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Homologous Recombination Between Overlapping Thymidine Kinase Gene Fragments Stably Inserted into a Mouse Cell Genome

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We have constructed a substrate to study homologous recombination between adjacent segments of chromosomal DNA. This substrate, designated λ tk^2, consists of one completely defective and one partially defective herpes simplex virus thymidine kinase (*tk*) gene cloned in bacteriophage λ DNA. The two genes have homologous 984-base-pair sequences and are separated by 3 kilobases of largely vector DNA. When λ tk^2 DNA was transferred into mouse LM*tk*$^-$ cells by the calcium phosphate method, rare Tk$^+$ transformants were obtained that contained many (>40) copies of the unrecombined DNA. Tk$^-$ revertants, which had lost most of the copies of unrecombined DNA, were isolated from these Tk$^+$-transformed lines. Two of these Tk$^-$ lines were further studied by analysis of their reversion back to the Tk$^+$ phenotype. They generated ca. 200 Tk$^+$ revertants per 10^8 cells after growth in nonselecting medium for 5 days. All of these Tk$^+$ revertants have an intact *tk* gene reconstructed by homologous recombination; they also retain various amounts of unrecombined λ tk^2 DNA. Southern blot analysis suggested that at least some of the recombination events involve unequal sister chromatid exchanges. We also tested three agents, mitomycin C, 12-*O*-tetradecanoylphorbol-13-acetate, and mezerein, that are thought to stimulate recombination to determine whether they affect the reversion from Tk$^-$ to Tk$^+$. Only mitomycin C increased the number of Tk$^+$ revertants.

Much of our current understanding of how recombination occurs between homologous DNA sequences stems from many elegant studies in bacteria and yeasts (for reviews, see references 29 and 44). These studies take advantage of gene mutants blocked in recombination and involve physical characterization of DNA-protein recombination intermediates. In mammalian somatic cells, homologous recombination also occurs, as evidenced by the production of recombinant viruses after infection of cultured cells with genetically (5, 8, 11, 53) or physically (11, 47) marked viruses and by the detection of recombination intermediates after viral infection (52). With recombinant DNA and gene transfer technology, homologous recombination has been detected and studied during the transfer of appropriately constructed DNA substrates into mammalian cells (7, 10, 17, 19, 37, 38, 43, 49). Surprisingly, recombination efficiencies are very high, approaching 10 to 20%. We have recently proposed a molecular model that can explain the recombination events occurring during the gene transfer process (F.-L. Lin, K. Sperle, and N. Sternberg, Mol. Cell. Biol., in press).

Although these successes have been encouraging, it remains to be determined whether conclusions drawn from viral and gene transfer studies, in which the recombining DNA may be entirely extrachromosomal, can be extrapolated to recombination between homologous segments stably integrated in mammalian cell chromosomes. Studies of chromosomal homologous recombination in somatic cells have been hampered by a paucity of assays to score the event and by its apparent inefficiency. For example, analyses of sister chromatid exchanges reveal that they occur at a frequency of about 10^{-6} per mammalian cell gene, or about one exchange per genome (18; R. P. Wagner, manuscript in preparation). This sort of recombination event has not provided much insight into the factors involved in and mechanisms of homologous recombination because it does not allow one to focus on specific sequences and events in the recombinations process. Attempts to detect recombination between distantly linked chromosomal markers in somatic cells have been unsuccessful (33).

An important question that emerges from these mammalian cell studies is whether the differences apparent between the inefficient chromosomal recombination and the more efficient extrachromosomal recombination reflect a basically different mechanism for the two events or whether they simply reflect the greater accessibility of extrachromosomal DNA, at least during gene transfer and viral infection, to recombination enzymes. To address this question, we have devised a system to quantitatively study chromosomal recombination between two defective herpes simplex virus (HSV) thymidine kinase (*tk*) genes inserted into the mouse cell genome by gene transfer techniques. Cells in which rare recombination events have occurred have been isolated based on their ability to grow in Tk$^+$ selection medium, and the associated physical rearrangement of the DNA has been assessed by Southern blot hybridization analysis. These results show that mammalian somatic cells can carry out chromosomal homologous recombination; the results also suggest which substrates might be used in that recombination.

MATERIALS AND METHODS

Chemicals. Mitomycin C and bromodeoxyuridine were obtained from Sigma Chemical Co. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and mezerein (13) were provided by N. Colburn, who confirmed that they were active as tumor promoters (2).

Animal cells and media. The mouse cell line used in these studies is the LM*tk*$^-$ *aprt*$^-$ line described by Wigler et al. (50). It was grown on plastic (B D Labware) in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum under an atmosphere of 5% CO_2. This medium was

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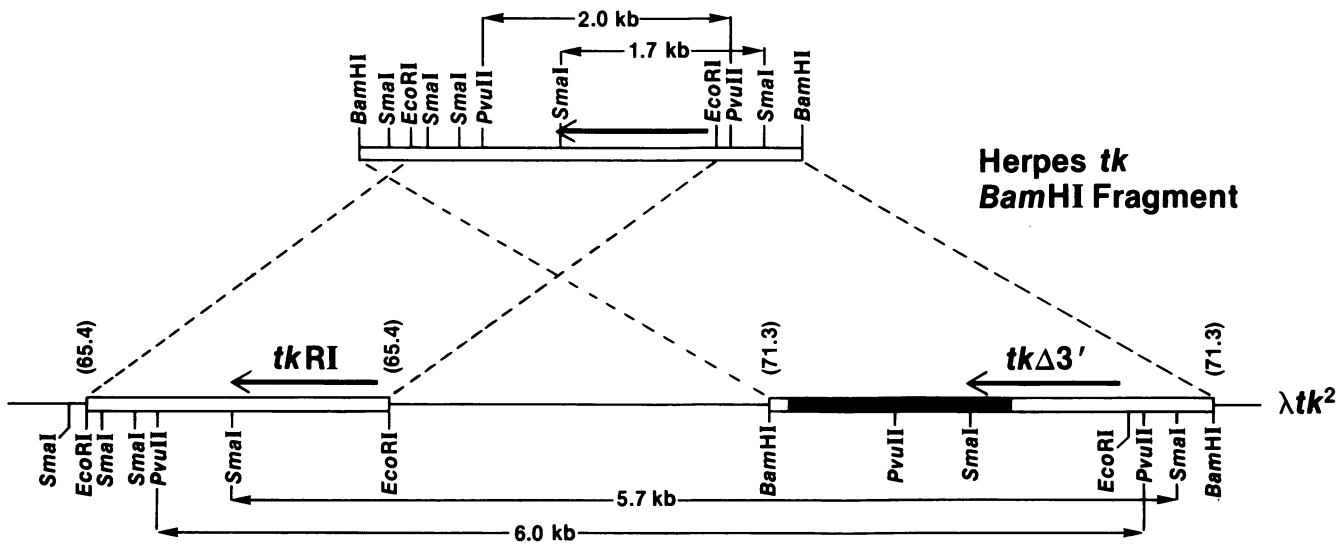

