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Modulating HIV-1 RNA processing and utilization

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1. ABSTRACT

Expression of the integrated HIV-1 provirus is achieved by overcoming multiple barriers to the processing, transport and utilization of the viral RNA. Some of the strategies involve viral encoded proteins (i.e. Rev, Gag). However, in large part it is host factors that play essential roles in the movement of HIV-1 RNA from the site of transcription to its ultimate encapsidation into new virions. Identifying these factors and their mechanism of action provides not only important insights into HIV-1 molecular biology but also that of the cell machinery itself. In this review, we highlight the viral and host factors regulating the splicing, polyadenylation, transport, and translation of HIV-1 RNA. The observations made underline the multiple fate decisions that must be made at each stage of the viral RNA metabolic pathway and highlight potential new avenues for controlling HIV-1 replication.

2. INTRODUCTION

Although current drug combinations have proven effective at blocking or slowing the progression to AIDS following HIV-1 infection, they are not a cure. The virus persists in the host and is able to overcome the current drugs should the patient fail to adhere to the treatment regimen. The detection of HIV-1 strains resistant to one or multiple classes of drugs calls for additional investigation of new targets and approaches to controlling infection (1, 2). With all of the viral enzymes (protease, reverse transcriptase and integrase) having already been targeted by small molecule inhibitors, current efforts have focused on identifying cellular processes that play critical roles in the virus lifecycle.

Following infection and integration of the HIV-1 provirus, the virus either commits to a state of latency or

active replication (3). In the latter case, replication is initiated by transcription of the integrated genome. The viral RNA must then undergo a multitude of fate decisions spanning alternative splicing, transport, translation and packaging that will dictate the number of infectious viral particles generated. Research over the last two decades has provided a detailed outline of the *cis* and *trans*-acting factors that impact each of these fate decisions. These findings with particular emphasis on the RNA-interacting factors that modulate viral RNA fates will be outlined in the following sections.

3. REGULATING HIV-1 RNA SPLICING: PUTTING THE PIECES TOGETHER

Following transcription, host transcripts undergo a number of processing steps, including splicing and polyadenylation. The process of splicing involves recognition of signals at the extreme ends of the intron. The 5' splice site (5'ss) is recognized through base pairing interactions with U1 snRNP, and the 3' splice site (3'ss) via interactions of U2 snRNP with the branchpoint (bpt) and U2AF with the polypyrimidine tract (ppt). The strength of the various interactions ultimately determines the efficiency of intron excision. However, the efficiency of splice site use can also be influenced by the action of members of the hnRNP and SR protein families binding to adjacent sequences.

Many cellular mRNAs undergo alternative splicing to produce multiple isoforms of the encoded protein. The requirement to generate nine proteins from the single 9 kb primary viral transcript necessitated that HIV-1 evolve mechanisms to regulate the conversion of this transcript into over 40 mRNAs (Figure 1A) (4, 5). This processing must happen in a balanced fashion to generate the necessary levels of both structural and regulatory viral proteins required for appropriate regulation of HIV-1 expression and assembly of replication competent viral particles. The four major 5'ss (SD1-4) were determined to have high activity. In addition to their role in intron excision, these 5'ss also function to promote recognition of upstream 3'ss by exon definition (6-8). Several examples of either positive or negative regulation of 3'ss recognition by an adjacent 5'ss have been observed in the context of HIV-1, mutation of which frequently results in dramatic alterations in viral RNA processing that ultimately impair virus replication (9-11).

In contrast to the high activity of HIV-1 5'ss, early studies indicated that the incomplete splicing of HIV-1 RNA was largely attributable to the inefficiency of the eight 3'ss (SA1-3, SA4c,a,b, SA5, SA7) distributed throughout the 9 kb transcript (12, 13) (see Figure 1A). More recent work has provided a more detailed understanding of the limited activity of the various HIV-1 3'ss. Kammler *et al.* (14) examined the efficiency of the individual 3'ss alone or in conjunction with adjacent exon sequences. They determined that when the region comprising only the branchpoint and polypyrimidine tract were tested, SA2 and SA3 were utilized to a high degree while the remaining 3'ss (SA1, SA4c,a,b, SA5 and SA7) were not active. When tested in the context of the adjacent exons, a very different picture emerged. The strongest 3'ss in this context were SA1, SA5 and SA7 and there was a reduction in activity of SA2 and SA3. These findings highlight the important role that exon sequences play in regulating the frequency of particular splice site use.

The role of exon sequences in 3'ss use has been attributed to the presence of exon splicing enhancers (ESEs) and exon splicing silencers (ESSs) distributed throughout the viral genome (Figure 1B,C) (15, 16). These elements act to enhance or suppress, respectively, the upstream 3'ss. ESSs have been shown to regulate use of SA2 (ESSV), SA3 (ESS2p, ESS2) and SA7 (ESS3a,b) (13, 17-23). In all cases except ESS2p, silencing is mediated by interaction of the hnRNP A/B group of proteins with the ESS to block assembly of the spliceosome complex on the adjacent 3'ss (19, 21, 24, 25). In contrast, ESS2p binds hnRNP H to achieve a similar effect (20). For both ESS2 and ESS3, *in vitro* footprinting experiments have shown that hnRNP A1 is able to oligomerize along the RNA from the primary binding site, competing for the binding of positive-acting factors (26, 27). ESS3 acts cooperatively with an intron splicing silencer (ISS) overlapping the branchpoint site to maximize repression of SA7 use (24, 28).

