of these clones also had a 2.23-kb *Dde*I restriction fragment characteristic of a noncrossover recombinant product (class III; Fig. 2B, lanes E and F). The other three clones did not have an identifiable 2.23- or 2.04-kb *Dde*I fragment (Fig. 2B, lanes C and G). Digestion with *Dde*I and *Sph*I revealed that two of the latter three clones (class II) had a 0.94-kb *Dde*I-*Sph*I fragment originating from the 5′ end of mp10*tk*Δ3′. Since these two clones are TK$^+$ and have no other *tk*-hybridizable bands, they probably contain crossover products in which the 5′ end of *tk* comes from mp10*tk*Δ3′ DNA and the 3′ end comes from p*tk*B8 DNA. Presumably, the latter has been rearranged so that it no longer contains the characteristic *Dde*I site. The remaining clone has the 1.10-kb *Dde*I-*Sph*I fragment coming from the 3′ end of p*tk*B8 and a rearranged 5′ end of the gene (class IV). This rearrangement makes it impossible to determine whether the 5′ end of the recombinant gene comes from p*tk*B8 DNA or mp10*tk*Δ3′ DNA and therefore whether the product is a crossover or noncrossover one. If we eliminate the single class IV recombinant, there were a total of 29 crossover events and one noncrossover event in 29 cell lines examined.

We have shown above that most of the TK$^+$ recombinants produced by cotransformation with *Bam*HI-digested p*tk*B8 and mp10*tk*Δ3′ DNAs were associated with the crossing over of the flanking DNA markers. This result is consistent with the prediction of the SSA model. However, since that model requires the two recombining molecules to have free ends with which to anneal, it cannot explain the generation of transformants obtained by using uncut mp10*tk*Δ3′ DNA unless that DNA is fortuitously broken in the transformation process. To gain insight into the structure of recombinant DNA in TK$^+$ transformants generated with *Bam*HI-cleaved p*tk*B8 DNA and uncleaved mp10*tk*Δ3′ DNA, we analyzed 14 TK$^+$ of these transformants by Southern hybridization (Table 3). Among the 14 TK$^+$ clones, 13 had a *Dde*I fragment (2.04 kb) consistent with the crossing over of flanking markers (classes I and III). One of the clones also had a 2.23-kb fragment expected of a noncrossover product. Only one clone had neither the 2.04-kb nor the 2.23-kb *Dde*I fragment. However, upon double digestion with *Dde*I and *Sph*I, this clone could be shown to contain a 0.94-kb fragment indicative of a crossover product. Overall, the ratio of crossover to noncrossover events was 14:1 when mp10*tk*Δ3′ DNA was not linearized. Since mp10*tk*Δ3′ was not linearized in these experiments and cells were transformed at higher DNA concentrations than in the experiment with the cut mp10*tk*Δ3′ DNA, 6 of the 14 TK$^+$ clones analyzed also carried the 1.9-kb *Dde*I fragment present in mp10*tk*Δ3′ DNA.

