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The Rex regulatory protein of human T-cell lymphotropic virus type I binds specifically to its target site within the viral RNA

(posttranscriptional gene regulation/AIDS/RNA transport)

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ABSTRACT **The Rex protein of human T-cell leukemia virus type I (HTLV-I) was expressed in bacteria and partially purified. Rex was shown to bind *in vitro* specifically to an RNA sequence located in the 3′ long terminal repeat of HTLV-I, named Rex-responsive element (RXRE). Rex also bound *in vitro* to the human immunodeficiency virus type 1 (HIV-1) Rev-responsive element (RRE), while purified HIV-1 Rev protein did not bind to the RXRE. The binding results obtained *in vitro* are therefore in agreement with the nonreciprocal function of Rev and Rex *in vivo*. Rex binds specifically to both RRE and RXRE and activates expression in both HIV-1 and HTLV-I, while Rev binds to RRE and activates only HIV-1. Binding of Rex to RRE deletion mutants previously shown to lack either the Rev-responsive or the Rex-responsive portion suggested preferential binding of Rex to a distinct target within the RRE. These results demonstrated that Rex, like Rev, acts by binding to a specific RNA target.**

Analysis of the genomic sequences and functional properties of lentiviruses, oncoretroviruses of the human T-cell leukemia virus (HTLV) family, and spumaviruses revealed that they express additional proteins, possess a complex splicing program, and, more importantly, regulate their own expression via viral factors. We have proposed the term complex retroviruses to distinguish these retroviruses from those that do not regulate their own expression (1, 2). HTLV and human immunodeficiency virus (HIV) are complex retroviruses that regulate expression of their structural proteins by specific viral factors called Rex and Rev, respectively (for recent reviews, see refs. 3–5). Both Rex and Rev are essential for viral replication.

Rev acts via a unique sequence named the Rev-responsive element (RRE) (6, 7) or CAR (8), located in the *env* region of HIV (6–13). The RRE is present only in the full-length and intermediate-sized mRNAs, which include those encoding Gag, Gag-Pol, Vif, Vpr, and Env proteins (14, 15). In contrast, due to splicing, the RRE is absent from the small multiply spliced mRNAs encoding regulatory proteins such as Tat, Rev, and Nef (16). Rev affects the transport of RRE-containing RNAs from the nucleus to the cytoplasm (6, 10–12, 17) and increases their half-lives (6). This results in higher levels of RRE-containing RNAs in the cytoplasm that are translated efficiently into structural viral proteins (6, 18) (D. M. Benko, B.K.F., J. Harrison, and G.N.P., unpublished data).

The sequence necessary for Rex function is named the Rex-responsive element (RXRE) and is located in the 3′ long terminal repeat of all members of the HTLV family (19–23); due to its location, RXRE is present in all viral mRNAs. The presence of Rex is essential for the accumulation of unspliced and singly spliced cytoplasmic viral mRNAs and for the production of the viral structural proteins (19, 20, 22, 24–27). The expression of the doubly spliced mRNAs encoding Rex and Tax is independent of Rex; nevertheless, these mRNAs contain RXRE.

The cis-acting RNA sequences RRE and RXRE are proposed to form complex secondary structures (28–31) but do not share significant sequence homology. Although Rex and Rev proteins have similar functions, they have little homology, except for the presence of an arginine-rich region that is important for nuclear localization and function (32–36). Interestingly, Rex can replace Rev function (20, 37) and acts via a sequence within the RRE (20, 21). On the other hand, Rev cannot substitute for Rex function (20, 21). The nonreciprocal activities of Rev and Rex suggested direct interaction of these factors with their respective RNA elements (20, 21). It was subsequently demonstrated that Rev binds specifically to the RRE *in vitro* (9, 28, 38–43).

Analysis of the sequence requirements within the RRE for Rev and Rex function revealed that the minimum continuous RRE regions necessary and sufficient for Rev and Rex function were different. While an element of 204 nucleotides (nt) (nt 7327–7530) in HXB2R provirus sequence) was sufficient for Rev function, a larger RRE element of 289 nt (nt 7307–7595 in HXB2R) was required for Rex function (44). The additional flanking sequences required by Rex may stabilize a specific RRE structure or may contain additional sequence-specific elements necessary for Rex binding. Analysis of internal RRE deletions revealed that the targets for Rev and Rex can be separated (31, 44). The Rev-responsive portion of RRE comprises hairpin loops 1 and 2 and the short stem, while the Rex-responsive portion is located in hairpin loops 4 and 5 and the long stem (see Fig. 4*A*).

