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Gene Conversion Between Unlinked Sequences in the Germline of Mice

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Manuscript received December 3, 1993
Accepted for publication March 15, 1994

ABSTRACT

Gene conversion between homologous sequences on non-homologous chromosomes (ectopic gene conversion) is remarkably frequent in fungi. It is thought to be a consequence of genome-wide homology scanning required to form synapses between homologous chromosomes. This activity provides a mechanism for concerted evolution of dispersed genes. Technical obstacles associated with mammalian systems have hitherto precluded investigations into ectopic gene conversion in the mammals. Here, we describe a binary transgenic mouse system to detect ectopic gene conversion in mice. Conversion events are visualized by histochemical staining of spermatids, and corroborated by polymerase chain reaction amplification of transgenes in spermatozoa. The results show that conversion between unlinked, hemizygous *lacZ* transgenes is frequent in the male germline, ranging from 0.1 to 0.7% of spermatids. Genomic location may affect the susceptibility to recombination, since the frequency varied between lines. The results suggest that homologous genes can undergo concerted evolution despite being genomically dispersed. However, mechanisms may exist to modulate this activity, enabling the divergence of duplicated genes.

GENE conversion—the non-reciprocal exchange of genetic information—is a potential outcome of most recombination events. First observed in fungi as the non-Mendelian segregation of alleles in individual meioses, it was realized that conversion and crossing over were alternative outcomes of recombination. Therefore, general models of recombination were developed that account for both reciprocal and nonreciprocal exchange (HOLLIDAY 1964; MESELSON and RADDING 1975; SZOSTAK *et al.* 1983). Elegant studies in fungal systems showed that gene conversion occurs not only between alleles, but intrachromosomally between duplicated sequences, and ectopically between homologous sequences on nonhomologous chromosomes (ERNST *et al.* 1981; JACKSON and FINK 1985; JINKS-ROBERTSON and PETES 1985, 1986; KLEIN and PETES 1981; LICHTEN *et al.* 1987; MIKUS and PETES 1982; PETES and HILL 1988).

It has been postulated that ectopic gene conversion is a consequence of genome-wide homology scanning required to form synapses between homologous chromosomes (ALANI *et al.* 1990; CARPENTER 1987; ENGEBRECHT *et al.* 1990; SMITHIES and POWERS 1986). According to this theory, either alleles or duplicated sequences on nonhomologous chromosomes undergo pairing and local synaptonemal complex formation during the first meiotic prophase, potentially undergoing gene conversion as a mechanism to determine whether the homology extends throughout both chromosomes (the case with chromosome homologs). In yeast, as little as 2.2 kb of homology is sufficient to catalyze ectopic recombination (LICHTEN *et al.* 1987).

Ectopic gene conversion has several implications for genome evolution and stability. It provides a potential mechanism for concerted evolution of genes that have been dispersed by chromosome duplications or amplification mechanisms. Conversely, novel hybrid genes can be created by conversion between gene subregions. In this scenario, ectopic gene conversion would be a mechanism for generating diversity and accelerating evolution (BALTIMORE 1981). Finally, since a considerable percentage of ectopic conversion events have an associated crossover (LICHTEN *et al.* 1987), chromosomal rearrangements can result, thereby providing a potential impetus for speciation (BUSH *et al.* 1977; WILSON *et al.* 1975). The most important parameter concerning significance of ectopic gene conversion during evolution is its frequency in the germline.

Evidence for gene conversion in mammals originated from sequence analysis of natural allele variants of human fetal globin genes (SLIGHTOM *et al.* 1980). A large body of supportive data from sequence comparisons of duplicated genes has since accumulated (ARNHEIM 1983; FITCH *et al.* 1990). More direct evidence was later obtained that mitotic gene conversion occurs in both mammalian cultured cells (LISKAY and STACHELEK 1983), and in the mouse germline (reviewed in GELIEBTER and NATHENSON 1987). Finally, an assay for detecting meiotic gene conversion between *lacZ* transgenes in mouse spermatids allowed efficient quantitation of this process in a whole animal (MURTI *et al.* 1992). To date, there have been no conclusive reports of gene conversion between unlinked DNA sequences in the mammalian genome. We report here a binary transgenic mouse system for

