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Molecular phylogeny of *Fv1*

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Abstract. Alleles at the *Fv1* gene of inbred mice confer resistance to infection and spread of vertically or horizontally transmitted murine leukemia viruses (MuLV). The nucleotide sequence of *Fv1* bears similarity to the *gag* of a human endogenous retrovirus, HERV-L, but is more closely related to the *gag*-coding sequence of a newly described class of HERV-L-related mouse endogenous retroviruses designated MuERV-L. Both observations suggest an origin of *Fv1* from endogenous *gag* sequences. The molecular definition of *Fv1* provided an opportunity to determine the phylogeny of the gene among wild mice and its relation to MuERV-L. PCR primers, chosen to include most of the coding region of *Fv1* for both the *n* and *b* alleles, were used to amplify sequences from animals of the genus *Mus*, which were then sequenced. Closely related products were obtained from almost all animals examined that evolved after the separation from *Rattus*, in which the homologous gene was shown to be absent. A phylogenetic tree generated with *Fv1* sequence data differs noticeably from that developed with sequence data from other genes. In addition, nonsynonymous changes were found to be present twice as frequently as synonymous changes, a fact that departs from the standard behavior of a structural gene. These observations suggest that the *Fv1* gene may have been subjected to possible horizontal transfers as well as to positive Darwinian selection.

Introduction

Fv1 is one of several genetic loci in inbred mice that restrict the replication and spread of various murine leukemia viruses (MuLV). Alleles at *Fv1* control the sensitivity of cells to different subgroups of MuLV (Lilly 1970; Pincus et al. 1971a, 1971b; Hartley et al. 1977). These viruses are classified as N-tropic if they replicate best in $Fv1^n$ cells or B-tropic if they replicate best in $Fv1^b$ cells. Alleles of *Fv1* are expressed codominantly. A phenotypically null allele, $Fv1^o$, was identified in certain wild mice sensitive to all retroviruses (Hartley and Rowe 1975; Lander and Chattopadhyay 1984; Kozak 1985). Two $Fv1^o$ cell lines are SC-1 cells from mice trapped in Bouquet Canyon, Calif. (Hartley and Rowe 1975) and dunni cells from *Mus dunni* (Lander and Chattopadhyay 1984). *Fv1 n* and *b* alleles act on these viruses at a point after entry into the cell and reverse transcription of the viral RNA genome but before entry into the nucleus and integration into the host genome (Jolicoeur and Baltimore 1976; Sveda and Soeiro 1976).

Recently, Best et al. (1996) reported the cloning of *Fv1*, which comprises a small intronless open reading frame (ORF). They suggested its origins lie within the *gag* region of an endogenous retrovirus with sequence similarity to the HERV-L family of human endogenous retroviruses (Cordonnier et al. 1995). The murine homolog of HERV-L, designated MuERV-L, has now been cloned and found to contain an ORF in the *gag* and *pol* genes (Bénit et al. 1997). The predicted Gag protein of MuERV-L shares 43% identity with the *Fv1* ORF product and bears a number of structural features. These studies suggest that MuERV-L may be the endogenous element that gave rise to the *Fv1* gene of inbred mice.

Best et al. (1996) also demonstrated that *Fv1*-related sequences were present in the genome of *Mus dunni* but not in the rat, suggesting that the putative integration event for this retrovirus-like sequence occurred early in *Mus* speciation. Here we present analyses of *Fv1*-related sequences of the genus *Mus*.

Materials and methods

DNA samples for analyses of Fv1. DNAs were prepared from established cell lines or tissues of mice, rats, and humans collected by Chattopadhyay and Lander (NIH) and described previously. Other samples were prepared from the collection of wild-derived mouse strains maintained in Montpellier or were purchased from The Jackson Laboratory (Bar Harbor, Me.; Table 1).

PCR amplification and determination of Fv1 sequences. PCR reactions were performed with the primers listed in Fig. 1A with 200 ng of cellular DNA and 0.05 mM primers and Taq polymerase. Denaturing was at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 45 s, and 72°C for 30 s with extension at 72°C for 7 min. Products were separated on 0.7% agarose gels. Amplified PCR products were cloned with the TA cloning kit (Invitrogen, San Diego, Calif.) and conditions recommended by the manufacturer. Sequencing of cloned *Fv1* products was performed manually and by machine with standard techniques.

Southern blot analysis of Fv1 sequences. Southern blot hybridization analyses of cellular DNA were performed with two probes: cloned sequences between the P1 primers amplified from the C57BL/6 (B6) *Fv1* gene, which was labeled with ^{32}P by random primer extension; and a synthetic probe corresponding to the coding sequences for the unique carboxyterminal 22 amino acids of the *b* allele of *Fv1*. Two oligonucleotides of 36 bases 5′ and 51 bases 3′ that overlapped for 14 bases were annealed and filled in with Klenow (Life Technologies, Gaithersburg, Md.) under conditions recommended by the supplier. The purified 66 bp fragment was end-labeled with ^{32}P. DNA separated on agarose gels was transferred to nitrocellulose, baked for 2 h at 80°C, and hybridized with probes at 65°C in 3 × SSC and 0.2% each Ficoll, PVP, and BSA, 5 mM EDTA, 0.1% SDS, 50 μg/ml sonicated herring sperm DNA, and 10% dextran sulfate for 18–20 h. Membranes were washed three times for 20 min at RT with 2 × SSC and once with 0.15 × SSC and 0.1% SDS at 65°C for 20 min.

