4

The Regulation of HIV-1 mRNA Biogenesis

Massimo Caputi
Charles E. Schmidt College of Medicine,
Florida Atlantic University
USA

1. Introduction

The machinery regulating the transcription and processing of the human immunodeficiency virus type 1 (HIV-1) genome has been extensively studied for the past two decades, leading to the characterization of a complex set of interactions between viral and cellular factors. Our understanding of the basic molecular mechanisms regulating the expression of cellular genes has been greatly advanced by the lessons learned from this virus. Studies aimed at the understanding of transcription, capping, polyadenylation, splicing and export of the viral transcripts have helped in modeling the mechanisms regulating transcriptional and post-transcriptional events in the cell. Recent developments in the field have also shown that, similarly to cellular genes, transcription and processing of the viral mRNAs are functionally coupled and can potentially be regulated by small non-coding RNAs.

HIV replication is a complex multistep process whereby, following the recognition of specific receptors and co-receptors on the host cell membrane, the virus enters the cell where the viral RNA genome is reverse transcribed and integrated into the cellular DNA. The integrated proviral genome is than transcribed by the host transcription machinery into a 9.2 kb primary transcript, which is alternatively spliced in mRNAs coding for the 9 viral genes. *Tat* and *rev* gene products are shuttled into the nucleus to aid the transcription process, the former, and export of unspliced transcripts, the later. Unspliced transcripts are packaged as viral genome into the nascent virions. *Gag* and *env* gene products code for the structural components of the new virions while the *pol* gene codes for key enzyme required for viral integration into the target host cell, which are than packaged within the virions. The products of the *Vif, Vpr, Vpu* and *Nef* genes are not essential for viral replication but are required for HIV pathogenesis and infectivity *in-vivo*.

HIV-1 has developed a number of strategies to regulate the transcription and processing of its primary transcript. Interactions between viral RNA sequences, the host cell and viral proteins are necessary to express the nine gene products required for its replication. Alteration of the delicate balance between spliced and unspliced RNAs, or disruption of the viral RNA export pathway, can dramatically affect HIV-1 infectivity and pathogenesis (Amendt, et al., 1994, Jablonski & Caputi, 2009, Pollard & Malim, 1998, Purcell & Martin, 1993, Stoltzfus & Madsen, 2006, Wentz, et al., 1997). A better understanding of the mechanisms regulating the transcription and processing of the viral RNA may provide us with novel therapeutic targets.

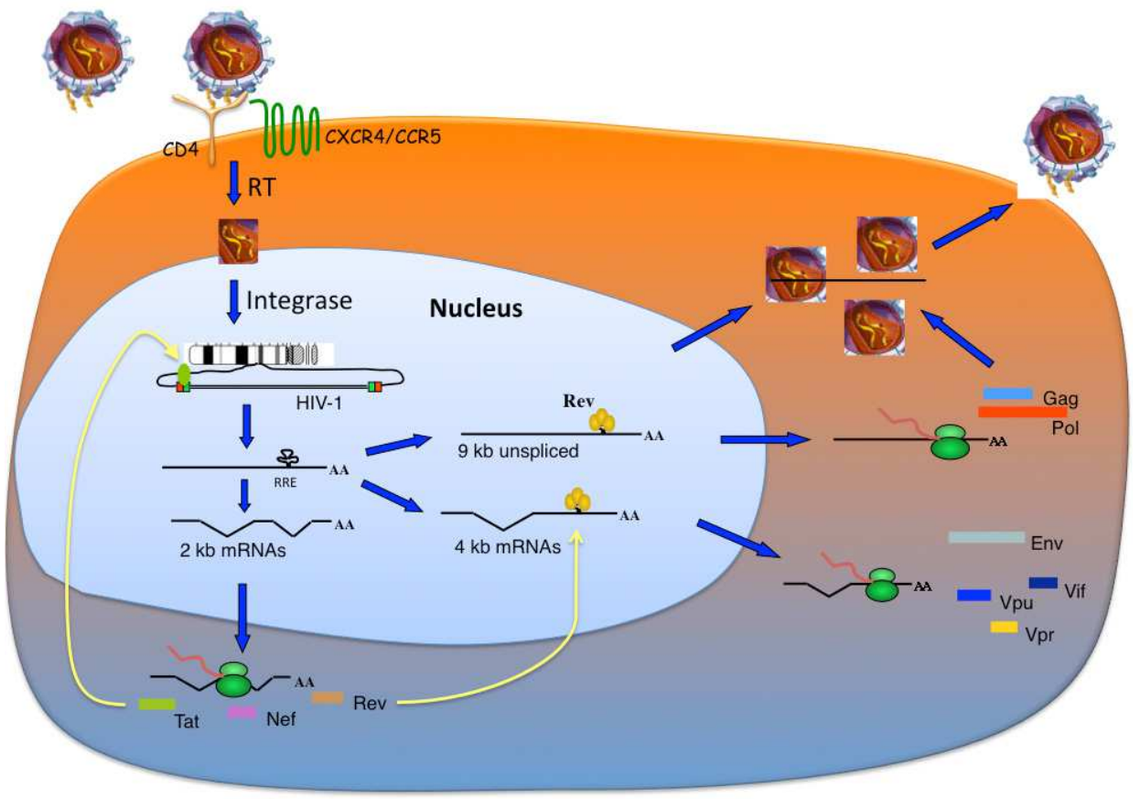

Fig. 1. The HIV-1 replication cycle. The key steps in the viral replication cycle: entry, integration, transcription, splicing export and assembly of the new virions are schematically shown together with the main viral gene products and their functions.

