

Translational control of retroviruses

Laurent Balvay^{*‡}, Marcelo Lopez Lastra[§], Bruno Sargueil^{||}, Jean-Luc Darlix^{*‡} and Théophile Ohlmann^{*‡}

Abstract | All replication-competent retroviruses contain three main reading frames, *gag*, *pol* and *env*, which are used for the synthesis of structural proteins, enzymes and envelope proteins respectively. Complex retroviruses, such as lentiviruses, also code for regulatory and accessory proteins that have essential roles in viral replication. The concerted expression of these genes ensures the efficient polypeptide production required for the assembly and release of new infectious progeny virions. Retroviral protein synthesis takes place in the cytoplasm and depends exclusively on the translational machinery of the host infected cell. Therefore, not surprisingly, retroviruses have developed RNA structures and strategies to promote robust and efficient expression of viral proteins in a competitive cellular environment.

Retroviruses are a unique family of RNA viruses that use virally encoded reverse transcriptase (RT) to replicate genomic RNA through a full-length viral DNA intermediate. These viruses have been shown to be present in the genomes of many vertebrates, including fish, rodents, birds, cats, ungulates, non-human primates and humans. Infection by retroviruses causes a wide variety of pathologies, most commonly cancers, such as leukaemias, sarcomas and mammary carcinomas, but retroviral infection can also cause immunodeficiencies, anaemias, arthritis and pneumonia¹. The retroviridae are classified into seven different genera that are named α through to ε retroviruses, as well as the lentiviruses and spumaviruses (see the [International Committee on Taxonomy of Viruses](#) database). Historically, retroviruses have been the source of many key discoveries in biology during the twentieth century, including cell transformation, viral and cellular oncogenes, RT and viral transduction, which paved the way to cDNA cloning and design of retroviral vectors for gene therapy¹.

This Review will focus on the mechanisms used by retroviruses to ensure the correct viral protein synthesis within the cytoplasm of the host infected cell with particular emphasis on α - and γ -retroviruses (whose prototypes are the avian leukosis virus (ALV) and murine leukaemia virus (MLV), respectively) and primate lentiviruses (HIV-1, HIV-2 and simian immunodeficiency virus (SIV)).

An overview of the retroviral life cycle. Retroviruses are enveloped RNA viruses that encapsidate two copies of the same capped and polyadenylated (positive sense) RNA molecule that ranges from 8,000 to 11,000

nucleotides in size. In the viral particle, the two RNA copies are linked by their 5'-end to form a dimeric RNA structure, and are coated by 1,500–2,000 molecules of nucleocapsid protein². These viruses are characterized by their unique property to reverse transcribe their RNA genome into double-stranded (ds) DNA and to integrate this dsDNA into the nucleus of the host cell (to become the proviral DNA)³. All retroviruses have a similar genetic organization consisting of the *gag*, *pol* and *env* genes, which are translated as polyprotein precursors and are later processed to yield internal structural proteins, enzymes and the envelope glycoproteins, respectively. In addition, lentiviruses code for regulatory genes that are required, among other functions, to coordinate viral gene expression at different levels, such as transcription, nuclear export and translational regulation.

The retroviral life cycle begins with the interaction of the infectious viral particle with the appropriate membrane receptors and co-receptors (FIG. 1a). This triggers membrane fusion and nucleocapsid entry into the cytoplasm of the cell (FIG. 1b). At this stage, the genomic RNA is reverse-transcribed into dsDNA by the virally encoded RT enzyme. Full-length viral DNA, in the form of a preintegration complex, is then imported to the nucleus where it integrates into the host chromosome (FIG. 1c). The viral long terminal repeats (LTRs), flanking the provirus, allow the synthesis of the viral genomic RNA by the cellular RNA polymerase II. As it is being synthesized, the primary transcription product interacts with the cellular RNA processing machinery and, similar to a typical cellular pre-mRNA, is spliced, exported to the cytoplasm, and translated by the host protein-synthesis machinery (FIG. 1d). A proportion of the pre-mRNA

^{*}Ecole Normale Supérieure de Lyon, Unité de Virologie Humaine, IFR 128, Lyon, F-69364 France. [†]Inserm, U758, Lyon, F-69364 France. [§]Laboratorio de Virología Molecular, Facultad de Medicina, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago, Chile. ^{||}Centre de Génétique, Moléculaire, CNRS UPR 2167, Avenue de la terrasse, 91190 Gif sur Yvette, France. Correspondence to T.O. e-mail: tohlmann@ens-lyon.fr doi:10.1038/nrmicro1599

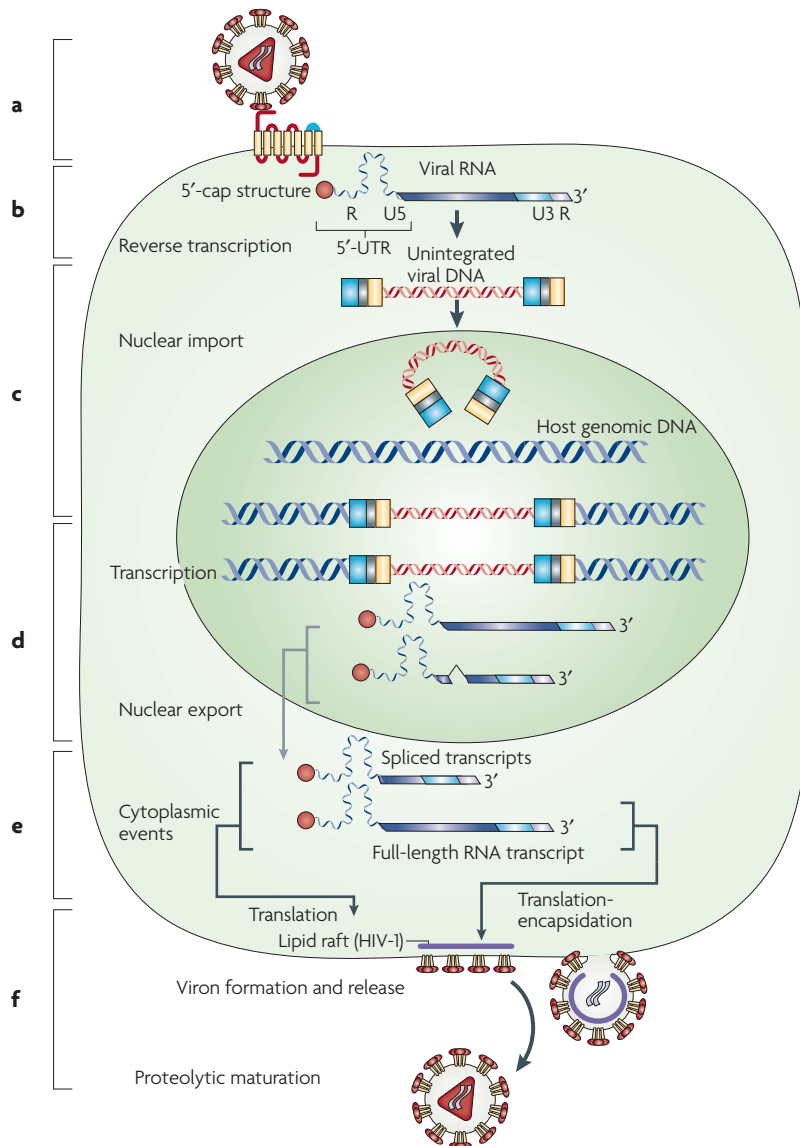


Figure 1 | An overview of the retroviral life cycle. The life cycle of retroviruses can be broadly divided into six essential steps, which are shown schematically in the figure: interaction, adsorption and entry into the cell (**a**); synthesis of reverse transcribed DNA (**b**); integration (**c**); transcription, splicing and nuclear export (**d**); translation and encapsidation (**e**); and viral assembly and budding (**f**).

