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Posttranscriptional Effector Domains in the Rev Proteins of Feline Immunodeficiency Virus and Equine Infectious Anemia Virus

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By systematically dissecting the Rev proteins of feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV), we have identified within each a short peptide that is functionally interchangeable with the effector domains found in Rev-like proteins from other retroviruses. The active sequences from FIV and EIAV differ in several respects from other known effectors and may represent a distinct class of effector domain.

Retroviruses that have complex, multi-intronic genomes face a predicament upon infecting a cell: the sequences encoding the major structural proteins (Gag, Pol, and Env) of these viruses are contained within potential introns and so would normally be spliced out of any nascent viral mRNA before it reached the cytoplasm. Replication of many complex retroviruses therefore is dependent upon the activity of a virally encoded regulatory protein, known as Rev or Rex, whose function is to allow specific viral RNAs to be exported from the nucleus into the cytoplasm (2, 18, 19). Proteins of this type are translocated into the nuclei of infected cells, where they bind selectively to singly spliced and unspliced viral transcripts. In each case, binding occurs at a discrete *cis*-acting sequence, known as the Rev (or Rex) response element (RRE), in the viral RNAs. As a result of this interaction, the bound nuclear RNAs are released into the cytoplasm before splicing is completed, enabling them to be translated to produce virion proteins and so transactivating viral replication. The exact mechanism of Rev or Rex action is unknown, but it is thought to involve specific effects on splicing and/or transport of RNA (1, 3, 4, 10, 11, 13).

The Rev protein from human immunodeficiency virus type 1 (HIV-1) was the first of these posttranscriptional transactivators to be identified and remains the best characterized protein of this type (reviewed in reference 2). Several laboratories have carried out systematic mutagenesis in order to identify essential functional domains in the HIV-1 Rev protein (for example, see references 7, 12, 15, 16, 22). The results indicate that sequences in the N-terminal half of HIV-1 Rev are sufficient for specific RNA binding, nuclear translocation, and protein-protein interactions among Rev monomers. However, biological activity of the protein also requires a C-terminal region known as the effector domain (7, 12, 15, 16). This effector domain is thought to mediate contacts between Rev and an unidentified cellular cofactor, which may be part of the cellular RNA splicing or transport apparatus.

Detailed mutational analysis of the HIV-1 effector domain (14) has disclosed that four critically spaced leucine residues clustered within a region spanning approximately 11 amino acids are essential for its activity, whereas all other residues in and around this region are dispensable. Moreover, sequence comparisons with Rev-like proteins from several other unrelated retroviruses reveal that each contains a similar short cluster of leucines or other hydrophobic amino acids (5, 14), and domain-swapping experiments indicate that each of these regions can functionally replace the effector domain of HIV-1 Rev (4a, 5, 14, 21). These findings have suggested that all Rev-like proteins might contain such leucine-rich effector regions.

However, the Rev-like proteins from two complex retroviruses—feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV)—have appeared to be exceptional in this regard. Although both these proteins exhibit biological properties similar to those of other Rev proteins (9, 17, 20), their published sequences do not include any recognizable clusters of leucines or other hydrophobic residues that might indicate effector activity. We therefore set out to determine whether the EIAV and FIV Rev proteins contain sequences that are functionally equivalent to the HIV-1 effector domain.

We measured Rev activity by using a transient transfection assay in which CV1 cells are cotransfected with an HIV-1 Rev expression vector along with a Rev-inducible chloramphenicol acetyltransferase (CAT) reporter plasmid (6–8). CAT induction in this assay is dependent upon sequences in the N-terminal half of Rev (which recognize an HIV-1 RRE in transcripts produced from the reporter plasmid) and also requires an intact effector domain. Thus, the CAT response is abolished when sequences encoding the native effector domain (residues 78 to 114) are deleted from the Rev vector but can be restored by replacing these sequences with sequences encoding functional effector regions from a number of other retroviruses (4a, 5).

