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Human Endogenous Retrovirus Type I-Related Viruses Have an Apparently Widespread Distribution within Vertebrates

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Retroviruses from lower vertebrate hosts have been poorly characterized to date. Few sequences have been isolated, and those which have been reported are all highly divergent when compared to the retroviruses known to be harbored by mammals and birds. Here we show that retroviruses with significant homology to the human endogenous retrovirus type I (HERV-I) are present within the genomes of fish, reptiles, birds, and mammals and that they may well be widespread within many vertebrates. Phylogenetic analysis of nucleotide sequences strongly supported the inclusion of viruses from each of these vertebrate classes into one monophyletic group. This analysis also demonstrated that the HERV-I-related viruses are more closely related to retroviruses belonging to the murine leukemia virus genus than to members of the other retroviral genera. The presence of HERV-I-related retroviruses in so many disparate vertebrate hosts suggests that other endogenous human retroviruses may also have a much wider distribution than is currently appreciated.

The *Retroviridae* are a family of RNA viruses possessing the ability to transcribe their genome into DNA by the process of reverse transcription (1, 27). Extensive research has been directed towards the isolation and characterization of retroviruses from mammals, especially humans, and birds have been the only other vertebrate class explored in any depth as retroviral hosts (3). There are, to date, few examples of retroviral sequences within the three other vertebrate classes, and those which have been reported are all highly divergent when compared to mammalian and avian isolates (9, 28, 29).

The murine leukemia-related viruses (MLVs, or mammalian C-type oncoviruses) form one of the seven currently recognized retroviral genera, and members of this genus comprise a large, well-studied group of endogenous and exogenous viruses associated with malignancies and numerous other diseases (3, 6, 33). Examples have been isolated from a wide variety of mammals and several species of birds, and viral particles with morphologies typical of C-type retroviruses have also been reported in reptiles (10, 24, 31).

There are a number of endogenous retrovirus-like elements (generally termed HERVs or ERVs) within the human genome which have a relatively close relationship to members of the MLV genus. Sequencing of these HERVs has shown that many of them, although they appear relatively distinct when regions of their *gag* and *pol* genes are compared, are nevertheless placed as sister taxa to the prototype MLV-related viruses in phylogenetic analyses (32). Consistent with this, several of these endogenous retroviruses (such as HERV-H, ERV-9, and HERV-I) are still loosely classified as members of the MLV genus (32).

One particular element, HERV-I (sometimes termed RTLV-I), so called because of homology between its primer binding site and the tRNA[Ile], is present at low copy number (up to 25, depending on which region of the viral genome is used for analysis) within humans, chimpanzees, gorillas, and Old World monkeys and is therefore likely to have been present within primates for at least 30 million years (11, 12, 23). HERV-I contains multiple in-frame stop codons and frameshift mutations, shows approximately 41% sequence identity to part of the murine leukemia virus reverse transcriptase gene, and also shares a similar genomic organization with this virus (12). Retroviruses with sequences similar to that of HERV-I, or to those of other endogenous human retroviruses, have not been isolated from outside of the placental mammals, and thus the diversity, origins, and host range boundaries of these elements are poorly understood at present.

Here we show that retroviruses closely related to HERV-I are present within marsupials, birds, reptiles, and fish and are likely to be abundant within many vertebrate classes. This raises the possibility that other endogenous human retroviruses, as well as those within other mammalian taxa, may also have a wide distribution within vertebrates.

MATERIALS AND METHODS

Amplification and characterization. Genomic DNA was extracted from a number of organisms, including the Komodo dragon, the rock wallaby, the house sparrow, and the lemon shark. DNA was extracted from a liver acetone preparation (SIGMA) in the case of lemon shark. An aliquot (~0.5 μg) of DNA from each of the above organisms was screened by PCR and two degenerate oligonucleotide primers (5′GTT/GTTIG/TTIGAT/CACIGGIG/TC and 5′ATIAGIA G/TA/GTCA/GTCIACA/GTA) designed against the active site motifs of retroviral protease and reverse transcriptase proteins. These primers are capable of amplifying a wide diversity of retroviruses and long terminal repeat (LTR) retrotransposons, as previously described (30).

