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Different effects of the TAR structure on HIV-1 and HIV-2 genomic RNA translation

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ABSTRACT

The 5'-untranslated region (5'-UTR) of the genomic RNA of human immunodeficiency viruses type-1 (HIV-1) and type-2 (HIV-2) is composed of highly structured RNA motifs essential for viral replication that are expected to interfere with Gag and Gag-Pol translation. Here, we have analyzed and compared the properties by which the viral 5'-UTR drives translation from the genomic RNA of both human immunodeficiency viruses. Our results showed that translation from the HIV-2 gRNA was very poor compared to that of HIV-1. This was rather due to the intrinsic structural motifs in their respective 5'-UTR without involvement of any viral protein. Further investigation pointed to a different role of TAR RNA, which was much inhibitory for HIV-2 translation. Altogether, these data highlight important structural and functional differences between these two human pathogens.

INTRODUCTION

Most eukaryotic mRNAs contain a 5'-terminal m⁷GpppN cap structure (where N is any nucleotide) that is incorporated during transcription (1). Cap-dependent translation begins with the recognition of the 5' cap structure by the eIF4F multimeric complex, which is composed of the cap binding protein eIF4E, the ATP-dependent RNA-helicase eIF4A and the scaffold protein eIF4G (2). Then, a 43S complex composed of the 40S ribosomal subunit and the tRNA initiator anchored to a set of initiation factors such as eIF2, eIF3, eIF1 and eIF1A is loaded onto the mRNA via multiple protein/protein and protein/RNA interactions. This results in the formation of the 48S pre-initiation complex, which scans the 5'-untranslated

region (5'-UTR) until the initiation codon (generally an AUG) is encountered (2–4). Several features within the 5'-UTR such as, upstream AUGs, nucleotide composition and the presence of secondary structures have a strong impact in 48S pre-initiation complex recruitment and, consequently, translational efficiency (5–7).

More than 20 years ago, study of Picornavirus translation has defined an alternative mechanism of ribosome recruitment, which is mediated by *cis*-acting RNA elements called Internal Ribosome Entry Sites (IRES) (8,9). These IRES elements allow cap-independent internal binding of the 43S pre-initiation complex onto the mRNA molecule independently from the cap structure (10). To date, IRES have been found in many viral and cellular mRNAs and were shown to be functional during inhibition of cap-dependent translation (10,11).

Human immunodeficiency viruses types-1 and -2 (HIV-1 and HIV-2) belong to the Lentivirus genus of the *Retroviridae* family and contain a capped and polyadenylated positive-stranded RNA molecule as genome. This genomic RNA (gRNA) is used both as template for translation of Gag and Gag-Pol precursors and as viral genome for viral particle assembly (12). The 5'-UTR present within the genomic RNA of both HIVs is organized in highly structured RNA motifs that contain signals required for most of the viral processes including *trans*-activation of transcription (TAR), reverse transcription (PBS), splicing (SD), dimerization (DIS) and packaging (ψ) (13–16). Interestingly, while the presence of these signals is conserved between HIV-1 and HIV-2, the length and folding of the 5'-UTR of both viruses strongly differs (13–15). These differences are particularly obvious at the level of the structure of the *trans*-activation response element (TAR), which is organized in a single stem-loop in HIV-1, while it is much longer and folded in a forked structure in HIV-2 (13,16–23). Although the TAR RNA element of HIV-1 was shown to impair

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ribosome entry at the 5'-end in cell-free *in vitro* systems, it was recently shown that ribosomal scanning along the 5'-UTR can take place in cultured cells (24–27). However, protein synthesis from the HIV-1 can also occur via an IRES element that functions under specific conditions such as G2/M phase of the cell cycle or during cellular stress (28–30). Interestingly, IRES elements have also been mapped within the Gag coding region of HIV-1 and HIV-2 indicating that genomic RNA translation is a complex process tightly controlled by different RNA motifs (31,32). In HIV-2, the Gag coding region was shown to contain three independent IRESes able to recruit three independent pre-initiation complexes in the complete absence of the 5'-UTR (32–34). This ability relies in the capacity of RNA structures present within the Gag coding region to directly recruit the 40S ribosomal subunit and eIF3 (35). Despite the fact that an IRES was also identified within the HIV-1 Gag coding region, the molecular mechanism of ribosome recruitment by this RNA element has not been investigated yet (31).

Given the strong differences in the structure of the 5'-UTR of both human immunodeficiency viruses and the ability to drive ribosome recruitment via multiple mechanisms, we have analyzed the translational properties of both gRNAs in cultured cells. Results presented here show that the HIV-2 5'-UTR and specifically the TAR RNA structure slow translation resulting in low levels of Gag production. This sharply contrasts with protein synthesis from the HIV-1 gRNA, which occurs very efficiently despite the presence of a structured 5'-UTR.