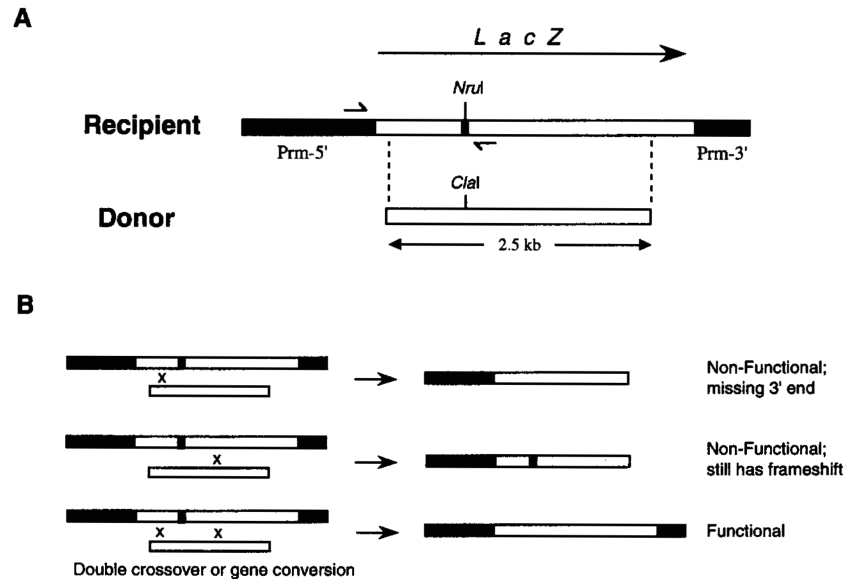

FIGURE 1.—Gene conversion constructs and possible recombination outcomes. (A) As indicated, the black boxes represent *Prm-1* sequences, and empty boxes represent *lacZ* sequences. Transcriptional orientation is indicated by the arrow. The black vertical stripe in the recipient *lacZ* gene is a 2-bp insertion mutation, created by cleavage at the wild-type *Cla*I site (present in "donor"), and filling the 2-bp overhang to create a *Nru*I site (MURTI *et al.* 1992). The vertical dashed lines indicate the portion of *lacZ*-coding sequence present in the donor; it is deleted for the first 36 and last 136 amino acids of the enzyme. The *Prm-1* promoter contains 1145 bp of 5'-flanking sequence and 95 bp of 5'-untranslated sequence ending precisely at the initiation codon, where it has been fused to the second amino acid of *lacZ*. The recipient construct is 5.0 kb in length. PCR primers used to specifically amplify the recipient gene from sperm DNA are indicated by half-arrowheads. (B) The results of possible recombination events between the recipient and donor and each outcome are shown. Only a double crossover or gene conversion will generate a function *lacZ* gene.

detecting and quantitating germline ectopic gene conversion in mice. Conversions between unlinked recipient and donor β-galactosidase (*lacZ*) templates are visualized by histochemical staining of spermatids, and a polymerase chain reaction (PCR) assay is used to detect converted molecules. The results demonstrate that ectopic gene conversion is a highly active process in the murine male germline, as it is during yeast meiosis. These data extend the paradox in yeast regarding how genomes maintain stability despite the presence of dispersed repetitive sequences that may serve as templates for illegitimate recombination.

MATERIALS AND METHODS

Transgenic mice: The "recipient" line used in this work has been described (MURTI *et al.* 1992), and was formerly called "mP1-LacFin." The donor construct is a 2.55-kb *Pvu*II fragment from the *lacZ* gene-coding region, purified from the plasmid pLacC. Microinjection of this DNA into C57BL/6J × SJL F_2 embryos was performed by standard methods (HOGAN *et al.* 1986). Transgenic lines were generated in the transgenic mouse facility at Case Western Reserve University and maintained in the Animal Resource Center there. Lines were expanded by crossing to CF1 mice (Charles River Laboratories), and *lacZ* activity staining (see below) was performed on animals produced in this fashion. The number of donor construct copies in each line was determined by PhosphorImager analysis of transgene junction fragments. Recipient mice contain three copies (MURTI *et al.* 1992). Donor/recipient double hemizygotes were produced by crosses between donor/+ and recipient/+ mice. Those offspring containing both transgenes were identified by Southern blot analysis of *Sac*I digested tail DNAs. The recipient and donor transgenes produce distinct fragments which are detected by hybridization to a radiolabeled *lacZ* probe. These lines were then imported to The Jackson Laboratory and bred into the C57BL/6J background. Sperm samples for PCR analysis (described below) were done at the N2 backcross generation.

