Long Inverted Repeats Are an At-Risk Motif for Recombination in Mammalian Cells

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Manuscript received June 1, 1999
Accepted for publication August 16, 1999

ABSTRACT

Certain DNA sequence motifs and structures can promote genomic instability. We have explored instability induced in mouse cells by long inverted repeats (LIRs). A cassette was constructed containing a herpes simplex virus thymidine kinase (*tk*) gene into which was inserted an LIR composed of two inverted copies of a 1.1-kb yeast *URA3* gene sequence separated by a 200-bp spacer sequence. The *tk* gene was introduced into the genome of mouse L*tk*$^-$ fibroblasts either by itself or in conjunction with a closely linked *tk* gene that was disrupted by an 8-bp *Xho*I linker insertion; rates of intrachromosomal homologous recombination between the markers were determined. Recombination between the two *tk* alleles was stimulated 5-fold by the LIR, as compared to a long direct repeat (LDR) insert, resulting in nearly 10^{-5} events per cell per generation. Of the *tk*$^+$ segregants recovered from LIR-containing cell lines, 14% arose from gene conversions that eliminated the LIR, as compared to 3% of the *tk*$^+$ segregants from LDR cell lines, corresponding to a >20-fold increase in deletions at the LIR hotspot. Thus, an LIR, which is a common motif in mammalian genomes, is at risk for the stimulation of homologous recombination and possibly other genetic rearrangements.

CELLULAR viability and propagation require that genomes be reasonably stable. Cancer is an example of a genetic disease often associated with and, in some cases, perhaps engendered by the loss of genomic stability. The potential cost of genomic instability resulting from homologous recombination is presumably mitigated by such benefits as generation of diversity and recombinational DNA repair functions. Because the ability to carry out homologous recombination appears to be ubiquitous in nature, it is important to gain a better understanding of the determinants of the rate and nature of recombination events to understand how genomic integrity is maintained.

Rates of genetic recombination can exhibit site specificity. For example, chi sites in *Escherichia coli* (reviewed in Smith 1988) or the *MAT* locus of *Saccharomyces cerevisiae* (reviewed in Haber 1998) are recombination hotspots due to specific interactions between specific nucleotide sequences and specialized proteins. In addition to particular sequences, certain DNA structures, motifs, or lesions can also have a significant influence on genetic exchange. For example, DNA double-strand breaks (DSBs) constitute a type of lesion that was originally proposed to stimulate homologous recombination via repair in yeast (Resnick 1976). In yeast, meiotic recombination is believed to be naturally initiated by DSBs (reviewed in Osman and Subramani 1998), and DSBs stimulate homologous recombination in mitotically dividing mammalian somatic cells (Rouet *et al.* 1994; Choulika *et al.* 1995; Brenneman *et al.* 1996; Moynahan and Jasin 1997; Sargent *et al.* 1997; Taghian and Nickoloff 1997) as well as in yeast (Haber 1995). It follows that regions of chromatin that possess structural features prone to strand breakage would be expected to be particularly active in homologous recombination.

DNA motifs that are predisposed to genetic change have been referred to as at-risk motifs (Gordenin and Resnick 1998). A long inverted repeat (LIR), *i.e.*, an inverted repeat in which the repeat unit is more than ~100 bp in length, is an example of an at-risk motif. LIRs that form perfect palindromes are extraordinarily unstable in *E. coli* and are deleted at a high rate (Collins 1981; reviewed in Leach 1994), while LIRs separated by a spacer sequence are more stable than perfect palindromes but are still prone to deletions (Albertini *et al.* 1982; Glickman and Ripley 1984). Inverted repeats have also been shown to induce deletions as well as recombination between homologous and diverged DNAs in yeast (Gordenin *et al.* 1992, 1993; Henderson and Petes 1993; Ruskin and Fink 1993; Nag and Kurst 1997; Tran *et al.* 1997; Lobachev *et al.* 1998). Stimulation of deletion and recombination was shown to be directly related to the size of the repeats and inversely proportional to the size of an intervening spacer (Lobachev *et al.* 1998). A perfect palindrome formed by two 1.0-kb inverted repeats stimulated interchromosomal

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homologous recombination in yeast by much as 17,000-fold (Lobachev *et al.* 1998), illustrating that recombinagenic effects can be profound. A defect in DNA polymerase δ augmented the destabilizing effects of LIRs in yeast, while the placement of a strong, bidirectional replication origin between inverted repeats attenuated the effects of LIRs (Lobachev *et al.* 1998). These results support a role for replication in LIR-stimulated recombination and deletion in yeast, possibly through the formation of duplex secondary structures in a DNA template that stalls nascent strand elongation and leads to genetic rearrangement (Gordenin *et al.* 1993; Tran *et al.* 1997; Lobachev *et al.* 1998).

The formation of unusual DNA secondary structure has also been linked to genetic instability in mammals. For example, unstable trinucleotide repeats are prone to expansion (so-called "dynamic mutations" reviewed in Sutherland and Richards 1995) and are associated with a variety of human diseases; these repeats can form hairpin structures *in vitro* (Chen *et al.* 1995; Gacy *et al.* 1995; Mitas 1997). It has been speculated that formation of hairpins during DNA replication might play a pivotal role in the expansion of trinucleotide repeats (Wells 1996; Gordenin *et al.* 1997; Mitas 1997; Gordenin and Resnick 1998) and may lead to DSBs, on the basis of results from yeast (Freudenreich *et al.* 1998). Palindromic sequences, which are proposed to adopt hairpin or cruciform structures, also exhibit instability resulting in high rates of rearrangements in both meiotic and mitotic cells when present in transgenes in mice (Collick *et al.* 1996; Akgun *et al.* 1997). Many of the observed rearrangements in transgenic mice involved an asymmetric deletion that eliminated the central axis of symmetry of the palindrome, leading to a stabilized structure. Gene conversions near the site of a large palindrome in a mouse transgene were also reported to be elevated (Akgun *et al.* 1997), although such events have not been characterized in detail and the effect of a palindrome on crossing over has not been explored. Thus, although inverted repeats may act as destabilizing elements within a mammalian genome, there has been no thorough study of the effects of LIRs on recombination in mammals.

