Interchromosomal Gene Conversion at an Endogenous Human Cell Locus

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ABSTRACT

To examine the relationship between gene conversion and reciprocal exchange at an endogenous chromosomal locus, we developed a reversion assay in a thymidine kinase deficient mutant, TX545, derived from the human lymphoblastoid cell line TK6. Selectable revertants of TX545 can be generated through interchromosomal gene conversion at the site of inactivating mutations on each *tk* allele or by reciprocal exchange that alters the linkage relationships of inactivating polymorphisms within the *tk* locus. Analysis of loss of heterozygosity (LOH) at intragenic polymorphisms and flanking microsatellite markers was used to initially evaluate allelotypes in TK^+ revertants for patterns associated with either gene conversion or crossing over. The linkage pattern in a subset of convertants was then unambiguously established, even in the event of prereplicative recombinational exchanges, by haplotype analysis of flanking microsatellite loci in $tk^{-/-}$ LOH mutants collected from the $tk^{+/-}$ parental convertant. Some (7/38; 18%) revertants were attributable to easily discriminated nonrecombinational mechanisms, including suppressor mutations within the *tk* coding sequence. However, all revertants classified as a recombinational event (28/38; 74%) were attributed to localized gene conversion, representing a highly significant preference ($P < 0.0001$) over gene conversion with associated reciprocal exchange, which was never observed.

THE resolution of mitotic homologous recombinational intermediates in mammalian cells can result in a localized gene conversion, which in some cases may be associated with a reciprocal exchange or crossover. Gene conversion affects highly localized regions of DNA and results in the nonequivalent distribution of parental alleles in the progeny cells. Reciprocal exchange, in contrast, results in a switch of the linkage relationships of all alleles from the point of the break to the telomere and therefore involves large tracts of DNA. When combined with an appropriate mitotic segregation, postreplicative reciprocal exchange events can produce loss of heterozygosity (LOH) for all markers distal to the reciprocal switch. Homologous recombination is an important pathway for the repair of double strand breaks (Liang *et al.* 1998; Thompson and Schild 1999) and can also result in the loss of functional alleles through LOH, providing a major mechanism for the expression of recessive phenotypes at tumor suppressor loci (Cavenee *et al.* 1983; Morley 1991; Hagstrom and Dryja 1999; Rousseau-Merck *et al.* 1999).

Tetrad analysis of recombination in yeast demonstrated that reciprocal exchange of flanking markers frequently accompanied gene conversion in meiotic cells (Fogel and Hurst 1967; Hurst *et al.* 1972). Gene conversion and reciprocal exchange are also observed to occur in conjunction in mitotic yeast, although the majority of mitotic gene conversions occur without associated crossover of flanking markers (Petes *et al.* 1991; Paques and Haber 1999). In contrast, relatively little is known about the prevalence and mechanisms of gene conversion in mammalian cells and the frequency of coordinate occurrence of gene conversion and reciprocal exchange in the repair of double strand breaks (DSBs). Most information on this topic has been obtained from analyses of artificial intrachromosomal recombination substrates, in which gene conversion is favored over reciprocal recombination by as much as two orders of magnitude (Nickoloff 1992; Sargent *et al.* 1997; Taghian and Nickoloff 1997). However, few reports have focused on rigorously discriminating between gene conversion and reciprocal recombination products occurring between allelic sequences on homologous chromosomes. One system used reversion analysis to study interchromosomal recombination in human lymphoblastoid cells (Benjamin and Little 1992), but the absence of nearby flanking markers precluded firm identification of gene conversion. A recent study from our laboratory determined the contribution of localized gene conversion to the overall yield of forward mutations at the heterozygous thymidine kinase (*tk*) locus in human lymphoblasts (Giver and Grosovsky 1997). Investigation of interchromosomal recombination has also been studied by using engineered recombinational substrates integrated into homologous positions within the retinoblastoma locus in mouse embryonic stem cells

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(Moynahan and Jasin 1997). In this study, induction of a targeted DSB into one of the integrated alleles using the rare cutting endonuclease I-*Sce*I resulted in an increase in interallelic recombination events by at least two orders of magnitude. All of these recombinational exchanges were due to localized gene conversion, although reciprocal exchanges were theoretically recoverable in this system (Moynahan and Jasin 1997). These data suggest that gene conversion is a potentially important recombinational mechanism in embryonic stem cells, although the applicability of this conclusion remains to be determined for interchromosomal allelic recombination in differentiated somatic cells.

Here, a reversion assay was used to enable examination of the relative frequencies of gene conversion occurring alone or in conjunction with reciprocal recombination. Revertants were derived from a previously characterized $tk^{-/-}$ mutant of TK6 called TX545 (Giver *et al.* 1995), and allelotypes could be determined using a set of five polymorphic markers within and closely flanking the *tk* locus. Due to the placement of heteroallelic inactivating mutations, selectable recombinational exchanges are restricted to the *tk* locus. Linkage relationships in a subset of individual convertants were unambiguously established, even in the event of prereplicative recombinational exchange, by identification of which alleles become coordinately homozygous at flanking microsatellite loci in $tk^{-/-}$ LOH mutants collected from the $tk^{+/-}$ parental convertant. Since none of the analyzed revertants were attributable to reciprocal exchange, this investigation provides direct evidence of a highly significant preference for interchromosomal gene conversion, without associated crossover, at an endogenous human cell locus.

MATERIALS AND METHODS

Cell lines and locations of polymorphic markers: The TK6 cell line is a well-characterized human B-lymphoblastoid cell line that is functionally heterozygous at the *tk* locus. The human *tk* locus is located on chromosome 17q23-25 (Fain *et al.* 1991). The gene is 12.9 kb long with 702 bp of coding region (Bradshaw and Deininger 1984; Flemington *et al.* 1987) and codes for a protein involved in a salvage pathway for thymidine triphosphate biosynthesis. Forward selection (TK^-) can be accomplished by growth in trifluorothymidine (TfT; Skopek *et al.* 1978), and reversion to TK^+ can be selected by colony formation in medium containing CHAT (cytidine, hypoxanthine, aminopterin, thymidine; Clive *et al.* 1973; Liber and Thilly 1982).

