JOURNAL OF VIROLOGY, July 1991, p. 3877–3881
0022-538X/91/073877-05$02.00/0

Vol. 65, No. 7

Conserved Functional Organization of the Human Immunodeficiency Virus Type 1 and Visna Virus Rev Proteins

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Received 18 January 1991/Accepted 26 March 1991

Visna virus encodes a posttranscriptional regulatory protein that is functionally analogous to the Rev *trans* activator of human immunodeficiency virus type 1. Here, we demonstrate that the known functional organization of the human immunodeficiency virus type 1 Rev *trans* activator is shared by the distantly related visna virus Rev protein. In particular, both Rev proteins contain an N-terminal domain marked by a highly basic core motif that determines RNA sequence specificity, as well as a second C-terminal domain containing an essential leucine-rich motif that functions as an activation domain. Chimeric proteins consisting of the binding domain of one Rev protein fused to the activation domain of the other were fully functional in the viral sequence context cognate for the binding domain. We also describe derivatives of visna virus Rev bearing a defective activation domain that displayed a *trans*-dominant negative phenotype in transfected cells. These visna virus Rev mutants may prove useful in the derivation of transgenic animals resistant to this agriculturally important retroviral pathogen.

Lentiviruses form a retroviral subfamily characterized by the ability to induce chronic, degenerative diseases marked by high levels of latently infected cells (5, 8). Phylogenetic analysis suggests that this retroviral family can be divided into two subgroups, here termed primate lentiviruses and ungulate lentiviruses (23). The prototypic primate lentivirus is the highly pathogenic human immunodeficiency virus type 1 (HIV-1), while the prototypic ungulate lentivirus is visna virus, a pathogen of sheep and goats. Although HIV-1 and visna virus share only limited primary sequence homology, they do share the characteristic lentivirus genomic organization, marked particularly by the presence of additional open reading frames between the viral *pol* and *env* genes (2, 17, 22). These open reading frames encode viral regulatory proteins that play a key role in orchestrating the complex pattern of viral gene expression observed in lentivirus-infected cells (2–4, 6, 10, 17, 21, 26).

Both HIV-1 and visna virus have been shown to encode a nuclear regulatory protein, termed Rev, that is required for the expression of the unspliced and singly spliced mRNAs that encode the viral structural proteins (6, 21, 24). Although the mechanism of action of these Rev proteins remains uncertain, both appear to activate the nuclear export of incompletely spliced viral mRNA species that are otherwise sequestered within the nucleus of expressing cells (7, 14, 24). While both visna virus and HIV-1 Rev act via structured RNA target sequences, termed Rev response elements (RREs), located in the viral envelope gene, neither Rev protein is able to functionally interact with the RRE of the other virus in vivo (14, 24).

Mutational dissection of the HIV-1 Rev protein has revealed the existence of two protein domains that appear comparable to the "binding domain" and "activation domain" described for several eukaryotic transcription factors (20). The RNA binding domain of Rev, located toward the protein N terminus, is an approximately 40-amino-acid (aa) sequence element encompassing a highly basic core that displays sequence homology to the arginine-rich RNA binding motif (11) (Fig. 1). Mutations within this domain affect the binding of Rev to the RRE and result in a recessive negative phenotype in vivo (9, 12, 13, 19). A second functional domain, located toward the C terminus of Rev, is characterized by an approximately 10-aa leucine-rich motif. Mutations within this motif have no effect on the interaction of Rev with the RRE in vitro yet result in a dominant negative phenotype in vivo (9, 12, 13, 16, 19, 25). It has therefore been proposed that this motif represents a site of interaction with a cellular protein(s) that regulates RNA fate within the nucleus (12).