MATERIALS AND METHODS

DNA constructs

pNL4-3-*Renilla* construct was obtained by inserting a HA-tagged *Renilla* luciferase-coding region in the SpeI site present in the Gag coding region (1507–1512) of the infectious HIV-1 molecular clone pNL4-3 (36). Similarly, pRod10-*Renilla* was obtained by inserting the HA-tagged *Renilla* luciferase-coding region in the HindIII site present in the Gag coding region (1457–1462) of the infectious molecular clone pRod10 (37). All p*Renilla*-derived constructs were obtained by inserting the selected region between the PvuII–BamHI sites of the p*Renilla* vector as was previously described (38–40). The 5'-UTR and the 5'-UTR-Gag sequences were obtained by PCR using the pNL4-3 and pROD10 proviral DNA as template (36,37). The human p*Renilla*-globin vector was previously described (40). cDNA sequences for the human GAPDH, NADH and cyclin D2 5'-UTR were obtained by RT-PCR using HeLa cells total RNA as template. The Line-1 5'-UTR was obtained from the pJM101f L1.3 vector (kindly provided by Dr Gael Cristofari, Université de Nice, France). HIV-1/HIV-2, HIV-2/HIV-1 and HIV-2 spliced constructs were obtained by a two-step PCR strategy. All constructed clones were verified by sequencing.

In vitro transcription

In vitro transcription was carried as previously described (38–40). Briefly, p*Renilla*-derived vectors containing a 100 nt long poly(A) tail were linearized by EcoRI digestion. Capped mRNAs were obtained using 1 µg of linear DNA template; 80 U of T7 RNA polymerase (Promega); 40 U of RNasin (Promega); 10 mM of rCTP, rUTP, rATP; 0.36 mM rGTP; 30 mM DTT in transcription buffer [40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine and 10 mM NaCl] and 1.24 mM of m⁷GpppG cap analogue (New England Biolabs). Transcription reaction was carried out at 37°C for 2 h and mRNAs were DNase treated and precipitated with 1 volume of ammonium acetate 5 M (2.5 M final concentration). The integrity of mRNAs was checked on agarose gel electrophoresis and their concentration was quantified by spectrophotometry at 260 nm using Nanodrop (NanoDrop Technologies, Wilmington, DE, USA).

Cell culture, DNA and RNA transfection

T-lymphocytes (Molt-4 cells) were maintained in RPMI growth media (Gibco, BRL) supplemented with 10% FCS and 1% L-glutamine at 37°C in a 5% CO₂ atmosphere. Cells were transfected with proviral-*Renilla* DNAs using the GeneJuice[®] Transfection Reagent (Novagen, EMD Chemicals Inc.) as indicated by the supplier. Cells extracts were prepared at 24 h post-transfection and used either for *Renilla* activity or RNA extraction and RT-qPCR. HeLa cells were maintained in DMEM growth media (Gibco, BRL) supplemented with 10% FCS at 37°C and 5% CO₂. DNA transfection was carried out using JetPEI[™] (PolyPlus Transfection) following supplier's indications. Cells or supernatants were recovered at 24 h post-transfection and used either for *Renilla* activity, RNA extraction and RT-qPCR, reverse transcriptase (RT) assay or western blot. For RNA transfection, HeLa, Cos7 and 3T3 cells were maintained in DMEM growth media (Gibco, BRL) supplemented with 10% FCS at 37°C and 5% CO₂. PBMCs-derived human macrophages were obtained and maintained as previously described (41). mRNA transfection was carried out using Mirus mRNA transfection system (Mirus Biosciences) as previously described (39). mRNA-transfected cells were incubated for 3 h at 37°C, in a 5% CO₂ atmosphere prior to *Renilla* activity analysis. When appropriate, cells were treated with 0.5 mM sodium arsenite for 30 min at 37°C and 5% CO₂ and cells extracts were prepared for western blot analysis.

RT assay

Supernatants from HeLa cells transfected with proviral DNA were spin down at 10 000 rpm for 30 s to eliminate cellular debris and then ultracentrifugated in a Beckman TL-100 rotor at 75 000 rpm during 1 h at 4°C. Viral particles were resuspended in RT buffer [60 mM Tris-HCl (pH 8.0), 180 mM KCl, 6 mM MgCl₂, 0.6 mM EGTA (pH 8.0), 0.12% Triton X-100, 6 mM DTT, 12 µg/ml poly rA and 6 µg/ml oligo dT] and RT activity was determined as described previously (42).

***In vitro* translation and complementation assays**

All mRNAs were translated in 50% of Untreated Rabbit Reticulocyte System (Promega Co., Madison, WI, USA) in the presence of KCl (75 mM), MgCl₂ (0.5 mM), 20 μM of each amino acid for 30 min at 30°C as previously described (40). When corresponded mRNAs were pre-incubated for 5 min at 37°C with hypotonic buffer [10 mM HEPES-KOH (pH 7.6); 10 mM KOAc; 0.5 mM MgOAc and protease inhibitor cocktail (Roche)] or HeLa cells extracts corresponding to 15 μg of total protein.

***Renilla* activity**

Renilla activity was measured using the *Renilla* Luciferase Assay System (Promega Co., Madison, WI, USA) in a Veritas Luminometer (TurnerBiosystems) as previously described (39).

Gag-*Renilla*-specific activity analysis

HIV-1 and HIV-2 Gag-*Renilla* fusion proteins were expressed in the Flexi[®] Rabbit Reticulocyte System (Promega Co., Madison, WI, USA) as described above in the presence of 0.6 mCi of [³⁵S]-methionine (Perkin Elmer) and *Renilla* activity was measured as indicated above. Equivalent Gag-*Renilla* activities were precipitated with 100% TCA (1 volume TCA for 4 volumes of protein) for 10 min on ice and washed three times with acetone. TCA-precipitated radioactivity was quantified using the FLA-5000 PhosphorImager (Fujifilm).

