An Examination of the Effects of Double-Strand Breaks on Extrachromosomal Recombination in Mammalian Cells

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ABSTRACT

We studied the effects of double-strand breaks on intramolecular extrachromosomal homologous recombination in mammalian cells. Pairs of defective herpes thymidine kinase (*tk*) sequences were introduced into mouse Ltk$^-$ cells on a DNA molecule that also contained a *neo* gene under control of the SV40 early promoter/enhancer. With the majority of the constructs used, gene conversions or double crossovers, but not single crossovers, were recoverable. DNA was linearized with various restriction enzymes prior to transfection. Recombination events producing a functional *tk* gene were monitored by selecting for tk-positive colonies. For double-strand breaks placed outside of the region of homology, maximal recombination frequencies were measured when a break placed the two *tk* sequences downstream from the SV40 early promoter/enhancer. We observed no relationship between recombination frequency and either the distance between a break and the *tk* sequences or the distance between the *tk* sequences. The quantitative effects of the breaks appeared to depend on the degree of homology between the *tk* sequences. We also observed that inverted repeats recombined as efficiently as direct repeats. The data indicated that the breaks influenced recombination indirectly, perhaps by affecting the binding of a factor(s) to the SV40 promoter region which in turn stimulated or inhibited recombination of the *tk* sequences. Taken together, we believe that our results provide strong evidence for the existence of a pathway for extrachromosomal homologous recombination in mammalian cells that is distinct from single-strand annealing. We discuss the possibility that intrachromosomal and extrachromosomal recombination have mechanisms in common.

THE ability to recombine homologous DNA sequences is a property shared by virtually all organisms, yet the mechanisms of recombination remain poorly understood. The repeated observation that double-strand DNA breaks can influence recombination has led to the development of several recombination models. The double-strand break repair (DSBR) model was proposed to explain certain stimulatory effects of DNA breaks on recombination in yeast (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; SZOSTAK *et al.*, 1983) and has been used to explain similar observations for recombination events in higher eukaryotes (reviewed in BOLLAG, WALDMAN and LISKAY 1989; SUBRAMANI and SEATON 1988). According to the DSBR model, recombination initiates at the site of a break via the action of an exonuclease that enlarges the break into a double-strand gap. The gap is then repaired by interaction with a strand of DNA from a second molecule that has homology to the sequences flanking the gap. This mechanism is somewhat different from earlier models proposed by HOLLIDAY (1964) and MESELSON and RADDING (1975) in which recombination initiates at a single-strand break and in which the broken molecule donates genetic information (reviewed in ORR-WEAVER and SZOSTAK 1985).

A recombination model that is strikingly different from the ones described above was proposed by LIN, SPERLE and STERNBERG (1984, 1990a,b) to account for certain effects of double-strand breaks on extrachromosomal recombination among sequences transfected into mouse Ltk$^-$ cells. This latter model is often referred to as *single-strand annealing* (SSA) and is essentially similar to an earlier proposal by CASSUTO and RADDING (1971) for recombination of bacteriophage λ. According to the SSA model, recombination is initiated at a double-strand break by the action of a strand-specific exonuclease. Single strands are digested bidirectionally from the break site until complementary sequences are exposed. Complementary strands then anneal to one another. The event is completed by cleavage of single-stranded unannealed sequences followed by single-strand gap repair or replication. A representation of the essential steps of SSA is depicted in Figure 1. The SSA model differs from the more "traditional" HOLLIDAY (1964), MESELSON and RADDING (1975) and DSBR (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; SZOSTAK *et al.*, 1983)

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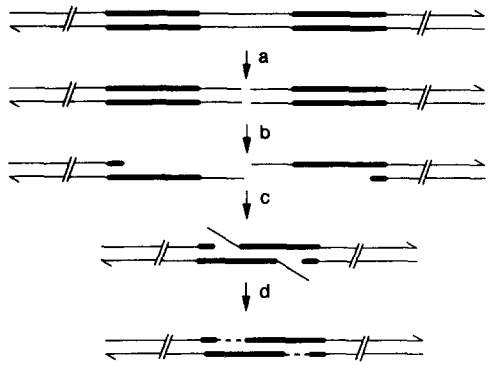

FIGURE 1.—The essential steps of the SSA model for homologous recombination. Recombination between two directly repeated homologous sequences (heavy lines) involves the following steps: (a) the introduction of a double-strand break at an appropriate location, (b) bidirectional strand-specific degradation from the break site to expose complementary sequences, (c) annealing of complementary sequences, and (d) removal of unpaired single-stranded DNA followed by single-strand gap repair and/or DNA replication.

models in at least four important ways. (i) There is no synapsis of duplexes, (ii) HOLLIDAY (1964) structures are not invoked as intermediates, (iii) recombination products are exclusively crossovers and (iv) recombination is viewed as being "nonconservative," that is, recombination proceeds with a loss of one copy of the recombining sequences.

The nonconservative nature of certain extrachromosomal recombination events has been confirmed in studies involving mammalian cells (CHAKRABARTI and SEIDMAN 1986; SEIDMAN 1987) as well as in recent studies involving Xenopus oocytes (JEONG-YU and CARROLL 1992; MARYON and CARROLL 1991a,b). It had been reported that extrachromosomal recombination in mammalian cells occurs predominantly through SSA (ANDERSON and ELIASON 1986). Although several studies of extrachromosomal recombination in mammalian cells appear to contradict the SSA model (BRENNER, SMIGOCKI and CAMERINI-OTERO 1985, 1986; SONG *et al.* 1985), it has recently been observed that, upon close scrutiny, no reported study of extrachromosomal recombination in mammalian cells is necessarily inconsistent with the SSA paradigm (see LIN, SPERLE and STERNBERG 1990a,b).

In contrast to extrachromosomal recombination, intrachromosomal recombination in mammalian cells does not appear to proceed via SSA. Recombination within mammalian chromosomes appears to be conservative (BOLLAG and LISKAY 1988) and gene conversion without exchange of flanking markers is a common occurrence (reviewed in BOLLAG, WALDMAN and LISKAY 1989). Additionally, intrachromosomal recombination in mammalian cells is more sensitive to heterology than is extrachromosomal recombination (WALDMAN and LISKAY 1987, 1988). Do such findings reflect a fundamental difference between mechanisms of intrachromosomal *vs.* extrachromosomal recombination in mammalian cells, or might these two types of recombination be accomplished by similar mechanisms subject to different constraints due to the particular characteristics of the DNA substrates?

In this work we begin to address this issue by reexamining the question as to whether all extrachromosomal recombination in mammalian cells must in fact occur via SSA. Evidence for alternate extrachromosomal recombination pathways might be difficult to obtain in the presence of high levels of SSA that could mask other mechanisms. We have used recombination substrates in which gene conversions or double crossovers, but not single crossovers, are recoverable. Using similar substrates, others (LIN, SPERLE and STERNBERG 1990a,b) have reported that extrachromosomal recombination is inefficient unless double-strand breaks are introduced at sites appropriate for the stimulation of two crossovers by two rounds of SSA. We took a different point of view and asked whether recombination that occurs in the *absence* of such appropriately placed breaks proceeds by a mechanism distinct from SSA.

By examining the effects of double-strand breaks on extrachromosomal gene conversion-like events in cultured mouse fibroblasts, we find that the frequency of recombination can be influenced indirectly by certain double-strand breaks in a manner that cannot be reconciled with the SSA paradigm. We also report on the efficient recovery of recombination events using a substrate configured in such a way that productive recombination should have been precluded by an efficient SSA machinery. An additional examination of the effects of DNA breaks on extrachromosomal recombination between imperfectly matched sequences reveals further inconsistencies with the SSA model. We feel that these studies present strong evidence that there is at least one operative pathway for extrachromosomal homologous recombination in mammalian cells that is distinct from SSA.

