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Palindrome Resolution and Recombination in the Mammalian Germ Line

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Genetic instability is promoted by unusual sequence arrangements and DNA structures. Hairpin DNA structures can form from palindromes and from triplet repeats, and they are also intermediates in V(D)J recombination. We have measured the genetic stability of a large palindrome which has the potential to form a one-stranded hairpin or a two-stranded cruciform structure and have analyzed recombinants at the molecular level. A palindrome of 15.3 kb introduced as a transgene was found to be transmitted at a normal Mendelian ratio in mice, in striking contrast to the profound instability of large palindromes in prokaryotic systems. In a significant number of progeny mice, however, the palindromic transgene is rearranged; between 15 and 56% of progeny contain rearrangements. Rearrangements within the palindromic repeat occur both by illegitimate and homologous, reciprocal recombination. Gene conversion within the transgene locus, as quantitated by a novel sperm fluorescence assay, is also elevated. Illegitimate events often take the form of an asymmetric deletion that eliminates the central symmetry of the palindrome. Such asymmetric transgene deletions, including those that maintain one complete half of the palindromic repeat, are stabilized so that they cannot undergo further illegitimate rearrangements, and they also exhibit reduced levels of gene conversion. By contrast, transgene rearrangements that maintain the central symmetry continue to be unstable. Based on the observed events, we propose that one mechanism promoting the instability of the palindrome may involve breaks generated at the hairpin structure by a hairpin-nicking activity, as previously detected in somatic cells. Because mammalian cells are capable of efficiently repairing chromosome breaks through nonhomologous processes, the resealing of such breaks introduces a stabilizing asymmetry at the center of the palindrome. We propose that the ability of mammalian cells to eliminate the perfect symmetry in a palindromic sequence may be an important DNA repair pathway, with implications regarding the metabolism of palindromic repeats, the mutability of quasipalindromic triplet repeats, and the early steps in gene amplification events.

Recombination rates are known to vary along chromosomes. A fixed genetic distance can correspond to widely different physical lengths depending on the location on the chromosome. Although certain sequences such as the χ site in phage λ and *Escherichia coli* are recognized by enzymes that promote recombination (43), growing evidence suggests that DNA structure and accessibility, rather than sequence per se, have a profound impact on recombination. During meiosis in mammals, for example, very different recombination rates can be observed at identical chromosomal regions in males and females (11; for a review, see reference 45). In *Saccharomyces cerevisiae*, where most, if not all, meiotic recombination is initiated by double-strand breaks (48), DNA sequence is not the primary determinant of the position of breaks; rather, breaks map to regions of chromatin that are nuclease sensitive (13, 49).

Recombination and genome rearrangements in mammalian mitotic cells are also influenced by interruptions to a continuous DNA duplex, including double-strand breaks. For example, an experimentally introduced double-strand break in the mammalian genome will stimulate homologous recombination between two repeats by more than 3 orders of magnitude (30). Unusual DNA structures assumed by simple sequence repeats have also been proposed to underlie the observed instability of the repeats (1, 22). For example, triplet repeats involved in human diseases are able to form hairpin structures as a result of their quasipalindromic nature (5, 15).

To begin to understand factors affecting germ line recombination and genetic instability in the mouse, we have developed a sperm fluorescence assay based on *lacZ* expression in the germ line of transgenic mice (21). In this assay, recombination of two defective *lacZ* gene fragments results in a $lacZ^+$ gene that is expressed postmeiotically in the male. β-Galactosidase-positive sperm are detected by flow cytometry after incubation with fluorogenic substrates (21, 33). In the course of our studies of recombination in the mouse germ line, we have discovered that a palindromic repeat, which has the inherent potential for intrastrand pairing, has a marked influence on gene conversion. We also found that the palindromic sequences, although transmitted at a Mendelian ratio, are rearranged in a large fraction of progeny. Analysis of rearrangements suggests that instability is due to the palindrome adopting a hairpin

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structure, which may lead to structure-specific nicking and repair, with resulting stabilization of the locus.

MATERIALS AND METHODS

Plasmid and transgenic mouse constructions. Plasmid a-2 encodes nucleus-localized *E. coli* β-galactosidase driven by the mouse *prm-1* promoter (21). (DNA for this construction was generously provided by J. Peschon and R. Palmiter.) The 3′ *prm-1* sequences of plasmid a-2 were replaced with a simian virus 40 (SV40) polyadenylation site, creating a-2/SV403′. This was done by replacing the downstream *Eco*RI fragments of a-2 with the 1.2-kb *Eco*RI fragment from pCH110 (17). The *lacZ4*Δ gene was created by cleaving a-2/SV3′ with *Sac*I, removing the overhangs with T4 DNA polymerase, and then religating the plasmid to create the 4-bp deletion. For the addition of the *inlacZ* repeat, a *Hin*cII fragment from the wild-type *lacZ* gene was cloned downstream of the SV40 polyadenylation site in the same orientation as the mutant *lacZ* gene. It was inserted into the *Apa*I site by blunt-end ligation, creating plasmid 1.8. The *Hin*cII fragment is 1,828 bp, with the *Sac*I site located 884 bp from the 5′ end of the fragment. For injection into fertilized eggs, the 7.7-kb *Pst*I fragment from plasmid 1.8 containing the *lacZ4*Δ and *inlacZ* genes was purified by CsCl centrifugation and injected into the pronuclei of fertilized (C57BL/6 × CBA/Ca)F_2 mouse eggs as described previously (20). Transgenic mice were identified by Southern analysis. Mouse lines were maintained by breeding with (C57BL/6 × CBA/Ca)F_1 mice.

Sperm analysis, tissue sections, and testicular cell preparations. Sperm preparations were similar to that previously described (21), with the following exceptions. Sperm were squeezed from the caudal epididymis of adult males into a phosphate-buffered saline (PBS) solution (PBS, 10 mM HEPES, 1% bovine serum albumin). Then 2×10^6 sperm were placed in 0.5 ml, washed twice, and resuspended in 0.2 ml. For staining, 20 μl of sperm (2×10^5) was added to 30 μl of the PBS solution and then 50 μl of 1 mM CM-FDG (5-chloromethylfluorescein-di-β-D-galactopyranoside [Molecular Probes]), the fluorogenic β-galactoside, was added. After 60 s, 1 ml of PBS solution was added. After 30 min, the sperm were analyzed by fluorescence-activated cell sorting (FACS) on a FACScan instrument (Becton Dickinson) with Lysis II software. Tissue sections were prepared as previously described (21). Spermatogenic cell suspensions were prepared as described previously (38). Round spermatids were identified by a uniform smooth appearance with a central area of greater density and the presence of an acrosomal cap forming on one pole of the nucleus; condensing spermatids were identified by the acquisition of a characteristic sickle shape.

DNA sequencing. Prior to amplification, tail tip DNA from mouse 364 was digested with *Hin*cII. The approximate locations of primers used to amplify the deletion junction are shown (see Fig. 8A). The 3′ end of primer P1 (5′CCCTGCTCATCAAGAAGCACT) hybridizes approximately 240 bp from the central *Pst*I site in a standard 78 mouse. The 3′ end of primer E4 (5′CCTGTAGTTTGCTAACACACC) is 3 bp away from the symmetry axis (see Fig. 8B). A single PCR product, reproducibly amplified in independent reactions, was visualized as a *Pst*I-sensitive band on agarose gels. No bands were present in an identically prepared *Hin*cII digest lacking tail tip DNA. A total of 31 cycles of amplification were performed in a PTC-200 thermal cycler (MJ Research). The PCR conditions were 94°C for 5 s, 63°C for 15 s, and 72°C for 15 s. The PCR products were cloned into a pUC-based vector (pJH298) with T-A overhangs, as described previously (2). Seven plasmids, representing five independent clones from two independent PCRs, were sequenced and found to be identical. For confirmation, a PCR was performed on nondigested line 364 tail tip DNA. After 31 cycles, no product was visible; presumably, snap-back hybridization within the inverted repeat limited the access of the primers to the template. A second round of PCR was performed with primer E4 and a third primer (5′GCAAAACAGGAGGCACTTTTCCCC), whose 3′ end is located roughly 190 bp from the *Pst*I site in a standard 78 mouse. A single band was detected, and DNA sequence analysis of clones yielded the same junction sequence as was previously found. No such product was obtained from a *Hin*cII digest lacking tail tip DNA that was likewise subjected to two rounds of PCR.

