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X Rays Induce Interallelic Homologous Recombination at the Human Thymidine Kinase Gene

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We have developed a human lymphoblast cell line for the study of interchromosomal homologous recombination at the endogenous thymidine kinase (*tk*) gene on chromosome 17 (M. B. Benjamin, H. Potter, D. W. Yandell, and J. B. Little, Proc. Natl. Acad. Sci. USA 88:6652–6656, 1991). This cell line (designated 6:86) carries unique heterozygous frameshift mutations in exons 4 and 7 of its endogenous *tk* alleles and can revert to TK+ by frame-restoring mutations, gene conversion, or reciprocal recombination. Line 6:86 reverts spontaneously to TK+ at a frequency of 10^{-7} to 10^{-8}, and exposures to X-irradiation or the frameshift mutagen ICR-191 induce increased reversion frequencies in a dose-dependent manner. Another cell line (designated 4:2) carries a homozygous exon 7 frameshift and is not expected to revert through mechanisms other than frame-restoring mutation. Line 4:2 reverts to TK+ at a lower spontaneous frequency than does 6:86 but can be induced with similar kinetics by ICR-191. In contrast to line 6:86, however, X rays did not induce detectable reversion of line 4:2. We have characterized a number of 6:86-derived revertants by means of restriction fragment length polymorphism analysis at *tk* and linked loci, single-strand conformation polymorphisms, and direct transcript sequencing. For X rays, most revertants retain both original mutations in the genomic DNA, and a subset of these frameshift-retaining revertants produce frameshift-free message, indicating that reversion is the result of reciprocal recombination within the *tk* gene. Frame-restoring point mutations, restoration of original sequences, and phenocopy reversion by acquisition of aminopterin resistance were also found among X-ray-induced revertants, whereas the ICR-191-induced revertants examined show only loss of the exon 7 frameshift.

Molecular genetic analysis has yielded evidence to support a role for somatic interchromosomal homologous recombination in the genesis and progression of a number of human tumors (8, 16, 33, 42, 54). Evidence has been presented recently for spontaneous somatic recombination in cells from patients with cancer-predisposing Bloom's syndrome (24, 36), and this process has also been implicated in the apparent polyclonal origin of some human tumors (65). The very low frequency with which human somatic chromosomes appear to undergo homologous recombination has made the study of this phenomenon particularly difficult. The use of plasmid-based extrachromosomal recombination assays (for a review, see reference 11; 39, 50, 60, 71, 72), recombination between tandemly arranged genes integrated into mammalian chromosomes (for reviews, see references 11 and 34; 3, 9, 10, 28–30, 69), or recombination between transfected and endogenous sequences (for a review, see reference 11; 1, 5, 47, 64) has proven to be effective for analyzing both the mechanisms and induction of mammalian somatic recombination.

Synthetic systems of this nature, however, are unable to accurately model the qualitative and quantitative homologous interactions that occur between mammalian somatic chromosomes. Studies of recombination between native mammalian chromosomes (for examples, see references 23, 46, and 70) have generally relied on the detection of homozygous segregants arising in culture from heterozygous parent lines. Densitometric analysis of Southern blots and an evaluation of restriction fragment length polymorphisms (RFLPs) are then used to distinguish hemizygotes from homozygotes and nondisjunctional loss from interchromosomal recombination. Only one product of the putative recombination event, the daughter cell carrying a partially homozygous chromosome, can be isolated with these systems.

In an effort to design an interchromosomal recombination system that relies only on endogenous native human DNA, the interaction between endogenous human chromosomes, and the retention of both recombination products, we have developed a cell line designated 6:86 (6). This line, developed by mutagenesis from the diploid B-lymphoblast cell line WIL-2 (37) (ATCC CRL 8155), carries unique frameshift mutations in each allele of the endogenous thymidine kinase (*tk*) gene, rendering it TK− and sensitive to cytidine-hypoxanthine-aminopterin-thymidine) medium (CHAT). Line 6:86 reverts spontaneously to TK+ and CHAT^r at a low frequency of 10^{-7} to 10^{-8}, but reversion is inducible by exposures to X-irradiation or the frameshift mutagen ICR-191. Line 4:2, which carries a homozygous frameshift mutation, does not show X-ray-inducible reversion in this manner. Through the analysis of chromosome 17-linked RFLPs, single-strand conformation polymorphisms generated by the heterozygous mutations in the gene, and direct sequencing of polymerase chain reaction (PCR)-amplified transcripts from revertants, we have been able to characterize reversion events at the molecular level. A large proportion of X-ray-induced revertants from line 6:86 retain both frameshifts in their genomic DNA, and a subset of these produce frameshift-free message. We present evidence for the involvement of reciprocal recombination between *tk* alleles in these reversion events.