2. HIV-1 transcription regulation

Once the virus is integrated into the host cell chromosome the viral genome is transcribed into a single pre-mRNA from a complex promoter located within the 5′ long terminal repeat (LTR) of the viral genome. The HIV-1 LTR promoter contains two Sp1 binding motifs and two nuclear factor NF-kB binding sites which serves to regulate basal HIV-1 transcription (Pereira, et al., 2000). The LTR also contains binding elements for positive nuclear factor of activated T cells, AP-1, and negative, YY1 and LSF, transcriptional regulators. Tumor necrosis factor-α (TNF-α) and other cytokines can induce NF-kb and activate HIV-1 transcription in infected cells (Van Lint, et al., 2004). Transcription regulation of the HIV-1 genome is mediated by RNA polymerase II (RNAP II) and a combination of basal and promoter specific factors (Fig. 2) (Brady & Kashanchi, 2005, Pereira, et al., 2000).

Shortly after transcription begins RNAP II activity is paused. Before transcription is halted a short (nucleotides +1 to +82) stem loop sequence, the transactivation responsive (TAR) RNA, is synthesized. Binding of the 101 amino acid viral regulatory protein Tat to TAR stimulates transcription elongation and possibly initiation of the viral transcription complex (Fig. 2) (Berkhout, et al., 1989, Gaynor, 1995, Raha, et al., 2005). The Tat-TAR interaction promote the recruitment of the cyclin T1 (CycT1) component of the human positive

transcription elongation factor b (P-TEFb) (Garber, et al., 1998, Wei, et al., 1998). CycT1 recruits Cdk9, the catalytic subunit of P-TEFb, which phosphorylates the C-terminal domain (CTD) of RNAP II to facilitate elongation of the viral transcript (Bres, et al., 2008, Peterlin & Price, 2006). The mammalian RNAPII CTD is composed of 52 tandemly repeated heptads with a consensus, Tyr-1-Ser-2-Pro-3-Thr-4-Ser-5-Pro-6-Ser-7. Ser-2 and Ser-5 are targets of phosphorylation and dephosphorylation during transcription (Hirose & Ohkuma, 2007). Phosphorylation of RNAPII CTD is essential for transcription. RNAPII CTD is phosphorylated at Ser-5 by TFIIH (CDK7) during transcription initiation through the promoter clearance stage (Hengartner, et al., 1998) and changes to Ser-2 phosphorylation during elongation when the polymerase is associated with the coding region (Komarnitsky, et al., 2000, Ni, et al., 2004). In addition to promoting Ser-2 phosphorylation, Tat modifies the activity of CDK9 to phosphorylate Ser-5 following release of TFIIH (Zhou, et al., 2000). Furthermore, P-TEFb targets cofactors such as the human homologoue of SPT5 (TAT-CT1), which together with SPT4 constitutes the 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) Rd protein (Yamaguchi, et al., 1999). The cooperative interaction of DSIF and NELF induce polymerase pausing near promoter start sites (Wada, et al., 2000, Wada, et al., 1998b). This pausing event can be reversed by a CDK9-dependent phosphorylation of SPT5 (Kim & Sharp, 2001) and the RD protein, which causes a dissociation of NELF from the stem of TAR RNA (Fujinaga, et al., 2004).

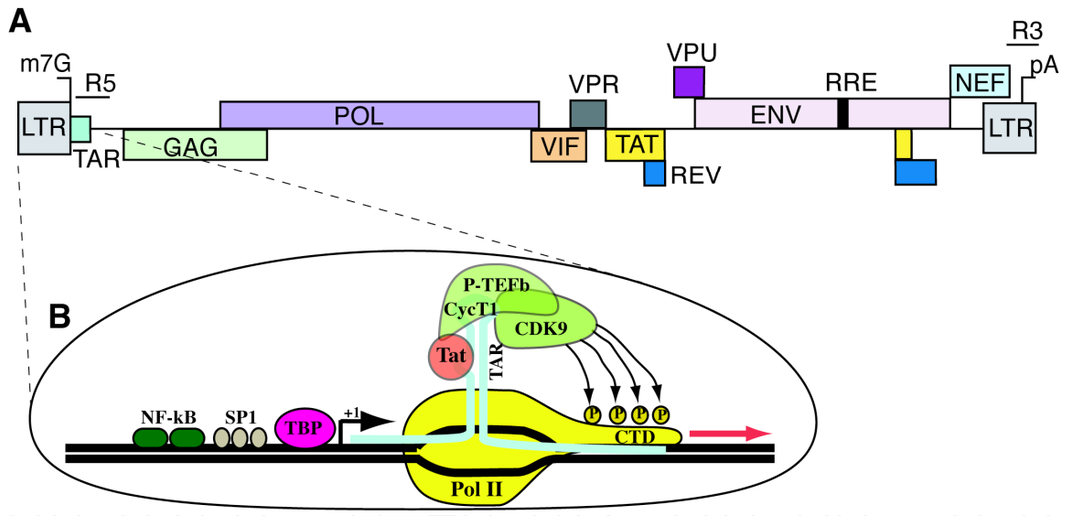

Fig. 2. A) The map shows the genomic organization of the HIV-1 provirus indicating the position of the various viral genes and key features of the viral genome. B) Schematic representation of viral transcription. A number of cellular transcription factors assemble onto the LTR promoter. Binding of Tat onto the TAR sequence promotes assembly of the components of P-TEFb (CycT1 and CDK9). The kinase activity of CDK9 phosphorylates the RNAPII CTD to facilitate elongation.

Tat role in viral transcription is not limited to the recruitment of RNAP II cofactors. Once integrated into the host cell genome, nucleosomes are deposited at specific positions within the viral promoter and exert a strong repression of transcriptional initiation. After Tat activates transcription, the chromatin associated with sequences immediately downstream of the transcription start site becomes accessible to nucleases (Verdin, et al., 1993). The