FIG. 1. Restriction maps of λ*tk* recombination substrates. Symbols: □, HSV *tk* DNA; ■, deleted HSV DNA; ——, λ DNA. The construction of λ *tk*, λ *tk*Δ3′, and λ tk^2 DNA is described in the text. A partial restriction map of the 3.5-kb HSV *tk* fragment is shown at the top, and a similar map of λ tk^2 DNA is shown at the bottom. The numbers in parentheses above the *Eco*RI and *Bam*HI sites in λ tk^2 DNA represent the λ map coordinates at which the HSV fragments are cloned. The Δ3′ deletion mutation arose spontaneously in the λ *tk* phage stock and was selected by using the *Dam* properties of the λ vector (42). The deletion removes 1.7 kb from the HSV *tk* fragment, including 330 bp from the 3′ end of the *tk* structural gene, the limits of which are designated by the arrow above the *Bam*HI *tk* fragment. The two defective *tk* genes in λ tk^2 DNA, the *Bam*HI Δ3′ *tk* fragment and the 2.4-kb *Eco*RI *tk* fragment, are oriented in the same direction, share 984 bp of homology, are separated by 3 kb of λ DNA, and are flanked by *Sma*I sites (generating a 5.7-kb fragment) and *Pvu*II sites (generating a 6.0-kb fragment). Recombination in the homologous region reconstructs an intact *tk* gene, producing a 1.7-kb *Sma*I fragment and a 2.0-kb *Pvu*II fragment. Just 3′ to the *Eco*RI *tk* gene of λ tk^2 DNA is a cluster of three *Sma*I sites (the last of which is in the λ DNA) which is retained in the gene reconstructed by recombination.

supplemented with 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.7×10^{-5} M thymidine (HAT medium) for selection of Tk^+ transformants and Tk^+ rerevertants and supplemented with hypoxanthine and 1×10^{-4} M bromodeoxyuridine (HBu medium) for selection of Tk^- revertants of Tk^+ transformants. The nonselecting medium (HT medium) is Dulbecco modified Eagle medium with hypoxanthine and thymidine. Under these conditions, the cell doubling time for the LMtk^- *aprt*$^-$ line at 37°C is about 24 h.

Construction of the substrate for recombination, λ tk^2. The construction and characterization of λ tk^2 DNA (Fig. 1) is described in detail elsewhere (Lin et al., in press). Briefly, a 3.5-kilobase (kb) *Bam*HI fragment from plasmid pX1 (9) containing the HSV *tk* gene was inserted into phage λ*D*$^-$ *srl*λ3 DNA (42) at map coordinate 71.3, and a spontaneous deletion mutation, designated *tk*Δ3′, that removed a portion of the 3′ end of the *tk* gene and the flanking HSV DNA was then isolated. By the DNA sequencing method of Sanger and Coulson (34), the rightward boundary of this deletion was placed between nucleotides 795 and 796 of the *tk* gene (48), indicating that the mutation removed 330 base pairs (bp) from the 3′ end of the gene. Into the λ *tk*Δ3′ DNA, we then cloned the 2.4-kb *Eco*RI *tk* fragment at λ map coordinate 65.4. A λ clone was selected that contained both the *Bam*HI Δ3′ fragment and *Eco*RI fragment in the same orientation, and this clone was designated λ tk^2. The *Eco*RI fragment contains the entire *tk* structural gene but lacks an essential component of the *tk* transcriptional control region (25). As a consequence, the *Eco*RI gene is largely defective and transforms Tk^- cells to Tk^+ at only 0.1% the efficiency of the intact gene. In λ tk^2, the two defective *tk* genes share 984 bp of homology. Recombination anywhere within this homologous region will reconstruct an intact gene.

DNA-mediated transformation of mouse LMtk^- cells. Calcium phosphate-mediated DNA transfer of mouse strain LMtk^- was performed by the method of Graham and Van der Eb (12). The DNA precipitate was prepared using either 10 or 100 ng of λ tk^2 DNA and 10 μg of LMtk^- carrier DNA. Only one Tk^+ transformant was selected from each transformation plate.

Selection of Tk^- revertants and Tk^+ rerevertants. Colonies were picked from the original transformation plates with cotton swabs and diluted in HAT medium, and individual cells were cloned by the limiting dilution method in 96-well dishes. Cells from wells with single colonies were transferred to T-75 flasks and grown in HAT medium for 50 days. A portion of the cells then was transferred directly to HBu medium to measure the fraction of Tk^- cells in the culture. From the remainder of the culture, portions consisting of 10^3 cells were transferred to 100-mm dishes containing HT medium and grown for 5 days. At the end of this period, the HT medium was replaced with HBu medium, and Tk^- colonies were scored by methylene blue staining after 18 days. Only one colony was selected from each plate and cloned as described above. After the clone was grown for 50 generations in HBu medium, a portion of the culture was transferred to HAT medium for measurement of the fraction of Tk^+ cells. With the remainder of the culture, portions consisting of 10^5 cells were transferred to 150-mm dishes in HT medium and grown for 5 days. The medium was then replaced with HAT medium, and Tk^+ rerevertants were scored after 18 days. Only one Tk^+ colony was picked from each plate and cloned as described above. To determine whether mitomycin C, TPA, and mezerein affect the number of Tk^+ rerevertants, we grew the Tk^- revertant lines in HT medium for 5 days and then in HT medium with either

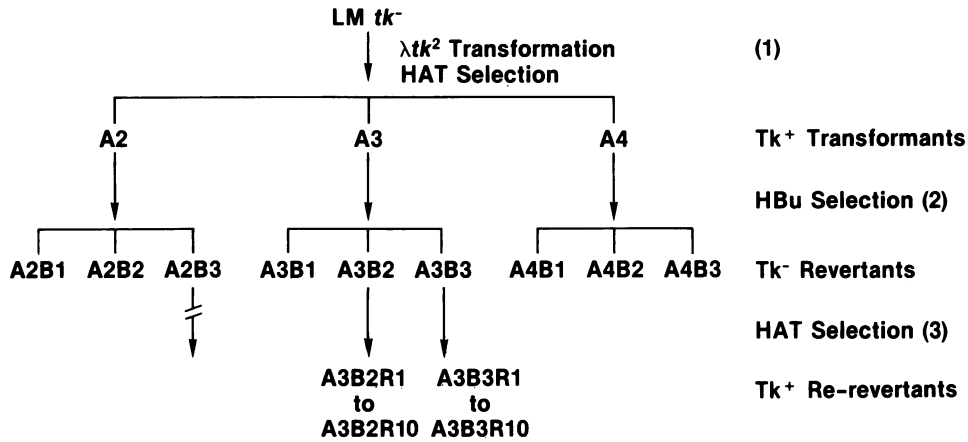

FIG. 2. Geneology of mouse L-cell lines used to study *tk* gene recombination. The original transformation of LM*tk*$^-$ cells to Tk$^+$ with λ *tk*^{2} DNA was carried out by the method of Graham and Van der Eb (12), using 2 μg of λ *tk*^{2} DNA and 10 μg of LM*tk*$^-$ DNA per 10^6 cells (step 1). The transformation efficiency was one transformant per 100 ng of λ *tk*^{2} per 10^6 cells, an efficiency of 0.1% that of an intact *tk* gene. Three transformants were selected (A2, A3, and A4) and processed as described in the text. The reversion from Tk$^+$ to Tk$^-$ (step 2) and the rereversion from Tk$^-$ to Tk$^+$ (step 3) were measured by plating cells in counterselective medium both immediately after growth in selection medium and after growth for 5 days in nonselection medium (see text). After growth in HAT medium, the fraction of Tk$^-$ cells in cell lines A2, A3, and A4 was 0.001 to 0.003, but after 5 days in HT medium, that fraction was 0.05 to 0.15. After growth in HBu medium, the fraction of Tk$^+$ cells in cell lines A3B2 and A3B3 was $<2 \times 10^{-8}$, but after 5 days in HT medium, that fraction was 2×10^{-6}.

mitomycin C, TPA, mezerein, or no additives for 2 days. The cultures were sampled to measure cell survival and then transferred to HAT medium to measure the number of Tk$^+$ rerevertants. Colonies were scored 2 to 3 weeks after the transfer to selection medium.

