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Genetics

A system for assaying homologous recombination at the endogenous human thymidine kinase gene

(human lymphoblast/interallelic recombination/gene conversion)

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ABSTRACT **A system for assaying human interchromosomal recombination *in vitro* was developed, using a cell line containing two different mutant thymidine kinase genes (*TK*) on chromosomes 17. Heteroalleles were generated in the $TK^{+/+}$ parent B-lymphoblast cell line WIL-2 by repeated exposure to the alkylating nitrogen mustard ICR-191, which preferentially causes +1 or −1 frameshifts. Resulting $TK^{-/-}$ mutants were selected in medium containing the toxic thymidine analog trifluorothymidine. Mutations were characterized by exon-specific polymerase chain reaction amplification and direct sequencing. In two lines, heterozygous frameshifts were located in exons 4 and 7 of the *TK* gene separated by ≈8 kilobases. These lines undergo spontaneous reversion to TK^+ at a frequency of $<10^{-7}$, and revertants can be selected in cytidine/hypoxanthine/aminopterin/thymidine medium. The nature and location of these heteroallelic mutations make large deletions, rearrangements, nondisjunction, and reduplication unlikely mechanisms for reversion to TK^+. The mode of reversion to TK^+ was specifically assessed by DNA sequencing, use of single-strand conformation polymorphisms, and analysis of various restriction fragment length polymorphisms (RFLPs) linked to the *TK* gene on chromosome 17. Our data suggest that a proportion of revertants has undergone recombination and gene conversion at the *TK* locus, with concomitant loss of frameshifts and allele loss at linked RFLPs. Models are presented for the origin of two recombinants.**

Molecular genetic studies suggest that somatic recombination and gene conversion may play an important role in the generation of certain human diseases. Of particular interest is the reduction to homozygosity of mutations at tumor-suppressor loci as the cause of a proportion of retinoblastomas, osteosarcomas (1–3), Wilm tumors (4–6), astrocytomas (7), and meningiomas and acoustic neuromas (8, 9). Previous analyses of recombination in cultured mammalian cells have used extrachromosomal plasmid-based systems or integrated markers in tandem array (10–17), but these may not be appropriate models for recombinational events between autosomal genes in their native chromosomal environment. The identification of interchromosomal homologous recombination in mammalian cells *in vitro* has been largely confined to detecting post-S-phase recombination and the reduction to homozygosity of large chromosomal regions where the result is loss of a dominant (usually wild-type) allele or the identification of recombination-induced cytogenetic changes (18–21).

We describe a system for the analysis of recombination between alleles of the endogenous human thymidine kinase gene (*TK*) on chromosome 17. The assay detects recombination events that are initiating within or migrate through this gene. TK-deficient ($TK^{-/-}$) lymphoblasts were generated from a near-diploid $TK^{+/+}$ line by repeated exposure to the frameshift mutagen ICR-191 (22). Frameshift mutations in three mutant lines were located and characterized by *TK* exon-specific polymerase chain reaction (PCR) amplification and direct DNA sequencing. Two of these $TK^{-/-}$ mutants contain a different frameshift in each allele, and so they can revert to a TK^+ phenotype only by interallelic recombination, gene conversion, or frame-restoring point mutation. Through the examination of restriction fragment length polymorphisms (RFLPs) linked to *TK* on chromosome 17 and the use of a single-strand conformation studies in conjunction with sequencing, we have characterized two TK^+ revertants that are the result of interallelic recombination.

MATERIALS AND METHODS

Mutagenesis. $TK^{-/-}$ cells were generated by sequential exposure of 400 ml (2×10^5 per ml) of the WIL-2 $TK^{+/+}$ line (ATCC no. CRL-8155) to 0.45 μg of ICR-191 per ml (Polysciences) in complete medium with fetal calf serum for 4 hr. The cells were then resuspended in fresh medium and allowed to recover for 5–6 days prior to any further exposure. After every other round of ICR-191 treatment, TK^- mutants were selected by plating ≈4×10^8 cells in microtiter wells with 2 μg of the thymidine analog trifluorothymidine (F_3dT) per ml. F_3dT-resistant (F_3dT^r) colonies appeared after ≈11 days of selection, at which time they were picked and expanded for analysis.

