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Translation initiation is driven by different mechanisms on the HIV-1 and HIV-2 genomic RNAs

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ABSTRACT

The human immunodeficiency virus (HIV) unspliced full length genomic RNA possesses features of an eukaryotic cellular mRNA as it is capped at its 5′ end and polyadenylated at its 3′ extremity. This genomic RNA is used both for the production of the viral structural and enzymatic proteins (Gag and Pol, respectively) and as genome for encapsidation in the newly formed viral particle. Although both of these processes are critical for viral replication, they should be controlled in a timely manner for a coherent progression into the viral cycle. Some of this regulation is exerted at the level of translational control and takes place on the viral 5′ untranslated region and the beginning of the gag coding region.

In this review, we have focused on the different initiation mechanisms (cap- and internal ribosome entry site (IRES)-dependent) that are used by the HIV-1 and HIV-2 genomic RNAs and the cellular and viral factors that can modulate their expression. Interestingly, although HIV-1 and HIV-2 share many similarities in the overall clinical syndrome they produce, in some aspects of their replication cycle, and in the structure of their respective genome, they exhibit some differences in the way that ribosomes are recruited on the gag mRNA to initiate translation and produce the viral proteins; this will be discussed in the light of the literature.

The human immunodeficiency viruses type I and II (HIV-1 and -2), the etiologic agents of the acquired immunodeficiency syndrome, are classified within the *Lentivirus* genus of the *Retroviridae* family. The viral particles contain two copies of the genomic RNA (gRNA) which codes for the structural (Gag and Env), enzyme (Pol) and accessory (Tat, Nef, Vpu/Vpx, Vif, Rev, Vpr) proteins. As for all viruses known to date, viral protein synthesis relies exclusively on the host cell translation machinery to provide ribosomes, tRNAs, amino acids and all required initiation, elongation and termination factors. The viral pre-mRNA is produced by the RNA polymerase II and, like almost all the cellular mRNAs, it is capped and polyadenylated. This pre-mRNA is then spliced at multiple sites to give rise to about 40 different mRNA species that are then exported to the cytoplasm by different export pathways (Stoltzfus, 2009; Suhasini and Reddy, 2009). Once in the cytoplasm, translation of these mRNAs involves numerous and different mechanisms (cap, scanning, internal ribosome entry site, frameshifting, leaky scanning) depending on the considered mRNA. In this review, we will pay a particular attention to the fate of the full length unspliced genomic RNA which codes for Gag and Gag-Pol and serves both for template for protein synthesis and genome for viral propagation. This genomic RNA offers an incredible combination of diverse translational mechanisms such as ribosomal scanning, internal ribosome entry sites (IRESes) and frameshifting signals. Due to this complexity, this review will focus solely on scanning and IRESes. Frameshifting has been reviewed by others (Brierley and Dos Ramos, 2006; Giedroc and Cornish, 2009). In addition to this, there are major differences at the level of translational control between the two types of HIV (Ricci et al., 2008b; Soto-Rifo et al., 2012a), which adds another layer of complexity to their understanding.

Abbreviations: 4E-BP, eIF4E-binding protein; BMH, branched multiple hairpins; CrPV, cricket paralysis virus; CSFV, Classical Swine Fever Virus; DIS, dimerization site; eIF, eukaryotic initiation factor; EMCV, encephalomyocarditis virus; FMDV, foot-and-mouth-disease virus; gRNA, genomic RNA; HCV, Hepatitis C Virus; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; HnRNP, heterogeneous nuclear ribonucleoprotein; HP, Hepaci- and Flavi- virus; HRV, human rhinovirus; IGR, intergenic rRegion; IRES, internal ribosome entry site; ITAF, IRES *Trans*-Acting Factor; LDI, long distance interaction; ORF, open reading frame; PABP, Poly(A) binding protein; PIC, pre-initiation complex; PV, poliovirus; RHA, RNA helicase A; RRL, rabbit reticulocyte lysate; SD, spliced donor; SIV, Simian Immunodeficiency Virus; SL1, stem loop 1; TAR, *trans*-activation response; UTR, untranslated region.

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Thus, this review will deal with the mechanisms used by HIV-1 and HIV-2 genomic mRNAs to recruit the host translation initiation machinery by highlighting their similarities and discussing their differences. In the first part, we will summarize the work that has been published over the last 20 years on this question and the last part of this manuscript will present novel data which further supports the fact that HIV-1 and HIV-2 exhibit differences at the level of translation initiation.

1. Introduction to translation initiation

Eukaryotic translation is a complex process which involves the concerted interaction of the 3 classes of ribonucleic acids (rRNA, tRNA and mRNA) with proteins and translation factors in a timely manner. To get a better understanding of eukaryotic protein synthesis, the overall process is broadly divided in four distinct stages. The initiation step begins with the recruitment of the small ribosomal subunit (40S subunit) on the mRNA and its movement until it reaches and localizes the initiation codon. The next step corresponds to the elongation phase in which the messenger RNA is decoded by the ribosomes that assembles the amino acids in the correct order to synthesize the protein. When the ribosome reaches one of three possible termination codons (UGA, UAA or UAG), the termination phase occurs with the hydrolysis of the ester bond of the peptidyl tRNA which induces the release of the nascent polypeptide (Alkalaeva et al., 2006). The fourth and final step is considered to be ribosome recycling in which each of the ribosome subunits is recycled for the next round of translation (Pisarev et al., 2007).

The initiation phase of protein synthesis is often considered to be the rate limiting step of the process. Translation initiation involves about a dozen eukaryotic initiation factors (eIFs) and associated proteins that control the recruitment of the ribosome to the mRNA and its migration to the AUG codon. The intrinsic RNA structures are important players in the overall process of 40S ribosomal subunit recruitment to the mRNA. Two major molecular mechanisms have been described: the 5′ cap-dependent and internal ribosome entry site (IRES)-mediated translation initiation.

1.1. The 5′ cap dependent initiation mechanism

Most cellular mRNAs are capped at their 5′ extremity and polyadenylated at their 3′ end. Both structural features are required for efficient mRNA translation by the 5′ cap-dependent initiation mechanism. This event requires at least 12 eIFs (Fig. 1A) (Jackson et al., 2010) and starts with the interaction of the eIF2·GTP·Met-tRNA$^{Met}_{i}$ ternary complex with the 43S pre-initiation complex (PIC), which corresponds to the 40S ribosome associated with eIF3, eIF1 and eIF1A (Fig. 1A). In parallel, the eIF4F complex, composed of eIF4E, eIF4A and eIF4G, interacts with the 5′ capped end of the mRNA (Fig. 1A). It is eIF4E that binds to the cap structure, which is composed of a guanosine methylated at position 7 and linked to the second nucleotide by an unusual 5′-5′ phosphodiester bond (m^7GpppN, N is any nucleotide; Shatkin, 1976). Attachment of the 43S preinitiation complex to the mRNA is scaffolded by eIF4G which is a large protein that interacts with multiple partners such as eIF4E, eIF4A, the poly(A) binding protein (PABP) and eIF3 (Prevot et al., 2003b). As such, eIF4G acts as the hinge between the 43S ribosomal subunit and the mRNA. The presence of the PABP in the PIC allows the pseudo-circularization of the mRNA by linking the 5′ extremity to the 3′ poly(A) end of the transcript (Fig. 1A). RNA pseudo-circularization enhances the efficiency of mRNA translation (Kahvejian et al., 2001; Martineau et al., 2008; Sachs, 2000). The last component of the eIF4F complex is the DEAD-box RNA helicase eIF4A which unwinds RNA structures close to the 5′ end preparing a track for the landing of the 43S PIC on the mRNA (Fig. 1A). The activity of the RNA helicase is ATP-dependent and is strongly enhanced by the binding to eIF4G but also by two other initiation factors that are namely, eIF4B and eIF4H which share a common binding site on eIF4A (Rogers et al., 2001). At this step, both eIF1 and eIF1A impose an open conformation of the 40S ribosomal subunit which is scanning permissive (Passmore et al., 2007; Pestova et al., 1998a). Both eIF1 and eIF1A are sufficient to promote ribosomal scanning on non-structured 5′-UTR but, in most cases, components of eIF4F are required for RNA unwinding of the 5′-UTR (Pestova and Kolupaeva, 2002); highly structured 5′-UTR may further require the activity of additional RNA helicases like the mammalian DExH box protein 29 (Pisareva et al., 2008), the RNA Helicase A (Hartman et al., 2006), or Ded1 in yeast (Berthelot et al., 2004; Chuang et al., 1997). In most cases, the presence of RNA secondary structures within the 5′-UTR inhibits ribosomal scanning and this effect is even stronger when localized at the very 5′ end of the mRNA as they impede initial ribosomal recruitment (Kozak, 1980, 1987; Pestova and Kolupaeva, 2002). In this respect a recent report describes that mRNAs that harbor complex RNA structures close to the 5′ termini require the recruitment of the DEAD box RNA helicase DDX3 to efficiently initiate translation (Soto-Rifo et al., 2012b). The recognition and selection of the initiation codon is coordinated by eIF1 which is able to discriminate between incorrect from correct codon-anticodon base pairing (Hinnebusch, 2011). Usually, the start site for polypeptide formation is the first AUG codon located in an optimum context GCC(A/G)CCAUGG with a purine at −3 and a guanine at the +4 positions relative to the A of the AUG codon (Kozak, 1987). These two positions have been reported to interact with the eIF2α subunit or with nucleotides AA$^{1818\text{-}1819}$ of helix 44 of the 18S rRNA and to participate in the AUG selection (Pisarev et al., 2006). Start site codon recognition occurs by the formation of a proper mRNA codon-tRNA anticodon base pairing (Fig. 1A). This induces displacement of eIF1 into the PIC and allows the 40S ribosomal subunit to adopt a close conformation which locks the mRNA into the 48S initiation complex (Maag et al., 2005; Passmore et al., 2007). The recognition of the initiation codon induces complete hydrolysis of the GTP bound to eIF2 which leads to the dissociation of the 43S complex and associated eIFs (Fig. 1A). The 60S large ribosomal subunit can then join to the 40S ribosomal subunit assembling create an 80S ribosome competent for the elongation step (Fig. 1A); this step requires GTP hydrolysis that is catalyzed by eIF5B (Pestova et al., 2000).