Data analyses. Sequence alignment was done visually with a standard text editor. Putative insertions and deletions were placed so as to minimize

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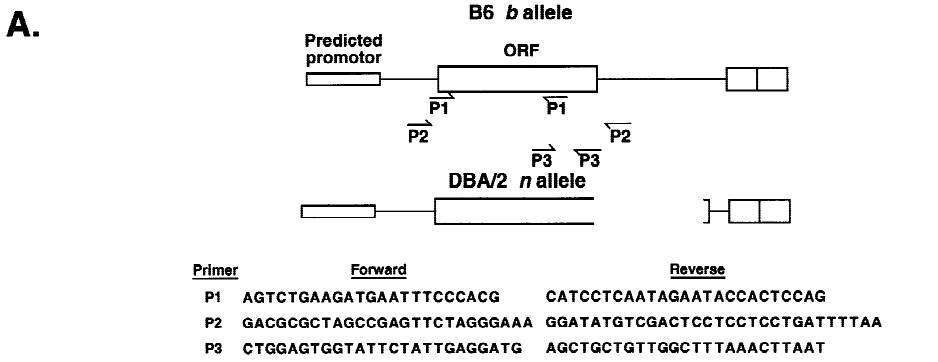
Table 1. Origins of DNA for *Fv1* studies.[a]

Source	
NIH	Inbred strains: AKR.M/SnJ, AKR/N, BALB.B/LiPt, BALB.K/LiPt, BALB/cAR, BALB/cJ, BALB/cORN; C57BL/6J, C57BL/KaPt, DBA/2Pt, DBA/2J Rat: FRE cell line Wild mice: *Mus m. domesticus:* ''praetextus'' (Giza and Faijum), Sc-1 cells (Bouquet Canyon, CA), Watkins Star (MD eastern shore), Queen Anne II (MD eastern shore), Centreville Lights (MD eastern shore), Lewes (DE), ''*poschiavinus*'' *Mus m. musculus;* Viborg, Skive, CZECH I, CZECH II, Vejrumbro, *Mus m. molossinus, Mus m. castaneus, Mus spretus* (Spain and Morocco) *Mus cookii, Mus cervicolor cervicolor, Mus cervicolor popaeus,* Mus dunni, Mus (*N.*) *minutoides, Mus (C.) pahari*
Montpellier	African murids: *Arvicanthus niloticus, Mastomys huberti* Field mouse: *Apodemus* Wild mice: *Mus. m. bactrianus, Mus spicilegus, Mus macedonicus, Mus* (*N.*) *setulosus, Mus* (*C.*) *famulus, Mus* (*C.*) *pahari, Mus* (*P.*) *platythrix, Mus* (*P.*) *saxicola*
Jackson Laboratory	Wild mice: *Mus m. castaneus* (CASA/Rk), *Mus m. domesticus* (PERA/Ei), *Mus spretus* (SPRET/Ei), *Mus spicilegus* (PANCEVO/Ei)

[a] Biologically determined genotypes where known; *Fv1*^{n}, dashed underline; *Fv1*^{b}, solid underline; *Fv1*^{o} (null), double underline.



