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Human Immunodeficiency Virus Rev Protein Recognizes a Target Sequence in Rev-Responsive Element RNA within the Context of RNA Secondary Structure

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Human immunodeficiency virus type 1 Rev protein modulates the distribution of viral mRNAs from the nucleus to the cytoplasm by interaction with a highly structured viral RNA sequence, the Rev-responsive element (RRE). To identify the minimal functional elements of RRE, we evaluated mutant RREs for Rev binding in vitro and Rev response in vivo in the context of a Gag expression plasmid. The critical functional elements fold into a structure composed of a stem-loop A, formed by the ends of the RRE, joined to a branched stem-loop B/B1/B2, between bases 49 and 113. The 5′ 132 nucleotides of RRE, RREDDE, which possessed a similar structure, bound Rev efficiently but were nonfunctional in vivo, implying separate binding and functional domains within the RRE. Excision of stem-loop A reduced Rev binding significantly and abolished the in vivo Rev response. The B2 branch could be removed without severe impairment of binding, but deletions in the B1 branch significantly reduced binding and function. However, deletion of 12 nucleotides, including the 5′ strand of stem B, abolished both binding and function, while excision of the 3′ strand of stem B only reduced them. Maintenance of the native RRE secondary structure alone was not sufficient for Rev recognition. Many mutations that altered the primary structure of the critical region while preserving the original RNA conformation were Rev responsive. However, mutations that changed a 5′ . . CACUAUGGG . . 3′ sequence in the B stem, without affecting the overall structure abolished both the in vitro Rev binding and the in vivo Rev response.

Human immunodeficiency virus type 1 (HIV-1) has at least two novel genetic regulatory mechanisms which are mediated by the interactions of two conserved RNA elements, the *trans*-acting-responsive (TAR) region and the Rev-responsive element (RRE), with the viral *trans*-activator proteins, Tat and Rev, respectively (5, 7, 12, 16, 19, 21, 27, 39). The 116-residue Rev protein, conserved in all HIV and simian immunodeficiency virus isolates, is a nuclear protein which is obligatory for the expession of viral structural proteins (2, 13, 19, 38). The functional target of Rev is a 244-nucleotide (nt) mRNA sequence referred to as the RRE in the *env* open reading frame at the junction of the gp120 and gp41 domains. In the absence of Rev, transport of RRE-containing RNAs from the nucleus to the cytoplasm is blocked; the RRE is either spliced out or the RNA is degraded (16, 17, 19, 21, 27). The RRE sequence is not highly conserved, and RREs from different primate immunodeficiency viruses can be functionally interchanged to a limited extent (25). The HIV-1 RRE can also be *trans* activated in a nonreciprocal manner by the Rex protein of the unrelated human retrovirus human T-cell lymphotropic virus type I (18, 22, 36).

The Zuker RNA suboptimal folding program (41, 44, 45) predicts a preferred secondary structure for the RRE consisting of four distinct stem-loops (A, C, D, and E) and one branched stem-loop (B/B1/B2) surrounding a central single-stranded bubble (27) (Fig. 1A). Genetic and molecular studies have identified distinct RNA-binding and functional domains within Rev (26). The RRE has been shown to bind directly to purified Rev expressed in *Escherichia coli* (11, 14, 43). Mutational analyses of the RRE have suggested that Rev function requires the presence of a core element composed of the 5′ stem-loop A linked to the branched stem-loop B/B1/B2 (15, 28, 33). It has further been suggested that the B/B1/B2 branched stem-loop is necessary and sufficient for binding Rev in the absence of other structures (28). These studies have indicated the region of RRE-Rev interaction but have not clarified the primacy of RNA sequence versus structure for the interaction. In addition, there is disagreement about the relative importance of the B1 or B2 stem-loop for the Rev response (15, 33).

RNA-protein interactions are fundamental to many biological processes (6, 24, 29, 34). Studies of the roles of the primary and secondary structure of RNA in the Rev-RRE interaction may serve as a paradigm for biochemical mechanisms related to mRNA splicing and transport. We report here that both the in vitro Rev binding and the biological response are dependent on the presence of at least one sequence of 12 nt within an RNA secondary structural context. These studies may help to define the critical elements of Rev-RRE interactions and may facilitate strategies to interfere with HIV-1 replication.

MATERIALS AND METHODS

Purification of HIV-1 Rev expressed in *E. coli*. HIV-1 Rev was expressed in *E. coli* from the thermally inducible coliphage 1 p_L promoter. The *rev* gene was constructed by fusing a chemically synthesized DNA fragment encoding the first 30 residues to an *AvaI-RsaI* fragment (positions 7970 to 8297 in the pNL432 provirus [1]) which encoded the C-terminal 86 residues of Rev. The *rev* fragment was exchanged in

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place of DNA encoding the Ner protein in plasmid pL-ner (4) to generate pL-Rev, which was used to transform *E. coli* MM294 (F⁻ *endA1 hsdR hsdM supE44 thi-1* λ⁻). The Rev protein was recovered by urea extraction (4) and purified to homogeneity by successive column chromatographic procedures. Purity was checked by amino acid composition and automated N-terminal sequencing. The purified protein migrated as a single species of 16 kDa under denaturing conditions. At neutral pH, Rev oligomerized to a stable tetramer at a concentration of ~1 mg/ml. Details of *E. coli* expression and biophysical studies will be described elsewhere.

Construction of RRE mutants. The 244-bp RRE sequence in the proviral plasmid pNL432 (positions 7749 to 7992) was amplified by the polymerase chain reaction (PCR) (30), using tagged DNA primers corresponding to the 20 nt at the 5′ and 3′ ends of the RRE, respectively. The primers were tagged with *Asp*I-*Eco*RI-*Xho*I (5′-3′) restriction sites. The PCR-amplified 292-bp RRE fragment was cloned into the lone *Asp*I site of the HIV-1 Gag reporter plasmid, pRMK4 (see below). The cloned RRE fragment was further amplified with primer pairs corresponding to the ends of the RRE, except that the 5′ primer was tagged with the bacteriophage T7 RNA polymerase promoter. The T7 promoter-tagged RRE fragment was used to synthesize T7 transcripts. The RRE mutants (Fig. 1) were generated by a two-step PCR amplification with mutant primer pairs. Initially, 5′ and 3′ half molecules extending from the mutated site were synthesized. In the second reaction, the mutated half molecules were reamplified with restriction site-tagged primer pairs corresponding to the ends of the mutant RREs. The mutant PCR fragments were inserted into the Gag reporter plasmid, pRMK4 (see below). The recombinants were verified by DNA sequencing. T7 promoter-tagged mutant RREs were then generated by PCR with a T7 promoter-tagged 5′ primer. 3′-Truncated RRE DNA fragments were generated by PCR, using primers corresponding to the 3′ ends of the respective truncations with T7 promoter-tagged 5′ primers starting at the 5′ end of the RRE. B/B1/B2 stem-loop RRE fragments were also generated by PCR except that the 5′ fragment was tagged with a T7 promoter upstream of position 48 and the 3′ fragment included a synthetic *Alu*I site at position 116. The PCR fragments were cleaved with *Alu*I and gel purified before T7 transcription.

