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A structurally plastic ribonucleoprotein complex mediates post-transcriptional gene regulation in HIV-1

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Abstract

HIV replication requires the nuclear export of essential, intron-containing viral RNAs. To facilitate export, HIV encodes the viral accessory protein Rev which binds unspliced and partially spliced viral RNAs and creates a ribonucleoprotein complex that recruits the cellular Chromosome maintenance factor 1 export machinery. Exporting RNAs in this manner bypasses the necessity for complete splicing as a prerequisite for mRNA export, and allows intron-containing RNAs to reach the cytoplasm intact for translation and virus packaging. Recent structural studies have revealed that this entire complex exhibits remarkable plasticity at many levels of organization, including RNA folding, protein–RNA recognition, multimer formation, and host factor recruitment. In this review, we explore each aspect of plasticity from structural, functional, and possible therapeutic viewpoints.

INTRODUCTION

HIV-1 transcribes a single 9-kb RNA in order to encode approximately 15 different proteins.[1] However, this RNA harbors multiple splice sites and complete processing of the RNA produces only three viral proteins, one of which is the viral accessory protein Rev. Rev facilitates the nuclear export of unspliced or partially spliced viral RNAs thereby allowing production of the remaining viral proteins, as well as supplying cytoplasmic full-length viral genomes that can be packaged into nascent particles (Figure 1).

In order to achieve this function, Rev relies on several other key players, most notably a viral RNA element known as the RRE (Rev-Response Element) and the host export factor Crm1 (Chromosome maintenance factor 1 also known as exportin-1 (XPO-1)). In brief, after Rev is translated, it is imported into the nucleus via the nuclear localization signal (NLS) present in its arginine-rich motif (ARM). Once in the nucleus, Rev oligomerizes along the RRE via two hydrophobic oligomerization domains (ODs), and this Rev-RRE ribonucleoprotein complex (RNP) then recruits the Crm1 receptor and its cofactor RanGTP via the multiple nuclear export sequences (NESs) present in the Rev oligomer (Figure 2(a)). This export-competent Rev-RRE-Crm1 RNP then guides the RNAs through the nuclear pore to the

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cytoplasm where they can be used in downstream processes essential for viral replication. Several reviews that provide broader and historical perspectives of Rev function are recommended.[2–7]

In this review, we examine the core functional unit of Rev-mediated export, the export-competent RNP, by detailing a set of simpler interactions that occur between each of its constituent parts: the RRE, the Rev oligomer, and Crm1 (Figure 2(b)). Remarkably, each type of interaction displays a high degree of structural plasticity that can tune both the affinity and activity of the entire complex, affecting viral replication and manifesting in disease progression in patients. Furthermore, the inherent plasticity of the system allows a high degree of mutational robustness, permitting this essential process to persist in the face of HIV's high mutation rate, and raising intriguing questions for therapeutic targeting.

THE RRE IS A FLEXIBLE RNP SCAFFOLD

The RRE is a large, multi-hairpin ~350-nucleotide (nt) RNA structure near the 3′ end of the HIV genome and is present in all RNA species exported by Rev.[2,8] The RRE functions as an assembly platform for the RNP, utilizing a combination of RNA structure and sequence elements to direct the formation of the Rev oligomer and consequently the entire export complex. Because the RRE serves this essential scaffolding role, its plasticity influences all subsequent steps of RNP assembly.[9]

Secondary Structure of the RRE

The RRE exists in multiple conformational states having either four or five stem loops (named Stems I–V) that all protrude from a central junction. Early chemical probing experiments together with RNA folding algorithms predicted a five-hairpin structure, or several four-hairpin structures in which Stems III and IV form a single stem (Stem III/IV) with alternative Stem V structures[10–15] (Figure 3). More recently, electrophoretic isolation of RRE conformers has shown that the RRE from the NL4-3 viral isolate exists as a mixture of the four and five hairpin conformations.[18] Functional studies demonstrated that the five-hairpin version has higher RNA export activity, suggesting that alterations in RNA conformation, whether arising from sequence changes or from natural conformational heterogeneity, can tune the export activity of the RNP complex.

Functionally Required Regions of the RRE

Mutational studies of the RRE have explored the minimal requirements for RRE function. Early studies established that the majority of Stem I can be truncated to produce a functional 242-nt element,[19,20] but recent studies have shown that this deleted region is able to form tertiary interactions with the central junction[12,16] (Figure 3). The most important region of Stem I (Stem IA) contains a purine-rich bulge that is required for full activity and is one of three well-characterized Rev-binding sites.[21]

Stem II harbors the other known Rev-binding sites. Like IA, Stem IIB also features a purine-rich bulge. This bulge serves as the nucleation site for Rev binding along the RRE[10,22] and widens the major groove of the mostly A-form helix to accommodate the α-helical ARM of Rev.[23,24] Adjacent to the Stem IIB bulge is a junction between Stems IIABC that opens the

RNA to form a second binding site for Rev.[25,26] Other discrete secondary binding sites likely exist along the RRE, but remain challenging to identify as their recognition may depend on the structural organization of the RNP instead of the sequence. The range of secondary structures proposed for the RRE and the existence of multiple RNA conformers further complicates site identification absent a three-dimensional (3D) structure of the full Rev-RRE complex.

Three-dimensional Structure of the RRE

A recent 3D model of the ~250-nt version of the RRE, determined from small angle x-ray scattering (SAXS), has filled some gaps in our structural understanding.[27] This model shows the RNA folding with a topology that resembles the letter `A' (Figure 4(b)), with Stem IIB and Stem IA close together in space. The proximity of these sites led to the hypothesis that a Rev dimer, described in detail below, may bind simultaneously to the Stem IIB and Stem IA sites. While AFM[29] and SHAPE[16] data are consistent with this overall RNA topology, the structure may be more dynamic than implied by the SAXS model. As noted above, there is evidence for at least two RRE conformers,[18] and the SAXS data may reflect the solution average of multiple structures. Furthermore, different RNA conformers may be stabilized by magnesium, a property that is observed in large folded RNA molecules,[30,31] which may explain why the SAXS density for Stem II was observed to decrease upon magnesium addition. Although other regions in the overall SAXS-derived model of the RRE were generated from samples in the absence of magnesium and are unlikely to undergo large conformational changes, the dynamic nature of the RRE makes detailed inferences about Rev-binding sites challenging. A better understanding of the arrangement of binding sites and the order of assembly of Rev on the RRE awaits the structure of the complete complex.