5'-cap structure

This is a methylated guanosine (methylated at position 7) that is added as the first nucleotide of all RNA pol II transcripts by a 5'-5' phosphodiester bond. In addition to its role in translation initiation, the presence of the cap structure protects the mRNA from exonucleases and has a role in splicing and mRNA export.

subverts typical RNA processing and interacts with viral and/or cellular nucleocytoplasmic shuttle proteins that activate nuclear export, despite the lack of intron removal⁴. In the cytoplasm, translation of the spliced, and incompletely-spliced, viral mRNAs depends exclusively on the host-cell translational machinery⁵ (FIG. 1e). The unspliced, genome-length RNA serves yet another function, as it is packaged into assembling virions as genomic RNA. In this respect, some simple retroviruses, such as MLV, contain two populations of unspliced viral RNAs that segregate either as mRNA for translation or as genomic RNA for encapsidation^{6,7}. Complex retroviruses, however, present a single RNA population that functions interchangeably as both mRNA and genomic RNA^{8,9}. After synthesis, the viral structural proteins are targeted

to the plasma membrane where assembly takes place. For HIV-1 this process is associated with cholesterol-rich membrane microdomains known as rafts¹⁰⁻¹³ (FIG. 1f). However, with some retroviruses, such as mouse mammary tumour virus (MMTV), Mason-Pfizer monkey virus (M-PMV) and spumaviruses, Gag-containing polyproteins assemble within the cytoplasm, forming stable particles that are then transported to the plasma membrane where the envelope is acquired during the budding process⁵. Interestingly, certain defective endogenous retroviruses bud exclusively from the endoplasmic reticulum (ER)⁵.

Genetic organization of the retroviral genomic RNA. The proviral DNA consists of three main open reading frames (ORFs) flanked by two LTRs that are involved in the integration and transcription processes^{2,3}. Transcription of proviral DNA generates genomic full length retroviral mRNA that functions as genomic and messenger RNA, and possesses well-defined functional domains, including the R region, which corresponds to the +1 site of transcription and the site of capping, the 5'-untranslated region (5'-UTR) or leader, the viral ORFs, the 3'-UTR and the 3'-poly (A) tail. The 5'-UTR of the genomic RNA contains *cis*-acting RNA structures that are crucially involved in various aspects of the viral life cycle¹⁴⁻¹⁸ (FIG. 2). These structures include: first, the transactivation response element (TAR; only found in lentiviruses), which transactivates viral transcription and modulates viral mRNA translation (see below); second, the poly adenylation stem loop, the structure responsible for the addition of the 3'-poly (A) tail; third, the primer binding site (PBS), which is complementary to the 3' portion of the cellular tRNA^{lys3} and functions as the recruitment point for the initiation of reverse transcription; fourth, the major splice donor (SD), which is the most upstream splice site used in the generation of viral subgenomic RNAs; fifth, the dimerization initiation site (DIS), which is involved in the establishment of the kissing-loop complex between two genomic RNA molecules²; and finally, the core packaging signals, necessary elements required for the selection and direction of the genomic RNA to the viral assembly machinery.

General mechanism of retroviral protein synthesis. Similar to all cellular mRNAs, retroviral mRNA is synthesized by RNA polymerase II, therefore it exhibits all the characteristics associated with eukaryotic mRNA, including a 5'-cap structure, a 5'-UTR, a 3'-UTR and a 3'-poly (A) tail⁴. Following nuclear export, structural proteins and enzymes are synthesized from the unspliced mRNA (Gag and Gag-Pol) on free ribosomes, whereas the precursor envelope protein is expressed from spliced viral mRNA (simple retroviruses) or incompletely spliced viral mRNA (complex retroviruses) on membrane-bound ribosomes⁵.

The overall expression of Gag and Pol proteins is regulated by a subtle mechanism. In all retroviruses, the *gag* gene is the first reading frame encoded on the genome and is located upstream of *pol*; as such, ribosomes synthesize the Gag protein until they encounter a stop codon. At this point, the strategy differs

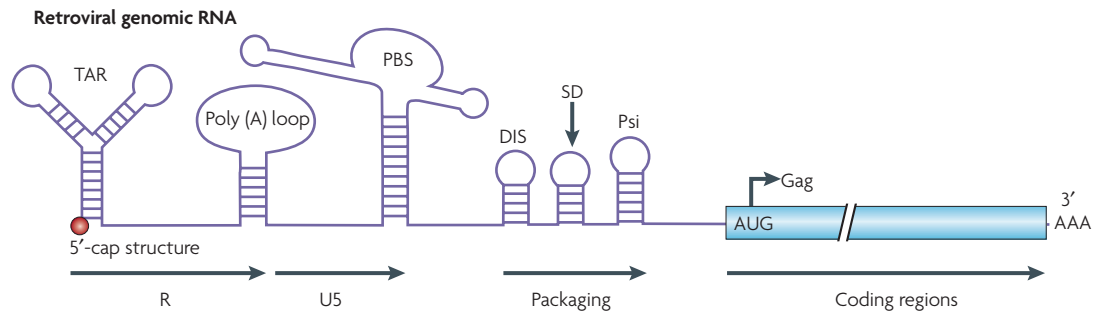


Figure 2 | Genetic organization of the 5'-untranslated region (UTR) of the retroviral genomic RNA: the example of SIVmac. The 5'-UTR (or leader) of the simian immunodeficiency virus (SIV) genomic RNA contains numerous *cis*-acting sequences, such as the TAR stem-loop, the poly(A) loop, the primer-binding site (PBS), the dimerization initiation site (DIS), the core packaging signals (Psi) and the major splice donor (SD). The R-U5, packaging and coding regions are indicated in the figure.

among different members of the retrovirus family. In γ -retroviruses, the stop codon is misread and there is a termination suppression process that results in the translation of the downstream *pol* gene. This mechanism occurs in Moloney MLV (MoMLV) by the suppression of an amber termination codon (UAG), occasionally decoded by a glutamine tRNA^{19,20}. The second mechanism, used by most retroviruses, is a frameshift mechanism whereby the ribosomes slip backward one nucleotide just before they encounter the stop codon of the *gag* gene^{21,22}. This mechanism is dependent on two specific features characteristic of the viral mRNA: a heptanucleotide 'slippery' sequence²¹ located upstream of an RNA pseudoknot²³. Recently, the molecular mechanism underlying '-1 frameshifting' was elegantly solved by cryoelectron microscopic imaging of ribosomes engaged in the coronavirus RNA pseudoknot. This analysis revealed that the 80S ribosome interacts with the RNA pseudoknot creating a physical block at the mRNA entrance channel. This results in the deformation of the P-site transfer RNA and this structural change impedes ribosomal translocation²⁴. As a result, ribosomes leave the *gag* reading frame and translate the *pol* gene. In HIV-1, the efficiency of such a mechanism is comparable to that of termination suppression and yields approximately 20 Gag proteins for each Gag-Pol molecule synthesized. Disruption of this ratio in HIV-1 reduces the infectivity of the virus by 250- to 1,000-fold, showing the importance of this evolutionary-conserved mechanism²⁵.

