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Evolution

Amplification of an ancestral mammalian L1 family of long interspersed repeated DNA occurred just before the murine radiation

(LINE sequence/murine evolution/transposons/taxonomy)

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ABSTRACT **Each mammalian genus examined so far contains 50,000–100,000 members of an L1 (LINE 1) family of long interspersed repeated DNA elements. Current knowledge on the evolution of L1 families presents a paradox because, although L1 families have been in mammalian genomes since before the mammalian radiation ≈80 million years ago, most members of the L1 families are only a few million years old. Accordingly it has been suggested either that the extensive amplification that characterizes present-day L1 families did not occur in the past or that old members were removed as new ones were generated. However, we show here that an ancestral rodent L1 family was extensively amplified ≈10 million years ago and that the relics (≈60,000 copies) of this amplification have persisted in modern murine genomes (Old World rats and mice). This amplification occurred just before the divergence of modern murine genera from their common ancestor and identifies the murine node in the lineage of modern muroid rodents. Our results suggest that repeated amplification of L1 elements is a feature of the evolution of mammalian genomes and that ancestral amplification events could provide a useful tool for determining mammalian lineages.**

All mammalian genomes studied to date contain 50,000–100,000 members of an L1 (LINE 1) family of mobile DNA elements (1–3). Full-length elements are 6–7 kilobases (kb) and contain two protein encoding regions [open reading frame (ORF) I and ORF II, see Fig. 1*A*], which are highly conserved among mammalian L1 families and are presumably important for L1 function (4–7). The putative protein encoded by ORF II is homologous to proteins encoded by transposable elements found in plants, fungi, as well as insects and other invertebrates (8). All of them contain sequence motifs typical of DNA polymerases (8), and at least one functions as a reverse transcriptase (9). Therefore L1 elements are apparently the mammalian counterpart of, or were derived from, a genetic element that arose very early in evolution.

The present-day L1 families evolved independently from an ancestral L1 element that predated the mammalian radiation 80–100 million years ago (3). However, in spite of their length of time in the genome each present-day family is rather new—i.e., the DNA sequences of most of the randomly sampled members are >90% identical, indicating that these members were generated within just the last few million years (7, 10–12). To account for this apparent paradox it has been suggested that either extensive amplification of L1 did not occur in the past (1, 13) or that old members are removed as new ones are generated (7, 11, 12).

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However, we report here that an ancestral rodent L1 family, which we call Lx, was extensively amplified ≈10 million years ago and that ≈60,000 copies of Lx are present in various modern murine genomes (Old World rats and mice). The occurrence of this amplification identifies those murine genera that shared a common ancestry and, therefore, defines the murine node in the lineage of modern muroid rodents. The possible mechanism and the implications of repeated amplification of L1 elements are discussed.

MATERIALS AND METHODS

General. The preparation of clones, genomic DNA, hybridization probes, agarose gels, nitrocellulose blots, and restriction enzyme digests was done using standard techniques (14). Hybridizations to blots were carried out at the temperatures indicated in the legend to Fig. 2 in a solution containing 0.5 mM Na_2EDTA, 0.2 M sodium phosphate (pH 6.8), 0.125% (wt/vol) SDS, denatured salmon sperm DNA at 50 μg/ml, 0.05% (wt/vol) Ficoll 400 (Pharmacia), and 0.05% (wt/vol) polyvinylpyrrolidone. The polymerase chain reaction (PCR) (15) was performed by using a kit from Perkin–Elmer/Cetus and conditions suggested by the supplier. The sequences of the primers used to amplify genomic L1 sequences from the rat or mouse genomes were as follows: rat L1RN C1, ATAGGATCCGCCCCACAGGTGGCCCAT, and L1RN C2, ATAGGATCCAGTGGCTTAGTCCCTGGA; mouse L1MD C1, ATAGGATCCATACACTAGCAAGATTTT, and L1MD C2, ATAGGATCCGTCAAGAGCTCCGGGGTA. The primers used to amplify Lx sequences from murine genomes were LX1B C1, ATAGGATCCCATCCAGAGACTACCTCACCT, and LX1 C2, ATAGGATCCTCTTCCTATGGGGTTGAAAAC. The first nine nucleotides of each primer are not complementary to either L1 or Lx and contained an ATA followed by a *Bam*HI site. This facilitated radiolabeling the ends of the amplified material by use of the Klenow polymerase after digestion with *Bam*HI endonuclease.