Electrophoresis of PCR products, through 1.3% agarose gels, revealed amplification of fragments with molecular sizes in the range of 800 to 1,000 bp. These fragments were ligated into a plasmid vector (pCRII-Invitrogen), cloned, transformed into competent cells, and propagated. A total of 11 clones containing inserts of approximately the correct size was obtained from the four species: 4 from each of the wallaby and Komodo, 2 from the lemon shark, and 1 from the sparrow. All clones were sequenced by using the double-stranded approach with an Applied Biosystems 373 automated DNA sequencer and a Taq FS kit (Perkin Elmer). Homology to other retroviruses was initially determined by screening against a computer database of retroelement sequences.

Phylogenetic analysis. Phylogenetic trees of the reverse transcriptase gene alone and in combination with the protease gene were generated using the neighbor-joining and maximum parsimony approaches with the computer packages PHYLIP and PAUP, respectively (7, 26). DNA sequences (with third codon positions deleted) were generally used in the input data sets, although some PAUP trees utilized amino acid data. Trees were also generated by using a

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variety of transition/transversion weighting ratios. The robustness of individual nodes was assessed by bootstrap resampling.

Southern hybridization. The origin of each of the viral fragments was confirmed, and its distribution and copy number were investigated by using standard Southern hybridizations (21). Aliquots (10 μg) of restriction-digested DNA from each species were electrophoresed through agarose gels and transferred onto nylon membranes. Membranes were hybridized for 12 to 16 h at 55 or 65°C with [α-^{32}P]dCTP-labelled retroviral probes. Filters were washed in 0.5% sodium dodecyl sulfate (SDS) and 0.5 or 1 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55 or 65°C.

Sequence sources. Sequences were obtained from computer databases with the original sources as follows: gibbon ape leukemia virus (GaLV) (4); bovine endogenous retrovirus (BoEV) (unpublished data); feline leukemia virus (FeLV) (5); human endogenous retrovirus type E (HERV-E) (19); human spumaretrovirus (HSV) (14); simian foamy virus 3 (SFVL3) (18); *Sphenodon* endogenous retrovirus (SpeV) (28); equine infectious anemia virus (EIAV) (25); simian immunodeficiency virus (SIVmac) (2); *Dendrobates* endogenous viruses types I, II, and III (DevI, II, and III) (29); Rous sarcoma virus (RSV) (22); lymphoproliferative disease virus (LDV) (8); mouse mammary tumor virus (MMTV) (15); human T-cell leukemia virus type I (HTLV-I) (13); bovine leukemia virus (BLV) (20); walleye dermal sarcoma virus (WDSV) (9); human endogenous retrovirus type I (HERV-I) (11).

Nucleotide sequence accession numbers. The sequences described here have been submitted to the EMBL/GenBank/DDJB databases and will appear with the following accession numbers: RV Komodo, Y07807; RV shark, Y07810; RV rock wallaby, Y07809; RV sparrow, Y07808.

RESULTS

We utilized oligonucleotide primers designed against two conserved motifs present within retroelement protease and reverse transcriptase genes to amplify genomic DNA from a number of widely disparate vertebrate hosts, including (i) the Komodo dragon (*Varanus komodoensis*, a reptile of the monitor lizard family, Varanidae), (ii) Godman's rock wallaby (*Petrogale godmani*, mammal, superorder Marsupialia), (iii) the house sparrow (*Passer domesticus*, bird, order Passeriformes), and (iv) the lemon shark (*Negaprion brevirostris*, fish, class, Chondrichthyes; subclass, Elasmobranchii). Eleven clones were characterized by sequencing 350 bp of the inserts before screening the resultant sequences against computer data banks. This analysis showed that 8 of the 11 clones contained significant homology to retroviral reverse transcriptase proteins. All eight sequences were more closely related to members of the MLV genus than to isolates belonging to other retroviral genera. Those derived from the same species were generally either identical or shared at least 95% nucleotide homology, and thus the sequence of only one of the fragments from each species was fully determined, as shown in Fig. 1. All four fragments extended from the active site of the protease gene to the YVDD motif within the reverse transcriptase gene and varied between 854 bp (RV Komodo) and 881 bp (RV shark). Two of the sequences, those obtained from the rock wallaby and sparrow, encoded both in-frame stop codons and frameshift mutations, whereas those obtained from the Komodo dragon and the lemon shark contained one uninterrupted open reading frame, which spanned the entire length of the fragment. We believe all of the fragments are likely to have been derived from endogenous elements.

