A Solution to Limited Genomic Capacity: Using Adaptable Binding Surfaces to Assemble the Functional HIV Rev Oligomer on RNA

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SUMMARY

Many ribonucleoprotein (RNP) complexes assemble into large, organized structures in which protein subunits are positioned by interactions with RNA and other proteins. Here we demonstrate that HIV Rev, constrained in size by a limited viral genome, also forms an organized RNP by assembling a homo-oligomer on the Rev response element (RRE) RNA. Rev subunits bind cooperatively to discrete RNA sites using an oligomerization domain and an adaptable protein-RNA interface, forming a complex with 500-fold higher affinity than the tightest single interaction. High-affinity binding correlates strongly with RNA export activity. Rev utilizes different surfaces of its α-helical RNA-binding domain to recognize several low-affinity binding sites, including the well-characterized stem IIB site and an additional site in stem IA. We propose that adaptable RNA-binding surfaces allow the Rev oligomer to assemble economically into a discrete, stable RNP and provide a mechanistic role for Rev oligomerization during the HIV life cycle.

INTRODUCTION

Several key macromolecular machines are composed of ribonucleoprotein (RNP) assemblies, including the ribosome, spliceosome, signal recognition particle, and telomerase (Ban et al., 2000; Hainzl et al., 2002; Shen and Green, 2004; Zappulla and Cech, 2004). To organize such assemblies, proteins use several strategies to bind RNA with high affinity and specificity. In some cases, such as the ribosome, many hetero-oligomeric subunits recognize discrete portions of an RNA and become organized further by protein-protein interactions (Ban et al., 2000). In other cases, such as the Sex-lethal splicing protein, multiple RNA-binding domains are tethered on a single polypeptide to enable the protein to bind to an extended RNA site (Lunde et al., 2007). Both of these strategies utilize substantial protein coding capacity to encode multiple subunits or multi-domain proteins, typically using gene or domain duplication, and subsequent evolution to generate new specificities. A more frugal coding strategy utilizes homo-oligomeric proteins to bind nucleic acids, most notably with DNA-binding proteins where each subunit typically recognizes a repeated or closely related binding site arrayed along the DNA (Marmorstein and Fitzgerald, 2003). Limiting the size of proteins is especially important to viruses, which often evolve mechanisms to cope with their limited coding capacity, such as organizing genes in overlapping reading frames. Here we describe a strategy used by HIV-1 to assemble a large RNP using a small, homo-oligomeric protein.

The 116 amino acid HIV-1 Rev protein binds to the ~350 nt Rev response element (RRE) RNA found in the introns of partially spliced and unspliced viral mRNAs, forming a large RNP that directs their transport to the cytoplasm before splicing is completed. These exported mRNAs are either translated into the viral structural proteins or packaged as genomic RNA into virions (Cullen, 1998, 2003; Pollard and Malim, 1998). To export its bound RNA cargo, Rev binds to the human nuclear export receptor, Crm1, through its nuclear export sequence (NES; Figure 1A), forming a ternary complex stabilized by GTP-bound Ran (Cullen, 2003; Fornerod et al., 1997). After the complex docks to and translocates through the nuclear pore, RRE-containing RNAs are released in the cytoplasm, and the protein components are shuttled back into the nucleus.