ESE2 and ESE3 regulate use of SA3 and SA7, respectively, through interaction with SR protein family members such as SRp40, SC35 and SF2/ASF (13, 29). The primary function of these elements is to counteract the adjacent or overlapping ESS. *In vitro* studies of ESE2 have determined that the element preferentially interacts with the SR proteins SRp40 and SC35 (29, 30). Interestingly, the binding sites for these factors overlap with the protection pattern seen for hnRNP A1 (29, 30). Consistent with the *in vitro* data, overexpression of either SRp40 or SC35 increased usage of SA3, resulting in increased Tat protein synthesis and a reduction in Nef expression (31). In the case of ESE3, multiple purine-rich regions in the terminal exon have splicing enhancer activity but only the first (encompassing +20 to +33 nt relative to SA7) was found to function in a SF2/ASF-dependent fashion (32, 33). *In vitro* footprinting mapped the SF2/ASF binding site to a region encompassing the sequence AUA $(GAA)_3$ which also serves as a primary site for hnRNP A1 interaction (Figure 1C) (27). Mutation of this sequence not only disrupted SF2/ASF binding but also disrupted interaction of additional hnRNP A1 proteins with the ISS and another region immediately downstream (ESS3b). This result suggests that the competition of SF2/ASF and hnRNP A1 for the AUA $(GAA)_3$ sequence may dictate the extent of SA7 usage (27).

The third exon splicing enhancer mapped within HIV-1 (designated GAR) is located immediately 3' of SA5 and has been implicated in enhancing both SA4c,a,b and SD4 recognition (Figure 1B) (34). Both SF2/ASF and SRp40 are able to interact with this sequence. Mutation of the GAR enhancer results in a marked perturbation of viral RNA processing whereby the joining of SD1 to SA7 is dramatically increased (34). This splicing event is rarely

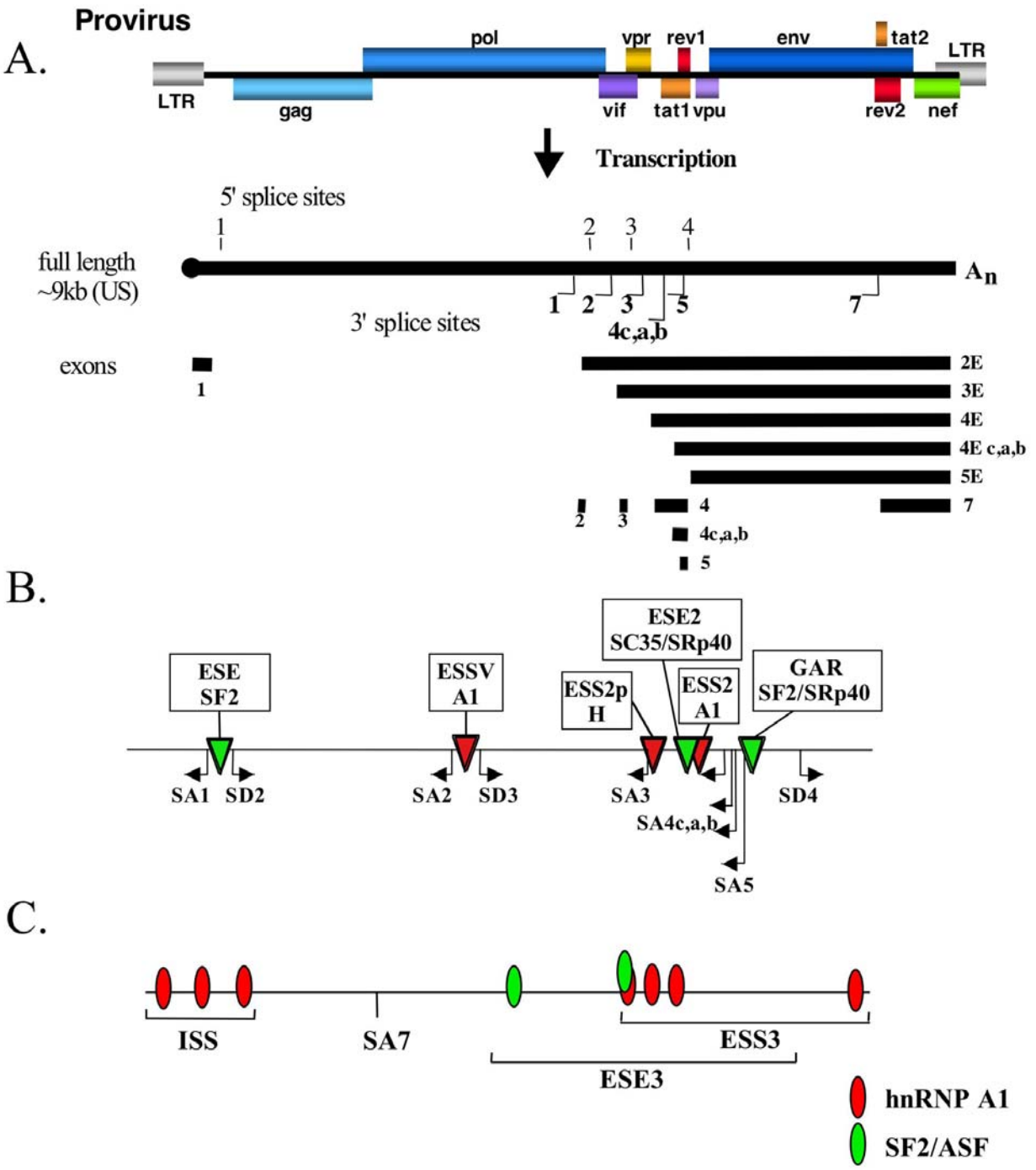

Figure 1. Cis- and Trans-Factors Regulating HIV-1 Splicing. A. Shown is the genomic organization of the HIV-1 provirus indicating the position of the various reading frames encoded. Below is the genomic RNA indicating the location of the splice donors (SD1-4) and splice acceptors (SA1-3, SA4c,a,b, SA5-7). Also illustrated are the various exons used in the generation of both 4 kb and 1.8 kb spliced forms of HIV-1 RNA (numbering of exons according to Purcell and Martin (4)). In the case of both exons 2 and 3, only partial inclusion is observed for both the 4 kb and 1.8 kb RNAs. For more detailed information on the structure of the HIV-1 RNAs generated, readers are referred to Purcell and Martin (4). B. Organization of *cis*-acting elements regulating use of SA1-SA5. Position of exon splicing enhancers (ESEs, green triangles) and exon splicing silencers (ESSs, red triangles) are indicated as well as the host proteins known to interact with each of the regulatory sequences. Factors interacting which each of the elements are indicated; SF2/ASF (SF2), SC35, SRp40, hnRNP A1 (A1) and hnRNP H (H). C. Position of the intron splicing silencer (ISS), exon splicing enhancer (ESE3) and exon splicing silencer (ESS3) regulating SA7 use. Also indicated are the mapped binding sites for hnRNPA1 (red) and SF2/ASF (green)