PCR Amplification. Exon-specific oligonucleotide primer pairs (19–22 bases) were synthesized, based on the human *TK* genomic sequence (23). Genomic DNA (0.1 μg) was amplified using a standard PCR protocol (24) in reaction buffer that contained 20 pmol of each primer, 200 μM dNTPs, 20 mM Tris (pH 8.4 or 8.6), 50 mM KCl, 2 μg of bovine serum albumin per ml, and a range of $MgCl_2$ concentrations from 1.25 mM to 5.0 mM; 0.75 unit of Taq polymerase (AmpliTaq, Perkin–Elmer/Cetus) was used per reaction. Initial denaturation for 4 min at 94°C was followed by 35 rounds of amplification with denaturation for 10 sec at 94°C and polymerization for 30 sec at 71°C. Annealing, for 10 sec, was carried out at 45–68°C, depending on the primer pair.

Sequencing. Amplification products were examined for specificity in a 2% agarose gel. Sequencing commonly made use of the same primers used in amplification. Primer (80–100 ng) was 5′ end-labeled with [^{32}P]dATP. Direct sequencing reactions using these primers were carried out by a modification of standard chain-termination techniques (25).

RFLP Analysis. Genomic DNAs were digested and blotted onto nitrocellulose, and hybridizations were performed using

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Abbreviations: F_3dT, trifluorothymidine; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; TK, thymidine kinase; ^{r}, resistant; CHAT, cytidine/hypoxanthine/aminopterin/thymidine.

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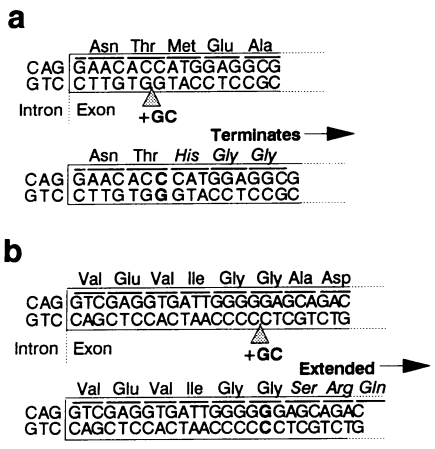

FIG. 1. Heterozygous sequence changes in lines 6:86 and 6:97. (*a*) Exon 4. The change is from GG to GGG at base pairs 4850–4851 of the *TK* genomic sequence; predicted to lead to incorporation of 31 incorrect amino acids and premature termination at residue 103. (*b*) Exon 7. The change is from GGGGG to GGGGGG at bases 12519–12523; predicted to lead to incorporation of 37 incorrect amino acids but extension of the protein by 131 residues, representing a 56% increase in monomer length.

standard techniques (26). The probe pTK11 containing full-length *TK* cDNA (27) was made available by P. Deininger (Louisiana State University). Probes pRMU3 and pTHH59 were obtained from Y. Nakamura (University of Utah) (28, 29). Probe pC63 was obtained from the American Type Culture Collection (no. 59030/1 and ref. 30).