The role of viral RNA structures on translation

IRES elements and simple retroviruses. MLV has been extensively studied and is considered the prototype for mammalian γ retroviruses. Although MLV is classified as a simple retrovirus, it encodes an additional longer glycosylated isoform of the Gag polyprotein (GCSA or glycoGag). GCSA is a membrane-bound protein that undergoes processing but is not incorporated into virions. Instead, the protein is exposed on the extra-cellular side of the plasma membrane, where it displays potential mitogenic activity^{26,27}. In the case of the Friend MLV (FMLV), GCSA is not necessary for virus replication in cell culture but is required for virus pathogenesis, that is, haemolytic anaemia and erythroleukaemia in mice^{27,28}.

The synthesis of GCSA displays some unique characteristics as translation is initiated at two CUG codons, 300 nucleotides upstream from the Gag AUG and in the same reading frame²⁶. Both Gag and GCSA are produced by two distinct internal ribosome entry segments (IRES) elements^{29,30} (BOX 1) that overlap the MLV and FMLV packaging signal³¹, indicating that the translation of the full-length mRNA and its recruitment as genomic RNA into newly formed virions could be functionally linked. Though possible, several reports suggest that simple retroviruses generate two independent pools of RNA, each predestined to a different fate: translation or encapsidation^{6,7}. Interestingly, IRES-mediated translation is not restricted to full-length mRNA in MLV, as a mechanism of internal initiation is also used for the production of the Env glycoprotein³².

Sequence analysis indicates that IRES elements are highly conserved among the murine Oncoretroviruses as they also occur in viruses that are distantly related to MLV, including the defective leukaemia virus, Harvey murine sarcoma virus (HaMSV)³³, and in retro-transposons such as mouse VL30 (REF. 34). HaMSV is a highly pathogenic virus that arose from multiple genetic recombinations between MoMLV, the VL30 rat retro-element and the *c-Ras* oncogene. As a result, the *v-Ras* protein is expressed by a potent IRES element, which is derived from the 5'-UTR of the VL30 element. In a manner similar to the MLV 5'-IRES element, this element also overlaps the HaMSV RNA dimerization and packaging signals³³. Finally, it is noteworthy that a human MLV-like retrovirus (called XMRV) was recently shown to be associated with prostate cancers³⁵. Furthermore, sequence analyses indicates that it is highly likely that the synthesis of XMRV Gag and Gag-Pol is directed by an IRES element closely resembling that of FMLV³⁵.

IRES elements have also been found in avian retroviruses, including the reticuloendotheliosis virus type A (REV-A)³⁶. REV-A has the genetic organization of a simple retrovirus, similar to that of MLV, yet this virus does not produce a glycosylated Gag isoform, but still exhibits an IRES activity in its 5'-UTR³⁶. However, in contrast to MLV, FMLV and HaMSV, this minimal IRES activity does not overlap with the main determinants of RNA encapsidation³⁶.

RNA pseudoknot

An RNA motif formed by the pairing of a loop (apical or internal) with a sequence located outside of this loop. These common structures are formed in the presence of divalent ions and have peculiar functions.

Reticulocyte lysate system

This is the most efficient *in vitro* translation system which is derived from immature red cells. As such, the system is devoid of any nuclear components but contains all the complete protein-synthesis machinery required to translate exogenously added mRNAs.

Bicistronic vectors

A construct that expresses two reading frames (cistrons) in tandem on the same transcriptional unit. The first cistron is translated by a normal cap-dependent mechanism, whereas the second cistron can only be expressed if an IRES structure is inserted upstream of its AUG initiation site. However, some proper controls must be undertaken in order to verify the integrity of the bicistronic RNA throughout the experiment.

Ribosomal scanning

This term describes the linear progression of the eukaryotic ribosomal subunit along the 5'-UTR in a 5' to 3' direction from its initial binding to the mRNA and up to the recognition of the AUG initiation codon.

Box 1 | The IRES mechanism

Internal ribosome entry segment (IRES) elements have been characterized within the genomic RNA of several retroviruses. IRES elements are defined RNA segments that allow translation initiation by direct ribosome binding to the 5'-untranslated region (UTR) of the mRNA^{128–130}. Internal translation initiation was first demonstrated for the picornavirus family but has since emerged as a general mechanism for both viral and cellular RNAs^{131,132}. The huge diversity of size, sequence and structure that has been reported for the 56 viral and 73 cellular IRES elements characterized so far indicates a polyphyletic origin^{133,134}. Despite their differences, all IRES elements can be defined as an RNA domain that has the ability to recruit ribosomes in a cap-independent manner. This process occurs by either promoting direct contact between the mRNA and the small subunit of the ribosome, as is the case for the classical swine fever virus, hepatitis C virus and cricket paralysis virus¹³⁵ or, more commonly, by the establishment of an RNA–protein–ribosome interaction through a set of initiation factors and/or by the use of specific IRES trans-acting factors¹²⁹. Following their binding to the IRES sequence, the ribosomes can initiate at their landing site, as is the case in type II IRES (prototype encephalomyocarditis virus), or they can scan the 5'-UTR in a 5' to 3' direction until they locate the AUG initiation codon as in type I IRES (prototype poliovirus). Despite the relatively large number of IRES sequences that have now been identified, a structural 'prototype' for the internal initiation mechanism is still not available, which suggests that there might be not one, but several, RNA motifs that enable ribosomal entry. In agreement with this idea, there is a broad range of unrelated genes that use an IRES-mediated mechanism of translation initiation. From a functional standpoint, internal initiation allows the translation process to bypass the control mechanisms that specifically regulate cap-dependent translation, especially those involving modification of the eIF4E/4E-BP complex¹³⁶. As a result, IRES-mediated translation occurs during many cellular stress conditions that are known to inhibit cap-dependent translation, including hypoxia^{137,138}, irradiation¹³⁹, apoptosis¹⁴⁰, angiogenesis¹⁴¹, amino-acid starvation¹⁴², continuous heat shock¹⁴³ and mitosis¹²⁷.

ALV is another example of an α -retrovirus that possesses an IRES element. The 5'-UTR of ALV is composed of 330 nucleotides and has many functions both in the early and later steps of virus replication. It is thought to fold into a stable secondary structure with four independent stem loops³⁷. Two of these structured regions encode small ORFs (uORFs) that have been shown to be crucial for Gag synthesis and virus replication^{38,39}. These two structured domains have also been shown to be part of an active IRES element directing the initiation of Gag synthesis⁴⁰.

Finally, it should be noted that two independent IRES elements have been found within the genomic RNA of the endogenous retrovirus *Gypsy* from *Drosophila melanogaster*⁴¹. The first element corresponds

to the 5'-UTR of the genomic RNA encoding *env* and is active both in the reticulocyte lysate system and in cultured cells. However, the second IRES can only drive translation with rabbit reticulocyte lysate and strongly represses translation in cells⁴¹.

Taken together, these data indicate that the presence of IRES elements is a mechanistic theme that is common to all simple retroviruses as they are found in avian, mammalian and insect viruses that are only distantly related to each other. This distribution across different species, together with the fact that they are often colocalized with viral packaging signals, indicates that IRES elements could have an important role in the regulation of viral replication.

IRES elements and lentiviruses. In primate lentiviruses, IRES elements have been located and characterized from in the 5'-UTR and in the coding region of the genomic RNA^{42–46}. Initially, the insertion of the 5'-UTR of HIV-1 into bicistronic vectors revealed that translation was extremely weak, both in the rabbit reticulocyte lysate and in transfected COS and HeLa cells, leading to the conclusion that this structure did not exhibit IRES element activity⁴⁷. In fact, their data show that the *cis*-acting replication elements that are present in the 5'-UTR structure are an important barrier for ribosomal scanning and strongly inhibit translation initiation⁴⁷.

