Viral RNA export

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Viruses can express intron-containing and intronless mRNAs, which are exported by alternative pathways. The study of the nuclear export of these unconventional mRNAs can provide key insights into the normal process of nuclear export and the alternative pathways provide an attractive target for the development of specific antiviral therapies.

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Introduction

Recent studies of viral gene expression have revealed that RNA export is a highly regulated process. Unlike most cellular mRNAs, which contain introns that are removed before an mRNA is exported to the nucleus, viruses often express unconventional mRNAs; these are either intronless or retain potential introns, but they are still efficiently exported to the cytoplasm. Viruses use endogenous cellular pathways to export their unconventional mRNAs, so understanding viral mRNA export can provide important insights into the process of nuclear export in normal cell function. Because many viruses depend on alternative pathways for mRNA export; these pathways provide an attractive target for the development of specific antiviral therapies. If the alternative pathways can be understood, all the human viruses which replicate in the nucleus — viruses ranging from the human immunodeficiency virus (HIV) and the human hepatitis B virus (HBV) to herpes viruses and influenza — may be vulnerable to therapeutic strategies which target viral RNA export.

The classical system for studying mRNA export is an assay in which radiolabeled RNAs are injected into the nuclei of *Xenopus* oocytes [1]. This technique allows direct monitoring of the movement of RNA from the nucleus to the cytoplasm. During the experiment, the microinjected oocytes are incubated and then separated into nuclear and cytoplasmic fractions. The RNA in each compartment is then analyzed by gel electrophoresis. Such microinjection analysis has revealed that RNA export is a carrier-mediated, energy-dependent, saturable process [1–3]. Competition studies with mRNAs, rRNAs, tRNAs, and the snRNAs suggest that they use four separate pathways which are dependent on distinct cellular factors [2].

Cellular mRNA export

Most of the mRNAs in higher eukaryotes contain introns. Because of the potential deleterious effects of translating intron-containing messages, such as premature termination or the generation of inappropriate polypeptides, all introns must be removed before an RNA is allowed to be exported from the nucleus. For example, it has been shown in the case of yeast that the assembly of splicing complexes without completion of the splicing reaction, can inhibit the export of RNA [4] — so splicing must be completed before the active process of retention in the nucleus is halted. In addition, the process of splicing appears to have a positive effect on RNA export. The first hints of a positive role for splicing, and therefore the involvement of an intron, in RNA export came from studies of Simian virus 40 (SV40) [5]. The analysis of a mutant derivative of SV40 that lacked an intron in the late region of the viral genome

demonstrated that although the transcription of SV40 late transcripts appeared to be normal, these transcripts failed to accumulate in the cytoplasm. The involvement of a functional intron in export has also been shown for β-globin [6,7], the ribosomal protein L32 [8], immunoglobulin μ [9], and several other mRNAs. In addition, in the expression of cellular cDNAs from expression vectors used in making transgenic mice and in the generation of stable expression in cell lines, the presence of an intron greatly increases gene expression, presumably by increasing the efficiency of the export of the cDNAs [7].

Viral export of intron-containing mRNAs

The requirement that intronic sequences must be deleted before an mRNA can be exported poses a serious problem for complex retroviruses such as HIV, which encodes nine proteins within a relatively small, 10,000 base pair genome. Three different classes of mRNAs are generated by the virus — unspliced, partially spliced, and completely spliced mRNAs. All viral mRNAs encode viral proteins. The unspliced RNA also functions as the genomic RNA, which must be present in the cytoplasm in order to be incorporated into virions which are assembling at the plasma membrane. Therefore, HIV must efficiently export incompletely spliced and unspliced RNA from the nucleus, even though normal nuclear checkpoints are designed to block the export of such transcripts. To bypass this checkpoint, HIV expresses a protein called Rev, which is responsible for the efficient export of unspliced and incompletely spliced viral mRNAs [10]. All complex retroviruses express proteins functionally related to Rev. In lentiviruses these proteins are known as Rev, whereas in the complex oncoviruses they are known as Rex. For the sake of simplicity, I refer to all these regulatory factors as Rev-like proteins.

Export by complex retroviruses

Rev-like proteins interact with a folded RNA structure that is contained in the viral RNA, thus facilitating the specific export of viral RNA. By expressing a Rev-like protein, complex retroviruses can specifically regulate the export and subsequent expression of incompletely spliced and unspliced RNA. This control of viral gene expression is likely to regulate the timing of protein production, thereby regulating levels of virion production.

Figure 1 shows the regulation of RNA export by complex retroviruses. The virus expresses early genes, which are encoded by conventionally spliced messages, using the normal pathway for mRNA export from the nucleus (Step 1). One of the early genes encodes a Rev-like post-transcriptional transactivator, which is imported into the nucleus through interactions with its nuclear localization signal (Step 2) [11]. When the Rev-like protein accumulates to the levels required, nuclear export of incompletely spliced and unspliced RNA is initiated (Step 3) [12,13]. This leads to the expression of viral late genes and subsequent virion assembly.

