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Effector Domains of Human Immunodeficiency Virus Type 1 Rev and Human T-Cell Leukemia Virus Type I Rex Are Functionally Interchangeable and Share an Essential Peptide Motif

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The human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex transactivators are posttranscriptional regulatory proteins that promote retroviral gene expression by interacting with specific viral mRNAs. Rev and Rex have markedly dissimilar amino acid sequences and RNA target specificities but are thought to act through the same cellular pathway. In this report, we demonstrate that short peptide domains which are required for effector activity in Rev and Rex are functionally interchangeable. Activity of these effector domains depends upon a previously unrecognized tetrapeptide motif that is present in both Rev and Rex and also in analogous proteins from other complex retroviruses. The conserved effector motif may mediate essential interactions of Rev, Rex, and other transactivators of this type with a common cellular cofactor.

The human immunodeficiency viruses (HIV) and human T-cell leukemia viruses (HTLV) are evolutionarily distinct retroviral families that differ in genomic organization, primary sequence, and pathogenic effects (29). Like other complex retroviruses (3), each encodes a posttranscriptional regulatory protein (designated Rev in HIV and Rex in HTLV) that transactivates viral replication by inducing the export of incompletely spliced viral mRNAs from the nucleus to the cytoplasm (reviewed in references 2 through 4 and 8). Rev and Rex are comparable in many respects: both are nuclear proteins that associate preferentially with nucleoli, and each acts by binding directly to its target transcripts at a specific, highly structured *cis*-acting element known as the Rev response element (RRE) or Rex response element (XRE), respectively (2, 4, 5, 7, 8, 15, 20, 27, 30). Indeed, Rex can functionally replace Rev under certain conditions (1, 9, 19, 24), suggesting that both proteins may act through a common pathway. Yet there is little apparent sequence similarity between these two proteins that could account for their similar effects. To search for peptide domains within Rev and Rex that serve related functions, we have constructed and characterized a series of fusion proteins containing sequences from both transactivators. In this report, we demonstrate that short effector domains which mediate the regulatory activities of Rev and Rex are functionally interchangeable and we identify a tetrapeptide present in both domains that may be a core effector motif for transactivators of this type.

MATERIALS AND METHODS

Plasmid constructions. All transactivator expression vectors (Fig. 1A) were derivatives of pRSV-Rev (10); fusions and oligonucleotide-directed mutations were verified by DNA sequence analysis. To construct Rex and Rev/Rex vectors, *rev* codon 116 in pRSV-Rev was mutated to a *Bgl*II site (Asp replaces Glu), and a Rex cDNA with appropriate flanking restriction sites was either inserted in place of the *rev* sequence (*Sac*I-*Bgl*II) or fused to the 3′ end of *rev* in frame (*Bgl*II-*Bgl*II). In all fusions, Leu replaces Met at *rex* codon 1. The RRE-containing reporter plasmid pDM128 has been described (10); to prepare the XRE reporter, a 1.2-kb *Stu*I-*Bsm*I fragment (HIV-1 nucleotides 6852 to 8066) containing the RRE was excised from pDM128 and replaced by a *Cla*I linker, and a 271-bp *Cla*I fragment bearing the XRE was inserted at this site (Fig. 1B). This XRE fragment had been prepared from cloned HTLV-I by polymerase chain reaction with primers 5′-CGCGGATCCATCGATATAAAC TAGCAGGAGTCTAT-3′ and 5′-CGCGGATCCATCGATC TCGAGAGTTGAGCAAGCAGGGTC-3′, which was followed by *Cla*I digestion.

Transfection and expression assays. Transfections, chloramphenicol acetyltransferase (CAT) assays, and immunoblots were performed as previously described (10, 11). Each CAT assay was normalized to the expression of a cotransfected internal control plasmid (10, 11), and all results shown were confirmed in three or more separate tests of each construct. Rev sequences were detected in immunoblots as previously described (11) by using a combination of two antipeptide sera (kindly provided by B. R. Cullen, Duke University) specific for the N-terminal half of Rev. Unfused Rex proteins were detected with an antipeptide serum (kindly provided by W. C. Greene, Duke University) against the Rex C terminus; because this Rex antiserum reacted with a background of cellular proteins in the 30- to 46-kDa range, the anti-Rev serum was used to detect fusion proteins.

