Different Sites of Interaction for Rev, Tev, and Rex Proteins within the Rev-Responsive Element of Human Immunodeficiency Virus Type 1

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We have analyzed the action of the Rev and Tev proteins of human immunodeficiency virus type 1 (HIV-1) and of the Rex protein of human T-cell leukemia virus type I (HTLV-I) on a series of Rev-responsive element (RRE) mutants. The minimum continuous RRE region necessary and sufficient for Rev function was determined to be 204 nucleotides. Interestingly, this region was not sufficient for Tev or Rex function. These proteins require additional sequences, which may stabilize the structure of the RRE or may contain additional sequence-specific elements. Internal RRE deletions revealed that the targets for Rev and Rex can be separated, since mutants responding to Rev and not Rex and vice versa were identified. Tev was active on both types of mutants, suggesting that it has a more relaxed specificity than do both Rev and Rex proteins. Although Rev and Rex targets within the RRE appear to be distinct, the trans-dominant mutant RevBL prevents the RRE interaction with Rex. RevBL cannot inhibit the function of Rex on RRE deletions that lack the Rev-responsive portion. These results indicate the presence of distinct sites within the RRE for interaction with these proteins. The binding sites for the different proteins do not function independently and may interfere with one another. Mutations affecting the RRE may change the accessibility and binding characteristics of the different binding sites.

Both human immunodeficiency virus (HIV) and human T-cell leukemia viruses (HTLV) regulate the expression of their structural proteins by specific viral factors called Rev and Rex, respectively (for recent reviews, see references 31 and 36). Both Rev and Rex are essential viral factors. Rev acts via a unique sequence, named the Rev-responsive element (RRE) (17), located in the *env* region of HIV (5, 9, 10, 13, 17, 20, 21, 26, 27, 37). The sequence necessary for Rex function has been named by analogy the Rex-responsive element and is located at the 3' long terminal repeat (LTR) of HTLV type I (HTLV-I) (11, 15, 22, 41, 43). It was shown that Rex can also act on HIV type 1 (HIV-1) via the same RRE region (15, 22, 35). An additional viral protein named Tev has been identified in some HIV-1-infected cells (3, 38). This protein has been shown to exhibit both Tat and Rev activities and can replace both of these essential HIV regulatory factors (3).

The RRE is present only in the full-length and intermediate-size mRNAs, some of which encode Gag, Gag-Pol, and Env proteins. In contrast, the RRE is absent, because of splicing, from the small multiply spliced mRNAs encoding regulatory proteins such as Tat and Rev. Rev affects the transport of RRE-containing RNAs from the nucleus to the cytoplasm (13, 14, 17, 21, 27) and increases their half-lives (17). This results in high levels of RRE-containing RNAs in the cytoplasm that are translated efficiently into structural viral proteins (17, 20).

Although Rev can be replaced by Rex, the activities of these factors are not reciprocal, since Rex of HTLV-I cannot be replaced by Rev (15, 22). This has been interpreted as evidence for direct interaction of these factors with their respective RNA elements. It was subsequently shown that

Rev binds specifically to the RRE and that binding is necessary for Rev function (5, 8, 23, 26, 30, 44). Binding alone is not sufficient for Rev function, since the trans-dominant Rev mutant RevBL (28) binds specifically to the RRE but does not function (D. M. Benko, R. Robinson, L. Solomin, M. Mellini, B. K. Felber, and G. N. Pavlakis, submitted for publication). Therefore, interaction with cellular factors may be necessary for function. Although the direct binding of Rex to the RRE or Rex-responsive element has not been demonstrated, structural and functional data (6, 7, 17, 22, 25, 28, 34, 42), such as the arginine-rich regions in both proteins, the nucleolar localization, the similar behavior of mutants, and the functional replacement of Rev by Rex, point to the conclusion that both are directly binding to specific RNA elements that promote mRNA transport and utilization (for reviews, see references 32 and 36).

Here we define the minimal elements within the RRE region that are necessary and sufficient for Rev, Rex, and Tev response. The results indicate that the binding targets for these molecules are distinct. The specificities of these proteins for different structural elements within the RRE are different and indicate that specific structures may be important for function. The contribution of the primary sequence of this region is unclear. Tev appears to have more relaxed specificity compared with both Rev and Rex.

MATERIALS AND METHODS

Constructs. The *gag* expression plasmid MVgag has been described previously (20). It was derived from the infectious proviral clone HXB2 (18) by removing a viral fragment from *Bal*I to *Xho*I (nucleotides [nt] 2165 to 8443) and replacing it with a polylinker. Various fragments containing the RRE were inserted into the polylinker and assayed for Gag protein production after introduction into human cells.