In vitro RNA synthesis and structure determination. T7 promoter-tagged PCR-amplified RRE DNA fragments (0.5 pmol) were used as templates to generate unlabeled or uniformly labeled ([α-^{32}P]UMP) RRE transcripts by using a commercial kit from Promega Biotec (Milwaukee, Wis.). 5′-End-labeled RNAs were synthesized in a 25-μl reaction mixture with [γ-^{32}P]GTP (12.5 mM, 400 Ci/mmol) and 0.2 pmol of DNA template. For 3′ end labeling, 1 pmol of the respective RRE RNAs was labeled with [^{32}P]pCP (3′,5′-cytidine bisphosphate; Amersham Corp.) with T4 RNA ligase according to the manufacturer (Pharmacia). All RNAs were purified by gel filtration on Sephadex G-100 columns. Purity was checked by electrophoresis on urea-acrylamide (10%) gels. Sequencing of the end-labeled RNAs with base-specific nucleases (RNase T_1, U2, Phy M, *Bacillus cereus*) was done according to a commercial protocol (Pharmacia). The secondary structure of selected RRE RNAs was determined by partial nuclease digestions with the above enzymes and the double-strand-specific V1 RNase at neutral pH under native (0.2 M NaCl) or denatured (7 M urea) conditions. RNase digestion products were resolved by urea-acrylamide (8, 12, and 20%) sequencing gels.

Nitrocellulose filter binding assay. Filter binding assays were done essentially by the method of Riggs et al. (35). Typically, 20 to 50 fmol of the labeled RRE RNAs was incubated at 25°C for 10 min in 10 μl of binding buffer (20 mM Tris hydrochloride [pH 7.5], 0.05 M KCl, 1 mM dithiothreitol, 5 mM spermidine, 20 μg of bovine serum albumin, 1 μg of yeast tRNA) with increasing concentrations of purified Rev. The samples were filtered through prewetted 25-mm nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell) at a flow rate of 5 μl/s. Filters were rinsed twice with 0.75 ml of binding buffer (without bovine serum albumin, spermidine, or tRNA) and air dried, and the bound radioactivity was determined. From the saturation curves of RRE-Rev binding, the fractions of free and bound Rev were computed and used to determine the average K_d of the wild-type (wt) RRE-Rev interaction, which was on the order of $\sim 5 \times 10^{-9}$ M. The RRE-Rev ratio at the saturation breakpoint was used to determine the stoichiometry of the reaction.

RNA gel mobility retardation assay. Uniformly labeled RRE RNAs (0.2 to 0.5 pmol) were incubated for 10 min at 25°C with constant amounts of purified HIV-1 Rev protein in 10 μl of binding buffer as above except that 40 μg of tRNA was used. Samples were electrophoresed through 4% polyacrylamide gels (cross-linked with 0.1% bisacrylamide) under native conditions in a buffer containing 16.5 mM Tris-borate (pH 8.3) and 0.4 mM EDTA for 1 to 2 h at 4°C at 350 V. The radioactive bands were visualized by dry-gel autoradiography.

Transient expression assay. To measure the Rev response, we constructed a *gag* expression plasmid, pRMK4, by excising the sequence between the *Asp*I sites at positions 3823 and 9003 of the infectious proviral plasmid pNL4-3 (1). *Asp*I-cleaved PCR DNAs corresponding to the wt or mutant RREs were then inserted between the above *Asp*I sites of pRMK4. HeLa cell lines constitutively coexpressing HIV-1 Tat and Rev were generated by electroporating HIV long terminal repeat-linked Tat and Rev expression plasmids (3) with pRSV-Neo. Clones were selected for neomycin resistance conferred by the expression of pRSV-Neo. Neomycin-resistant cell lines were then evaluated for Rev expression by their ability to support virus production from a Rev-defective provirus (2). Individual *gag* plasmids (5 μg) were coelectroporated with pHIV-Cat (2 μg) at 250 V into the cell lines expressing both Tat and Rev (Bio-Rad). Cells were harvested after 48 h and disrupted by three freeze-thaw cycles, and the extracts were clarified by low-speed centrifugation. *gag* expression was monitored by immunoblotting (40) and quantitated by p24 enzyme-linked immunosorbent assay (ELISA) of cell extracts and clarified culture fluid (Coulter Diagnostics). $p24^{gag}$ expression levels were normalized to constant values of chloramphenicol acetyltransferase activity expressed from HIV long terminal repeat-linked *cat* (2, 20). None of the Rev-responsive RRE mutants exhibited any disparity in the ratios of cell-associated and secreted Gag. Each value represented a mean from six or eight independent transfections with multiple preparations of plasmid DNA.

RESULTS

Construction of RRE mutants. The RRE RNA associated with the pNL4-3 HIV-1 virus differed from the HXB2 RRE at positions 106 (A→G), 181 (C→U), and 184 (G→A) but folded into a thermodynamically favored structure similar to that reported previously (27). Base-specific partial RNase