chromatin remodeling induced by Tat is dependent on the recruitment of enzymes with histone acetyl transferase (HAT) activity, which modify chromatin conformation (Marcello, et al., 2001). These factors include the transcriptional co-activators p300 and the cAMP-responsive binding protein (CREB)-binding protein (CBP) (Marzio, et al., 1998), the p300/CBP-associated factor (P/CAF) (Benkirane, et al., 1998), the general control non-derepressible-5 (GCN5) factor (Col, et al., 2001), the transcription factor TAFII250 (Weissman, et al., 1998) and the TIP60 protein (Kamine, et al., 1996). HATs acetylate the N-terminal tails of histones to stimulate chromatin remodeling and modify specific lysines of transcription factors to modulate DNA-binding affinity (Li, et al., 2007, Yang & Seto, 2008). In addition to histones, Tat itself is a substrate for acetylation by HATs. Lysines at positions 50 and 51 are major substrates for acetylation by p300 and hGCN5 (Col, et al., 2001, Deng, et al., 2001, Kiernan, et al., 1999). Acetylation of lysine 50 of Tat promotes the dissociation of Tat from TAR RNA during early transcription elongation and recruitment of the SWI/SNF chromatin-remodeling complex (Treand, et al., 2006), which synergize with p300 acetyltransferase and acetylated Tat to remodel the nucleosome at the HIV promoter in order to activate transcription (Mahmoudi, et al., 2006). Furthermore, acetylation of lysine 50 triggers the recruitment of P/CAF to the elongating RNA Pol II (Dorr, et al., 2002). P/CAF acetylates Tat on Lys28 (Kiernan, et al., 1999), which enhances the Tat-CycT1 interaction (Bres, et al., 2002). Besides acetylation other post-translational modifications appear to regulate Tat activity. Phosphorylation of Tat Ser16 and Ser46 by the cell cycle regulator Cdk2/cyclin E appears to be required for efficient HIV-1 transcription and replication (Ammosova, et al., 2006). Methylation of Tat on arginine residues by PRMT6 has been shown to inhibit transcriptional activity (Boulanger, et al., 2005), while non-proteolytic ubiquitination of Tat by Hdm2 appears to enhance viral transcription (Bres, et al., 2003).
In addition to its role in viral transactivation, Tat has also been shown to regulate the rate of transcription and expression of host cellular genes (Caldwell, et al., 2000, Gibellini, et al., 2002, Huang, et al., 1998, Ott, et al., 1998, Secchiero, et al., 1999). Furthermore, Tat function appears not to be restricted to infected cells, which actively secrete large amounts of Tat in the bloodstream (Ensoli, et al., 1990). Extracellular Tat can generate a wide array of cell responses ranging from T-cell activation (Wu, et al., 2007) to stimulation of cytokine secretion, cell death in neurons and cell proliferation in endothelial and T-cells (Huigen, et al., 2004, King, et al., 2006, Rubartelli, et al., 1998). The mechanism by which Tat exerts these pleiotropic effects is still unclear.

3. HIV-1 mRNA processing

Before a gene transcript is ready to be transported out of the nucleus it has to be processed by acquiring a cap structure at the 5′ terminus, introns have to be spliced out and a 3′ end is generated by adding a poly(A) tail. Although these reactions are biochemically distinct processes, they are interlinked and influence one another's specificity and efficiency. Most mechanisms regulating the processing of viral transcripts are common to most cellular mRNAs, nevertheless some processes, such as the export of the unspliced and partially spliced mRNAs to the cytoplasm, are unique for the virus.

3.1 Capping

mRNA capping is carried out by a series of enzymatic reactions in which the 5′ triphosphate terminus of the pre-mRNA is cleaved to a diphosphate by a RNA triphosphatase (RTP), then

capped with GMP by RNA guanylyltransferase (GT), and methylated by RNA (guanine-N7) methyltransferase (MT). Targeting of Cap formation to transcripts made by RNAP II is achieved through the interaction of the capping enzymes with the phosphorylated RNAPII CTD. The Cap structure is recognized by the Cap binding complex (CBC), which contains the proteins, CBP20 and CBP80 (Proudfoot, et al., 2002, Shatkin & Manley, 2000). HIV-1 capping takes place during the transition from transcription initiation to elongation when the nascent pre-mRNA is only 20–40 nucleotides long. Tat promotes viral mRNA Cap formation by inducing TAR-dependent phosphorylation of RNAPII CTD (Chiu, et al., 2002, Zhou, et al., 2003) Phosphorylation of the CTD Ser-2 and Ser-5 residues has differential effects on recruitment and activation of capping enzymes (Bentley, 2002, Proudfoot, et al., 2002). Although Ser-2 phosphorylation of CTD heptads is sufficient for mammalian GT binding, its activation requires Ser-5 phosphorylated CTD (Ho & Shuman, 1999).

3.2 3' end formation

In higher eukaryotes, with the exception of histone genes, all protein encoding mRNAs contain a uniform 3′ end consisting of 200-400 adenosine residues. The poly(A) tail regulates degradation of the mRNA and translation. The formation of the poly (A) tail is directed by sequences present on the pre-mRNA and the mammalian polyadenylation machinery. Prior to the addition of poly(A), the pre-mRNA must be cleaved. The site of cleavage, in most pre-mRNAs, lies between a highly conserved AAUAAA hexamer and a downstream sequence element (DSE), which is a U- or GU-rich motif. The cleavage/ poly(A) complex consists of cleavage factors (CPSF, CstF, CF) and the poly(A) polymerase (PAP). RNA is first cleaved ~10 to 30 nts 3' to AAUAAA, predominantly at a CA dinucleotide, than the PAP synthesizes a polyA tail using ATP as substrate (Proudfoot, et al., 2002, Shatkin & Manley, 2000).

