Mutational Definition of the Human Immunodeficiency Virus Type 1 Rev Activation Domain†

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Replication of human immunodeficiency virus type 1 requires the functional expression of the virally encoded Rev protein. The binding of this nuclear *trans* activator to its viral target sequence, the Rev-response element, induces the cytoplasmic expression of unspliced viral mRNAs. Mutation of the activation domain of Rev generates inactive proteins with normal RNA binding capabilities that inhibit wild-type Rev function in a *trans*-dominant manner. Here, we report that the activation domain comprises a minimum of nine amino acids, four of which are critically spaced leucines. The preservation of this essential sequence in other primate and nonprimate lentivirus Rev proteins indicates that this leucine-rich motif has been highly conserved during evolution. This conclusion, taken together with the observed permissiveness of a variety of eukaryotic cell types for Rev function, suggests that the target for the activation domain of Rev is likely to be a highly conserved cellular protein(s) intrinsic to nuclear mRNA transport or splicing.

The pathogenic retrovirus human immunodeficiency virus type 1 (HIV-1) displays a pattern of gene expression that is markedly complex (25). Following the initial activation of viral transcription from the integrated provirus, a family of multiply spliced ~2-kb viral mRNAs is expressed. Once the level of these early mRNAs has reached a critical point, a shift in the pattern of viral mRNAs in the cytoplasm occurs and the singly spliced ~4-kb and unspliced ~9-kb RNAs are expressed. These late RNAs comprise the genomic RNA and the mRNAs that encode the viral structural proteins Gag, Pol, and Env (25) as well as the viral Vif, Vpu, and Vpr gene products (15, 45).

The ~2-kb class of early mRNAs encodes three nonstructural viral proteins. Two of these, Tat and Rev, are *trans* activators essential for viral replication in culture (7). The Tat protein is a potent activator of the expression of all sequences linked in *cis* to the viral long terminal repeat promoter element and is therefore required for efficient expression of all viral gene products. In contrast, the Rev protein acts posttranscriptionally to mediate the qualitative switch in viral gene expression noted above (12, 48). Specifically, Rev activates the cytoplasmic expression of the incompletely spliced late viral mRNAs that are otherwise sequestered within the cell nucleus (10, 13, 18, 32). These viral mRNAs have been shown to contain the *cis*-acting RNA target for Rev, the Rev-response element (RRE) (32, 43). Rev binds directly and with high specificity to the RRE in vitro, and it is believed that this interaction targets the unspliced viral mRNAs for nucleocytoplasmic export in vivo (9, 20, 33, 38, 55). Whether Rev functions to inhibit the assembly of splicing complexes on viral mRNA (3) or to activate the transport of viral mRNA directly (13, 32) is currently unclear. The critical nature of Rev function has, however, been underscored by the recent demonstration that low or subcritical levels of Rev activity can lead to the establishment of a latent HIV-1 infection (40).

The HIV-1 Rev protein comprises 116 amino acids, is serine phosphorylated, and is localized to the nuclei of expressing cells (4, 8, 19). The most notable feature of the protein sequence is an arginine-rich stretch of amino acids, extending from positions 35 to 50, that has been shown to serve as the nuclear localization signal (29, 39). Previous studies have identified two domains within Rev that, when mutated, give rise to inactive proteins (21, 22, 29, 34, 37, 39, 54). The first and more N-terminal domain includes the arginine-rich motif, extends from approximately residues 18 to 60 (21, 22, 29, 39), and has been shown to participate in Rev multimerization and RRE binding (30, 37). Nonfunctional proteins mutated within this domain do not significantly inhibit wild-type Rev function in *trans* and are termed recessive negative mutants (29).