1.2. The internal ribosome entry sites

Picornaviridae are a family of small single stranded positive RNA viruses that are epitomized by the poliovirus (PV) and the encephalomyocarditis virus (EMCV). The genomic RNA is composed of a large unique open reading frame (ORF) that encodes a single polyprotein product which is then cleaved and processed into several structural proteins and enzymes (Daijogo and Semler, 2011). The 5′ end of the genomic RNA is not capped but harbors a virally encoded protein, Vpg, covalently bound to the end of the ribonucleic acid (Nomoto et al., 1977). The 5′-UTR of the genomic RNA is long (between 600 and 1200 nucleotides), highly structured and contains multiple non-conserved and silent AUG triplets upstream from the original initiation start site. All these structural features are difficult to reconcile with the eukaryotic ribosomal scanning model for mRNA translation. Furthermore, picornaviral infection is associated with the sequestration of eIF4E by the eIF4E binding protein (4E-BP) and/or the cleavage of eIF4G, PABP and eIF5B by picornaviral proteases (de Breyne et al., 2008a; Roberts et al., 2009). These modifications of eIFs integrity contribute to a rapid and strong shut-off of host cellular mRNA translation (Daijogo and Semler, 2011). Interestingly, despite the lack of a 5′ cap structure and all the mentioned constraints targeting normal eukaryotic

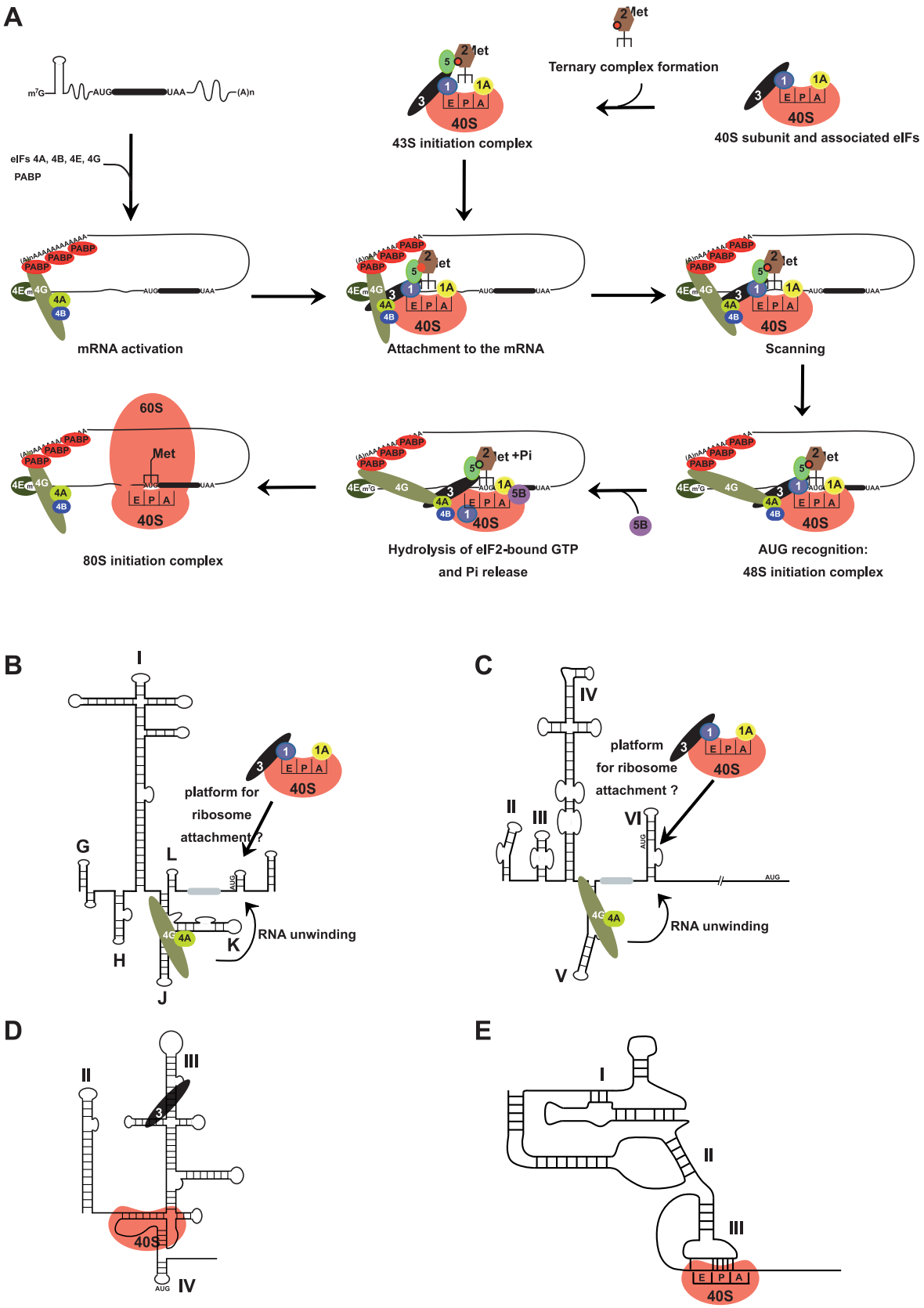

Fig. 1. Model for eukaryotic translation initiation. (A) cap-dependent translation initiation is divided into several steps which are the eIF2·GTP·Met-tRNA$^{Met}_{i}$ ternary complex formation; the formation of the 43S preinitiation complex composed of the 40S subunit, eIF1, eIF1A, eIF3, eIF2·GTP·Met-tRNA$^{Met}_{i}$ and eIF5; mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F with eIF4B; attachment of the 43S complex to the 5′ cap of the mRNA; the scanning of the 5′-UTR in a 5′ to 3′ direction by 43S complexes; recognition of the initiation codon (AUG); hydrolysis of eIF2-bound GTP; the eIFs are released from the ribosomal complex and the 60 S can be associated. (B) Model of the secondary structure of the EMCV IRES which is composed of domains G to L. The couple eIF4G/eIF4A binds to domains J and K to allow RNA unwinding of the downstream hairpin from the polypyrimidine track (grey box). This probably allows a landing track for the PIC. (C) Model of the secondary

mRNA translation, in infected cells the production of picornaviral proteins occurs at very high rate which initially suggested that picornaviral mRNAs were using an alternative translation initiation mechanism.

1.2.1. The study of IRES

The ability of the picornaviral 5′-UTR to promote initiation in a 5′ end-independent manner was initially demonstrated by using a bicistronic mRNA assay. In this assay the viral 5′-UTRs were inserted between two reporter genes and the efficiency of translation of the two cistrons was monitored (Jang et al., 1988; Pelletier and Sonenberg, 1988). Internal initiation takes place when the expression of the second cistron occurs independently from the first one, e.g. repression of translation of the first cistron does not affect production of the downstream product. In addition, several controls are required to validate the results from the bicistronic assay in order to avoid any artifacts that would lead to data misinterpretation (Kozak, 2001, 2003; Shatsky et al., 2010). Indeed, cryptic promoters and splice sites have been identified both in some bicistronic reporter vectors and in some sequences that were initially proposed to exhibit IRES activity (Kozak, 2005; Shatsky et al., 2010). To resolve this question, a siRNA interference test against the first cistron should be carried out. If the expression of both cistron relies on the same mRNA, the siRNA would interfere both expression. If the second cistron is translated from an mRNA resulting from a spliced or a cryptic promoter, this siRNA test would not affect its expression. Internal initiation is highly dependent of specific RNA structures and motifs present within the 5′-UTR sequence. In fact, small deletions or even point mutations of the picornaviral sequence can cause a dramatic reduction in translation (Fernandez-Miragall et al., 2009; Martinez-Salas, 2008). This effect has been attributed to either changes in the RNA folding of the IRES that affect its function or to the loss of an interaction site with some specific cellular proteins that are important for IRES activity (see below). Apart from the bicistronic assay, IRES activity can also be assessed by using synthetic circular mRNAs (Chen and Sarnow, 1995) or monocistronic mRNAs under specific conditions (Ohlmann et al., 2000). These include a series of assays that have been developed to specifically inhibit cap-dependent translation initiation but that do not alter IRES mediated initiation. For instance, ribosomal scanning from the 5′ end can be impaired by introduction of a stable hairpin (Kozak, 1980, 1989; Pestova and Kolupaeva, 2002), by hybridization of 2′O-methyl oligoribonucleotide (Ohlmann et al., 2000) or even by addition of upstream AUG codons (Berkhout et al., 2011). Ribosome attachment at the cap structure can be inhibited by addition of cap analog (competitors for eIF4E binding) or by processing the eIF4G by picornaviral proteases (Ricci et al., 2008b). Finally, modification of cell homeostasis can also be achieved by adding the immunosuppressant rapamycin, inducing a G2/M cell cycle arrest, a heat shock or an oxidative stress which results mostly in the specific inhibition of cap-dependent initiation. Despite these conditions, various mRNAs, some of which are regulated by an IRES, remain efficiently translated (Beretta et al., 1996a,b; Duncan et al., 1987; Pham et al., 2000; Pyronnet et al., 2001).