Western blot analysis

Cells extracts were subjected to 10% SDS-PAGE and transferred to PVDF membrane (Amersham Biosciences, UK Ltd). The membrane was blocked for at least 2 h with 5% dried milk in TBST 1× and incubated overnight at 4°C with a 1/1000 dilution of antibodies directed against SIVmac Gag (43), eIF2α-P (US Biological) or actin (Santa Cruz Biotechnologies). After three washes with TBST 1×, an antirabbit horseradish peroxidase (HRP)-coupled secondary antibody was added and incubated for 1 h at RT. The membrane was washed three times with TBST and HRP was revealed by using SuperSignal[®] West Pico Chemiluminiscent Substrate (Pierce, Rockford, IL, USA).

RNA extraction and RT-qPCR

RNA extraction and RT-qPCR were performed exactly as previously described (44). Briefly, HeLa cells transfected with *Renilla* based *in vitro* transcribed mRNAs were washed intensively with PBS and lysed with 200 μl of RLNa buffer [10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 0.5% NP40 and 15 U/ml of RNaseOUT (Invitrogen Co.)]. Whole-cell extracts were recovered and RNA extraction was carried out by adding 1 ml of TRIzol[®] Reagent (Invitrogen Co.) as indicated by the manufacturer. Extracted RNAs (200 ng) were reverse-transcribed using the qScript[™] Flex cDNA kit (Quanta Biosciences). For quantitative PCR, a 20 μl reaction mix was prepared with 5 μl of template cDNAs (previously diluted to 1/10), 10 μl of MESA green SYBR

premix (Eurogentec), 0.2 μM of sense and antisense primers and subjected to amplification using a fluorescence thermocycler (Applied Biosystems 7000 Real-time PCR, Foster City, CA, USA). The housekeeping gene GAPDH was amplified in parallel to serve as a control reference. Relative copy numbers of *Renilla* luciferase cDNAs were compared to GAPDH using x^{-DCt} (where x corresponds to the experimentally calculated amplification efficiency of each primer couple). Primers used to quantify *Renilla* and GAPDH mRNAs by qPCR were described previously (44).

RNA secondary structure predictions

Secondary structures were predicted online using the mfold software (45) available at the unfold server (<http://mfold.rna.albany.edu/>).

RESULTS

Low Gag synthesis from the HIV-2 provirus in human cells

The Gag polyprotein is the major structural component of the retroviral particle and around 5000 molecules of this polyprotein are contained in an immature virion (46). However, up to 80% of the Gag polyprotein produced could undergo degradation in the cytoplasm (47), placing efficient Gag synthesis as a critical step to ensure viral production and the spread of infection. Although HIV-1 and HIV-2 are closely related, they strongly differ in their replication rates and viral production (48,49). These data prompted us to study the levels of Gag expression from the HIV-1 pNL4-3 (36) and HIV-2 pRod10 (37) molecular clones in cultured cells (Figure 1). As a first approach, we analyzed the intracellular levels of Gag polyprotein in extracts derived from HIV-1 and HIV-2 expressing HeLa cells by using an antibody raised against the matrix portion of the SIVmac Gag (43). As observed, this antibody recognized both Gag proteins and its processed forms (Figure 1A, see western blot). However, results obtained showed that cells expressing HIV-1 accumulate much more intracellular Gag than cells expressing HIV-2 suggesting that HIV-2 Gag could be produced at lower levels. Similar results were obtained with another antibody recognizing the capsid region of Gag (data not shown). Then, to rule out the possibility that lower intracellular levels of HIV-2 Gag reflect a higher rate of viral particles release, we measured the RT activity in the supernatant of transfected cells. This comparison could be possible as both recombinant viral enzymes were shown to possess similar specific activities (50). Results obtained showed that cells transfected with the HIV-1 molecular clone produced more than threefold RT activity than cells transfected with the HIV-2 clone suggesting that lower Gag production in HIV-2 expressing cells is not related to the release of higher amounts of HIV-2 Gag to the supernatant (Figure 1A, left graph). However, these results could reflect a different affinity of the SIV Gag antibody or even differences in the specific activities of the virion-associated RT enzymes rather than differences at the transcriptional or post-transcriptional