Because LIRs are common motifs in mammals, we initiated a systematic study of LIR effects on intrachromosomal homologous recombination between closely linked sequences in the genome of mouse fibroblasts. The quasipalindrome LIR used in this work was composed of two inverted copies of a 1.1-kb yeast *URA3* gene sequence separated by a 200-bp spacer sequence. The LIR was inserted into the coding region of a herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene, and this gene was introduced into the mouse genome alone or with a closely linked HSV-1 *tk* gene that was disrupted by an 8-bp *Xho*I linker insertion. DNA transactions were monitored by selecting for *tk*-positive segregants. The LIR motif did not appear to be intrinsically unstable, in that spontaneous deletions of the LIR in the absence of a recombination partner were not detectable. However, the LIR stimulated intrachromosomal homologous recombination between closely linked sequences by ~5-fold overall. Strikingly, the rate of gene conversions leading to the deletion of the LIR from the genome was increased by >20-fold. Thus, the LIR motif can stimulate intrachromosomal homologous recombination in mammalian cells, which suggests that LIRs could be important genetic elements with regard to the stability and evolution of genomes of higher eukaryotes.

MATERIALS AND METHODS

Cell culture and derivation of experimental cell lines: Mouse L cells deficient in thymidine kinase (L*tk*$^-$ cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.1 mm minimal essential medium nonessential amino acids (GIBCO-BRL, Gaithersburg, MD), and 50 μg gentamicin sulfate/ml. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2.

Plasmid DNA was cleaved with *Cla*I and introduced into mouse L*tk*$^-$ cells by electroporation (Lukacsovich *et al.* 1994) or by syringe-mediated mechanical loading (Waldman and Waldman 1998). Stable transformants were isolated after selection in G418 (200 μg active drug/ml) as described previously (Waldman and Liskay 1987). An estimate of the number of copies of plasmid integrated in the genome of a transfectant was made using Southern blots to evaluate hybridization signal intensities and the number of junctions between plasmid and chromosomal DNA. The copy number for a given transfectant was ultimately confirmed by Southern blotting analysis of recombinant segregants, because only a single copy of integrated plasmid is expected to be altered by recombination.

Plasmid descriptions: All the plasmids used are based on vector pJS-1, which is equivalent to pSV2neo (Southern and Berg 1982), with restriction site modifications as described previously (Liskay *et al.* 1984). Mutant 26 and mutant 153 HSV-1 *tk* genes (Liskay and Stachelek 1986) each contain an 8-bp *Xho*I linker insertion after nucleotide 737 or 1410, respectively [*tk* gene nucleotide numbering according to Wagner *et al.* (1981)].

Plasmids containing a *tk* gene disrupted by an LIR comprised of two inverted copies of the 1.1-kb yeast *URA3* gene sequence were constructed as follows. The 1.1-kb *Bgl*II fragment from pFL34 (Bonneaud *et al.* 1991) containing the yeast *URA3* gene was inverted, creating plasmid pFL34′. The *Bgl*II fragment from FL34 was cloned into the unique *Bam*HI site of pFL34′, 200 bp upstream and facing the opposite direction from the *URA3* sequence already on pFL34′ to create p4. Plasmid p4 contained an LIR of two 1.1-kb *URA3* sequences separated by a 200-bp spacer sequence. Plasmid p4 was cleaved with *Xba*I and *Hin*dIII, which cut within the multiple cloning site between the two inverted *URA3* sequences. The ends were filled in with Klenow fragment and ligated together to destroy the *Hin*dIII site and preserve the *Xba*I site, producing plasmid p183. The 2.4-kb *Nsi*I fragment from p183 containing the *URA3* LIR was cloned between the *Pst*I sites of pUC-4K (Vieira and Messing 1982) to produce p184. The 2.4-kb *Sal*I fragment from p184, which contained the *URA3* LIR, was cloned into the *Xho*I site of the mutant 153 HSV-1 *tk* gene. The LIR was thus immediately flanked by a 4-bp direct repeat of the sequence of the *Xho*I sticky ends. The LIR-disrupted *tk* gene was present on a 2.5-kb *Bam*HI fragment that was cloned into the *Bam*HI

site of vector pJS-1 to produce plasmid pLIR-m or into the *Bam*HI site of pJS-3 (Liskay *et al.* 1984) to produce pLIR-r. Plasmid pLIR-r was identical to pLIR-m, except that pLIR-r also contained the mutant 26 *tk* gene inserted on a 2.0-kb fragment at the *Hin*dIII site of the vector.

A plasmid containing a *tk* gene disrupted by a long direct repeat (LDR) of *URA3* sequences was constructed as follows. A 2.4-kb *Sal*I fragment (from construct C in Tran *et al.* 1997) containing two direct repeats of *URA3* separated by 200 bp was cloned into the *Xho*I site of the mutant 153 HSV-1 *tk* gene. This LDR-disrupted *tk* gene was cloned into the *Bam*HI site of pJS-3 to produce pLDR-r.

The plasmids are diagrammed in Figure 1.

Determination of the rate of appearance of tk^+ segregants: Rates of intrachromosomal homologous recombination and the rate of deletion of the LIR were determined by selecting for tk^+ segregants by hypoxanthine-aminopterin-thymidine (HAT) selection and by performing fluctuation analyses (Yang and Waldman 1997). For each fluctuation analysis, 10 independent subclones of a cell line were propagated to the appropriate number of cells and then plated separately into HAT medium. After a 2-wk incubation, colonies were counted and rates were determined in terms of the number of events per cell generation per integrated copy of recombination substrate by solving for a in the equation $r = aN_t\ln(N_tCa)$, where r is the average number of recombinants recovered per subclone, a is the rate of recombination, N_t is the average number of cells per subclone at the time of selection, and C is the number of subclones examined (Luria and Delbruck 1943). Rate calculations were facilitated by using the data of Capizzi and Jameson (1973).

DNA preparation and Southern hybridization analysis: Genomic DNA was prepared from cultured cells and analyzed by Southern hybridization with a ^{32}P-labeled probe specific for HSV-1 *tk* sequences as described previously (Letsou and Liskay 1987).

PCR amplification and sequencing of PCR-amplified DNA: To assay for the retention of the *URA3* LDR in recombinants, PCR reactions were carried out using primer 1 (5′-GGGAAGG GATGCTAAGGTAG-3′) and primer 2 (5′-CGGTGGGGTATC GACAGAGT-3′). Primer 1 corresponds to nucleotides 720–739 of the coding region of the *Saccharomyces cerevisiae URA3* gene; primer 2 corresponds to nucleotides 1786–1767 of the noncoding strand of the HSV-1 *tk* gene. To amplify *tk* sequences from recombinants for nucleotide sequence determinations, primer 3 (5′-TGCCGAGCCCCAGAGCAACG-3′) and primer 4 (5′-TTTGTCCAAACTCATCAATGTATC-3′) were used. Primer 3 corresponds to nucleotides 1328–1347 of the coding sequence of the HSV-1 *tk* gene, while primer 4 is the pJS1 vector sequence mapping from 38 through 15 bp downstream from the unique *Bam*HI site on the vector.

