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)
models in at least four important ways. (i) There is no synopsis 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 (Chakraborti 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 Eliasen 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% CO₂.

**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 × 10⁵ cells per 75-cm² 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
are oriented in a counterclockwise fashion. In plasmid 3' ends of the coding region of the pAL5cc HincII-SmaI fragment of the HSV-1 pAL5i, the is oriented clockwise. A 2.5-kb BamHI fragment inserted into the unique BamHI site on the vector. Plasmid pAL5 also contains a 1.2-kb linker inserted at nucleotide 1215 of the HSV-1 tk gene, numbering according to Wagner, Sharp and Sum-

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 XhoI 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 BamHI fragment inserted into the unique BamHI site on the vector. Plasmid pAL5 also contains a 1.2-kb HincII-Smal fragment of the HSV-1 tk gene inserted into the HindIII site on the vector, after attachment of HindIII 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 BamHI fragment is oriented counterclockwise while the tk gene on the HindIII fragment is oriented clockwise. The 2.3-kb HindIII fragment of the bacteriophage λ genome was inserted into the unique SalI 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 EcoRV-StuI fragment of the herpes simplex virus type 2 (HSV-2) tk gene (Kt et al. 1983; Swain and Galloway 1983) is inserted at the HindIII site. This fragment of the HSV-2 tk gene is missing the 30% of the coding region that maps upstream from the EcoRV site as well as the polyadenylation signals downstream from the StuI 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 BamHI fragment. Plasmid pDY1 is identical to pAL5 except that the neo gene has a clockwise orientation. To construct pDY1, pAL5 was digested with BamHI and NdeI. The 2.9-kb BamHI-NdeI fragment containing the neo gene under the control of the SV40 early promoter/enhancer was isolated. The BamHI end of the fragment was converted to an NdeI end by ligating the BamHI end to a BamHI/NdeI adaptor (5'GATCGGTCAATGAC3'). The original NdeI end of the fragment was converted to a BamHI end by filling-in the sticky end and subsequently attaching a 12-mer BamHI linker (Boehringer Mannheim). The processed fragment was then digested with BamHI plus NdeI and ligated to pAL5 that had been cleaved with BamHI and NdeI to produce pDY1. DNA preparation and Southern hybridization analysis:

**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 (III). An HSV-1 tk gene (■) mutated by a XhoI linker insertion ("X") is contained within a BamHI (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 HindIII (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 Clal (C), NdeI (N), SalI (S), SalI (Sc), and Hpal (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.
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 intramolecular rather than intermolecular 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 XhoI 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).

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

Similar results were obtained when we used pAL5cc
sequences are separated by about 6.2 kb; when pAL5 is cut with NdeI, the tk sequences are separated by only 3.3 kb. The shorter distance between the tk genes following NdeI 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 NdeI. As shown in Table 1 (lines 8–13), the effect of cutting pAL5L or pAL5ccL with Clal or NdeI 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 Clal or NdeI 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 Clal or NdeI 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 NdeI and the sticky ends were filled-in by treatment with the Klenow fragment and deoxyribonucleotides. Such treatment of NdeI ends had no effect on recombination frequencies compared to DNA molecules with untreated NdeI 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 recombinant substrates prior to transformation 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 Clal or NdeI 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