Although a second discrete functional domain of Rev is known to be located toward the C terminus, near residue 78, the essential features of the domain have remained undefined. Mutations in this domain give rise to nonfunctional proteins that display wild-type nuclear localization, multimerization, and RRE binding activity (29, 30, 37, 39). Importantly, these mutants have been shown to inhibit wild-type Rev function in a dose-dependent manner and are referred to as dominant negative or *trans*-dominant repressors (22, 29, 34, 37, 54). These observations have led to the suggestion that the C-terminal essential domain of Rev is likely to be operationally equivalent to the activation domains described for a number of DNA sequence-specific transcriptional activators (29, 41). Here, we report a detailed mutational analysis of the Rev activation domain. We demonstrate that three or four closely grouped leucine residues are critical for Rev function and suggest that this domain may participate in an interaction with a cellular protein that has been conserved during evolution.

MATERIALS AND METHODS

Construction of molecular clones. The pgTAT, pcREV, pBC12/CMV, pHIVΔRev, and pBC12/RSV/SEAP plasmids have been described previously (1, 5, 29, 31), as have the construction and phenotypic analysis of the pcREV mutants

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† This work is dedicated to the memory of our friend and colleague David F. McCarn.

FIG. 1. Description and phenotypic analysis of HIV-1 Rev mutants. The primary amino acid sequence of HIV-1 Rev between residues 67 and 92 is shown. The changes in the amino acid sequence for each mutant are indicated below, with the standard abbreviations for amino acids. A dash (-) indicates that an amino acid has been deleted. The arrowheads (>), representing mutants Δ22/14 and Δ23/14, indicate that all sequences up to residue 112 (located at the *Bgl*II site in M14; see reference 29) have been deleted; in effect, these two mutants are C-terminal truncations. To assess the in vivo phenotypes of the various Rev mutants, two indicator plasmids, pgTAT and pHIVΔRev, were used in cotransfection experiments with COS cells (29, 31). With pgTAT, Rev activity was scored as the ability of a given mutant to induce expression of the 72-amino acid form of Tat (see Fig. 2 and text for details). ++, full activity; +, partial activity; -, no activity. With pHIVΔRev (the replication-defective provirus clone), the extent of provirus rescue for each mutant is given as a percentage of the rescue provided by wild-type (wt) Rev (see Materials and Methods for details).

M4, M9, M10, M11, and M14 (29). Oligonucleotide-directed mutagenesis with a bacteriophage M13 system (Amersham Corp., Arlington Heights, Ill.) was used to introduce a number of further point mutations into the coding region of the Rev cDNA present in pcREV. All mutations were confirmed by dideoxynucleotide sequencing. The precise amino acid sequence changes in each mutant are shown in Fig. 1. Because a unique *Bgl*II restriction site was introduced at the mutations in M9, M14, M18, M19, M22, and M23, these vectors were used for the subsequent generation of a series of deletion (Δ) mutants (Fig. 1). The Rev-responsive chloramphenicol acetyltransferase (CAT) expression vector pDM128/CMV contains the simian virus 40 origin of replication, the cytomegalovirus immediate-early promoter, and the bacterial CAT gene inserted into an intron regulated by Rev. It was constructed by ligating the ~1.7-kb *Xba*I-*Bgl*II fragment from pDM128 (21) between the *Sal*I site and the more distal *Bgl*II site of pgTAT. pBC12/CMV/CAT contains the cytomegalovirus immediate-early promoter driving expression of the CAT gene and was constructed by inserting the *Hind*III-*Bam*HI CAT coding fragment (16) between the *Hind*III and *Bam*HI sites of pBC12/CMV.

Cell culture and DNA transfection. The monkey cell lines COS and CV-1, mouse cell line Ltk⁻, and quail cell line QCl-3 were maintained as described previously (5). The mouse cell line NIH 3T3 was maintained in Dulbecco's modified Eagle medium with high glucose, 10% calf serum, and 50 μg of gentamicin sulfate per ml. Cells were transfected with DEAE-dextran and chloroquine (6, 27).

