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Information transfer between duplicated chromosomal sequences in mammalian cells involves contiguous regions of DNA

(homologous recombination/gene conversion/repeated sequences/herpes simplex virus thymidine kinase gene)

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ABSTRACT **We have investigated the nature of information transfer that appears to occur nonreciprocally between duplicated chromosomal sequences in cultured mouse L cells. We have studied gene conversion between two different defective thymidine kinase genes derived from two closely related strains of type 1 herpes simplex virus and that share a silent restriction site polymorphism. Our results demonstrate that this silent site can be coconverted along with the selected mutant sites. The findings are consistent with a mechanism of gene conversion that involves contiguous blocks of DNA differing in length, position, or both. An additional finding is that the products of coconversion events involving the silent site are unequally recovered although the rates of conversion observed at four different selected sites are similar.**

Gene conversion, a nonreciprocal form of information transfer, was originally described in fungi some 30 years ago. In 1970, Edelman and Gally suggested that a mechanism similar to gene conversion could be active in maintaining sequence homogeneity in mammalian multigene families (1). Since then, numerous DNA sequence comparisons of related genes have reinforced the idea that genetic information could be transferred between related mammalian genes, at least when viewed on an evolutionary time scale. In one early study (2), the process responsible for such transfer of information was proposed to be gene conversion.

We have previously described studies designed to systematically examine homologous recombination events occurring between a pair of genes lying in close proximity on the same chromosome in cultured mammalian cells (3, 4). We observed that events consistent with both nonreciprocal and reciprocal exchange could occur in the same parent line containing a single gene duplication (4). Events consistent with nonreciprocal information transfer, or gene conversion, were found to make up a majority (80–85%) of the total recombination events (4). In light of these findings, we have undertaken a detailed inquiry into the mechanism of gene conversion between artificially created gene duplications in mammalian cells. Studies such as these should be of interest both in terms of understanding how repeated sequences interact and in terms of providing information on homologous recombination between chromosomal sequences.

In fungi, gene conversion can act in a single event on two or more markers—that is, the markers can be "coconverted." Coconversion of separate markers suggests that conversion events in fungi involve regions of DNA more extensive than simply a single site within a gene (for a review see ref. 5). In this report, we present experiments designed to answer several related questions about the nature of information transfer between duplicated Herpes virus thymidine kinase gene sequences in mammalian cells. Do the events involve regions of DNA as in fungi, or might the process act only on the mutation itself? Assuming that regions are involved, is information transferred as a contiguous block or in a discontinuous manner so that transfer is "patchy"? Are these tracts of conversion fixed or can different regions of information be transferred?

In brief, the conversion events studied appear to involve transfer of a contiguous block of information. In addition, the findings indicate that conversion tracts between duplicated elements sharing 1.2 kilobase pairs (kbp) of homologous sequence can involve as much as 358 bp of information, and that these tracts are of variable length, position, or both. Finally, another outcome of these studies is that products of coconversion events are unequally recovered, although the rates of conversion *per se* are similar within the duplication.

MATERIALS AND METHODS

Cell Culture and Transformation. Mouse L cells lacking thymidine kinase (TK) (LTK^- cells) were cultured and transformed with DNA as previously described (3, 4).

Plasmid Construction. The plasmid vector is a derivative of pSV2-neo (6) with restriction site alterations as previously described (4). *Xho* I linker insertion mutations of the herpes simplex virus (HSV) type 1 (strain F) TK gene were a generous gift from David Zipser and Jesse Kwoh. The four "recipient" *Xho* I mutant TK genes used in these studies were inserted on a 2.4-kbp fragment into the unique *Bam*HI site of the pSV2-neo plasmid after attachment of synthetic *Bam*HI linkers (New England Biolabs). The 1.2-kbp "donor" fragment was originally isolated from the wild-type TK gene from strain 101 of HSV type 1 by a *Hin*cII/*Sma* I double digestion, and was inserted into the *Hin*dIII site of our pSV2-neo plasmid derivative (4) by using synthetic *Hin*dIII linkers (New England Biolabs). This fragment encodes most of the coding region but lacks the normal HSV TK promotor region and polyadenylylation signals.

