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A 5'UTR-spliced mRNA isoform is specialized for enhanced HIV-2 *gag* translation

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Abstract

Full-length unspliced genomic RNA plays critical roles in HIV replication, serving both as mRNA for the synthesis of the key viral polypeptides Gag and Gag-Pol and as genomic RNA for encapsidation into assembling viral particles. We show that a second *gag* mRNA species is produced during HIV-2 replication in cell culture and in infected patients that differs from the genomic RNA molecule by the absence of an intron in the 5' untranslated region (5'UTR). We developed a co-transfection system in which epitopically tagged Gag proteins can be traced back to their mRNA origins in the translation pool. We show that a disproportionate amount of Gag is translated from 5' UTR intron-spliced mRNAs, demonstrating a role for the 5'UTR intron in the regulation of *gag* translation. To further characterize the effects of the HIV-2 5'UTR on translation, we fused wild type, spliced, or mutant leader RNA constructs to a luciferase reporter gene and assayed their translation in reticulocyte lysates. These assays confirmed that leaders lacking the 5'UTR intron increased translational efficiency compared to the unspliced leader. In addition, we found that removal or mutagenesis of the C-box, a pyrimidine-rich sequence located in the 5'UTR intron and previously shown to affect RNA dimerization, also strongly influenced translational efficiency. These results suggest that both the splicing of the 5'UTR intron and the C-box element have key roles in regulation of HIV-2 *gag* translation *in vitro* and *in vivo*.

Keywords

5'UTR; translation; splicing; C-box; HIV-2

INTRODUCTION

Unspliced genomic-length RNA plays at least two central roles in HIV replication. It serves both as messenger RNA for the synthesis of the key viral proteins Gag and Gag-Pol, and as genomic RNA for encapsidation into virus particles. Regulation of the use of this full-length viral RNA as either mRNA or genomic RNA (or both) is likely to be critical for efficient viral replication. In fact, regulation of many viral RNA functions, including translation and

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encapsidation, occurs through the 5' untranslated region (UTR), a highly conserved region in HIV¹.

Previous research has shown that the 5'UTR influences translation of HIV-1 mRNAs. The overall length of the HIV-1 5'UTR was shown to contribute to a decrease in initiation efficiency by increasing the requirement for ribosomal scanning in advance of the AUG start codon². In addition, the position and stability of the highly structured transactivation responsive element (TAR) at the very 5' end of the UTR plays a role in impeding 5' cap accessibility and ribosomal scanning³.

HIV-2 and HIV-1 share a common overall organization of their 5'UTRs, thus it is likely that HIV-2 mRNA translation is influenced by the 5'UTR. However, differences between the HIV-1 and HIV-2 UTRs may necessitate alternative modes of translational regulation. First, the HIV-1 (LAI isolate) leader region is significantly shorter at 335 nucleotides (nt) long compared to the HIV-2 (ROD isolate) leader region, which is 545 nt long. Second, HIV-1 contains a 59 nt TAR structure consisting of one major stem loop, while HIV-2 contains a larger 123 nt TAR structure consisting of three stem loops⁴⁻⁶. Third, unlike HIV-1, the HIV-2 5'UTR contains a predicted intron⁷. Splicing of this intron results in the removal of 140 nucleotides of 5' leader sequence including half of the large TAR structure sequence, the entire poly(A) signal domain, and the C-box region (compare Fig. 1A and 1B). Evidence of 5'UTR intron splicing was first revealed in a simian immunodeficiency virus (SIV_{mac239}), which is a phylogenetic relative of HIV-2⁸. Most viral mRNA species found in SIV_{mac239} infected cells were present in both the 5'UTR intron spliced and unspliced forms^{8,9}. In HIV-2, evidence of 5'UTR splicing was revealed in multiply-spliced mRNA species, but its occurrence on *gag/gag-pol* coding mRNA species was not investigated⁷.

The effects of 5'UTR intron presence or absence on translation were first studied using partial leader SIV_{mac239} constructs truncated at the major splice donor site thus mimicking the 5'UTR of multiply-spliced mRNA species⁹. Translation of a downstream reporter gene was found to be more efficient if the 5'UTR intron was not present⁹. However, a direct examination of the role of the HIV-2 5'UTR and its splicing in *gag* (and *gag-pol*) translational regulation has not been conducted and is of unique interest for several reasons. First, the 5'UTR spliced *gag* is singly, rather than multiply-spliced at a site that does not employ the otherwise ubiquitous major splice donor site (SD). Second, it is plausible the 5'UTR splicing could regulate translation by shortening and removing secondary structure in the 5'UTR. Finally, this splicing event could regulate translation by removal of RNA signals that have importance in other regulatory events, including the long range interaction between the 5'UTR element known as the C-box and the G-box that overlaps the *gag* translation initiation codon^{10,11}.