1.2.2. Classification of IRESes

Since their seminal discovery in 1988, IRESes have been reported in the mRNA of all studied picornaviral mRNAs and some of other viral families like the *Flaviridae*, *Dicistroviridae* and *Retroviridae* (Balvay et al., 2009). IRESes have but been characterized in some cellular mRNAs that generally code for proteins involved in development, apoptosis, cell cycle regulation and differentiation (Stoneley and Willis, 2004). Translational activity of IRESes is both due to the RNA architecture that is adopted to constitute functional domains and to the presence of some cellular proteins that can bind to them (Balvay et al., 2009; Fitzgerald and Semler, 2009). These proteins that bind and modulate IRES activity were called IRES *trans*-acting factors (ITAFs) and some of them will be described later. Based on their structural features and the functional use of both eIFs and ITAFs, IRESes have been classified into 4 major groups:

Group 1: It contains the picornaviral IRESes and was initially subdivided into 2 major subgroups: the entero-/rhinovirus (type I, epitomized by PV (Fig. 1C)) and the cardio-/aphthovirus (type II, epitomized by EMCV (Fig. 1B) and by the foot-and-mouth-disease virus (FMDV)). Both types of IRES have distinct RNA structure with some conserved motifs such as the pyrimidine tract (Fig. 1B and C, grey box). From a functional point of view, initiation does not need eIF4E but requires, at least, the carboxy terminal fragment of eIF4G (p100) in which binding sites for eIF4A and eIF3 are present (Ohlmann et al., 1996; Prevot et al., 2003a). Such a p100 fragment is generated by the cleavage of eIF4G by the FMDV L or PV 2A proteases, and interacts with a domain located upstream of the pyrimidine tract in both types of IRES (de Breyne et al., 2009; Pestova et al., 1996). Binding of eIF4G to this RNA domain and its association with eIF4A induces RNA remodeling of the downstream hairpin (de Breyne et al., 2009; Kolupaeva et al., 2003) and was proposed to ensure adequate conditions for ribosome attachment to the IRES at its 3′ border (Fig. 1B and C). Canonical eIFs are usually not sufficient to allow efficient ribosome recruitment and the event requires ITAFs (Fitzgerald and Semler, 2009; Lopez-Lastra et al., 2005). The hepatitis A virus represents a minor type III subgroup with a structurally distinct IRES. This cap-independent initiation requires the complete eIF4F complex but the molecular mechanism used remains to be determined (Borman et al., 2001; Brown et al., 1994). Recently, a type IV subgroup of IRES has been proposed and is composed of picornaviral IRESes that have sequence, RNA structure and functional homologies with the Hepaci- and Pestivirus (HP) IRESes (Bakhshesh et al., 2008; Chard et al., 2006a,b; de Breyne et al., 2008b; Hellen and de Breyne, 2007; Liu et al., 2011a; Pisarev et al., 2004; Willcocks et al., 2011).

Group 2: The Hepatitis C virus (HCV) and the Classical Swine Fever Virus (CSFV) IRESes form the HP-IRESes which consist of three RNA domains (II, III and IV) relatively conserved and organized around a pseudo-knot structure (Fig. 1D) (Pestova et al., 1998b). Initiation differs from all eukaryotic mRNAs or picornaviral IRESes because 40S subunit attachment on the mRNA does not require any eIFs, and only needs the eIF2 ternary complex together with eIF3 to yield the 48S PIC (Fig. 1D) (Pestova et al., 1998b). The eIF4G/eIF4A association is not required for basic activity but their presence can, in some cases, enhance ribosomal recruitment (Bakhshesh et al., 2008; de Breyne et al., 2008b).

Group 3: This group contains the dicistrovirus epitomized by the cricket paralysis virus (CrPv). The intergenic region has a highly complex structure composed of three pseudoknot domains (Fig. 1E)

structure of the PV IRES which is composed of domains II to VI. The couple eIF4G/eIF4A binds domains V and allows RNA unwinding of domain VI. The initiation codon located in domain VI is not recognized and ribosomes have to reach the downstream initiation codon. (D) Model of the secondary and tertiary structure of the HCV IRES which consists in three domains (II, III and IV) organized around a pseudoknot. eIF3 binds to the apical loop of III domain. The 40S subunit binds to the core domain at the vicinity of the AUG codon and requires addition of the ternary complex and eIF3 to yield 48S complexes. (E) Model of the secondary and tertiary structure of the CrPV IRES composed of three pseudoknot domains. This IRES allows direct recruitment of the two ribosomal subunits at domain III which mimics base pairing between the codon and anti-codon of the tRNA. Initiation does not require any eIF.

Adapted from Balvay et al. (2009).

in which the RNA motif of domain III mimics the conformation of the $tRNA^{Met}_i$ and can bind to the P site of the ribosome by base-pairing between the codon and anticodon (Fig. 1E). This results in a very unique way of ribosomal recruitment, and so initiation, from the A site of the ribosome in the absence of any eIFs and without initiator tRNA (Deniz et al., 2009; Wilson et al., 2000).

Group 4: IRESes from cellular mRNAs differ from viral IRESes mainly because the mRNA is capped and translation may involve both cap- and IRES-dependent mechanisms. The ratio between cap/IRES is probably regulated by the physiological conditions of the cell which may favor one or the other mode of initiation. For instance, the X-chromosome linked inhibitor of apoptosis, XIAP, is encoded by two distinct mRNAs that differ by the length of their 5′-UTR. The shorter 5′-UTR promotes cap-dependent initiation during normal physiological conditions, and the less abundant but longer 5′-UTR contains an IRES element which is active during stress (Riley et al., 2010). The p110 (PITSLRE) protein kinase and ornithine decarboxylase are both expressed by a cell cycle regulated IRES which is active at the G2/M transition (Cornelis et al., 2000; Pyronnet et al., 2000) when cap-dependent translation is inhibited (Pyronnet et al., 2001). Cellular IRESes also require a different set of ITAFs which bind to the IRES during mRNA passage from the nucleus to the cytoplasm. These ITAFs belong to the heterogeneous nuclear ribonucleoproteins (HnRNP) family which are able to shuttle between the nucleus and the cytoplasm (Komar and Hatzoglou, 2011; Lewis and Holcik, 2008).

1.3. Ribosomal shunting

Some mRNAs have developed others alternative cap-dependent strategies to initiate translation such as the ribosomal shunting. The 43S ribosomal subunit is recruited to the 5′cap, starts to scan, but at a defined donor site translocate to an internal acceptor site which is located close to the start codon. Ribosomal shunting has been originally described in viral mRNA, including those of cauliflower mosaic virus (Futterer et al., 1993), rice tungro bacilliform pararetrovirus (Pooggin et al., 2006), Sendai paramyxovirus (Curran and Kolakofsky, 1988, 1989; de Breyne et al., 2003; Latorre et al., 1998), duck hepatitis B virus (Cao and Tavis, 2011), the prototype foamy virus (Schepetilnikov et al., 2009) and the adenovirus (Yueh and Schneider, 2000), but also in cellular mRNAs, including those of Hsp70 (Yueh and Schneider, 2000), cIAP2 (Sherrill and Lloyd, 2008) and ß secretase (Rogers et al., 2004). For more details, see these reviews (Firth and Brierley, 2012; Ryabova et al., 2002).

2. Translational control of HIV

2.1. A broad view of translational control on HIV-1

The lentiviral genomic RNA is about 9000 nucleotides in length and consists of 3 major ORFs gag, gag-pol and env flanked by a 5′ and a 3′ untranslated region. As a product of the cellular RNA polymerase II, the HIV genomic RNA harbors a m^7-GTP cap structure at its 5′ end and a poly(A) tail at the 3′ extremity. Following nuclear export, the unspliced genomic RNA is translated on free ribosomes in the cytoplasm of the host cell to produce the Gag and Gag-Pol polyproteins. The protein synthesis of Gag-Pol occurs by a programmed-1 ribosomal frameshift, process that has been extensively reviewed by others (Brierley and Dos Ramos, 2006; Giedroc and Cornish, 2009) and will not be further discussed in this manuscript. Although many different players have been involved in the control of HIV translation over the past years, such as the Gag, Tat and Rev (Anderson and Lever, 2006; Arrigo and Chen, 1991; Bannwarth and Gatignol, 2005; Clerzius et al., 2011; Groom et al., 2009; Kimura et al., 1996), most of the regulation occurs at the initiation step and implies the viral 5′-UTR and the beginning of the gag coding region. This is mostly due to the fact that the genomic lentiviral RNA is used both as a template for viral proteins synthesis and as genome within newly formed virions. As such, these two events must be timely regulated and these controls are mainly exerted at the level of the 5′-UTR which is used for translation and viral assembly.

The viral 5′-UTR is long and composed of several structural features (Fig. 2) involved in almost all steps of the replication cycle: (i) The *trans*-activation response element (TAR) is the binding site for the viral protein Tat which strongly stimulates transcription from the viral promoter (Roebuck and Saifuddin, 1999). (ii) The poly-adenylation signal (poly(A)) is present both in the 5′ and 3′ end of the viral transcripts but only the latter is involved in 3′ end processing (Cochrane et al., 2006). (iii) The primer binding site (PBS) domain contains the complementary sequence of the cellular $tRNA^{Lys3}$ required for viral reverse transcription (Arts and Le Grice, 1998). (iv) At the dimerization site (DIS), the kissing loop hairpin facilitates the packaging of the two copies of genomic RNAs (Paillart et al., 2004). Finally, the last two domains are (v) the 5′ splice donor site (SD) which is used to generate the subgenomic transcripts (Stoltzfus, 2009) and (vi) the packaging signals (Ψ) which are critical for RNA encapsidation (D'Souza and Summers, 2005).

The mechanism of translation initiation of the genomic HIV mRNA has been the subject of intense debate over the past years. Indeed, at first, initiation on the HIV genomic mRNA was defined to be strictly cap-dependent when assayed in the rabbit reticulocyte system (Miele et al., 1996). Such a hypothesis was later revisited with data showing the presence of functional IRESes both within the 5′-UTR and the gag coding region of HIV-1 (Brasey et al., 2003; Buck et al., 2001). Very recently, two studies showed that scanning can also take place on both the HIV-1 and HIV-2 5′-UTRs in cultured cells (Berkhout et al., 2011; Soto-Rifo et al., 2012a). Thus, the actual tendency is that both mechanisms (cap- and IRES-driven) can be alternatively used and that these apparent controversies may reflect the complexity of the different controls and constraints that are exerted on the HIV genomic RNA. Therefore, the remaining of the review will focus on the description of our current knowledge of the translation initiation mechanisms that take place on both HIV-1 and HIV-2 genomic mRNA.

2.2. HIV-1

2.2.1. The 5′ end dependent initiation

Translation of the full length unspliced mRNA of HIV-1 has been more studied than that of HIV-2. This capped and poly-adenylated transcript contains a 335nt long 5′-UTR (in pNL4.3) which harbors several structured RNA domains. The first AUG codon is the authentic initiation codon which is located in a good and conserved context GAGAG*AUG*GCT (analysis of the sequences related in the HIV-1 database: http://www.hiv.lanl.gov/). Initiation at this site can occur by a 5′ end initiation mechanism (Berkhout et al., 2011; Miele et al., 1996; Ricci et al., 2008b; Sharma et al., 2012; Soto-Rifo et al., 2012a,b) and this is consistent with the fact that addition of cap analog or the FMDV L protease resulted in the inhibition of translation mediated by the viral 5′-UTR in the rabbit reticulocytes lysate (RRL) (Ricci et al., 2008b). Introduction of upstream AUG within the viral 5′-UTR confirmed that ribosomal scanning occurs in transfected cells, which is in favor of a cap-dependent mechanism of translation (Berkhout et al., 2011).