Immunoprecipitation. COS cell cultures (35 mm) cotransfected with 0.25 μg of pgTAT and 0.25 μg of each mutant Rev expression vector were metabolically labelled with [³⁵S] cysteine (31). Labelled Tat and Rev proteins were then immunoprecipitated with rabbit anti-peptide sera, resolved

by electrophoresis through 14% sodium dodecyl sulfate-polyacrylamide gels, and visualized by autoradiography (31).

Analysis of HIV-1 provirus replication. To determine the phenotypes of the Rev mutants (Fig. 1), 0.125 μg of each mutant Rev expression vector was transfected into a COS cell culture (35 mm) together with 0.25 μg of pHIVΔRev and 0.0125 μg of the internal control plasmid pBC12/RSV/SEAP. Supernatants were harvested after 72 h, and viral replication was monitored by using an enzyme-linked immunoassay system for soluble p24^{gag} expression (Dupont, NEN, Inc., Billerica, Mass.) (29). The values obtained for secreted alkaline phosphatase activity in the supernatants served as controls for transfection efficiency, and all p24 values were adjusted accordingly (29). To quantitate the *trans* dominance of nonfunctional Rev mutants, COS cell cultures (35 mm) were cotransfected with 0.025 μg of pHIVΔRev, 0.05 μg of pcREV, 0.5 μg of the mutant Rev expression vector, and 0.0125 μg of pBC12/RSV/SEAP. Provirus replication was then assayed as above.

CAT assays. Cultures (60 mm) were transfected with DEAE-dextran (6, 27). COS and QCl-3 cells were transfected with either 1.6 μg of pBC12/CMV/CAT, 1.2 μg of pDM128/CAT plus 0.4 μg of pcREV, 1.2 μg of pDM128/CAT plus 0.4 μg of pBC12/CMV, or no DNA (mock transfection). CV-1, Ltk⁻, and NIH 3T3 cells were transfected with the same ratios of plasmid DNAs, except that the total amounts of DNA were 2, 6.7, and 6.7 times greater, respectively. Protein extracts were prepared from cells 48 h after transfection, and levels of CAT activity were determined as described previously (36). Relative protein concentrations (2) were used to normalize CAT activity.

RNA isolation and S1 nuclease protection assays. Cultures (100 mm) were transfected with a 3:1 ratio of pgTAT to either pcREV or pBC12/CMV. Transfections into COS,

QCI-3, or Ltk⁻ cells required a total of 4, 4, or 20 μ g of DNA, respectively. Nuclear and cytoplasmic RNAs were isolated from COS cells 48 h after transfection and from Ltk⁻ and QCI-3 cells 72 h after transfection (32). A previously described DNA probe end labelled within the first exon of Tat (31) was used for the S1 nuclease protection assays. The input probe is 798 nucleotides in length, while the fragments rescued by the unspliced and spliced Tat mRNAs are predicted to be 560 and 202 nucleotides, respectively.

RESULTS

Scanning mutagenesis of the HIV-1 Rev protein between amino acids 67 and 92. The aim of this study was to define the activation domain of the HIV-1 Rev *trans* activator. We have previously described the derivation of a missense mutant of Rev that possesses a disrupted activation domain (29). This *trans*-dominant mutant of Rev, termed M10, has the leucine and glutamic acid residues at positions 78 and 79 exchanged for aspartic acid and leucine, respectively (Fig. 1). Mutations introduced at positions 67 and 68 (M9) or at positions 91 and 92 (M11) are functional, as is a deletion mutant lacking all residues between 91 and 112 (Δ 11/14). This led us to believe that the activation domain of Rev was likely to be located between residues 69 and 90. We therefore introduced a series of scanning missense mutations into the Rev expression vector pcREV that sequentially changed all amino acids between positions 69 and 90 (M15 to M25, Fig. 1).