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TABLE 1. Identification of the minimal continuous RRE for Rev, Rex, and Tev function (arrows) by a collection
of 5' and 3' deletion mutants[a]

DELETION	Rev	Rex	Tev
(-100)-422	210	10	27
1-330	133	23	34
1-265	60	1	1
1-190	1	1	1
14-330	159	10	26
14-311	134	3	12
14-292	155	4	24
24-330	94	3	7
42-330	121	4	9
62-330	136	1	1
62-273	63	nd	nd
62-244	1	1	3
62-194	2	1	1
86-765	1	nd	nd
104-765	1	nd	nd

[a] Fold activation obtained by the p24[gag] antigen capture assay in the presence of each protein is indicated. Results are averages of two to six experiments. nd, Not determined.

The pMVgag-RRE plasmid constructs are shown in Table 1. For convenience, the numbering system used here for the various RRE fragments defines as nt 1 the first nucleotide of the StyI site at nt 7266 of HXB2 (where +1 is the RNA start site of the corrected HXB2 sequence). The differences identified between our sequence and the HXB2 sequence given in the acquired immunodeficiency syndrome data base (29, 33) are as follows: insertion of 3 nts after position 223, GAG-226; deletion of 2 nt after position 260, GCGCCCGC to GCGCGC, which restores the BssHII site; C-7369 to G; A-7501 to G; A-7513 to G; A-7549 to G; and C-7636 to T. The names of the different constructs indicate the coordinates of the RRE inserts. p1-330 and p62-330 contain the StyI fragments (nt 7266 to 7595 and nt 7327 to 7595 in HXB2, respectively) inserted into pMVgag. 3' RRE deletions were constructed in p62-330 by BAL 31 digestions and resulted in endpoints at nt 273, 244, and 194, respectively. Polymerase chain reaction amplification was used to generate the other deletion mutants. The amplified fragments were first cloned into the EcoRV site of Bluescript vector KS(-) (Stratagene) and then inserted into the pNLgag vector, resulting in p1-265, p1-190, p14-330, p14-311, p14-292, p14-265, p24-330, and p42-330. pNLgag is a vector similar to pMVgag, constructed by insertion of a BssHII-to-XhoI fragment of pMVgag (nt 265 to 2165 of HXB2 containing the gag region) between the LTRs of a different HIV-1 molecular clone, pNL43 (1). pNLgag contains the pUC replication origin and grows better in Escherichia coli. Internal deletions in the RRE contained in plasmid p1-330 were constructed by using site-directed mutagenesis in M13 vectors (Mutator kit; Stratagene); the mutated RRE fragments were then cloned into the pNLgag expression vector. The internal RRE deletion mutants lack the following sequences: DL12s, nt 89 to 155; DL45, nt 183 to 238; DL345, nt 156 to 238; and DL345s, nt 147 to 238. All mutations were confirmed by sequencing. pL3rex contains the HTLV-I rex cDNA (15) from the rex

initiation codon to the ApaI site (nt 7927 in reference 40) linked to the HIV-1 LTR promoter in pL3pA (28). pNL1.4.6D.7 is an HIV-1 cDNA expressing only the p28[rev] protein of HXB2 (3). pL3rev and pLBL express the HIV-1 Rev (17) and the Rev trans-dominant mutant RevBL (28), respectively, from the HIV-1 LTR promoter. pRSV-L contains the luciferase gene linked to the Rous sarcoma virus LTR promoter (11).

Transfections. HLtat, a cell line constitutively expressing HIV-1 Tat (39), was transfected by the calcium phosphate precipitation technique as described previously (16, 19). All transfection mixtures contained 2 μg of pRSV-L as an internal standard. At 20 to 48 h posttransfection, cells were washed twice with phosphate-buffered saline and scraped off the plate in 400 μl of 100 mM Tris (pH 7.4)–0.5% Triton X-100. The cell lysate was frozen and thawed once, centrifuged for 5 min in an Eppendorf centrifuge, and used in all subsequent assays.