The RRE Scaffold Tunes Export Activity

While high-resolution crystallographic or EM structures could resolve some of these questions, they are likely to provide incomplete snapshots of functional export complexes, which may involve more than a single discrete RRE scaffold. Besides the conformational variation described above, different HIV-1 clades show subtle differences in the RRE that can substantially alter its structure, and mutations can accrue during viral replication that alter the functional fitness of the RRE. For example, Stem III/IV/V is a poorly characterized region that can tolerate deletions in experiments monitoring proviral gene expression,[8] but recent work exploring the longitudinal evolution of the Rev-RRE system has demonstrated the importance of this region in tuning Rev function. Experiments tracking changes in the RRE over a short course of infection from a small number of patients found several mutations that led to differential levels of Gag production.[32] Although the correlation between export and viral loads did not reach statistical significance due to the relatively small sample size, these data suggest that changes in the RRE can modulate the level of viral replication and possibly effect disease progression. However, the most striking example of the importance of the RRE scaffold comes from a study examining adaptive mutations in the context of a defective NES. In this study, an RRE mutant, RRE61, acquired G–A mutations (Figure 3) that changed the RRE fold[17] and partially compensated for the dominant negative effect of the otherwise defective NES.[17,33] Taken together, these studies suggest that the sequence and structural plasticity of the RRE accommodates a wide range of functional

activities, that this range may be tuned in a clinically relevant manner, and that RNA plasticity can influence subsequent protein assembly, such as Crm1 recruitment, on the RNP.

Selective Pressures Acting on the RRE

While the plasticity of the RRE may be useful in tuning the viral life cycle, additional selection pressures resulting from the genomic organization of the virus make such a flexible and robust organization even more advantageous. The RRE is located within the *env* gene, where it overlaps with the C-terminal end of gp120 and the N-terminal segment of gp41. This coding/noncoding overlap potentially constrains the evolution of the RRE, as mutations must maintain both Env and RRE function.[34] Furthermore, host immunity or Env-specific therapeutics can indirectly shape the evolution of the RRE. For example, antibodies have been isolated from infected patients that recognize Env epitopes that overlap with the RRE.[35] Additionally, Env escape mutations arising against the entry inhibitor enfuvirtide have been found to affect RRE activity.[36] Thus, the overlapping organization of the HIV genome creates multiple selective forces to constrain and drive RRE evolution. In this light, RRE plasticity might serve a dual role: first to tune function, and second to provide functional robustness in the face of mutations and positive selection.

PLASTIC ASSEMBLY OF THE REV OLIGOMER

Rev is the virally encoded protein component of the RNP. The majority of structural studies have been conducted with 116-amino acid versions of the protein found in standard lab isolates. In patients, Rev length can vary from 96 to 129 amino acids, with the majority of viruses harboring either 116- or 123 - amino acid versions and most other variants having changes C-terminal to the NES (hiv.lanl.gov). All known functional Revs contain a bipartite OD, an ARM, and an NES. One of the key features of RNP assembly is the manner in which this small subunit can adopt multiple configurations to recognize a wide variety of RNA sites and specifically recruit the host export machinery.

Variation in RNA Recognition

RNA–protein contacts in the Rev-RRE RNP are mediated through Rev's α-helical ARM.[37] As mentioned above, the specificity of Rev for the RRE is driven by a high-affinity interaction between Stem IIB and the Rev ARM.[23,38] This interaction serves as an anchor point for the recruitment of additional Rev molecules that ultimately form an oligomer on the RRE.[12,39,40] This oligomeric complex has been reconstituted as a Rev hexamer on the 242-nt RRE.[41] Although each subunit of the Rev oligomer contains identical ARMs, the mode of RNA recognition at each binding site in the RRE is unique.[21,26] Structural and thermodynamic studies of the three well-characterized binding sites (Stem-loop IIB, Stem IA and Junction IIABC) have revealed that different sets of Rev residues make varied contacts to the RNA in each case (Figure 4(a)). At the IIB site, the unpaired bases widen the major groove and the helical ARM orients to allow residues R35, R39, N40, and R44 to make base-specific contacts,[23,25] while several other ARM residues, notably arginines, contact the phosphate backbone. At the secondary binding sites (Junction IIABC and Stem IA), the RNA–protein contacts are largely electrostatic and utilize arginines on different faces of the helix to contact the backbone. Interestingly, the IIABC site buries the same face

of the ARM as IIB but makes sequence-independent phosphate contacts, whereas the IA site uses an entirely different helical face in which R38, R41, and R46 bind the RNA (Figure 4(a)). The electrostatic nature of these contacts may explain why identifying secondary Rev-binding sites by sequence analysis has been challenging: recognition at these sites appears to be largely dependent on the structure of the RNA and orientation of the ARM, not on the nucleotide sequence. Indeed, studies with ARM peptides and RNA fragments show much greater dynamics at the secondary sites, suggesting that the structural constraints imposed by the initial stable binding to IIB sets the orientation and consequent specificity at the secondary sites.[26] The arrangement of binding sites on the RNA scaffold explains the high cooperativity seen in Rev-RRE binding, with the full RRE having 500-fold tighter affinity than the ARM-IIB interaction,[21] whereas removing IIB reduces affinity at the secondary sites by 130-fold.[42] While these three established sites vary in their modes of recognition, they likely represent only a subset of possible Rev-recognition modes, with other recognition features at the remaining cryptic sites yet to be established (Figure 4(a)).

Order of Assembly

It is clear that assembly of the Rev oligomer begins with binding at IIB but subsequent steps of assembly are not as well understood. One model, based on the SAXS RRE structure discussed above, utilizes the proximity of the IIB and IA sites across the legs of the `A' in the SAXS model to position the second subunit of a Rev dimer.[27,43] In contrast, time-resolved SHAPE studies have shown that the initial-binding event at IIB is rapidly followed by binding at the nearby IIABC site,[16] consistent with the cocrystal structure of the Rev dimer-RNA Stem II complex.[25] One reasonable model is that the first Rev subunit binds at IIB, forms a dimer to bind at IIABC, and then oligomerization continues across the bridge to IA. The time resolution of these events suggests that intermediate-binding states may act as specificity checkpoints,[16] ultimately leading to an RNA conformation that supports bridging to Stem I sites (Figure 4(b)). Another late-binding region in Stem I was identified by SHAPE experiments[16] which is present only in the longer ~350-nt RRE; this region may participate in later bridging interactions to fully stabilize the RNP.