We have previously described a transfection assay for Rev function based on immunoprecipitation of the HIV-1 Tat protein (31). COS cells transfected with the genomic *tat* gene expression vector pgTAT exclusively express cytoplasmic Tat mRNA that is fully spliced and encodes an 86-amino acid, two-exon form of Tat (Fig. 2, bottom panel, lane 1). In contrast, cells cotransfected with pgTAT and the wild-type Rev expression vector pcREV additionally contain unspliced cytoplasmic Tat mRNA that directs the synthesis of a 72-amino acid, one-exon form of the Tat protein (Fig. 2, bottom panel, lane 2). The induced expression of the 72-amino acid Tat protein was therefore used to determine the relative biological activity of each Rev mutant (Fig. 2, bottom panel). These data, summarized in Fig. 1, reveal that of these novel Rev mutants, two (M21 and M22) had no activity, one (M18) had severely impaired activity, and the rest retained essentially full activity. As previously reported, M10 was inactive, whereas M9 and M11 were fully active. The relative expression levels of each of the various Rev mutants in these transfected cell cultures were measured in parallel by immunoprecipitation (Fig. 2, top panel). As previously reported, the wild-type HIV-1 Rev protein migrates as a single band of ~19 kDa (Fig. 2, top panel, lane 2). Because each of the mutants (except Δ 22/14 and Δ 23/14, which both lack cysteine residues) was detected at a comparable level, these mutations apparently affected neither the expression nor the stability of Rev.

The ability of Rev mutants to rescue structural gene expression from an HIV-1 provirus in which the *rev* gene has been inactivated provides a quantitative measure of Rev activity in vivo (29). We therefore assayed the ability of the various Rev mutants to complement the Rev-defective provirus expression vector pHIV-1 Δ Rev after cotransfection into COS cells. The levels of provirus replication were determined by measuring the amount of p24^{gag} antigen present in the culture supernatants and are presented as a

FIG. 2. Immunoprecipitation of HIV-1 Rev and Tat proteins. COS cell cultures were cotransfected with pgTAT and either the negative control plasmid pBC12/CMV (lane 1), the wild-type Rev expression vector pcREV (lane 2), or selected mutant (M or Δ) Rev expression vectors (lanes 3 to 12). The relative migrations of known protein molecular mass markers are indicated to the left of each panel. Top, wild-type Rev migrates as a single band of ~19 kDa, whereas the various mutants exhibit minor variations of both increased and decreased relative migration. Bottom, the 86- and 72-amino acid forms of Tat migrate as single bands of ~15.5 and ~14 kDa, respectively.

percentage of the rescue afforded by the wild-type Rev expression vector pcREV (Fig. 1). These data fully confirm the results obtained with the pgTAT-based assay and again demonstrate that M21 and M22 had lost all biological activity, whereas M18 had reduced Rev activity.

Inspection of the data obtained with these scanning mutants indicated that the leucine residues at positions 78, 81, and 83 were likely to be critical for function. Any perturbation of these amino acids (M10, M21, and M22) resulted in the loss of Rev activity. As a more rigorous test of their importance, we substituted each of these leucines with alanine (M27, M28, and M29). Since leucine and alanine both have aliphatic side chains, these substitutions affect neither charge nor polarity. Phenotypic analysis revealed that each mutant was completely devoid of Rev activity in both the pgTAT and provirus rescue assays (Fig. 1 and 2). Not surprisingly, a triple alanine substitution (M32) was also inactive.

Because of the nature of the introduced mutations (Fig. 1), the data presented to this point have not addressed the importance of the two residues in this Rev domain that bear acidic side chains, namely, the glutamic acid at position 79 and the aspartic acid at position 84. We therefore substituted these residues for either the amino acid with the corresponding acid amide side chain (M33 and M35) or valine (M34 and M36). The introduction of an acid amide maintains hydrophilicity but eliminates charge, whereas the valine substitutions introduce an amino acid with a hydrophobic side chain. Although neither of these mutations had any effect at position 84, the introduction of valine at position 79 (M36) did

TABLE 1. *trans*-dominant capabilities of negative Rev mutants^a

Clone tested	% Inhibition of wild-type Rev function
pBC12/CMV	0
M4	32
M10	93
M21	97
M22	93
M27	92
M28	92
M29	91
M32	97

^a The inhibition of HIV-1 provirus replication induced by expression of each mutant Rev protein is shown as the percent decrease in supernatant p24^{gag} expression relative to the standard (pBC12/CMV-transfected) culture.

result in a marked decrease in Rev activity (Fig. 1 and 2). However, it should be noted that residual Rev function was still readily detectable.