Cell line TX545 is a $tk^{-/-}$ mutant of TK6. The structure of the *tk* locus in TX545 is shown in Figure 1. Parental TK6 cells contain a single base insertion in exon 4 of allele B of the *tk* locus, within a run of three C's at position 4864 of the genomic DNA sequence. In addition, there is a phenotypically silent frameshift insertion on allele A in a run of four G's at position 12690 in exon 7 (Grosovsky *et al.* 1993). A number of informative microsatellite polymorphisms exist on 17q, both centromeric and telomeric of *tk* in the TK6 cell line (Giver and Grosovsky 2000). TX545 has an inactivating tandem base substitution at positions 12441–2 in exon 6 of allele A of the *tk* gene (Giver *et al.* 1995) resulting in a change of a threonine codon (ACC) to lysine (AAG; Figure 1). Cell lines were maintained in RPMI 1640 with 10% iron-supplemented calf serum, and 1% L-glutamine and 1% penicillin/streptomycin mix, and were cultured at 37° with 5% CO_2, with cell density kept between 2 and 10×10^5 cells/ml.

Collection of TX545 independent revertants: Independent flasks of TX545 were pretreated with 2 μg/ml TfT for 2 days to remove preexisting revertants and expanded to 1×10^8 cells/flask. Up to 2×10^9 cells were used for each experiment. Cells were treated with either 200cGy γ-irradiation (^{137}Cs source; J. L. Shepard and Associates) or 0.3 μM BPDE [benzo(a)pyrene-r-7,t-8,dihydrodiol-t-9,10-epoxide(+/−) (anti); Midwest Research Institute, Kansas City, MO]. Untreated cultures were run in parallel. The cultures were allowed 3 days postirradiation to express TK before selection at 40,000 cells per well in medium containing CHAT [1×10^{-5} M cytidine, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine; 100× HAT was purchased from GIBCO/BRL (Gaithersburg, MD) and cytidine was purchased from Sigma Chemical Co. (St. Louis)]. The BPDE-treated cells (10 flasks with 10^8 cells per flask) were exposed for 24 hr, washed, and then placed in new media. After a 3-day expression period they were selected in CHAT-containing medium as described above. Spontaneous revertants were also collected after similar treatment. Revertant colonies were scored on day 14, but the 96-well dishes were additionally scored at day 21 to identify potential slow growing revertants. Slow growing colonies, attributable to recombinational exchange with LOH tracts extending to the telomere, are readily scored in forward mutational analyses in TK6 cells (Liber *et al.* 1989; Amundson and Liber 1992; Dobo *et al.* 1995; Giver and Grosovsky 2000). A single revertant was picked from each independently treated culture for further analysis.

Genomic and cDNA analysis of revertant clones: DNA purification was performed either by a phenol:chloroform extraction (Nelson *et al.* 1994) or by the use of a spin column (QIAamp blood kit; QIAGEN, Valencia, CA). [^{32}P]ATP end labeling, PCR amplification of the intragenic polymorphisms, and single C and G lane sequencing of the exon 4 and 7 polymorphisms were each performed as previously described (Grosovsky *et al.* 1993; Giver *et al.* 1995). The products were run and visualized on 6% polyacrylamide gels and examined for loss of the frameshift insertions. The exon 6 tandem substitution was amplified in the same fragment as the exon 7 polymorphism and was analyzed by PCR sequencing (fmol kit; Promega, Madison, WI) for loss of the tandem substituted bases. PCR amplification and electrophoresis of genomic DNA for visualizing flanking microsatellite markers D17S937 and D17S802 were performed as described in Giver and Grosovsky (1997). Primers were obtained from Research Genetics (Huntsville, AL).

For cDNA sequencing of the exons 4, 6, and 7 polymorphisms, total cytoplasmic RNA was isolated using commercial spin columns (RNeasy plant mini kit; QIAGEN). Production and amplification of the cDNA by reverse transcriptase (RT)-PCR were performed as previously described (Grosovsky *et al.* 1993). RT-PCR of *tk* cDNA results predominantly in recovery of allele A message, presumably due to rapid degradation of the allele B message, which carries an inactivating frameshift in exon 4 (Grosovsky *et al.* 1993). As a result only the sequence of the allele A cDNA is visible when pooled cDNA is used for DNA sequence analysis (Grosovsky *et al.* 1993; Giver *et al.* 1995; Giver and Grosovsky 1997). PCR single-lane sequencing of the cDNA was performed for the three polymorphic sites as described above.