Protein measurements. For Western immunoblots, 50 μl of cell lysate was adjusted to 0.5× RIPA by addition of 5 μl of 5× RIPA buffer (20). Proteins were separated on 12.5% denaturing acrylamide gels (24) and blotted onto nitrocellulose filters. Gag proteins were visualized by using human patient serum, followed by incubation with ^{125}I-labeled protein A. For p24[gag] antigen capture assays (Dupont, NEN), 50 μl of lysate was diluted into 150 μl of dilutant solution (provided by the kit; Dupont, NEN), and serial 10-fold dilutions thereof were analyzed as instructed by the manufacturer. For luciferase assays (4, 12), 25 μl of cell lysate was mixed with 1 volume of luciferase lysis buffer (1.5% Triton X-100, 50 mM glycylglycine [pH 7.8], 30 mM MgSO4, 8 mM EGTA, 2 mM dithiothreitol) and 180 μl of luciferase reaction buffer (25 mM glycylglycine, 15 mM MgSO4, 4 mM EGTA, 15 mM KPO4 [pH 7.8], 1 mM dithiothreitol, 2 mM ATP). A substrate mixture of 100 μl containing 0.2 mM luciferine dissolved in 1× luciferine

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buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM dithiothreitol; stored in the dark at $-70°C$ as a $10\times$ stock solution) was added to the above-described mixture by injection, and luminescence was measured for 20 s in an LKB-Wallac 1251 luminometer. Luciferase assays were performed within 1 to 2 days after cell lysis. Total protein was measured from 2 to 4 μl of lysate, using the Bio-Rad protein determination assay.

RESULTS

It has been reported that Rev of HIV-1 can be replaced either by Rex of HTLV-I (15, 35) or by Tev, a hybrid protein with both Tat and Rev activities that is produced by some HIV-1 strains (3). It was also shown that Rev and Rex act on HIV-1 through the same RRE region (15). To define the minimal RRE for Rev, Rex, and Tev response, a series of 5', 3', or internal deletion mutants was generated by using convenient restriction sites, BAL 31 digestion, polymerase chain reaction amplification, or site-directed mutagenesis. These RRE fragments were inserted into plasmid pMVgag (or pNLgag), which contains the HIV-1 LTRs and the gag region (20). After transfections into human cells, pMVgag produced very low levels of Gag and did not respond to Rev. We have shown that efficient production of Gag requires the presence of both the RRE and Rev (17, 20). The constructed pMVgag-RRE plasmids were transfected either alone or in the presence of the Rev-expressing plasmid pL3crev, the Rex-expressing plasmid pL3rex, or the Tev-expressing plasmid p1.4.6D.7 into HLtat cells, which constitutively express Tat. One or two days later, the cells were harvested and analyzed for Gag production by immunoblotting, $p24^{gag}$ antigen capture assay, or both. The results of some immunoblotting experiments are shown in Fig. 1. Quantitations of the levels of Gag were obtained by the $p24^{gag}$ antigen capture assay and are summarized in Table 1. All transfection mixtures contained 2 μg of pRSV-L as internal standard. It was found that the presence of Rev, Tev, or Rex consistently inhibited the levels of luciferase expression by two- to fivefold. Different plasmid preparations gave similar results. Additional internal controls were performed by using a human cytomegalovirus early promoter-chloramphenicol acetyltransferase gene, with similar results. This finding may reflect inhibition of some nonviral expression by these factors. Despite this, Gag production by the RRE-containing plasmids was induced by up to 200-fold, indicating the specific function of Rev, Tev, or Rex. Because of these observations, the data were not normalized to the luciferase standard.

Definition of the minimal continuous RRE for Rev response. We have previously reported that a fragment within env from nt 7242 to 7687 is necessary and sufficient for full Rev function (15). To define the 5' and 3' borders of RRE for Rev response in more detail, two StyI fragments comprising nt 7266 to 7595 and nt 7327 to 7595 of HXB2 were inserted into pMVgag, generating plasmids p1-330 and p62-330, respectively. The names of the different constructs indicate the coordinates of the RRE inserts. The numbering system used here for the various RRE fragments defines as nt 1 the first nucleotide of the StyI site at nt 7266 from the RNA start site of HXB2.

Rev induced Gag expression from both p1-330 and p62-330 to a high extent (Fig. 1A, lanes 2 and 14). 5' deletion mutants to nt 86 (pMVgag012 or p86-765) or 104 (pMVgag218 or p104-765) have been shown to be inactive in the presence of Rev (15). These data define the 5' border of RRE at nt 62. To

establish the 3' border, deletions of the RRE in p1-330 were constructed by using either BAL 31 or polymerase chain reaction amplification of portions of the RRE (see Materials and Methods). RRE fragments 62-330, 62-273, 14-265, 62-244, 62-194, and 1-190 were inserted into pMVgag (or pNLgag) and analyzed as described above. While the 3' deletion to nt 265 resulted in a minor decrease in activation (twofold), further deletions to nt 244, 194 (Fig. 1A, lanes 9 and 10), or 190 (Fig. 1A, lanes 5 and 6) completely abolished Rev responsiveness (Table 1). Therefore, the 3' border for full Rev response was assigned to nt 265. These experiments defined the minimal continuous RRE element required for full Rev response as a 204-bp fragment from nt 62 to 265 (nt 7327 to 7530 in HXB2). Any further deletions severely affected or abolished Rev response.

