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High-Frequency Germ Line Gene Conversion in Transgenic Mice

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Gene conversion is the nonreciprocal transfer of genetic information between two related genes or DNA sequences. It can influence the evolution of gene families, having the capacity to generate both diversity and homogeneity. The potential evolutionary significance of this process is directly related to its frequency in the germ line. While measurement of meiotic inter- and intrachromosomal gene conversion frequency is routine in fungal systems, it has hitherto been impractical in mammals. We have designed a system for identifying and quantitating germ line gene conversion in mice by analyzing transgenic male gametes for a contrived recombination event. Spermatids which undergo the designed intrachromosomal gene conversion produce functional β-galactosidase (encoded by the *lacZ* gene), which is visualized by histochemical staining. We observed a high incidence of *lacZ*-positive spermatids (~2%), which were produced by a combination of meiotic and mitotic conversion events. These results demonstrate that gene conversion in mice is an active recombinational process leading to nonparental gametic haplotypes. This high frequency of intrachromosomal gene conversion seems incompatible with the evolutionary divergence of newly duplicated genes. Hence, a process may exist to uncouple gene pairs from frequent conversion-mediated homogenization.

Gene (or DNA) duplication is a major molecular mechanism in the evolution of higher organisms (38). It recruits preexisting genetic material as a substrate for the formation of novel functional units, thereby catalyzing saltatory genetic changes. Extra gene copies created through duplication may ultimately diverge to perform related but specialized developmental and biochemical function. The mammalian globin family, which has evolved a highly coordinated process of tissue- and stage-specific expression of developmentally specialized genes from a single ancestral gene, exemplifies this process. Duplication of sub-gene fragments, followed by exon shuffling, is thought to have created the present-day pool of genes from only 1,000 to 7,000 exons (12). The homeodomain is an example of a widely used functional motif which spread by duplication (1, 46).

Immediately following duplication via unequal recombination, a new gene or DNA fragment would be identical to the preexisting genetic information. These duplicated sequences are susceptible to gene conversion, a recombination mechanism which can involve any homologous DNA sequences.

Gene conversion has been studied primarily in fungi, from which all of the products from a meiosis can be isolated (39, 40). In mammals, much of the evidence for germ line gene conversion is based on comparative sequence analysis of duplicated genes. A duplication unit containing a patch of near sequence identity within a larger stretch of considerable divergence is the kind of data best explained by gene conversion. The classic example of such evidence exists in the human fetal globin genes, G_γ and A_γ, which arose via duplication of a 5-kb DNA sequence about 34 million years ago. While regions flanking the genes have diverged by 14%, an internal 1.5-kb region is virtually identical, leading to the conclusion that a recent gene conversion event had occurred at this locus (49).

Gene conversion can play paradoxical roles in the evolution of a gene family. On the one hand, related gene family members can undergo sequence homogenization by gene conversion, thereby inhibiting divergence and potential adaptation. Several theoretical studies have addressed the confounding effects of gene conversion-mediated homogenization upon the evolutionary analysis of repeated genes, presenting mathematical models on the role of gene conversion in evolution (13, 18, 27, 34, 53). On the other hand, micro-gene conversions (events involving portions of a gene as small as about 50 bp) can generate diversity by introducing multiple, simultaneous sequence changes into a member of a gene family (3). In either case, the significance of gene conversion during evolution is directly related to its frequency in the germ line.

A comprehensive investigation into mammalian gene conversion frequency faces two major technical and logistical difficulties. The first is that large numbers of progeny must be scored. Second, the events must be readily detectable. These obstacles have limited observation of gene conversion in mice to labor-intensive DNA sequence analysis or serological assays of graft rejection (reviewed in reference 16).

We have devised a novel strategy to detect germ line gene conversion which eliminates these constraints. The crux of the system is to generate transgenic mice in which a planned conversion event can be visualized in sperm, rather than offspring, by simple histochemical staining. This strategy allows the rapid quantification of thousands of meiotic events for gene conversion at the transgene locus. The results indicate that germ line gene conversion is a highly active process. This has important implications for the evolution of duplicated genes.

MATERIALS AND METHODS

Constructs. The mP1-LacFin construct was built as follows. Plasmid pLacF (provided by Jacques Peschon), which contains the entire *lacZ* gene upstream of the mouse protamine 1 (mP1) coding and 3′ untranslated sequences, was linearized at a *Cla*I located in the *lacZ* gene, Klenow filled, self-ligated, and transformed into bacteria. This resulted in a 2-bp insertion at amino acid 280 of the *lacZ* gene, causing a frameshift mutation. The insertion was verified by sequencing. This clone was digested with *Nco*I (which encompasses the initiation codon of *lacZ*) and *Sma*I, which is located 5′ of