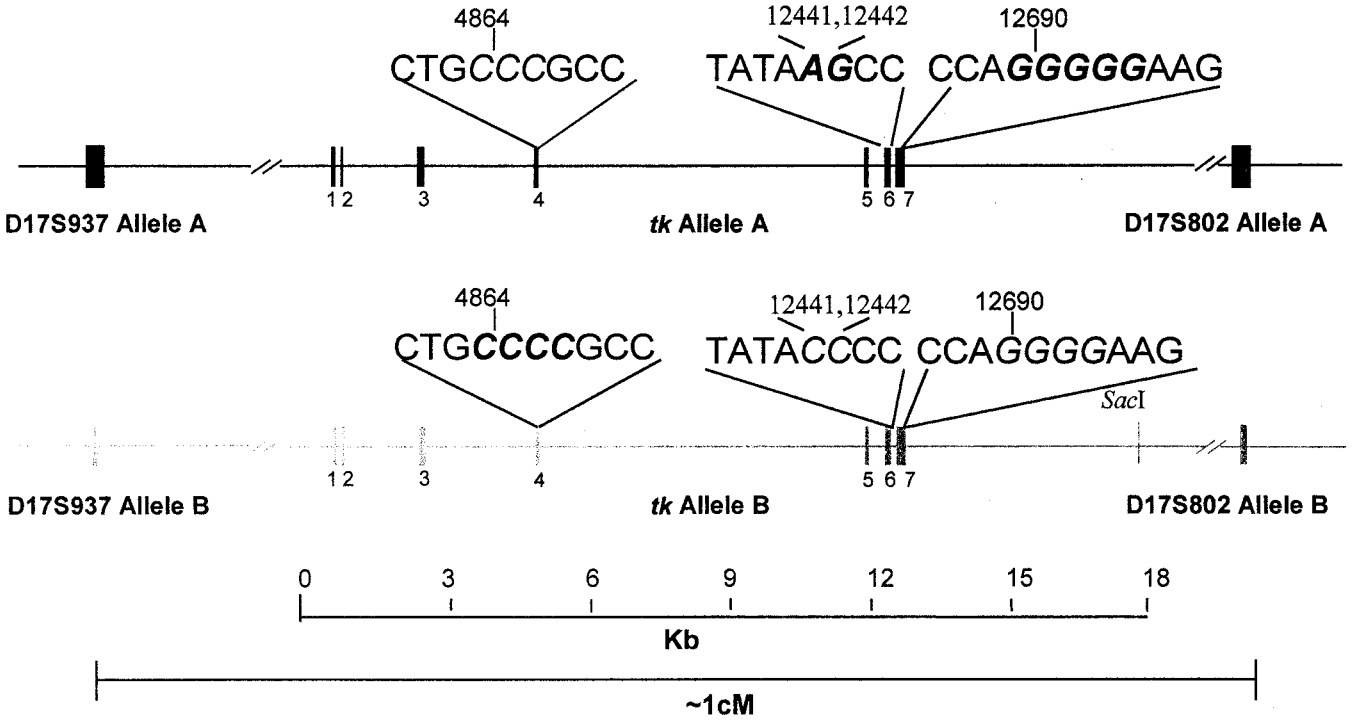

FIGURE 1.—Map of the human thymidine kinase (*tk*) gene in cell line TX545. Three polymorphisms in the coding portion of the gene were used for analysis: an inactivating frameshift (fs) insertion at exon 4 on allele B, an inactivating tandem substitution (td) in exon 6 on allele A, and a silent fs insertion in exon 7 on allele A. TX545 differs from the parental cell line TK6 only at the position of the exon 6 tandem base substitution. Polymorphisms at the centromeric and telomeric polymorphic microsatellites D17S937 and D17S802 depicted here were also analyzed to determine the extent of LOH. These microsatellites map to within a region of 1 cM. Exons are shown to scale and are labeled 1–7. The locations of the mutations in the genomic sequence of the coding strand of the *tk* gene are indicated using the numbering system of FLEMINGTON *et al.* (1987).

Collection and microsatellite analysis of TK$^-$ LOH mutants from *tk*$^{+/-}$ convertants: For each convertant clone, three 96-well plates were seeded with 4×10^4 cells per well in media containing 2 μg/ml TfT. Plates were scored and colonies were picked at 14 and 21 days after seeding; a minimum of 12 TK$^-$ mutant clones were collected for LOH analysis for each convertant. Colonies were expanded for DNA isolation using the QIAMP Dneasy tissue kit. Microsatellite analysis used procedures identical to the analysis of revertant clones as described above. TK$^-$ clones were initially screened for LOH encompassing microsatellite locus D17S937. A single TK$^-$ clone exhibiting LOH at D17S937 was selected from each set of 12 mutants for LOH analysis at D17S802. In rare cases clones exhibiting LOH at D17S937 remained heterozygous at D17S802, and additional clones were screened to obtain one exhibiting coordinate LOH at both loci.

RESULTS

Genotypes associated with gene conversion and recombinational loss of heterozygosity: Spontaneous, γ-irradiated, and BPDE-induced revertants of TX545 were analyzed for LOH using three intragenic and two flanking microsatellite markers at the *tk* locus (Figure 1; Table 1). Revertants demonstrating LOH involving only a portion of the *tk* locus and not extending to flanking microsatellite markers were classified as localized gene conversions, which could occur with or without reciprocal exchange (Figure 2, a–f). In contrast, any revertant involving LOH of intragenic and flanking telomeric microsatellite polymorphisms would be classified as recombinational LOH (Figure 2g). It is also possible to recover a reciprocal exchange that directly results in linkage of wild-type sequences on one allele without concomitant LOH (Figure 2, h and i). Direct restoration of TK function by mutational reversion of the tandem base substitution in exon 6 is a low probability event requiring specific substitutions at both positions (Figures 1 and 2, a and d); coincident mutational reversion involving exons 6 and 7 (Figure 2, b and e) has an even lower probability. LOH at the inactivating frameshift insertion in exon 4 (Figures 1 and 2, c and f) could occur by localized gene conversion or by a reverting frameshift deletion within the run of four guanines in the exon 4 sequence (Figure 1). However, the probability of a frameshift mutation within a specific four-base sequence is quite low (BENJAMIN and LITTLE 1992; GIVER *et al.* 1995; GIVER and GROSOVSKY 1997) and unlikely to account for a substantial fraction of all recovered revertants involving LOH at exon 4. Although this possibility cannot be fully excluded, the distribution of revertants between exons 4 and 6 is equivalent (Table 1), suggesting that this additional potential mechanism for generating exon 4 revertants is not contributing significantly to the overall yield of revertants.

TABLE 1

Patterns of loss of heterozygosity among TK$^+$ revertants of TX545

Revertant category	No. of revertants[a]	Genomic DNA (allelotype)				
		D17S937	*tk* exon 4	*tk* exon 6	*tk* exon 7	D17S802
1. Exon 4 conversion	13 (0.34)	A/B	A	A/B	A/B	A/B
2. Exon 6 Conversion	3 (0.08)	A/B	A/B	B	A/B	A/B
3. Exon 6 and 7 conversion	12 (0.32)	A/B	A/B	B	B	A/B
4. No LOH[b]	6 (0.16)	A/B	A/B	A/B	A/B	A/B
5. Suppressor mutation[c]	4 (0.11)	A/B	A/B	A[c]/B	A/B	A/B

Analyses at polymorphic sites were conducted as described in MATERIALS AND METHODS. Allele A includes the *tk* wild-type sequence at the exon 4 polymorphism, and allele B includes the *tk* wild-type sequence for exons 6 and 7. The alleles are listed as they appear on autoradiographs obtained from the analysis of each mutant.