MATERIALS AND METHODS

Cell culture: Thymidine kinase-deficient mouse L cells (Ltk^- cells) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 0.1 mM MEM nonessential amino acids (GIBCO), and 50 μg/ml of gentamicin sulfate. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2.

DNA transfections and determination of extrachromosomal recombination frequency: Cells were transfected by the calcium phosphate coprecipitation method essentially as described by GRAHAM and VAN DER EB (1973). Briefly, cells were plated at a density of 5×10^5 cells per 75-cm^2 flask (or 100-mm dish) on day 1 and on day 2 each flask was transfected with plasmid DNA plus sufficient salmon sperm carrier DNA to bring the total amount of DNA to 20 μg per flask. Plasmids were introduced as supercoiled molecules or were linearized by restriction enzyme digestion prior to transfection. Cells were treated with the calcium phosphate/DNA coprecipitate for 5 h after which the precipitate was

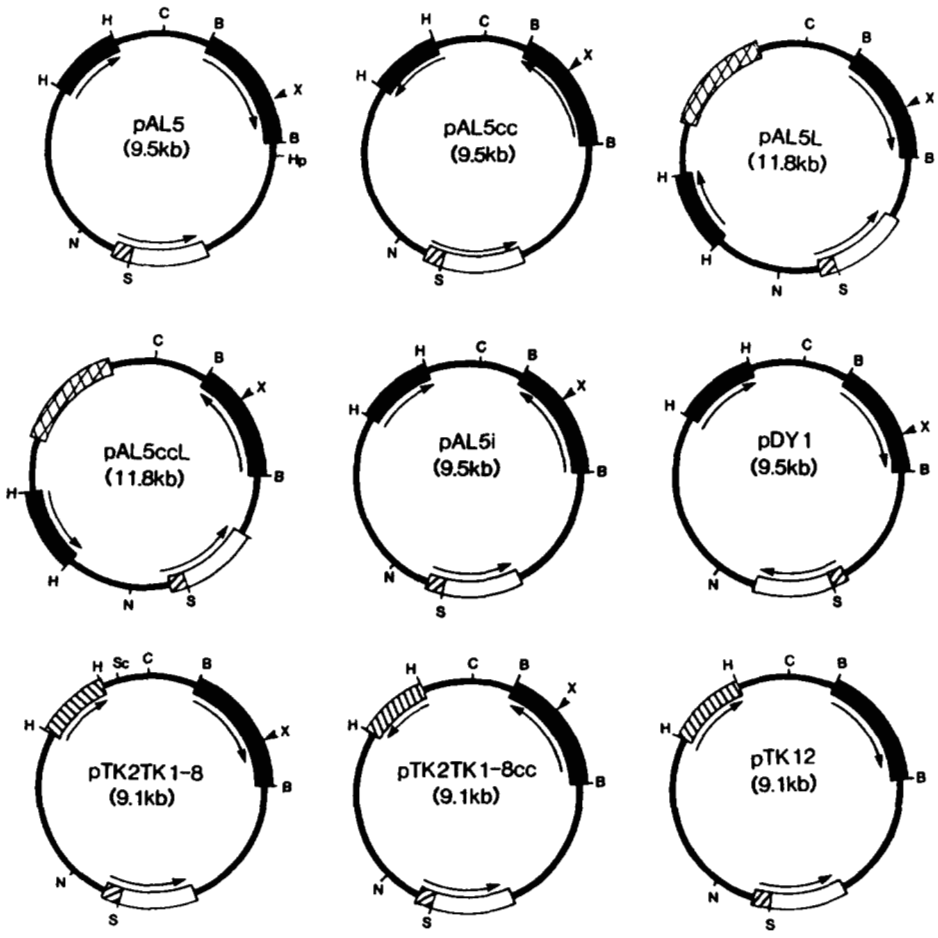

FIGURE 2.—DNA substrates used to study extrachromosomal recombination between defective *tk* sequences. All constructs are based on a pSV2neo-derived vector, indicated by the thin lines. The vector contains a *neo* gene transcribed from the SV40 early promoter (▨□). An HSV-1 *tk* gene (■) mutated by a *Xho*I linker insertion ("X") is contained within a *Bam*HI (B) fragment on each plasmid except pTK12 which contains a wild-type HSV-1 *tk* gene. Either an HSV-2 *tk* gene with a 5′ deletion of the coding region (▨) or an HSV-1 *tk* gene with 5′ and 3′ deletions (■) is contained within a *Hin*dIII (H) fragment of each plasmid. Plasmids pAL5L and pAL5ccL contain an insertion of 2.3 kb of the λ genome (▤). Also shown are cleavage sites for *Cla*I (C), *Nde*I (N), *Stu*I (S), *Sca*I (Sc), and *Hpa*I (Hp). Arrows depict the direction of transcription of the *tk* and *neo* sequences. Figures are drawn approximately to scale. See MATERIALS AND METHODS for construction details.

washed off and cells were fed with nonselective medium. Twenty hours later, the cells were refed with medium containing hypoxanthine/aminopterin/thymidine (HAT) (SZYBALSKI, SZYBALSKA and RAGNI 1962). After 10–14 days, cells were fixed with methanol, stained with Geimsa stain, and HAT-resistant (HAT^r) colonies were counted as a measure of the level of extrachromosomal homologous recombination.

Plasmid constructions: All plasmids are based on vector pJS-1 which is a derivative of pSV2neo (SOUTHERN and BERG 1982) with restriction site modifications as previously described (LISKAY, STACHELEK and LETSOU 1984). Maps of all plasmids are illustrated in Figure 2. The #8 mutant herpes simplex virus type 1 (HSV-1) *tk* gene has been described (WALDMAN and LISKAY 1987). This gene contains a *Xho*I linker inserted at nucleotide 1215 of the HSV-1 *tk* gene, numbering according to WAGNER, SHARP and SUMMERS (1981). Plasmid pAL5, described previously (WALDMAN and LISKAY 1987), contains the #8 mutant *tk* gene on a 2.5-kb *Bam*HI fragment inserted into the unique *Bam*HI site on the vector. Plasmid pAL5 also contains a 1.2-kb *Hin*cII-*Sma*I fragment of the HSV-1 *tk* gene inserted into the *Hin*dIII site on the vector, after attachment of *Hin*dIII linkers. This 1.2-kb fragment has truncations of both the 5′ and 3′ ends of the coding region of the *tk* gene. On pAL5, both defective *tk* sequences are oriented in a "clockwise" fashion with respect to transcription direction. Plasmid pAL5cc is identical to pAL5 except that the *tk* sequences are oriented in a counterclockwise fashion. In plasmid pAL5i, the *tk* gene on the *Bam*HI fragment is oriented counterclockwise while the *tk* gene on the *Hin*dIII fragment is oriented clockwise.

The 2.3-kb *Hin*dIII fragment of the bacteriophage λ genome was inserted into the unique *Sca*I sites of pAL5 and pAL5cc to produce plasmids pAL5L and pAL5ccL, respectively.

Plasmid pTK2TK1-8 has been described (WALDMAN and LISKAY 1987) and is identical to pAL5 except that the 800-bp *Eco*RV-*Stu*I fragment of the herpes simplex virus type 2 (HSV-2) *tk* gene (KIT *et al.* 1983; SWAIN and GALLOWAY 1983) is inserted at the *Hin*dIII site. This fragment of the HSV-2 *tk* gene is missing the 30% of the coding region that maps upstream from the *Eco*RV site as well as the polyadenylation signals downstream from the *Stu*I site. The *tk* sequences on pTK2TK1-8 were flipped into a counterclockwise orientation to produce pTK2TK1-8cc. Plasmid pTK12 (WALDMAN and WALDMAN 1990) is identical to pTK2TK1-8 except that a wild-type (HSV-1) *tk* gene is contained on the 2.5-kb *Bam*HI fragment.