The function of the RNP relies on the binding of several Rev monomers to the RRE. This oligomeric assembly requires the RNA-binding arginine-rich motif (ARM) and flanking oligomerization domains of Rev (Figure 1A) in addition to most of the highly structured RRE (Figure 1B) (Hope et al., 1990; Huang et al., 1991; Malim and Cullen, 1991; Mann et al., 1994; Pollard and Malim, 1998). Although essential for in vivo function, the mechanistic role of oligomerization has been unclear due to the lack of a clear biochemical function in RNA binding or RNA export. Early studies identified stem-loop IIB (Figure 1B) as the primary specific binding site (Cook et al., 1991; Heaphy et al., 1991; Huang et al., 1991; Iwai et al., 1992; Kjems et al., 1991; Malim and Cullen, 1991; Tiley et al., 1992), and a minimized IIB RNA hairpin binds a single Rev monomer with high specificity although it is insufficient for export activity (Kjems and Sharp, 1993; Mann et al., 1994). Previous biochemical studies showed that 6–10 subunits can bind the RRE, but curiously, Rev binds to the isolated IIB hairpin with similar affinity as to the full RRE even though fewer subunits are bound (Cook et al., 1991; Heaphy et al., 1991; Huang et al., 1991; Iwai et al., 1992; Kjems et al., 1991; Malim and Cullen, 1991; Tiley et al., 1992).
et al., 1992). Furthermore, an isolated ARM peptide, properly stabilized as an α-helix, has been shown to recapitulate the affinity and specificity of the full complex but does not oligomerize (Tan et al., 1993). The Rev peptide-IIB interaction has been well characterized: the peptide inserts deeply into a wide RNA major groove and makes numerous specific contacts, most notably forming hydrogen bonds between Asn40 and both partners of the G47:A73 base pair (Battiste et al., 1996; Tan et al., 1993; Tan and Frankel, 1994). Although other regions of the RRE have been suggested to be involved in Rev binding (Kjems et al., 1991; Mann et al., 1994; Zemmel et al., 1996), no other specific binding sites have yet been uncovered, leading to models in which binding to IIB nucleates oligomerization along the RNA (Heaphy et al., 1991; Jain and Belasco, 2001; Malim and Cullen, 1991; Mann et al., 1994), analogous to the coating of viral DNAs or RNAs during genome packaging (Harrison et al., 1996). Mutation of residues important for oligomerization have shown no loss in primary RNA-binding affinity (Edgcomb et al., 2008; Jain and Belasco, 2001; Malim and Cullen, 1991), and thus, the functional role of oligomerization in the HIV life cycle has remained uncertain. It has been postulated that the high concentrations of Rev needed for oligomerization might set an expression threshold such that Rev function would occur only during the later stages of viral replication (Malim and Cullen, 1991; Mann et al., 1994).

The Rev ARM, like ARMs of other RNA-binding proteins, is highly adaptable to different nucleic acid targets. Selection experiments have identified both RNAs and DNAs with imperfect double helices that bind the Rev ARM with high affinity and specificity (Bayer et al., 2005; Landt et al., 2005; Xu and Ellington, 1996), utilizing different amino acid side chains to contact the RNA and, in some cases, different surfaces of the helical ARM or different peptide backbone conformations (Landt et al., 2005; Ye et al., 1999). This "chameleon" binding behavior of ARMs in principle can allow a protein to readily adapt to a new RNA site, particularly in a rapidly evolving virus (Bayer et al., 2005; Smith et al., 2000), but no natural example has been described. Inspection of the RRE reveals numerous intriguing bulge structures and other helical disruptions suggestive of the types of sites found in the selection experiments (Figure 1B). Indeed, these disruptions appear to be important for higher order Rev assembly on the RRE (Kjems et al., 1991; Zemmel et al., 1996), while amino acids in the ARM other than those used for IIB recognition are highly conserved among viral isolates (Figure 1A), suggesting that other binding sites and perhaps alternative binding modes may be used in assembling the functional Rev RNP.

Here we present evidence that Rev forms a cooperative, high-affinity (~100 pM) oligomeric complex with the RRE, nearly three orders of magnitude tighter than the single binding site interaction previously reported. Assembly requires the Rev oligomerization domains and multiple specific RNA sites that bind Rev with moderate affinities, including IIB and an additional site in stem IA. Interestingly, Rev shows chameleon-like binding behavior in which different Rev monomers of the oligomer bind in different modes to at least one other site. We suggest that, to overcome a limited genome size, the virus has evolved a mechanism to

![Figure 1. Rev Protein and RRE RNA](image-url)
form a large, defined RNP using only a single protein with an ARM domain flexible enough to adapt to each site. Further, we have shown a strong correlation between the affinity of the oligomer and RNA export, suggesting that Rev oligomerization is needed to create a stable export-competent RNP that helps regulate the timing of the HIV life cycle.

