Integration of a Vector Containing a Repetitive LINE-1 Element in the Human Genome

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Mammalian cells contain numerous nonallelic repeated sequences, such as multigopy genes, gene families, and repeated elements. One common feature of nonallelic repeated sequences is that they are homeologous (not perfectly identical). Our laboratory has been studying recombination between homeologous sequences by using LINE-1 (L1) elements as substrates. We showed previously that an exogenous L1 element could readily acquire endogenous L1 sequences by nonreciprocal homologous recombination. In the study presented here, we have investigated the propensity of exogenous L1 elements to be involved in a reciprocal process, namely, crossing-overs. This would result in the integration of the exogenous L1 element into an endogenous L1 element. Of over 400 distinct integration events analyzed, only 2% involved homologous recombination between exogenous and endogenous L1 elements. These homologous recombination events were imprecise, with the integrity of the vector being flanked by one homologous and one illegitimate junction. This type of structure is not consistent with classical crossing-overs that would result in two homeologous junctions but rather is consistent with one-sided homologous recombination followed by illegitimate integration. Contrary to what has been found for reciprocal homologous integration, the degree of homology between the exogenous and endogenous L1 elements did not seem to play an important role in the choice of recombination partners. These results suggest that although exogenous and endogenous L1 elements are capable of homologous recombination, this seldom leads to crossing-overs. This observation could have implications for the stability of mammalian genomes.

MATERIALS AND METHODS

Cell lines and vectors. The cell line used in all experiments was GM00637E, a normal line of human fibroblasts transformed with simian virus 40, obtained from NIGMS Human
added. After 2 weeks, individual colonies were picked or mixed in pools of 10 to 20 colonies.

**Plasmid rescue.** Genomic DNA digested with *BglII* was ligated and electroporated into *Escherichia coli* DH10B (4). Selection was performed with ampicillin (50 μg/ml).

**DNA sequencing.** DNA sequencing was performed by the dideoxy method (26), using the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Sequences were analyzed with the DNasis program (Pharmacia LKB Biotechnology AB).

**Comparison with human L1 elements.** L1 elements used for sequence comparison with the vector (see Fig. 6C) were identified by Jurka (19) and obtained from GenBank. They are HUMHBBB (two elements, bases 27115 to 26126 and 35885 to 36874), HUMFIXG (712 to 1711), HUMIGHAD (1883 to 2499), HUMMHDBR1 (418 to 1382), HUMRSKP04 (1 to 1023), HUMIFNAG (4389 to 5216), HUMRSKPT (45 to 504), HUMRSKP1 (63 to 259), HUMRSKPE (1 to 969), HUMRSSA1A (1689 to 731), HUMHBEG (3864 to 4871), HUMRSKP08 (906 to 1597), HUMRSKPNA (1547 to 2228), HUMRSKP03 (1 to 510), HUMPDP16 (1 to 101), and HUMRSKP07 (5 to 353) (L1 fragments on the complementary strand are listed in the 3'-5' order).

## RESULTS

**Experimental strategy.** To investigate the propensity of cellular L1 elements to undergo crossing-overs, we studied the integration of exogenous L1 sequences into the human genome. Two types of events were expected: homologous integrations resulting from crossing-overs between the exogenous and endogenous L1 elements and illegitimate integrations.

The exogenous human L1 sequences were taken from cD11 (28) as a 2.5-kb fragment (X-Y in Fig. 1A) and cloned in a vector containing a selectable *neo* gene. Two plasmids, pL1HSIV1.2 and pL1HS1.2del, that differed one from the other by a 23-bp deletion at the *BamHI* (B) linearization site, *neo* and *tk* refer to the *E. coli* *neo* gene and the herpes simplex virus type 1 *tk* gene, respectively. Boxes X and Y identify the L1 sequences on each side of the linearization site. The thick line represents plasmid sequences. The open box with a question mark represents the integration site. (C) Structures of integrated vectors. Probe P is illustrated by a thick line. Numbers represent lengths in kilobases. Bg, *BglII*; K, *KpnI*.

Two closely related plasmids, pL1HSIV1.2 and pL1HS1.2 del, were constructed. First, the *Neo1-Eag1* fragment of pMC1 *neo* (32) was replaced by the same fragment from pSV2neo (29) to augment the G418<sup>+</sup> activity (35). Then the 2.5-kb *EcoRI* fragment from the human L1 cDNA cD11B (28) was cloned into the *BamHI* site of the modified pMC1neo vector. The herpes simplex virus type 1 *tk* gene, obtained as a 2.0-kb restriction fragment from pAGO (10), was added at the *HincII* site. The resulting vector was called pL1HSIV1.2 (Fig. 1). This vector was further modified by deleting a 23-bp *Nhel-BamHI* fragment in the middle of the L1 sequences, resulting in pL1HS1.2del.

