Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7757–7761, October 1990
Genetics

Differential distribution of long and short interspersed element sequences in the mouse genome: Chromosome karyotyping by fluorescence *in situ* hybridization

(B1 and B2 sequence elements/L1md sequence element/gene mapping/chromosome structure/G and R banding)

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Communicated by Sherman M. Weissman, July 18, 1990

ABSTRACT **Fluorescence *in situ* hybridization has been used to demonstrate the differential distribution of interspersed repetitive elements in the genome of *Mus musculus domesticus*. Hybridization with a mouse long interspersed element sequence results in a sharp, highly reproducible banding pattern on metaphase chromosomes, which is quite similar to Giemsa banding for all chromosomes except 7 and X. The families of short interspersed elements, B1 and B2, preferentially cluster in the R, or reverse, bands. There is no evidence of any interspersed repeat present in the centromeric heterochromatic regions. Both the long interspersed element and B2 probes give banding patterns suitable for karyotype analysis. Simultaneous hybridization of the biotinylated long interspersed element probe and a digoxigenin-labeled cosmid to metaphase spreads allows rapid localization of a probe of interest to a particular cytogenetic band on a chromosome.**

Mammalian genomes contain both unique and repetitive DNA sequences. In addition to the highly abundant satellite DNA, which occurs in tandem arrays clustered mostly in the the centromeric heterochromatin region of each chromosome, the genome also contains repeated sequences that are interspersed among single-copy sequences. The two classes of such repeats are short interspersed repetitive elements (SINEs) and long interspersed repetitive elements (LINEs).

The mouse genome contains three predominant families of interspersed repetitive sequences: the L1, B1, and B2 elements (1). The full-length L1md repeat [LINE-1 (L1) of *Mus musculus domesticus*] is ≈6–7 kilobase pairs in length; however most members are variably truncated at the 5′ end (for reviews on L1, see refs. 1 and 2). It is estimated that sequences at the 3′ end are represented 100,000 times in the genome, while there are fewer than 10,000 copies of the full-length sequence (1). Among the mammalian L1 families are subregions that display some degree of homology. Both mouse and human L1 elements contain two open reading frames. One of the most conserved regions between the L1 families of these two species lies in the longer open reading frame, which bears regions of homology with retroviral reverse transcriptases (3). This resemblance provides a clue as to the origin of the L1 sequence and supports the theory that the L1 interspersed elements arose from the reintegration of reverse-transcribed transcripts (4).

Both the B1 [130 base pairs (bp)] and the B2 (190 bp) sequences are SINEs (5). These sequences are abundantly transcribed and give rise to a fraction of heterogeneous nuclear RNA (6). From 130,000 to 180,000 copies of the B1 repeat and from 80,000 to 120,000 copies of the B2 repeat are present in the mouse genome and are found on all chromosomes (7). Experiments by Kramerov *et al.* (6) indicate that the members of the B1 family are nearly identical to one another. Individual members within the B2 family also show a high degree of homology, displaying only 3–5% deviations from the B2 consensus sequence (5). B1 and B2 sequences do share some general organizational features and display short regions of homologies, including segments that are homologous to the consensus RNA polymerase III promoter (5). Furthermore, B1 shows sequence homology to the most abundant human SINE, the *Alu* sequence (for reviews, see refs. 1, 4, and 8). A major structural difference between the two is that B1 is present as monomeric units, whereas *Alu* is a dimer. Both are thought to be derived from 7SLRNA, a component of the signal-recognition particle (9). There have been no reports of B2-like sequences in human.

Hybridization of probes for mouse and human interspersed repeats to genomic DNA fractionated by density-gradient techniques indicates that the distribution of these repeats is nonuniform and conserved (10). Pulsed-field gel electrophoresis analysis reveals that human L1 and *Alu* sequences reside on mutually exclusive DNA fragments larger than a chromosome loop (60,000–120,000 bp) (11). Human *Alu* and L1 probes hybridized to metaphase chromosomes further show that the human *Alu* sequences reside predominantly in R bands, whereas the LINE sequences are in G bands (12–14). G bands, which are stained dark by the Giemsa dye, contain late-replicating DNA and are more A+T rich. Furthermore, there is evidence that many of the tissue-specific genes examined reside in G bands. In contrast, R bands (i.e., *reverse* G bands) replicate early and are more G+C-rich. All housekeeping genes tested to date have been found in R bands (for review of G and R bands, see ref. 15).