**RESULTS**

**A High-Affinity, Cooperative Oligomeric Rev-RRE Complex**

Previous in vitro binding experiments have shown that the number of Rev monomers bound to the RRE increases as the RNA is lengthened from the IIB hairpin alone to a complete 250–350 nt element but that its affinity remains approximately constant at 20–100 nM (Cook et al., 1991; Heaphy et al., 1991; Huang et al., 1991; Iwai et al., 1992; Malim and Cullen, 1991; Mann et al., 1994; Tiley et al., 1992). The affinity of a minimal Rev ARM peptide-IIB complex is also similar (Tan et al., 1993), leading to models in which one Rev subunit binds to the IIB site and then nucleates binding of additional subunits through protein-protein and nonspecific RNA interactions (Heaphy et al., 1991; Jain and Belasco, 2001; Malim and Cullen, 1991; Mann et al., 1994). In contrast to those previous studies, we have found that Rev purified under native conditions, in which Rev is maintained bound to RNA throughout the purification, has much higher affinity for the full RRE. As the RRE is lengthened from the IIB hairpin to an IIABC three-helix junction to a 242 nt RRE, we observe a large, progressive increase in Rev binding affinity, with 500-fold higher affinity for the full RRE element versus IIB (Figures 2A, 2B, and 2E). Moreover, distinctly cooperative behavior is seen with the larger fragments, with a >3-fold increase in the Hill coefficient and little monomer or dimer observed with the full RRE.

Mutation of the Rev oligomerization domains disrupts function, but not its ability to recognize IIB (Edgcomb et al., 2008; Jain and Belasco, 2001; Malim and Cullen, 1991), and we wished to determine whether the loss of function might reflect a loss of high affinity, cooperative REV binding. We first compared full-length REV binding to the 17 amino acid ARM alone, expected to bind noncooperatively, by fusing the Rev ARM peptide to the B1 domain of
streptococcal protein G (GB1) to increase its molecular mass to ~11 kDa and thereby allow us to observe a mobility shift with the full RRE. The GB1-fused Rev ARM binds to the isolated IIB hairpin with similar affinity (30–50 nM) as Rev (data not shown), consistent with previous observations with the ARM peptide. However, in marked contrast to Rev, the ARM binds noncooperatively to the full RRE and with little increase in binding affinity (Figures 2C and 2D). The small increase in binding affinity likely reflects the entropic benefit of an increased number of binding sites for individual subunits or small differences in the folding of the IIB hairpin in the larger RRE context. Interestingly, previously described single point mutations in the oligomerization domains, L18Q and L60R (Jain and Belasco, 2001), show similarly deficient binding characteristics (Figures 2C and 2D and data not shown), underscoring the importance of proper protein-protein interactions for cooperative, high-affinity binding to the RRE.

Even a minimized binding site, such as an extended IIB hairpin, can cooperatively bind two Rev subunits, provided that the RNA sites are correctly juxtaposed and the oligomerization domains are intact. Placing a second helical disruption in an extended IIB hairpin permits binding of a second Rev subunit (IIB 40-mer, Figure 2A) as has been observed previously (Zemmel et al., 1996); however, the high affinity for the full RRE compared to IIB alone suggested that regions outside of IIB also may contribute to Rev recognition. Indeed, we find that introducing an A73G mutation into the isolated IIB hairpin reduces Rev affinity 100-fold and ablates specificity relative to an antisense hairpin (Figures 3A and 3C) as previously observed (Jain and Belasco, 1996), whereas in the context of the full RRE, affinity is reduced only 40-fold and still is 100-fold tighter than an antisense control (Figures 3B and 3C). Moreover, the affinity for the mutated RRE is still 10-fold higher than for the wild-type IIB hairpin alone (Figure 3C) and shows reduced levels of monomer and dimer accumulation (see below and Figure S2). Importantly, the extended RRE not only permits high-affinity binding but also allows the Rev protein to distinguish the viral RNA from other RNAs, with a 40-fold increase in specificity compared to IIB alone. Thus, binding to IIB accounts for only a fraction of the RRE affinity and specificity, suggesting that Rev may also recognize additional sites.