RESULTS

Previous studies have delineated at least two essential functional domains in the 116-amino-acid HIV-1 Rev protein. Sequences in the N-terminal half of Rev provide signals for nuclear and nucleolar localization and also mediate high-affinity binding of the RRE, whereas a discrete C-terminal region known as the Rev effector domain (approximately residues 78 to 88) is believed to interact with unidentified cellular factors required for transactivation (10, 11, 13,

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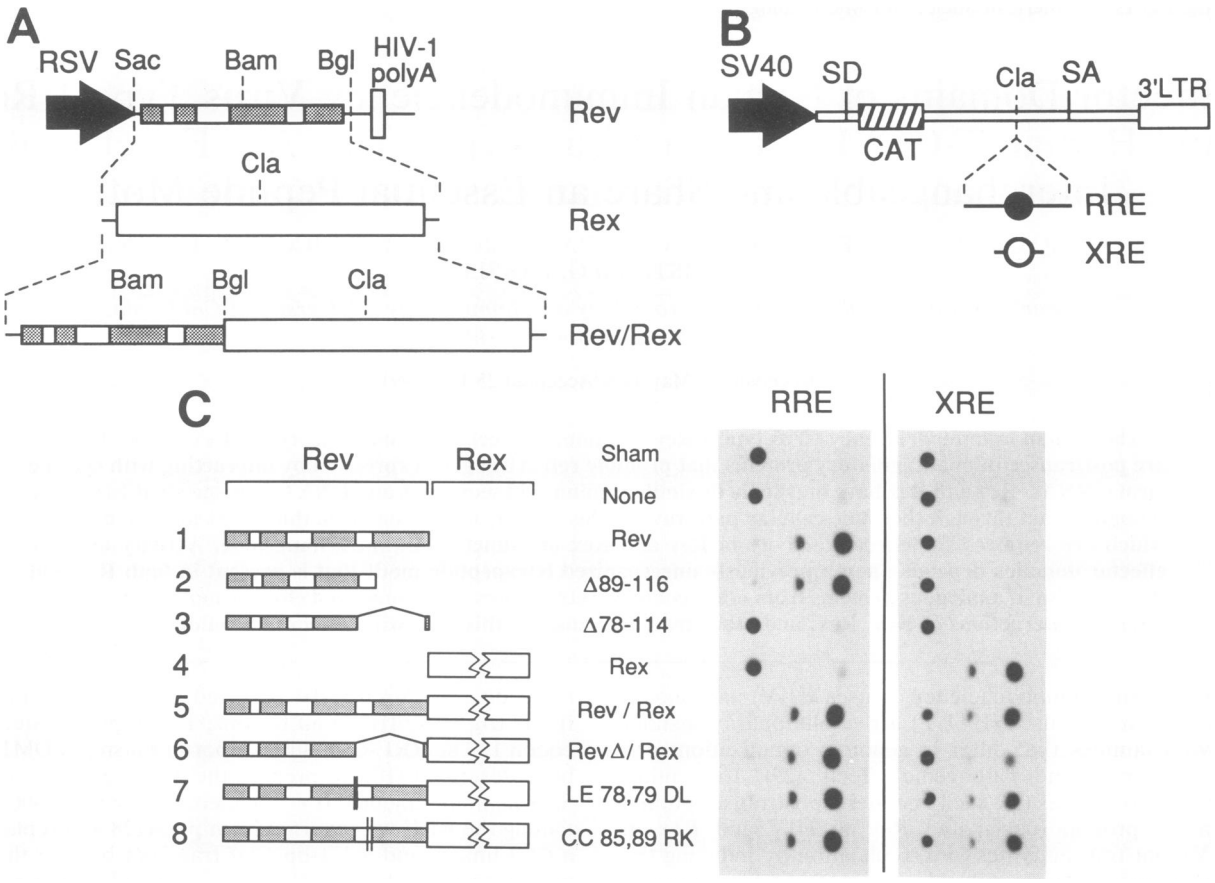
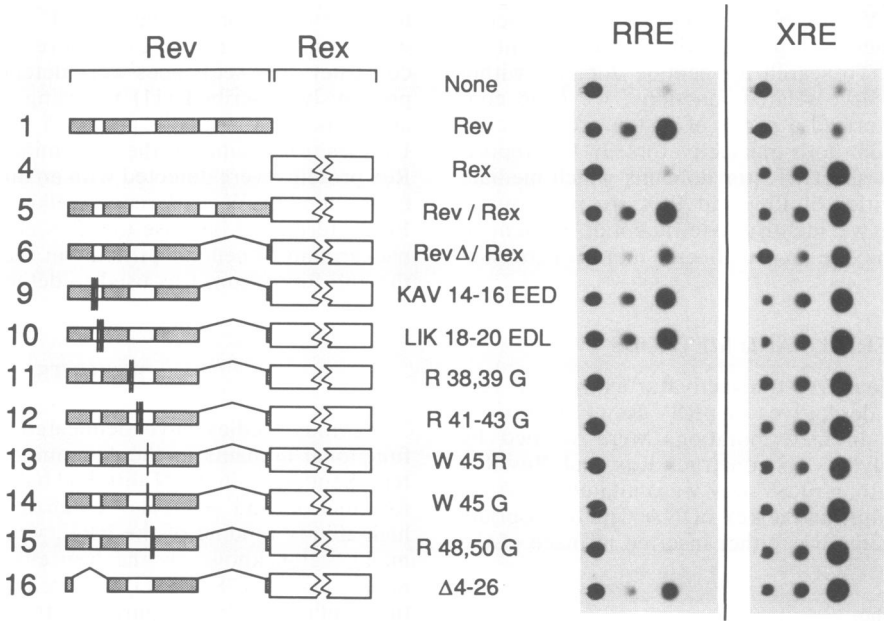