Derivation of Parental Cell Lines and Determination of Plasmid Copy Number. LTK^- cells were transformed by plasmid DNA (linearized by digestion with the restriction enzyme *Cla* I) plus LTK^- carrier DNA. Subsequent selection in the presence of the antibiotic G418, isolation of G418-resistant transformants, and determination of plasmid copy number and sequence arrangement were performed as described (4). Copy number was initially determined by single restriction enzyme digestions with *Hin*dIII and *Bam*HI. Candidates for single-copy lines were those that displayed only two bands in each digest—i.e., either the 1.2-kbp band plus a junction fragment in the case of *Hin*dIII digestion or a 2.4-kbp band plus one junction fragment in digestions with *Bam*HI. As a better criterion for the identification of parent lines possessing only one recipient gene, TK^+ recombinants

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; bp, base pair(s).

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were examined by *Bam*HI digestion, either with or without *Xho* I. In single-copy lines all the material at 2.4 kbp—that is, the recipient sequence—should be resistant to *Xho* I digestion. The four parent lines containing donor plus *Xho* I mutants 29/8, 8, 153, or 29 each maintain a single duplication without detectable partial copies. In the two lines with mutant 28, line 1 contains two complete copies, whereas line 2 has one complete copy in addition to a partial copy that has the recipient sequence but no detectable donor. In the line with two complete copies the rates of conversion were adjusted by division of the overall rate by 2. In both cases extra sequences do not have any effect on the determination of coconversion frequencies.

Isolation of Independent Recombinants and Determination of Recombination Rates. Isolation of independent recombinants was performed as previously described (4). Rate determinations were made by measuring the total number of hypoxanthine/aminopterin/thymidine-resistant colonies arising in each of 10–15 independent subcultures for each parent line. Rates per cell generation were then calculated by a modified form of the Luria–Delbruck equation (7).

Molecular Hybridization Analysis. After isolation and purification, DNAs were digested to completion with the appropriate restriction endonucleases, electrophoresed through 0.8% agarose (Sigma) gels, transferred to nitrocellulose, and hybridized with an HSV TK-specific probe as previously described (3).

RESULTS

Experimental Rationale. In a previous study we examined intrachromosomal recombination in two mouse cell lines each containing a single pair of defective HSV TK genes. The majority of the events observed (80% overall) were consistent with either nonreciprocal information transfer (gene conversion) or double unequal reciprocal exchange between sister chromatids. The remaining events were readily explained by single reciprocal exchange. Because it seems unlikely that double sister chromatid events should be more prevalent than single events, we believe that the simplest explanation for the majority class is gene conversion. Also relevant are similar studies performed in yeast that indicate conversion events not associated with reciprocal exchange are prevalent in both meiosis and mitosis (8–11).

The experiments described here are designed to inquire into the nature of this apparent nonreciprocal information transfer between closely linked repeated sequences in cultured mammalian cells. Two main issues are addressed. First, does the gene conversion mechanism involve contiguous regions of DNA or does "patchwork" correction occur? Second, are the same or different blocks of information transferred from event to event?

To address these issues, we have constructed cell lines containing a duplication of HSV type 1 TK gene sequences. The system is designed so that information transfer in only one direction is productive. The "donor" sequence used in each case is a 1.2-kbp internal fragment of the wild-type TK gene of HSV type 1 strain 101. This fragment lacks its normal promoter and essential 3′ sequences and is therefore defective; this fragment does not revert at a detectable frequency (unpublished observations). The donor fragment does, however, include most of the coding region for HSV TK (Fig. 1). The recipient sequence is a full-length gene from strain F of HSV type 1 with either one or two insertions positioned at a different location in each cross. The inserted DNA consists of an 8-bp synthetic oligonucleotide containing an *Xho* I restriction site. Conversion of the *Xho* I mutation to the normal sequence by the donor fragment results in a TK^+ gene and growth by that cell in selective medium. The two HSV TK genes from strain 101 and F are very similar, differing where sequenced in only 3/500 bp (Jesse Kwoh, personal communication, and our unpublished observations). Importantly, these two genes display a *Sac* I site polymorphism at position 961 according to the numbering system of Wagner *et al.* (12); the strain F recipient gene contains the *Sac* I site.