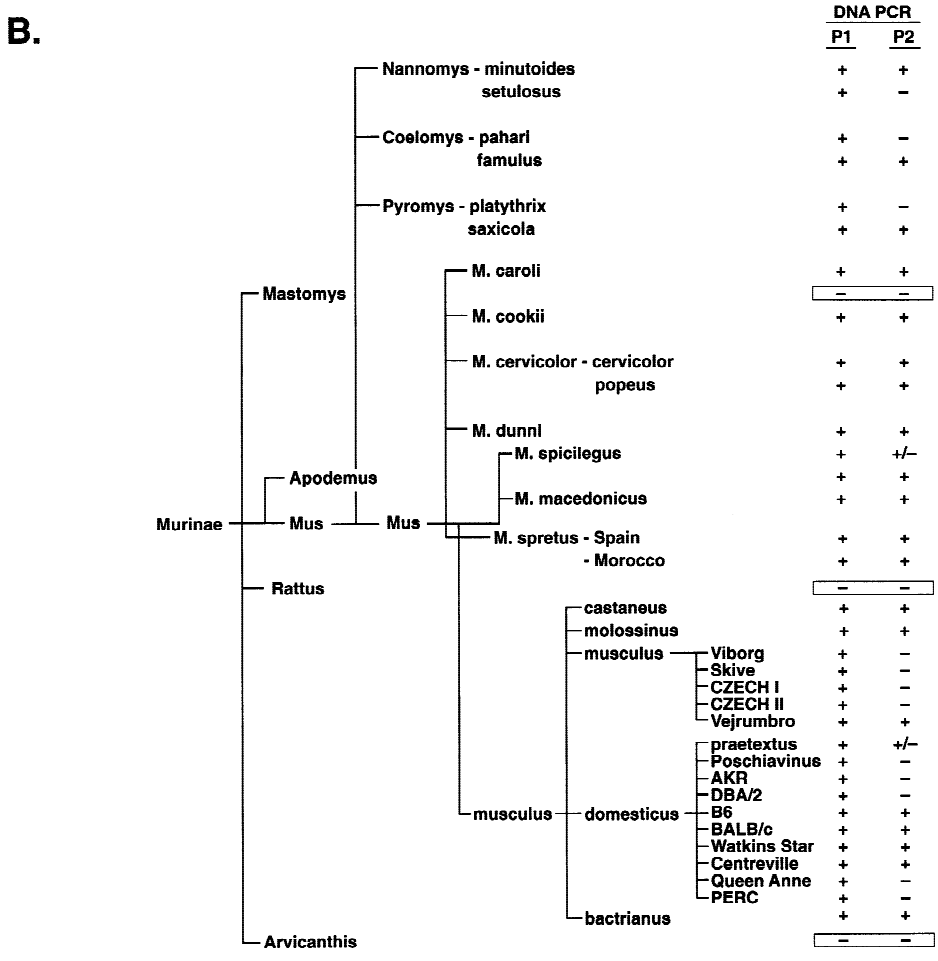


Fig. 1. PCR amplification of *Fv1*-related sequences. **A.** Genomic structure of B6 and DBA/2 *Fv1* alleles (Best et al. 1996) and characteristics of P1, P2, and P3 primer pairs used to amplify DNA sequences. The structure of other *Fv1*^{n} alleles differs from that of DBA/2 but not in ways known to affect the potential for amplification by P1 primers. **B.** Characteristics of PCR amplifications using P1 and P2 primer pairs and rodent DNAs. The structure of the tree is generally modeled after the molecular phylogeny tree published by Boursot et al. (1993). +, PCR product of appropriate size amplified with P1 or P2 primers; −, no PCR product with P1 or P2 primers; ±, some mice positive and some negative for amplification with P2 primers. No mice were heterogeneous for P1 primer products. Rodents with more than one animal tested for P2 primers, number positive/total: *M. caroli,* 2/2; *M. cervicolor,* 4/4; ''*praetextus,*'' 4/5; *Mus* (*C.*) *famulus,* 2/2; *Mus* (*C.*) *pahari,* 0/3; *M. spicilegus,* 1/2; *M. spretus,* 4/4.

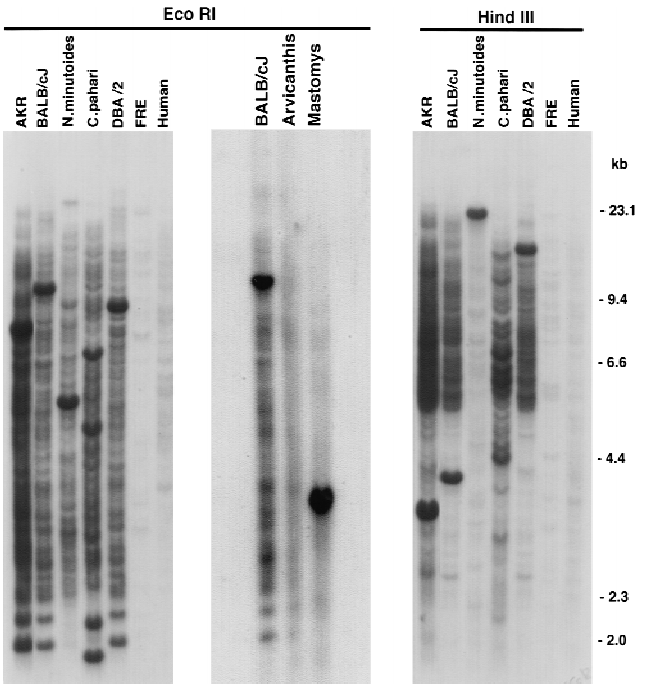


Fig. 2. Southern blot analyses of *Fv1*-related sequences in rodent DNA. DNAs were digested with the indicated restriction enzymes and hybridized with labeled P1 probe.

their number. The corresponding file was imported into the MEGA package (Kumar et al. 1994) to obtain statistics on the sequences [transitions/transversions, number of synonymous (d_S) and non-synonymous (d_N) substitutions]. From there, they were exported into the PHYLIP package (Felsenstein 1989, 1992) for phylogenetic treatment in a standard fashion. A neighbor-joining tree was produced on nucleotide divergence matrices computed with the Kimura two-parameter model with the default options of the program. To evaluate the robustness of the topology, bootstrap values were obtained on 100 replicate data sets. The minimum number of substitution steps involved was estimated according to maximum parsimony. Additional analyses were performed with the MACAW program (Karlin and Altschul 1990; Schuler et al. 1991).

