Title: The different pathways of HIV genomic RNA translation

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Abbreviations used: eIF, eukaryotic initiation factor; CrPV, Cricket Paralysis Virus; FIV, feline Immunodeficiency Virus; FMDV, Foot and Mouth Disease Virus; gRNA, genomic RNA; HIV, Human Immunodeficiency Virus; HCV, Hepatitis C Virus; IRES, Internal Ribosome Entry Site; ITAF, IRES *trans*-acting factor; ORF, Open Reading Frame; PABP, poly(A) binding protein; PV, poliovirus; RhPV, *Rhopalosiphum padi* virus; SIV_{MAC}, Macaque Simian Immunodeficiency Virus; UTR, untranslated region; 40S, eukaryotic small ribosomal subunit

Abstract

Lentiviruses, the prototype of which is HIV-1, can initiate translation either by the classical cap-dependent mechanism or by internal recruitment of the ribosome through RNA domains called IRESes (Internal Ribosome Entry Sites). Depending on the virus considered, the mechanism of IRES-dependent translation widely differs. It can occur by direct binding of the 40S subunit to the mRNA, necessitate a subset or most of the canonical initiation factors and/or ITAF (IRES *trans*-acting factors). Nonetheless, a common feature of IRESes is that ribosomal recruitment relies, at least in part, on IRESes structural determinants. Lentiviral genomic RNAs present an additional level of complexity as in addition to the 5'-UTR IRES, the presence of a new type of IRES, embedded within Gag coding region was recently described. This IRES, conserved in all three lentiviruses examined, presents conserved structural motifs that are crucial for its activity thus reinforcing the link between RNA structure and function. However, there are still important gaps in our understanding of the molecular mechanism underlying IRES-dependent translation initiation of HIV among which, the determination of the initiation factors required, the dynamic of initiation complex assembly as well as the dynamic of the RNA structure during initiation complex formation. Finally, the ability of HIV gRNA to initiate translation through different pathways questions the possible mechanisms of regulation and their correlation to the viral paradigm, i.e, translation versus encapsidation of its genomic RNA.

Introduction

Lentiviruses are complex retroviruses, the replication of which involves retro-transcription of their genomic RNA (gRNA) into a proviral intermediate that is permanently integrated into the host-cell genome. Transcribed by the host RNA polymerase II, the genomic RNA is capped and polyadenylated and can either serve as messenger RNA for the production of the retroviral proteins or as a propagated genome to be encapsidated into newly formed virions. Depending on the virus considered, the dual roles of the Gag genomic RNA are mutually exclusive or temporally regulated. While HIV-1 gRNA can fulfill both functions interchangeably and independently [1], HIV-2 gRNA translation is a prerequisite for genome encapsidation [2]. The transition between translation and encapsidation is critical to the virus cycle and partly relies on the presence of structural elements in the 5'-untranslated region (5'-UTR). Indeed, the 5'-UTR is long and contains many conserved sequences and structures that are critical to the coordination of the different phases of the viral cycle. The presence of these structural signals sets the 5'-UTR as a predictable site for regulation of the translation-encapsidation transition; but also clearly interferes with translation and affects the production of the Gag polyprotein by impeding ribosomal scanning from the cap [3-5]. While a cap-dependent translation initiation has been demonstrated *ex vivo* and *in vitro* for HIV-1 [6, 7] and FIV [8], Gag translation can also rely on Internal Ribosomal Entry Sites (IRES) present in the 5'-UTR of HIV-1 [9] and SIV [10] or within Gag Open Reading frame (ORF) of HIV-1, HIV-2 and SIV_{MAC} [11, 12] (Figure 1). It thus appears that lentiviral RNAs have the possibility to switch from a cap-dependent to a cap-independent mechanism of translation initiation, depending on the host cell environment modifications that follow infection [13].

Cap-dependent translation initiation

Cap-dependent translation relies on the recognition of the cap structure at the 5'-UTR to recruit the 43S complex. This is mediated by the eIF4F complex that allows cap recognition through eIF4E, unwinding of mRNA secondary structures by the eIF4A helicase and contains the eIF4G platform which coordinates 40S and mRNA association through its connection with both eIF4E and eIF3 [14]. Cap-dependent translation of HIV-1 gRNA has been demonstrated *ex vivo* [4] and more recently confirmed *in vitro* [6]. Competition experiments were performed in a synergistic competitive reticulocyte lysate using either

high amounts of free cap analogue that competes with capped mRNA for the recruitment of eIF4F or by addition of the L protease (FMDV) that cleaves eIF4G and specifically impairs cap-dependent translation. The results indicate that the synthesis of the full length Gag polyprotein of HIV-1 relies mostly on a cap-dependent mechanism in this system; contrary to HIV-2 and SIV_{Mac} which rely exclusively on IRES mediated translation [6, 13].