observed in the wild type context and results in reduction of Tat and Rev expression. Current evidence favours the hypothesis that GAR functions to enhance recruitment and/or stabilize interaction of U1 snRNP to SD4. In light of earlier data highlighting the role of SD4 in the stabilization of unspliced HIV-1 RNA (35, 36), the GAR element appears to play multiple roles in regulating the metabolism of the viral RNA.

Several observations suggest that additional splicing regulatory elements exist elsewhere in the viral genome. The finding that SF2/ASF overexpression leads to a dramatic enhancement of SA2 and to a lesser extent SA1 use suggests the presence of additional of ESEs within the adjacent exons (31). Consistent with this conclusion is recent data demonstrating that inactivation of SF2/ASF binding sites 3' of SA1 resulted in reduced SA1 use and increased use of SA2 (14).

In light of the complex network of *cis* elements and *trans*-acting factors implicated in regulating HIV-1 RNA splicing, it is of interest to question how essential such control is to the replication of the virus. Two recent findings highlight the requirement for tight control of HIV-1 splicing. Inactivation of ESSV or optimization of SD2 both result in a dramatic inhibition of HIV-1 replication stemming from a significant reduction in the abundance of 9 kb unspliced RNA with a corresponding decrease in *gag* protein synthesis and virion assembly (10, 11). Therefore, it would appear essential that the virus evolve mechanisms to limit the extent of HIV-1 RNA splicing. In addition to the control exerted by both ESS and ESE elements, HIV infection also limits the activity of SR proteins which stimulate 3'ss use. Work by Fukuhara *et al.* has shown that HIV-1 infection induces alterations in SR protein subcellular distribution, as well as reduction in SR protein phosphorylation and abundance of SC35 and SF2/ASF (37). The ability of SR protein kinase II overexpression to reverse the effects on SR protein phosphorylation suggests that the changes in SR protein modification/abundance might be achieved through modulating the activity of these SR kinases.

4. REGULATING HIV-1 POLYADENYLATION: CUTTING AT THE RIGHT PLACE

In addition to regulating the extent of RNA splicing, HIV-1 also has evolved mechanisms to modulate recognition of its polyadenylation signals. Cleavage and polyadenylation occur at the 3' end of the majority of RNA polymerase II transcripts and are important for downstream mRNA processing events such as export, translation and stability (38-43). In HIV-1, the AAUAAA polyadenylation signal and the downstream GU-rich element are duplicated at the 5' and 3' ends of the viral transcript due to their location within the R region (Figure 2). Given that use of the promoter proximal polyadenylation signal would be detrimental to viral gene expression, HIV-1 has evolved mechanisms to alter the relative efficiencies of each of the signals. Early studies indicated that RNA secondary structures formed by the 5' R-U5 sequences block access by the polyadenylation machinery (44, 45). Subsequent work has also shown that the major 5'ss (SD1) has a role in suppressing the promoter proximal polyadenylation signal. Loss of U1 snRNP binding to SD1 resulted in increased cleavage of the 5' polyadenylation site (Figure 2) (46-48). Inhibition of cleavage could be restored by compensatory mutations in U1 snRNP indicating that binding of U1 snRNP itself inhibits cleavage or recognition of the cleavage site. U1 snRNP likely inhibits cleavage by interaction of the 70K component of U1 snRNP with polyA polymerase (46, 49). Our lab has recently exploited this mechanism to suppress HIV-1 gene expression by retargeting U1 snRNP to the 3' terminal exon of HIV-1 transcripts (50). Retargeting was achieved via substitution of the first 10 nt of U1 snRNA with sequences complimentary to highly conserved regions in the terminal exon of HIV-1 and resulted in a marked reduction in viral RNAs levels putatively through effects on HIV-1 polyadenylation (50).

There is emerging evidence that cellular proteins can play a role in regulating cleavage and polyadenylation of HIV-1 RNA. Given the essential role that polyadenylation of the viral RNAs plays in their stability and transport to the cytoplasm (39), factors that alter HIV-1 RNA cleavage and polyadenylation could be important for viral replication. One example is the STAR (signal transduction and activation of RNA) protein family member Sam68 (Src-associated during mitosis of 68kD). Overexpression of Sam68 enhances HIV-1 gene expression (51-55). This effect may be due in part to Sam68's ability to stimulate cleavage of unspliced viral RNA (Figure 2) (51). However, Sam68 overexpression did not affect the level of unspliced HIV-1 RNA in the cytoplasm (51). These results and others (56) suggest that Sam68 may mark unspliced HIV-1 RNA in the nucleus and thereby promote its translation in the cytoplasm. Complementing the overexpression studies, results from two groups indicated that endogenous Sam68 is essential for HIV-1 structural protein expression (57-59). However, our own studies have found little to no effect of Sam68 depletion on HIV-1 gene expression or 3' end processing, suggesting considerable redundancy might exist to ensure appropriate metabolism of the viral RNAs (M. McLaren and A. Cochrane, unpublished results).