**Transfection.** Prior to transfection, pL1HSIV1.2 and pL1HS1.2del were linearized with *BamHI*. Then 1 μg of vector DNA was electroporated in 5 × 10<sup>6</sup> GM00637E cells with a gene Zapper 450/2500 apparatus (IBL, New Haven, Conn.) as described by Chu et al. (9) except that incubations were at 4°C. At 72 h after electroporation, 200 μg of G418 per ml was

![Fig. 1. (A) L1 element. Stippled boxes are the two open reading frames. Boxes X and Y represent the L1 sequences that were present in vectors pL1HSIV1.2 and pL1HS1.2del. Thick lines represent probes 3′ and L1. (B) Insertion vectors pL1HSIV1.2 and pL1HS1.2del, which differ one from the other by a 23-bp deletion at the *BamHI* (B) linearization site. *neo* and *tk* refer to the *E. coli* *neo* gene and the herpes simplex virus type 1 *tk* gene, respectively. Boxes X and Y identify the L1 sequences on each side of the linearization site. The thick line represents plasmid sequences. The open box with a question mark represents the integration site. (C) Structures of integrated vectors. Probe P is illustrated by a thick line. Numbers represent lengths in kilobases. Bg, *BglII*; K, *KpnI*.](http://mcb.asm.org/)

Mutant Cell Repository, Corfell Institute for Medical Research, Camden, N.J.

To determine the number of integration events per clone, 38 independent G418<sup>+</sup> clones were analyzed by *BglII* digestion, which cuts once in the vector (Fig. 1C), followed by Southern analysis using the *neo* gene as a probe. Over 90% of the clones analyzed contained only one integrated vector, while the remainder contained two or three (Fig. 2). Homologous recombination between L1 elements does not lead to a selectable phenotype. Thus, to determine the nature of the integration events, homologous or illegitimate, we cloned the junctions. Genomic *BglII* fragments containing plasmid DNA of the integrated vector plus the flanking cellular sequences present on the right side (Fig. 1C) were rescued by ligation followed by transformation in bacteria. Analysis of the integration events rescued from individual clones indicated that the length of each fragment rescued in bacteria corresponded to the length expected from Southern analysis of the genomic DNA. Thus, the plasmid rescue procedure did not produce rearrangements.
Characterization of the integration events. Rescued integration events were analyzed by restriction enzyme mapping, hybridization, and sequencing. The restriction enzymes used were BglII and KpnI, while hybridization was done with probes L1, 3', and P (Fig. 1). The scheme for analysis of the rescued junctions is illustrated in Fig. 3. Four types of junctions were expected. First, homologous junctions would have (i) the 4.1-kb BglII-KpnI plasmidic fragment that would hybridize with the P probe, (ii) the 1.2-KpnI-KpnI L1 fragment (X) that would hybridize with the L1 probe, and (iii) KpnI-BglII cellular sequences that would hybridize with the L1 and 3' probes because of the presence of endogenous L1 sequences (Fig. 3A). The second type of junction expected consisted of the illegitimate ones, whereby the distinction with the homologous junctions would be made on the basis that the cellular sequences would not hybridize with the L1 and 3' probes (Fig. 3B). However, there could also be illegitimate junctions whereby the cellular sequences would hybridize with the L1 and 3' probes because integration would have occurred near an endogenous L1 element (Fig. 3C). Discrimination between these and the homologous junctions would rely on the mapping of the position of the endogenous L1 element in relation to the vector sequences as well as on sequencing of the vector-cell junction to determine the presence of a homologous junction point. Finally, the fourth type of junctions would represent vector-vector junctions resulting from vector-vector recombination (Fig. 3D). These would be discriminated from bona fide integration junctions by the fact that the sequences that should have corresponded to cellular sequences would hybridize with the P probe.

Examples of the different types of rescued integration events obtained are shown in Fig. 4A. Lanes 2 to 5 correspond to rescued integration events, while lane 1 contains pL1HS1.2del. The 4.1-kb BglII-KpnI fragment is present in all lanes, while the 1.2-kb KpnI fragment is present in lanes 2 to 4 but absent in lane 5. In the latter case, sequencing indicated that this absence was due to loss of sequences at the end of the vector inclusive of the KpnI site. Thus, BglII-KpnI digestion did not produce the usual KpnI 1.2-kb fragment but instead produced a fusion fragment containing both the remaining vector X sequences and the flanking cellular sequences. The additional bands of various lengths seen in lanes 2 to 4 correspond to cellular DNA fragments. They sometimes number more than
TABLE 1. Type and relative incidence of integration events

| Exp | No. of distinct rescued integration events obtained | Illegitimate | Homologous |
|-----|--------------------------------------------------|--------------|------------|
|     | Without L1 | With L1 |                  |            |
| 1   | 27        | 2       | 0               |
| 2   | 71        | 1       | 2               |
| 3   | 87        | 2       | 2               |
| 4   | 40        | 1       | 2               |
| 5   | 174       | 2       | 1               |
| Total | 399 | 8 | 7 |

one per rescued integration event because of the presence of cellular KpnI sites (lanes 2 and 3).