In this study, we demonstrate the presence of both 5'UTR spliced and unspliced *gag* mRNA species in transfected and infected cells and in PBMCs isolated from HIV-2 infected patients. We show in transfected cells that a 5'UTR unspliced construct yielded less Gag compared to its 5'UTR spliced counterpart. To further characterize the effect of the HIV-2 5'UTR on translation, we tested the translation of a luciferase reporter gene fused to various 5'UTR leader constructs in reticulocyte lysates. We find that leaders lacking the 5'UTR intron increased translational efficiency compared to constructs harboring the unspliced leader. Furthermore, our *in vitro* and cell culture studies implicate the C-box, which is part of the intronic sequence removed by 5'UTR splicing, as a contributor to translational regulation. Taken together, our results underscore the importance of the 5'UTR in the coordinated regulation of several essential viral replicative functions *in vitro* and *in vivo*.

RESULTS

5'UTR splicing of *gag* mRNA in cell culture

In HIV-1, there is only one type of *gag* and *gag-pol* mRNA, the unspliced genome-length RNA species. Because HIV-2 RNA contains a predicted intron in the 5'UTR⁷, we sought to detect the presence of a second 5'UTR-spliced *gag* mRNA species in transfected and infected cells. RNAs derived from peripheral blood mononuclear cells (PBMCs) from HIV-2 seropositive but asymptomatic patients in Senegal¹² were also analyzed using nested RT-PCR for the presence of 5'UTR spliced RNA (Fig. 1D).

COS-7 (monkey kidney fibroblast) cells were transfected with full-length HIV-2 proviral DNA to produce viral particles. We examined the 5'UTR of *gag* mRNA species in both the intracellular RNA fraction and extracellular viral particles of transfected cells by RT-PCR. The intracellular RNA fraction yielded two distinct products on an agarose gel (Fig. 1C). The primer asECO561 allowed us to amplify only the leader region of *gag* mRNA molecules that were not spliced at the major splice donor site (SD). Sequencing of the gel-purified bands revealed that the larger band corresponded to the leader region of the unspliced genomic RNA species while the smaller band corresponded to a leader region with nucleotides 61-202 missing. The smaller band was clearly the 5'UTR spliced *gag* mRNA, since the missing sequence corresponds exactly to the predicted 5'UTR intron and mutation of the splice donor or splice acceptor site resulted in the disappearance of the smaller band (data not shown). Furthermore, RT-PCR analysis of the viral particles produced from COS-7 cells revealed that the unspliced genomic RNA was encapsidated, while the 5'UTR spliced variant was excluded (Fig. 1C).

Viral particles produced from the transfected COS-7 cells were used to infect permissive C8166 (human T lymphocyte) cells. As with the COS-7 cells, both spliced and unspliced *gag* mRNA species were found inside the infected C8166 cells, while only the unspliced genomic RNA was seen in C8166-produced viral particles (Fig. 1C), suggesting that the 5'UTR spliced *gag* mRNA species lacks a functional packaging signal.

5'UTR splicing of *gag* mRNA in PBMCs from HIV-2 infected persons

Lysates from PBMCs from five HIV-2 asymptomatic seropositive patients were subjected to nested RT-PCR wherein the first PCR primer set was designed to amplify *gag* mRNAs, and the second primer set to further amplify a fragment within the 5'UTR containing the predicted intron. Three of five samples produced bands corresponding to HIV-2 leader RNA. HIV-2 RNA was not seen in all samples, perhaps due either to sequence variation in these isolates that prevented primer hybridization, or the level of viral mRNA was below our detection limit. Of the three samples that yielded PCR product, two samples produced obvious bands indicating 5'UTR-spliced RNA was present; a third sample had lower or no obvious 5'UTR-spliced RNA (Fig. 1D). The PCR products were sequenced and it was determined that the *gag* mRNAs were derived from sources other than the stock HIV-2 ROD isolates used in our laboratory (data not shown). The presence of the 5'UTR spliced *gag* mRNA in samples derived from human patients confirms that this splicing event is not limited to experimental infections or transfections in cell culture.

Effect of the 5'UTR intron on Gag production in cell culture

Since two *gag* mRNA species are found in the cytoplasm of both transfected and infected cells, we wanted to assess their relative contributions to the overall Gag protein production. To this end, we co-transfected COS-7 cells with two HIV-2 DNA plasmids. One plasmid provided only the unspliced *gag* mRNA species because of a mutation in the 5'UTR splice donor site that prevents splicing (called SD(-)) (Fig. 2A). The other plasmid provided the *gag* mRNA species lacking the 5'UTR intron through the deletion of nucleotides 61-202 in the proviral

DNA (called Δ intron) (Fig. 2B). To differentiate which Gag proteins came from which *gag* mRNA species, we inserted a FLAG-tag at the end of the *gag* gene of one or the other of the co-transfected plasmids (Fig. 2).