Results

Fv1-related sequences in Mus. DNAs prepared from members of the subfamily *Murinae* were screened for the presence or absence of *Fv1*-related sequences by use of two sets of primers (Fig. 1A). P1 primers were chosen to amplify sequences from inbred mice bearing either the *n* or *b* allele of *Fv1,* whereas P2 primers were chosen to amplify sequences from inbred mice bearing the *b* but not the *n* allele. P1 primers amplified sequences from all the laboratory mouse strains listed in Table 1 (Fig. 1B and data not shown) and all other rodents examined except for Fischer inbred rats, *Mastomys,* and *Arvicanthis.*

A probe generated by cloning sequences included between the P1 primers was used for Southern blot analyses of DNAs digested with *Eco*RI or *Hin*dIII restriction endonucleases (Fig. 2). Under the conditions employed, very weak signals, if any, were obtained with human, *Arvicanthis,* or Fischer rat (*Rattus*) DNA. Prominent strong signals and ladders of weaker hybridizing bands were obtained with the other samples. For these DNAs, there was a single, strongly hybridizing band in DNAs restricted with either enzyme; DNA from *Mus pahari* exhibited two bands of moderate intensity in the *Eco*RI digest and several bands of comparable intensity in DNA cut with *Hin*dIII. Of interest, DNA from *Mastomys,* which could not be amplified with P1 primers, contained a single intensely hybridizing band after digestion with *Eco*RI. Thus, sequences related to *Fv1* can first be detected in *Murinae* after the separation of *Rattus* and *Arvicanthis* from the other family members and are present throughout subsequent speciation.

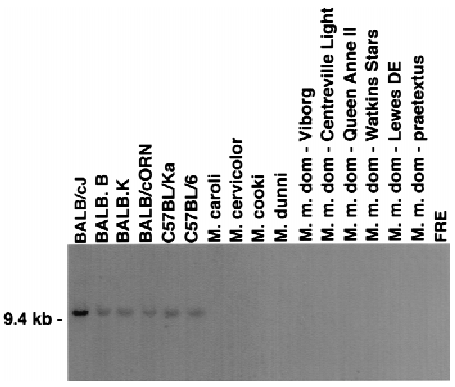


Fig. 3. Southern blot analyses of *Fv1*-related sequences in DNAs from mice with possible *b* alleles. DNAs were digested with *Eco*RI and hybridized with a 66-bp probe encoding the carboxy-terminal 22 amino acids of the B6 $Fv1^b$ allele.

The P2 primers were found—as expected—to amplify sequences from the DNAs of C57BL/6 (B6) and BALB/c, but not from AKR or DBA/2 inbred mice (Fig. 1B). Amplified bands of similar intensity were obtained from almost half of the remaining DNAs. In addition, one of five DNAs prepared from different *Mus m. domesticus* mice referred to as ''*praetextus*'' and one of two *M. spicilegus* DNAs contained sequences that amplified with the P2 primers. These findings suggest that sequences resembling both the *b* and *n* alleles of inbred mice are present among wild populations and were segregating within at least two subspecies.

To compare the *b* allele of inbred mice and the *b*-like alleles of wild animals, we used a synthetic 66-bp probe with the sequence coding for the allele-specific carboxyterminal 22 amino acids of the inbred mouse *b* allele (Fig. 3). The probe hybridized with DNAs from B6 and BALB/c mice, but not with DNA from a rat, a human, or ten different species and subspecies of wild *Mus* predicted to have *b*-like alleles based on the amplification of sequences using P2 primers. These results and those with the P2 primers most likely demonstrate gapping patterns in this region (Best et al. 1996) rather than marked sequence differences and, thus, are not useful phylogenetic characters.

Sequence comparisons of Fv1 genes. The sequence of the B6 *Fv1* locus amplified by the P1 primers, covering bp 2138 through 3232 of the published sequence (Best et al. 1996), was cloned and sequenced and found to be identical to the published sequence. The same procedure was performed with amplified products from 19 other mice including two ''*praetextus*'' samples.

The predicted protein products of these sequences are compared in Fig. 4. The first eight sequences, including all the *Mus musculus* animals, form a remarkably homogeneous group differing by no more than two amino acids. *M. macedonicus* and *M. spretus* comprise a small but distinct second subset. The remaining ten sequences segregate into two groups most clearly distinguished by the location of a three-amino acid difference relative to the B6 sequence preceding position 30. Only *M. dunni* and *M. cookii* exhibit major truncations; the terminal two amino acid changes in other mice may be caused by sequencing artifacts, as they lie at the end of the 3′ primer.