HIV-1 encodes the polyadenylation signal within the repeat (R) region, which is present at the extreme 5' and 3' end of the viral transcript. Thus the 5' polyadenylation signal needs to be repressed while usage of the 3′ one needs to be enhanced. Usage of the 3' polyadenylation site is promoted by an upstream enhancer (USE) motif, which stabilizes binding of the cleavage polyadenylation specificity factor (CPSF) to the AAUAAA (Gilmartin, et al., 1995). The 5' HIV-1 polyadenylation site is repressed because it is positioned too close to the transcription initiation site and polyadenylation factors have not yet gained access to the nascent transcript through the RNAP II complex (Cherrington & Ganem, 1992, Weichs an der Glon, et al., 1991). Moreover, binding of U1 snRNP to the major splice donor site, which is located downstream of 5' R, represses polyadenylation at the 5' polyadenylation signal (Ashe, et al., 1995, Ashe, et al., 1997). Cellular proteins can play a role in regulating cleavage and polyadenylation of HIV-1 RNA. hnRNP U has been shown to be involved in the post-transcriptional regulation of viral RNA via interactions with the 3′ UTR (Valente & Goff, 2006). The STAR (signal transduction and activation of RNA) protein family member Sam68 enhances HIV-1 gene expression and this effect may be due in part to Sam68's ability to stimulate cleavage of unspliced viral RNA (McLaren, et al., 2004, Reddy, et al., 1999). Experimental evidence also indicates a role for viral proteins in regulating the host polyadenylation machinery to render the cell more supportive of virus replication. The accessory protein Vpr induces polyA polymerase dephosphorylation and its subsequent activation (Mouland, et al., 2002), while Tat increases the expression of the cleavage and polyadenylation specificity factor (CPSF) (Calzado, et al., 2004).

3.3 mRNA Splicing

The removal of intronic sequences in the nascent transcript is carried out by large multicomponent ribonucleoprotein complex, the spliceosome, constituted by five small nuclear ribonucleoprotein complexes (snRNPs, U1, U2, U4, U5, U6), which assembly onto the pre-mRNA requires auxiliary proteins called splicing factors (Moore, et al., 1993). The process of splicing involves recognition of short loosely conserved sequences flanking the introns. The U1 snRNP recognizes the 5′ splice site (5′ss) while the U2 snRNP in combination with the splicing factor U2AF65/35 recognizes sequences at the 3′ splice site (3′ss). 5' and 3' splice sites are required for splicing but alone are not sufficient for the proper recognition of exonic and intronic sequences.

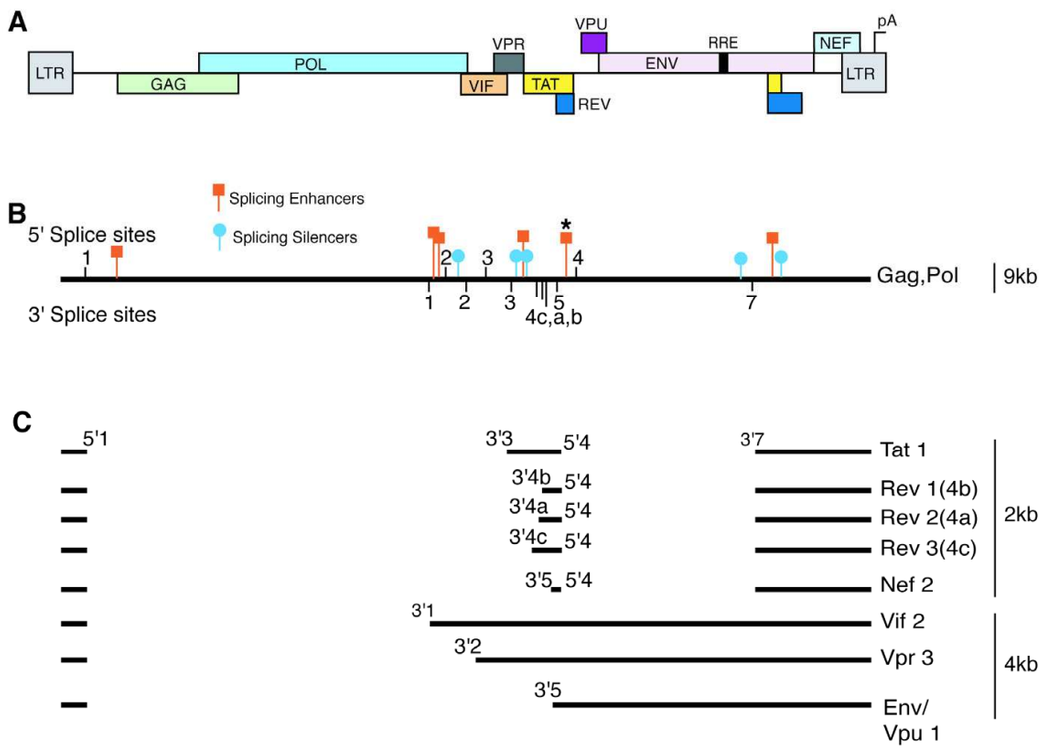

Fig. 3. A) The map shows the HIV-1 open reading frames. B) A single pre-mRNA of 9.2 kb is transcribed by the virus. 5′ and 3′ splice sites are indicated The unspliced viral mRNA codes for the Gag/Pol gene products. 5′ and 3′ splice sites are indicated. Splicing silencers (intronic and exonic) and splicing enhancers (intronic and exonic) are indicated. (*) marks the location of the GAR splicing enhancer (see Fig 5). C) Prevalent spliced viral mRNAs. Over 40 alternatively spliced mRNAs are originated by the alternative usage of the multiple 5′ and 3′ splice sites, the most abundant mRNA isoforms are indicated with their approximate size and the splice site utilized to generate them.

Additional regulatory elements are classified as either exonic and intronic splicing enhancers (ESE and ISE) or exonic and intronic splicing silencers (ISS and ESS). These sequences can interact with factors that promote proper recognition of the splice sites and regulate splicing in response to physiological stimuli. Among the best-characterized ESEs are purine-rich sequences that recruit members of the serine/arginine-rich (SR) family of

splicing activators (Blencowe, 2000). SR proteins regulate splicing by binding enhancer elements and recruiting and stabilizing components of the core splicing machinery to nearby splice sites (Graveley, 2000). Recent work implicates SR proteins in additional steps of gene expression, including mRNA export, stability, quality control and translation (Huang & Steitz, 2005). The best-known ESSs are dependent on interactions with members of the heterogenous ribonucleoprotein A/B family (hnRNPs A/B) (Krecic & Swanson, 1999). Positive and negative *cis*-acting sequences are often organized in multipartite control elements where SR proteins and hnRNPs often play counteracting roles (Caceres, et al., 1994, Han, et al., 2005, Zahler, et al., 2004).