IRES mediated translation initiation

IRES-dependent translation initiation has been well documented in recent years and allows a direct recruitment of the initiation complex near or at the AUG codon, without requiring a 5'-cap. This mechanism relies on RNA motifs and structures called IRESes, generally present in the 5'-UTR of a given mRNA, which drive internal ribosome recruitment [15]. Although numerous IRESes have been identified in both viral and cellular mRNAs, they differ from one another, leading to the hypothesis that many different mechanisms of ribosome internal entry exist. Indeed, IRES-dependent initiation can occur by direct binding of the 40S subunit to the mRNA, or may be mediated by a subset of the canonical initiation factors, and in some cases necessitate specific IRES *trans*-acting factors (ITAFs [16]).

IRESes in the 5'-UTR have been described for HIV-1, SIV_{MAC} and FIV and their activity has been shown to be modulated by the host cell environment such as the arrest of cells in G2/M (in the case of HIV-1) or the stress caused by the infection [8, 9]. Secondary structures models for HIV-1 and FIV 5'UTRs have been established [17-21], however, the RNA determinants, the proteins and the mechanisms involved in the process leading to the initiation remain unknown. While no IRES activity has been detected to date in HIV-2 gRNA 5'-UTR, we previously reported that the HIV-2 Gag coding region is endowed with an IRES activity. This new type of IRES, embedded within Gag coding region, has also been described for HIV-1 and SIV; and is responsible for the synthesis of alternative Gag isoforms [11, 12, 22, 23]. These shorter Gag isoforms are translated from in frame initiation codons located within the Gag coding region both *in vitro* and *ex vivo*. In addition to Gag p57, HIV-1 produces a p40 isoform while HIV-2 produces additional p50 and p44 Gag isoforms. The function of these isoforms is not elucidated but their deletion affects HIV-1 replication [22] and the shorter isoforms of HIV-2 have been shown to be incorporated within the immature virion [11] arguing for their possible physiological relevance. Interestingly, Gag shorter isoforms

translation appears to be exclusively the results of ribosomal entry from IRESes located within the coding region [11, 12, 23]. Furthermore, we recently described that up to three initiation complexes could be assembled on a single HIV-2 Gag RNA molecule, most probably on each of the initiation codons. This property of the Gag IRES is surprising considering that for the well documented cases of Hepatitis C Virus (HCV) and Cricket Paralysis Virus (CrPV) IRESes most of the surface of the three dimensional structure of the RNA is covered by the ribosome, rendering sterically impossible the recruitment of a second initiation complex [16, 24, 25]. This peculiarity clearly distinguishes Gag HIV IRES from other described IRESes, questioning the mode of initiation complex recruitment and the nature of the determinants involved.

Defining the IRES determinants

A peculiar characteristic of viral IRESes is the absence of a consensus or motif in their primary sequence which precludes any *in silico* identification [26, 27]. Nevertheless, viral IRES elements often share several common structural characteristics such as long and structured 5'-UTR and several AUG triplets located before the authentic start codon. The *Dicistroviridae* Intergenic Region IRESes (class I) fold into a highly compact structure that is sufficient to bind the 40S and initiate translation without the help of any other factors (for a review see [28]). The HCV IRES is the archetype of class II IRESes that can directly bind the 40S subunit as well as eIF3 and requires eIF2 and eIF5 to initiate translation [29]. Class III and IV IRESes originate mostly from picornaviruses (such as Foot and Mouth Disease Virus and Poliovirus, respectively). These IRESes do not bind directly to the 40S subunit but rather it is eIF4G binding to the IRES that provides a scaffold for the 40S subunit recruitment. In addition, all eIFs but eIF4E and often ITAFs are necessary to initiate translation [30, 31]. Concerning HIV Gag IRES, initiation complex determination as well as direct interaction evaluation indicate that this IRES does not fall into any of these classes. Indeed, it displays properties of class II IRES (direct and independent binding to the 40S and eIF3) as well as properties of class III and IV by necessitating all eIFs but 4E to initiate translation (Locker et al, in preparation). Of note, other viral IRESes do not belong to any of the defined classes, as for example the *Rhopalosiphum padi* virus IRES which binds simultaneously the 40S and eIF3 and is mostly devoid of RNA structure [32].

Concerning the definition of the Gag IRES determinants, initial work showed that the HIV-2 Gag isoforms are produced by an IRES element that lies downstream from the authentic AUG (AUG₁) initiation site and spans to the third AUG (AUG₃). Interestingly, this RNA sequence allows the delivery of an initiation complex upstream from its core sequence to produce the Gag p57 polyprotein from AUG₁. Further work performed by Ricci and colleagues [33] indicated that not only a sequence downstream of AUG₁ but also sequences downstream of AUG₂ were endowed with an IRES activity leading to the hypothesis that two distinct IRES activities were relying on independent sequences. In order to evaluate the role of structural determinants in Gag IRES activity, we elaborated secondary structure models for HIV-1, HIV-2 and SIV_{MAC} Gag open reading frames. To this end, we used several chemical and enzymatic probes, and reveal that the Gag IRES of the three species display conserved stable structural motifs (P2, P3, P4 and P5, see Figure 2). Furthermore, HIV-2 Gag IRES mutations that destabilize a conserved structural element (P2), but not compensatory mutations that restore the base pairing, severely inhibit IRES mediated translation of all three Gag isoforms [12]. These results led to the attractive hypothesis that recruitment on the three AUG relies on common determinants. We cannot exclude at this stage that these structures are not the primary determinants for ribosome recruitment but instead allow the maintenance of the global architecture of the IRES. Further work is necessary to discriminate between these hypotheses among which the systematic determination of the mutants structure.