Single-Strand Conformation Polymorphisms (SSCPs). SSCP analysis was carried out as described (31, 32). Primers S1 (5′-CACGCTCTGGCTTTCTCTTC-3′) and S2 (5′-ATGCCAAGACAAGCCAACTT-3′) were used for PCR amplification of *TK* exon 4, priming amplification from just upstream and just downstream of the exon. Primers S3 (5′-TCCTTCCTGTCCTGGCCCTT-3′) and S4 (5′-CAGTTC-TCTTTGTTGTCCGG-3′) are specific for the upstream 90 base pairs (bp) of *tk* exon 7 and adjacent intron DNA. Genomic DNA was isolated as described above and utilized as template in PCR amplification. PCR reactions include 0.5–1.0 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 1 Ci = 37 GBq; New England Nuclear), which is incorporated during amplification. The labeled PCR product (5 μl) was mixed with SSCP buffer (10% SDS/10 mM EDTA), denatured at 85°C for 5 min, and loaded onto a nondenaturing polyacrylamide gel (6% polyacrylamide/0.3% bisacrylamide/10% glycerol) run at 30 W constant power at room temperature (exon 7) or 4°C (exon 4) for 6–7 hr. Gels were dried and standard autoradiography was performed.

RESULTS

Generation of $TK^{-/-}$ Lines. ICR-191 was chosen as the mutagen as it tends to produce single base additions and deletions rather than large-scale rearrangements (33–35). Plating for F_3dT^r (TK^-) followed rounds 2, 4, 6, and 8 of ICR-191 treatment. F_3dT^r cells were not detectable after round 2 but were present at frequencies of 3.6×10^{-8} after round 4, 1.7×10^{-6} after round 6, and 1.4×10^{-5} after round 8.

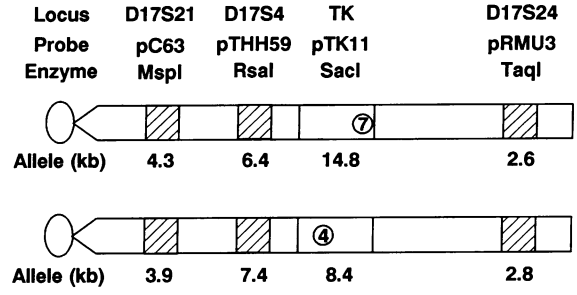

FIG. 2. Allelic maps of the q arms of the chromosome 17 homologs present in the WIL-2, B-lymphoblast line and its derivatives 6:86 and 6:97. The circled 4 and 7 represent frameshift mutations in these exons in the 6:86 and 6:97 lines. Loci are indicated along with enzymes used and RFLP allele sizes in kb.

Characterization of $TK^{-/-}$ Lines. Three TK^- lines were selected for further study: line 4:2, isolated after round 4 of ICR-191 mutagenesis, and lines 6:86 and 6:97, isolated after round 6 of ICR-191 mutagenesis. Exon-specific PCR amplification and direct sequencing revealed heterozygous frameshifts in exons 4 and 7 at the same locations in lines 6:86 and 6:97, indicating that they were apparently isolates of the same double mutant. Line 4:2 carried a homozygous exon 7 frameshift mutation at the same location as the exon 7 frameshift in 6:86 and 6:97 (data not shown). Fig. 1 shows sequence and amino acid changes predicted in $TK^{-/-}$ mutant lines 6:86 and 6:97. Although it was difficult to obtain good sequencing data for a few regions of the coding sequence, especially in exon 1, the likelihood of multiple mutations in a given *TK* allele is very low. Thus, we have assumed that the heterozygous exon 4 and 7 mutations shown in Fig. 1 are responsible for the TK^- phenotype of lines 6:86 and 6:97.

To characterize potential recombinants it was important to have a map of polymorphisms on 17q so that the fate of neighboring markers could be followed in reverting lines. The TK^- line 4:2 is homozygous for all polymorphisms we have studied on 17q (Table 1). Since karyotypic analysis confirms the presence of two chromosomes 17 in this line, it presumably arose through either nondisjunction and reduplication or somatic recombination close to the centromere following mutation in exon 7 of one *TK* allele. This cell line therefore shows us how the frameshift and polymorphisms are apportioned between the two chromosomes in the parent line (Fig. 2). The TK^- lines 6:86 and 6:97 are heterozygous for the exon 7 frameshift found in 4:2 and became $TK^{-/-}$ through the acquisition of an additional frameshift in exon 4. The allelic maps shown in Fig. 2 were derived from these data and are consistent with deletion mapping of these loci in WIL-2-derived lines carried out in this laboratory (S. Amundson and C.-Y. Li, personal communications).