The 5′-UTR presents a high degree of RNA structures (Fig. 2) which is known to interfere the ribosomal scanning in the rabbit reticulocyte lysate (Parkin et al., 1988; Soto-Rifo et al., 2012a; Svitkin et al., 1994). The major hurdle for ribosomal scanning is most probably imposed by the TAR domain, a highly stable stem-loop structure (Fig. 2A; $\Delta G = -29.6$ kcal/mol) which is located at

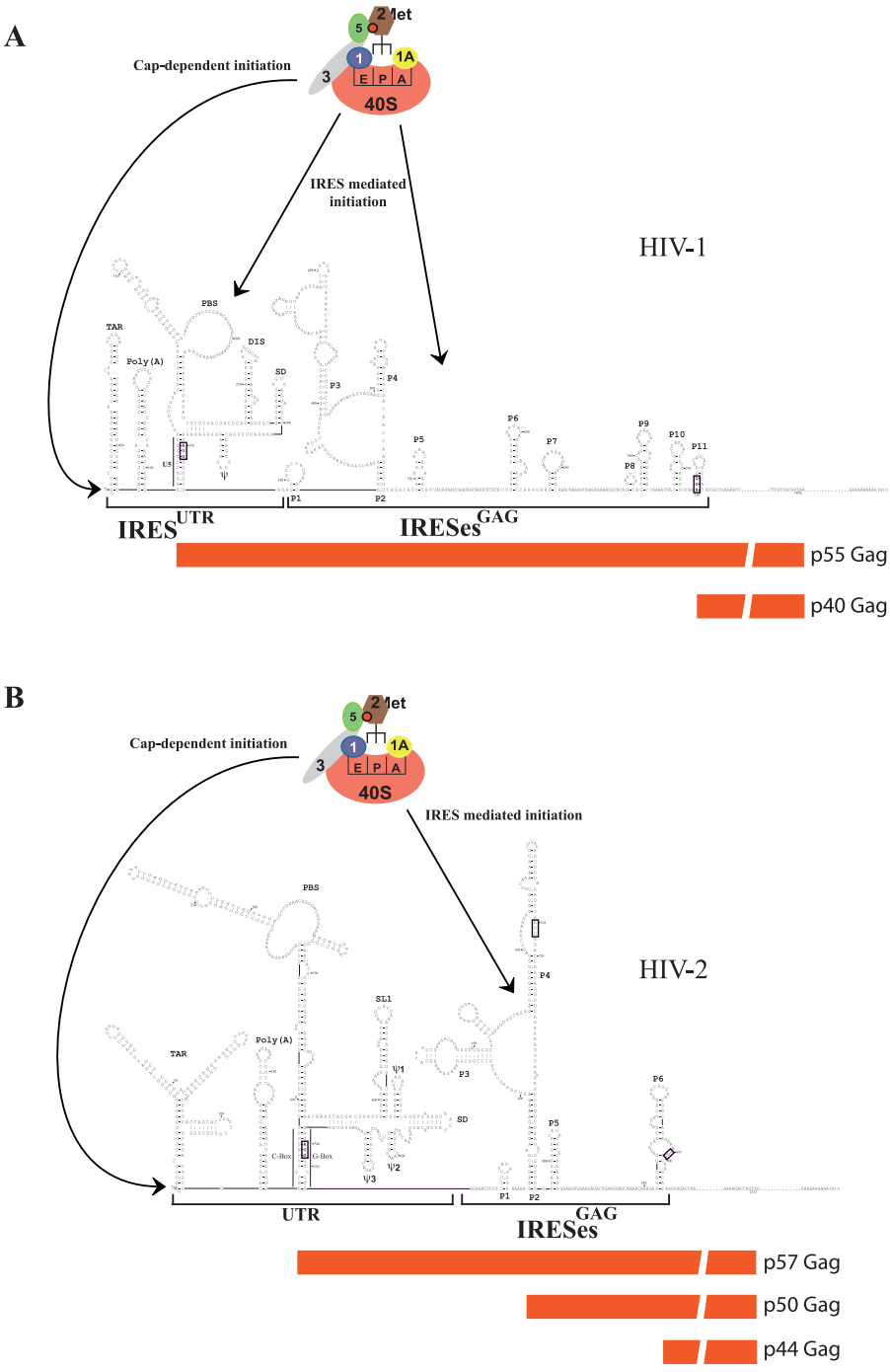

Fig. 2. Ribosome recruitment on HIV. (A) Model of the secondary structure of the HIV-1 genomic RNA from nucleotide +1 up to nucleotide 759 (Adapted from Abbink et al. (2005), Weill et al. (2010)). The 5′-UTR spanning the +1 of transcription to nucleotide 335 is composed of the TAR, the poly(A), the PBS, the DIS, the SP and the Ψ domain. The gag coding region is schematized by domains P1 to P11, which possess an IRES activity. Ribosomal complexes can attach to the mRNA either by the 5′ cap structure, via the 5′-UTR IRES or via the IRES in the coding region. Full-length p55 and its N-truncated p40 isoform of HIV-1 Gag are represented below the RNA structure. (B) Model of the secondary structure of the HIV-2 genomic RNA from nucleotide +1 up to nucleotide 906 (Adapted from Purzycka et al. (2011), Weill et al. (2010). The 5′-UTR is spanning from +1 to the initiation codon (nucleotide 545) and is composed of the TAR, Poly(A), PBS, SL1, Ψ1, SD, and Ψ3 domains. The coding region from domain P1 to P6 contains an IRES. Ribosome binding on the mRNA can occur at the 5′ end and in the gag coding region. Full-length p57 and its N-truncated p50 and p44 isoform of HIV-2 Gag are represented below the RNA structure.

the most 5′ end of the RNA spanning from nucleotide +1 to +57 (Soto-Rifo et al., 2012a). Presence of a weaker stable stem-loop ($\Delta G = -13.6$ kcal/mol) at the 5′ end of a synthetic mRNA is sufficient to inhibit ribosome recruitment at the 5′ end (Pestova and Kolupaeva, 2002). Therefore, it is not a surprise to observe that the TAR structure inhibits translation, alone or in the context of the HIV-1 5′-UTR, in the RRL or in *Xenopus* oocytes (Dugre-Brisson et al., 2005; Parkin et al., 1988; Soto-Rifo et al., 2012a; Svitkin et al., 1994). RNA stem loops can be destabilized by the scanning PIC with the assistance of components of eIF4F complex (Kozak, 1980, 1989; Pestova and Kolupaeva, 2002). So, addition of the *trans*-dominant negative eIF4A^{R362Q} mutant (Pause et al., 1994) into the RRL strongly repressed expression mediated by the HIV-1 5′-UTR indicating that the couple eIF4G and eIF4A is required

for bypassing the RNA structures present in the HIV-1 5′-UTR (de Breyne et al., 2012). Interestingly, translational inhibition mediated by TAR is less pronounced in cells (Soto-Rifo et al., 2012a). In agreement with this, it was also shown that translation driven by the HIV-1 5′-UTR was dramatically enhanced by addition of HeLa cell extracts in the RRL (Soto-Rifo et al., 2012a; Vallejos et al., 2011) suggesting that cellular factors, like RNA helicases which are missing in the RRL, can overcome this translational blockage. The first described was the RNA-binding protein lupus autoantigen La (Chang et al., 1994) which the binding to the TAR relieves the translational repression imposed by this structure in an *in vitro* system (Svitkin et al., 1994); this requires the dimerization domain of La (Craig et al., 1997). Huhn et al. (1997) reported that La possesses a dsRNA unwinding enzyme activity which could potentially be used to destabilize the TAR structure (Huhn et al., 1997); however, the role of La on HIV-1 expression in cell culture or its role on HIV-1 replication has not been further investigated. The DHX9/RNA helicase A (RHA) is able to interact with the HIV-1 5′-UTR which acts like a post-transcriptional control element (Bolinger et al., 2010). The knock-down of RHA by siRNA specifically reduced Gag expression without altering the level and the cellular localization of the viral mRNA. Interestingly, expression can be rescued by exogenous expression of wild type RHA but not a mutant in which the ATP hydrolysis domain was altered (Bolinger et al., 2010). The human DDX3 is a DEAD (Asp-Glu-Ala-Asp)-box RNA helicase involved in the HIV-1 replication. DDX3 shuttles between the nucleus and the cytoplasm, and is involved in several RNA metabolic processes like transcription, splicing, export and translation (Schroder, 2010). DDX3 is also a Rev-cofactor and participates to the regulation of the HIV-1 full-length transcript nuclear export via the CRM1 pathway (Yedavalli et al., 2004). In addition, works from our lab have shown that DDX3 is required for translation of mRNAs with high RNA structures at the vicinity of the 5′ end like the TAR element in HIV-1 (Soto-Rifo et al., 2012b). Indeed, down regulation of the DDX3 expression by siRNA did not modify the distribution of the HIV-1 genomic mRNA in the cytoplasm but strongly inhibited the expression driven by the HIV-1 5′-UTR. This was imposed by the localization of the TAR structure at the 5′ end. Deletion of the TAR hairpin or the upstream introduction of the unstructured (CAA)n repetition (Tzareva et al., 1994) are sufficient to relieve the DDX3 dependence (Soto-Rifo et al., 2012b). Moreover, DDX3 can bind to the HIV-1 5′-UTR, and interacts with both eIF4G and PABP. This multiple interactions complex could promote local RNA double strand separation to allow the entry of the eIF4F complex. Indeed, the entry of eIF4F to mRNAs is greatly facilitated by the presence of a free 5′ end in yeast (Rajagopal et al., 2012). Works from Reed's lab have shown that DDX3 interacts with eIF3 and could also promote translation (Lee et al., 2008). So in cells and in contrast with the RRL system, these RNA helicases and maybe others, like DHX29, can participate for efficient scanning on long and structured 5′-UTRs (Dmitriev et al., 2009; Pisareva et al., 2008; Soto-Rifo et al., 2012a,b).