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lacZ in the plasmid polylinker. Into this was forced cloned an mP1 1.2-kb *Stu*I-*Nco*I fragment representing 1,145 bp of 5′ flanking and 95 bp of 5′ untranslated sequence ending precisely at the initiation codon. This fragment was isolated from the plasmid clone pmP1-P3.2 (provided by Jacques Peschon) after complete digestion with *Stu*I and a partial *Nco*I digest. The *Nco*I site includes the mP1 initiation codon, and thus the hybrid construct (pmP1-LacFin) is a precise joining of the mP1 5′ untranslated sequences and the *lacZ* amino acid coding region. The insert was excised from the vector with the enzymes *Kpn*I and *Hin*dIII for microinjection. To construct mP1-LacFin-COR and mP1-LacFin-OPP, this clone was linearized at a *Bgl*II site in the polylinker distal to the mP1 3′ sequences, Klenow filled, and ligated to a 2.55-kb *Pvu*II fragment representing the *lacZ* gene truncated for its first 36 and last 136 amino acids. The fragment was inserted in both orientations to yield the two constructs. The inserts were excised for microinjection with the enzymes *Hin*dIII and *Kpn*I.

Transgenic mice. Purified fragments were microinjected into C57BL/6 × SJL F2 embryos by standard methods (21). Founder animals were mated to CF1 mice, as were subsequent progeny. The transgene was detected by Southern blotting and hybridization to a ^{32}P-labelled 2.5-kb *lacZ Pvu*II fragment.

Testes cell isolation and separation. Eight to twelve hemizygous transgenic male mice (60 to 90 days old) were sacrificed by cervical dislocation. The testes were stripped of their outer tunic, and germ cells were dispersed with trypsin and collagenase as described previously (41). Various cell types were fractionated through a 2 to 4% bovine serum albumin (BSA) gradient at unit gravity in a Cel-Sep device. A series of 10-ml fractions was collected in the portion of the gradient containing round and elongating spermatids. These were then stained for *lacZ* expression by a rapid microwave fixation protocol as described previously (33).

Preparation of cryosections. Freshly dissected testes from proven transgenic animals were placed on a precooled sectioning block and immersed in 1 ml of OCT medium (Miles, Inc.). The sample-mounted block was frozen at −20°C for at least 30 min in the precooled cryomicrotome, and 10-μm sections were cut. The cryosections were allowed to dry briefly at room temperature and then washed gently but extensively with SpM buffer (2 mM $MgCl_2$–0.3% glucose–0.3% fructose in 10 mM phosphate-buffered saline) to remove the excess OCT medium prior to microwave fixation and *lacZ* activity staining.

Examination of OPP transgene structures. The OPP-2 and OPP-3 lines were examined by Southern blotting with four restriction enzymes (*Bam*HI, *Eco*RI, *Pvu*II, and *Sac*I) and two probes (a 2.55-kb *Pvu*II internal *lacZ* fragment and an mP1 3′ fragment). Fragment size predictions were made for (i) tandem head-tail repeats, (ii) recombination between two circularized molecules as in Fig. 2D and E, and (iii) recombination between the *lacZ* genes in a stem-loop structure which could form between the two inverted genes of a single molecule. Although each enzyme yielded Southern patterns consistent with a simple tandem repeat, the results were clearest with *Pvu*II. The recipient gene contains three *Pvu*II sites: one near the 5′ end of the mP1 promoter, and two separated by 2.55 kb within the *lacZ* gene. The donor gene, which is entirely the same 2.55-kb *Pvu*II fragment, has no sites (they were lost in the blunt-end ligation to a Klenow-filled *Bgl*II site). A tandem array would have prominent 3.4-kb (diagnostic of the repeat) and 2.55-kb bands when probed with the 2.55-kb *Pvu*II fragment. Only the 3.4-kb fragment would hybridize with the mP1 3′ probe. This was indeed the case. Recombinant structures would create hybrid genes containing one *Pvu*II site within their *lacZ* genes, resulting in anomalously sized bands hybridizing with either one or both probes. These were not seen.

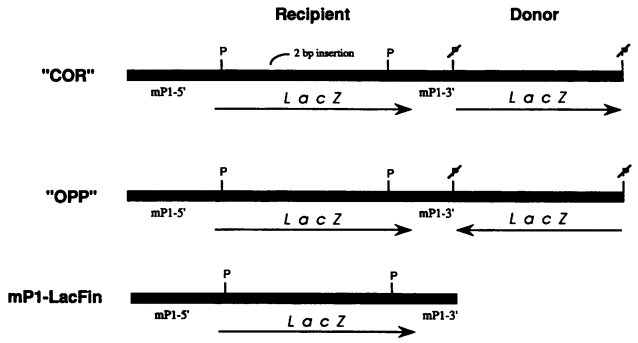

FIG. 1. Structures of the microinjected constructs. Transcriptional orientations of the *lacZ* genes are indicated by arrows. The black stripe in the recipient *lacZ* gene is a 2-bp insertion mutation (see Materials and Methods). P, *Pvu*II. The crossed-out P's correspond to *Pvu*II sites which were destroyed during cloning. The donor *lacZ* gene is deleted for the first 36 and last 136 amino acids of the enzyme. The mP1 promoter contains 1,145 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 mP1-LacFin-COR (COR) and mP1-LacFin-OPP (OPP) constructs are 7.5 kb long, while the mP1-LacFin construct is 5.0 kb long. The drawings are to scale.

