Δ(*HHT1-HHF1*); MSY157: *MATa ura3-52 lys2-801 ade2-101* Δ(*HHT2-HHF2*); MSY159: *MATa ura3-52 lys2-801 ade2-101*; MX4-22A: *MATa ura3-52 leu2-3,112 lys2Δ201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) (requires a plasmid with H3 and H4 genes). The construction of strains MSY155, MSY157, and MSY159 has been described (*8*).
12. Samples from early exponential growth cultures were diluted in 1% saline, and sonicated 5 s to disperse single cells. Cell volume histograms were collected with a Coulter Counter model ZM particle counter and Coulter Channelyzer C256 pulse height analyzer.
13. Cells for light microscopy were fixed in methanol: acetic acid (3:1) and stained in 1 μg/ml (4′,6-diamidino-2-phenylindole (DAPI) [D. Williamson and D. Fennell, *Methods Cell Biol.* **12**, 335 (1975)].
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21 August 1989; accepted 14 November 1989

Secondary Structure Is the Major Determinant for Interaction of HIV *rev* Protein with RNA

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A region in the human immunodeficiency virus (HIV) *env* message, with the potential to form a complex secondary structure (designated RRE), interacts with the *rev* protein (Rev). This interaction is believed to mediate export of HIV structural messenger RNAs from the nucleus to the cytoplasm. In this report the regions essential for Rev interaction with the RRE are further characterized and the functional significance of Rev-RRE interaction in vivo is examined. A single hairpin loop structure within the RRE was found to be a primary determinant for Rev binding in vitro and Rev response in vivo. Maintenance of secondary structure, rather than primary nucleotide sequence alone, appeared to be necessary for Rev-RNA interaction, which distinguishes it from the mechanism for cis-acting elements in DNA. Limited changes within the 200 nucleotides, which preserved the proper RRE conformational structure, were well tolerated for Rev binding and function. Thus, variation among the RRE elements present in the diverse HIV isolates would have little, if any, effect on Rev responsiveness.

THE GENOME OF HIV IS MUCH more complex than other retroviruses. In addition to the *gag*, *pol*, and *env* genes present in all retroviruses, it encodes at least seven regulatory proteins. Two of these proteins, encoded by *tat* (*1*) and *rev* (*2*) (referred to as Tat and Rev, respectively) are absolutely required for virus replication (*3*) and, thus, represent attractive targets for therapeutic intervention. The mechanisms proposed for Tat function are diverse, with both transcriptional (*4, 5*) and posttranscriptional components being considered (*5, 6*). In contrast, there is general agreement that Rev exerts its effect at the posttranscriptional level to facilitate export of structural mRNAs, entrapped in the nucleus, to the cytoplasm (*7, 8*). Export is most likely mediated through a cis-acting element, referred to as RRE [originally referred to as CAR (*9*)] (*8*), which is present in *env*.

We and others have recently shown that Rev interacts specifically with RNA that contains the RRE sequence (*10, 11*). The predicted structure formed by base-pair interactions within the RNA that comprise the RRE element is shown in Fig. 1. Solution mapping of the RRE RNA indicates that the structure depicted is likely to be correct (*12*). Through RNA annealing experiments, we have shown that the large base-paired "stem structure" does indeed form and that its formation is required for Rev binding (*10*). This finding could be interpreted in several ways: (i) base-pairing interactions in this region are required for the appropriate formation of the other hairpin structures, one or a combination of which may comprise the site for Rev interaction; (ii) Rev interacts directly with the stem structure itself; or (iii) Rev interacts with the large stem structure and other elements of secondary structure simultaneously. To distinguish between these possibilities, we introduced nucleotide changes into the stem

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Table 1. Activity of the RRE mutations in a Rev-response assay. The mutations shown were cloned into the Cla I–Xba I site of plasmid pIIIAR depicted in Fig. 4 and CHOZip-tat_{III} cells were transfected with 100 ng of each plasmid and 100 ng of the Rev expression vector pSVRev. CAT assays were run for 30 mins, a time found to give a linear response. The percent conversion of [^{14}C]chloramphenicol to acetylated products was determined by liquid scintillation counting of the spots cut from the thin-layer chromatography plate. The percent conversion per minute is given. The relative activity of each mutant is compared to that of pIIIAR, which was assigned a value of 1.0. The low to barely detectable level of CAT activity obtained in the absence of Rev did not allow for quantitation. Rev binding was assessed by visual inspection of the gel retardation autoradiograms obtained from at least three independent experiments. The scoring ranges from best binding (+++) to no binding observed (−).