**Identification of a Second Specific Rev Binding Site in the RRE**

In vitro selection experiments have identified nucleic acid motifs other than IIB that bind Rev ARM peptides with high affinity and specificity (Landt et al., 2005; Xu and Ellington, 1996). Bulge, internal loop, and stem junctions generally appear to be favorable for binding, and inspection of the RRE reveals many potential binding sites (Figure 1B). Some of these regions are protected from nuclease upon Rev binding (Kjems et al., 1991), and the junctions formed along stem I are important for Rev oligomerization (Mann et al., 1994; Zemmel et al., 1996), but no specific sites have been characterized. To identify such additional specific
binding sites, we generated small RNA models attempting to mimic the junction regions between stems I, II, III/IV, and V (Figure 1B) and found that an internal loop region in stem IA bound the Rev ARM with only 5-fold lower affinity than IIB (Figure 4A). We observed conformational rearrangements in a three-helix model but no tight binding to the stem IIC, III/IV, or V models (data not shown). It is likely that some of these molecules fold incorrectly in the small RNA contexts and do not accurately mimic the binding conformations present in the full RRE.

Like IIB, the stem IA binding site contains an asymmetric purine-rich internal loop that we surmised would be important for binding, and indeed, deleting the loop reduced binding of the Rev ARM 5-fold to nonspecific levels (Figure 4A). This reduction is not due to a gross perturbation of IA structure, as conservative mutations that preserve the purine content but change their sequence either on the 5’ or 3’ side, or both sides reduces binding to the same extent as the deletion (Figure 4A and data not shown). The same 5’ side substitution engineered into the full RRE significantly reduces affinity of the Rev oligomer even though the IIB site is completely intact (Figures 4B and 4C). Similar to the IIB binding site, the 5-fold decrease in affinity of Rev for the IA-mutated RRE mirrors the 5-fold decrease observed with minimal ARM peptide binding to the mutated IA hairpin. Thus, the stem IA site represents the first characterized specific Rev binding site outside of stem IIB and is needed in conjunction with IIB, and probably other sites, to generate the high-affinity Rev oligomeric RNP.

The Rev Peptide Binds to Stem IA Differently Than to Stem IIB

ARM peptides generally can adapt to their nucleic acid binding sites, and the Rev peptide in particular has been shown to use different amino acids and, in some cases, different surfaces of the α-helix to make essential contacts to binding sites selected in vitro (Landt et al., 2005; Xu and Ellington, 1996; Ye et al., 1999) (S.G. Landt, M.D.D., A. Ramirez, and A.D.F., unpublished data). This binding mode adaptability has not been functionally characterized in a biological setting, but the presence of at least one other specific binding site in the RRE raised the possibility that Rev may bind to IA in a manner different than IIB.

When binding IIB, Rev uses the carboxamide moiety of Asn40 to hydrogen bond to the two purines of the A73:G47 base pair, and mutation at that position causes a 40-fold reduction in binding affinity (Tan et al., 1993). Given the purine-rich internal loop of IA, we asked if this amino acid also is essential for IA binding. Interestingly, mutation of Asn40 had no effect on IA binding. Conversely, mutation of Arg41, which does not affect IIB binding (Tan et al., 1993), showed a reproducible 2-fold decrease in IA affinity (Figure 5A). R38A and R46A mutants showed similar 2-fold reductions in IA affinity but had no effect on IIB, while R43A, R44A, and W45A mutants bound IA like the wild-type peptide. Helical wheel projections clearly show that different surfaces of the helix are used to recognize IIB and IA RNAs (Figure 5B).

We further characterized the differences in IA and IIB binding modes using amide proton chemical shifts from 15N HSQC NMR spectra to monitor changes in the peptide-RNA interfaces. There is little peak dispersion of peptide resonances in the absence of RNA (Figure 5C), indicative of a largely unstructured molecule. However, we observe substantial amide peak dispersion in the presence of either IIB (red) or IA (blue) RNAs (Figure 5C), including the Hc protons of arginine side chains.
The overall upfield shifts observed in both $^1$H and $^{15}$N dimensions is consistent with stabilization of peptide α-helical structure upon binding (Wang and Jardetzky, 2002). Strikingly, the chemical shift patterns are quite different when bound to stem IA or IIB, including peaks that are perturbed in the IA, but not in the IIB, complex. Most notable is H$_3$ of Trp45, which is positioned opposite the binding interface in the IIB complex (Battiste et al., 1996) and, thus, is not shifted, while it probably is at or near the interface in the IA complex. This face of the α-helix also includes Arg38, Arg41, and Arg46, identified by mutagenesis as important for the IA interaction (Figure 5B), further highlighting the chameleon-like binding behavior of the Rev peptide when bound to the two sites.