Quantitation of transgene copy number. Genomic DNA from each line was digested with *Sac*I or *Eco*RI, yielding fragments corresponding to the tandem repeats, an internal fragment, and two additional fragments corresponding to the junction of the transgene array and the host genome (see the *Eco*RI digest in Fig. 2C for an example). Dilutions of the digested DNA were Southern blotted and probed to determine how many fold more intense the tandem array or internal bands were compared with the junction bands. The number of nucleotides detected in each fragment detected by the probe (a 2.55-kb *lacZ* fragment) was taken into account in order to normalize molecule copy number to band intensity.

RESULTS

Experimental design. We generated transgenic mice carrying the constructs diagrammed in Fig. 1. The COR and OPP constructs each contain two differentially mutated *lacZ* genes. The recipient gene contains an internal 2-bp frameshift mutation and is driven by the mP1 promoter. Situated downstream is the mP1 3′ untranslated region, which provides a polyadenylation signal and sequences presumably responsible for restricting translation to elongated spermatids (8). The donor *lacZ* gene lacks a promoter and is truncated for 36 and 136 amino acids at the amino and carboxy termini, respectively. The recipient and donor genes share 2.5 kb of homology: 730 bp to the left of the recipient mutation and 1.8 kb to the right.

The three-mutation design precludes a single intrachromosomal crossover recombination between the recipient and donor genes, either within an individual construct unit or between tandemly duplicated copies of the construct in the transgenome, from restoring *lacZ* function. This strategy of

selecting for gene conversion has been used successfully in mammalian tissue culture systems (28, 30). Male mice carrying this construct should transcribe the recipient gene specifically in haploid germ cells but will not produce functional *lacZ* unless a spontaneous mutation or recombination event corrects the frameshift mutation.

These constructs were microinjected into one-cell mouse embryos in the standard fashion (21). This procedure typically yields a head-to-tail array of multiple construct copies integrated into a single location in the genome. Since we initially expected the conversion frequency to be low, we reasoned that the multiple copies would increase the chance for recombination, facilitating detection and quantitation. However, as described below, copy number had no relationship to recombination frequency.

Structure of transgene arrays. One mP1-LacFin (LacFin), three mP1-LacFin-COR (COR), and two mP1-LacFin-OPP (OPP) transgenic lines were established. Each transcribed the transgene, as detected by Northern (RNA) blot analysis, and all but one (COR4, which had low amounts in liver) appeared to do so testes specifically (data not shown).

Since transgenes usually integrate into the genome in a head-to-tail fashion, containing up to several hundred copies at a single integration site, it is thought that recombination between injected molecules must occur to create such an ordered configuration (9). To determine whether our injected constructs underwent recombination between the donor and recipient genes prior to integration, we performed Southern blot analysis of each line to check for the presence of restriction fragments characteristic of simple head-tail integrations or recombinant genes. The restriction patterns of the OPP2 and OPP3 lines were consistent with a nonrecombinant tandem array of construct units (see Materials and Methods). The COR3 line contains a single construct unit of the expected structure. However, the COR1 and COR4 lines appear to have incorporated recombinant arrays of the microinjected DNA. Restriction sites and fragment sizes predicted from normal and three potential recombinant structures are presented in Fig. 2. Figure 2B shows the product of recombination between two linear molecules, whereas Fig. 2D and E show recombination between two circularized molecules. Only the recombinant form in Fig. 2B, which is the product of recombination between the donor and recipient genes of two linear molecules, is completely consistent with the observed Southern blot pattern (Fig. 2C; see the legend for a detailed analysis of the data leading to this conclusion).

Although the recombinant configuration creates a hybrid recipient-donor gene which is still defective, it creates the opportunity to restore *lacZ* function through reciprocal intrachromosomal recombination between the hybrid gene and a recipient gene. However, this would be possible only if the hybrid contained the unmutated (donor) version of the recipient frameshift mutation, which depends on the location of the initial crossover following microinjection. Regardless, data from other systems indicate that gene conversion would still be the predominant mechanism responsible for *lacZ* correction in these two lines (23, 25, 26, 30).

The structures of the COR2 and COR4 transgenic insertions indicate that one-cell embryos are efficient at mediating homologous recombination between linear molecules. Such an activity may underlie the reconstruction of large DNA segments from smaller, overlapping coinjected fragments (47). Because of their inverted orientations, recombination between the donor and recipient *lacZ* genes of linear OPP molecules would not result in the creation of a multimolecular recombinant arrays. Therefore, the tandem arrays in the OPP lines must have arisen by other mechanisms.