DNA Melting Curves. An ≈230-base-pair (bp) portion of L1 or Lx DNA beginning ≈100 bp 3′ of ORF II (see Fig. 1*A*) was amplified from various genomes by PCR using oligonucleotides specific for each family. A small portion of each DNA was radiolabeled at the 3′ end with the Klenow polymerase.

Abbreviations: ORF, open reading frame; PCR, polymerase chain reaction; t_m, melting temperature (of double-stranded nucleic acid). L1 or LINE 1, family of long interspersed repeated DNA elements; L1Rn, rat (*Rattus norvegicus*) L1 family; L1Md, mouse (*Mus domesticus*) L1 family; Lx, ancestral rodent L1 family.

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After denaturation by boiling for 10 min each DNA was hybridized in 0.3 M sodium phosphate buffer, pH 6.8, at either 55°C or 65°C until at least 10 times the $C_0t_{1/2}$ was attained. C_0t is the product of the initial concentration of DNA (in mol of nucleotide per liter) and the time of hybridization (in sec). The $C_0t_{1/2}$ is that value by which 50% of the starting DNA has formed duplex (16). The samples were diluted to 0.12 M sodium phosphate buffer and applied to a jacketed hydroxyapatite column equilibrated in the same buffer at 50°C. The temperature was then raised in 3°C increments, and the amount of eluted DNA was determined at each step. At least 95% of the radioactivity that bound to each column was recovered.

RESULTS

Identification of an Ancestral L1 Element. In studying different genomic clones of the rat (*Rattus norvegicus*) L1 family (L1Rn), we determined the DNA sequence of several DNA elements (unpublished work) that were homologous to L1Rn but were not typical members of the L1Rn family. Typical L1Rn members are at least 90% identical with each other, whereas these elements, which we've called Lx elements, were only ≈65% or 85% identical to L1Rn members, depending on the region compared.

Fig. 1*A* shows a diagram of an Lx element (Lx1) compared with a typical rat L1 element. Lx1 is 1.5 kb, and the first 770 bp of Lx is homologous to the last one-fifth of the ORF II region of the rat or mouse (*Mus domesticus*) L1 elements (L1Rn and L1Md, respectively; see Fig. 1*B*). An ancestral relationship between Lx and both the L1Rn and L1Md elements is evident when the DNA sequences 3′ of the termination codon of ORF II are compared. Lx1 is more closely related to L1Rn 3 or L1Md A2 than either is to each other (see Fig 1*B* *3*, *4*, and *1*, respectively). In terms of percent similarity, the Lx sequence 3′ of ORF II is ≈65% similar to either L1Rn or L1Md, whereas the corresponding regions of these sequences are only 55% similar to each other (see legend to Fig. 1). Fig. 1*B2* shows the relationship between two typical members of the L1Rn family, and a similar relationship is found when typical members of the L1Md family are compared (12).

In the remainder of this paper we shall show that Lx1 belongs to a family of sequences amplified much earlier than the present-day L1 families of rat or mouse. Therefore, the Lx family is an old or ancestral rodent L1 family from which the present-day rat and mouse L1 families descended. To facilitate discussion we shall refer to this ancestral L1 family and its members as Lx and to the present-day rat or mouse L1 family and its members as L1Rn or L1Md, respectively.