PCR was accomplished using Ready-To-Go PCR beads (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's specifications, using 0.5 μg genomic DNA per PCR reaction. A touchdown PCR protocol was used as follows. Samples were initially held at 95° for 5 min. This was followed by an amplification cycle consisting of 1 min denaturation at 95°, 1 min annealing at 72°, and 3 min elongation at 72°. This cycle was repeated once, followed by two similar cycles in which the annealing temperature was decreased to 70°. Cycling continued in this fashion, in which annealing temperature was decreased by 2° every other cycle, until annealing temperature was reduced to 58°. At this point, an additional 24 cycles were performed with no further decrease in annealing temperature.

Nucleotide sequences of PCR products were determined directly using Sequenase version 2.0 (Amersham Life Science, Cleveland, OH) following treatment of PCR products using a PCR product presequencing kit (Amersham Life Science) according to manufacturer's specifications and suggestions. Primer 3, described above, was used as a sequencing primer.

RESULTS

Establishing cell lines to study the effect of an LIR on genetic recombination: To assess the impact of an LIR on intrachromosomal homologous recombination in mammalian cells, we designed DNA constructs that contained an HSV-1 *tk* gene disrupted by a quasipalindromic LIR composed of two inverted copies of the 1.1-kb yeast *URA3* gene separated by a 200-bp spacer sequence (Figure 1A). Construct pLIR-m (monocopy of the *tk* gene; Figure 1B) contained the LIR-disrupted *tk* gene alone, whereas pLIR-r (repeated copy of the *tk* gene; Figure 1B) contained the LIR-disrupted *tk* gene and a second *tk* gene disrupted by an 8-bp *Xho*I linker insertion. The *tk* genes were separated by 4.4 kb. As a control, we also constructed pLDR-r (Figure 1B), which contained a *tk* gene disrupted by an LDR composed of direct repeats of two *URA3* genes separated by a 200-bp spacer sequence. (The intragenic distance between the *URA3* and *Xho*I inserts in pLIR-r and pLDR-r was 673 bp.) The various constructs were cleaved with *Cla*I and introduced into mouse Ltk^- cells. For each construct, several G418^R clones were isolated that contained one or a few stably integrated copies of the particular construct.

Spontaneous reversion of an LIR mutation in a mammalian genome is an infrequent event: Due to the manner in which our DNA constructs were made, the *URA3* LIR used in these studies was flanked by a 4-bp direct repeat. Because LIRs are known to be unstable in yeast and subject to deletion at a high rate via interaction between flanking small direct repeats, we first measured the rate of spontaneous deletion of the *URA3* LIR in three cell lines containing pLIR-m (Figure 1B). This construct contained no *tk* recombination partner, and restoration of *tk* function required loss of the LIR cassette in the *tk* gene. As shown in Table 1, the average rate of appearance of HAT-resistant (HAT^R) segregants was $<2.5 \times 10^{-8}$ events per cell per generation per locus. HAT^R segregants were recovered from only one of three cell lines tested (cell line 186-5). If small, partial deletions of the LIR had occurred, they would not be recovered, because only events leading to restoration of *tk* function could be detected. However, these results did indicate that spontaneous deletion of the entire *URA3* LIR from the genome is rare.

Genomic DNA was isolated from the three HAT^R segregants arising from cell line 186-5 and subjected to Southern blotting analysis (Figure 2). Each HAT^R clone displayed a 4.9-kb *Bam*HI fragment, as seen in the parental cell line (Figure 2, lanes 1, 2, 4, and 6), and each *Bam*HI fragment was cleavable with *Xba*I into 2.8- and 2.1-kb fragments (Figure 2, lanes 3, 5, and 7). On the

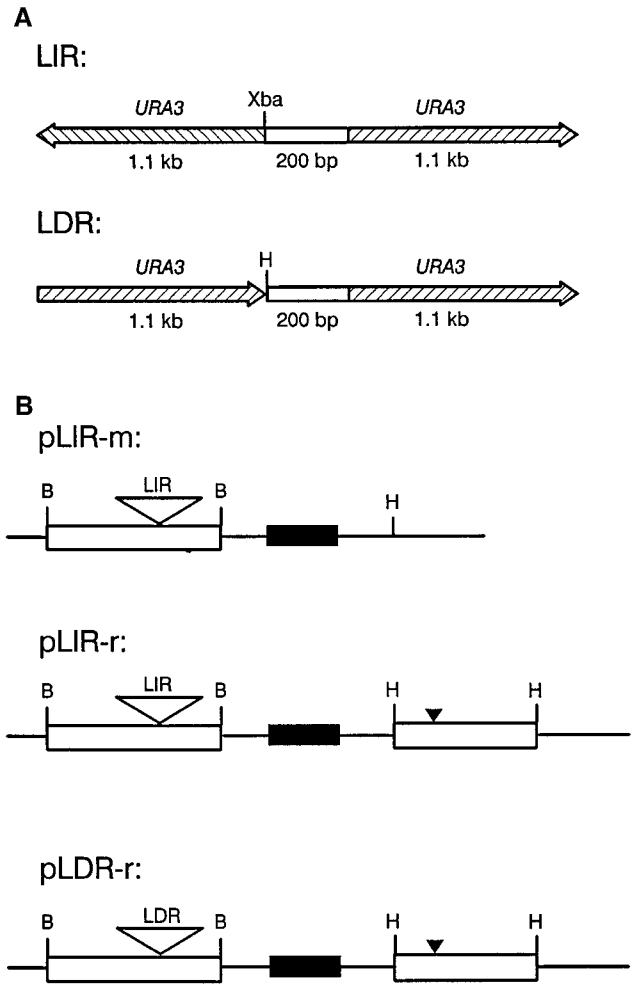

Figure 1.—LIR and LDR DNA substrates. (A) Schematic presentation of the LIR and LDR motifs composed of two 1.1-kb yeast *URA3* sequences. The LIR and LDR contain *Xba*I and *Hin*dIII (H) sites in the 200-bp spacer as indicated. (B) Plasmids pLIR-m, pLIR-r, and pLDR-r. *tk* sequences are indicated as open rectangles inserted between *Bam*HI (B) sites or *Hin*dIII (H) sites. The *Bam*HI insert is 2.5 kb in length, while the *Hin*dIII insert is 2.0 kb, and these two *tk* inserts are separated by 4.4 kb. The solid rectangle denotes the *neo* gene contained on the vector. The *tk* gene flanked by *Bam*HI sites in each construct is disrupted by the insertion of either the *URA3* LIR or LDR, as indicated, while the *tk* gene flanked by *Hin*dIII sites in pLIR-r and pLDR-r is disrupted by a *Xho*I linker insertion (solid triangle). The direction of transcription of *tk* genes is from left to right as drawn.

basis of the restriction map of the LIR-disrupted *tk* gene (see Figure 3C), the Southern blotting analysis indicated that, surprisingly, the three HAT^R segregants contained *tk* genes that retained most or all of the LIR insertion. These clones were not studied further (see discussion).