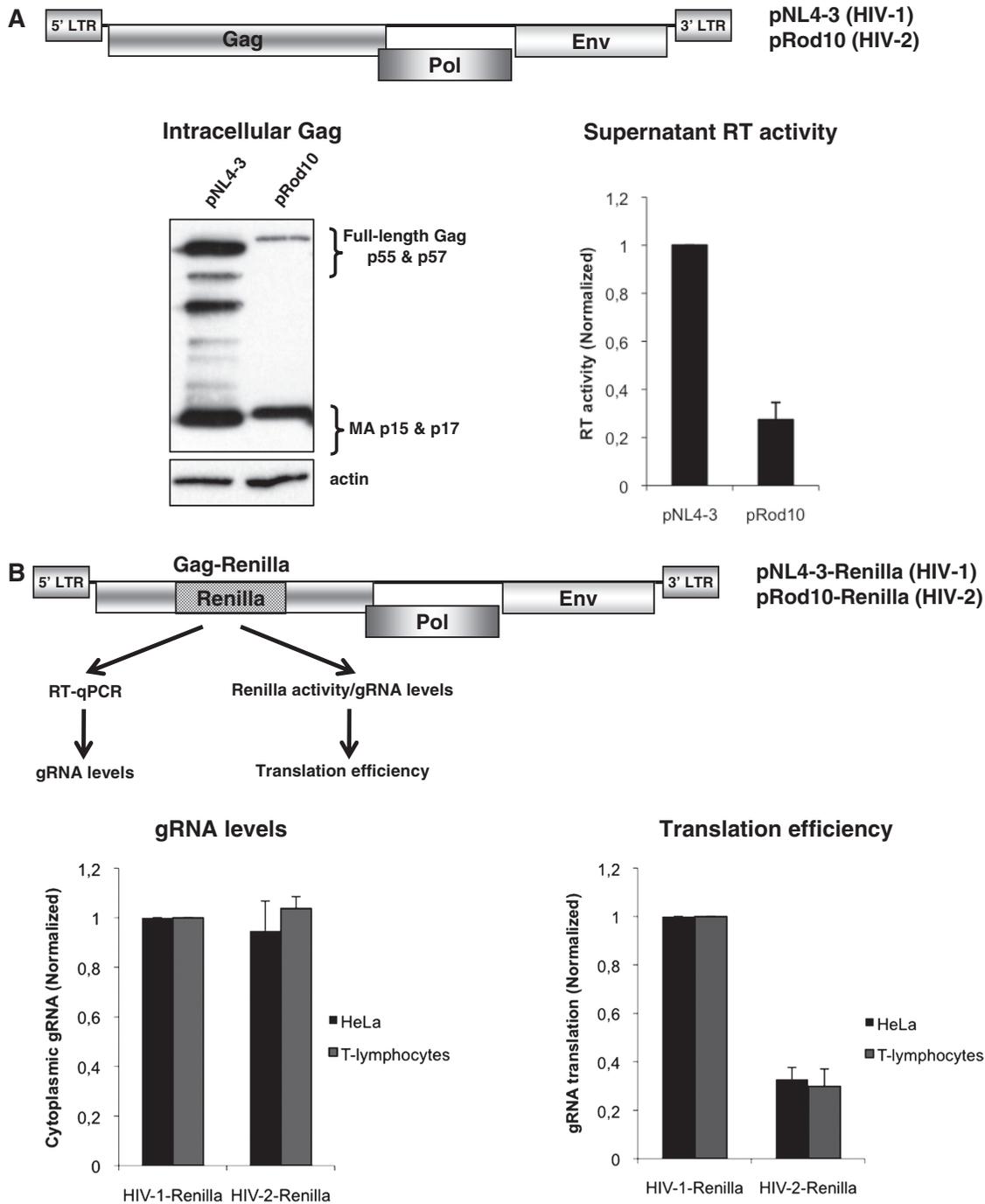


Figure 1. Low HIV-2 Gag production in human cells. (A) Wild-type pNL4.3 (HIV-1) and pROD10 (HIV-2) proviral plasmids (upper scheme, regulatory and accessory proteins were omitted for simplicity) were transfected in HeLa cells and cells extracts from cells expressing HIV-1 and HIV-2 were prepared and probed against Gag by western blot as described in ‘Materials and Methods’ section. In parallel, viral production was determined by measuring the RT activity present in supernatant of transfected cells as described in ‘Materials and Methods’ section (left graph). (B) Schematic representation of the HIV-1 and HIV-2 proviral-*Renilla* DNA constructs used in this study (regulatory and accessory proteins were omitted for simplicity). They correspond to pNL4.3 (HIV-1) and pROD10 (HIV-2) proviral plasmids in which the *Renilla* reporter gene was inserted in frame within the Gag coding region as described in ‘Materials and Methods’ section. gRNA levels (left graph) and the translation efficiency from the gRNA (right graph) were determined in T-lymphocytes (black bars) and HeLa cells (gray bars) at 24 hpt as indicated. Results are normalized to values obtained for pNL4.3-*Renilla* (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three duplicate independent experiments.

level. Thus, to avoid any of the bias mentioned above and to perform a direct comparison between these two viruses, we have developed a quantitative system allowing us to directly analyze Gag production and gRNA levels by

constructing HIV-1 and HIV-2-derived proviral molecular clones in which the *Renilla* luciferase reporter gene was inserted in frame within the Gag coding region (Figure 1B and ‘Materials and Methods’ section). These modified

proviruses are transcribed, spliced, exported and translated as the wild-type proviruses but they are defective in budding (data not shown). Thus, by using these *Renilla* proviruses we were able to compare side by side both viruses under similar conditions and have a better estimation of translational efficiency from the gRNA by measuring the enzymatic activity of the Gag–*Renilla* fusion normalized to the amount of gRNA (determined by RT–qPCR against the *Renilla* ORF). Transfection of these modified proviral DNAs in human T-lymphocytes or HeLa cells revealed that translational efficiency (Gag–*Renilla* activity normalized by gRNA) from the HIV-1 gRNA was three- to fourfold higher than that of HIV-2 gRNA (Figure 1B, left graph). Quantification of the gRNA levels by RT–qPCR showed no significant differences between HIV-1 and HIV-2 indicating that they could be present at similar levels within the cells (Figure 1B, right graph). It should be noted that differences in translation efficiency were similar in T-lymphocytes and HeLa cells suggesting that lower HIV-2 gRNA translation with respect to HIV-1 occurs in human cells that are permissive for viral replication.