Here we present an analysis of the chromosomal distribution of LINE and SINE sequences in the mouse genome by fluorescent *in situ* hybridization. Both B1 and B2 probes reveal a banding pattern similar to R bands, while the L1md probe highlights G bands. Chromosome regions rich in, or devoid of, these repetitive elements are readily observed, and deviations from classical G and R bands can be identified. Furthermore, the clarity and reproducibility of the L1md banding make it a valuable cytogenetic tool, permitting rapid chromosome identification and, in conjunction with a differentially labeled DNA probe, gene localization on simultaneously banded chromosomes.

MATERIALS AND METHODS

DNA Probes. pSP6-4 plasmids containing entire mouse B1 and B2 sequences were provided by Karen Bennett (University of Missouri, Columbia). Probe KS13A containing the 1.3-kilobase (kb) *Eco*RI fragment from the middle of the mouse L1 sequence was obtained from Thomas Fanning

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Abbreviations: SINE, short interspersed repetitive element; LINE, long interspersed repetitive element; L1, LINE-1.

(National Institutes of Health). David Housman (Massachusetts Institute of Technology) provided the cosmid MαG-6, containing 40 kb of the Na^+/K^+ ATPase α1 subunit gene (16).

Mouse Metaphase Preparations. Female mouse metaphase spreads from spleen cells were prepared using a modification of the method described by Sawyer *et al.* (17). Spleen cells were cultured in RPMI 1640 medium/20% fetal bovine serum/Con A at 6 μg/ml/2-mercaptoethanol at 150 μg/ml for T-cell stimulation. After 48 hr, ethidium bromide (final concentration of 25 μM) and colcemid (0.1 μg/ml) were added for 30 min. Standard techniques for hypotonic treatment, methanol/acetic acid fixation, and slide preparation were used (18).

Probe Labeling. Probes B1, B2, and KS13A were labeled by nick translation with biotin-11–dUTP (19). Cosmid MαG-6 was labeled by nick translation using a mixture of digoxigenin-11–dUTP and dTTP in a ratio of 1:3 (20). Unincorporated nucleotides were removed using a Sephadex G-50 medium spin column equilibrated with 10 mM Tris·HCl/1 mM EDTA/0.1% SDS, pH 8.0.

***In Situ* Hybridization.** (*i*) Forty to one hundred nanograms of a biotinylated repetitive sequence probe and 7 μg of DNase-treated salmon sperm DNA were ethanol-precipitated together and redissolved in 10 μl of hybridization mixture [50% (vol/vol) deionized formamide/2× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/10% dextran sulfate]. After denaturation at 75°C for 5 min, the probes were placed on ice before application to denatured metaphase spreads. Slides were denatured in 70% deionized formamide/2× SSC at 70°C for 2 min and then dehydrated through cold 70%, 90%, and 100% ethanol, 5 min in each. Probe and specimen were incubated under a sealed coverslip in a moist chamber at 37°C overnight for hybridization. Posthybridization blocking steps and washes were as described by Lichter *et al.* (21). Biotinylated probes were detected using fluorescein isothiocyanate–avidin DCS (Vector Laboratories) at 5 μg/ml. Chromosomes were counterstained with propidium iodide (200 ng/ml), which was added to the antifade mounting solution (22).

(*ii*) In double-label experiments, biotinylated KS13A probe (30 μg/ml) in 5 μl of hybridization mixture was denatured at 75°C for 5 min and placed on ice. Digoxigenin-labeled cosmid MαG-6 (10 μg/ml), salmon sperm DNA (700 μg/ml), and mouse genomic competitor DNA (200 μg/ml) in 5 μl of hybridization mixture were denatured at 75°C for 5 min and then allowed to partly hybridize at 37°C for 10 min (see ref. 21 for details of suppression hybridization). The two probe mixtures were combined and immediately added to the denatured chromosome preparation. Detection of the KS13A probe was as described above. Digoxigenin-labeled MαG-6 was detected by incubation with sheep anti-digoxigenin Fab fragments (4 μg/ml) (Boehringer Mannheim) and then with Texas red-conjugated donkey anti-sheep antibodies (15 μg/ml) (Jackson ImmunoResearch). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (200 ng/ml).