Part of the reverse transcriptase gene from each of the four isolates was then compared to a variety of other retroviruses (Fig. 2) and it was apparent that all shared a number of sequence motifs with the human endogenous retrovirus HERV-I. HERV-I is known to be related to the MLV genus, and consistent with this, there appeared to be more similarity between the viruses described here (now termed HERV-I-related retroviruses) and members of the MLV genus (GaLV, FeLV, and HERV-E in Fig. 2), than to members of the other retroviral genera. This was confirmed when average percentage similarities across the region, shown in Fig. 2 (excluding residues between the domains), were calculated (Table 1). The HERV-I-related retroviruses shared an average of approximately 62% amino acid identity with each other, 48% with members of the MLV genus, and between 32 and 43% with other retroviral sequences.

To investigate the relationships of these viruses in greater detail, we constructed phylogenetic trees using both distance and maximum parsimony based approaches (7, 26). Numerous trees, based on the alignment shown in Fig. 2 (33), were generated using different transversion/transition ratios, and a variety of character weighting and sequence addition options (Fig. 3a). The trees were largely consistent with each other in that all placed the HERV-I-related viruses into a monophyletic group and positioned this group next to the MLV genus. Bootstrapping by both the neighbor-joining and maximum parsimony approaches suggested that these relationships were well supported. A minimum of 97 of 100 replicates supported the inclusion of the Komodo dragon, shark, wallaby, sparrow, and HERV.I sequences into one closely related group, with at least 80 of 100 replicates suggesting that this group had the closest relationship to the MLV-related retroviruses.

These trees, however, failed to demonstrate strong support for any phylogenetic relationships within the HERV-I group. To attempt to provide greater resolution we generated trees which included information from both the protease and reverse transcriptase regions (regions 18 to 252 and 394 to 866 in Fig. 2). The protease gene shows greater variability than the reverse transcriptase gene (6) and it was therefore not possible to include the full range of retroviral sequences within the data set, due to the difficulty of aligning homologous amino acid positions. Members of the MLV genus, however, could be accurately aligned, and they were therefore included as outgroups. Despite the extra sequence information, many of the relationships within the HERV-I group remained unresolved, although there was reasonable support for placing the sparrow sequence in a basal position when compared to the other isolates (Fig. 3b).

We next performed Southern blot analyses, both to confirm the origins of the retroviral fragments and to determine whether related sequences were present in additional taxa. All four retroviral fragments were present at low to medium copy numbers in their hosts, as shown in Fig. 4. Furthermore, hybridization of the rock wallaby isolate to genomic DNA obtained from several different families of marsupials and monotremes revealed the presence of similar viruses in the koala, stripe-faced dunnart, Tammar, common brushtail possum, brindled bandicoot, opossum, short-beaked echidna, and platypus (Fig. 5).

DISCUSSION

We have been screening the genomes of fish, amphibians, and reptiles for endogenous retroviruses, in an effort to understand their diversity and distribution within these vertebrate classes and thereby gain insights into the origins and evolution of the mammalian retroviruses. The small number of retroviral sequences available, to date, from lower vertebrate hosts have all been quite distinct from mammalian and avian examples. None can be easily placed with any of the mammalian genera and only in the case of SpeV, from the reptile tuatara, is there some bootstrap support for the placement of one of these viruses next to a single retroviral genus (generally 60 to 80 of 100 bootstrap replicates place SpeV as a sister taxon to the spumavirus genus) (28).