RESULTS

Inverted transgene repeat in mouse line 78. To examine gene conversion in the mouse germ line, a *lacZ* reporter substrate consisting of two defective *lacZ* genes was constructed (Fig. 1A). The upstream gene, *lacZ4*Δ, contains a 4-bp deletion which generates a frameshift and disrupts a *Sac*I site. The downstream gene, designated *inlacZ*, is a 1.8-kb internal fragment of *lacZ* with a wild-type sequence at the *Sac*I site. Upstream of the *lacZ4*Δ gene is a promoter specific for postmeiotic cells, derived from the protamine 1 (*prm-1*) gene. The *prm-1* promoter is active only in the male germ line (36). *inlacZ* is cloned in the same orientation as *lacZ4*Δ, 3′ to an SV40 polyadenylation signal. The *lacZ4*Δ gene contains an SV40

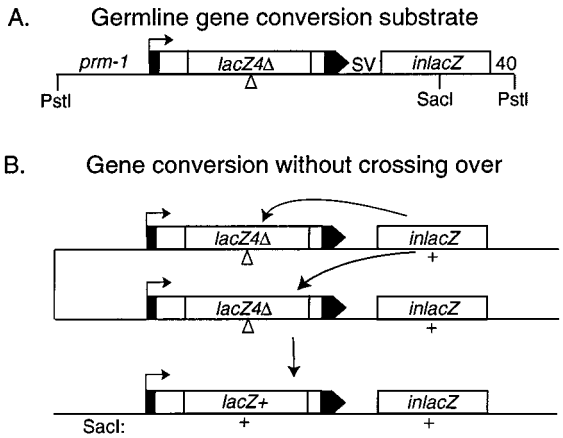


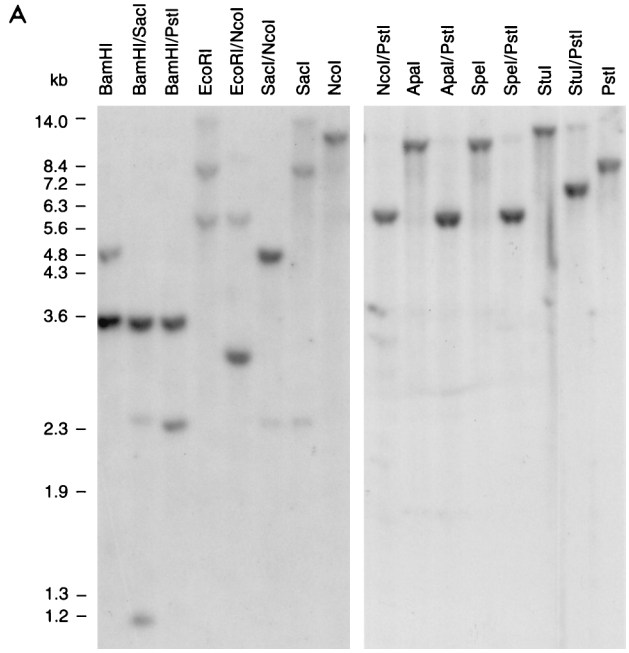
FIG. 1. *lacZ* conversion substrate. (A) *Pst*I restriction fragment injected into fertilized mouse eggs. The upstream *lacZ4*Δ gene is intact except for a 4-bp deletion (Δ) within the coding region. The 1.8-kb internal *lacZ* fragment, *inlacZ*, is in the same orientation as *lacZ4*Δ and contains a wild-type *Sac*I site at the position of the 4-bp deletion. The promoter for *lacZ4*Δ is derived from the postmeiotic specific mouse *prm-1* gene. The polyadenylation site (SV) is upstream of the *inlacZ* fragment and is derived from SV40. SV40 sequences are also present downstream of *inlacZ* (40). (B) Sister chromatid or intrachromatid gene conversion of the *lacZ4*Δ gene. The *inlacZ* gene acts as the donor of information to correct the 4-bp deletion in the *lacZ4*Δ gene, restoring the *Sac*I site.

nuclear localization signal at the 5′ end of the *lacZ* coding region, for greater retention of β-galactosidase in mature sperm (20a, 21).

Gene conversion of the upstream *lacZ4*Δ gene from the downstream *inlacZ* donor fragment in the male germ line restores a functional $lacZ^+$ gene (Fig. 1B). Intrachromatid or sister chromatid gene conversion occurring throughout germ cell development can be scored, because the *prm-1* promoter drives the expression of the *lacZ* transgene at the final stages of germ cell development in spermatids. To quantitate recombination, β-galactosidase activity is detected by flow cytometry in mature sperm stained with fluorescent β-galactosides (21, 33). Further, the delayed expression of the *lacZ* reporter gene makes it possible to infer the timing of gene conversion during germ cell development. This is because 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of testis sections reveals whether stained cells are clustered or isolated.

Transgenic mice containing the gene conversion substrate were constructed by injecting fertilized mouse eggs with a 7.7-kb *Pst*I fragment (Fig. 1A). Four founder mice were obtained, as determined by Southern blot analysis of tail tip DNA. One founder, mouse 78, appeared to have integrated two transgene copies in an unusual tail-to-tail repeat of the injected DNA (Fig. 2). A *Pst*I site was regenerated at the junction of the two transgene copies, at the center of symmetry of the inverted repeat that was thereby generated. Apparently, the two copies of the injected DNA had become precisely ligated to each other prior to integration, forming a 15.3-kb palindrome. Extensive restriction mapping of the locus (Fig. 2 and data not shown) was fully consistent with this structure. Northern blot analysis of tissues from line 78 mice demonstrated that, as intended, transcription of the transgene was restricted to the testes (data not shown).

The other three founders contained multiple copies of the transgene integrated in the more usual direct-repeat orientation (3). In each case, one or more copies had apparently undergone recombination within the *lacZ* repeats prior to integration (data not shown). In this, our experience was not



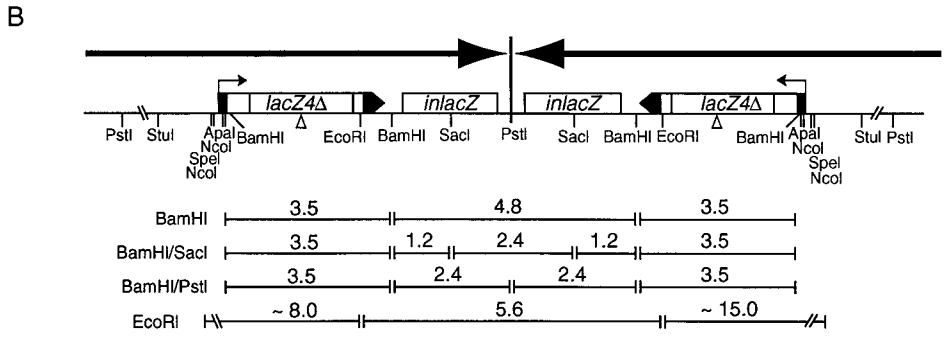


FIG. 2. Palindromic structure of the transgene locus in mouse line 78. (A) Southern blot analysis of tail tip DNA from a mouse with the standard line 78 palindrome. The probe is the 3.5-kb *lacZ Bam*HI fragment. (B) The tandem, inverted orientation of the *lacZ* gene conversion substrates is indicated by the convergent arrows. The *Pst*I site from the injected transgene fragment lies at the center of the inverted repeat, indicating a precise tail-to-tail ligation. The sizes of the fragments generated by restriction enzymes used in the analysis are given in kilobase pairs.

unusual; we and others have previously detected recombination of DNA injected into fertilized eggs (33, 34). Because the transgenic sequences in these other founders were already recombinant, the mice were not further analyzed for gene conversion.

Germ line gene conversion in males with the standard 78 palindrome. Flow cytometry of sperm was performed to detect gene conversion between the *lacZ* genes in line 78 mice. Sperm were isolated from the caudal epididymis and stained with the fluorogenic β-galactoside CM-FDG. The substrate is retained within cells and will fluoresce when hydrolyzed by *E. coli* β-galactosidase present in the mature sperm.

Sperm from three transgenic mice from line 78 and a nontransgenic littermate were tested (Fig. 3). The bulk sperm population from each of the transgenic mice (78A, 78B, and 78C) gave a profile very similar to that for sperm from the nontransgenic mouse (NEG). The mean fluorescence intensities of the sperm from these four mice were very similar (Table 1). A small portion of sperm from the transgenic mice, however, had a significantly greater fluorescence intensity than did the bulk population (insets, Fig. 3). The highly fluorescent sperm comprised approximately 0.41 to 0.70% of the sperm from the transgenic mice versus 0.06% of the sperm from the nontransgenic mouse (Table 1).