A more recent study has implicated hnRNP U in the post-transcriptional regulation of viral RNA (Figure 2) (60). A peptide comprising the N-terminal 86 amino acids of hnRNP U was found to render a cell unable to support HIV-1 replication. Resistance correlated with a decrease of unspliced and singly spliced viral RNAs in the cytoplasm, suggesting this protein fragment may act at the level of viral RNA export. The authors further show that resistance to HIV-1 infection is dependent on the 3' UTR (containing the AAUAAA polyadenylation signal and GU-rich element) and that this region is sufficient to confer inhibition by the hnRNP U N-terminal fragment in a heterologous reporter. Together the data suggests that the N-terminal fragment of hnRNP U may compete with endogenous hnRNP U for HIV-1 RNA binding and may act via changes in HIV-1 transcription termination, polyadenylation or export.

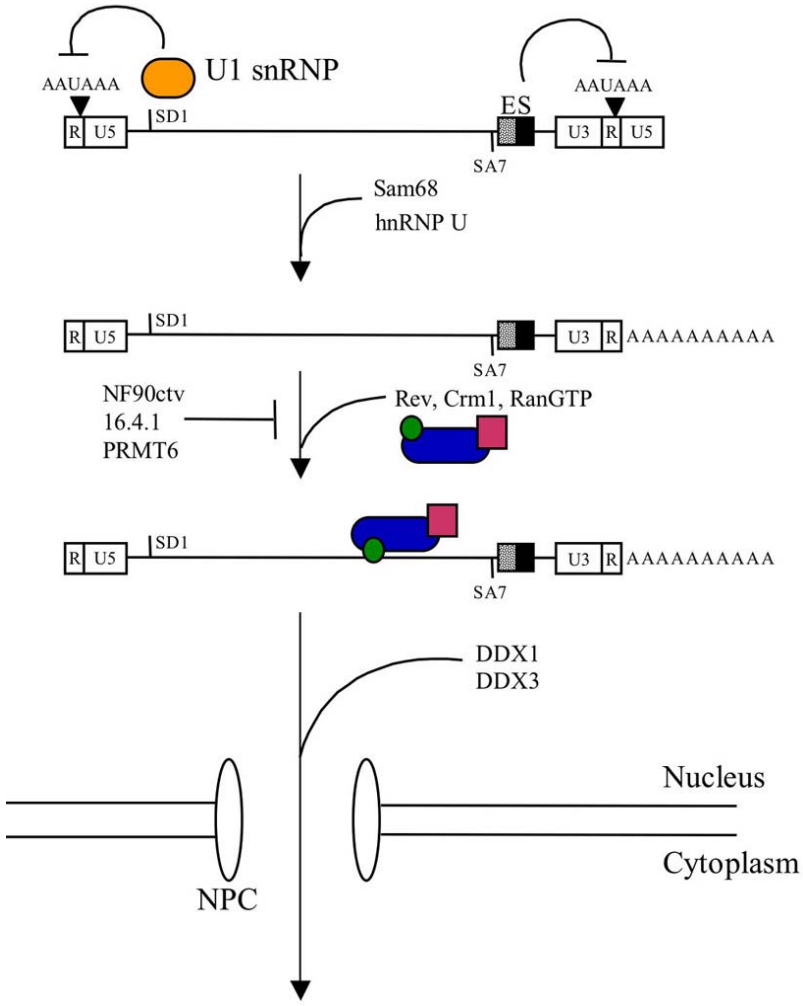

Figure 2. Regulation of HIV-1 RNA Polyadenylation and Transport to the Cytoplasm.Following transcription, use of the 5' polyadenylation signal is suppressed by interaction of U1 snRNP with SD1. Use of the 3' polyadenylation site is regulated by the competing inhibitory action of ESS3 (S) and the action of ESE3 (E). Once polyadenylation occurs, the viral RNA becomes a substrate for Rev-mediated export through interaction of Rev/Crm1/RanGTP complex with the RRE within the RNA. Interfering with formation of the export complex are the host factors NF90ctv, PRMT6 and 16.4.1. Movement of the Rev-HIV RNA complex to the cytoplasm through the nuclear pore complex (NPC) is facilitated by the factors DDX1 and DDX3.

In addition to *trans*-acting factors, *cis*-acting elements may control use of the 3' polyadenylation signal. ESE3 and ESS3 act in a competitive fashion to regulate use of SA7 (13, 26, 27, 32, 61), but further work suggested that ESS3 can also function to block 3' cleavage and transport of unspliced viral RNA (Figure 2) (24, 51, 62). Removal of the ESE3 alone resulted in loss of viral RNA polyadenylation and export to the cytoplasm. Deletion of both ESE3 and ESS3 restored 3' end formation and nuclear export to wild type levels. These observations suggest that antagonism between these two elements is also important for regulating viral RNA polyadenylation (24, 51, 62). As these experiments were carried out using HIV-1 reporter constructs, it will be important to determine what role ESS3 plays in splicing, 3' end processing and export in the context of the full virus.

In addition to its effects on splicing factors, HIV-1 may also alter the host polyadenylation machinery to render the cell more supportive of virus replication. The accessory protein Vpr causes dephosphorylation and subsequent activation of polyA polymerase (63). In addition, the viral transcriptional activator protein, Tat, increases the expression of the cleavage and polyadenylation specificity factor (CPSF) (64). It remains to be determined whether these alterations affect HIV-1 RNAs directly or cause a more general increase in cellular RNA polyadenylation.