***LacZ* activity staining:** Elongated spermatids were purified from testes by collagenase/trypsin treatment and unit gravity sedimentation. The procedures for this and *lacZ* activity staining were performed exactly as described (MURTI and SCHIMENTI 1991; MURTI *et al.* 1992). For examination of intact seminiferous tubule regions, limited collagenase/trypsin treatment was performed and stained as above.

PCR amplification of transgenic sperm DNA: About 2,000 epididymal sperm were used as substrate for PCR as described (CUI *et al.* 1989). The primers (Figure 1A) generate a 1.2-kb amplified fragment. The *lacZ* primer was radiolabeled using polynucleotide kinase and [γ-^{32}P]dATP prior to PCR. The amplified fragments were purified from agarose gels, followed by digestion with either *Cla*I or *Nru*I. The digested DNA was electrophoresed on a 12% acrylamide non-denaturing gel, which was dried and exposed to X-ray film (shown) or imaged on an AMBIS blot analysis system for direct quantitation of radioactive emissions.

RESULTS

Transgenic system for detecting ectopic gene conversion: Transgenic mice carrying two different *lacZ* constructs, a "recipient" and "donor," were generated (Figure 1A). The recipient *lacZ* gene is under the transcriptional control of the spermatid-specific mouse protamine-1 (*Prm-1*) promoter and has an internal 2-bp frameshift mutation. The donor *lacZ* gene is truncated

TABLE 1

Quantitation of ectopic conversion events

Genotype	NonTg + double Tg/total	No. cells scored	Transgene copies	No. blue	Percent blue
Recip/+ donor A/+	28/45	2,700	2	19	0.7
Recip/+ donor B/+	12/20	9,872	3	13	0.13
Recip/+ donor D/+	6/13	1,842	4	4	0.22
Recip/+ +/+		32,000[a]	3	2	0.006

For each transgenic genotype shown, purified elongated spermatid populations were stained for *lacZ* activity, and the indicated number of positives (No. Blue) were observed in a screen of the indicated number of elongated spermatids (No. cells scored). The "NonTg + double Tg/total" column displays the ratio of [pups inheriting neither or both transgenes] to [total offspring] produced by each double hemizygote when mated to a wild-type mouse.

[a] The quantitation of this line is taken from our previous report (MURTI *et al.* 1992).

at both termini, has no promoter, but is unmutated at the position corresponding to the recipient's lesion. One recipient line and three donor lines (donors A, B and D) were used in these studies. Mice containing recipient and donor transgenes in non-homologous positions of the same genome were produced by matings between recipient and donor animals. In these double hemizygotes, a gene conversion that transfers the donor's unmutated internal sequence to the recipient generates a functional *lacZ* gene, allowing the host spermatid to produce β-galactosidase (Figure 1B). The three mutation design (insertion in the recipient, double truncations of the donor) does not allow single reciprocal crossovers to generate a functional *lacZ* gene (Figure 1B). Since recombination studies with analogously mutated thymidine kinase gene templates in cultured mammalian cells indicate that double crossovers are never seen (LETSOU and LISKAY 1987; LISKAY and STACHELEK 1986), any functional correction of the recipient is presumably caused by gene conversion.