An LIR modestly stimulates homologous recombination in a mammalian chromosome: To determine the effect of an LIR on the rate of homologous recombination between closely linked genomic sequences, fluctuation tests were performed on three independent cell lines containing pLIR-r (Figure 1B). The rate of recombination for these three cell lines ranged from 5 to 15.7 $\times$ 10^{-6} events per locus per generation, with an average rate of 8.7 $\times$ 10^{-6} (Table 1). In comparison, the average rate of recombination for three independent cell lines containing the *URA3* direct-repeat construct pLDR-r (Figure 1B) was approximately fivefold lower (1.7 $\times$ 10^{-6} events per locus per generation, Table 1). The difference in the average rates for the pLIR-r *vs.* the pLDR-r cell lines is statistically significant ($P < 0.041$ by a *t*-test). We also note that the recombination rate recorded for each LIR-r cell line was greater than the rate for any LDR-r line. Because the sole difference between the two plasmids is the arrangement of the *URA3* sequences as an LIR *vs.* an LDR, we concluded that the LIR modestly stimulates recombination.

Analysis of LIR-induced recombination events: Illustrated in Figure 3 are the expected products of several types of simple homologous recombination events that reconstruct a functional *tk* gene. The different types of events, namely a reciprocal crossover (or single-strand annealing) between defective *tk* genes (Figure 3A), gene conversion leading to correction of the LIR (or LDR) mutation (Figure 3B), or gene conversion correcting the *Xho*I linker mutation (Figure 3, C or D) produce distinct restriction maps.

DNA samples were isolated from HAT^R segregants from cell lines containing pLIR-r and subjected to Southern blotting analysis (Figure 4) to characterize the nature of the rearrangements that had occurred. In total, 20 independent clones from line 187-11, 24 independent clones from line 187-17, and 12 independent clones from 187-T3 were examined. Among the clones studied, 19 (34%) of the clones generated a pattern similar to that shown in Figure 4, lanes 1 and 2. These clones displayed 4.9- and 2.0-kb fragments upon digestion with *Bam*HI plus *Hin*dIII, and neither fragment was cleavable with *Xho*I. We inferred that these clones arose from gene conversions correcting the *Xho*I linker mutation (see Figure 3C). A total of 29 (56%) of the analyzed clones displayed the pattern shown in Figure 4, lanes 3–5. This group of clones displayed a 2.5-kb fragment upon digestion with *Bam*HI plus *Hin*dIII (lane 4), and this fragment was resistant to *Xho*I (lane 5). We inferred that this group of clones arose from crossovers (see Figure 3A). Eight (14%) of the clones generated the pattern shown in Figure 4, lanes 6–8. These clones displayed a 2.5-kb fragment upon digestion with *Bam*HI (lane 6) and displayed a 2.0-kb *Hin*dIII fragment (lane 7) that was cleavable with *Xho*I into 1.5- and 0.5-kb fragments (lane 8). This pattern was consistent with a gene conversion eliminating the LIR (Figure 3B). These results are summarized in Table 2. The *tk* genes were PCR amplified from four of these latter recombinants, and sequence analysis of the region surrounding the original LIR site revealed that the LIR had been precisely removed and the wild-type *tk* sequence had been restored in each case (data not shown). Because in experiments

TABLE 1

Rate of appearance of HAT-resistant segregants

DNA substrate[a]	Cell line	Copy number[b]	No. of cells tested ($\times 10^{-6}$)	No. of HAT^R colonies	Rate ($\times 10^6$)[c]
pLIR-m	186-5	1	65.8	3	0.043
	186-7	1	77.2	0	<0.013
	186-9	1	54.9	0	<0.018
	Mean				<0.025
pLIR-r	187-11	1	50.1	1386	5.0
	187-17	1	60.0	1817	5.3
	187-T3	1	60.0	6475	15.7
	Mean				8.7
pLDR-r	225-T1	1	54.6	735	2.7
			39.0	396	2.3
	225-T5	1	56.6	301	1.2
			62.0	384	1.4
	225-7	2	60.0	839	1.1
	Mean				1.7

[a] Illustrated in Figure 1.
[b] The number of copies of DNA substrate stably integrated in the particular cell line and determined by Southern blotting (not shown).
[c] Each line presents data from an independent fluctuation test. Rate is in terms of number of HAT^R clones produced per cell per generation per locus (normalized for copy number). The difference in the mean rates for pLIR-r *vs.* pLDR-r cell lines is statistically significant ($P < 0.041$ by a *t*-test).

involving cell lines containing pLIR-m we failed to recover any HAT^R clones in which the LIR had been deleted from the genome (see above), it was clear that the deletion of the LIR from the genomes of 14% of the HAT^R clones recovered from pLIR-r cell lines resulted from gene conversion (*i.e.*, deletion of the LIR was dependent on interaction between homologous *tk* sequences).

We also noted some exceptional recombinants that arose from cell line 187-11. Two recombinants in which the *Xho*I linker appeared to have been converted (*e.g.*, Figure 4, lanes 15 and 16) and five apparent crossover recombinants (*e.g.*, Figure 4, lanes 9–11 and 12–14) had undergone additional rearrangements as evidenced by novel hybridizing bands. Although we have not determined the precise nature of these rearrangements, each of these exceptional cases appeared to involve a partial duplication of the recombination substrate.