RNA and proteins were harvested from the co-transfected cells, with half of the intracellular fraction processed for protein analysis and the other half for RNA analysis. Because of the eight amino acid addition in the FLAG-tagged proteins, an electrophoretic migration difference could be discerned between FLAG-tagged and untagged Gag proteins (Fig. 2C, top panel). Those with a FLAG-tag migrated slightly slower, and all proteins were recognized by an antibody directed against the capsid domain (anti-capsid antibody). As a control, we probed the western blot with an anti-FLAG antibody to directly verify the presence and position of the FLAG-tagged proteins (Fig. 2C, bottom panel). The 5'UTR Δ -intron mRNA produced more Gag protein than the 5'UTR SD(-) mRNA (Fig. 2C, lanes 4 and 5 in top panel). We also confirmed that Gag expression was not significantly affected by the presence or absence of the FLAG-tag (Fig. 2C, lanes 4 and 5 in top panel). Moreover, RNase protection assay (RPA) revealed that Gag expression levels varied according to whether or not the 5'UTR was spliced, and not simply with mRNA concentrations (Fig. 2D). The protein yields (corrected for mRNA concentrations) were quantified and are reported in Figure 2E. To determine whether the Gag (FLAG-tagged or untagged) translated from mRNAs containing or missing the 5'UTR intron was incorporated into virions, aliquots of the cell culture media were centrifuged ($21,000 \times g$ for two hours) and the viral pellets were subjected to protein analysis as with the intracellular fractions. Gag derived from all mRNA sources was found in the virion fraction, and, as above, the mRNA species lacking the 5'UTR intron was consistently the predominant source of the protein (Fig. 2F). These results strongly support our hypothesis that the 5'UTRs of the two *gag* mRNA species lead to different translational efficiencies of the downstream open reading frame.

***In vitro* translation of luciferase mRNA harboring 5'UTR spliced or unspliced HIV-2 leaders**

We next tested the translation of HIV-2 leaders fused to a Renilla luciferase reporter gene in reticulocyte lysates. This enabled us to specifically examine the effect of the 5'UTR on translation independently of other viral functions. Briefly, two luciferase constructs were built containing either the first 569 nucleotides of the HIV-2 genomic RNA sequence (unspliced), or the first 569 nucleotides minus 61-202 nts (spliced) fused to the second codon of the Renilla luciferase reporter gene (Fig. 3A). Following transcription, 5' capping, and polyadenylation, the luciferase RNA constructs were introduced in equimolar amounts into individual rabbit reticulocyte lysate translation reactions. Aliquots were taken at set time points and luciferase activity at each time point was quantified using a microplate luminometer. The spliced HIV-2 5'UTR luciferase construct translated approximately five times more efficiently than the unspliced HIV-2 5'UTR luciferase construct, illustrating that the removal of the 5'UTR intron significantly increased the efficiency of HIV-2 translation (Fig. 3B). All mRNAs demonstrated similar stability in the translation reactions as assayed by RPA (Fig 3C).

***In vitro* translation of luciferase mRNAs harboring serially truncated HIV-2 leaders**

Several structural elements are affected by the 5'UTR intron splicing including the TAR and poly(A) signal domains and the C-box (compare Fig. 1A and 1B). To probe the influence of these structural elements on translation, we constructed chimeric luciferase reporter genes fused with the HIV-2 5'UTR containing serial truncations of the 5' HIV-2 leader. The constructs produced mRNAs by T7 RNA polymerase transcription beginning at nucleotide 1, 186, 198, or 486 (HIV-2 ROD numbering). The 186 truncation lacked the entire TAR sequence as well as the poly(A) signal domain. The 198 truncation was similar to the 186 truncation with just twelve additional nucleotides corresponding to the C-box deleted¹³. The 486 truncation lacked

TAR, the poly(A) signal domain, the C-box, and the PBS domain. Translation efficiencies of these constructs were analyzed as described above.

The truncation constructs exhibited progressively higher translational efficiencies than the full-length 5' leader construct, but notably the 198 construct, which lacked the C-box, showed a disproportionately higher translational efficiency than the incrementally longer 186 construct, suggesting that the C-box sequence exerts a specific effect on translation (data not shown). To test this, we made mutations in the C-box, described below.

***In vitro* translation of luciferase mRNAs harboring a mutated HIV 5'UTR leader**

We next sought to determine whether the observed increased translational efficiency caused by removal of the C-box could be recapitulated by substitution of nucleotides within this element. Two Renilla luciferase constructs were built containing substitutions that would test the effect of mutating the C-box sequence in the context of a full-length leader background (Fig. 4A). The cytidine nucleotides in the 189 to 196 sequence of mutants A and G were substituted with adenine and guanine, respectively. Both C-box mutants exhibited higher translational efficiencies than the wild-type 5' HIV-2 leader driven luciferase construct, suggesting the C-box sequence itself exerts translational regulation (Fig. 4B).

HIV-2 C-box mutants in cell culture

After identifying a role of the C-box region in translational regulation *in vitro*, we investigated whether mutations in the C-box could affect HIV-2 replication in cell culture. To obtain viral particles, COS-7 cells were transfected with full-length HIV-2 plasmid DNA containing the A and G substitutions in the C-box (Fig. 4C).