**Binding Mode Adaptability Is Important for Rev RNA Export Activity**

Based on the in vitro peptide binding behavior, we wished to test whether Rev acts as a chameleon in which a combination of binding modes is used to assemble its oligomeric RNP. If so, we would expect that amino acids apparently located at the IA interface (Figure 5B) might be important for oligomeric binding and Rev function, while other residues important for IIB binding would not be as crucial as previously thought. Indeed, R41A, R42A, and W45A mutations in the full-length Rev context, which do not affect IIB binding, reduce oligomeric Rev affinity 5- to 100-fold (Figure 6A), while N40A still binds the RRE with 6 nM affinity even though it is essential for IIB recognition (Jain and Belasco, 1996; Tan et al., 1993). Interestingly, the N40A mutant, like the IIB A73G mutant, shows reduced levels of monomer and dimer species (Figure S2), suggesting that these are intermediate Rev-IIB complexes that are not absolutely required to form the complete Rev-RRE RNP.

Given earlier studies showing that oligomeric binding is essential for RNA export (Malim and Cullen, 1991; Pollard and Malim, 1998), we asked whether formation of the high-affinity complex in vitro correlates with formation of an export competent complex in vivo. Using a Rev reporter assay in which the RRE has been engineered in the intron of a chloramphenicol acetyltransferase (CAT) gene such that Rev-mediated export of the unspliced mRNA is required for expression (Huang et al., 1991), we observed a striking correlation between oligomeric RNA-binding affinity in vitro and Rev function in vivo (Figures 6B and 6F). Mutation at the crucial N40 position, which binds IIB with 80- to 350-fold lower affinity (Jain and Belasco, 1996), in fact retains ~40% activity, a value similar to the R41A mutant that binds IA weakly. Moreover, the W45A mutant that forms the lowest affinity oligomeric complex is functionally the most deficient. We next tested the activities of these mutants in a more native RRE context, using a pCMV-GagPol-RRE reporter in which HIV-1 Gag expression requires RRE-mediated mRNA export (Srinivasakumar et al., 1997). Western blot analyses of p55 and
Gag (Figure 6D) show similar effects with the Rev mutants as the CAT assays and correlate well with in vitro binding affinities (Figure 6F). These data are consistent with the hypothesis that Rev binds the RRE using multiple binding modes.

As further confirmation that high-affinity, oligomeric binding is relevant to Rev function, we found that oligomerization mutations (L18Q and L60R) that ablate high-affinity complex formation also have lower (3-fold) export activities using CAT and Gag reporters (Figures 6B and 6D). Moreover, a mutation in IIB still retained 30% activity (Figures 6C and 6E), as previously observed (Jain and Belasco, 1996), suggesting that other binding sites still support partial function. A mutant with purine substitutions in the 3' side of the stem IA internal loop showed a slight decrease (10%) in activity (Figure 6C), consistent with its slight decrease in affinity (see Figure 3), and a double mutant in both IIB and IA showed a slight additive effect, consistent with a functional role for both sites. Thus, mutations in RNA-binding residues of the ARM, the oligomerization domains, and the two identified RNA-binding sites all show an excellent correlation between high-affinity, oligomeric RNA binding in vitro and activity in vivo (Figure 6F), supporting the hypothesis that Rev oligomerization is needed to create a stable, export-competent RNP.