5. HIV-1 RNA EXPORT: DELIVERING THE mRNA TO THE CYTOPLASM

Once processing is complete, the viral RNAs must be transported to the cytoplasm for subsequent

translation. Most cellular mRNAs must be capped, spliced and 3'end processed before they are competent for export to the cytoplasm (65). Recognition of fully processed mRNAs for export may occur by assembly of the exon junction complex (EJC) during splicing or by direct binding of factors to sequence elements within the RNA (65-67). However, HIV-1 has evolved strategies to overcome cellular mechanisms preventing transport of unspliced RNAs. Early in infection, only the 2 kb class of viral RNAs is exported to the cytoplasm (leading to synthesis of Tat, Rev and Nef) while members of the 9 and 4 kb classes of RNA are retained in the nucleus (3). Nuclear retention of unspliced and incompletely spliced HIV-1 RNAs has been attributed to either partial spliceosome assembly (68) or specific nuclear retention sequences in the RNA (69-77). Following its synthesis, the HIV-1 Rev protein is imported into the nucleus and binds to a structured region (the Rev-response element or RRE) found in the unspliced and incompletely spliced HIV-1 RNAs (78, 79). After multimerization of Rev on the RRE, the nuclear export factor Crm-1 and Ran-GTP are recruited to the complex and association of Crm-1 with the nuclear pore complex (NPC) allows passage of the Rev-bound viral RNA into the cytoplasm (Figure 2). Dissociation of the Rev-Crm1-viral RNA complex occurs upon conversion of Ran-GTP to Ran-GDP (78, 79). A number of host cell factors have been implicated in HIV-1 Rev function (57-59, 62, 80, 81). Due to space limitations, we will only discuss those identified in the past few years.

Upon movement of RNA from the nucleus to the cytoplasm, a significant alteration occurs in the composition of the ribonucleoprotein particle (RNP) consistent with the involvement of RNA helicases (82, 83). Recent work has demonstrated the requirement for the DEAD-box helicase DDX1 for HIV-1 gene expression and proper nuclear/nucleolar distribution of Rev protein (Figure 2) (84). DDX1 binds Rev and the RRE, suggesting that this factor may be important for initial binding of Rev to the RRE in the nucleus or subsequent dissociation of the complex in the cytoplasm (84). It was further demonstrated that both DDX1 and Rev show aberrant cytoplasmic localization in astrocytes (85), a cell type known to be restrictive to HIV-1 replication (86). These results support the hypothesis that DDX1 is important for Rev nuclear/nucleolar localization and subsequent export activity.

Another RNA helicase, DDX3, has also been shown to be required for Rev function (87). DDX3 shuttles between the nucleus and cytoplasm, is localized to the cytoplasmic face of the nuclear membrane at steady state and binds Crm-1, Rev and nucleoporins (87). Although it is unclear whether DDX3 modulates Rev subcellular distribution, the results suggest that DDX3 may act in export of the Crm1-Rev-RRE complex through the nuclear pore or in release of viral RNAs from this complex once in the cytoplasm (87). Further support for DDX protein involvement in HIV-1 replication comes from the finding that expression of several DDX proteins is upregulated when latently infected cells are induced to undergo active replication (88).

Cells may also have evolved mechanisms to inhibit Rev function. Recently, it has been reported that a C-terminal variant of nuclear factor 90 (NF90ctv) can regulate Rev activity (Figure 2) (89). In this case, overexpression of NF90ctv moderately reduced Rev-dependent viral protein expression and this effect was attributed to multiple domains of NF90ctv: the nuclear export signal (NES), double-stranded RNA binding domain (DRBD2) and C-terminal arginine/glycine deficient domain (RG-). Inhibition of Rev function correlated with NF90ctv's ability to bind and partially relocalize Rev to the cytoplasm. These results are similar to those observed upon depletion of DDX1, emphasizing the importance of steady state Rev nuclear/nucleolar localization for function. NF90ctv has also been implicated in inhibition of Tat-dependent transcription, suggesting this protein acts at multiple stages of the HIV-1 lifecycle (90).

A yeast two-hybrid screen recently identified a novel binding partner of unknown function for Rev, termed 16.4.1. (Figure 2) (91). Interaction between 16.4.1 and Rev was confirmed by a mammalian two-hybrid assay and the two proteins colocalized in the nucleolus. Given that 16.4.1 localization is predominantly cytoplasmic in the absence of Rev, these results suggest that Rev can recruit 16.4.1 to the nucleolus. Overexpression of 16.4.1. inhibited expression of a Rev-dependent reporter and, importantly, siRNA knockdown of 16.4.1. resulted in increased Rev-dependent expression. Further studies will likely focus on the mechanism by which 16.4.1 modulates Rev activity and the cellular role of 16.4.1.

Another way to regulate Rev activity is through covalent modification. Although Rev phosphorylation is not essential for function (92), more recent work has indicated that Rev is asymmetrically methylated by the arginine methylase PRMT6 in its arginine rich NLS (93). This modification reduces Rev binding to the RRE. Rev-dependent reporter expression was also reduced upon co-transfection with PRMT6, suggesting a block in viral RNA export. Whether this effect is a consequence of reduced unspliced HIV-1 RNA export to the cytoplasm was not shown directly, and remains to be determined.

6. TRANSLATION OR PACKAGING: MAKING THE CHOICE

6.1. Controlling viral RNA movement and utilization in the cytoplasm

Once transit through the nuclear pore has occurred, multiple fates remain for HIV-1 RNA. However, several pieces of data indicate that movement away from the perinuclear space does not occur by simple diffusion. In the first instance, depletion of the host factor hRIP (human Rev interacting protein) results in accumulation of unspliced viral RNA in the perinuclear space and loss of structural protein expression (94). An analogous phenotype is observed upon overexpression of a deletion mutant of Sam68 that removes the last 100 a.a. of the protein (Sam68ΔC) (54). Together, these findings suggest the viral RNP must undergo some remodeling following export to allow it to continue on the path of translation or packaging