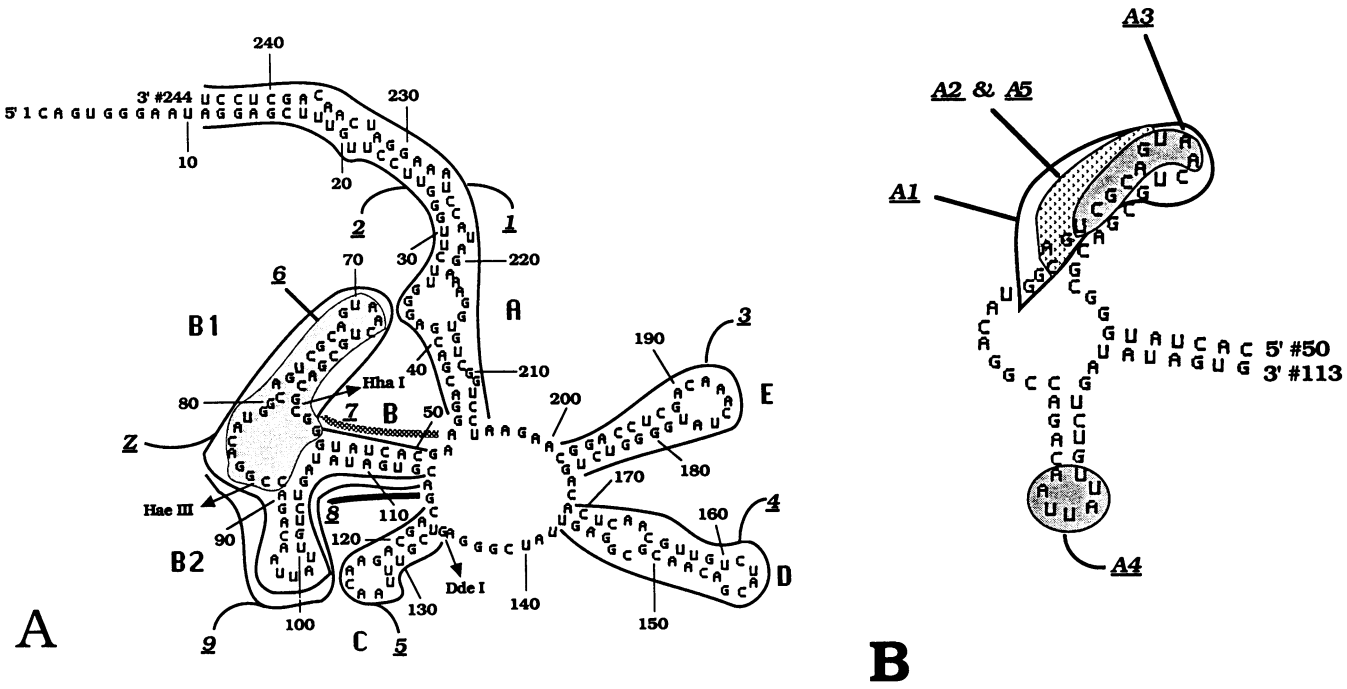

FIG. 1. Illustrations of the RRE structure and deletions used in this study. Sequence coordinates for the deletions indicated are given in Table 1. (A) Secondary structure prediction of the 244-nt RRE. The individual stem-loops are labeled A to E, respectively, and specific restriction endonuclease cleavage sites are indicated. Deletions RRE1 to RREZ, designed to disrupt or excise individual stem-loops, are indicated by the respective curves wrapping the RRE structure. Mutants that removed more than one stem-loop (e.g., RRE34 lacking stem-loops D and E) or excised stem-loop A (RRE12) are described in the text. (B) Branched stem-loop B/B1/B2 (nt 50 to 113). The enclosed areas indicate the deletions made.

digestions of the RRE RNA were performed. We did not demonstrate the singly bulged bases C237, U233, A227, U222, A218, and A217 in the A stem and A78 in the B1 stem, and we did find G220 in an unpaired state. The remainder of the structures, in particular the B stem and the B1 stem-loop, were confirmed under native conditions to be as predicted (data not shown).

Mutations were engineered to specifically remove the individual stems and/or loops (designated A to E in Fig. 1A). Deletions RRE1 through RREZ, designed to excise or disrupt the individual stem-loops, are also illustrated in Fig. 1A. Figure 1B shows the coordinates of deletions (RREA1 through RREA5) limited to the branched stem-loop, B/B1/B2. Every mutant RRE used in this study was analyzed by the suboptimal RNA folding algorithm of Zuker (41, 44, 45). The structures of selected mutants were additionally verified by RNase digestions. Mutant RRE RNAs were evaluated for ribonucleoprotein (RNP) formation by gel mobility retardation and filter binding experiments. To correctly evaluate the relative affinities of mutant RREs for Rev by gel retardation, we deliberately chose Rev concentrations which gave >50% saturation of wt RRE in filter binding assays. Mutants were also transferred to a *gag* reporter plasmid to measure their Rev response in vivo.

In vitro Rev binding and in vivo Rev response of major stem-loop deletions of RRE. RRE mutants (RRE1 through RREZ; Fig. 1A) could be grouped into three sets according to Rev binding results: those that bound almost as well as wt RRE, those that did not bind, and a group exhibiting intermediate levels of activity. Under the conditions used for binding, the wt RRE transcript bound Rev at a stoichiometric ratio of 1:8 with an apparent K_d of $\sim 5 \times 10^{-9}$ M. Antisense RRE RNA (ERR) and HIV-1 TAR RNA were used as negative controls. Under the conditions used for filter binding, less than 10% of these two RNAs was bound by Rev even at a 200-fold molar excess (10 fmol of RNA versus 2,000 fmol of Rev). Removal of stem-loop C, D, or E (RRE5, RRE4, RRE3) only resulted in marginal reduction in Rev RNP formation by filter binding or gel retardation (Fig. 2A and B). These mutants also competed efficiently with wt RRE for Rev binding (data not shown). Rev-dependent *gag* expression from this group of mutant RRE plasmids was slightly less than that of wt RRE (Table 1). Although the separate excision of the three 3′ RRE loops was well tolerated, their combined excision (e.g., stem-loops D and E, RRE34) resulted in greater than 50% reduction in binding and in vivo response (Table 1).

In contrast to the above mutants, deletions that eliminated the entire branched stem-loop B/B1/B2 (RREZ), included the B1 branch (RRE6), or disrupted stem B (RRE7) were severely impaired for in vitro Rev binding (Fig. 2A and B) and were not responsive to Rev in vivo (Table 1). These mutated RRE elements failed to compete with wt RRE for Rev binding even at a 20-fold molar excess (data not shown).

A third set of deletions was intermediate in Rev binding. Deletions which excised the 3′ strand of stem B (RRE8) or stem-loop B2 and the 3′ strand of stem B (RRE9) were ~20% as efficient as wt RRE in RNP formation (Fig. 2A and B) and in vivo Rev response (Table 1). Rev recognition of mutants that had lost either the 3′ strand of stem-loop A (RRE1) or both strands of stem-loop A (RRE12) was more severely impaired and could not be readily visualized by gel retardation (data not shown). The mutant RRE2, which lacked the 5′ strand of stem-loop A, was better in this regard and was able to complex with Rev in a gel retardation assay (Fig. 2B). When the RNP formation was evaluated by a filter binding