Alignment of the primary amino acid sequence of the HIV-1 and visna virus Rev proteins reveals minimal sequence identity. However, the two protein sequence elements defined above as critical for HIV-1 Rev function, i.e., the basic motif and the leucine motif, do appear to be moderately conserved (Fig. 1). To test the hypothesis that these lentivirus regulatory proteins have maintained a similar functional organization through evolution, we asked whether hybrid Rev proteins bearing the basic domain of one Rev protein joined to the leucine domain of the other Rev protein would be functional in vivo. Chimeric proteins were constructed using the M9 mutant of HIV-1 Rev, which contains a missense mutation at aa positions 67 and 68 (SA→DL) that introduces a *Bgl*II site (12), and a mutant of visna Rev that contains a missense mutation at aa positions 98 to 100 (FYP→LDL) that introduces a *Bam*HI site (Fig. 1). These two introduced restriction enzyme sites are in the same reading frame and share identical cohesive ends, thus facilitating the construction of plasmids expressing chimeric HIV-1–visna virus *rev* gene products. The pL/Rev vector is predicted to encode a hybrid protein consisting of the 97 N-terminal aa of visna virus Rev and the 47 C-terminal aa of HIV-1 Rev separated by a 3-aa linker region (N′-IPSQ/ldl/EPVP-C′), where lower-case letters indicate introduced linker amino acids. Similarly, the pRev/L vector is predicted to encode a hybrid protein bearing the 66 N-terminal aa of the HIV-1 Rev protein and the 67 C-terminal aa of visna virus Rev separated by a single introduced aa (N′-YLGR/d/

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VISNA   1- MASKESKPSRTTWRDMEPPLRETWNQVLQELVKRQQQEEEEQQGLVSGLQ -50
HIV-1   1- MAG.............................RSGDSDEE...LIRTVR -17

                                  BASIC DOMAIN
VISNA  51- ASKADQIYTGNSGDRSTGGIGGKTKKKRGWYKWLRKLRAR....EKNIPS -96
HIV-1  18- LIK..LLYQSNPPPNPEGT...RQARRNRRRRWRERQRQIHSISER.ILG -61

                  LEUCINE DOMAIN
VISNA  97- QFYPN.....MESNMVGMENLTLETQLEDNALYNPATH.IG..DMAMDRG -138
HIV-1  62- T.YLGRSAEPVPLQLPPLERLTLDCN.EDCG..TSGTQGVGSPQILVESP -107

VISNA 139- EWMEWRESAQKEKRKGLSGQRTNAYPGK                      -166
HIV-1 108- TVL...ESGTKE                                      -116
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FIG. 1. Alignment of the predicted amino acid sequences of the Rev proteins of visna virus and HIV-1. Sequences (3, 12) were aligned by computer to maximize the incidence of amino acid identities and of conservative changes. The basic domain and the leucine domain fulfill essential but distinct functions in the HIV-1 Rev protein (see text for details). Exon junctions are indicated by black triangles.

PNME-C'). The pcRev and pcL plasmids express the wild-type HIV-1 and visna virus Rev proteins, respectively (14, 24).

To assay the in vivo activity of these chimeric proteins, we used previously described transient transfection assays for the detection of HIV-1 or visna virus Rev activity (14, 24). In the presence of the HIV-1 Rev protein, the HIV-1-based indicator construct, pgTat, gave rise to an unspliced cytoplasmic mRNA that encodes the single-exon, 72-aa form of the viral Tat protein (Fig. 2, lane 1). This protein was not expressed in the absence of HIV-1 Rev or in response to the visna virus Rev protein (Fig. 2, lane 2). Similarly, the visna virus indicator construct pENV responded to the visna virus Rev protein by expressing an unspliced cytoplasmic mRNA that encoded the viral envelope (Env) proteins (Fig. 2, lane 6). Again, no visna virus Env expression was detected in the absence of visna virus Rev or in response to the HIV-1 Rev *trans* activator (Fig. 2, lane 5).

The hybrid protein bearing the basic domain of the visna virus Rev protein and the leucine domain of HIV-1 Rev, termed L/Rev, proved fully able to *trans* activate visna virus structural protein expression but lacked any activity in the HIV-1 system (Fig. 2, lanes 3 and 7). Conversely, the hybrid protein bearing the basic domain of HIV-1 Rev and the putative leucine domain of visna virus Rev proved fully active in the HIV-1 context but was inactive when tested on the visna virus pEnv indicator construct (Fig. 2, lanes 4 and 8). These results therefore demonstrate that the N-terminal domains of HIV-1 and visna virus Revs are fully sufficient to confer RNA sequence specificity, while both C-terminal protein domains are able to provide similar or identical activation functions.