Alternative splicing is a process common to most cellular mRNAs by which exons from a primary transcript (pre-mRNA) can be spliced in different arrangements to yield mRNAs that will produce functionally different protein variants (Black, 2003). The primary viral transcript undergoes a complex series of splicing events to generate over 40 mRNA isoforms, thus, the same viral protein is encoded by multiple mRNAs that vary for their 5′ and 3′ untranslated regions. Spliced viral mRNAs can be classified in a group of approximately 4kb in length, coding for the Env, Vpu, Vpr and Vif proteins, and a group of approximately 2 kb in length, coding for the Tat, Rev, Vpr and Nef proteins (Fig. 3C) (Purcell & Martin, 1993). Furthermore, approximately 50% of the viral pre-mRNAs leave the nucleus without being spliced. The unspliced 9 kb mRNA codes for the Gag and Gag-Pol polyprotein and is packaged within the nascent virions as viral genome. Alteration of this complex splicing pattern can have profound effects on viral replication and infectivity (Amendt, et al., 1994, Jablonski, et al., 2008, Jacquenet, et al., 2005, Purcell & Martin, 1993). HIV-1 splicing regulation relies on the presence of multiple viral regulatory sequences as well as cellular splicing factors that interact with these elements. To date, 4 exonic splicing silencers (ESS), 1 intronic splicing silencer (ISS), 1 intronic splicing enhancer (ISE) and 6 splicing exonic enhancers (ESE) have been identified (Fig. 3B) (Exline, et al., 2008, McLaren, et al., 2008, Schaub, et al., 2007). Several SR proteins (SC35, SF2, SRp40, 9G8) have been shown to bind the viral splicing enhancers and regulate splicing, while members of the hnRNP A/B (A1, A2 and A3) family have been shown to inhibit the usage of viral splice sites by binding viral spicing silencer elements and counteracting the activity of SR proteins (McLaren, et al., 2008). A third group of proteins interacts with both, enhancer and silencer sequences, is the hnRNP H family (H′, F, 2H9 and GRSF1). These are highly homologous and ubiquitously expressed factors, which regulate splicing, polyadenylation, capping, export and translation of cellular and viral mRNAs (Fogel & McNally, 2000, Han, et al., 2005, Jablonski & Caputi, 2009, Min, et al., 1995, Schaub, et al., 2007).

Cis-acting splicing regulatory elements within the HIV genome are highly heterogenous, redundant and provide for ample regulation of viral genome expression. In addition, the virus appears to directly regulate the relative amount and activity of cellular splicing factors in infected cells. HIV-1 infection has been shown to induce alteration in SR protein subcellular distribution, and activity via modificaton of their phosphorylation state by SR-specific kinases (Fukuhara, et al., 2006). Furthermore, data obtained from infected monocyte-derived macrophages demonstrate that a peak in viral production results in down-regulation of members of the hnRNP A/B, H and SR family, thus confirming the critical role that these proteins may play in viral replication. (Dowling, et al., 2008).

3.4 mRNA export and stability

In the early phase of viral infection the 2 kb class of viral RNAs (translated into Tat, Rev and Nef) is exported to the cytoplasm while 9 and 4 kb viral RNAs are retained in the nucleus.

Nuclear retention of 9 and 4 kb HIV-1 RNAs has been attributed to either partial spliceosome assembly (Chang & Sharp, 1989) or to a series of of poorly characterized sequences called the instability (INS) or *cis*-acting repressor (CRS) sequences (Maldarelli, et al., 1991, Mikaelian, et al., 1996, Nasioulas, et al., 1994, Olsen, et al., 1992) present within the viral mRNA. These sequences restrict the expression of the 9 kb and 4 kb mRNA species. The mechanistic details of inhibition of gene expression by INS/CRS remains obscure, it has been proposed to involve increased splicing efficiency, prevention of nuclear export and degradation of INS/CRS containing RNAs or a combination thereof (Boris-Lawrie, et al., 2001, Reddy, et al., 2000, Wodrich & Krausslich, 2001). The fully spliced 2 kb mRNAs are exported from the nucleus to the cytoplasm with a mechanism similar to the one utilized by the assembly of a protein complex at the junctions between exons (exon junction complex, EJC) during splicing (Rodriguez, et al., 2004). Additionally, factors directly binding to sequences within the RNA may contribute to efficient export (Huang & Steitz, 2005).