We first examined 5'UTR splicing of *gag* mRNA species in both the intracellular fraction and extracellular viral particles of transfected COS-7 cells. RT-PCR of the total intracellular RNA yielded only one distinct band for each mutant, instead of the two bands observed with wild type HIV-2 transfections (compare Fig. 4D to 1C). Sequencing of the gel-purified bands confirmed the lack of 5'UTR splicing for each mutant (data not shown). In addition, RT-PCR analysis of the extracellular RNA confirmed the presence of the unspliced genomic RNA in viral particles (Fig. 4E). These results demonstrated that there is only one species of intracellular *gag* mRNA for both C-box mutants.

Viral particles produced from the transfected COS-7 cells were used to infect permissive C8166 cells. Viral replication of both C-box mutated viruses was attenuated compared to wild type (Fig. 4F) demonstrating that in addition to affecting translation, the C-box also plays an important role in viral replication.

DISCUSSION

The impetus for studying the use of different *gag* mRNA species and their roles in HIV-2 replication came from several observations. First, the long and highly structured leader RNA in HIV-2 would not be expected to be efficiently translated by a cap-dependent scanning mechanism. Second, Lever and coworkers have proposed that the specificity of HIV-2 genomic RNA packaging depends on regulated *gag* translation in which viral RNA selection is mediated by a co-translational interaction of the genomic RNA with its nascent Gag protein product¹⁴. Third, the absence of a 5'UTR intron was shown to favor translation of model SIV RNAs, although the 5'UTR constructs used in this prior study were truncated ~150 nt upstream of the *gag* AUG start codon⁹. Here we show that two *gag* mRNA species exist in HIV-2 in culture and in patients, differing only by an intron in the 5'UTR. The species missing this intron is a better template for *gag* translation (Fig. 5).

Introns entirely contained within the 5'UTR occur in about 25% of metazoan mRNAs and average between 100-200 nucleotides in length^{15,16}. For example, removal of a 5'UTR intron has been shown to have a significant translational stimulatory effect for various cellular and viral mRNAs including platelet-derived growth factor B chain/c-*sis* and β 1,4-galactosyltransferase¹⁷⁻¹⁹. 5'UTR splicing can affect translation in several ways. First, removal of secondary structures within the 5'UTR may increase translation efficiency at several stages of the translation initiation process, including scanning by the initiating ribosome and AUG recognition²⁰. In HIV-2, splicing of the 5'UTR intron removes half of the TAR sequence, the entire poly(A) signal, and the C-box region, thus decreasing the size of the 5'UTR spliced leader RNA to a size similar to that of the HIV-1 leader (from 545 to 405 nts, as compared to 335 nts for HIV-1) (Fig. 1B). No internal 5'UTR splicing has been shown to exist in HIV-1, where the most proximal splicing event is initiated using the major SD site²¹. We observed in the course of this study that translational efficiency of a reporter construct fused to an HIV-1 full-length leader was comparable to the 5'UTR Δ -intron HIV-2 leader construct (data not shown), thus the translationally inefficient HIV-2 leader can be converted to one with higher efficiency (i.e. comparable to HIV-1) through 5'UTR splicing. Splicing may also contribute to translational efficiency through increased accessibility of the 5' methyl-G cap in HIV-2 mRNA, since the 3' half of the TAR structure (nts 61 to 123), which normally base pairs to the 5' end of the mRNA, is intronic. A more accessible 5' cap structure would increase translation initiation efficiency²² and *in vitro* studies using HIV-1 5'UTR have shown that TAR has a significant inhibitory effect on translation^{3,23-25}.

It is notable that 5'UTR splicing removes a pyrimidine-rich sequence known as the C-box. The C-box was previously shown to influence HIV-2 RNA dimerization through base pairing with the G-box, an area encompassing the *gag* start codon^{26,27}. A similar base pairing interaction has been demonstrated in HIV-1, where it affects the equilibrium between two proposed 5' UTR conformations. It was demonstrated to influence genomic RNA dimerization both *in vitro*²⁸ and in cell culture²⁹, although evidence for 5'UTR conformational control of HIV-1 translation was not observed using a transfected reporter construct³⁰. In the present study, we showed that viral replication of C-box substitution mutants is impaired. Our attempts to make compensatory mutations in the G-box were confounded by the overlap of the G-box with a consensus Kozak sequence surrounding the *gag* AUG start codon as well as the obligatory myristoylation signal at the subsequent codons of *gag* (data not shown). Interestingly, the same C-box mutations increased translational efficiency of 5'UTR unspliced RNA constructs *in vitro*, suggesting a role for the C-box in translational regulation of the downstream gene. In addition to their effects on translation, the C-box substitution mutants do not undergo 5'UTR splicing in transfected cells. Examination of the C-box region and surrounding sequence revealed that it encompasses the conserved polypyrimidine tract of the splice acceptor site whose disruption likely explains the absence of 5'UTR splicing in these mutants (Fig. 4C) 31-33.