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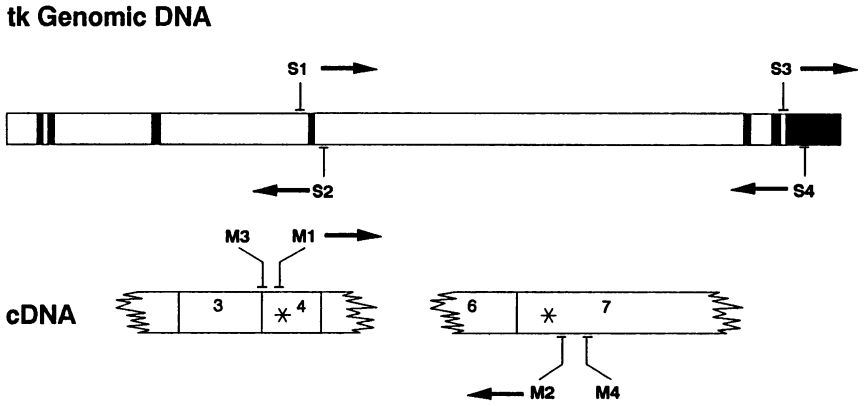

FIG. 1. Diagram of *tk* genomic DNA and cDNA indicating the relative locations of primers used for PCR-SSCP analysis and sequencing. For *tk* genomic DNA, exons 1 to 7 are shown as dark panels. For *tk* cDNA, those regions encompassing exons 3 and 4 and exons 6 and 7 are shown. Arrows indicate primer extensions during PCR. *, site of frameshift mutation.

MATERIALS AND METHODS

Mutagenesis. (i) X-irradiation. For each X-ray exposure, 50 ml of cells at approximately 5×10^5 cells per ml was exposed in a T75 culture flask centered in the irradiation field of a G.E. Maximar X-ray source operated at 220 keV and 15 mA, yielding a dose rate of 78 cGy/min. Cells were allowed to recover and were plated to measure cloning efficiency and reversion frequency on day 3 after exposure.

(ii) ICR-191 exposure. For each dose of ICR-191, 25 ml of cells at approximately 5×10^5 cells per ml was incubated with ICR-191 for 3.5 h. At the end of this period, cells were centrifuged at 2,000 rpm and resuspended in 25 ml of prewarmed medium supplemented with 10% fetal calf serum. Cells were plated for cloning efficiency and reversion on day 3 after exposure.

(iii) Plating. Cells from each experiment were seeded in 96-well microtiter dishes. For cloning efficiency and toxicity, cells were plated at either 1 or 100 cells per well, depending on dose, in RPMI 1640 medium supplemented with 10% fetal calf serum. For reversion frequencies, cells were seeded at either 5×10^3 or 2×10^4 cells per well, depending on cell line, in medium supplemented with CHAT and 15% fetal calf serum.

Molecular genetic analysis. (i) RFLP analysis. Genomic DNAs were digested with *Msp*I, *Sac*I, or *Taq*I, using the conditions recommended by the supplier (New England Biolabs). Blotting onto nitrocellulose and hybridizations were performed by using standard techniques (41). Probe pTK11 (12) was made available by P. Deininger (University Medical Center, New Orleans, La.). This probe contains full-length *tk* cDNA. Probes pRMU3 (48, 49) and pTHH59 (27, 49) were obtained from Y. Nakamura (University of Utah, Salt Lake City). Probes pC63 (32) and p128-E5 (40) were obtained from the American Type Culture Collection (no. 59030/1 and 59318/9, respectively).

(ii) Reverse transcription. Cell lysis and reverse transcription were carried out by using a modification of the procedure of Yang et al. (78). A volume containing 10^6 cells in exponential growth was centrifuged at 2,000 rpm, and the cell pellet was resuspended in 5 ml of ice-cold phosphate-buffered saline (PBS). Cells were washed with PBS in this manner three times and resuspended in 1 ml of PBS. For 10^3 or 10^4 cells, aliquots from this resuspension, of 1 or 10 μl, respectively, were used for reverse transcription. For 10-μl aliquots, cells were pelleted for 5 min at 14,000 rpm in a bench-top microcentrifuge, and 9 μl was aspirated from the pellet. To the 1 μl of pelleted cells (either 10^3 or 10^4 cells) was added 5 μl of a lysis-reverse transcription reaction mix containing 1× reverse transcription buffer (Bethesda Research Laboratories), 10 mM dithiothreitol, 500 μM each deoxynucleoside triphosphate (dNTP), 100 μg of bovine serum albumin (BSA) per ml, and 0.025% Nonidet 40; 1 μl of RNasin (10 U/μl; U.S. Biochemical) and 0.5 μl (100 U) of murine mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories) were added. Primer for reverse transcription was 50 ng of the 20-base oligonucleotide M4 (5′-TTCCCCTGGCTTTCCTGGCA-3′), which specifically primes from exon 7 of the *tk* mRNA (Fig. 1). This reaction mix was incubated for 90 min at 37°C.