Taken together, these results indicate that HIV-2 produces lower Gag levels than HIV-1 during a single round replication cycle probably due to a lower translational efficiency of its gRNA.

Translation of an HIV-2 gag mRNA is inefficient

Although results present above suggest that HIV-2 gRNA translation is less efficient than that of HIV-1 during a single round replication cycle, the molecular processes that precede gRNA translation (i.e. transcription, 3'-end processing and nuclear export) are complex and controlled by several viral and cellular proteins (51). These processes are not fully understood and it is unknown whether they occur at similar rates in both viruses making very difficult to compare gRNA translation in the context of a full-length provirus. Thus, an important challenge at this stage was to determine the most suitable experimental strategy to better evaluate the gRNA translational control in a cellular context. To focus exclusively on translation, we decided to use an mRNA transfection strategy that allowed us to study different *in vitro* generated transcripts directly in the cytoplasm of transfected cells. Compared to other mRNA transfection strategies, our system was very efficient with a wide range of mRNAs and allowed us to measure protein product as early as 3 h post-transfection using very low levels of transfected mRNAs (0.125 pmol, corresponding to 134 ng of the larger RNA used in this study). This is important as it allowed us to have a rapid estimation of protein synthesis rather than a cumulative effect of mRNA and protein product accumulation and/or degradation.

Thus, we first designed transcripts that contained all the viral elements involved in translation; these are the complete viral 5'-UTR (starting at the +1 site of transcription, HIV-2 Rod10 and HIV-1 NL4-3 strains) followed by the entire Gag coding region, which were appended in frame to the *Renilla* luciferase reporter gene which serves as a readout of Gag protein expression. These *in vitro*

transcribed gag mRNAs contained an m⁷GpppG cap structure at their 5'-end and a poly (A) tail of 100 residues at the 3' extremity as was previously described (40) (see cartoon in Figure 2A). Equimolar levels of HIV-2 and HIV-1 gag mRNAs were transfected in HeLa cells and Gag expression was measured by reading *Renilla* activity at 3 h post mRNA transfection. This revealed that Gag expression from the HIV-2 gag mRNA was much lower when compared to HIV-1 (Figure 2A, left graph). RT–qPCR quantification of transfected mRNAs revealed no major differences in their intracellular levels indicating that differences in Gag expression might not reflect differences in transfection efficiency or stability of both gag mRNAs (Figure 2A, right graph). This is not surprising as the HIV-1 and HIV-2 genomic RNAs were shown to be stable for several hours in the cytoplasm of expressing cells (52). To rule out the possibility that lower Gag–*Renilla* levels from cells transfected with the HIV-2 gag mRNA were due to a lower specific activity of the HIV-2 Gag–*Renilla* fusion protein, we generated HIV-1 and HIV-2 ³⁵S-labeled Gag–*Renilla in vitro* and volumes corresponding to equivalent activities for each Gag–*Renilla* product were precipitated with TCA and the associated radioactivity was quantified. As observed, no major differences were observed indicating that both Gag–*Renilla* fusions have similar specific activities (Figure 2B). As an additional control, we added two HA tags at the C-terminus of each Gag–*Renilla* fusion and we detected proteins by western blotting after mRNA transfection (data not shown). In agreement with results obtained using the *Renilla* activity as a measure, while HIV-1 Gag–*Renilla* product was easily detected by western blot, HIV-2 Gag–*Renilla* was barely detected and only a faint band was only observed at longer expositions (data not shown). Then, to rule out the possibility that differences in HIV-1 and HIV-2 Gag expression could reflect a faster degradation of the HIV-2 Gag–*Renilla* product, we analyzed the stability of both Gag–*Renilla* fusion proteins (Figure 2C). For this, HIV-1 and HIV-2 gag mRNAs were transfected as in Figure 1A and the translation inhibitor cycloheximide (CHX) was added at 3 h post mRNA transfection. We observed that both Gag–*Renilla* fusion proteins decayed at similar rates and thus presented the same stability indicating that differences in Gag expression occur at the level of translation. We were then interested to determine whether lower expression from the HIV-2 gag mRNA compared to HIV-1 could occur in other cell types. For this, we transfected both gag mRNAs into African green monkey fibroblasts (Cos7), murine fibroblasts (3T3) or peripheral blood mononuclear cells (PBMC)-derived human macrophages and Gag–*Renilla* expression was determined (Figure 2D). As it could be observed, no cellular tropism was evidenced and lower HIV-2 Gag–*Renilla* expression was reported in all cell types tested including primary human macrophages which are natural hosts for both human viruses. Interestingly, these differences were not observed in the untreated rabbit reticulocyte lysate (URRL) *in vitro* system (40), suggesting that host factors and/or the cellular environment rather than differences in the specific activity