This assertion was later revisited and it was shown that the 5'-UTR from the closely related SIV (Mac) was capable of internal translation initiation both *in vitro* and *in vivo*⁴⁶. Nevertheless, the bicistronic expression driven by the 5'-UTR of SIV was relatively weak when compared with encephalomyocarditis virus (EMCV) IRES-positive control. In addition, some cap-dependence was observed when monocistronic constructs were used in the study. Another study that focused on the 5'-UTR of HIV-1 did reveal the presence of a cell-cycle-regulated IRES element that is expressed preferentially during the

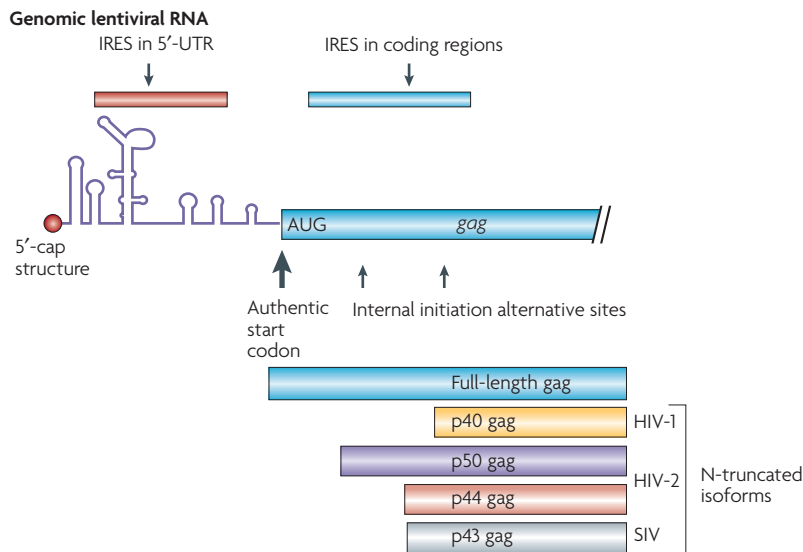


Figure 3 | IRESs in Lentiviruses. Schematic diagram of the position of the different IRESs within the lentiviral genomic RNAs. The N-truncated Gag isoforms that result from alternative translation initiation from the coding region are shown.

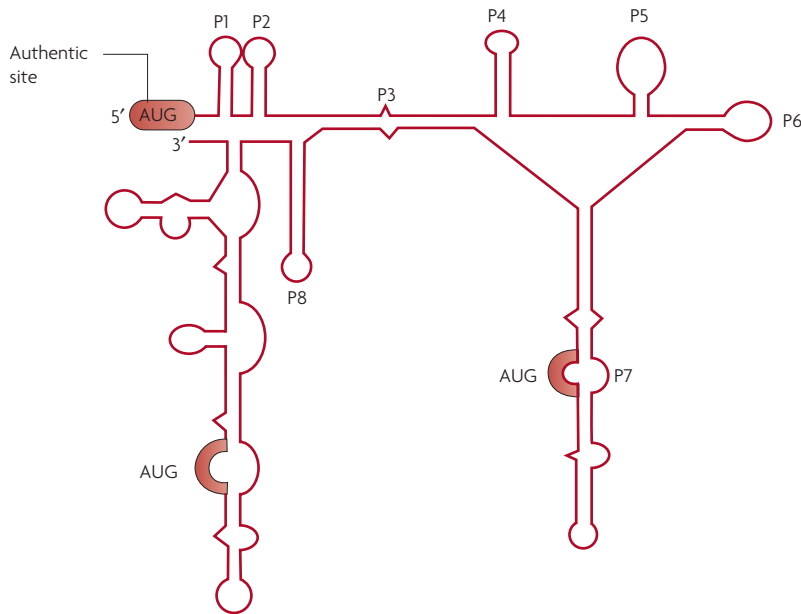


Figure 4 | Schematic representation of the secondary structure adopted by the first 450 nucleotides of the HIV-2 Gag coding region. Secondary structure model deduced from chemical and enzymatic probing data (adapted from REF. 44). Helices are numbered from P1 to P8, structures after P8 were not named and are not conserved in HIV-1 and simian immunodeficiency virus (SIV)mac. The P2 helix is also absent from HIV-1. The three initiation codons are highlighted in red.

G2/M phase, both in HeLa cells and in translational extracts derived from the same cells⁴³. These conflicting results indicate that lentiviruses might use alternative initiation strategies throughout the viral replication cycle. This could be explained by the existence of alternative RNA structures of the 5'-leader (see below) that would be favourable for either ribosomal scanning or IRES-dependent translation. Therefore, the physiological status of the cell, and/or the expression of viral proteins, would favour one or the other alternative conformations. This would, in turn, determine whether ribosomal binding occurs at the 5'-end of the genomic RNA or at an internal position within the untranslated region of the RNA. Moreover, as pointed out recently by Yilmaz *et al.*, these inconsistencies could also reflect a disadvantage of the bicistronic reporter system, which does not fully recapitulate the RNA structures that can be found in the genomic mRNA⁴⁸. In agreement with this possibility, it should also be noted that the assessment of the role of lentiviral internal initiation has not yet been carried out in the context of a complete replicating provirus in CD4⁺ primary cells.

By using chemical and enzymatic probing techniques, it was recently shown that the 5'-UTR of HIV-1 can fold into two alternative conformations⁴⁹. The first conformation exposes the TAR, 3'-poly (A) tail, PBS, DIS and SD structures (as defined above), and one or several packaging signals (FIG. 2). This conformation, termed BMH (branched multiple hairpins), exists in equilibrium with another, more stable conformation, in which the 3'-poly (A) tail, DIS and SD structures and the packaging signals are paired with each other through a long distance interaction (LDI). The transition from the LDI to the BMH

conformation is promoted by the presence of magnesium ions, high RNA concentrations and, most relevant to the physiological situation, by the presence of the nucleocapsid, a lentiviral protein that exhibits RNA chaperone activity⁵⁰⁻⁵². Even though it is highly attractive to hypothesize that these changes in conformation are sufficient to explain the molecular switch that would determine the usage of genomic RNA as either mRNA for translation or as genomic RNA for the generation of new virions, new evidence indicates that this might not be the case⁵³.

Although the characterization of IRES elements in the 5'-UTR of primate lentiviruses has been controversial, there is greater consensus regarding the discovery of IRES elements in the Gag coding region. In fact, alternative N-terminal truncated isoforms of the Gag polypeptide precursor have been observed in cells infected with HIV-1, HIV-2 and SIV^{42,44,45}. Further investigation revealed that these isoforms are synthesized by IRES-mediated translation in which the element is located entirely within the gag coding region. Therefore, alternative translation initiation gives rise to one shorter isoform of Gag in the case of both HIV-1 and SIV (named p40 and p43, respectively), whereas two isoforms are produced from the HIV-2 coding region (named p50 and p44) (FIG. 3). The loss of these alternative Gag isoforms by mutagenesis of the AUG codons has a significant impact on viral infection, suggesting an important role for these proteins in pathogenicity^{42,45}. Interestingly, production of the truncated Gag isoforms of HIV-1, HIV-2 and SIV occurs exclusively by internal entry of the ribosomes to the Gag coding region^{42,44,45} (FIG. 3).