(iii) PCR amplification. For PCR amplification of genomic DNA, exon-specific oligonucleotide primer pairs (19 to 22 bases) were synthesized on the basis of the human *tk* genomic sequence (21) (Fig. 1). Genomic DNA (0.1 μg) was amplified by using an Ericomp temperature cycler (Ericomp Inc., San Diego, Calif.) and conditions modified from those established by Saiki et al. (61). PCR buffer contained 20 pmol of each primer, 200 μM dNTPs (Pharmacia), 20 mM Tris (pH 8.4 or 8.6), 50 mM KCl, 2 μg of BSA per ml, and a range of $MgCl_2$ concentrations from 1.25 to 5.0 mM. *Taq* polymerase (AmpliTaq; Perkin Elmer Cetus) was used at 0.75 U per reaction. Initial denaturation for 4 min at 94°C was followed by 35 rounds of amplification with denaturation for 10 s at 94°C and polymerization for 30 s at 71°C. Annealing, for 10 s, was carried out at 45 to 68°C, depending on the primer pair.

For cDNA, reverse transcription reaction mix (see above) was used as the template for round 1 of PCR amplification within 30 min of the initial 90-min incubation. PCRs (50 μl) were carried out as described for genomic DNA except for the use of different primers. For round 1 of amplification, primers M3 (5′-ACAGAGTTGATGAGACGCGT-3′) and M4 (see above), which generate a 542-bp fragment, were used. Only extremely faint bands of the expected size could be visualized after loading of 10% of this initial reaction mix in ethidium-stained agarose gels. One microliter of this initial product was then used as the template in a second round of PCR in which nested primers M1 (5′-ATGAGTCGACACA CATGACCGGAA-3′) and M2 (5′-AAGTGTCGACGGAGT GGTACTTGT-3′) were used. The relative locations of these primers in *tk* cDNA are shown in Fig. 1. Examination of

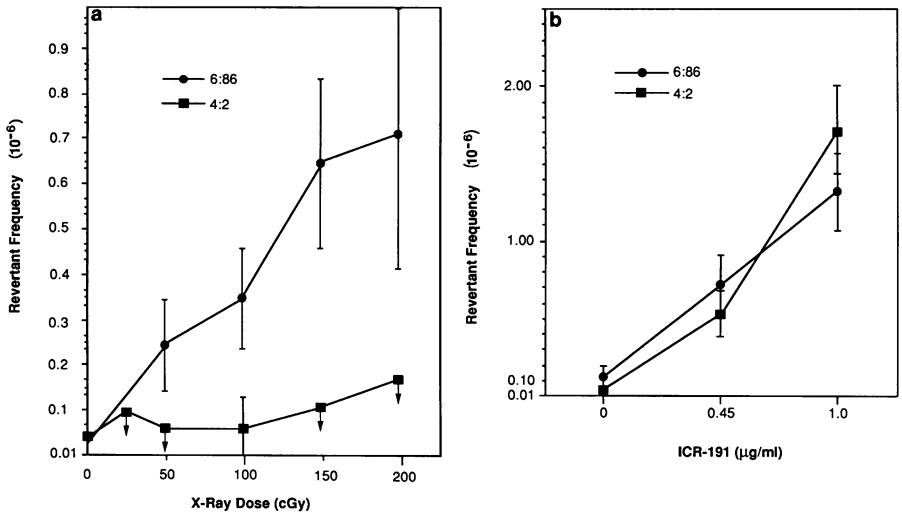

FIG. 2. Reversion frequencies for the heterozygous line 6:86 and homozygous line 4:2 as a function of mutagen exposure. (a) X-irradiation. 4:2 reversion frequencies were below detectable limits at 0, 25, 50, 150, and 200 cGy and are represented as arrowheads, based on the estimated frequency if a single revertant had been observed. Datum points and error bars are based on results obtained from five separate experiments. Survival declined in a dose-dependent manner to 9 and 29% at 200 cGy for lines 6:86 and 4:2, respectively. (b) ICR-191 exposure. Survival declined in a dose-dependent manner to 49 and 80% at 1.0 μg/ml for lines 6:86 and 4:2, respectively.

product from this second round of PCR shows the expected fragment of 370 bp. To limit the ability of these primers to use genomic DNA as a template, primer M3 spans the exon 3-exon 4 junction in *tk* cDNA.

(iv) SSCP analysis. Single-strand conformation polymorphism (SSCP) analysis was carried out as described by Orita et al. (53). 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′-CAGTTCTCTTTGTTGTCCGG-3′) are specific for the upstream 90 bp of *tk* exon 7 and adjacent intron DNA (Fig. 1). Primers M1 and M2 (see above) are specific for the portion of *tk* cDNA carrying both the exon 4 and exon 7 frameshift mutations (Fig. 1). Genomic DNA or cDNA was isolated as described above and used as the template in PCR amplification. PCR mixes included 0.5 to 1.0 μl of [^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear), which is incorporated during amplification. The labeled PCR product (5 μl) was mixed with SSCP buffer (10% sodium dodecyl sulfate, 10 mM EDTA), and 2 μl of this mixture was mixed 1:1 with Sequenase stop solution (U.S. Biochemical). This final mix was denatured at 85°C for 5 min. The single-stranded PCR products were loaded onto a nondenaturing polyacrylamide gel (6% polyacrylamide, 0.3% bisacrylamide, 10% glycerol) and run at 30-W constant power at room temperature (exon 7) or 4°C (exon 4) for 6 to 7 h. Gels were dried, and autoradiography was performed for 6 to 10 h at −80°C in the presence of an intensifying screen.

