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Evidence for an Early Evolutionary Origin and Locus Polymorphism of Mouse VL30 DNA Sequences

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The VL30 sequences of mouse DNA are a family of sequences with retrovirus-like structure which code for a 30S RNA transcript that can be packaged into the virions of murine leukemia viruses and thereby transmitted from cell to cell. A Southern blot analysis of these sequences revealed that multiple copies are present in the DNA of all mice examined, regardless of species or geographic origin. Considerable locus polymorphism was also apparent, and at least one of these polymorphisms appeared to reflect the differing chromosomal location of a complete VL30 sequence. These data indicated that VL30 elements are not recent additions to the mouse genome and suggested that the evolution of the VL30 multigene family has been accompanied by duplication and dispersion of VL30 sequences to diverse genomic sites. In addition, we reexamined the issue of genetic relatedness between mouse VL30 sequences and a physically similar family of virus-like elements in the rat genome. We found that many, if not all, rat and mouse VL30 loci contain regions of sequence homology. These data suggested that rodent VL30 sequences have evolved from a common ancestral sequence.

The mouse VL30 family of DNA sequences derive their name from the fact that their transcripts can be packaged into either infecting or activated endogenous retrovirus particles and transmitted between cells by pseudotype infection (1, 10, 21, 22), making them virus-like, and from the fact that these transcripts are 30S. These VL30 sequences are organized as a family of related structures which are present in multiple copies and dispersed throughout the mouse genome (4, 14). VL30 DNA sequences are 5.5 kilobases (kb) long and contain 0.4-kb direct terminal repeats (13). This type of structure is analogous to those of both integrated retrovirus proviruses (5, 23, 24, 29) and procaryotic and eucaryotic transposable genetic elements (2, 15). The structural similarities among proviruses, transposable elements, and VL30 sequences suggest an evolutionary relationship (30). Retroviruses could thus be regarded as transposable elements which have evolved in such a way as to extend their repertoire of reintegration beyond the genome of origin. VL30 sequences may be additional examples of this process, being capable of virion packaging, cell-to-cell transmission, and copying by reverse transcriptase but lacking a functional set of replicative genes. An alternative explanation is that VL30 sequences represent defective retroviral genomes which have arisen by infection of the germ line. This could have occurred either early in rodent evolution or relatively recently. A third possibility is that the VL30 sequences originated from a cellular mRNA which fortuitously was able to be packaged and transcribed by the RNA-dependent DNA polymerase of a murine leukemia virus. Successive cycles of transmission of this cellular RNA would select for variants able to be more efficiently packaged and transcribed as well as introduce multiple copies of these sequences at dispersed sites in the mouse genome.

Early studies with cDNA probes provide evidence that multiple copies of VL30 sequences are also present in the DNA of two Asian *Mus* species, *M. caroli* and *M. cervicolor* (22). Although alternative explanations are possible, one interpretation of these results is that VL30 sequences arose in the germ line of mice before *Mus* speciation. In the present study we examined the geographical distribution, locus variation, and sequence conservation of mouse VL30 sequences in a variety of different laboratory strains and feral subspecies of *Mus musculus* and other *Mus* species. Our findings confirmed that VL30 sequences are not limited to mice of restricted geographic origin, as is the case with the endogenous ecotropic AKR-type provirus genome (27), and also suggested that VL30 DNA did not arise from relatively recent insertions into the germ line.

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MATERIALS AND METHODS

Mice. The sources for the mice used are shown in Table 1.

Cells and cell culture. The cell lines and culture conditions used were those described previously (7, 8).

DNA preparation. The isolation of DNA from mouse livers and mouse embryos was as described previously (26). The isolation of DNA from culture cells and the preparation of phage were performed according to standard procedures as previously described (4).

Restriction endonucleases. Restriction endonucleases were obtained from New England Biolabs, Boehringer Mannheim Corp., and Bethesda Research Laboratories. Generally, the digestion conditions recommended by the supplier were used. Reaction completion was monitored by adding a sample of each incubation at time zero to 1 μg of wild-type phage λ DNA and assaying for complete digestion of phage λ DNA by electrophoresis.

Southern blots. The method used for Southern blots was that of Southern (25) with the modifications of Jeffreys and Flavell (12). Probes were prepared by preparative gel electrophoresis (18) and labeled in vitro by nick translation (17). Hybridizations were performed in thermally sealed plastic bags as previously described (4).