To examine β-galactosidase-positive sperm progenitors, X-Gal staining was performed on testis sections and testicular cell preparations. Blue spermatids were detected in both preparations from line 78 mice. In testis sections, most of the seminiferous tubules were unstained (Fig. 4G). However, clusters of blue haploid spermatids were present near the lumen of some of the seminiferous tubules (Fig. 4A to D). The clusters could be monitored in consecutive testis sections (Fig. 4B to D). In addition, isolated but well-stained blue spermatids were seen near the lumen of some of the seminiferous tubules (Fig. 4E and F). The clusters of cells most probably arose from gene conversion events occurring prior to meiosis, whereas the isolated blue cells may have arisen from late (meiotic) events. Similarly stained cells, either isolated or in clusters, were not observed in testes of nontransgenic mice.

To quantify the X-Gal staining in the testes, testicular cell populations were prepared from two line 78 mice. Blue cells were counted microscopically. Spermatids, which made up the majority of the cells in the preparation, were identified by morphology (38). In one mouse, 0.7% of the spermatids were blue (340 of 48,000 spermatids). In the second, approximately 0.3% of the spermatids were positive. In nontransgenic mice, only occasional blue-green, rather than deep blue, cells were observed. Thus, both flow cytometry of sperm and X-Gal stain-

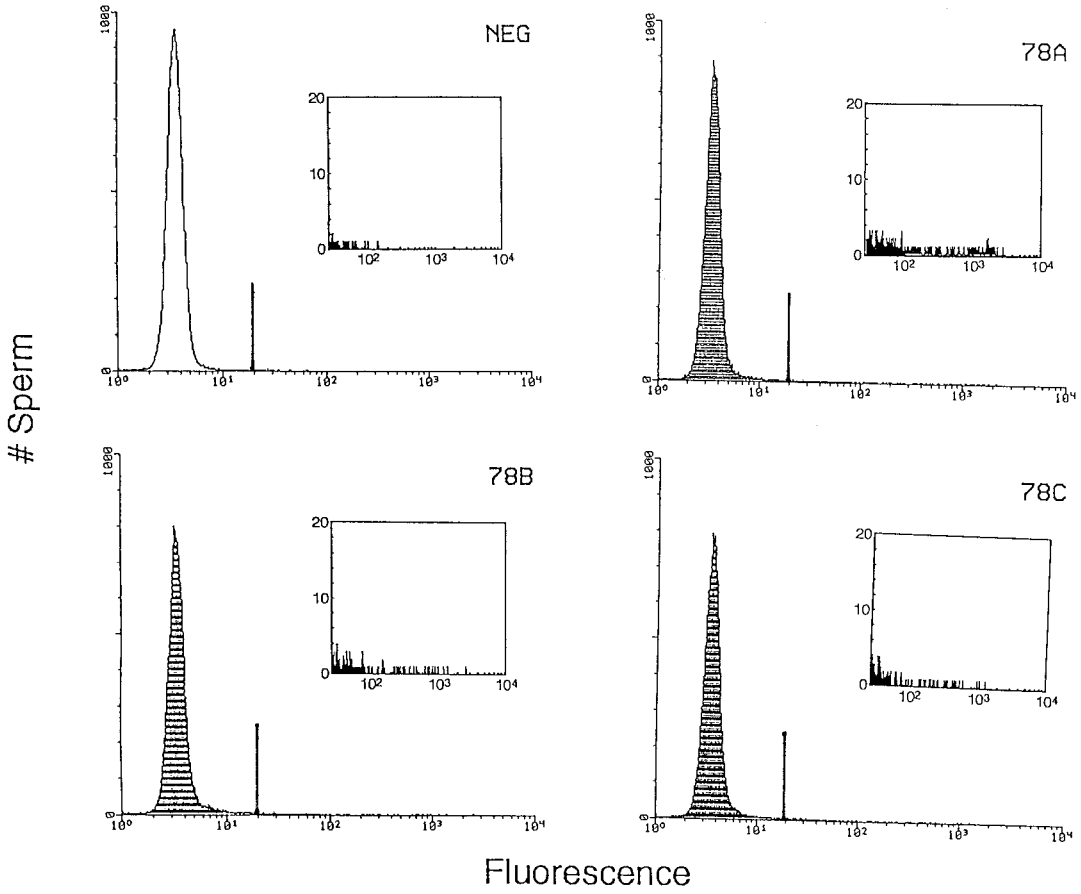


FIG. 3. Flow cytometric analysis of sperm to detect β-galactosidase activity. Sperm from the caudal epididymis were stained with the fluorogenic substrate CM-FDG. (Insets) The *y* axis was set to a maximal sperm count of 20 to better view the small number of sperm with increased fluorescence. The vertical line indicates the marker that was set to delimit the *x* axis in the insets. NEG, nontransgenic mouse sperm; 78A to 78C, sperm from three mice from line 78.

ing of spermatids gave similar quantitative results. It is of note that levels of β-galactosidase-positive cells from different line 78 mice fell within a small range (two- to threefold), demonstrating little mouse-to-mouse variation.

Instability of the inverted repeat in mouse line 78. In our routine maintenance of line 78, transgenic progeny were identified by Southern blot analysis of tail tip DNA. *Bam*HI-digested samples were probed with *lacZ* sequences. Two bands of 3.5 and 4.8 kb are diagnostic of the founder transgene arrangement (Fig. 2). The 4.8-kb band is from the central region of the inverted repeat and contains the *inlacZ* gene fragments. The 3.5-kb band is from the two outer regions of the inverted repeat and contains identical *lacZ4*Δ genes. Although the founder mouse appeared to be mosaic for the transgene insertion, progeny carrying the transgene transmitted it at roughly a normal Mendelian ratio (Table 2).

TABLE 1. Flow cytometric analysis of sperm from mice with the standard line 78 palindrome

Sperm source[a]	Mean fluorescence[b]	Total no. of sperm	No. of fluorescent sperm	% Positive
NEG	3.49	14,343	8	0.06
78A	3.28	13,987	98	0.70
78B	3.19	13,154	81	0.62
78C	3.28	13,504	56	0.41

[a] The four mice were littermates sired by mouse 919. NEG, nontransgenic mouse; 78A to 78C, three mice from line 78.

[b] Mean fluorescence is for the entire sperm population. The marker for fluorescent sperm was set at 19.46 (arbitrary fluorescence units [Fig. 3]).

Despite the normal frequency of transmission, novel arrangements of the transgene locus were frequently detected in progeny from mice containing the standard line 78 palindrome (Fig. 5). Each of the variants had a band at 3.5 kb, indicating that one or both flanks of the transgene locus were intact. Most of the variants lacked the central 4.8-kb *Bam*HI band but instead had bands that were either larger or smaller. Some of the new bands were faint, appearing to be present at less than single copy in intensity (e.g., the 2.7-kb band in variant 1495), suggesting that the individual mouse sampled was mosaic. From these analyses, it appeared that the central region of the inverted repeat was particularly susceptible to rearrangement. The fact that the 4.8-kb fragment was highly variable indicated that illegitimate (nonhomologous) rearrangements had occurred. In addition, one new band of 2.7 kb was repeatedly found in several variant mice, as would be predicted for a homologous crossover (see below).

Mice with rearrangements typically constituted 15 to 56% of the progeny from any one breeder (Table 2). Breeding for line 78 was limited almost exclusively to males. However, the one female breeder also produced progeny with rearrangements