As the goal of this work is measure gene conversion between sequences on non-homologous chromosomes, we performed backcross analysis to determine whether any of the three independent donor insertions were linked to the recipient insertion. Each of the donor and recipient lines have a single site of integration as reflected by Mendelian transmission (data not shown). Since microinjected transgenes insert randomly into the genome, and none of the insertions showed sex linkage, there is a 1/19 chance (mice have 19 autosomes) that a donor insertion has an integration site on the same chromosome as the recipient. If recipient and donor genes were linked, most offspring produced by doubly hemizygous F_1 parents would inherit either the recipient or donor transgene, but not both. As shown in Table 1, about half of the offspring from double hemizygotes of each recipient/donor combination inherited neither or both of the transgenes. This observation of independent assortment indicates that each of the donor transgenes resides on a separate chromosome from the recipient. There remains a small possibility, however, that one or more of the donors is on the same chromosome as the recipient, but genetically unlinked by hyper-recombination or physical distance.

Quantitation of conversion events: The recipient line was mated to each of three independent donor lines to create three sets of doubly transgenic males. Purified elongated spermatids from males of each genotype were stained for *lacZ* activity. Recipient hemizygotes yielded a negligible number of blue spermatids (Table 1; MURTI *et al.* 1992). However, all three types of double hemizygotes produced a substantial proportion of *lacZ* positive sperm, ranging from 0.13% to 0.7% (Table 1).

In accordance with our previous studies on intrachromosomal gene conversion in the male germline, transgene copy number was unrelated to the conversion frequency (Table I). These results agree with investigations of ectopic gene conversion in yeast (HABER *et al.* 1991). In contrast to yeast, however, genome location may affect ectopic recombination frequency in mice; the donor A line yielded 3–4-fold more *lacZ*-positive cells than the other two lines.

Identification of converted *lacZ* templates by PCR analysis of sperm DNA: To obtain molecular corroboration that the presumed gene conversions responsible for generating *lacZ*-positive cells indeed involve the planned sequence transfer, PCR amplification of the recipient gene from recipient/donor A sperm DNA was performed using the primers shown in Figure 1. The amplified fragments were digested with either *Nru*I or *Cla*I. Cleavage with *Cla*I is indicative of a gene conversion event, and *Nru*I cleaves the vastly predominant nonrecombinant products. The results are shown in Figure 2. Whereas amplified material from a mixture of recipient/+ and donor A/+ sperm DNA showed no digestion with *Cla*I (Figure 2), approximately 0.5% (determined by direct quantitation of radioactive emissions–see MATERIALS AND METHODS and Figure 2 legend) of the product from double hemizygotes was cleaved to the expected size. To normalize this percentage for comparison to the *lacZ* activity staining results, it must be divided by 2, since only half the sperm contain an amplifyable recipient template, and multiplied by three, since only one recipient transgene copy (of the three in each sperm) needs to be converted in order for

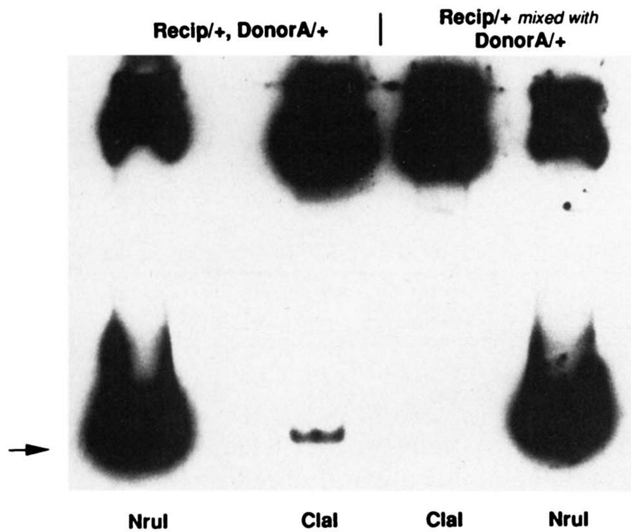

FIGURE 2.—Molecular identification of converted spermatids. The recipient gene from sperm DNA was PCR-amplified using the primers shown in Figure 1A, end-labeled, and digested with either *Cla*I or *Nru*I. The first two lanes represent a control in which sperm from recipient and donor A hemizygotes was mixed. The last two lanes represent sperm from [recipient/+, donor A/+] doubly hemizygous animals. Liberation of the 50-bp fragment (indicated by an arrow) by cleavage with *Nru*I reflects non-recombinant transgenes, whereas *Cla*I cleavage represents the converted material.

a sperm to become *lacZ*-positive. The resulting percentage of 0.75% is in remarkable agreement with that determined by *lacZ* staining (0.7%).