Plasmid	Conversion (%/min)	Activity	Rev/RRE interaction
pIIIAR	1.46	1.00	+++
HS-1	0.59	0.41	++
HS-2	0.37	0.26	++
HS-1,2	1.13	0.80	+++
HB-1	1.96	1.34	+++
HB-2	1.45	1.00	+++
HSL-6	0.73	0.50	++
HSL-7	0.08	0.05	−
HSL-8L	0.10	0.07	−
HSL-8R	0.08	0.06	−
HSL-8RL	1.83	1.25	++
HSL-9	1.63	1.10	+++
HSL-1	1.81	1.23	+++
HSL-2	1.16	0.80	+++
HSL-3	0.26	0.20	+
HSL-4	1.23	0.80	++
HSL-5	0.07	0.05	−

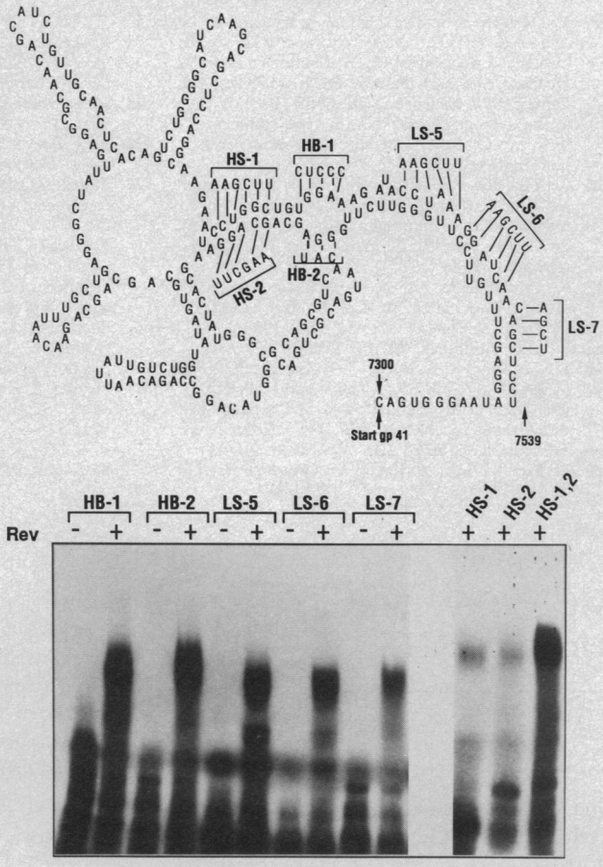


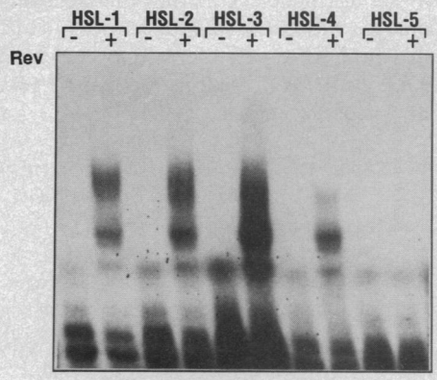
Fig. 1. Schematic illustration of nucleotide changes created in the HIV-1 RRE element and the effect on Rev-RRE interactions. The predicted secondary structure assumed by the HIV-1 RRE is shown (*7*). The nucleotide changes were created by site-directed mutagenesis (*21*) and confirmed by sequencing of DNA. All RRE elements were present in a Bluescript vector (Stratagene) such that the RRE RNA could be produced in vitro by transcription with T7 polymerase. In vitro transcribed RNAs were incubated with purified Rev protein (*10*) and binding was assessed by a nuclease resistance gel retardation assay (*22*). RNAs that failed to form a complex with Rev are digested to oligonucleotides and small fragments, while those bound to protein will be partially protected from ribonuclease (RNase) T1 digestion and remain bound to the Rev protein. Reaction mixtures were analyzed on 4% nondenaturing polyacrylamide gels after RNase T1 digestion. RRE RNA was incubated in the absence (−) and presence (+) of Rev protein.