Nucleotidic divergence. A 20 × 20 matrix of nucleotide percentage divergence between mouse *Fv1* gene sequences encompassed by the P1 primers was derived from the DNADIST program of PHYLIP according to the Kimura two-parameter model, which

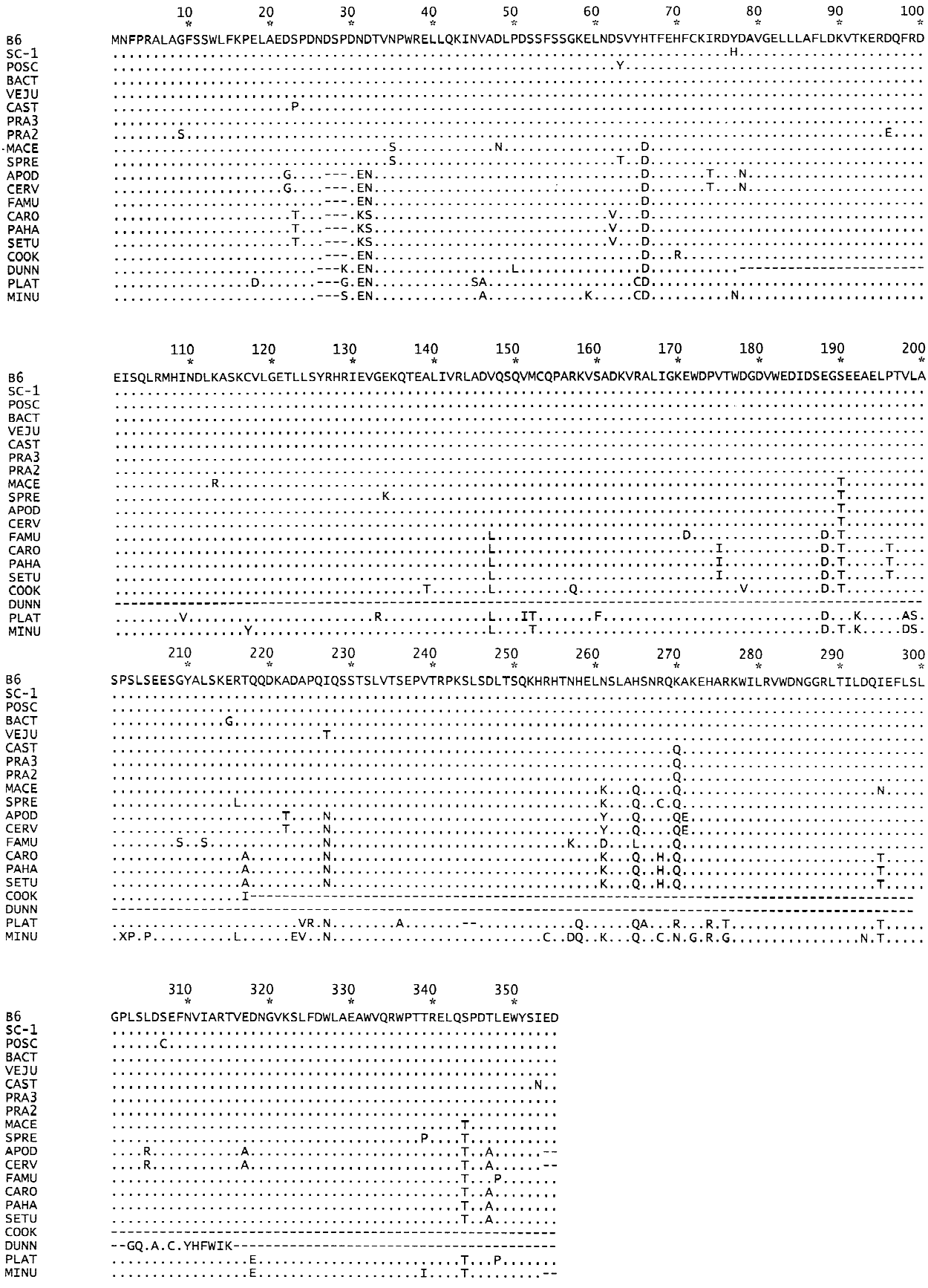


Fig. 4. Deduced amino-acid sequences of *Fv1*-related sequences amplified by P1 primers.