The nature of the information transferred is determined by selecting for conversion at the *Xho* I sites followed by testing for the presence of the *Sac* I site in the recipient gene. For example, if conversion involves regions of DNA, we would expect that in some fraction of recombinants the recipient gene would not only lose its *Xho* I site (the mutation) but also gain information from the donor at the *Sac* I polymorphic site and hence be resistant to digestion by *Sac* I. Alternatively, if conversion affects only the mutant site then the loss of the *Xho* I site would not be accompanied by the loss of the *Sac*

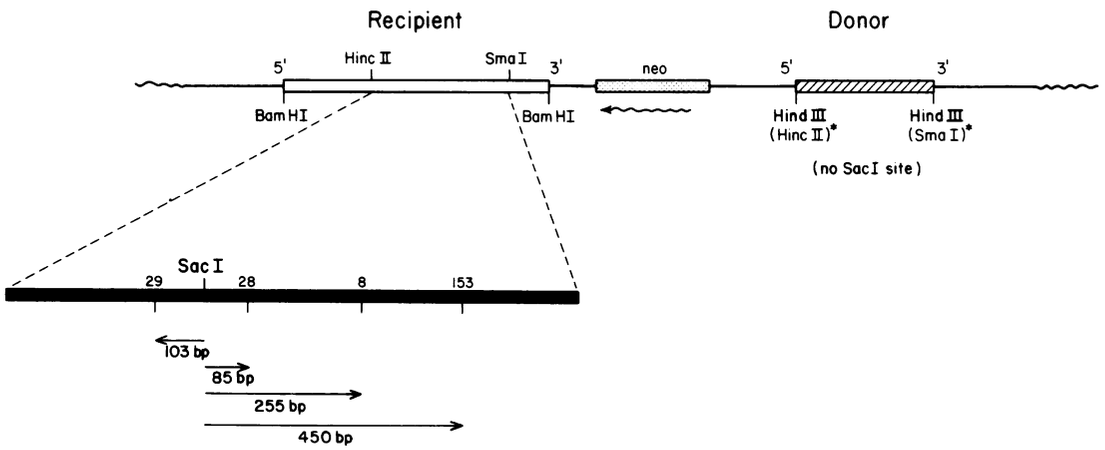

FIG. 1. Substrate used for examining gene conversion events between directly repeated HSV TK sequences that appear to be integrated into a chromosome. The donor sequence in each construction is a defective internal 1.2-kbp fragment of the normal TK gene from HSV type 1 strain 101. This internal fragment includes most of the coding region of the HSV TK gene. The recipient sequences derived from HSV type 1 strain F are each located on a 2.4-kbp *Bam*HI fragment and contain the *Xho* I oligonucleotide linker insertion mutation at different locations in the coding region of the TK gene of HSV type 1 strain F. The wild-type HSV TK gene does *not* contain an *Xho* I restriction site. * indicates previous *Hinc*II and *Sma* I restriction "ends" modified with *Hin*dIII linker addition. At the lower left, the region of the recipient gene that shares homology with the donor sequence is expanded, showing the location and the designation number of the four different *Xho* I mutations and the location of the "silent" polymorphic *Sac* I restriction site. The region indicated as neo encodes a drug resistance peptide used in selecting for cells carrying the plasmid sequences.

I site. Thus, events that alter the *Sac* I site provide evidence for coconversion.

