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Dominant-Negative Mutants Are Clustered in a Domain of the Human T-Cell Leukemia Virus Type I Rex Protein: Implications for *trans* Dominance

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The 27-kDa Rex *trans*-acting protein appears to be essential for replication of human T-cell leukemia virus type I. Mutations introduced outside of the Rex RNA-binding domain–nucleolar localization signal display either wild-type activity or, conversely, yield dominant-negative proteins. We generated missense mutations in a particular domain of the Rex protein (amino acid residues 54 to 69) which is characterized by a cluster of dominant-negative mutants. Our results indicate that amino acids 57 to 67 are critically important for Rex function mediated through the RxRE *cis*-acting RNA sequence. Within this domain, only amino acids 61 to 63 could be mutated without loss of function. All other missense and deletion mutants yielded dominant-negative proteins. In vitro RNA-binding studies performed with glutathione S-transferase–Rex fusion proteins demonstrated that all of the mutant Rex proteins interacted specifically with RxRE RNA. Analysis of chimeric Rex-Rev proteins suggests that this Rex domain is important for oligomerization.

The genomes of human retroviruses are distinguished by their relative complexity (17, 56). In addition to the Gag, Pol, and Env proteins, these retroviruses encode regulatory proteins that are essential for viral replication (51, 53). At least two regulatory proteins appear to be required for replication of human T-cell leukemia virus type I (HTLV-I) (46), the causative agent of adult T-cell leukemia (43, 55), tropical spastic paraparesis (18), and HTLV-I-associated myelopathy (42). The *trans*-activator protein Tax increases transcription of all retroviral genes via indirect effects on host factors that bind to the long terminal repeat (16, 21, 48). A second regulatory gene product, Rex (31), acts at a posttranscriptional level by selectively augmenting the cytoplasmic accumulation of genomic-length and singly spliced HTLV-I *env* mRNA (25–27). Translation of these retroviral transcripts into structural and enzymatic proteins is required for virion assembly and formation of infectious particles. Rex is a 27-kDa nuclear protein which is concentrated in the nucleoli (47). A stretch of basic amino acid residues at the amino terminus (nuclear-nucleolar localization signal) is responsible for this subcellular localization (39, 47). Rex function depends on specific binding of the protein to its target RNA sequence (Rex response element [RxRE]; 2, 4, 7, 14, 20, 22, 50). The RxRE is encoded in the 3′ long terminal repeat and thus is present on all primary HTLV-I transcripts (2, 22). This *cis*-acting sequence has the potential to form a stable secondary structure (2, 4, 50) which is required for HTLV-I mRNA polyadenylation (1). A very similar situation is observed in the HTLV-II system (28, 40, 54).

In the replicative cycle of human immunodeficiency virus type 1 (HIV-1), the Rev protein serves similar functions (11, 51). Rev corresponds to a 19-kDa nuclear-nucleolar phosphoprotein (9, 23) that binds to a highly structured RNA target sequence termed the Rev response element (12, 13, 24, 29, 37). Like Rex, Rev promotes cytoplasmic accumulation of incompletely spliced HIV-1 mRNA species (15, 21, 35). Mutational analysis of the HIV-1 Rev protein has revealed a modular organization (5, 33, 36) reminiscent of many DNA-binding transcription factors (44). Mutations in the nuclear-nucleolar localization signal notably result in altered subcellular localization, as well as loss of RNA binding. These findings indicate that this arginine-rich basic domain serves at least two functions: specific RNA recognition and nuclear-nucleolar localization (5, 56). Mutations in this region yield recessive-negative proteins (33). Recent evidence suggests that Rev oligomerization is also required for function in vivo and that the domain responsible for oligomerization flanks the RNA-binding domain (10, 34, 41, 56). It was also suggested that Rev interferes with HIV-1-specific mRNA splicing (8, 30) and contributes to the translation efficiency of HIV-1-specific RNA (3, 32). Mutations in the Rev activation domain, which is postulated to interact with a cellular factor(s), lead to dominant-negative Rev proteins which interfere with wild-type function in *trans*. This functional Rev domain is located near the carboxy terminus of Rev (33, 36, 38).