Observation of *lacZ* correction events. The seminiferous tubules from adult male nontransgenic and transgenic hemizygotes were dispersed into single cells by collagenase-trypsin treatment of whole testes (41). The mixture, which contains cells at various stages of spermatogenesis, was fractionated in a BSA gradient at unit gravity. Fractions enriched for different spermatogenic cell types were fixed in a glutaraldehyde solution by microwaving (33) and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

No *lacZ* staining was observed in nontransgenic control males; however, blue cells were readily detectable in each transgenic line (Fig. 3A). Although we expected the mP1 3′ untranslated sequences to restrict translation to elongated spermatids (8), round spermatids also displayed *lacZ* staining. The presence of *lacZ*-positive cells cannot be explained by spontaneous reversion of the frameshift mutation in the recipient *lacZ* gene; the transgenic line mP1-LacFin, which contains a construct identical to COR except that it lacks the donor gene (Fig. 1), transcribed the recipient gene but produced a negligible number of *lacZ*-positive cells (Table 1). Therefore, interaction between donor and recipient sequences must be responsible for the gene correction.

Clusters of *lacZ*-positive cells: mitotic gene conversion versus intercellular leakage. The existence of intercellular bridges connecting haploid cells is thought to represent a conduit for functional equilibration of genetically nonidentical germ cells (14). The first experimental test of this question demonstrated that a composite human growth hormone gene product or RNA indeed diffuses across the bridges (7). In those experiments, nearly all of the sperm in a hemizygous male stained positive for human growth hormone. Additional histological and functional evidence for this process has been obtained in studies of the Myk-103 transgenic mice (54) and mice hemizygous for an mP1 locus (10). If the *lacZ* gene product were to behave similarly in our system, we would expect that a single correction event would result in multiple blue cells, depending on the number of cells connected in a syncytium (about 128 [19]) and the distance to which diffusion would detectably occur. This could complicate the quantitation of conversion events.

To investigate this issue, we stained cryosections of seminiferous tubules from transgenic animals (Fig. 3B). This analysis indicates that *lacZ* RNA or protein does not diffuse significantly, if at all, from a converted spermatid. Although a minor degree of blue staining over adjacent cells is detectable in some cases, this may represent diffusion of the chromogen over the section rather than β-galactosidase. In a second approach, portions of X-Gal-stained seminiferous tubules were scanned for blue cells in order to gain a three-dimensional perspective. This analysis revealed the presence of solitary blue cells surrounded by negative cells (Fig. 3C and D). These data suggest that intercellular leakage is not a significant in vivo factor and does not significantly interfere with quantitation of gene conversion frequency in these experiments.

In contrast, large clusters of positive cells in seminiferous tubules (Fig. 3D) were occasionally observed. The opposing observations can be explained by contribution of both meiotic and mitotic recombination events. A single conversion event in a mitotically proliferating spermatogonium (or precursor) would be inherited by its progeny, resulting in a cluster of positive cells (Fig. 3D), whereas a meiotic conversion event results in a solitary *lacZ*-positive cell.