Table 1. Genetic constitution of cell line chromosome 17s

Probe	Locus	Location	Enzyme	Allele, kb: Lines 6:86 and 6:97	Allele, kb: Line 4:2	Ref(s).
C63	*D17S21*	17q23-qter	*Msp* I	3.9, 4.3	4.3	34
THH59	*D17S4*	17q23-25.3	*Rsa* I	7.4, 6.4	6.4	33
TK11	*TK*	17q23.2-25.2	*Sac* I	8.4, 14.8	14.8	31
RMU3	*D17S24*	17q (distal)	*Taq* I	2.8, 2.6	2.6	32, 33
Frame-shifted *TK* exons				4 and 7	7	

kb, Kilobases.

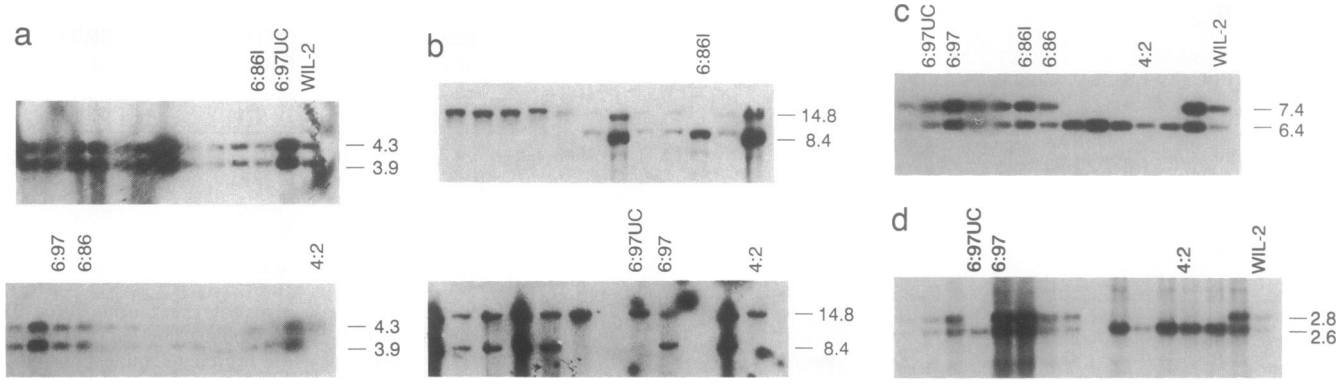

FIG. 3. Representative RFLP analyses of $TK^{-/-}$ lines and TK^+ revertants. All hybridizations were performed using nitrocellulose membranes. Allele sizes are indicated in kb. (*a*) *D17S21* locus. Genomic DNA was digested with *Msp* I and probed with pC63. Lanes are designated for the cell line DNA digested and probed. (*b*) *TK* locus. Genomic DNA was digested with *Sac* I and probed with pTK11. The first five lanes (from the left) in the upper panel are cell lines homozygous for the same allele as line 4:2. (*c*) *D17S4* locus. Genomic DNA was digested with *Rsa* I and probed with pTHH59. Lanes 8–10 and 12 are also homozygous lines. (*d*) *D17S24* locus. Genomic DNA was digested with *Taq* I and probed with pRMU3. Lanes 10–12 and 14 are homozygous lines.

Characterization of TK^+ Revertants. We have used the heteroallelic $TK^{-/-}$ lines 6:86 and 6:97 to look for recombination between the *TK* alleles. For both cell lines, the frequency of cytidine/hypoxanthine/aminopterin/thymidine-resistant ($CHAT^r$) TK^+ revertants was $\approx 10^{-7}$. The nature of these revertants was initially investigated in two ways: (*i*) RFLP analysis of the heterozygous markers on chromosome 17 as a screen for allele loss that might be the result of recombination and (*ii*) DNA sequencing to determine the fate of the exon 4 and 7 frameshifts.

