Contributions of Individual Domains to Function of the HIV-1 Rev Response Element

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ABSTRACT The HIV-1 Rev response element (RRE) is a 351-base element in unspliced and partially spliced viral RNA; binding of the RRE by the viral Rev protein induces nuclear export of RRE-containing RNAs, as required for virus replication. It contains one long, imperfect double helix (domain I), one branched domain (domain II) containing a high-affinity Rev-binding site, and two or three additional domains. We previously reported that the RRE assumes an “A” shape in solution and suggested that the location of the Rev binding sites in domains I and II, opposite each other on the two legs of the A, is optimal for Rev binding and explains Rev's specificity for RRE-containing RNAs. Using small-angle X-ray scattering (SAXS) and a quantitative functional assay, we have now analyzed a panel of RRE mutants. All the results support the essential role of the A shape for RRE function. Moreover, they suggest that the distal portion of domain I and the three crowning domains all contribute to the maintenance of the A shape. Domains I and II are necessary and sufficient for substantial RRE function, provided they are joined by a flexible linker that allows the two domains to face each other.

IMPORTANCE Retroviral replication requires that some of the viral RNAs transcribed in the cell nucleus be exported to the cytoplasm without being spliced. To achieve this, HIV-1 encodes a protein, Rev, which binds to a complex, highly structured element within viral RNA, the Rev response element (RRE), and escorts RRE-containing RNAs from the nucleus. We previously reported that the RRE is “A” shaped and suggested that this architecture, with the 2 legs opposite one another, can explain the specificity of Rev for the RRE. We have analyzed the functional contributions of individual RRE domains and now report that several domains contribute, with some redundancy, to maintenance of the overall RRE shape. The data strongly support the hypothesis that the opposed placement of the 2 legs is essential for RRE function.

KEYWORDS HIV, RNA structure, Rev response element, gene expression, small-angle X-ray scattering

The great majority of transcripts from the chromosomal DNA of mammalian cells are spliced before leaving the nucleus. However, the genomic RNA in a retrovirus particle is an intact, unspliced transcript of the proviral DNA. As the DNA is in the cell nucleus but the RNA is packaged into assembling virus particles in the cytoplasm, successful replication requires a mechanism for evading the splicing machinery, so that some transcripts are exported to the cytoplasm without being spliced. Members of the lentivirus genus of retroviruses, such as HIV-1, fulfill this requirement by encoding a protein called Rev. Unspliced (and singly spliced) viral transcripts, which also function

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FIG 1 RRE activity as a function of Rev concentration. Cotransfection of 1 μ g pCMV-Gag-RRE plasmid or pCMV-Gag-A131G-RRE plasmid (12) with different amounts of pCMV-Rev plasmid. Gag production was quantified by Western blotting and normalized to actin levels (loading control) and *Gussia* luciferase levels (transfection efficiency control) as described previously (15).

in the cytoplasm as mRNAs for structural viral proteins, contain an RNA element, the Rev response element (RRE); Rev binds to the RRE in the nucleus, and this complex is exported to the cytoplasm (1, 2).

Why does Rev bind specifically to the RRE, despite the great excess of other RNAs in the nucleus? The RRE is a complex structure of 351 bases, comprising several domains (2). It contains, within stem-loop IIB, a high-affinity binding site for Rev (3–11); but this does not fully explain its specificity for the RRE, as successful export from the nucleus requires the binding of multiple Rev molecules to the RRE (2, 12–14). Another relatively high-affinity binding site has also been identified on stem I, which is a very long, largely double-stranded component of the RRE (12). We recently used small-angle X-ray scattering (SAXS) to obtain a low-resolution 3-dimensional structure of a truncated RRE (15). Interestingly, we found that the entire structure is shaped like the letter “A”; the binding sites on stem-loop IIB and on stem I are opposite each other on the two legs of the A. Further, the distance between these sites is ~ 55 Å. As this is the span of a dimer of Rev (13, 16), we suggested that this architecture can explain the specificity of Rev for the RRE. Presumably (despite the fact that single Rev molecules can bind the RRE [17, 18]), a Rev dimer first binds these two sites; this initial binding is then followed by cooperative binding of additional Rev dimers to the RRE. Using an optimized assay for RRE function (15), we found that RRE-derived constructs containing only one or the other of the “legs” of the A were almost completely nonfunctional. We also tested constructs in which the “crossbar” of the A was extended: these RNAs, in which the distances between the two Rev binding sites were somewhat greater than 55 Å, showed reduced Rev binding and impaired RRE function. Taken together, these results strongly supported the overall hypothesis that the A shape, specifically, the spacing between the two primary Rev binding sites, is the key to the specific effect of Rev on RRE-containing RNAs, leading to their export from the nucleus.