(v) Direct cDNA 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 to 100 ng) was end labelled with [γ-^{32}P]dATP (New England Nuclear) and polynucleotide kinase (New England Biolabs). Direct sequencing reactions using these primers were carried out by a modification of standard chain termination techniques (75).

RESULTS

Two cell lines have been used in this analysis; both are described in detail elsewhere (6). Line 6:86 is a tk−/− cell line carrying different heterozygous +1 frameshift mutations in each *tk* allele. Heterozygous frameshifts are located at monotonous GC base pair runs in exon 4 (bp 4850 to 4851) and exon 7 (bp 12519 to 12523) separated by approximately 8 kb of genomic sequence. Line 6:86 represents combined data for lines previously identified as 6:86 and 6:97 (6), since our analysis shows these lines to be isolates of the same heteroallelic mutant. Line 4:2 is homozygous for all RFLPs analyzed on chromosome 17q; it has no exon 4 mutations but carries a homozygous frameshift mutation in exon 7 at the same location as that (bp 12519 to 12523) in the heterozygous line 6:86 (6).

Reversion frequencies. Figure 2a shows reversion frequencies for the 6:86 and 4:2 lymphoblast lines as a function of X-ray exposure. The homozygote 4:2 shows little or no induction by X rays at any dose, whereas the heterozygote 6:86 demonstrates a dose-dependent increase in reversion frequency which reaches 20-fold over spontaneous levels with 200 cGy. Figure 2b shows reversion frequencies for these two lines as a function of exposure to the frameshift mutagen ICR-191. In contrast to the X-ray response, 6:86 and 4:2 show similar dose-response kinetics when exposed to this agent.

RFLP analysis of revertants. Of 37 independently derived spontaneous and induced revertants analyzed for RFLP changes at the *tk* locus and linked loci on chromosome 17, none showed novel Southern banding patterns and only two showed any loss of heterozygosity. These two revertant lines, 6:86I and 6:97UC, neither the result of X-irradiation, appear to be the result of interchromosomal recombination events which occurred in the vicinity of *tk* but were resolved outside of the gene. They are described in detail elsewhere (6).

TABLE 1. SSCP genotypes for 6:86 revertant lines

Type of revertant	No. (%) with SSCP genotype[a]:					Total
	FS/+ FS/+[b]	+/+ FS/+[c]	FS/+ +/+[d]	N/+ FS/+[e]	FS/+ N/+[f]	
X ray induced	14 (52)	3 (11)	4 (15)	4 (15)	2 (7)	27
ICR-191 induced	0	0	6	0	0	6
Spontaneous	0	1	0	0	1	2
Total	14	4	10	4	3	35

[a] For exon 4 (top line) and exon 7 (bottom line).
[b] Retention of heterozygous frameshifts in both exons 4 and 7.
[c] Loss of frameshift (reversion to homozygous wild type) at exon 4, retention of heterozygous frameshift in exon 7.
[d] Retention of heterozygous frameshift in exon 4, loss of frameshift (reversion to homozygous wild type) at exon 7.
[e] Novel banding pattern (likely second-site mutation) in exon 4, retention of heterozygous frameshift in exon 7.
[f] Retention of heterozygous frameshift in exon 4, novel banding pattern (likely second-site mutation) in exon 7.

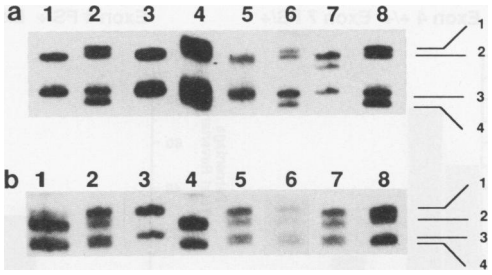

FIG. 3. SSCP analysis of TK+ revertants. (a) Exon 4; (b) exon 7. Lanes: 1, line WIL-2 (TK+ wild type); 2, line 6:86 (tk−/− heterozygote); 3, line 4:2 (tk−/− homozygote); 4, line 6:86I (TK+ revertant, loss of exon 7 mutation); 5, line 6:97UC (TK+ revertant, loss of exon 4 mutation); 6, line 6:86X3C+1 (TK+ revertant, retaining both frameshifts); 7, line 6:86 X2C+1 (TK+ revertant, new pattern in exon 4); 8, line 6:86X2B+2 (TK+ revertant, new pattern in exon 7). For exons 4 and 7, bands are labelled 1 to 4. Empirical data suggest that for exon 4 (a), bands 2 and 3 represent wild-type coding and noncoding strands. This is most clearly seen in lane 1. Exon 4 frameshifted coding and noncoding strands appear in the autoradiograph as bands 1 and 4 and are evident along with wild-type coding and noncoding bands from the 6:86 heterozygous mutant in lane 2. For exon 7 (b), wild-type coding and noncoding strands are represented by bands 2 and 4, with frameshifted strands appearing as bands 1 and 3. This is particularly evident in the homozygous mutant line 4:2 (lane 3), which demonstrates only bands 1 and 3.