Once the RRE is fully bound by six Rev subunits, it is unclear if additional subunits bind along the viral RNA or if there is a mechanism to cap further RNA binding. Indeed, the final oligomerization state *in vitro* appears to be a property driven by the length of the RNA and Rev concentration[12,41] and may explain the wide variety of reported nonhexameric Rev stoichiometries.[44–46] In the cellular context, it appears likely that the viral RNA is decorated with a variety of host factors such as hnRNP proteins, and these interactions may prevent further nonspecific binding of Rev along the RNA.[47] Furthermore, Rev concentration levels are regulated via negative feedback—as successful export of Rev-dependent RNAs decreases the pool of short Rev-encoding RNAs—and this regulation may prevent runaway oligomerization[48] and correctly time complex assembly in a replication context. Future CLIP-SEQ studies of Rev and host RNA-binding proteins will likely shed light on these questions.

Rev Oligomerization

Although the Rev ARM has the ability to contact RNA in a variety of different ways, the orientation in which the ARM is presented to the RNA is dependent on the construction of the Rev oligomer. Recent structural work has revealed that, just as the ARM has variable modes of RNA recognition, the Rev oligomerization surface has multiple modes of interaction that impart the Rev oligomer with a large degree of pliability.

Rev-Rev interactions are driven by a bipartite, hydrophobic OD having two distinct surfaces: a core dimerization surface, also known as the tail, and a higher order, multimeric surface, also known as the head.[49,50] Elegant biochemical studies showed that mutations in the multimeric surface trap Rev in a dimeric state on the RRE while mutations in the dimeric surface lead to monomeric binding, suggesting that the dimer unit forms first.[49,50] Crystal structures of each interface solved in the non-RNA-bound state further elucidated the mechanism of oligomerization.[43,51] In both interfaces, Rev monomers cross each other in a splayed fashion (Figure 5). The multimeric `head' interface is composed of hydrophobic contacts near the termini of the oligomerization helices: L12, L13, V16, I19, and L60. As a result of the terminal interactions, these monomers have a relatively `open' 140° crossing angle[51] (Figure 5). In contrast, the core dimer, or tail, interface is maintained by hydrophobic interactions from comparatively interior residues L18, F21, L22, I55, and I59. In the dimer structure, monomers cross at a ~120° angle—slightly less open then the multimer surface[43] (Figure 5). Together these structures demonstrate that the organization of monomers via oligomerization propagates the variation in the relative orientation and distance between each monomer's ARM. Most recently, a crystal structure of the Rev dimer (tail) interface bound to two RRE sites (IIB and Junction IIABC) demonstrated that additional variation occurs via the plastic rearrangement of these surfaces.[25] In this structure, the affinity gained through RNA binding allows further rearrangement of the dimer surface to produce a novel crossing angle of ~50° resulting in a relatively `closed' dimer conformation (Figure 5). This remodeling demonstrates that this flexible and `greasy' hydrophobic surface is capable of drastic rearrangements. This rearrangement is likely not specific to the dimer surface, as the multimer surface is also anticipated to rearrange and stabilize its conformation upon RNA binding (Figure 5).

Together, these three orientations of Rev-Rev complexes demonstrate that the Rev oligomer can be used to generate a wide variety of possible assemblies. The flexibility allows the Rev-RRE complex to take full advantage of, and amplify, the plasticity observed for ARM-RNA recognition. In this way, the initial nucleation event can direct secondary binding events on the RRE scaffold via both ARM and oligomeric plasticity. This plasticity, which is an inherent property of hydrophobic surfaces that can pack in multiple ways, is likely helpful not only in recognizing a wide variety of possible RRE conformations, but also in allowing several possible intermediates during RNP assembly. Indeed, the three currently observed dimer conformations are likely only a subset of the possible and biologically relevant conformations. For instance, a novel contact that occurs in the RNA-bound dimer (between Q51 residues) does not appear to be essential in the context of the complete complex (as viruses harboring a Q51 A mutation demonstrate no major defect in replication), suggesting

that there are multiple assembly pathways and conformations that can occur, depending on the presence or absence of specific contacts.[25]

Understanding the Rev-RRE RNP as a Single Cofolded Unit

Ultimately, the formation of the Rev-RRE RNP is driven by the contributive affinities of each ARM in the oligomer as they contact the tight-binding IIB site and other more cryptic sites in the RNA. The linkage between RNA binding and oligomerization can be viewed as a `coupled folding' process in which specific RNA-binding events remodel the Rev oligomer by repacking oligomerization surfaces to recognize a 3D-RNA scaffold. The resulting large homo-oligomeric RNP is the functional, fully folded viral macromolecule that recruits Crm1. In this light, Rev-RRE RNP formation can be compared to DNA-binding proteins that, upon nucleic acid binding, achieve sequence-specific conformations that determine their function.[52] Indeed, experiments that replace the RRE with tandem repeats of the high-affinity IIB site fail to recapitulate wild-type Rev-RRE activity,[53] suggesting that overall complex architecture is a key component for Rev's cellular functions.

Finally, the notion of coupled folding is further reinforced by single-molecule studies demonstrating that the RNA initiates specific Rev oligomerization, despite the propensity of Rev to oligomerize on its own. These studies show that Rev is recruited to the RRE in several discrete subunit steps, and not as a fully formed oligomer.[42,54] While Rev appears to be recruited to the RRE as a monomer, the stability of the core dimer interface[43,50] and the temporal proximity of binding events in kinetic SHAPE studies[16] suggest that core dimer assembly on the RRE occurs relatively quickly. Regardless, Stem IIB binding is the first event necessary to trigger this assembly, demonstrating a hierarchical assembly pathway that directs Rev oligomerization.

Additional Selection Pressures on Rev

Similar to the genomic overlap between the RRE and Env, Rev also shares coding information with other HIV proteins. The first exon of HIV *rev* overlaps entirely with *tat*, while the second exon of *rev* overlaps with both *tat* and *env*. These additional selection pressures may help explain the plastic and robust architecture of the Rev oligomer: the same structural flexibility that allows a wide range of RRE recognition may also allow Rev to accommodate selection pressures from overlapped reading frames. These confounding pressures, combined with Rev's own robustness, make it difficult to infer functional importance from simple sequence conservation.

