Mus spretus LINE-1 Sequences Detected in the *Mus musculus* Inbred Strain C57BL/6J Using LINE-1 DNA Probes

Brad A. Rikke,[1] Yingping Zhao, Lorrie P. Daggett,[2] Rosario Reyes[3] and Stephen C. Hardies

Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7760

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ABSTRACT

The inbred mouse strain, C57BL/6J, was derived from mice of the *Mus musculus* complex. C57BL/6J can be crossed in the laboratory with a closely related mouse species, *M. spretus* to produce fertile offspring; however there has been no previous evidence of gene flow between *M. spretus* and *M. musculus* in nature. Analysis of the repetitive sequence LINE-1, using both direct sequence analysis and genomic Southern blot hybridization to species-specific LINE-1 hybridization probes, demonstrates the presence of LINE-1 elements in C57BL/6J that were derived from the species *M. spretus*. These *spretus*-like LINE-1 elements in C57BL/6J reveal a cross to *M. spretus* somewhere in the history of C57BL/6J. It is unclear if the *spretus*-like LINE-1 elements are still embedded in flanking DNA derived from *M. spretus* or if they have transposed to new sites. The number of *spretus*-like elements detected suggests a maximum of 6.5% of the C57BL/6J genome may be derived from *M. spretus*.

A variety of biochemical techniques have indicated that the inbred mouse strains developed at the beginning of this century are really recombinant inbreds between subspecies of the *Mus musculus* complex, specifically *M. musculus domesticus* and *M. musculus musculus* (reviewed in BONHOMME and GUENET 1989). None of these techniques discovered any contribution from the closely related species *M. spretus*. In this study, however, *M. spretus* LINE-1 sequences were found in the inbred strain C57BL/6J by using species-specific LINE-1 probes.

LINE-1 elements are interspersed, repetitive DNA sequences present in mammalian genomes (reviewed in SKOWRONSKI and SINGER 1986; EDGELL *et al.* 1987; HUTCHISON *et al.* 1989). In mice, LINE-1 is present in ~100,000 copies per haploid genome. LINE-1 elements achieve their high copy numbers because they are retrotransposons; however, most copies are incomplete and incapable of further transposition. During the evolution of LINE-1, progenitor elements acquire mutations and then spread subfamilies of progeny elements sharing that sequence variation. Oligonucleotide hybridization probes have been designed that detect shared sequence variants. Probes that detect subfamilies that spread in one mouse species but not another are called species-specific LINE-1 probes (RIKKE *et al.* 1991; RIKKE and HARDIES 1991).

Corresponding author: Stephen C. Hardies, Department of Biochemistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760.
E-mail: hardies@thorin.uthscsa.edu

[1] *Present address:* Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309-0447.

[2] *Present address:* Sibia, 505 South Coast Blvd., La Jolla, CA 92037

[3] *Present address:* Department of Biology, Institute of Neuroscience, University of Oregon, Eugene, OR 97403.

Previous hybridization studies have indicated that two such LINE-1 probes, oMs496 and oMs416C, are specific for a *M. spretus*-LINE-1 subfamily (RIKKE *et al.* 1991). While determining the copy number of this subfamily in a library probing experiment, we identified two positively hybridizing plaques in a C57BL/6J library that was intended to be a negative control (RIKKE and HARDIES 1991). One of the clones, which we now name λEL111, also hybridizes to another *M. spretus*-specific probe named oMs475. The hybridization of these probes to λEL111 suggested that it contains *M. spretus* sequences, in spite of the fact that C57BL/6J was not expected to have a *M. spretus* component to its genome. The presence of multiple *M. spretus*-specific shared sequence variants in the same LINE-1 element was argued to exclude the alternative explanation of coincidental mutation to non-*spretus* LINE-1s. In this study, we confirm the presence of the *M. spretus*-specific variants by direct sequencing and extend the number of *M. spretus*-specific shared sequence variants found in λEL111.