Imaging. Chromosome spreads were imaged by fluorescence using either a wide-field microscope [Zeiss Axioskop; 63× 1.25 numerical aperture Plan Neofluar oil-immersion objective equipped with a cooled charge-coupled device camera (Photometrics CH220)] or by means of a confocal laser scanning microscope (Bio-Rad MRC-500 scanner; Nikon Optiphot microscope; 60× 1.4 numerical aperture Plan Apochromat oil-immersion objective). Charge-coupled device image-acquisition and processing employed an Apple Macintosh IIx computer running custom software developed by Marshall Long (Department of Chemical Engineering, Yale University). Images were recorded sequentially for the banding probe, cosmid probes (when used), and counterstain. 4′,6-Diamidino-2-phenylindole could be imaged with wide-field instrument only. The wide-field microscope was equipped with precision bandpass filters (Zeiss) to reduce image displacement to less than ±1 pixel (approximately ±0.05 μm) as filter cubes are switched. Grey-scale manipulation/thresholding of the 8-bit source images was performed using either the MRC-500 software for confocal images or the Enhance software package (MicroFrontiers, De Moines, IA) for wide-field images. Merging and 24-bit pseudocoloring were done on the Macintosh IIx computer by using software developed in this laboratory (T. Rand, M. Ferguson, S.G.B., unpublished work). Final images were photographed from the computer monitors.

RESULTS

When a biotinylated L1 probe, KS13A, derived from the middle region of L1md was hybridized to mouse metaphase spreads, a sharp highly reproducible banding pattern was seen (Fig. 1*A*). The chromosomes can be easily paired, and the banding pattern is consistent with Giemsa bands for virtually every chromosome. Fig. 1*F* compares a published Giemsa banding for chromosomes 1–7 with the L1 banding. The full karyotype of this metaphase spread is shown in Fig. 1*B*. A suitable DNA counterstain, such as propidium iodide, is used both to delineate the entire lengths of the chromosomes and to enhance the contrast of the bands, permitting better visualization of some minor bands. Several features of the hybridization banding warrant mention. Most Giemsa banding protocols also stain the centromeric heterochromatin, but the L1 probe does not hybridize at detectable levels to this region on any chromosome. A second difference between the banding patterns produced by the L1 probe and Giemsa stain concerns band intensities. Variations in Giemsa band intensities have proven useful in identifying mouse chromosomes (17, 24, 25). Although some L1 bands have the expected lower intensity (e.g., cytogenetic bands 1H2, 1H4, 10C2, and 18E2), most stain quite brightly. The most dramatic example of unexpected band intensities is found on chromosome 7. Giemsa or quinacrine-stained chromosome 7 is characterized by two equally heavy middle bands, designated 7C and 7E, on a background of lightly staining, low-intensity bands. At first glance, the L1-banded chromosome 7 is barely recognizable. Closer inspection shows that there are indeed two equally stained bands, 7C and 7E, but these are not the only intense bands. Bands A3 and B4 are also very prominent, and bands 7D2 and 7E3 are clearly visible (Fig. 1*D*). For a comparison of Giemsa-banded and L1-banded chromosome 7, see Fig. 1*F*. Thus, the position of the bands is similar to G bands, but some exhibit different intensities.

The X chromosome is also noteworthy. While some preparations do indicate bands, especially the heavy E band (see Fig. 1*E*), no significant G-negative bands appear on the X chromosome by the hybridization method. Thus, the X chromosome is exceptionally rich in LINE sequences.

The L1 hybridization banding patterns were also compared with quinacrine banding (25, 26) after the chromosomes were subjected to *in situ* hybridization (data not shown). Such chromosome preparations do not routinely band as sharply or reproducibly with quinacrine as nondenatured chromosomes. For this reason, as well as others mentioned in the discussion, we find L1 hybridization to be the method of choice.