DNA isolation and filter blot hybridization. High-molecular-weight (>50-kb) mouse DNA was prepared from the various cultured cell lines by the method of Corsaro and Pearson (3). A 5- or 10-μg amount of that DNA was digested overnight with *Sma*I or *Pvu*II under conditions specified by the vendor (Bethesda Research Laboratories) and was then subjected to electrophoresis in 1% agarose gels in 0.09 M Tris–0.08 M borate–0.002 M EDTA buffer for 4 to 6 h at 50 V. The gel was then stained with ethidium bromide and examined under UV radiation to ensure that there were no gross discrepancies in the amount of DNA added to each lane. Transfer of the DNA from the gel to BA85 nitrocellulose filter paper (0.45-μm pore size) was performed as described by Southern (39). The filter was then prehybridized at 68°C for 1 h in 100 ml of a solution containing 0.6 M NaCl, 0.06 M sodium citrate, 5× Denhardt solution (1× Denhardt solution is 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), and 100 μg of sonicated calf thymus DNA per ml. The filter was hybridized at 68°C overnight in sealed bags with 10 ml of hybridization solution containing 0.6 M NaCl–0.06 M sodium citrate, 1× Denhardt solution, 40 μg of sonicated denatured calf thymus DNA per ml, 10% dextran sulfate, and 2×10^7 cpm of an α-^{32}P-labeled probe consisting of the 2.0-kb *Pvu*II *tk* fragment (Fig. 1) cloned in plasmid pBR322. The DNA was labeled by nick translation (24) to a specific activity of 2×10^8 to 5×10^8 cpm/μg with [α-^{32}P]dCTP and [α-^{32}P]TTP. After hybridization, the filter was washed with a 5× 200-ml solution of 0.1% sodium dodecyl sulfate and 0.015 M NaCl–0.0015 M sodium citrate at 68°C and then exposed to Kodak XAR-5 film with Cronex intensifying screens (Du Pont Co.). The intensities of particular bands on the film were measured by densitometer tracing with a Quick Scan densitometer from Helena Laboratories. The copy number represented by that amount of DNA was then determined by comparison with variable amounts of marker DNA, which was subjected to electrophoresis in adjacent gel lanes and analyzed in a similar manner. If we assume that the haploid DNA content of mouse L cells is 3.0×10^6 kb (21), then 2 pg of the *Sma*I 1.7-kb *tk* fragment or the 2.0-kb *Pvu*II *tk* fragment per 4 μg of genomic DNA is approximately equivalent to one copy of the *tk* gene per haploid mouse genome.

RESULTS

Construction and analysis of a mouse cell line to study homologous recombination. A two-step process was used to construct a cell line to study recombination between regions of DNA stably inserted into a mouse cell genome. A flow diagram outlining these steps and the strains used in this report is shown in Fig. 2. In the first step, the DNA of a recombination substrate constructed in vitro, λ *tk*^{2}, was transferred into LM*tk*$^-$ cells by the calcium phosphate method, and Tk$^+$ transformants were selected in HAT medium. The structure of λ *tk*^{2} DNA is shown in Fig. 1, and its construction is described above. Its most salient feature is that it contains two defective HSV *tk* genes (*tk*RI and *tk*Δ3′) cloned into phage λ DNA and separated by 3 kb of vector DNA. Both of the defective *tk* genes are oriented in the same direction in *tk*^{2} DNA, and recombination in the 984-bp sequence common to both of the genes excises the DNA between them and reconstructs a functional gene. That event can be assayed physically by digestion of the DNA with either *Sma*I or *Pvu*II. The DNA rearrangement converts a 5.7-kb *Sma*I fragment to a 1.7-kb fragment and a 6.0-kb *Pvu*II fragment to a 2.0-kb fragment (Fig. 1).

The efficiency of transformation of LM*tk*$^-$ cells with λ *tk*^{2} is 0.1% that observed with a λ phage containing an intact *tk* gene (one transformant per 10^6 cells per 100 pg of λ *tk* DNA). We believe that the low level of Tk$^+$ transformation with λ *tk*^{2} is a result of the rare incorporation of many copies of the *Eco*RI *tk* fragment into the genome of the mouse cell. Presumably, inefficient expression of *tk* from many copies of the defective *Eco*RI gene produces enough kinase to grow in