In contrast to Rev, the HTLV-I Rex protein appears to be organized in a less modular manner. Only one recessive-negative mutant outside of the nuclear-nucleolar localization signal has been described in the Rex protein (45). Dominant-negative mutants are dispersed throughout the entire coding sequence of HTLV-I Rex, but four of these mutants were clustered between amino acid residues 58 and 65 (6, 45). To define a functional domain within this region, we generated a series of additional point and deletion mutations between amino acid residues 54 and 70 of the Rex protein (Fig. 1A and B). The HTLV-I *rex* coding sequence was subcloned

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A

aa	52																			71
wt	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
M6	G	D	**L**	V	R	P	A	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
M6*	G	D	**P**	V	R	P	A	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
M32	G	D	**D**	**L**	R	P	A	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
M35	G	D	Y	V	R	**D**	**L**	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
M7*	G	D	Y	V	R	P	**D**	**P**	I	V	T	P	Y	W	P	P	V	Q	S	I
M7**	G	D	Y	V	R	P	**S**	**S**	I	V	T	P	Y	W	P	P	V	Q	S	I
M36	G	D	Y	V	R	P	A	**D**	**L**	V	T	P	Y	W	P	P	V	Q	S	I
M37**	G	D	Y	V	R	P	A	Y	**S**	**S**	T	P	Y	W	P	P	V	Q	S	I
M38	G	D	Y	V	R	P	A	Y	I	**D**	**L**	P	Y	W	P	P	V	Q	S	I
M39	G	D	Y	V	R	P	A	Y	I	V	**D**	**L**	Y	W	P	P	V	Q	S	I
M8*	G	D	Y	V	R	P	A	Y	I	V	T	**D**	**P**	W	P	P	V	Q	S	I
M40	G	D	Y	V	R	P	A	Y	I	V	T	P	**D**	**L**	P	P	V	Q	S	I
M42**	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	**C**	**S**	**S**	V	Q	S	I
M43	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	W	P	**D**	**L**	Q	S	I
M44	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	W	P	P	**D**	**L**	S	I
M9*	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	W	P	P	V	**D**	**P**	I

B

aa	52																			71
wt	G	D	Y	V	R	P	A	Y	I	V	T	P	Y	W	P	P	V	Q	S	I
Δ1	G				**D**	**L**			I	V	T	P	Y	W	P	P	V	Q	S	I
Δ2	G							**D**	**L**					W	P	P	V	Q	S	I
Δ3	G									**D**	**L**									I
Δ4	G	D	Y	V	R	P				**D**	**L**			W	P	P	V	Q	S	I
Δ5	G	D	Y	V	R	P							**D**	**L**						I
Δ6	G	D	Y	V	R	P	A	Y	I	V	T					**D**	**L**			I
Δ1*	G				**D**	**P**			I	V	T	P	Y	W	P	P	V	Q	S	I
Δ2*	G							**D**	**P**					W	P	P	V	Q	S	I
Δ3*	G									**D**	**P**									I
Δ4*	G	D	Y	V	R	P				**D**	**P**			W	P	P	V	Q	S	I
Δ5*	G	D	Y	V	R	P							**D**	**P**						I
Δ6*	G	D	Y	V	R	P	A	Y	I	V	T					**D**	**P**			I

FIG. 1. (A) Summary of Rex point mutants used. The amino acid sequence of the wild-type Rex protein between residues 52 and 71 is indicated at the top. The various Rex mutants are listed below, and the altered amino acid residues are in boldface. One asterisk indicates introduction of a *Bam*HI recognition site (***D P***), while two asterisks indicate introduction of an *Xho*I recognition site (***S S***). Mutants with no marks indicate introduction of a *Bgl*II site (***D L***). aa, amino acids; wt, wild type. (B) Summary of Rex deletion mutants used. The Rex deletion mutants used are listed on the left, and deleted amino acid residues are not shown (compare with wild-type Rex sequences at the top). New amino acids introduced are in boldface.