FIG. 1. A RevΔ/Rex fusion protein transactivates through either the RRE or the XRE. (A) Transactivator plasmids. The expression vector pRSV-Rev (10) transcribes a Rev cDNA by using the Rous sarcoma virus (RSV) promoter and HIV-1 polyadenylation signals. Open rectangles in the *rev* sequence denote the essential N-terminal region, arginine-rich tract, and C-terminal effector domain (11, 13, 16). Sac, *Sac*I; Bam, *Bam*HI; Bgl, *Bgl*II; Cla, *Cla*I. (B) Reporter plasmids. RRE-containing reporter pDM128, derived from HIV-1, encodes no functional viral proteins (10); locations of the CAT coding sequence and of unique splice donor (SD) and acceptor (SA) sites are shown. SV40, simian virus 40; LTR, long terminal repeat. (C) Functional characterization of wild-type, mutant, and chimeric transactivators in a cotransfection assay. CV1 cells were transfected with indicated constructs and assayed for CAT activity 36 to 40 h later. Δ denotes in-frame deletion of indicated *rev* codons; missense mutations in constructs 7 and 8 are named as described in the legend to Fig. 2. Mutant Δ78-114 has been described (11); Δ89-116 contained a stop codon at position 89; LE 78,79 DL is the mutation designated M10 by Malim et al. (13). Sham, pUC118 only; None, reporter only.