Hybridization of DNA probes for either of the two mouse SINE families, B1 and B2, also gives banding patterns on mouse chromosomes (Figs. 1*C* and 2*A*). While the B2 banding is not as sharp and detailed as the L1 pattern, it is amenable to karyotyping (see Fig. 2*B*). The major bands are consistent with the major early replicating bands described by Somssich *et al.* (i.e., similar to R bands) (27). A number of chromosomes, such as chromosomes 3, 6, and 10, do not display all of the expected bands, but they do show faint B2

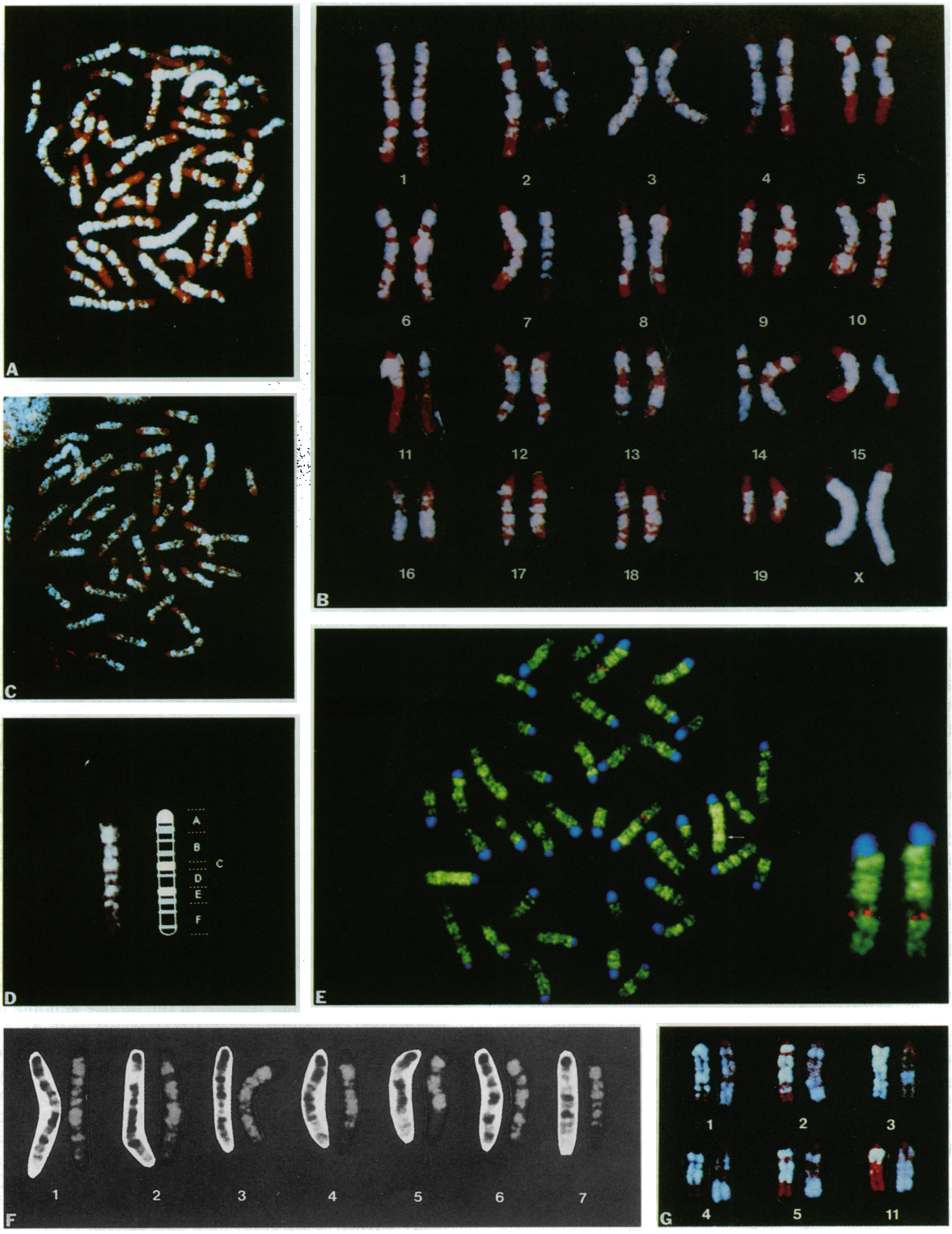

FIG. 1. Hybridization of interspersed repetitive sequence probes to female mouse metaphase chromosome spreads. Except where indicated, the fluorescein isothiocyanate signal is displayed white and the propidium iodide counterstain red. (*A*) Hybridization with biotinylated KS13A, a clone containing a 1.3-kb DNA fragment from the midregion of the L1 repeat. (*B*) Karyotype prepared from the metaphase spread in *A*; note the absence of centromere labeling. (*C*) Hybridization of biotinylated B1 sequence to mouse chromosomes. (*D*) Digitized image of chromosome 7 from *B* aligned with an idiogram of chromosome 7; only major bands are indicated. (*E*) Simultaneous hybridization of