In this report we demonstrate that Rex of HTLV-I, produced in *Escherichia coli* and partially purified, is able to bind specifically to both the RXRE and the RRE. Therefore, Rex, like Rev, acts by binding specifically to its RNA target. In contrast, purified Rev of HIV-1 does not bind to the RXRE. This explains the nonreciprocal activities of Rex and Rev *in vivo* and indicates that specific binding to the RNA target is necessary for the function of these factors.

MATERIALS AND METHODS

Protein Expression and Purification. The *rex* cDNA was isolated from plasmid pϕ10-DD-HTRex as a 600-base-pair

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Abbreviations: HTLV-I, human T-cell leukemia virus type I; HIV-1, human immunodeficiency virus type 1; RXRE, Rex-responsive element; RRE, Rev-responsive element; nt, nucleotide(s); RXREs, RXRE in the sense orientation; RXREas, RXRE in the antisense orientation.

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Nco I/*Apa* I fragment and inserted into the *Nco* I and *Sma* I sites of the bacterial expression plasmid pUC12N (45, 46). The resulting plasmid can express Rex but not Tax protein. The bacterially expressed Rex has two extra amino acid residues (methionine and glycine) at the amino terminus. The bacterial expression of Rev using the same vector has been described elsewhere (47). The Rev and Rex proteins were expressed in the lon protease mutant *E. coli* strain BU8049 (48). The purification of Rev has been described (39). Rex was purified by a similar protocol, followed by an additional chromatographic step. Briefly, bacteria were harvested at stationary phase and lysed by sonication. The extract was treated with polyethyleneimine (0.3%) and subjected to centrifugation. The supernatant was loaded onto a carboxymethyl-Sepharose Fast Flow (Pharmacia) cation-exchange column. Elution was performed with a linear gradient of 0.25–2.0 M NaCl. Fractions containing Rex (0.5–1.0 M) were further purified on a Bio-Gel HPHT hydroxylapatite column (Bio-Rad), which was eluted with a linear gradient of 0.01–1.0 M sodium phosphate (pH 6.8). Rex was eluted at 0.6–0.8 M sodium phosphate, and these fractions were subjected to size-exclusion chromatography on a Superose-12 (Pharmacia) column. The purification of Rex was followed by Western blot analyses of the different column fractions with an anti-Rex rabbit antiserum raised against a Rex peptide (amino acids 98–111). The same fractions were also assayed by gel mobility shift for RXRE binding. Specific binding activity was found only in the fractions containing Rex protein. The concentration of Rex in this extract was still low as judged from Coomassie blue-stained gels. This enriched Rex preparation was used for the *in vitro* binding assays.

***In Vitro* RNA Synthesis.** pGEM-RRE330 contains the intact RRE (nt 7266–7595 in HXB2R) ligated downstream of the T7 promoter (39). pGEM-DL12s and pGEM-DL345 contain internal deletions of the RRE330, inserted downstream of the T7 promoter (39). These mutations deleted the Rev-responsive and Rex-responsive portions within the RRE, respectively (44). pBS-RXRE contains the RXRE sequence of HTLV-I [nt 8241–8493 (30)], cloned into the *Eco*RV site of pBluescript KS− (Stratagene) after PCR amplification. Transcription of the sense RNA strand (RXREs) is directed by the T7 promoter and that of the antisense RNA strand (RXREas) is directed by the T3 promoter. The different template plasmids carrying RXRE sequences were linearized with *Hin*dIII or *Eco*RI, respectively, and resulted in transcripts of 333 nt (RXREs) and 323 nt (RXREas). [α-^{32}P]UTP-labeled RNA transcripts or unlabeled transcripts for competition experiments were synthesized by using T3 or T7 RNA polymerase under standard conditions (49). The labeled and unlabeled RNAs were analyzed by electrophoresis on denaturing 5% polyacrylamide gels.