The presence of a *M. spretus*-like LINE-1 in C57BL/6J implies that a *M. spretus* mouse bred to (at least) one of the ancestors of the C57BL/6J line. This raises the question of how much *spretus* DNA might remain in this strain. In this study, we estimate the amount of *spretus* DNA in C57BL/6J using a genomic Southern blot probed with a *spretus*-specific LINE-1 oligonucleotide.

MATERIALS AND METHODS

Isolation of λEL111: λEL111 was isolated from a genomic DNA library from a C57BL/6J-Tr^{J}/+ Trembler mouse whose construction was previously described (RIKKE and HARDIES 1991). It was isolated as 1 of 2 plaques out of 17,700 plaques which hybridized to the *M. spretus*-specific oligonucleotide

probes oMs496 and oMs416C. The probes and procedures for this library screening also were described previously (Rikke and Hardies 1991).

Sequencing of L1EL111: A L1EL111-containing 2.27-kb restriction fragment from λEL111 was subcloned into the vector M13mp19. L1EL111 was sequenced on both strands by the dideoxy chain termination method (Sanger *et al.* 1977) using LINE-1 internal primers. The LINE-1 element into which L1EL111 was inserted also was sequenced partially on one strand. L1EL111 was inserted into a portion of the canonical LINE-1 sequence that L1EL111 itself does not have such that there was no internal repetition within the restriction fragment that was sequenced.

Construction of the LINE-1 molecular phylogenetic tree: The tree was constructed by the method of maximum parsimony (Fitch 1977). The sequences and calibration method used are described elsewhere (N. C. Casavant and S. C. Hardies 1994b).

Southern blot hybridization: *Eco*RI-digested genomic DNA (20 μg) was loaded onto a 1.2% agarose gel and subjected to electrophoresis at 2 V/cm overnight. A *Hin*dIII digest of bacteriophage lambda DNA was run as a size marker. The gel was photographed with a ruler after staining in 0.5 μg/ml ethidium bromide. The DNA then was vacuum transferred from the gel to a Zetaprobe nylon membrane. This included 10 min of transfer using 0.25 M HCl for depurination followed by 2 hr of transfer in 0.4 M NaOH. The membrane was washed in 6× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7) and dried 30 min at 80° under vacuum. The membrane was prehybridized for 2 hr at room temperature in 50 ml of 6× SSC, 5× Denhardt's reagent, 0.5% sodium pyrophosphate, 0.1 mg/ml boiled salmon sperm DNA, 5% sodium dodecyl sulfate. The oMs475 probe (Rikke *et al.* 1991, 5′-GGAGCC-GACATGAAA-3′) was end labeled to >2,000 Ci/mmole with T4 polynucleotide kinase. Unincorporated label was removed using a DE52 column. Hybridization was carried out overnight at room temperature in 50 ml of 6× SSC, 1× Denhardt's reagent, 0.1 mg/ml tRNA, 0.1 mg/ml polyriboadenylate, and 3 pmol of radiolabeled probe. After hybridization, the filter was washed four times in 6× SSC, 0.05% sodium pyrophosphate, 0.1% sodium dodecyl sulfate at 47°. The filter was exposed at −75° to Kodak AR X-ray film for 14 days with an intensifying screen.

RESULTS

L1EL111 contains a large number of shared sequence variants defining it as belonging to a *M. spretus*-specific LINE-1 subfamily: Preliminary characterization of λEL111 indicated that it actually contained several LINE-1 elements (data not shown). By identifying a $496^+/416C^+/475^+$ restriction fragment and then sequencing outward, we confirmed that all of these shared sequence variations occurred in a single LINE-1 insert, which we call L1EL111. L1EL111 had an inverted bipartite structure (Figure 1), which is a common characteristic of LINE-1 insertions (Hutchison *et al.* 1989; N. C. Casavant and S. C. Hardies, unpublished results). Ms496, Ms416C and Ms475 fell in the same arm of the bipartite structure. Although inserted within another LINE-1 element, L1EL111 clearly was defined as a single bipartite insertion by a 14-bp target-site duplication, a common characteristic of LINE-1 inserts (Hutchison *et al.* 1989). The complete sequence