Export by simple retroviruses

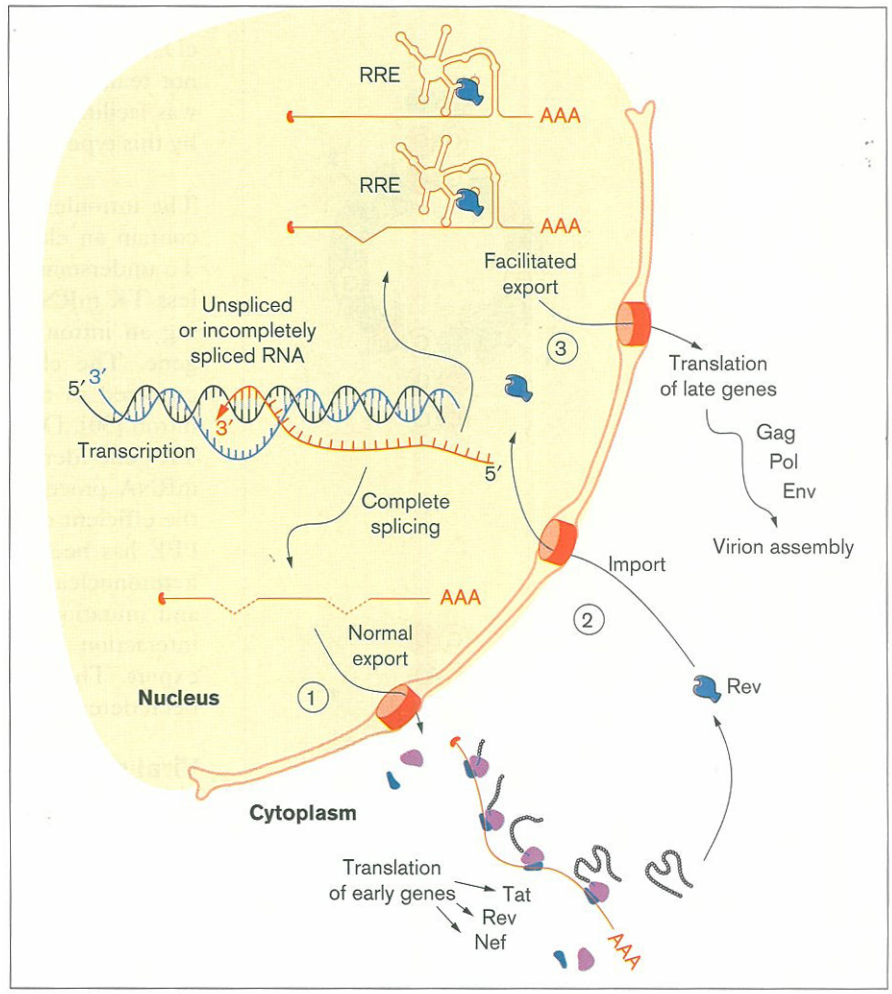
The simple retroviruses contain a single potential intron and therefore encounter the same dilemma faced by the complex retroviruses: they must export unspliced RNA which retains an intron [14]. But, unlike the complex retroviruses, the simple retroviruses do not express a Rev-like protein. Instead, they contain a binding site for cellular factors which mediate the nuclear export of unspliced RNAs. Such binding sites for cellular factors, required for the cytoplasmic localization of unspliced RNAs, have been found in the type D retroviruses [15] and avian leukosis viruses (ALVs) [16]. A critical consequence of using cellular factors is that nuclear export is constitutive: in other words, because the factors mediating export are always present, there is no differential expression of early and late genes.

The constitutive transport element (CTE) found in type D retroviruses is the best characterized of the elements that bind cellular factors in order to mediate export of unspliced RNA. The secondary structure of this CTE has recently been determined by mutational and biochemical analysis [17,18]. It consists of an extended, imperfect stem–loop with two large non-base-paired regions, or 'bubbles'. Remarkably, the two bubble regions are composed of identical sequences which are rotated by 180° relative to each other [17]. The structure of the type D retrovirus CTE and the related structure of the two bubble regions is shown in Figure 2. Point mutations in either bubble region have been shown to abrogate CTE function. These regions of primary sequence have therefore been proposed to act as binding sites for the cellular factors that mediate RNA export. A related RNA element, which contains two bubble regions identical to those found in the CTEs of type D retroviruses, has been identified in the intracisternal-A particle retroelement [19].

It has recently been proposed that the nuclear protein RNA helicase A is the cellular factor that mediates the function of the CTE of type D retroviruses [20]. RNA helicase A was initially identified in nuclear extracts as a cellular protein that bound to CTE RNA but not to a mutant form of the CTE RNA that is non-functional *in vivo*. In addition, RNA helicase A became relocated to the cytoplasm when RNAs containing wild-type CTE sequences were expressed. This observation is consistent with what one might expect for a cellular equivalent of the viral Rev-like proteins. RNA helicases are also known to be involved in the cytoplasmic localization of maternal RNAs within *Drosophila melanogaster* oocytes [21]. Although the observations outlined here are consistent with the involvement of RNA helicase A in the export function of the type D retrovirus CTE, the exact role of RNA helicase A remains unknown.