Plasmid pDY1 is identical to pAL5 except that the *neo* gene has a clockwise orientation. To construct pDY1, pAL5 was digested with *Bam*HI and *Nde*I. The 2.9-kb *Bam*HI-*Nde*I fragment containing the *neo* gene under the control of the SV40 early promoter/enhancer was isolated. The *Bam*HI end of the fragment was converted to an *Nde*I end by ligating the *Bam*HI end to a *Bam*HI/*Nde*I adaptor (5′GATCGTCATATGAC3′). The original *Nde*I end of the fragment was converted to a *Bam*HI end by filling-in the sticky end and subsequently attaching a 12-mer *Bam*HI linker (Boehringer Mannheim). The processed fragment was then digested with *Bam*HI plus *Nde*I and ligated to pAL5 that had been cleaved with *Bam*HI and *Nde*I to produce pDY1.

DNA preparation and Southern hybridization analysis:

Genomic DNA was prepared from cultured cells and analyzed by Southern hybridization using a ^{32}P-labeled probe specific for the HSV-1 *tk* sequence as described (WALDMAN and LISKAY 1987).

RESULTS

Experimental system: We study extrachromosomal homologous recombination between two defective HSV *tk* sequences introduced into mouse Ltk$^-$ cells by the calcium phosphate coprecipitation method. The number of colonies recovered after HAT selection is a measure of the frequency of extrachromosomal homologous recombination that reconstructs a functional *tk* gene (WALDMAN and LISKAY 1987). Formation of a colony ostensibly requires that the reconstructed gene integrate into the mouse genome since the introduced constructs appear incapable of autonomous replication. Reducing random integration of transfected DNA into the mouse genome in fact causes a proportional decrease in recovery of recombinant colonies (WALDMAN and WALDMAN 1990).

Based on earlier studies, we surmised that recombination events that we recover are primarily *intra*molecular rather than *inter*molecular events when we use a plasmid harboring two defective *tk* genes. This conclusion is based largely on the observation that when defective *tk* genes were placed on two different molecules so that intermolecular events were obligatory, the frequency of recombination events per microgram of construct was reduced about 10-fold (WALDMAN and LISKAY 1987). Characterization of our experimental system also revealed that the recovery of recombinants is a linear rather than second order function of the amount of plasmid DNA transfected, consistent with an intramolecular mechanism. Data illustrating this latter point are presented in Figure 3. The intramolecular nature of the recovered recombination events is pertinent to interpreting several of the experiments presented in this work.

Stimulatory and inhibitory effects of double-strand breaks on the frequency of extrachromosomal gene conversion-like events: We monitored extrachromosomal homologous recombination in mouse Ltk$^-$ cells using pAL5 and its derivatives (Figure 2). The construct pAL5 contains an HSV-1 *tk* gene mutated by an 8-bp *Xho*I linker insertion as well as a defective fragment of the HSV-1 *tk* coding region with 5′ and 3′ deletions. Because the defective *tk* fragment has truncations of both the 5′ and 3′ ends of the coding region, only gene conversions (or double crossovers) are recoverable with pAL5; a single crossover would always produce a nonfunctional *tk* gene with a 5′ or 3′ deletion. Reconstruction of a functional *tk* gene on pAL5 could in theory be accomplished by either *two* rounds of SSA (see LIN, SPERLE and STERNBERG 1990a,b) or a *single* execution of any of a number of conservative recombination mechanisms (reviewed in ORR-WEAVER and SZOSTAK 1985).

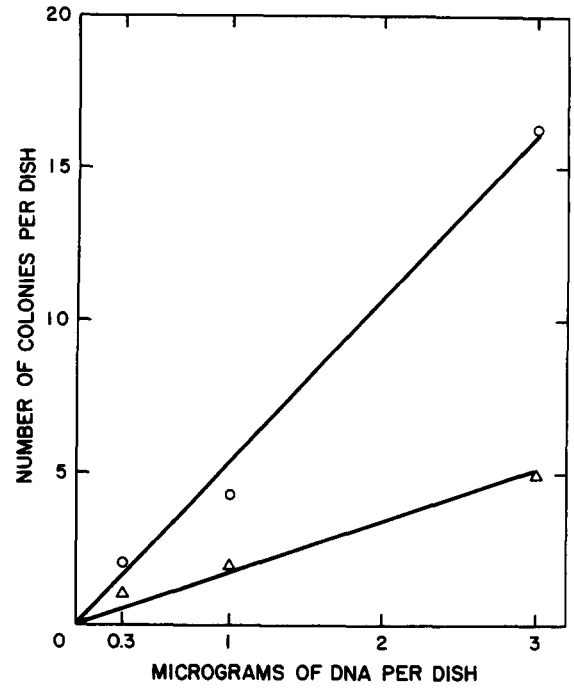

FIGURE 3.—Number of HAT^r colonies recovered is a linear function of the amount of DNA transfected. Mouse L cells (5×10^5 cells per 100 mm dish) were transfected with 0.3, 1.0 or 3.0 μg of uncut pAL5 (○) or pTK2TK1-8 (△). Sufficient carrier DNA was used to bring the total amount of DNA to 20 μg per dish. The number of HAT^r colonies were counted 14 days after selection was applied. Each point represents the average of four dishes.

In earlier work (WALDMAN and LISKAY 1987), we determined that recombination between the *tk* sequences on pAL5 could be stimulated 5–10-fold by placing a break at the *Xho*I linker insertion. Placing a break within homology allows the DNA termini, at least in theory, to participate directly in either SSA or DSBR and so it was not surprising that cleavage with *Xho*I prior to transfection had a stimulatory effect. To further examine the possible influence of double-strand breaks, with the hope of gaining insight into mechanism, we decided to determine what effect breaks in sequences outside of homology might have on recombination.

Interestingly, digestion of pAL5 with *Nde*I (see Figure 2) prior to transfection reproducibly had a mild stimulatory effect on extrachromosomal recombination while digestion with *Cla*I had a mild inhibitory effect (Table 1, lines 1–3). The recombination frequency for pAL5 cut with *Nde*I was nearly 4-fold that of pAL5 cut with *Cla*I. Transfections with pTK12, which contains a wild-type *tk* gene (Figure 2) was insensitive to cutting with either *Cla*I or *Nde*I (Table 1, lines 23–25), indicating that cutting of pAL5 with *Cla*I or *Nde*I influenced extrachromosomal recombination rather than random integration or expression of the reconstructed *tk* gene.