DISCUSSION

In this report, we have endeavored to distinguish between two prevailing models, the DSBR and SSA models, that have been used to account for extrachromosomal recombination in mammalian cells. We have accomplished this by varying the nature of the pairs of recombination substrates that are introduced into those cells and by analyzing the products of the recombination reaction. If L*tk*$^-$ cells are transformed with DNA from the *Bam*HI linker mutant (p*tk*B8) and a mutant containing deletions at both ends of the *tk* gene (mp10*tk*Δ3′Δ5′), the DSBR model predicts efficient reconstruction of the *tk* gene in p*tk*B8 DNA if that DNA is linearized at the *Bam*HI site (34; Fig. 3A). This can occur efficiently even if the mp10*tk*Δ3′Δ5′ DNA is circular. In contrast, the SSA model (Fig. 4A) predicts that these DNAs should not efficiently reconstruct an intact *tk* gene since that pathway always generates crossover products (products with rearranged flanking markers), and in this case crossover products would not produce an intact *tk* gene. Our results indicate that mp10*tk*Δ3′Δ5′ DNA cannot recombine efficiently with *Bam*HI-cleaved p*tk*B8 DNA to produce a functional *tk* gene and that this inability is most pronounced when the mp10*tk*Δ3′Δ5′ DNA is circular. When p*tk*B8 and mp10*tk*Δ3′ DNAs were used to transform L*tk*$^-$ cells, the efficiency of recombination was very high if the p*tk*B8 DNA was linearized at the *Bam*HI linker site and the mp10*tk*Δ3′ DNA was linearized at the *tk* deletion junction (Fig. 4B; 15). Indeed, recombination levels with this set of DNAs were 2 to 3 orders of magnitude higher than levels of recombination with the p*tk*B8-mp10*tk*Δ3′Δ5′ set of DNAs (Tables 1 and 2). If one assumes that the Holliday intermediate generated in the DSBR model (Fig. 3B) is resolved with equal efficiencies into crossover and noncrossover products, then the elevated levels of recombination seen with *Bam*HI-linearized p*tk*B8 and mp10*tk*Δ3′ DNAs cannot be accounted for by the DSBR model. According to that model, only a twofold increase in TK$^+$ recombination (due to crossover products) should occur when mp10*tk*Δ3′Δ5′ is replaced by mp10*tk*Δ3′ DNA. These arguments, and three additional results, suggest that the SSA model more adequately explains our results. First, there is no a priori reason according to the DSBR model for the reaction to be stimulated by linearizing the mp10*tk*Δ3′ DNA. Yet at low DNA concentrations (10 ng; Table 2), *Bam*HI cleavage of mp10*tk*Δ3′ DNA stimulated recombination 30- to 80-fold. The SSA model predicts that cleaving mp10*tk*Δ3′ DNA is necessary for efficient recombination. Second, transformants generated using *Bam*HI-digested p*tk*B8 and mp10*tk*Δ3′ DNAs hardly ever contain either of the two substrate DNAs intact. This result is more consistent with a degradative, nonconservative recombination pathway in which substrates are destroyed in generating recombinants than it is with the conservative DSBR pathway. Finally, the DSBR model predicts that recombination with the p*tk*B8-mp10*tk*Δ3′ set of DNAs should be associated with the crossover of flanking markers only 50% of the time (Fig. 3B), whereas the SSA model predicts that all of the recombinants should have the crossover configuration (Fig. 4B). We found that 29 of 30 independent recombinants tested had flanking markers crossed over. The failure to detect significant levels of gene conversion products in these recombination experiments does not by itself rule out the DSBR pathway if one argues that recombination intermediates, e.g., the double Holliday structure of Fig. 3B, must always be resolved to produce crossover products. However, in light of the need to cleave the mp10*tk*Δ3′ substrate and in light of the data supporting a degradative pathway, a DSBR model with a strong bias in the resolution step is an unlikely possibility. This conclusion is strongly supported by the experiment of Chakrabarti and Seidman (9).

Why might extrachromosomal recombination occur so much less efficiently by the DSBR pathway than by the SSA pathway? We can think of at least two reasons. First, one or