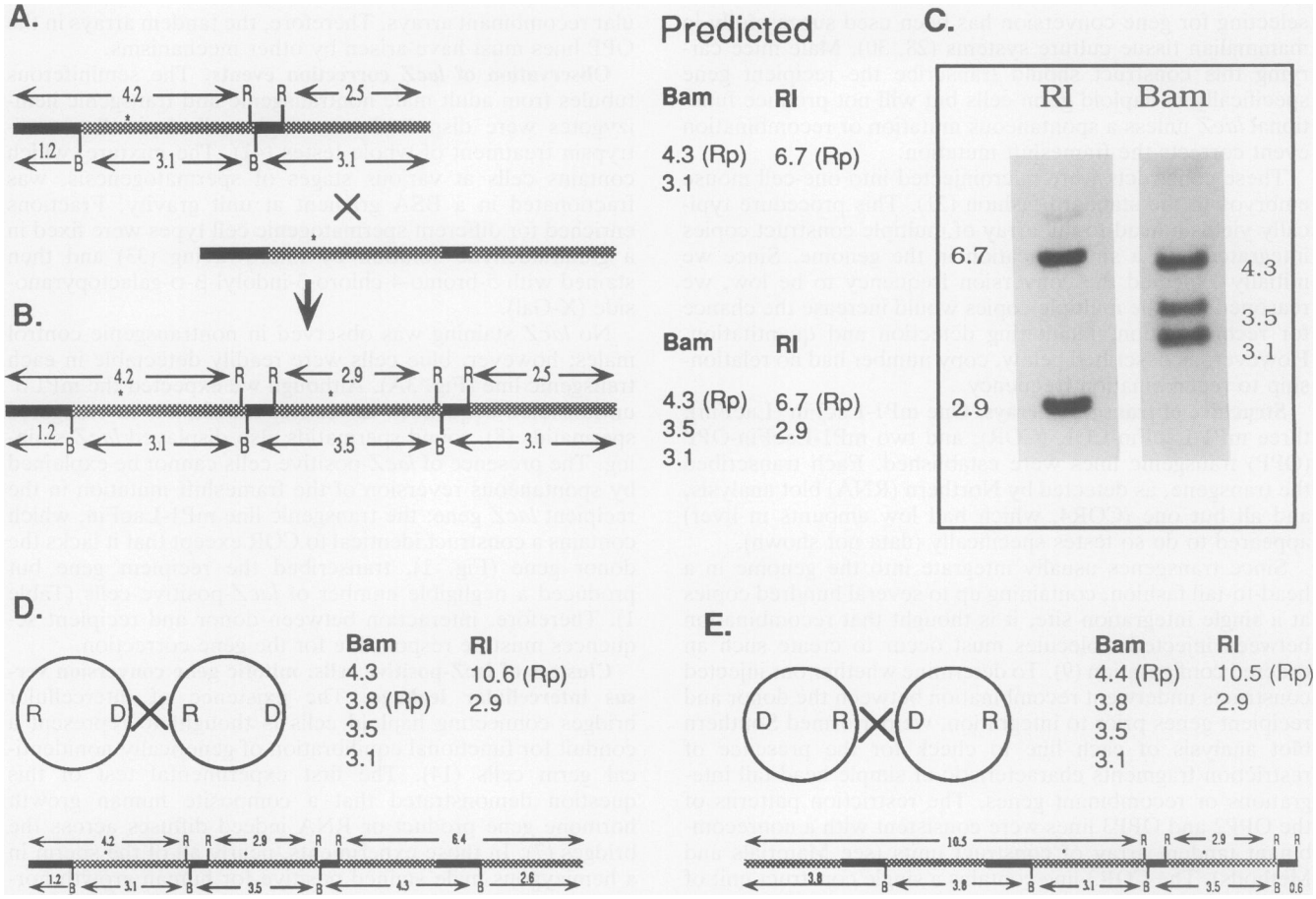

FIG. 2. Possible structures of COR transgenic constructs. The stippled boxes represent *lacZ*, and the solid black boxes represent mP1 sequences. The longer black box corresponds to the 5′ promoter region, and the smaller black box represents 3′ mP1 sequences. In panel A, the longer stippled box with an asterisk above is the recipient gene, and the smaller box is the donor. The structure in panel A is identical to the microinjected construct mP1-LacFin-COR shown in Fig. 1. All numbers represent distances in kilobases. Predicted restriction fragment sizes of each structure are shown. Rp next to a fragment size indicates that a fragment of this size would be present if the relevant structure were repeated head-to-tail in the genome. For example, the 4.3-kb *Bam*HI fragment predicted in panel A is the sum of 3.1 and 1.2. The asterisk above the recipient gene indicates the location of the frameshift mutation (see text). (A) The nonrecombinant COR construct. (b) The product of recombination between two linear COR molecules in the region indicated by the X. (C) Southern blots of *Eco*RI- or *Bam*HI-digested COR1 DNA probed with a radiolabelled 2.55-kb internal *Pvu*II *lacZ* fragment. The COR4 line had an identical pattern of repeated bands (the ones with indicated sizes) but different junctions bands (not shown). (D and E) D and R represent donor and recipient, respectively. Recombination between two circularized COR molecules in the region indicated by an X creates the indicated structures. Although the diagrams indicate a recombinant unit beginning and ending precisely with a gene, the recombinant molecule would linearize randomly prior to chromosomal integration. R, *Eco*RI; B, *Bam*HI. In a simple head-tail tandem array (A) 3.1- and 4.3-kb *Bam*HI fragments would be detected by an internal *lacZ* probe, but an additional multicopy 3.5-kb fragment is detected (B and C). This correlates with the presence of a hybrid recipient-donor gene, which could have been created by any of the models. The 2.9-kb repeated *Eco*RI fragment (C) is also compatible with any rearrangement type. Only the recombinant form in panel B is completely consistent with the observed Southern blot pattern. The presence of a repeated 6.7-kb *Eco*RI band, which is the size predicted for tandem copies of this unit, is particularly diagnostic for this structure.

Frequency of correction events. To determine the percentage of corrected spermatids produced by hemizygotes of the transgenic lines, testicular fractions enriched for elongated spermatids were stained with X-Gal, placed on a hemocytometer, photographed (Fig. 3A), and tallied for blue versus nonblue spermatids. Cells with punctate, localized staining, which could arise as an artifact of either the dispersion method or partial leakage of gene product from a true corrected spermatid to a syncytial neighbor, were counted as negative.

Approximately 2% of the elongated spermatids in each transgenic line are *lacZ* positive (Table 1). The frequency was unrelated to transgene copy number (Table 1), which is most clearly demonstrated by the single-copy COR3 line. If the recipient gene is converting the donor at the same frequency, which would not produce a functional *lacZ* gene, the overall percentage of intrachromosomal conversion would be twice the observed values (1.8 to 5.2%). In this system as well as in cultured mammalian cells, the orientation of the recipient and donor genes with respect to one another (the COR versus OPP constructs) does not appear to affect intrachromosomal recombination frequency (50).