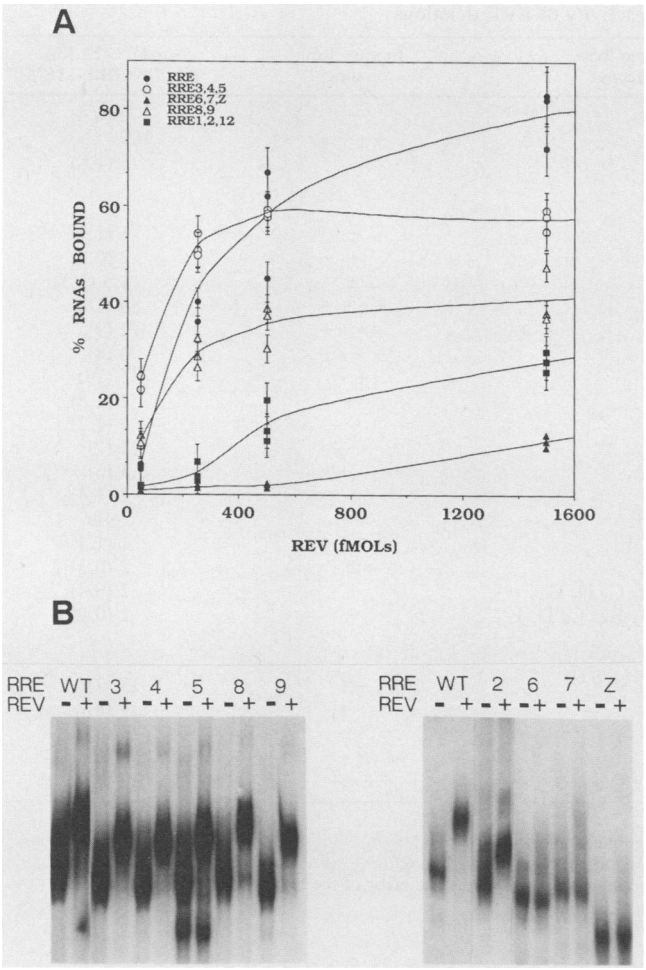

FIG. 2. Rev binding to major RRE deletion mutants. (A) Results of filter binding assays with the three classes of major RRE deletions described in the text and shown in Fig. 1A. Fixed amounts (5×10^{-9} M) of uniformly labeled mutant or wt RRE were incubated with increasing concentrations of Rev to obtain saturation breakpoints. Each point was determined in triplicate, and each mutant RRE was assayed three times. Each plot portrays the Rev binding representative of the individual class. The members of each class are identified next to the respective plot symbol. Rev binding data for the members in each class did not vary by more than the standard deviation for any one member. (B) Results of gel shift assays with most of the above mutants. For this assay, 0.5 pmol of each mutant RRE was incubated with 10 pmol of Rev. Under these conditions, all the wt RRE was converted to RNP. Each mutant RNA was electrophoresed pairwise with (+) or without (−) Rev. The lane pairs are identified at the top by the named mutants.

assay, all three stem-loop A mutants (RRE1, RRE2, RRE12) were less efficient than RRE8 and RRE9 (Fig. 2A). Stem-loop A mutants RRE1 and RRE12 were also negative for Rev-dependent *gag* expression (see Fig. 4). These gel retardation, filter binding and *gag* expression results suggested that the minimal functional unit of RRE should include the stem-loop A and the branched stem-loop B/B1/B2.

Rev response of deletions in B1 and B2 branches. To identify the putative Rev recognition elements in the B1 and B2 branches of stem-loop B, we studied the behavior of five discrete deletions in these regions (Fig. 1B and Table 1). Excision of the small loop at the end of B2 (RREA4) reduced in vitro filter binding only slightly (Fig. 3A). However, this RRE mutant shifted poorly in the gel retardation assay, requiring more Rev than wt RRE (Fig. 3B). In vivo, the Rev response of a plasmid containing the RREA4 mutation was only 20% compared with wt (Table 1).

The Rev binding of the B1 stem-loop mutants (RREA1, RREA2, RREA3, and RREA5) was even more impaired; they behaved like the intermediate group of RRE mutants in filter binding assays (Fig. 3A). Removal of most of the B1 stem (RREA1) significantly reduced binding as monitored by gel retardation, requiring significantly more Rev than wt RRE to effect only partial shift (Fig. 3B). Among the B1 mutants, excision of the terminal loop (RREA3) had the least effect in gel retardation assays (Fig. 3B). The two remaining B1 deletion mutants (RREA2 and RREA5) shifted poorly at low Rev concentrations; but at a twofold-higher concentration of Rev, some bound Rev (Fig. 3B). *gag* expression from all four B1 stem-loop lesions was significantly reduced (Table 1).

Rev recognition of 3′-truncated RREs. Since stem-loops C, D, and E could be excised without severely affecting either binding or activation, we examined whether 3′-truncated RREs which retained the branched stem-loop B/B1/B2 were Rev responsive. Three 3′-truncated RRE fragments were studied: a 132-nt fragment, RREDDE (extending up to the *Dde*I site at position 132 of RRE); an 89-nt fragment, RREHAE (shortened to the *Hae*III site); and a 58-nt fragment, RREHHA (trimmed to the *Hha*I site; Fig. 1A). The 132-nt RREDDE RNA was predicted to fold into a structure that preserved the branched stem-loop B/B1/B2 and stem-loop C of wt RRE but with a modified branched stem A. The modified branched stem A consisted of two hairpin structures formed between nt 1 and 49 of the RRE. The distal (3′) hairpin of the RREDDE branched stem A was immediately adjacent to the B/B1/B2 stem-loop, forming a structure similar to wt RRE. RREDDE bound Rev as well as wt RRE in filter binding (Fig. 3C) and gel retardation (data not shown) assays. In RNA competition experiments, RREDDE exhibited four times more affinity for Rev than wt RRE (Fig. 3D). RREHAE RNA, which folded into a structure lacking the B2 branch of the B/B1/B2 stem-loop, bound Rev less efficiently (Fig. 3C). The 58-nt RREHHA RNA structure lacked the B/B1/B2 stem-loop and was not recognized by Rev (Fig. 3C). Rev binding of RREDDE was reduced when a 34-nt deletion, equivalent to the RRE2 deletion (Fig. 1), excised the modified stem-loop A (RRE2DDE, Fig. 3C). Rev also bound the B/B1/B2 stem-loop RNA (see below) but with a lower affinity than that for RREDDE. Although RREDDE interacted with Rev by several criteria in vitro, *gag* reporter plasmids containing RREDDE were consistently unresponsive to Rev in transfection assays (Table 1).

Rev recognition of structure-disrupting and structure-preserving base substitutions in B/B1/B2 stem-loops. The above experiments localized the stem-loops B/B1/B2 as the critical subdomain required for in vitro Rev binding. Therefore, we analyzed the effects of base substitutions within this domain of the RRE RNA using the mutants described in Table 2. Uncompensated mutations that altered the sequences of stem B (RRE J23ACG, RRE X23ACG, and RRE C41) or stem-loop B2 (RRE D23 and RRE Z23) disrupted the predicted RRE structure in the respective regions. However, mutations that altered the 5′ . . GGG . . 3′ sequence beyond the 5′ strand of stem B (RRE 3G3C and RRE 3G3U) had no effect on the predicted folding in this region. Base substitutions in the B2 stem (RRE D23 and RRE Z23) resulted in modest decreases in the in vitro Rev binding (Fig. 4A) but