Figure 1

Viral RNA export in complex retroviruses. The expression of early and late genes by complex retroviruses is illustrated (HIV is used in this example). After the transcription of viral RNA begins, only completely spliced mRNAs are exported to the cytoplasm and translated (Step 1). One of the early gene products, Rev, returns to the nucleus (Step 2) where it interacts with an RNA structure located within the second intron of viral transcripts. Interaction of Rev with the Rev-response element (RRE) leads to the export of unconventional mRNAs (Step 3) and the expression of the viral late genes.



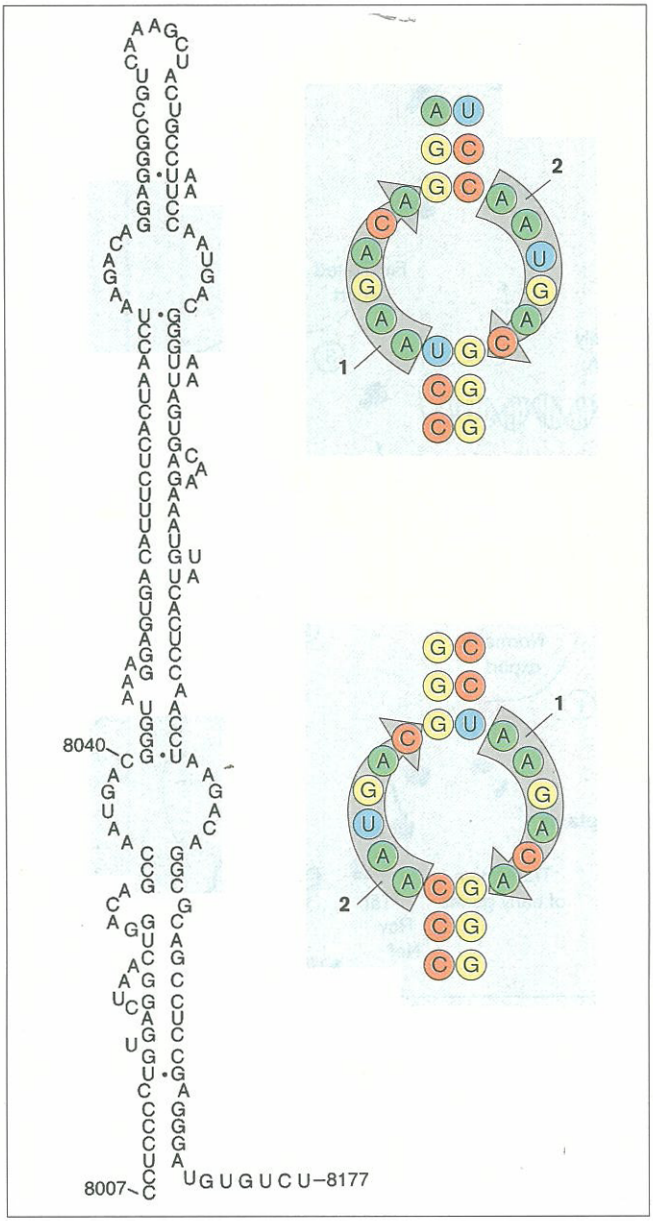
Viral export of intronless mRNAs

The presence of specific RNA export elements in retroviruses indicates that facilitated export is required for the export of intron-containing RNAs. Functionally related RNA export elements have also been identified in the intronless RNAs of HBV and in the intronless thymidine kinase (TK) gene of the human herpes simplex virus (HSV). Furthermore, evidence suggests that the intronless late genes of human papillomavirus (HPV) contain cell-type specific RNA export elements. The expression of HPV late genes is limited to terminally differentiated keratinocytes, but late-gene expression can be induced in fibroblasts when Rev is expressed, and the RRE is contained in intronless late-gene mRNAs [22]. The presence of these elements in intronless viral RNAs suggests that the facilitation of RNA export may be required for the efficient cytoplasmic localization and subsequent translation of intronless mRNAs. As for the RNA export elements in simple retroviruses, the expression of viral proteins is not required for the function of the elements found in HBV, HSV, and HPV. Therefore, the function of these elements is believed to be mediated by cellular proteins.

Studies of the enhancer I region of HBV by Vannice and Levinson [23] indicated that some of the enhancement of gene expression mediated by this region was dependent on its orientation. More recent studies of this region have identified a posttranscriptional regulatory element (PRE) that is required for the cytoplasmic localization and expression of HBV mRNAs encoding the HBV Surface protein. The PRE may also be involved in the expression of all the intronless transcripts of HBV [24,25].