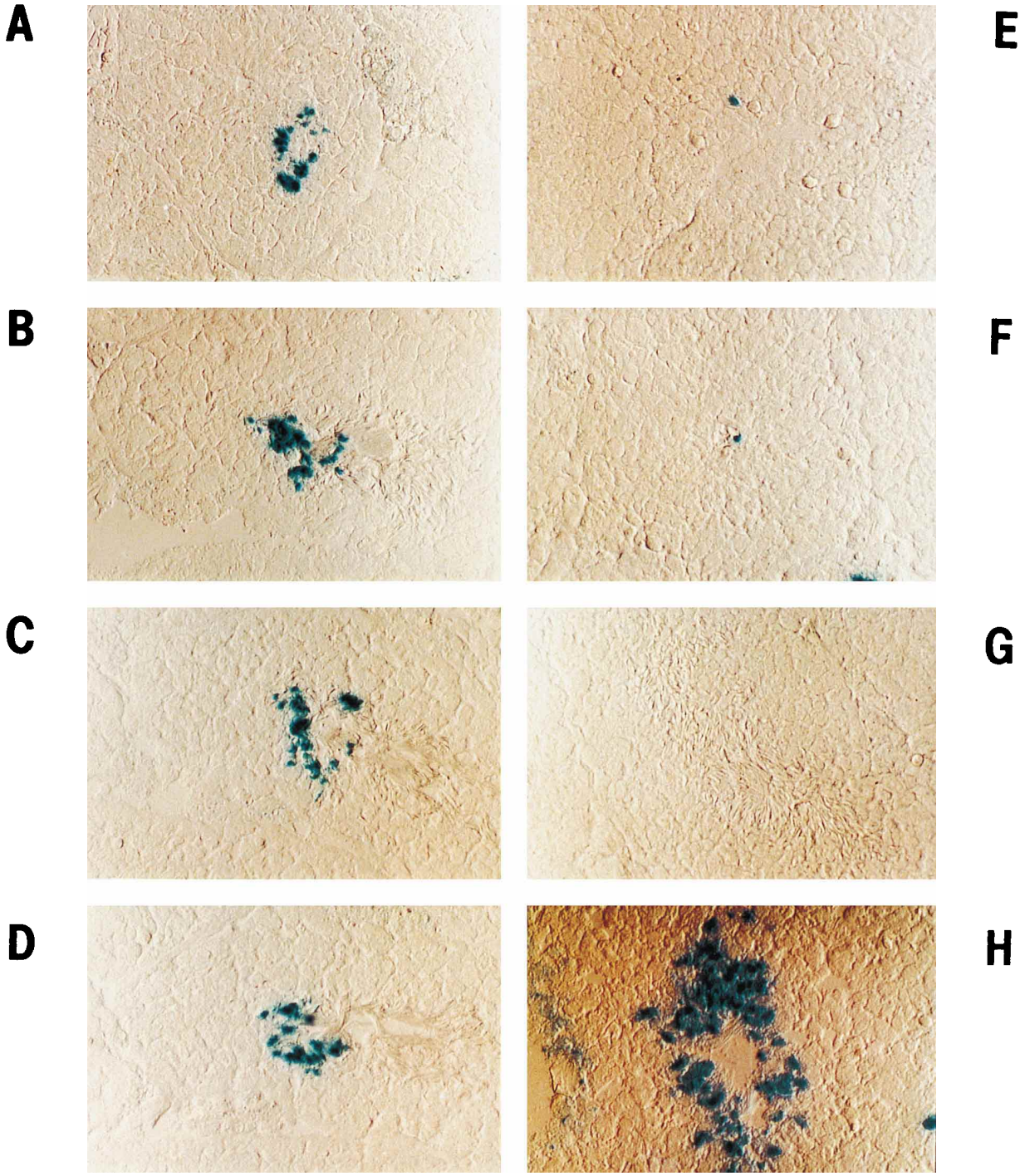


FIG. 4. X-Gal staining of adult mouse testis sections. (A to G) Line 78 mouse. Panels B to D are from adjacent sections of the same tubule. (H) Control mouse which contains multiple copies of a *lacZ*$^+$ transgene. All sections are at the same magnification (×36).

(Table 2, mouse 1157), suggesting that transgene instability was not restricted to the male germ line and was unrelated to *prm-1* promoter activity. In previous work, more than 20 other transgenic mouse lines containing various *prm-1*/*lacZ* transgene insertions had been generated, without exhibiting any similar gross transgene instability (references 21 and 33 and data not shown). Thus, a unique property of line 78—either the locus of integration or the unusual palindromic arrangement of the transgene insertion—was responsible for the instability of the introduced sequences.

To investigate further, several variants of the transgene locus from line 78 were mapped in detail by Southern analysis of tail tip DNA samples. In certain cases, mice containing rearrangements were bred to distinct sublines. Both classes of recombinants described above were analyzed: illegitimate recombination events in which the transgene locus was centrally

TABLE 2. Breeding analysis of mice with the standard line 78 palindrome

Breeder[a]	Genotype	No. of progeny	No. (%) transgenic	No. (%) of variants[b]	Variant examples[b]
78[c]	Mosaic	113	13 (12)	2 (15)	364/354
919	+/−	98	51	16	1129, 1148, 1347, 1456, 1495, 1497, 1678/1733
1137	+/−	62	28	4	1485, 2275, 2279/2280, 2240
1157	+/−	20	9	3	1387
2241	+/−	48	21	6	2548, 2688
2426	+/−	32	9	5	2774
2781	+/−	19	8	4	2941/2965, 2966
Total		279	126 (46)	38 (30)	
1333	+/+	52	52 (100)	8 (15)	1722
1127/1128 × 1135[d]	+/− × +/−	22	16 (73)	11 (68)	1338/1491, 1594

[a] Breeders had the standard 78 palindrome (Fig. 2), as determined by Southern analysis. Breeders were male except 1157, 1127, and 1128.
[b] Variants were determined by *Bam*HI digestion and in some cases by *Bam*HI-*Pst*I digestion. Numbers for variant mice discussed in the text are indicated in the last column. Variants separated by a slash appeared to have identical transgene patterns.
[c] Founder mouse. The transgene intensity of mouse 78 appeared roughly half that of transgenic progeny.
[d] Breeders that were kept in the same cage. In addition to variants 1338 and 1491, seven other mice from these breeders had the same Southern pattern as 1338 and 1491.

deleted, and homologous recombination events between transgene *lacZ* repeats.

Small central deletions. The most frequently observed variations were small deletions in the central region of the inverted repeat. An example was mouse 364. The *Bam*HI and *Eco*RI restriction patterns of the transgene locus in this mouse were nearly identical to the standard line 78 pattern (Fig. 6A). However, a deletion estimated to be about 0.1 to 0.2 kb in extent, near the symmetry axis, was revealed by *Bam*HI-*Sac*I and *Bam*HI-*Pst*I digestion. It was apparent that the deletion was located near the central *Pst*I site but did not include the exact point of symmetry, because the *Pst*I site was still present (Fig. 7). A littermate, mouse 354, appeared to have the identical deletion (data not shown). The occurrence of a deletion limited to one side of a palindrome was unusual, since in yeast and bacterial systems, palindrome deletions extend into both sides, spanning the symmetry axis (12, 26).

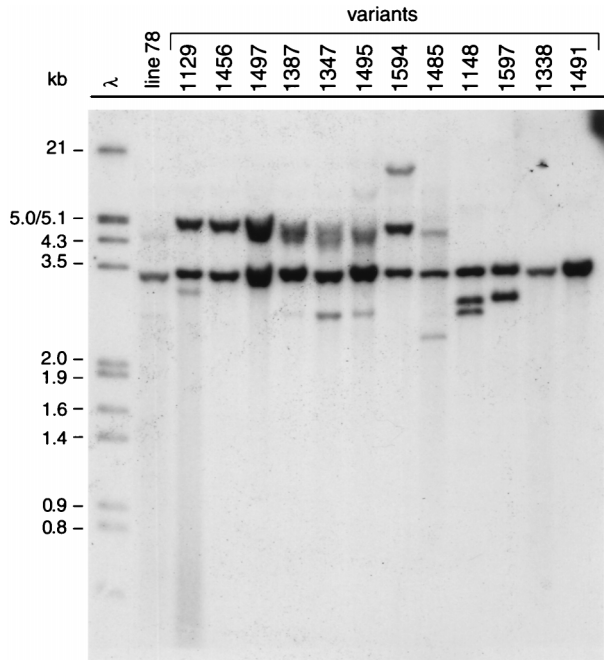


FIG. 5. Variant arrangements of the transgene locus in mice from line 78. Southern analysis was performed on *Bam*HI-digested tail tip DNA with the 3.5-kb *Bam*HI *lacZ* fragment as a probe. The lane labeled line 78 contains tail tip DNA from a mouse with the standard line 78 palindrome. The 4.8-kb band is less intense than expected, presumably due to snap-back of the inverted sequences during neutral transfer, prior to hybridization with the probe. The sample in this lane was also slightly underloaded. The lanes labeled variants contain tail tip DNA from mice with altered transgene configurations. Except for mouse 1597, the variants are progeny of mice with the standard line 78 palindrome. Mouse 1597 was derived from mouse variant 1148. See Table 2, footnote *a*, for derivation of the variant mice.

Southern blot analysis of mouse 364 was sufficiently detailed that it was possible to amplify the new central junction. The template was pretreated with a restriction enzyme, *Hin*cII, which selectively digested the intact copy of the inverted repeat (Fig. 8). By this means, self-annealing of the template, which otherwise interferes with PCR, was largely eliminated. Primers were designed to amplify the unique central region across the recombinant junction (Fig. 8A). The DNA sequence is shown in Fig. 8B. The endpoints of the 137-bp deletion were within several base pairs of the original symmetry axis, located at a 4-bp repeat that overlapped the *Pst*I site.