In the present work, we have dissected the RRE, with the aim of determining the functional roles of specific substructures within it. Our results suggest that the A shape is metastable and that a principal function of the very long domain I and three smaller “crowning” domains is to contribute to the maintenance of this overall conformation. They also provide additional strong support that the A shape, allowing opposed, rather than coaxial, placement of the two primary Rev binding sites, is a crucial determinant of RRE function.

RESULTS

Assay for RRE function. The assay for RRE activity (15) is described in detail in Materials and Methods. An essential feature of this assay is that the signal gives a linear response for Rev-RRE interaction. We assessed the effects of increasing Rev doses upon the level of Gag protein synthesized in the assay. The results are shown in Fig. 1. It can be seen that the Gag signal is strictly linear only at quite low ratios of the Rev plasmid to the Gag-RRE reporter plasmid: at higher Rev plasmid doses, the assay saturates and the signal is no longer an accurate reflection of the Rev-RRE interaction. As an example, we tested the “IIB mutant” described by Daugherty et al. (mutation A73G in their numbering system, A131G in the full-length RRE) (12). As shown in Fig. 1, the Gag signal

is ~4% of that obtained with wild-type RRE in the linear range, although it is ~24% at a higher Rev/RRE ratio. Thus, this mutant possesses only ~4% of the RRE activity of wild-type (WT) RRE.

Domain I function. The secondary structure of the RRE (17) is shown in Fig. 2. It consists of an extremely long stem, “domain I”; an opposing domain, “domain II,” which contains the primary high-affinity Rev binding site; and three (or two, in some studies [19]) smaller upper domains, “domains III to V” (the sequence in this region is compatible with two alternative structures, one with two and one with three upper domains; in fact, the 2- and 3-stem structures may well be in equilibrium with each other [19]). Domain I is nearly 100 nucleotides (nt) on each side and is largely double stranded, although it is interrupted by several bulges. It contains a site (“site IA”) with modest affinity for Rev (12). How does the very long stem contribute to RRE function? Possibilities include the ideas that it provides an extended surface to which many Rev molecules can bind, that it presents specific RNA sequences important for Rev binding or other functions, that portions engage in long-range tertiary interactions, and that it somehow provides structural support for the entire RRE structure.

The role of domain I has previously been analyzed with a series of truncations (14). We have confirmed and extended these findings using our optimized assay, as shown in Fig. 3. We found that removal of ~60 nt from each strand of the stem (producing “Tr3,” the RNA previously analyzed by SAXS [15]), reduced the activity only ~2.5-fold relative to that of the full-length (FL) RRE; thus, a large fraction of this very long stem apparently is not required for efficient function of an otherwise intact RRE. However, removal of an additional ~12 nt from each side of the stem (resulting in Tr4) reduces this remaining activity ~6-fold, leaving only ~7% of FL activity.

It seemed possible that the “A” shape of the intact RRE, constructed by virtue of complex tertiary interactions, is under substantial strain and that the long domain I is important because its ~70 base pairs impart stability to the structure. In support of this possibility, we found, using SAXS data and *ab initio* modeling, that Tr4 models do not exhibit an A shape (Table 1; Fig. 4B [green envelope] and C). The 5′ side of Tr4 begins with nt 71 of the RRE, while the 3′ side ends with nt 279. The 12 bp at the end of domain I in Tr4 include 7 A·U or G·U base pairs. We replaced all of these with G·C base pairs (and also replaced an unpaired U and an unpaired A with C and G, respectively), so as to stabilize the short residual domain I (Fig. 4A). The resulting construct, “Tr4GC,” had evidently recovered the A shape: its SAXS-derived model was considerably more compact than that of Tr4 and closely resembled that of Tr3 (Table 1; Fig. 4B and C). Taken together, these results argue strongly that the base pairing in domain I is crucial for maintenance of the A-shaped structure of the RRE.

We also tested Tr4GC for RRE function. It was found (Fig. 4D) to have recovered all of the activity lost in shortening Tr3 to Tr4; thus, while it is as short as Tr4, it is as active as (or somewhat more active than) Tr3. Introducing the same mutations into the FL RRE, producing “FL-GC,” had no significant effect on RRE activity (Fig. 4D). These results lend further support to the idea that the A shape of the RRE is essential for its function.