biotinylated L1 probe, detected with fluorescein isothiocyanate–avidin and digoxigenin-labeled MαG-6, detected with Texas red-conjugated antibodies. Fluorescein isothiocyanate is shown as green, Texas red as red, and the 4′,6-diamidino-2 phenylindole counterstain as blue. Arrow indicates the prominent E band on chromosome X. The homologous chromosomes 3, each with a signal on both chromatids, are enlarged in the lower right corner. (*F*) Comparison of Giemsa (left)- and L1 (right)-banding patterns for chromosomes 1–7. Band positions are nearly identical in each case, although band intensities on chromosome 7 differ when using the two methods. Giemsa-banded chromosomes were reproduced with permission from Evans (ref. 23; copyright Oxford University Press). (*G*) Chromosomes 1–5 and 11. The left member of each pair is hybridized with L1 probe, and the right chromosome is decorated with B2 probe. Note the reverse banding patterns, which are especially evident on chromosome 11.

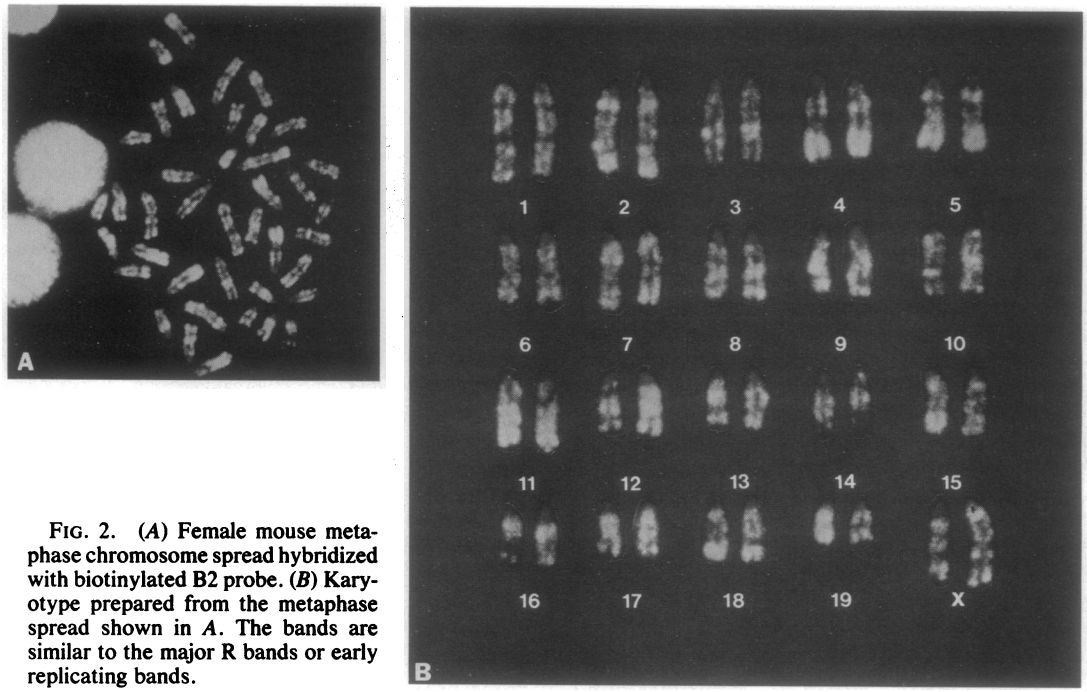

FIG. 2. (*A*) Female mouse metaphase chromosome spread hybridized with biotinylated B2 probe. (*B*) Karyotype prepared from the metaphase spread shown in *A*. The bands are similar to the major R bands or early replicating bands.