However, the most surprising and unexpected finding is that these IRES elements can also drive translation of the full-length polypeptide from the upstream authentic AUG codon^{42,44}. This is particularly interesting as it implies that ribosomes can be recruited by determinants downstream of the first initiation codon, and gives rise to the possibility that the genomic RNA lacking the whole 5'-UTR sequence upstream of the AUG codon could be efficiently translated. Indeed, this property leads to the provocative suggestion that ribosomes can, in some contexts, scan backwards for a short distance. The presence of an IRES element in the coding region supports the idea that the *gag* gene can be translated independently despite the structural rearrangements and the various interactions that occur in the 5'-UTR. A structural model of the HIV-2 RNA coding region has been established by enzymatic and chemical analysis and is characterized by a collection of seven or eight hairpins (P1 to P8) and an LDI conformation termed P3 (FIG. 4). The region between the P8 and the 3'-end of the coding region is not conserved and is marginally structured⁴⁴. Most of this structural model, derived for HIV-2, can be applied to the coding regions of HIV-1 and SIV, although no motifs of predictable function have emerged from a comparative analysis of the three structures. However, in several locations, all three structures have stretches of single-stranded purine nucleotides, a structural motif that is known to promote ribosome binding⁵⁴. Although the precise molecular determinants required for ribosome recruitment are unknown, sucrose gradient analysis has indicated that

three 43S, or 80S, ribosomal complexes can bind to each of the AUG initiation sites on the same HIV-2 mRNA molecule (Sargueil *et al.*, unpublished data).

The presence of an IRES element both in the 5'-UTR and in the coding region is likely to impact on the activity of each IRES element. In cells that were transfected with bicistronic constructs containing the HIV-1 IRES element in the 5'-UTR followed by segments of the gag coding region, Brasey *et al.* demonstrated that the IRES activity of the 5'-UTR was significantly inhibited when the structure was immediately upstream of gag coding region⁴³. The converse is also true; interestingly, the HIV-2 coding region is less efficient as an IRES element when preceded by its 5'-UTR⁴⁴. In both cases, this is likely to reflect an interaction between the 5'-UTR and the coding region, resulting in alternative folding of the two IRES elements. In agreement with this proposal, two LDI conformations have been predicted to occur in the 5'-UTR region of HIV-1, a prediction which can be extrapolated to HIV-2 and SIV. The first LDI is predicted to occur between the R-U5 region and the sequence in close proximity to the authentic AUG codon, resulting in the occlusion of the initiation site^{49,50,55,56}. Site-directed mutagenesis analysis revealed that this interaction is crucial to regulate the conformational switch from BMH to LDI, but does not modulate translation efficiency⁵³.

The interpretation of these experiments is hindered by the fact that translation could be initiated through different mechanisms. A second LDI conformation occurs between the 3'-poly (A) loop and a sequence separated by hundreds of nucleotides in the gag-coding region⁵⁷. No functional information is available about this structure. In addition, neither of these LDI conformations have been detected either *in vivo* or from *in vitro* experiments in which the 5'-UTR and the coding region were probed together and/or independently^{44,58}. Nevertheless, taking into account the dynamic nature of the RNA structure of HIV, it is conceivable that an interaction between the 5'-UTR and the gag-coding region could be one of several alternative conformations used by the genomic RNA. Therefore, the transition from one folding state to another might depend on the binding of one, or several, viral proteins to RNA signals located in the leader region. Consistent with this proposal is the recent finding that the Gag polyprotein was shown to modulate its own translation⁵⁹ (FIG. 5). In the model described by Anderson and Lever, there is a bimodal effect of Gag in which low amounts of the protein initially stimulates translation. The resulting increasing local concentration of Gag then results in the oligomerization of the protein and coating of the 5'-UTR of HIV-1 (REF. 59).

Together, these results strongly indicate that the initiation of translation of the full-length Gag polyprotein can occur by three distinct mechanisms: first, ribosomal assembly, entry and scanning from the 5'-capped end of the genomic RNA; second, internal ribosome entry through the IRES element located within the 5'-UTR; third, internal entry of the ribosome through the IRES located in the coding region (FIG. 5). Future work will determine how the virus uses these alternative translation-initiation mechanisms during the course of infection.

Additional RNA structures involved in the initiation of translation. The genomic RNA of Rous sarcoma virus (RSV) possesses a long and structured 5'-UTR that contains three ORFs that are phylogenetically conserved in size and position⁶⁰. In RSV, the packaging signal localizes to the third ORF and the integrity of this structure is crucial for viral replication. The introduction of point mutations to change the AUG initiation codons of the first and third reading frames leads to a dramatic reduction in RNA packaging (50- to 100-fold) indicating a functional link between translation and packaging^{39,61}. A model for the packaging of RSV has emerged, in which the synthesis of Pr76^{gag} represses translation from its cognate genomic RNA by binding to the packaging sequence⁶². This creates an autoregulatory mechanism that stops viral protein synthesis when the local concentration of p76^{gag} to RSV genomic RNA reaches a ratio of about 2000:1 to allow packaging and viral assembly. However, studies have also mapped the minimal region for RSV packaging to a 160 nucleotide segment located downstream of the PBS that spans to the 5'-end of the gag coding sequence^{63,64}. Therefore, the presence of this 160-nucleotide region (which contains the third ORF) on an RSV-based vector confers the ability to be encapsidated *in trans*; this is consistent with the use of RSV as a retroviral vector for gene-therapy purposes.

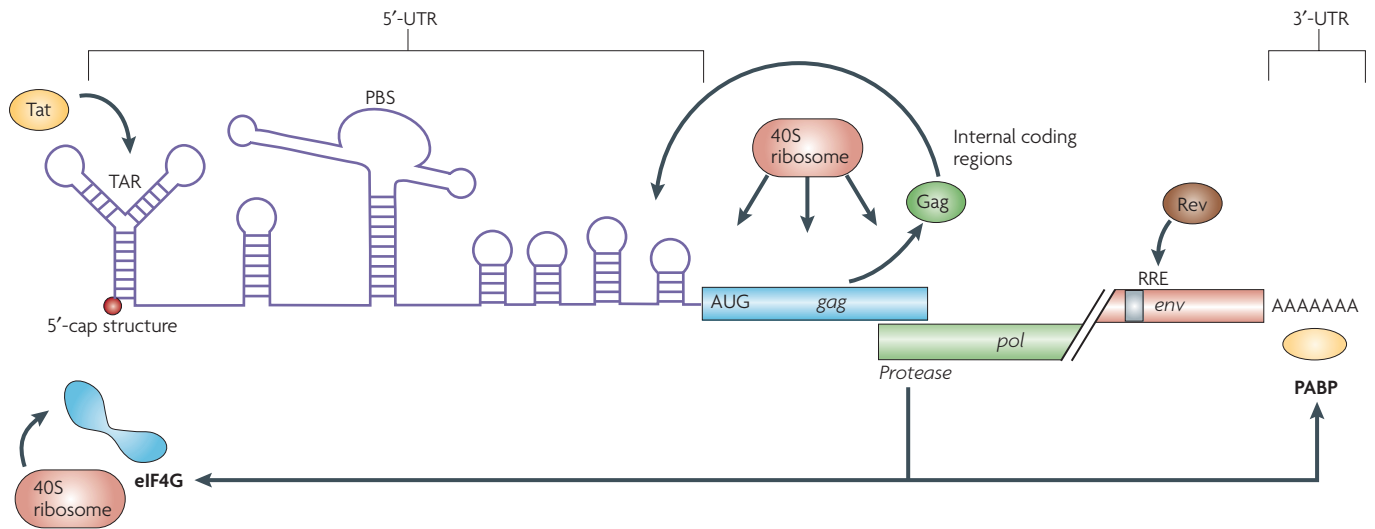
Another mechanism of translational enhancement mediated by an RNA structure was described in two retroviruses: M-PMV and the avian spleen necrosis virus (SNV). In these viruses, the region of genomic RNA between the 5'-LTR and the R-U5 domain was shown to enhance the translation of SNV genomic RNA and was subsequently termed the post-transcriptional control element (PCE). Interestingly, this effect was also demonstrated when this PCE was inserted upstream of both the main reading frame of the HIV-1 RNA and a Luciferase reporter gene⁶⁵⁻⁶⁷. Quantitative RNA analysis, together with ribosomal sedimentation profile analysis, has established that this translational enhancement is due to increased ribosome loading of the mRNA. RNA transfection assays, together with competition experiments, indicates that the R-U5 region interacts with nuclear proteins thereby creating a ribonucleoprotein complex that becomes actively translated in the cytoplasm⁶⁸. Finally, it was recently shown that cellular RNA helicase A was involved in this process as it recognizes and binds specifically to the PCE in both the nucleus and the cytoplasm. This interaction probably induces structural changes at the level of RNA-RNA and/or RNA-protein that ultimately results in the enhanced translation of PCE-containing RNAs⁶⁹.