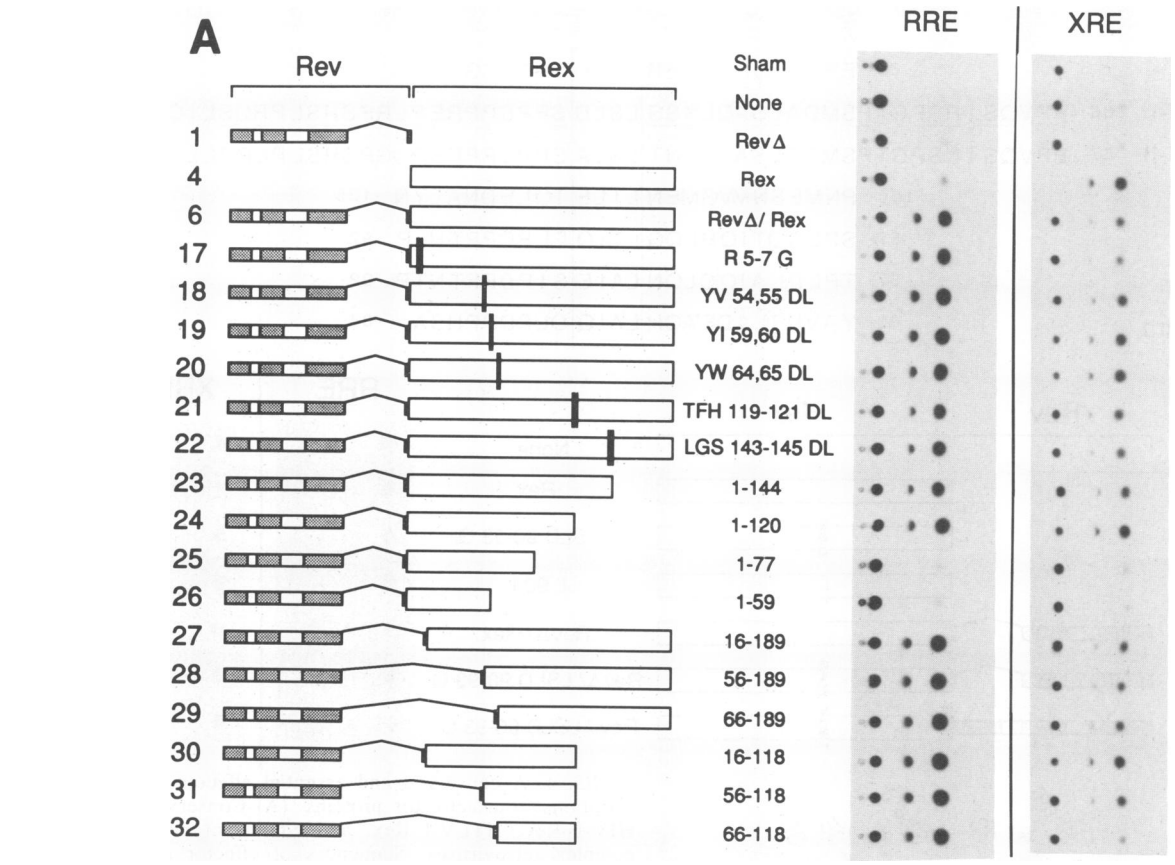


FIG. 3. Mutational analysis of the Rex moiety in RevΔ/Rex. (A) Functional analysis. CAT enzyme activity was assayed in CV1 cells that had been transfected with indicated transactivator constructs together with CAT reporter plasmids containing either the XRE or the RRE. Mutations were prepared by oligonucleotide-directed mutagenesis of a cloned Rex cDNA and were then subcloned into expression vectors. Missense mutations in the Rex moiety are named as described in the legend to Fig. 2; each introduces a unique *Bgl*II site. The mutations in constructs 17 and 19 to 22 are similar or identical to those designated M1, M6, M7, M13, and M15, respectively, in a previous study (18). Truncated mutants (constructs 23 to 32) were prepared by cleaving *rex* at the *Bgl*II site introduced by a missense mutation or at an endogenous *Cla*I site (codons 78 and 79) and are named according to the *rex* amino acids present. (B) Immunoblot analysis of mutant proteins. Extracts (60 μg of total protein per lane) of transfected COS7 cells were fractionated on a denaturing sodium dodecyl sulfate–14% polyacrylamide gel and probed with antipeptide antisera specific for the N-terminal half of Rev. Sham, pUC118 only; None, reporter only.