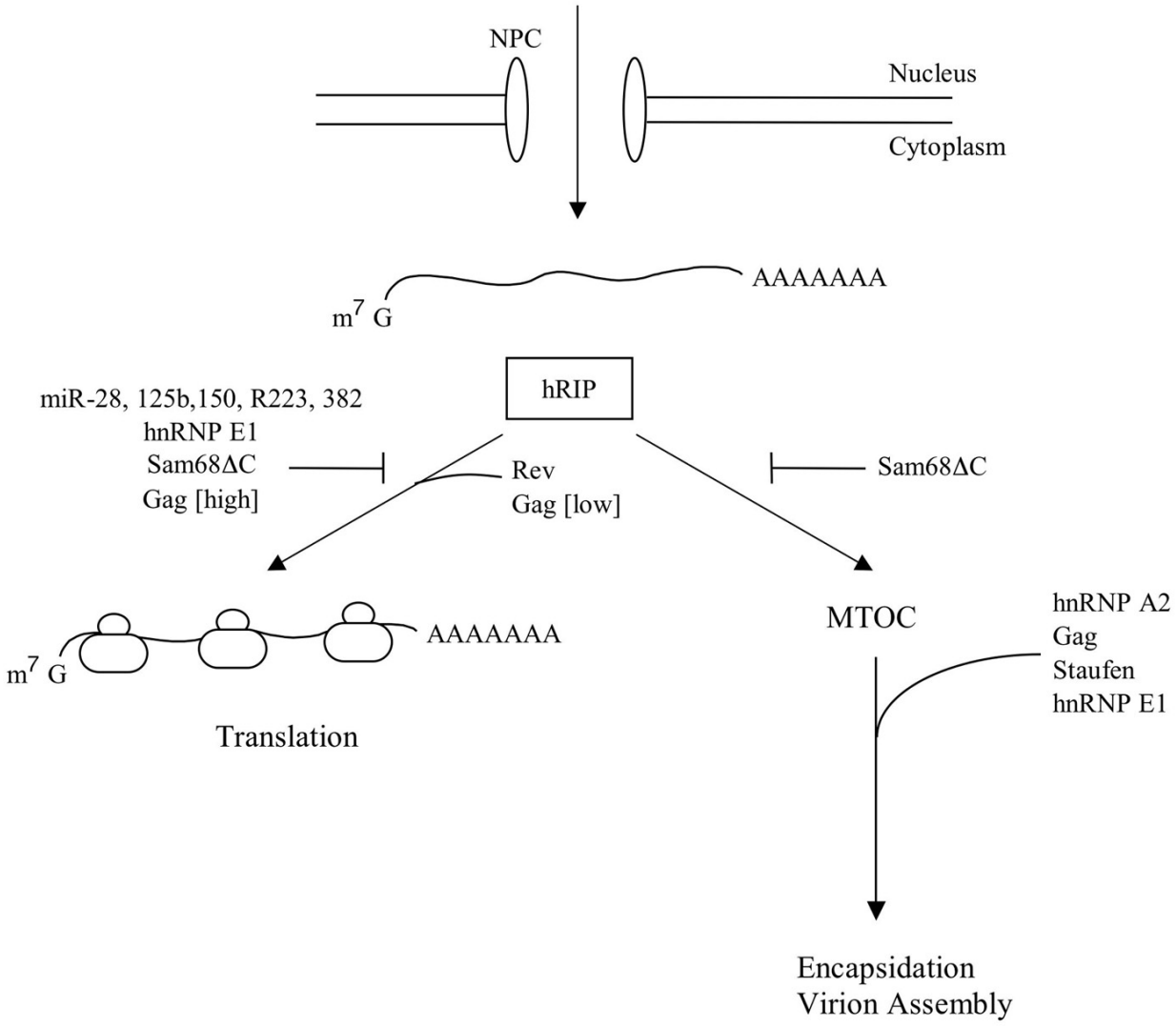

Figure 3. HIV-1 RNA Cytoplasmic Trafficking and Translation. Following export to the cytoplasm through the nuclear pore complex (NPC), movement of viral RNA from the nuclear periphery is dependent on the action of hRIP. Movement from the nuclear periphery is inhibited by the action of Sam68ΔC as is engagement with the translation apparatus. Genomic HIV-1 RNA can engage in translation to generate Gag and Gagpol protein but this activity is blocked by the action of hnRNP E1, miRNAs (miR-28, miR-125b, miR-150, mi-R223, miR-382) or high concentrations of Gag (Gag (high)). In contrast, genomic RNA translation is enhanced by the action of Rev and low concentrations of Gag (Gag (low)). Alternatively, genomic RNA migrates to the MTOC where it interacts with hnRNP A2, hnRNP E1, Staufen and Gag to traffic to the site of virion assembly and is encapsidated in new virions.

into assembling virions. If this is the case, hRIP appears to be obligatory for the remodeling step while the Sam68 mutant prevents it (Figure 3).

Once freed from the perinuclear space, two major fates remain for HIV-1 genomic RNA; translation or packaging (15). In contrast, the singly spliced and multiply spliced viral RNAs are committed to translation which, in the case of *env* mRNAs, is done in association with the endoplasmic reticulum (15). Unspliced HIV-1 RNA serves as both template for synthesis of Gag and Gagpol and substrate for packaging through the interaction of the NC portion of Gag with the packaging signal (psi) within the RNA. Therefore, it is anticipated that after activation of proviral DNA transcription, the bulk of the unspliced viral RNA would be committed to translation but as Gag protein levels increase there would be a shift to packaging (Figure 3, Gag (low) versus Gag (high)). Consistent with this expectation, Gag has a bimodal effect on viral RNA translation; at low levels of Gag, translation of viral RNA is enhanced while at higher concentrations, it is inhibited (95). Stimulation of translation maps to the MA domain of Gag while inhibition requires the NC sequence (95). The inhibitory effect is dependent on the integrity of the packaging signal within the RNA, supporting the hypothesis that it is the result of direct interaction between

Gag and the RNA. Research to date supports the model that, in the case of HIV-1, there are not distinct pools of unspliced viral RNA committed to either encapsidation or translation (96). Rather, Gag is able to select from RNAs either actively engaged in translation or within the free RNP pool.