[a] A total of 9 spontaneous, 7 γ-ray-induced, and 22 BPDE-induced revertants were collected. Of these 38, 31 were ultimately confirmed to be convertants, including 3 no LOH revertants with localized reciprocal switch. Analysis of the occurrence of conversion alone, conversion in association with reciprocal exchange, or other categories of reversion, was performed using pooled data from spontaneous and induced treatment groups. There was no statistically significant difference ($P = 0.53$) in the distribution of conversion events among the treatment classes; 6/8 spontaneous convertants, 4/7 γ-ray-induced convertants, and 8/16 BPDE-induced convertants involved exon 6 with the remaining events involving exon 4. Therefore, convertants in each treatment class included events at both sites, and these distributions did not significantly deviate ($P = 0.61$) from the null hypothesis of equivalent occurrence of conversion at exons 6 and 4.

[b] Three of the no LOH revertants involved a localized reciprocal switch of allelotypes at exons 6 and 7 (Table 3, d and e; Table 5).

[c] A function-restoring point mutation was identified in exon 6 for these mutants and is labeled as a suppressor mutation. See Table 3 for further details.

Gene conversion without reciprocal exchange is the predominant mechanism for generation of revertants: A total of 38 revertant clones were collected, and genomic DNA was used for analysis of allelotypes (Table 1). A large fraction of recovered revertants (28/38, 0.74) demonstrated one of several genomic allelotypes consistent with localized gene conversion (Figure 2, a and c; Table 1, categories 1–3). These events were similarly distributed at the two opposed inactivating mutations; 15 revertants exhibited LOH at exon 6, and another 13 revertants involved LOH at exon 4. Most of the LOH revertants involving exon 6 (12/15, 0.80) also extended to the phenotypically silent frameshift polymorphism in exon 7 located 247 bp away (Figure 1; Table 1, category 3), suggesting that recoverable conversion tracts are rarely less than several hundred bases in length.

In addition to the revertants exhibiting localized LOH, four clones were collected that contained a function-restoring single base alteration affecting one of the tandem-substituted bases at positions 12441–2 in exon 6 (Table 2; Table 1, category 5). Three of these revertants are suppressor mutations that encode asparagine, an amino acid that represents a conservative change from the wild-type sequence and that permits TK function to be restored. The remaining revertant is also a single base suppressor mutation, which due to coding degeneracy restored the wild-type threonine at the mutated codon (Table 2).

Six of the 38 revertants had no LOH in the genomic DNA at any of the polymorphic markers, yet grew in selective medium containing CHAT (Table 1, category 4). This pattern could be the result of gene conversion associated with a local reciprocal switch (Figure 2i), reciprocal recombination (Figure 2h), or a compensating mutation elsewhere in the genome such as amplification of the DHFR gene (SHARMA and SCHIMKE 1994). To further examine the mechanisms involved in generating the clones that exhibited no LOH, the *tk* cDNA from these revertants was amplified and sequenced. Due to differences in allele-specific expression this analysis reflects only the sequence of the message transcribed from *tk* allele A (GROSOVSKY *et al.* 1993), thus permitting the establishment of intraallelic linkage relationships among the sequences at polymorphic positions within the *tk* locus. These data are summarized in Table 3. The cDNA from three revertants retained the inactivating tandem base substitution in a pattern that is unchanged from the parental TX545 cells (Table 3c). Since the CHAT resistance in these three revertants does not appear to result from a molecular alteration within the *tk* locus, these are tentatively categorized as second site compensating alterations. On the other hand, single-lane cDNA sequencing analysis demonstrated the restoration of the wild-type sequence at exon 6 in three other revertants that remained heterozygous in analysis of genomic DNA (Table 3, d and e). These observations suggest a restoration of TK expression through a localized allelic exchange, resulting in linkage of wild-type sequences on one allele without concomitant LOH (Figure 2i). Considered alone, the results for these re-