into M13 DNA and mutagenized as described by Taylor et al. (49). Subsequently, the wild-type and mutant Rex-coding sequences were ligated into a eukaryotic expression plasmid containing the human cytomegalovirus immediate-early promoter (6). Transfection of these Rex mutants into COS cells and subsequent immunoprecipitation with a Rex-specific rabbit antiserum (6) revealed that each mutant expression plasmid directed the synthesis of an immunoreactive polypeptide with an apparent molecular weight similar to that of the wild-type Rex protein (data not shown). In terms of function, cotransfection with the Rex-responsive HIV-1 proviral DNA (4) into human SW480 cells demonstrated that mutations amino terminal of residue 59 or carboxy terminal of residue 65 resulted in Rex function comparable to that of the wild-type protein (Fig. 2). Similarly, mutations at amino acid positions 61 to 63 also resulted in wild-type activity (RexM38 and M39; Fig. 2), whereas overlapping mutations were clearly reduced in their *trans* activation potential (RexM37** and M8*; Fig. 2). The biological activities of all of the other mutants in this domain (RexM7*, M7**, M36, and M40) were markedly impaired (Fig. 2). The deletion mutants from this region (shown in Fig. 1B) completely failed to induce detectable HIV p24 production (data not shown). Next, we analyzed the nonfunctional missense and all deletion mutants for the ability to interfere with the function of wild-type Rex protein in *trans* (Fig. 3A). Each of these mutants displayed a dominant-negative phenotype. Specifically, a 10-fold molar excess of either Rex mutant M37** or M8* diminished wild-type Rex function by 70% while mutants RexM7* and M7** reduced wild-type Rex activity by approximately 80% (Fig. 4A). RexM36 and M40 proved the most potent missense mutants, suppressing Rex activity by >95% (Fig. 3A). The deletion mutants proved to be even more powerful inhibitors. The least effective dominant-negative deletion mutants, RexMΔ3 and RexMΔ6*, reduced Rex function by 80%, while mutants RexMΔ1, MΔ6, MΔ3*, and MΔ5* suppressed Rex activity by ≥90%. All of the other deletion mutants reduced the wild-type Rex protein function to levels below detection (Fig. 3B).

We next tested the RxRE-binding activities of select mutant *rex* genes which were cloned into prokaryotic expression plasmid pGEX-2T (Pharmacia). The resultant GST-Rex fusion proteins were partially purified (6) and analyzed for RNA binding activity in vitro in gel mobility shift assays. Decreasing fivefold serial dilutions of GST-RexM32, M7*, M36, and M40 were incubated with radiolabeled RxRE RNA. The GST fusion protein derived from the functional RexM32 gene formed discrete RNA-protein complexes with RxRE RNA (Fig. 4) which were similar to the pattern observed with the GST-Rex wild-type protein (Fig. 4). The GST-Rex fusion proteins derived from dominant-negative mutants RexM7*, M36, and M40 also interacted with the RxRE target RNA, generating RNA-protein complexes similar to that generated by wild-type Rex protein (Fig. 4). These experiments indicate that each dominant-negative mutant Rex protein retains RNA-binding activity comparable to that of the Rex wild-type fusion protein. However, these *trans*-dominant proteins differ in subcellular localization from wild-type Rex. They can be detected in the nuclear compartment but appear to be excluded from the nucleoli (data not shown). However, this aberrant subcellular localization is not responsible for the dominant-negative phenotype of these Rex proteins, as has been suggested for HTLV-I Tax (19). Our experiments indicate that the dominant-negative proteins cannot prevent the nucleolar localization of wild-type Rex, even at a 10-fold molar excess (data not shown).

Recently, multimerization has been shown to be important for biological activity of the HIV-1 Rev protein (10, 34, 41, 56). Therefore, mutations in the activation domain, as well as the multimerization domain, could lead to dominant-negative Rev proteins. It remains unclear whether multimerization is also important for HTLV-I Rex function. To test whether the Rex domain located between amino acids 58 and 65 might be involved in multimerization, we constructed chimeric Rex-Rev proteins by inserting the putative Rev multimerization domain (amino acid residues 19 to 28) into a Rex gene deleted between residues 57 and 66 (Fig. 5). *Nhe*I (5′) and *Asp*718 (3′) restriction sites were inserted into the Rex gene and ligated by using adaptor molecules (5′-CTAGCTCGCGGG-3′ and 5′-GTACCCCGCGAG-3′) to generate RexΔ57/66 (Fig. 5). Oligonucleotides that encode the indicated amino acid residues were subsequently inserted into the *Nhe*I and *Asp*718 restriction sites to construct the chimeric Rex-Rev proteins. The mutant chimeric protein (RexΔRev19/28) revealed nearly full restoration of biological activity on both the HTLV-I RxRE and HIV-1 RRE target sequences (Fig. 6A and B, lanes 5). In contrast, the same