Hybridization of the rescued integration events with probes P, L1, and 3' is shown in Fig. 4B to D. With probe P (Fig. 4B), the 4.1-kb plasmidic band can be seen as expected, as well as other hybridizing bands in lanes 1 and 2. The additional band in lane 1 corresponds to the remaining fragment of pL1HS1.2del that contains plasmidic sequences. Hybridization of the additional fragment with probe P in lane 2 indicates that this rescued junction is of the type vector-vector junction illustrated in Fig. 3D. About 4% of the rescued junctions were of this type. They were not included in the tabulation of the distinct rescued junctions analyzed (see below) since they represented a vector-vector rather than a vector-cell recombination event. With probe L1 (Fig. 4C), the KpnI 1.2-kb fragments can be seen as expected except in lane 5. As explained above, in this case, the KpnI 1.2-kb fragment has been replaced by a plasmid-cell fusion fragment that hybridizes with probe L1 because of the remaining X sequences. There are also additional hybridizing bands in lanes 1, 2, and 4. In the case of lanes 1 and 2, this is due to the presence of vector L1 sequences in these bands as explained above. The additional band in lane 4 hybridizes with probe L1, which indicates the presence of endogenous L1 sequences in the rescued cellular sequences. Finally with probe 3' (Fig. 4D), there are bands hybridizing in lanes 1, 2, and 4. Again, the reason for the bands in lanes 1 and 2 is as explained above. The fact that the cellular sequences in lane 4 hybridize with probe 3' raises the possibility that the rescued integration junction is of the homologous type (Fig. 3A) or alternatively that integration occurred near an endogenous L1 element (Fig. 3C). Restriction enzyme analysis and sequencing were used to discriminate between these two possibilities. Note that the absence of bands hybridizing with probe 3' in lanes 3 and 5 indicates that the rescued integration events in these lanes are of the illegitimate type (Fig. 3B).

More than 400 distinct rescued integration events were analyzed as described above (Table 1). The presence of endogenous cellular L1 sequences in the rescued junctions was detected in 4% of them, and half of these represented homologous junctions. The variable degree of homology of the L1 elements rescued compared with the probes used for their detection (see below) indicated that the hybridization conditions used for analysis enabled us to detect the vast majority of L1 elements that could have been present in the rescued plasmids.

Analysis of homologous junctions. The seven homologous integration junctions that were rescued are presented in Fig. 5. All seven junctions had distinct lengths, and restriction maps indicating that they represented distinct integration events. In six of seven cases, the BamHI site used for linearization had been regenerated at the expected position. This BamHI site had most likely been reconstituted by the endogenous L1 element, which indicated that the endogenous L1 elements were located at the expected position for a homologous junction. To ensure further that these were truly homologous junctions, we sequenced the L1 sequences present in the rescued plasmid (data not shown). In all cases, we found that the 23 bp that had been deleted from the vector were present at the expected location. Furthermore, we found in all cases a switch from vector L1 sequences to cellular L1 sequences, the latter being identified by the variation in sequences compared with the vector L1 sequences. These switches occurred at different positions for the different homologous junctions. All seven endogenous L1 sequences were unique compared one with the other and with the vector L1 sequences.

If these homologous junctions were the result of classical crossing-overs, the left junction should also have been homologous. We rescued and analyzed the left junction of three of
these homologous integration events. In all cases, the left junction was found to be illegitimate and did not involve endogenous L1 sequences (data not shown). Analysis of the left junctions of other integration events indicated that the left side of the vector could undergo homologous recombination with endogenous L1 elements. However, this also resulted in one homologous and one illegitimate junction.

**Homology of the endogenous L1 elements.** The overall degree of homology between the endogenous L1 sequences present at the homologous junction downstream from the vector X sequences and the L1 sequences originally present in the vector at that position (Y sequences in Fig. 1) ranged from 98 to 94% (Fig. 6A). This distribution is similar to the one found when the L1 sequences present in the vector are compared with the endogenous L1 elements which were found near illegitimate recombination junctions (Fig. 6B) and also when the L1 sequences of the vector are compared with human L1 elements obtained from GenBank (Fig. 6C) (19). Thus, there was no indication that the homologous recombination events involved a marked selection for endogenous L1 elements with a higher degree of homology to the L1 sequences present in the vector.