To address whether the putative leucine motif of visna virus Rev indeed represented the core of an activation domain, we next constructed a series of mutants targeted to this protein sequence (Table 1). From the precedent of HIV-1 Rev (12), we predicted that these visna virus Rev mutants might display a dominant negative phenotype in vivo. The ability of these mutant visna virus Rev proteins to activate visna virus structural protein expression was first tested with the pEnv indicator construct. As shown in Fig. 3A, the L-M1 and L-M2 mutants retained partial Rev activity, while the L-M3 and L-M4 missense mutants failed to induce any detectable visna virus Env protein expression. The more extensive LΔM14 mutant also proved phenotypically inactive in this assay (data not shown).

We next assayed the ability of these mutant visna virus Rev proteins to inhibit the wild-type visna virus Rev protein in *trans*. In this experiment (Fig. 3B), the pEnv construct was cotransfected with a small amount of the wild-type visna virus Rev expression plasmid pcL together with an 18-fold molar excess of each mutant visna virus Rev expression plasmid. Under similar conditions, HIV-1 Rev mutants bearing a defective leucine motif have been shown to inhibit wild-type HIV-1 Rev activity by >90% (12). As predicted, the pL-M1 and pL-M2 mutants, which displayed partial

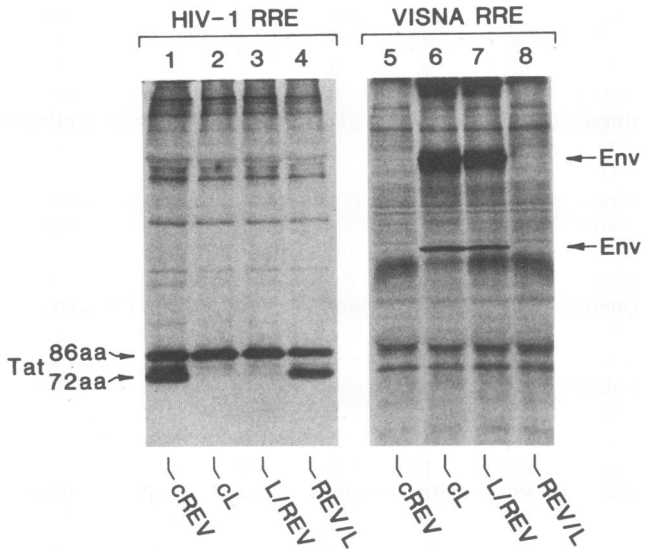

FIG. 2. Biological activity of wild-type and hybrid forms of the visna virus and HIV-1 Rev proteins. COS cell cultures were transfected (1) with the HIV-1-based indicator construct pgTat (lanes 1 to 4) or the visna virus-based construct pEnv (lanes 5 to 8) as well as the indicated Rev expression vectors (14, 24). At 72 h after transfection, cultures were labeled with [^{35}S]cysteine (lanes 1 to 4) or [^{35}S]methionine (lanes 5 to 8) as previously described (1) and were then subjected to immunoprecipitation with a 1:140 dilution of a rabbit polyclonal antiserum specific for the HIV-1 Tat protein (1, 14) (lanes 1 to 4) or of a sheep antiserum specific for the visna virus envelope outer membrane protein (a gift from Ashley Haase) (lanes 5 to 8). Precipitated proteins were resolved on discontinuous 14% (lanes 1 to 4) or 10% (lanes 5 to 8) sodium dodecyl sulfate-polyacrylamide gels and visualized by autoradiography. In the HIV-1 system (lanes 1 to 4), Rev function is detected as expression of the one-exon, 72-aa form of the viral Tat protein (14). In the visna virus system (lanes 5 to 8), Rev induces the expression of the viral envelope (Env) proteins (24). The full-length visna virus Env protein migrates at ~135 kDa, while the outer membrane component migrates at ~70 kDa (22).