Translational control by viral proteins

The TAR structure and the Tat protein. The TAR element is a stable 60-nucleotide stem-loop structure located at the 5'-extremities of all lentivirus transcripts that binds the virally encoded Tat protein with high affinity⁷⁰⁻⁷². Efficient production of viral mRNA from integrated proviral DNA results in the coordinated action of TAR, the cellular transcriptional machinery and the viral Tat protein, which functions as an adaptor protein for cellular

R-U5 domain

The long terminal repeat (LTR) sequences are located at both the 5' and 3' termini of the retroviral genome. The LTRs can be broadly divided into three RNA sequences: U3, which contains promoter and enhancer regions for RNA synthesis; R (for repeat), which corresponds to the start (+1) of the genomic RNA and spans up to the end of the poly (A) loop; and U5, which starts from the end of the poly (A) loop, comprising the primer binding site and extends up to the packaging signal.



	IRES		Gag isoforms	Cleavage		
	5'-UTR	Coding region		eIF4GI	eIF4GII	PABP
Simple retroviruses						
MLV	Yes (2)	Yes (in env)	2	Unknown	Unknown	Unknown
ALV	Yes	Unknown	1	Unknown	Unknown	Unknown
<i>Drosophila gypsy</i>	Yes (2)	Unknown	1	Unknown	Unknown	Unknown
MoMLV	Yes	Unknown	1	Yes	Yes	No
MMTV	Unknown	Unknown	1	Yes	Yes	Yes
Complex retroviruses						
HIV-1	Yes	Yes	2	Yes	No	Yes
HIV-2	Unknown	Yes	3	Yes	No	Yes
SIV	Yes	Yes	2	Yes	Yes	No

Figure 5 | **Translational control of lentiviral genomic RNAs.** The figure summarizes the events that modulate and control the translation of the lentiviral genomic RNA. The table summarizes the location of the IRESs, the number of Gag isoforms and the effect of the protease on the translational apparatus for all the retroviruses described in the manuscript. ALV, avian leukosis virus; eIF4G, eukaryotic initiation factor (eIF) 4G (isoform I and II); MLV, murine leukaemia virus; MoMLV, Moloney MLV; MMTV, mouse mammary tumour virus; PABP, poly(A) binding protein; PBS, primer binding site; Rev, regulator of expression of virion proteins; RRE, Rev response element; SIV, simian immunodeficiency virus; TAR, transactivation response element.

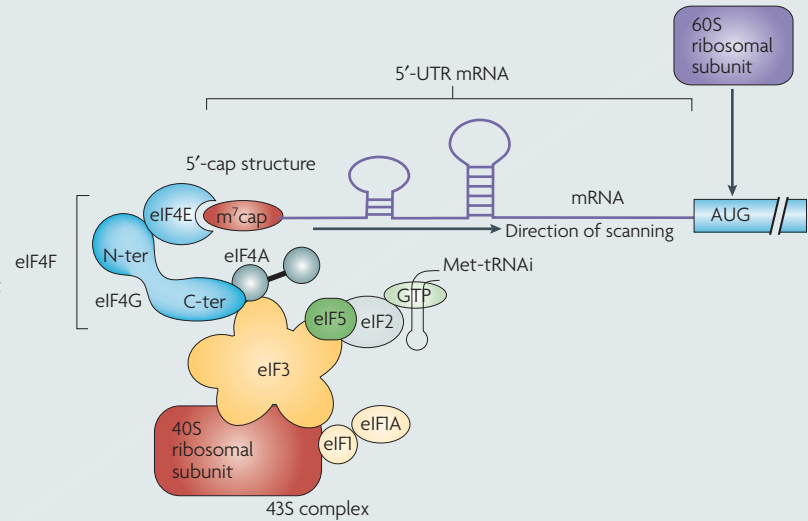
cofactors⁷³. Initially it was shown that, owing to its location immediately downstream of the cap structure, TAR could inhibit protein synthesis by impairing ribosome binding to the 5'-end of the mRNA⁷⁴. However, further experiments revealed that TAR is capable not only of modulating translation in *cis* but also in *trans*⁷⁵. This phenomenon was shown to be related to the ability of TAR to induce phosphorylation of the α subunit of eukaryotic initiation factor (eIF)-2 by the activity of the interferon (IFN)-induced, dsRNA-dependent, serine/threonine protein kinase, PKR^{76,77}. Phosphorylation of eIF2 α blocks the recycling of eIF2B-GDP into eIF2-GTP^{77,78}, which virtually shuts off protein synthesis. In this context, the TAR element has the intrinsic ability to activate both PKR and the 2-5A-synthetase — two enzymes associated with the IFN-mediated antiviral response^{76,77,79,80}. Structural studies revealed that PKR binds TAR and undergoes a

conformational change owing to PKR dimer formation on the TAR stem loop^{81,82}.

To counteract PKR activation that is induced by TAR, HIV-1 has evolved the ability to evade the subsequent antiviral IFN response through the action of the viral protein Tat, which binds to PKR. Tat functions as a substrate homologue for PKR, competing with eIF2, and inhibiting the translational blockage induced by eIF2 α phosphorylation^{76,79,80,83}. Interestingly, the Tat-PKR interaction occurs directly and is not mediated by the presence of dsRNAs^{83,84}. The action of Tat on PKR is not limited to the inhibition of its activation, as Tat can reverse the inhibition of translation that is induced by activated PKR^{84,85}. Together, these findings lend support to a model whereby Tat functions as an inhibitor of PKR by competing with its natural substrate (eIF2) and by blocking the autophosphorylation of PKR.

Box 2 | Translation initiation in eukaryotes

In the model for initiation (see figure), translation begins by the attachment of the 40S ribosomal subunit to the 5'-capped end of the transcript, followed by a linear scanning process, until it reaches an initiation codon. This process is mediated by a number of proteins called initiation factors that allow both efficient binding of the ribosome to the mRNA and migration to the initiation codon. Among these proteins, the eIF4F complex has a pivotal role¹⁴⁴. It is composed of the eIF4E cap-binding protein, which drives the complex to the 5'-capped end of the eukaryotic mRNA, the eIF4G scaffolding protein and the eIF4A RNA helicase¹⁴⁵. Two different isoforms of eIF4G (eIF4GI and eIF4GII) can connect eIF4E with the 43S ribosome complex, which is composed of the 40S ribosomal subunit associated with eIF3, eIF4A, eIF5, eIF1, eIF1A and eIF2-GTP bound to an initiator tRNA¹¹¹ (see figure). The initiation factor eIF4G has been found to also interact with the poly (A) binding protein (PABP) which binds to the 3'-poly (A) tract of the eukaryotic mRNA, lending support to a closed-loop model for translation initiation^{146,147}. After its attachment to the capped end of the mRNA, the 43S complex scans in a 5' to 3' direction until it reaches an initiation codon¹⁴⁸. At this point, eIF5 promotes the dissociation of the preinitiation complex, GTP hydrolysis, and the 60S ribosomal subunit can assemble with the 40S to form the 80S ribosome.