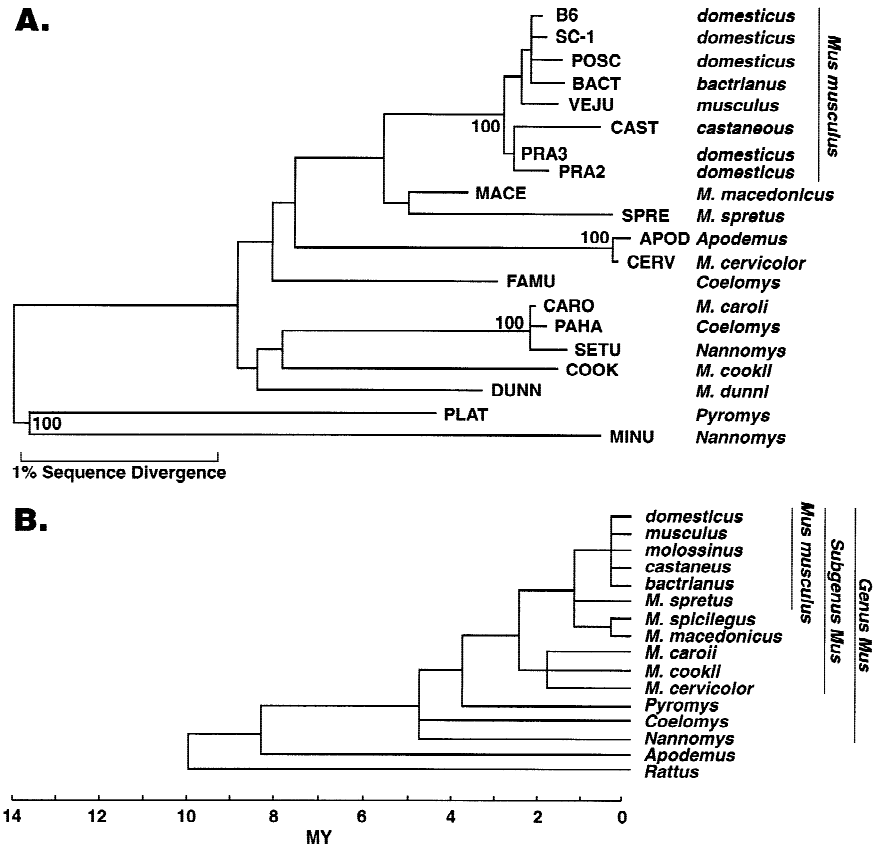


Fig. 5. Phylogenetic relationships of rodents. **A.** Neighbor-joining tree of *Fv1* relationships of genus *Mus* with *Apodemus* as an outlier. Numbers indicate bootstrap values (%) at the indicated nodes. **B.** Molecular phylogeny of genus *Mus* with *Rattus* and *Apodemus* as outliers: modified from Boursot et al. (1993).

allows differences in the rate of transition vs. that of transversion. The largest distance found corresponded to a divergence of 6.2% between *M.* (*N.*) *minutoides* on one side and *Apodemus* spp. or *M. m. castaneus* on the other.

The same distance matrix was used to construct a molecular phylogeny of the *Fv1* gene in the genus *Mus* (Fig. 5A). This neighbor-joining tree is fairly stable, as indicated by the rather large number of nodes displaying bootstrap values of 90% and above. When looked at by the maximum parsimony criterion, the tree implies 188 mutational steps distributed among 90 sites that varied in a single taxon, and 66 sites that were phylogenetically informative and underwent 98 mutations. This indicates a high level of reversions or convergence events affecting a subset of sites allowed to vary. The distribution of mutated sites along the molecule was not homogeneous (Fig. 4). As estimated from the average of all pairwise comparisons, the actual transition:transversion ratio was 1.28.

When the tree-building algorithm was applied to the reconstructed 358-amino acid data set rather than to DNA sequences, a very similar tree was obtained (not shown). The number of variable amino acids was 85, while the number of informative sites amounted to 39. When these figures are compared to the nucleotide statistics, one can readily see that amino acid substitutions are not rare and that they affect a relatively larger fraction of sites than the nucleotide substitutions. This is because the number of base substitutions that result in amino acid changes (d_N) are more frequent than the number of silent changes (d_S), as shown by the pairwise sequence statistics of the MEGA program that yielded a ratio of d_N to d_S sites of 2.1. This is far from the average of 0.2 observed for structural genes undergoing mostly purifying selection. We recognize that the evidence suggesting amino acid selection is not conclusive, as the d_N/d_S does not exceed the drift value. Nonetheless, even a cursory examination of the highly conserved regions yields a d_N/d_S of around 1.6.

Relations between Fv1 and MuERV-L sequences. MuERV-L sequences are thought to be recently amplified mouse retrovirus-like elements that could supply appropriate Gag coding sequences for generation of the *Fv1* gene (Bénit et al. 1997). Using the MACAW program (Karlin and Altschul 1990; Schuler et al. 1991), we compared the most divergent *Fv1* sequences, B6 and *M.* (*N.*) *minutoides*, with the MuERV-L protein (Fig. 6). Five blocks of highly significant homology ($p < 10^{-4}$) were identified and are shown as black bars; the amino-terminal box encompasses the myristylation sequence of each protein. The highest scores among these five blocks were associated with the 98-amino acid sequence from position 58 to 155, the 63-amino acid stretch from position 254 to 316, and the 26-amino acid peptide from position 159 to 184. Amino acid identity in these regions was 44%, 51%, and 88% respectively (Fig. 6, bottom panel). The regions highly conserved between MuERV-L and B6 *Fv1* are seemingly exempt from the high rate of amino acid change that so strikingly marks *Fv1* phylogeny.