Table 1

<table>
<thead>
<tr>
<th>Recombination substrate</th>
<th>Number of independent experiments</th>
<th>HAT* colonies per flask</th>
<th>Relative recombination frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pAL5</td>
<td>8</td>
<td>18.9 ± 2.3</td>
<td>1.00</td>
</tr>
<tr>
<td>2. pAL5/NdeI</td>
<td>13</td>
<td>32.5 ± 1.9</td>
<td>1.72 (3.6)</td>
</tr>
<tr>
<td>3. pAL5/Clal</td>
<td>3</td>
<td>9.0 ± 1.9</td>
<td>0.47</td>
</tr>
<tr>
<td>4. pAL5/NdeI + Clal</td>
<td>2</td>
<td>3.5 ± 0.3</td>
<td>0.18</td>
</tr>
<tr>
<td>5. pAL5cc</td>
<td>2</td>
<td>12.0 ± 1.9</td>
<td>0.63</td>
</tr>
<tr>
<td>6. pAL5cc/NdeI</td>
<td>3</td>
<td>18.5 ± 2.2</td>
<td>0.98 (4.0)</td>
</tr>
<tr>
<td>7. pAL5cc/Clal</td>
<td>2</td>
<td>4.6 ± 1.4</td>
<td>0.24</td>
</tr>
<tr>
<td>8. pAL3L</td>
<td>2</td>
<td>6.0 ± 0.6</td>
<td>0.82</td>
</tr>
<tr>
<td>9. pAL5L/NdeI</td>
<td>3</td>
<td>21.3 ± 1.9</td>
<td>1.12 (4.6)</td>
</tr>
<tr>
<td>10. pAL5L/Clal</td>
<td>2</td>
<td>4.6 ± 0.5</td>
<td>0.24</td>
</tr>
<tr>
<td>11. pAL5cc</td>
<td>3</td>
<td>7.8 ± 0.8</td>
<td>0.41</td>
</tr>
<tr>
<td>12. pAL5ccL/NdeI</td>
<td>3</td>
<td>14.7 ± 1.1</td>
<td>0.78 (5.9)</td>
</tr>
<tr>
<td>13. pAL5ccL/Clal</td>
<td>2</td>
<td>2.5 ± 0.6</td>
<td>0.13</td>
</tr>
<tr>
<td>14. pAL5/StuI</td>
<td>5</td>
<td>1.4 ± 0.3</td>
<td>0.07</td>
</tr>
<tr>
<td>15. pAL5/StuI + NdeI</td>
<td>3</td>
<td>0.5 ± 0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>16. pAL5/HpaI</td>
<td>3</td>
<td>3.4 ± 1.3</td>
<td>0.18</td>
</tr>
<tr>
<td>17. pDY1</td>
<td>3</td>
<td>116.6 ± 1.0</td>
<td>61.2</td>
</tr>
<tr>
<td>18. pDY1/NdeI</td>
<td>5</td>
<td>22.0 ± 1.1</td>
<td>11.6 (5.2)</td>
</tr>
<tr>
<td>19. pDY1/Clal</td>
<td>2</td>
<td>4.2 ± 0.6</td>
<td>0.22</td>
</tr>
<tr>
<td>20. pDY1/StuI</td>
<td>2</td>
<td>0.5 ± 0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>21. pAL5i</td>
<td>2</td>
<td>21.3 ± 5.4</td>
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</tr>
<tr>
<td>22. pAL5i/NdeI</td>
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<td>26.0 ± 4.6</td>
<td>1.38</td>
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<tr>
<td>23. pTK12</td>
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<td>72.0 ± 10.5</td>
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<td>24. pTK12/NdeI</td>
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<td>76.0 ± 4.2</td>
<td>9.22</td>
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<tr>
<td>25. pTK12/Clal</td>
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<td>79.0 ± 7.0</td>
<td>9.22</td>
</tr>
<tr>
<td>26. pTK12/StuI</td>
<td>2</td>
<td>68.5 ± 2.3</td>
<td>9.22</td>
</tr>
</tbody>
</table>

* Three micrograms of plasmid plus 17 μg of salmon sperm DNA were used per 75 cm² flask except only 50 ng of pTK12 (lines 23–26) were used per flask.
* Each independent experiment involved transfection of at least three flasks each containing 5 × 10⁶ cells.
* Average ± the standard error of the mean for all flasks counted.
* Number of colonies per flask divided by number of colonies per flask obtained with pAL5L uncut. Numbers in parentheses indicate the recombination frequency for the particular plasmid cut with NdeI divided by recombination frequency for the plasmid cut with Clal.

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 NdeI compared with pAL5cc cut with Clal (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 Clal or NdeI might have influenced recombination frequencies by merely altering the amount of DNA that separated the tk sequences. When pAL5 is cleaved with Clal, the homologous nucleotides within the tk sequence are separated by about 5.6 kb when either plasmid is cleaved with NdeI. As shown in Table 1 (lines 8–13), the effect of cutting pAL5L or pAL5ccL with Clal or NdeI 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 Clal or NdeI was not related to the resulting distance between the tk sequences.

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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 \textit{NdeI}. The complementary \textit{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 \textit{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 \textit{NdeI} places both \textit{tk} sequences downstream from the SV40 early promoter/enhancer which drives transcription of the \textit{neo} gene. In contrast, when the constructs are linearized with \textit{ClaI} the SV40 promoter/enhancer is positioned between the \textit{tk} sequences. By analogy to studies done in yeast (Keil and Roeder 1984; Voeckel-Meman, Keil and Roeder 1987; Thomas and Rothstein 1989) and mammalian (Nickoloff and Reynolds 1990) systems we wondered if transcription from the \textit{tk} 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 \textit{NdeI}. 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 \textit{NdeI} 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 \textit{tk} gene.