For the present study, we first inserted a full-length FIV Rev cDNA into the HIV-1 Rev expression vector in place of the native effector domain. As indicated in Fig. 1A, we found that this insertion restored function of the effectorless HIV-1 Rev mutant, implying that FIV Rev contains sequences that can complement the loss of HIV-1 Rev effector function. By testing progressively truncated FIV sequences in this manner, we mapped the minimal active sequences to amino acids 95 to 121. Insertion of this 27-residue sequence into the effectorless HIV-1 Rev yielded levels of CAT induction nearly equal to

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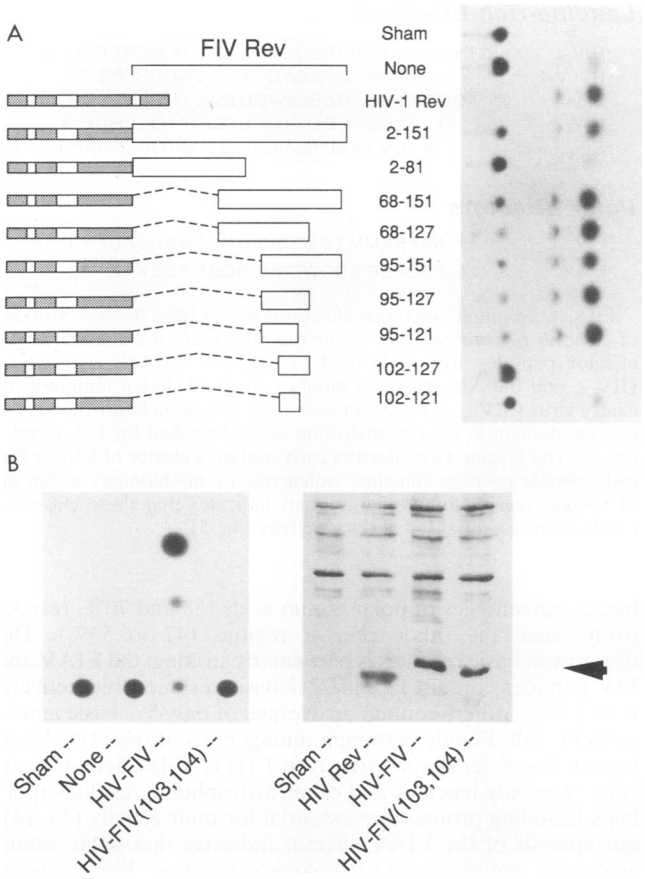

FIG. 1. Mapping the minimal effector peptide in FIV Rev. (A) Functional characterization of HIV-FIV fusion proteins. The autoradiogram on the right indicates the levels of CAT enzyme expression in CV1 cells that had been cotransfected with the Rev-inducible CAT reporter plasmid pDM128, along with derivatives of the HIV-1 Rev expression vector pRSV-Rev encoding each of the constructs shown schematically at left. Small open rectangles in the 116-amino-acid HIV-1 Rev protein (shaded rectangle) represent the essential N-terminal, arginine-rich, and C-terminal effector regions, from left to right (7, 12, 14). Fusion constructs were created by replacing HIV-1 *rev* codons 78 to 114 by a *Bgl*II linker and then inserting the indicated codons from FIV *rev* into this *Bgl*II site. FIV *rev* sequences (large open rectangle) were subcloned by PCR. (B) Mutational inactivation and immunoblot analysis of an HIV-FIV Rev fusion protein containing the minimal FIV effector domain (FIV residues 95 to 121). (Left panel) CAT expression in cells tranfected with this minimal construct (HIV-FIV) or with a mutant of the same construct in which FIV residues 103 and 104 (Asp and Leu) were replaced by alanines. (Right panel) Immunoblot detection of HIV-1 Rev and of the indicated fusion proteins (arrow) in transiently transfected cells, using an antibody specific for HIV-1 Rev (a gift from M. L. Zapp). Plasmid construction, mutagenesis, and transfection assays were performed as described elsewhere (5–7). All CAT assays were normalized to β-galactosidase activity expressed from a cotransfected internal control plasmid. Sham, sham-transfected cells; None, cells transfected with pDM128 alone.