The folded structure of the RNA export element in HBV (HBV PRE) has not been determined, although initial mapping studies indicate that it is composed of two distinct regions, or sub-elements, which are required for efficient function [26]. The sub-elements appear to be functionally equivalent, because the duplication of either element activates RNA export to levels similar to those

Figure 2



The structure of the type D retrovirus constitutive transport element (CTE). The secondary RNA structure of the CTE is shown (derived from the structure determined by Ernst *et al.* [17]) The structures of the two conserved loop regions are shown on the right. The repeats are indicated by 1 and 2 within the light blue shading. The colors denote different nucleotides.

found for the native HBV PRE. Cellular factors which interact with the HBV PRE have recently been identified using *in vitro* RNA-binding assays [27], but their involvement in RNA export remains to be demonstrated. Studies by Huang and Yen [28] have shown that an intron of the β-globin gene can replace the HBV PRE in facilitating the export of the HBV Surface protein mRNAs. This is consistent with the HBV PRE providing a role analogous to the export activity associated with RNA splicing. Further evidence for the export function of the HBV PRE comes from the use of the 3′ end of HBV in vectors used for cDNA expression in transgenic mice [29]. Although it was not realized at the time, the presence of the HBV PRE was facilitating the export of intronless cDNAs generated by this type of vector.

The intronless TK gene in HSV has also been shown to contain an element which facilitates RNA export [7,30]. To understand the cytoplasmic localization of the intronless TK mRNA, chimeric RNAs were generated containing an intron from the β-globin gene fused to the TK gene. The chimeric RNA was found to be efficiently exported to the cytoplasm while retaining the β-globin intron [30]. Detailed analysis of the export activity of the TK gene identified an RNA element, known as the pre-mRNA processing element (PPE) which could mediate the efficient nuclear export of a β-globin cDNA [31]. The PPE has been shown to interact with the cellular factor heteronuclear RNA-binding protein L (hnRNP L) [31], and mutational analysis of the PPE has shown that this interaction correlates with PPE's mediation of nuclear export. The structure of the PPE element has not yet been determined.

Viral proteins which mediate unconventional mRNA export

Studies of the Rev-like proteins of complex retroviruses provides most of the knowledge on the structure and function of the proteins which mediate the export of viral RNAs. The most studied of these proteins is HIV Rev. At least two essential functions have been assigned to Rev and the Rev-like transactivators: a specific RNA-binding activity which directs specific interactions with viral mRNAs; and the cytoplasmic localization of unconventional viral mRNAs by an effector domain [11,32]. Rev-like proteins therefore act as adapters that impart a specificity for viral RNA with the cellular export pathway. For many years the exact mechanism of action of Rev was controversial: in one model, Rev inhibited splicing, leading to cytoplasmic localization; and in the other model, Rev directly facilitated the export of intron-containing viral mRNAs [10,33]. Recently, HIV Rev has been shown to directly facilitate RNA export [34–36].

The first direct evidence that Rev was facilitating RNA export came from studies of Rev function in *Xenopus* oocytes [34]. When Rev was injected into oocytes with an RNA substrate containing the second intron of HIV (which contains the RRE), unspliced substrate RNA became cytoplasmically localized in a Rev-dependent manner. Surprisingly, the excised product generated by the splicing of the injected RNA substrate, also became cytoplasmically localized. It was difficult to envision a model in which the inhibition of splicing by Rev would

lead to the cytoplasmic localization of the excised product of the splicing reaction. But the cytoplasmic localization of the excised product was consistent with a model in which Rev would directly export any RNA containing an RRE.

The proof that the mechanism of Rev function was direct nuclear export came when it was determined that the effector domain of HIV Rev was, in fact, a nuclear export signal (NES) [35,36]. The ability of the effector domain of HIV to act as an NES was shown directly using microinjection analysis. It was shown that when peptides corresponding to the effector domain of HIV-1 Rev were cross-linked to bovine serum albumin (BSA), they could facilitate the cytoplasmic localization of BSA–peptide (BSA–NES) conjugates that had been microinjected into the nucleus of *Xenopus* oocytes and mammalian fibroblasts. Conjugates containing a peptide corresponding to a non-functional effector-domain mutant of Rev were not exported from the nucleus. The export of the BSA–NES conjugates was energy-dependent (export was completely blocked at 4°C); the NES-mediated export pathway was saturable; and the injection of excess unlabeled BSA–NES conjugates could block the export of labeled conjugates, indicating that export of this class of NES is mediated by a factor present in limiting amounts in the nucleus. Wheat germ agglutinin (WGA) can inhibit the import function of the nuclear pore by interacting with complex sugars on proteins within the nuclear pore [37]. WGA can also inhibit the export of BSA–NES conjugates, suggesting that the export of these signals is mediated by the nuclear pore [38].

Because the Rev-like proteins contain both a nuclear localization signal (NLS) and an NES they move continuously between the cytoplasm and the nucleus [39]; for this reason they are known as shuttle proteins. By shuttling, it is possible for a single Rev protein to mediate the export of many viral RNAs. Like that of several of the hnRNPs, the shuttling of the Rev-like proteins is dependent on active transcription [40]; if cellular transcription is inhibited by a drug such as actinomycin D or 5,6-dichlorobenzimidazole riboside (DRB), shuttle proteins accumulate in the cytoplasm.