This report is therefore the first, supported by molecular data and phylogenetic analysis, showing that retroviruses present within highly disparate vertebrate hosts can all cluster

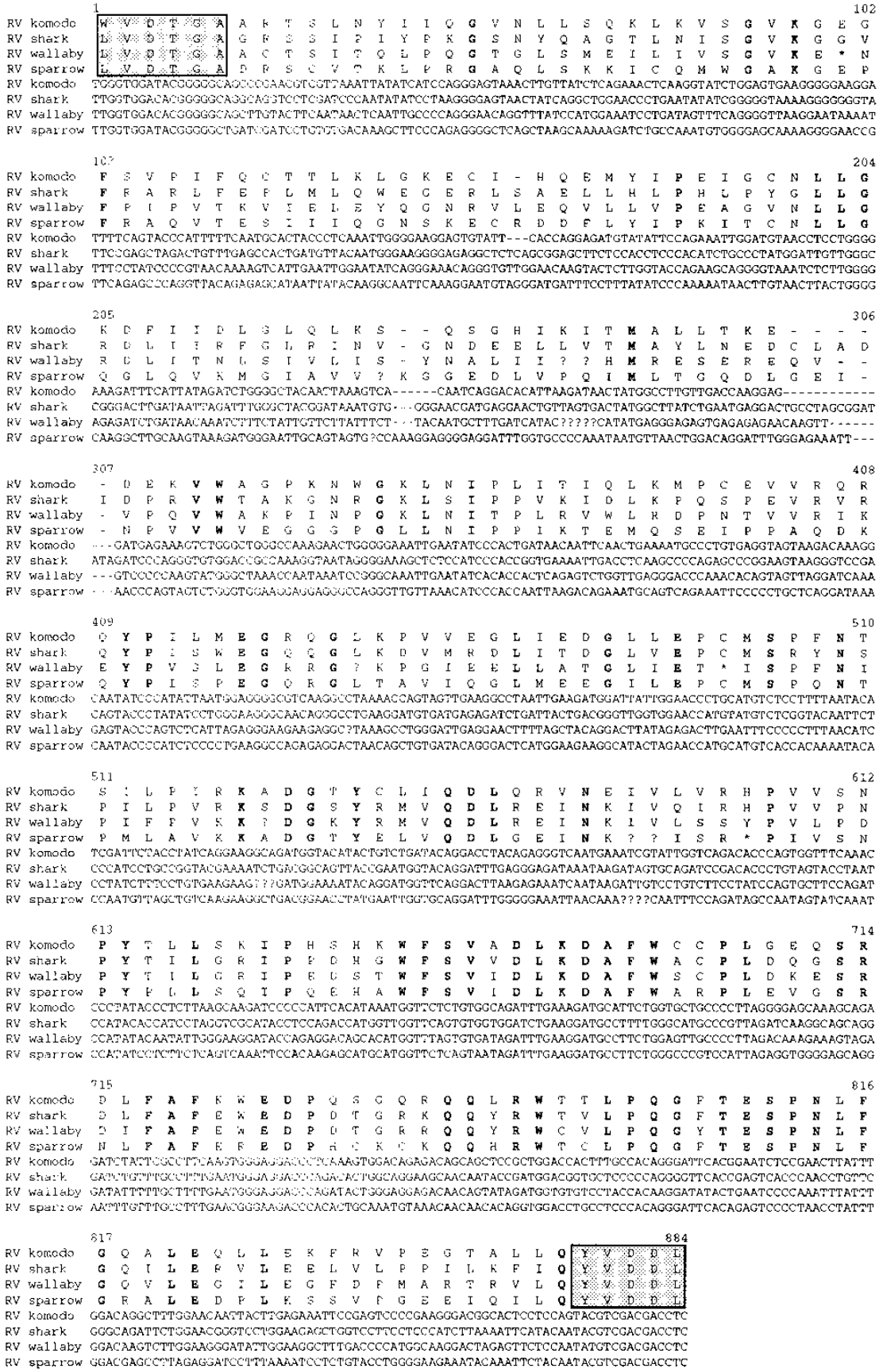

FIG. 1. Amino acid and nucleotide sequence alignment of four endogenous retroviral fragments isolated from different vertebrate classes. The conserved motifs (the active sites of the protease protein and reverse transcriptase protein) encoded by all characterized retroviruses, and from which PCR primers were designed, are shown boxed at each end of the alignment. Amino acid residues conserved between all four isolates are shown in boldface type. Asterisks indicate in-frame stop codons; question marks indicate probable frameshift mutations.