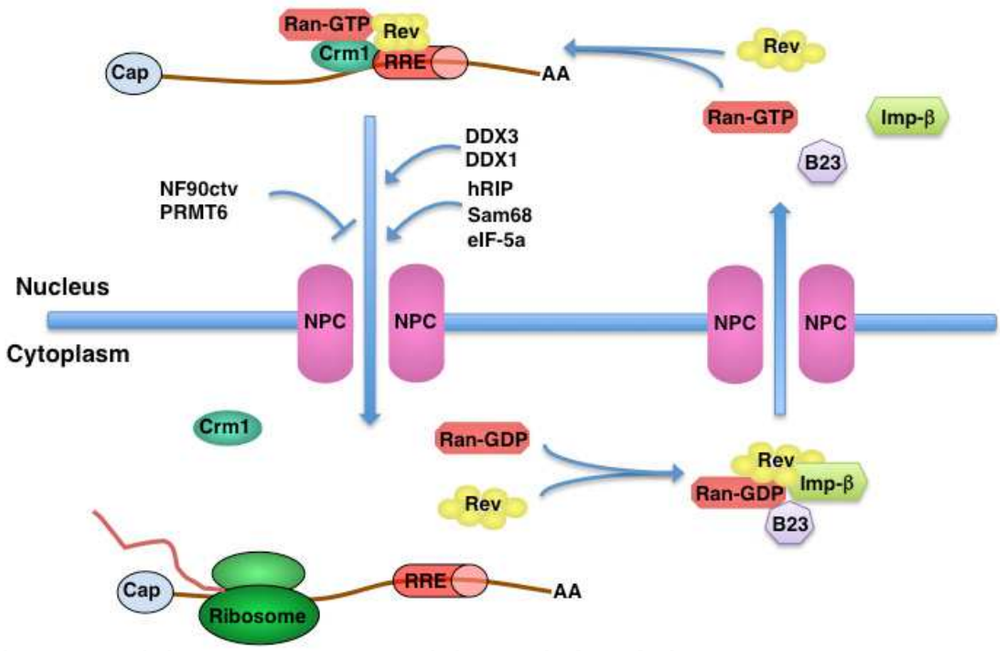

Fig. 4. HIV-1 RNA export to the cytoplasm. 9 and 4 kb viral RNAs are exported to the cytoplasm upon interaction of the Rev/Crm1/RanGTP complex with the RRE sequence within the RNA. The DDX1 and DDX3 helicases facilitate translocation of the Rev-RNA complex to the cytoplasm through the nuclear pore complex (NPC). Other host cell factors have positive (hRIP, SAM68, eIF-5a) or negative (NF90ctv, PRMT6) effects on the Rev-dependent export of the viral RNA but their role is less understood. Importin-b, RanGDP and B24 are required for import of Rev into the nucleus.

Nuclear retention of the unspliced and partially spliced viral mRNAs (9 and 4 kb) is overcome by the viral protein Rev, which is imported into the nucleus and binds to an RNA element within the *env* gene, called the Rev Responsive Element (RRE), and mediates nuclear export and efficient expression of its target RNAs (Fig. 4) (Cullen, 2000, Cullen, 2003). All the 4kb and 9kb viral mRNA species contain the RRE element and in the absence of Rev are poorly expressed in the cytoplasm. Rev import from the cytoplasm into the

nucleus is aided by importin-β and the nuclear phosphoprotein B23. Following Rev binding to the RRE, the nuclear export factors CRM1 and Ran-GTP are recruited to the complex to promote export of the viral RNA into the cytoplasm. The Rev-RRE-CRM1-RanGTP RNA complex is finally dissociated in the cytoplasm upon conversion of RanGTP to RanGDP (Suhasini & Reddy, 2009).

Little is known of cellular co-factors required by Rev-RRE other than the general nuclear export factors exportin CRM1 and RAN-GTP. RNA helicases DDX1 and DDX3 are known to associate with the Rev-CRM1-RRE complex and are postulated to aid the egress of the viral RNA through the nuclear pore by remodeling its structure (Fang, et al., 2004, Yedavalli, et al., 2004). Other factors such as the eukaryotic initiation factor eIF-5A (Bevec, et al., 1996, Ruhl, et al., 1993), the human Rev interacting protein (hRIP) (Bogerd, et al., 1995, Fritz, et al., 1995) and Sam68 (Reddy, et al., 1999) have also been shown to enhance Rev activity although their mechanism is still unclear. Cellular factors may also play an inhibitory role in Rev-dependent RNA export. The C-terminal variant of nuclear factor 90 (NF90ctv) has been shown to reduce Rev function by binding and partially relocalizing Rev to the cytoplasm (Urcuqui-Inchima, et al., 2006), while Rev methylation by the arginine methylase PRMT6 reduces Rev binding to the RRE and blocks viral RNA export (Invernizzi, et al., 2006).

4. Transcription and mRNA processing coupling

In recent years our view of gene expression has changed significantly. While a growing number of genetic studies have revealed functional links between the factors that carry out the different steps in the gene expression pathway, conventional biochemical approaches and large-scale mapping of protein-protein interaction networks have uncovered physical interactions between the various machineries (Orphanides & Reinberg, 2002, Proudfoot, et al., 2002). The transcriptional apparatus plays an active role in recruiting the machinery that processes the nascent RNA transcript (Bentley, 2002, Bentley, 2005). The RNAPII CTD operates as a binding platform for components of the RNA processing machineries and its phosphorylation regulates the activity of the capping enzymes, assembly of the spliceosome and the binding of the cleavage/polyadenylation complex (Buratowski, 2003, Fong & Bentley, 2001, Hirose & Ohkuma, 2007, Komarnitsky, et al., 2000, Proudfoot, et al., 2002).

The mechanism coupling 5′ RNA capping with transcription has been well studied. Binding of the DSIF factor (Wada, et al., 1998a, Wada, et al., 1998b) to RNAPII shortly after initiation recruits NELF (Yamaguchi, et al., 1999), which arrests transcription. The cdk7 subunit of the initiation factor TFIIH phosphorylates the RNAPII CTD Ser-5 between initiation and arrest (Woychik & Hampsey, 2002). The paused RNAP II is then joined by the capping enzymes through interactions with the Ser-5 phosphorylated CTD and DSIF (Wada, et al., 1998b, Wen & Shatkin, 1999). Following the addition of the Cap, the kinase activity of P-TEFb phosphorylates DSIF (Ivanov, et al., 2000, Kim & Sharp, 2001), this neutralizes the repressive action of NELF and allows the polymerase to resume elongation. Similary 3' End formation is also linked to transcription. The cleavage/polyadenylation factors CPSF and CstF are transferred by the RNAP II CTD to their specific pre-mRNA-binding sites to produce the mRNA 3' end (Buratowski, 2005). Splicing and 3′ end formation machineries are also connected since repression of the polyadenylation signal within the R5 region is dependent on proximity to the promoter and recognition of the major 5′ splice site by the U1 snRNP (Ashe, et al., 1995, Ashe, et al., 1997).

Several studies have also shown that transcription and splicing are closely connected processes. The rate of elongation, the promoter type, transcriptional activators and the chromatin remodeling factors nearby can all affect splicing of a pre-mRNA (Batsche, et al., 2006, de la Mata, et al., 2003, Kornblihtt, 2005, Kornblihtt, 2007). Again, the RNAPII CTD assumes a central role in the regulation of RNA splicing. Phosphorylation at Ser-2 position of the RNAPII CTD stimulates pre-mRNA splicing (Hirose, et al., 1999, Misteli & Spector, 1999). Studies have identified several splicing factors that interact either directly or indirectly with the transcription machinery (Kameoka, et al., 2004, Kwek, et al., 2002). Many of the works published indicate processes that link the transcription machinery to pre-mRNA splicing. However, a "reverse coupling" mechanism, whereby pre-mRNA splicing exerts an influence on transcription has also been described. Indeed, the SR protein SC35 has been shown to affect transcription elongation (Lin, et al., 2008) and promoter-proximal 5´ splice sites increase transcription initiation via recruitment of the transcription preinitiation complex (PIC) (Damgaard, et al., 2008).