The deletion in subline 364 created a 137-bp stretch of unique sequence between the remaining 7.6 kb of inverted sequences, interrupting the palindrome. Instability in the transgene locus of mouse 364 was tested by analysis of tail tip DNA of its progeny and by the sperm fluorescence assay. Fortuitously, none of the *lacZ* or transgene regulatory sequences had been deleted in mouse 364, since this would affect the sperm fluorescence recombination assay in either of the repeat units. More importantly, since the entire sequence of one repeat unit was intact, this subline allowed us to determine whether the instability seen in the standard line 78 transgene was due to the specific sequence of the transgene or to the fact that it is arranged as a symmetrical repeat.

Breeding of mouse 364 was limited because he was sacrificed for sperm collection. However, all 15 transgenic progeny from him, as well as 9 progeny from mouse 354, which appeared to contain the identical deletion, demonstrated no further rearrangement of the transgene locus (Table 3). These breeding results suggest that the relatively small interruption of symmetry in the palindrome had a stabilizing effect on the locus.

Flow cytometric analysis of sperm from several mice derived from mice 364 and 354, as well as from mouse 364 himself,

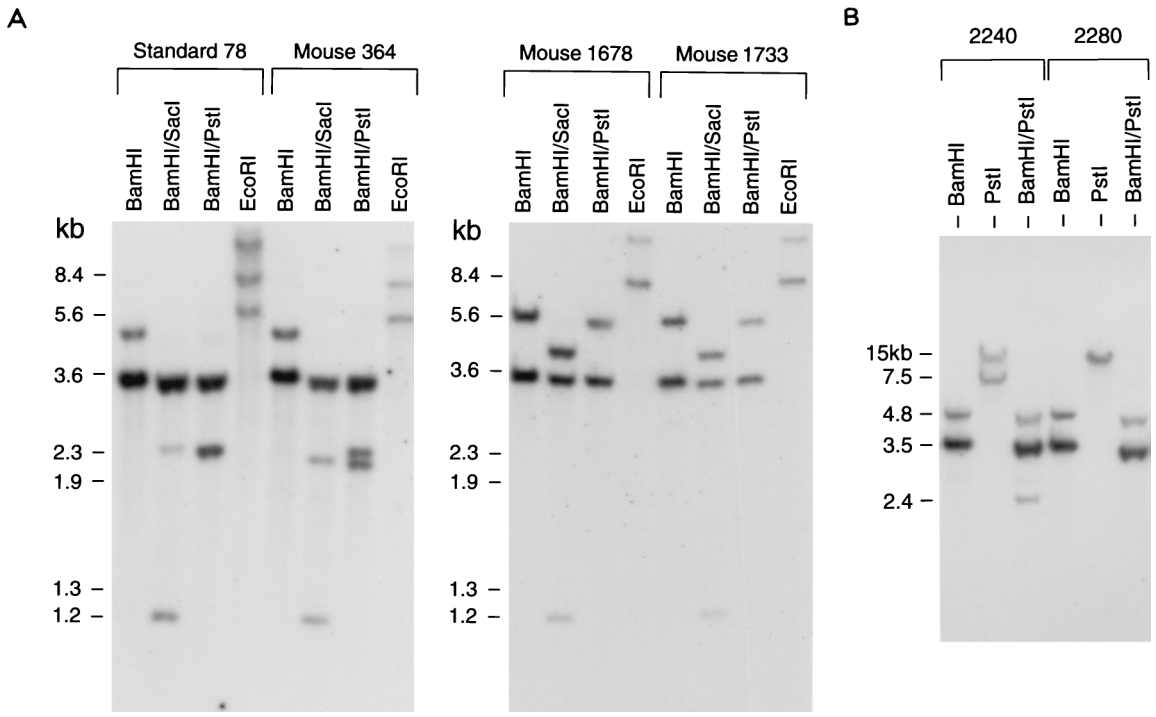


FIG. 6. Southern analysis of selected line 78 variants. Tail tip DNA was digested with the indicated restriction enzyme and probed with the 3.5-kb *Bam*HI *lacZ* fragment.

revealed that the frequency of *lacZ* homologous recombination had also been affected by the deletion. Unlike mice with the standard line 78 palindrome, only background levels of β-galactosidase positive sperm were detected from sublines 364 and 354 (data not shown). This represents at least a 10- to 20-fold reduction in recombination. Thus, both illegitimate and homologous recombinations were substantially reduced in sublines 364 and 354, even though one perfect copy of the original transgene remained and no *lacZ* sequences, or sequences affecting *lacZ* expression, were deleted.

Other mice of the line 78 lineage had small deletions nearby or including the *Pst*I site, which interrupted the perfectly palindromic structure of the standard line 78 transgene (Fig. 7). Mouse 2774 had a deletion of approximately 200 bp that again spared the central *Pst*I site. Another small central deletion of approximately 200 bp was found in mice 2279 and 2280. In these mice, derived from a single litter, the deletion included the *Pst*I site (Fig. 6B). This type of small deletion was consistently found in every breeder analyzed that had the standard line 78 palindrome. As with sublines 364 and 354, FACS analyses of sperm from mouse 2774 and progeny from mouse 2279 indicated that gene conversion was depressed to background levels (data not shown). When bred, mice 2279 and 2280 stably transmitted the new transgene configuration (Table 3); no further rearrangement of the locus was detected in 22 transgenic progeny.

For some mice, it was possible to infer the timing of the illegitimate deletion events. For example, in mouse 2240, the deletion appeared to have occurred during early embryogenesis (Fig. 6B). Roughly half of the transgene DNA from his tail tip had no rearrangement and was cleaved by *Pst*I at the center of symmetry, whereas the other half containing the deletion was not cleaved. Digestion of brain DNA from mouse 2240 gave the same result (data not shown). When mice with a similar mixed pattern were bred, the two transgene patterns segregated. Thus, a portion of cells from these mice had the standard 78 palindrome, but the other portion contained a small deletion at the symmetry center, indicating that deletion occurred within the first cell divisions of the embryo.

Large central deletions. Mice with larger deletions at the center of symmetry were also detected, although less frequently than those with small central deletions. Three large deletions were characterized with sizes estimated to be 1.3, 2.7, and 3.1 kb. In each case, the deletion was asymmetric, which effectively interrupted the palindromic structure.

For the 1.3-kb deletion, found in mice 1338 and 1491 (Fig. 5), one copy of the *inlacZ* gene was intact or nearly so whereas the other *inlacZ* gene suffered a deletion (Fig. 7). This created a unique central region in the transgene locus of approximately 1.1 kb, flanked by 6.5 kb of inverted repeat. As with the smaller central deletions discussed above, the asymmetric 1.3-kb deletion conferred stability on the transgene locus, as detected by either breeding analysis or the sperm fluorescence assay. In contrast to breeders with the standard line 78 palindrome, where 15 to 56% of progeny had a rearranged locus (Table 2), the 1338 and 1491 sublines yielded only 1 offspring with a rearranged locus in 76 analyzed (Table 3). Scoring the frequency of β-galactosidase-positive recombinants by FACS analysis of sperm was also informative for these sublines, because one copy of the gene conversion substrate was virtually intact. In both the 1338 and 1491 sublines, only background levels of fluorescent sperm were detected (data not shown). Thus, small (≥200-bp) and large (1.3-kb) interruptions of the palindrome correlated with a decrease in both illegitimate and homologous recombinations.