Nevertheless, the HIV-1 infection is associated with modifications of the cellular homoeostasis. The viral protein Vpr alters cell cycle progression by arresting the cells in the G2/M phase (Andersen et al., 2008; Sharma et al., 2012; Zhao et al., 2011). Interestingly, the G2/M phase is associated with hypophosphorylation of both eIF4E and 4E-BP that lead to disruption of the eIF4F complex and the sequestration of eIF4E by 4E-BP. As a consequence, cap-dependent initiation is reduced (between 50 and 60%) due to the lack of functional eIF4E (Pyronnet et al., 2001; Sharma et al., 2012). Despite of this condition and others (see below), viral protein synthesis persists during this stage of the cell cycle (Brasey et al., 2003; Sharma et al., 2012). One possibility is that the unspliced HIV-1 mRNA can still be translated by cap-dependent initiation. Indeed, some mammalian mRNAs with long and structured 5′-UTR can be expressed by cap-dependent initiation even under the 4E-BP inhibition (Andreev et al., 2009).

The HIV-1 mRNAs are composed of a mixture of monomethylated capped (MG) and trimethylated (TMG)-capped mRNAs (Yedavalli and Jeang, 2010). TMG-capped-RNAs are known to translate poorly (Darzynkiewicz et al., 1988), due to the inefficient recognition of TMG-caps by eIF4E (Rutkowska-Wlodarczyk et al., 2008). In contrast, TMG-capping of viral HIV-1 mRNAs increases the expression of HIV-1 structural proteins (Yedavalli and Jeang, 2010). The nuclear cap binding complex composed of CBP80 and CBP20 is involved in the first round of mRNA translation to ensure the quality of gene expression as exemplified by nonsense-mediated mRNA decay (Maquat et al., 2010). CBP80/20 is bound to the cap structure and also interacts either with eIF3g (Choe et al., 2012) or with the CTIF (CBP80/20-dependent translation initiation factor) (Kim et al., 2009) to promote recruitment of the PIC. Interestingly, during infection, the 5′ end of cytoplasmic HIV-1 unspliced mRNAs stay preferentially bound to the CBP80/20 (Sharma et al., 2012). In addition, biophysical measurements have determined that CBP80/20 have a higher affinity for the TMG-cap than eIF4E (Worch et al., 2005). These data suggest that the unspliced HIV-1 mRNA could be translated by a 5′ end initiation mechanism which does not involve eIF4E.

Others cellular factors have been reported to interact with the TAR domain. The Staufen1 protein is TAR-interacting protein which contains a dsRNA-binding domain that was initially shown to induce the de-repression of *oskar* mRNA translation in oocytes (Micklem et al., 2000). Staufen1 has been reported to stimulate translation of TAR-containing mRNAs (Dugre-Brisson et al., 2005). The TAR domain is also a site of multiple levels of regulation by the protein kinase RNA-activated (PKR). Indeed, PKR was shown to contact TAR via its dsRNA binding domain which leads to its autophosphorylation and activation to trigger the phosphorylation of the eIF2α subunit ultimately inhibiting global cellular protein synthesis. eIF2 is used to deliver the initiator $tRNA^{Met}$ to the ribosome (Fig. 1A); when inactive by phosphorylation of its α subunit, eIF2 remains associated with GDP, losing its capacity of recruiting the initiator $tRNA^{Met}$ to the translation initiation complex leading to an inhibition of the global translation (Proud, 2005; Wek et al., 2006). Interestingly, activation of PKR can be antagonised by the Tat protein and the TAR RNA binding protein (Bannwarth and Gatignol, 2005; Clerzius et al., 2011). Although all these factors can alleviate translational inhibition imposed by TAR, the contribution of each one remains to be determined.

2.2.2. IRESes-mediated translation initiation

2.2.2.1. Several IRESes are present within the genomic RNA. IRES elements have been reported in several members of the *Retroviridae* family from both simple (Berlioz and Darlix, 1995; Berlioz et al., 1995; Deffaud and Darlix, 2000a,b; Lopez-Lastra et al., 1997; Vagner et al., 1995) and complex retroviruses (see below; (Attal et al., 1996; Brasey et al., 2003; Buck et al., 2001; Camerini et al., 2008; Herbreteau et al., 2005; Ohlmann et al., 2000) and also from endogeneous retroelements (Lopez-Lastra et al., 1999; Meignin et al., 2003; Ronfort et al., 2004).

The HIV-1 genomic mRNA harbors two IRES elements: the first one, and best characterized, lies within the viral 5′-UTR, while the second one is found within the gag coding region (Fig. 2A) (Brasey et al., 2003; Buck et al., 2001). The HIV-1 IRES was identified by cloning the 5′-UTR (nucleotides 1–336) of the laboratory adapted HIV-1 infectious recombinant proviral clone NL4.3 (pNL4.3; AF 324493) into the intercistronic region of a dual luciferase (dl) reporter construct (Brasey et al., 2003). The dl reporter construct contained the *Renilla* luciferase gene (RLuc) upstream and the firefly luciferase gene (FLuc) downstream. To ensure that the two cistrons were independently translated, a defective EMCV IRES (ΔEMCV),

known to inhibit ribosome reinitiation and read-through, was inserted upstream of the HIV-1 5′-UTR (Brasey et al., 2003). In this experimental setting the translational activity of the HIV-1 5′-UTR was monitored using FLuc activity as the readout, while the RLuc reporter gene served as an upstream translational control. This approach demonstrated that, when in the context of a bicistronic mRNA the HIV-1 5′-UTR of pNL4.3 was capable of driving efficient translation initiation (Brasey et al., 2003). Most recently, the presence of a competent IRES was also reported within the 5′-UTR of the CXCR4 (X4)-tropic primary isolate HIV-LAI and viral RNA isolated from clinical samples (Gendron et al., 2011; Vallejos et al., 2012). Both of these studies based their experimental approach on the use of bicistronic mRNAs. As mentioned above studies to identify internal initiation in isolated viral or cellular UTR segments utilizing the transfection of bicistronic reporter plasmids have been strongly criticized. It is recognized that a caveat to the bicistronic approach is false-positive IRES activity attributable to cryptic promoter activity or splicing of the tested sequence. Alternative splicing would potentially bypass the first cistron while allowing the expression of the second cistron from the mRNAs promoter. However, in the studies of Gendron et al. (2011) and Vallejos et al. (2012) several controls were added to exclude the possibility that results were due to the generation of spliced RNAs or due to the activity of a cryptic promoter. Gendron et al. (2011) used a shRNA targeting the first coding sequence of the bicistronic mRNA (Van Eden et al., 2004),to show that the second cistron is not expressed from a cryptic promoter or by spurious splicing. Vallejos et al. (2012) on the other hand quantified the amount of bicistronic mRNA generated in cells by individually amplifying each cistron using a quantitative RT-qPCR assay and demonstrating the [RNA (second cistron) in pmol/mRNA(first cistron) in pmol] ratio is one or close to one. Additionally in Vallejos et al. (2012) discarded the presence of a cryptic promoter by removing the promoter from the DNA bicistronic constructs to show that in its absence no additional form of mRNA encoding the reporter proteins was generated. Together, these studies indicate that the capacity of the HIV-1 5′-UTR to drive cap-independent translation initiation. Furthermore, the study of Vallejos et al. (2012) shows that HIV-1 IRES activity is not restricted to the laboratory adapted HIV-1 clone pNL4.3 (Brasey et al., 2003) or to the single primary HIV-1 isolate, pLAI (Gendron et al., 2011).

The region spanning the 5′-UTR from nucleotides 104 to 336 was shown to contain the minimal IRES activity (Brasey et al., 2003). Interestingly, this same region contains several RNA structural elements such as the PBS domain and the SD, DIS and Ψ that are critical for different stages of the viral replication cycle (Fig. 2A). This HIV-1 5′-UTR IRES was inefficient in promoting translation in RRL *in vitro* translation system (Miele et al., 1996). Poor activity of the HIV-1 5′-UTR IRES in RRL was due to the requirement of additional cell factors present in HeLa cell extracts prepared with G2/M arrested cells (Vallejos et al., 2011). Thus, similar to PV and human rhinovirus (HRV) IRES elements (Borman et al., 1993; Brown and Ehrenfeld, 1979; Dorner et al., 1984), the HIV-1 5′-UTR IRES requires additional cell factor for optimal activity (discussed below). The HIV-1 5′-UTR IRES is active in RRL supplemented with G2/M HeLa extracts (Rivas-Aravena et al., 2009; Vallejos et al., 2011), HeLa based translation extracts (Brasey et al., 2003), cells (HeLa, U20S, 293-T, and Jurkat T cells) (Brasey et al., 2003; Gendron et al., 2011; Liu et al., 2011b; Monette et al., 2009; Wilker et al., 2007), and in *Xenopus laevis* oocytes (Rivas-Aravena et al., 2009; Vallejos et al., 2012, 2011). Nonetheless, the molecular mechanism driving ribosome recruitment by the HIV-1 5′-UTR IRES remains largely unknown. Cellular factors could act like chaperone proteins to promote an active conformation of the IRES as it has been proposed for the cellular Apaf1 IRES (Mitchell et al., 2003) or like an intermediate between the IRES and the preinitiation complex. Based on the reports of Vallejos et al. (2011, 2012) at the time authors favor the latter possibility (discussed below).