TABLE 1. In vitro and in vivo activity of RRE deletions

RRE mutant[a]	Sequence coordinates	Stem-loop affected[b]	In vitro Rev binding[c]	p24^{gag} ELISA (% of wt RRE [SEM])[d]
Mock			−	2 (0.4)
wt RRE	1–244	NA[e]	++++	100
ERR, reverse RRE	244–1	NA	−	3 (1.8)
Major deletions				
RRE1	Δ207–244	A	±	3 (1.2)
RRE2	Δ11–44	A	+	2 (0.8)
RRE12	Δ11–44 + Δ207–244	A	±	1.5 (1.1)
RRE3	Δ174–199	E	++++	80 (18)
RRE4	Δ144–170	D	++++	72 (9)
RRE5	Δ117–134	C	++++	90 (8)
RRE34	Δ144–170 + Δ174–199	D, E	++	35 (13)
RRE6	Δ57–89	B1	−	2 (1.8)
RRE7ACG	Δ47–58	B	−	3 (1.2)
RRE8	Δ107–113	B	+++	22 (7)
RRE9	Δ90–114	B	++	15 (6)
RRE Z	Δ49–113	B/B1/B2	−	2 (1.4)
RRE B/B1/B2	Δ1–49 + Δ117–244	A, C, D, E	++	ND[f]
RREDDE	Δ132–244	A (3′), D, E	++++	1 (1.1)
RRE2DDE	Δ11–44 + Δ132–244	A, D, E	±	2 (0.3)
RREHAE	Δ87–244	A (3′), B2, C, D, E	+	2 (0.4)
RREHHA	Δ61–244	A (3′), B1, B2, C, D, E	−	1 (0.2)
B/B1/B2 deletions				
RREA1ACG	Δ65–81	B1	++	13 (7)
RREA2ACG	Δ72–79	B1	++	15 (8)
RREA3ACG	Δ68–76	B1	++	15 (8)
RREA5ACG	Δ71–78	B1	++	14 (5)
RREA4	Δ94–100	B2	+++	20 (10)

[a] All RRE sequences are 5′..(62)AGC(64)..3′ except where noted as ACG.
[b] The stem-loops of the RRE structure (Fig. 1) disrupted by the deletions are identified.
[c] The results of RRE RNA filter binding and gel mobility shifts are symbolically denoted from − (no binding) to ++++ (wt binding).
[d] The normalized p24^{gag} ELISA values represent averages from six or eight experiments with the standard error of the mean shown in parentheses.
[e] NA, Not applicable.
[f] ND, Not done.

marked reductions in the in vivo Rev response (Table 2). In contrast, mutations that altered the six bases of stem B (RRE J23ACG, RRE X23ACG, and RRE C41) or the three G residues at positions 56 to 58 (3G3U) resulted in complete loss of in vitro Rev binding (Fig. 4) and Rev response in transfections (Table 2). It is of interest that the 9-base sequence 5′ . . CACUAUGGG . . 3′ altered in the stem B mutants forms a subset of the 12 nt deleted in RRE7, which was also unresponsive to Rev both in vitro and in vivo. Because the sequence of the RRE B1 region is somewhat heterogeneous in the 5′ . . (56)GGGCGCAGC(64) . . 3′ tract, with the 3′ AGC (underlined) replaced by ACG in some isolates, we constructed several mutants with either AGC or ACG in this position (Tables 1 and 2; all constructs are AGC except where noted). Changes in this site could influence stem-loop B1 structure or the unpaired conditions of the guanine residues at positions 57 and 58.

To evaluate the role of primary versus secondary structure of the B/B1/B2 stem-loops, we introduced base substitutions into both strands of the respective stems such that the secondary structure was preserved. The location and composition of these mutants are given in Table 2. In the 23X RRE mutants, the two strands of wt stem B were exchanged, while in the 23J RRE mutants, the strands were exchanged and inverted. In 41C RRE, a 5′ . . CCCGGG . . 3′ *Sma*I site and its complement were substituted for the wt sequence of stem B. The two strands of the B1 stem were also exchanged and inverted with respect to each other to generate the compensatory mutant 23B RRE. Structure-compensated stem B2 mutants included inversion of the 5′ and 3′ strands as in 23Z RRE and substitution of a base-paired 5′ . . CCGC . . 3′ sequence in 23D RRE (Table 2). The predicted structures of 23D RRE and 23Z RRE were verified by nuclease analysis to correspond to the wt RRE structure. The compensatory mutants were evaluated for in vitro Rev binding and Rev-dependent *gag* expression in vivo. All the stem B compensatory mutants, 23J RREACG, 23J RREAGC, 23X RREACG, 23X RREAGC, and 41C RRE, lost in vitro Rev binding (Fig. 4) and in vivo Rev response (Table 2). In contrast, structure-preserving mutations of stem B2 (23D RRE and 23Z RRE) were indistinguishable from wt RRE in binding studies (Fig. 4B). In transfections, 23Z RRE was consistently better than wt RRE in Rev response, while 23D RRE was somewhat less (Table 2). The structure-compensated B1 stem mutant, 23B RRE, had intermediate levels of Rev binding (Fig. 4B) and moderately reduced in vivo Rev response (Table 2).

The above studies suggested that the primary structure of the B stem was crucial for Rev recognition. However, it was possible that the changes in the B stem destabilized the overall secondary structure of RRE and caused a loss of Rev binding. To examine this possibility, we evaluated the Rev binding of *Dde*I fragments corresponding to the 5′ 132 nt of wt, RRE X23ACG, and 23X RREACG RNAs. Neither the uncompensated 6-nt substitution (RRE X23DDE) nor the compensated substitution (23X RREDDE) was recognized by Rev in vitro, under conditions in which wt RREDDE was readily bound (Fig. 5A). We then examined the Rev-binding