Discussion

Comparison with the species phylogeny. The consensus molecular phylogeny of the genus *Mus* (Boursot et al. 1993, 1996; Din et al. 1996) is given in Fig. 5B. This structure (Fig. 5B) embodies several layers of quasisynchronous speciation resulting in the ordered nestings of subgenera within genus and species within subgenus. Note in addition the close relation of *M. spicilegus* with the geographically apposed *M. macedonicus* and the distinct trio formed by the Asian radiation of *M. caroli*, *M. cookii*, and *M. cervicolor*. Aside from the conserved associations within *Mus musculus*, the *Fv1* sequence-based tree (Fig. 5A) is at great variance with this scheme. First, there is no clear separation between alleles carried by mice of the subgenus *Mus* from those of the other subgenera,

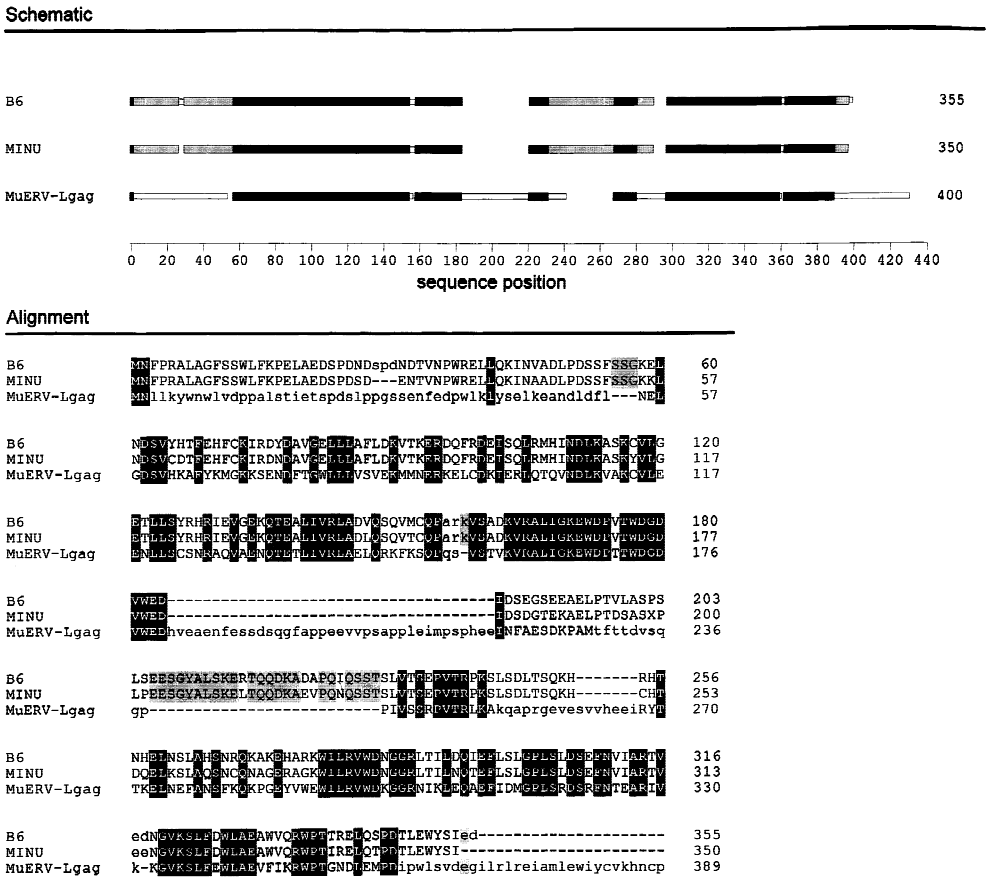


Fig. 6. Sequence comparisons of *Fv1* alleles and MuERV-L: relations of deduced amino acid sequences of B6 and *M.* (*N.*) *minutoides Fv1* genes within the P1 amplicon with that of MuERV-L Gag. **Upper panel:** Schematic presentation of sequence similarities among the *Fv1* alleles of B6 and *M.* (*N.*) *minutoides* (MINU) and MuERV-L. Regions of high homology among the three sequences are indicated as solid black bars. Sequences with high homology between B6 and MINU only are indicated in gray. Unique sequences are indicated by smaller white boxes, and breaks indicate that gaps have been inserted as the result of linking. **Lower panel:** Sequence alignment of the three genes presented schematically in the upper panel. Regions of high homology among all three sequences are given in black, with upper case letters indicating the regions that have been linked. Regions with significant similarity for two sequences are given in gray. Dashes indicate gaps that have been inserted as a result of linking. Schematic and alignment comparisons were generated by MACAW.