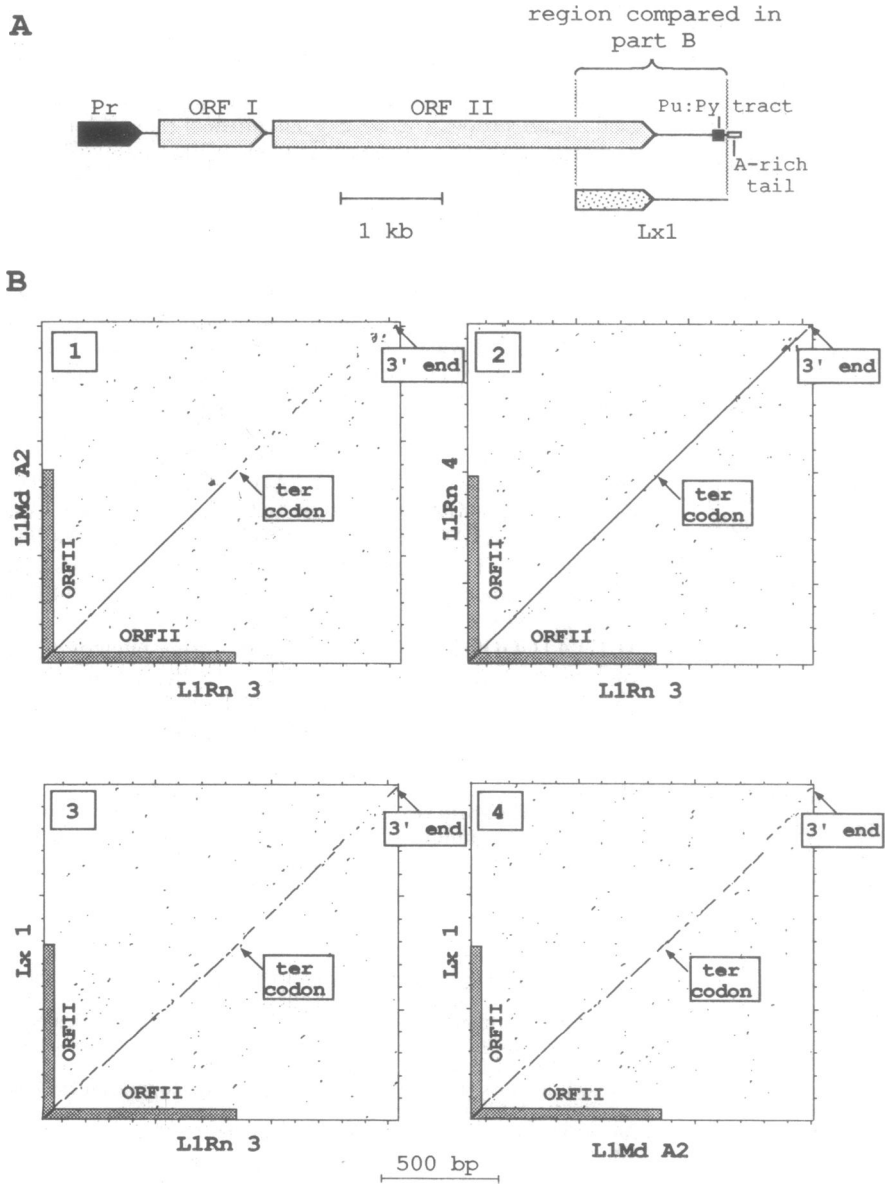

FIG. 1. Dot-matrix analysis of the rat L1 (L1Rn), mouse L1 (L1Md), and Lx nucleotide sequences. (*A*) Schematic representation of an archetypical full-length rat L1 element and of the Lx1 sequence shown below. Pr, promoter; Pu:Py tract, polypurine:polypyrimidine region; A-rich tail, adenine-rich region. Although most L1Rn elements are full-length as depicted, ORFs of all rat elements sequenced so far are interrupted by one or more genetic defects. An L1Md element with full-length ORF I and ORF II has been sequenced, however (6). (*B*) Matrices *1–4* are dot-matrix comparisons between Lx1, rat L1, and mouse L1 nucleotide sequences. ter, Termination codon of ORF II. Matrix *1*, sequence comparison of that portion of rat [L1Rn 3, positions 5514–7049 (4)] and mouse L1 element [L1Md A2, positions 5873–7356 (6)] that corresponds to the Lx1 sequence shown in *A*; Matrix *2*, comparison between two L1Rn members, L1Rn 3 and L1Rn 4, positions 1132–2670 (4); Matrices *3* and *4*, comparison of Lx1 sequence with L1Rn and L1Md, respectively. Sequences were compared in every 21-bp register, and a dot was recorded when 14 of 21 nucleotides matched. Percent similarity between Lx1 ORF II and that of rat or mouse L1 ORF II sequences is ≈85%. The region of Lx1 3′ of the termination codon of ORF II is ≈65% similar to the corresponding region of either rat or mouse L1 sequence. Percent similarity between rat and mouse L1 sequences is ≈88% for ORF II and 55% for the region 3′ of ORF II and for the two rat L1 sequences the similarity is ≈97% for ORF II and 94% for the region 3′ of ORF II.