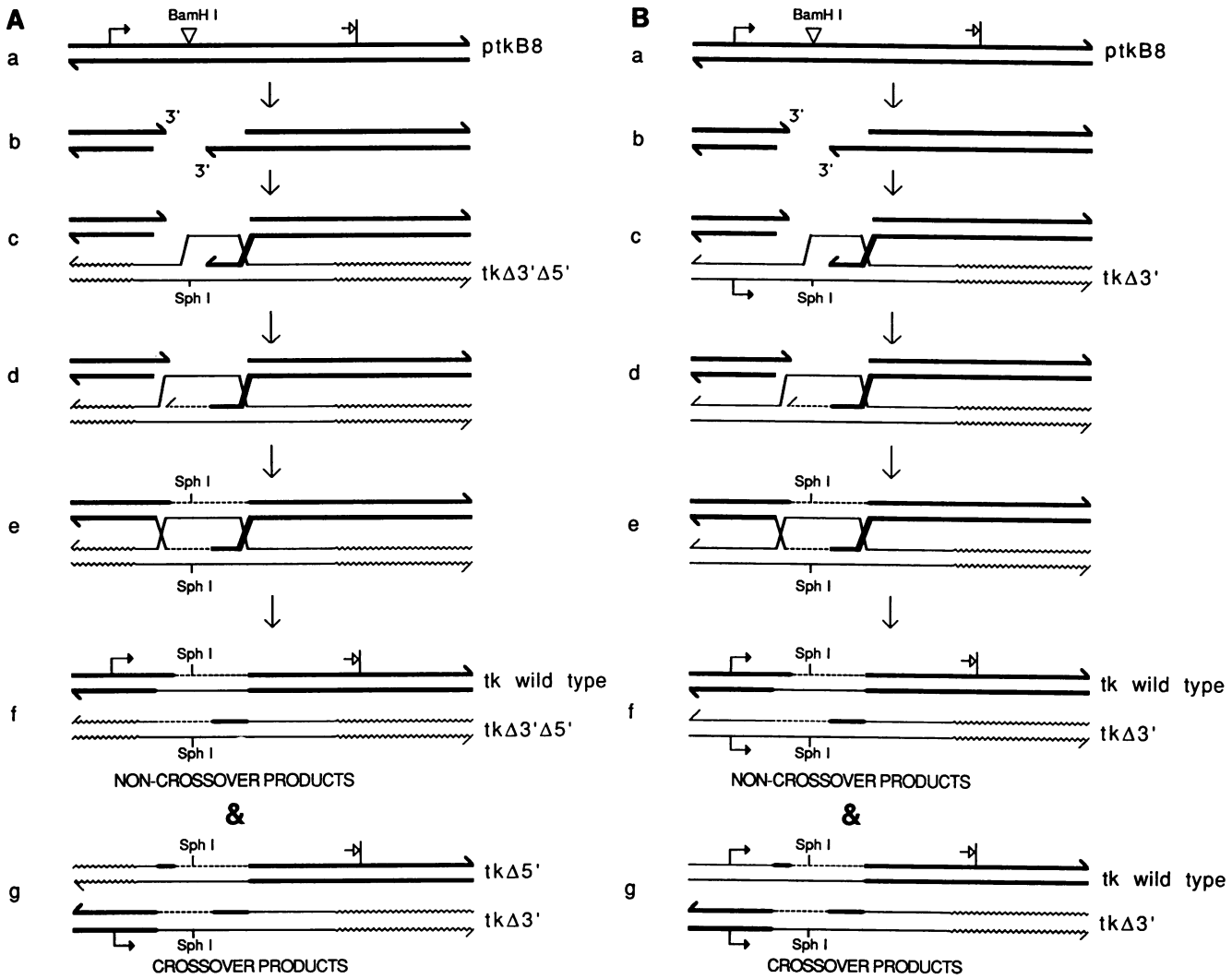

FIG. 3. DSBR model demonstrating how *Bam*HI-cleaved p*tk*B8 DNA can be efficiently converted to generate an intact *tk* gene by using a supercoiled mp10*tk*Δ3′Δ5′ DNA (A) or a supercoiled mp10*tk*Δ3′ DNA (B) as an information donor. Diagrams are drawn according to Szostak et al. (34), with thick lines representing the recipient (p*tk*B8) DNA strands and thin lines representing the donor DNA strands. (a) A double-strand break is made at the *Bam*HI site in p*tk*B8. (b) A gap flanked by 3′ single strands is formed by the action of a 5′ exonuclease. (c) One 3′ single-stranded end invades the homologous duplex in mp10*tk*Δ3′Δ5′ or mp10*tk*Δ3′ to form a D loop. (d) The D loop is extended by repair synthesis until the other 3′ single-stranded end can anneal to the complementary single-stranded sequences in the D loop. (e) Repair synthesis from the second 3′ end completes the process of gap repair; ligation and branch migration result in the formation of two Holliday junctions. Resolution by cutting both junctions horizontally leads to two possible noncrossover configurations (f), and resolution by cutting the left junction vertically and the right junction horizontally leads to two possible crossover configurations (g). The wild-type *tk* gene is reconstructed only in the noncrossover configuration with *tk*Δ3′Δ5′ DNA as the donor in panel A, whereas it is reconstructed in noncrossover and crossover configurations with *tk*Δ3′ DNA as the donor in panel B. Symbols: ┌→, start site of *tk* transcription; →|, stop codon of the *tk* gene; – – –, newly synthesized DNA generated by gap repair. The pBR322 cloning vector sequence for p*tk*B8 is not shown, and only part of the mp10 sequence for mp10*tk*Δ3′Δ5′ is shown (∿∿∿).