However, the effect of Tat on protein synthesis goes beyond its ability to inhibit PKR activity; indeed, Tat can stimulate *in vitro* translation of a TAR-containing reporter gene independently of its effect on both PKR and the (2'-5')-oligoadenylate synthetase activation pathways⁸⁶ (FIG. 5). Similar effects can be mediated by other TAR-binding proteins, including RNA helicase A, Staufen-1, and the La autoantigen⁸⁷⁻⁹¹. Therefore, it seems that protein interaction with TAR can alleviate translational repression in *cis* by disrupting the stability of the TAR element. Interestingly, eIF2 can also interact with TAR⁹², allowing one to speculate that the interaction of both PKR and eIF2 with contiguous regions in TAR could facilitate the phosphorylation of eIF2 by PKR⁹². Finally, it should be noted that a cellular TAR-binding protein (TRBP) binds strongly to TAR, and transactivates the HIV-1 LTR synergistically with Tat function⁸⁷. Moreover, similar to Tat, TRBP associates with PKR and inhibits its activity.

The RRE/ Rev interaction. The Rev (regulator of expression of virion proteins) protein is a 116 amino-acid protein that induces the transport of incompletely spliced lentiviral RNAs harbouring the *cis*-acting Rev response element (RRE) between the nucleus and the cytoplasm⁹³⁻⁹⁶. The complete RRE of HIV-1 consists of 351 nucleotides and is a highly ordered RNA stem-loop structure located in the *env* gene⁹³⁻⁹⁷. Initially, the Rev protein binds with high affinity at a site located at the apex of the central stem of the RRE, where it can recruit additional Rev molecules through cooperative protein-protein and protein-RNA interactions⁹⁸⁻¹⁰². As a consequence, RRE-containing transcripts are compartmentalized differently, increasing their association with cytoskeleton polyribosomes¹⁰³⁻¹⁰⁵.

Therefore, it is plausible that Rev not only helps in the transportation of viral RNA out of the nucleus but is also responsible for the localization of the RNA to polyribosomes, enhancing their use by the cellular translational machinery. In agreement with this suggestion, a number of reports have described a discordant relationship between the Rev-induced increase in cytoplasmic RRE-containing RNA levels and the dramatic increase reported for protein production from these same RNA transcripts. In one report, D'Agostino *et al.* (1992) used a reporter plasmid encoding the RRE-containing *gag* gene to show a 4- to 16-fold increase in the concentration of *gag* mRNA in the presence of Rev, a finding that cannot account for the more than an 800-fold increase in the production of the Gag protein¹⁰⁵. A similar effect has been reported for the *env* mRNA, in which Rev expression results in a 1.6-fold increase in *env* mRNA transport, whereas translation from this mRNA was increased more than 100-fold¹⁰⁶. Further studies confirmed the increased association of viral RNA with polyribosomes in response to the presence of the Rev protein^{104,105}. In the absence of Rev or RRE, there is no association of viral RNA with polyribosomes and the RNA is found in 40S to 80S complexes¹⁰⁴. Additional evidence to support a direct role for the Rev protein in mRNA translation was generated using a non-viral plasmid expression system capable of generating an intron-RRE-containing reporter RNA. This RNA lacks the viral nuclear retention sequences, thereby allowing the unspliced transcript to be exported from the nucleus to the cytoplasm. In this experimental setting, protein levels increased 3.5-fold in response to the viral Rev protein whereas the level of cytoplasmic mRNAs remained stable¹⁰⁷.

Viral protease activity and the host-cell translational apparatus. In the retrovirus life cycle, different proteolytic events take place during the assembly of infectious virions (FIG. 1). The virally encoded aspartyl protease processes the Gag and Gag-Pol polyprotein precursors into mature proteins. The retroviral protease is a product derived from the *pol* gene and is one of the key targets in anti-HIV-1 therapies^{108,109}. In addition to its function in the retroviral life cycle, this endopeptidase can also cleave certain cellular proteins, resulting in a cytotoxic effect¹¹⁰. One such protein is the initiation factor, eIF4GI, which is a scaffolding protein that has a key role in the assembly of the ribosomal pre-initiation complex¹¹¹ (BOX 2). The cleavage of eIF4GI was first observed in CD4⁺ cells infected with a laboratory strain of HIV-1, and this activity could be correlated with a global decrease in protein synthesis¹¹². Expression of the HIV-1 protease (encoded on a plasmid) in COS-7 cells resulted in the proteolytic processing of eIF4GI and could be prevented by the addition of a specific HIV protease inhibitor¹¹². Similar results were obtained by the addition of recombinant HIV-1 protease to HeLa-derived S10 extracts¹¹² and to the rabbit reticulocyte system¹¹³. Sequence analysis from mass spectrometry revealed that the eIF4GI protein was cleaved twice between the eIF4E binding site and the first of

the eIF4A binding sites (in fact, the two cleavage sites are only three amino acids apart). The protein was also cleaved further downstream in the region of the second eIF4A-binding domain^{112,113}. The recombinant HIV-2 protease was also shown to be capable of cleaving eIF4GI at the same sites. However, in this case, the proteolytic activity occurred by a two-step reaction. First, a 100-kDa fragment (Ch-1 fragment) was generated as a result of proteolysis attack at the two sites located between the eIF4E- and eIF4A-binding sites. Second, at higher protease concentrations, the Ch-1 fragment was further digested into two 50-kDa C-terminal segments¹¹⁴. Interestingly, the Ch-1 fragment is only 40 amino acids shorter than the main cleavage product that results from the proteolysis of eIF4GI by the picornaviral protease, L/2A (REF. 115). However, whereas the cleavage of eIF4GI by the picornaviral proteases results in a strong stimulation of virtually all IRES elements identified so far^{116–118}, proteolysis mediated by the HIV-2 protease results in a strong inhibition of type I IRES-mediated translation with little or no effect on translation by type II IRES. This finding strongly indicates that the 40 amino-acid region between the L/2A and the HIV-2 protease cleavage sites (amino acids 641–681), which has RNA binding properties, has a role in ribosomal scanning¹¹⁴.