Surprisingly, despite the conservation of the 5'UTR splice sites among HIV-2 and SIV strains, abrogation of 5'UTR splicing by mutagenesis of the UTR splice acceptor or donor sites did not strongly affect replication of HIV-2 in immortal C8166 cells (data not shown). This suggests either that translational efficiency is not the replication rate-limiting factor in viruses grown in this cell line (which is used because it is one of the few cell lines that supports robust HIV-2 replication), or that 5'UTR splicing becomes important only during a *bona fide* infection in a host. This prompted us to examine whether 5'UTR splicing was evident in clinical isolates. The observation of 5'UTR splicing in the PBMCs of HIV-2 infected individuals (Fig. 1D) attests to its physiological relevance, and the role(s) and regulation of 5'UTR splicing will continue to be explored. It is also possible that several mechanisms of translational activation are available to HIV-2/SIV, including the use of internal ribosome entry sites (IRESs) as recently reported^{34,35}. In the case where UTR splicing was inhibited by splice-site mutations,

it is possible that increased IRES activity could compensate. The interplay between these two proposed mechanisms may well represent another level of regulation of translation and replication.

Unspliced genome-length HIV RNA can serve either as mRNA or as packageable genomic RNA, or both³⁶ (Fig. 5). The present study suggests a specialized translation-only role for HIV-2 *gag* RNA following 5'UTR splicing since it augments translation, is not encapsidated to a detectable level (Fig. 1), and co-expression of homologous 5'UTR spliced and unspliced *gag* mRNAs showed a strong translational bias toward the spliced species (Fig. 2). Previous research has shown that recognition of the packaging signal (Ψ) by Gag is necessary for initiation of HIV genomic RNA encapsidation and suggests that the transition between translation and packaging of the HIV genomic RNA relies on a switch-like mechanism that affects packaging signal presentation³⁷. Removal of secondary structure in the 5'UTR by splicing could result in an improperly presented packaging signal. In fact, the C-box, through its interaction with the G-box, has been shown to influence the presentation of the encapsidation and dimerization signals *in vitro*^{10,26}. Taken together, our results suggest a role for the 5'UTR splicing and the C-box in the concomitant regulation of *gag* translation and packaging during the HIV-2 replication cycle.

MATERIALS AND METHODS

Plasmid construction

To engineer mutations in the 5' untranslated region or to insert a tag in the C-terminal domain of the Gag protein, we used plasmids derived from the modified pROD10 containing the full-length HIV-2 ROD³⁸⁻⁴⁰. The 12,828 bp-long modified plasmid pROD10 was provided by the EU Programme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK (Grant Number QLK2-CT-1999-00609 and GP828102). The *AatII-XhoI* fragment of modified pROD10 digestion was sub-cloned in pGEM-7Zf+ (Promega). This plasmid, pGRAXS, contains the upstream long terminal repeat (LTR) and most of the Gag coding region, up to the *XhoI* site⁴¹. The numbering is based on the sequence of the HIV-2 ROD isolate (GenBank N° M15390) which is contained within modified pROD10. Mutations were introduced in pGRAXS using either a ligation-PCR protocol (to delete the 5'UTR intron, nts 61-202) or the QuikChange II XL site-directed mutagenesis kit (Stratagene) and the appropriate primers (to mutate the 5'UTR splice donor site in the TAR structure or the C-box). The 5'UTR SD(-) mutation removes the splice donor site while keeping the second TAR stem structure intact (nts 61-62 GG changed to CC and nts 78-79 CC changed to GG). The *AatII-XhoI* fragment of plasmids bearing the mutations was then reinserted in the modified pROD10 backbone.

To introduce the same mutation (5'UTR SD(-)) in both LTR, the mutation was separately introduced in the unique upstream LTR of pGRAXS as described above and in the unique downstream LTR of the pAVR plasmid using the QuikChange II XL kit. The pAVR plasmid corresponds to modified pROD10 missing an internal *AvrII-AvrII* fragment encompassing the upstream LTR and *gag* region and thus contains only the downstream LTR. After mutagenesis, the *AvrII-AvrII* fragment containing the mutated downstream LTR in a pGRAXS backbone was reinserted in the *AvrII* site of pAVR mutated in the upstream LTR. To insert the FLAG-tag coding sequence (5'-gat tac aag gac gac gac gac aag-3', N-terminus-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C-terminus) near the end and in frame with the *gag* gene, we used a single PCR step following by replacement of the FLAG tag-containing *XhoI-Bsu36I* fragment in wild type and mutated HIV-2 plasmids. All constructs were checked by DNA sequencing.

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal calf serum, penicillin, and streptomycin (Invitrogen). Transient transfection of COS-7 cells was performed using Trans-IT-COS transfection kit (Mirus). Cells and media were harvested two days post-transfection. HIV-2 capsid protein levels in the media were quantified by a p27 enzyme-linked immunosorbent assay (SIV p27 ELISA; Zeptometrix).