RESULTS

We used the Southern blot technique to study the organization of VL30-related sequences in DNA isolated from various cell lines, inbred laboratory animals, and wild isolates representative of widely diverse geographic origins (Table 1). Figure 1 illustrates a partial restriction map of a VL30 clone used in these studies. This clone, termed BVL-1, was isolated from a BALB/c Charon 4A λ library and contains a complete 5.5-kb VL30 sequence (4). In addition, it contains non-VL30 flanking sequences harboring both single-copy and highly repetitive sequences. The repetitive sequences were detected by annealing fragments of the clone to total

TABLE 1. Mouse strains and cell lines

Mouse strain	Tissue	Origin	Source
M. musculus			
AKR/J	Embryo	American laboratory strain	Jackson Laboratory
AKR-2B	Cultured fibroblasts	American laboratory strain	H. L. Moses
C3H/Fg	Embryo	American laboratory strain	Massachusetts Institute of Technology Center for Cancer Research Animal Colony
C3H/An	Liver	American laboratory strain	Massachusetts Institute of Technology Center for Cancer Research Animal Colony
C3H/HeJ	Liver	American laboratory strain	Jackson Laboratory
C3H/10T½ (C3H/He)	Cultured fibroblasts	American laboratory strain	C. Heidelberger
DBA/2J	Liver	American laboratory strain	Massachusetts Institute of Technology Center for Cancer Research Animal Colony
FM	Cultured fibroblasts	Japanese laboratory strain	H. Yoshikura
RR	Cultured fibroblasts	Japanese laboratory strain	H. Yoshikura
subsp. *mollosinus*/Jackson	Liver	Japan	Jackson Laboratory
subsp. *domesticus* MIT no. 1	Liver	Cambridge, Mass. (originated in western Europe)	R. Weinberg
subsp. *domesticus* CLW	Liver	Boston, Mass. (originated in western Europe)	R. Weinberg
subsp. *musculus* (Vejrumbo)	Liver	Denmark	V. Chapman
subsp. *castaneus*	Liver	Southeast Asia	V. Chapman
subsp. *brevirostris*	Liver	California (originated in western Europe)	R. Callahan
M. cookii	Liver	Southeast Asia	R. Callahan
M. spraetus	Liver	Spain	R. Sage

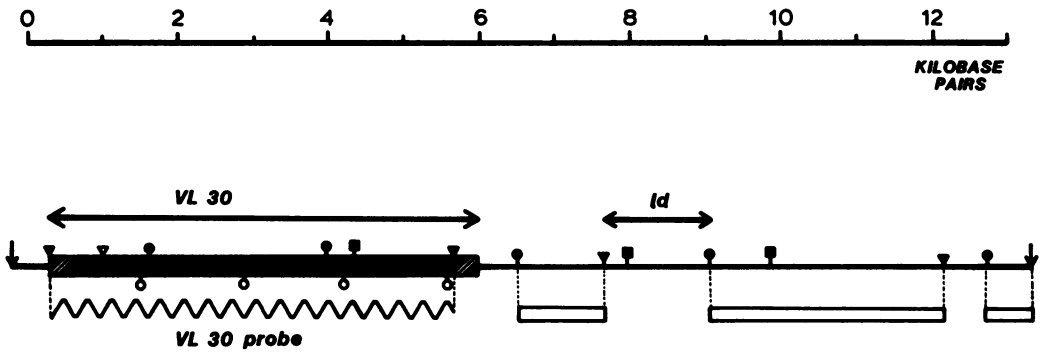

FIG. 1. Map of VL30 clone BVL-1. Orientation is defined with respect to the phage λ Charon 4A vector arms. The solid bar represents the internal portion of the VL30 sequence, the hatched bars represent VL30 long terminal repeats, and the open bars represent highly reiterated DNA. Restriction enzyme cleavage sites shown are *Eco*RI (↓), *Hin*dIII (◇), *Xho*I (▼), *Kpn*I (▽), *Pst*I (●), and *Bam*HI (■). The locus-defining (ld) sequence lies between the indicated *Xho*I and *Pst*I sites. The VL30 probe (wavy line) used was a mixture of the VL30 internal fragments derived from a *Hin*dIII-*Xho*I digest and isolated by preparative gel electrophoresis. This probe was determined to be representative of the entire VL30 sequence, including one copy of the long terminal repeat, and was not detectably contaminated by non-VL30 sequences as determined by analytical gel electrophoresis.

mouse DNA under conditions in which only repetitive sequences hybridize (9).