Definition of the minimal continuous RRE for Rex response. It has been shown that the Rex protein of HTLV-I acts on HIV-1 via an RRE-containing fragment from -25 to $+422$ (15). To investigate whether Rex requires a minimal continuous RRE fragment similar to Rev, the RRE deletion mutants discussed above were cotransfected with the Rex-producing plasmid pL3rex. To define the 5' border of RRE, plasmids p1-330, p14-330, p24-330, p42-330, and p62-330 were tested (Table 1). Rex activated Gag expression from p1-330 by 23-fold, which was 6-fold less than the activation by Rev. While a 5' deletion to nt 14 lowered the Rex response about twofold, further deletions to nt 24 and 42 led to a severe drop in Rex response. Mutant 62-330 could not be activated by Rex, while activation by Rev was not affected. To define the 3' border, we analyzed and compared plasmids p1-330, p14-311, p14-292, and p1-265. While deletion from nt 330 to 311 or 292 reduced Rex activation by 80 to 90%, deletion to nt 265 resulted in a complete loss of Rex function. These results demonstrated that the region necessary for full Rex response lies within nt 14 to 330. The smallest element displaying low activity was nt 42 to 330. Interestingly, RRE fragment 62-265 could not be activated by Rex but retained full Rev response. These experiments demonstrated that both the 5' and 3' borders of RRE necessary for Rev or Rex function are distinct (nt 62 to 265 versus nt 42 to 330).

Tev also requires additional RRE sequences. The interaction of Tev with the RRE was also examined with use of the same RRE mutants by cotransfecting the different gag constructs containing RRE deletions with a Tev-expressing plasmid and measuring Gag expression (Table 1). Tev activated Gag expression from constructs containing RRE fragments 1-330 and 14-330. Activation by Tev was four- to eightfold lower than that by Rev, which is in agreement with previously published studies in which the function of Rev and Tev was investigated on the complete rev-negative provirus (3). 5' deletions to nt 24 or 42 lowered the Tev response, while a further deletion to nt 62 abolished Tev function. Additional experiments using 3' RRE deletion mutants showed that removal of sequences to nt 311 or 292 affected activation by Tev only minimally. Removal of additional sequences to nt 265 (p1-265) or 190 resulted in a complete loss of Tev response. All smaller mutants were not activated by any of the three proteins (Table 1 and Fig. 1A). The minimal continuous sequence necessary for Tev function was thus defined as nt 42 to 292. It is interesting that Tev and Rex require additional sequences for their function compared with Rev. Rev requires the smallest RRE element for function (nt 62 to 265), while Rex and Tev require larger continuous RRE fragments (nt 42 to 330 and nt 42 to 292, respectively).