Competition studies with BSA–NES conjugates in *Xenopus* oocytes revealed that Rev-mediated export relies on the export pathway used by 5S ribosomal RNA and small nuclear RNAs, rather than that typically used by mRNAs [35]. These observations indicate that the previously observed Rev-dependent inhibition of splicing is indirectly caused by the export of unspliced RNA [41]. Export thus removes a potential substrate (unspliced RNA) for the splicing reaction. It is known that the transcription factor TFIIIA activates the expression of the 5S RNA; TFIIIA can also interact with 5S RNA to form a complex, TFIIIA–5S RNA, which is then exported to the cytoplasm in *Xenopus* oocytes. Export of this complex is mediated by an NES in TFIIIA which is structurally and functionally related to the NES present in HIV Rev [42]. It is possible functionally to replace the NES in HIV Rev with the TFIIIA NES. Furthermore, the Rev NES can block export mediated by the TFIIIA NES by competing for common factors in *Xenopus* oocyte injection assays. In addition, alignment of the Rev and TFIIIA NES sequences reveals that the positioning of several amino acids is conserved. The related nature of the NESs in TFIIIA and Rev supports a model in which Rev directs the export of intron-containing HIV RNAs along a pathway not typically used by cellular mRNAs.

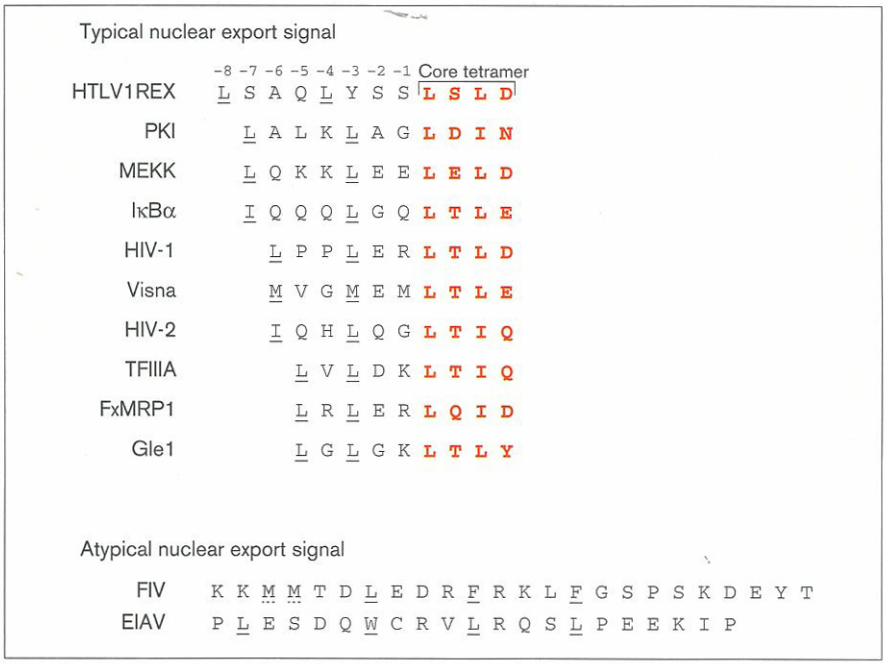
Nuclear export signals in cellular proteins

Structurally and functionally equivalent NESs have been identified in cellular proteins involved in RNA metabolism. The fragile X mental retardation protein type 1 (FxMRP1) contains an NES [43,44] which can be used to complement the function of an NES mutant of Rev [44]. It has been shown that the NES of FxMRP1 is located in the region encoded by exon 14, and is essential for the proper localization of FxMRP1. When the NES of FxMRP1 is mutated, the normally cytoplasmically localized protein becomes completely nuclear. A recessive-negative form of FxMRP1 results from the exclusion of exon 14 and, unlike wild-type FxMRP1 which is localized in the cytoplasm, this form is localized in the nucleus. It is believed that FxMRP1 first moves to the nucleus where it interacts with an unidentified cellular RNA; the FxMRP1–RNA complex is then exported to the cytoplasm where the complex is involved in cellular translation [44].

An NES has also been identified in the Gle1 protein of *Saccharomyces cerevisiae* [45]. Mutants of the GLE1 are defective in the nuclear export of polyadenylated mRNAs. The Gle1 NES is required for the Gle1 function, and the related structures of the yeast-derived and higher eukaryotic NESs indicate that factors involved in mediating nuclear export are conserved in all eukaryotes

The same nuclear export pathway is also used by cellular proteins that are not involved in RNA metabolism. The inhibitor of the cAMP-dependent protein kinase (PKI) has been shown to contain an NES which is structurally and functionally related to those found in the Rev-like proteins [36]. The NES of PKI can restore the function of an NES mutant of HIV-1 Rev [46]. In addition, the PKI NES can block the export of the Rev NES when present in excess in microinjection analysis [36]. Related NESs have also been identified in Ran-binding protein 1 (RanBP1) [47], MAP kinase kinase (MEKK) [48], and the inhibitor of nuclear factor kappa B alpha (IκBα) [49]. Finding NESs in proteins that have no involvement in RNA metabolism indicates that this pathway of RNA export is used by a variety of cellular proteins.