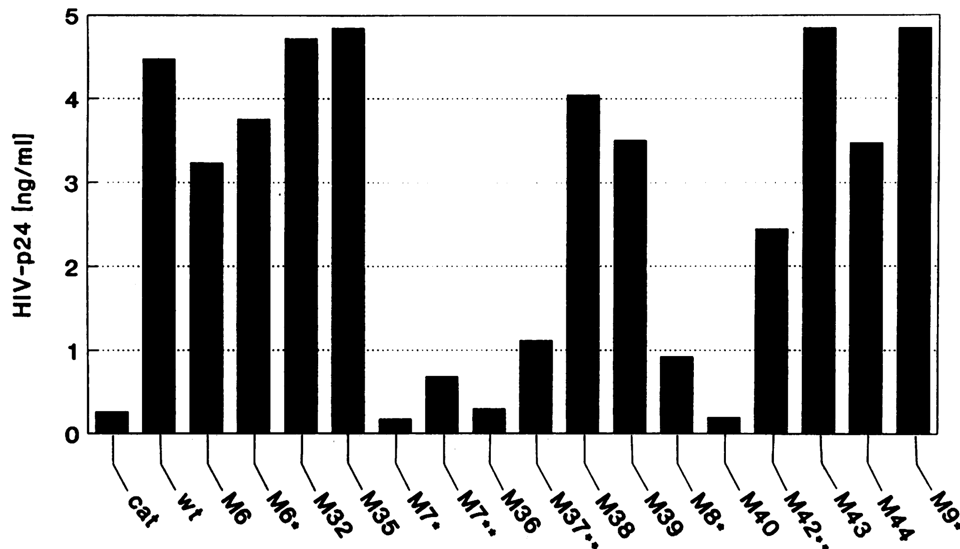

FIG. 2. In vivo analysis of Rex mutant proteins on the HTLV-I RxRE target sequence. The rex expression plasmids were cotransfected with a Rex-dependent HIV-1 proviral plasmid, and HIV-1 p24 protein synthesis was determined in the culture supernatants (Coulter enzyme-linked immunosorbent assay). A chloramphenicol acetyltransferase expression plasmid (cat) was transfected to determine background levels of HIV-1 p24 production in the absence of Rex. The Rex mutants are indicated at the bottom. The Rex activities displayed were obtained in one of several transfection experiments using two independent plasmid preparations. wt, wild type.

Rev-derived amino acids inserted in a rearranged order (RexΔRev19/28rv) failed to restore biological activity. It is of note that mutations in the Rev-derived amino acid residues altered the biological potential of these chimeric proteins. Introduction of two asparagine residues rendered Rev wild-type protein nonfunctional (data not shown) and abrogated biological activity of the chimeric protein (RexΔRev19/28D22D23) on both *cis*-acting sequences (Fig. 6A and B, lanes 8). The previously reported RevM4 mutation (33) destroys apparent multimerization and function of the Rev protein. The corresponding chimeric protein (RexΔRev19/28M4) was still functional on HTLV-I RxRE but not on