(Fig. 1). All mutations were designed to alter the primary nucleotide sequence. The individual changes produced small bulges in the stem structure, removed bulges from the stem, or maintained a different secondary structure. RRE RNAs with mutations designed to create small bulges in the stem (LS-5, LS-6, LS-7, HS-1, and HS-2) all bound to Rev, albeit some apparently to a lesser extent than others (Fig. 1 and Table 1). Mutations HS-1 and HS-2, both predicted to destabilize base-pairing at the top of the stem, bound weakly to Rev. However, RNA HS-1,2, which includes both the HS-1 and HS-2 mutations and thus restores the stem by compensatory mutation, showed a better interaction with Rev, similar to that obtained with the authentic RRE element (Fig. 1). Mutations HB-1 (which increases base-pairing in the stem) and HB-2, both of which affect the bulge, did not affect REV-RRE interaction. The above results suggest that Rev-RRE interaction is not conferred by a primary nucleotide sequence present in the stem structure.

Since small changes in base-pairing in the stem had little effect on Rev-RRE interaction, we reasoned that the lack of Rev binding obtained when the RREs had major deletions in the stem (*10*) could reflect the formation of alternate secondary structures that lack the structural elements required for Rev binding. This possibility was examined by creating the mutations illustrated schematically in Fig. 2. Nucleotides that make up several of the smaller hairpin loop structures could be deleted without affecting Rev-RRE interaction (Fig. 2; HSL-1 and HSL-2). RNA HSL-4, designed to delete three of the hairpin loops, gave a weak interaction with Rev, which was evident after long exposure of the gels. RNAs with deletions illustrated in HSL-5 failed to interact with Rev; no Rev-RRE complexes were detected even after prolonged exposure of the gels.

The importance of the structure deleted in HSL-5 was examined further by creating the mutations pictured in Fig. 3. Removal of the hairpin loop, shown for HSL-6, greatly reduced the Rev-RRE interaction; whereas removal of the other hairpin, shown for HSL-7, completely abolished Rev binding. The above findings suggested that nucleotides deleted in the HSL-7 hairpin loop are most important for Rev-RRE interaction. The contribution of primary nucleotide sequence to Rev binding was examined by introduction of additional nucleotide changes in this hairpin structure. Altering the nucleotide sequence in the loop (HSL-

Fig. 2. Effect of hairpin loop deletions of Rev RRE interaction. Deletions created in the RRE hairpin loop structures are schematically illustrated. The secondary structure depicted is only meant to serve as a point of reference for the deletions and most probably does not assume the exact folding pattern shown. All mutations were made within the complete RRE structure (Fig. 1). Analysis of Rev-RRE interactions was done as described in the legend to Fig. 1. Bold letters indicate nucleotide changes introduced into the RRE.