hybridization signals, possibly reflecting a low number of B2 elements in these areas. The high degree of homology displayed among individual members of either the B1 or B2 families suggests that the differences between the observed hybridization banding and the expected R banding pattern are not from weak homology between the probe and target sequences. For each chromosome, the L1 and B2 sequences clearly occupy distinct chromosomal domains that are, in fact, the reverse of one another. Direct comparison of chromosomes 1–5 after hybridization with L1 and B2 probes is shown in Fig. 1*G*; chromosome 11 is also included as an extreme example of their differential distribution.

Although the B1 family is one of the most abundant interspersed repeats in the genome, it gives the weakest signal of the three repeats (Fig. 1*C*), and the bands are grainy and indistinct. We have been unsuccessful in preparing a complete karyotype using B1. Comparison of some of the more recognizable chromosomes, such as 1–5, 11, and 16–19 with the corresponding B2-banded chromosome (data not shown), indicates that the two SINE families are distributed similarly in the genome. However, a thorough analysis was not possible due to the overall poor quality of the B1 banding.

Because of its sharp, strong, and reproducible banding, the L1 probe provides a useful method for karyotyping mouse chromosomes by fluorescent *in situ* hybridization. Thus, this probe can be used to facilitate the high-resolution mapping of unique sequence DNA clones. Fig. 1*E* shows a mouse metaphase spread hybridized with both the biotinylated L1 probe and digoxigenin-labeled genomic cosmid clone (MαG-6) for the Na^+/K^+ ATPase Gα1 subunit. The red cosmid signal is clearly visible on both chromatids of both homologs (see Fig. 1*E*, *Inset*). Hybridization efficiencies also were similar to those achieved by Lichter *et al.* (20) and Landegent *et al.* (28). The fact that the blue 4′6-diamidino-2-phenylindole counterstain does not show in the G negative bands is due solely to limitations of the current computer program used to merge images from the charge-coupled device camera. The MαG-6 clone maps to band 3F3 on chromosome 3, in agreement with the previously reported localization achieved by Southern blot analysis of somatic-cell hybrid panels and restriction fragment length polymorphism analysis of crosses between *Mus musculus* and *Mus spretus* (16).

DISCUSSION

We have presented cytological evidence that the major interspersed repeat families of the mouse, the LINE L1 element and the SINEs, B1 and B2, occupy discrete positions on metaphase chromosomes, which correspond to G bands and R bands, respectively. These findings are consistent with results obtained with human interspersed repeats, by both *in situ* hybridization (12–14) and pulsed-field gel analysis (11). In view of the fact that the SINE and LINE families appear to have amplified throughout the genome after divergence of the primate and rodent lineages (29), it is interesting to note that these two interspersed repeat families seem to have preferentially inserted into the same discrete subregions of the genome during their independent evolution.

None of these repeats hybridize at any detectable level to the centromeric heterochromatin regions, the known location of the major satellite DNA (30), indicating that there are few or no interspersed repetitive sequences in these regions of the chromosomes. Other regions of the mouse genome lacking a significant accumulation of L1 elements include the distal two-thirds of chromosome 11 and distal segments of chromosomes 5 and 15 (see Fig. 1*B*). In contrast, the X chromosome is so rich in L1 that Giemsa-negative bands are virtually obscured; there are, however, regions on X chromosome that have sufficient concentrations of the B2 sequence to be detectable by *in situ* hybridization. Thus, at the level of resolution afforded by this technique, the LINEs and SINEs on the X chromosome are not necessarily clustered in mutually exclusive chromosomal subregions.