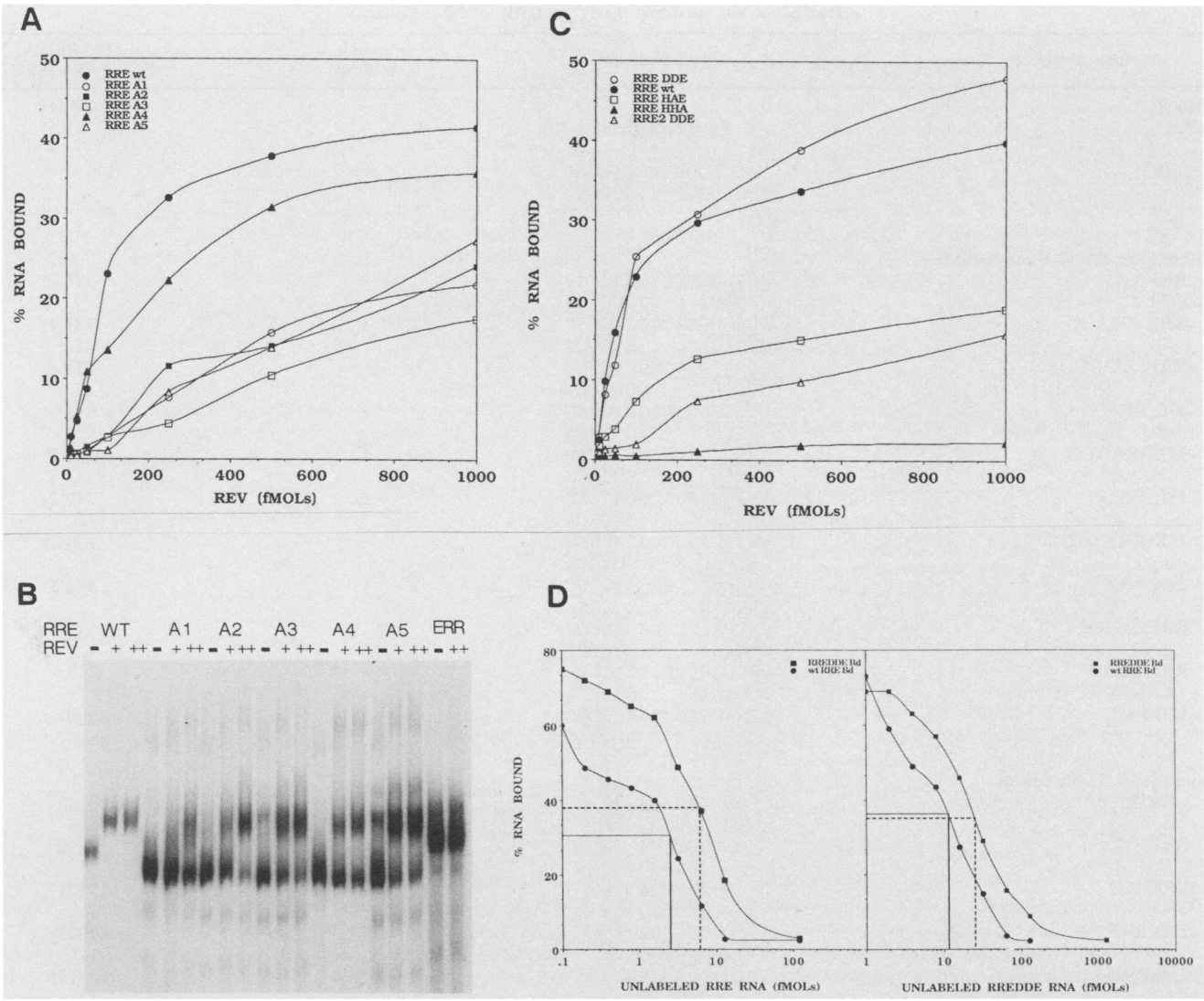

FIG. 3. Rev binding of the branched stem-loop deletions and the 3′-truncated RRE RNAs. (A and C) Filter binding results obtained with the branched stem-loop deletions and the 3′ truncations, respectively. The mutants used in each experiment are identified in the respective panel. The conditions were similar to those described in the legend to Fig. 2, except that lower concentrations of Rev were used to evaluate the relative affinities of different mutants. (B) Gel shift assay of the mutant RREs shown in panel A. A 0.5-pmol sample of the respective mutant RREs was analyzed without (−) or with (+ [10 pmol] or ++ [20 pmol]) Rev. (D) Results of competitive inhibition of wt RRE-Rev binding by RREDDE and vice versa analyzed by filter binding. In each case, 40 to 50 fmol of the respective labeled RNA was incubated with 0.5 pmol of Rev and the indicated amounts of the cold competitor RNAs.

potential of isolated B/B1/B2 stem-loop RNAs synthesized from wt and selected mutant RRE templates (Fig. 5B). The B2-compensated mutant 23Z RRE B/B1/B2 bound Rev better than the wt fragment. B/B1/B2 stem-loop RNAs of two RRE deletions that had either lost the 3′ strand of stem B (RRE8) or lost the 3′ strand of stem B and the entire stem B2 (RRE9) also bound Rev better than wt RRE. However, the B/B1/B2 RNAs carrying the X23 and 23X stem B mutations remained negative in this assay (Fig. 5B).

DISCUSSION

Our studies show that Rev binding to the RRE RNA is a prerequisite for Rev function and that a minimal unit of the RRE composed of the branched stem-loop B/B1/B2 is sufficient for Rev binding. Although this is essentially in agreement with other reports (15, 28, 33), some of our findings differ. Zapp and Green (43) were unable to demonstrate RNase protection after binding of Rev to an RRE RNA truncated at the *Dde*I site. Although we were able to demonstrate RREDDE protection from RNase T_1 degradation in the presence of Rev (data not shown), we did not attempt this experiment with the addition of RNase A as did Zapp and Green (43). Dayton et al. (15) suggested that sequence-specific binding information was most likely to reside in stem-loops B2 and A. We and others (28) have shown that binding is influenced by but not dependent on the A stem. The B2 stem-loop appears to be dispensable for both binding and biological function, as shown by our mutants RRE8 and RRE9 and by Olsen et al. (33).

Specific recognition of nt 50 to 58 represents a critical step in RRE-Rev RNP formation. Filter binding analysis allowed us to evaluate the relative affinities of Rev for wt and mutant

TABLE 2. In vitro and in vivo activity of RRE mutants

RRE construct[a]	Relevant sequence[b]	In vitro Rev binding[c]	$p24^{gag}$ ELISA (% of wt RRE [SEM])[d]
Mock		−	2 (0.4)
ERR	Reverse RRE	−	2 (1.5)
	50 66		
wt RRE	CACUAUGGGCGCAGCGU	++++	100
	GUGAUG.....GUCGCA		
	113 108 77 72		
Stem-loop B and B1 mutations			
RRE C41	CCCGGGGGGCGCAGCGU	−	2 (1.2)
	GUGAUA.....GUCGCA		
RRE J23ACG	AUAGUGGGGCGCACGGU	±	3 (1.4)
	GUGAUA.....GUCGCA		
RRE X23ACG	GUGAUAGGGCGCACGGU	−	2 (1.4)
	GUGAUA.....GUCGCA		
41C RRE	CCCGGGGGGCGCAGCGU	−	6 (3)
	GGGCCC.....GUCGCA		
23J RREACG	AUAGUGGGGCGCACGGU	±	3 (1.2)
	UAUCAC.....GUCGCA		
23J RRE	AUAGUGGGGCGCAGCGU	±	7 (2.2)
	UAUCAC.....GUCGCA		
23X RREACG	GUGAUAGGGCGCACGGU	−	3 (1.2)
	CACUAU.....GUCGCA		
23X RRE	GUGAUAGGGCGCAGCGU	±	3 (1.5)
	CACUAU.....GUCGCA		
RRE 3G3C	CACUAUCCCCGCAGCGU	ND[e]	5 (3)
	GUGAUA.....GUCGCA		
RRE 3G3U	CACUAUUUUCGCAGCGU	−	5 (3)
	GUGAUA.....GUCGCA		
23B RRE	CACUAUGGGCGCACGCUG	++	25 (9)
	GUGAUA.....UGCGAC		
Stem-loop B2 mutations			
wt RRE	89 CAGACA 94	++++	100
	104 GUCUGU 101		
RRE D23	CCCGCA	+++	27 (10)
	GUCUGU		
RRE Z23	CUCUGA	+++	18 (7)
	GUCUGU		
23D RRE	CCCGCA	++++	67 (11)
	GGGCGU		
23Z RRE	CUCUGA	+++++	140 (20)
	GAGACU		

[a] All RRE sequences are 5'..(62)AGC(64)..3' except where noted as ACG.
[b] The numbered sequence across from wt RRE denotes the RRE subsequence that was mutated. The mutant bases are underlined. The dots denote unpaired regions.
[c] The results of RRE RNA filter binding and gel mobility shifts are symbolically denoted from − (no binding) to ++++ (wt binding).
[d] The normalized $p24^{gag}$ ELISA values represent averages from six or eight experiments with the standard error of the mean shown in parentheses.
[e] ND, Not done.