In our initial analysis, DNAs from various strains and subspecies of mice were digested with restriction endonuclease *Eco*RI, electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized to a VL30 sequence probe (Fig. 2). This probe was derived from the BVL-1 clone (4) and consists of all of the VL30 sequences, including one copy of the

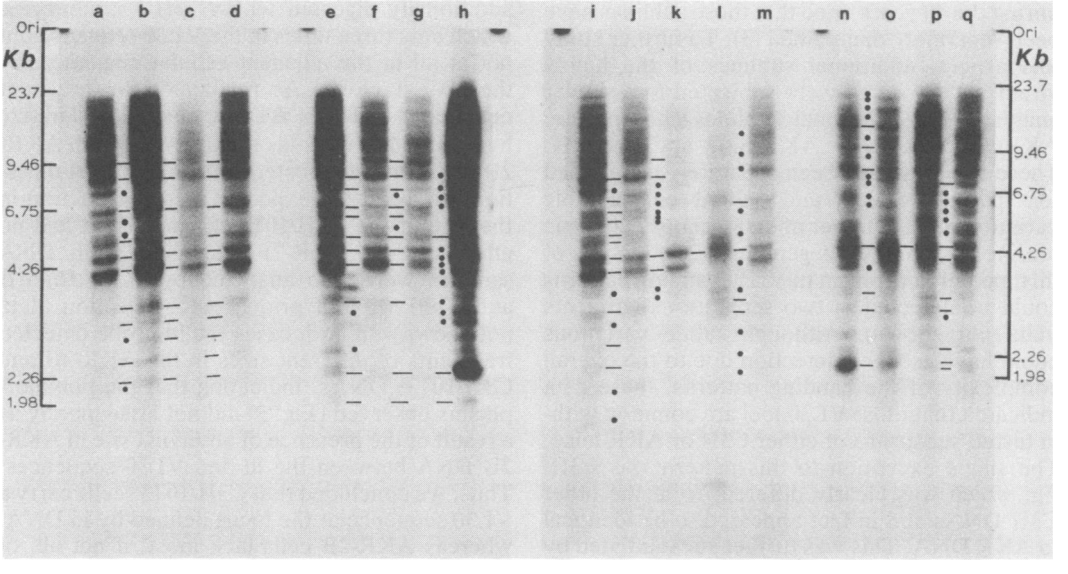

FIG. 2. VL30 sequence organization in various mouse strains. Samples were digested to completion with *Eco*RI, electrophoresed (10 μg per lane) through 0.7% agarose gels for 36 h at 25 V, transferred to nitrocellulose, and hybridized to ^{32}P-labeled VL30 probes. *Hin*dIII-cut wild-type phage λ DNA fragments served as size markers. As a guide, pairwise comparisons were made between adjacent lanes. Certain fragments which appeared to be common were joined with a line; those different were joined with a dot. Some of the apparently common fragments may represent coincidental comigration of different DNA sequences. DNA samples were from AKR-2B (a); C3H/10T½ (b); C3H/An (c); C3H/HeJ (d); C3H/Fg (e); FM (f); RR (g); *M. musculus* subsp. *mollosinus* (h); *M. musculus* subsp. *domesticus* MIT no. 1 (i); *M. musculus* subsp. *domesticus* CLW (j); *M. spraetus* (k); *M. cookii* (l); DBA (m); *M. musculus* subsp. *musculus* (Vejrumbo) (n); *M. musculus* subsp. *castaneus* (o); *M. musculus* subsp. *brevirostris* (p); and AKR/J (q).

long terminal repeat (Fig. 1). Internal heterogeneity within the VL30 sequences results in the presence of *Eco*RI sites in some, but not all, of these sequences. As a consequence, the sizes of the DNA fragments detected by the Southern technique are a function of the distribution of *Eco*RI sites within each VL30 sequence as well as the positions of these sequences within the mouse genome.

VL30 sequences were present in multiple copies in the genomes of all mice analyzed (Fig. 2). In spite of this uniform geographic distribution, however, considerable polymorphism in the organization of these VL30 sequences was observed. This is apparent when different species, different subspecies, different individuals of the same subspecies, and different inbred American laboratory strains, e.g., AKR, C3H, and DBA, are compared (Fig. 2). Our impression is that more distantly related mice have a greater number of differences from one another, but the complexity of the pattern makes this conclusion less certain.