A larger deletion of 2.7 kb was found in the central symmetry region of the transgene locus in mice 1678 and 1733 (Fig. 6A). In these mice, the central asymmetry created by the deletion was at least 2 kb. The Southern patterns of the two mice were identical and were consistent with a deletion extending from the 3′ end of the *inlacZ* gene in one transgene copy to the

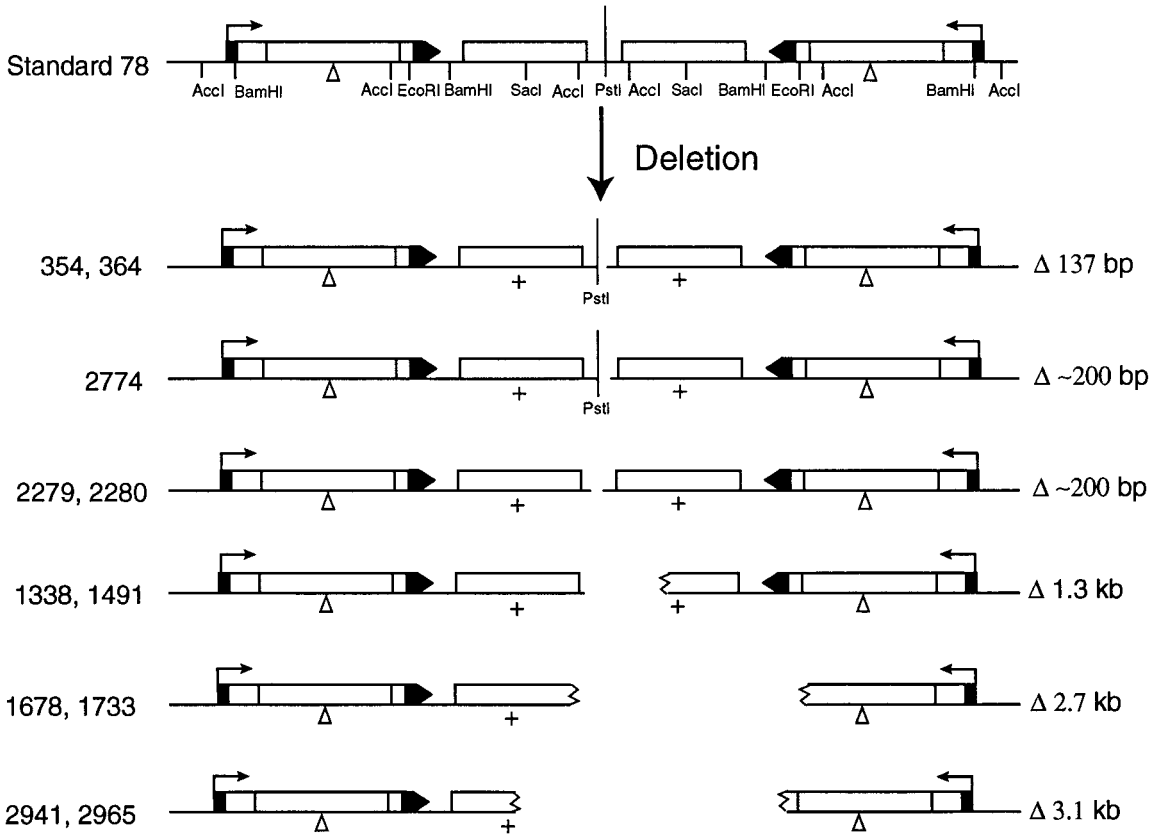


FIG. 7. Structure of the transgene locus in variants with central deletions. Note that mouse pair 364 and 354 and mouse 2774 have deletions just to one side of the original axis of symmetry and thus retain one complete copy of the repeat. Deletion endpoints are mapped by using the indicated restriction enzymes. The status of the *Sac*I site in the *lacZ* genes is indicated by + or Δ. In most cases, it is not possible to identify the side on which each deletion occurred.

3′ end of the *lacZ4*Δ gene in the other transgene copy (Fig. 7). A third large central deletion of 3.1 kb was found in mice 2941 and 2965. Here, the central asymmetry created by the deletion was at least 1.6 kb. The Southern pattern, also identical in these two mice, was consistent with a deletion extending from the 3′ end of the *inlacZ* gene in one transgene copy to the 3′ end of the *lacZ4*Δ gene in the other transgene copy (Fig. 7 and data not shown). Because these deletions disrupted the *lacZ* repeats, which precluded analysis of *lacZ* gene conversion, sperm from mice 1678 and 1733 and mice 2941 and 2965 were not analyzed, nor were these mice bred.

Large central deletions gave unique junction fragments that provided a convenient sublineage marker. As mentioned above, each of the large deletions was found in multiple progeny from the same breeder. For example, both 1678 and 1733, which were born 2 months apart, were derived from mouse 919. Mouse 919 had other offspring with what appeared to be the same deletion, yet had still other progeny with rearrangements that were unique (e.g., mouse 1148 [Fig. 5]). These data indicated that although mouse 919 and the other breeders had inherited the standard line 78 palindrome, large central deletions, as with smaller deletions, could occur prior to meiosis in a germ cell precursor or spermatogonial stem cell.

Homologous recombinants. The most frequently observed transgene variants from breeders with the standard line 78 palindrome contained central deletions, as described above. However, a faint band of 2.7 kb was also frequently observed in *Bam*HI-digested tail tip DNA from mice with the standard line 78 palindrome. The 2.7-kb band was present at less than 1/10 of the intensity but was of the size predicted to arise from a homologous crossover between one *inlacZ* fragment and the oppositely oriented *lacZ4*Δ gene. This recombinant transgene configuration could be recovered in some offspring of mice with the standard line 78 palindrome. One example was mouse 2275. A *Bam*HI digest of tail tip DNA from this variant revealed the 2.7-kb band, as well as a larger 5.6-kb band (Fig. 9A). Further digests confirmed that a crossover had inverted the inner region of the transgene locus, forming two new *lacZ* gene fragments that contained the 5′ and 3′ portions of the *lacZ* gene (referred to as *lacZ5′* and *lacZ3′*, respectively [Fig. 9B]). Crossing over was accompanied by a gene conversion of the participating *lacZ4*Δ gene, so that a *Sac*I site was present in both the *lacZ3′* and *lacZ5′* gene fragments (Fig. 9).

Inversion of the transgene locus brought about by a homologous recombination between a *lacZ4*Δ gene and an *inlacZ* fragment will reduce but not eliminate the palindrome in the transgene. By comparison to the 15.3-kb palindrome of the original line 78 mouse, the perfectly palindromic region in mouse 2275, after inversion, was 4.2 kb long. Thus, it was possible to determine whether, in a fixed chromosomal context, a reduction in the size of the palindrome would suffice to stabilize the locus. To investigate this point, mouse 2275 was bred with a nontransgenic female. It was evident, by Southern blot analysis that illegitimate rearrangement continued to occur; transgenic progeny from this mouse were again variant (Table 3). As with the standard line 78 palindrome, rearrangements of the transgene locus included those that had small deletions at the center of symmetry. Furthermore, FACS anal-

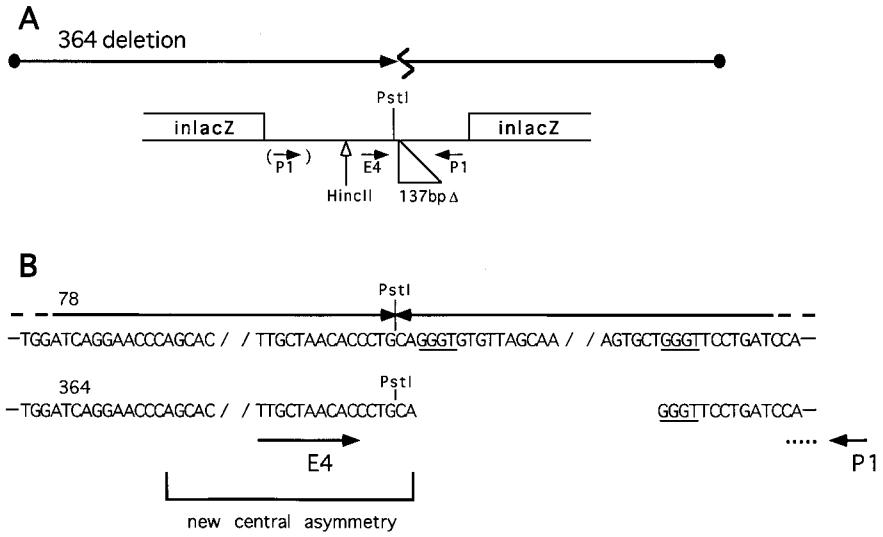


FIG. 8. Deletion junction in mouse 364. (A) Map of the deletion in mouse 364 (arbitrarily drawn to the right) and positions of the primers used for amplification of the junction. (B) Sequences around the *Pst*I site at the symmetry axis of the inverted repeat as inferred for mouse 78 and as determined for mouse 364. The underlined GGGT sequence is present on both sides of the deletion in the precursor transgene and may be derived from either. The *Pst*I site is CTGCAG. Slash marks indicate sequences that are not shown.

ysis performed on sperm from a line 2275 mouse indicated that fully 2% of the sperm from this mouse resolved as a distinct highly fluorescent peak (i.e., they were homologous *lacZ* recombinants [data not shown]). Progeny of a second, independently derived mouse with an inversion of the transgene locus gave similar results. Thus, transgene variants which maintain a perfect, uninterrupted palindrome, even one reduced in size, maintain a high degree of genetic instability.