Function of domains III to V. We also investigated the functions of domains III to V, which contain neither of the “primary” Rev binding sites. We constructed one plasmid with a deletion of domains III and IV; another with a deletion of domain V; and a third lacking all three domains, yielding an RNA containing intact stems I and II, connected by the 16 nucleotides at the bases of domains III to V that are unpaired in the secondary structure of WT RRE. As shown in Fig. 5, even the construct lacking all three domains retained ~33% of the RRE activity of FL WT RRE. Thus, in the context of the FL domain I, domains III to V make a relatively minor contribution to RRE activity. Somewhat similar results have been reported previously (6, 9, 20, 21).

As shown above (Fig. 3), the loss of 120 bases, including 39 bp, from domain I (“Tr3”) reduces the activity of the WT RRE only ~2.5-fold. We combined this truncation with the deletion of domains III to V. Interestingly, the RRE containing both of these changes, Tr3/ΔIII-V(16) (Fig. 6A), possessed almost no activity (Fig. 6B). Stabilizing the truncated

FIG 2 Secondary structure of the full-length, 351-nt HIV-1 RRE. The secondary structure of the 5-domain conformation is shown (17, 19). Domains are labeled with Roman numerals. The sequence corresponds to the region between nucleotides 7701 and 8051 in the pNL4.3 sequence. The structure was drawn using the VARNA software (28).

FIG 3 Functional analysis of truncations in domain I. (A) Schematic of the secondary structure of the 351-nt RRE. Domain I was progressively truncated at the positions indicated with red dashed lines. The following regions were deleted in each truncation: 1 to 26/334 to 351 (Tr1), 1 to 46/312 to 351 (Tr2), 1 to 59/293 to 351 (Tr3), 1 to 69/280 to 351 (Tr4), and 1 to 89/263 to 351 (Tr5). (B) Functional analysis of the RRE truncation mutants as described in Materials and Methods. pCMV-Gag-RRE plasmids were transfected in the absence or presence of pCMV-Rev (indicated by – or +, respectively). “Mock” refers to transfection with pcDNA3.1. Error bars are based on data from independent experiments ($n = 2$ for Tr1, Tr2, and Tr5; $n = 4$ for Tr3; $n = 8$ for Tr4). NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

stem I within Tr3 by replacing A-U and G-U base pairs (as in Tr4GC) did not restore RRE activity to the Tr3/ Δ III-V double mutant (Fig. 6B). Thus, the data indicate that FL domain I and domains III to V are functionally redundant: neither is required for substantial activity, provided that the other is present. In fact, Tr3/ Δ III-V(16) does not appear to share the “A” shape found in WT Tr3, as indicated by SAXS analysis (Fig. 6) (15).

We further explored this aspect of the function of domains III to V by deleting them individually in Tr3; these assays showed that domains III and IV are each required for RRE function in the Tr3 context, while significant function is retained in a Tr3/domain V deletion double mutant (Fig. 7).

All of the results presented thus far are consistent with the overarching hypothesis that RRE function requires that domains I and II be opposite each other in the RRE. This would require not only the presence of domains I and II but also that the sequence separating them be flexible enough to bend over, so that domains I and II can be opposed rather than coaxial. In order to test this prediction, we varied the number of bases between domains I and II in constructs lacking domains III to V (here denoted Δ III-V) (Fig. 8A). As shown in Fig. 8B, reduction of this number to 10 or fewer reduced the activity to only 3 to 4% of the WT level. The relationship between length and activity is not completely monotonic; we do not know the reason for this.

As a further test of the idea that flexibility of the stretch between domains I and II is a key aspect of the structure, we also tested a construct in which domains III to V had been deleted and there were 16 bases between stems I and II, but these bases were all

TABLE 1 Molecular parameters derived from SAXS

Sample	Mean $R_g^a \pm SD$ (Å):			Mean $D_{max} \pm SD$ (Å) ^d
	From Guinier ^b	In reciprocal space ^c	In real space ^c	
Tr3	52 ± 1	54.4	54.5 ± 0.3	198 ± 2
Tr4	54.6 ± 0.4	57.2	57.2 ± 0.3	195 ± 3
Tr4GC	44.4 ± 0.4	44.9	44.9 ± 0.2	156 ± 2
Tr3/ Δ III-V(16)	47 ± 1	50.0	50.1 ± 0.4	175 ± 2

^a R_g , radius of gyration.