It is also of interest to note that within a given inbred strain, e.g., C3H/He, C3H/An, and C3H/10T½, all DNAs generally appeared to be identical. This finding was of interest since it could indicate that VL30 sequences have not undergone extensive rearrangements or transpositions during the 40 years or so that these sublines have been separately maintained (3). To further study this aspect, additional sublines of the highly viremic AKR strain were subjected to a similar analysis. The additional sublines chosen were AKR/Gs, AKR/Fu, AKR/Ski, and AKR/N. These sublines have been separately maintained for several decades and exhibit considerable variation in the number of endogenous ecotropic murine leukemia virus genomes (28). In spite of this, no polymorphism in VL30 banding patterns could be detected in two separate experiments (data not shown). Although subtle variations could have escaped detection due to the overall complexity of the banding patterns, these data indicated that most VL30 loci are common within tested substrains of either C3H or AKR mice. The single exception to this pattern was C3H/Fg, which was clearly different from the other C3H DNAs and in fact appeared to be identical to AKR DNA. This was further substantiated by the BVL-1 locus analysis described below and may indicate genetic impurity in the derivation of the C3H/Fg substrain. This may be particularly significant in view of the fact that C3H/Fg animals are highly viremic and leukemic, quite unlike other C3H lines but very similar to the AKR strain.

It was important to determine whether the observed differences in the Southern blot profiles (Fig. 2) reflected simple restriction site polymorphisms, perhaps due to single base changes, or different locations of VL30 elements in the various genomes. To investigate this question, we studied the organization of DNA at a specific locus defined by a piece of single-copy DNA (ld) located adjacent to the VL30 sequence in clone BVL-1 (Fig. 1).

Initially, we hybridized the ld nucleic acid probe to DNA from AKR-2B and C3H/10T½ cells digested with various restriction endonucleases (Fig. 3A). Digestion of C3H/10T½ DNA with *Eco*RI produced a single hybridizing DNA fragment of approximately 13 kb (lane a). This is the same size as the BVL-1 clone, which was also derived from an *Eco*RI digest, indicating that a VL30 sequence is present at the locus defined by the ld probe in the C3H/10T½ genome. *Eco*RI digestion of AKR-2B DNA produced a single hybridizing fragment of approximately 8 kb. Since the difference in size between the fragments from AKR-2B DNA and C3H/10T½ DNA (5 kb) is about the size of the VL30 sequence present in the BVL-1 clone, this result suggested that the difference observed between these two DNAs might be due to the presence of a VL30 sequence in the locus defined by ld in the C3H/10T½ genome and the absence of a VL30 sequence at this locus in the AKR-2B genome. To test this hypothesis, these two DNAs were additionally digested with *Hin*dIII, an enzyme which cuts three times in the VL30 sequence but not at all in the adjacent cellular sequences of the BVL-1 clone. As predicted, the fragment detected in C3H/10T½ DNA was reduced in size by about 5 kb, whereas the fragment from AKR-2B DNA was unaffected. Similarly, *Kpn*I digestion produced the expected 1.5-kb reduction in the size of the C3H/10T½ fragment but had no effect on the AKR-2B fragment. Both DNA samples were also analyzed by using *Hin*dIII and *Kpn*I without prior *Eco*RI digestion (data not shown). In both cases the ld probe detected fragments of different sizes in the AKR-2B and C3H/10T½ DNAs, indicating that the polymorphisms observed (Fig. 3) did not arise merely as a result of the presence of an *Eco*RI site in AKR-2B DNA between the ld and VL30 sequences. Thus, we concluded that C3H/10T½ cells carry a VL30 sequence at the locus defined by ld DNA, whereas AKR-2B cells lack most, if not all, of such a sequence.