Because full-length members of the various mammalian L1 families are 6–7 kb (1–3), we presume that the Lx element shown in Fig. 1 is a 5′ truncated member of the Lx family. The process by which L1 families are amplified produces both full-length and 5′ truncated members, and in most genera, but not rat, the number of truncated members far exceeds the number of full-length members (1, 2, 4).

The Copy Number of Lx in Rat and Mouse Genomes. In experiments not shown here we found that the ORF II region of Lx hybridized to the corresponding region of L1. This would be expected because the ORF II region of Lx is ≈85% similar to the ORF II region of either L1Rn or L1Md. However, the region of Lx that is 3′ of ORF II is only ≈65% homologous to the corresponding region of L1Rn or L1Md and does not hybridize to these sequences at even low stringency (see legend to Fig. 2). Therefore, in the rest of the paper we confined our analysis to sequences 3′ of ORF II because only in this region could we distinguish Lx and L1 sequences by hybridization.

Fig. 2 *Upper* shows that Lx is repeated to about the same extent in two species of rat (*R. norvegicus* and *Rattus rattus*) and in mouse (*M. domesticus*) (*Left*) and that Lx is almost as prevalent in the rat genome as L1 (*Right*). In addition, the Lx probe hybridized to 25 or 30% of the plaques of a λ phage

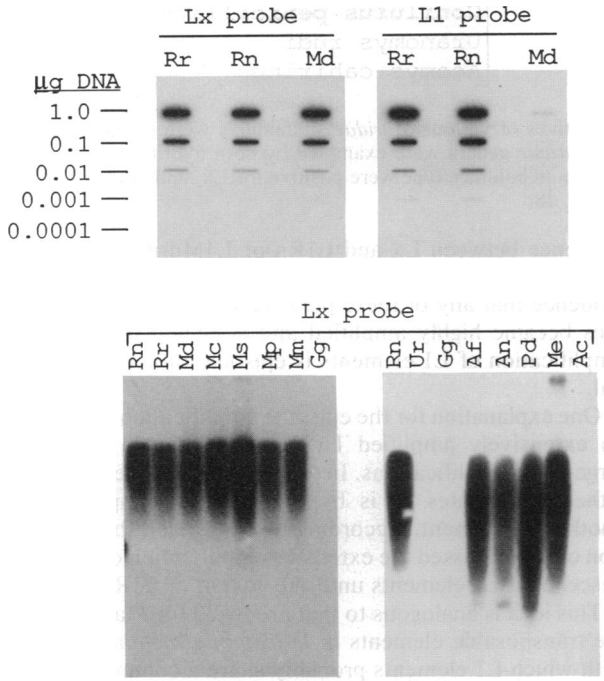

FIG. 2. Hybridization of the region of Lx or L1 3′ of ORF II to the DNA of various rodents. (*Upper*) Slot blots containing various concentrations of *R. rattus* (Rr), *R. norvegicus* (Rn), and *M. domesticus* DNA (Md) DNA were hybridized at 65°C with either radioactive Lx or L1Rn DNA. Both probes were ≈450 bp and were amplified by PCR from the region of the cloned Lx1 or L1Rn 3 element just 3′ of the termination codon of ORF II. The DNA sequence of this region of Lx is ≈65% identical to the corresponding region of L1Rn (or L1Md), and these sequences do not cross-hybridize even when the hybridization temperature is 55°C or 45°C (data not shown). (*Lower*) Blot hybridizations of various rodent DNAs. Approximately equal amounts of DNA (≈100 ng) were digested with *Eco*RI and electrophoresed on 1% agarose gel. After being blotted to nitrocellulose, the DNA was hybridized to the Lx probe described above at 55°C. In each case, the hybridization pattern paralleled the pattern of ethidium bromide-stained DNA fragments, which ranged in size from ≈0.2 kb to ≈23 kb. Rn, *R. norvegicus*; Rr, *R. rattus*; Md, *M. domesticus*; Mc, *Mus caroli*; Ms, *Mus saxicola*; Mp, *Mus pahari*; Mm, *Mus minutoides*; Gg, *Gerbillus gerbillus*; Ur, *Uranomys rudi*; Af, *Apodemus flavicollis*; Pd, *Praomys daltoni*; Me, *Mastomys erythroleucus*; Ac, *Acomys cahirinus*.

library of *R. norvegicus* or *M. domesticus* genomic DNA, respectively (data not shown). Because the genomes of these animals are ≈2.7 × 10^6 kb and the average insert in our λ clones is ≈12 kb, this corresponds to 56,000 and 65,000 copies of Lx in the rat and mouse genomes, as compared with 74,000 and 90,000 copies of L1 detected in a parallel experiment by using hybridization probes derived from the corresponding region of L1Rn or L1Md DNA.