Similar results were obtained when we used pAL5cc

TABLE 1

Effect of double-strand breaks on extrachromosomal homologous recombination

Recombination substrate[a]	Number of independent experiments[b]	HAT^r colonies per flask[c]	Relative recombination frequency[d]
1. pAL5	8	18.9 ± 2.3	1.00
2. pAL5/*Nde*I	13	32.5 ± 1.9	1.72 (3.6)
3. pAL5/*Cla*I	3	9.0 ± 1.9	0.47
4. pAL5/*Nde*I + *Cla*I	2	3.5 ± 0.3	0.18
5. pAL5cc	2	12.0 ± 1.9	0.63
6. pAL5cc/*Nde*I	3	18.5 ± 2.2	0.98 (4.0)
7. pAL5cc/*Cla*I	2	4.6 ± 1.4	0.24
8. pAL5L	2	6.0 ± 0.6	0.32
9. pAL5L/*Nde*I	3	21.3 ± 1.9	1.12 (4.6)
10. pAL5L/*Cla*I	2	4.6 ± 0.5	0.24
11. pAL5ccL	3	7.8 ± 0.8	0.41
12. pAL5ccL/*Nde*I	3	14.7 ± 1.1	0.78 (5.9)
13. pAL5ccL/*Cla*I	2	2.5 ± 0.6	0.13
14. pAL5/*Stu*I	5	1.4 ± 0.3	0.07
15. pAL5/*Stu*I + *Nde*I	3	0.5 ± 0.4	0.03
16. pAL5/*Hpa*I	3	3.4 ± 1.3	0.18
17. pDY1	3	11.6 ± 1.0	0.61
18. pDY1/*Nde*I	5	22.0 ± 1.1	1.16 (5.2)
19. pDY1/*Cla*I	2	4.2 ± 0.6	0.22
20. pDY1/*Stu*I	2	0.5 ± 0.2	0.03
21. pAL5i	2	21.3 ± 5.4	1.13
22. pAL5i/*Nde*I	2	26.0 ± 4.6	1.38
23. pTK12	3	72.0 ± 10.3	
24. pTK12/*Nde*I	2	76.0 ± 4.2	
25. pTK12/*Cla*I	1	79.0 ± 7.0	
26. pTK12/*Stu*I	2	68.5 ± 2.3	

[a] Three micrograms of plasmid plus 17 μg of salmon sperm DNA were used per 75 cm^2 flask except only 50 ng of pTK12 (lines 23–26) were used per flask.

[b] Each independent experiment involved transfection of at least three flasks each containing 5×10^5 cells.

[c] Average ± the standard error of the mean for all flasks counted.

[d] Number of colonies per flask divided by number of colonies per flask obtained with pAL5 uncut. Numbers in parentheses indicate the recombination frequency for the particular plasmid cut with *Nde*I divided by recombination frequency for the plasmid cut with *Cla*I.

in which the defective *tk* sequences are oriented in a counterclockwise fashion (Figure 2). Again, about 4-fold higher recombination frequencies were obtained with pAL5cc cut with *Nde*I compared with pAL5cc cut with *Cla*I (Table 1, lines 5–7). The position of a double-strand break in relation to the orientation of the *tk* sequences and/or the *tk* promoter had no bearing on the effect of the break.

Effect of an introduced double-strand break is not due to the distance separating the *tk* genes: A series of experiments was performed to characterize the possible mechanisms by which restriction digestion might influence recombination frequency. Because the recovered recombination events were intramolecular (see above), we reasoned that cleavage with *Cla*I or *Nde*I might have influenced recombination frequencies by merely altering the amount of DNA that separated the *tk* sequences. When pAL5 is cleaved with *Cla*I, the homologous nucleotides within the *tk* sequences are separated by about 6.2 kb; when pAL5 is cut with *Nde*I, the *tk* sequences are separated by only 3.3 kb. The shorter distance between the *tk* genes following *Nde*I digestion might have enhanced recombination.

To address this issue, plasmids pAL5L and pAL5ccL were constructed (Figure 2). In these plasmids, a 2.3-kb fragment of the phage λ genome was inserted to increase the distance between the *tk* genes to about 5.6 kb when either plasmid is cleaved with *Nde*I. As shown in Table 1 (lines 8–13), the effect of cutting pAL5L or pAL5ccL with *Cla*I or *Nde*I was nearly the same as in experiments using pAL5 or pAL5cc (Table 1, lines 1–7). These results indicated that the effect of cleavage with *Cla*I or *Nde*I was not related to the resulting distance between the *tk* sequences.

The nature of the DNA ends does not determine recombination frequency: We considered the possibility that the nature of the DNA ends at an introduced break was important in determining recombination frequency. The nature of DNA termini might affect the loading of recombination proteins or the accessibility of the DNA ends to a nuclease, for example. Digestion with either *Cla*I or *Nde*I leaves sticky ends, 2 bp in length and of the same polarity, and yet these digestions exerted different influences on recombination frequency. The nature of ends therefore did not appear to be of paramount importance. As a further test of the importance of the nature of DNA termini, plasmid pAL5 was cleaved with *Nde*I and the sticky ends were filled-in by treatment with the Klenow fragment and deoxyribonucleotides. Such treatment of *Nde*I ends had no effect on recombination frequencies compared to DNA molecules with untreated *Nde*I ends (data not shown).

We were additionally concerned that certain restriction enzymes may remain bound to the ends of our DNA molecules after cleavage (GUSEW, NEPVEU and CHARTRAND 1987) and that these DNA-bound proteins may have influenced our results. Phenol extraction of recombination substrates prior to transfection to remove any residual restriction enzymes had no effect on the outcome of experiments (data not shown).

Recombination appears to occur prior to substantial processing of DNA termini: In both the SSA and DSBR models, double-strand breaks serve as sites for strand degradation. It was quite unlikely that double-strand gap formation from the cut sites was involved in productive recombination since a gap originating at the *Cla*I or *Nde*I site would not be flanked by homology and would therefore not be a suitable substrate for DSBR. As a means of assessing how much *strand-specific* degradation (as in SSA) might have occurred from an introduced break, we constructed

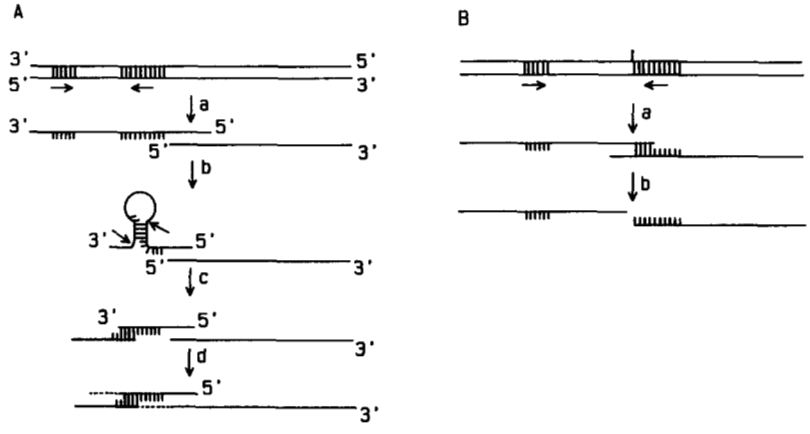

FIGURE 4.—Intrachromosomal recombination between inverted repeats. Homologous sequences are depicted as ▥ in this simplified drawing. (A) Productive recombination by (one or more rounds of) SSA involves the following steps: (a) single-strand degradation to expose complementary sequences on the same strand, (b) annealing of complementary sequences to form a stem-loop structure, (c) cleavage of unpaired single strands, and (d) completion of round of SSA by gap repair or replication. (B) Representation of pAL5i linearized with *Nde*I. Homologous sequences on pAL5i are more centrally located than in the theoretical substrate at left. The midpoint of pAL5i sequences is indicated on top strand. (a and b) Bidirectional degradation of single strands from the *Nde*I site converges at midpoint of pAL5i (assuming equal rates of degradation in both directions). Complementary sequences on a single strand are destroyed before stem-loop can form, precluding the production of a functional *tk* gene.

pAL5i (Figure 2) in which the *tk* genes reside as inverted repeats. If degradation of single strands in both directions from break sites occurred with high efficiency, then the recovery of a functional product by rounds of SSA should have been precluded when pAL5i was cleaved with *Nde*I. The complementary *tk* sequences, which are on the same DNA strand of pAL5i, would be destroyed before they could anneal (see Figure 4) and continued degradation of strands would likely totally destroy the *tk* sequences. Recombinants were in fact recovered as efficiently with pAL5i (Table 1, lines 21 and 22) as with pAL5 (Table 1, lines 1 and 2). Results with pAL5i therefore indicated that recombination likely occurred prior to substantial bidirectional strand degradation from the break site.