A CRM1 DIMER RECOGNIZES THE REV-RRE COMPLEX IN MULTIPLE CONFIGURATIONS

Crm1 is the major host component of the export complex and is a karyopherin that is responsible for the nuclear export of a wide range of host substrates, predominantly proteins but also ribosomal RNAs and a specific subset of mRNAs.[55–57] Rev repurposes this normal cellular pathway to facilitate viral replication.

The Rev NES Interacts with Crm1 in a Noncanonical Manner

Rev hijacks the host nuclear import machinery by binding to Crm1 through its NES. In its GTP-bound form, the small G protein Ran acts as a cofactor for Rev binding by allosterically activating Crm1 to bind the Rev NES.[58–60] Crm1 recognizes a variety of NESs that have a motif of leucines, or other large hydrophobic residues, spaced 3–4 amino acids apart.[61] This degenerate motif allows Crm1 to recognize a variety of protein substrates in different orientations,[62] with different efficiencies of nuclear export.[63]

Similar to NESs found in host cargo proteins, the Rev NES contains regularly spaced leucine residues. However, there are key differences from host NESs that allow Rev to utilize a comparatively short peptide to establish contacts with Crm1 (Figure 6(a)). The Rev NES positions two proline residues between the first two leucines to effectively remove the first turn of the ∝-helix.[61] Additionally, the N-terminal proline establishes a hydrophobic contact typically made by a leucine in a canonical NES. In some HIV isolates, the Rev NES appears even more severely impaired for Crm1 binding, where the prolines are substituted with polar or charged residues.[66,67] It appears likely that these NESs are able to function by utilizing other compensating molecular contacts to facilitate complex formation.[66,67] Overall, Rev utilizes unconventional NES recognition to mimic a host Crm1 substrate.

RNP Assembly Increases the Affinity of Rev for Crm1

Although the Rev NES is able to mimic host substrates, the Rev NES binds to Crm1 with lower affinity. Studies of chimeras with the Rev NES fused to a host protein have determined the importance of each residue for binding,[61] and are consistent with previous studies showing that the Rev NES is indeed weaker than a typical host NES.[68] The RRE helps overcome this weak affinity, presumably by scaffolding a Rev oligomer to increase the local concentration of NESs for Crm1 to bind,[28] and thereby prevent the reduced Crm1 affinity from decreasing the level of nuclear export.[63]

Upon binding the Rev-RRE complex, Crm1 forms a dimer that further enhances NES recognition, as shown by negative stain EM and supporting biochemical experiments.[28]

Previously, Crm1 had only been observed to bind cargo proteins as a monomer. An EM reconstruction of the entire export complex unambiguously fits two subunits of Crm1/RanGTP, with extra density corresponding to the Rev-RRE particle spanning across the Crm1 dimer interface (Figure 6(b)). The docking surface of Rev-RRE is different than those observed with host cargoes such as Snurportin 1, and may represent a novel interface used by the virus when hijacking the host machinery or may mimic the binding of host cargoes yet to be observed. The arrangement of Crm1 subunits in the dimer has, in fact, been observed as crystal contacts in a previously determined structure.[64] It had been previously established that murine Crm1 does not support Rev-dependent Gag production to the same degree as human Crm1,[65,69–71] and the differences between these proteins map to seven amino acids located precisely at the Crm1 dimer interface (Figure 6(c)). The interface further enhances complex formation by ~30-fold compared to binding a single nuclear export sequence.[28] Thus, HIV takes advantage of the underlying biophysical property of Crm1 dimerization to enhance nuclear export of HIV RNA.

Because the Rev oligomer presents multiple NESs for the Crm1 dimer to recognize, there may be multiple functionally equivalent configurations for the overall nuclear export complex. The disordered carboxyl terminus[67] that contains the NES allows further opportunities for Crm1 to bind the NES in different ways. Despite the seemingly wide array of binding possibilities,[62] the large size of Crm1 sterically limits the plausible binding orientations of the Rev oligomer, since binding of one NES in some orientations may not allow binding of a second NES to the adjacent Crm1 subunit.[28,43] Such binding is reminiscent of statistical models derived by McGhee and Von Hippel,[72] and Epstein[73] that detail how large ligands bind a two-dimensional array of small sites. These models emphasize that saturating an array of sites generally involves configurations of the large ligand that occlude a significant fraction of possible binding sites. Cooperative interactions between the large ligands favor formation of complexes at lower ligand concentrations. Although binding to the Rev-RRE complex may be further complicated by its flexible 3D architecture, these models still suggest that the cooperative interaction of the Crm1 dimer interface facilitates recognition of a larger fraction of Rev-RRE complexes.[28]

Crm1 Recruitment Can Modulate Export Activity

Viewing the assembly of Crm1 and Rev-RRE through a statistical framework such as the one described above suggests plausible models for explaining how HIV may modulate the nuclear export activity of the Rev-RRE complex. In principle, mutations in the RRE, or different RNA conformers, could cause different numbers of Rev subunits to bind and thereby increase (or decrease) the local concentration of NESs for Crm1 recognition. It also has been observed that subtle mutations in the RRE can increase Gag production with little effect on the number of Rev subunits bound[17,32] and that different RRE conformations can have different activities.[18,32] In such cases, the RNA scaffold may dictate the orientation of Rev dimers thereby positioning the Rev NESs to enhance (or weaken) Crm1 binding.[25] At a structural level, a more open Rev dimer conformation that positions the two NESs further apart would potentially result in less steric hindrance of the Crm1 dimer (Figure 7). Thus, in principle the RRE could act as an allosteric rheostat to fine-tune Crm1 binding.[25] In thermodynamic terms, mutations in the RRE could increase the entropy of Crm1 binding by offering a greater multiplicity of bound states.

Evolutionary History of Crm1

As with other viral-host protein interactions, understanding the evolutionary history of the host cofactor adds another facet to understanding the Crm1-Rev interaction. The Crm1 dimer interface appears to be specific to the simian primate lineage[65] but is unlikely to have evolved to assist a pathogenic virus. It is possible that dimerization may facilitate Crm1 function during processes such as mitosis or the nuclear export of other cargoes where oligomeric assemblies that present multiple NESs may be involved.[74,75] Another intriguing possibility is that Crm1 has evolved to facilitate the function of a beneficial endogenous retrovirus. Recent work has shown that the human endogenous retrovirus HERV-K encodes a Rev-like protein, Rec, that is active during embryo-genesis to potentially activate innate immunity.[76] Because Rec, like Rev, forms an oligomeric assembly on its cognate RNA element,[77] Rec activity in developing cells may also depend on Crm1 dimerization, although this remains to be established. If Crm1 dimerization is used in this case, the host may face

interesting trade-offs that support the function of beneficial endogenous retroviruses yet increase susceptibility to pathogenic exogenous retroviruses.