**Density of L1 elements in rescued cellular sequences.** To verify that the density of L1 elements present in the rescued cellular sequences was representative of the density found in the genome, we calculated the ratio of L1 elements per kilobase of cellular DNA rescued in experiments 1 and 2 (Table 1). There were five distinct elements in a total of 251 kb of cellular DNA. This corresponds to a density of one endogenous L1 element per 50 kb of rescued cellular DNA, which is comparable to the density found in random genomic DNA (one per 30 to 60 kb [14, 18]). Thus, it seems that the exogenous L1 sequences had access to representative genomic DNA in terms of endogenous L1 element density.

**DISCUSSION**

In this study, we examined the integration of an exogenous L1 element into the genome of human fibroblasts. We analyzed over 400 independent integration events and found that only 2% had involved homologous recombination with endogenous L1 elements. Although this frequency is in the upper limit of what has been observed for single-copy genes, one might have expected it to be even higher considering the very high copy number of L1 elements in the genome. It was shown previously that in mammalian cells, increasing the number of homologous targets did not result in an increase in the ratio of homologous to illegitimate integration events (33, 36). In these studies, the targets were localized at very few distinct loci and numbered less than $10^3$. The results presented here indicate that even with $10^4$ potential homologous targets and a density of one homologous target per 30 to 60 kb, the vast majority of integration events were still not homologous.

Since the endogenous L1 sequences were homologous to the exogenous L1 sequences, their sequence divergence could have affected the frequency of homologous integration. Previous studies showed that the frequency of homologous integration for single-copy genes was extremely sensitive to any mismatch between the exogenous and endogenous sequences. Divergence of less than 1% resulted in a more than 10-fold reduction in the frequency of homologous integration (11, 31). Furthermore, when competing homologous targets were present, divergence of less than 5% resulted in at least a 15-fold preference for the homologous versus homologous target (23). In the study reported here, the exogenous L1 sequences had access to targets with a very wide range of homology, yet homologous recombination did not seem to favor endogenous L1 sequences with a higher degree of homology to the exogenous sequences. The various degrees of homology of L1 sequences involved in homologous recombination were what would be expected if the endogenous L1 sequences had been selected randomly. This was also found to be the case when endogenous L1 sequences were rescued by exogenous L1 vectors with various degrees of homology (6). One possibility for the discrepancy between our results and those cited above (11, 23, 31) is that the latter case involved assays that looked specifically at classical crossing-overs with two homologous junctions, one on each side of the integrated vector. Classical crossing-overs were not observed in the present study. Instead, we found that only one integration junction was homologous, the other being illegitimate.

Integration events with one homologous and one illegitimate junction have been observed previously in gene targeting experiments involving perfectly homologous sequences (1, 2, 8, 13). When both ends of the vector were homologous to the cellular target, as was the case here, integration events with one homologous and one illegitimate junction were associated with a two-step process involving nonreciprocal homologous recombination with the cellular target followed by illegitimate integration elsewhere in the genome. This process left the cellular target unmodified. When only one end of the vector was homologous, integrations with one homologous and one illegitimate junction directly in the cellular target (8) as well as integration by the two-step process described above (2) were reported. Integration events with one homologous and one
illegitimate junction are not consistent with the double-strand break repair model, which predicts products with two homologous junctions (30), but could be explained by the one-sided invasion model (5, 7). In the present study, we cannot differentiate between integration into an endogenous L1 element and the two-step process. Nevertheless, despite the presence of homologous ends on both sides of the vector, classical crossing-overs were not observed, contrary to what is seen with perfectly homologous sequences.

The considerations presented above raise the possibility that in mammalian cells, homology interferes with the outcome of the homologous recombination process such as to favor non-reciprocal homologous recombination without crossing-over. This is the case in yeast cells; in this case, it has been observed that homologous sequences undergo gene conversion but are rarely associated with crossing-overs, contrary to homologous sequences (24, 34). This observation is also consistent with data from our laboratory indicating that the reduced homology between two L1 sequences does not interfere with the frequency of homologous recombination but favors the production of recombinants with one homologous and one illegitimate junction rather than two homologous junctions (6). We do not know to what extent exogenous-endogenous interactions seen in transformation experiments reflect the normal behavior of genomic DNA. However, if genomic nonallelic repeated sequences interact among themselves as they do with exogenous sequences, homologous recombination could contribute to their concerted evolution with minimal risk of genome instability resulting from crossing-overs.

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