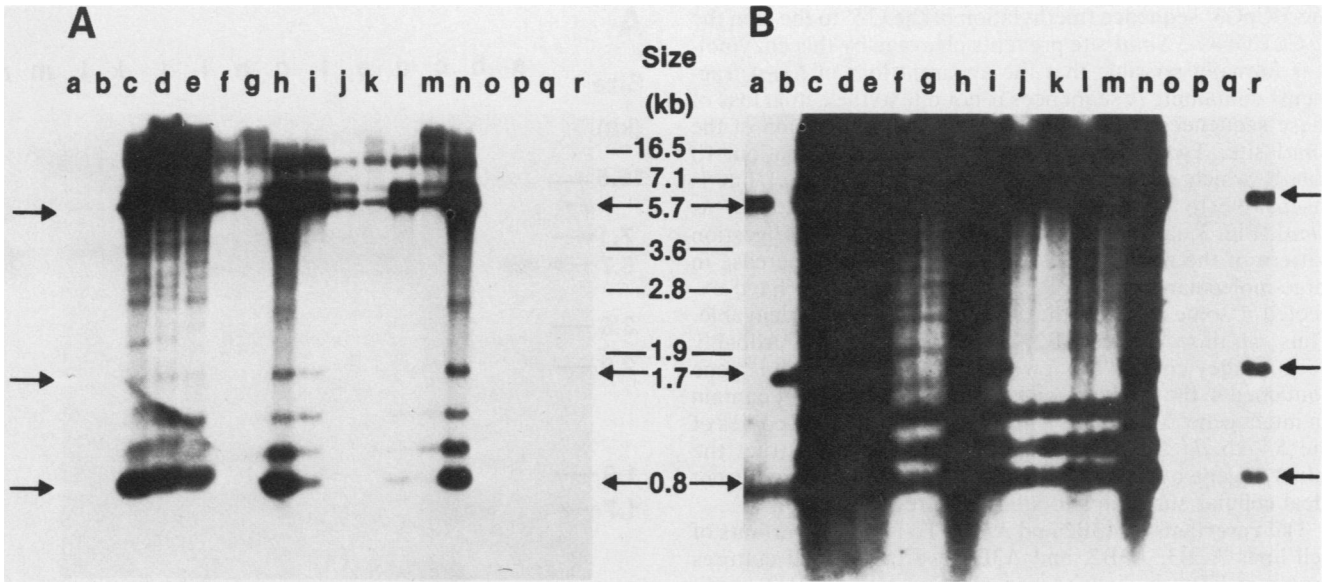

FIG. 3. Southern analysis of *Sma*I and *Xma*I digests of the DNA from Tk$^+$ transformants (A2, A3, and A4) and their Tk$^-$ revertants. The DNAs of the original Tk$^+$ transformant lines, digested with *Sma*I, are shown in lanes c (A2), h (A3), and n (A4). The DNAs of Tk$^-$ revertants of line A2 (A2B1, A2B2, and A2B3), digested with *Sma*I, are shown in lanes d to f, respectively. The DNAs of Tk$^-$ revertants of lines A3 (A3B1, A3B2, and A3B3), digested with *Sma*I, are shown in lanes i, j, and l, respectively. The DNAs of Tk$^-$ revertants of lines A4, (A4B1, A4B2, and A4B3), digested with *Sma*I, are shown in lanes o to q, respectively. DNAs digested with *Xma*I are those from lines A2B3 (lane g), A3B2 (lane k), and A3B3 (lane m). Lanes c to q contain 4 μg of genomic mouse DNA. The control lanes contain 50 pg of *Sma*I-digested λ *tk*^{2} DNA (lanes a and r) and 50 pg of *Sma*I-digested λ *tk* DNA (lanes b and r). These DNAs contain the 5.7-kb *Sma*I *tk*^{2} fragment and the 1.7-kb *Sma*I *tk* fragment, respectively. Both of the DNAs also contain a 0.8-kb *Sma*I fragment that comes from the 3′ end of the *tk* gene and the 3′ untranslated DNA (Fig. 1). The two bands between the 0.8- and 1.7-kb fragments (lanes c to e, h, i, and n) are due to incomplete enzyme digestion and represent DNA immediately 3′ to the *tk* gene. Two gel exposure times are shown; that of (A) is 1/10 that of (B). The size standards used (from an *Eco*RI digest of bacteriophage P1 DNA [42]) are shown between the gels; the arrows indicate the positions of pertinent *tk* fragments. The copy number of 5.7- and 1.7-kb fragments from mouse DNA digests was determined by densitometer tracing, using as a standard the signal intensity of DNAs in lanes a and b and, when necessary, additional control dilutions of *Sma*I-digested λ *tk*^{2} and λ *tk* DNAs. The copy number of the *tk* fragments in the control lanes corresponds to about one per haploid mouse genome per 4 μg of mouse DNA. The probe was the 2.0-kb *Pvu*II *tk* fragment.

HAT medium. Support for this explanation comes from two observations. (i) The efficiency of transformation of LM*tk*$^-$ with λ DNA containing just the 2.4-kb *Eco*RI *tk* fragment is the same as that of λ *tk*2, 0.1% that of λ *tk*. In contrast, transformation with λ DNA containing just the Δ3′ defective gene has never, in our studies, produced a Tk$^+$ transformant (efficiency, <0.001% that of λ *tk*). (ii) Southern hybridization analysis of three independent Tk$^+$ transformants (A2, A3, and A4, Fig. 3) demonstrated that all of the transformants contain about 40 to 100 copies of a 5.7-kb *Sma*I fragment that includes both of the defective *tk* genes (Fig. 1 and 3). These transformants also contain, at a low copy number (two to six copies per haploid mouse genome), a 1.7-kb *Sma*I fragment present in the intact *tk* gene. The presence of this fragment indicates that the intact *tk* gene has been reconstructed by homologous recombination either during the gene transfer process or after the incorporation of the many copies of *tk*^{2} DNA into the mouse genome. The ability of mammalian cells to carry out homologous recombination during the gene transfer process has been demonstrated recently by several laboratories (5, 7, 10, 17, 37, 38, 43). It is unclear whether the Tk$^+$ phenotype of the three transformants results from inefficient expression of each of the many copies of the defective *Eco*RI *tk* genes or from efficient expression of each of the few copies of the intact *tk* genes.

The second step in the construction of a cell line to study homologous recombination (Fig. 2, step 2) is the isolation of Tk$^-$ revertants from the three Tk$^+$ transformants. This was accomplished by transferring Tk$^-$ cells growing in HAT medium to nonselecting growth medium for ca. 5 days and then to Tk$^-$ selection medium containing bromodeoxyuridine. Three Tk$^-$ revertants (designated B1, B2, and B3) were isolated from each of the Tk$^+$ cell lines, and their DNAs were analyzed by Southern analysis after digestion with *Sma*I (Fig. 3). Based on this analysis, three classes of Tk$^-$ revertants were distinguishable. The first class, represented by A2B1, A2B2, and A3B1 (Fig. 3, lanes d, e, and i), has much the same DNA composition as the original Tk$^+$ transformants. The reason for the Tk$^-$ phenotype of these strains is not known, but possible reasons include the shutoff of gene expression, caused by DNA methylation, and changes in the nucleosome structure. Both mechanisms have been reported previously for the Tk$^+$-to-Tk$^-$ reversion event (1, 6, 41). The reversion to Tk$^-$ in these strains is probably not due to a mutation in the *tk* gene(s), because the level of Tk$^-$ reversion was high; 5 to 15% of the cells after 5 days of growth in HT medium were Tk$^-$ (see legend to Fig. 2 and above). The second class of Tk$^-$ revertants, represented by the three A4 revertants (Fig. 3, lanes o, p, and q), has lost all of the *tk* gene sequences. The third class of Tk$^-$ revertants, represented by A2B3, A3B2, and A3B3 (Fig. 3, lanes f, j, and l), has lost a large number of 5.7-kb *Sma*I *tk*^{2} fragments and all of the 1.7-kb *Sma*I fragments. We estimate that 10 to 20 copies of the 5.7-kb fragment remain in these revertants (see legend to Fig. 3 and 5).

Since digestion by *Sma*I is modulated by methylation of

the 5'CpG3' sequence (methylation of the C 5' to the G in the 5'-CCCGGG-3 *Sma*I site prevents cleavage by this enzyme), it is formally possible that the apparent loss of *Sma*I fragments containing *tk* sequences is not due to the actual loss of these sequences but rather is due to the methylation of the *Sma*I site. Two results argue against this explanation: (i) *Xma*I, which cleaves the same sequence as *Sma*I but is insensitive to CpG methylation, gave the same result as *Sma*I (Fig. 3, lanes g, k, and m); and (ii) the *Sma*I digestion pattern of the revertant DNAs did not show an increase in large-molecular-weight fragments that one would have expected if some or all of the *Sma*I sites were not cleavable. Thus, strains A2B3, A3B2, and A3B3 are Tk^- probably because they contain too few copies of the defective *tk* gene contained in the 2.4-kb *Eco*RI *tk* fragment and do not contain an intact gene. Moreover, since they retain 10 to 20 copies of the 5.7-kb tk^2 *Sma*I fragment, which can reconstruct the intact *tk* gene by homologous recombination, they represent ideal cellular substrates to study that recombination.

Tk^+ revertants of A3B2 and A3B3. To isolate revertants of cell lines A2B3, A3B2, and A3B3, we transferred cultures grown in HBu medium to nonselecting medium (HT medium) for 5 days and then to HAT selection medium to isolate Tk^+ colonies. From cell lines A3B2 and A3B3 we obtained 200 and 250 Tk^+ colonies, respectively, from 10^8 cells. From cell line A2B3 we were not able to obtain any Tk^+ revertants from 10^8 cells. Because of the latter result, we discontinued studying line A2B3.