Table 1 shows RFLPs employed in the analysis of TK^+ revertants. Fig. 3 shows the banding patterns for these RFLPs. A total of 63 $CHAT^r$ revertant lines was analyzed for RFLP changes. Two revertants (6:97UC, which arose spontaneously, and 6:86I, which had been exposed to ICR-191) showed banding patterns indicating a reduction to homozygosity at the *Sac* I RFLP just downstream of *TK* (Fig. 3*b*). Line 6:97UC also showed allele loss at the distal D17S24 RFLP. No change at any other RFLP was found for any other revertant examined (Fig. 3).

PCR and direct sequencing of exons 4 and 7 from the lines 6:86I and 6:97UC revealed that each had regenerated a TK^+ allele through the loss of one of the two frameshifts. Line 6:86I had reverted to wild type at the exon 7 frameshift but retained the frameshift in exon 4, whereas 6:97UC had reverted to wild type at the exon 4 frameshift but retained the exon 7 frameshift (data not shown).

Models for the Two Recombination Events. In each revertant the specific event leading to $CHAT^r$ was apparently the generation of a TK^+ allele through the restoration of wild-type sequence at the site of one of the original frameshift mutations rather than the occurrence of a second, restorative mutation within the gene. Since each revertant showed homozygosity at one or more of the RFLPs on chromosome 17, we have generated models based on these data to describe the reversion events in terms of interchromosomal recombination with Holliday junction migration, mismatch repair at the *TK* locus, and segregation. These models are presented in Figs. 4 and 5.

The remaining 61 TK^+ revertants showed no allele loss on chromosome 17 and could be the result either of frame-restoring point mutations or of pre-S-phase reciprocal recombination events occurring in the 8 kb between the exon 4 and 7 frameshifts. Such reciprocal events are expected to move both frameshifts to the same allele, producing a double-mutant allele and a wild-type active allele, producing a double-mutant allele and a wild-type active allele. The revertant phenotype would be TK^+, although both frameshifts would be retained (Fig. 6). These revertants would not be expected to show changes at any linked RFLP. It was therefore important to find a rapid way to determine the fate of the frameshifts and the surrounding *TK* sequence in revertant lines. SSCPs were found to be a rapid and simple way to analyze PCR-amplified TK^+ revertant DNA.

SSCP Analysis of Frameshifts. Under appropriate conditions the conformation of short stretches of single-stranded DNA is very sensitive to minor changes in sequence and will generate SSCP (31, 32). We have used SSCP analysis to determine the retention or loss of the exon 4 and 7 heterozygous mutations in revertants. SSCP banding patterns for wild-type and heterozygous frame-shifted exon 4 and 7 PCR products are shown in Fig. 7. Although the origin and content of each band are unknown, it is likely that the SSCP bands of wild-type DNA represent the coding and noncoding strands and that heterozygosity in a stretch of DNA will produce two additional bands.

SSCP analysis has been carried out on the DNA from a total of 38 $CHAT^r$ revertants. Results for 11 representative lines are shown in Fig. 7. Of 36 $CHAT^r$ revertants showing no change at any linked RFLP, 15 x-ray-induced and 2 UV-light-induced revertants showed new bands in either the exon 7 region or exon 4 region, suggesting a second site, frame-restoring mutation. The remaining 12 revertants showed loss of one or other frameshift mutation. The 17 TK^+ lines retaining both frameshifts with no obvious new mutation in *TK* may have undergone reciprocal recombination as outlined in Fig. 6. Detailed characterization of all of these revertants will be reported elsewhere.