Evidence for mitotic gene conversion: Yeast undergo mitotic ectopic gene conversion about 1000 times less often than during meiosis (ERNST *et al.* 1981; HABER *et al.* 1991; JINKS-ROBERTSON and PETES 1985; LICHTEN *et al.* 1987; MIKUS and PETES 1982; SCHERER and DAVIS 1980). Examination of *lacZ* staining patterns in seminiferous tubules of recipient/donor hemizygotes provided evidence that some portion of the conversions occur in the mitotically proliferating germline. As with our previous investigations of intrachromosomal gene conversion (MURTI *et al.* 1992), this is inferred by the presence of *lacZ*-positive spermatid clusters in seminiferous tubules (Figure 3). The portion of converted spermatids that arose from pre-meiotic recombination events could not be determined in this system; the converted cells can only be visualized once they have matured to spermatids, the time at which the *Prm*-1 promoter becomes active.

DISCUSSION

Nature of the ectopic correction events: The transgene constructs used in this study were designed such that gene conversion is the most plausible explanation for *lacZ* correction. That *lacZ* activity was only observed when both recipient and donor transgenes were present in the same genome indicates that the appearance of positive spermatids is due to interaction of the transgenes, not spontaneous mutation or suppression of the frameshift mutation in the recipient. Additionally, the appearance of clusters of *lacZ*-positive cells is indicative of a single *genetic* event that is inherited clonally, rather than a leaky, biochemical phenocopy that appears in a certain percentage of cells. Finally, the predicted molecular change in the *lacZ* recipient sequence was detected by a PCR assay.

Another possible recombination mechanism that could restore *lacZ* function is double reciprocal crossover between the donor and recipient transgenes, with crossover points on either side of the recipient gene's mutation. However, this is highly unlikely for such a small stretch of DNA, unless negative interference is involved. Similar experiments performed in cultured mammalian cells revealed no such exchange (LETSOU and LISKAY 1987; LISKAY and STACHELEK 1986). Ectopic recombination in yeast occurs primarily by gene conversion, and sometimes by reciprocal translocation (single crossovers), but double reciprocal crossing over is not observed (SUGAWARA and SZOSTAK 1983; JINKS-ROBERTSON and PETES 1985; LICHTEN *et al.* 1987; MIKUS and PETES 1982). Although gene targeting events in mouse ES cells are usually portrayed as double crossovers, it is unknown whether a gene conversion or reciprocal crossover mechanism is responsible, since the resultant structure of the donor (transfected DNA) cannot be recovered. Furthermore, in contrast to ectopic recombination between chromosomal sequences, gene targeting involves a non-chromosomal DNA fragment with two free ends. In conclusion, while either gene conversion or double reciprocal recombination can underlie the molecular correction of *lacZ* activity, and the two cannot be distinguished in this system, existing data from yeast and mammalian systems indicate that gene conversion would be exclusively or predominantly responsible for the correction events.

Comparison of ectopic gene conversion in yeast and mice: Ectopic gene conversion during yeast meiosis can be remarkably frequent, occurring at rates comparable to that between allelic sequences or adjacent sequences on the same chromosome (HABER *et al.* 1991; JINKS-ROBERTSON and PETES 1985; KLEIN and PETES 1981; LICHTEN *et al.* 1987; PETES and HILL 1988). Haber and colleagues found that the ectopic conversion frequency of a *LEU2* recipient marker gene was independent of donor sequence copy number, and that an unlinked *leu2* donor was just as likely to convert the recipient as was the allele of the recipient (HABER *et al.* 1991). Based on these data, they argued that conversion frequency is strictly dependent upon "activation" of a recipient gene to undergo recombination, and that the genomic search process for homologous sequences is not rate-limiting. We also report that the conversion of a recipient gene was unrelated to the number of donor templates present in the genome. However, one of the donor lines (donor A) converted the recipient at a greater frequency than