To study the revertants of A3B2 and A3B3, we isolated 10 independent Tk^+ clones, designated A3B2R1, A3B2R2, etc., from each of the two strains and analyzed their DNAs by Southern hybridization analysis with restriction enzymes *Sma*I and *Pvu*II. All 20 revertants contain the 1.7-kb *Sma*I fragment indicative of *tk* gene reconstruction by homologous recombination (Fig. 4). The copy number of this fragment is about 0.5 to 2 per haploid mouse genome (see legend to Fig. 4). To rule out the remote possibility that the recombinant 1.7-kb *Sma*I fragment present in all of the recombinant lines represents an unmasking of the original fragment that is present in the A3 line but is somehow masked in lines A3B2 and A3B3, we took advantage of the *Pvu*II digestion pattern of these DNAs. The *tk* gene is contained within a 2.0-kb *Pvu*II fragment (Fig. 1). Since each defective *tk* gene in λ tk^2 DNA is missing one *Pvu*II site, λ tk^2 DNA contains, instead of the 2.0-kb *Pvu*II fragment, a 6.0-kb *Pvu*II tk^2 fragment. Transformant A3 contains about 40 copies of the 6.0-kb *Pvu*II fragment, about 3 copies of a 1.9-kb *Pvu*II *tk* fragment, but no intact 2.0-kb fragment (best seen in Fig. 5B, lanes b and c). Since this transformant has an intact 1.7-kb *Sma*I *tk* fragment that contains the *Pvu*II site 5' to the *tk* gene, the lack of a 2.0-kb *Pvu*II fragment probably indicates a rearrangement of the DNA sequences 3' to the *tk* gene between the *Sma*I and *Pvu*II sites (Fig. 1). As observed for *Sma*I-digested DNA, the Tk^- revertants of line A3 (A3B2 and

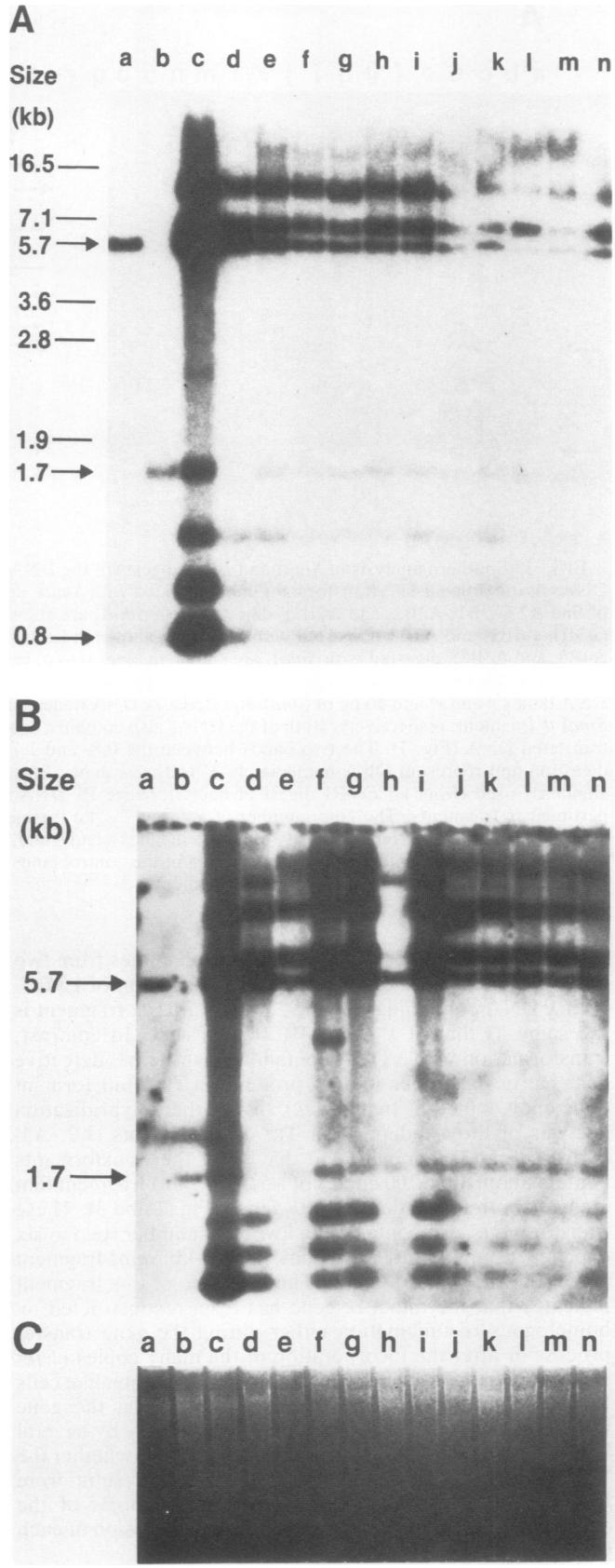

FIG. 4. Southern analysis of *Sma*I digests of DNAs from Tk^+ rerevertants of Tk^- lines A3B2 and A3B3. (A) contains 4 μg of *Sma*I-digested DNAs from lines A3 (lane c), A3B2 (lane d), and A3B2R1 to A3B2R10 (lanes e to n). Control lanes a and b are the same as those in Fig. 3. The probe used is also the same as that in Fig. 3. (B) contains 4 μg of *Sma*I-digested DNAs from lines A3 (lane c), A3B3 (lane d), and A3B3R1 to A3B3R10 (lanes e to n). Note that the 1.7-kb fragment of the intact gene (arrow) is present in all lanes except those containing DNAs from cell lines A3B2 and A3B3. (C) shows an ethidium bromide staining of the gel containing the A3B3 DNAs of (B).

A3B3) have fewer copies of the 6.0-kb *Pvu*II tk^2 fragment and completely lack the 1.9-kb recombinant *tk* fragment (Fig. 5A and B, lanes c and d). When the 20 Tk^+ rerevertant DNAs were analyzed with *Pvu*II, all were shown to contain the intact 2.0-kb *Pvu*II *tk* fragment (Fig. 5A and B). This result indicates that the conversion of Tk^- cell lines A3B2 and A3B3 to Tk^+ is likely due to a homologous recombination event that reconstructs an intact *tk* gene from two defective overlapping parts during the rereversion step. The Tk^+ rereversion could not be due to a segment of *tk* DNA present in the original A3 transformant, since the recombinant 2.0-kb *Pvu*II fragment present in the rerevertant was not present in the original transformant.

The copy number of the unrecombined 6.0-kb *Pvu*II fragment (Fig. 5A) in all of the A3B2 recombinants is about the same as the copy number of the 6.0-kb *Pvu*II fragment in the A3B2 Tk^- parent strain. In contrast, the copy number of this fragment in the A3B3 recombinants is more variable (Fig. 5B). In some recombinants (A3B3R3 and A3B3R10; Fig. 5B, lanes g and n) the copy number is similar to that found in the A3B3 Tk^- parent strain (11 copies per haploid genome); in other recombinants (A3B3R2 and A3B3R5; Fig. 5B, lanes f and i) the copy number is two times greater than that found in the Tk^- parent strain; and in other recombinants (A3B3R1 and A3B3R4; Fig. 5B, lanes e and h) the copy number is much lower (0.5 to 3) than that found in the Tk^- parent strain. The data obtained with *Sma*I-digested DNA (Fig. 4) are entirely consistent with the *Pvu*II data.

Reversion of A3B2 and A3B3 to Tk^+ is stimulated by mitomycin C. If the rereversion of A3B2 and A3B3 is due to recombination, then agents that stimulate recombination, such as mitomycin C (22, 27, 51), should stimulate reversion. The effect of mitomycin C on the rereversion frequency is shown in Fig. 6. For both of the Tk^- cell lines, there was a modest but steady increase in the number of Tk^+ rerevertants with increasing drug concentrations. At 30 μg/ml, there was about a three- to fourfold stimulation of rereversion, a value comparable to the stimulation of sister chromatid exchange seen with this drug (22, 51). In contrast, treatment of line A3B2 with the tumor promoters TPA and mezerein, which have been suggested to be recombinogenic agents (18, 26, 27), had no effect on the number of Tk^+ rerevertants (Fig. 6). Moreover, similar experiments performed with strain A2B3, which shows no detectable Tk^+ rereversion, failed to demonstrate a stimulation of rereversion with mitomycin C, TPA, or mezerein (data not shown). Analysis of the DNA of several mitomycin C-induced rerevertants by *Sma*I and *Pvu*II yielded results identical to those observed without the drug (data not shown).