14, 16, 17, 31). The functional organization of the 189-amino-acid Rex protein of HTLV-I is less well characterized, but protein localization signals have been identified at the N and C termini (18, 23), and mutations at several other sites within the protein have been found to abolish function (1, 9, 18). We previously described variants of the mammalian expression vector pRSV-Rev that encode wild-type or mutant forms of HIV-1 Rev (13, 17); for the present study, we prepared additional derivatives coding for HTLV-I Rex or for various Rev/Rex chimeras (Fig. 1A). The biological activities of these proteins were then assayed by transient transfection into CV1 cells along with each of two reporter plasmids (Fig. 1B). One reporter generated transcripts containing both the CAT coding sequence and the RRE of HIV-1 within a single intron; we have shown that Rev transactivates CAT expres-

FIG. 2. Fusion with N-terminal Rev sequences confers RRE specificity onto Rex. CAT reporter plasmids containing the RRE or the XRE were cotransfected with vectors encoding Rev, Rex, Rev/Rex, or various derivatives of RevΔ/Rex. Naming of missense mutations in the Rev moiety of RevΔ/Rex indicates native and mutant amino acids (in single-letter code) at the affected positions; thus, W 45 G denotes replacement of tryptophan by glycine at position 45. Δ4-26 denotes in-frame deletion of *rev* codons 4 to 26. Mutant vectors were prepared by replacing the 5′ *Sac*I-*Bam*HI fragment of construct 5 (Fig. 1C) with corresponding fragments from pRSV-Rev mutants described previously (11). None, reporter only.