SSCP analysis of revertants. The exon 4 and 7 frameshift mutations generate SSCPs which are readily resolved in nondenaturing polyacrylamide gels and were used as a rapid screen for the retention or loss of the exon 4 and 7 frameshifts in TK+ revertants. To optimize conditions for the detection of novel SSCPs caused by compensating base additions or deletions, small amplification products (approximately 150 bp) were required (53). Primers S1 and S2 (see above and Fig. 1) were designed to amplify a 140-bp genomic fragment containing the exon 4 frameshift, and primers S3 and S4 (see above and Fig. 1) were designed to generate a 150-bp fragment containing the exon 7 mutation. Using these primer pairs, it was possible to determine the retention or loss of either frameshift and to detect second-site mutations which arise within these 140- to 150-bp amplification fragments.

Data from SSCP analysis of 35 revertants are shown in Table 1, and examples of SSCPs formed by the exon 4 and 7 fragments in mutants and revertants are shown in Fig. 3.

X-ray-induced revertants. As determined by SSCP analysis, all classes of reversion event are represented among the 27 X-ray-induced revertants (Table 1 and Fig. 3). These include the retention of both heterozygous frameshifts, reversion to wild type at exon 4 (Fig. 3a) or exon 7 (Fig. 3b), and new mutations (novel SSCP bands) lying close to the original frameshifts. Retention of both frameshifts is the most common genotype of X-ray-induced revertants (Table 1). Figure 4 shows a breakdown of each SSCP genotype as a function of dose. Retention of both frameshifts forms an increasing proportion of revertant genotypes with increasing X-ray dose from 25% (one of four) at 50 cGy to 78% (seven of nine) at 200 to 250 cGy. All other SSCP genotypes show a general decline as a proportion of total revertants with increasing dose (Fig. 4).

ICR-191-induced revertants. In contrast to our results with ionizing radiation, all six ICR-191-induced revertants analyzed by SSCP show loss of the exon 7 frameshift mutation (Table 1). One of these revertants (6:86I), thought to have arisen as the result of an interchromosomal recombination event resolving outside of *tk*, is described elsewhere (6).

Spontaneous revertants. Because of the very low spontaneous reversion frequency seen in these experiments, few spontaneous revertants were available for study. Of the two subjected to SSCP analysis in this study, one showed a novel exon 7 banding pattern suggesting a second-site mutation close to the exon 7 frameshift. The second revertant is line 6:97UC, which lost the exon 4 mutation and is thought to have occurred as a result of interchromosomal recombination resolving outside of *tk* as described elsewhere (6).

Analysis of revertants which retain both frameshifts in their DNA. The lack of any change in SSCP banding pattern in a $CHAT^r$ revertant would likely indicate one of the following events, as shown diagrammatically in Fig. 5: (i) a phenocopy, i.e., generation of $CHAT^r$ without restoration of *tk* function in a line that is still tk−/− (Fig. 5a); (ii) the presence of a frame-restoring, second-site mutation in one allele, not revealed by SSCP analysis, which would lead to the restoration of TK function in a *tk*+ allele containing a stretch of frameshifted sequence (Fig. 5b); or (iii) a reciprocal recombination event with crossing over occurring within the 8 kb of genomic DNA separating the exon 4 and 7 allelic mutations, which would displace both frameshift mutations to one double-mutant *tk*− allele, while the reciprocal product would be a functional and frameshift-free *tk*+ allele (Fig. 5c).

A proportion of revertants demonstrating retention of both frameshifts do appear to be TK+ phenocopies. These cell lines, although $CHAT^r$ (apparently TK+), remain trifluorothymidine resistant (F_3TdR^r) (apparently TK−). Subcloning of these lines from single cells showed that they represent not a mixed population of TK+ and TK− cells but a clonal population of cells each of which is simultaneously $CHAT^r$ and F_3TdR^r. Further analysis revealed that these cell lines survived plating in aminopterin alone, so that their apparent $CHAT^r$ is in fact due to aminopterin resistance (Am^r), not the presence of TK activity (data not shown). We conclude that these revertants are TK+ phenocopies and in fact represent TK− lines that have developed Am^r (see Discussion). Phenocopy revertants, however, represent a small proportion of the total revertants induced by X rays (3 of 27, or 11%).