DISCUSSION

The studies described in this report clearly demonstrate that mammalian cells can promote homologous recombination, albeit inefficiently, between adjacent DNA segments stably inserted into a mouse cell genome. We chose to study homologous recombination between two defective HSV *tk* genes with homologous 984-bp sequences that are separated by 3 kb of λ vector DNA. This DNA substrate was inserted into the genome of mouse cells by gene transfer techniques,

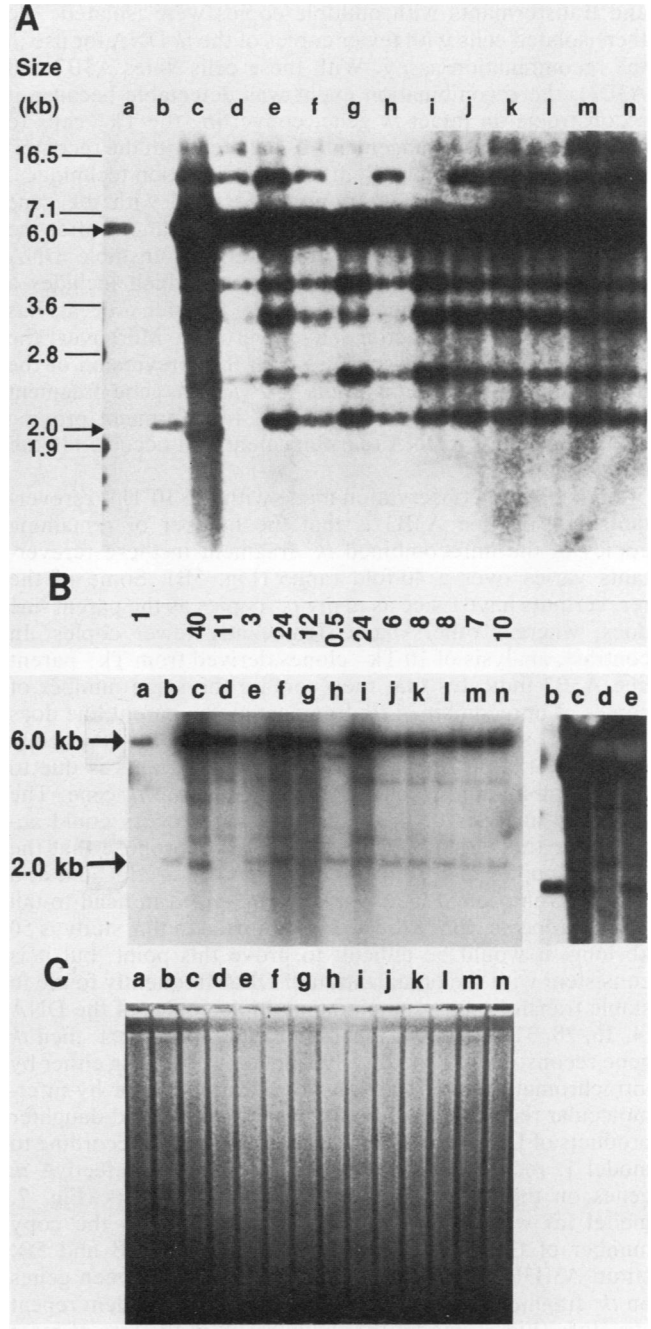

FIG. 5. Southern analysis of *Pvu*II digests of DNAs from Tk^+ rerevertants of Tk lines A3B2 and A3B3. (A) contains 4 μg of *Pvu*II-digested DNAs from lines A3 (lane c), A3B2 (lane d), and A3B2R1 to A3B2R10 (lanes e to n). The control lanes contain 50 pg of *Pvu*II-digested λ tk^2 DNA (lane a) and 50 pg of *Pvu*II-digested λ *tk* DNA (lane b). The latter DNAs contain a 6.0-kb *Pvu*II tk^2 fragment and a 2.0-kb *Pvu*II *tk* fragment, respectively, whose copy number corresponds to one *tk* gene equivalent per haploid mouse genome per 4 μg of mouse DNA. (B) contains 4 μg of *Pvu*II-digested DNAs from lines A3 (lane c), A3B3 (lane d), and A3B3R1 to A3B3R10 (lanes e to n). A longer exposure of lanes b to e is shown on the right. Lanes a and b contain the same control digests as (A). The number of copies of the 6.0-kb *Pvu*II fragment for each of the A3-derived lines relative to that in the control lane (B, lane a) is indicated above each lane in (B). The copy numbers of these bands in the gel were determined by densitometer tracing of signal intensities and normalized to those in the control lanes or in lanes containing various amounts of *Pvu*II-digested λ tk^2 or λ *tk* DNA (data not shown). The probe used is the same as that in Fig. 3 and 4. (C) shows the ethidium bromide staining of the gel containing the A3B3 DNAs.

and transformants with multiple copies were isolated. We then isolated cells with fewer copies of the *tk* DNA for use in the recombination assay. With these cells (lines A3B2 and A3B3), the recombination event was detectable because it reconstructs an intact *tk* gene, converting the Tk^- cells to Tk^+. The DNA rearrangements associated with the recombination were analyzed by Southern hybridization techniques. The recombination event is not associated with the gene transfer process itself. It occurs many generations after the completion of that process and after the unstable DNA present in the initial Tk^+ transformant, which includes a recombinant gene produced during the transfer process, has been lost by backselecting Tk^- revertants. Moreover, the recombination event associated with the rereversion of the Tk^- cells to Tk^+ reconstructs a *Pvu*II *tk* gene fragment clearly absent from the original Tk^+ transformant, proving that it represents a DNA rearrangement that occurred in the rereversion step.