Movement of genomic RNA from the nuclear pore throughout the cytoplasm appears to be an active process facilitated in part by interaction with hnRNP A2. Earlier work had mapped two hnRNP A2 binding sites (A2RE-1, A2RE-2) within HIV-1 RNA, either of which could confer axonal RNA transport in oligodendrocytes (97). Subsequent studies confirmed the interaction of hnRNP A2 with HIV-1 genomic RNA and, in support of a role in viral RNA trafficking, mutation of A2RE-2 induced a marked accumulation of the RNA in the nucleus (98). Experiments examining the effect of hnRNP A2 depletion on HIV-1 RNA metabolism lend additional support for a primary role of hnRNP A2 in cytoplasmic trafficking. While depletion of hnRNP A1 was found to have little or no effect on viral RNA processing or subcellular distribution, reduced hnRNP A2 expression induced perinuclear accumulation of HIV-1 genomic RNA in and around the microtubule-organizing centers (MTOC, Figure 3) (99). Accumulation at the MTOC is also induced by overexpression of Rab7 interacting lysosomal protein (RILP), consistent with movement of the viral RNA being dependent upon dynein-dynactin motors (99). There is also evidence that Gag-genomic RNA interaction first occurs within the MTOC and perinuclear space to initiate the packaging process (100). Together, the information suggests that HIV-1 genomic RNA is transported as an RNP along microtubules to the site of virion assembly in a manner dependent, in part, on the presence of hnRNP A2 in the HIV-1 genomic RNP.

Other factors known to affect HIV-1 RNA utilization and processing within the cytoplasm include hnRNP E1 (PCBP-1) (101) and Staufen (102-104). Both proteins are found within the virion suggesting that they traffic along with HIV-1 genomic RNA to sites of virion assembly on the plasma membrane (Figure 3) (102, 105). In the case of hnRNP E1, overexpression of the factor resulted in a marked reduction in Gag and Env synthesis while depletion of the protein had the opposite effect (101). This ability to suppress viral protein synthesis does not involve any change in viral RNA abundance, splicing or extent of cytoplasmic accumulation. Therefore, hnRNP E1 may be regulating translation initiation on the viral RNA to indirectly influence the accessibility of the genomic RNA for packaging. Staufen appears to play a more direct role in HIV-1 RNA metabolism given its direct and selective association with viral genomic RNA and that modulation of its levels (increased or decreased) result in reduced infectivity of the virus (102-104). Part of the reduced infectivity might be attributed to alterations in the amount of viral RNA within the capsid or Staufen's interaction with Gag and its effects on Gag multimerization (102-104).

6.2. Translation of HIV-1 mRNAs

In general, translation and stability of mRNAs are enhanced by the terminal modifications of the RNA: the presence of a 5' cap and the 3' poly A tail. During its synthesis, the 5' cap of an mRNA is found associated with the nuclear cap binding complex (CBC, composed of CBP20/80). However, current hypotheses suggest that, following a pioneering round of translation, the CBC is exchanged for the eIF4E component of the translation initiation complex eIF4F (82, 83). A similar exchange of factors occurs at the 3' end, the initial polyA binding protein 2 (PABP-2) being replaced by PABP-1 at some point in the transport of the RNA to the cytoplasm (82). The presence of both eIF4E and PABP-1 on a mRNA is believed to enhance translation by the interaction of both proteins with eIF4G, effectively circularizing the RNP and recycling terminating ribosomes to the site of translation initiation (106, 107). The 43S preinitiation complex, composed of Met-tRNAi_i, 40S ribosome and eIF2, is thought to interact with the eIF4F complex at the 5' end of the mRNA and subsequently scan down the RNA until it interacts with an AUG start codon. The 60S ribosomal subunit is then recruited to form the 80S initiation complex (108). Translational control is mainly exerted at initiation, where there are two major control points. First, there is a limiting amount of eIF4E in the cell, and its activity is controlled by phosphorylation (109). Second, the formation of the ternary complex (eIF2-GTP-Met-tRNA$_i$) is inhibited by phosphorylation of the eIF2□ subunit. The eIF2□ protein kinase family phosphorylates the □-subunit of eIF2 in response to a variety of cell stresses (such as amino acid starvation or viral infection) (110).

HIV-1 uses the scanning mechanism in the translation of many of its mRNAs, however recent studies have highlighted additional strategies. In the case of HIV-1 genomic RNA, scanning from the 5' cap is particularly problematic given the extensive secondary structure within the 5' untranslated region (5'UTR) that includes the signals for primer binding (PBS), Tat transactivation (TAR), control of polyadenylation, viral RNA dimerization (DIS) and packaging (psi sequence). Normally, such extensive secondary structure would negatively impact on the translational efficiency of any mRNA. HIV-1 has circumvented this problem by encoding an internal ribosome entry site (IRES) between +104 to +336 nt of the RNA. The presence of the IRES allows HIV-1 to bypass cap-dependent translation initiation. Although initial reports suggested that the HIV-1 5'UTR had no IRES activity *in vitro* or *in vivo* (111), subsequent experiments have determined that the HIV-1 IRES functions in a cell cycle dependent fashion, being most active during the G2/M phase of the cell cycle (112). Such a property is advantageous given that HIV-1 Vpr is known to arrest cells in this state (113). It may also permit viral protein synthesis under conditions of cell stress. A second IRES has also been mapped within the coding region of HIV-1 Gag (114). This IRES is unique as it is located within the Gag ORF and is able to stimulate translation from the upstream full-length Gag AUG start site as well as induce expression of an N-terminal truncated Gag isoform (p40) from a downstream AUG start site.