Reciprocal recombinants produce wild-type message. Reverse-transcribed mRNAs isolated from frameshift-retaining

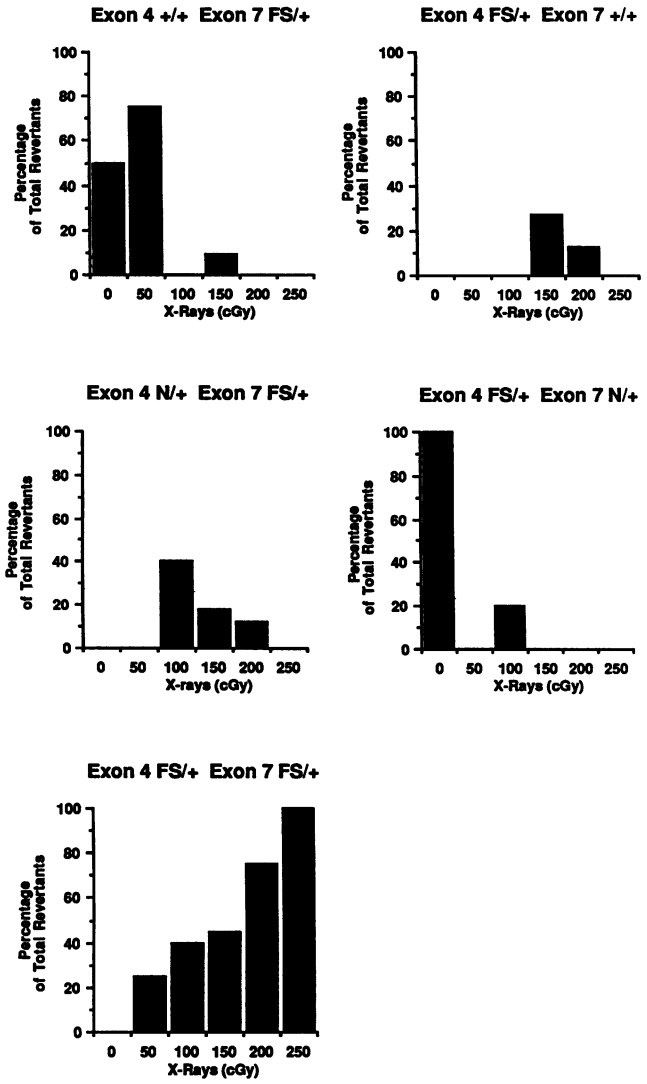

FIG. 4. Histograms showing SSCP revertant genotype as a function of X-ray dose. In each histogram, vertical bars represent the proportion of revertants exhibiting the SSCP genotype described arising at that X-ray dose. SSCP genotypes: FS/+, heterozygous frameshift; +/+, homozygous wild type; N/+, new banding pattern, likely second-site mutation. Bars at 0 cGy represent spontaneous revertants.

revertants, which were not phenocopies, were sequenced directly from PCR products. The expected complications from simultaneously sequencing two different messages (if a true recombinant were being analyzed, we would expect a wild-type message and a double-mutant message; Fig. 5) were not encountered. In all true revertants that retain both frameshifts, only one message was present; cDNAs representing the exon 4 mutant message were not detectable and appear inaccessible to reverse transcription in these lines (see Discussion). Single cDNAs showing wild-type sequences at exon 4 were always obtained following reverse transcription. The absence of frameshifted cDNA sequences in exon 7 therefore became the principal determinant of reciprocal recombination in revertant lines which had retained both genomic frameshifts.

Four independently isolated, X-ray-induced revertant

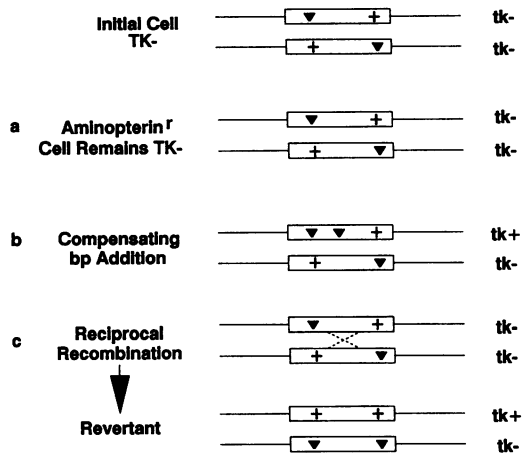

FIG. 5. Proposed mechanisms of reversion in 6:86 TK+ revertant lines. In each case, frameshifts are represented by inverted triangles. Wild-type sequences are represented by +. (a) Phenocopies; cell is an apparent revertant due to Am^r but remains TK−. (b) A compensating frameshift mutation in either allele; restores the reading frame to permit TK protein synthesis and leaves an undefined region of coding sequence out of phase. (c) A reciprocal recombination event in the 8 kb separating the two mutations; leads to displacement of both mutations to the same allele.

lines (6:86X3C+1 and three others) which showed heterozygous frameshifts at exons 4 and 7 in genomic DNA produced a single message which is wild type for exon 4 and exon 7 sequences. Figure 6 shows cDNA sequences obtained from exon 7 of line 6:86X3C+1. This revertant, induced by