^bProduced from Guinier plot.

^cProduced from GNOM (indirect transform program for small-angle scattering data processing).

^d D_{max} values were obtained from the pair distance distribution functions.

FIG 4 Stability of domain I is necessary for the structural integrity and functional activity of the RRE. (A) Schematic of the secondary structure of the 351-nt RRE. The red X indicates the truncation of domain I up to the point indicated by a red dashed line (Tr4 in Fig. 2). The region that was mutated in Tr4GC and FL-GC is indicated with a shaded rectangle, and its secondary structure is shown below the schematic. The nucleotides that were mutated are colored in green and were replaced in Tr4GC with the nucleotides colored in blue. (B) SAXS envelopes of Tr3 (magenta mesh) (15), Tr4 (green mesh), and Tr4GC (dark blue mesh). Envelopes were visualized and edited in PyMol. (C) Pair distance distribution function versus distance *R* between pairs of atoms in RRE truncation mutants: Tr3 (magenta), Tr4 (green), and Tr4GC (blue). (D) Functional analysis of mutants. Error bars are based on data from independent experiments ($n = 4$ for Tr3 and Tr4GC; $n = 8$ for Tr4; $n = 3$ for FL-GC). NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$.

adenines: runs of adenines are known to be more rigid than other nucleic acid sequences (22, 23). As shown in Fig. 8B, this change in the sequence of the linker caused an ~ 4 -fold drop in RRE activity relative to the Δ III-V RRE. This result provides support for the general hypothesis outlined above.

DISCUSSION

Interaction between the HIV-1-encoded protein Rev and the RRE, a 351-base, highly structured region in the viral RNA, is necessary for the export of unspliced and partially spliced viral RNAs from the nucleus to the cytoplasm (2). Such export is absolutely

FIG 5 Contribution of domains III through V to RRE activity. (A) Schematic of the secondary structure of the 351-nt RRE. The domains marked with a red X were deleted: nucleotides 164 to 213 were deleted in Δ III-IV, nucleotides 223 to 246 were deleted in Δ V, and both regions 164 to 213 and 223 to 246 were deleted in Δ III-V. (B) Functional analysis of the RRE mutants from independent experiments ($n = 2$ for Δ III-IV; $n = 4$ for Δ V; $n = 5$ for Δ III-V). *, $P < 0.05$; ***, $P < 0.001$.

FIG 6 Domains III to V are necessary for RRE function in the absence of a long domain I. (A) Schematic of the secondary structure of the 351-nt RRE. The domains marked with a red X were deleted (deletions in domains III to V are the same as in Fig. 5), and domain I was truncated up to the point indicated by the red dashed line (this truncation was designated “Tr3” above). (B) Functional analysis of RRE mutants. The number 16 in parentheses indicates that 16 nucleotides remain between domains I and II when domains III, IV, and V are deleted. The mutant Tr3GC/ΔIII-V(16) has the same mutations in domain I as Tr4GC (shown in detail in Fig. 4). Error bars are based on data from independent experiments: $n = 5$ for ΔIII-V(16); $n = 4$ for Tr3; $n = 3$ for Tr3GC/ΔIII-V(16); and $n = 2$ for Tr3/ΔIII-V(16). NS, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Pair distance (R) distribution function of Tr3 and Tr3/ΔIII-V(16). (D) SAXS envelopes of Tr3(15) (blue mesh) and Tr3/ΔIII-V(16) (magenta mesh).

essential for viral replication. Consequently, the Rev-RRE interaction has been the subject of intensive study for over 25 years.

The RRE contains five (or, in some structures, four [19]) domains. One of these, “domain I,” is a long, imperfect double helix, with approximately 95 bases in each strand and nearly 70 bp (14). Domain II contains a unique site, to which Rev binds with particularly high affinity; it seems likely that binding to this site initiates the Rev-RRE interaction (3–11, 24). There is also a lower-affinity Rev binding site in domain I (12). Numerous observations indicate that, although single Rev molecules can bind to an unoccupied RRE (3, 17, 18), the interaction of multiple Rev molecules with a single RRE is required for productive Rev-RRE interactions (14, 25, 26).