Double-strand breaks, transcription and recombination: As illustrated in Figure 5, we noted that linearization of the recombination substrates with *Nde*I places both *tk* sequences downstream from the SV40 early promoter/enhancer which drives transcription of the *neo* gene. In contrast, when the constructs are linearized with *Cla*I the SV40 promoter/enhancer is positioned between the *tk* genes. By analogy to studies done in yeast (KEIL and ROEDER 1984; VOELKEL-MEMAN, KEIL and ROEDER 1987; THOMAS and ROTHSTEIN 1989) and mammalian (NICKOLOFF and REYNOLDS 1990) systems we wondered if transcription from the SV40 promoter/enhancer might stimulate recombination between downstream *tk* sequences, perhaps by read-through transcription.

We digested pAL5 with *Stu*I which cleaves 556 bp from the *Nde*I site but 65 bp downstream from the TATA box of the SV40 promoter (Figure 2). Cleavage with *Stu*I places a break between the SV40 promoter and the *tk* genes. As shown in Table 1 (line 14), *Stu*I cleavage severely reduced recombination within pAL5. Recovery of HAT^r colonies using a construct with a wild type *tk* gene was not effected by *Stu*I digestion (Table 1, compare lines 23 and 26). Digestion of pAL5 with both *Stu*I and *Nde*I also resulted in a low recovery of recombinants, indicating the effect of *Stu*I digestion was "dominant" to *Nde*I digestion (Table 1, line 15). Digestion of pAL5 with *Hpa*I, which also places a break between the SV40 promoter and the *tk* genes (Figure 2), reduced colony recovery in relation to cleavage with *Nde*I (Table 1, line 16).

We next studied recombination using pDY1 in which the entire *neo* transcription unit was oriented in a clockwise fashion (Figure 2). If read-through transcription from the SV40 promoter upstream from the *tk* sequences was important in recombination, we expected that cleavage of pDY1 with *Nde*I, which places a break between the SV40 promoter and the *tk* genes, would have an inhibitory effect on recombination. As shown in Table 1 (lines 17–20), inverting the *neo* gene had no effect on recombination frequency and the relative effects of cleavage with *Cla*I and *Nde*I were unchanged. This indicated that read-through transcription from the SV40 promoter was not influential in recombination between the *tk* sequences. However, when pDY1 was cleaved with *Stu*I (Table 1, line 20), recombination was once again greatly reduced, suggesting that a sequence important for recombination was located at or near the *Stu*I site.

Southern analysis of recombinant *tk* genes: To ascertain that HAT^r colonies that arose following transfection of cells with recombination substrates contained reconstructed HSV-1 *tk* genes, DNA samples were isolated from several HAT^r colonies and subject to Southern analysis. As displayed in the Southern blot in Figure 6, hybridization with an HSV-1 *tk*-specific probe revealed that all putative recombinants contained HSV-1 *tk* sequences and most exhibited a 2.5-kb *Bam*HI fragment that was resistant to cleavage by *Xho*I. Such a fragment is predicted if the

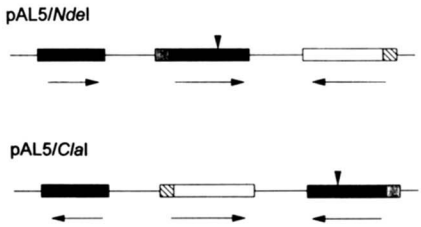

FIGURE 5.—Position of the *neo* transcription unit relative to *tk* sequences in linearized pAL5-derived constructs. Cleavage of a recombination substrate with *Nde*I places both *tk* sequences downstream from the SV40 promoter/enhancer (▨) controlling the *neo* gene (□). *Cla*I digestion positions the SV40 promoter/enhancer between the *tk* sequences. The truncated and insertion mutant *tk* genes are denoted as ■ and ■ with ▼, respectively. Shaded box linked to the insertion mutant *tk* gene represents the *tk* promoter. Direction of transcription is indicated by arrows.

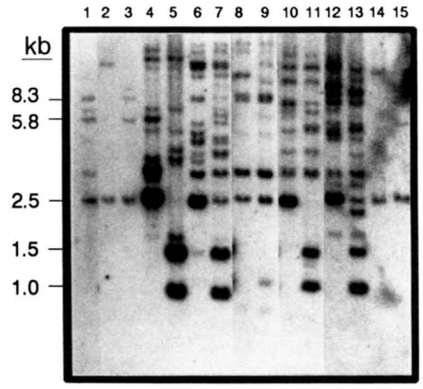

FIGURE 6.—Southern analysis of representative *tk* genes produced by extrachromosomal recombination. DNA was isolated from putative recombinants and hybridized with a probe specific for HSV-1 *tk* sequences. Lane 1 contains molecular weight markers. Lanes 2–15 show the analysis of DNA isolated from 7 HAT^r colonies. Samples in even numbered lanes were cleaved with *Bam*HI, samples in odd numbered lanes were cleaved with *Bam*HI plus *Xho*I. Colonies arose following transfection of cells with pDY1 cut with *Nde*I (lanes 2–5), pAL5i cut with *Nde*I (lanes 6 and 7), pAL5 cut with *Nde*I (lanes 8–11), pAL5 uncut (lanes 12–15). Most samples displayed a 2.5-kb *Bam*HI fragment that was resistant to *Xho*I cleavage, indicative of a gene conversion correcting the *Xho*I linker insertion mutant *tk* gene. Some samples displayed 1.5- and 1.0-kb bands upon cleavage with *Bam*HI plus *Xho*I, indicative of copies of uncorrected linker insertion mutant *tk* genes.

*Xho*I linker insertion mutant *tk* gene on the recombination substrate had been corrected by gene conversion or a double crossover. Samples lacking such a fragment (*e.g.*, Figure 6, lanes 5 and 11) probably lost one of the *Bam*HI sites flanking a corrected *tk* gene. This simple analysis ruled out the unlikely possibility that HAT^r colonies were merely mouse L cell variants that survived an ineffective selection regimen and strongly suggested that the colonies arose from homologous recombination events. A more detailed analysis was not feasible because of the multiple bands in most samples and because of the lack of restriction site differences between the recombining sequences that could be used as markers.

Stimulatory and inhibitory effects of double-strand breaks on the frequency of recombination between imperfectly matched *tk* sequences: We were curious as to whether cleavage with *Cla*I or *Nde*I would have similar effects on extrachromosomal *homeologous* recombination, that is, recombination between imperfectly matched sequences. We previously constructed (WALDMAN and LISKAY 1987) plasmid pTK2TK1-8 (Figure 2) containing a *Xho*I linker insertion mutant HSV-1 *tk* gene as well as a defective fragment of the HSV-2 *tk* gene. The defective fragment had a 5′ deletion of *tk* coding sequence and *tk* promoter as well as a 3′ deletion of polyadenylylation signals. Both gene conversions and single crossovers are recoverable using pTK2TK1-8. [The construct pTK2TK1-8 was originally designed for recovery of gene conversions only, but the deletion of the polyadenylylation sequences from the HSV-2 *tk* fragment was found to be unimportant for *tk* expression (WALDMAN and LISKAY 1987; A. WALDMAN, unpublished results). The HSV-2 *tk* fragment therefore functionally displayed only a 5′ deletion and the recovery of productive single crossovers was an unintended result.] We previously noted that the placement of a double-strand break at the site of the *Xho*I linker in pTK2TK1–8 stimulated recombination about 5–10-fold, similar to observations made for pAL5 (WALDMAN and LISKAY 1987).