RNA–Protein Binding and Gel Mobility Shift Assays. *In vitro* binding experiments were performed in a vol of 20 μl, containing 10 ng of labeled RNA (1–2 × 10^5 cpm) and 0.1–1 μg of the partially purified Rex preparation or ≈1 μg of the purified HIV-1 Rev protein (39). The binding buffer consisted of 6.25 mM Tris·HCl, pH 7.4/325 mM NaCl/0.5% Triton X-100/0.5% sodium deoxycholate/2 μg of yeast RNA (as nonspecific competitor)/2 μg of heparin (unless specified otherwise)/10 mM dithiothreitol/40 units of RNasin (Promega). In competition experiments, 0.01–5 μg of unlabeled RXRE RNA, RRE330 RNA or *E. coli* RNA (Boehringer Mannheim) was included in the reaction mixtures. The binding reaction mixtures were incubated for 15 min at room temperature and immediately subjected to electrophoresis on 4% nondenaturing polyacrylamide gels for 2.5 hr at 140 V and room temperature. The gels were prerun for 1 hr at the same voltage and temperature. Gels were dried and analyzed by autoradiography.

RESULTS

Expression and Purification of Rex. For the *in vitro* analysis of RNA-binding properties of Rex, the *rex* cDNA was inserted in the bacterial expression vector pUC12N and expressed in *E. coli* BU8049. The total lysate was analyzed by immunoblotting with an anti-Rex antiserum (Fig. 1, lane 2) and compared to a bacterial lysate containing only the parental pUC12N vector (lane 1). The expressed polypeptide had the expected size of 27 kDa and migrated with the same electrophoretic mobility as Rex produced in eukaryotic cells (data not shown). Staining of the gel with Coomassie blue revealed that Rex represented <1% of the protein in the crude lysate. Crude *E. coli* lysates were prepared by different procedures (35, 39) and were tested in *in vitro* binding experiments. All crude lysates contained high levels of RNase activity or showed nonspecific binding activity to RNA templates. Therefore, the Rex-containing bacterial lysate was further purified following a protocol similar to the one used previously to purify Rev (39). In contrast to Rev, Rex was found to aggregate with several bacterial proteins, which complicated its purification. We found that sequential carboxymethyl Sepharose, hydroxylapatite, and size-exclusion chromatography yielded a Rex fraction that was free of nonspecific binding and RNase activity (lane 3). This Rex-enriched fraction was used for further binding experiments.

Rex Binds Specifically to RXRE RNA. To examine whether Rex acts by binding to its RNA target, the Rex-enriched fraction was used in an *in vitro* RNA gel mobility shift assay. The RXRE of HTLV-I, contained in a fragment located between the poly(A) signal and the poly(A) site in the 3′ long terminal repeat, was amplified by PCR and inserted into the pBluescript KS− vector. Radiolabeled RNA transcripts containing the RXREs (333 nt) or RXREas (323 nt) orientation (Fig. 2*A*) were incubated with the Rex-enriched fraction. The resulting complexes were analyzed on a 4% nondenaturing gel (Fig. 2*B*). The presence of Rex resulted in the formation of stable complexes with the RXREs RNA that migrated slower than the free RNA probe (lanes 1 and 2). Rex did not bind to RXREas under identical binding conditions (lanes 3 and 4).

Although the RXREs and RXREas probes migrated as single bands during electrophoresis under denaturing conditions, both RXREs and RXREas migrated as two bands under the nondenaturing conditions used here (Fig. 2*B*, lanes 1 and 3). We have previously reported a similar finding with

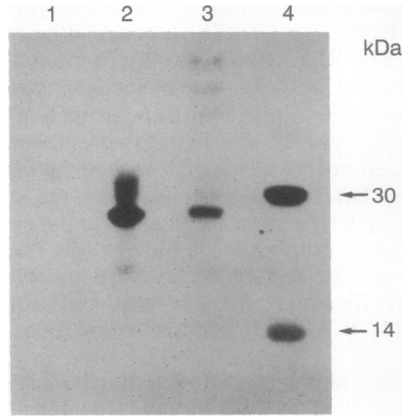

FIG. 1. Western blot analysis of Rex in the crude bacterial lysate and in the partially purified Rex fraction. Lanes: 1, 15 μl of a bacterial lysate containing the parental plasmid pUC12N (≈50 μg of protein); 2, 15 μl of a bacterial lysate containing the Rex-producing plasmid pUC12N-Rex; 3, 15 μg of column-purified Rex preparation; 4, marker proteins.