As outlined above, we previously analyzed the 3-dimensional structure of a truncated, 233-base RRE by SAXS (15). We found that it folds into an A shape. The two preferential binding sites are opposite each other on the two legs of the A, separated by ~55 Å. As this is the distance between the two ARMs in a Rev dimer, we suggested that this architecture is responsible for the selectivity of Rev for RRE-containing RNAs.

FIG 7 Individual contributions of domains III, IV, and V to RRE function in Tr3. Individual deletions of domain III, IV, or V in Tr3 RRE included the following regions: nucleotides 164 to 174 (ΔIII), 190 to 218 (ΔIV), 164 to 174 and 190 to 218 (ΔIII-IV), and 221 to 246 (ΔV). Results of functional assays from independent experiments of these mutants are shown ($n = 3$ for ΔIII, ΔIV, and ΔV; $n = 2$ for ΔIII-IV). NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 8 Contribution of a flexible region between domains I and II to RRE activity. (A) Schematic of the secondary structure of the 351-nt RRE (plotted as the 4-domain conformer [19]). The domains marked with a red X were deleted. The secondary structure of the region within the shaded rectangle is shown below the schematic. Deleted nucleotides are shown in blue, and nucleotides remaining between domains I and II are shown in magenta. (B) Functional analysis of RRE mutants in which the linker region is shortened or mutated. The numbers in parentheses indicate the numbers of nucleotides remaining in the linkers. The regions remaining in each construct are the following: ACUCAGCAAGAA (14 nt), ACACAGCAAGAA (12 nt), ACAGCAAGAA (10 nt), ACAAGAA (7 nt), and AAA (3 nt). Δ III-V (16A) has 16 nucleotides between domains I and II, but these nucleotides are all adenines. Error bars are based on data from independent experiments: $n = 5$ for Δ III-V(16); $n = 4$ for Δ III-V(12), Δ III-V(10), and Δ III-V(16A); $n = 3$ for Δ III-V(14); and $n = 2$ for Δ III-V(7) and Δ III-V(3). *, $P < 0.05$; ***, $P < 0.001$. The differences between Δ III-V(14) and Δ III-V(12) and that between Δ III-V(12) and Δ III-V(10) are significant at a P level of <0.05 , while that between Δ III-V(14) and Δ III-V(10) is significant at a P level of <0.01 .

We analyzed both RRE function and binding of Rev protein in a series of mutant RREs lacking one or the other leg of the A or in which the distance between the legs was increased. All the results were consistent with this basic proposal (15).

In the present work, we have examined the contributions of these RRE domains to RRE function. Our results can be briefly summarized as follows. The results suggest that one major function of domain I is to stabilize the RRE. It was reported many years ago that truncations of domain I reduce RRE function (14). However, while an RRE with a severely truncated domain I possesses very little RRE activity (Fig. 3), we found that most of the RRE function can be recovered if the remnant of domain I in this truncation mutant is stabilized by replacing A-U and G-U with G-C base pairs (Fig. 4). The fact that strengthening the short domain I stem of Tr4 with these mutations restores RRE activity suggests that domain I contributes to the maintenance of the functional structure. Indeed, SAXS analysis showed that the truncated version had lost the "A" shape of

wild-type RRE, but the introduction of the stabilizing GC mutations had apparently restored this structure (Fig. 4). It seems plausible that the entire RRE structure is under strain and that one role of the FL domain I, containing nearly 70 bp, is to lend stability to the overall structure. Data indicating that FL domain I bends back on itself as a result of long-range interactions between bases in bulges in different positions on the stem have recently been presented (27). These interactions occur in the lower part of domain I; however, according to our analysis (Fig. 3), this lower region does not make a major contribution to RRE function.

We also investigated the function of domains III to V. Previous investigators have reported that even large deletions within this region of the RRE do not completely destroy RRE function (6, 9, 20, 21), and little or no binding of Rev to these stem-loops was detected by nuclease protection and electrophoretic mobility shift assays (14). We confirmed this general conclusion; in fact, deletion of all the base-paired stems and apical loops in this part of the RRE reduced RRE function only 3-fold (Fig. 5 and 8). However, the combination of this deletion of domains III to V with a truncation in domain I to “Tr3” (involving the removal of ~60 bases from each side of stem I [Fig. 3]) virtually eliminated RRE function (Fig. 6). In fact, deletions of individual stems III and IV each had a severe deleterious impact on RRE function in the Tr3 background (Fig. 7). We also found that stabilizing the truncated domain I by replacing A-U and G-U pairs with G-C pairs failed to rescue RRE function in the Tr3/ Δ (III-V) context (Fig. 6). Thus, domains III to V are functionally important in the absence of the FL domain I; there is functional redundancy between domains III to V and the distal portion of domain I. As our other data suggested that one important role of this region of domain I is to stabilize the overall RRE structure (Fig. 4), the redundancy suggests that domains III to V may also contribute to this stabilization.