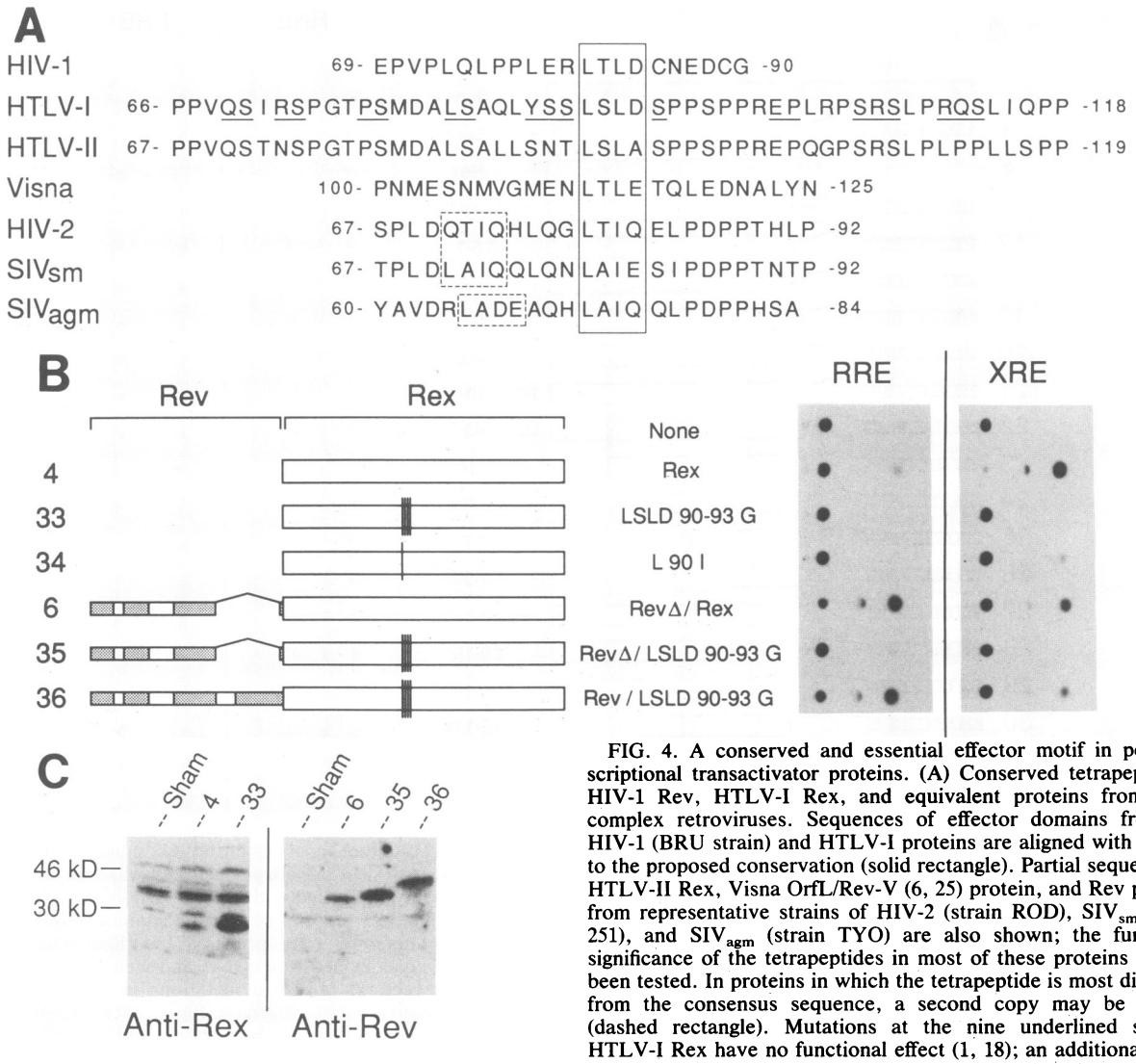


FIG. 4. A conserved and essential effector motif in posttranscriptional transactivator proteins. (A) Conserved tetrapeptide in HIV-1 Rev, HTLV-I Rex, and equivalent proteins from other complex retroviruses. Sequences of effector domains from the HIV-1 (BRU strain) and HTLV-I proteins are aligned with respect to the proposed conservation (solid rectangle). Partial sequences of HTLV-II Rex, Visna OrfL/Rev-V (6, 25) protein, and Rev proteins from representative strains of HIV-2 (strain ROD), SIV_{sm} (strain 251), and SIV_{agm} (strain TYO) are also shown; the functional significance of the tetrapeptides in most of these proteins has not been tested. In proteins in which the tetrapeptide is most divergent from the consensus sequence, a second copy may be present (dashed rectangle). Mutations at the nine underlined sites in HTLV-I Rex have no functional effect (1, 18); an additional mutation at residues 100 and 101 has been reported to inactivate Rex, but the mutant protein appeared grossly anomalous on gel electrophoresis (18). (B) Mutation of the tetrapeptide in Rex eliminates function and is complemented by the effector domain of Rev. CAT assays with CV1 cells transfected with the indicated transactivator and reporter plasmids were performed. (C) Immunoblot analysis of transfected COS7 cells expressing the indicated constructs. Extracts (100 μg of total protein per lane) were fractionated on denaturing sodium dodecyl sulfate–14% polyacrylamide gels and detected with the indicated antisera. Sham, pUC118 only; None, reporter only.

sion from this reporter more than 100-fold by inducing cytoplasmic expression of the unspliced transcripts (10). The second reporter, which was identical except that the RRE had been replaced by the XRE from HTLV-I (8), yielded 30- to 60-fold CAT induction in response to Rex.

Under the conditions of our assay, each wild-type transactivator functioned efficiently only through its cognate response element (Fig. 1C, constructs 1 and 4); in particular, Rex yielded little or no CAT induction through the RRE. This relatively strict specificity of Rex contrasts with findings in some other transfection systems (19, 24) but is in accord with the observations of at least one other group (22). Although the technical features of an assay that maintain such specificity have not been identified, we speculate that our use of nonreplicating expression vectors may be contributory. As expected, we found that deletion of residues 89 to 116 from Rev had no functional effect (construct 2), but that a slightly larger deletion (residues 78 to 114) which removed the effector domain abolished activity completely (construct 3). By fusing the full-length Rex sequence to the C terminus of Rev, we then created a chimeric protein (construct 5) that could transactivate efficiently through either the RRE or the XRE. Remarkably, the dual activity of this fusion protein was not affected by deletion of the Rev effector domain (construct 6, designated RevΔ/Rex), nor by point mutations that are known (11, 13) to inactivate this domain (constructs 7 and 8). These findings indicated that fusion with the N-terminal portion of Rev could confer RRE specificity onto Rex in our assay and also implied that fusion with Rex could complement loss of the effector domain from Rev.