Studies on the association between the viral transcription and splicing machinery are still in their infancy. Research indicates that the cellular factor Tat-SF1, which is required for efficient transcriptional transactivation of the viral genome (Parada & Roeder, 1999, Zhou & Sharp, 1996), is also interacting with spliceosomal components (Fong & Zhou, 2001). The association with both elongation and splicing factors has led to the suggestion that Tat-SF1 can couple these two processes. Tat-SF1 also binds to another transcription-splicing coupling factor, CA150 (TCERG1) (Smith, et al., 2004). Over-expression of CA150 has been shown to reduce the ability of Tat to mediate viral transcription (Sune & Garcia-Blanco, 1999). This function is dependent on the association of CA150 with pre-mRNA splicing factors and RNAPII CTD (Carty, et al., 2000, Goldstrohm, et al., 2001) and may bridge splicing complexes to actively transcribing RNAPII (Sanchez-Alvarez, et al., 2006). The cellular protein c-Ski-interacting protein, SKIP, has been shown to regulate Tat-dependent viral transcription and interact with the splicing associated U5 snRNP and the tri-snRNP 110K protein (Bres, et al., 2005). Studies also indicate the presence of a reverse coupling mechanism in HIV-1. U1 snRNA binding to a specific 5′ splice site within the viral genome appears to overcome a checkpoint for elongation present in the *env* gene intron (Alexander, et al. 2010).

The viral transactivator Tat has also been shown to regulate viral splicing through the ASF/SF2 inhibitor p32 (Berro, et al., 2006). p32 is recruited to the HIV-1 promoter by the acetylated form of Tat, suggesting a mechanism by which acetylation of Tat promotes binding of p32 and thereby inhibits HIV-1 splicing, thus increasing the amounts of unspliced transcripts available for being translated into *gag/pol* gene products and packaging into the nascent virions as viral genome. More recently we have shown that Tat is also a selective mediator of HIV-1 splicing (Jablonski, et al., 2010) via the recruitment of the cellular co-transcriptional splicing activators Tat-SF1 and CA150 (Fig. 5). The Tat-transcription-splicing complex activates a distal splicing enhancer (GAR), which is required for *env* mRNA expression. In the context of the full-length viral genome, this mechanism promotes an autoregulatory feedback that decreases expression of Tat-coding mRNAs and favors expression of Env-specific mRNAs. Tat-mediated splicing does not appear to be dependent on its transcriptional activity. Substitution of the LTR promoter for the heterologous *β-globin*, promoter or down-regulation of Tat transcription co-factors SKIP and Tat-CT1 blocks Tat-mediated transactivation but not splicing. Tat appears to modulate splicing independent of its ability to functionally engage the transcription machinery and alter phosphorylation of the RNAP II CTD, defining a novel mechanism that couples

transcription and RNA processing via the direct recruitment of splicing factors through transcription activators. We have also shown that, like Tat, mammalian transcriptional co-activators of the Torc family can also activate splicing of endogenous genes and that this activity is independent from their role in transcription (Amelio, et al., 2009). These observations provide support for a general mechanism whereby transcriptional activators, viral or cellular, can selectively regulate splicing processes.

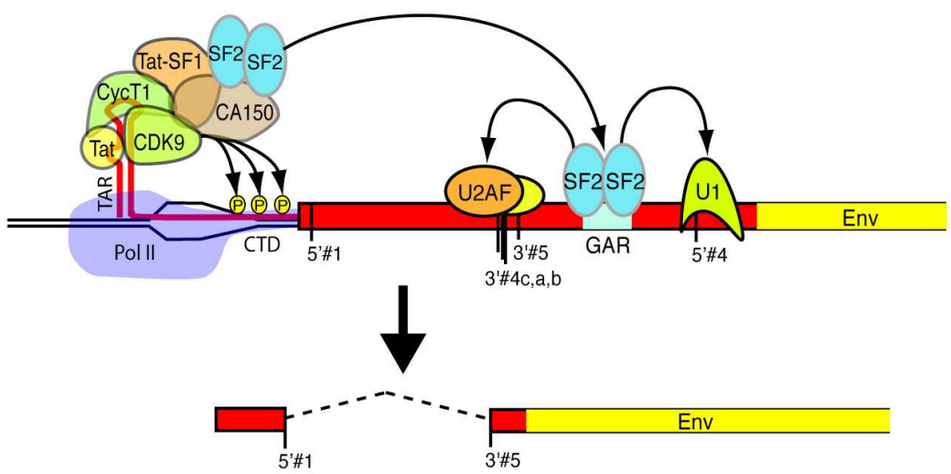

Fig. 5. Model for Tat-mediated splicing. Tat binding to TAR helps the recruitment of Tat-SF1 and CA150, which stimulates the assembly of the splicing factor SF2 onto the GAR enhancer (see Fig. 2). SF2 interaction with GAR promotes the upstream 3'ss and recruitment of U1 snRNP to the downstream 5'ss, which promotes expression of the *env* specific mRNA.