An interesting observation made with the 10 Tk^+ rerevertants of Tk^- line A3B3 is that the number of remaining copies of the unrecombined tk^2 fragment in these rerevertants varies over a 40-fold range (Fig. 5B). Some of the rerevertants have twice as many tk^2 copies as the parent line does, whereas others have significantly fewer copies. In contrast, analysis of 10 Tk^- clones derived from Tk^- parent line A3B3 indicates that they contain the same number of copies of unrecombined tk^2 fragment as the parent line does (data not shown). These results suggest that the copy number variation seen with the Tk^+ rerevertants is due to the recombination event that reconstructs the *tk* gene. The following analysis of the recombination process could account for this variation (Fig. 7). First, we propose that the multiple copies of λ tk^2 DNA in line A3B3 are located at a single chromosomal locus and are organized in head-to-tail tandem repeats. Since the λ tk^2 DNA used in this study is 50 kb long, it would be difficult to prove this point, but it is consistent with the organization of DNA frequently found in stable transformants containing multiple copies of the DNA (4, 16, 28, 31, 32, 36). If this organization is correct, then *tk* gene reconstruction can be envisioned as occurring either by intrachromatid recombination (Fig. 7, model I) or by intermolecular recombination between sister-chromatid-daughter products of DNA replication (Fig. 7, model II). According to model I, recombination between overlapping defective *tk* genes on the same or on adjacent tk^2 fragments (Fig. 7, model Ia) would result in very little change in the copy number of the unrecombined tk^2 DNA (Fig. 4B and 5B, strain A3B3R3). In contrast, recombination between genes on tk^2 fragments present at the termini of the tandem repeat structure (Fig. 7, model Ib) would result in the loss of most of the unrecombined DNA, assuming that the excised DNA between the recombining genes is not subsequently reinserted into the mouse genome. Possible examples of such a class of recombinants are strains A3B3R1 and A3B3R4 (Fig. 4B and 5B). These two classes of recombinants can also be produced by unequal sister chromatid recombinations (Fig. 7, models IIa and b). However, unlike intrachromatid recombination, sister chromatid exchange should also be able to generate recombinant lines in which the number of unrecombined tk^2 fragments increases to a maximum of twice that in the the nonrecombinant parent line (Fig. 7, model IIc). Possible examples of such a class of recombinants are lines A3B3R2 and A3B3R5 (Fig. 4B and 5B). The existence of this last class of recombinants suggests that at least some of the gene reconstruction events described in this report are interchromatidal in origin. A similar sort of unequal intermolecular recombination event between units of tandemly repeated DNA has been implicated recently as a step in the process of gene amplification (30). Unlike results with line A3B3, the copy number of the unrecombined tk^2 fragment in the recombinants derived from line A3B2 is constant and about the same as that in A3B2 (Fig. 4A and 5A). We do not know the reason for the difference in behavior of the two sister lines.

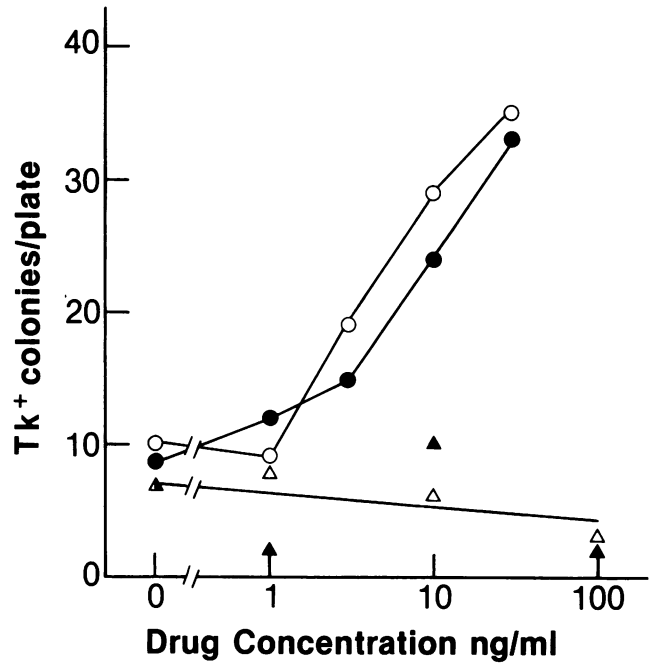

FIG. 6. Effect of mitomycin C, TPA, and mezerein on the number of A3B2 or A3B3 Tk^+ rerevertants. Symbols: ○, line A3B2 treated with mitomycin C; △, line A3B2 treated with TPA; ▲, line A3B2 treated with mezerein; ●, line A3B3 treated with mitomycin C. The effect of the drugs in nonselective medium was determined as described in the text. First, the cells were grown for 5 days in HT medium without the drug and then for 2 days in HT medium with the drug before being placed in HAT selection medium. Each point represents the number of Tk^+ rerevertants per 150-mm dish of 5×10^6 cells. During the 2-day growth period in the presence of the drug, the growth rate of the cells could not be distinguished from a control culture that received no drug. Moreover, we could see no differential effect of the drugs on the growth of Tk^- lines or Tk^+ rerevertants in HT medium. Treatment of lines A3B2 and A3B3 with mitomycin C for 5 h had no effect on the observed number of Tk^+ rerevertants, and treatment for 1 day had an intermediate effect (data not shown).

Recently, Liskay and Stachelek (23) have shown that *tk* gene reconstruction events similar to those described here between two defective *tk* genes containing DNA linker insertions can be accounted for either by a nonreciprocal exchange of genetic information or by gene conversion. Gene conversion can also be invoked to account for the recombinants observed in this report (Fig. 8). For example, a single *Eco*RI *tk* fragment could provide information to correct the *tk* gene defects in a tk^2 unit containing *Eco*RI *tk* and Δ3′*tk* without itself undergoing any detectable DNA rearrangement. If the defective *tk* genes that are corrected are adjacent to each other on the same tk^2 unit, there will be no apparent change in the number of copies of the unrecombined tk^2 units detected by Southern analysis, but if they are on different units, then the unrecombined tk^2 copy number

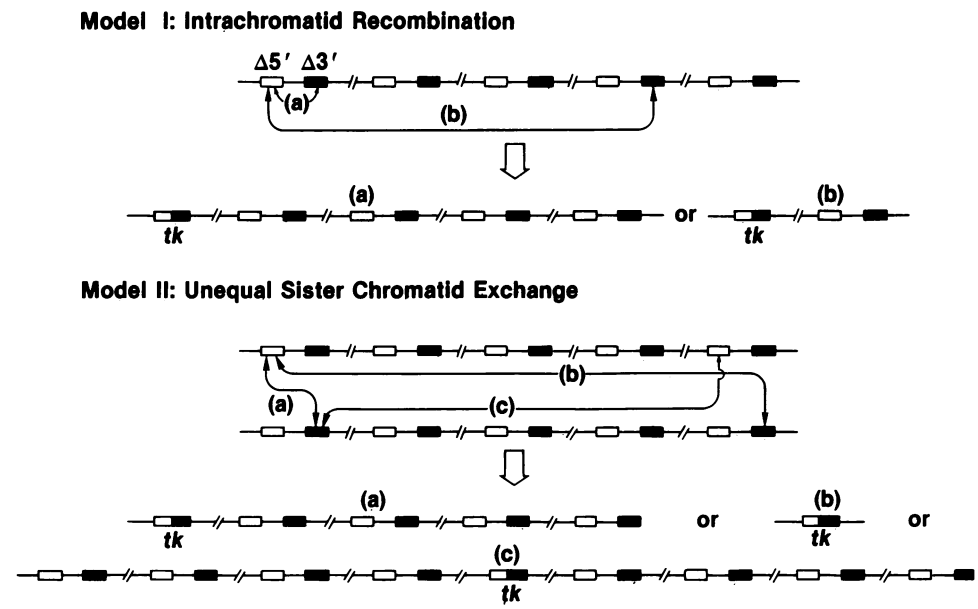

FIG. 7. Models for intrachromatid recombination and unequal sister chromatid exchange (interchromatid recombination) in *tk* gene reconstruction. We postulate that if the multiple copies of tk^2 DNA in cell lines A3B2 and A3B3 are tandemly repeated, then both intrachromatid and interchromatid recombination can generate an intact *tk* gene. In both models I and II, the difference between recombination events (a) and (b) is that in (a) most of the unrecombined DNA is retained, whereas in (b) most of it is lost. Examples of each recombinant type are found among the 10 isolated Tk^+ rerevertants of line A3B3 (Fig. 5B). In contrast, model IIc should result in an increase in the number of unrecombined tk^2 fragments not exceeding a factor of 2. Rerevertants A3B3R2 and A3B3R5 (Fig. 5B) satisfy this model.

will appear to go down. The gene conversion model, however, cannot account for the increase in copy number of the tk^2 fragments seen in cell lines A3B3R2 and A3B3R5. The recombination frequencies observed by Liskay and Stachelek (23) are generally 10 times higher than those observed here. This difference likely reflects the different substrates used in the two cases and perhaps the different chromosomal locations of those substrates.