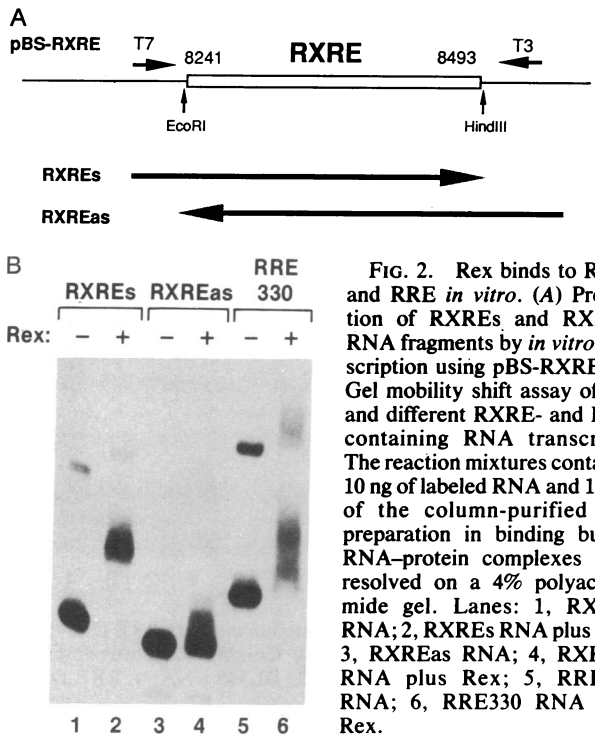

FIG. 2. Rex binds to RXRE and RRE *in vitro*. (*A*) Production of RXREs and RXREas RNA fragments by *in vitro* transcription using pBS-RXRE. (*B*) Gel mobility shift assay of Rex and different RXRE- and RRE-containing RNA transcripts. The reaction mixtures contained 10 ng of labeled RNA and 1.5 μg of the column-purified Rex preparation in binding buffer. RNA–protein complexes were resolved on a 4% polyacrylamide gel. Lanes: 1, RXREs RNA; 2, RXREs RNA plus Rex; 3, RXREas RNA; 4, RXREas RNA plus Rex; 5, RRE330 RNA; 6, RRE330 RNA plus Rex.

different RRE probes (39). Both the RRE and RXRE are proposed to form strong secondary RNA structures (28, 29, 31), which could explain their migration properties in nondenaturing gels.

To further test the specificity of the observed Rex–RXRE interaction, excess amounts of unlabeled RXREs RNA (specific competitor) or *E. coli* rRNA (nonspecific competitor) were included in the binding reaction mixture (Fig. 3*A*). Increasing amounts of unlabeled RXREs RNA effectively competed for binding of the labeled RXREs probe to Rex in a dose-dependent manner (lanes 3–6). A 100-fold excess of unlabeled RXREs completely abolished complex formation (lane 5). In contrast, the presence of increasing amounts of rRNA did not affect the Rex–RXREs complex formation (lanes 7–10). A second nonspecific competitor, total yeast RNA, was also used in the place of rRNA with similar results (data not shown). Thus, the Rex preparation bound only to RXREs RNA, and this binding was not affected by nonspecific competitor RNAs. Therefore, we concluded that Rex binds specifically to its RNA target.

Rex Binds Specifically to the HIV-1 RRE. Although both the RRE and RXRE are RNA regions with strong predicted secondary structures, there is no discernible sequence homology between the two elements. However, it has been demonstrated that Rex can replace the Rev protein of HIV-1 *in vivo* by acting on the RRE (20, 37). Detailed analyses of internal deletions of the RRE have demonstrated that Rev and Rex have different target sites within the RRE (31, 44). These results strongly suggested that there is a direct interaction of Rex with elements in the RRE. To study this interaction *in vitro*, RNA probes containing the complete RRE sequence required for Rex response (RRE330) were generated. *In vitro* binding experiments showed that the RRE330 RNA probe formed a complex with Rex (Fig. 2*B*, lanes 5 and 6). The Rex–RRE330 complexes migrated as a smear, while the Rex–RXRE complexes migrated as distinct bands (compare lanes 2 and 6). This smear could be the result of limiting amounts of Rex, as suggested by subsequent experiments (see Fig. 3*B*, lane 2), in which a higher concentration of Rex was used. This resulted in better resolved bands of Rex–RRE330 complexes.