An LIR significantly stimulates gene conversions leading to elimination of the LIR: To further explore the influence of an LIR on recombination in mammalian chromosomes, we compared the nature of LIR-induced events with recombination events associated with an LDR. DNA samples isolated from 12 independent HAT^R segregants from each of cell lines 225-T1 and 225-T5 were subjected to Southern blotting analysis (Figure 5). Among all HAT^R segregants analyzed, 10 displayed the pattern shown in Figure 5, lanes 1–4, for isolates from 225-T1. These clones displayed a single 2.5-kb fragment upon triple digestion with *Bam*HI, *Hin*dIII, and *Xho*I (see lanes 2 and 4), which is consistent with crossovers (Figure 3A). Fourteen clones displayed the pattern seen in Figure 5, lanes 5–8. For these clones, *Bam*HI digestion generated a 4.9-kb fragment (lanes 5 and 7), while a *Bam*HI, *Hin*dIII, and *Xho*I triple digest produced a 2.8-kb fragment and two fragments comigrating at ~2.0 and 2.1 kb (lanes 6 and 8). This pattern is consistent with conversion of the *Xho*I linker insertion mutation (Figure 3D).

Surprisingly, none of the 24 clones derived from LDR lines that were initially examined by Southern blotting appeared to have arisen from conversion of the LDR mutation. An additional 24 independent HAT^R segregants from each of two LDR lines were therefore screened to more accurately determine the percentage of recombinants that may have arisen from gene conversion eliminating the LDR. Each of the 48 additional HAT^R segregants was initially screened for resistance to G418. In all, 9 clones from cell line 225-T5 and 6 clones from 225-T1 were sensitive to G418. We deduced that these 15 G418-sensitive HAT^R clones had arisen from a crossover, because a crossover event would result in the loss of the *neo* gene (Figure 3A). Southern blotting analysis (not shown) of representative G418-sensitive, HAT^R segregants confirmed this conclusion. The remaining 33 HAT^R clones were subjected to PCR analysis in which one primer mapped within the URA3 sequence of the LDR while the other mapped in *tk* sequence downstream from the LDR (see Figure 6). For any clone that retained the LDR, we expected a 465-bp PCR product to be generated. Any clone that lost the LDR should have produced no PCR product. Analysis of 18 samples

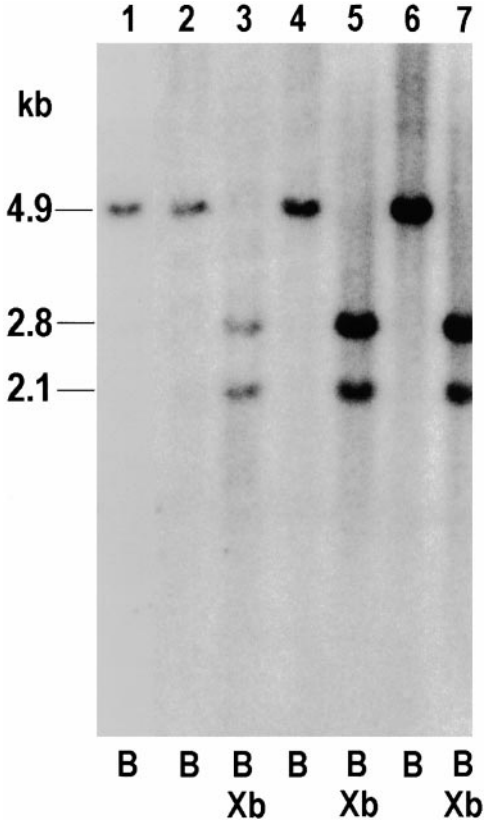

Figure 2.—Southern blotting analysis of HAT^R segregants arising from a cell line containing pLIR-m. Samples of genomic DNA (8 μg) isolated from cell line 186-5 (lane 1) or three HAT^R segregants derived from cell line 186-5 (lanes 2–7) were digested with *Bam*HI (B) or *Bam*HI plus *Xba*I (Xb), as indicated below the lanes, and analyzed using a probe specific for *tk* sequences. Each HAT^R segregant is displayed in a pair of adjacent lanes. Each HAT^R segregant retained the parental 4.9-kb *Bam*HI fragment, which is cleavable with *Xba*I, indicating retention of the LIR.

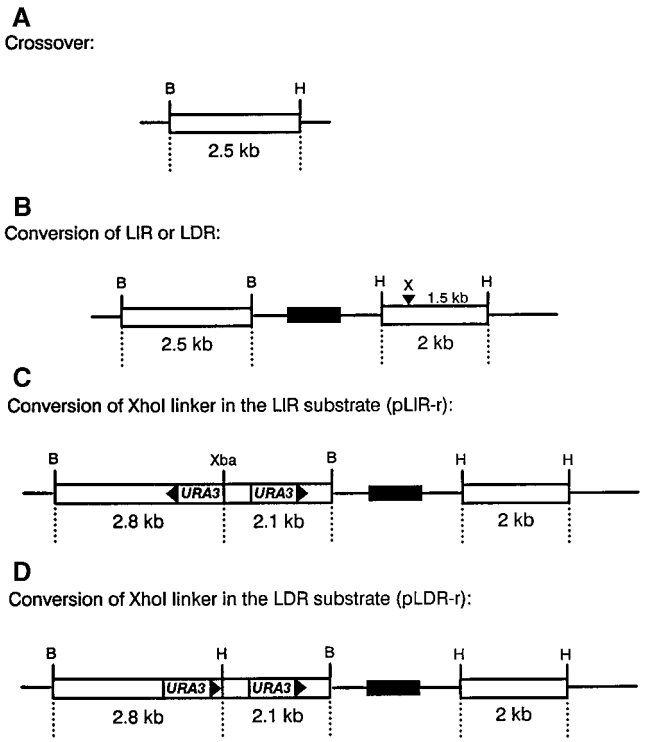

Figure 3.—Products of homologous recombination events. Shown are schematic maps and predicted restriction fragment lengths for several products of recombination anticipated to be recovered from cell lines containing pLIR-r or pLDR-r. Symbols are as used in Figure 1. See text for further discussion.

is shown in Figure 6. Southern blotting analysis of representative samples corroborated the PCR analysis (data not shown). In total, only two HAT^R clones, one from cell line 225-T5 and one from cell line 225-T1, failed to produce a PCR product. Sequence analysis revealed that the LDR had been precisely removed and that the wild-type *tk* sequence had been restored in both cases (data not shown). Thus, only 2.9% (2/72) of recombinants from the LDR cell lines arose from gene conversion of the LDR mutation (Table 2), which was markedly lower than the 14% conversion frequency among the LIR recombinants. Relative to the LDR control, the LIR brought about a >20-fold increase in the rate of gene conversion at the site of the LIR (Table 2). The relative percentages (and rates) for the different types of recombination events presented in Table 2 are significantly different ($P < 0.003$ by a *G*-test for heterogeneity; Sokal and Rohlf 1981) for the set of HAT^R clones recovered from LIR cell lines *vs.* the set of clones recovered from LDR cell lines.