The organization of the RNA architecture of the viral 5′-UTR was initially suggested to play a major role in translation mediated by the 5′-UTR (Brasey et al., 2003). The 5′-UTR can adopt two spatial conformations which are the branched multiple hairpins (BMH) and the long distance interaction (LDI) structures (Huthoff and Berkhout, 2001; Lu et al., 2011). The BMH conformation (Fig. 2A) was shown to expose the dimerization and packaging signals and to favor encapsidation and viral replication (Abbink et al., 2005; Berkhout et al., 2002; Lu et al., 2011; Ooms et al., 2004). This riboswitch may occur during virus infection as it is enhanced both by the RNA concentration and the NC protein (Huthoff and Berkhout, 2001; Lu et al., 2011). However, despite the fact that the BMH structure occludes the AUG start site codon (Abbink and Berkhout, 2003), the structure by itself does not modify the translation rate of the HIV-1 5′-UTR (Abbink et al., 2005; Vallejos et al., 2011). In fact the introduction of mutations designed to alter the LDI-BMH equilibrium does not significantly impact on the ability of the HIV-1 5′-UTR to drive translation initiation in both the context of a mono or bicistronic mRNA (Abbink et al., 2005; Vallejos et al., 2011). These findings suggest that both the BMH and LDI RNA structures, as well as several intermediate RNA structures (Abbink et al., 2005; Vallejos et al., 2011), are capable of driving protein synthesis. Additionally, translation activity from the HIV-1 5′-UTR IRES was resistant to mutations designed to alter specific RNA structural element within the functional region (Vallejos et al., 2011). These observations suggest that structure-function relationship for the HIV-1 5′-UTR IRES is not as rigid as that described for other viral IRESes (Fernandez-Miragall et al., 2009; Fitzgerald and Semler, 2009; Fraser and Doudna, 2007; Kieft, 2008; Martinez-Salas, 2008; Pfingsten et al., 2010). These observations have been recently confirmed when analyzing the translational activity of HIV-1 5′-UTR regions of natural isolates recovered from clinical samples (Vallejos et al., 2012).

Studies of the genetic drift of infectious RNA viruses that initiate translation via an IRES reveals that substitutions within the IRES region causes compensatory changes destined to support the assembly of the secondary/tertiary structure of the IRES (Fitzgerald and Semler, 2009; Kieft, 2008; Martinez-Salas, 2008). Small deletions or insertions, and even point mutations in the IRES can have a profound effect on internal initiation (Barria et al., 2009; Jubin et al., 2000; Martinez-Salas et al., 1993). Unexpectedly, the work of Vallejos et al. (2011 and 2012) reveals that these RNA structure/IRES function constrains, described for most viral IRESes, do not seem to apply in the case of the HIV-1 5′-UTR IRES. Based on these findings Vallejos et al. (2011, 2012) proposed that the HIV-1 5′-UTR IRES functions as a cellular more than as a viral IRES.

In sharp contrast to viral IRESes, cellular IRES elements share no obvious structure conservation, even when comparing IRESs from closely related mRNAs (Baird et al., 2007, 2006; Hellen and Sarnow, 2001; Stoneley and Willis, 2004). Furthermore, point mutation or deletions within many cellular IRESs hardly alter translation initiation, suggesting that the structure–function relationship in cellular IRESes is not as rigid as what is described for viral IRESes (Le Quesne et al., 2001). Strikingly, individual sections of cellular IRESs are able to promote internal initiation, albeit not as efficiently as the entire IRES (Baird et al., 2007; Chappell and Mauro, 2003). This strongly suggests that, in contrast to viral IRESes which are a well defined RNA structure (Filbin and Kieft, 2009; Kieft, 2008; Lukavsky, 2009; Martinez-Salas, 2008; Pfingsten et al., 2006), cellular IRESes may be composed of multiple short modules and do not correspond to a unique RNA structure (Baird et al., 2007). Therefore, the combined effect of these individual modules promotes internal initiation for cellular IRES elements (Baird et al., 2007; Chappell et al., 2000; Chappell and Mauro, 2003). Another major difference between IRES

found in most RNA viruses and cellular mRNAs that harbor IRES elements is that the latter also possess a 5′cap structure (Hellen and Sarnow, 2001; Stoneley and Willis, 2004). Therefore, in cellular mRNAs that harbor an IRES, translation initiation can occur both by a cap- and an IRES-dependent initiation mechanism. This dual mechanism for 40S ribosome subunit recruitment suggests that the structure of the IRES elements present in cellular mRNA is dynamic and its function could be regulated by cellular proteins that could act like RNA chaperones to maintain a correct conformation the RNA structural features and so the IRES function (King et al., 2010; Komar and Hatzoglou, 2011; Stoneley and Willis, 2004).

As for cellular IRESes, the function of the HIV-1 5′-UTR IRES seems to be highly regulated by both cellular and viral proteins. Expression of the Gag protein inhibits its own synthesis while stimulating the conformation of the BMH structure (Anderson and Lever, 2006; Lu et al., 2011). As the BMH structure does not by itself alter IRES activity (Vallejos et al., 2011), it may be the presence of the Gag protein on the viral mRNA that affects ribosome attachment or scanning along the 5′-UTR. This possibility is given credence by the observation that the Gag protein exerts a bimodal effect on its own production with enhancement of expression at low concentration and inhibition at high concentration (Anderson and Lever, 2006). In this model, translation repression would be dependent on the establishment of the packaging signal, RNA structure required for genomic RNA encapsidation; the duplex formed by the genomic RNA and the Gag protein via the NC domain probably establishes a strong hindrance for scanning or recruitment of the PIC. Therefore, at high concentrations the Gag protein would be directly responsible for inhibiting translation from the viral mRNA favoring its packaging into *de novo* synthesized viral particles (Anderson and Lever, 2006).

Regulation of translation initiation driven by the HIV-1 5′-UTR is also controlled by RNA *cis*-acting elements located in a region upstream from the primer binding site (PBS), named IRENE, that negatively impacts on activity of the HIV-1 IRES but the molecular mechanism remains to be solved (Gendron et al., 2011).

While studying translation initiation from the genomic HIV-1 mRNA, Buck and colleagues identified an IRES within the gag ORF (Fig. 2A) (Buck et al., 2001). These sequences have the characteristics to modulate negatively the activity of the IRES from the 5′-UTR (Brasey et al., 2003) probably by reducing the amount of free ribosomes. This IRES was shown to mediate expression of both the full length Gag polyprotein and a shorter N-truncated isoform of Gag (p40) in which the entire matrix domain is missing. Although the function of Gag p40 is unknown, it is produced during infection and its deletion impaired the growth kinetics of HIV-1 in culture (Buck et al., 2001). The presence of shorter N-truncated isoforms that result from alternative initiation at codons located in the gag coding region is conserved in HIV-2 and the simian homolog (SIV) (Herbreteau et al., 2005; Nicholson et al., 2006). A potential function for the simian Gag isoforms was proposed to prevent premature processing of the Gag polyprotein by serving as decoy substrate to the viral protease (Nicholson et al., 2007).

In our laboratory, we have been very much interested in the production of this p40 isoform of Gag and we observed its expression in all clinical HIV-1 isolates from a rather large cohort of patients ($n = 100$). Interestingly, we also noted major differences in the ratio of expression of p40 to p55 Gag amongst patients suggesting that their relative production may be subjected to fine regulation (unpublished observations). Investigation into the translational mechanism used for production of p40 revealed the exclusive use of an IRES located in the gag ORF and whose activity is totally independent from the cap structure, eIF4E, and is resistant to the FMDV L protease treatment (Ricci et al., 2008b). However, the most peculiar characteristics of this IRES lies in its ability to promote expression on the upstream AUG located at its 5′ border (Buck et al., 2001; Weill et al., 2010). This allows expression from synthetic leaderless mRNAs in which the AUG codon is located right at the 5′ end of the mRNA. Surprisingly, translation mediated by this synthetic mRNA is highly efficient (de Breyne et al., 2012; Weill et al., 2010) and occurs at the authentic AUG codon with no associated leaky scanning indicating that ribosome recruitment on the gag coding region IRES is mediated by an unconventional mechanism. This contrasts with all other IRESes described to date that promote translation from the AUG codon located at the 3′ border of the IRES. In addition, the gag coding region (up to the initiation codon of p40) has the ability to bind alone both eIF3 and the 40S ribosome (Locker et al., 2011). This characteristic is reminiscent with a mode of initiation described for the IRESes of the group #2 (HCV, CSFV, HCV-like. . .) (Bakhshesh et al., 2008; Chard et al., 2006a,b; de Breyne et al., 2008b; Liu et al., 2011a; Pestova et al., 1998b; Pisarev et al., 2004; Willcocks et al., 2011) and is conserved in HIV-2 and in SIV (Herbreteau et al., 2005; Locker et al., 2011; Weill et al., 2010). Such a peculiar biochemical feature suggests that the gag coding region contains two binding sites for ribosome attachment which are dedicated to each initiation codon.

2.2.2.2. Rational for IRES. Although cap-dependent translation can take place on the HIV-1 5′-UTR (Berkhout et al., 2011), it is not known whether this mechanism can be fully operational during viral infection due to changes of the cell homeostasis and the modification of several components of the host translational apparatus. For instance the expression of the Vpr viral protein, which is used to regulate both reverse transcription, nuclear transport, transcription and apoptosis also promotes cell cycle arrest in the G2/M phase (Zhao et al., 2011) which is associated with an hypophosphorylation of both eIF4E and 4E-BP that leads to disruption of the eIF4F complex and the sequestration of eIF4E by 4E-BP (Sharma et al., 2012). As a consequence, the global cap-dependent initiation is reduced up to 60% due to the lack of functional eIF4E (Pyronnet et al., 2001; Sharma et al., 2012) whereas some cap-dependent initiation and IRES-driven expression is barely affected (Andreev et al., 2009; Brasey et al., 2003; Cornelis et al., 2000; Pyronnet et al., 2000).