TABLE 1. Description of visna virus Rev mutants[a]

Name	Mutation	Biological activity
pcL	Wild type	+
pL-M1	ME→DL (aa 109–110)	±
pL-M2	NLT→QDL (aa 111–113)	±
pL-M3	LTL→ASS (aa 112–114)	−
pL-M4	LE→DL (aa 115–116)	−
pLΔM14	MENLTLE→DL (aa 109–116)	−

[a] The location of mutations is given using the amino acid coordinates presented in Fig. 1. Vectors encoding mutant forms of the visna virus Rev protein were derived from the previously described visna virus Rev expression plasmid pcL (24) using the polymerase chain reaction (18). Flanking primers spanned unique restriction enzyme sites located in the cytomegalovirus immediate-early promoter (*Sac*I) or in the 3′ non-coding region present in the vector (*Xho*I). The polymerase chain reaction primers utilized for mutagenesis contained mismatches that introduced the indicated mutations. These primers also introduced unique restriction enzyme sites into the visna virus *rev* gene (*Bgl*II in L-M1, L-M2, and L-M4; *Nhe*I in L-M3). The deletion mutant pLΔM14 was constructed by inserting a *Sac*I-to-*Bgl*II *rev* gene fragment derived from pL-M1 in place of the similar *Sac*I-to-*Bgl*II fragment present in pL-M4. Biological activity of these visna virus Rev mutants was assayed as described in the text: +, fully active; ±, partially active; −, inactive.

activity in vivo (Fig. 3A), were unable to inhibit the wild-type Rev protein when expressed in *trans* (Fig. 3B, compare lanes 2 and 4 with positive control lane 1). Unexpectedly, the phenotypically negative visna virus Rev missense mutants pL-M3 and pL-M4 exerted only a moderate inhibitory effect on wild-type visna virus Rev function, leading to an ~60% drop in the synthesis of the viral Env proteins (Fig. 3B, compare lanes 3 and 5 with lane 1). Phenotypically negative visna virus Rev proteins bearing more extensive carboxy-terminal deletion mutations also proved unable to exert a significant *trans* inhibitory effect in this assay system (Fig. 4A and data not shown).

One possible explanation for the lack of a marked *trans*-dominant negative phenotype is that the mutations introduced into visna virus *rev* resulted in reduced expression of the mutant proteins. As an initial test of this hypothesis, we used immunoprecipitation to compare the level of synthesis of the wild-type and mutant visna virus Rev proteins in transfected cell cultures (Fig. 3C). This experiment suggested that the level of expression of the various missense mutants of visna virus Rev was comparable. Of note, immunofluorescence analysis of transfected cultures revealed that the phenotypically negative L-M3 protein was appropriately localized to the cell nucleus (24) and appeared to be expressed at levels comparable to those of the wild-type visna virus Rev protein (data not shown). Therefore, it does not appear that the lack of a marked *trans*-dominant negative phenotype evidenced by these negative mutants of visna virus Rev can be explained by low expression.

The data presented in Fig. 2 showed that an HIV-1 Rev protein lacking the essential C-terminal leucine motif could be rescued by fusion to an apparently similar protein motif present within the visna virus Rev protein. However, mutation of this same motif within the context of the full-length visna virus Rev *trans* activator resulted in proteins that either retained partial activity (L-M1 and L-M2) or displayed only weak *trans* dominance (L-M3 and L-M4). We therefore next examined the role of the leucine motif in the context of the hybrid Rev proteins described above. We have described elsewhere a *trans*-dominant negative mutant of HIV-1 Rev, termed M32, in which three leucines present in the leucine motif have been changed to alanine (15). This same mutation was introduced into pL/Rev to give the pL/Rev-M32 expression plasmid. Similarly, the M3 missense mutation (Table 1)