Figure 3



Alignment of the sequences of nuclear export signals. Both the typical and atypical NESs are shown. The core tetramer regions are shown in red. Leucines and chemically related amino acids which are involved in NES function are underlined. Two methionines, which are potentially involved in the function of the feline immunodeficiency virus (FIV) Rev NES, are underlined with a dotted line.

Structure of nuclear export signals

Mutational analysis of several different NESs has implicated leucines, or chemically related amino acids, as being essential for export function (for the sake of simplicity, these conserved, leucine-related amino acids will be referred to here as leucines). The sequences of a number of known NESs are shown in Figure 3. Typical NESs contain a conserved motif (LXLY) consisting of two leucines spaced by a single amino acid [32], known as the core tetramer. The Y position is usually occupied by a charged amino acid, whereas the X position is highly variable. Two other leucines that are required for export function are found within an eight amino acid sequence located upstream of the core tetramer. The exact position of the two upstream leucines is highly variable (as seen in Figure 3; leucines that are important in NES function are underlined).

The functional flexibility of the upstream leucines was demonstrated directly when it was shown that the positions of the upstream leucines could be shifted within the NES of human lymphotrophic virus 1 (HTLV-1) Rex without revoking function [38,50]. These studies also showed that it is possible to replace the leucines typically found in NESs with any large, hydrophobic amino acid including isoleucine, valine, methionine, cysteine, phenylalanine, and tryptophan. The NESs found in the Rev proteins of equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) have atypical sequences [51,52], as shown in Figure 3. Although the function of these NESs is mediated by hydrophobic amino acids, as for the typical NESs, the spacing of important amino acids is distinct. Because the injection of excess NESs of either typical and atypical sequence will block the function of NESs of the other type [53], the two classes of NESs are believed to interact with common factors or to use the same export pathway.

Cellular factors which interact with NESs have now been identified. The nucleoporin known as Rab or Rip has been shown to interact with the NESs of HIV Rev, visnamaedi virus Rev, EIAV Rev, HTLV-1 Rex, Gle1, and IκBα [54–56]. In fact, the NESs have been shown to interact with domains found in many nucleoporins, which contain many copies of the phenylalanine–glycine repeat (FG repeat) [49,57]. The cellular protein eIF5A has also been shown to interact specifically with the NES of HIV-1 Rev [58]. Dominant-negative forms of eIF5A have been generated which can block HIV-1 Rev function [59]. In addition, eIF5A has been shown to be specifically exported from the nucleus after injection into the nuclei of fibroblasts [59]. This observation is consistent with the possibility of a direct role for eIF5A in HIV-1 Rev function. The exact involvement of eIF5A and RAB/RIP in mediating the export of proteins containing NESs remains to be determined.

RNA binding by HIV-1 Rev

It is clear that all the Rev-like proteins contain structurally related and functionally equivalent NESs, and it appears that they can interact with RNA using a variety of mechanisms. This is demonstrated best by the observation that

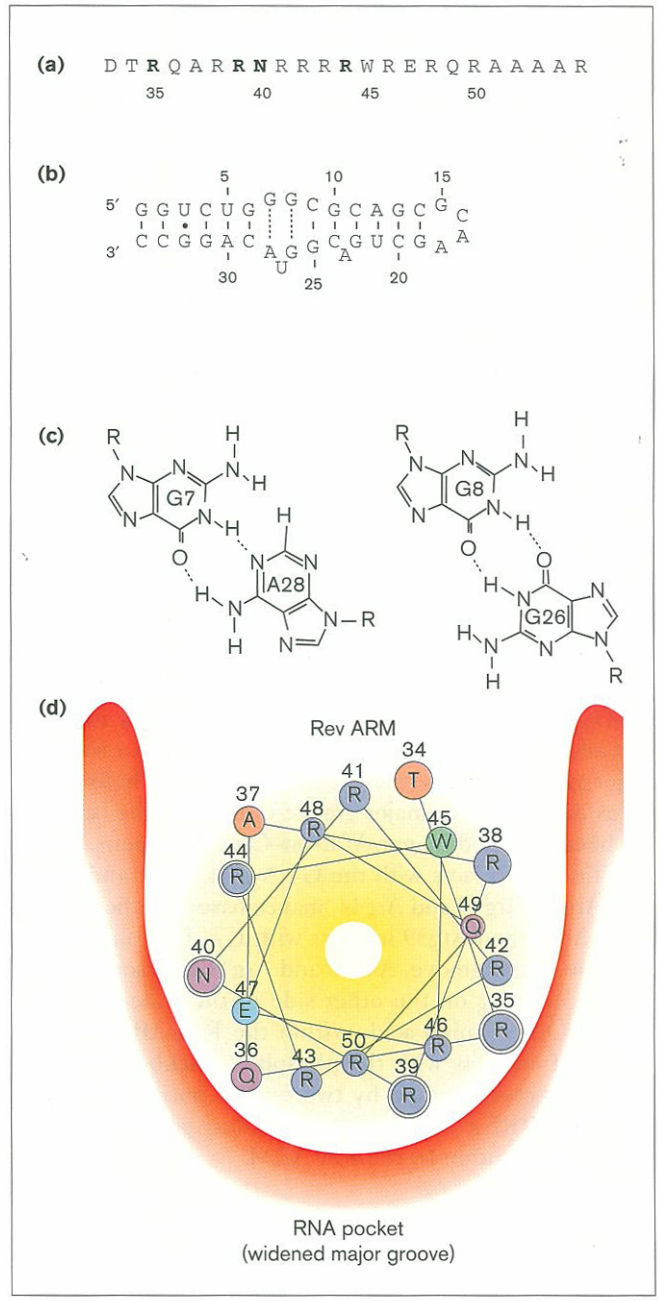
it is possible to target HIV Rev and HTLV Rex to interact with RNA using the RNA-binding specificity of the bacteriophage MS2 coat protein [60,61]. Fusion of the coat protein to Rev or Rex generates export proteins which can direct the cytoplasmic localization of intron-containing RNA when multiple copies of the binding site for the MS2 coat protein are present. This result shows that all that is required for the export of RNA is the tethering of proteins which contain NESs.