In addition to the single amino acid substitutions described above, we also tested a series of expression vectors carrying deletions within the Rev gene (Fig. 1 and 2). The 10 residues deleted from Δ 9/19 do not, when mutated in pairs, result in the loss of Rev activity (M9 and M15 to M19). They are, however, collectively important because their removal yielded a protein with no detectable Rev activity in either assay system. The deletion of residues 76 and 77 in Δ 18/19 resulted in no further inhibition of Rev function compared with that in the partially active parental mutant M18. The removal of the three critical leucines by gross deletion (Δ 18/23) rather than by amino acid substitution (M32) predictably yielded a mutant with no activity. The lack of function of Δ 22/14 was expected given the established inactivity of the point mutant M22 (see above). Interestingly, Δ 23/14 retained a level of activity, at least in the pgTAT assay, indistinguishable from wild-type Rev (Fig. 2, bottom panel, lanes 2 and 11). These observations therefore indicate that all residues extending from position 84 to the C terminus of Rev are dispensable for function.

Analysis of inactive mutants for *trans* dominance. The purpose of the mutagenesis described above was to define the activation domain of HIV-1 Rev. As stated earlier, mutants that have had this domain inactivated are predicted to inhibit wild-type Rev function in a dominant negative fashion. To assess whether these newly derived nonfunctional mutants of Rev were indeed *trans* dominant, we cotransfected COS cells with the replication-defective provirus clone pHIV Δ Rev together with the wild-type Rev expression vector pcREV and a 10-fold excess either of the negative control plasmid pBC12/CMV or of each of the inactive Rev expression vectors. At 72 h after transfection, the culture supernatants were assayed for p24^{gag} expression (Table 1). As previously reported (29), these experimental conditions reveal M4, a defective Rev protein that is mutated in the N-terminal RRE binding and multimerization domain, to be a mild (approximately 30%) inhibitor of wild-type Rev, whereas M10 scored as a potent (more than 90%) inhibitor of Rev function. These two mutant forms of Rev therefore constitute prototypic recessive negative and dominant negative Rev mutants. The analysis shown in Table 1 demonstrates that all the novel Rev mutants tested exhibited a degree of *trans* dominance, i.e., in excess of 90%, fully comparable to M10. We therefore conclude that each of these mutants lacks a functional activation domain. In HIV-1 Rev, this domain appears to comprise a six-amino acid core

TABLE 2. Relative *trans* activation by HIV-1 Rev in transfected cell lines^a

CAT expression vector	Level of CAT expression per cell line (cpm)					
	COS	CV-1	Ltk ⁻	NIH 3T3	QCI-3	
pBC12/CMV/CAT	9,347	469	16,599	4,833	23,523	
pDM128/CMV + pBC12/CMV	1,496	123	694	114	1,627	
pDM128/CMV + pcREV	14,326	1,338	8,809	826	19,627	
Fold <i>trans</i> activation	10	11	13	7	12	

^a Values represent the relative levels of CAT expression measured (36) over a period of time when the change in transacetylation was linear with respect to time (this point was determined empirically) and have been corrected for background (mock) activity. The values for relative *trans* activation represent the ratio of CAT expression of the pDM128/CMV + pcREV- and pDM128/CMV + pBC12/CMV-derived samples.

in which the leucines at positions 78, 81, and 83 are absolutely essential for biological activity.