EVENTS PRE- AND POST-RNP ASSEMBLY

Although assembly of the Rev-RRE-Crm1 RNP is well studied at the structural level, the cellular fate of the export complex as it traverses the pore and the cytoplasm remains largely unknown. At some point in the cytoplasm, Rev must disassemble from the RNA for proper translation and genomic RNA encapsidation. The mechanisms of disassembly are unknown but several basic models are plausible: (1) The cellular Ran GTP/GDP gradient drives disassembly of the complex when Crm1 is ejected; (2) The cellular import machinery competes with the RNA for Rev's ARM, which serves as both the RNA-binding motif and NLS; (3) The ribosome forcibly ejects Rev during translation and/or Gag binds the RRE to eject Rev during packaging; and (4) Other host factors, such as helicases, participate in complex disassembly. Suggestive evidence exists for each of these models: (1) The Rev-RRE-Crm1 interaction is mediated by Ran only in its GTP state,[28,78] however, the Rev-RRE interaction is stable even in the absence of Ran and Crm1,[41] so this model appears least likely; (2) Binding of nuclear import factors such as B23 are mutually exclusive with RRE binding and could provide a competitive displacement mechanism[79]; (3) CLIP-SEQ data has shown that the viral matrix protein binds the RRE,[80] offering indirect evidence for Gag-mediated ejection; and (4) A large number of Rev-binding proteins have been implicated in Rev-mediated export, including a variety of presumed RNA helicases.[81–84] Moreover, displacement mechanisms may differ between the 4.5- and 9-kb mRNA classes, which contain the RRE in different locations.

Cytoplasmic Fate of the RNA

Once in the cytoplasm, Rev-dependent RNAs have two basic fates: viral RNAs engage ribosomes to translate late-stage proteins such as Gag and Env, or the full-length genomic RNAs dimerize and are packaged in viral particles. Although HIV-1 does not appear to have distinct reservoirs of RNA for each fate,[85] the RNAs do not need to be translated to be packaged,[86] suggesting some independence between the functions. Although Gag binding to the RNA in the cytoplasm is thought to be a critical determinant for trafficking RNA to the cell membrane,[87–89] it is clear that the route of nuclear export also has consequences for viral RNA encapsidation.[90] Nuclear fusion studies in cells infected with engineered viruses using either RRE or CTE (constitutive transport element) mediated export,[91] have shown that recombination between CTE–CTE genomes and RRE–RRE genomes is more common than heterologous RRE–CTE recombination.[92] These data suggest that the export pathway used (Crm1/RRE vs. Nxf1/CTE) influences the timing or spatial location of dimerization and packaging (or alternatively that RRE and/or CTE play a role in dimerization). It is also possible that the Rev-RRE RNP directly influences RNA encapsidation,[93] or is marked with a specific Rev-RRE-dependent host factor such as DDX24.[94]

Additional Host Factors and Functions

Despite Rev's relatively small size, a large number of host factors have been implicated in Rev function and may be part of pre- or post-export RNP complexes. An extensive

description of putative Rev interactions is beyond the scope of this review, but the inherent flexibility present in the RNP raises the possibility that host factors and cellular localization can remodel the RNP for alternative functions. Indeed, many of the factors already implicated in nuclear export may also contribute to other functions, as most of the reporter assays used, such as measurements of Gag production, encompass other steps of expression, such as translation. Although no currently identified host factor has been definitively shown to alter the Rev oligomer for a different function, remodeling of viral oligomers to achieve distinct function has been observed in the Ebola virus protein VP40 which exists in dimeric, hexameric, and octameric forms. In the VP40 case, each structure is dependent on unique interactions with additional factors such as the cell membrane or RNA, and each structure performs a distinct function appropriate for its context.[95] The same may be true for the Rev oligomer with cytoplasmic or nuclear host factors remodeling the oligomer into different architectures for diverse functions. From this viewpoint, RNA helicases have been of particular interest to the Rev-RRE community as these factors commonly remodel RNPs and regulate RNA metabolism. The host helicase DDX3 has been shown to interact with Crm1 and affects Rev-mediated export in a manner suggesting that DDX3 remodels the RNP for proper transit through the pore.[96] DDX3 is a particularly intriguing target as it has additional cytoplasmic roles that affect RNA translation and also can act as nucleic acid sensor.[90,97] Another helicase, DDX1, has also been implicated in Rev function, with several studies demonstrating that DDX1 interacts with the Rev OD and may prevent unproductive and nonspecific intra-cellular multimerization of Rev.[54,82,98,99] DDX24, mentioned previously, has been shown to interact with Rev and influence RNA packaging.[82,94]

These interactions represent only a small fraction of the host factors reported to interact with Rev. Indeed, both targeted and unbiased studies have reported a wide variety of putative Rev cofactors.[81–84,100–102] Multiple regulatory processes, including transcription, splicing, and translation, all can affect Rev function, directly or indirectly. Indeed, mathematical modeling of Rev concentrations and viral replication rates has shown that Rev plays a critical role in timing the expression of other viral factors.[103] While this biological complexity and crosstalk explains why such a small protein might interact with multiple factors, establishing the specificity of interactions, their functional relevance, and mechanism remains a challenge for the field. In light of the structural progress on the export RNP, additional detailed structural and mechanistic work may reveal which host factors or other RNAs further rearrange the Rev oligomer for a different function, potentially revealing new aspects of Rev-RRE architectural plasticity.