As mentioned above, we determined the DNA sequence of several Lx elements in addition to Lx1 (unpublished work). Two of these were ≈1 kb and included both ORF II sequence and the region 3′ of it. Pairwise comparisons between these sequences and Lx1 showed that each differed from the other by ≈20% in both ORF II and the region 3′ of it (data not shown). At the time of their amplification Lx sequences would be nearly identical to each other. If Lx elements have accumulated base changes since then at the neutral rate determined for rodents (≈1% per 10^6 years) (17), then in 10 million years they will each have diverged ≈10% from the starting sequence or ≈20% from each other. This hypothesis would place the amplification of Lx ≈10 million years ago, which is some time after divergence of the subfamily *Murinae* from the other major *Muridae* subfamilies (e.g., *Sigmodontinae*, *Gerbillinae*, *Dendromurinae*, etc.; see Fig. 3 and ref. 18) and at about the time when the modern murine genera (e.g., *Rattus*, *Mus*, *Apodemus*, etc.) began to diverge (19). Therefore, we might expect Lx to be amplified in various *Murinae* genera but not in the genera of other *Muridae* subfamilies.

Distribution of Lx Family in Muroid Rodents. We determined in which genera Lx was amplified by hybridizing an Lx probe to the DNA of various rodents. Fig. 2 *Lower* shows some of these data, and Fig. 3 summarizes all our results. Lx is repeated to about the same extent in all but two of the genera (*Acomys* and *Uranomys*) currently classified as *Murinae*. Lx is undetectable in *Acomys* and *Uranomys* and in representatives of the other *Muridae* subfamilies. Genera that were negative for Lx by hybridization were also negative when examined by PCR (data not shown). Lx was not detectable (by hybridization) in other distantly related rodents—e.g., *Caviidae* (guinea pig), other mammalian orders including *Lagomorpha* (rabbit), *Carnivora* (dog), *Artiodactyla* (sheep, cow), and *Primates* (human), and in an avian species, the chicken (data not shown).

Sequence Divergence of the Lx Family in Various Genera. If the Lx family was amplified in the murine ancestral genus ≈10 million years ago, then the age of the Lx family in each present-day genus should be the same—i.e., each should be ≈20% divergent, as for the rat Lx family. To test this prediction we determined the melting temperature (t_m) of hybridized Lx sequences of at least one representative of each of the Lx-positive genera, as described in *Materials and Methods*. As Fig. 4 shows the Lx melting curves are almost the same with a t_m of ≈75°C. This is ≈17°C below the t_m of a perfectly matched hybrid (Fig. 4 *Top*) and indicates that the Lx family in each genus is ≈17% divergent. [The t_m is lowered by ≈1°C for each 1% mismatch (20)]. By contrast the t_m of the hybrids of the present-day L1 families in *R. norvegicus* (*Top*) and *M. domesticus* (*Middle*) is ≈85°C, indicating that these families are ≈7% divergent, which agrees with the results from DNA sequence data (4, 7, and 12). There is only a small difference between the melting curves obtained for Lx duplexes hybridized at 65°C and 55°C. Therefore, most members of each Lx family comprise a relatively discrete cohort, as would be expected if most of the amplification occurred over a relatively short time period (e.g., 1–3 million years), as for the present-day L1 families in *Mus* (11). In addition, the Lx sequences amplified from either the mouse or rat genome by PCR hybridize poorly, or not at all, with the L1 sequences amplified from these genomes