The ld sequence was also used to study the distribution of alleles among geographically dispersed mice (Fig. 3B). DNA from *Mus musculus* subsp. *brevirostris* and *Mus musculus* subsp. *musculus* generated 13-kb fragments, indicating that they carry VL30 sequences at this locus, whereas DNA from AKR/J, DBA/2J, and *Mus musculus* subsp. *domesticus* generated an 8-kb band, suggesting that they lack a VL30 sequence

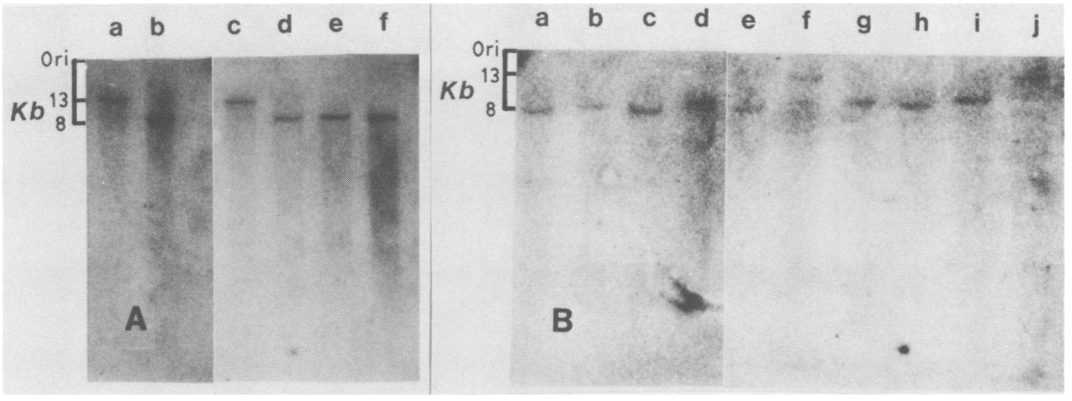

FIG. 3. Polymorphism at the BVL-1 locus. A portion (10 μg) of each appropriately digested DNA sample was run on 1% agarose gels at 30 V for 20 h, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled ld sequence (see Fig. 1). This probe was prepared by cutting the sequence from between the regions of reiterated DNA with *Pst*I and *Xho*I and was purified by preparative gel electrophoresis. DNA samples in (A) were from C3H/10T½ digested with *Eco*RI (a); AKR-2B digested with *Eco*RI (b); C3H/10T½ digested with *Eco*RI-*Kpn*I (c); AKR-2B digested with *Eco*RI-*Kpn*I (d); C3H/10T½ digested with *Eco*RI-*Hin*dIII (e); and AKR-2B digested with *Eco*RI-*Hin*dIII (f). Samples in (B) were all digested to completion with *Eco*RI. These were from *M. musculus* subsp. *domesticus* MIT no. 1 (a); *M. spraetus* (b); AKR/J (c); *M. cookii* (d); DBA/2J (e); *M. musculus* subsp. *musculus* (Vejrumbo) (f); *M. musculus* subsp. *castaneus* (g); C3H/Fg (h); *M. musculus* subsp. *mollosinus*/ Jackson (i); and *M. musculus* subsp. *brevirostris* (j).

at this locus. Interestingly, DNAs from the other mice examined appeared to generate bands slightly larger than 8 kb but smaller than 13 kb. This may have resulted from slight technical problems in the gels; alternatively, it may indicate additional perturbations at this locus, perhaps involving the highly repetitive sequences in the flanking region.

Rat genomes contain a family of virus-like elements (21, 31) which are physically similar to the mouse VL30 sequences. To determine whether the rat and mouse VL30 elements were related by nucleotide sequence, we hybridized cloned rat and mouse VL30 sequences to restriction enzyme digests of rat, mouse, and cloned rat VL30 DNAs. Direct hybridization between the rat and mouse VL30 clones is shown in Fig. 4, lane e. An intensely hybridizing band was detected, demonstrating sequence homology between rat and mouse VL30 elements. This band represents an internal 2.5-kb *Eco*RI fragment of rat clone 27A (6). More extensive mapping experiments, to be presented in detail elsewhere, have established the presence of additional mouse-related sequences in an approximately 1-kb *Kpn*/*Bgl*II fragment corresponding to 27A linear map coordinates 0.2 to 1.2 (6). No other mouse-related sequences were detectable in this clone. Complementary experiments also established approximate locations for the rat-related sequences in clone BVL-1. These were restricted to a *Pst*I/*Kpn*I fragment that mapped between coordinates 1.0 and 1.7 and a *Hin*dIII/*Pst*I fragment that mapped between coordinates 4.3 and 5.6 (Fig. 1). These results contrast with previous experiments which suggest a lack of homology between mouse and rat VL30 elements (21, 31). This discrepancy might be explained by the greater sensitivity afforded by representative cloned probes, which were used here.