more of the steps in the DSBR pathway is inefficient. Candidates for such steps include the pairing of single strands of DNA with their complementary DNA duplexes (an early step in the DSBR pathway) and the resolution of Holliday structures (a late step in the DSBR pathway). Neither of these steps is required in the SSA pathway. A second possible explanation for the inefficiency of the DSBR pathway deals with the nature of the assay system used to measure recombination. In the calcium phosphate-mediated transfer procedures that we used to introduce DNA into cells, it has long been known that cells frequently take up many copies of the DNA added to the medium (42). If this is the case here, it is possible that the DSBR pathway is compromised by the fact that the two ends of the *Bam*HI-cleaved p*tk*B8 DNA frequently associate with different donor molecules (mp10*tk*Δ3′Δ5′ or mp10*tk*Δ3′). In such a circumstance, an intact *tk* gene would be generated only if the lost p*tk*B8 end were replaced by the same end from a second p*tk*B8 molecule (Fig. 5). Thus, most successful recombination reactions involving a DSBR pathway in this system might require the participation of three molecules. In contrast, the generation of an intact *tk* gene by the SSA pathway would require only the annealing of the two ends containing complementary portions of the defective *tk* genes. The other ends could be destroyed or sequestered without affecting the outcome of the reaction. In the accom-

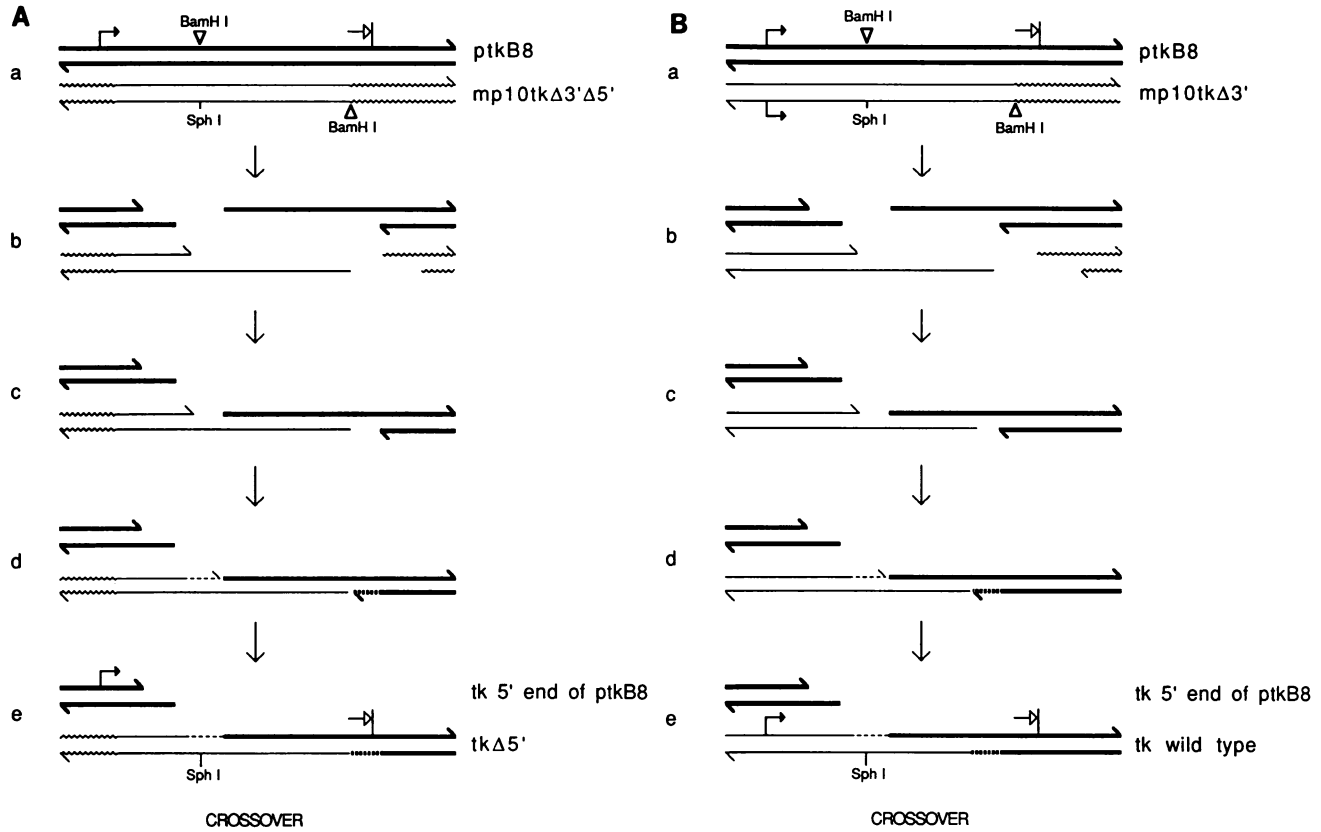