These observations lead to interesting questions concerning chromosomal architecture. If the LINEs and SINEs are retroposons that were dispersed through the genome by reintegration of reverse transcriptase products, why are some chromosomes more receptive to one element or the other? For example, what is different about the structures of chromosomes 11 and X, such that many LINEs were readily reintegrated into DNA throughout the X chromosome but were reintegrated only in limited regions of chromosome 11, in contrast to the high levels of SINEs found in the distal two-thirds of chromosome 11? Another puzzling issue concerns the differences in the B1 and B2 distribution. Although both exhibit banding patterns that are similar and coincide

with R bands, the B1 "banding" is much more diffuse, such that sharp, clear bands are not as readily distinguished. The B1 elements appear more dispersed throughout the genome, suggesting that reintegration of B1 sequences was not as tightly restricted to R bands as were B2 sequences. Whether this is from an inherent property of the B1 element itself or reflective of differential levels of timing or expression of B1 and B2 transcripts is unclear. The answers to these and other questions concerning the functions, distributions, and origins of the LINEs and SINEs require additional investigation.

Comparison of gene loci as well as chromosome banding patterns can reveal homologies among species that lead to insights into evolutionary relationships between the species. Extensive linkage and synteny maps exist between mouse and human (31). Sawyer and Hozier (32) compared syntenic regions for several Giemsa-banded mouse and human chromosomes and noted extensive similarities on the subset of chromosomes they examined. We also see a gross correlation of L1-rich and L1-deficient regions in the two species when we compare our mouse L1 distribution with that published for the human L1 (13). One notable exception is the region on mouse chromosome 7, mapping roughly from 7A3 to 7B2, which is syntenic with human 19q13.1–13.4. More than 10 genes are assigned to this syntenic group. Although this region on mouse chromosome 7 usually stains very lightly with Giemsa, as does the distal part of human 19q, this region is unusually rich in L1 sequences. In contrast, the published human L1 distribution indicates that this syntenic region of 19q is relatively deficient in L1 sequences, as would be predicted from its Giemsa-staining pattern. The significance of this finding is unclear. Perhaps it reflects more recent reintegration events of L1 elements in this region of mouse chromosome 7 or maybe differences in the evolutionary history of the two species.

We have also demonstrated that the chromosome-banding pattern achieved with the L1 probe facilitates the physical mapping of mouse clones by fluorescence *in situ* hybridization, the latter being a technique that has been used extensively for mapping human DNA clones (20, 27, 33–36). The use of *Alu* hybridization banding in combination with gene mapping has been reported (20). Several factors have hampered large-scale efforts to map mouse genes by fluorescent *in situ* hybridization. The mouse chromosomes are among the most difficult to karyotype. The 40 mouse chromosomes are all telocentric and size variation between them is minimal, thus making an initial chromosome identification based on morphology nearly impossible. Hybridization by using multiple known marker clones to tag chromosomes is possible; however, this technique requires several successive experiments, and suitable tags for each mouse chromosome are not readily available. While numerous protocols for gene mapping on banded chromosomes have been reported (e.g., refs. 37–39), most require two photographic steps (before and after hybridization) and pre- or posthybridization staining with fluorophores or dyes for karyotyping. Not only are two photographic steps extremely time-consuming, but one runs the risk of losing documented spreads during the *in situ* hybridization procedure. None of these prior approaches complements the ease and speed of fluorescence *in situ* hybridization mapping as readily as the L1-hybridization banding approach. Giemsa staining protocols reduce the fluorescence intensity of the labeled probe. Quinacrine banding is commonly used for mouse karyotyping (25, 26) but suffers from low-contrast bands and rapid photobleaching. Unlike quinacrine, the L1 bands obtained by *in situ* hybridization do not fade as readily, thereby allowing both prolonged inspection at the microscope and long-term storage of the slide without significant signal loss. Furthermore, because we had demonstrated (40) that the L1 sequence probe is species specific, this method can be used to selectively band chromosomes of murine origin in mouse–hamster hybrid lines.

We thank Karen Bennett, Thomas Fanning, and David Housman for providing clones; Dimitrina Pravtcheva, Peter Lichter and Joan Menninger for very helpful discussions; and Alan Weiner for critically reading this manuscript. We thank Dorothy Greenberg for preparing this manuscript. This research was supported by Grants GM41596, GM40115, and HG00272 from the National Institutes of Health.

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