FLEXIBILITY AND INHIBITION

The essential role of the Rev-RRE RNP in HIV replication makes it an attractive therapeutic target. In another lentivirus, EIAV, modulating Rev activity has been shown to correlate with disease states, suggesting that Rev inhibition can have a therapeutic effect.[104,105] While no simple correlation is observed between disease state and Rev-RRE activity in HIV-1, several studies have shown that activity fluctuates over the course of infection.[32,106,107] Interestingly, several long-term nonprogressors harbor Rev alleles with mutations in the NES or C-terminus that have diminished activities.[108,109] From the host perspective, the restriction of HIV replication in mouse cells, in part due to Crm1 differences, further

indicates that controlling Rev activity may be an effective inhibition strategy. To that end, several small molecule screens using Rev or HIV-1 RNA processing assays have yielded promising lead compounds.[110–114] Additionally, drugs that inhibit Crm1 binding to NESs, including leptomycin B, have already been developed as putative therapeutics for blood cancers and may be applicable to viruses.[115–117]

The theme of this review has focused on the remarkable flexibility of the Rev-RRE system with respect to protein structure, RNA structure, and Crm1 interactions. This plasticity is likely an evolutionary feature, allowing the virus to build a sophisticated and regulated RNP out of a relatively small protein and maintain its function in the face of a high viral mutation rate and the constraints of overlapping genes. On the surface, plasticity appears an insurmountable challenge for inhibitor design. However, it is precisely this plasticity that appears to be at the heart of Rev function, allowing it to bind RNA in multiple modes to assemble a large RNP, rearranging its subunit structure to adapt to the RNA scaffold, and using appropriate oligomeric arrangements to adapt to the Crm1 dimer scaffold. Less well-understood facets of Rev function in the cytoplasm may require further rearrangements to adapt to other host partners. The apparent flexibility of the RRE to tune Rev function provides another dimension to the problem. While this level of Rev-RRE plasticity appears daunting, each functional state provides a potential opportunity for inhibition. Rev inhibitors ultimately may be useful in further developing combination treatments that target multiple steps of the virus life cycle, for example, using a late-stage Rev inhibitor as part of a `shock-and-kill' strategy[118] and reserving early stage inhibitors for use in pre-exposure prophylaxis.[119] How the virus might adapt to inhibitors via escape mutations, like the RRE mutation arising against the Rev M10 dominant negative inhibitor,[17,33] will provide fascinating evolutionary glimpses and undoubtedly uncover interesting new functions for this pliable RNP.

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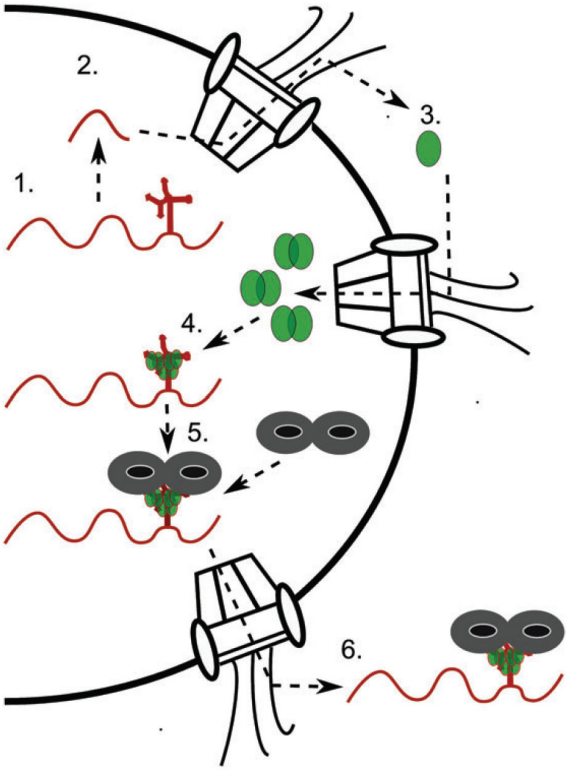

FIGURE 1.

Rev transports partially spliced and unspliced viral RNAs from the nucleus to the cytoplasm. (1) HIV-1 transcription produces a single 9-kb RNA from its promoter; (2) The RNA is spliced into 2-kb mRNAs that are exported to the cytoplasm via the nuclear pore; (3) The Rev-encoding 2-kb mRNAs are translated and Rev is imported into the nucleus via its nuclear localization sequence; (4) Multiple Rev molecules bind the RRE (Rev-Response Element) before splicing occurs; (5) The Rev-RRE complex recruits a Crm1 (Chromosome maintenance factor 1)/RanGTP dimer to the unspliced or incompletely RNAs; (6) The RNAs are exported through the nuclear pore to the cytoplasm.

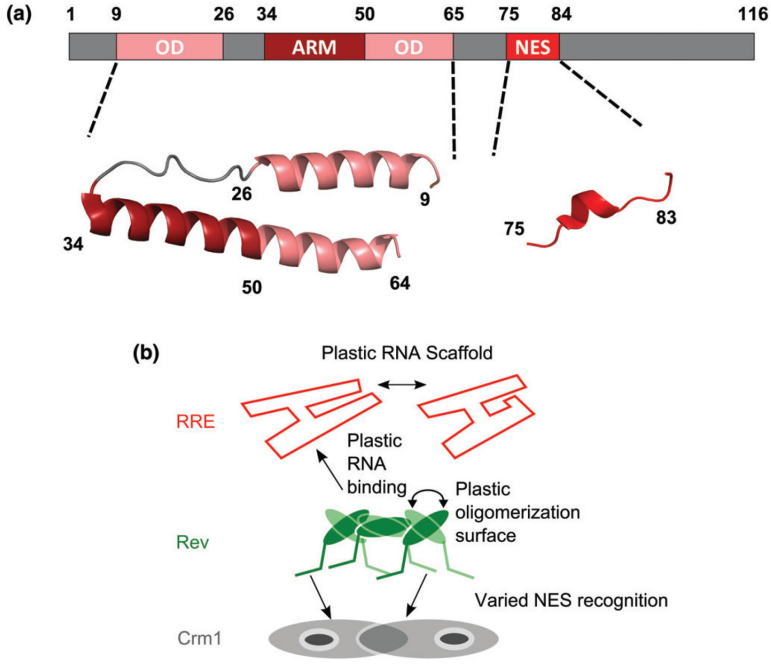

FIGURE 2.

Domain Organization of Rev and Plastic Assembly of the Export RNP. (a) Domain organization of Rev. Partial crystal structures of Rev from PDB codes 3LPH and 3NBZ are shown and colored accordingly to the labeled domains: OD, oligomerization domain; ARM, arginine-rich motif; NES, nuclear export sequence. (b) Points of Rev-RRE-Crm1 export complex assembly with demonstrated plasticity. The RRE (Rev-Response Element) can adopt multiple conformations through alternative secondary or tertiary structures or mutation. The Rev oligomer recognizes these scaffolds using multiple modes of RNA recognition and the entire oligomer can rearrange as directed by the RNA scaffold. Finally, the Rev-RRE complex recruits a Crm1 (Chromosome maintenance factor 1) dimer using multiple Rev NESs from its flexible C-terminal region.