Nannomys, *Coelomys*, and *Pyromys*. The scrambled relations of the Asian species with *M.* (*N.*) *setulosus* and *M.* (*C.*) *pahari* are remarkable in this regard. Second, and perhaps more unusual, is the pairing of *Apodemus* with *M. cervicolor* almost equally distant from mice at the extremes of our study, B6 and *M.* (*N.*) *minutoides*. These discrepancies between the reconstructed and conventional trees suggest that polymorphisms of this gene evolved as the result of evolutionary pressures distinct from those usually placed on structural genes. The discrepancies between the conventional and reconstructed trees suggest horizontal gene transfer as a possible cause. It is also possible, but we believe it is less likely, that the polymorphisms of *Fv1* predate the divergence of the mice examined.

Are amino acid changes positively selected for? Analyses of the *Fv1* phylogeny demonstrated that d_N outnumbered d_S changes by a ratio of more than two. For structural genes undergoing negative selection, the ratio of d_N to d_S is usually on the order of 1:5 or approximately 10-fold less than seen for *Fv1*, and such an inverted ratio is generally taken as a sign of positive selection (Endo et al. 1996). Of course, a ratio of 2:1 is smaller than the value of 3:1 expected for neutrally drifting pseudogenes. We can most likely exclude the possibility that our sequences are functionless, since stop codons were found for only three of the sequences. Given the fact that roughly one twentieth (3/64) of the possible mutations will produce a stop codon in a randomly drifting sequence, we should have detected 10–15 in our sequences rather than the 3 observed. The high rate of amino acid turnover seen with *Fv1* may be a characteristic of gene products involved in molecular interactions with a rapidly evolving counterpart. In the mouse, such a case has been made for *Abpa*, the gene encoding the α subunit of salivary androgen-binding protein that is possibly implicated in mate recognition selection. For this gene, a d_N to d_S ratio of 5:1 was found (Hwang et al. 1997).

Sequences on which positive selection may operate comprised only 0.45% of all the gene groups examined by Endo and associates (1996). Of interest, 9 of the 17 candidate genes affected by positive selection were surface proteins of parasites and viruses. The areas of these genes with the highest ratio of d_N to d_S lay in discrete hypervariable regions that corresponded to antigenic epitopes, thereby suggesting that selection occurred to avoid detection by the immune system. Avoidance of immune recognition is an unlikely basis for the high ratio of d_N to d_S within *Fv1*. One could hypothesize that owing to its role in regulation of MuLV, *Fv1* was likely to have co-evolved with viral mutants favoring new amino acid changes in key positions. Exogenous infections acquired at transition zones between murid populations might mold specific changes within the common background of invariant *Fv1* sequences. This model cannot account, however, for the identity of silent site changes shared by remotely related species that was

observed multiple times in our data set and leads us to postulate more than an orthologous evolution of *Fv1* sequences within each mouse lineage.

Horizontal transfers. The demonstration of an abrupt appearance of *Fv1* sequences within *Murinae* and the relation of these sequences to endogenous retroviral elements such as MuERV-L suggest infection from without as the source. The prominent discrepancies between the 'true' phylogeny of *Mus* and the *Fv1* gene phylogeny, mostly outside of the *M. musculus* group, also suggest horizontal transfers as a way to reconcile the data. The idea is particularly appealing in considering the closeness of *Fv1* sequences in the distantly related *Apodemus* sp. and *M. cervicolor*. In considering this possibility, it is worth remembering that MuERV-L sequences were amplified in *Mus* after their introduction. A comparison of MuERV-L DNA sequences with Moloney ecotropic virus in the 351-bp portion of the viral genome (Ψ) known to encode packaging sequences (Mann et al. 1983) revealed a 58% identity over the whole region and 87% identity over an internal 109-bp fragment. If MuERV-L sequences could be packaged in virions, alone or with a retrovirus RNA, the conditions for horizontal spread would be met along with opportunities for recombination between the RNA species. Recombination, horizontal transfer, and subsequent mutation could explain the gene conversion-like events that would be required to generate new *Fv1*-related sequences from MuERV-L and endogenous retroviruses. These events would also explain how the *Fv1* phylogeny is at such great variance with the consensus phylogeny.

Although the data are limited, we feel that the results of Southern blot analyses of Murid sequences with the P1 probe are inconsistent with the concept of *Fv1* as a member of a multigene family. The absence of hybridizing bands of either strong or moderate intensity in *Rattus* and *Arvicanthis spp.* clearly delimits the introduction of the gene to the murids, with all later evolving members having strongly hybridizing, usually single, bands. The bands that hybridize with low intensity in *Mus* samples can be understood as reflecting the blocks of conserved sequences among B6, *M.* (*N.*) *minutoides*, and MuERV-L-related sequences that are amplified a hundredfold or more (Bénit et al. 1997). The heterogeneity of MuERV-L-related sequences is unknown, but members of this family may provide a rich source of material for gene conversion-like events. Of note, a MuERV-L-related cDNA sequence has recently been identified in *Rattus norvegicus* (Bonaldo et al. 1996). A clearer understanding of the evolutionary relations of *Fv1* and retrovirus-related elements will likely emerge as more data on these sequences are uncovered.

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