DISCUSSION

We have developed cell lines that permit the detection of recombination events occurring within, or migrating through, an 8-kb stretch of DNA between the exon 4 and 7 mutations representing the two alleles of the human *TK* gene. Our results indicate that the retention of both frameshift mutations, unaccompanied by any RFLP changes, is a frequent genotype of revertants.

Line 4:2 is homozygous for the exon 7 mutant chromosome 17 but reverts spontaneously at a 5- to 10-fold lower frequency than either 6:86 or 6:97 and shows lower induced reversion rates following x-irradiation but a similar ICR-191-induced rate (data to be presented elsewhere). Because of its homozygosity, line 4:2 is not expected to revert by recombination. This difference in spontaneous and x-ray-induced

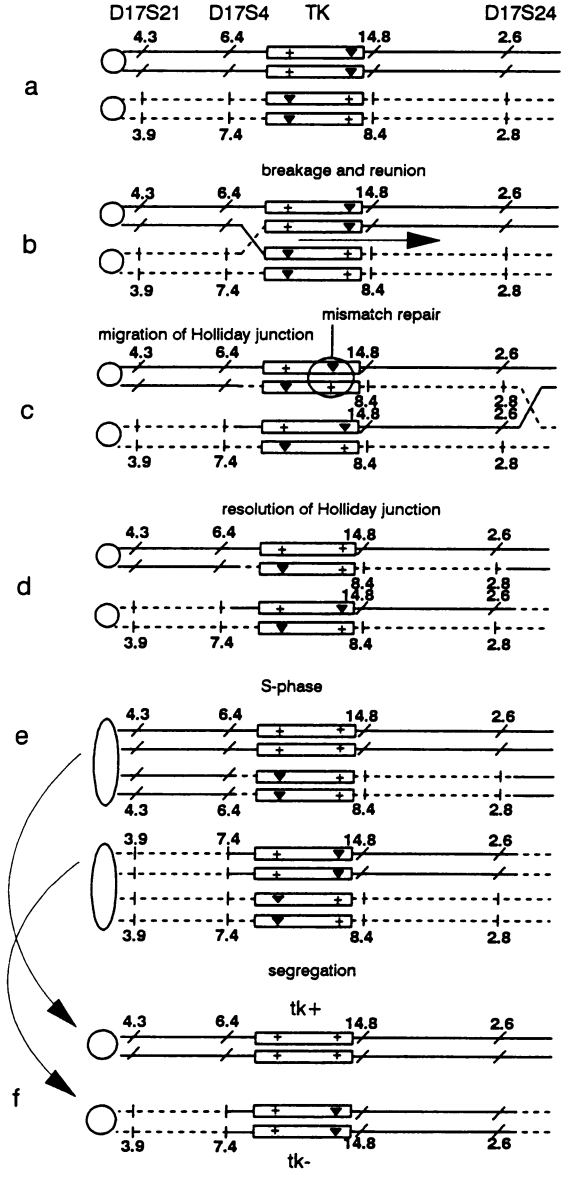

FIG. 4. Model describing the events leading to the 6:97UC revertant genotype. Loci are listed in *a* along with allele sizes (in kb). Map distances are not drawn to scale. The inverted filled triangle represents the frameshift; "+" represents wild-type sequences. In 6:97UC, sequence changes may have occurred as part of a mismatch repair tract formed during a single recombination event (*a–e*). Segregation (*f*) would then be responsible for the pattern of allele loss seen in the revertant. The 6:97 $TK^{-/-}$ genotype is shown in *a*. This model requires that the initial point of exchange took place proximal to *TK* (*b*) and that the Holliday junction formed then migrated through *TK* to the distal portion of the chromosome (*c*), bringing together a large stretch of heteroduplex DNA, probably containing several mismatches. The junction would then be resolved beyond D17S24 (*d*), possibly at the telomere, with the large expanse of heteroduplex DNA serving as a substrate for mismatch repair enzymes. This repair would lead to mismatch correction at the exon 7 mutation. The *Sac* I RFLP at *TK*, which lies ≈4 kb outside of the gene, appears not to have been included in the repair tract. After S phase (*e*), independent segregation of chromatids at mitotic anaphase would generate a line carrying the two chromosomes 17 found in the $TK^{+/-}$ 6:97UC revertant (*f*). The active *TK* allele would therefore be the product of exon 7 mismatch repair.