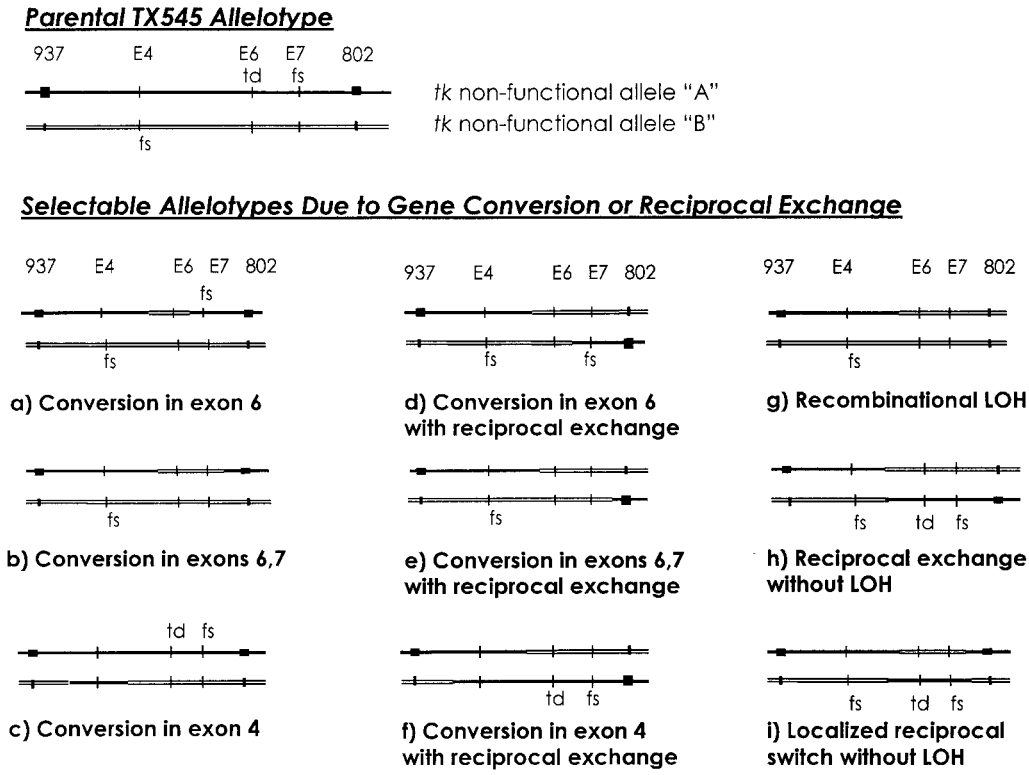

FIGURE 2.—Possible allelotypes of TK^+ revertant clones due to gene conversion or reciprocal exchange. Distances are not represented to scale. Polymorphisms are those detailed in Figure 1. The allelotype of the parental TX545 cell line is shown at the top left. Selectable modification of allelotypes may be due to localized gene conversion with or without associated reciprocal exchange (a–f), or by reciprocal exchange with or without associated LOH (g and h). In the case of reciprocal exchange an associated localized gene conversion at the site of the exchange may also occur, but cannot be ascertained if it occurs in a region without polymorphic markers. The localized gene conversion allelotypes (a–c) involve LOH for only a portion of the *tk* locus, without associated crossover. Parallel gene conversion events with associated distal crossover are depicted in the center column (d–f). Note that in all of the cases represented in a–f flanking microsatellite markers remain heterozygous. The reciprocal exchange allelotypes (g and h) indicate that associated loss of heterozygosity is expected in 50% of postreplicative crossovers, depending on the mitotic segregation patterns of the recombinational partners following resolution of the exchange intermediate.

vertants are also compatible with reciprocal recombination with no LOH (Figure 2h), but this explanation is excluded by subsequent allelotype analysis of TK^- LOH mutants (see Table 5). These revertants may be straightforwardly explained by mismatch repair of heteroduplex DNA on both alleles following branch migration through the polymorphic position (GIVER and GROSOVSKY 1997) or by replication through heteroduplex DNA in conjunction with mitotic segregation of the appropriate chromosomes (MARIANS 2000). Alternatively, these events could theoretically be double crossovers, but since not even single crossovers were observed in association with any other gene conversion event the probability of double crossovers within such a small intragenic region seems quite remote.

Taken as a whole, these analyses demonstrate that no revertant exhibited the expected genomic pattern for recombinational LOH (Figure 2g). In contrast, 28 revertants (Table 1) could be definitively classified as interchromosomal gene conversion. The difference in recovery of gene conversion and recombinational LOH is highly significant ($P < 0.0001$; Table 4). Random mitotic segregation patterns following postreplicative recombinational exchange should result in equivalent recovery of reciprocal switches that either produce LOH or remain heterozygous at distal markers. However, if prereplicative resolution of recombinational intermediates involved reciprocal exchange, these could not be detected by analysis of LOH at distal markers (Figure 2). This possibility was addressed by allelotype analysis in $tk^{-/-}$ LOH mutants derived from $tk^{+/-}$ convertants.

The allelotype of selectable $tk^{+/-}$ convertants, with or without reciprocal exchange at distal flanking markers, is shown in Figure 3. Isolation of derivative $tk^{-/-}$ mutants with flanking marker LOH provides coordinate homozygosis of polymorphic markers from the haplotype that includes the nonfunctional *tk* allele. Therefore, allelotype analysis of microsatellite markers in these mutants permits the parental linkage relationship to be unambiguously established, thereby determining whether a crossover occurred in the parental convertant (Figure 3). To perform this analysis we collected a set of $tk^{-/-}$ mutants from each convertant and screened for flanking marker LOH, permitting establishment of haplotype for a subset of 20 convertants (Table 5). All of these LOH mutants exhibited microsatellite allelotypes that indicate a local gene conversion without associated crossover in the parental convertants (Table 5; $P = 0.0004$). Three additional convertants affecting only exon 6 (Table 1, category 2) and one reciprocal switch revertant (Table 3d) were also found to have occurred without an associated crossover by using cDNA sequence

TABLE 2

Suppressor point mutations in revertant clones

TK6 sequence[a]	TX545 sequence[a]	Revertant sequences[a]	No. of revertants
TAT ACC AAG Tyr Thr Lys	TAT A**AG** AAG Tyr **Lys** Lys	TAT AA**C** AAG Tyr **Asn** Lys	1
		TAT AA**T** AAG Tyr **Asn** Lys	2
		TAT A**C**G AAG Tyr **Thr** Lys	1

[a] Sequences shown are from exon 6 of the *tk* locus (see Figure 1). DNA sequences and amino acids shown in boldface and underlined type are changed from the predecessor cell line.

analysis to demonstrate the conservation of parental linkage relationship with the exon 7 frameshift polymorphism allele. These findings (Tables 4 and 5) provide strong evidence that interchromosomal gene conversion in human lymphoblastoid cells is rarely associated with crossing over.

DISCUSSION

Extensive studies in yeast indicate that localized interallelic gene conversion predominates over reciprocal exchange in analysis of mitotic recombinational products (Paques and Haber 1999). A substantial preference for recovery of mitotic gene conversion rather than reciprocal exchange was also reported in mammalian cells, largely by analysis of intrachromosomal tandem arrays carried on integrated plasmid constructs (Liskay and Stachelek 1983; Godwin and Liskay 1994; Sargent *et al.* 1997; Taghian and Nickoloff 1997; Yang and Waldman 1997; Liang *et al.* 1998; Lin *et al.* 1999; Lukacsovich and Waldman 1999; Dronkert *et al.* 2000). These investigations suggest that, although reciprocal exchange may occur without a detectable gene conversion due to the absence of local polymorphisms, all recombinational exchanges may proceed by a mechanism that would necessarily produce a conversion within an appropriate sequence context. However, investigation of systems capable of detecting interchro-