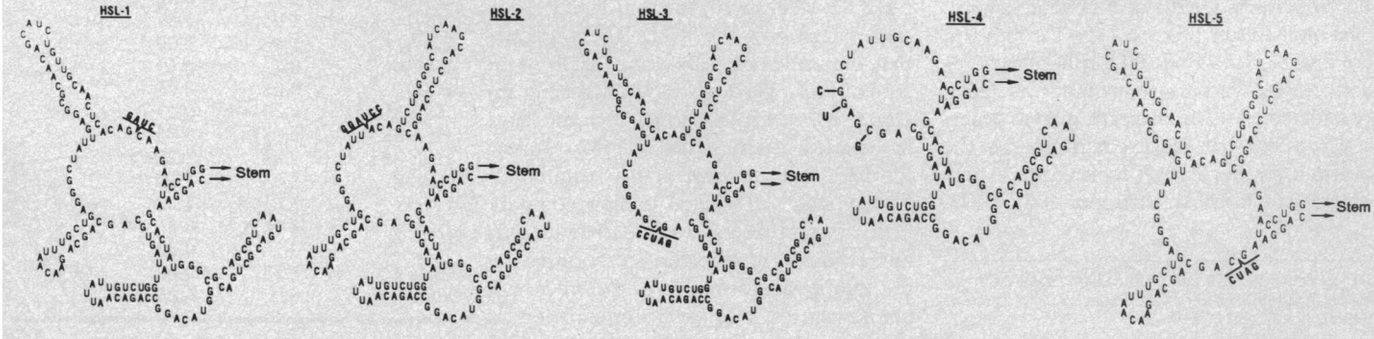
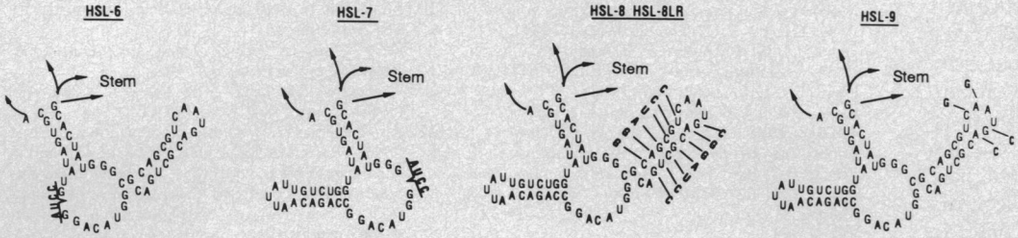
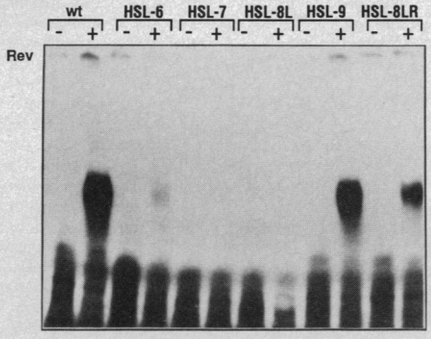


Fig. 3. Localization of the major determinant for Rev-RRE interaction. The nucleotide deletions and insertions (bold letters) made in the lower hairpin loop structure of the RRE (region removed in the HSL-5 deletion shown in Fig. 2) are illustrated. All mutations were made within the complete RRE structure. RRE element HSL-8L contains only those changes present on the left side of the hairpin structure; whereas HSL-8LR contains those changes shown on the left and right half of the hairpin. wt, wild type.





9) had no obvious effect on Rev-RRE interaction (Fig. 3). In contrast, mutation HSL-8L, designed to disrupt formation of the hairpin, prevented Rev binding. Similar results were obtained with mutation HSL-8R (Table 1). Mutation HSL-8R (not shown) is the same mutation as HSL-8L except that it is present on the opposite side of the stem structure. Mutation HSL-8LR, which is a compensatory mutation for HSL-8L and is predicted to maintain secondary structure, restored Rev-RRE interaction, even though the primary nucleotide sequence is quite distinct from that of the original hairpin loop structue. In summary, we have not, as yet, found a single region of the RRE where mutation dramatically alters Rev binding while maintaining secondary structure of the region.

We examined the ability of the mutant RREs to function in vivo by replacing the wild-type RRE in plasmid pIIIAR with the individual RRE mutations and by measuring their ability to complement gene expression in a Rev-dependent heterologous gene expression assay (*9*). This assay has been described previously and has been used to identify the Rev-responsive element (*9*). The HIV *env* sequences, harboring the sequences that mediate nuclear retention of viral RNA as well as the Rev response element, are positioned 3′ to the stop codon of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene such that the viral sequences are incorporated into the 3′ untranslated portion of the CAT gene transcript. The result of this modification is that CAT expression becomes subject to regulation by Rev. As has been shown previously, gene expression, directed by plasmid pIIIAR, is repressed [relative to pU3R-III (*13*), which lacks the HIV *env* sequence] unless Rev protein is supplied in trans (Fig. 4 and Table 1). In brief, RRE mutations with impaired Rev-RRE interaction in vitro also had an impaired Rev response. Mutations that failed to interact with the RRE (that is, HSL-8R, HSL-8L, HSL-7, and HSL-5) were not Rev-responsive (Fig. 4). Of greatest significance is the compensatory mutation HSL-8LR in which the stem structure is completely different from that in the original hairpin in the RRE. This compensatory mutation was still Rev-responsive, in sharp contrast to the noncompensatory mutations, which failed to maintain secondary structure. Mutants LS-5, LS-6, and LS-7, when tested in a different Rev-response assay, were all found to be functional (*14*).