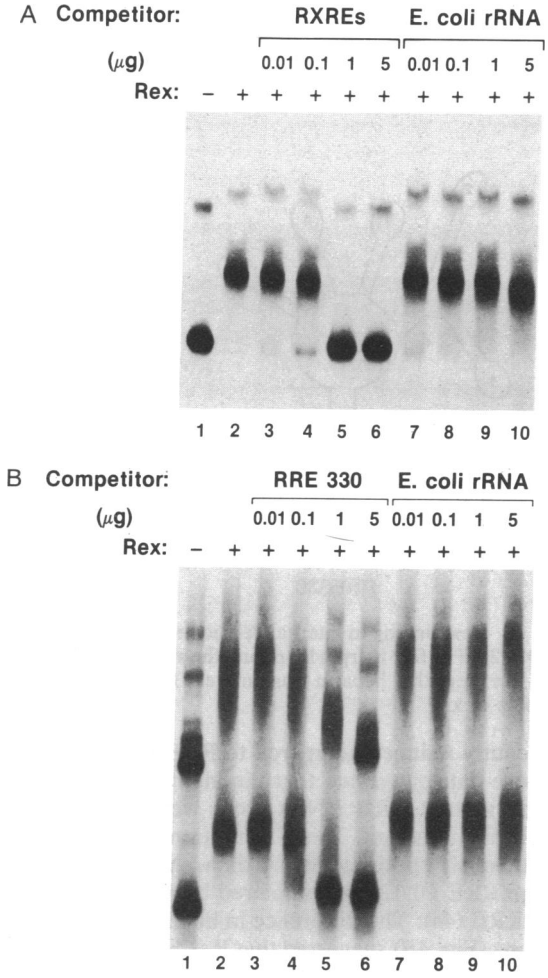

FIG. 3. Binding specificity of Rex to RXRE (*A*) and RRE (*B*) studied by competition analyses *in vitro*. Assay conditions were as described in Fig. 2. (*A*) Lanes: 1, free RXREs RNA; 2–10, RXREs RNA plus Rex preparation; 3–6, competition with 0.01, 0.1, 1.0, and 5.0 μg of unlabeled RXREs RNA, respectively; 7–10, competition with 0.01, 0.1, 1.0, and 5.0 μg of unlabeled rRNA, respectively. (*B*) Lanes: 1, free RRE330 RNA; 2–10, RRE330 RNA plus Rex; 3–6, competition with 0.01, 0.1, 1.0, and 5.0 μg of unlabeled RRE330 RNA, respectively; 7–10, competition with 0.01, 0.1, 1.0, and 5.0 μg of unlabeled rRNA, respectively. In this experiment the Rex concentration was 2-fold higher compared to Fig. 2.

The specificity of the Rex–RRE interaction was further tested in an *in vitro* competition experiment (Fig. 3*B*). While the presence of increasing amounts of unlabeled RRE330 RNA competed efficiently for complex formation (lanes 3–6), the presence of increasing amounts of rRNA did not affect complex formation (lanes 7–10). These results demonstrated the specificity of the Rex–RRE interaction.

To further study the Rex–RRE interaction, two internal deletion mutants, RRE DL12s and RRE DL345, were tested for binding (Fig. 4). RRE DL12s contains a deletion of hairpin loops 1 and 2 and the short stem, which are essential for Rev function *in vivo* (Fig. 4*A*). RRE DL345 contains a deletion of hairpin loops 3, 4, and 5, which are essential for Rex function *in vivo* (Fig. 4*A*). Binding experiments performed under the same conditions as described for the intact RRE330 RNA revealed markedly reduced binding affinity of Rex to both RNA targets (data not shown). Complex formation between Rex and the RRE DL12s RNA could be visualized only by omitting the heparin in the binding reaction mixture (Fig. 4*B*, lanes 1 and 2). Under the same conditions, the complex formation between Rex and the RRE DL345 RNA was