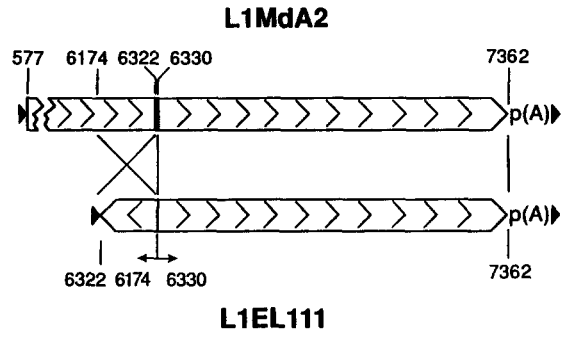

Figure 1.—Structure of L1EL111. Drawing (top) illustrates the canonical LINE structure using the L1MdA2 coordinate system. (LOEB *et al.* 1986). The symbol p(A) represents the 3′ polyadenylated tail. The other drawing (bottom) illustrates the inverted bipartite structure of L1EL111. In L1EL111, the 5′ end up to 6174 is missing, the region from 6174 to 6322 is inverted relative to the 3′ end, and seven base-pairs (6323–6329) are deleted. According to the cDNA synthesis model proposed by Hutchison *et al.* (1989), this structure represents reverse transcription primed at the polyadenylated tail and terminated at 6174, self-priming of second-strand synthesis at 6330, loop cleavage at 6329 and nuclease digestion from 6329 to 6322. The black arrowheads represent a 14-bp target site duplication created by the L1EL111 insertion.

of L1EL111 and its target-site duplication is shown in Figure 2.

The L1EL111 sequence was analyzed to determine if it contained additional shared sequence variants specific to the Ms496 subfamily. The Ms496 subfamily has been subjected to detailed characterization in *M. spretus* (Casavant and Hardies 1994a,b). It developed as a series of successively smaller subfamilies each characterized by additional shared sequence variants (Figure 3). The Ms496 subfamilies split into two clades (distinctive subgroups), one of which is referred to as the Ms475 subfamily. The Ms475 subfamily is defined by hybridization to oMs475 (Rikke *et al.* 1991), which detects a pair of closely spaced shared sequence variants (6852 and 6855 on Figures 2 and 3), and thus is a highly specific hybridization probe even when used in isolation (Herman *et al.* 1992). There are a total of eight shared sequence variants that have been ordered in the LINE-1 lineage springing from the Ms496 progenitor and branching into the Ms475 subfamily. EL111 contains all eight of these *M. spretus*-specific shared sequence variants.

Comparison of L1EL111 with other LINE-1 sequences reveals no base variations that would be inconsistent with its assignment to the *M. spretus*-specific Ms475 LINE-1 subfamily. It contains, as would be expected, a number of shared sequence variations exemplified by positions 7146 and 6920. These variations occurred early in the development of the Ms496 subfamily, and their time of origin relative to the speciation is uncertain. However, L1EL111 should contain all such variants, and it does. Finally, L1EL111 contains only