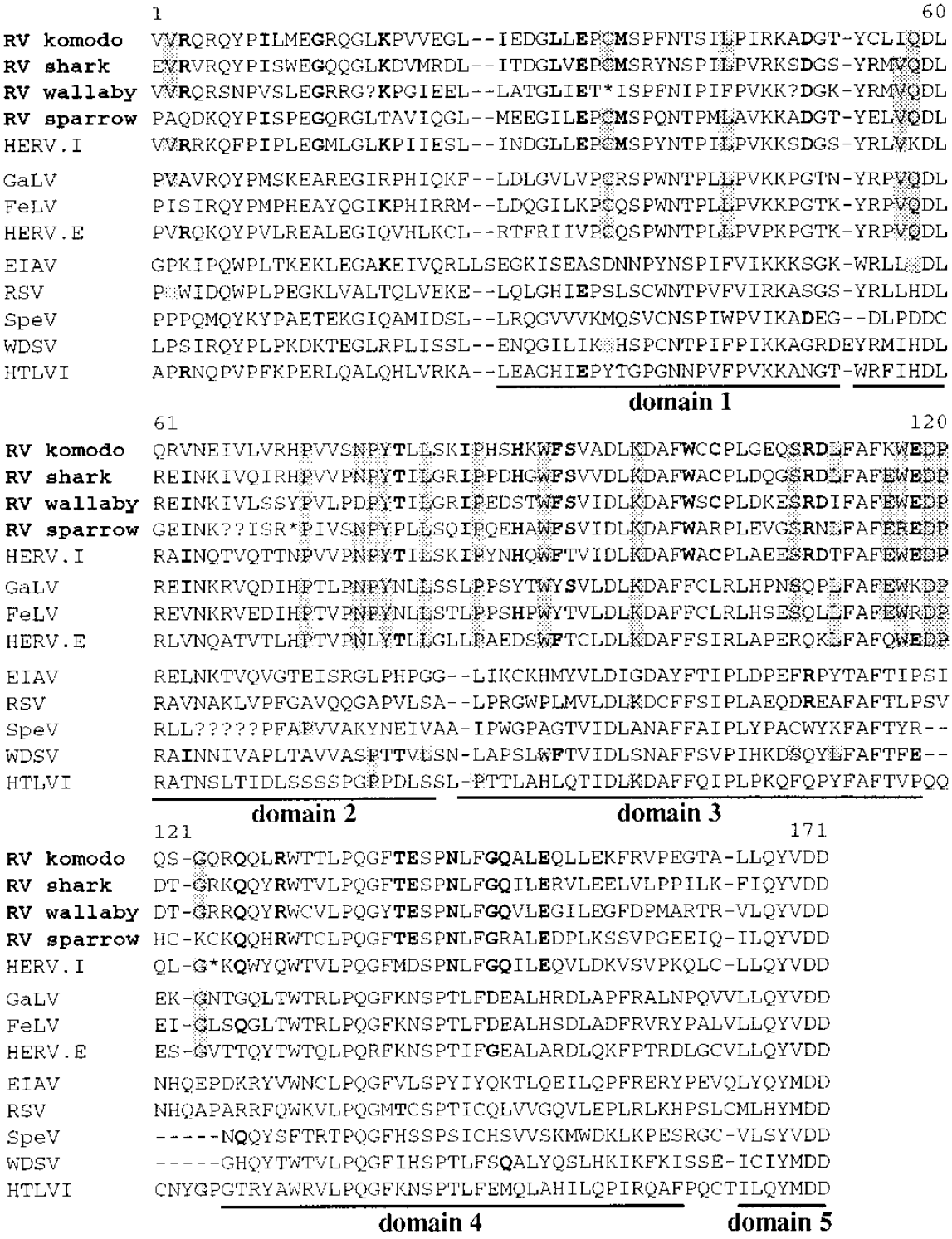

FIG. 2. Alignment of a 171-amino-acid region of retroviral reverse transcriptase proteins. Residues conserved between at least four of the HERV-I-related retroviruses and absent from the majority of other retroviral sequences are shown in boldface type. Those present between members of both the HERV-I and MLV groups, but generally absent from other retroviral sequences, are shaded. Gaps, indicated by dashes, were introduced in order to align the five conserved reverse transcriptase domains.