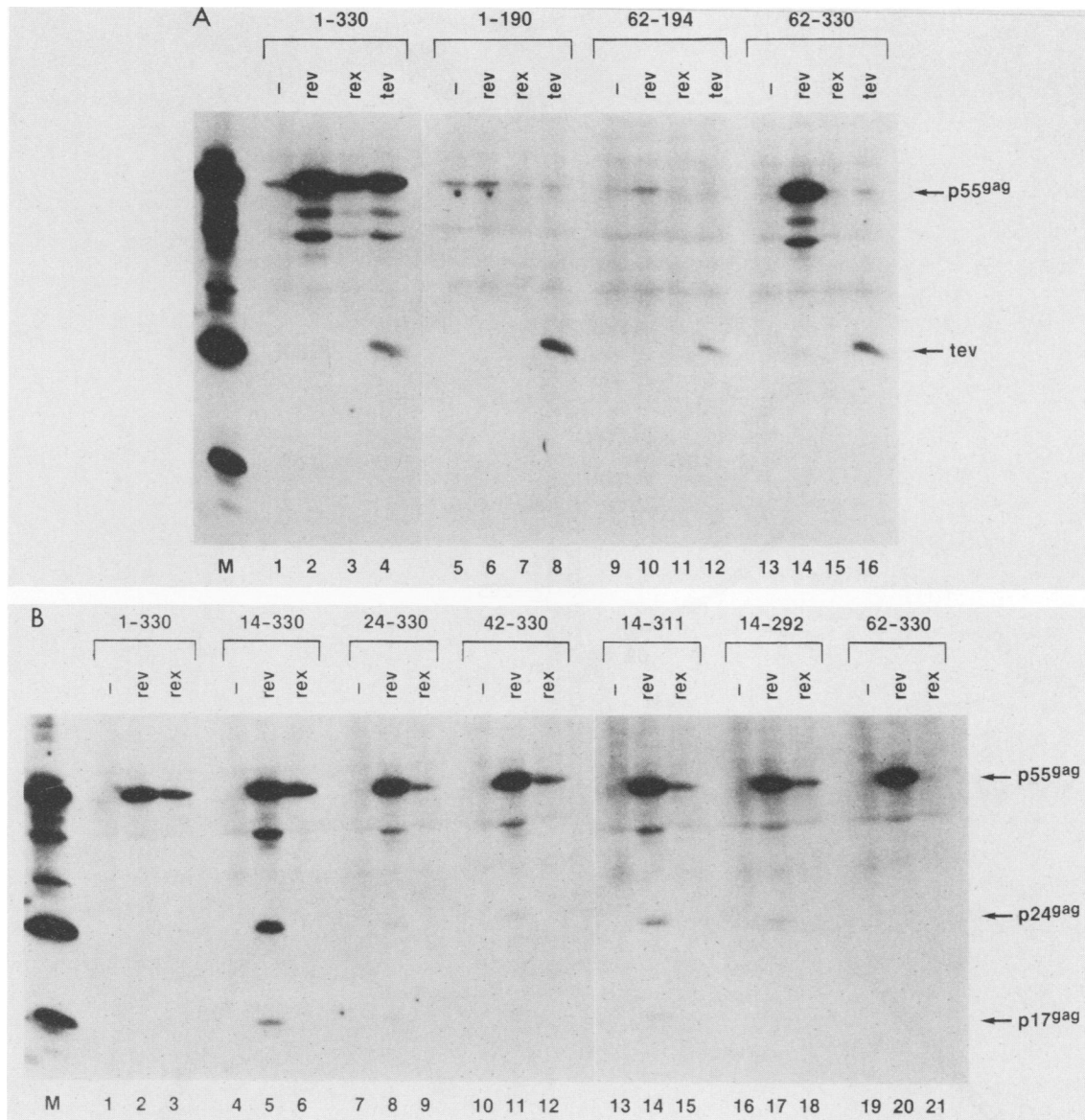**Internal deletions demonstrate that Rev, Rex, and Tev**

FIG. 1. Activation of pMVgag-RRE constructs by Rev, Rex, and Tev. HLtat cells were transfected with the indicated pMVgag-RRE constructs in the absence (−) or presence of pL3crev (Rev-producing), pL3rex (Rex-producing), or p1.4.6D.7 (Tev-producing) plasmid, as indicated. One to two days later, the cells were harvested, and a sample of the lysate was analyzed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose filters. The viral proteins were visualized by using HIV-1 patient serum and ^{125}I-labeled protein A. Positions of the Gag proteins (p55, p24, and p17) are indicated. Lane M, Cell extract from a H9/HXB2-infected cell line.

targets are distinct. These results suggested that the sites of interaction of the three factors within the RRE may be different. To further examine whether Rev, Rex, and Tev interact with different sites within the RRE, we generated four internal deletions of the RRE-containing fragment 1-330 and tested their ability to be activated by the different factors. The RRE was proposed to have extensive secondary structure (27) comprising five hairpin loop structures numbered 1 to 5 in Fig. 2, a long-stem (LS) structure, and a short-stem (s) structure. The plasmid names indicate the deleted hairpin loops as well as the deletion endpoints. pDL12s(89/155) contains a deletion of nt 89 to 155 comprising the first two hairpin structures and the short stem (loop II in reference 27). Plasmids DL345s(147/238), DL345(156/