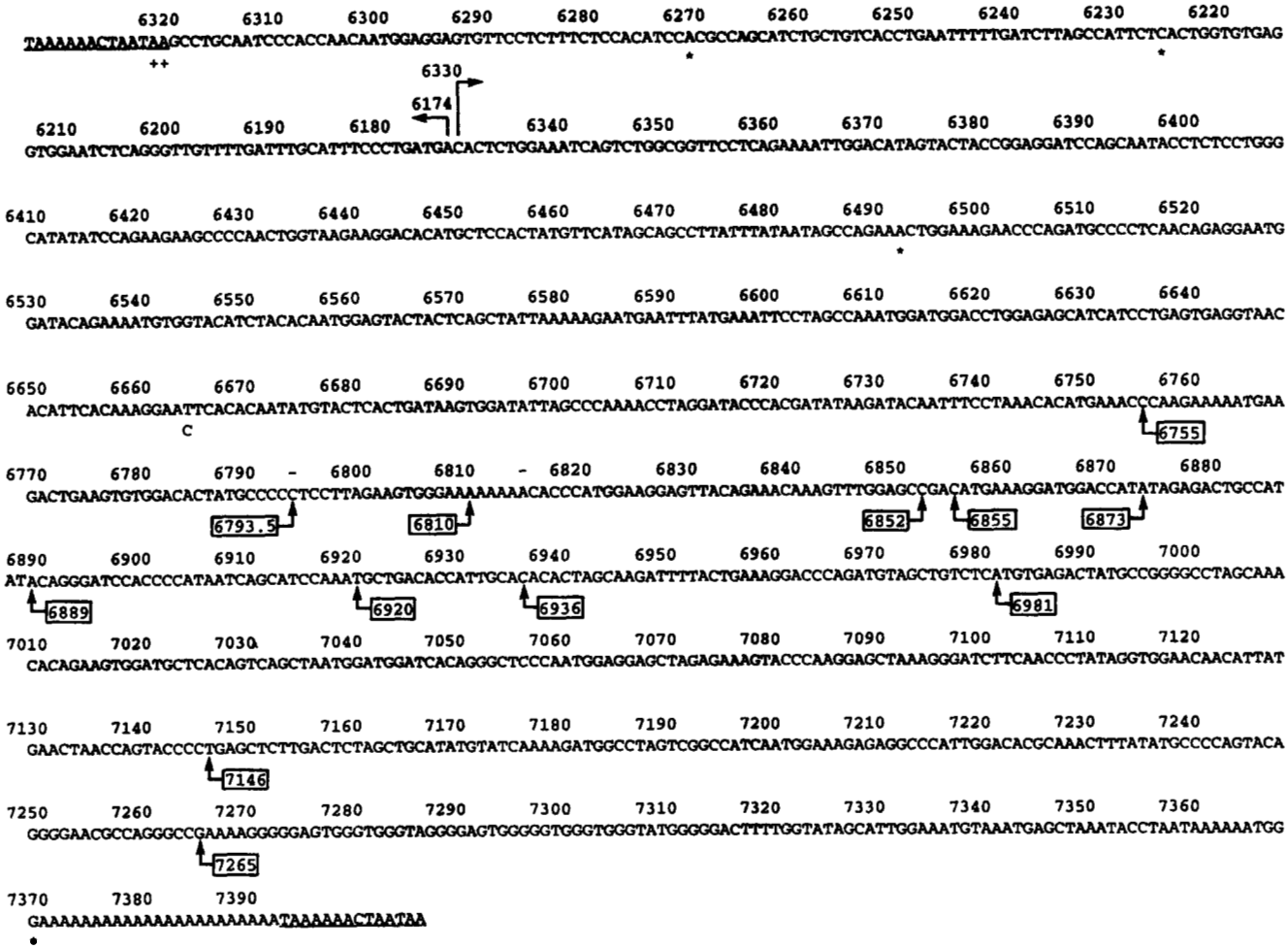

FIGURE 2.—Sequence of L1EL111. Numbering is according to the L1MdA2 coordinate system (LOEB *et al.* 1986). The underlined regions represent the target site duplication. The plus signs indicate a 2-bp overlap between the left target site and LINE-1 sequence. The arrows at 6174 and 6330 indicate the 5 to 3′ direction of the canonical LINE-1 structure. Boxes indicate shared sequence variants that define the *M. spretus*-specific Ms475 subfamily lineage. The C under the T at 6664 indicates that, whereas L1EL111 has a T at this position, other Ms475 members have a C. The caret under the A at 6815 indicates that the A in L1EL111 is a one base insertion relative to other Ms475 members. Dashes above the sequence indicate that EL111 has an insertion relative to L1MdA2. Asterisks indicate nucleotides discussed in the text because of their potential for further characterization of the Ms475 subfamily.

two individualistic base variations: a C-to-T substitution at 6664 and a one base insertion at site 6815. This very low burden of private mutations is consistent with a recent derivation from the Ms475 subfamily (see below). Taken together, these results rule out any possibility that L1EL111 is an old LINE-1 element that has accumulated a large number of sequence mutations of which a couple happen to match *M. spretus*-specific shared sequence variants. Instead, L1EL111 clearly inherited its shared sequence variants as a *bona fide* member of the *M. spretus* Ms475 subfamily.