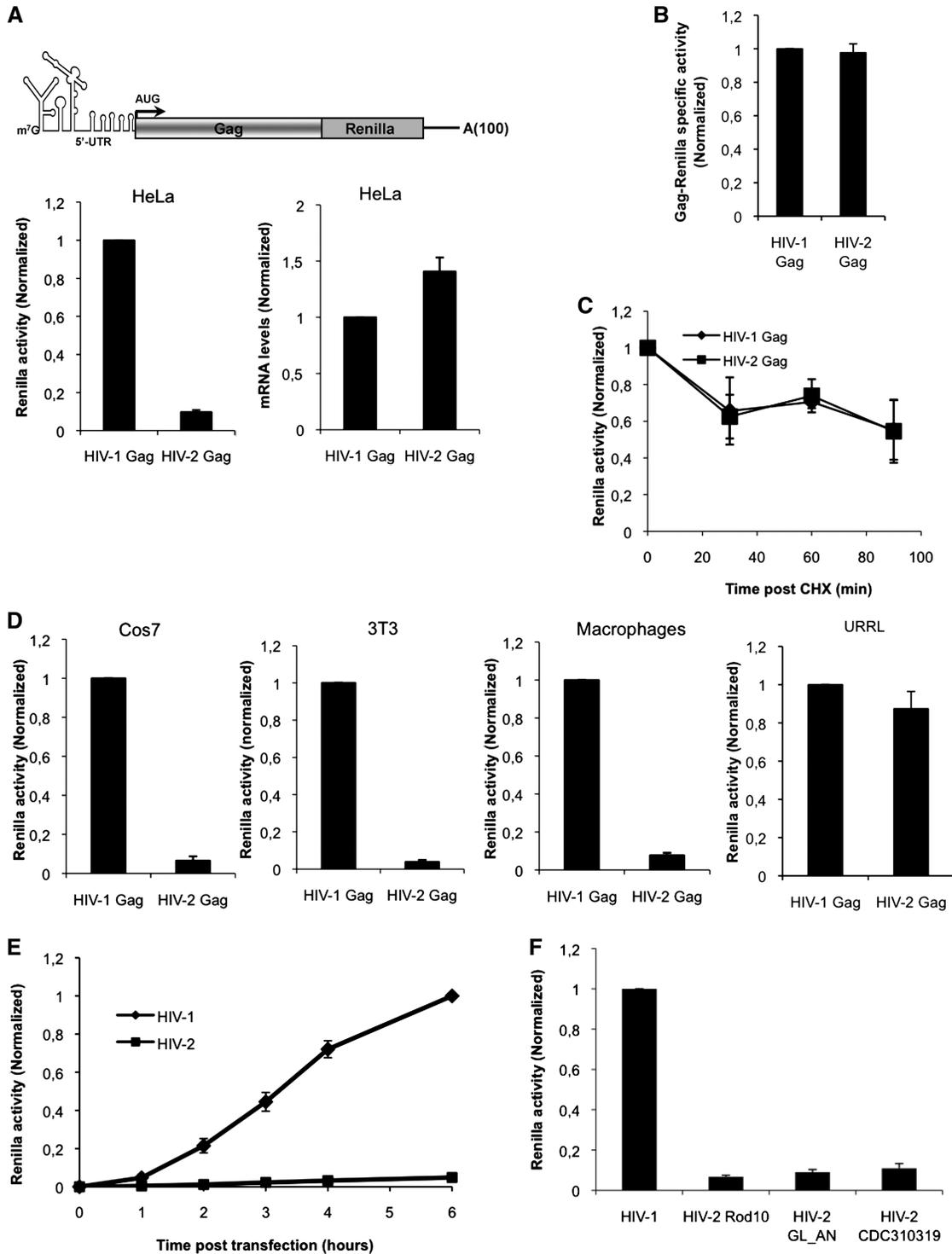


Figure 2. Translation of the HIV-2 gag mRNA is inefficient in cells. (A) About 0,125 pmoles of *in vitro* transcribed HIV-2 and HIV-1 gag mRNAs (see cartoon on the graph) were transfected into HeLa cells as described in ‘Materials and Methods’ section. *Renilla* activity (graph on the left) and transfected mRNA levels (graph on the right) were measured at 3 hpt. Results were normalized to values obtained for HIV-1 gag mRNA (HIV-1 Gag, arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (B) Specific activities of *in vitro* translated ³⁵S-labeled HIV-1 and HIV-2 Gag–*Renilla* fusion proteins were analyzed as described in ‘Materials and Methods’ section. Results were first normalized by the methionine content and then to values obtained for HIV-1 Gag–*Renilla* (HIV-1 Gag, arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (C) About 0.125 pmol of HIV-2 and HIV-1 gag mRNAs were transfected in HeLa cells and 3 hpt CHX was added at 100 μ g/ml. *Renilla* activity was analyzed at 0, 30, 60 and 90 min post CHX treatment as indicated in the figure. Results are normalized to values obtained at time 0 (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (D) About 0.125 pmol of HIV-2 and HIV-1 gag mRNAs were transfected in Cos7 cells, 3T3 cells or human macrophages or translated *in vitro* in the URRL system and *Renilla* activity was measured at 3 hpt (transfected cells) or 30 min (*in vitro* translation). Results were normalized to values obtained for HIV-1 gag mRNA (HIV-1 Gag, arbitrary set to 1) and expressed as

(continued)

of the Gag–Renilla fusion proteins are critical in regulating such differences (see below).