*Eco*RI digestion of total rat DNA produced a complex pattern of bands detectable with a rat VL30 probe, including two high-intensity bands with apparent lengths of 2.9 and 2.7 kb, respectively (Fig. 4, lane a). These presumably resulted from *Eco*RI cleavage within many of the rat sequences. Whether either band corresponds to the 2.5-kb internal *Eco*RI fragment of rat clone 27A is not clear. These same fragments were detected, albeit with less sensitivity, with a mouse VL30 probe (lane b), confirming the notion that the rat and mouse VL30 sequences represent homologs. *Eco*RI digestion of mouse DNA produced a complex pattern of fragments detectable with a mouse VL30 probe (lane c). A different pattern of fragments was detected with a rat VL30 probe. The complexity of the patterns made it difficult to determine the exact relationship between them, and, as a consequence, we do not fully understand this result. Nevertheless, it is apparent that mouse DNA contains many sequences at least partially homologous to sequences present in a rat VL30 clone.

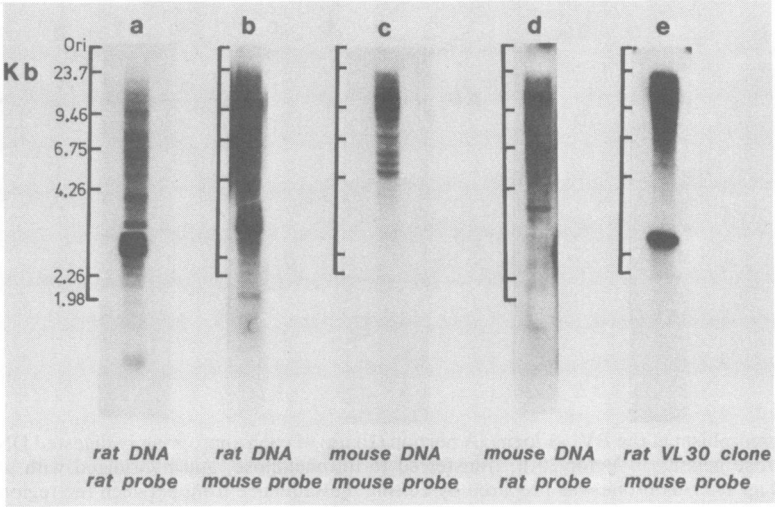

FIG. 4. Homology between rat and mouse VL30 sequences. *Eco*RI-digested rat and mouse DNAs (10 μg per sample) were electrophoresed through 0.8% agarose gels for 36 h at 20 V, transferred to nitrocellulose, and hybridized to either ^{32}P-labeled rat VL30 (6) or mouse VL30 probes. Samples were: NRK DNA (exposure time, 2 h) hybridized to a rat probe (a); NRK DNA (exposure time, 20 h) hybridized to a mouse probe (b); AKR-2B DNA (exposure time, 2 h) hybridized to a mouse probe (c); AKR-2B DNA (exposure time, 20 h) hybridized to a rat probe (d); and clone 27A rat VL30 DNA (exposure time, 2.5 h) hybridized to a mouse probe (e). All filters were washed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before development. Stringent washing (0.1× SSC) reduced the signal, but most, if not all, fragments remained hybridized (data not shown).

DISCUSSION

VL30 sequences were present in multiple copies in the genomes of mice of diverse geographic origins (Fig. 2). The DNAs studied included samples from various inbred strains and feral subspecies of *M. musculus* and from two other mouse species, *M. spraetus* and *M. cookii*. Two other species, *M. cervicolor* and *M. caroli*, have previously been shown to contain multiple copies of VL30 sequences in their genomes (22). These findings are in contrast to the distribution of the endogenous mouse mammary tumor virus sequences in mice (3), the endogenous Rous-associated virus-0-related sequences in chickens (11), and the ecotropic AKR-type murine leukemia provirus (27), all of which are absent from some members of their host species. The presence of VL30 sequences in mice of diverse geographic origins implies that they are present in all mice and argues that they did not arise from recent insertions into the germ lines but were present much earlier in the evolution of the mouse, possibly before speciation.