DISCUSSION

We have demonstrated that perfect inverted repeats (palindromes) in the mouse genome promote genetic instability and are consequentially converted to a more stable form. Although the palindromic transgene is transmitted in a normal Mendelian ratio, rearrangement of the inverted repeat is frequent, being detectable in 15 to 56% of transgenic progeny by Southern blot analysis of tail tip DNA. Many of the rearrangements involve illegitimate (nonhomologous) recombination. Homologous events are also detected, as monitored by a *lacZ* sperm fluorescence assay. In approximately 0.3 to 0.7% of sperm, gene conversions between defective *lacZ* repeats had produced a functional *lacZ* gene.

Illegitimate events within the transgene locus often produce an asymmetric deletion at the center of the palindrome. In some cases, one complete half of the palindromic repeat is maintained, resulting in a "one-sided" deletion. These asymmetric transgene deletions allowed us to determine the effect of perfect symmetry of an inverted repeat on both illegitimate and homologous recombinations of the transgene. Whereas perfectly symmetrical palindromes were unstable, lineage analysis, coupled with the sperm fluorescence assay, indicated that central asymmetries stabilize the locus with regard to both illegitimate and homologous recombination events. With regard to illegitimate events, another transgene palindrome of unrelated sequence has also been reported to be frequently rearranged in progeny and to be stabilized upon the formation of central asymmetry (7) (see below).

Mosaicism of the transgene locus, as detected in tail tip DNA and by breeding analysis, suggests that illegitimate events occur soon after fertilization and during germ cell development. Gene conversion events also appeared to occur at multiple developmental stages, in both mitotic and meiotic cells, with the overall contribution to variation in sperm genotypes being potentially much greater for mitotic events. Notably, one other study of gene conversion in the mouse germ line has been undertaken, and it was reported that as many as 2% of spermatids were recombinant (34). Comparable substrates in our study, the stabilized transgenes with interrupted palindromes, undergo recombination at least at a 20-fold-reduced rate. Although details of the recombination substrates differ, the strain background might well influence gene conversion frequencies (41a). In the previous study, recombinants were analyzed on a random-bred CF1 background (34), whereas our studies were performed on a mixed C57BL/6 × CBA/Ca inbred genetic background.

In *E. coli*, palindromes in the size range of our transgene insertion are profoundly unstable, leading to inviability of the replicon on which they reside (8, 32). Thus, a key difference between mammalian cells and *E. coli* exists in the level of genetic instability caused by large palindromes: although unstable, a large palindrome can be maintained in a mammalian genome without major deleterious effects on the chromosome

TABLE 3. Breeding analysis of line 78 variants[a]

Subline	No. of progeny	No. transgenic	No. of new variants
Variants with central deletions			
364/354	35	24	0
2279/2280	47	22	0
1338/1491	131	76	1
Variant with an inversion			
2275	26	14	8

[a] All of the breeding results were obtained from hemizygous transgenics. Mice 364, 354, 2279, 2280, and 2275 were males and were the sole breeders for their sublines. Mouse 1338 was female, and the breeding analysis included her and four of her sons. Mouse 1491 was male, and the breeding analysis included him and four of his sons.

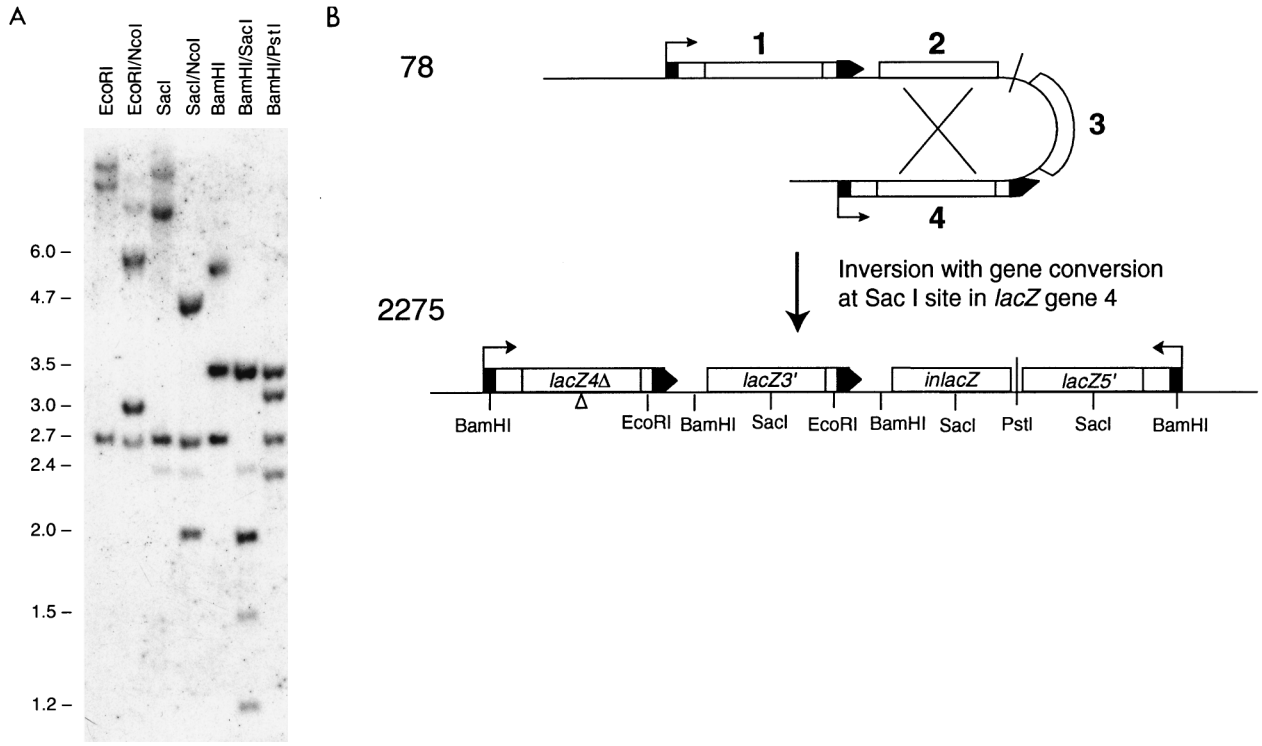


FIG. 9. Inversion of the transgene locus in mouse 2275. (A) Southern blot analysis of tail tip DNA from mouse 2275. The probe is the 3.5-kb *lacZ Bam*HI fragment. (B) Map of the inversion found in mouse 2275. A reciprocal exchange between repeats 2 and 4 occurred, with an associated gene conversion at the *Sac*I site in repeat 4.

carrying it. This suggests either that DNA structures associated with large palindromes are less likely to form in mammalian cells or that such structures do not interfere as profoundly with processes such as replication.

Palindrome processing in *E. coli* and yeast. In prokaryotic systems, replicons containing smaller palindromes of a few hundred base pairs or less can be propagated, although deletions of the palindromes occur readily (for reviews, see references 12 and 26). The deletion products are "two-sided" in that each repeat unit of the palindrome is substantially reduced in size or completely eliminated. These products are consistent with a model in which the palindrome undergoes intrastrand base pairing to form a hairpin structure, hindering DNA replication (Fig. 10A). When DNA polymerase is prevented from replicating through the hairpin, the terminal bases of the nascent strand dissociate from the template and anneal to a short stretch of complementary sequence on the other side of the hairpin. The hairpin is thus bypassed, and DNA replication proceeds unhindered. Palindrome deletions in yeast are consistent with this model (18, 35, 41). Support for this replication "slippage" or "bypass" model comes from in vitro studies in which DNA polymerase has been observed to stall shortly after entering a palindromic region in the template DNA (4, 47).

One-sided deletions suggest an alternative palindrome-processing pathway. Large central deletions found in the transgene palindrome in mouse pairs 1338 and 1491, 1678 and 1733, and 2941 and 2965 (Fig. 7) could result from a replication slippage type of mechanism. In these cases, replication would proceed well into one side of the inverted repeat and then "slip" to the other side of the repeat. This would occur if less than the entire 15.3-kb inverted repeat undergoes intrastrand pairing, so that replication encounters hairpin structures at variable distances from the center of symmetry.

Central to replication slippage models for palindrome deletion is a hairpin obstruction to polymerase progression. Polymerase stalls before it reaches the center of the hairpin, and then synthesis begins again, having bypassed the secondary structure. Not predicted by the model are one-sided deletions, where one side of the inverted repeat is completely intact (Fig. 10B). These types of deletions have not been reported for bacterial and yeast palindrome deletions but are present in mouse pair 364 and 354 and mouse 2774. One-sided deletion events in mammalian systems are apparently not rare. An apparently perfect inverted repeat at another transgene locus in mice has yielded similar products (7). In this case, the transgene locus was composed of human minisatellite repeats embedded in mouse gamma-satellite DNA. As with the line 78 transgene, some of these deletions were small and asymmetric and were located on one side of the central symmetry axis. Another example of a one-sided palindrome deletion has been described in the human genome. In an individual with hereditary persistence of fetal hemoglobin, a 48.5-kb deletion endpoint was mapped to within 4 bp of the axis of symmetry of what had been a 160-bp perfect palindrome (19).