TABLE 3

Genomic and cDNA analysis of no LOH revertant clones

	tk locus genomic DNA[a]			*tk* locus cDNA[a]		
	Exon 4	Exon 6	Exon 7	Exon 4	Exon 6	Exon 7
Parental cell lines						
a. TK6	A/B	A/B	A/B	A (wt)	A (wt)	A (fs)
b. TX 545	A/B	A/B	A/B	A (wt)	A (td)	A (fs)
No LOH TX545 revertant clones category (no. of clones)						
c. Second site alteration[b] ($n = 3$)	A/B	A/B	A/B	A (wt)	A (td)	A (fs)
d. Conversion with local switch in exon 6[c] ($n = 1$)	A/B	A/B	A/B	A (wt)	B (wt)[c]	A (fs)
e. Conversion with local switch in exons 6 and 7[d] ($n = 2$)	A/B	A/B	A/B	A (wt)	B (wt)[d]	B (wt)[d]

[a] cDNA status of revertants that exhibited no LOH in genomic DNA analysis (Table 1, category 5). RT-PCR and cDNA sequencing were conducted as described in materials and methods. Alleles A and B indicate sequences shown in Figure 1, with the exception of TK6 cells, which are homozygous wild type at the position of the inactivating exon 6 tandem base substitution. The symbols in parentheses indicate wild type (wt), tandem base substitution (td), or frameshift (fs).

[b] No change of any type is detected within the *tk* locus. Therefore, a second site change is suggested, such as amplification of the *dhfr* locus.

[c] This event is classified as a gene conversion involving a localized switch of sequences within *tk* exon 6 due to mitotic segregation. Wild-type function is restored on allele A and the tandem base substitution is transferred to allele B.

[d] This event can be explained as a gene conversion with a localized sequence switch within *tk* exons 6 and 7 due to mitotic segregation (Figure 2i). Considered alone these data are also compatible with reciprocal recombination with no LOH (Figure 2h), but this explanation is excluded by allelotype analysis of TK^- LOH mutants (Table 5).

TABLE 4

Recovery of recombinational LOH and gene conversion among revertants

Category	No. of recovered events	Statistical significance
Gene conversion	28	$P < 0.0001$
Recombinational LOH	0	
Additional recovered events[a]	10	Not applicable

[a] These events include six revertants without LOH (Table 1, category 4; Table 3c) and four suppressor mutations (Table 1, category 5; Table 2).

mosomal allelic gene conversion in human and other mammalian cells was restricted to only a few studies (Grist *et al.* 1992; Shulman *et al.* 1995; Giver and Grosovsky 1997; Moynahan and Jasin 1997). The data presented here for TX545, a human lymphoblastoid cell line, indicate that a highly significant preference for gene conversion without associated reciprocal exchange may be a general feature of interchromosomal recombination, extending to more differentiated cell types and to human cells.

Previous evidence that homologous recombinational repair of DSBs is associated with gene conversion rather than crossover has primarily been derived from studies of I-*Sce*I-induced cleavage of plasmids integrated within the genome of mouse embryonic stem cells (Moynahan and Jasin 1997; Donoho *et al.* 1998; Elliott *et al.* 1998; Richardson *et al.* 1998). Recent reports used targeted knockouts to further examine pathways of double strand break repair in embryonic stem cells and other differentiated adult cell types. Targeted knockout of DNA-PKcs, which is essential for nonhomologous end joining (NHEJ), does not result in hypersensitivity to ionizing radiation exposure in embryonic stem cells (Gao *et al.* 1998). On the other hand, radiosensitivity of embryonic stem cells is associated with knockout of *RAD54*, which is involved in homologous recombinational repair but not NHEJ (Essers *et al.* 2000). In adult tissues the situation appears to be reversed; adult mice are not radiosensitized by *RAD54* deficiency (Essers *et al.* 2000), but loss of DNA-PKcs function results in significant radiosensitivity (Biedermann *et al.* 1991; Gao *et al.* 1998). Further evidence of high levels of homologous recombinational repair activity in embryonic stem cells is provided by the relatively high frequency at which gene targeting can be achieved by homologous recombinational mechanisms (Fung-Leung and Mak 1992; te Riele *et al.* 1992; Rathjen *et al.* 1998). Taken as a whole, these studies suggest that embryonic stem cells may not be representative of most mammalian cell types since homologous recombinational repair, rather than nonhomologous end joining, appears to be the predominant cellular response to double strand breaks. Therefore, extended analysis in additional cell types is essential to establish the generality of the conclusion that gene conversion without crossover is the predominant mode of homologous recombinational repair in a wide range of mammalian cell types.

Molecular analysis of TK^+ TX545 revertants initially utilized analysis of loss of heterozygosity in distal markers as an indicator of crossing over associated with localized gene conversion within the *tk* locus (Table 1). If gene conversion occurs in conjunction with crossover, there is a 50% chance of associated LOH that would

TABLE 5

Microsatellite allelotypes in $tk^{-/-}$ LOH mutants derived from $tk^{+/-}$ convertants: localized gene conversion is not associated with crossover

	Microsatellite allelotypes[a] (D17S937/D17S802)			
Site of conversion	AA/AA (No crossover)	BB/BB (No crossover)	BB/AA (Crossover)	
Exon 4	9	NA	0	
Exon 6[b]	NA	1	0	
Exons 6 and 7[b]	NA	10	0	
Total	9	11	0	$P = 0.0004$[c]

[a] Microsatellite analysis was performed on $tk^{-/-}$ LOH mutants derived from $tk^{+/-}$ convertants. The alleles at D17S937 and D17S802 that are coordinately reduced to homozygosity indicate whether linkage relationships in parental convertants were altered by a crossover occurring in conjunction with a localized gene conversion at the indicated site. Although two alleles are listed at each locus, some TK^- LOH mutants could be attributable to deletion; this would not influence the use of these data in inferring the haplotype of parental convertants. NA, not applicable.

[b] Conversion of the inactivating tandem base substitution within exon 6 generally involved coconversion of the nearby frameshift polymorphism within exon 7. Data for conversion at exons 6 and 7 include one switch clone that did not involve local LOH.