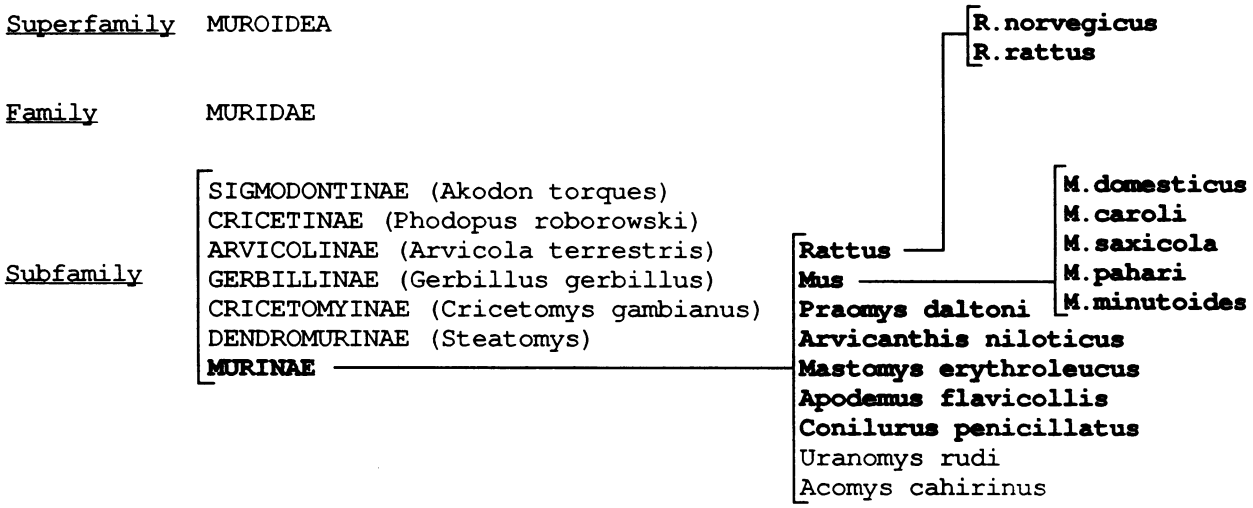

FIG. 3. Taxonomic distribution of the Lx family. The indicated representatives of various *Muridae* subfamilies were examined by blot hybridization (described in legend to Fig. 2). *G. gerbillus* and the indicated *Murinae* genera were examined by both hybridization and PCR reaction with oligonucleotides specific for Lx DNA (data not shown). The genera in boldface type were positive for Lx, and in every case the results from hybridization and PCR agreed. The taxonomic tree is based on ref. 18.

(data not shown). Both results indicate that there is not a continuum of sequences between the Lx family and either present-day L1 family.

DISCUSSION

We have identified in modern murine genomes the relics of an ancestral L1 family that we have called Lx. The Lx family was amplified ≈10 million years ago, and ≈60,000 copies of Lx are present in a diverse group of murine genera. Therefore, Lx was amplified before these genera diverged from their common ancestor 8–12 million years ago (19). The age of the Lx family in the genera tested (Fig. 4) and its absence from other subfamilies of *Muridae* (Fig. 3) indicate that the amplification of Lx occurred close to the time of the murine radiation.

The absence of Lx from two genera (*Acomys* and *Uranomys*), which were previously classified as *Murinae* by morphological criteria (18), indicates that these genera were probably incorrectly classified. This conclusion agrees with biochemical (21) and DNA hybridization (22) studies that indicate that *Acomys* is as distantly related to the other murine genera as are some of the other muroid subfamilies shown in Fig. 3. Our results indicate that the relics of previous L1 amplifications could serve as useful markers for determining the lineage of modern genera. While intergeneric or horizontal transfer of L1 sequences would decrease their usefulness for this purpose, there is no evidence that this occurs (3).

The amplification of Lx in the murine ancestor and the amplification of L1Rn or L1Md in the rat or mouse were separated by ≈8 million years. Sequence comparisons between these DNA elements indicate that during this time the L1Rn and L1Md elements were evolving from Lx under selective pressure (Fig. 1, and unpublished work). Therefore, we presume that functional L1 elements intermediate in sequence between Lx and L1Rn or L1Md must have been present in the rat and mouse lineages. However, we found no evidence that any of these putative evolutionary intermediates became highly amplified and conclude that extensive amplification of L1 elements is episodic rather than continual.

One explanation for the episodic amplification of L1 is that an extensively amplified L1 family represses subsequent large-scale amplifications. In time, the repressive mechanism either degenerates or is bypassed by the appearance of another L1 element. According to this model, the amplification of Lx repressed the extensive amplification of functional descendant L1 elements until the advent of L1Rn or L1Md.

