Isolation of Markers from Recently Transposed LINE-1 Retrotransposons

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ABSTRACT

Many previous techniques for the isolation of endogenous retroelements such as LINE-1 retrotransposons have produced major sampling bias or required laborious procedures. These problems led to the isolation of only older elements in some cases. In other cases, specialized systems were required for the isolation of recently transposed elements. We report here a system for the easy isolation of markers from a wide range of LINE-1 elements and the screening of recently transposed elements from that population. This is accomplished by the use of PCR with degenerate primers specific for conserved regions of the reverse transcriptase gene, a modified screening vector, and a refined blue/white colony assay that screens for amplified DNA containing open reading frames. This method should be applicable to searches for endogenous retroviruses.

INTRODUCTION

Eukaryotic genomes contain a range of retroelements such as LINE-1s (L1s) and endogenous retroviruses (2,6,8). A common complicating factor in studies of these elements is their large copy number, ranging in L1 elements up to greater than 100 000 copies per mammalian genome and composing up to 20% of these genomes. Random sampling of retroelements can lead to inaccurate conclusions about their nature and activity because the vast majority may be inactive pseudogenes that inserted long ago and have collected mutations over great periods of time. For example, random sampling of L1 elements in two species of rodents resulted in the erroneous conclusion (10,20), which was later refuted (4,16), that L1 transposition had slowed dramatically or ceased in these species. Previous techniques aimed at analysis of recently inserted L1 elements suffered from problems such as the need for sequencing extensive numbers of elements, the requirement of a chance encounter with a recent phenotypically observable insertion event, or laborious approaches targeting specific subfamilies (4,5,11,18). In this paper, we describe a technique that allows easy isolation of a portion of L1s from a broad range of mammalian species, followed by identification of those elements that have recently inserted. This general method should be applicable to studies of any other retroelements that contain coding regions.

The method is based on two observations. First, regions of the reverse transcriptases of retroelements are highly conserved so that the design of appropriate degenerate primers allows PCR am-

plification of a wide range of these elements (19). Second, these elements collect random mutations as time passes after insertion, so more recently inserted (younger) elements will have fewer mutations and should be more likely to retain their open reading frames (ORFs).

For this paper, we designed degenerate PCR primers based on conserved amino acid sequences in the L1 reverse transcriptases from a wide range of mammalian L1s because the reverse transcriptase reading frame is the most highly conserved region in L1 elements. We then modified a standard bacterial cloning vector such that it does not contain a functional *lacZ'* reading frame but can produce a functional LacZ' fusion peptide if an L1 ORF amplified with the degenerate primers is inserted appropriately. By careful choice of the recipient bacterial strain and modification of *lacZ* induction conditions, we have produced a blue/white colony screen for delineation of blue colonies containing L1s that have transposed so recently that they still contain ORFs. White colonies can be used as a source of old elements (ones that have transposed long ago).

MATERIALS AND METHODS

Degenerate Primer Design and PCR Amplification

A typical full-length L1 element and the region amplified are shown in Figure 1. The upstream primer was designed to include the highly conserved Y/FXDD box from homology domain 5 in a published alignment of reverse transcriptases from viruses and trans-

posable elements (22). The downstream primer, 9Rdeg, was designed to a region within the L1 ORF 2 but outside of the reverse transcriptase domain. Final design of primers was based on our alignments of L1s from human, gorilla, mouse, rat, deer mouse, slow loris, rabbit, and dog. Figure 2A shows the pertinent regions from an alignment of L1s from six of these species, while Figure 2B shows the degenerate primers used for PCR amplification aligned with the amino acid sequence upon which they are based. Even though the alignment in Figure 2A shows only Fs at amino acid number 707, the 8FDeg primer was designed for the possibility of Fs or Ys at this position because a large number of reverse transcriptases contain Ys at this position. The E rather than a D in the dog sequence at position 710 was deemed to be due to a mutation after insertion because the two Ds at this location are the most highly conserved amino acid pair in reverse transcriptases and have been found in the vast majority of all functional reverse transcriptases (22). Degeneracy was kept under 200-fold by limited use of inosines. 8FDeg and 9Rdeg degeneracies are 32- and 192-fold, respectively. Inosines were placed as far from the 3' ends of primers as possible because 3' ends are important for annealing specificity, and although inosine can base pair with any of the four nucleotide bases, it does not exhibit the same binding affinity for each base and can therefore introduce a bias for some sequence subsets (13,17). Two restriction sites were added at the 5' end of each primer

to increase cloning flexibility and to allow higher annealing temperatures after the initial PCR cycles.