In the Δ (III-V) deletions discussed above, the RRE consists solely of domains I and II; the 3' sides of these two domains are linked together by the 16 bases that are unpaired at the bases of stems III to V in the two-stem form of the intact RRE (Fig. 8, magenta bases). If, as we have proposed (15), RRE function requires that domains I and II be placed in an opposed configuration, rather than coaxially, then in these Δ (III-V) deletions, this linker must also be able to bend over to bring the two domains into opposition. We made stepwise deletions in this linker and found that Δ (III-V) deletions with shorter linkers were functionally impaired compared to that with the 16-base linker (Fig. 8). We also replaced the 16-base heteropolymeric sequence with (A)₁₆, thus increasing its rigidity rather than decreasing its length (22, 23); this change also interfered with RRE function (Fig. 8). This result strongly supports the idea that RRE activity requires that the connection between domains I and II permit them to face each other.

Taken together, our data show a strong correlation between changes that disrupt (or are expected to disrupt) the spatial opposition of domains I and II and changes impairing RRE function. Compensating changes, restoring the opposition, restored RRE function. This correlation argues that domains I and II must be spatially opposed for RRE function, underscoring the significance of the “A” shape for the RRE. They also imply that the long stem of domain I and the crowning stems III to V all support RRE function by lending stability to the overall structure, and that this stabilizing activity is somewhat redundant in these regions of the RRE. Further analysis of the forces responsible for the “A” shape is now under way.

MATERIALS AND METHODS

Plasmid construction. Mutations in the RRE were introduced into a pCMV-Gag-RRE reporter plasmid, which is described below. All deletions were introduced by PCR, and successful candidates were sequenced throughout the pCMV-Gag-RRE region to ensure fidelity. For structural studies of the RNA, equivalent mutations were introduced in a Gag-RRE plasmid that also contains the T7 promoter to allow *in vitro* transcription (15).

RRE reporter assay. Functional assays were performed as described previously (15). The reporter plasmid, pCMV-Gag-RRE, encodes only the HIV-1 Gag protein, and the N-terminal glycine codon has been replaced with an alanine codon. This change blocks the addition of myristate to the protein and thus prevents it from assembling into budding virus-like particles. The Gag-coding region (derived from the

BH10 HIV-1 isolate, GenBank [AH002345](#)) is followed immediately by HIV-1 nucleotides 6977 to 7830 (numbers corresponding to the BH10 HIV-1 sequence), where the RRE represents nt 7066 to 7416. The RRE sequence is that of NL4-3 HIV-1, GenBank [AF324493](#). Briefly, in the assay pCMV-Gag-RRE was transfected into 293T cells in the presence or absence of the pCMV-Rev plasmid (a gift of Barbara Felber, NCI), at a ratio of 1 ng Rev plasmid to 40 ng reporter plasmid, and cell lysates were collected 24 h posttransfection. All transfections also included a plasmid expressing *Gaussia* luciferase under the control of the cytomegalovirus (CMV) promoter. Gag production in the cells was then measured by quantitative Western blotting using anti-p24 antibody and an Odyssey imager (Li-Cor Biosciences). The p24 signal was normalized to actin levels, measured in the same lane of the same Western blot as Gag, and to transfection efficiency as determined by *Gaussia* luciferase levels. A standard curve in each assay confirmed that the imager responded linearly to the amount of Gag in the sample and that the experimental values were within the linear range. Bar graphs show means and (where appropriate) standard deviations of the RRE activity as a percentage of full-length WT RRE activity. *P* values were obtained by 2-tailed Student *t* tests; where not indicated otherwise, *P* values represent comparisons between an RRE mutant and full-length WT RRE, both in the presence of pCMV-Rev.

RNA preparation. RNA was transcribed *in vitro* and purified using native gel electrophoresis as described previously (15). Samples were prepared for SAXS as described previously (15), except that the buffer contained 0.5 mM Mg²⁺ rather than 1 mM. Sample homogeneity and monodispersity were assessed by dynamic light scattering for quality assurance prior to SAXS data collection. SAXS and data analysis were performed as described previously (15).

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