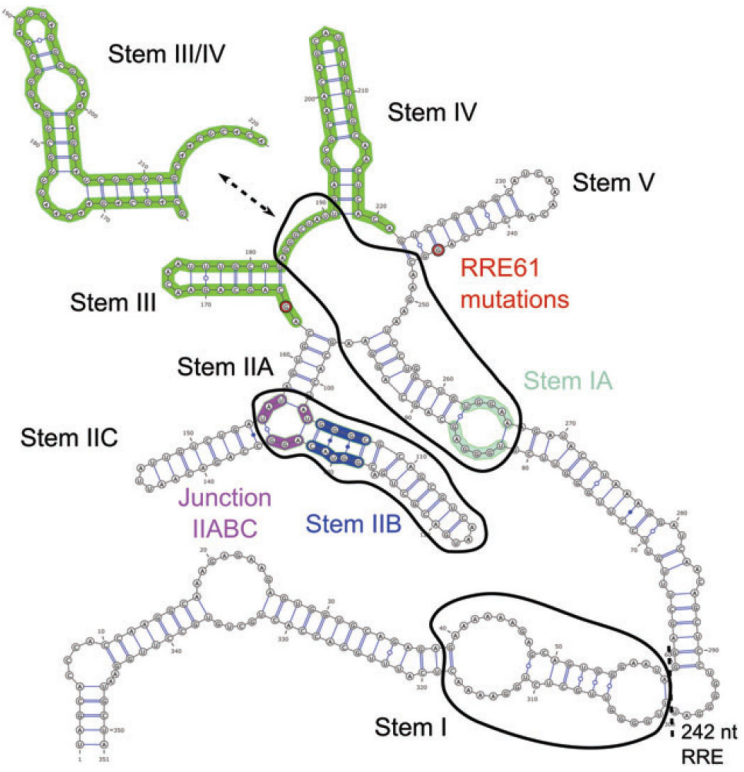

FIGURE 3.

Secondary structures of the RRE (Rev-Response Element). The RRE predominantly adopts either a five stem-loop structure (pictured) or a four stem-loop structure (the Stem III/IV rearrangement is highlighted in green). The three well-characterized Rev-binding sites are indicated (Stem IA: cyan, Stem IIB: blue, Junction IIABC: purple). Regions that show the greatest protection upon addition of Rev in SHAPE experiments[16] are circled in black. Two mutations found in RRE61 which change the RRE secondary structure and rescue function in the context of a Rev dominant negative nuclear export sequence (NES) mutant (M10)[17] are shown in red. The portion of Stem I dispensable for Rev activity in reporter assays is to the left of the dashed line.

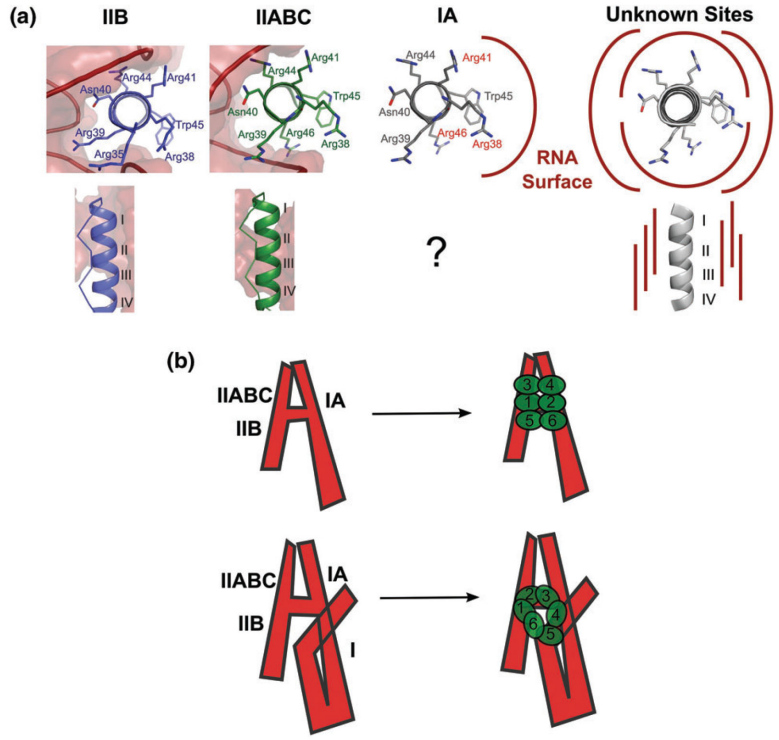

FIGURE 4.

Rev-RRE (Rev-Response Element) interactions of the arginine-rich motif (ARM). (a) Plasticity in Rev-RNA recognition. The ∝-helical Rev ARM is shown with each of three well-characterized binding sites. The structures of complexes with IIB and IIABC have been solved by NMR and crystallography,[23,25] while the IA-binding region is inferred from mutational data.[21] Each site uses a different set of residues to contact the RNA and buries different faces and sections of the ARM helix. Other sites yet to be identified may utilize other portions of the helix, which is surrounded with arginine residues, for binding. (b) Models for order of assembly. A small angle x-ray scattering (SAXS) model of the RRE[27] juxtaposes the IIB- and IA-binding sites such that a Rev dimer (subunits 1 and 2 in the upper diagram) might bridge the two sites to initiate oligomer assembly. SHAPE and biochemical data[16] and the cocrystal structure of a dimer-RNA complex[25] suggest that dimer binding of the IIB and IIABC sites nucleates Rev oligomerization across the bridge of the 'A'-shaped RNA structure. (Reprinted with permission from Refs 25 and 28. Copyright 2014 Creative Commons Attribution License)

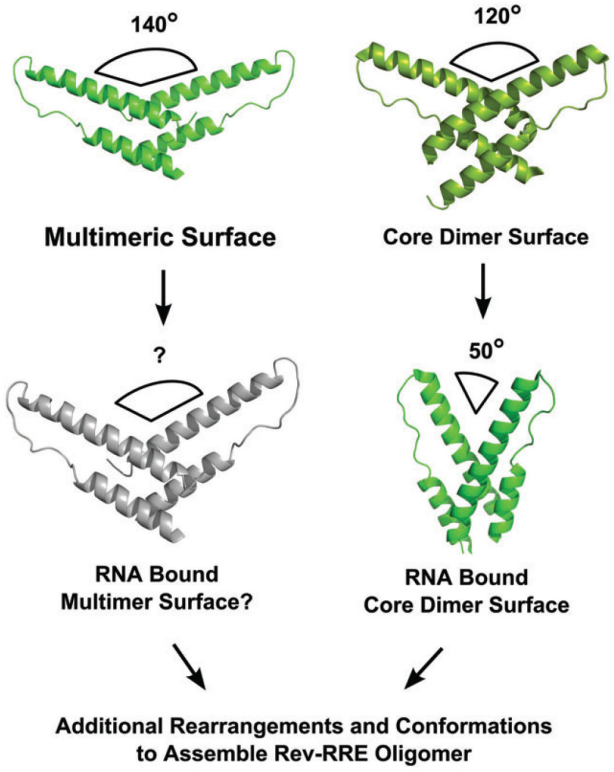

FIGURE 5.