Our data suggest that complex base-pairing interactions within the HIV RRE element generate hairpin loop structures that are essential for Rev-RRE interaction. Structure, rather than simply primary sequence, appears to be a major determinant for Rev-RRE interaction. If a specific primary sequence is required, the nature of the mutations made suggests that the determinant is small in size and that its recognition is heavily dependent on its context within the secondary structure of this region. The interaction of Rev with RRE RNA contrasts significantly with many DNA-protein interactions where primary nucleotide sequence, rather than structure, is the primary determinant of protein recognition. The interaction of Rev with RRE RNA also differs from other well-characterized protein-RNA interactions in which the nucleotide sequence and the secondary structure of the hairpin loops are equally important for protein recognition (*15*).

Since alteration of the nucleotide sequence, present in the HSL-5 hairpin loop, had little effect on Rev-RRE interaction, it

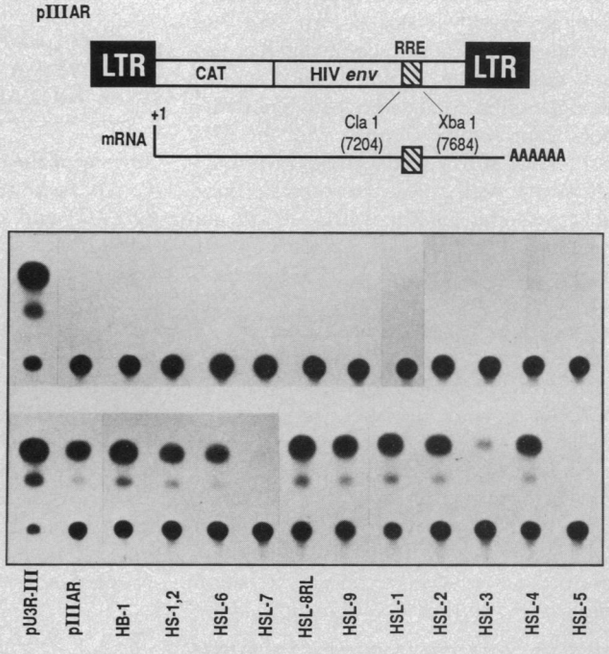


Fig. 4. Functional analysis of the altered RRE element in a Rev-dependent heterologous gene expression assay. The mutated RREs shown were used to replace the authentic RRE present in plasmid pIIIAR (*9*). Exchange of fragments was facilitated by site-directed mutagenesis to create unique Cla I and Xba I sites at the 5′ and 3′ terminal ends of the RRE element. Mutated RREs containing Cla I and Xba I cohesive ends were then inserted into this vector. Transfection of CHOZip-tat_{III} cells (*23*) was done in the presence (+) (1 μg) or absence (−) of plasmid pIIIRev [originally referred to as pHart (*9*)]. CAT assays were performed 48 hours after transfection (*24*). All transfections were repeated a minimum of three times. Similarly, the results obtained in the absence and presence of Rev should only be compared among the individual plasmids, as comparison among them is difficult to interpret because each mutation has the potential of creating plasmids with different basal levels of gene expression. The actual percent conversion of [^{14}C]chloramphenicol to acetylated products for these and other mutants is given in Table 1.

is likely that Rev has several contact points within the RRE. Our previous observation that formation of the large stem structure is required for Rev interaction (*10*), taken together with the findings reported here, suggests that Rev has contact points on both the large stem and the HSL-5 hairpin loop that are in close proximity. By deletion analysis of the RRE, Dayton *et al.* (*16*) have also found that maintenance of secondary structure is required for Rev response. In other experiments with the RRE elements obtained from the evolutionarily conserved simian immunodeficiency virus (SIV) and HIV-2 viruses, we find that both interact with the HIV-1 Rev protein (*17*). However, the hairpin loop structures required for this interaction, which are also in close proximity to the stem structure, bear little sequence similarity to the HIV-1 hairpin loop recognized by Rev. This observation strengthens our contention that structure, rather than simply primary sequence, determines Rev-RRE interactions. This may also explain how the human T cell lymphotropic virus type 1 (HTLV-1) Rex protein, which is unrelated to HIV-1 Rev, can substitute for Rev function (*18*). Furthermore, the strong correlation between the alteration of the binding in vitro and the functional characteristics of the mutant RREs in vivo supports the hypothesis that binding of Rev to the RRE is of physiological relevance.