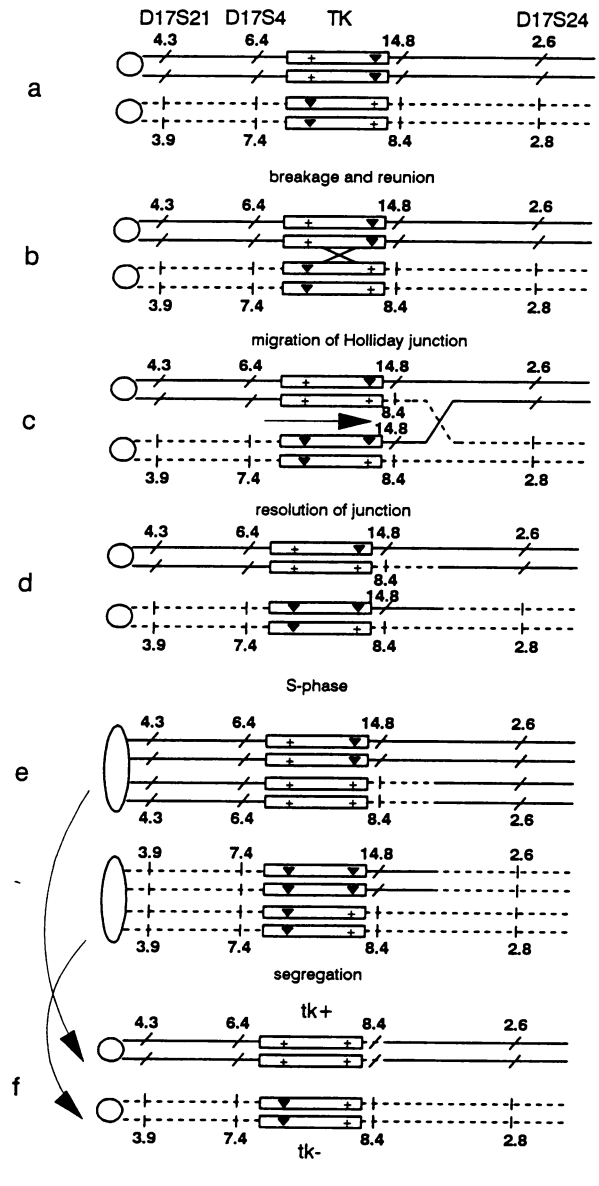

FIG. 5. Model describing the events leading to the 6:86I revertant genotype. Loci are listed in Fig. 4. For the 6:86I revertant, recombination may have occurred without accompanying mismatch repair, even though heteroduplex DNA was formed. The 6:86 $TK^{-/-}$ genotype is shown in *a*. This model requires a crossing-over event to have occurred in the 8-kb region between the exon 4 and 7 mutations (*b*). The Holliday junction formed then underwent limited migration through the gene (*c*). In this case the Holliday junction is resolved beyond the *Sac* I RFLP but proximal to D17S24 (*d*), resulting in a stretch of heteroduplex DNA much shorter than that formed during the generation of 6:97UC. There was no apparent mismatch repair at the *Sac* I RFLP. S phase (*e*) and independent segregation of chromosomes at anaphase would then lead to the generation of a $TK^{+/-}$ line carrying the 6:86I revertant genotype (*f*).

reversion but similar responses to ICR-191 suggests that recombination plays a role in a large proportion of the spontaneous and x-ray-induced reversion events in 6:86 and 6:97.