RREs and to examine the importance of structure versus sequence for this reaction. In general, there was a good correlation between in vitro Rev binding and biological response among the RRE mutants. However, the 5' 132-nt RRE fragment (RREDDE) bound Rev more avidly than the wt RRE but did not function in vivo. RREDDE folded into a structure similar to that of a biologically active mutant that had lost the 3' stem-loops C, D, and E (15, 28, 33) except for a modified stem A, consisting of hairpin structures immediately 5' to the B/B1/B2 branched stem-loop. When the modified stem A was excised from RREDDE, Rev binding was significantly reduced. Therefore, the presence of stem A, even in a modified form as in RREDDE, appears to enhance the Rev binding of the branched stem-loop B/B1/B2. The importance of stem A in RRE binding was further demonstrated by comparison of RREDDE with RRE1. RREDDE had better Rev-binding characteristics than wt RRE, whereas RRE1, a 3' truncation of RRE at base 206, had only trivial Rev binding. RRE1 has lost the central bubble, and as a consequence, the nearest hairpin structure in stem A begins 16 nt 5' to the B/B1/B2 branched stem-loop. Since the B/B1/B2 structure alone is capable of binding, there must be some feature of the RRE1 structure which is unstable or inhibitory. The inability of RREDDE to function in vivo implied separate binding and activation domains within the RRE. Since Rev function may require spliceosome formation (9), the missing component(s) of the Rev-RRE functional unit may be small nuclear RNPs or other host nuclear factors. RREDDE RNA may lack the target sequences for these putative factors, or they may not be correctly exposed after complexation with Rev.

Although the RRE core domain composed of B/B1/B2 was necessary and sufficient to elicit in vitro Rev recognition, it has been difficult to deduce the contribution of sequence

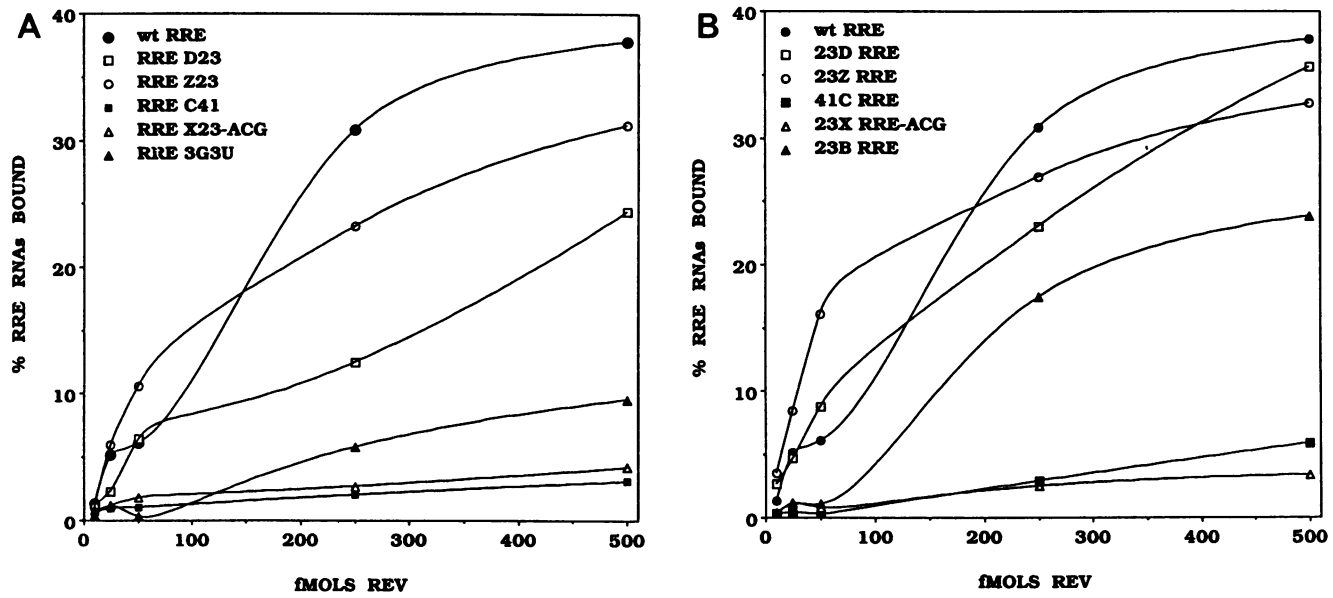

FIG. 4. Filter binding assay of uncompensated, structure-disrupting substitution mutants (A) and compensated, structure-preserving mutants (B) in the B/B1/B2 stem-loop. The conditions are similar to those described in the legend to Fig. 3, and the values represent averages from three experiments. The specific bases substituted are shown in Table 2.

elements in the B1 and B2 stem-loops (15, 28, 33). Fine-structure deletion mapping of B1 and B2 stem-loops gave ambiguous results. A deletion mutant (RRE9, positions 90 to 114) that removed the B2 stem-loop and the 3′ strand of stem B did not eliminate Rev binding or in vivo Rev response. Excision of the loop at the end of B2 (RREA4) only marginally affected filter binding to Rev, although it reduced the in vivo response significantly. Therefore, the B2 stem-loop may not play a critical role in the initial steps of Rev binding but may stabilize the RNP once it is formed and augment the in vivo Rev response. In contrast, the B1 stem-loop appears to contain important recognition sequences. The RRE6 deletion mutant, which removed stem-loop B1, lost the Rev response. Somewhat smaller deletions which disrupted the predicted B1 stem (RREA1, RREA2, RREA3, and RREA5) markedly reduced but did not eliminate Rev binding. The lack of a completely negative phenotype of the structure-disrupting B1 stem-loop mutants suggests that this region is not recognized exclusively on the basis of secondary structure, at least in vitro.

In contrast to the above mutants, a 12-nt deletion of the main stem B (RRE7, positions 47 to 58) gave a negative phenotype. The sequences absent in this mutant (residues 49 to 56) normally base pair with the sequences 107 to 113 in the wt RRE. However, removal of sequences 107 to 113 (RRE8) preserved the Rev binding and the in vivo response, and a