Rev can function in primate, murine, and avian cell lines.

As noted above, the cellular components of the pathway through which Rev operates to induce HIV-1 structural gene expression remain undefined. However, it is almost certain that a number of host cell factors would be intrinsic to such a pathway. To assess the ubiquity of such factors among different species, we examined the ability of Rev to function in a variety of tissue culture cell lines. For this study, we chose two primate cell lines (COS and CV-1), two murine cell lines (Ltk⁻ and NIH 3T3), and one avian (quail) cell line (QCI-3).

The indicator plasmid initially employed for these experiments, pDM128/CMV, has the CAT gene placed within an intron bordered by functional HIV-1 splice sites and containing the HIV-1 RRE. Hope et al. (21) have shown that this expression cassette is responsive to Rev in cotransfection experiments. Rev function is manifested as increased CAT protein expression resulting from the appearance of unspliced CAT containing mRNA in the cell cytoplasm. We therefore cotransfected pDM128/CMV into the above cell lines either with or without pcREV; parallel transfections with the constitutive CAT expression vector pBC12/CMV/CAT served as positive controls for transfection efficiency and cytomegalovirus immediate-early promoter function (Table 2). All the pDM128/CMV-transfected cultures expressed levels of CAT activity reproducibly above background (Table 2). In the absence of Rev, this is presumably caused by the inefficient nucleocytoplasmic transport of unspliced CAT mRNA. Nevertheless, in the presence of Rev a clear induction of CAT expression was observed for each cell line tested. We therefore conclude that *trans* activation by Rev is as efficient in murine and avian cells as in primate cell lines. These findings contrast with a recent report suggesting that murine cell lines such as NIH 3T3 are unable to support Rev function (53). While the reasons for these differences are unknown, we did observe Rev function to be diminished in NIH 3T3 cells relative to the other cell lines tested.

We, and others, have previously used subcellular fractionation to show that Rev activation of viral structural gene expression correlates with a redistribution of HIV-1 mRNA within transfected cells (13, 18, 32). In particular, unspliced HIV-1 transcripts that would ordinarily be restricted to the nucleus are expressed in the cytoplasm in the presence of Rev. To address whether Rev function in murine and avian cells is comparable to that previously observed with primate cells, we transfected the indicator plasmid pgTAT into COS,

FIG. 3. S1 nuclease protection analysis of HIV-1 Tat mRNAs in transfected cells. Nuclear (N) and cytoplasmic (C) RNAs isolated from cells transfected with pgTAT in the presence (+) or absence (-) of pcREV were examined by S1 nuclease protection. The positions of the input probe (I) and the fragments rescued by the unspliced (U) and spliced (S) Tat mRNAs are indicated.

Ltk⁻, and QCl-3 cells in the presence or absence of pcREV. Following transfection, the cells were fractionated and total nuclear and cytoplasmic RNAs were isolated. The pattern of unspliced and spliced Tat mRNA expression in each fraction was then determined by S1 nuclease protection (Fig. 3). As previously reported (32), the cytoplasmic Tat mRNA in COS cells is fully spliced in the absence of Rev and predominantly unspliced in the presence of Rev (compare lanes 3 and 4). In contrast, the majority of nuclear Tat mRNA is unspliced regardless of Rev expression (lanes 1 and 2). Similarly, in both Ltk⁻ and QCl-3 cells, Rev had minimal effect upon the pattern of Tat mRNA expression in the nucleus (compare lanes 5 and 6 and lanes 9 and 10). It is, however, worth noting that splicing of the Tat pre-mRNA appeared to be significantly more efficient in both the murine Ltk⁻ and avian QCl-3 cells compared with COS cells (compare lanes 5 and 9 with lane 1). As predicted, there was a marked shift toward unspliced Tat mRNA expression in the cytoplasmic fractions of both of these cell types in the presence of Rev (compare lanes 7 and 8 and lanes 11 and 12). However, we did note a low but readily detectable level of unspliced Tat mRNA in the cytoplasm of QCl-3 cells (lane 11). Although we believe that this may reflect low-level nuclear contamination of the cytoplasmic fractions derived from these avian cells, we cannot formally eliminate the possibility that Rev function is manifested differently in avian cells. Nevertheless, these results do support the notion that the mechanism by which Rev activates the cytoplasmic expression of unspliced HIV-1 mRNAs is likely to be similar in primate, murine, and avian cells. This implies that any host factors necessary for Rev function must have been conserved during evolution. The demonstration of reconstituted Rev function in cultured insect (*Drosophila melanogaster*) cells (23), but not in the budding yeast *Saccharomyces cerevisiae* (30a), suggests that this conservation is extensive, but not absolute, among eukaryotes.