**DISCUSSION**

The Rev protein has been intensively studied for many years, and while much has been learned about the interaction of the helical ARM peptide with the RRE IIB site, the mechanism of assembly and role of the larger oligomeric complex have been less well defined. Previous results suggested that the ARM-IIB interaction contributed most of the affinity and specificity to complex formation, leading to models in which homo-oligomerization is required for steps in RNA export other than assembly of the Rev-RRE RNP, such as enhancing the Rev-Crm1 interaction (Askjaer et al., 1999). However, we now provide evidence that oligomerization on the RRE is required to assemble a complex nearly three orders of magnitude tighter than the IIB interaction, cooperatively assembling a few weakly binding subunits (KD > 20 nM) at specific sites on the RNA scaffold, including stem IIB and a previously uncharacterized site in stem IA. It also was shown previously that a large portion of the RRE is required for RNA export, as well as Rev subunit interactions (Hope et al., 1990; Huang et al., 1991; Jain and Belasco, 2001; Malim and Cullen,
Molecular Cell
Assembly of a High-Affinity Rev-RNA Complex

Figure 7. Strategies for Assembling Protein-RNA Complexes
Schematic representation of three strategies for forming protein-RNA assemblies and aspects of each that contribute to forming the homo-oligomeric Rev-RRE RNP. Solid blue lines represent RNA, with protein binding sites highlighted in red. Different shading of the binding sites indicates that the sequence or structural motifs differ between sites. Colored ovals represent different proteins or protein domains. In the Rev-RRE complex, different shading of the Rev subunits represents different binding modes at several sites in the RRE.

Disease Virus Tat ARM domain, where the RNA-binding domains are able to adopt different conformations in the context of different RNA sites, arguing that adaptable binding may be well suited to rapidly evolving new RNA specificity in viruses (Amarasinghe et al., 2000; Smith et al., 2000). In the Rev case, this property has been biologically exploited such that different portions of the RRE structure may have been “selected” during viral evolution to bind different surfaces of the ARM, which overlaps with the coding frame of the env gene, much like how in vitro selection can identify a variety of tight-binding aptamers. The large RRE scaffold, which also is constrained by the env gene, presumably then presents these multiple binding sites in an arrangement compatible with the subunit orientations defined by the oligomeric protein-protein interfaces (Jain and Belasco, 2001). The importance of a proper binding site arrangement is underscored by the observations that concatenated arrays of an IIB hairpin or aptamer do not function as well as the wild-type RRE despite the high affinity of the individual sites (Kjems and Sharp, 1993; Symensma et al., 1999). Thus, the proper configuration of binding sites within a larger RNA context allows the Rev oligomer to recognize the RRE and form an organized RNP that exports RRE containing viral RNAs over non-RRE containing RNAs with a very high degree of specificity.

The homo-oligomeric Rev complex demonstrates that even a small, 14kDa protein, constrained by the limited genome size of a virus, can generate a rather substantial RNP (200–300 kDa) with the diversity of protein-RNA contacts more characteristic of multidomain or hetero-oligomeric complexes (Figure 7). It long has been known that covalently tethering RNA-binding domains, such as RRMs, within a single protein can increase RNA-binding affinity and specificity (Lunde et al., 2007). Individual contributions can be dissected for each RRM (Perez et al., 1997; Shamoo et al., 1995), and the entropic benefits and costs of tethering may be determined by domain orientations that are fixed in some cases by protein-protein contacts and in others only when bound to an RNA scaffold. Similar effects are seen with DNA-binding proteins, especially for dimeric arrangements in which subunits are spatially oriented to match the array of binding sites on the DNA, such as with nuclear receptors or GAL4-related proteins (Marmorstein and Fitzgerald, 2003). In contrast, large RNP assemblies, such as the ribosome, typically...
rely on more complex sets of protein-protein interactions between hetero-oligomers, as well as a more complex RNA scaffold (Ban et al., 2000; Hainzl et al., 2002; Shen and Green, 2004). Rev appears to have achieved a rather extraordinary level of structural organization by flexibly utilizing its 116 amino acid chain to recognize multiple RNA sites in a homo-oligomeric RNP context.

**EXPERIMENTAL PROCEDURES**

**Plasmids, RNAs, and Peptides**

Proteins were expressed in *E. coli* using pHGB1, a plasmid derived from pHis-GB1-parallel1, which introduces an N-terminal Hiss tag, followed by the B1 domain of streptococcal protein G (GB1) to increase solubility, followed by a TEV cleavage site before the coding region (Harper et al., 2003). Details of pHGB1 construction can be found in the Supplemental Experimental Procedures. Full length Rev (1–116) and the Rev ARM (34–50, with a C-terminal pentapeptide AAAAR to stabilize the ζ-helix) were cloned into pHGB1 immediately downstream of the TEV site. Mutants were generated using standard site-directed mutagenesis (Stratagene).