In addition, the HIV-1 viral protease, required for processing of the two major polyproteins Gag and Gag-Pol, has several cellular targets (Adams et al., 1992; Alvarez et al., 2006; Jager et al., 2012; Nie et al., 2002; Riviere et al., 1991; Shoeman et al., 1991; Solis et al., 2011; Ventoso et al., 2001) amongst which are eIF4G, PABP and eIF3d (Alvarez et al., 2006; Jager et al., 2012; Ohlmann et al., 2002; Ventoso et al., 2001). The effect of the cleavage of eIF3d on translation was not determined but would rather affect the stage of reverse transcription (Jager et al., 2012). *In vitro* studies on HeLa cell extracts reveals that the recombinant HIV-1 protease cleaved the PABP in the RNA Recognition Motif 3 and in the carboxy-terminal domain. These cleavage products are consistent with those observed during infection (Alvarez et al., 2006). The cleavage of eIF4G was observed during infection and determined both in HeLa cell extracts and in RRL using recombinant protease or RNA coding for the viral protease. Two cleavage sites yielding three distinct fragments have been described: the central domain with the eIF3/eIF4A binding sites from the amino-terminal part which interacts with eIF4E and PABP and from the carboxy-terminal part and its second binding domain for eIF4A (Ohlmann et al., 2002; Ventoso et al., 2001). Experiments have been done in RRL, cell free extracts and cells, by monitoring expression of a reporter gene which is under the control of a strictly cap-dependent initiation, EMCV IRES or HIV-1 (5′-UTR or 5′-UTR-GAG) mediated initiation. This leads to repression of cap-dependent translation in RRL, in cell free extracts and in cells (Castello et al., 2009; Ohlmann et al., 2002; Perales et al., 2003; Ventoso et al., 2001); however, cap-independent translation driven by the EMCV- and PV-IRESes remained unaffected both in cell free extracts and in cells (Castello et al., 2009; Perales et al.,

2003). Interestingly, the HIV-1 protease inhibits expression mediated by the viral 5′-UTR alone (de Breyne et al., 2012; Perales et al., 2003) which would be consistent with the use of a cap-dependent mechanism. However, the effect of the viral protease has not been studied when the cells were arrested in the G2/M phase. Nevertheless, addition of the gag coding region downstream to the viral 5′-UTR provokes a relative resistance of expression to the viral protease (Castello et al., 2009; Ventoso et al., 2001). This indicates that the gag coding region confers alternative mechanisms for ribosome recruitment on the genomic mRNA when cap-dependent initiation is reduced, and this will be discussed below.

At least, the viral protein Vpr can activate the apoptosis and caspases (Zhao et al., 2011). Interestingly, activation of caspase leads to the cleavage of eIF4G in HeLa cells (Marissen et al., 2000; Marissen and Lloyd, 1998) and in lymphoid cells (Bushell et al., 1999, 2000; Clemens et al., 1998).

2.2.2.3. ITAFs for HIV-1. The study of cellular mRNAs harboring a dual initiation mode such as that exhibited by the HIV-1 mRNA shows that normal physiological conditions favor cap-dependent translation. However, under various cellular and environmental stresses, global protein translation declines and production of diverse stress-responsive factors driven by IRESes is preferentially upregulated (Komar and Hatzoglou, 2011; Lewis and Holcik, 2008; Lopez-Lastra et al., 2005; Sonenberg and Hinnebusch, 2009). For these examples of cellular mRNAs the switch from cap-dependent translation to cap-independent translation has been proposed to function as an adaptive response of stress resistance (Komar and Hatzoglou, 2011; Lewis and Holcik, 2008; Lopez-Lastra et al., 2005; Sonenberg and Hinnebusch, 2009). Thus, we propose that this is also the case for the regulation of translation initiation from the HIV-1 mRNA. The HIV-1 5′-UTR has been shown to drive cap-dependent translation initiation under normal physiological conditions (Berkhout et al., 2011; Ricci et al., 2008b), and to mediate IRES-dependent protein synthesis during oxidative and osmotic stress (Gendron et al., 2011; Monette et al., 2009). During infection, HIV-1 induces an endogenous cellular increase of reactive oxygen intermediates. This oxidative stress has been shown to facilitate NF-kappa B-dependent activation of HIV transcription, however, oxidative stress impairs cap-dependent translation initiation (MacCallum et al., 2006). Thus, an increased transcription is not sufficient to assure the synthesis of the viral structural proteins. However, the activity of the HIV-1 5′-UTR IRES is stimulated under these stress conditions favoring Gag protein synthesis (Gendron et al., 2011). The infection by HIV-1 also induces a G2/M cell cycle arrest, event that also increases synthesis of viral mRNA (Goh et al., 1998; Thierry et al., 2004). Again viral protein synthesis is persistent during this stage of the cell cycle, in part, due to an active 5′-UTR IRES (Brasey et al., 2003; Vallejos et al., 2011). The complex nature of HIV-1 mRNA translation under different physiological conditions suggests that there may be multiple levels of regulation modulating IRES-mediated initiation. Based on this possibility and on the observation that the HIV-1 5′-UTR IRES showed to have marginal activity in RRL and RRL supplemented with G1 HeLa cell extracts when compared to its activity in RRL supplemented with G2/M HeLa extracts, Vallejos et al. (2011) set out to determine the RNA structures of HIV-1 5′-UTR in different stages of the cell cycle. At the time it was assumed that most likely the 5′-UTR need to be remodeled from an inactive structure in G1 to active conformation in G2/M in order to be competent for initiation (Brasey et al., 2003; Vallejos et al., 2011). In agreement with this possibility two distinct RNA protection patterns, for G1 and G2/M, were characterized (Vallejos et al., 2011). In this study, Vallejos et al. (2011) established a model structure of the HIV-1 5′-UTR by probing the HIV-1 5′-UTR recovered from the dl HIV IRES construct described by Brasey et al. (2003), using DiMethyl sulfate (DMS), N-Cyclohexyl-N-[N-Methylmorpholino)-ethyl]-Carbodiimide-4-Toluolsulfonate (CMCT) and RNAse V1 (Brasey et al., 2003; Vallejos et al., 2011). The initial probing data were further validated by a Selective 2′Hydroxyl Acylation analysis by Primer Extension (SHAPE) analysis using 1-methyl-7-nitroisatoic anhydride (1M7) as a modifying agent (Vallejos et al., 2011). The same RNA region corresponding to the HIV-1 5′-UTR was then probed using 1M7 in the presence of cytoplasmic cell extract generated from cells arrested in G1 or in G2/M phase of the cell cycle. Results showed that significant RNA protections in the TAR apical loop, within the PBS and in the DIS element were observed upon addition of cell extracts independently of the stage where the cells were blocked (Vallejos et al., 2011). However, specific 1M7 accessibility RNA patterns for each used condition were also identified. In the presence of G2/M extracts reactivity modifications mapped to the poly(A) loop, the PBS structure, the SD loop and the Psi stem loop immediately upstream from the initiation codon (Vallejos et al., 2011). Noteworthy, the PBS structure, the SD loop, and the Psi Stem loop are regions previously identified as crucial for HIV-1 IRES activity (Brasey et al., 2003). In the presence of G1 extracts specific alterations of the modification pattern exclusively consisted of positions that become highly reactive (Vallejos et al., 2011). Most interestingly, sequences modeled as double stranded in the presence of G2/M extracts reacted as unpaired nucleotides when protection assays were carried out in the presence of G1 extract addition (Vallejos et al., 2011).

A mutational analysis was then conducted to directly associate changes in RNA structure with IRES activity. Despite the large number of tested mutations, no dependency between RNA structure and IRES function could be established (Vallejos et al., 2011). In consequence the most plausible explanation for these findings is that the distinct RNA protection patters described for G1 and G2/M most probably corresponds to a mixture of the footprint of proteins and protein induced structural rearrangements that shift the RNA from a translational inactive structure to an active IRES. This would suggest that the on/off switch of the HIV-1 5′-UTR IRES is regulated by cellular proteins. This conclusion is based on the observations that the described experiments were conducted in the absence of viral proteins (Vallejos et al., 2011).

IRES mediated protein translation requires in most cases the assistance of ITAFs. When interacting with IRES, ITAFs appear to facilitate translation initiation by recruiting eIFs and ribosomes, or by assisting the RNA to undertake a structure with the capacity of recruiting the initiation complex. Among the RNA binding proteins that have been characterized as functional ITAFs for viral IRESes in general, we find the Polypyrimidine Tract Binding Protein (PTB), Poly r(C) Binding Protein 1/2 (PCBP1/2), La autoantigen, *U*pstream of N-ras (unr), Heterogeneous Nuclear Ribonucleoproteins C1 and C2 (hnRNPC1/C2), Heterogeneous Nuclear Ribonucleoproteins A1 (hnRNP A1), Death Associated Protein 5 (DAP5), Embryonic Lethal Abnormal Vision/protein HuR (ELAV/HuR) and Nucleolin (Fitzgerald and Semler, 2009; King et al., 2010; Komar and Hatzoglou, 2011; Lopez-Lastra et al., 2005; Semler and Waterman, 2008; Stoneley and Willis, 2004). One or several ITAFs can bind to an IRES to regulate protein synthesis. Recent observations strongly suggest that in the case of cellular mRNAs the activity of ITAFs in IRES-dependent translation initiation is regulated by the subcellular distribution of these proteins (King et al., 2010; Komar and Hatzoglou, 2011; Lewis and Holcik, 2008). Furthermore, the nucleocytoplasmic trafficking of ITAFs may be particularly relevant in the light of the observation that exposure to the nuclear compartment is essential for efficient internal initiation of some cellular IRESes (Lewis and Holcik, 2008; Semler and Waterman, 2008; Stoneley et al., 2000).

Using surface plasmon resonance (BIAcore) technology and *in vitro* translation assays Waysbort et al. (2001) showed that