DISCUSSION

The purpose of this study was to define the activation domain of the HIV-1 Rev *trans* activator. The data we have

presented clearly demonstrate that the leucine residues at positions 78, 81, and 83 are absolutely required for Rev function and suggest that this essential functional domain comprises a minimal core of six amino acids, three of which are leucines. Other residues such as the leucine at position 75 and glutamic acid at position 79 are also important, though not critical, for activity. The precise nature of the residues located between the hydrophobic leucines appears to be less constrained, though a clear preference for hydrophilicity is apparent. These observations are generally in good agreement with those made in a number of laboratories (34, 39, 54); one exception is, however, the effect of mutations of the leucine at position 75. We (M18, Fig. 1) and Perkins et al. (39) obtained mutants with partial activity, whereas Venkatesh et al. (54) observed similar mutants to be inactive. Nevertheless, it does seem reasonable to extend the N-terminal boundary of this domain to include the leucine at position 75. So defined, this nine-amino-acid domain contains four leucine residues and will now be referred to as the leucine-rich domain of HIV-1 Rev. Of interest, this small domain is highly sensitive not only to specific single amino acid changes but also to minor changes in amino acid order. For example, mutant M22, in which the leucine and glutamic acid residues at positions 83 and 84 are simply reversed, displays a fully *trans*-dominant phenotype. Indeed, all the nonfunctional mutants in the leucine-rich domain were shown to be *trans* dominant over wild-type Rev, thus defining this stretch of amino acids as the activation domain of Rev.

HIV-1 is regarded as the prototypic member of the lentivirus family of retroviruses. These pathogenic retroviruses appear to share a number of characteristics, including tropisms for cells of the monocyte/macrophage lineage, disease courses marked by prolonged periods of latency, and patterns of gene expression that are both complex and temporally regulated (11, 17). As discussed earlier, the HIV-1 Rev protein regulates the temporal aspects of viral gene expression and may be instrumental in allowing the virus to establish and maintain latent infections (40). It therefore seems likely that all lentiviruses encode a Rev protein and that these proteins share conserved features. Figure 4 shows the optimal alignment of the leucine-rich domain of HIV-1 Rev with the amino acid sequences of the identified Rev proteins of HIV-2 (28), simian immunodeficiency virus (28), and visna virus (51) and the predicted Rev equivalents of caprine arthritis-encephalitis virus (44) and ovine maedi-visna virus (42). The most striking feature of this alignment is that the leucines at positions 4, 7, and 9 (residues 78, 81, and 83 in HIV-1 Rev) are tightly conserved. In some instances, a highly conservative substitution of a leucine with isoleucine or methionine is observed. Other positions within this region also display some conservation; the amino acid at position 1 is generally hydrophobic, those at positions 5, 6, and 10 are hydrophilic, and a threonine is usually found at position 8. Notably, the most highly conserved amino acids within this domain are precisely those defined as critical by the mutational analysis reported here. We therefore suggest that a consensus Rev activation domain will consist, minimally, of four hydrophobic amino acids, usually leucines, at positions 1, 4, 7, and 9 interspersed with amino acids that are less well conserved and generally hydrophilic. This proposal is strongly supported by recent data (52) showing that mutations within this domain of the visna virus Rev protein can yield nonfunctional proteins that display a *trans*-dominant phenotype.