Two questions that arise from the results of these studies and that deserve further consideration are whether the sequences undergoing recombination are present in mouse chromosomes and what factors might affect recombination efficiency. With regard to the first question, we have not definitively shown that the unrecombined tk^2 DNA is integrated into a mouse chromosome; however, several arguments can be made in favor of this possibility. First, studies by others using identical gene transfer techniques have shown that stable *tk* transformation can be correlated with the physical linking of the *tk* gene and mouse chromosomal DNA (35, 36). In the few cases analyzed, transferred DNA can be associated with specific chromosomes by in situ hybridization (31, 32, 36). Second, the cell lines (Tk^- revertants A3B2 and A3B3) that are the immediate precursors of the recombinant Tk^+ rerevertants contain the unrecombined *tk* sequences in a stable form. We have detected no change in the *tk* hybridization pattern of DNA from lines A3B2 and A3B3 during growth in HBu medium for 50 days (data not shown). Finally, Hirt extraction procedures (14), which separate small extrachromosomal DNA from larger chromosomal DNA, indicate that the *tk*-hybridizable DNA in cell lines A3B2 and A3B3 is associated with the nonextrachromosomal fraction (data not shown).

Any discussion of the factors that affect the efficiency of the recombination must, at this stage of our analysis, be purely speculative, since we do not yet have enough different examples of these events to draw any firm conclusions. In the recombination events described here, the unrecombined DNA substrate was transferred to the mouse genome by gene transfer techniques and was present at a high copy number (about 10 copies per cell) just before recombination. One wonders whether either or both of these circumstances affected the results. For example, it is has been suggested that the insertion of DNA into chromosomes by gene transfer techniques may be nonrandom, perhaps favoring regions that are susceptible to DNA rearrangement (31, 32). This possibility seems unlikely, at least in the cases studied here, in light of the low recombination levels observed. It is also possible that substrate chromosomal location will affect the efficiency of recombination. In this respect, it is interesting that from the two original Tk^+ transformants, A2 and A3, only the Tk^- revertants of the A3 line show any substantial level of rereversion to Tk^+ by recombination. Thus, lines A3B2 and A3B3 contain about 200 Tk^+ rerevertants per 10^8 cells, whereas line A2B3 contains none. The fact that *Sma*I-digested DNAs from all three Tk^- cell lines show virtually the same pattern of *tk* hybridization would seem to rule out the possibility that the differences observed reflect a gross difference in *tk* DNA content or structure. They possibly reflect differences in the location of the unrecombined *tk* DNAs in the mouse genome. For example, the rereversion of line A2B3 by recombination may be lower than that of lines A3B2 and A3B3 because the unrecombined *tk* DNA in the former case is integrated in a region of the mouse genome less able to perform a step in the recombination process than the region into which the *tk* DNA has been integrated in the latter two strains. Effects of the chromosomal location on the efficiency of DNA rearrangements have been documented extensively in eucaryotic systems (15, 20, 22; Wagner, in preparation). Another possibility, is that recombination per se in strain A2B3 is as high or higher than it is in the

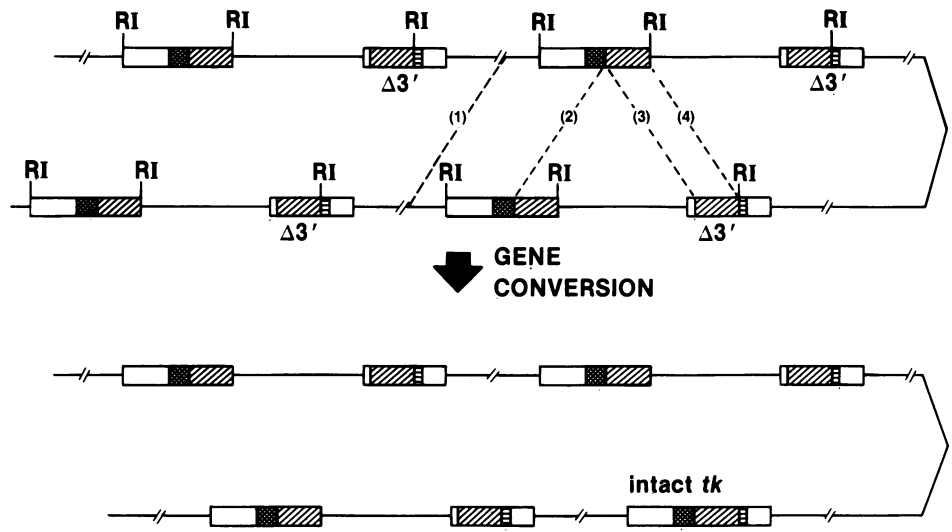

FIG. 8. Gene conversion model for *tk* gene reconstruction. Symbols: ——, λ DNA; □, HSV DNA; ⊡, DNA at the 3′ end of the *tk* gene present in the *Eco*RI *tk* fragment but deleted in the Δ3′ *tk* fragment; ▤, *tk* DNA at the 5′ end of the *tk* gene present in the Δ3′ *tk* fragment but absent in the *Eco*RI *tk* fragment; ▨, *tk* DNA shared by both the RI and Δ3′ defective *tk* genes. The model shown depicts a gene conversion event in which the *Eco*RI *tk* DNAn one sister chromatid acts to convert to the *tk* wild type two defective *tk* genes, *Eco*RI *tk* and Δ3′ *tk*, on the other sister chromatid. The conversion event requires two genetic exchanges: one within the λ or HSV DNA, between dashed lines 1 and 2, and the other in the homologous DNA sequences shared by both the *Eco*RI *tk* gene and the Δ3′ *tk* gene, between dashed lines 3 and 4.

A3 strains, but it is undetectable by our assay because the reconstructed gene cannot function in the region of the mouse genome containing the A2B3 DNA. This possibility seems unlikely because the *tk* DNA present in the original Tk^+ A2 line is functional at that genomic location. To address some of these questions, we are presently inserting unrecombined tk^2 DNA at various chromosomal sites in a single step by cotransformation with the *neo* gene (40) and using the resulting stable transformants to study the effect of chromosomal location and substrate copy number on recombination efficiency. However, if the data obtained from these studies are to be extrapolated to similar events between natural constituents of mouse chromosomes, those events will have to be measured directly. It is interesting that the incidence of sister chromatid exchange reported by others (22, 51) is about 10^{-6} per gene per cell, a value for recombination quite comparable to that observed with strains A3B2 and A3B3.

Mitomycin C is capable of both cross-linking DNA strands and producing DNA monoadducts (21, 51), and it has been shown to stimulate mitotic recombination in bacteria, and yeasts and sister chromatid exchange in mammalian cells (15, 22, 27, 52; Wagner, in press). The latter effect is only about three- to fourfold. The existing data for tumor promoters such as TPA and mezerein are equivocal; there are data both supporting (18, 26) and failing to support (45, 46) a stimulation of recombination by these agents. In this report, we observe a stimulation of Tk^- to Tk^+ rereversion by mitomycin C in strains A3B2 and A3B3 but not by TPA or mezerein in strain A3B2. None of these agents stimulates rereversion of strain A2B3. The mitomycin C result supports the recombination model for the A3 strains and suggests that these strains may be useful in screening chemicals for recombinogenic activity.

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