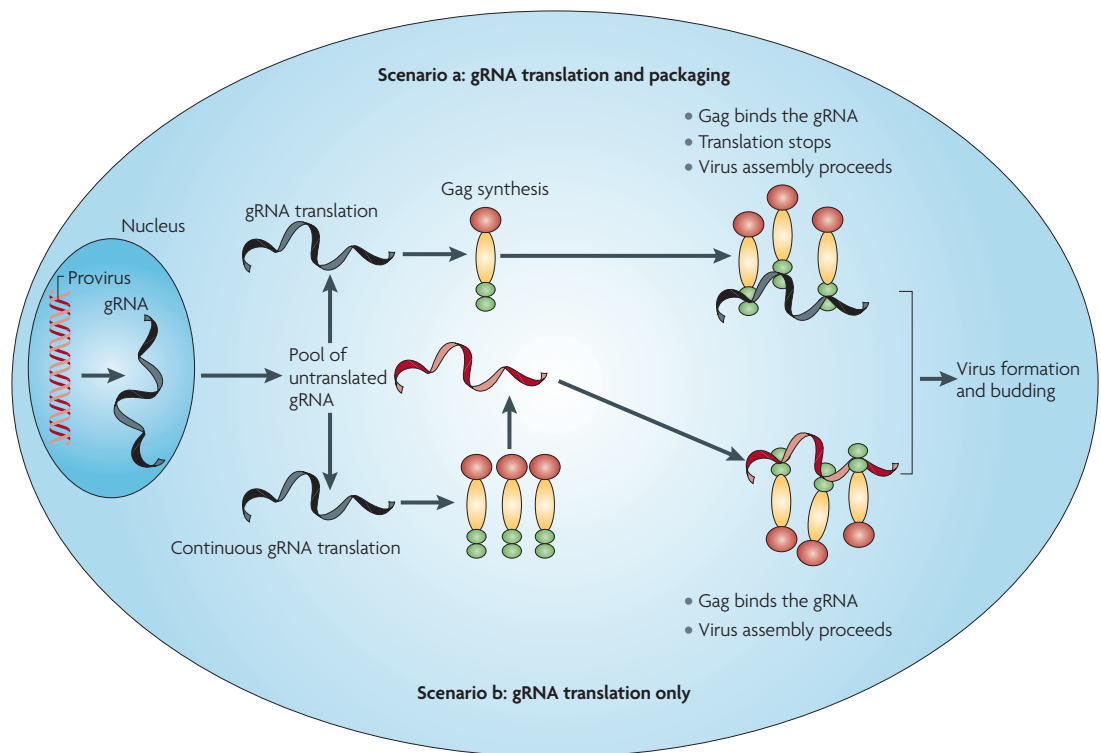


Figure 6 | Translation and encapsidation of the retroviral genomic RNA. Transcription of the integrated provirus generates the genome length RNA (gRNA). A fraction of the gRNA is spliced to give the spliced viral RNAs (not shown here). The gRNA is exported to the cytoplasm where it is recruited by active ribosomes (scenarios **a** and **b** with gRNA in black) or eventually accumulates as a pool of non-translated RNA (gRNA in red). According to scenario **a**, the gRNA is translated to give Gag molecules and Gag-Pol (not shown here). Once Gag molecules accumulate, there is a switch from translation to packaging as Gag binds to the gRNA at the 5'-IRES-Psi signal. Virus formation and release by budding follow. In scenario **b**, the gRNA is continuously translated. Newly formed Gag molecules bind the gRNA from the non-translated pool (red gRNA). Virus formation follows.

The cleavage of eIF4GI by retroviral proteases has been extended to those from HTLV-1, MMTV and MoMLV¹¹⁹ (FIG. 5). Interestingly, with the exception of MoMLV, none of these proteases were able to cleave eIF4GII, the functional homologue of eIF4GI, despite the fact that eIF4GII harbours one of the consensus cleavage sites^{113,119}.

Recently, the Poly (A) binding protein 1 (PABP1), which is required for mRNA circularization during translation initiation through its interaction with eIF4G and the mRNA poly (A) tail was also found to be a target for retroviral proteases¹²⁰ (FIG. 5). This was shown both in the context of infected cells and in *in vitro* extracts derived from HeLa cells, and was demonstrated using proteases derived from MMTV, HIV-1 and HIV-2. However, the implications of PABP1 cleavage by retroviral proteases on the overall translation process deserves further investigation.

Following HIV-1 infection, protein synthesis by the infected cell is decreased; however, despite the fact that both eIF4GI and PABP1 are cleaved by the viral protease, translation still occurs in these cells^{112,120,121}. This contrasts with the complete translational shut-off observed in host cells infected with picornaviruses, which is also a result of the cleavage of eIF4G (eIF4GI and eIF4GII) and PABP¹²². There are several explanations that could be used to explain these differences between picornaviral and retroviral infections: first, proteolysis of eIF4GI and PABP by retroviral proteases occurs at a much later time-point during the viral replication cycle; secondly, the HIV-1 protease is significantly less active on eIF4GI compared with the L picornaviral protease from the foot-and-mouth-disease virus (FMDV)¹²³; third, eIF4GII is not cleaved by the retroviral proteases and therefore this protein could support translation in the absence of eIF4GI (as has been shown for picornaviral infection)^{124,125}.

However, it is unknown how the translation of retroviral genomic RNA can benefit from these proteolytic events given that they occur at time when most, if not all, of the viral structural proteins and enzymes have been synthesized. The physiological role of eIF4GI cleavage by the retroviral proteases remains to be addressed in the context of viral infection. However, this might be very difficult to assess owing to the toxicity of the enzyme on the cellular apparatus¹¹⁰. Based on the *in vitro* data, it is tempting to speculate that the expression of low levels of the retroviral protease might sustain HIV translation at times when cellular protein synthesis is already severely affected^{112,114}. However, at higher levels of protease, it is likely that the damages inflicted to the cell would result in polysomal dissociation and the inhibition of viral and cellular translation, which would favour viral packaging, assembly and the release of progeny virions^{110,113}.

Perspectives and discussion

Over the past decade, retroviruses and lentiviruses have been the focus of intense research interest primarily as a result of the AIDS epidemic. Characterization of the IRES elements of several retroviruses has emphasized the importance of translation in viral replication. The

potential use of several mechanisms, such as a combination of IRES elements located both in the 5'-UTR and the coding region, together with ribosomal entry at the 5'-capped end, suggests that viral gene expression is highly regulated at the translational level. An additional layer of complexity is supplied by the interplay of viral proteins that directly bind to the genomic RNA or that modify the host cell translational apparatus. The coordinated action of all these elements creates a selective translational advantage for viral genomic RNA, resulting in the recruitment of ribosomes and initiation factors in a competitive cellular environment (FIG. 5).

A second important issue relating to retroviral translation concerns its close relationship with the viral assembly process that leads to packaging of the genomic RNA into newly formed virions. It should be remembered that the full-length, unspliced, genomic retroviral RNA has a dual role in that it functions as a template for protein synthesis and ensures the flow of genetic information⁶. As a consequence, the same RNA molecules are either translated or packaged into newly formed retroviral particles (FIG. 6). However, the two mechanisms are believed to be mutually exclusive. Encapsidation of the unspliced RNA is dependent on the interaction between the Gag polyprotein and the RNA packaging motifs within the 5'-UTR. These Gag protein-RNA interactions result in a conformational change of the 5'-UTR as an increasing number of nascent Gag molecules progressively bind to the structure⁵⁹. Therefore, it is conceivable that the use of multiple translational mechanisms could reflect the ability of the virus to adapt to these conformational changes in order to ensure continuous viral protein synthesis. As such, the early steps of infection might be characterized by the exclusive use of a 5'-cap-dependent mechanism as it is the most efficient way to rapidly build a stockpile of structural proteins and enzymes. At this stage, production of the Gag polyprotein could stimulate its own translation from the genomic RNA, as was recently proposed by Anderson and Lever⁵⁹. Further progression in the viral cycle results in the production of high levels of Gag and Gag-Pol molecules that bind to the RNA packaging signals located within the 5'-UTR. This process results in the progressive occlusion of the 5'-UTR as the ribonucleoprotein scaffold is formed. Therefore, translation initiation switches to an 'all IRES'-based mechanism of ribosome entry at the AUGs that are located in the gag coding region. In the case of lentivirus replication, further support for this hypothesis comes from the fact that the Vpr protein probably blocks the cell cycle in a G2/M phase that is not favourable for cap-dependent translation initiation^{126,127}. As such, this mechanism is a method to continue viral protein synthesis during the early steps of viral assembly. Finally, the accumulation of Gag and Gag-Pol in the cytoplasm of the infected cell ultimately triggers the maturation of the active protease that will, in turn, cleave the cellular translation initiation factor eIF4GI and PABP. This process results in a progressive inhibition of cellular and viral translation thereby promoting assembly and the release of new infectious particles.

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Competing interests statement

The authors declare no competing financial interests.

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