We and others have shown that, in both the HIV-1 and

HIV-1	L	P	P	L	E	R	L	T	L	D
HIV-2	I	Q	H	L	Q	G	L	T	I	Q
SIV _{mac}	I	Q	Q	L	Q	N	L	A	I	E
SIV _{agm}	V	Q	A	I	D	Q	L	V	L	D
VISNA	M	V	G	M	E	N	L	T	L	E
CAEV	L	G	A	L	A	E	L	T	L	E
OMVV	C	A	G	L	E	N	L	T	L	G
Consensus	Z	-	-	□	J	J	□	T	□	J

FIG. 4. Amino acid sequence alignment of the proposed activation domains of lentivirus Rev proteins. The coordinates of the amino acid sequences derived from the Rev proteins shown in this alignment are as follows: 75 to 84 of the HXB-3 isolate of HIV-1 (35); 73 to 82 of the ROD isolate of HIV-2 (35); 73 to 82 of the MAC-142 isolate of simian immunodeficiency virus (SIV_{mac}) (35); 63 to 72 of the AGM3 isolate of simian immunodeficiency virus (SIV_{agm}) (35); 106 to 115 of visna virus (VISNA) (49); 93 to 102 of caprine arthritis-encephalitis virus (CAEV) (44), and 107 to 116 of ovine maedi-visna virus (OMVV) (42). The consensus derived from these sequences is shown at the bottom of the figure. The three conserved leucines are boxed; Z, hydrophobic residue; J, hydrophilic residue; -, nonconserved residue.

visna virus systems, wild-type Rev and *trans*-dominant mutants of Rev exhibit indistinguishable RRE binding and protein multimerization properties *in vitro* (30, 37, 51a). It therefore appears that the defect in these *trans*-dominant proteins is likely to be in their ability to interact with a host cell factor(s) necessary for Rev activity. Here, we provide two pieces of evidence to suggest that such a primary cellular target for Rev is likely to be either a protein or a complex of proteins. First, the conserved leucine-rich motif disrupted in the *trans*-dominant mutants bears no similarity to known nucleic acid binding motifs and therefore seems unlikely to be involved in such an interaction. Second, reiterated leucine-rich motifs somewhat similar to the unique motif of the lentivirus Rev proteins, i.e., in which leucines are repeated at every second or third position, have been described for a number of proteins. Examples of proteins shown to contain such leucine-rich repeats include the mammalian U2 small nuclear ribonucleoprotein particle (snRNP) A' protein (14), yeast adenyl cyclase (24), trypanosome variant surface glycoprotein (47), mouse CD14 (46), and human serum leucine-rich α_2 -glycoprotein (50) (for more complete listings, see references 14 and 47). The most intriguing of these is the U2 snRNP A' protein, which contains a region of leucine repeats essential for its incorporation into the U2 snRNP, a cellular component critical for pre-mRNA splicing. All these leucine-rich motifs are clearly distinct from the well-characterized "leucine zipper" heptad repeat found in a number of DNA sequence-specific *trans*-activators (26). However, in each case, the leucine-rich sequence element has either been proposed or shown to be important for protein-protein interactions. By analogy, we would predict that the protein-protein contact between the leucine-rich activation domain of Rev and its cellular target is likely to be initiated by long-range ionic and electrostatic interactions followed by highly specific close-range hydrophobic van der Waals interactions mediated by the conserved leucines. The apparent evolutionary conservation of

this target protein(s) suggests that it is an integral component of a nuclear pathway conscripted by the lentivirus Rev proteins for the regulation of viral mRNA expression. While the identity of this pathway is unknown, factors involved in either pre-mRNA splicing or mRNA transport currently appear to be the most promising candidate targets for binding by this novel regulatory protein domain.

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