into one strongly supported monophyletic group. Indeed the lemon shark is the most primitive organism from which a retroviral sequence has been isolated, yet its reverse transcriptase protein displays 65% homology (across the region shown in Fig. 2) with the rock wallaby sequence, despite the estimated date of divergence of the two hosts of approximately 400 million years (17). The broad host range of this group of retroviruses suggests that they may well be ubiquitous within many

TABLE 1. Percentage similarity between the HERV-I group and other retroviruses

Group	Similarity (%)						
	HERV-I group	MLV group	EIAV	RSV	SpeV	WDSV	HTLV-I
HERV-I[a]	62.0	48.2	31.7	33.5	34.7	43.2	35.7
MLV[b]	48.2	66.4	32.9	33.3	35.1	42.5	37.4

[a] Includes RV Komodo, RV sparrow, RV shark, RV wallaby, and HERV-I.
[b] Includes GaLV, FeLV, and HERV-E.

vertebrate classes. HERV-I (the only previously characterized isolate of this group) is present within several primates and Old World monkeys. Furthermore, Southern hybridizations with the rock wallaby retroviral fragment as a probe suggest similar

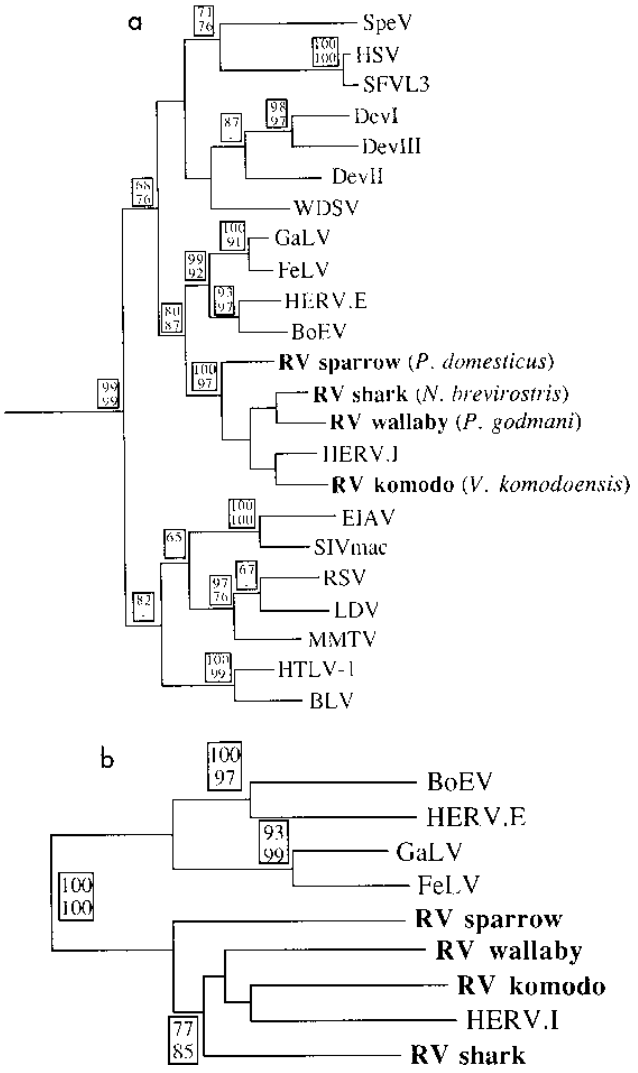

FIG. 3. (a) Neighbor-joining tree of retroviral reverse transcriptase sequences generated from a nucleotide sequence-based data set aligned identically to the amino acids shown in Fig. 2. The tree was rooted on several *gypsy* LTR-retrotransposon sequences. Third codon positions were excluded, and transversions/transitions were weighted at a 2:1 ratio. Boxed figures indicate the percentage support for a particular node after 100 bootstrap replicates made by using the neighbor-joining (upper figure) and maximum parsimony (lower figure) approaches. Horizontal branch lengths are proportional to the similarity between the sequences. (b) As above, except that data from both the protease and reverse transcriptase gene were included in the dataset. The tree is unrooted.