This idea is analogous to that proposed for *I* factors which are transposable elements in *Drosophila melanogaster* and with which L1 elements probably share a common ancestry (8). *I* factors do not replicate or transpose in genomes that contain functional *I* factors because in sufficient number they repress their own replication. An interesting parallel with L1 is that all genomes of *D. melanogaster* contain the degenerate relics of a previous *I*-factor amplification that occurred in an ancestor of the present-day species. These relics are incapable of replication but are thought to retain some repressive activity (23).

The successive amplification of Lx and the present-day L1 families alone has generated 10–20% of the mass of the rat and mouse genomes. In addition to causing insertional mutations (24), we have shown in other studies (ref. 25 and unpublished observations) that L1 DNA can profoundly affect the structural and regulatory properties of neighboring DNA sequences. Therefore, amplification of an L1 family might greatly increase the genetic diversity of a population. This could predispose subsets of the population to genetic isolation, which is a prerequisite for speciation. It would be of interest to see whether the radiation of other rodent

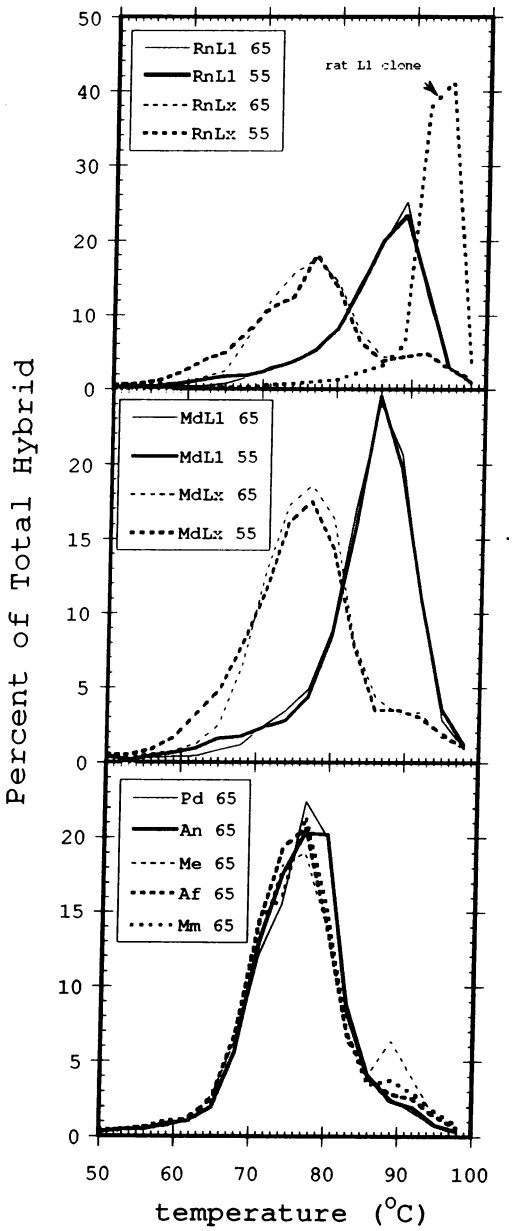

FIG. 4. Thermal elution of hybridized L1 or Lx sequences from hydroxyapatite. L1 or Lx sequences from the indicated genomes were radiolabeled, denatured, and hybridized at either 55°C or 65°C, as described. For clarity, only the thermal elution curves for DNA hybridized at 65°C are shown (*Bottom*). The melting curves of these DNAs showed the same small difference between 55°C and 65°C samples as did the Lx curves of rat (*Top*) or mouse (*Middle*) DNAs. Rn, *R. norvegicus*; Md, *M. domesticus*; Pd, *P. daltoni*; An, *Arvicanthis niloticus*; Me, *M. erythroleucus*; Af, *A. flavicollis*; Mm, *M. minutoides*. The t_m was taken to be that temperature by which 50% of radioactivity in the major peak of 65°C hybridizations was eluted, and the values we obtained were as follows: perfectly matched duplexes (cloned L1 and two different cloned Lx sequences), 92.1 ± 0.57°C; L1Rn and L1Md, 84.9 ± 0.35°C; all of the Lx hybridizations shown, 74.6 ± 0.39°C.

subfamilies is as closely related in time to the amplification of ancestral L1 family as was the murine radiation.

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