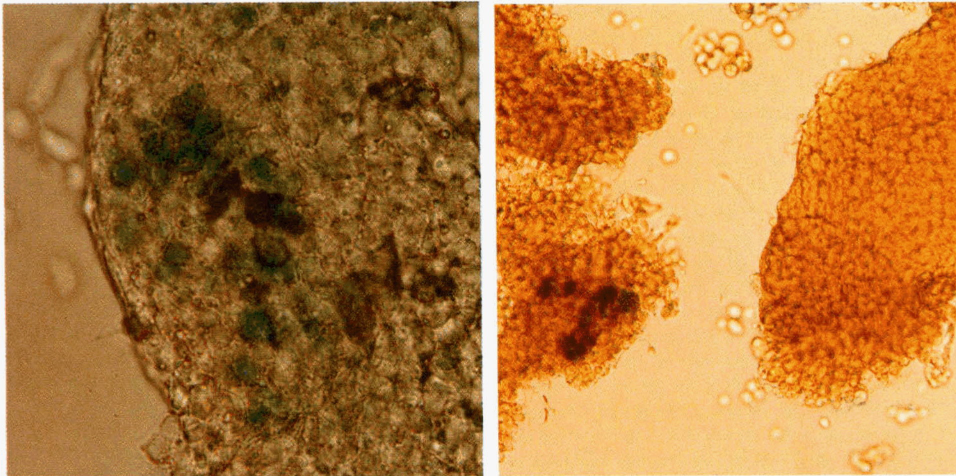

FIGURE 3.—Mitotic conversion events. The photomicrographs show portions of seminiferous tubules from different mice stained for *lacZ* activity (MATERIALS AND METHODS). They represent examples of clustered *lacZ* positive cells that are occasionally observed. Magnification of the left panel is 1000×, and the right panel 500×.

two other donor lines. This suggests that genomic location may affect the ability of two sequences to interact, and that conversion is not strictly dependent upon recipient "activation" in mice. However, one caveat is that the relative orientations of the donor and recipient *lacZ* genes relative to the centromere are unknown. It is possible that the donor lines with a lower recombination frequency (donors B and D) might be in the opposite centromeric orientation relative to the recipient, and that if crossovers were associated with a significant proportion of conversion events, the resulting acentric and dicentric chromosomes might lead to cell death, effectively reducing the observed conversion frequency.

Another potential difference with yeast is that the frequency of ectopic conversion measured here was up to 10-fold lower than intrachromosomal conversion levels (about 2%) we previously reported, in which the recipient and donor substrates were identical to those shown in Figure 1 (MURTI *et al.* 1992). If the limiting step in recombination is indeed recipient gene activation, then the ectopic and intrachromosomal gene conversion frequencies in mice should be similar. One possible cause of this disparity is that the larger mammalian chromosome and genome size decreases the chance for unlinked sequences to interact before homologous chromosomes undergo proper alignment. Another possibility is that the recipient gene in these studies happens to be at a location in the genome that lowers its relative propensity to become recombinationally active.

This ability of mammalian germ cells to undergo ectopic recombination is consistent with the hypothesis that indicate a genome-wide homology search of nuclear DNA is conducted as a mechanism to pair homologous chromosomes prior to the first meiotic division (CARPENTER 1987; SMITHIES and POWERS 1986). The availability of numerous recombination and meiosis mutants in yeast have been utilized to gather strong support for this theory (ALANI *et al.* 1990; BISHOP *et al.* 1992; ENGEBRECHT *et al.* 1990). It may be some time before the tools are available to address these questions similarly in mammals. However, it is likely that the mechanisms for homolog recognition are conserved, since meiosis is such a fundamental cellular process.

Why mitotic ectopic recombination?: Whereas ectopic recombination appears to reflect a mechanism for homologous chromosome pairing at meiosis, pairing is not required for mitotic division. The fact that yeast and mice perform ectopic recombination during mitotic growth, somatic crossing over (in mammals), and homologous recombination of exogenously introduced sequences with chromosomal homologs may reflect another function, possibly that of DNA repair (PADMORE *et al.* 1991). It may be useful to measure the ectopic conversion rate in "knockout" mice deficient in DNA repair to address this question.