238), and DL45(183/238) contain deletions from nt 147, 156, and 183, respectively, to nt 238. These deletions are located in the region of the short-stem and hairpin structures III to V (27). These constructs were transfected into HLtat cells as described above in the absence and presence of the Rev-, Rex-, or Tev-producing plasmids. Gag production was measured by p24^{gag} antigen capture assay (Table 2) and compared with that of p1-330. Analysis of these RRE deletion mutants also showed that the requirements for Rev, Tev, and Rex action were distinct. pDL12s was almost completely refractory to activation by Rev, in agreement with previously published data (10, 26, 30). Interestingly, the Rex response was minimally affected. pDL12s was activated by Tev to a low but significant extent. On the contrary, mutants
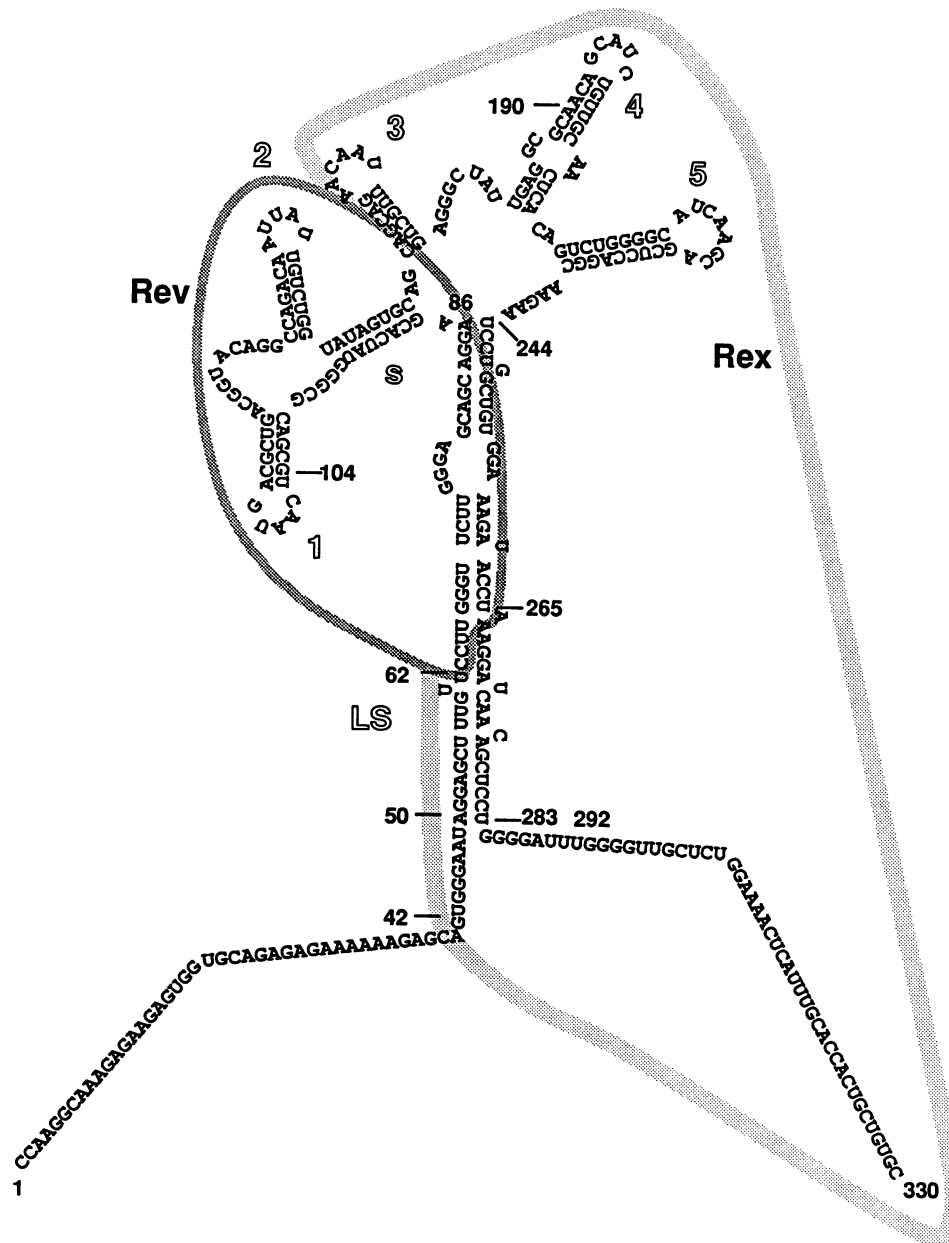
FIG. 2. Interactions of Rev and Rex with the RRE of HIV-1. The proposed secondary structure of the RRE is shown, and the regions important for Rev and Rex interaction are outlined. The RRE is proposed to contain five stem-loop structures numbered 1 to 5, a long stem (LS), and a short stem (s). Nucleotide numbering starts at the StyI restriction site at position 7266 of the HIV-1 molecular clone HXB2.