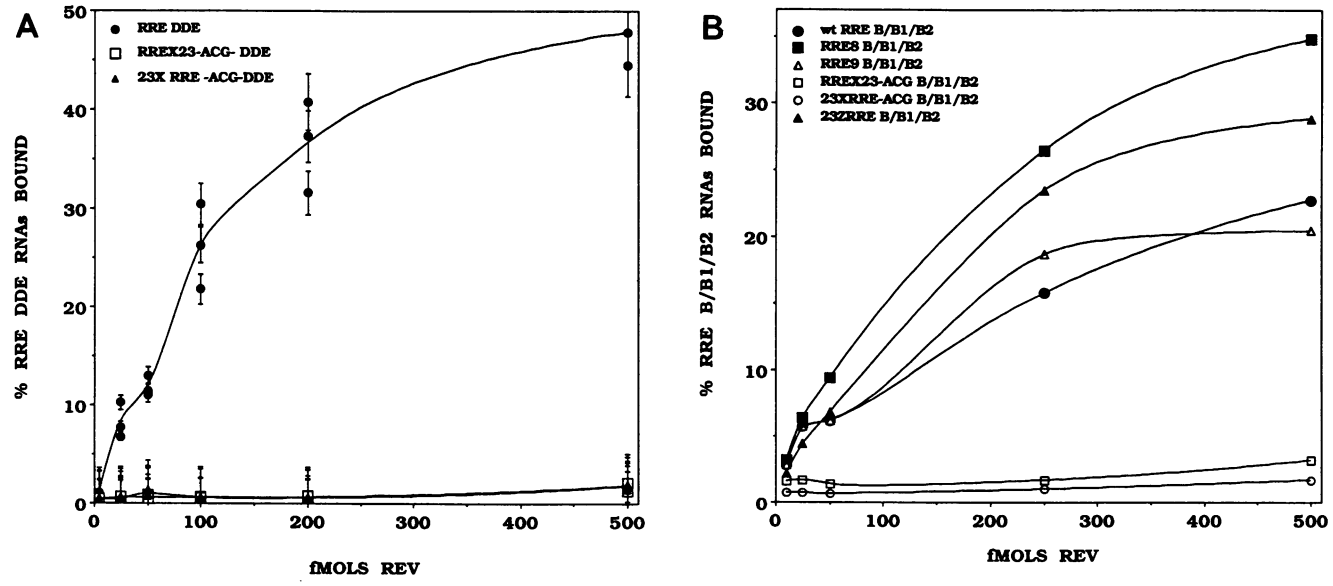

FIG. 5. Filter binding assays of 3′-truncated mutant RREs (A) and B/B1/B2 stem-loop RNAs (B) derived from several mutant RREs. The mutants used are identified in each panel. Conditions for in vitro Rev binding are similar to those described in the legend to Fig. 3.

larger deletion mutant (RRE9, positions 90 to 114) also did not lose the Rev response. Interestingly, RRE7, RRE8, and RRE9 folded into different structures, each of which lacked the B/B1/B2 stem-loop arrangement. Moreover, truncated RNAs corresponding to the core B/B1/B2 domains of RRE8 and RRE9 mutants bound Rev efficiently, although these core domains had markedly different structures from the wt counterpart. The differential Rev response of RRE7, RRE8, and RRE9 mutants led us to suspect that the nucleotides 47 to 58 lost in the RRE7 deletion may represent the primary structural motif for Rev recognition. Indeed, the 12 nt between nt 47 and 58 may not even have to be partly base paired for Rev binding. For instance, the 89-nt RREHAE RNA bound Rev, albeit inefficiently, despite lacking the downstream sequence that could have base paired with the bases 49 to 56 (Fig. 1A). Further, the verified structures for the Rev-responsive RRE8 and RRE9 mutants showed the 12 nt between 47 and 58 to be unpaired.

Previous analysis of the stem-loops A, B1, and B2 has shown that base changes in these regions are tolerated, if the individual stem-loops are preserved. For instance, structure-preserving mutations in the A and B2 stems resulted in a wt phenotype (15, 28, 33). Our compensatory mutants, 23D RRE and 23Z RRE, lay in the B2 stem and behaved similarly. Structural disruption of the B2 stem caused by mutating only the 5′ strand (RRE D23 and RRE Z23) led to only a modest decrease in Rev binding and in vivo Rev response. Mutations in the B1 stem which left the base pairing intact (23B RRE) reduced but did not abolish the Rev recognition. Therefore, the argument that the recognition of the RRE of HIV and its close relatives by Rev and human T-cell lymphotropic virus type I Rex is dependent on the secondary rather than the primary structure of RNA seemed plausible. However, we identified a sequence-specific Rev interaction in the B stem of RRE. Three mutants (23J RRE, 23X RRE, and 41C RRE) that altered the 6 bp of stem B (nucleotides 50 to 55 and 108 to 113) while preserving the wt secondary structure were unresponsive to Rev both in vitro and in vivo. These effects of the 23X and X23 mutations carried down to the *Dde*I and the B/B1/B2 fragments. The Rev recognition sequence probably includes the three G residues beyond the 5′ . . (50)CACUAU(55) . . 3′ sequence since mutation of the three Gs to UUU or CCC (RRE 3G3U and RRE 3G3C) abolished binding and biological response although the predicted secondary structure was identical to that of the wt RRE. Two of the 3′ Gs in this region are in a bulge sequence, and these bulged G residues may have a protein-binding role analogous to the roles envisioned for the bulged A residues in the RNA bacteriophage translational operators (37, 42) and the 5, 16, and 23S rRNAs (10, 32).

Recently, Rev binding has been shown to specifically shield a 33-nt RRE RNA fragment, including nt 47 to 58, from nuclease digestion (23). In this region of RRE, a 12-nt 5′ . . CACUAUGGGCGC . . 3′ sequence is conserved in all HIV-1 isolates except for the substitution of G for U at position 4. Although this may reflect the strong codon bias in this region of the *env* open reading frame in HIV-1, a similar but somewhat degenerate 5′ . . (U/G)GC(A/G)AUGGG . . 3′ sequence is also found in the RREs of HIV-2 and simian immunodeficiency virus isolates, which use a different codon scheme in this region. Computer-predicted RNA folding of all HIV-1 RREs showed the 5′ . . CACUAUG . . 3′ sequence to be base paired to an equally conserved 3′ . . GUGAUAU . . 5′ sequence at positions 113 to 107. The latter sequence is also conserved in the HIV-2 and simian immunodeficiency virus RREs, which are responsive to HIV-1 Rev. Unlike HIV-1, the above two 7-nt elements are not base paired with each other in the HIV-2 or simian immunodeficiency virus RREs. Therefore, it is likely that a primary structure motif between nt 47 and 58 of the HIV-1 RRE represents an optimal contact point for Rev within the context of the B/B1/B2 stem-loop. The exact sequence requirements for Rev recognition must, however, await binding studies with short oligonucleotides and exhaustive mutational analysis.

While it is possible that similar sequence-specific interactions occur in other regions of the minimal RRE structural motif, none have so far been discovered. Sequences located in stem-loops A and B1 may be somewhat degenerate and not be readily identified by in vitro studies but could play crucial roles in vivo. Alternatively, stem-loops A, B1, and B2 may simply provide the secondary structural context for optimal Rev recognition both in vitro and in vivo. There is some precedence for the context-sensitive stabilization of RNA-protein interactions. The R17 coat protein recognition of the bulged A residue and the loop sequence 5′ . . AUUA . . 3′ in its target RNA was stringent for the requirement of a stem structure (8, 37, 42). On the other hand, although U2 snRNP can bind its target 5′ . . CACC AAC . . 3′ sequence near the splice junction branchpoint out of the natural RNA context, the magnitude of this interaction was enhanced when the same sequence was presented in the context of an authentic intron (31).

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