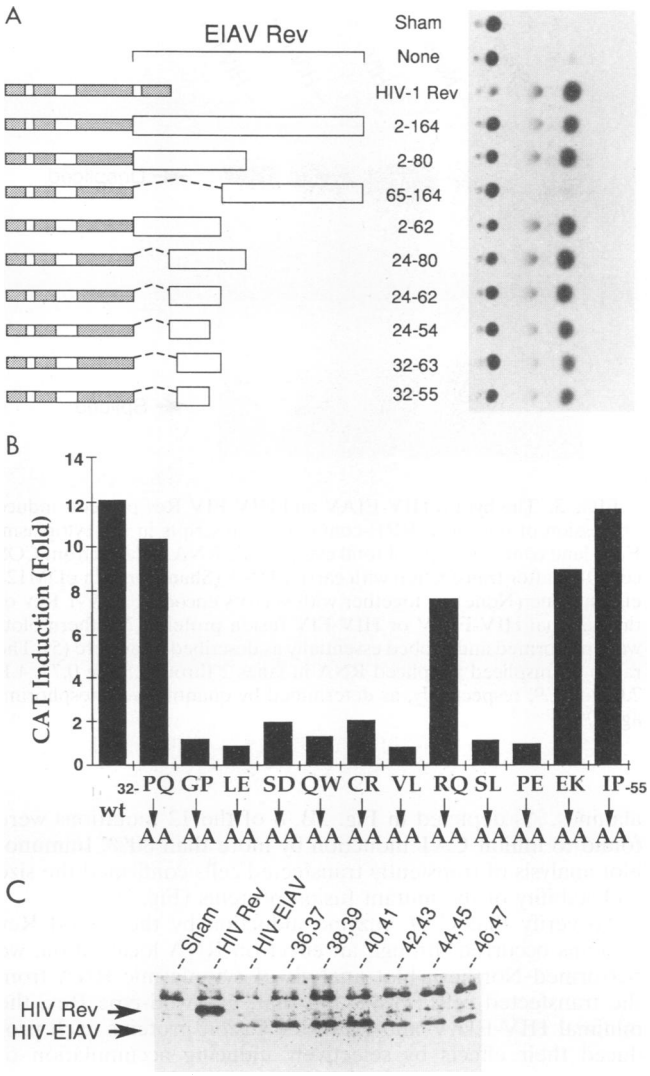

FIG. 2. Mapping the minimal effector peptide in EIAV Rev. (A) Functional characterization of a series of HIV-EIAV fusion proteins. Large open rectangles represent sequences from the 164-amino-acid EIAV Rev. (B) Mutational analysis of the minimal EIAV effector region (EIAV Rev residues 32 to 55) in the context of the HIV-EIAV fusion. Fusion proteins that contained either the wild-type EIAV peptide (wt) or dicodon mutations that replaced each of the indicated dipeptides with alanine pairs were constructed. CAT activity induced by each construct is expressed as a multiple of CAT activity in cells receiving pDM128 alone, and each value is the mean of duplicate determinations. (C) Immunoblot detection of HIV-1 Rev, HIV-EIAV containing the minimal EIAV effector domain, and mutants of the latter in which alanines replace the indicated dipeptides in EIAV. Methods were as described for Fig. 1.

those obtained with wild-type HIV-1 protein. Specificity of the activity was confirmed by the findings that other regions from FIV Rev were inactive in the fusion assay (Fig. 1A) and that introduction of a dicodon missense mutation (AA replacing DL at positions 103 and 104) into the minimal FIV sequence abolished its effector activity (Fig. 1B).

By constructing an additional series of fusion proteins, we then tested sequences from EIAV Rev for effector activity (Fig. 2A). In this case, maximal activity was found to reside at amino acids 24 to 55, though a somewhat smaller region (residues 32 to 55) also retained substantial activity. To determine which residues within this 24-amino-acid sequence were required for effector function, we also constructed 12 different plasmids encoding mutant forms of the HIV-EIAV fusion protein in which consecutive pairs of native residues within the EIAV effector region were replaced by pairs of

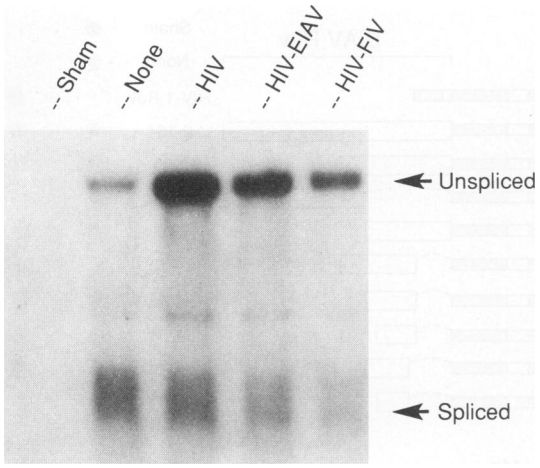

FIG. 3. The hybrid HIV-EIAV and HIV-FIV Rev proteins induce expression of unspliced RRE-containing transcripts in the cytoplasm. Each lane contains 15 μg of total cytoplasmic RNA isolated from COS cells 48 h after transfection with carrier DNA (Sham) or with pDM128 either alone (None) or together with vectors encoding HIV-1 Rev or the minimal HIV-EIAV or HIV-FIV fusion proteins. Northern blots were performed and probed essentially as described elsewhere (5). The ratios of unspliced to spliced RNA in lanes 2 through 5 are 0.77, 4.1, 2.6, and 1.9, respectively, as determined by quantitative phosphorimaging.

alanines. As depicted in Fig. 2B, 8 of the 12 mutations were found to inhibit CAT induction by more than 80%. Immunoblot analysis of transiently transfected cells confirmed the size and stability of the mutant fusion proteins (Fig. 2C).