To map the sequences required to confer RRE specificity, we introduced selected missense mutations into the Rev moiety of RevΔ/Rex (Fig. 2, constructs 9 to 15). Each mutation had previously been found to inhibit binding of Rev to the RRE in vitro (14, 17, 31) and to inactivate Rev in vivo (11, 13). In the context of the fusion protein, five of these

TABLE 1. Transactivation by RevΔ/Rex chimeras through the RRE or the XRE

Construct[a]	Induction (fold) of CAT activity[b]			
	RRE		XRE	
	Expt 1	Expt 2	Expt 1	Expt 2
3		2.8		1.8
4	2.1	4.1	38.0	40.4
6	44.2	89.6	10.9	4.7
17	52.9	102	5.1	2.6
18	52.3	131	9.5	7.7
19	57.7	137	17.6	8.9
20	59.1	97.3	17.5	10.4
21	36.9	53.5	8.1	6.0
22	34.7	58.4	5.8	4.8
23	29.4	58.3	8.8	2.7
24	43.7	83.3	12.4	7.3
25	1.4	1.9	0.8	0.6
26	1.7	2.2	1.3	1.0
27	58.7	97.2	6.3	2.9
28	54.1	110	7.0	4.9
29	59.0	116	9.6	3.7
30	54.0	120	11.2	9.8
31	53.3	118	5.4	4.1
32	50.8	84.0	1.3	1.7

[a] Constructs are numbered as in Fig. 3A.
[b] Data are from two independent experiments, derived and expressed as described in Table 2, footnote *b*.

mutations selectively eliminated transactivation through the RRE; the latter mutations each involved sequences within an arginine-rich tract in Rev (amino acids 35 to 50) that has been shown to be critical for RRE binding (14, 17, 31). By contrast, two other mutations (involving Rev amino acids 14 to 20) had no detectable effect, suggesting that the function of these sequences was complemented by Rex. Indeed, we found that large simultaneous deletions could be introduced at both Rev termini (amino acids 4 to 26 and 78 to 114) without diminishing RRE-specific transactivation (construct 16). Parallel testing confirmed that all seven mutants could transactivate through the XRE, implying that the proteins were stably expressed and that signals in the intact Rex moiety could ensure appropriate localization within the nucleus. Fusion with a 51-amino-acid region that includes the arginine-rich tract of Rev is therefore necessary and sufficient to confer RRE specificity onto Rex in this assay.

We next performed mutational analysis of the Rex moiety in RevΔ/Rex to map the sequences that could complement deletion of the Rev effector domain. Representative functional data are shown in Fig. 3A and Table 1; the size and stability of selected mutant proteins were confirmed by immunoblot analysis of the transfected cells (Fig. 3B). We found that missense mutations involving known protein localization signals (18, 23) at the N and C termini of Rex (constructs 17 and 22) did not inactivate RevΔ/Rex, presumably because equivalent signals are present in the Rev moiety (10, 13). Three other missense mutations that have been reported (1, 18) to abolish effector function in Rex failed to inactivate the fusion protein (constructs 19 to 21). Progressive truncations of the Rex moiety (constructs 23 to 29) revealed that up to 79 C-terminal or 65 N-terminal amino acids could be deleted without eliminating function, although simultaneous deletion of both these regions profoundly inhibited XRE-specific transactivation (construct 32). Most importantly, two chimeras that lacked the central portion of Rex failed to transactivate through either response element (constructs 25 and 26), whereas a fusion protein comprising only amino acids 66 to 118 of Rex (construct 32) transactivated through the RRE as efficiently as wild-type Rev.

These findings indicated that a 52-amino-acid internal segment from Rex could functionally replace the effector domain of Rev. Upon comparing the sequences of these two regions (Fig. 4A), we noticed that each includes a single copy of the tetrapeptide $Leu^{Thr}_{Ser}LeuAsp$. Moreover, we found related (though not identical) tetrapeptides at comparable locations in all other known retroviral transactivators of this type, including HTLV-II Rex, Rev proteins from two related families of primate immunodeficiency lentiviruses (simian immunodeficiency virus type sm [SIV_{sm}]/HIV-2 and SIV_{agm}), and the functionally equivalent OrfL/Rev-V protein of Visna, an ungulate lentivirus (6, 25). In the HTLV-I and Visna proteins, this motif encompasses the site of mutations that have been reported (18, 26) to inhibit transactivation.