Degenerate PCR amplifications of 50 μ L contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μ M each dNTP, 100 pmol each primer, 200 ng genomic DNA, and 2.5 U AmpliTaq[®] DNA polymerase (Applied Biosystems, Foster City, CA, USA). Hot starts were performed by combining all components except the genomic DNA and the polymerase, heating to 70°C, and then adding these final two components before cycling.

Amplification conditions were designed with a low initial annealing temperature to maximize the range and age of L1s that would be amplified. This temperature of 41.8°C is based on only the portion of each primer that is homologous to L1 sequences and is 5°C below the melting temperature (T_m) of that region when it has the maximum A + T content within the degenerate mixture ($T_mATmax-5^\circ$) (15). A slow ramp of 1°C/5 s was used for the first five cycles to increase annealing of L1s with higher T_m s. After the first five cycles, the annealing temperature was raised to the $T_mATmax-7^\circ$ for the total length of each primer (including bases beyond the region of L1 homology). Thermal cycling was performed in a GeneAmp[®] PCR System 9600 (Applied Biosystems) as follows: hot start at 70°C, 1.5 min denaturation at 94°C, 5 cycles of 94°C for 30 s, 41.8°C for 30 s, 2.5 min ramp to 72°C, and 72°C for 1 min, followed by 23 cycles of 94°C for 30 s, 56.6°C for 30 s, and 72°C for 1 min, followed by a final elongation at 72°C for 7 min. In

some cases, with either very divergent species or when dealing with only exceptionally old L1s, the T_m for the first five cycles was reduced to 37.8°C, the ramp was kept at 1°C/5 s, and the number of cycles at the higher temperature was increased from 23 to 28 to increase the amount of product and potentially collect more divergent sequences.

Construction of Cloning Vector and Libraries

The plasmid pBluescript[®] II KS+ (Stratagene, La Jolla, CA, USA) was modified to produce the plasmid vector pKSW, which does not contain a functional *lacZ'* ORF, but which can produce a functional LacZ alpha peptide fusion protein if an appropriate fragment is inserted to recreate an ORF. The pBS II KS+ plasmid was digested with *EcoRI* and *NotI*. The complementary oligonucleotides, 5'-AATTCACCCG GGGGATCCTTCTAGAGC-3' and 5'-GGCCGCTCTAGAAGGATCCCCCG GGTG-3', were then annealed and ligated into the digested plasmid, producing pKSW. This new vector allows the directional insertion of PCR products produced with the 8FDeg and 9Rdeg primers after digestion with either *EcoRI* or *XhoI* for the 5' end of the insert plus *BamHI* for the 3' end of the insert. The additional option of a *PstI* cloning site is available for the 3' end of the insert when the pKSW vector is prepared from a clone that contains an 8FDeg/9Rdeg-generated L1. When an amplification product from an L1 element with an ORF is inserted, a fusion protein is produced from the *lacZ'* initia-

Figure 1. Map of a typical full-length L1 element. Some major features of a typical full-length L1 element with nucleotide numbering based on the mouse element described by Loeb et al. (12), GenBank[®] accession no. M13002. ORF 2 is the reverse transcriptase/endonuclease reading frame. The numbers defining the amplified region include only the regions of homology in the 8FDeg and 9Rdeg primers.

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tion codon upstream of the multiple cloning site, containing 205 amino acids of L1 reverse transcriptase sequence fused with the LacZ alpha peptide.

Amplified DNA was purified with the QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA) before extensive digestion with *EcoRI* and *BamHI*. The 630-bp digestion product was then isolated following gel electrophoresis by standard techniques (1). The DNA was ligated into pKSW, also cut with *EcoRI* and *BamHI*, and subsequently electroporated into *E. coli* strain TG1 (Stratagene).

Colony Screening Assay

Plasmids to be screened were electroporated into *E. coli* strain TG1. Cells were then plated onto LB plates containing 100 µg/mL ampicillin, 40 µg/mL X-gal, and 0.05 mM isopropyl-β-D-thiogalactoside (IPTG) (the last two from Life Technologies, Rockville, MD,

USA) and incubated for 16–18 h at 37°C before scoring. It was essential to use plates within one week of pouring (stored at 4°C) and to score only isolated colonies incubated as described above. Scoring was most reproducible when colonies were observed on a background of light blue paper, type PIMP-2201-BE (Boise Cascade, Boise, ID, USA). Positive controls used during development of the colony screening assay consisted of a clone containing pBlue-script II KS+ and a number of clones containing the pKSW vector with the appropriate regions of previously isolated *Peromyscus* ORF⁺ L1s inserted. Negative controls were a clone containing pKSW plus a number of clones containing pKSW constructs that had received the appropriate regions of ORF⁻ L1s.