Examples of molecular hybridization analyses of a TK^- parent line and two recombinants can be seen in Fig. 2. Both an event showing coconversion and an event without coconversion are presented. Note that in both recombinants the potential donor fragment remains unchanged, that is, it has gained neither the *Xho* I mutant site nor the *Sac* I site. Such observations are consistent with intrachromatid gene conversion but not with double-reciprocal exchange occurring within a chromatid. We cannot, however, be certain that any of these events resulted from an *intrachromatid* interaction. It is formally possible that all the events resulted from interaction between sister chromatids after unequal pairing. If so, in each case the donor sequence would have been segregated to the daughter cell and hence not recovered. We realize that this is an important issue; it may be resolved by conducting experiments with inverted repeats designed to determine the frequency of intrachromatid reciprocal exchange.

Events involving single reciprocal exchanges between the donor and recipient sequences should *not* be productive because they generate either 5′ or 3′ deletions, depending on the position of the reciprocal exchange or crossover. Correction of the donor fragment to wild type is not likely because terminal nonhomologous sequences flank this sequence.

Conversions of At Least 358 bp of Information Occur in Contiguous Blocks Within the HSV TK Gene. We have constructed an HSV TK recipient gene carrying two different *Xho* I insertions, each of which confers a mutant TK phenotype. Correction of this double mutant to a functional gene requires conversion at both *Xho* I sites. If conversion acts only at a mutant site, then correction to wild type would require, a priori, two independent events each of which, on the basis of previous work (unpublished observations) and experiments presented below, would occur at a rate of $\approx 10^{-6}$ per cell generation. Therefore, correction of the double mutant to wild type should take place at a rate of $\approx 10^{-12}$ per cell generation and hence not be detectable.

As shown in Table 1, conversion of the 29/8 double mutant gene to wild type was observed at a rate of 5×10^{-8} per cell generation, or well above that expected for two independent events. This rate suggests that the conversion of the two *Xho* I sites is due to at least associated events if not a single event encompassing both *Xho* I sites. As expected, all 22 independent recombinants showed loss of both *Xho* I sites (mutations) in the recipient gene.

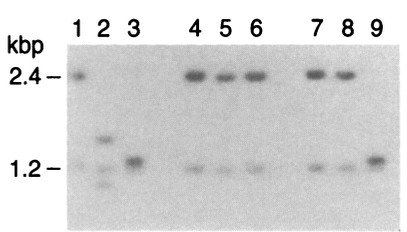

FIG. 2. Hybridization analysis of a TK^- parent (line 2) and two TK^+ recombinants from a cross involving mutant 8. The 2.4-kbp *Bam*HI fragment of HSV TK was used as probe. The donor sequence and the recipient gene are visualized on a 1.2-kbp *Hin*dIII fragment and a 2.4-kbp *Bam*HI fragment, respectively. Shown are the same three multiple digestions: *Hin*dIII/*Bam*HI, *Hin*dIII/*Bam*HI/*Xho* I, and *Hin*dIII/*Bam*HI/*Sac* I for the *parent* (lanes 1, 2, and 3), a *coconversion recombinant* (lanes 4, 5, and 6) and a *no-coconversion recombinant* (lanes 7, 8, and 9). Note that the recipient gene is *Xho* I resistant *only* in the recombinants (lanes 5 and 8) and is *Sac* I resistant *only* in the coconverted recombinant (lane 6).

Table 1. Coconversion results

Parent line (donor plus *Xho* I mutant)	Rate of conversion at *Xho* I site,* events per cell division	Frequency of coconversion at *Sac* I site
29/8 (double)	4.7×10^{-8}	22/22
28 line 1	1.2×10^{-6}	8/19
28 line 2	0.95×10^{-6}	6/11
8	0.8×10^{-6}	4/18
153	1.7×10^{-6}	0/23
29	1.5×10^{-6}	1/32