The interactions between Rev and the RRE have been extensively characterized and the structure of the RNA-binding site of REV and a peptide encompassing the RNA-binding domain has been solved by nuclear magnetic resonance (NMR) analysis. The RNA-binding domain of Rev is an arginine-rich motif (ARM). Mutations within this region have been shown to block Rev function *in vivo* and RNA-binding activity *in vitro* [32,62]. Peptides encompassing the region of amino acids 34–50 of Rev have been shown to interact specifically with the RRE [63]. The sequence of the Rev ARM is shown in Figure 4a. The ARM also encompasses the nuclear and nucleolar localization signals of Rev [64,65]. Circular dichroism (CD) analysis of peptides encompassing the Rev ARM indicates that this region can form an α-helical structure [66].

Although the RRE is a 250 base pair, highly structured RNA, the sequences required for Rev binding are located within a small stem–loop structure [10]. This structure, known as the high-affinity Rev-binding site, consists of a small bubble located within a stem–loop [67,68]. It has been shown that eight Rev monomers form a stable complex with the RRE [69]. The binding specificity of Rev for the RRE is determined by direct interactions between the Rev RNA-binding domain and the high-affinity binding site within the RRE. This complex is stabilized by a combination of less specific RNA–protein interactions and protein–protein interactions between Rev monomers [70]. Consistent with this model, Rev exists as an oligomer, most likely a tetramer, in solution [62,71].

The high-affinity binding site of HIV Rev is shown in Figure 4b. Two unconventional base-pair interactions between purines take place within the high-affinity binding site. The hydrogen-bonding arrangement of these unique base pairing interactions is shown in Figure 4c (the numbering of the nucleotide positions is based on the structure in Figure 4b). First, a G_7–A_{28} base pair is located at the base of the mismatch region in the stem–loop structure. In addition, a G_8–G_{25} base pair is located within the high-affinity binding site on the loop side of the stem–loop structure. This G_8–G_{25} base pair can be replaced with an A_8–A_{26} base pair without perturbing Rev binding [72]. A single, non-base-paired U_{27} residue is located between these two mismatched base pairs. NMR analysis has revealed that the unique base

Figure 4



Key structures involved in Rev binding to the RRE. **(a)** Amino acid sequence of the Rev arginine-rich motif (ARM). The numbering corresponds to the positions within HIV-1 Rev. Residues in bold are involved in specific interactions with bases within the Rev high-affinity binding site. **(b)** Secondary RNA structure of the Rev high-affinity binding site. Non-Watson–Crick interactions between purine base pairs are illustrated with dotted lines. This sequence is derived from the region encompassing nucleotides 7808–7846 of HIV clone HXB2 (GenBank accession number: K03455). **(c)** Hydrogen bonding stabilizes the two purine base pairs found in the Rev high-affinity binding site. **(d)** Schematic representation of the α-helical Rev ARM within the widened major groove of the Rev high-affinity binding site. The amino acids within double circles are involved in base-specific interactions.

pairing interactions within the Rev high-affinity binding site cause the major grove of the stem–loop to be widened. Widening of the major groove is essential for specific RNA–protein interactions within an RNA helix. This is because the major groove of a type A RNA helix is too narrow and deep to allow amino acid residues to reach into the RNA helix and 'feel' the specific nucleotides contained within.