DISCUSSION

DNA sequence motifs that are prone to genetic change have been referred to as at-risk motifs (ARMs; Gordenin and Resnick 1998). We have demonstrated that an LIR is an at-risk motif for homologous recombination in a mammalian genome. Although this work was restricted to studies of a repeat of 1.1-kb yeast *URA3* sequences separated by a 200-bp spacer, our results provide strong evidence that DNA structural motifs can stimulate homologous recombination within mammalian chromosomes. Cell lines containing integrated copies of pLIR-m produced HAT^R colonies at an average rate of $<0.025 \times 10^{-6}$ events per cell per generation, compared to an average rate of 8.7×10^{-6} for cell lines containing pLIR-r (Table 1). Both constructs contain an LIR-disrupted *tk* gene, but pLIR-r also contains an *Xho*I linker insertion mutant *tk* gene (Figure 1B). Because previous studies (Waldman and Liskay 1987) had shown that the rate of reversion of an *Xho*I linker insertion mutant *tk* gene is $<10^{-9}$, it was clear that the 350-fold greater rate of recovery of HAT^R segregants was due to opportunities for recombination between the pair of defective *tk* genes contained in pLIR-r. This inference was confirmed by Southern blotting analysis (Figure 4). The pLIR-r and pLDR-r plasmids are identical, except that the repeated *URA3* sequences in pLDR-r

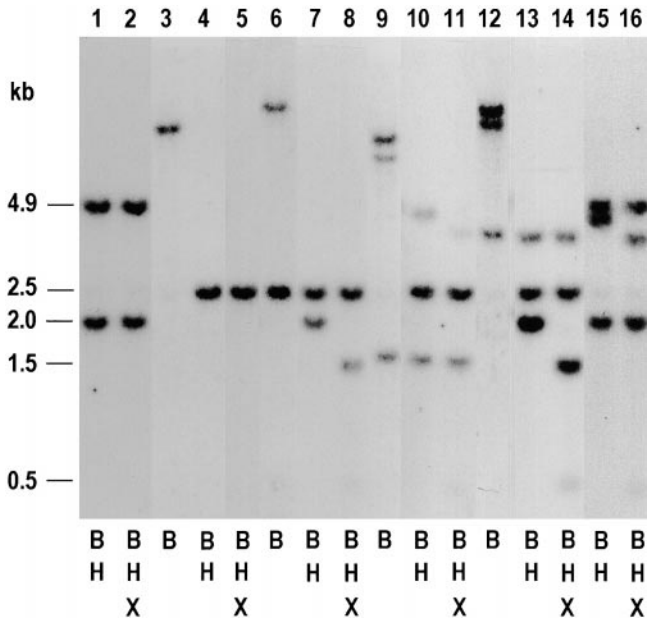

Figure 4.—Southern blotting analysis of representative HAT^R segregants arising from a cell line containing pLIR-r. Samples of genomic DNA (8 μg) isolated from HAT^R segregants derived from cell line 187-11 were digested with *Bam*HI (B) alone, *Bam*HI plus *Hin*dIII (H), or *Bam*HI plus *Hin*dIII plus *Xho*I (X), as indicated below the lanes, and displayed on a Southern blot using a *tk*-specific probe. In total, 56 HAT^R segregants from three different cell lines (187-11, 187-17, and 187-T3) containing pLIR-r were analyzed. See text for details.

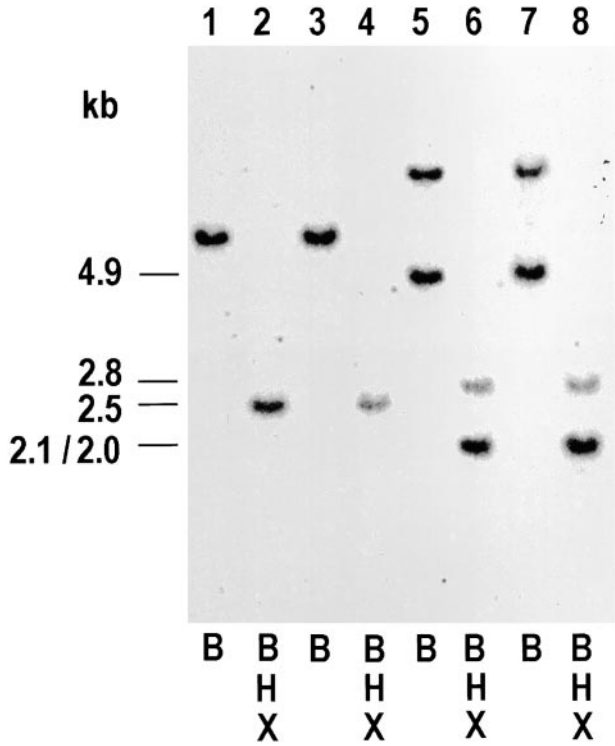

Figure 5.—Southern blotting analysis of representative HAT^R segregants from a cell line containing pLDR-r. Samples of genomic DNA (8 μg) isolated from HAT^R segregants from cell line 225-T1 were digested with *Bam*HI (B) alone or with *Bam*HI plus *Hin*dIII (H) plus *Xho*I (X), as indicated below the lanes, and displayed on a Southern blot using a *tk*-specific probe. In total, 24 HAT^R segregants from two cell lines (225-T1 and 225-T5) were analyzed. See text for details.

are arranged as a direct repeat. The differences in recombination rates and spectra of recombinants for these two constructs demonstrate that sequence motif can indeed influence intrachromosomal homologous recombination and that an LIR is a recombinagenic hotspot in mammalian cells.

The overall rate of recombination of 1.7×10^{-6} measured for the *URA3* LDR-containing cell lines (Table 1) is in strong agreement with the previously reported (Godwin and Liskay 1994) value of 1.1×10^{-6} for the rate of intrachromosomal homologous recombination

TABLE 2

Spectra of recombination events

DNA substrate	Cell line	No. of crossovers	No. of LIR or LDR conversions	No. of *Xho*I linker conversions
pLIR-r	187-11[a]	10	1	9
	187-17	15	3	6
	187-T3	4	4	4
	Total (%)	29 (52)	8 (14)	19 (34)
	Rate[b] ($\times 10^6$)	4.5	1.2	3.0
pLDR-r	225-T1	10	1	25
	225-T5	15	1	20
	Total (%)	25 (35)	2 (3)	45 (62)
	Rate ($\times 10^6$)	0.66	0.05	0.75

[a] Five of the crossover events and two of the *Xho*I conversions were associated with additional genetic rearrangements (see text).