While use of an IRES overcomes problems of secondary structure within the 5'UTR of HIV-1 genomic

RNA, additional strategies have been suggested to allow high level synthesis of Env (gp160). In all mRNAs encoding Env, the AUG for the *env* reading frame is not the first AUG after the 5' cap. Rather, it is preceded by the AUG for Rev and/or Vpu depending on the 3'ss used in generating the singly spliced RNA (SA4c,a,b versus SA5) (4, 5). Whereas one might anticipate that the presence of a 5' AUG might negatively impact use of 3' reading frames, recent evidence indicates that this is not the case for any of the *env* mRNAs. Using constructs representing individual forms of *env* mRNA, it was found that the level of gp160 synthesis was constant regardless of the number of upstream AUG codons (115). One mechanism to explain this observation is that initiation proceeds by a leaky scanning mechanism (116, 117). In support of this hypothesis is the finding that mutation of the upstream weak Vpu AUG to a canonical AUG suppresses Env translation, while deletion of the weak Vpu AUG enhanced Env expression, indicating that the Vpu AUG is inefficiently recognized (118). Evidence also supports the possibility that Env translation initiation occurs via discontinuous ribosome scanning from the 5'end, or a ribosome shunt (115). A third model implicates translation termination and re-initiation. Directly upstream of the Vpu AUG is a highly conserved AUG codon, followed immediately by a stop codon, which overlaps with the weak Vpu AUG (118). Inactivation of the highly conserved upstream AUG was found to negatively affect Env synthesis.

6.3. Host and viral proteins influence translation

In addition to the multiple *cis*-acting elements impacting on the efficiency of viral mRNA translation, multiple factors (both viral and cellular) also act to regulate synthesis of HIV-1 proteins. A subset of these are briefly summarized below.

Along with its crucial role in transcription, Tat plays an important role in translational regulation. First and foremost, Tat acts as a pseudosubstrate for PKR, one of the components involved in the cellular antiviral response (119-123). HIV-1 TAR RNA has been shown to activate PKR, resulting in eIF2α phosphorylation and translational shutoff (124). Tat competes with eIF2α for binding to the C-terminus of PKR and can reverse the inhibition of translation mediated by active PKR. The importance of this activity cannot be overstated as PKR activation by HIV-1 TAR RNA, if unchecked, would block HIV-1 replication (124). The second role postulated for Tat in modulating translation is through binding to the elongation factor (EF)-1δsubunit. EF-1 is required for delivery of aminoacyl tRNAs to the ribosome. The interaction of Tat with EF-1δsubunit was shown to inhibit cellular but not viral RNA translation (125).

The TAR RNA binding protein (TRBP) binds to the TAR RNA and synergistically activates transcription with Tat. TRBP has also been found to bind to PKR and inhibit its kinase activity (126-128). It has been reported that TRBP binding to TAR RNA may help to sequester this dsRNA element away from PKR, thereby inhibiting the antiviral response induced by PKR binding to viral RNA.

In addition to Rev's role in the export of viral RNA to the cytoplasm, it is also believed to enhance the translation of these mRNAs. It has been shown to promote the loading of Gag-Pol and Env mRNA on to polysomes (129). Rev elevates the amount of Gag RNA in the cytoplasm by only 8-16 fold while the amount of Gag protein increases by over 800 fold (129). In addition, experiments using cytoplasmically transcribed Env RNA show that Rev is still able to enhance translation (130). RNA must contain the RRE for Rev-induced stimulation, but the RRE alone is not sufficient, indicating the requirement for additional *cis*- or *trans*-acting factors.

The HIV-1 protease is encoded by the *pol* gene and cleaves the Gag and Gagpol polyproteins into their mature forms. In addition, it cleaves host proteins chief among which are the translation factors eIF4GI and PABP1 (131-134). It cleaves eIF4GI in 3 places: twice between the eIF4E and first eIF4A binding site, and once in the second eIF4A binding site (134, 135). HIV-1 protease cleaves PABP1 twice: once in RRM3 and once in the C-terminal domain (132). These effects are similar to the poliovirus 2A protease which cleaves eIF4GI, eIF4GII, and PABP1 (136-141), resulting in rapid host translational shutoff and enhancement of IRES mediated translation. It is possible that HIV-1 protease cleavage of host factors similarly influences IRES-mediated translation, but further investigation is required. However, while HIV-1 protease cleavage of these translation factors causes a global decrease in cellular translation, there is not a complete shutoff of cap-dependent translation (133).

Recent work by Huang *et al.* (142) has suggested that the host cell (in the form of resting CD4+ T cells) may actively suppress HIV-1 gene expression at the level of translation. The group observed that resting CD4+ T cells had reduced ability to support expression of constructs containing the terminal exon of HIV-1. This activity was associated with increased expression of several microRNAs (miR-28, miR-125b, miR-150, miR-223, miR-382) in resting CD4+ T cells that could bind to regions in the HIV-1 terminal exon. Selective inhibition of these miRNAs was associated with increased HIV-1 expression. Given that no change in viral RNA abundance or processing was observed, it is probable that these miRNAs are acting to inhibit translation. However, HIV-1 may have already evolved a strategy to minimize the effect of such innate antiviral defenses. Recent work (143) has demonstrated that TAR RNA is able to modulate these antiviral activities through its interaction with TRBP, a component of the RNA-inducing silencer complex (RISC). Overexpression of TAR RNA was observed to reduce Dicer-RISC activity. Given the role of Dicer-RISC in the generation and activity of miRNAs, TAR RNA may act by reducing the levels of anti-HIV miRNAs/siRNAs within the cell to permit a high level of viral gene expression.

7. SUMMARY

In this review, we have highlighted a subset of the modifications and interactions that HIV-1 RNA undergoes to achieve viral protein expression and assembly

of new virions. As our understanding of the molecular biology of HIV-1 increases, it is likely that we will uncover a greater complexity of HIV-1 RNA metabolism. But such knowledge also identifies new points at which intervention strategies can be directed to achieve a more profound suppression of HIV-1 replication. The observations may prove advantageous as interventions directed at viral-host interactions should limit the ability of HIV-1 to evolve resistance (due to the low rate of host mutation).

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