pDL345s, pDL345, and pDL45 were not activated by Rex but were activated by Rev and Tev. It is interesting that these mutants showed a gradual decrease in Rev responsiveness (50 to 8% of the full response), while Tev was less affected (100 to 47%). Hairpin 3 and the short stem clearly contributed to the overall Rev activation (compare DL45 with DL345 and DL345s). The analysis of Tev function on internal RRE deletions revealed that Tev was only marginally affected by deletions that removed the Rex-responsive portion of RRE (DL345s, DL345, and DL45), suggesting that the targets of Tev resemble those of Rev. On the other hand, removal of the Rev-responsive portion (DL12s) lowered (by sevenfold) but did not abolish Tev function. Activation of

DL12s by Tev was low, while its activation by Rev was virtually abolished.

The function of Rex can be abolished in the presence of the *trans*-dominant mutant Rev protein. Analysis of the RRE deletion mutants revealed that Rev and Rex may interact with different parts of the RRE structure. We next examined whether there is any interference between Rex and Rev binding to the RRE. We have previously shown that the presence of *trans*-dominant mutant Rev proteins can abolish both Rev (28) and Tev (3) function. Here, we tested whether the function of Rex can also be inhibited in the presence of the *trans*-dominant mutant Rev (RevBL). p1-330 was transfected in the presence of the Rex-producing plasmid pL3rex

TABLE 2. Analysis of the sites of interaction of Rev, Rex, and Tev by using internal RRE deletions[a]

Rev	Rex	Tev	
132	23	34	RRE
2	14	5	DL12s
11	1	16	DL345s
26	1	15	DL345
72	2	33	DL45

[a] Fold activation obtained by the p24^{gag} antigen capture assay in the presence of each protein is indicated. Results are averages of two to six experiments.

or the Rev-producing plasmid pL3crev and increasing amounts of pLBL, which expressed RevBL (28). One day later, the cells were harvested and the cell lysates were subjected to immunoblot analysis (Fig. 3). Transfection of the Rex-producing plasmid pL3rex with increasing amounts of RevBL (lanes 1 to 4) demonstrated that Rex function was inhibited in the presence of RevBL. Similarly, the presence of increasing amounts of RevBL led to a gradual decrease in Rev-activated p55^{gag} production (lanes 5 to 8). Rex function was abolished in the presence of equimolar amounts of RevBL protein. This ratio is lower than the fourfold excess of RevBL necessary to abolish Rev function (28). These results suggest that the interaction of Rex with RRE is weaker than that of Rev. We have made a similar observation in studies of the interaction of Tev with RRE (3), suggesting that both Tev-RRE and Rex-RRE interactions are weaker than the Rev-RRE interaction. The inhibition on Rex-induced activation by RevBL was quantitated by p24^{gag} antigen capture assays using plasmids p1-330 and pDL12s. The results of three experiments were similar, and those of a
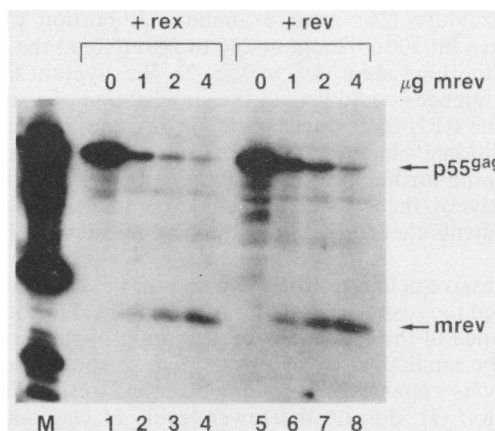

FIG. 3. Inhibition of Rex and Rev function by trans-dominant mutant RevBL. HLtat cells were cotransfected with p1-330 and 2 μg of the Rex-expressing plasmid pL3rex (lanes 1 to 4) or 1 μg of the Rev-expressing plasmid pL3crev (lanes 5 to 8). Increasing amounts (1, 2, and 4 μg, as indicated) of a plasmid expressing RevBL were included in the transfections (lanes 2 to 4 and 6 to 8). One day later, the cells were harvested and analyzed as described for Fig. 1. The positions of p55^{gag} and trans-dominant mutant RevBL (mrev) are indicated. Lane M, Cell extract from a H9/HXB2-infected cell line.

typical experiment are shown in Table 3. Interestingly, while RevBL inhibited Rex function on p1-330, it did not inhibit Rex function on pDL12s, which lacks the binding site for Rev and RevBL. The presence of increasing amounts of RevBL lowered Gag expression of DL12s by 1.5- to 4-fold (three experiments). This was not considered significant, since a similar decrease in the internal standard expression (luciferase) was observed in the presence of RevBL.