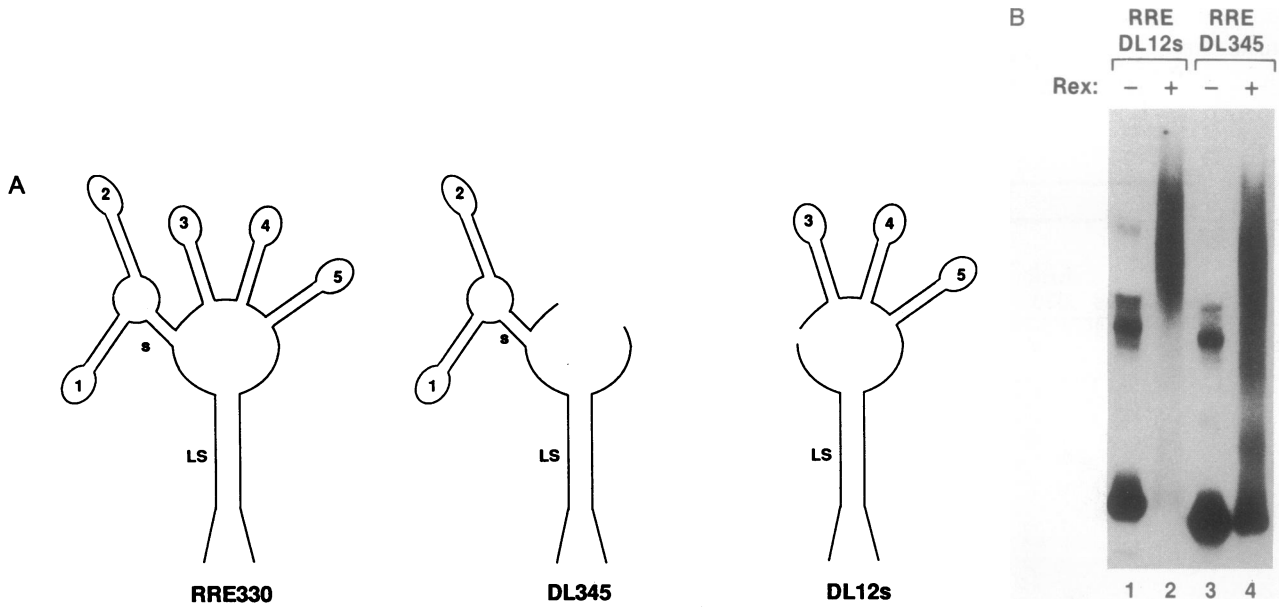

FIG. 4. Rex binding to internal deletion mutants of HIV-1 RRE. (*A*) Secondary structures of the RRE and deletion mutants RRE DL345 and RRE DL12s. The five hairpin loops and the short (s) and long (LS) stems are indicated. (*B*) Gel mobility shift assay. Conditions were as described in Fig. 2, except that heparin was omitted. Lanes: 1, RRE DL12s RNA; 2, RRE DL12s RNA plus Rex; 3, RRE DL345 RNA; 4, RRE DL345 RNA plus Rex.

significantly reduced compared to RRE DL12s (lanes 3 and 4). These data suggested that the interactions between Rex and the mutant RRE RNAs were weak compared to the Rex–RRE interaction. This observation is in agreement with our previously published *in vivo* data, showing that activation through RRE DL12s was impaired by 40% compared to the intact RRE (44). The difference in binding of Rex to the two deletions (Fig. 4*B*) suggested that Rex binds preferentially to an RNA target within the RRE similar to the one revealed by the *in vivo* experiments.

HIV-1 Rev Does Not Bind to RXRE RNA. The functions of Rev and Rex are not reciprocal (20, 21, 37). While Rex of HTLV-I acts on both HTLV-I and HIV-1, Rev does not act on the HTLV-I RXRE. The most probable explanation for this finding is that Rev fails to bind to the RXRE. To confirm this hypothesis, the purified Rev protein was incubated with the labeled RXREs RNA transcript using the binding conditions that resulted in specific complex formation between the RXREs and Rex (see Fig. 2*B*). The gel mobility shift assay revealed no complex formation between the Rev and RXREs RNA (Fig. 5, lanes 3 and 4) while the same Rev preparation bound specifically to RRE (lanes 1 and 2), as also demonstrated previously (39). These data agree with the *in vivo* findings and support the conclusion that interaction of Rev with its RNA target is necessary for function.

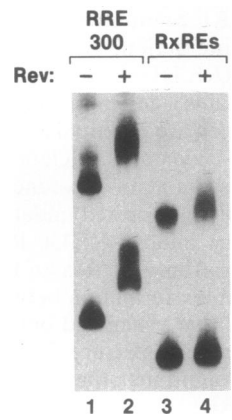

FIG. 5. Rev does not bind to RXRE *in vitro*. Assay conditions were as described in Fig. 2. Lanes: 1, RRE330 RNA; 2, RRE330 plus purified HIV-1 Rev; 3, RXREs RNA; 4, RXREs RNA plus purified HIV-1 Rev.