Then, we measured the kinetics of Gag expression from HIV-2 and HIV-1 gag mRNAs over time (Figure 2E). Once again, a much lower level of protein synthesis from the HIV-2 gag mRNA was observed at each time point suggesting that differences in Gag expression between HIV-2 and HIV-1 were not due to a delayed kinetics of HIV-2 translation.

Finally and in order to rule out the possibility that inefficient translation could reflect a specific feature of the HIV-2 strain used in these experiments (HIV-2 Rod10), we analyzed translation from HIV-2 gag mRNA constructs carrying sequences derived from other isolates. For this, we cloned the 5'-UTR-Gag region from the HIV-2 molecular clone GL-AN, which is able to replicate in a wide variety of lymphocytic cell lines of human and simian origins (53). We also used sequences from the primary isolate HIV-2 CDC₃₁₀₃₁₉ (54) that were amplified by using proviral DNA obtained from infected T-lymphocytes (our unpublished results). As observed with the Rod10 strain, low Gag expression was reported with these two HIV-2 isolates indicating that inefficient Gag synthesis (with respect to HIV-1 NL4-3) is a conserved feature among HIV-2 isolates (Figure 2F).

Taken together, these results indicate that differences in HIV-1 and HIV-2 Gag synthesis may reflect intrinsic differences between both gag mRNA sequence.

The 5'-UTR determines lower Gag expression from the HIV-2 gag mRNA

Then, we went on to characterize the RNA determinants that could account for low translation of the HIV-2 gag mRNA. Interestingly, we have previously shown that an HIV-2 gag mRNA lacking the entire 5'-UTR (leaderless RNA) showed enhanced Gag protein expression *in vitro* in a mechanism driven by the IRES located within the Gag coding region (32–34). These previously published observations suggest that ribosomal entry from the Gag coding region can be competed with ribosomal entry from the 5'-UTR. To address this hypothesis, we designed chimeric gag mRNAs in which the 5'-UTR of each type of virus was appended to the complete Gag coding region of the other (see cartoons in Figure 3). These 5'-UTR chimeric gag mRNAs were transfected in HeLa cells and Gag expression was determined by measurement of the Renilla activity at 3 h post mRNA transfection. Interestingly, when the HIV-2 5'-UTR was driving HIV-1 Gag synthesis, translation was dramatically reduced (by about 6-fold) compared to the wild-type HIV-1 gag mRNA (Figure 3). Conversely, when the HIV-1 5'-UTR was driving HIV-2 Gag synthesis,

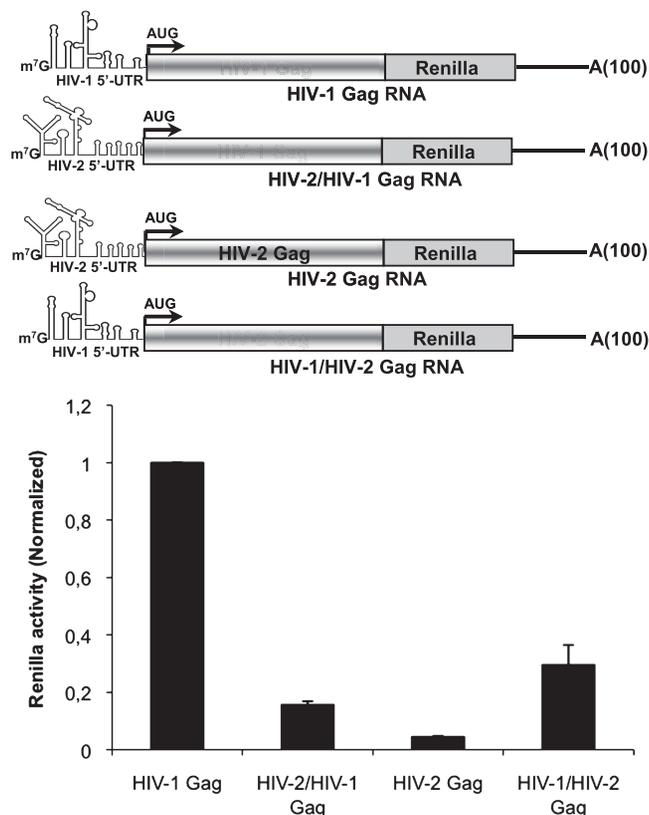


Figure 3. The HIV-2 5'-UTR impairs Gag expression. About 0.125 pmol of chimeric gag mRNAs in which the 5'-UTR of one virus was appended to the Gag coding region of the other (see cartoon) were transfected in HeLa cells and Renilla activity was measured at 3 hpt. In the cartoon, HIV-2/HIV-1 Gag refers to the RNA that harbors the HIV-2 5'-UTR followed by the HIV-1 Gag coding region. HIV-1/HIV-2 RNA refers to the Renilla RNA that harbors the HIV-1 5'-UTR followed by the HIV-2 Gag coding region. Results were normalized to values obtained for wild-type HIV-1 gag mRNA (HIV-1 Gag, arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments.

translation was strongly increased (by 6-fold), suggesting that specific sequences and/or RNA structures present within the HIV-2 5'-UTR may interfere with translation. It should be noted that replacement of the HIV-2 5'-UTR was not sufficient to reach a level of Gag synthesis that would be comparable to HIV-1 suggesting that the HIV-2 5'-UTR might compete with the IRESes present within the Gag coding region for ribosome recruitment (see below).