Implications of high frequency ectopic recombination for genome stability and evolution: The frequencies observed imply that the mammalian genome, considering its highly repetitive nature, should be chaotic as a consequence of frequent ectopic recombination. In yeast, a substantial proportion of ectopic gene conversions are associated with crossing over, resulting in translocation (LICHTEN *et al.* 1987). The paradox between high frequency ectopic recombination and relative genome stability has been considered previously (JINKS-ROBERTSON and PETES 1985), with the suggestion that illegitimate reciprocal crossovers are somehow largely suppressed, possibly due to selection against recombinationally active sequences which yield inviable products (LICHTEN *et al.* 1987). Interestingly, a yeast mutant has been described which specifically lowers recombination within the rDNA and *CUP1* gene loci, but not others (KEIL and MCWILLIAMS 1993). It is possible, then, that cells may be able to selectively regulate the recombination of particular sequences.

It is unknown whether the high levels of ectopic recombination observed between the *lacZ* transgenes applies to endogenous, dispersed sequences in the mammalian genome. It is important to consider three factors about homologous recombination in mammalian cells

when considering this question. (1) A pair of sequences must be highly homologous for efficient recombination (LISKAY *et al.* 1987; RIELE *et al.* 1992), (2) recombination rates decrease linearly with size of the shared homologies from 2 kb down to 295 (LISKAY *et al.* 1987) and (3) at least 134–232 bp of perfect, uninterrupted homology is required for efficient initiation of recombination (WALDMAN and LISKAY 1988). The conversion substrates in this study have a single difference embedded within 2500 bp of identity, and therefore meet all the above criteria for efficient recombination.

In general, related but unlinked mammalian genes display significant divergence, suggesting that they manage to escape the homogenizing influence of gene conversion. L1 sequences in house mice are a large family of highly similar, dispersed sequences. They underwent a recent expansion about 2 million years ago (about 50,000 new copies created), and all members are at least 95% identical (CASAVANT *et al.* 1988). Nevertheless, the low degree of divergence may be enough to violate criteria (1) and (3) above. Small interspersed repeats (such as B1 and B2 elements) may not be long enough to serve as efficient substrates for ectopic recombination [criterion (2)]. Alternatively, sequences such as L1's may indeed catalyze significant levels of ectopic recombination, and those events which create inviable rearrangements could underlie, for example, spontaneous abortions. Viable rearrangements may be responsible for the initiation of species divergence (BUSH *et al.* 1977; WILSON *et al.* 1975) and karyotypic differences among isolated mouse populations.

The results reported here indicate that sequences cannot be recombinationally isolated during mammalian evolution simply by genomic dispersal, despite the large genome size. The data suggest that immediately after duplication, genes or larger chromosomal regions would undergo concerted evolution in the absence of events that disrupt conversion. Since genes do in fact diverge, there must be mechanisms to uncouple homologs from a co-evolutionary mode and enable divergence (WALSH 1987). Some possibilities are the disruption of homology by insertion of transposable elements (BRUNNER *et al.* 1986; HESS *et al.* 1983; SCHIMENTI and DUNCAN 1984), modulation of recombination between specific genes by genetic mechanisms (KEIL and MCWILLIAMS 1993), and stimulation of rapid sequence divergence by "ripping," or *r*epeat *i*nduced *p*oint mutations (KRICKER *et al.* 1992).

We thank TRACY MOURTON for performing the microinjections, EVA EICHER and DERRY ROOPENIAN for critical reading of the manuscript, and appreciate the helpful discussions with CRAIG DUNCAN and MARSHALL EDGELL. This work was supported by grants to J.C.S. from the Searle Scholars Program/The Chicago Community Trust, the National Institutes of Health (GMR01-45415), and the National Science Foundation (Presidential Young Investigator Award). M.B. was supported by a National Institutes of Health Molecular Biology training grant at Case Western Reserve University, and the transgenic facility there was supported by a grant from The Lucille Markey Charitable Trust. J. R. M. and M. B. contributed equally to this work.

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Communicating editor: R. E. GANSCHOW