Although the OPP2 line had a lower percentage of blue cells than did the others, a prescreening against "jackpot" mitotic events was performed prior to quantitation. The testes of 10 mice were individually processed for the Cel-Sep

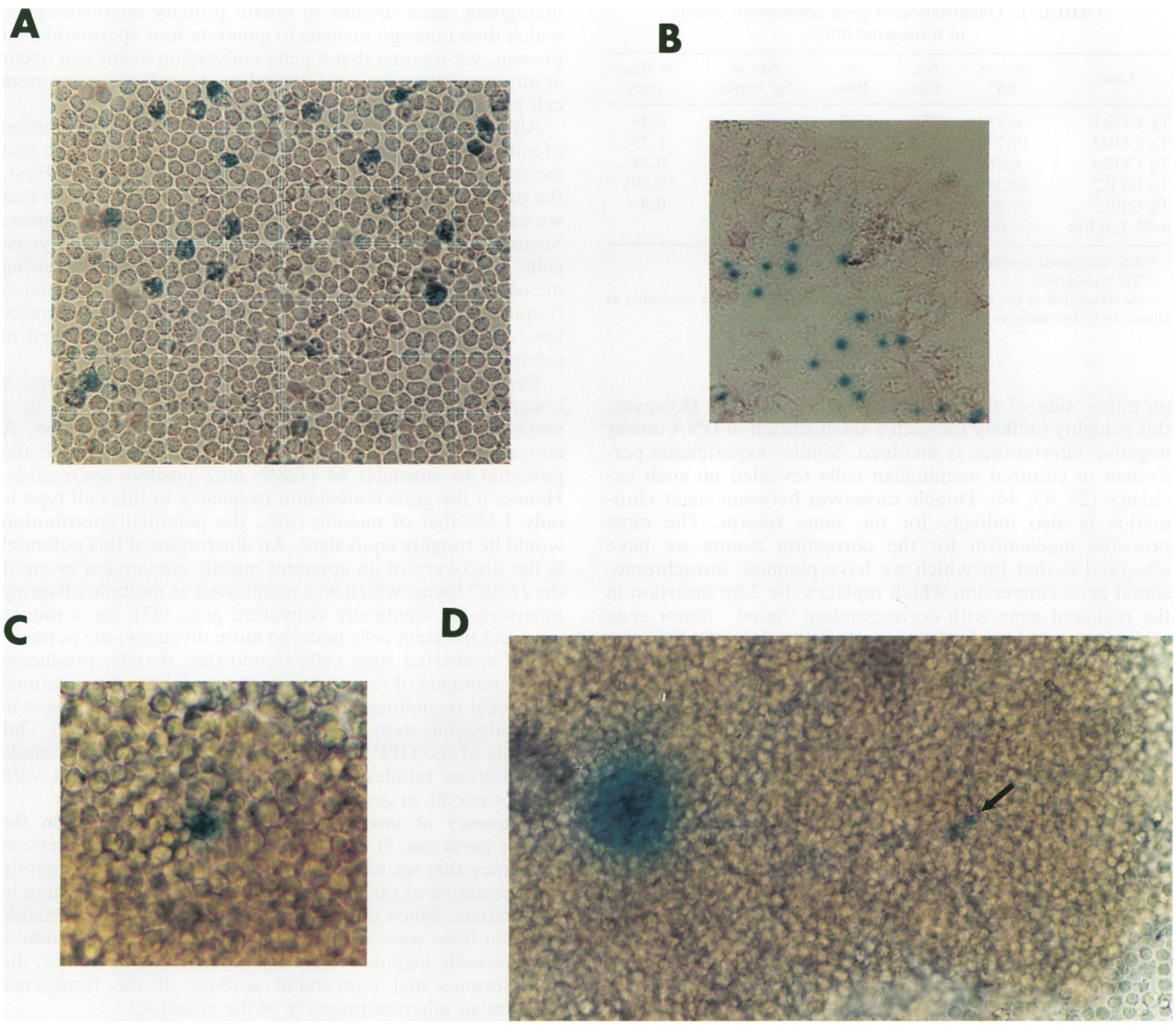

FIG. 3. Histochemical staining of testicular cells for *lacZ* activity. (A, C, and D) Samples from transgenic hemizygotes; (B) sample from a homozygote. (A) Fractionated testicular cells packed onto a hemocytometer for quantitation. (b) Cryosection through a seminiferous tubule of a COR1 homozygote. Approximately one-fourth of the cross section is shown. (C) Portion of a seminiferous tubule containing an isolated blue cell. The tubules were mechanically and enzymatically disrupted to view cells nearest the lumen. (D) Cluster of *lacZ*-positive cells within a seminiferous tubule. Also present is a solitary blue cell indicated by the arrow.

procedure. For each, a portion of a testicle was used for whole tubule staining prior to pooling. Three of the mice showed significant blue regions in some of the tubules, indicating the occurrence of an early mitotic conversion event. These mice were excluded from the Cel-Sep procedure and quantitation. This result suggests that mitotic gene conversion plays a significant role in generating corrected spermatids, even though meiotic events may be much more frequent (see Discussion).

Recombination on the Y chromosome. The transgene insertion in the OPP2 line appears to be on the Y chromosome, since only males inherit the transgene. Although we have not cytologically localized the transgene insertion site, the strict male inheritance indicates that it lies outside the pseudoautosomal region and therefore in a location which does not undergo crossing over with the X chromosome. This line exhibits a percentage of blue cells (Table 1) similar to that in lines with autosomal insertions (note that the percentage for this line is somewhat lower because of the aforementioned prescreening for mitotic events).