The similarity of organization within inbred strains and derived cell lines suggests that the VL30 loci are presently stable within the genome and would seem to argue against the possibility that VL30 sequences are currently highly active transposable elements. The two C3H strains studied here were derived from a cross between a Bagg albino and a DBA mouse 60 years ago and have been separately maintained for approximately 40 years (3). The lack of any detectable differences among C3H/An, C3H/HeJ, and the cell line C3H/10T½, which was derived from C3H/He mice 8 years ago (16), argues in favor of the stability of VL30 sequences over this time period. Further evidence of this stability is the apparent identity among DNAs from the various AKR sublines and from the embryo-derived cell culture line AKR-2B, which was established approximately 10 years ago (19). Thus, if VL30 sequences are mobile, rearrangement mechanisms such as transposition must be taking place at a low rate over a longer period of time. Similarly, these observations argue against the frequent and widespread introduction by retrovirus-associated transmission of new VL30 elements into the germ lines of the contemporary laboratory breeding populations we examined. The various AKR sublines used are uniquely suited for detecting such an occurrence since two of them, AKR/Gs and AKR/Ski, appear to have recently acquired additional AKV-type murine leukemia proviral sequences in their germ line DNA (28).

The studies of polymorphism at the BVL-1 locus indicated that at least some of the variation observed among the VL30 sequences of different mice derives from different locations of the VL30 sequences in the mouse genome. Also, the

presence of a VL30 sequence at the locus defined by the ld probe occurred only in certain laboratory strains of *M. musculus*, *M. musculus* subsp. *brevirostris*, and *M. musculus*, subsp. *musculus*. Based on the distribution of polymorphic proteins, *M. musculus* subsp. *brevirostris* probably does not constitute a separate subspecies; it appears, rather, to be indistinguishable from *M. musculus* subsp. *domesticus* (20). The *M. musculus* subsp. *musculus* individual used for these studies came from a colony which derives from mice trapped in the town of Vejrumbo, Denmark, which lies in the hybrid zone between *M. musculus* subsp. *domesticus* and *M. musculus* subsp. *musculus*. Mice from this colony appear to be 90% *M. musculus* subsp. *musculus* and 10% *M. musculus* subsp. *domesticus* in their genetic makeup (A. Wilson, personal communication). Therefore, it would seem most likely a priori that this mouse derived its VL30 sequence at the ld locus from its *M. musculus* subsp. *musculus* background. If this were true, the VL30 sequence at the ld locus would have been introduced before the separation of *M. musculus* subsp. *domesticus* and *M. musculus* subsp. *musculus* 2,000,000 years ago. To explain the heterogeneity among individuals of *M. musculus* subsp. *domesticus*, it would be necessary to postulate that the filled BVL-1 locus was still segregating at the time of the divergence of *M. musculus* subsp. *musculus* and *M. musculus* subsp. *domesticus* or that VL30 sequences were able to leave the BVL-1 locus after this divergence. An alternative explanation, however, is that in the Vejrumbo mouse, the VL30 sequence at the ld locus may have been derived from its 10% *M. musculus* subsp. *domesticus* background. If this is the case, then, within the limits of our study, all mice carrying a VL30 sequence at the ld locus are of or are related to *M. musculus* subsp. *domesticus*, indicating that the VL30 sequence at the ld locus was introduced within the last 2,000,000 years, after the separation of *M. musculus* subsp. *domesticus* and *M. musculus* subsp. *musculus*, and is still segregating.

In summary, the results of this study clearly differ from several recent analyses of endogenous retrovirus genomes. In contrast to the endogenous mouse mammary tumor proviruses, the endogenous Rous-associated virus-0-related proviruses of chickens, and the endogenous AKV-type murine leukemia proviruses, VL30 sequences are ubiquitous among tested animals of the host species. We conclude that VL30 sequences have an earlier evolutionary origin than these proviruses. In spite of this, however, VL30 sequences exhibit a degree of locus polymorphism, as do the aforementioned endogenous retroviruses. Thus, either many of the VL30 sequences were introduced recently enough so that several alleles are still segregating in the population (presumably inconsistent with their uniform and broad geographic distribution), or, alternatively, VL30 elements are more likely than cellular genes to undergo marked genetic alterations such as rearrangements, duplications, transpositions, or unequal crossover. It is also possible that retrovirus-assisted transmission via mixed pseudotype virions is an important source of locus polymorphism, although we were unable to find evidence for this. The existence of genetic relatedness between mouse and rat VL30 sequences is compatible with the thesis that these elements are quite ancient and evolved from a common sequence present in a primordial rodent ancestor. This early sequence could have been of either cellular or retroviral origin.

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