PTB is not a determinant ITAF for HIV-1 5′-UTR IRES function. Waysbort et al. (2001) adapted the BIAcore technology, mainly to assess protein–protein interactions, to characterize and quantify RNA–protein interactions. GST-La and GST-PTB recombinant proteins were directly used to evaluate their interactions with picornaviral and retroviral 5′-UTR RNAs immobilized on streptavidin chips bearing a biotinylated DNA with a 3′ end complementary to the different RNAs. Results from these assays showed that PTB interacts with the EMCV (K_D = 4 pM), poliovirus (K_D = 5 pM) and HTLV-I (K_D = 5.3 pM) RNAs, but not with the HIV-1, HIV-2 and SIV RNAs. The same report describes that La autoantigen binds to the HIV-1 5′-UTR (K_D = 0.3 nM) yet its function as an ITAF was not established (Waysbort et al., 2001). Using pulldown experiments Vallejos et al. (2011) identified a series of proteins that present in G2/M HeLa extracts that interact with the viral 5′-UTR. Other reports have also evaluated the role of ITAFs on translation initiation of the HIV-1 5′-UTR IRES, here results strongly suggest that translation initiation is modulated by cellular proteins (Liu et al., 2011b; Monette et al., 2009; Rivas-Aravena et al., 2009). By transfecting a dual luciferase bicistronic construct harboring the HIV-1 5′-UTR in the intercistronic space in combination with overexpression and knockdown experiments, Liu et al. showed that the eIF5A, the human Rev-interacting protein (hRIP), and DDX3 stimulate HIV-1 5′-UTR IRES activity (Liu et al., 2011b). The same report discarded Sam68, a member of the signal transduction and activation of RNA (STAR) protein family, as a possible ITAF for the HIV-1 5′-UTR IRES (Liu et al., 2011b). Using a similar strategy, Monette et al. (2009) showed that hnRNP A1 is a cellular factor that enhances HIV-1 5′-UTR IRES activity. Interestingly, this report shows that HIV-1 mRNA loads hnRNP A1 in the cell nucleus and upon association the RNA-protein complex shuttles to the cytoplasm (Monette et al., 2009). As nascent cellular mRNAs, the HIV-1 mRNA is expected to first encounter RNA-binding proteins in the nucleus. In fact, the full length viral mRNA reaches the cytoplasm as part of a distinct ribonucleoprotein (RNP) complex with nuclear RNA-binding factors (Cochrane, 2009; Cochrane et al., 2006). Therefore, it is plausible that both viral and cellular proteins such as Rev and hnRNP A1 (D'Agostino et al., 1992; Monette et al., 2009), loaded onto the viral mRNA within the nuclear environment would destine viral RNA towards translation. These and other RNA-binding proteins of nuclear origin might be part of an IRES RNP-specific signal that is further modified by interaction with cytoplasmic proteins prior to associating with the translation apparatus (Cochrane, 2009; Semler and Waterman, 2008). Should this possibility be true it would imply that like cellular IRESes (Lewis and Holcik, 2008; Semler and Waterman, 2008; Stoneley et al., 2000), the HIV-1 5′-UTR IRES needs a nuclear experience, for the assembly of a translation specific RNP complex in order to be fully active.

2.3. HIV-2

The 5′-UTR of the HIV-2 genomic RNA shares with HIV-1 many of the functional domains required for viral replication such as TAR, Poly(A), PBS, SL1 (for Stem Loop 1 equivalent to DIS), SD and Ψ (Fig. 2B). However, the HIV-2 5′-UTR is significantly longer (544 nt instead of 335 nt) and much of this difference can be attributed to the structure of the TAR domain which is constituted of three branched hairpins of which the RNA stability is also stronger (Fig. 2B, $\Delta G = -60.7$ kcal/mol; (Soto-Rifo et al., 2012a). The HIV-2 packaging signal is composed of three hairpins located to both sides of the SD domain (Griffin et al., 2001; McCann and Lever, 1997) and implicated in RNA dimerization with the SL1 signal (L'Hernault et al., 2007). These structural modifications tend to render the HIV-2 5′-UTR even more structured than that of HIV-1 which could be a potential impediment to ribosomal scanning (compare Fig. 2A with 2B). In addition, bioinformatic determination of the RNA structure revealed that the HIV-2 5′-UTR can fold in different RNA domains in which the AUG codon can be occluded between a C-box (pyrimidine rich) and a G-box (located at the AUG codon); such an interaction was shown to affect RNA dimerization (Lanchy et al., 2003a, 2003b) and could exert an influence on the translation rate (Strong et al., 2011). In agreement with these data, expression mediated by the HIV-2 5′-UTR was found to be very low in comparison with that from the HIV-1 5′-UTR. Soto-Rifo et al. (2012a) placed the 5′-UTR of both types of HIV upstream a reporter gene coding for a luciferase and measured the luciferase activity in both transfected HeLa and T cells. Translation mediated by the HIV-1 5′-UTR showed to be more efficient than the one driven by the HIV-2 5′-UTR. Interestingly, deletion of the TAR domain from the 5′-UTR of HIV-2 alleviated the translational inhibition indicating that the TAR motif probably inhibits ribosome attachment at the 5′ end and/or scanning of the RNA (Soto-Rifo et al., 2012a,b). The presence of a very stable TAR structure at the 5′ end of the HIV-2 genomic RNA suggests that IRES elements could be present in the genomic RNA to allow viral protein expression. However, in contrast with HIV-1, no IRES activity has been yet demonstrated within the 5′-UTR. Instead, HIV-2 seems to use an alternative strategy to overcome the translational blockage imposed by the TAR structure. For this, it uses a splice donor site located at position 60 (within TAR)(Chatterjee et al., 1993) and splicing results in the removal of a 140 nucleotide-long RNA segment that partially includes TAR, the entire poly(A) signal and the C-box region (Strong et al., 2009) and could modify the translation efficiency of Gag. Indeed, a comparison of expression between the unspliced and spliced mRNAs was performed both in RRL and in transfected cells and showed that the alternatively spliced HIV-2 RNA translates at higher efficiency than the unspliced one (Soto-Rifo et al., 2012a; Strong et al., 2009).

As highlighted for HIV-1, the HIV-2 gag coding region spanning from nucleotide 556 to 899 (in Rod10) harbors several distinct IRES elements which allow expression of two shorter N-truncated Gag isofoms of p57 Gag that are namely p50 and p44 (Fig. 2B); these IRESes are also at use to express the full length Gag p57 from the upstream AUG codon as it was shown for HIV-1 (Herbreteau et al., 2005; Ricci et al., 2008a). In fact, delineation of the boundaries of the IRESes contained in the HIV-2 coding region revealed the presence of three distinct and independent IRESes able to initiate at 3 distinct AUG codons to produce p57, p50 and p44 (Ricci et al., 2008a). Such a peculiar mechanism of expression was confirmed by Weill et al. (2010) who further showed that three independent PICs can simultaneously assemble and can co-exist on the same mRNA molecule. These IRESes in the HIV-2 gag region can recruit and load ribosomes on the 3 AUG sites in a way completely independent from any initiation events that would take place on the viral 5′-UTR. As such, a synthetic mRNA, in which the entire viral 5′-UTR has been deleted, was shown to translate at high efficiency from the 3 AUG sites to produce full length Gag together with p50 and p44 (Herbreteau et al., 2005; Locker et al., 2011; Weill et al., 2010). The role of these shorter Gag isoforms in HIV-2 viral replication has not been further investigated but their presence in HIV-1, 2 and SIV suggest a conserved, yet unidentified, function for these proteins in viral replication. The ratio of production of the HIV-2 Gag isoforms is also modulated by the cleavage of eIF4G by the HIV-2 protease and/or by addition of the Gag protein in an *in vitro* system (Ricci et al., 2008a) and by the interaction between the C- and G-boxes (Strong et al., 2011). On the leaderless mRNA, the core domain of HIV-2 IRES composed of the P2 to the P5 domains has the ability to directly bind to both the naked 40S ribosome and eIF3 (Fig. 2B) (Locker et al., 2011). This mode of ribosomal attachment is reminiscent to the Hepaci- and Pestivirus-IRES (Pestova et al., 1998b). Redistribution of the PIC from this initial binding site to the three initiation codon could be regulated by the availability of eIFs or by RNA remodeling or both as the function of the DEAD-box helicase

eIF4A is required for expression of all Gag isoforms (Locker et al., 2011) suggesting that RNA unwinding is needed.

Finally, although the presence of IRES elements is conserved between both types of HIV, it is not sure that they operate in a similar way and that they require the same factors (either eIFs or ITAFs).

3. Discussion

There is no doubt that translation from the genomic RNA is essential during the HIV replication cycle as this viral transcript codes for the major structural protein (Gag) and the viral enzymes (Pol). HIV infection is associated with important modifications of the cell homeostasis that can have dramatic impact on global cellular translation. In consequence, the virus has adapted strategies to ensure continue and robust viral protein production under these conditions. As such, it has evolved to utilize both a cap-dependent and several IRESes that appeared to be regulated throughout viral replication. Thus, expression of the HIV-1 genomic RNA is mediated both by a cap-dependent and two IRES elements in the 5′-UTR and the coding region (Berkhout et al., 2011; Brasey et al., 2003; Buck et al., 2001; Gendron et al., 2011; Ricci et al., 2008b). On the HIV-2 unspliced RNA, ribosomes are driven from the 5′ end by a cap-dependent mechanism and several IRESes located exclusively in the coding region. It is important to note that the overall efficiency of translation from the HIV-2 genomic RNA is significantly weaker than that of HIV-1 (Soto-Rifo et al., 2012a). For both HIV-1 and HIV-2, the gag coding region plays an important role in translational control as it allows expression of N-truncated isoforms of Gag (p40 for HIV-1 and p50 and p44 for HIV-2) and their expression is not sensitive to the destabilization of cap-dependent initiation for both HIV types (Buck et al., 2001; Ricci et al., 2008a,b) which is consistent with the use of IRES mediated initiation from this region.

On the other hand, our lab has also shown that HIV-1 gRNA translation can occur very efficiently by a cap-dependent mechanism in the cap- and poly (A)-dependent untreated RRL *in vitro* system (Ricci et al., 2008b). Cap-dependent ribosomal scanning on the highly structured 5′-UTR was recently confirmed in cultured cells by the Berkhout's lab (Berkhout et al., 2011). Consistent with this, we have also shown that despite the presence of secondary structures, translation driven by the HIV-1 5′-UTR was very efficient and comparable to that of short and simple 5′-UTRs derived from cellular transcripts (Soto-Rifo et al., 2012a). Here, eIF4A and most probably RNA helicase A (RHA) contributes to resolve structural constraints during ribosomal scanning (Bolinger et al., 2010; de Breyne et al., 2012). However, how the eIF4F complex recognizes the cap structure that is embedded within the TAR stem loop was a mysterious issue that was recently solved by our group. In fact, we identified that the DEAD-box RNA helicase DDX3 was required to specifically unwind TAR in a very early step of translation initiation that occurs prior ribosomal scanning takes place (Soto-Rifo et al., 2012b).

Thus, to summarize, the use of a dual cap-dependent and IRES-driven mechanism of translation initiation on HIV may be at use in the course of viral infection. The ability to translate the gRNA by a dual mechanism under the control of specific host factors might provide to HIV-1 a way to quickly respond to changes in the cellular environment ensuring proper levels of Gag synthesis during infection. Thus, while cap-dependent translation is probably responsible for most of the Gag production, the activation of the IRES-driven mechanism provide a way to compensate for reductions in cap-dependent translation under adverse conditions such as G2/M cell cycle arrest and oxydative stress that are induced during viral replication.

Future work should determine the molecular mechanism that allows such a switch in the course of viral infection.

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