FIG. 4. SSA model. (A) Model illustrating that *Bam*HI-cleaved p*tk*B8 DNA and linearized mp10*tk*Δ3'Δ5' DNA cannot reconstruct an intact *tk* gene. (B) Model showing how *Bam*HI-cleaved p*tk*B8 DNA and linearized mp10*tk*Δ3' DNA can generate an intact *tk* gene in which flanking markers are rearranged. Symbols are as for Fig. 3. Wavy lines represent nonhomologous mp10 DNA. The pBR322 DNA in p*tk*B8 is not shown. (a) A double-strand break is made at the *Bam*HI site in p*tk*B8 and at the *Bam*HI site of either mp10*tk*Δ3'Δ5' (A) or mp10*tk*Δ3' (B) DNA. (b) DNA ends are degraded by a strand-specific exonuclease to expose the complementary single strands. Here a 3' exonuclease is used. (c) Annealing of the single-stranded *tk* DNAs. (d) Repair synthesis from the 3' ends of the *tk* DNA. (e) A *tk*Δ5' gene (A) or a wild-type *tk* gene (B) is generated after ligation. The 5' end of the *tk* gene from p*tk*B8 DNA is eventually discarded.

panying paper (17), we directly measure the efficiency of an SSA pathway that requires the participation of three molecules and the annealing of four ends to generate an intact tk^+ gene. Whereas the efficiency of that reaction is 10-fold lower than the efficiency of a reaction requiring the participation of two molecules and the annealing of only two ends (compare Table 1, line 12 with Table 2, line 8), it is 150-fold higher than the efficiency of the reaction between *Bam*HI-digested p*tk*B8 DNA and undigested mp10*tk*Δ3'Δ5' DNA (see Table 1 of reference 17). These results suggest that although the requirement for a trimolecular interaction may in part account for the inefficiency of the DSBR pathway, it cannot completely account the inefficiency of that pathway.