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

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

Southern analysis of recombinant \textit{tk} genes: To ascertain that \textit{HAT'} colonies that arose following transfection of cells with recombination substrates contained reconstructed HSV-1 \textit{tk} genes, DNA samples were isolated from several \textit{HAT'} colonies and subject to Southern analysis. As displayed in the Southern blot in Figure 6, hybridization with an HSV-1 \textit{tk}-specific probe revealed that all putative recombinants contained HSV-1 \textit{tk} sequences and most exhibited a 2.5-kb \textit{BamHI} fragment that was resistant to cleavage by \textit{XhoI}. Such a fragment is predicted if the
sequences in linearized PALS-derived constructs. Cleavage of a recombination substrate with Ndel places both tk sequences downstream from the SV40 promoter/enhancer (bold) controlling the neo gene (2). Clal 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 5.—Position of the neo transcription unit relative to tk sequences in linearized pAL5-derived constructs. Cleavage of a recombination substrate with Ndel places both tk sequences downstream from the SV40 promoter/enhancer (bold) controlling the neo gene (2). Clal 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+ colonies. Samples in even numbered lanes were cleaved with BamHI, samples in odd numbered lanes were cleaved with BamHI plus XhoI. Colonies arose following transfection of cells with pDY1 cut with NdeI (lanes 2–5), pAL5 cut with NdeI (lanes 6 and 7), pAL5 cut with NdeI (lanes 8–11), pAL5 uncut (lanes 12–15). Most samples displayed a 2.5-kb BamHI fragment that was resistant to XhoI cleavage, indicative of a gene conversion correcting the XhoI linker insertion mutant tk gene. Some samples displayed 1.5- and 1.0-kb bands upon cleavage with BamHI plus XhoI, indicative of copies of uncorrected linker insertion mutant tk genes.

XhoI 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 BamHI sites flanking a corrected tk gene. This simple analysis ruled out the unlikely possibility that HAT+ 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 Clal or NdeI 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 XhoI 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 XhoI 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 2, with Table 1, line 1). Digestion of pTK2TK1-8 with NdeI prior to transfection resulted in a 30-fold higher recombination frequency compared with the frequency measured when pTK2TK1-8 was cut with Clal (Table 2, lines 2 and 3). Digestion with ScaI, which cuts near the site of Clal cleavage (Figure 2), had an effect similar to Clal.

| Table 2 | Effect of double-strand breaks on extrachromosomal homeologous recombination |
|-----------------|-----------------|-----------------|-----------------|
| Recombination substrate | Number of independent experiments | HAT+ colonies per flask | Relative recombination frequency |
| 1. pTK2TK1-8   | 3               | 1.5 ± 0.3       | 1.00            |
| 2. pTK2TK1-8/NdeI | 3               | 19.0 ± 2.3      | 12.67 (31.7)    |
| 3. pTK2TK1-8/Clal | 3               | 0.6 ± 0.2       | 0.40            |
| 4. pTK2TK1-8cc  | 2               | 0.8 ± 0.3       | 0.53            |
| 5. pTK2TK1-8cc/NdeI | 3              | 2.5 ± 0.3       | 1.67 (6.2)      |
| 6. pTK2TK1-8cc/Clal | 3              | 0.4 ± 0.2       | 0.27            |

a Three micrograms of plasmid plus 17 μg of salmon sperm DNA carrier were used per 75-cm² flask.

b Each independent experiment involved transfection of at least three flasks each containing 5 × 10⁶ 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 NdeI divided by recombination frequency for the plasmid cut with Clal.
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 NdeI had a mild stimulatory effect while digestion with ClaI 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 initiation 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 HindIII 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
strand gap starting from ClaI or NdeI 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 NdeI and ClaI 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 ClaI and pTK2TK1-8 cut with NdeI (Table 2, lines 2 and 3) vs. only a 3.6-fold difference for pAL5 cut with ClaI and pAL5 cut with NdeI (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+ 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 XhoI (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 NdeI and ClaI cleavage of pTK1TK2-8 could, at least in principle, be reconciled with the SSA model since degradation from only the NdeI 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 NdeI 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 NdeI was in fact dimin-
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 Clai site (panel A) or at the NdeI site (panel B). The $tk$ sequences are depicted as $D_1$. 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 Clai site leads to a promoterless, nonfunctional $tk$ gene whereas initiation of SSA from the NdeI site leads to a functional $tk$ gene.

Our previous intrachromosomal studies have always involved cutting a construct with Clai prior to transfection so the substrate would integrate using its Clai 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 NdeI termini. Work in progress indicates that the enhancement of recombination observed extrachromosomally for constructs cut with NdeI 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 unfinished (see Table 2, lines 4–6). Significantly, however, NdeI cleavage was still stimulatory relative to Clai 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 XhoI 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 intrachromosomal recombination in mammalian cells (Anderson and Elias 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 Clai prior to transfection so the substrate would integrate using its Clai 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 NdeI termini. Work in progress indicates that the enhancement of recombination observed extrachromosomally for constructs cut with NdeI 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 StuI may reveal a starting point for determining an underlying mechanism. Digestion of pAL5 constructs with StuI, 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 NdeI 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 StuI site. It is possible that binding of a transcription factor(s) to the SV40 promoter region played a role in synopsis of tk sequences and that StuI 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 (Shenk, 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 NdeI, stimulated recombination to a greater extent than binding between the tk sequences, such as after ClaI 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 ClaI site destabilizes (or if a break at the NdeI site stabilizes) paired complexes, it would not be entirely unexpected that recombination between mismatched tk sequences would be most affected 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 synopsis 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 discrepancies 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 our 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 extrachromosomal 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 Eliaon 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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