L1EL111 had a *M.spretus* ancestor within the last 0.2 Myr: We have so far established that L1EL111 had an ancestor in *M. spretus,* but appears in the *M. musculus musculus/M. musculus domesticus* hybrid C57BL/6J. The question arises as to when this transfer may have occurred relative to the traditional estimates of the speciation of *M. spretus* from the subspecies of the *M. musculus* complex. Studies of biochemical divergence have indicated that the speciation of *M. spretus* and *M. domesticus* occurred 1–5 myr ago (SAGE 1981; FERRIS *et al.* 1983b; BONHOMME *et al.* 1984; BONHOMME and GUENET 1989; HAMMER and SILVER 1993), which we indicate as ~3 myr on Figure 3. Those studies suggest that most of the genome stopped exchanging between the two species at that time, although the current result suggests not all of it. The Ms475 subfamily itself has 2000 members in *M. spretus* (RIKKE and HARDIES 1991), but only a handful in C57BL/6J (see below). Therefore, the Ms475 subfamily and the progenitor to L1EL111 came into existence after most exchange between the species had stopped. Similarly, Ms496 (the parent family to Ms475) has 7500 copies in *M. spretus,* but few copies in C57BL/6J (RIKKE and HARDIES 1991). The nine shared sequence variants on Figure 3 between Ms496 and the birth of L1EL111 indicates that much time had

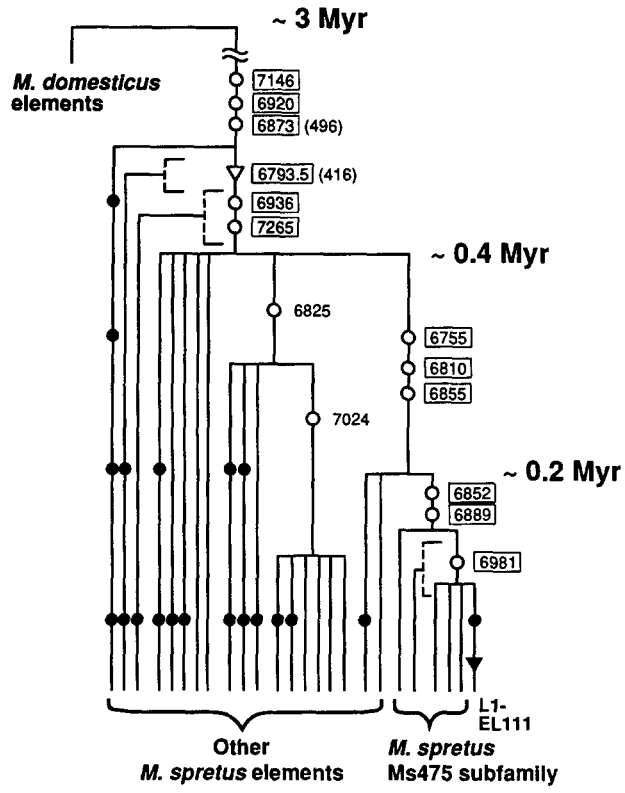

FIGURE 3.—LINE-1 molecular phylogenetic tree analysis of L1EL111. The tree and its calibration was taken from CASAVANT and HARDIES (1994b). The tree illustrates the relationship between L1EL111, previously characterized members of the Ms475 subfamily lineage and *M. m. domesticus* LINE-1 elements. Shared sequence variants, ○ and ▽; unshared sequence variants, ● and ▼; nucleotide substitutions, ○ and ●; nucleotide insertions, ▽ and ▼. The shared sequence variants 6873 and 6793.5 are followed by 496 or 416 in parentheses to indicate the variants which are detected by the *M. spretus*-specific probes oMs496 and oMs416C, respectively. Shared sequence variants also shown on Figure 2 are boxed in.