DISCUSSION

Nature of the correction events. In the COR3, OPP2, and OPP3 lines, the construct design is such that intrachromosomal gene conversion is the most plausible explanation for *lacZ* correction. Nevertheless, other recombination mechanisms could conceivably correct the mutation in the recipient gene. One is intrachromosomal double-reciprocal crossover between the donor and recipient, with crossover points

TABLE 1. Quantitation of gene conversion events in transgenic mice

Line	No. of ES[a]	No. blue	% Blue	No. of Tg[b] copies	% Blue/copy
Tg COR1	6,834	132	1.93	~5	0.39
Tg COR3	19,700	340	1.73	1	1.73
Tg COR4	6,569	112	1.7	~5	0.34
Tg OPP2[c]	40,280	366	0.91	~10	0.091
Tg OPP3	24,806	643	2.59	3	0.9
mP1-LacFin	32,000	2	0.006		

[a] ES, elongated spermatids.
[b] Tg, transgene.
[c] As described in the text, quantitation of this line involved a screening to eliminate testes samples containing early mitotic events.

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 (29, 43, 44). Double crossover between sister chromatids is also unlikely for the same reason. The most probable mechanism for the correction events we have observed is that for which we have planned: intrachromosomal gene conversion which replaces the 2-bp insertion in the recipient gene with corresponding "good" donor gene sequences. Although we cannot determine whether the conversion is occurring between sister chromatids or intramolecularly, interchromatid recombination predominates in cultured mouse cells (5). This type of genetic transfer makes it impossible to prove that a meiotic correction event was nonreciprocal; even if converted animals (or sperm) are recovered and analyzed, the sister chromatid is lost. However, there is a potential to perform analogous experiments in females, in which the meiotic partners of oocytes remain in physical proximity, distinct from the products of other meioses.

The two lines with rearranged transgene arrays, COR1 and COR4, could correct *lacZ* function by two mechanisms, intrachromosomal gene conversion and intrachromosomal reciprocal recombination. Data from other systems indicate that gene conversion would still be the predominant mechanism responsible for *lacZ* correction. In yeast cells, meiotic recombination between duplicated *LEU2* genes was strictly via gene conversion in one experiment (26). Similar results were obtained at the *HIS4* locus (22, 23, 25), leading to a proposal that a mechanism exists to suppress intrachromosomal reciprocal exchanges (23). Studies of recombination in mammalian cells also show that gene conversion is five times more prevalent than reciprocal recombination for intrachromosomal gene corrections (30, 31, 50). Since the percentage of blue spermatids in these lines is similar to that in the unrearranged lines, it is likely that the predominant recombination mechanism for *lacZ* correction was gene conversion.

Mitotic versus meiotic events. The numbers that we have observed must be interpreted with caution. It is important to realize that the percentage of converted spermatids does not simply reflect the frequency of gene conversion events. This is a consequence of the contribution of mitotic gene conversion in the germ line. Males possess a self-renewing pool of spermatogonial stem cells (type A_s spermatogonia). They undergo division both to renew themselves and to give rise to cells destined for meiosis (type A_1 spermatogonia). After six additional mitotic divisions producing type B spermatogonia, each divides to create primary spermatocytes, which then undergo meiosis to generate four spermatids. At present, we assume that a gene conversion event can occur at any step along the way, including the self-renewing stem cell population and earlier germ line precursors.

Although we have not determined the relative contribution of mitotic and meiotic events, there is reason to believe that meiotic conversion occurs with a greater frequency. First, the particular set of seminiferous tubules cross sections that we examined contained independent blue cells, not clusters. Second, the frequency of mitotic gene conversion in yeast cells is about 100- to 1,000-fold less frequent than during meiosis (22, 39). Third, intrachromosomal gene conversion frequency in cultured (mitotic) mammalian cells is extremely low, on the order of 10^{-6} per cell generation (reviewed in reference 6).

Even though its frequency per cell division may be much lower, mitotic gene conversion in the germ line might be a more significant process than meiotic gene conversion. A conversion in a spermatogonial stem cell would have the potential to engender 64 (128/2) *lacZ*-positive spermatids. Hence, if the gene conversion frequency in this cell type is only 1.5% that of meiotic cells, the potential contribution would be roughly equivalent. An illustration of this potential is the discovery of an apparent mitotic conversion event at the *H-2K*^{b} locus, which was manifested as multiple offspring inheriting an identically converted gene (17). As a mouse ages and the stem cells undergo more divisions, the percentage of converted stem cells should rise, thereby producing higher numbers of recombinant offspring. Intrachromosomal reciprocal recombination at the MyK-103 transgene locus in spermatogenic stem cells exhibits such a pattern (54). Our analysis of the OPP line, in which a prescreening of whole seminiferous tubules was performed, was consistent with jackpot events in germ line precursors.