5. HIV-1 derived microRNAs

MicroRNAs (miRNAs) are short non-coding RNA molecules encoded by most eukaryotic life forms ranging from plants to higher order mammals. miRNAs have multiple pre- and post-transcriptional roles in the regulation of gene expression. The first step in the biogenesis of miRNA is the nuclear processing of primary RNA transcripts (pri-miRNAs) approximately 80 nucleotide long into shorter (~60nt) pre-miRNAs, which are exported to the cytoplasm where pre-miRNA are further processed into mature miRNAs and assembled into a ribonucleoprotein complex named RISC (RNA-induced silencing complex) (Chua, et al., 2009, Perron & Provost, 2008, Winter, et al., 2009). The RISC complex and the associated miRNA often contain sequences complementary to the 3' UTR region of the target mRNAs. Perfect complementarity between the target sequence and the miRNA triggers degradation of the target RNA. However, when miRNA and target mRNA sequences are only partially complementary the mRNA is translationaly repressed. Furthermore, miRNA can also operate at chromatine level. miRNAs have been shown to associate with the RNA-induced initiation of transcriptional silencing (RITS) complex and be recruited to complementary sequences in the chromosomal DNA. This promotes the activity of histone modifying enzymes, which alter the chromatin structure and induce transcriptional silencing (Buhler & Moazed, 2007, Verdel, et al., 2004).

Several HIV-1 encoded non-coding RNAs have been identified. In particular, the stem-loop TAR sequence is structurally similar to a pre-miRNA and it has been shown to be processed

into two functional miRNAs, the 5′ stem (miR-TAR-5p) and the 3′ stem (miR-TAR-3p) (Klase, et al., 2007, Klase, et al., 2009, Ouellet, et al., 2008). miR-TAR-3p acts as an inhibitor of cellular gene expression targeting genes regulating stress induced cell death (Klase, et al., 2009). The downregulation of the host machinery by viral miRNAs is part of the viral strategies to prolong the life span of an infected cell and allows for efficient viral replication and the emergence of latently infected cells. Additionally, it has been shown that the TAR-derived miRNA can downregulate gene expression by recruiting chromatin remodeling components, thus inducing transcriptional silencing via the RITS mechanism (Purzycka & Adamiak, 2008).

A second miRNA is coded within the viral gene *nef*. Nef is a viral protein required for productive viral infection. It has been shown that miRNAs derived from *nef* transcripts are present in HIV-1 infected cells (Yamamoto, et al., 2002). Nef derived miRNAs appear to down regulate expression of the *nef* gene and they appear to be present in higher amounts in HIV-1 patients that are long time non-progressors and display low viremia.

A third miRNA generated by HIV-1, named miR-H1, is an 81 nucleotide stem loop structure present downstream of the two NF-kB sites in the LTR (Bennasser, et al., 2004). MiR-H1 has been shown to degrade the apoptosis antagonizing transcription factor (AATF) (Kaul, et al., 2009), which leads to lowered cell viability, thus counteracting the anti-apoptotic effect of the TAR-derived miRNAs. Furthermore, miR-H1 down regulates expression of the cellular miRNA miR149, which targets the Vpr gene encoded by HIV-1 (Kaul, et al., 2009). Studies on HIV-1 miRNA variability in different viral isolates have also shown that there is a strong correlation between specific miR-H1 sequences and the development of HIV-1-associated dementia and AIDS related lymphoma (Lamers, et al., 2009).

In addition to generating several viral miRNAs, HIV also regulates the cellular machinery that process small non-coding RNAs. The viral protein Tat appears to act as a generic suppressor of the activity of Dicer (Bennasser, et al., 2005), a key enzyme required for the maturation of small non-coding RNAs. Furthermore, the viral proteins Vpr and Nef have been shown to suppress the cellular miRNA machinery by suppressing production of Dicer (Coley, et al., 2011). Vertebrates have developed RNAi-based antiviral mechanisms. Given the presence in the HIV-1 genome of multiple regions that produce interfering RNAs, the anti-RNA silencing function of several viral proteins appears to be required to sustain viral replication in infected cells.

6. Future prospective

Different aspects of viral replication have been the targets of therapeutics; nevertheless, few efforts have been aimed at the disruption of the mechanism regulating viral RNA biogenesis. Formation of the HIV-1 transcript provides an important model for human RNA processing pathways and can be crucial in the isolation of novel therapeutic targets to block viral replication. Cellular factors regulating HIV RNA are expressed in most cell types and regulate a multitude of cellular splicing events theoretically making them less ideal therapeutic target candidates. Nevertheless, several drugs inhibiting different aspects of Tat transactivation are currently being tested. Different classes of compounds have been shown to specifically inhibit viral transcription by: (i) binding the TAR sequence, (ii) binding Tat, (iii) inhibiting PTEF-b components and (iv) generally inhibiting transactivation by mechanisms not yet well defined (Giacca, 2004). Small molecules that inhibit the splicing activity of SR proteins have also been shown to efficiently repress viral replication in

peripheral blood mononuclear cells (PBMC) with little cell toxicity (Bakkour, et al., 2007). Furthermore, the possibility of utilizing delivery systems that specifically target cells infected by the virus (Neff, et al., 2011, Peretti, et al., 2006) suggest that cellular factors regulating the making of the viral RNA can be efficiently targeted to inhibit viral replication.

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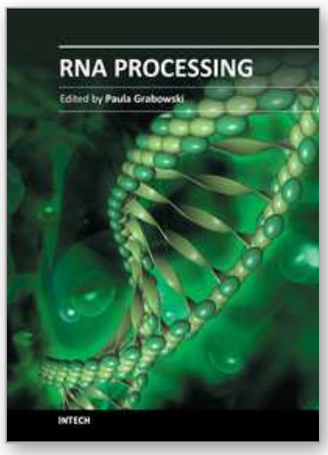

RNA Processing

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RNA functions broadly as informational molecule, genome, enzyme and machinery for RNA processing. While these functions reflect ancient activities, they also remain vital components of contemporary biochemical pathways. In eukaryotic cells RNA processing impacts the biogenesis of RNA molecules of essentially every shape and function. The collection of articles in this volume describes the current state of understanding of the broad array of RNA processing events in animal and plant cells, key unanswered questions, and cutting edge approaches available to address these questions. Some questions discussed in this volume include, how viruses subvert the RNA processing machinery of the host cell, how the coordination of co-transcriptional RNA processing is regulated at the level of chromatin, the status of RNA processing in plant organelles, and how micro RNA machinery is biosynthesized and regulated.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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