passed between the isolation of most of the genome and the transfer of L1EL111 or its progenitor from *M. spretus*. Our best estimate of the time of origin of the Ms475 subfamily is based on totaling up the private mutations of all LINE-1 pseudogenes descended from this lineage (CASAVANT and HARDIES 1994b). This method places the origin of Ms475 at ~0.2 myr ago and the birth of L1EL111 at <0.1 myr. Similarly, the single private substitution in L1EL111 is consistent with an age of 0.1–0.2 myr [1 substitution/1070 bp divided by 0.005 substitutions/bp-myr neutral rate (LI *et al.* 1981, 1987)]. Consequently, either L1EL111 or its progenitor must have transferred from *M. spretus* to an ancestor of C57BL/6J long after the majority of the *M. spretus*/*M. musculus* genomes became isolated.

L1EL111 provides for additional characterization of the Ms475 subfamily: L1EL111 was compared with two other young *M. spretus* elements and several non-*spretus* LINE-1 elements revealing additional useful variants as follows: the C at 6226 was present in 3/3 Ms475 *spretus* elements, but not 7/7 non-*spretus* LINE-1 elements. At position 6271, the A was present in 3/3 Ms475 elements, but only one of the non-*spretus* elements. These variants arose either after or just before the speciation of *M. spretus* and the *M. musculus* complex. The A at 6492 is present in (3/4) members of the closely related L1Md-4 subfamily but not in other non-*spretus* elements. The L1Md-4 subfamily joins the LINE-1 tree in the midst of the speciation process and its exact position is currently unclear. Finally, the G at 7369 is present in one other Ms475 member. In contrast, there tends to be an A at this position in non-*spretus* elements.

Other members of the Ms475 subfamily are present in C57BL/6J: The probe oMs475 is specific for two shared variations (sites 6852 and 6855) and therefore can be used in isolation to detect members of this subfamily. It was used in a Southern blot analysis to determine if there might be other Ms475 members besides L1EL111 in the C57BL/6J genome. As a positive control, we used a congenic mouse in which the *M. spretus pearl* locus had been transferred to a C57BL/6J background (RIKKE *et al.* 1993).

The Southern blot analysis was conducted by digesting DNA from *M. spretus*, C57BL/6J, and the congenic mouse with *Eco*RI. Probe oMs475 maps near the 3′ end of LINE-1, and there are no *Eco*RI sites between this position and the 3′ end. Therefore, digestion with *Eco*RI results in fragments each containing the 3′ end of a LINE-1 element and a different length of 3′ flanking region. Consequently, each oMs475 LINE-element will appear on a Southern blot as a single copy band of a unique size. Copy number controls were included to guard against inadvertently identifying repetitively produced fragments as single LINE-1 elements.

As seen in Figure 4, hybridization of oMs475 produced an intense smear in *M. spretus*, six bright and seven faint bands in C57BL/6J, and an additional eight bands in the congenic mouse. The intense smear from *M. spretus* is consistent with the 2000 oMs475-positive copies estimated for this genome by library probing (RIKKE and HARDIES 1991). The number of additional bands visible in the congenic mouse relative to C57BL/6J was somewhat less than, but not inconsistent with, the number of additional bands (20) expected due to the theoretical size (16 cM) calculated for the *M. spretus pearl* locus (RIKKE *et al.* 1993).