To verify that CAT enzyme induction by the hybrid Rev proteins occurred through an effect on RNA localization, we performed Northern blot analysis of cytoplasmic RNA from the transfected cells. We found that, like wild-type Rev, the minimal HIV-EIAV and HIV-FIV fusion proteins each produced their effects by selectively inducing accumulation of unspliced RRE-containing reporter transcripts in the cytoplasm (Fig. 3).

Our studies demonstrate that the FIV and EIAV Rev proteins each contain a region that can functionally replace the effector domain of HIV-1 Rev. This, together with results of earlier studies of other evolutionarily unrelated retroviruses (4a, 5, 14, 21), establishes that effector regions are present in all known Rev-like proteins; indeed, the presence of such domains may be regarded as a defining feature of this class of regulatory proteins. In every protein tested thus far, full effector activity maps to a region no more than 31 amino acids long which behaves as an autonomous domain. The finding that all such domains are functionally interchangeable is consistent with the view that all Rev proteins may act through the same regulatory pathway, perhaps by interacting with a common cellular cofactor.

Figure 4 depicts the minimal active effector peptides from all retroviral Rev proteins tested to date. Upon comparing these primary sequences, it is apparent that the EIAV and FIV domains differ in several respects from previously characterized effector regions. First, although multiple leucines and other hydrophobic residues are present in the EIAV and FIV effectors and may be required for function (Fig. 1B and 2B), these are not tightly clustered as in other proteins of this type. Second, the EIAV and FIV domains both contain somewhat

Leucine-rich Effectors

HIV-1	61- STYLGRSAEPVPLQLPPLERLTLDCNEDCG	-90
HTLV-I	80- DALSAQLYSSLSLDSPPS	-98
Visna	93- ADLQWFYPNMESNMVGMEMLTLETQLEDNA	-122
HIV-2	64- PADSPLDQTIQHLQGLTIQELPDP	-87
SIV_{agm}	58- QVYAVDRLADEAQHLAIQQLPDPPHSA	-84

Polar Effectors

FIV	95- KAFKKMMTDLEDRFRKLFGSPSKDEYT	-120
EIAV	32- PQGPLESDQWCRVLRQSLPEEKIP	-55

FIG. 4. Minimal functional effector domains from the Rev proteins of complex retroviruses. The sequences shown are the minimal active effector peptides from HIV-1, HTLV-I, FIV, EIAV, visna virus, HIV-2, and the African green monkey strain of simian immunodeficiency virus (SIV_{agm}). Each can substitute functionally for the HIV-1 effector domain in the cotransfection assay described for Fig. 1 and 2 (4a, 5). The leucine-rich effectors each contain a cluster of four or five hydrophobic residues (leucine, isoleucine, or methionine) within an 11-residue region; mutational analysis indicates that these clustered residues are essential for effector activity (4a, 5).

higher percentages of polar amino acids (58 and 70%, respectively) than the other effector regions (47 to 53%). The disparity in basic residues is particularly striking: the EIAV and FIV peptides contain 13 and 22% basic residues, respectively, whereas the others contain an average of only 5% basic amino acids overall. Finally, although mutagenesis of the HIV-1 and human T-cell leukemia virus type I (HTLV-I) effectors indicates that only leucines and other hydrophobic residues (perhaps including proline) are essential for their activity (4a, 14), our analysis of the EIAV effector indicates that polar amino acids also contribute to its biological function. For example, mutations in polar dipeptides 38,39 and 42,43 of the EIAV effector each greatly diminish its activity (Fig. 2B).

Taken together, these data suggest that the EIAV and FIV sequences constitute a separate class of effector regions that are distinct at the level of primary sequence but are nevertheless functionally interchangeable with the conventional leucine-rich effectors. It must be emphasized that, despite their divergent amino acid sequences, all of these effectors might conceivably fold into comparable three-dimensional structures that could be responsible for their similar biological activities. However, alternative possibilities are (i) that polar effectors of the type found in EIAV and FIV might interact with the putative cofactor used by the leucine-rich effectors but at a different site and (ii) that they might interact with an entirely different factor in the same regulatory pathway. Comparative studies of these novel effector regions may therefore provide new insights into the mechanism of Rev protein function and might also suggest new approaches to interdicting this essential pathway of retroviral transactivation.

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