To generate RRE RNAs, a 242 nt portion of the RRE from pDM128 (Huang et al., 1991) was cloned between the NotI and EcoRI sites of a pBluescript-KS+ vector downstream of the T7 promoter. RNAs were produced by T7 RNA polymerase in vitro run off transcription from plasmid templates linearized by EcoRI or, for shorter fragments, using synthetic DNA templates as described in the Supplemental Experimental Procedures. For radiolabeling, RNAs were dephosphorylated using alkaline phosphatase, 5’ end-labeled using T4 polynucleotide kinase and γ-32P ATP (New England BioLabs), and separated from unincorporated ATP using NucAway spin columns (Ambion). Long and short RNAs were purified on denaturing 4% and 10% or 12% polyacrylamide/8 M urea gels respectively and annealed by heating at 95°C for 2 min and slow cooling to room temperature in renaturating buffer (20 mM Tris pH 7.5, 100 mM NaCl).

The Rev ARM peptide (residues 34–50), capped at the N terminus by a succinyl group and at the C terminus by AAAAR at the C terminus of streptococcal protein G (GB1) to increase solubility, followed by a TEV cleavage site on Applied Biosystems Model 433A peptide synthesizer and standard Fmoc chemistry. Wild-type peptide (succ-TROQARRNRRRRWRERQAAAAR) was synthesized using Rink amide resin on a Applied Biosystems Model 433A peptide synthesizer and standard Fmoc chemistry. Wild-type peptide (succ-TROQARRNRRRRWRERQAAAAR) was synthesized using Rink amide resin and was cleaved from the resin, deprotected and purified by HPLC on a C18 reverse-phase column using an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA).

**Rev Protein and Peptide Purification**

Proteins were expressed in *E. coli* strain BL21/DE3 from pHGB1-derived vectors as N-terminal fusions with a Hiss tag, GB1 domain and a TEV-protease cleavage site. Cells were grown to OD600 of 0.8 at 37°C in LB medium with 100 μg/ml ampicillin. Isopropyl-β-D-thiogalactopyranoside was added to 1 mM to induce expression by shaking for 4 hr at 37°C, and harvested cells were resuspended in lysis buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 0.1% Tween -20, 2 mM [i-mercaptoethanol (i-ME), 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail [Roche]]. The suspension was incubated on ice for 20 min with 1 mg/ml lysozyme and frozen in liquid nitrogen. After thawing and sonication, cell debris was removed by centrifugation at 14,000 rpm for 30 min.

Full-length Rev was purified by Ni-NTA affinity chromatography and cleaved by TEV using standard procedures. Briefly, supernatant from the cell lysate was applied to Ni-NTA superflow resin. (QIAGEN) equilibrated with buffer A+ (50 mM Tris 8.0, 250 mM NaCl, 0.1% Tween-20, 2 mM i-ME, and 10 mM imidazole). The resin was rinsed with buffer A+ then buffer A (buffer A+ without Tween-20). A stepwise elution was performed using buffer A with increasing concentrations of imidazole. Fractions were analyzed by SDS-PAGE, pooled, dialyzed in buffer B with 2 mM DTT, aliquoted, and frozen. The resulting protein retains the N-terminal Hiss-GB1 tag, and any uncleaved protein. Full length Rev, with an N-terminal GA dipeptide, was collected in the flow-through, DTT was added to 2 mM, and protein was quantified by SDS-PAGE (generally 0.05–0.2 mg/ml) and immediately aliquoted and frozen in liquid nitrogen. A significant absorption peak at 260 nm and bands observed on ethidium bromide stained agarose gels indicated that large amounts of nonspecific *E. coli* RNA copurified with Rev under these native conditions.