Insights into the details of the binding of Rev to the high-affinity binding site of the RRE have been revealed by several studies. Two groups have published NMR-derived structures of a peptide encompassing the Rev ARM bound to a small RNA containing a high-affinity binding site for Rev [73,74]. One study used sequences found in the RRE [73], while the other used an RNA aptamer which had been selected from a randomized population and shown to interact efficiently with Rev *in vitro*. This aptamer contains the A_8–A_{26} base pair in place of the G_8–G_{26} base pair in the Rev high- affinity binding site, as described previously [72,74]. The studies resulted in nearly identical structures and they showed that the Rev peptide assumes an α-helical structure, as predicted by CD analysis. The phosphate backbone adjacent to the G_8–G_{26} (or A_8–A_{26}) base pair adopts an unusual structure that allows the Rev peptide deep access within a widened major groove, as illustrated in Figure 4d. The helix penetrates deep into the major groove of an RNA helix which has been widened by non-Watson–Crick base pairs. Asn40 specifically interacts with the G_7–A_{28} base pair and, along with Arg35, Arg39 and Arg44, makes base-specific interactions. Arg35 and Arg39 interact with U_{21}, G_{22}, and G_{25} on one side of the groove. Asn40 and Arg44 interact with U_5, G_6, G_7, and A_{28} on the other side of the widened major groove. Other amino acids within the Rev ARM make a variety of contacts with the phosphate backbone. Therefore, a structure formed by two purine–purine base pairs within the RRE creates a distinct binding pocket, allowing specific recognition by the Rev ARM.

The NMR structure of the Rev ARM binding to the Rev high-affinity binding site was validated by a study which identified supressor mutants of Rev that restored specific interactions with a mutant form of the Rev high-affinity binding site [75]. When A_{28} of the Rev high-affinity binding site was modified to G_{28}, Rev could no longer bind. This modification caused both purine base pairs in the Rev high-affinity binding site to be G_8–G_{26} interactions. Using an assay in *Escherichia coli* in which RNA–protein interactions can be selected, a specific mutant of Rev, which interacted efficiently with the A_{28}→G mutant of the Rev high-affinity binding site, was identified. The modified versions of Rev interacted with the mutated binding site to allow RNA export in transfection studies. This A_{28}→G mutant contained a modification of Asn40 to a glutamine. The observation that the change Asn40→Glu in Rev could suppress the A_{28}→G mutation is consistent with the NMR structures which show key interactions between Asn40 and the G_7–A_{28} base pair.

Viral RNA export as a target for antiviral therapies

It is now apparent that although viruses can exploit the typical cellular mRNA export pathway, they often use alternative export pathways. The challenge of developing a new antiviral therapy involves identifying an agent that negatively affects the pathogen but does not cause deleterious side effects for the host or affect the endogenous use of, in this case, the export pathway. RNA export elements are promising potential targets for antiviral therapy because they are virus-specific RNA entities that are required for viral gene expression. There are currently several therapeutic strategies being developed to block HIV replication by inhibiting Rev function. These approaches can be separated into two groups: the first focuses on perturbing Rev function and the second disrupts or blocks the Rev high-affinity binding site.

Several approaches to disrupting Rev function depend on gene therapy to allow the expression of inhibitory proteins and RNAs within a cell. For example, it has been shown that the expression of decoy RNAs, which will compete with the RRE for the Rev protein, can inhibit HIV replication [76]. Dominant-negative versions of Rev can also be expressed within a cell, thereby blocking HIV replication [77]. These dominant-negative mutants inactivate wild-type Rev monomers through the formation of non-functional oligomers [71]. Dominant-negative mutants of the eIF5A protein can also block HIV replication by inactivating Rev function [78]. Finally, intracellular immunization with single-chain anti-Rev antibodies cause Rev to be retained within the cytoplasm [79]. Unfortunately, these promising approaches cannot be realized or even tested until techniques for efficient gene delivery are developed.

Other strategies focus on disrupting the Rev high-affinity binding site and the ability of Rev to interact with viral RNA. Antisense oligonucleotides which alter RNA folding and the formation of the Rev high-affinity binding site have shown some promise in cell culture [80,81]. But before the therapeutic use of antisense-based drugs can be realized, obstacles of oligonucleotide delivery and stability must be overcome. It has also been shown that certain aminoglycosides, like neomycin, can bind tightly to the Rev high-affinity binding site efficiently blocking Rev–RRE interactions [82]. These types of drug-based therapies which disrupt the site-specific RNA–protein interactions required for viral replication show more promise in the short-term future because the development of efficient gene delivery for gene therapy is a complex problem.

Drug-based therapies which disrupt the RNA-binding site for export proteins may also be useful for perturbing the function of viral RNA-export elements which bind cellular factors. Therapies that disrupt protein function in these cases will not work because the normal cellular function of these factors, presumably required for RNA export, will also be disrupted. But if a drug-based therapy to disrupt the binding site in viral RNA for cellular Rev-like proteins can be developed, this approach could be used to inhibit the replication of most, if not all, viruses which replicate in the nucleus. Therefore, the future potential of therapies which disrupt the nuclear export of unconventional viral RNAs holds great promise in the ongoing battle between man and many virally induced diseases.

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