Cell culture and infection

C8166 cells⁴² were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (Invitrogen). Media from transfected COS-7 cells containing 10 ng of HIV-2 p27 capsid protein, as determined by ELISA, was used to infect 2×10^4 C8166 cells (NIH AIDS reagent, N° 404). After 12 hours, cells were washed twice and resuspended RPMI/FBS. A media aliquot was then taken and served as a reference (ELISA p27). Aliquots of media were taken daily, spun to remove cells, and the supernatant was frozen at -80°C. The viral replication was followed for one or two weeks by quantifying the level of CAp27 protein in the supernatant. The CAp27 protein concentration was determined by enzyme-linked immunosorbent assay (Retro-Tek SIV p27 Antigen ELISA kit from Zeptometrix).

Protein isolation and analysis

Intracellular COS-7 RNA and proteins were harvested 48 hours after transfection by washing and scraping cells in phosphate-buffered saline (PBS). One half of the cells was harvested in RNA lysis buffer for genomic RNA quantitation (see below) while the other half was lysed in radioimmunoprecipitation (RIPA) assay buffer (Santa Cruz Biotechnologies Inc.; Tris-HCl, 50 mM, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, with protease inhibitors). After lysis on ice, cells were passaged six times through a 27-gauge needle, centrifuged for 10 minutes at $21,000 \times g$, 4°C and supernatants transferred to new tubes. Samples were fractionated on sodium dodecyl sulfate-8% polyacrylamide gels. After electrotransfer onto polyvinylidene difluoride (PVDF) membranes, Gag proteins were visualized by western blotting with one of the following primary antibodies: biotin-labeled anti-capsid antibody (p27 ELISA kit from Zeptometrix) or goat anti-FLAG tag antibody (sc-807G from Santa Cruz Biotechnologies, Inc.). Bound primary antibodies were detected using either streptavidin-horseradish peroxidase (p27 ELISA kit from Zeptometrix) or chicken anti-goat IgG-horseradish peroxidase (sc2953 from Santa Cruz Biotechnologies) by electrochemiluminescence (ECL Plus Western Blotting Detection Reagents from GE Healthcare) on a Fujifilm LAS-3000.

For virion fractions, media from transfected or infected cells was carefully removed without disturbing cell layer, filtered through 0.45µm filters to remove cells and debris, and centrifuged at $21,000 \times g$ for two hours at 4°C to pellet virus. The pellet was gently washed and resuspended in RIPA buffer. Aliquots were then treated as above for electrophoresis, blotting and visualization.

PBMC sample acquisition

The peripheral blood mononuclear cells of HIV-2 infected individuals were obtained from a cohort of commercial sex workers in Senegal that has been followed since 1985. Detailed clinical and epidemiological data concerning this cohort have been published elsewhere^{43,12}. The patients were all antiretroviral therapy-naïve, were asymptomatic, and had CD4⁺ T-cell counts above 200 per microliter at the time of sample acquisition. All subjects signed informed consent documents and participated in protocols approved by the Conseil National de Lutte

Contre le SIDA Comité Ethique et Juridique (Senegal) and the Harvard School of Public Health Human Subjects Committee.

RNA isolation and analysis

The total cellular RNA from transfected COS-7 or infected C8166 cells and the extracellular viral RNA fractions were purified using Stratagene's Absolutely RNA mini-prep and micro-prep kits, respectively. To pellet the viral particles, a fraction of the media was centrifuged for two hours at 4°C, at $21,000 \times g$. Purified RNAs from cells and viruses were used for RT-PCR (OneStep RT-PCR kit, Qiagen), or RNase protection assay (RPA III assay, Ambion), as described by the manufacturers. Briefly, the RNase protection assay was done using an antisense RNA probe complementary to the 1988-2137 region of HIV-2 ROD isolate containing the FLAG-tag insertion at the end of the *gag* gene. The antisense region was cloned into the pGEM7Zf(+) vector (Novagen) so that the T7 transcript has 41 nucleotides of vector origin at its 5' end. This non-HIV-2 tail is used as a marker of the RNase's digestion efficiency during the RPA experiment. Up to three protected bands are expected: one 174-nt long band corresponding to the FLAG-tagged *gag* RNA and two protected bands corresponding to the untagged *gag* RNA, 49- and 101-nt long.

For RNA derived from human PBMCs, RNA was extracted as described in MacNeil et al.¹². PolyA⁺ RNA was reverse transcribed using random hexamer primers. cDNAs were amplified first using primers P1 and P2. The P2 primer selectively targets and amplifies HIV-2 *gag* species, since its binding site is located downstream of the major splice donor site. The P1/P2 PCR products encompassed nts 2 to 555. A second, nested PCR was performed on an aliquot of the first P1/P2 PCR reaction using the P3/P4 primer pair. The P3/P4 PCR products encompassed nts 40 to 400. The final products are predicted to be either 361 bp-long DNA, for the unspliced *gag* species, or 219 bp-long DNA, if the 142 nts-long 5'UTR intron was spliced out (Fig. 1D). These PCR products were cloned into pGem7-Zf(+) and sequenced to verify their identities.