[b] The rate (events per cell per generation per locus) for each specific type of recombination event was calculated by first determining the fraction of the total number of analyzed events represented by each type of event and then multiplying this fraction by the average overall recombination rate for the appropriate cell lines.

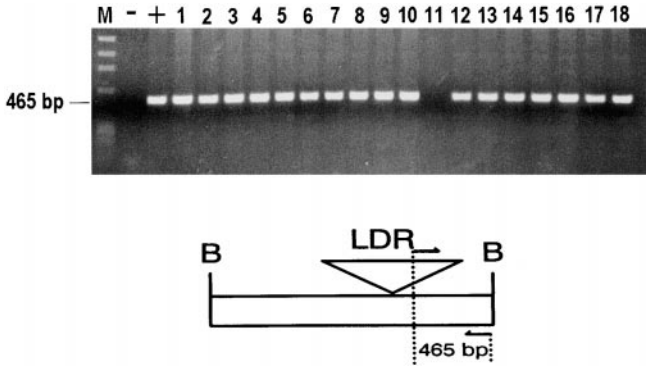

Figure 6.—PCR analysis to assay for the retention of the *URA3* LDR in HAT^R segregants derived from a cell line containing pLDR-r. Shown in lanes 1–18 are the products of PCR reactions performed on DNA samples isolated from G418^R, HAT^R segregants derived from cell line 225-T1 using one primer mapping within the *URA3* LDR, and one primer mapping within *tk* sequence downstream from the LDR. As illustrated below the gel, HAT^R segregants retaining the LDR are expected to yield a 465-bp PCR product, while clones lacking the LDR should produce no product. Among the samples displayed, only one (lane 11) failed to produce a product. DNA isolated from parental cell line 225-T1 served as a positive control (+), and a PCR reaction with no added template served as a negative control (−). Molecular weight markers are displayed in lane M. Fifteen additional HAT^R segregants from cell line 225-T1 were similarly analyzed, with only one additional clone failing to yield a product.

in mouse L*tk*$^-$ cells between an *Xho*I linker insertion mutant *tk* gene and a closely linked *tk* gene disrupted by a 1.5-kb nonrepetitive insertion (unrelated to *URA3*). The percentages of crossovers (35%, Table 2) *vs.* gene conversions (65%) recovered in the presence of the *URA3* LDR are also very similar to the percentages (30% crossovers, 70% gene conversions) recovered in the presence of the 1.5-kb nonrepetitive insertion mutation (Godwin and Liskay 1994). Moreover, the rates of gene conversion leading to correction of the large insert are comparable for the two studies (0.05×10^{-6} in the current study *vs.* 0.09×10^{-6}). The collective data provide strong evidence that neither the *URA3* nucleotide sequence itself nor the LDR structural motif exerted any specific influence on intrachromosomal homologous recombination. Thus, we infer that the *LIR* motif stimulates recombination.

Analysis of the spectra of recombination events revealed that the LIR had a unique effect. The rate of each type of recombination event depicted in Figure 3 was increased in the presence of the LIR, yielding an overall 5-fold rate increase relative to LDR-containing lines (Table 1). However, the rate for gene conversion leading to correction of the LIR mutation was particularly stimulated with such events occurring at a >20-fold greater rate than conversion of the LDR (Table 2). Sequence analysis of representative samples indicated that the LIR was precisely removed during gene conversion, confirming that the LIR motif strongly stimulated accurate gene conversions leading to removal of the LIR from the genome. This finding was particularly striking because previous reports (Letsou and Liskay 1987) had established that the rate of correction of a mutation via intrachromosomal gene conversion is greatly reduced with increasing size of the mutation, presumably reflecting a reduced likelihood of formation of heteroduplex DNA (hDNA) spanning a large heterology (Letsou and Liskay 1987). Indeed, an increase in the rate of crossing over relative to gene conversion has been observed in mouse cells and other eukaryotic organisms as the size of a heterologous insert is increased (Hilliker *et al.* 1988; Langin *et al.* 1988; Vincent and Petes 1989; Godwin and Liskay 1994). We propose that LIR-stimulated gene conversion resulting in its own elimination is due to provocation of homologous recombination by an event in which hDNA formation does not involve the LIR.

At least two models have been proposed that may explain the recombinagenic effect of LIRs. In one of the models, LIR-associated deletions and recombination in yeast arise through replication blockage (Gordenin *et al.* 1993; Tran *et al.* 1997; Lobachev *et al.* 1998). In this model, a stem-loop secondary structure forms during replication of an LIR if the LIR is located within a domain of single-stranded DNA. This would be particularly likely to occur on the lagging strand. Such duplex secondary structures in the DNA template can block progression of DNA polymerase, stalling strand elongation (Weaver and Depamphilis 1984; Canceill and Ehrlich 1996). After polymerase stalling, replication slippage between short direct repeats at the base of the secondary structure would lead to deletion of the LIR. Alternatively, stalled replication may generate a potentially recombinagenic 3′ end of the nascent strand, which may initiate recombination by invading a homologous sequence. Other possibilities are that blockage of replication leads to formation of DSBs (Michel *et al.* 1997) or extended regions of single-stranded DNA, either of which may provoke recombination.

A somewhat different model was proposed to explain the high rate of occurrence of one-sided deletion of large palindromes in transgenes in mice (Akgun *et al.* 1997). This particular model invokes an endonucleolytic hairpin-nicking activity acting at the tip of the hairpin, which could lead to DSBs if both strands of a palindrome (or quasipalindrome) were nicked or if a singly nicked sequence were replicated. Palindromes have been shown to be sites of cleavage by the sbcCD gene products in *E. coli.* Interestingly, palindromes are extremely recombinagenic in yeast (Lobachev *et al.* 1998). We recently demonstrated that quasipalindrome-stimulated recombination in yeast requires gene products known to be involved in recombination, some of which are homologous to sbcCD (K. S. Lobachev, D. A. Gordenin and M. A. Resnick, unpublished results).