Recombination frequencies for uncut pTK2TK1-8 were about 10-fold lower than frequencies for uncut pAL5 (compare Table 2, line 1, with Table 1, line 1). Digestion of pTK2TK1-8 with *Nde*I prior to transfection resulted in a 30-fold higher recombination frequency compared with the frequency measured when pTK2TK1-8 was cut with *Cla*I (Table 2, lines 2 and 3). Digestion with *Sca*I, which cuts near the site of *Cla*I cleavage (Figure 2), had an effect similar to *Cla*I

TABLE 2

Effect of double-strand breaks on extrachromosomal homeologous recombination

Recombination substrate[a]	Number of independent experiments[b]	HAT^r colonies per flask[c]	Relative recombination frequency[d]
1. pTK2TK1-8	3	1.5 ± 0.3	1.00
2. pTK2TK1-8/*Nde*I	3	19.0 ± 2.3	12.67 (31.7)
3. pTK2TK1-8/*Cla*I	3	0.6 ± 0.2	0.40
4. pTK2TK1-8cc	2	0.8 ± 0.3	0.53
5. pTK2TK1-8cc/*Nde*I	3	2.5 ± 0.3	1.67 (6.2)
6. pTK2TK1-8cc/*Cla*I	3	0.4 ± 0.2	0.27

[a] Three micrograms of plasmid plus 17 μg of salmon sperm DNA carrier were used per 75-cm^2 flask.

[b] Each independent experiment involved transfection of at least three flasks each containing 5×10^5 cells.

[c] Average ± the standard error of the mean for all flasks counted.

[d] Number of colonies per flask divided by number of colonies per flask obtained with pTK2TK1-8 uncut. Numbers in parentheses indicate the recombination frequency for the particular plasmid cut with *Nde*I divided by recombination frequency for the plasmid cut with *Cla*I.

(data not shown). These experiments indicated that digestion with *Nde*I and *Cla*I had qualitatively similar effects for both homeologous and homologous extrachromosomal recombination but that the effects were more dramatic for homeologous recombination.

Experiments using pTK2TK1-8cc (Figure 2) in which the *tk* genes were oriented in a counterclockwise fashion indicated that digestion with *Nde*I was again stimulatory relative to digestion with *Cla*I (Table 2, lines 5 and 6). Because of the restriction site differences between the HSV-1 and the HSV-2 *tk* genes, recombinants obtained with pTK2TK1-8 are more amenable to mapping than recombinants obtained with pAL5. In previous work (WALDMAN and LISKAY 1987) we had analyzed several recombinants arising from pTK2TK1-8. We previously deduced restriction maps expected for gene conversions of varying conversion tract length as well as single crossovers displaying a variety of crossover points.

DISCUSSION

In this paper, we have reported on extrachromosomal homologous recombination in mammalian cells using several substrates in which single crossovers were not recoverable. We examined recombination using circular substrates or substrates broken at sites within vector sequences. We asked whether the recovered events were products of two rounds of SSA or instead were products of a different mechanism. As discussed below, our data indicate that the recovered events were not produced by multiple rounds of SSA. Additional experiments involving recombination between imperfectly matched sequences corroborated this conclusion.

Our experiments are interpreted in terms of intramolecular recombination events. Our inference that the recovered events are intramolecular is based on two observations: (i) obligatory intermolecular events occurred at a frequency 10-fold lower than that recorded with pAL5 (or pTK2TK1-8) (WALDMAN and LISKAY 1987) and (ii) the number of recombinants recovered with pAL5 or pTK2TK1-8 is a linear function of the amount of construct transfected (see Figure 3). We cannot rigorously rule out the possibility that recombination involves multiple steps, some of which may involve intermolecular interactions. However, using the best of our ability to make measurements in our system, we surmise that at least the rate-limiting step is intramolecular. Therefore, it seems likely that double-strand breaks influence recombination frequency in our system by influencing an intramolecular interaction.

Our work deals primarily with the effects of double-strand breaks on recombination frequency. We can imagine two very general modes by which a double-strand break in a transfected molecule may influence extrachromosomal recombination. A break may act *directly* by providing a terminus needed for strand invasion, as in conservative recombination models (see ORR-WEAVER and SZOSTAK 1985), or by serving as an initiation site for strand degradation (LIN, SPERLE and STERNBERG 1984) or unwinding (WAKE, VERNALEONE and WILSON 1985) in nonconservative recombination. Alternatively, a break may act *indirectly* by, for example, altering the binding of a recombination protein to the substrate.

The SSA model was founded on the observation that appropriately placed breaks can significantly stimulate or inhibit extrachromosomal recombination by a *direct* mechanism by serving as a start site for degradation or unwinding. We may infer that a rate-limiting step of SSA for a circular substrate is the random placement (by cellular machinery) of a break at a location appropriate for a productive outcome. Our determinations of the effects of double-strand breaks within vector sequences of pAL5-related constructs are not easily interpretable by such a paradigm. Experiments using pAL5, pAL5cc, pAL5L, or pAL5Lcc (Table 1, lines 1–13) revealed that digestion with *Nde*I had a mild stimulatory effect while digestion with *Cla*I had a mild inhibitory effect on recombination. There was no relationship between the effects of the breaks and either the distance separating the recombining *tk* sequences or the distance between a break and any particular *tk* sequence, including the *tk* promoter. Had the breaks acted directly as inititation sites for strand degradation or unwinding, a distance dependency might have been expected. A dependency of recombination frequency on the distance between a break and the recombining sequences was observed in the development of the SSA model (LIN, SPERLE and STERNBERG 1984, 1990a,b).

Using constructs similar to pAL5, others have found it necessary to place a break at the insertion mutation in order to recover any recombinants (LIN, SPERLE and STERNBERG 1990a,b). The break internal to the *tk* sequence was viewed as providing DNA termini needed for two rounds of SSA to produce two crossovers flanking the mutation site. Breaks at both ends of the defective internal *tk* fragment (equivalent to breaks at the *Hin*dIII sites of pAL5) further stimulated SSA by presumably generating single-stranded tails complementary to those generated at the break at the insertion mutation site. The fact that we can measure recombination in the absence of an introduced break (Table 1, line 1) suggests that either recombination is initiated in our cells in the absence of strand breakage or that our cells make breaks in the DNA constructs prior to recombination. The cells presumably do not extensively break transfected molecules prior to recombination since wholesale breakage would make the effects of introduced breaks

indiscernible. Additionally, cleavage of pAL5 with both *Nde*I and *Cla*I prior to transfection reduced colony yield about 10-fold relative to cutting with *Nde*I alone and about 3-fold relative to cutting with *Cla*I alone (Table 1, compare lines 2, 3 and 4). These results indicate that the transfected DNA is not usually broken by the cells into two or more pieces prior to recombination.

According to the SSA scheme, bidirectional degradation from a break site positioned like the *Nde*I site or the *Cla*I site on pAL5 should not be helpful since either break by itself should initiate a nonproductive single crossover. Therefore, had bidirectional degradation from break sites been efficient, cleavage of pAL5 with either *Nde*I or *Cla*I should have presumably yielded very few or no recombinants. This was not observed; *Nde*I digestion was in fact mildly stimulatory.

Efficient recombination between inverted repeats on pAL5i cut with *Nde*I (Table 1, lines 21 and 22) provided additional evidence that introduced breaks in fact did not serve as inititation sites for significant bidirectional strand degradation on molecules that yielded recombinant *tk* genes. Strand-specific degradation in both directions initiating at the *Nde*I site and proceeding into *tk* sequences would have destroyed complementary *tk* sequences on the same strand (Figure 4) which leads to the prediction that pAL5i cut with *Nde*I should have displayed a very low colony yield. This prediction was not confirmed. This is not to say that strand degradation from the *Nde*I (or *Cla*I) site did not occur. The results obtained with pAL5i indicate that recombination events occurred before much bidirectional degradation occurred. (This particular experiment does not rule out the possibility that DNA strands were unwound starting from the break site.)