Somatic recombination in human cells has previously been examined through the use of exogenous or chromosomally integrated substrates. But there is reason to believe that neither is a good indicator of interchromosomal mammalian recombination. Evidence from prokaryotic studies indicates that extrachromosomal DNA is recombined and repaired by pathways that differ from those involved in chromosomal recombination (for example, see ref. 36). Studies of extrachromosomal and integrated recombination substrates in mouse cells (37) show that DNA sequence heterologies existing between substrate molecules dramatically reduce the frequency of intermolecular homologous recombination

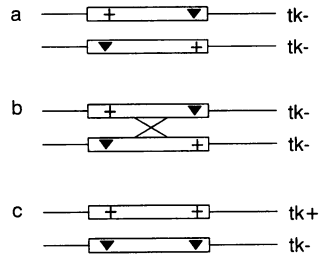

FIG. 6. Retention of heterozygous frameshifts in a reciprocal recombinant. (*a*) Genotype of the 6:86 and 6:97 $TK^{-/-}$ mutant lines showing the heterozygous nature of the two frameshift mutations. Frameshifts are represented as filled inverted triangles and wild-type sequences are represented as "+". (*b*) Simple reciprocal recombination without migration is shown occurring in the 8 kb of DNA between the two mutations. (*c*) Resulting $TK^{+/-}$ line has an active allele with no mutations and a double-mutant TK^{-} allele. Both frameshifts remain heterozygous.

when the substrates are integrated in the mouse genome. When extrachromosomal substrates are used, only a minor reduction in recombination is seen, suggesting that mismatch repair is more efficient for chromosomal DNA than for extrachromosomal sequences (38). In addition, integrated exogenous DNA shows inherent instabilities (39, 40), which might complicate those recombination studies that involve tandemly integrated substrates. Furthermore, tandemly integrated genes are unable to model recombination between genes in their native configuration and are often subject to unequal sister chromatid exchange (38, 41, 42).

The lymphoblast cell lines and selection system we describe permit the detection of very low frequency events; 10^8 or more cells can be treated and selected for reversion with relative ease. SSCP analysis reveals new mutations around exons 4 and 7, which can be confirmed by sequencing, and analysis of RFLPs on chromosome 17 will show recombinants that result in multilocus changes. The two recombinants analyzed in this paper show that human spontaneous recombination at this locus occurs in this cell line at a frequency of $\approx 10^{-9}$. An additional 61 revertants await analysis for sequence changes at exons 4 and 7; preliminary data suggest that they consist of second site point revertants and reciprocal recombinants. Reciprocal recombinants are expected to possess wild-type and double-mutant alleles (Fig. 6) and to produce wild-type and double-mutant mRNA. cDNA analysis is necessary to further characterize those revertants that retain both frameshifts and are potential reciprocal recombinants.

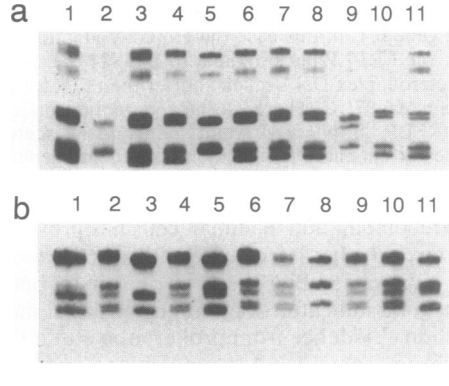

FIG. 7. Single-strand conformation analysis of exon 4 and 7 mutations analyzed by nondenaturing acrylamide gel electrophoresis. (*a*) Exon 4 DNA PCR products: 6:86 DNA (lane 4), 4:2 (lane 7), 6:86I (lane 1), and 6:97UC (lane 2). (*b*) Exon 7 DNA PCR products: 6:86 DNA (lane 2), 4:2 (lane 8), 6:86I (lane 3), 6:97UC (lane 5), WIL-2 (lane 1), and 6:97 (lane 4).

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