Regulation of translation initiation

We previously showed that HIV-1, HIV-2 and SIV_{MAC} Gag gRNA are more efficiently translated as leaderless RNAs indicating that the 5'-UTR exerts an inhibitory effect on Gag translation [11, 12]. Although Gag leaderless RNA was not described *in vivo*, one can envisage that the observed phenomenon reflects an *in vivo* regulation by cellular proteins that are absent from our *in vitro* assay. Furthermore, the conservation of an IRES activity in Gag ORF that relies on conserved structural determinants and that is regulated by the 5'-UTR argue in favor of a physiological role for this IRES. It is thus tempting to speculate that the 5'-UTR directly or indirectly interferes with the active folding of the IRES. Interestingly, a minor splicing event in HIV-2 stimulates Gag translation [34]. This could reflect relieving of cap-dependent inhibition by Tar, or else activation of an IRES within the 5'-UTR or Gag coding

region induced by a structural rearrangement upon splicing. It is worth noting that translation occurs in the cytoplasm of host infected cells and is often accompanied by modification of the physiological conditions and/or the production of viral proteins. It has been shown that the Gag polyprotein can associate with the 5'-UTR of its cognate messenger RNA forming a gRNA-Gag complex that creates a platform for multimerization of additional Gag molecules [35]. Building such a complex could progressively block ribosomal scanning which ultimately would result in translation extinction and in the selection of the genomic RNA for encapsidation and viral particle assembly [33, 36]. In addition, the viral protease can modulate the host-cell translation machinery by targeting eIF4G and impeding eIF4E recognition or PABP (Poly(A) binding protein) interaction. Cleavage of eIF4G results in a host-cell deficient in cap-dependent translation thus favoring IRES-dependent translation of viral RNAs [37, 38]. The cross-talk between viral RNAs and cellular proteins can also be exemplified by the IRES element within HIV-1 5'-UTR that is only active in G2/M phase of the host cell [9] although the identity of the proteins involved is to be determined. A host protein, RNA helicase A has been shown to stimulate Gag translation [39], however, its mode of action and the pathway on which it may act remain unclear. In this context, deciphering the interactions between Gag ORF and its cognate 5'-UTR as well as exploring protein/RNA interactions appear as an important step towards our understanding of the mechanism(s) underlying translation of the different Gag isoforms and how it is regulated; but could also shed light on their role during the viral cycle.

Concluding remarks

Lentiviral RNAs rely on several mechanisms for translation initiation (Cap-dependent, 5'-UTR IRES and IRES in the coding region, Figure 1) that need to be coordinated to the lentiviruses paradigm, i.e. translation versus encapsidation of their genomic RNA; and to take into account the modifications of the host cell environment following infection. Furthermore, the same viral molecule can be the site of numerous interactions along the viral cycle. Importantly the specificity and the coordination of these interactions rely, at least in part, on the dynamic of the viral RNA architecture. Translation in lentiviruses is thus an extremely dynamic system, tightly regulated and our understanding of the exact mechanisms involved is at its early steps. In a broad perspective, each new insight into this

non-conventional mechanism of translation offers a prospective target for drug development / chemotherapy.

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Legends to the figures

Figure 1: Translational control of HIV genomic RNA. The figure schematically represents HIV-1 gRNA and depicts the different modes of translation initiation as well as regulation of IRESes activity. The 5'-UTR contains numerous *cis*-acting elements such as the TAR stem loop, the poly(A) loop, the primer binding site (PBS), the dimerization initiation site (DIS), the major splice donor (SD) and the core packaging signals (Ψ). The 5'-cap structure is indicated by a closed circle, the structural elements of Gag IRES conserved in primate lentiviruses are boxed and shaded in grey and initiation codons are indicated .

Figure 2: Conserved structural elements of the Gag IRES present in HIV-1, HIV-2 and SIV_{MAC}. ORFs. This figure is a schematic representation adapted from [12]. Structural models of Gag ORF of HIV-2 (from AUG₄₄₅₋₄₄₇ to G₉₆₅), HIV-1 (from AUG₃₃₆₋₃₃₈ to G₇₈₈) and SIV_{MAC} (from AUG₅₃₇₋₅₃₉ to U₉₅₂) are depicted in the figure. Pairings are designated by Pn in order of appearance from 5' to 3'. The conserved P2, P3, P4 and P5 structural elements are boxed and shaded in grey. In frame AUG codons leading to the different Gag isoforms are indicated and numbered.

5'-UTR Inhibition