One-sided palindrome deletions found in mammalian systems suggest that an initiating event for illegitimate recombination may be localized at the center of symmetry of the palindrome, the hairpin tip. One such initiating event that could be postulated is an endonucleolytic nick. Nicking at hairpin tips would focus deletion events at the center, rather than the sides, of the palindromic sequence. Hairpin nicks would result in double-strand breaks if both strands of the palindrome are nicked or if a singly nicked strand is replicated. Cellular break repair after nucleolytic digestion would result in deletions in the palindrome, from one or both sides, with the potential to protect the locus from further rearrangement. In

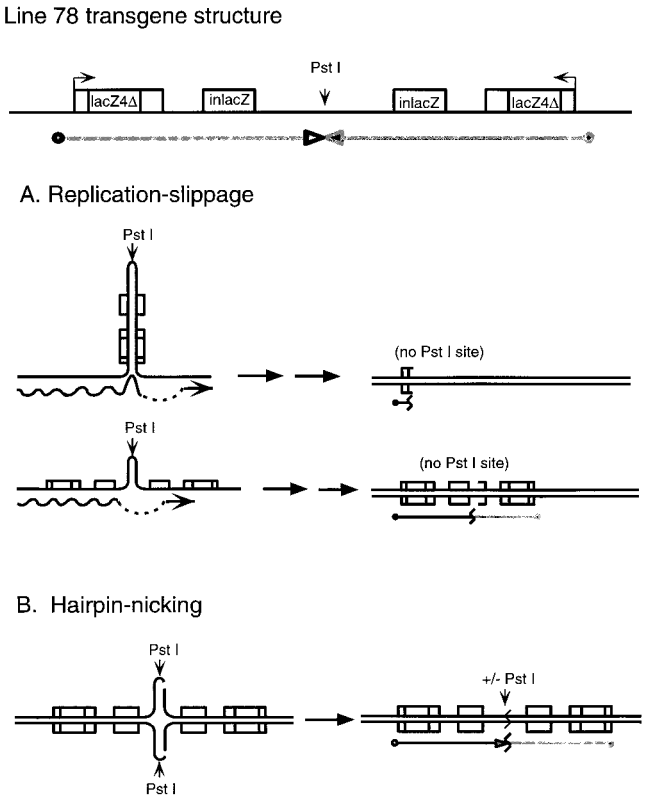


FIG. 10. Potential models of the generation of palindrome deletions. Both models rely on an initial step of intrastrand base pairing to form a hairpin structure on at least one of the strands. (A) Replication slippage to generate two-sided palindrome deletions. In this model, replication stalls once it encounters the hairpin. The nascent strand melts off the template and then slips to a short repeat on the other side of the hairpin, allowing replication to resume. The next round of replication creates a product with a deletion in the palindrome. Different amounts of intrastrand base pairing result in deletions of different sizes, as shown. Deletions by this model always span the central symmetry axis and have been found to arise from small palindromes in bacteria and yeast. (B) Hairpin nicking to generate one-sided or two-sided palindrome deletions. An initiating event for these deletions is postulated to occur at the symmetry center of the hairpin. One such event may be an endonuclease nick at the tip of the hairpin structure. The broken strands are then rejoined by cellular repair mechanisms to create different-sized deletions. In some cases, repair would result in a deletion in only one of the repeat units, adjacent to the symmetry center (one-sided deletions). In other cases, repair would result in deletions in both of the repeat units, on both sides of the symmetry center (two-sided deletions). Both one-sided and two-sided deletions arise from deletion events in palindromes in mammalian genomes. In the case of the line 78 transgene palindrome, the presence or absence of the central *Pst*I site is diagnostic for one-sided or two-sided palindrome deletions, respectively.

addition to accounting for one-sided deletions, a hairpin-nicking mechanism would satisfactorily explain the occurrence of insertions at the center of symmetry (7).

Double-strand break repair and hairpins. A nuclease capable of introducing nicks at or near the tip of hairpin DNA has been postulated to be responsible for opening hairpin intermediates formed during V(D)J recombination (for a review, see reference 28). Although the hairpin-nicking activity in V(D)J recombination has not been biochemically characterized, the metabolism of hairpin DNA has been examined in vivo. Through analysis of the fate of linear hairpin-ended vectors transfected into cells, it was shown that a significant fraction of the hairpin termini were opened by a single-strand nick in both fibroblastoid and lymphoid cells (27). These studies did not address whether such an activity exists in germ line cells.

The proposed involvement of strand breaks in initiating palindrome deletions relies on the fact that the illegitimate rejoining of double-strand breaks in mammalian cells is a relatively efficient process (39). In mammalian cells, chromosomal deletion junctions formed at double-strand breaks occur at fortuitous short repeats or at positions where there is no apparent base-pairing potential and with the occasional incorporation of novel sequences at the junction (27, 30, 39, 40). The joints formed between two hairpin-terminated DNA ends have all of these properties, with the added feature that a short stretch of sequences past the "turnaround" point (P nucleotides) are often present in the junction as well (27), as is seen in the mouse 364 junction.

The relatively efficient illegitimate rejoining of double-strand breaks in mammalian cells is in striking contrast to what is seen in *E. coli* (see, e.g., reference 25). In *E. coli*, a double-strand break will rapidly result in destruction of a replicon by nucleases, an observation that has been central to mammalian break repair assays that involve bacterial transformation (39; for a recent example, see reference 29). This leads to the speculation that the initial processing step of long palindromes in mammalian cells and *E. coli* could be similar (i.e., hairpin nicking) but that mammalian cells are able to heal such breaks whereas *E. coli* destroys the replicon containing them. Palindromes which are bypassed in *E. coli* by replication slippage do not present this type of damage to the cell.

The inviability of replicons containing long palindromes can be alleviated in *E. coli* by *sbcCD* mutations (for a review, see reference 26). Since the SbcCD protein has both endo- and exonuclease activities (9), it is a candidate for either introducing nicks into hairpins or processing the broken ends. Interestingly, it has been noted that two yeast proteins essential for meiotic recombination and double-strand break formation, Rad50 and Mre11 (23), share homology with the SbcCD nuclease (42). Mammalian homologs have recently been identified (10, 37).

Inverted duplications with a structure very similar to our asymmetrically deleted transgenes are found at gene amplifications in mammalian cells (14). Since hairpin structures may be formed at the initial stages of gene amplification (6, 31, 46), it is possible that some gene amplifications begin as perfect palindromes and are then resolved to interrupted palindromes, as is the case for our transgene. Hairpin DNA structures are not restricted to being formed at inverted repeats. Hairpins form in solution from the quasipalindromic sequences that expand in triplet repeat diseases (5, 15). Although triplet repeat expansion has been suggested to be due to replication slippage at a hairpin structure, it is also conceivable that the creation of breaks at the hairpin tips contributes to their instability. Chromosomal breakage would provide a fairly simple explanation for deletions with endpoints in triplet repeat sequences in patients (24). Interestingly, as is seen for palindrome instability, mosaicism is frequently observed in individuals exhibiting triplet repeat instability (1).

Additional consequences of instability. Elevated intrachromosomal homologous recombination, in addition to illegitimate events, is observed in the palindromic transgene. Crossovers create inversions of the locus, and gene conversions without crossing over result in $lacZ^+$ transgenes. No offspring with inversions were obtained from mice that possessed a central asymmetry in the transgene locus. The sperm fluorescence assay further correlated a high level of homologous recombination with the existence of perfect symmetry in the transgene locus.

It has not previously been noted that the frequency of homologous recombination is elevated at a palindrome. Interrupted palindromes enhance homologous crossovers in yeast (16) but have not been associated with elevated gene conver-

sion in transgenic mice (34). Models for homologous recombination generally involve a double-strand break at the recipient of genetic information (44), which in our design is the *lacZ4*Δ gene. We have no evidence for double-strand breaks at this off-center position, since deletion endpoints are not found central to one repeat. However, the correlation of a palindromic structure with an elevation in both homologous and illegitimate recombination suggests a possible linkage between these two processes. Further analysis of recombination products and the identification of components involved in the instability will resolve this issue.

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