A feature shared by the replication slippage and hair-

pin nicking models is the potential for DSB formation at the site of an LIR. Our data fit nicely with the hypothesis of the formation of a DSB at the site of the LIR. DSBs are known to be recombinagenic lesions (reviewed in Osman and Subramani 1998) and, as described by the DSB repair model for homologous recombination (Resnick 1976; Szostak *et al.* 1983), often lead to gene conversions in which the broken sequence serves as a recipient of genetic information. This scenario concurs with our observation of the particularly strong enhancement of gene conversion leading to the accurate removal of the LIR from the mouse genome. Also of relevance to this discussion is the reported ability of a 140-bp palindromic insertion mutation to stimulate gene conversion during meiosis in the yeast *S. cerevisiae* due to the generation of a DSB at the site of the palindrome (Nag and Kurst 1997). In this latter report, a model was presented in which the palindrome is extruded into a cruciform that is then cleaved by a structure-specific endonuclease, generating a recombinagenic DSB.

Although we consider our data to be consistent with DSB formation at the LIR, we have no way at this time of determining whether a DSB is induced by replication blockage, endonucleolytic cleavage, or possibly by some other pathway. Our observations (Y. Lin and A. S. Waldman, unpublished results), as well as work by others (Taghian and Nickoloff 1997), indicate that artificial induction of a DSB in a mammalian chromosome by the endonuclease I-*Sce*I preferentially induces intrachromosomal gene conversions in which the broken sequence receives information, lending credence to our hypothesis that LIR-stimulated recombination may be mediated by a DSB. It is interesting that DSB-induced recombination is sometimes associated with amplification of the broken locus (Y. Lin and A. S. Waldman, unpublished results), which is reminiscent of the sequence duplications we observed in some of the LIR-induced recombinants (*e.g.*, Figure 4, lanes 9–16).

While gene conversions eliminating the LIR are the most strongly stimulated recombination events in the LIR cell lines, we also see a stimulation of crossovers and, perhaps more surprisingly, gene conversions removing the *Xho*I linker insertion mutation (Table 2). This suggests that in addition to the proposed induction of the DSB repair pathway for homologous recombination (see above), a more general provocation of recombination occurs in the vicinity of the LIR. Such provocation may be brought about by recruitment of recombination proteins to the LIR locus, possibly caused by affinity of proteins for the LIR structural motif itself or for the putative strand discontinuities that may be generated.

Our findings regarding the effect of an LIR on intrachromosomal homologous recombination in mammalian cells are qualitatively similar to the findings made in yeast. In *S. cerevisiae*, LIRs stimulate both intra- and interchromosomal recombination, and gene conversions eliminating the LIR are preferentially induced (Gordenin *et al.* 1993). The degree to which an LIR may stimulate recombination in yeast varies from severalfold to >17,000-fold (Lobachev *et al.* 1998). The particular LIR we used in the current work was demonstrated to enhance recombination between homologous sequences flanking the LIR by ~9-fold in yeast (Tran *et al.* 1997). In general, LIR stimulation of homologous recombination in yeast is related to the size of the repeat sequences and is inversely related to the size of intervening sequences (Lobachev *et al.* 1998). As noted above, a perfect 1.0-kb palindrome produced the highest degree of stimulation. Defects in DNA polymerases also tend to enhance the effect of an LIR in yeast (Gordenin *et al.* 1993; Ruskin and Fink 1993; Tran *et al.* 1997; Lobachev *et al.* 1998). How and to what degree factors such as repeat length, spacer length, and replication status influence the recombinagenic effects of LIRs in mammalian chromosomes are important issues that await further study. An LIR alone or in conjunction with altered replication has also been shown to strongly stimulate recombination between highly diverged DNA sequences in yeast (Tran *et al.* 1997). Whether LIR motifs can overcome the barrier to homeologous recombination in higher eukaryotes remains to be determined.

It has been established that LIRs as well as long palindromes are inherently quite unstable in bacteria (Collins 1981; Leach 1994) and yeast (Gordenin *et al.* 1993; Henderson and Petes 1993; Ruskin and Fink 1993; Lobachev *et al.* 1998), where such DNA elements are often efficiently deleted from the genome. In our system, the *URA3* LIR was not deleted from the genome at a detectable rate (see data for cell lines containing pLIR-m; Table 1). One plausible explanation for this is that, due to the manner in which our substrates were constructed, the LIR in our constructs was flanked by direct repeats only 4 bp in length. In yeast, LIR deletion via replication slippage requires flanking direct repeats that are >4 bp in length. Although the rate was low, we were able to recover three HAT^R clones from cell lines containing pLIR-m that apparently retained most or all of the LIR (Figure 2). We noticed that the intensities of the bands for at least two of these recovered clones (Figure 2, lanes 4–7) were increased relative to the intensity for the parental line (Figure 2, lane 1). An intriguing and potentially testable hypothesis is that the recovered clones survived HAT^R selection due to transcriptional slippage, *i.e.*, exclusion of the LIR from mRNA due to slippage of RNA polymerase past the LIR during transcription. Transcriptional slippage has been demonstrated over small distances in mammalian cells (Linton *et al.* 1997). If transcriptional slippage occurs only very rarely, perhaps selection for HAT^R reveals clones that have amplified the LIR-disrupted *tk* gene, resulting in an expression level of *tk* sufficient for survival.

The possibility of LIR-induced transcriptional slippage would not appear to be conceptually very different from LIR-induced replication slippage and may represent an additional mechanism for altered gene expression.

Genomes of higher eukaryotes, including humans, contain an abundance of repetitive elements, such as LINES and Alu sequences. The most abundant of these genomic elements are present, on average, once every few kilobases, and many such repetitive sequences with high levels of homology, particularly Alus, are indeed positioned in the genome in configurations equivalent to LIRs (Jurka 1998; J. Stenger, J. Jurka, D. A. Gordenin, K. S. Lobachev and M. A. Resnick, unpublished results). Understanding genetic transactions involving LIRs is thus of biological relevance. Our studies suggest that LIRs may play important roles in both the shaping of eukaryotic genomes through evolution as well as the genesis of genomic instability in genetic disorders, such as cancer, by acting as hotspots for recombination. It is noteworthy that a DNA motif such as an LIR is at risk for genetic change in higher and lower eukaryotes as well as prokaryotes, and this is likely a reflection of evolutionary conservation of at least certain mechanistic aspects of DNA transactions. Further study of at-risk motifs may help us to better understand these vital transactions.

The authors thank Dmitry Gordenin for helpful discussions and critical review. The authors also thank Tim Mousseau for help with statistical analysis. This work was supported by Public Health Service grant GM47110 from the National Institute of General Medical Sciences and Research Project grant NP-949 from the American Cancer Society to A.S.W., and by Interagency Agreement DE-A105-94ER61940 from the Department of Energy.

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Communicating editor: M. Lichten