An argument may be constructed that degradation from the *Nde*I break site in pAL5i might proceed much more rapidly in one direction compared to the other, thereby allowing a stem-loop structure as depicted in Figure 4A to form. Multiple rounds of such a process might enable the recovery of recombinants. If this were the case, however, then recovery of recombinants using pAL5 cut with *Nde*I should have been very inefficient since similar asymmetric strand degradation would destroy complementary *tk* sequences on the complementary strands of pAL5 before annealing. The main point is that the SSA paradigm predicts that, all else being equal, inverted repeats should exhibit a substantially different recombination frequency than direct repeats when strand degradation is initiated from a common site on the vector. This prediction was not corroborated in our experiments.

It is not feasible that the formation of a double-strand gap starting from *Cla*I or *Nde*I should lead to productive DSBR since DSBR requires that the gap be flanked by homology. It also seems unlikely that the breaks were initiation sites for a single-strand invasion mechanism since the breaks were placed outside of the recombining sequences. We conclude that the breaks did not act *directly* in recombination.

Perhaps the influence of digestion with *Nde*I and *Cla*I reflected *indirect* effects on SSA. How might such effects be mediated? Perhaps the introduced breaks influenced the rate of formation of secondary breaks which then served as initiation sites for efficient strand degradation or unwinding and subsequent SSA. We do not find this scenario plausible because of a series of additional observations, discussed below, that do not appear compatible with SSA.

Restriction enzyme cleavage seemed to have a qualitatively similar yet more pronounced influence on recombination between mismatched sequences compared with recombination between homologous sequences. We recorded a 30-fold difference in recombination between pTK2TK1-8 cut with *Cla*I and pTK2TK1-8 cut with *Nde*I (Table 2, lines 2 and 3) *vs.* only a 3.6-fold difference for pAL5 cut with *Cla*I and pAL5 cut with *Nde*I (Table 1, lines 2 and 3). It therefore appears that the degree of homology between the recombining sequences played a role in mediating the effects of the introduced breaks. It is difficult to imagine how the degree of homology could alter the influence an introduced break would have on the formation of a second break site for initiation of strand degradation and SSA. Homology recognition does not come into play in the SSA pathway prior to the annealing step.

In experiments using pTK2TK1-8, both gene conversions and single crossovers were recoverable. This means that pTK2TK1-8 could in theory produce HAT^r colonies through a single round of SSA. The near-equal frequency of recovery of conversions and crossovers recovered with pTK2TK1-8 uncut or cut with *Xho*I (WALDMAN and LISKAY 1987) appears inconsistent with SSA but is consistent with conservative mechanisms.

Because single crossovers were recoverable with pTK2TK1-8, the respective stimulatory and inhibitory effects of *Nde*I and *Cla*I cleavage of pTK1TK2-8 could, at least in principle, be reconciled with the SSA model since degradation from only the *Nde*I site yields a productive crossover (see Figure 7). However, the SSA model makes the strong prediction that the relative effects of the breaks should be reversed if the orientations of the *tk* genes were to be inverted since degradation from the *Nde*I site would then lead to destruction of the *tk* promoter (Figure 7). When the *tk* genes were inverted in pTK2TK1-8cc, the stimulatory effect of cleavage with *Nde*I was in fact dimin-

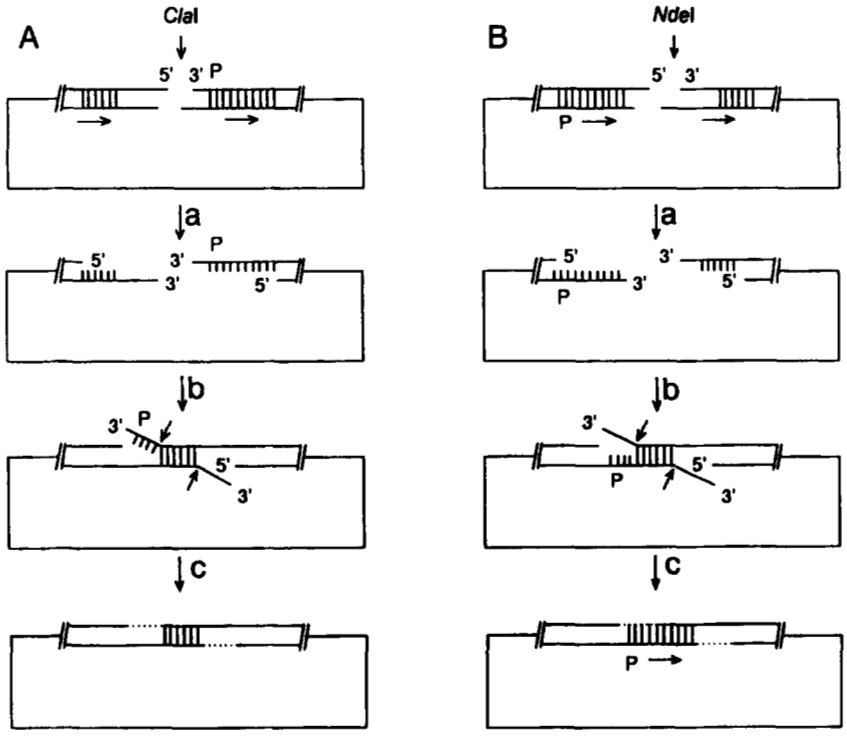

FIGURE 7.—Recombination between *tk* sequences within pTK2TK1-8 according to the SSA model. Shown is a simplified schematic diagram of pTK2TK1-8 (see Figure 2) cleaved either at the *Cla*I site (panel A) or at the *Nde*I site (panel B). The *tk* sequences are depicted as ▥. One *tk* sequence on pTK2TK1-8 has a promoter, designated "P," the other lacks a promoter due to a 5′ deletion. Major steps of the SSA model are illustrated and are as described in the legend to Figure 1. Initiation of SSA from the *Cla*I site leads to a promoterless, nonfunctional *tk* sequence whereas initiation of SSA from the *Nde*I site leads to a functional *tk* gene.

ished (see Table 2, lines 4–6). Significantly, however, *Nde*I cleavage was still stimulatory relative to *Cla*I cleavage. These experiments provide strong evidence that recombination on pTK2TK1-8 did not occur predominantly by SSA.

We previously reported that when pTK2TK1-8 was placed into the genome of L cells the rate of intrachromosomal recombination between the HSV-1 and HSV-2 *tk* sequences was reduced more than 1000-fold compared to the intrachromosomal rate of recombination between two HSV-1 *tk* sequences placed into the genome on pAL5 (WALDMAN and LISKAY 1987, 1988). In contrast, the extrachromosomal recombination frequency for pTK2TK1-8 (uncut or *Xho*I cut) was only about 10-fold lower than the corresponding frequency for pAL5 (this study; WALDMAN and LISKAY 1987). We suggested (WALDMAN and LISKAY 1987) that this differential sensitivity to heterology may reflect a mechanistic difference between intra- *vs.* extrachromosomal recombination. Based on our reading of literature on extrachromosomal recombination in mammalian cells (ANDERSON and ELIASON 1986; CHAKRABARTI and SEIDMAN 1986; LIN, SPERLE and STERNBERG 1984, 1990a,b; SEIDMAN 1987), we recently entertained the notion that extrachromosomal recombination in mammalian cells is accomplished exclusively via SSA while intrachromosomal recombination is accomplished by a conservative mechanism with a stringent homology search. One may imagine that annealing of complementary DNA strands during SSA might not be terribly stringent. Our current work shows, however, that extrachromosomal recombination of *tk* sequences within pTK2TK1-8 is often not accomplished by SSA. This leads us to consider the possibility that extrachromosomal and intrachromosomal recombination might both be accomplished by fundamentally similar mechanisms involving identical or overlapping sets of gene products as players. The recombination rate or frequency we measure may be determined by a complex interplay between the degree of homology of the recombining sequences, the positioning of other sequences near the recombining sequences, as well as the nature of the substrate (chromatin *vs.* naked DNA).