A Hiss–GB1 fused Rev ARM peptide was purified similarly, with a modification to remove nonspecific RNA. RNase A (50 μg/ml) and T1 (50 U/ml) (Roche) and NaCl to 2 M were added to the cleared lysate, and buffer A+ contained 2 M NaCl. Buffer A and elution buffers contained 250 mM NaCl as above. Following elution, fractions were analyzed by SDS-PAGE, pooled, dialyzed in buffer B with 2 mM DTT, aliquoted, and frozen. The resulting protein retains the N-terminal Hiss-GB1 tag, GAMMA appended to the N-terminus of the Rev ARM, followed by AAAAR at the C terminus. The binding affinity for RRE IIB was comparable to the synthetic Rev ARM peptide described above (data not shown).

For NMR experiments, the Hiss–GB1 fused Rev ARM peptide was grown in M9 minimal media supplemented with trace minerals, thiamine, and 15N,14C as the sole nitrogen source. The protein was purified as above and incubated with TEV protease for 1 hr. The resulting peptide (GAMATRQARRNRRRWRERQAAAAR) was purified by HPLC as above.

**RNA-Binding Gel Shift Assays**

Electrophoretic mobility shift assays were performed in binding buffer (10 mM HEPES pH 7.5, 100 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 10% glycerol, and 50 μg/ml yeast tRNA [Sigma-Aldrich]). Full-length Rev proteins and His5–GB1 tagged Rev ARM were diluted serially in buffer (5 mM Tris pH 8.0, 20 mM NaCl, and 200 μg/ml bovine serum albumin) and combined with an equal volume of <10 pM radiolabeled RNA. Reactions were incubated at room temperature for 20 min and loaded onto continuously running 8% or 10% polyacrylamide (37:1:1:5 mono:bis, 0.5 x TBE) gels. Gels were run at room temperature for 1–4 hr depending on the RNA size, dried, and exposed to a Phosphorimaging plate for >12 hr. Binding assays of synthetic Rev ARM to stem IA were performed similarly except that the peptide was serially diluted in water, and binding reactions and gel separation were performed at 4°C. Bands were quantified with a Molecular Dynamics Phosphorimager and Imagequant software. Binding constants were calculated by measuring the fraction of all bound RNA species compared to total RNA, fitting the data to binding curves using Kaleidagraph software (Synergy Software, Reading, PA), and are reported as the mean ± standard deviation of two or more replicates.

**NMR**

All 15N-resolved HSQC NMR experiments were conducted at 283K on a Bruker BioSpin DRX 800 MHz spectrometer equipped with a cryoprobe. All samples contained 200 μM HPLC-purified uniformly 15N labeled Rev ARM peptide in 25 mM HEPES pH 6.5, 100 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, and 10% D2O, and contained stem IA or IIB RNAs at 200 μM. Spectra were processed with NMRPIPE (Delaglio et al., 1995) and analyzed with SPARKY (Goddard and Kleyler, 2007).

**RNA Export Reporter Assays**

For CAT assays, HEo cells were cotransfected with 24-well plates with Polyfect (QIAGEN) and 25 ng pDM128 CAT reporter plasmid (Huang et al., 1991), 5 ng pSV2-Rev expression vector, and 2.5 ng pRL-CMV luciferase plasmid (Promega) as a transfection control. After 48 hr, cells were lysed with passive lysis buffer, and CAT activity was measured and normalized to luciferase levels (Promega). Relative fold activation was calculated as CAT values normalized to the luciferase values, relative to reporter without Rev, and setting the activation level of wild-type Rev and RRE to 100. Assays were performed in triplicate, and data are presented as the mean ± standard deviation.

For Gag western blot analyses, HEoLa cells were cotransfected with 100 ng pCMV-GagPol-RRE reporter plasmid (Srinivasakumar et al., 1997) and 100 ng pSV2-Rev expression vector. After 48 hr, cells were lysed and samples were separated by SDS-PAGE, transferred to nitrocellulose, blocked overnight with a mouse anti-p24 monoclonal antibody (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) or mouse anti-β-actin followed by goat anti-mouse IgG-HRP (Santa Cruz Biotechnology), and developed with SuperSignal West Pico chemiluminescence (Thermo Scientific).
SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supple-
mmental References, and two figures and can be found with this article online
at http://www.molecule.org/cgi/content/full/31/6/824/DC1/.

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