[c] Statistical significance is calculated using Fisher's exact test by comparing the total of no crossovers (20) and crossovers (0) to the null hypothesis of equivalent recovery for each category.

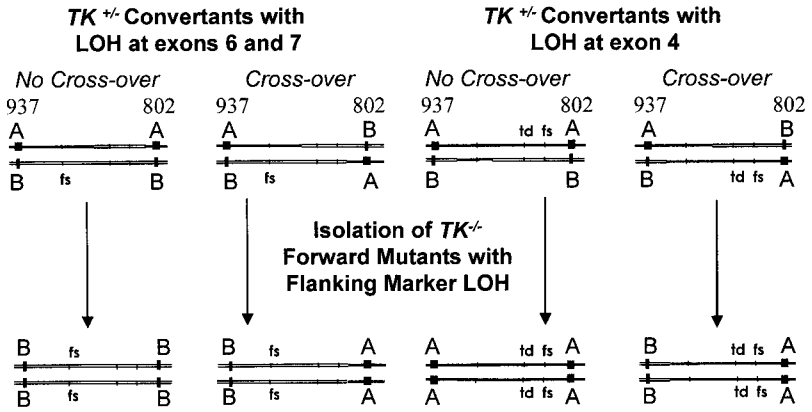

FIGURE 3.—Haplotype analysis using $tk^{-/-}$ LOH mutants for detection of reciprocal exchange in parental $tk^{+/-}$ convertants. The anticipated allelotypes of $tk^{+/-}$ convertants at either exons 6 and 7 (top left) or exon 4 (top right) are depicted with or without crossover. The flanking marker allelotypes in convertants restricted to exon 6 alone would be identical to conversion tracts encompassing exons 6 and 7, and only the latter are shown for simplicity. A set of 12 $tk^{-/-}$ mutants were isolated from each convertant and screened for LOH encompassing flanking microsatellite markers. The linkage relationship in the parental convertant can be established by identification of which alleles become coordinately homozygous at the flanking microsatellite loci. Conservation of the parental linkage confirms the absence of crossover associated with the localized gene conversion within the *tk* locus. Using this approach, no crossovers were observed in haplotype analysis of 20 convertants, indicating a highly significant ($P = 0.0004$) preference for conversion without associated reciprocal exchange.

produce a distinguishing allelotype (Figure 2g) if the exchange involved DNA that had already been replicated. In contrast, distal heterozygosity would be retained in an equal number of postreplicative crossovers if the two homologous chromosomes involved as recombination partners are cosegregated during mitosis, but this allelotype cannot be distinguished from the parental pattern (Figure 2h). LOH was determined to be restricted to a portion of the *tk* locus in 28 independent revertants (Table 1), which were thus classified as localized gene conversions. These data alone do not exclude the possibility that all or some of these had undergone a reciprocal exchange in conjunction with the gene conversion if in each case the mitotic segregation pattern resulted in retention of heterozygosity in the distal markers. Furthermore, in the event of recombinational exchange occurring in G1 or early S phase, there would be no possibility for LOH to serve as a marker for crossover. This possibility was unambiguously excluded for a subset of 20 independent convertants by allelotype analysis of flanking microsatellite loci in $tk^{-/-}$ LOH mutants collected from the $tk^{+/-}$ parental convertant (Figure 3; Table 5). This sample represents convertants occurring in *tk* exon 4 as well as clones undergoing coconversion of the inactivating tandem base substitution in exon 6 and the silent frameshift polymorphism in exon 7 (Figure 1; Table 5). These results provide strong evidence ($P = 0.0004$) that interchromosomal gene conversion in human lymphoblastoid cells is rarely associated with crossing over. Interestingly, since coconversion tracts are a minimum of several hundred bases in length (Figure 1), these findings also extend to relatively long conversion tracts. Indeed, 4/10 coconvertants were found to encompass the *Sac*I restriction polymorphism situated ~5.5 kb downstream of the tandem base substitution in exon 6 (data not shown).

The reversion assay in TX545 cells requires a selectable recombinational exchange to occur between the sites of inactivating mutations in each *tk* allele (Figure 1), permitting a direct comparison of the relative recovery of gene conversion alone and in association with reciprocal exchange within an individual endogenous locus. A complementary estimate can be derived from consideration of forward mutation data at the *tk* locus in parental TK6 lymphoblasts. In selection of forward TK^- mutants, gene conversion is restricted to the local region surrounding an inactivating heterozygous frameshift mutation within exon 4 of the *tk* locus (GIVER and GROSOVSKY 1997), whereas recombinational LOH can include tracts extending >40 cM proximal to *tk* (DOBO *et al.* 1995; GIVER and GROSOVSKY 2000). By considering the overall TK^- mutation frequency and the percentage of each class of event determined by molecular analyses of mutant collections (LIBER *et al.* 1989; AMUNDSON and LIBER 1992; GIVER *et al.* 1995; GIVER and GROSOVSKY 1997), the frequency of recombinational LOH can be estimated to be ~70-fold higher than that of gene conversion (Table 6). However, this apparent discrepancy with the reversion analysis can be resolved by factoring in the available target size for the initiation of selectable recombinational exchanges. Target size approximations were used to calculate a yield per kilobase for gene conversion and recombinational LOH, resulting in a size-normalized estimate that gene conversion is 650- to 1000-fold more frequent than recombinational LOH (Table 7). This approach was extended (Table 8) to calculate that gene conversion was recovered ~700-fold more frequently than was recombinational LOH, when frequency was considered as a function of the estimated number (LOBRICH *et al.* 1995) of radiation-induced double strand breaks within each target region. This calculation indicates that a minimum of 6% of double strand breaks result in a recoverable gene conversion (Table 8), although this figure represents only selectable outcomes and is therefore expected to underestimate the number of double strand breaks that are processed by homologous interchromosomal recombinational repair.