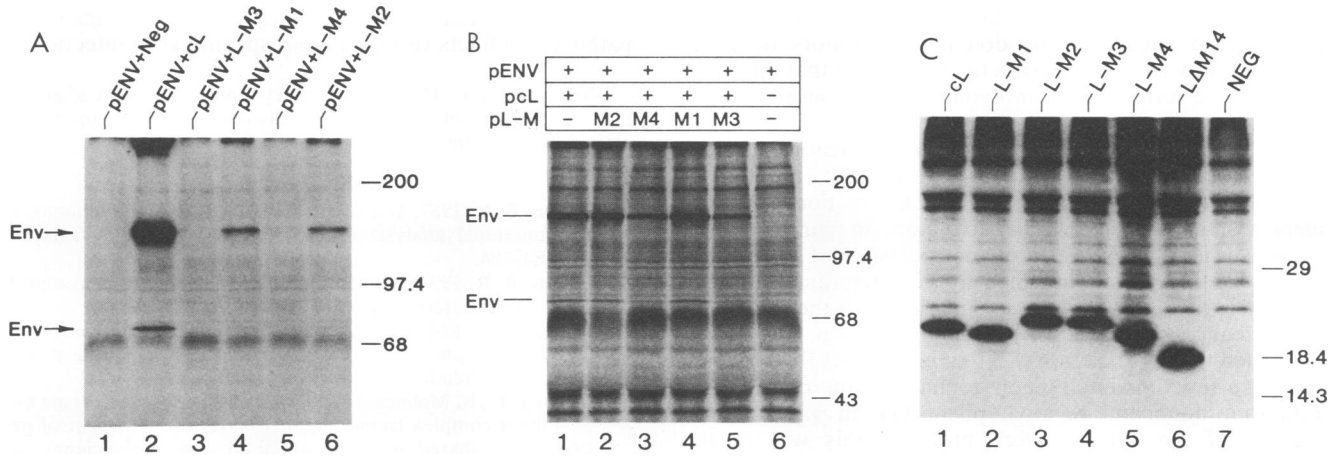

FIG. 3. Biological activity of mutants of the visna virus Rev protein. (A) COS cell cultures were transfected (1) with the indicator construction pEnv together with either the negative control plasmid pBC12/CMV (14) (Neg) or the indicated wild-type or mutant visna virus Rev expression vector. Rev activity, detected as expression of the visna virus envelope proteins, was assayed by immunoprecipitation as described in the legend to Fig. 2. (B) COS cell cultures were transfected (1) with 25 ng of pEnv and (with the exception of negative control lane 6) 25 ng of pcL. Cultures were also transfected with 450 ng of the indicated visna virus Rev mutant expression plasmid or with the negative control plasmid pBC12/CMV. If the mutants exhibit a dominant negative phenotype, then inhibition of the expression of the visna virus Env protein is predicted (12). Env expression was assayed by immunoprecipitation as described in the legend to Fig. 2. (C) COS cell cultures were transfected with the indicated wild-type or mutant visna virus Rev expression vectors. At 72 h after transfection, cultures were labeled with [^{35}S]methionine (1) and visna virus Rev protein expression levels were determined by immunoprecipitation with a 1:140 dilution of a sheep anti-visna virus Rev antibody (a gift from Robert Vigne). The visna virus Rev protein has been shown to migrate at ~21.5 kDa on sodium dodecyl sulfate-polyacrylamide gels (3). The lane designated NEG represents a culture transfected with the negative control vector pBC12/CMV (14). In all three panels, the numbers at right indicate the relative migration of marker proteins of the indicated sizes in kilodaltons.