The results obtained with the p*tk*B8-mp10*tk*Δ5' pair of DNAs (Table 2, lines 14 to 17) largely mimic those obtained with the p*tk*B8-mp10*tk*Δ3' pair of DNAs and provide additional support for the SSA model. However, a comparison of recombination frequencies between *Bam*HI-cleaved p*tk*B8 DNA and either uncleaved mp10*tk*Δ3'Δ5', uncleaved mp10*tk*Δ5', or uncleaved mp10*tk*Δ3' DNA (Table 2) indicates that the mp10*tk*Δ3' donor DNA is 100 times more efficient than are the other two DNAs. Since the overall lengths of *tk* homology between the three mp10*tk* DNAs and p*tk*B8 are nearly the same (1,344 bp for mp10*tk*Δ3', 1,582 bp for mp10*tk*Δ5', and 885 bp for mp10*tk*Δ3'Δ5'), differences in homology are probably not responsible for the effect. Although mp10*tk*Δ3' has three times more homologous DNA upstream of the *Bam*HI linker insertion than do the other two mp10*tk* DNAs (695 bp versus 236 bp), this difference probably also cannot account for the difference in recombination frequencies observed, since recombination frequency varies only linearly with the extent of homology in the range used (2, 18, 25). Another possibility arises from the observation that of all donor DNAs used here, only mp10*tk*Δ3' DNA contains a promoter (the herpesvirus *tk* promoter) that can promote transcription into the *tk* gene in mammalian cells. It is possible that transcription from this promoter stimulates recombination with circular DNA. The positive effects of transcription on recombination have been documented in both yeast and mammalian cell systems (4, 38). To test this transcription hypothesis, the avian Rous sarcoma virus long terminal repeat promoter was inserted at the *Sac*I site of mp10*tk*Δ3'Δ5' DNA to promote transcription of the doubly deleted *tk* gene. This construct recombines no better than does mp10*tk*Δ3'Δ5' with *Bam*HI-cleaved p*tk*B8 DNA. At present, we have no good explanation for the high recombination frequencies seen with the uncleaved mp10*tk*Δ3' DNA.

The results reported here strongly support the contention that recombination between DNAs introduced into mammalian cells occurs primarily by a nonconservative pathway that leads to nonreciprocal crossover products. This conclusion is completely consistent with the elegant studies of Seidman (28) and Chakrabarti and Seidman (9), which were

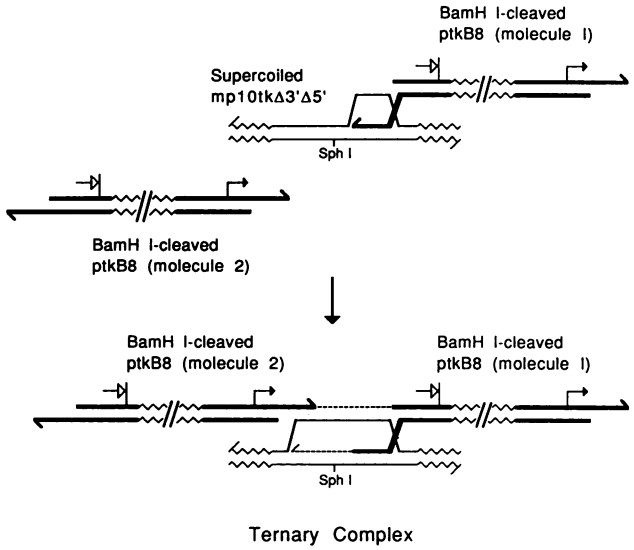

FIG. 5. Diagram showing how gene conversion can occur between a supercoiled mp10*tk*Δ3′Δ5′ DNA molecule and two *Bam*HI-linearized p*tk*B8 DNA molecules according to the DSBR model of recombination. The two *Bam*HI-linearized p*tk*B8 DNAs are shown with 3′ single-stranded ends after exonuclease degradation. All symbols are as for Fig. 3. Wavy lines represent nonhomologous pBR322 vector DNA in p*tk*B8 and mp10 vector DNA in mp10*tk*Δ3′Δ5′.

carried out in a very different way. First, we used a *tk* gene substrate that was transferred, along with carrier DNA, into mouse L cells by the calcium precipitate method, and we measured recombinants as hypoxanthine-aminopterine-thymidine-resistant stable transformants or by Southern analyses of genomic DNA. In contrast, Seidman (28) and Chakrabarti and Seidman (9) used either a T-antigen or *neo* gene substrate transferred into African green monkey kidney cells by DEAE-dextran without carrier and assayed nonintegrated recombinant DNA 48 h after transfer by Southern analyses of Hirt extract DNA. The largely similar conclusions drawn in these studies despite differences in substrate DNA, mode of DNA introduction into cells, cell type, and recombinant substrate analyzed argue strongly for the generality of the conclusions and indicate that none of the mentioned variables significantly influenced or biased the basic observations.