The six bright bands in the C57BL/6J pattern were consistent between the two C57BL/6J lines and appear at single copy intensity. The bright 3.3-kb band seen in the C57BL/6J pattern corresponds in size to a 3.2-kb oMs475-positive, *Eco*RI fragment from the λEL111 clone (not shown). The seven faint bands in the C57BL/6J pattern are at the limit of sensitivity; therefore, they appear inconsistently among experiments, and their origin has been difficult to ascertain. Nevertheless, these results indicate that other members of

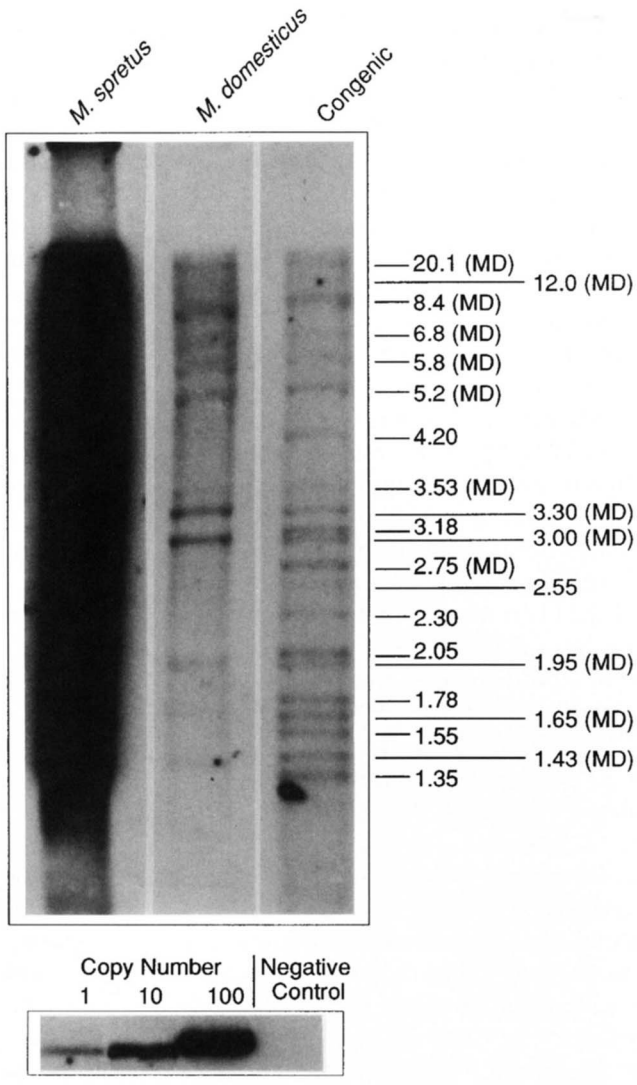

FIGURE 4.—Southern blot analysis of mouse genomic DNAs probed with the LINE-1 oligonucleotide oMs475. Each lane was loaded with 20 μg of *Eco*RI-digested genomic DNA from the source indicated. The congenic mouse was B6-*spretus* $pe^{+Pin}/^{+Pin}$ (RIKKE *et al.* 1993). The copy number controls were constructed from S22, a cloned LINE-1 element known to perfectly match oMs475 (RIKKE *et al.* 1991). The negative control contained 100 copies of S4, which is known to mismatch oMs475 only at the two diagnostic bases (sites 6852 and 6855 of Figures 2 and 3; RIKKE *et al.* 1991). Control DNAs were linearized with *Bgl*II, subjected to electrophoresis on the same gel, blotted and hybridized in the same bag as the genomic lanes. The three genomic lanes were all from the same gel, but were juxtaposed from different parts of the autorad by aligning the slots. Sizes were estimated from an ethidium bromide stained marker lane on the original gel (not shown).

the Ms475 subfamily besides L1EL111 are probably also present in the C57BL/6J genome.

DISCUSSION

The major conclusion of this paper is that *M. spretus* DNA has transferred in recent times across a species barrier into C57BL/6J, which is a representative of the *M. musculus* complex. This was demonstrated by observing members of a *M. spretus*-specific LINE-1 subfamily in the genome of C57BL/6J. A number of studies have indicated that gene transfer has occurred among *M. m. domesticus, M. m. musculus* and *M. m. castaneus* of the *M. musculus* complex before the development of laboratory inbred strains (FERRIS *et al.* 1983a; BISHOP *et al.* 1985; MORIWAKI *et al.* 1985; BLANK *et al.* 1986; BONHOMME and GUENET 1989). However, this is the first report that the genome of at least one such inbred strain also contains sequences derived from *M. spretus*.