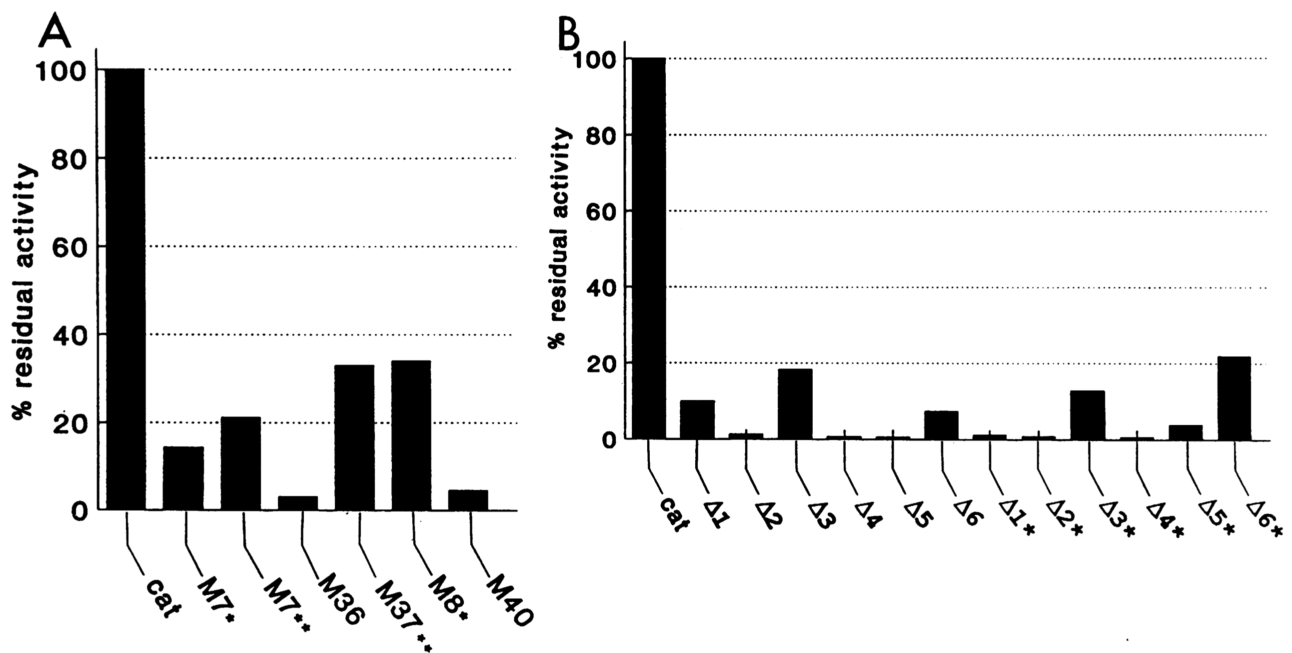

FIG. 3. *trans* dominance of Rex missense (A) or deletion (B) mutants over Rex wild-type activity. A 10-fold molar excess of Rex mutant expression plasmids over wild-type Rex was transfected into SW480 cells. The p24 activity obtained in the presence of the control plasmid (cat) represents 100%. The activity obtained in the presence of the Rex mutant expression plasmids (bottom) is indicated as percent residual wild-type Rex activity. The results of typical experiments are shown. All transfection studies were performed at least three times with two independent plasmid preparations.

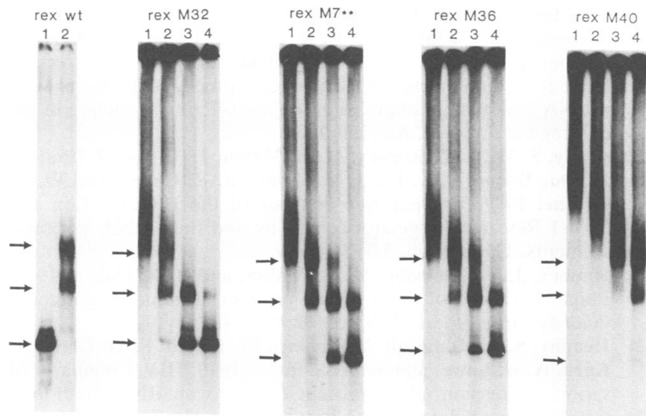

FIG. 4. Mutant Rex-RxRE RNA gel shift experiments. The RNA gel shifts of four different GST-Rex mutant proteins (indicated at the top) are shown. The band obtained in the absence of added Rex protein is demonstrated in lane 2 (left panel). The RxRE-Rex complexes obtained with undiluted GST-Rex (lanes 1), a 1:5 dilution (lanes 2), a 1:25 dilution (lanes 3), and a 1:125 dilution (lanes 4) of the GST-Rex protein preparations are depicted. The arrows indicate the positions of the free RxRE RNA and distinct RxRE–GST-Rex complexes. wt, wild type.

HIV-1 RRE (Fig. 6A and B, lanes 9). Introduction of amino acid residues derived from the Rev activation domain (RexΔRev78/87), which were able to restore a mutant Rex protein deleted in the activation domain (52), also failed to restore biological activity. We have previously identified amino acid residues 79 to 99 of Rex as an activation domain sufficient to restore function to a mutant of Rev from which the activation domain was deleted (52). These results indicate that amino acid residues 57 to 67 constitute a functional HTLV-I Rex domain different from the activation domain. Our current studies with Rex-Rev chimeric proteins suggest that amino acid residues 54 to 69 of Rex are important for oligomerization of this viral *trans* activator. Insertion of the putative multimerization domain of Rev into Rex, which had