The table shows rates of conversion per duplication and frequencies of coconversion in the six parent lines studied. Each parent line, except line 1 for mutant 28, contains a single intact duplication of HSV TK sequences. Line 1, mutant 28, harbors two duplications. A duplication is composed in each case of the donor sequence plus a different *Xho* I linker insertion mutant serving as the recipient sequence. One double mutant (as recipient) which contains two *Xho* I insertion mutations (29 and 8) was also tested. In the case of mutant 28 two different parent lines were examined. The rates of conversion represent $TK^- \rightarrow TK^+$ events per cell division and are corrected for duplication copy number in the case of line 1 for mutation 28 (see *Materials and Methods* for copy number determination). The frequency of coconversion is the number of independent TK^+ recombinants that show loss of the *Sac* I restriction site in the recipient gene, divided by the total number of independent recombinants examined. A Fisher's exact test was performed to determine whether the coconversion frequencies differ significantly for the various pairwise combinations. The difference between the 29 and 28 coconversion data is highly significant, with a P value of 0.00001. For comparisons of 28 vs. 8 and 153 the P values are 0.005 and 0.0003, respectively. For 8 vs. 29 and 153 the P values are 0.03 and 0.05, respectively. The data for the double-mutant cross, 29/8, are significantly different from the four single-mutant crosses, with P values ranging from 10^{-4} to 10^{-8}. For the comparison of 29 and 153 the difference is not statistically significant.

*The total numbers of cells tested to determine the rates were as follows: for mutants 8, 28, 29, 153, and the double 29/8, they were 4.8×10^7, 1.2×10^8, 4.4×10^7, 1.1×10^8, and 8.9×10^8, respectively.

The presence or absence of the *Sac* I site in the converted recipient gene was tested. For all 22 independent recombinants examined, the recipient gene was now resistant not only to digestion by *Xho* I but also to digestion by *Sac* I, indicating coconversion at the *Sac* I site. Concomitant and consistent loss of the *Sac* I site from the converted sequence lends further support to the idea that in each case conversion at the two *Xho* I mutant sites and the intervening *Sac* I site represents a single event spanning at least 358 bp of the HSV TK gene, the distance separating the two *Xho* I mutant sites. Furthermore, loss of the *Sac* I site information in all 22 independent events suggests that in individual conversion tracts (events) information transfer occurs predominantly as a contiguous block.

The rate of conversion of the double mutant is $\approx$1/20th of the conversion of the 29 or 8 mutant sites alone. This reduced rate indicates that most conversion events do not encompass both mutant sites. To better assess the average length and position of conversion tracts we have performed additional crosses using single *Xho* I mutations in the recipient sequence.

Conversion Tracts Are Not of Fixed Length and Position, Nor Are Coconversion Events Randomly Distributed Within the TK Sequence. Using the experimental design described above, we performed four additional crosses, selecting for conversion at one of four different *Xho* I sites and subsequently scoring for coconversion at the *Sac* I site. The three *Xho* I mutations 28, 8, and 153 lie, respectively, 85, 255, and 450 bp 3′ to the *Sac* I site. The fourth *Xho* I mutation, 29, lies 103 bp to the 5′ side of the *Sac* I site.

The rates of conversion at the four different mutant sites are similar and are shown in Table 1. The rate of conversion is not affected significantly by the position of the mutations within the gene. Furthermore, in one case (crosses involving mutant 28) the rates of conversion for two different parent lines each containing the same gene pair do not vary significantly. We have not observed any obvious effects of genomic position on the rates of recombination in crosses with other mutant sequences (4, 13). Therefore, the rate of recombination seems to be determined primarily by the nature of the gene pair itself, rather than its position within the genome.

The frequencies of coconversion at the *Sac* I site for each of the four crosses are presented in Table 1. In the case of mutant 28, similar results were obtained for two parental lines, thus suggesting a lack of "position effect" on coconversion frequencies. The data for mutants 28, 8, and 153 lying 3′ to the *Sac* I site demonstrate that, as the selected site is positioned further to the right of the *Sac* I site, coconversion becomes less likely. In addition, these results also indicate that conversion tracts are not of fixed length *and* position—i.e., different blocks of information can be transferred.