Therefore, binding of the RevBL mutant appears to be necessary for inhibition of the Rex function. These results taken together confirm the conclusion that Rev and Rex targets within the RRE are distinct. However, the interactions of the different proteins on the complete RRE are not independent and may interfere with one another.

DISCUSSION

Analysis of the activation of RRE mutants by Rev, Rex, and Tev revealed that their sites of interaction are distinct. While Rev requires the shortest continuous element (nt 62 to 265), Tev and Rex need additional sequences for function (nt 42 to 294 and nt 42 to 330, respectively). It was shown that the RRE has a potential to form a strong secondary structure including five hairpin structures flanked by a long stem (Fig. 2) (10, 26). The positions of the sequences from nt 42 to 62 and from nt 265 to 330 within the proposed model for the

TABLE 3. Inhibition of Rex function by the trans-dominant mutant RevBL on the complete RRE but not on RRE deletion DL12s

Plasmid	p24^{gag} (pg/ml)[a]				
	Rex	Rex + RevBL			
		1 μg	2 μg	4 μg	10 μg
p1-330	1751 (44)	39 (1)	18 (<1)	10 (<1)	
pDL12s	416 (32)		294 (23)		130 (10)

[a] Results of a representative experiment are shown. Fold activation in the presence of Rex or of Rex and RevBL proteins is shown in parentheses.

RRE structure (26) were examined. A portion of these sequences (nt 50 to 62 and nt 266 to 283) extend the bottom part of the long stem (LS in Fig. 2). We hypothesize that these sequences could lead to the stabilization of a structure within the RRE that is necessary for Tev and Rex function. The additional flanking sequences from nt 14 to 49 and nt 284 to 330 could further contribute to the structure stabilization. Alternatively, these sequences could contain sequence-specific elements that form part of the binding sites for Tev and Rex.

Progressive deletions from both ends of the RRE gradually decreased the response to all three activators. The biological significance of the low levels of activation of some mutants should be studied on virus production. It should be noted that Tev is capable of replacing Rev and results in virus production (3), despite the lower levels of Gag activation (four- to eightfold lower in our experiments). Therefore, we estimate that activation at 10% of the wild-type Rev levels may result in virus production and is biologically significant.

Internal RRE mutants revealed that Rev, Tev, and Rex require different parts of the RRE for function. While hairpins 1 and 2 (Fig. 2) are clearly part of the Rev recognition signal, hairpins 4 and 5 are necessary to allow interaction with Rex. Hairpin 3 appears to contribute to Rev, Tev, and Rex function. It is interesting that DL345s shows a severely impaired activation by Rev. This mutant RRE retains the hairpins 1 and 2 necessary for Rev function but lacks part of the sequence forming the small stem. This result suggests that the small-stem structure may contribute to Rev function. The results obtained for Rev activation of the internal RRE mutants are in good agreement with other reports (10, 26, 30). The internal deletion mutants fall into two classes: one which has lost Rev and retained Rex response, and one which has retained Rev and lost Rex response. Tev, a variant Rev protein produced by some HIV-1 strains, contains all of the functionally necessary domains of Rev and can replace Rev (3). Interestingly, the requirements for interaction with the RRE by Rev and Tev are distinct. We hypothesize that the Tat or Env portion at the amino terminus of Tev may affect the structure and binding properties of this protein. Therefore, Tev may have a relaxed specificity and may recognize both the Rev target and, to a low extent, the Rex target within the RRE.

Study of the internal RRE mutants revealed that the sites for Rev and RevBL binding within RRE were the same (Benko et al., submitted). While the function of Rex on the complete RRE was abolished in the presence of mutant RevBL, Rex function on a deletion of the Rev-responsive part of the RRE, mutant DL12s, was not affected by RevBL. These data strongly suggest that the trans-dominant mutant RevBL must bind efficiently to the RRE to inhibit either Rev or Rex function. These results are in agreement with the observation that Rev and RevBL bind in vitro to the RRE with the same affinity (Benko et al., submitted). Competition experiments using RevBL revealed that although Rev and Rex targets within the RRE are distinct, Rev and Rex interfere with the binding of each other. After the completion of this work, similar conclusions about the different targets for Rev and Rex within RRE were reported (2). The work described here identifies further the different minimal elements necessary for the functions of Rev and Rex.

ACKNOWLEDGMENTS

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