The mechanisms by which Rev-RRE interactions facilitate export of HIV structural mRNAs from the nucleus to cytoplasm remain to be determined. Whether Rev acts by itself or in concert with host proteins to facilitate export is not known. We have recently purified a transdominant Rev protein described by Malim *et al.* (*19*). Although nonfunctional in vivo, as reported, it does form a stable complex with the RRE (*20*). Thus, if Rev mediates export through interaction with a host transporter protein, the transdominant Rev is likely to lack this function. As an alternative explanation, the interaction of Rev with the RRE may facilitate a conformational change in the RRE secondary or tertiary structure, which permits access of a host "transporter" factor that mediates export of HIV structural mRNAs from the nucleus to cytoplasm. If such a mechanism for control of HIV gene expression does exist, it is envisioned that this factor also plays a role in the normal posttranscriptional events that govern cellular gene expression. Thus, HIV may have usurped a normal host control mechanism and added a further level of complexity.

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22. Rev-RRE binding was assessed as follows. Plasmid DNAs were linearized at a unique Xba I site, and in vitro transcription with T7 RNA polymerase was performed according to manufacturer's specifications (Promega Biotec). The integrity of the transcribed products was examined by analysis on 8% denaturing polyacrylamide gels. Labeled RNAs were purified through a G-50 Sephadex spin column and used directly for the in vitro Rev binding assay. Approximately 50 ng of purified Rev protein was resuspended in binding buffer [5 m*M* Hepes (*p*H 7.6), 25 m*M* KCl, 2 m*M* $MgCl_2$, and 3.8% glycerol] containing 10 μg of nonspecific carried transfer RNA. The reaction was incubated for 15 min at room temperature at which time labeled RRE RNA (0.4 to 1 ng, 10^4 to 2×10^5 cpm) was added in a final volume of 20 μl. Incubation was continued an additional 15 min at 30°C at which time five units of ribonuclease T1 (Pharmacia) was added. Reactions were further incubated at 37°C for 10 min then adjusted to 10% glycerol and applied to a 4% nondenaturing polyacrylamide gel containing 4% glycerol. Electrophoresis was carried out at a constant voltage of 250 volts at 4°C in 0.5× tris-borate (TBE) buffer. The gel was fixed in 10% acetic acid, dried, and exposed to x-ray film.
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25. Supported, in part, with funds from a National Cooperative Drug Discovery Award.

15 November 1989; accepted 24 January 1990

Expanded HIV-1 Cellular Tropism by Phenotypic Mixing with Murine Endogenous Retroviruses

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In view of the current interest in in vivo murine models for acquired immunodeficiency syndrome (AIDS), the interaction between human immunodeficiency virus type 1 (HIV-1) and endogenous murine leukemia virus (MuLV)–related retroviruses was investigated with a human leukemic T cell line (PF-382$_x$) that acquired xenotropic MuLV (X-MuLV) after in vivo passage in immunosuppressed mice. Despite similar levels of membrane CD4 expression and HIV-1 ^{125}I-labeled gp120 binding, a dramatic acceleration in the time course of HIV-1 infection was observed in PF-382$_x$ compared to its X-MuLV–negative counterpart (PF-382). Moreover, PF-382 cells coinfected by X-MuLV and HIV-1 generated a progeny of phenotypically mixed viral particles, enabling HIV-1 to productively infect a panel of CD4$^-$ human cells, including B lymphoid cells and purified normal peripheral blood CD4$^-$/CD8$^+$ T lymphocytes. Mixed viral phenotypes were also produced by human CD4$^+$ T cells coinfected with an amphotropic MuLV-related retrovirus (A-MuLV) and HIV-1. These data show that endogenous MuLV acquired by human cells transplanted into mice can significantly interact with HIV-1, thereby inducing important alterations of HIV-1 biological properties.

IT IS WELL ESTABLISHED THAT HIV IS the causative agent of AIDS and related disorders (*1*). The need for analytical approaches to the study of AIDS pathogenesis and for large-scale testing of new drugs and vaccines has prompted the search for experimental animal models of HIV infection. On the basis of phylogenetic related-