Finally, a curious aspect of this study is the relative lack of coconversion at the silent *Sac* I site when selection was applied 5′ to this site at the nearby 29 mutation. The distances between either the 29 or the 28 mutant site and the *Sac* I site are similar, and yet the coconversion frequencies are strikingly different: 14/30 in the case of 28 and only 1/32 for mutant 29.

DISCUSSION

We are using mouse cell lines carrying a pair of closely linked defective HSV TK genes to study intrachromosomal recombination. A recent report of ours (4) demonstrated the usefulness of this system for detecting both nonreciprocal (gene conversion) events and reciprocal (crossover) events. In that study, events consistent with nonreciprocal information transfer were responsible for the majority of recombination events (80–85%) and single reciprocal events accounted for the remainder. Other investigators working with mammalian cells have reported on similar systems designed to detect intrachromosomal recombination events (14–16). In one of these reports (15), results consistent with reciprocal and nonreciprocal events were obtained from cell lines with one copy of the gene duplication, although the total number of events examined was small.

Here we describe experiments designed to determine the amount and nature of information transfer occurring during intrachromosomal gene conversion events. Plasmids were constructed that harbor different combinations of two defective HSV TK genes plus a dominant marker that facilitates introduction of the plasmid into TK^- mouse L cells. The two defective HSV TK sequences were engineered so that one acts as donor and one as recipient of information in the desired gene conversion events. The defective donor fragment is in each instance a 1.2-kbp internal fragment from the wild-type TK gene of HSV type 1 strain 101. This donor fragment encodes most of the coding region of the HSV TK gene (Fig. 1). The mutant HSV TK genes serving as recipients of information are derived from strain F of HSV type 1 and carry different *Xho* I linker insertion mutations, which each abolish TK gene activity (17). The important features of this system are as follows: (*i*) Simple gene conversion events not associated with reciprocal exchange are recoverable. Single reciprocal events are not productive because they produce truncated TK genes bearing deletions of essential 5′ or 3′ genetic information. (*ii*) Productive information flow should occur only from the internal fragment of strain 101 to correct the *Xho* I mutation in the full-length strain F gene, hence the terms "donor" and "recipient" sequences. (*iii*) Finally and importantly, the strain F thymidine kinase gene contains a *Sac* I site not found in the strain 101 gene. The general experimental approach was to select for gene conversion events at the mutant *Xho* I sites and to test by molecular hybridization for coconversion at the *Sac* I site. Disappearance of the *Sac* I site in the recipient gene with concomitant loss of the *Xho* I mutant sites provides a signal for coconversion, that is, events that also encompass the *Sac* I site. Four of the parent lines used in this study each contain only a single copy of the duplication, one line contains two copies, and another line has a single copy plus a partial copy (see *Materials and Methods*), thus making hybridization analysis straightforward.

The initial experiment was designed to determine if gene conversion involves regions of DNA and whether information is transferred as one contiguous block. To this end, a double *Xho* I linker insertion mutant was used as the recipient in gene conversion. The conversion rate at either of the two *Xho* I mutations alone was $\approx 10^{-6}$ per cell generation. If conversion acts only on the mutations themselves and conversion at the two sites is independent, then conversion of the double mutant should occur at a rate of $\approx 10^{-12}$ per generation and not be detectable. Wild-type convertants of the double mutant were observed at a rate of 5×10^{-8} per cell generation, suggesting that conversion of the two mutant sites represents at least associated events. In each of 22 independent recombinants the *Sac* I site of the converted gene was lost, indicating coconversion at this site. The rate of conversion of the double mutant and the consistently observed coconversion at the *Sac* I site can be most easily explained by conversion that spans the two *Xho* I mutations and involves transfer of a contiguous block of genetic information from the donor sequence to the recipient. Additionally, the fact that for all 22 independent recombinants the *Sac* I site showed coconversion supports the notion that the individual conversion events studied involve blocks of information and not some type of "patchy" transfer or correction.