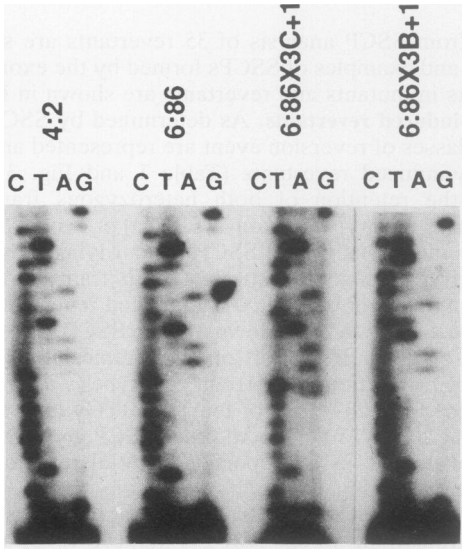

FIG. 6. Examples of a directly sequenced cDNA exon 7. cDNAs isolated from lines 4:2 (TK−; the homozygous exon 7 mutant), 6:86 (TK−; the heterozygous mutant), 6:86X3C+1 (a reciprocal recombinant retaining both genomic frameshifts), and 6:86X3B+1 (an exon 7 revertant) are shown. In lines 4:2 and 6:86, the frameshift mutations appear as six C residues, whereas the wild-type sequences seen for lines 6:86X3C+1 and 6:86X3B+1 exhibit only five. Line 6:86X3C+1 retains both genomic frameshifts, whereas 6:86X3B+1 demonstrates the loss of the exon 7 mutation in genomic DNA.

exposure of line 6:86 to 200 cGy of X-irradiation, retains both genomic frameshifts (Fig. 3). We believe that these lines represent revertants which arose through reciprocal interchromosomal homologous recombination within the *tk* gene (Fig. 5c). The retention of heterozygous genomic frameshifts is predicted in a reciprocal recombinant (Fig. 5c), and we believe that the presence of wild-type message in a line which clearly demonstrates retention of both frameshifts is strong evidence for reciprocal recombination. All cDNAs analyzed, as mentioned above, including these four lines, show wild-type sequence in exon 4.

X-ray-induced reversion frequencies for line 6:86 at 150 and 200 cGy were 6×10^{-7} and 7×10^{-7}, respectively. From the fraction of revertants retaining both frameshifts that produced wild-type message, we calculate that reciprocal recombinants make up 18% of reversion events at 150 cGy and 12.5% of reversion events at 200 cGy, yielding reciprocal recombination frequencies of approximately 10^{-7} at both doses.

DISCUSSION

The data presented here demonstrate the inducibility of reciprocal interchromosomal recombination at the endogenous *tk* gene by exposure of the cell line 6:86 to X rays. We believe that this is the first demonstration of inducible interchromosomal recombination between the alleles of an endogenous human gene. In contrast to X-irradiation, the alkylating nitrogen mustard ICR-191 appears to induce reversion by exon 7 frame-restoring mutations only.

The exon 7 frameshift, which was originally generated by ICR-191 exposure (6), is a +1 change from GGGGG to GGGGGG. It is therefore a likely target for ICR-191 mutagenesis, which has a preference for GC base pair additions or deletions in monotonous runs of these nucleotides in *Escherichia coli* (7, 13), *Salmonella* (2, 52), and mammalian (66) cells. The deletion of any one of these six consecutive GC pairs would restore the reading frame at this site. The exon 4 frameshift is a GG-to-GGG +1 mutation and is thus a smaller target for ICR-191 deletion-reversion. No revertants were seen at this site. In light of this, the generally similar ICR-191-induced reversion frequencies in lines 4:2 and 6:86 are not unexpected, since both lines carry the exon 7 frameshift mutation. Line 4:2 showed a higher level of induction (40-fold versus 10-fold over spontaneous-reversion frequencies for line 6:86; Fig. 2b) with 1.0 μg of ICR-191 per ml, consistent with the presence of two exon 7 mutations in this homozygous line.

The marked difference in the inducibility of reversion by X rays in lines 4:2 and 6:86 (Fig. 2a) suggests a role for changes other than frame-restoring mutations in the reversion of 6:86. Since the loss of the exon 4 frameshift is responsible for few X-ray-induced 6:86 revertants (3 of 27; Table 1 and Fig. 4), this mutation does not appear to be a primary factor in the higher X-ray-induced reversion frequency of this cell line. We believe that this differential dose response suggests a major role for heterozygous *tk* DNA in the reversion event and is consistent with our data for induced recombination in these lines.

Ionizing radiation has been shown to induce DNA single- and double-strand breakage (for recent studies, see references 4, 17, 18, 43, 44, 57, and 74). There is evidence for DNA strand breakage as an initiating step in yeast meiotic (58, 67) and mitotic (51, 63) recombination as well as in intermolecular recombination in mammalian cells (35). Thus, ionizing radiation would appear to be a good candidate as a mammalian recombinogen. X rays do behave as recombinogens in yeast cells (for examples, see references 19, 45, and 59) and appear to induce intermolecular homologous recombination in *Xenopus* oocytes (68) and in *Drosophila* species (56).