RESULTS AND DISCUSSION

Before using degenerate primers to

amplify part of the reverse transcriptase gene from a broad range of L1s, we inserted that same region from a number of previously isolated L1s into the screening vector, pKSW, such that only those clones containing ORFs should give rise to blue colonies upon screening with X-gal and IPTG. Tests for ORFs (i.e., blue vs. white colonies) in the *E. coli* strain DH5α revealed an unacceptable percentage of both false-positive and false-negative clones even when the scoring conditions were carefully controlled. Modifications of growth time and X-gal/IPTG concentrations, combined with tests of different bacterial strains, were essential to produce the assay described in Materials and Methods in which greater than 98% of the colonies from any of the test constructs were of the predicted color. It is extremely important that the conditions described be maintained, particularly when comparisons are made between species.

The degenerate primers (Figure 2)

Figure 2. Design of degenerate PCR primers. (A) Alignment of six mammalian L1 reverse transcriptases showing the region used for primer design and 10 amino acids upstream and downstream of each primer. Numbering of amino acids is based on the second ORF of the mouse sequence (14), GenBank accession no. U15647. Accession numbers for other sequences are: rat, M13100; rabbit, X15965; dog, AB012223; human, M19503; and gorilla, AFO36235. Xs in the rat sequence represent a deletion in that sequence. (B) Diagram of the 8FDeg and 9RDeg degenerate primers used to amplify 575 bp of L1 elements. Restriction enzyme sites added near the 5' end of each primer are shown. The 3' portion of each primer homologous with L1 reverse transcriptase genes is aligned with the corresponding amino acid sequence (shown in bold). The broken bar indicates the approximately 575-bp ORF between the primers that is amplified from the L1 reverse transcriptase genes.

were designed, primarily, to allow amplification of L1 sequences from a wide range of rodent species and, secondarily, to allow amplification from a wide range of other mammalian species. They were initially used to amplify genomic DNA from the deer mouse, *Peromyscus maniculatus*. The major amplification products of approximately 630 bp from two completely independent amplifications were inserted into pKSW, giving rise to two libraries. Each of these libraries showed a similar percentage of blue clones. Thirty-one percent of the clones were blue in one library and 27% in the other library. A number of blue and white colonies were randomly selected from each of these libraries for further analysis, and 100% of them were found to contain the predicted L1 region. Eighteen elements from blue clones and 14 from white clones were sequenced. ORFs were found in the majority of the blue clones (94%) but only in a minority of the white clones (21%). This contrasts

greatly with previous studies of L1 elements in a range of mammals. In some studies, only elements with interrupted ORFs have been identified, while in other studies, elements with ORFs have been identified only after sequencing a relatively large number of clones or using laborious approaches tailored for identification of such elements (4,5,10,11,18,20). The high incidence of ORFs among clones isolated after this degenerate PCR, even if no color screen had been used, underscores the power of this PCR alone to enrich for recently inserted elements. However, the enrichment due to degenerate PCR alone varies between species. Blue/white screening will be most useful to characterize species in which recent L1 activity is reduced or absent.

The presence of ORF⁻ blue clones and ORF⁺ white clones might initially seem puzzling, but ORF⁻ blue clones were found to contain internal in-frame restart codons, which were in at least some cases preceded by fortuitous

Figure 3. Sequence divergences between L1s. (A) Pairwise distances between L1s from blue clones. (B) Pairwise distances between L1s from white clones. The Y-axis gives the percent of the total pairwise interactions between any two elements that fall within the indicated pairwise distances, or percent sequence differences, shown on the X-axis.

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Shine-Dalgarno signals. ORF⁺ white clones presumably exist because they produce lower levels of functional β -galactosidase (β -gal) fusion protein. Reasons for this could include the presence of L1 variants or mutations that reduce the translation elongation rate or fusion proteins that do not fold properly. Upon restreaking, blue colonies reproducibly gave rise to blue colonies and white colonies gave rise to white colonies irrespective of their ORF content.