We therefore asked whether this tetrapeptide was required for Rex effector activity. As shown in Fig. 4B and Table 2, we found that Rex was completely inactivated when all four residues in the tetrapeptide were replaced by glycines (construct 33) and that even a highly conservative mutation (isoleucine replacing leucine) at the first position of the tetrapeptide strongly inhibited function (construct 34). In contrast, data from previous studies (1, 18) indicate that mutations at nine nearby sites (Fig. 4A, underlined) have no effect on Rex activity, suggesting that the tetrapeptide is the only essential sequence in the Rex effector domain. We also found (Fig. 4B and Table 2) that fusion with full-length Rev partially restored the ability of a Rex tetrapeptide mutant to transactivate through the XRE (construct 36) but that deletion of the Rev effector domain prevented this complementation (construct 35). Immunoblot analysis confirmed that these effects were not due to differences in protein stability (Fig. 4C). Thus, the tetrapeptide in Rex is essential for transactivation and is interchangeable with a region from Rev that contains this same peptide motif.

TABLE 2. Effects of mutations in the conserved tetrapeptide motif on the ability of Rex and of Rev/Rex chimeras to transactivate through the XRE

Construct[a]	Induction (fold) of CAT activity[b]				
	Expt 1	Expt 2	Expt 3	Expt 4	Mean ± SEM
4	50.6	30.6	29.5		36.9 ± 9.7
33	1.1	1.6	1.1	0.6	1.1 ± 0.4
34	1.2	1.7	2.0	1.4	1.6 ± 0.3
6	9.7	9.4	10.1	6.8	9.0 ± 1.3
35			0.4	0.4	0.4
36			3.9	6.2	5.1 ± 1.2

[a] Constructs are numbered as in Fig. 4B.
[b] Ratio of CAT expression from the reporter plasmid in CV1 cells transfected with and without each indicated construct. Acetylation of chloramphenicol was assayed by thin-layer chromatography and quantified by scintillation counting. All data were corrected for the trace acetylation produced by extracts from untransfected cells and were normalized within each experiment relative to the expression of an internal control plasmid, as previously described (10, 11).

DISCUSSION

The studies reported here extend earlier mutational analyses and provide new insights into the functional architecture of Rev and Rex. Under the conditions of our in vivo assay, in which each of these proteins can transactivate only

through its cognate response element, we find that sequences in and around the arginine-rich tract of Rev are sufficient to confer RRE specificity onto Rex. This observation is compatible with earlier evidence (14, 17, 31) that mutations throughout this region in Rev inhibit RRE binding in vitro and that similar arginine-rich tracts together with their flanking sequences dictate the target specificities of several other RNA-binding proteins (12, 21, 28). Our data suggest, however, that the structural determinants of XRE specificity may be very different: although an arginine-rich tract is present at amino acids 1 to 15 in Rex, several RevΔ/Rex mutants which lacked this region (constructs 27 to 31) remained fully competent for transactivation through the XRE. A similar interpretation has recently been suggested by Hofer et al. (9), who found that the arginine-rich sequence in Rex could be replaced by that of Rev without affecting recognition of the XRE. Sequences critical for target discrimination must therefore be located in the remainder of Rex, which includes relatively few arginines or other basic residues but can nevertheless confer XRE specificity onto Rev in vivo (e.g., construct 27). Further mutational analysis will be needed to map these sequences precisely.

Our studies also reveal that amino acids 66 to 118 of Rex comprise an autonomous effector domain that is functionally interchangeable with the effector domain of Rev. The shared function of these two domains appears to depend upon a previously unrecognized tetrapeptide motif found in both Rev and Rex, as well as in all other known retroviral transactivators of this type (Fig. 4A). Identification of this conserved effector motif provides a structural basis for the view (3, 8) that, while the posttranscriptional transactivators of various complex retroviruses have markedly different sequences and target specificities, they may nevertheless act through a common effector pathway. Although the details of this pathway are presently unknown, transactivation is believed to result from specific interactions of the viral protein not only with the target mRNA but also with one or more cellular components of the RNA splicing or transport apparatus (2–4, 13, 18). The existence of a conserved and essential effector motif suggests that posttranscriptional transactivators from diverse retroviruses may exert their effects through contact with a common cellular factor.

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