Attempts to induce recombination between homologous sequences in mammalian cells by exposures to various sources of ionizing radiation have proven largely unsuccessful. Recombination between duplicated herpes simplex virus *tk* genes integrated into the mouse genome could be induced by a number of agents but not ^{60}Co-generated gamma radiation (73). Similarly negative results were obtained with X rays by using integrated *neo* genes in Chinese hamster ovary cells (30). In vivo studies in mice have yielded conflicting results; by use of twin spot formation as an indication of somatic recombination, irradiation of mouse fetuses with 150 cGy led to little or no induction (20), but studies using the loss of dominant allele expression at the mouse W locus suggest that somatic recombination was inducible in vivo by ionizing radiation (55). X-irradiation of the human TK6 tk+/− heterozygote generated a large number of mutants showing loss of heterozygosity (LOH) for *tk* and chromosome 17-linked loci, which, the authors suggest, might represent the products of somatic recombination (76, 77).

Recent data collected in this laboratory indicate that X-ray-induced tk− mutants derived from the TK6 heterozygote show concurrent LOH at unlinked loci, implying that X-ray-induced LOH might be an untargeted event in the mammalian nucleus (38). LOH was associated with homozygosity; that is, two copies of the remaining allele were present as determined by densitometry. If this LOH represents somatic recombination, such radiation responses might be inducible cellular events as opposed to direct interactions of radiation with DNA. The target size for somatic recombination would therefore be considerably reduced and would approach that for X-ray-induced point mutations (25).

A large body of information regarding recombination mechanisms, frequencies, and responses to DNA damaging agents exists in the literature, derived primarily from studies of extrachromosomal and intrachromosomal recombination substrates (for a review, see reference 14). Extrachromosomal recombination assays using nonreplicating plasmid substrates have shown that recombination frequencies may be extremely high, up to one event per molecule (22), whereas intrachromosomal recombination assays using chromosomally integrated tandem markers have shown recombination frequencies in the range from 2.3×10^{-8} to 1.2×10^{-4} (11). However, the use of different substrates, cell lines, and species and the random integration of intrachromosomal substrates into the host genome make a meaningful comparison between different intrachromosomal recombination assays or between intrachromosomal assays and the interchromosomal recombination assay used in this study impossible.

The Am^r phenocopy revertants seen in the experiments presented in this report are most likely the result of dihydrofolate reductase gene amplification (see reference 62 for a review). This well-characterized phenomenon has been demonstrated in radiation-exposed mammalian cell lines (26) and appears to be restricted to X-irradiated 6:86 revertants in our experiments. Notably, gene amplification of this nature has been proposed to occur through recombinational mechanisms (62).

Of the 14 X-ray-induced revertants that retain both frameshifts in their DNA, four demonstrated wild-type message

and appear to be the result of reciprocal recombination. Three were Am^r phenocopies, possibly the result of gene amplification. Two have reverted to $CHAT^s$, indicating either unstable reversion or misassignment. Five yield mRNA containing an exon 7 frameshift mutation and probably represent revertants carrying a compensating mutation in the exon 7 mutant allele, outside of the region examined by SSCP analysis. Two lines were subsequently lost to contamination and could not be analyzed for message production. We are currently sequencing complete message from the exon 7 mutant message-producing lines to characterize second site mutations.

Our finding that the original heterozygous line 6:86 produces only the exon 7 mutant message, although it also carries an exon 4 mutant allele, was surprising. The exon 4 mutation is not expected to interfere with message synthesis but is expected to lead to a truncated protein 45% of normal length (6). The possibility that premature termination of protein synthesis could lead to destabilized and shorter-lived message has been explored in considerable detail by others (14, 15, 31). Single-base deletions leading to premature termination in the reading frame of the human β-globin gene in β-o-thalassemia leads to appropriate splicing and polyadenylation but rapid turnover of the β-globin message and to smaller, although polyadenylated, mRNAs (31).

Similar effects have been seen with triosephosphate isomerase mRNAs encoding proteins whose synthesis terminates prematurely (15). Cheng et al. (14) have reported that translation into the penultimate exon of the triosephosphate isomerase mRNA was required for mRNA stability. In the present investigation, the absence or very low level of exon 4 mutant message in cell lines in which this mutation is clearly present in the DNA suggests that a similar phenomenon might be taking place in human *tk* mRNA maturation. The exon 4 frameshift, which is predicted to lead to dramatically premature protein termination, might be responsible for the lack of this mutant message during reverse transcription. The exon 7 mutation occurs close to the distal end of the *tk* message and, contrary to the effect of the exon 4 frameshift, is expected to lead to protein extension. Any double-mutant message generated by reciprocal recombination would carry the exon 4 mutation and terminate protein synthesis well in advance of exon 7. This would explain the apparent absence of double mutant message in the reciprocal recombinants.

Line 6:86 should prove to be a useful tool for examining interallelic recombination in response to environmental genotoxins and will permit evaluation of the relative role of frameshifts and recombination in reversion events. Line 4:2, which is homozygous for all of chromosome 17, including the exon 7 frameshift, is a suitable control for frameshift reversion events since it cannot revert to TK+ by recombination or gene conversion.

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