Do blue colonies indeed contain a more recently inserted (younger) set of L1 sequences than white colonies? Younger L1s should show less sequence divergence between elements than older L1s because they have had less time to accumulate mutations than those copies that were inserted at an earlier time. We therefore looked at pairwise distances (percent sequence difference) between L1s from the predominant L1 lineage in *P. maniculatus*. We only used L1s from Lineage 1, or those which predated Lineage 1, because some of the differences in L1s from different lineages (i.e., produced from different active master elements) would be due to divergence in the masters from which they were derived rather than mutations that accumulated after they inserted into the genome [see Casavant et al. (4) for an explanation of L1 lineages in *P. maniculatus*]. Figure 3 shows the ranges of pairwise distances obtained between L1 sequences from blue clones (3A) and between L1 sequences from white

clones (3B). Thirty-seven percent of the pairwise distances between L1s from blue clones show less than 1% difference in their sequences, while none of the white clones yield L1s with such small pairwise distances. The same trend is seen upon comparison of average pairwise distances for all Lineage 1 L1s. The average pairwise distance between all L1s derived from blue clones is 2.6%, while the average pairwise distance between L1s derived from white clones is 9.2%. This suggests that use of the color screen indeed leads to identification of a substantially younger set of elements. Phylogenetic analysis (not shown) also indicates that elements derived from blue clones tend to be substantially younger than elements derived from white clones.

When we compared the L1s isolated with this technique to previously isolated L1s from *P. maniculatus*, we found that the new technique allows us to identify younger elements and to identify them much more easily, even though some of the previously isolated elements were identified in searches specifically tailored for isolation of young elements (4). How young are the most recently inserted elements? Among the 136 pairwise comparisons between blue clones here, 10 show a pairwise distance of only 0.2% (a single base pair difference) and two pairs are identical, suggesting that they represent extremely recent insertion events. When analyzing such elements, one should bear in mind some of the basic problems associated

with all PCR-based approaches. Clones this similar may in some cases be artifacts produced by PCR errors, while identical clones can arise as multiple amplification products from a single element. There are times when older elements, rather than younger ones, are desired for complete analysis of the evolutionary history of L1s within a species. The availability of such elements from this screen is demonstrated by the extreme sequence differences of up to 36% between white clones.

We have seen variation in the populations of elements amplified in independent experiments, but a broad spectrum of elements appears to be obtained from every amplification. For example, *P. maniculatus* is known to contain two major L1 lineages, with Lineage 1 being present at a substantially higher copy number in the genome than Lineage 2 (4). Phylogenetic analysis (not shown) indicates that we recovered both of these lineages during independent amplifications. Even though the percentage of clones belonging to each lineage varied between amplifications, Lineage 1 appeared more commonly in both. In addition, elements from one specific amplification do not cluster into particular clades but interdigitate with elements from separate amplifications, as would be expected. Nonetheless, during amplification of mixed populations, one should always bear in mind that the amplified population does not necessarily reflect the genomic population because some sequences will always be preferentially amplified over others because of such factors as concentration of specific primer subsets, amplification conditions that favor certain sequences, the possibility of target divergence from the primer set, and stochastic effects. Younger elements in particular will be preferentially amplified because they have not suffered the mutational divergence from an active element that occurs with time in older elements.

We have found that this technique allows rapid, easy isolation of this region of L1 elements from a broad phylogenetic range of mammals and has the possibility of giving a quick preliminary test for L1 activity in these animals. With every species amplified, we have obtained L1s. Figure 4 shows a selection of some of these species, indicating that

Figure 4. ORF screening allows rapid scanning of L1 activity from a range of mammals. Deer mouse, *P. maniculatus*; cotton rat, *Sigmodon mascotensis*; vole, *Microtus arvalis*; rice rat, *Oryzomys palustris*; marsh rat, *Holochilus brasiliensis*; Hartmann's mountain zebra, *Equus zebra*; Mexican fruit bat, *Artibeus jamaicensis*; and human, *Homo sapiens*.

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we see a wide range for the percent blue clones produced. When we have seen the level of blue clones reduced to less than 15%, as with many species of rodents of the closely related genera *Oryzomys* and *Holochilus*, we have been unable to find any L1s with ORFs in spite of extensive searches by this and other procedures. See Casavant et al. (3) for further characterization of blue clones from *Oryzomys*. These observations on the *Oryzomys* and *Holochilus* species are part of the data supporting the surprising suggestion that these rodents are the first identified mammals in which L1 activity has ceased (3).

This technique for easy isolation and delineation of a portion of young and old L1 elements using minute amounts of DNA from a wide range of mammals will greatly facilitate the use of these elements as extremely powerful markers to determine the evolutionary history of mammals (7,21). The use of endogenous retroviral sequences for constructing mammalian phylogenies (9) should also be simplified by using

this technique with modified primers. Since it allows the easy isolation of elements from such a wide range of mammals, this method should be extremely useful for studies into the mode and rate of retroelement evolution throughout much of the mammals.

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