Luciferase template construction for *in vitro* transcription

The full length and truncated HIV-2 inserts (5'UTR plus the first 8 codons of *gag*) were generated using PCR (polymerase chain reaction). A sense primer (sNHERod) containing a *NheI* site and an antisense primer (asBsoBIrod) containing a *BsoBI* site were used to amplify the first 1-569 nucleotides of the HIV-2 genomic RNA, ROD isolate (GenBank: M15390; genomic RNA sequence starts at 1) and the first 1-569 nucleotides minus the 5'UTR intron (61-202) of HIV-2 genomic RNA¹³, respectively. Sense primers (sNHE186, sNHE198, sNHE486) containing a *NheI* site in combination with an antisense primer (asBsoBIrod) containing a *BsoBI* site were used to amplify 186-569, 198-569, and 486-569 of HIV-2 genomic RNA, ROD isolate, respectively. The PCR products were digested and then inserted via *NheI*/*BsoBI* sites into the phRL-CMV vector (just upstream of the second codon of the hRLuc reporter gene)(Promega). The C-box mutants were generated using the sNHERod primer and asBsoBIrod primer to amplify the first 569 nucleotides of the mutant A and the mutant G constructs. All constructs were checked by DNA sequencing.

In vitro transcription

The plasmids were linearized via *BamHI* digestion. The linearized DNA templates were then used in mScript (Epicentre) transcription reactions to synthesize RNA. Following incubation, the transcription reactions were treated with RNase-free DNase followed by ammonium acetate precipitation. The RNAs underwent 5' capping and polyadenylation (mScript mRNA Production System, Epicentre) followed by ammonium acetate precipitation.

***In vitro* translation**

Equimolar amounts (46nM) of the various mRNAs were used in comparison translation reaction sets. A 25.2 μ L volume of mRNA in water was denatured at 65°C for 3 minutes followed by snap cooling on ice for 3 minutes. The translation mix (70 μ L rabbit reticulocyte lysate, 1 μ L amino acid mixture minus leucine, 1 μ L amino acid mixture minus methionine, and 2.8 μ L KCl 2.5M; Flexi Rabbit Reticulocyte Lysate System, Promega) was added to the mRNA sample to initiate the reaction. The translation reactions were incubated at 30°C for 1 hour. A 22.0 μ L aliquot was taken from each sample at various time points and immediately placed on ice. The aliquot tubes stayed on ice until the luciferase assays were performed.

Luciferase assays

Twenty microliters of each translation reaction aliquot were plated (in sets) on a 96-well plate. A Tropic TR717 microplate luminometer was used to auto-inject each sample well with 100 μ L of Renilla Luciferase Assay Reagent (Promega) and quantify/record the resulting chemiluminescence product.

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Figure 1.

Analysis of HIV-2 *gag* mRNA species in cell culture. Schematic of the two forms of HIV-2 *gag* (and *gag-pol*) mRNAs, 5'UTR-unsliced (A) and 5'UTR-spliced (B). TAR, poly(A) signal, C-box, PBS, major SD, G-box, *gag*, and *pol* represent the trans-activation region, the poly(A) signal domain, the core of the C-box, the tRNA primer binding site, the major splice donor site, the core of the G-box, the Gag protein coding region, and the Pol protein coding region, respectively. The absent 5'UTR intron (nts 61-202) is represented by the dotted lines in (B). (C) Gel electrophoresis analysis of RT-PCR products from COS-7 cells transfected with wild type HIV-2 plasmid DNA (lanes 2-5) or from C8166 cells infected with wild type HIV-2 (lanes 6-9). The origin of the RNA used in the RT-PCRs, intracellular or extracellular (*i.e.* viral particles), is indicated below the gel. The primers used for the RT-PCRs are represented by arrows in (B). Lane 1 contains a 100 b.p. DNA ladder. (D) Gel electrophoresis analysis of RT-PCR products from HIV-2 seropositive patients (lanes 2-6). After reverse transcription, a first PCR was performed with primers P1 and P2. An aliquot of the P1/P2 PCR product was then used as template for a second, nested PCR with primers P3 and P4 (lanes 2-6). Sample identification numbers from the original reference⁴⁴ are indicated. Primers and expected products are described in the right panel.

Figure 2.