In contrast to the results discussed above, the experiments described by Brenner et al. (6), Song et al. (30), and Waldman and Liskay (41) largely argue for a conservative gene conversion recombination mechanism that is more consistent with a DSBR model. By using substrates containing two defective genes on the same or different plasmids, recombination products were observed that could be explained by the repair of one of the defective genes (the recipient) using information from the other (the donor) without a loss of donor sequences. The problem with conclusions drawn from these experiments, however, is the difficulty of determining the fate of the donor molecules used for any one recombination event. Failing in this, one could just as well propose that apparent conservative recombination products actually arise by nonconservative pathways. Indeed, Seidman (28) has shown that a recombinant heterodimer generated by what would appear to be a conservative intermolecular reciprocal exchange can, in fact, be generated by a nonconservative mechanism if one of the recombination substrates is a dimer. Degradation of one of the monomers of that dimer during recombination with a second substrate molecule could produce an apparent conservative heterodimer by a nonconservative pathway. By the same means, intramolecular recombination between two defective genes on the same plasmid could generate an apparent conservative product by a nonconservative pathway if the substrate plasmid happens to be a dimer. Alternatively, multiple rounds of recombination of the sort that frequently generates tandemly repeated DNA after the introduction of foreign DNA into mammalian cells could also produce apparent conservative products by a nonconservative pathway. The experiments of Folger et al. (11) lend credence to the latter possibility. They observed that stable transformants generated by the microinjection of two defective genes into L cells contain a small number of direct tandem arrays containing 3 to 15 copies of various rearranged forms of the substrate gene and at least one copy a functional recombinant gene. These workers noted the lack of reciprocal recombination products in their experiments, a result that they argue is consistent with gene conversion but one that is equally consistent with the nonconservative pathways described here.

An issue that needs to be considered is whether the assay system we use, detection of recombinants as stable transformants, biases the results in favor of crossover products. Since recombinant products need to be captured in the chromosome by nonspecific integration to be assayed, factors that affect capture could bias the conclusions. For example, if free DNA ends were required for the integration of foreign DNA and crossover recombination products were more likely to have free ends than noncrossover products, the conclusion regarding the relative abundance of the two recombination products could be biased. We believe this possibility unlikely for two reasons. First, Chakrabarti and Seidman (9) observed a similar bias in favor of crossover products when recombinant products were assayed before integration. Second, we have not seen a significant difference in transformation efficiency regardless of whether DNA containing an intact *tk* gene is introduced into cells as a linear or a circular molecule (16). If free ends are needed for integration, this result must mean that they can be easily generated when circular DNA is introduced into cells.

On the basis of studies of sequences from multigene families, it has been proposed that gene conversion can occur in mammalian chromosomes (21, 22, 24, 36). It has also been demonstrated with selectable markers that intrachromosomal gene conversion events can occur in mammalian cells (19, 26, 33). In addition, it has been inferred that gene conversion can occur when introduced DNA recombines with its chromosomal homolog (3, 10, 13, 31, 37). In all of these cases, it remains to be proven that the recombination is due to a gene conversion rather than a double-reciprocal event. The failure to detect chromosomal nonconservative recombination events (deletions, rearrangements, etc.) more frequently than conversion or double-reciprocal events may mean that the former events are more frequently lethal to cells or that chromosomal recombination occurs by a pathway that is different than the major extrachromosomal recombination pathway. The latter possibility was proposed by Waldman and Liskay (41) to account for differences in the efficiencies of extrachromosomal and chromosomal recombination between *tk* genes with sequence mismatches. However, if the SSA model describes most of the recombination events in mammalian cells, then DNA breaks in both the input DNA and its chromosomal target will be needed. Since

it has not been possible to specifically cleave chromosomal targets efficiently, the efficacy of the SSA model for gene targeting and for recombination between repeat elements has not yet been tested.

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