Plasticity of the Rev oligomer. Structures of the core dimer and multimer surfaces of Rev[43,51] reveal different crossing angles. In addition to the different states of unbound Rev, the core dimer surface rearranges upon RNA binding by pivoting around hydrophobic residues in the dimer interface,[25] likely one of many possible angles depending on the structure of the RNA as well as the energetic stability of the bound dimer. The multimer surface may also rearrange to accommodate RNA binding but this complex has not yet been observed. Each dimer arrangement may orient the helical subunit arginine-rich motifs (ARMs) to bind RNA in slightly different ways during oligomer assembly.[25]

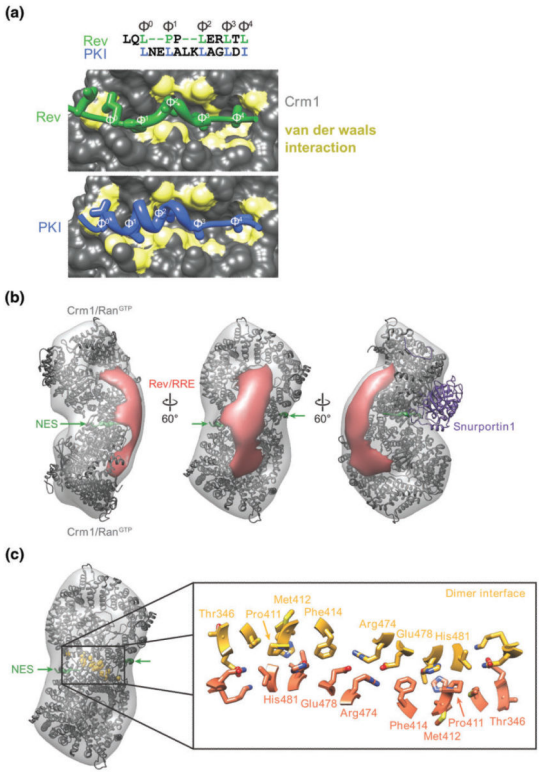

FIGURE 6.

The Rev nuclear export sequence mimics host cargoes to bind a Crm1 (Chromosome maintenance factor 1) dimer. (a) The spacing of hydrophobic residues (Φ) in an NES within the Crm1-binding pocket is shown for Rev (green) and protein kinase A inhibitor (PKI; purple), which has a canonical NES. The Rev NES establishes similar hydrophobic contacts with Crm1 (yellow) but uses a shorter and less-ordered peptide (PDB 3NBZ) than the PKI NES (PDB 3NBY). (b) Crm1 forms a dimer interface that enhances Rev-RRE (Rev-Response Element) complex formation. Two Crm1 monomers were unambiguously fit into the EM density (gray) with an additional, weak density that corresponds to the position of the Rev-RRE complex (red) and sits between the binding sites for the Rev NES (green). The location of the Rev-RRE differs from the known binding site of Snurportin1 (purple). (c) The interface of the Crm1 dimer was observed as a crystal contact in the structure of human Crm1.[64] The residues (annotated) in this interface that differ between the human and murine proteins were previously recognized to be important for Rev-RRE nuclear export.[65] (Reprinted with permission from Refs 25 and 28. Copyright 2014 Creative Commons Attribution License)

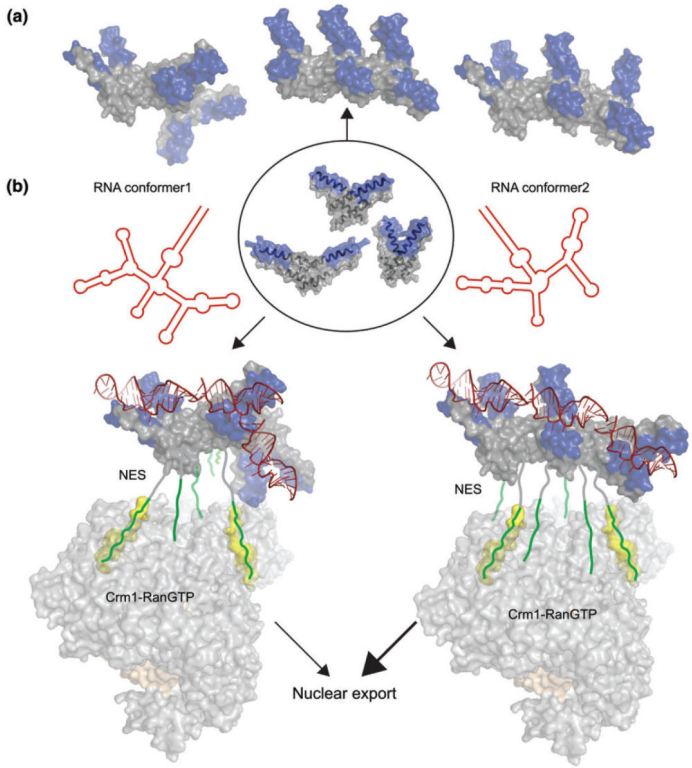

FIGURE 7.

Model for how Rev-RRE (Rev-Response Element) plasticity can lead to different export outputs. Multiple conformations of the Rev oligomer are seen in existing crystal structures and in principle can be mixed and matched to generate different arrangements of the oligomer. The RRE scaffold, and its possible different conformers, can present the Rev nuclear export sequences (NESs) in different ways to the Crm1 (Chromosome maintenance factor 1) dimer and thereby influence nuclear export activity, most likely by increasing the number of viable configurations for Crm1 binding. (Reprinted with permission from Refs 25 and 28. Copyright 2014 Creative Commons Attribution License)