Effects of the 5'UTR intron on HIV-2 *gag* mRNA and protein species in cell culture. (A) The unique *gag* mRNA species produced upon transfection of HIV-2 5'UTR SD(-) plasmid DNA in COS-7 cells. The HIV-2 5'UTR SD(-) DNA contains mutations designed to abrogate the underlined 5'UTR splice donor site. The substitutions are in lower case letters. (B) The unique *gag* mRNA species produced upon transfection of HIV-2 5'UTR Δ-intron plasmid DNA in COS-7 cells. The HIV-2 5'UTR Δ-intron DNA contains a deletion of nucleotides 61-202, which corresponds to the 5'UTR intron. Two versions of 5'UTR SD(-) and Δ-intron plasmid DNA were used, one without a tag and one with a FLAG-tag inserted downstream of the *XhoI* site in the *gag* gene. (C) Western blot analysis of intracellular proteins from COS-7 cells co-transfected with equimolar amounts of 5'UTR SD(-) FLAG-tagged or untagged DNA and 5'UTR Δ-intron FLAG-tagged or untagged DNA. Blots were probed with either an anti-capsid primary antibody (top panel) or with an anti-FLAG primary antibody (bottom panel). Lane 1 contains the mock-transfected sample. DNA templates used for each co-transfection are designated below each respective lane (lanes 2-5). (D) (Top panel) Schematic of the radioactive probe and predicted protected fragments based on presence or absence of the FLAG-tag. (Bottom panel) RNase protection assay of intracellular RNAs. Lane 1 contains the mock-transfected sample. DNA templates used for each co-transfection are designated below each respective lane (lanes 2-5). Lanes 6 and 7 represent yeast RNA used as a non-specific target RNA control, incubated with and without RNases, respectively. (E) Quantitation of Gag protein species produced in the cytoplasm of cells co-transfected with 5'UTR SD(-) and Δ-intron HIV-2 plasmid DNA, with the FLAG-tag inserted in one or the other plasmid. The levels of the Gag protein species produced, either FLAG-tagged or untagged, were quantified from a western blot assay (C, lanes 4 and 5) and then corrected for the level of their corresponding mRNA (D, lanes 4 and 5). Three experiments were used to determine the fraction of Gag protein species in each cotransfection. A schematic of the cotransfected plasmid DNAs used is shown below the graph. (F) Western blots of intracellular and extracellular (vireon fraction centrifuged from cell culture media) Gag protein. A schematic of the cotransfected plasmid DNAs used is shown below the blots. The blots show both the intracellular and extracellular fractions contain a majority of Gag derived from the Δ-intron construct, and that both the FLAG-tagged and untagged Gag are present without discrimination in the virus fraction.

Figure 3.

Effects of the 5'UTR intron on translation of HIV-2 luciferase chimeras *in vitro*. (A) Wild type unspliced (top panel) or 5'UTR Δ -intron (bottom panel) HIV-2 sequences were fused to a Renilla luciferase open reading frame. The absent 5'UTR intron is represented by dotted lines. (B) Luciferase assays of translation reactions using the wild type unspliced luciferase construct (open squares) or the 5'UTR Δ -intron luciferase construct (closed circles). The y-axis represents the Renilla luciferase activity resulting from translation of the full-length or 5'UTR spliced constructs. The x-axis represents sample incubation times of the complete translation reactions. (C) RNase protection assay of translation reactions to determine relative mRNA stabilities. Aliquots of the same reactions used to quantify luciferase activity in panel (B) were extracted to isolate RNA, which was then subjected to RPA. Decay profiles unspliced (lanes 1-5) or spliced (lanes 6-10) were similar for these RNAs. Lanes 11 and 12 represent yeast RNA used as a non-specific target RNA control, incubated with and without RNases, respectively.

Figure 4.

Effects of C-box mutations on *in vitro* HIV-2 luciferase translation and HIV-2 replication. (A) RNA constructs containing C-box mutations used in the *in vitro* translation assays. The substitutions are in lower case letters. (B) Luciferase activity of *in vitro* translation reactions using the wild type luciferase construct (open squares), or C-box mutated constructs including mutant G (closed circles) or mutant A (closed triangles). (C) Gag mRNA produced following COS-7 transfection of HIV-2 DNA mutant A or G. The substitutions are in lower case letters with the splice acceptor site underlined. Predicted C-G-box wild-type base pairing is shown, with mismatches introduced in the A and G mutants shown for comparison. (D) Gel electrophoresis analysis of intracellular RT-PCR products from COS-7 cells transfected with either mutated A (lanes 2-3) or mutated G (lanes 4-5) DNA plasmids. Lane 1 contains a 100 b.p. DNA ladder. (E) Gel electrophoresis analysis of virus particles RT-PCR products from COS-7 cells transfected with either mutated A, mutated G (lanes 3-4), or wild-type (lanes 5-6) DNA plasmids. Lane 7 contains a 100 b.p. DNA ladder. (F) Replication of either wild-type (open squares), mutant A (closed circles), or mutant G (open circles) viruses in permissive C8166 cells. Viral production was measured by ELISA quantitation of viral p27 capsid protein.

Figure 5.

Model of the roles of the two forms of *gag* (or *gal-pol*) RNA species during HIV-2 replication. Both 5'UTR-spliced and —unspliced *gag* HIV-2 RNA molecules are exported from the nucleus into the cytoplasm where they serve as mRNA for Gag and Gag-Pol protein expression (small arrowheads). One functional difference between the two RNA species is that the 5'UTR spliced molecule is a better substrate for *gag* translation (this work). However, only the 5'UTR unspliced molecule is a substrate for packaging into assembling viral particles (this work). Furthermore, prior translation of the unspliced *gag* HIV-2 RNA appears to be necessary for its selection for packaging (in dimeric form)¹⁴. A co-translational binding event (double arrowhead) between the unspliced *gag* mRNA and the nascent Gag protein helps to select this RNA species for packaging¹⁴.