Our previous intrachromosomal studies have always involved cutting a construct with *Cla*I prior to transfection so the substrate would integrate using its *Cla*I termini. Based on the work presented here, we were motivated to make cell lines containing pAL5 or pTK2TK1-8 integrated into the L cell genome through *Nde*I termini. Work in progress indicates that the enhancement of recombination observed extrachromosomally for constructs cut with *Nde*I is mirrored in intrachromosomal recombination (data not shown). This result provides further support for the notion that a similar recombination machinery may act both intra- and extrachromosomally. This also suggests that the particular linear arrangement of sequences on our constructs, rather than DNA termini themselves, is responsible for influencing recombination rate since a construct has no termini once it integrates into the genome.

Precisely how did the introduction of breaks into

recombination constructs influence recombination in our system? A detailed answer to this question remains to be determined, but experiments involving cleavage of constructs with *Stu*I may reveal a starting point for determining an underlying mechanism. Digestion of pAL5 constructs with *Stu*I, 65 bp downstream from the SV40 TATA box, reduced recombination over 10-fold relative to uncut substrate and this inhibitory effect was dominant to the stimulatory effect of a nearby break at the *Nde*I site (Table 1, lines 14 and 15). Experiments with plasmid pDY1 (Table 1, lines 17–20) ruled out the possibility that breaks served as blockades against read-through transcription from the SV40 promoter and suggested that a sequence important for recombination maps at or close to the *Stu*I site. It is possible that binding of a transcription factor(s) to the SV40 promoter region played a role in synapsis of *tk* sequences and that *Stu*I digestion interfered with such binding by disrupting the binding site. [Evidence for transcription-independent enhancement of recombination by binding of transcription factors has been described by others studying recombination in mammalian cells (SHENKAR, SHEN and ARNHEIM 1991) and yeast (SCHULTES and SZOSTAK 1991).] Furthermore, it is conceivable that binding of the factor to one side of the recombining *tk* sequences, such as in constructs linearized with *Nde*I, stimulated recombination to a greater extent than binding between the *tk* sequences, such as after *Cla*I digestion. The precise orientation or distance of the binding site from the *tk* sequences may be irrelevant (a "recombinational enhancer"). This possibility warrants further investigation; studies of recombination occurring in the absence of SV40 sequences are underway.

In summation, we offer the following sketch for how double-strand breaks influenced recombination in our studies. Homologous pairing of *tk* sequences in DNA duplexes is an early step in recombination, possibly occurring prior to strand breakage. The breaks introduced by restriction digestion influenced the step of duplex pairing/synapsis indirectly by affecting the manner in which recombination proteins (or a transcription factor) interacted with the recombination substrates. These effects are due to DNA topology and/or disruption of specific binding sequences. Paired synaptic complexes formed between mismatched *tk* sequences may be intrinsically less stable than complexes between homologous sequences. Therefore, if a break at the *Cla*I site destabilizes (or if a break at the *Nde*I site stabilizes) paired complexes, it would not be entirely unexpected that recombination between mismatched *tk* sequences would be most effected by the breaks.

Although our above scenario might seem "ad hoc," three points that we want to stress are: (i) the introduced breaks affected recombination *indirectly*, (ii) our collective data are inconsistent with SSA and (iii) our data are not inconsistent with any recombination model that invokes a protein-driven homology search and synapsis of duplexes as early steps. Therefore, our data are consistent with either the HOLLIDAY (1964), MESELSON and RADDING (1975) or DSBR (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; SZOSTAK *et al.* 1983) models, among others. Although we did not (and cannot) determine whether the events we recovered are conservative or nonconservative (that is, we do not know if sequences were lost during recombination), the execution of an homology search among transfected duplexes would obviate the "need" to conduct extrachromosomal recombination in a nonconservative fashion.

Apparent discrepencies between our work and work supporting the SSA model in mammalian cells might arise from the use of different cell types or subclones of cell lines, differences in culture conditions or transfection techniques, or the use of different vectors containing sequences that influence recombination. We routinely use over 30-fold more plasmid DNA in our transfections of mouse L cells than do LIN, SPERLE and STERNBERG (1990a,b) and yet we recover smaller numbers of colonies than those investigators. The amount of DNA that we use is dictated by the number of colonies we recover. We work with an amount of DNA in the range where colony number is sensitive to the amount of DNA used, that is, the DNA is not saturating (see Figure 3). If we used much lower amounts of DNA, we would have difficulty obtaining colonies. It is not clear why we must use more plasmid DNA than the other investigators. Perhaps our cells are less efficiently transfected. Alternatively, it may be argued that out low colony recovery reflects a great inefficiency of non-SSA processes that we are selectively recovering with the pAL5-related substrates. In recent studies we transfected mouse L cells with a substrate that is identical to pAL5 except that it contains two insertion mutant *tk* genes, allowing for the recovery of single crossovers (SSA events). With such a substrate, we measured only about a 4-fold increase in extrachromosomal recombination (data not shown). Preliminary Southern analysis has revealed that at least 3 out of 21 recombinants examined to date appear to have arisen from gene conversions or double crossovers (not shown). These data suggest that non-SSA events might in fact make up a significant fraction of the total number of extrachromosmal recombination events occurring in our system.

We do not wish to imply that the SSA mechanism is not a legitimate model for extrachromosomal recombination in mammalian cells. A body of literature (ANDERSON and ELIASON 1986; CHAKRABARTI and SEIDMAN 1986; LIN, SPERLE and STERNBERG 1984, 1990a,b; SEIDMAN 1987) suggests that SSA is a viable

mechanism for extrachromosomal recombination when a mammalian cell is presented with a variety of substrates. Our work adds to a body of evidence (BRENNER, SMIGOCKI and CAMERINI-OTERO 1985, 1986; SONG *et al.*, 1985) that SSA is not the exclusive mechanism by which transfected DNA can undergo extrachromosomal recombination in mammalian cells. Multiple extrachromosomal recombination pathways may exist and the pathway used may be the one kinetically favored for the particular substrate. (SSA may even occur among chromosomal sequences but at greatly reduced efficiency because of the faster kinetics of competing processes.) Strategic positioning of double-strand breaks and/or special sequences may reveal different extrachromosomal recombination mechanisms by altering the relative kinetics of product formation by competing pathways. NUSSBAUM, SHALIT and COHEN (1992) recently reported, for example, that alternate recombination mechanisms are revealed in *Escherichia coli* when breaks are placed in different regions of recombination substrates. In agreement with our conclusions in principle, these authors argued that breaks placed within homologous sequences affected recombination *directly* whereas breaks placed outside the homology influenced recombination *indirectly*.

It was recently reported (FISHMAN-LOBELL, RUDIN and HABER 1992) that the speed of strand-specific degradation from break sites limits the rate of SSA in yeast and that placing a break suitably far from homologous sequences allows recovery of DSBR in favor of SSA. Similarly, in our system a slow rate of strand degradation from the break site may cause SSA to be overshadowed by a competing alternate mechanism. In any event, we feel that our work collectively provides strong evidence for the existence of a mechanism for extrachromosomal homologous recombination in mammalian cells that is distinct from SSA. This finding in turn suggests the possibility that recombination in mammalian cells can be accomplished inside or outside of the genome by overlapping pathways.

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