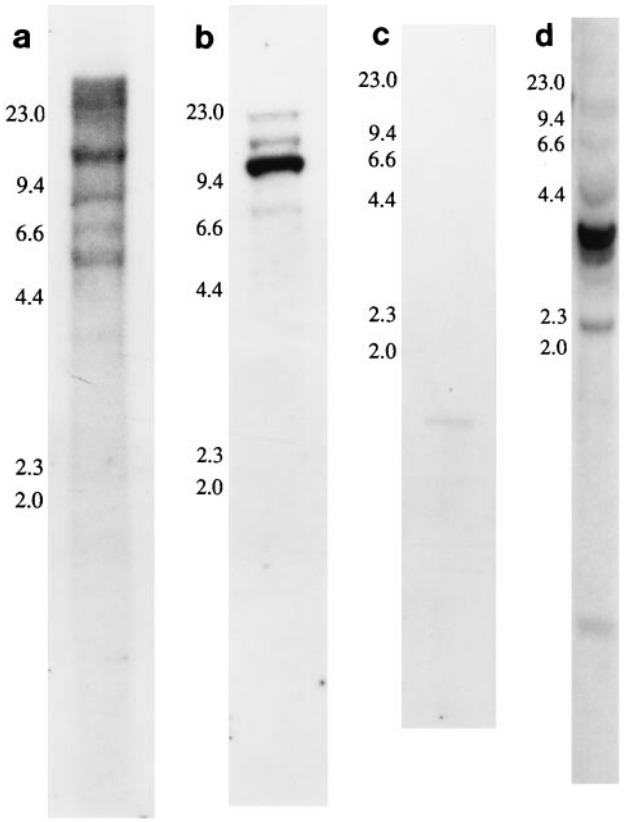

FIG. 4. Southern blot analyses of host genomic DNA by using the retroviral fragments as probes. Hybridization was performed at 65°C, and filters were washed in 0.5 or 1 × SSC–0.5% SDS also at 65°C. Sizes are indicated in kilobases: (a) *Eco*RI-digested lemon shark DNA (*Negaprion brevirostris*) hybridized to the lemon shark fragment, (b) *Eco*RI-digested sparrow DNA (*Passer domesticus*) hybridized to the sparrow fragment, (c) *Hin*dIII-digested Komodo dragon DNA (*Varanus komodoensis*) hybridized to the Komodo fragment, and (d) *Eco*RI-digested Godman's rock wallaby DNA (*Petrogale godmani*) hybridized to the rock wallaby fragment.

viruses are also present within the genomes of many marsupials and monotremes (Fig. 5). We have also partially characterized members of this retroviral group from the dogfish (*Scyliorhinus*), the slowworm (*Anguis fragilis*), and two birds, the partridge (*Perdix perdix*) and great tit (*Parus major*) (unpublished results). The integration of HERV-I into the germ line of a common ancestor of apes and Old World monkeys suggests that this retroviral group is at least 30 million years old (23). However, their widespread distribution within vertebrate genomes indicates that they first arose significantly earlier than this, and therefore the high degree of similarity between the sequences is best explained by the proposal of Doolittle et al. (6) that exogenous retroviruses exist only briefly and are transmitted as endogenous elements for long periods.