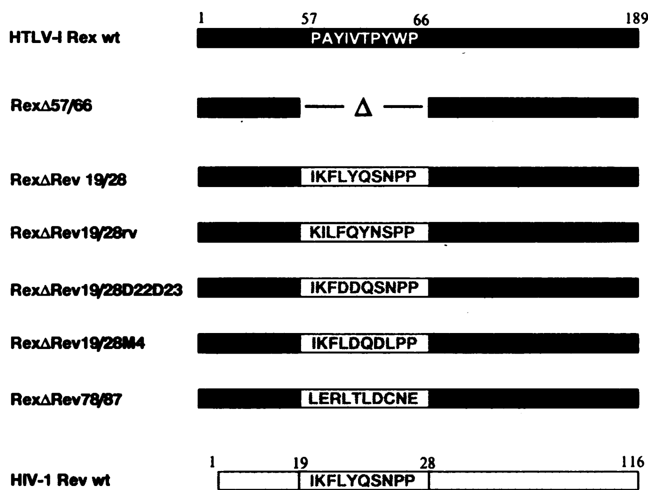

FIG. 5. Construction of Rex-Rev chimeric proteins. HTLV-1 Rex (black bar at the top) and HIV-1 Rev (grey bar at the bottom) wild-type (wt) proteins are schematically depicted. Rex amino acid residues 57 to 66 were deleted to generate RexΔ57/66. Letters indicate Rev-derived amino acid residues inserted into RexΔ57/66.

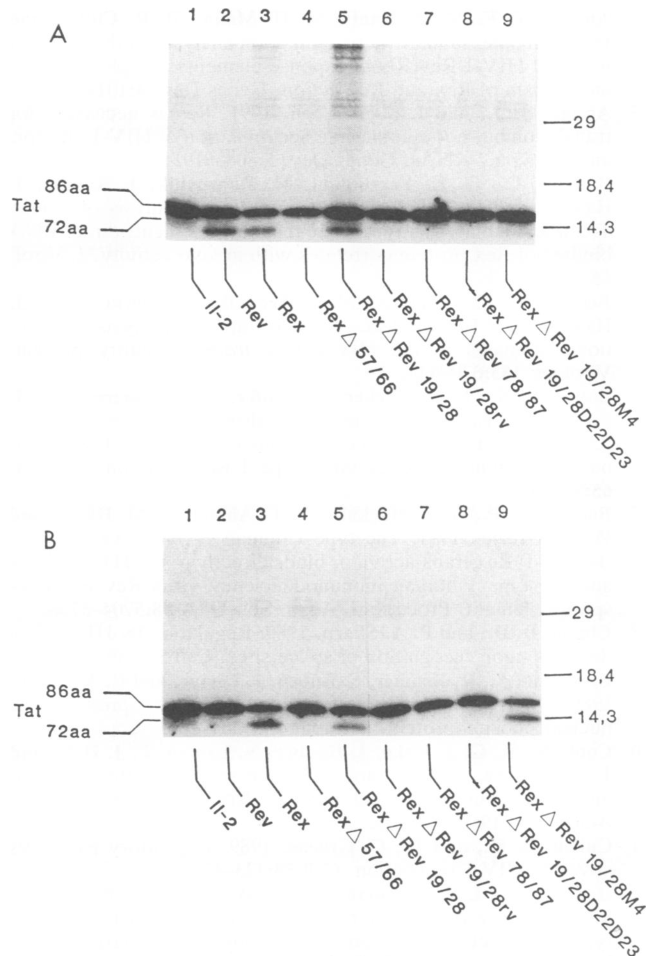

FIG. 6. Functional analysis of the Rex-Rev chimeric proteins on the HIV-1 RRE (A) and HTLV-I RxRE (B) target sequences. The gTat (A) and gTat-RxRE (B) vectors were cotransfected with the indicated expression plasmids as previously described (7). Molecular size standards (in kilodaltons) and the two HIV-1 Tat protein forms (86aa and 72aa) are indicated. The *trans*-activating proteins are indicated at the bottom. aa, amino acid.

amino acid residues 57 to 66 deleted, restored biological activity. In contrast, insertion of the Rev activation domain or various mutant forms of the Rev multimerization domain did not reconstitute biological activity. Together, our experiments suggest that the functional domain between amino acids 54 and 69 of Rex are involved in multimerization. It further implies that the dominant-negative phenotype of proteins mutated in this region reflects their failure to multimerize. Studies are under way to test directly the potential involvement of this Rex domain in multimerization by inserting these Rex-derived amino acids in the HIV-1 Rev protein in lieu of residues 19 to 28.

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