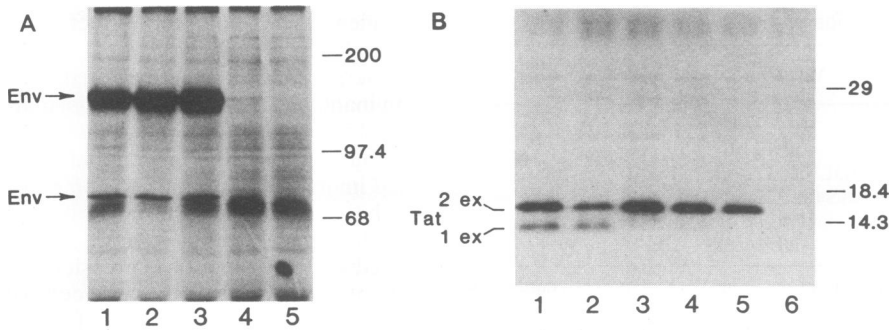

FIG. 4. Hybrid HIV-1–visna virus Rev proteins lacking a functional leucine motif display a *trans*-dominant negative phenotype. The ability of various hybrid HIV-1–visna virus Rev protein mutants to inhibit the wild-type HIV-1 and visna virus Rev proteins was assayed with the indicator constructs pgTat and pEnv, as described in the legends to Fig. 2 and 3. (A) The visna virus-based indicator construct pENV (25 ng) was cotransfected into COS cells together with the following expression plasmids: lane 1, 475 ng of pcL (positive control); lane 2, 25 ng of pcL plus 450 ng of pBC12/CMV (positive control); lane 3, 25 ng of pcL plus 450 ng of pLΔM14; lane 4, 25 ng of pcL plus 450 ng of pL/Rev-M32; lane 5, 475 ng of pBC12/CMV (negative control). (B) The HIV-1-based indicator construct pgTat (250 ng) was cotransfected into COS cells together with the following expression plasmids: lane 1, 250 ng of pcRev (positive control); lane 2, 25 ng of pcRev plus 225 ng of pBC12/CMV (positive control); lane 3, 25 ng of pcRev plus 225 ng of pM32; lane 4, 25 ng of pcRev plus 225 ng of pRev/L-M3; lane 5, 250 ng of pBC12/CMV (negative control). The culture analyzed in lane 6 was transfected with 500 ng of pBC12/CMV only (i.e., not with pgTat) and therefore represents a negative control for two-exon Tat expression. In both panels, the numbers at right indicate the relative migration of marker proteins of the indicated sizes in kilodaltons.

was introduced into the putative visna virus leucine motif present in pRev/L to give the expression plasmid pRev/L-M3. Analysis of the ability of these mutant, hybrid Rev proteins to inhibit in *trans* the wild-type visna virus Rev or HIV-1 Rev proteins revealed that the chimeric protein encoded by the pL/Rev-M32 vector was a highly effective inhibitor of visna virus Rev function (Fig. 4A, compare lanes 2 and 4). Similarly, the hybrid protein encoded by pRev/L-M3, which contains a mutated visna virus Rev leucine motif fused to the basic domain of HIV-1 Rev, was an effective inhibitor of the HIV-1 Rev *trans* activator (Fig. 4B, compare lanes 2 and 4). The observation that mutation of the proposed leucine motif of both HIV-1 and visna virus Revs can give rise to effective *trans*-dominant inhibitors of either wild-type Rev protein supports the hypothesis that this motif does indeed form part of an evolutionarily conserved activation domain.

In this report, we provided evidence indicating that the known functional organization of the HIV-1 Rev protein (13) is conserved in the distantly related but functionally equivalent visna virus Rev *trans* activator. In particular, we demonstrated that hybrid proteins consisting of the binding domain of one protein attached to the activation domain of the other are fully active in conjunction with the viral RRE target sequence cognate for the binding domain. This observation led to the prediction (12) that visna virus Rev proteins lacking a functional activation domain leucine motif would exhibit a dominant negative phenotype in vivo. In the context of the chimeric Rev proteins, this was indeed observed to be the case (Fig. 4). However, similar leucine motif mutations, when introduced into the full-length visna virus Rev protein, resulted in only a weakly dominant negative phenotype (Fig. 3). This phenotype could reflect an impaired ability to bind to the visna virus RRE, a reduced ability to form functional Rev multimers (13, 19), or reduced in vivo stability. While we are currently unable to distinguish among these possibilities, our results do argue against the latter hypothesis.

In this report, we described the derivation of a highly effective dominant negative mutant of visna virus Rev generated in the context of a hybrid visna virus–HIV-1 Rev protein. In fact, the L/Rev-M32 protein appears able to essentially totally ablate visna virus Rev function in transfection assays when expressed in *trans* (Fig. 4A). This result therefore raises the possibility of using the pL/Rev-M32 construct to generate transgenic sheep or goats resistant to visna virus and possibly also to closely related ungulate lentiviruses such as caprine arthritis-encephalitis virus. These diseases represent economically important pathogens of domestic sheep and goats (8), so resistant breeds could be of significant value. In addition, this might well represent a useful model to test the hypothesis that *trans*-dominant mutants of essential viral regulatory proteins could be useful in protecting cells, and hence whole animals, against the pathogenic effects resulting from specific viral infections.

We thank Ashley Haase and Robert Vigne for the gift of antisera specific for visna virus proteins. We also thank Sharon Goodwin for secretarial assistance.

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