HERV-I is one of several human endogenous retroviruses which are placed, by phylogenetic analysis, on the MLV lineage of the retroviral family tree (32). Despite this, these viruses are only distantly related to the prototype MLV isolates (such as GaLV, FeLV, MuLV, and HERV-E). It thus appears that the MLV lineage has a complex evolutionary history and HERV-I, rather than simply representing one of a number of divergent isolates from the MLV genus, has been evolving independently from them as part of a large group of closely related retroviruses for a considerable period of time. This suggests that at least some other human retroviruses (such as

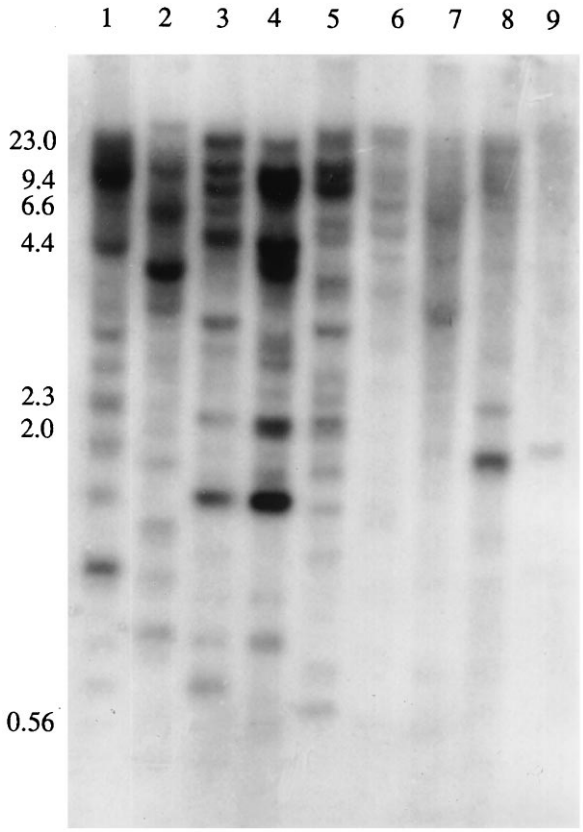

FIG. 5. Southern blot analysis of *Bgl*II-digested genomic DNA hybridized to the rock wallaby fragment. Hybridization was performed at 55°C, and filters were washed down to 1 × SSC–0.1% SDS at 55°C. Lane 1, koala (*Phascolarctos cinereus*); lane 2, stripe-faced dunnart (*Sminthopsis macroura*); lane 3, tammar (*Macropus eugenii*); lane 4, Godman's rock wallaby (*Petrogale godmani*); lane 5, common brushtail possum (*Trichosaurus vulpecula*); lane 6, brindled bandicoot (*Isoodon macrourus*); lane 7, opossum (*Monodelphis* sp.); lane 8, short-beaked echidna (*Tachyglossus aculeatus*); lane 9, platypus (*Ornithorhynchus anatinus*). Size is indicated in kilobases.

HERV-H and ERV-9) which are also distantly related to, but members of, the MLV lineage may also have counterparts with a wide distribution. Consistent with this, we have recently isolated an ERV-9 related retroviral fragment from the stripe-faced dunnart (*Sminthopsis macroura*—a carnivorous marsupial of the family Dasyuridae) (unpublished results).

Although all of the HERV-I-related retroviruses described here are endogenous, we think it is probable that there are exogenous members of this group in some organisms. Two of the isolated sequences (from the lemon shark and Komodo dragon) do not encode any frameshift mutations or stop codons within the region examined and are therefore likely to have been exogenous in the recent past. Thus, it may be possible to infer much about the original biological properties of some human endogenous retroviruses, such as HERV-I, by studying related exogenous viruses in other species.

Now that retroviral sequences have been identified within sharks (which belong to the Elasmobranchii, a primitive subclass of fish), it is interesting to speculate on other organisms in which these elements may also be present. Retroviral branch elements have not been isolated from insects, plants, or invertebrates and with the well-characterized nature of insect, and to a lesser extent plant, retroelements this suggests that they are likely to be absent (or at least very rare) within these host taxa. Retroviral sequences have also yet to be identified within invertebrates, but there have been reports of retroviral-like particles within the soft-shelled clam (*Mya arenaria*), which may be associated with the high level of disseminated sarcoma which has been observed in this species (16). These, and the fact that both of the fish retroviral sequences characterized to date (the lemon shark sequence and the walleye dermal sarcoma virus) do not appear to be particularly basal to other known retroviruses in phylogenetic analyses, suggest that the host range boundary of the retroviral branch elements has not yet been identified.

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