DISCUSSION

In vitro binding experiments using gel mobility shift analysis to detect protein–RNA complex formation revealed that Rex binds specifically to RXRE and to RRE. Two types of highly structured RNA molecules (tRNA and rRNA) were used to test the specificity of interaction of Rex with its RNA targets. The interaction of Rex to the RXREs and RRE RNAs was not affected by the presence of either tRNA (present in all reactions) or rRNA (used in the competition experiments shown in Fig. 3). The formation of specific protein–RNA complexes was inhibited only in the presence of specific competitor RNAs such as RXREs and RRE330.

In contrast, Rev was shown not to bind to RXRE *in vitro*. This observation is in agreement with the inability of Rev to activate expression from RXRE-containing RNAs *in vivo* (20, 21). Therefore, the *in vitro* binding conditions used here reflect to a large extent the *in vivo* specificities of these factors. RNAs found to be nonfunctional *in vivo* did not form strong complexes *in vitro*. These experiments suggest that specific high-affinity binding of the activators to their RNA targets is essential for function.

Heparin has been shown to be an effective nonspecific competitor for binding to RNA by reducing specific as well as nonspecific protein–RNA interactions (50). The presence of heparin was required to demonstrate specific binding of Rex to RXREs in our *in vitro* binding assay. The presence of heparin also reduced the binding of Rex to the intact RRE330 RNA. These results suggested that Rex has a higher affinity for the RXRE than for the RRE. *In vivo* studies have shown that Rex can activate expression from RXRE-containing mRNAs to a greater extent than from RRE-containing mRNAs, which correlates well with the binding activities. Rex activates expression of RRE-containing mRNAs ≈23-fold, while it activates RXRE-containing RNAs 100- to 200-fold (ref. 44; V. Ciminale, G.N.P., and B.K.F., unpublished data). These studies demonstrated that the activation of the internal RRE deletion mutants by Rex is further impaired [i.e., 14-fold activation of DL12s by Rex compared to 23-fold activation of RRE330 (44)]. The presence of heparin in the *in vitro* binding experiment using RRE DL12s RNA completely abolished complex formation, suggesting

weaker binding affinity to the deletion mutant. Omission of heparin allowed efficient complex formation. Interestingly, in the absence of heparin the mutant RRE DL345 RNA, which is not activated by Rex *in vivo* (44), allowed a low extent of complex formation *in vitro*. The residual binding to RRE DL345 may be the result of weak specific or nonspecific interactions. As shown previously (44), the activation of RRE-containing RNAs by Rex requires an extended long stem and flanking sequences, in addition to hairpin loops 3, 4, and 5. We had speculated that these sequences could contribute to the stabilization of the RRE structure. Alternatively, these sequences could contain sequence-specific elements that form part of the binding site with Rex. However, the putative binding of Rex to these sequences in the context of RRE DL345 RNA does not result in functional RNA–protein complexes *in vivo*.

Analysis of binding of two different Rev trans-dominant mutants to RRE RNA has demonstrated that binding alone is not sufficient for Rev function *in vivo* (35, 39). Experiments with the trans-dominant mutant RevBL suggested that it was unable to perform a step that closely followed formation of the RNA–protein complex and preceded Rev-dependent rescue from splicing and degradation pathways (39). Several lines of evidence strongly indicate that this step involves interaction of the Rev–RRE complex with cellular factors. Similarly, mutagenesis of the Rex protein has revealed mutants displaying a trans-dominant phenotype (36). By analogy to Rev, these mutants are thought to interfere with Rex function by binding to the RXRE, which would result in the formation of an inactive RNA–protein complex.

The results presented here demonstrate direct and specific binding of Rex to specific RNA targets *in vitro*. Thus, both Rex and Rev proteins interact directly with their RNA targets. The similar properties of Rev and Rex *in vivo* and *in vitro* strongly suggest that regulation by Rev and Rex involves similar cellular pathways. It is therefore anticipated that cellular factors will be necessary for Rex-mediated function. It remains to be determined whether the cellular factors involved in the Rex and Rev activation are the same.

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