It is not presently clear whether or not the transfer of *M. spretus*-specific LINE-1s into C57BL/6J involved transposition. The LINE-1s could be passively hitchhiking on a segment of *M. spretus* DNA migrating through the *M. musculus* population. Alternatively, these inserts could represent transposition out of such a *M. spretus* DNA segment that may have been subsequently lost. It is also not yet clear if the transfer occurred as a single event or as a history of multiple introgressions.

In either case, the presence of the *M. spretus*-specific LINE-1s in C57BL/6J requires that an interspecific hybridization occurred. Breeding between these two species is certainly feasible considering that *M. spretus* and *M. domesticus* can be crossed in the laboratory to produce fertile females (BONHOMME *et al.* 1978). Interestingly, however, studies of hundreds of wild caught *M. spretus* and *M. m. domesticus* mice living together in Southern France and Spain have indicated that such hybridization does not presently occur in nature (BRITTON and THALER 1978; SAGE 1978); although, in Southern France, there was actually one *M. spretus* mouse that was heterozygous for the *M. spretus* and *M. domesticus* alleles at the *Adh*-1 locus (BRITTON and THALER 1978). We would like to interpret our results to mean that interspecific hybridization does occur to some extent in nature. However, we cannot yet rule out the alternative possibility that interspecific hybridization occurred at the hands of European or Japanese mouse fanciers who provided the mice that founded the laboratory inbred strains (MORSE 1981). Whether or not hybridization occurred in nature might be resolved by analyzing wild-caught *M. domesticus* strains with *M. spretus*-specific LINE-1 probes. In particular, the Southern blot method is extremely sensitive to small numbers of *M. spretus*-specific LINE-1 elements and does not require prior knowledge of which loci to examine.

The Southern blot of Figure 4 shows that there could be as many as 13 members of the Ms475 *M. spretus* LINE-1 subfamily in C57BL/6J. This same subfamily has expanded to 2000 members in *M. spretus* itself. If these LINE-1s have moved passively into C57BL/6J, then this would correspond to a transfer of approximately 6.5% (13/2000) of the *M. spretus* genome. Such a small fraction of *M. spretus* DNA easily could have gone undetected in previous studies. For example, the

most extensive survey of the relationship among *M. spretus*, wild *M. domesticus*, and C57BL/6J found no *spretus* alleles in C57BL/6J (SAGE 1978, 1981). However, out of 56 protein encoding loci examined, only 29 were potentially informative (*i.e.*, showed allelic variation). Therefore, the probability of at least one of those loci falling within a given 6.5% region of the genome would be 0.86 ($1-0.935^{29}$), leaving room to have missed a small *spretus* component. In contrast, the oMs475 LINE-1 probe allowed us to survey ~2000 informative loci (*i.e.*, *M. spretus*-specific LINE-1 elements). It would seem, therefore, that species-specific LINE-1 DNA probes provide a powerful means of detecting genetic exchange.

In conclusion, our characterization of *M. spretus*-specific LINE-1 elements in C57BL/6J illustrates several useful properties of LINE-1 subfamilies for the further study of gene flow. One is that members of a subfamily that dispersed in a particular place (or race or subspecies) may be identified by a hybridization probe. Therefore, one can directly search for DNA from that source rather than having to look at a set of preconceived polymorphic loci. Second, because the subsequent generations springing from a particular LINE-1 progenitor each acquire their own distinctive sequence variations, probes might be designed to monitor not only the place, but also the time of origin of a chromosomal segment. Finally, if the element is using transposition to spread after crossing the species barrier, then the probes may offer a rare opportunity to observe the dynamics of a LINE-1 variant's invasion of a new genome.

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