That the rate of coconversion of two *Xho* I sites separated by 358 bp in the double mutant is $\approx$1/20th the conversion rate at either of these sites alone indicates that most conversion events do not encompass both *Xho* I sites. In studies with the single mutants, selection for conversion at four different mutant sites was performed, with subsequent testing for coconversion at the silent *Sac* I site. The results with the single mutants indicate that conversion tracts are of variable length, position, or both and therefore involve transfer of different segments of the HSV TK sequence.

There is one peculiarity in the observed coconversion frequencies with the single mutants. For the three markers lying to the right (3′) of the *Sac* I site the findings are relatively straightforward; the frequency of coconversion at the *Sac* I site decreases with distance, as if events are randomly located or of variable length. For example, in crosses involving mutant 28, which maps 85 bp 3′ to the *Sac* I site, coconversion occurred 50% of the time. The unexpected result was obtained for crosses involving mutant 29, which lies only 103 bp to the left (5′) of the *Sac* I site. Of 32 independent convertants, only one case of coconversion at the *Sac* I site was observed. In the simplest sense, the observed coconversion events appear to be distributed unequally within the TK gene. This observation is *not* easily explained by unequal distribution of conversion, *per se*, because the individual rates of conversion at the four *Xho* I mutant sites are similar (see Table 1). We cannot in the case of coconversion between the *Sac* I site and the 29 mutation rule out either an allele-specific effect or a cell line effect (since we have scored these events in only one parental line). One possible explanation is that the initiation of conversion events occurs randomly within the duplication, but that

propagation of these events is not the same in the vicinity of the 29 mutation as compared to that in the region of the other mutations—e.g., propagation is asymmetric for the 29 mutation but symmetric for the other mutations. Another possibility is that recombination is initiated nonrandomly and propagates in only one direction. One could envision conversion initiating primarily at the *Xho* I linker mutations in each cross and then always propagating to the left as drawn in Fig. 2. Such a model can account for both the rarity of *Sac* I/29 coconversions and the graded coconversion observed with the *Xho* I mutations lying 3′ to the *Sac* I site.

From these data we cannot distinguish between conversion tracts of different lengths and tracts of the same length that occur at different positions. We feel that the most plausible explanation of the results is that the tracts are of various lengths and positions. On the basis of this assumption, the average length of conversion tracts deduced from the experiments described above appears short when compared to coconversion in fungi (for review see ref. 5). However, these studies in yeast involved genes occupying their normal position on homologous chromosomes, thereby facilitating pairing over extensive regions. In the system presented here the shared homology is limited to 1.2 kbp of sequence. Coconversion studies analogous to ours using artificially created gene duplications have not, to our knowledge, been performed in fungi. It would be of interest to increase the size of the homologous regions in our system and determine whether more information is subsequently transferred in a given event. Deduction of the "length" of putative conversion events from most of the sequence comparison studies in mammals mentioned earlier is difficult because the number of individual events cannot be readily determined. In two cases in which only one conversion event is likely to have been involved, the two proposed tracts of conversion are rather short, ≈50 bp (18, 19).

The major finding of our study is that information transfer appears to occur as a contiguous block of information. When selection was applied for conversion at two sites separated by 358 bp, a polymorphic site lying between those two sites was always coconverted in the same direction. Therefore, in our system we did not observe "patchy" events as have been proposed in two investigations comparing DNA sequences of the human α-globin genes (20) and fetal globin genes (21). It is possible because of the design of the experiments presented here that we are looking only at a subset of conversion events.

In summary, we have studied gene conversion events between duplicated HSV TK sequences in mouse L cells and have found that these events appear to involve contiguous regions of DNA that are often less than 358 bp in length. The data also indicate that products of coconversion events are unequally recovered within the HSV TK sequence. A more complete analysis of the issues addressed in this study should be possible by using a pair of more divergent gene sequences.

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