Frequency of intrachromosomal gene conversion in the murine germ line. It is unclear whether the gene conversion frequency that we have observed in this system is generally representative of random duplicated sequences elsewhere in the genome. Since the percentages of corrected spermatids between lines were similar, it is unlikely that the numbers are unusually high due to chromosomal context. Rather, the intrachromosomal conversion activity of the transgenes must be an inherent property of the construct.

It has been suggested that transcriptional activity may enhance recombination by organizing the expressed DNA into a conformation especially susceptible to recombination machinery (51). Correlation between gene activity and meiotic recombination have been observed at the *ARG4* locus in *Saccharomyces cerevisiae* (37), at an immunoglobulin variable region gene (4), and between extrachromosomal sequences in mammalian cells (36). Although the mP1 gene is first detectable by Northern blotting postmeiotically in round spermatids (24), which is after the time either the meiotic or mitotic conversions would be occurring, it is possible that the mP1 locus is poised for transcription in a conformation conducive to recombination. If so, the frequency of converted cells measured in this system might be higher than in other transcriptionally silent genes.

Gene conversion and the evolution of gene families. The percentage of converted gametes that we have observed greatly exceeds values used in theoretical studies on the influence of this process upon the evolution of duplicated genes (35, 53). Such studies are biased toward lower conversion rates reported in mammalian cultured cells, in part to account for the divergence which extant gene pairs display.

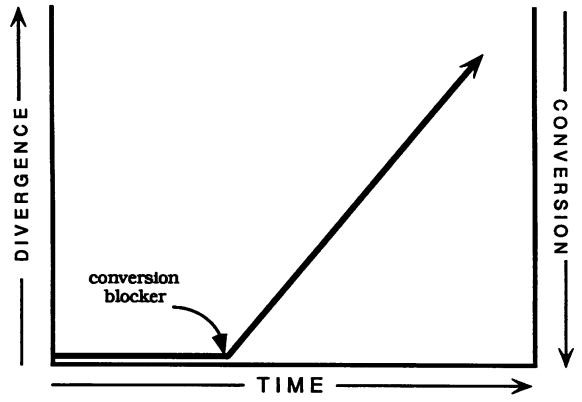

FIG. 4. Model for evolution of duplicated genes.

If a newly duplicated gene pair is converted in 2% of gametes, it is unlikely they would ever diverge. This interpretation concurs with the formula derived by Walsh (53), which states that a pair of identical genes would never escape conversion (they would never diverge) if $2\mu/\lambda << 0.1$, where μ is the per nucleotide mutation rate and λ is the conversion frequency. Assuming a generous mutation rate of 10^{-6}, then $2\mu/\lambda = 10^{-4}$ when the conversion frequency is 2%.

Assuming a generation turnover time of 3 months, a duplicated chromosomal locus would undergo gene conversion once every 300 months, or 12.5 years, if 2% of the gametes underwent gene conversion. These data stand in contrast to the observed rates of divergence in mice of about 1% per million years (35). Hence, it is paradoxical that genes do in fact diverge despite being susceptible to such frequent homogenization. Selection against conversion should not be a factor immediately following a duplication event, since the newly created gene would not have a unique, critical function. One explanation, as discussed above, is that not all newly duplicated genes undergo such frequent conversion. Factors affecting recombination may include sequence composition and distance between the homologs. Another possibility is that mechanisms exist to diminish unabated gene conversion. Although the frequency of recombination decreases as the length and degree of homology decreases (2, 29, 42, 48, 52), how do duplicated genes escape conversion in the first place? It has been suggested that structural events which interrupt homology and heteroduplex formation, such as insertion of repetitive elements, could substantially inhibit gene conversion between duplicated genes (11, 15, 20, 32, 45), allowing them to diverge freely. As random events occur to interrupt stretches of perfect or high homology between duplicated gene pairs, the gene conversion frequency would decline locally. This would increase the average time between conversion events and hence permit the accumulation of base changes. This scenario is illustrated in Fig. 4. Eventually, this process could reach a point where substantial portions of a gene are relatively immune to conversion and may actually diverge to a point where differential function has been acquired. This would then impose an additional selection against conversions.

Utility of this methodology and practical significance. These experiments represent a significant conceptual and technological breakthrough in the study of gene conversion in the germ line of mammals. The previous obstacles of sample size and lack of a visible conversion phenotype are surmounted in this experimental paradigm. Our results indicate that gene conversion is a potentially powerful force in the genome. This has several practical implications. For example, independent isolates of inbred mouse strains may actually differ because of gene conversion. Furthermore, apparent double crossovers in recombinant inbred mouse lines may instead be gene conversions.

We anticipate that refinements of this general approach will be achieved by introducing analogous constructs into distinct genomic regions by homologous recombination in embryonic stem cells. This approach would eliminate the problem of multiple copy inserts and allow tests of interchromosomal gene conversion. Variations of the system can be used to understand particular properties of gene conversion, such as conversion tract lengths, effect of varying nonhomologies, and interaction between homologous sequences on nonhomologous chromosomes.

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