TABLE 6

Recovery of recombinational LOH and gene conversion among γ-ray-induced and spontaneous forward mutations: Gross yield

Category of forward mutation	Radiation-induced mutation frequency	Spontaneous mutation frequency
Overall TK$^-$ mutation frequency[a]	93×10^{-6}	7.5×10^{-6}
Recombinational LOH[a]	75×10^{-6}	5×10^{-6}
Gene conversion[b]	1.3×10^{-6}	0.12×10^{-6}

[a] The overall TK$^-$ mutation frequency in TK6, including normal and slow growth mutants, was reported by Liber *et al.* (1989). The slow growth mutants contributing to this frequency were classified as recombinational LOH events (Amundson and Liber 1992; Xia *et al.* 1994; Grosovsky *et al.* 1996).

[b] These frequencies were estimated by multiplying the TK$^-$ normal growth mutation frequency in TK6 cells (Liber *et al.* 1989; Giver *et al.* 1995) by the percentage of gene convertants in the spectrum of TK$^-$ mutants (Giver and Grosovsky 1997).

TABLE 7

Recovery of recombinational LOH and gene conversion among γ-ray-induced and spontaneous forward mutations: Yield per kilobase

Category	Estimated target size (kb)	Gross yield		Yield per kilobase	
		Radiation induced	Spontaneous	Radiation induced	Spontaneous
Recombinational LOH[a]	4×10^4	75×10^{-6}	5×10^{-6}	2×10^{-9}	0.12×10^{-9}
Gene conversion[b]	1	1.3×10^{-6}	0.12×10^{-6}	1300×10^{-9}	120×10^{-9}

[a] Target size for recombinational LOH was estimated from the 40 cM recombinational distance between *tk* and the most centromeric marker (D17S806) recovered in contiguous LOH tracts on chromosome 17q in TK$^-$ mutants (Giver and Grosovsky 2000). In this calculation an equivalence of 1 Mb per cM was used, which was chosen as a conservative underestimate of the actual physical distance available for recombinational breakpoints.

[b] An estimate of 1 kb was used for the target size of a gene conversion event. The actual length of gene conversion tracts is poorly defined and probably variable.

TABLE 8

Recovery of recombinational LOH and gene conversion among γ-ray-induced and spontaneous forward mutations: Yield per radiation-induced double strand break

Category	Gross yield	Target size (kb)	DSBs per target[a]	Yield per DSB
Recombinational LOH	78×10^{-6}	4×10^4	0.928	8.4×10^{-5}
Gene conversion	1.3×10^{-6}	1	2.3×10^{-5}	0.06

[a] The estimate of 5.8×10^{-3} DSBs/Mb/Gy of ionizing radiation (Lobrich *et al.* 1995) was used in this analysis. The relevant target was considered to be a 40-Mb tract within each copy of chromosome 17q.

It was recently estimated (Liang *et al.* 1998) that 30–50% of I-*Sce*I-induced double strand breaks in mouse embryonic stem cells were repaired by homologous recombination, primarily through sister chromatid exchange (Liang and Jasin 1999), since the frequency of homologous recombination is 100- to 1000-fold less for substrates requiring interchromosomal or ectopic interactions. The data presented in this study (Tables 1 and 4) indicate that, in addition to endonuclease cleavage sites, gene conversion without crossing over is the preferred recombinational outcome induced by exposure to radiation and BPDE. It remains possible that these gene conversion events are not directly induced by intragenic damage within the target gene but rather are secondary effects of an overall increase of recombination in exposed cells. Nevertheless, the analyses presented here suggest that homologous recombinational repair in human lymphoblastoid cells may frequently involve interactions between homologous chromosomes (Tables 4, 6–8) and is not restricted to postreplicative interactions between sister chromatids. Since the spectrum of DNA damage induced by these agents is quite

broad and the biochemical structure of strand break ends may be highly variable (Moran and Ebisuzaki 1991; Bigger *et al.* 1994; MacLeod, *et al.* 1994; Ward 1995, 1990; Milligan *et al.* 1999), these recombinational events may not be fully modeled by endonuclease cleavage sites. For example, recombination may be initiated at sites of radiation-induced clustered damage or at breaks associated with excision repair of closely opposed DNA adducts (Vamvakas *et al.* 1997). Recombinational exchange at multiply damaged sites could potentially be linked to the observation of coincident DNA sequence alterations (Strathern *et al.* 1995; Giver and Grosovsky 1997) in the vicinity of gene conversion events.

Efforts to explain the strong bias for recovery of mitotic gene conversion without associated crossing over have led to a fundamental reconsideration of recombinational mechanisms in recent years. Earlier models for double strand break-induced homologous recombinational repair (Szostak *et al.* 1983) involved the formation of Holliday intermediates. Equivalent resolution of Holliday junctions as localized conversion or reciprocal exchange is generally expected, primarily due to studies of meiotic yeast (Paques and Haber 1999) and early studies of mitotic recombination (Szostak *et al.* 1983; Willis and Klein 1987). In contrast, more recently developed models for homologous recombinational repair, including synthesis-dependent strand annealing (SDSA) and break-induced replication (Paques and Haber 1999), do not require formation of a Holliday intermediate. Therefore, most variations of these models exclude the possibility of crossing over (Paques and Haber 1999). Alternatively, if a mitotic mechanism existed to suppress structural isomerization of Holliday intermediates prior to their resolution (Meselson and Radding 1975; Hastings 1992; Foss *et al.* 1999), this pathway could also significantly contribute to homologous recombinational repair and the generation of gene conversion.

In summary, the data presented here demonstrate that localized conversion without associated crossover is the predominant pathway for interchromosomal homologous recombinational repair at an endogenous chromosomal locus in human B-lymphoblastoid cells. The scarcity of reciprocal exchanges may reflect the low probability of completing all of the events necessary for establishment and successful resolution of a crossover intermediate without diversion to simpler related configurations that can only be resolved in gene conversion. For example, SDSA structural intermediates (Paques and Haber 1999) may represent an earlier stage of formation of the Szostak intermediate; only if formation of the Szostak intermediate is completed would a crossover be possible. Alternatively, the differential recovery of these homologous recombinational products may suggest the necessity for a regulatory mechanism that actively controls the potential for formation of reciprocal exchange intermediates in a manner appropriate to cell type, cell cycle position, and developmental stage.

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