Taken together, these results strongly indicate that the 5'-UTR of HIV-2 interferes with efficient ribosome recruitment onto the gag mRNA.

Figure 2. Continued

mean \pm SD corresponding to values obtained in three independent duplicate experiments. (E) About 0.125 pmol of HIV-2 and HIV-1 gag mRNAs were transfected in HeLa cells and Renilla activity was measured at 0, 1, 2, 3, 4 and 6 hpt. Results are normalized to values obtained for HIV-1 gag mRNA (arbitrary set to 1 at 6 hpt point time) and expressed as mean \pm SD corresponding to values obtained in three independent duplicated experiments. (F) About 0.125 pmol of gag mRNAs derived from different HIV-2 strains were transfected in HeLa cells and Gag–Renilla expression was compared to HIV-1. Renilla activity was measured at 3 hpt. Results are normalized to values obtained for HIV-1 (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments.

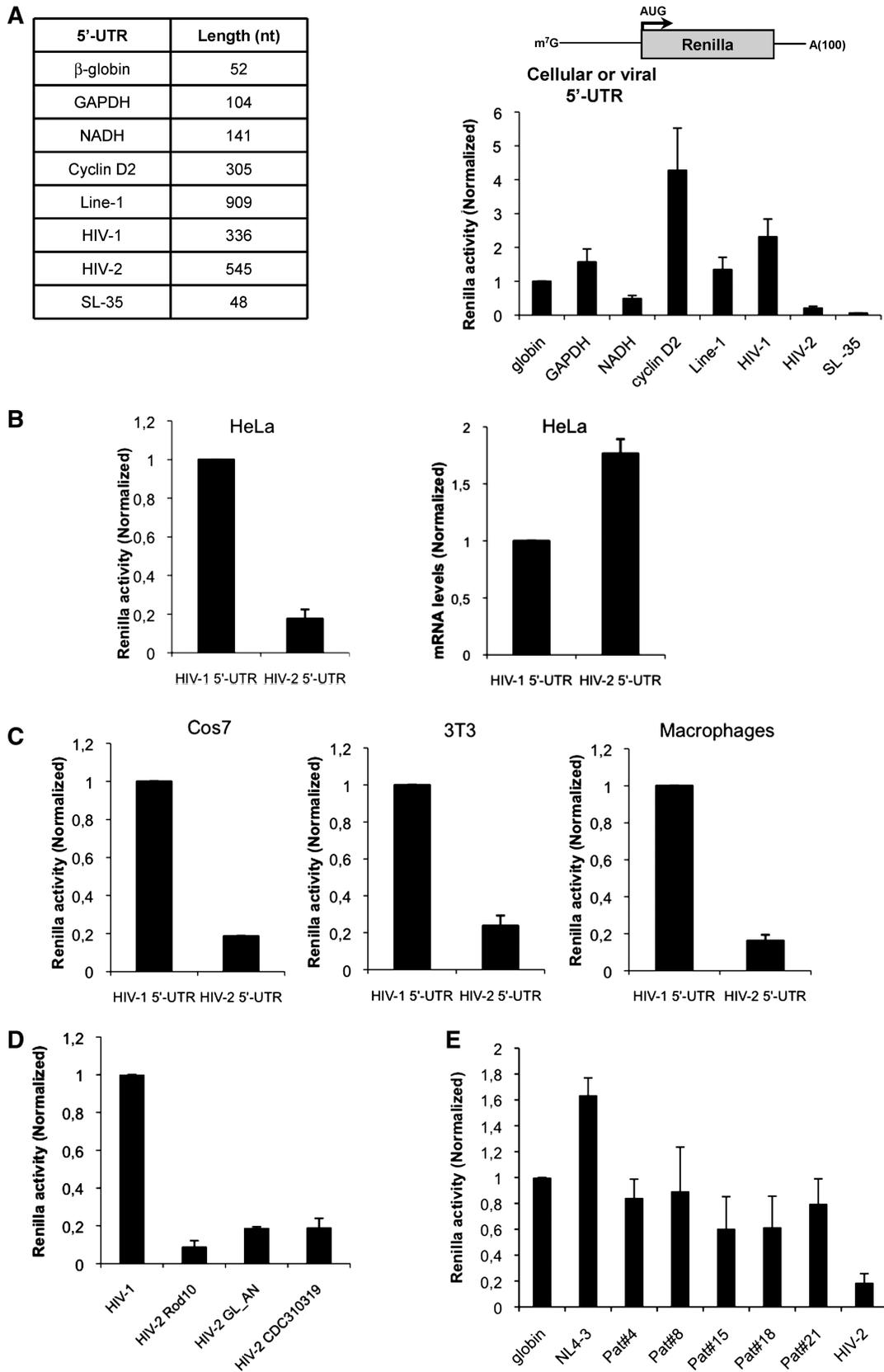


Figure 4. The HIV-2 5'-UTR interferes with ribosome recruitment. (A) About 0.125 pmol of *Renilla* RNAs in which translation was driven by different 5'-UTR (see table on the left) were transfected in HeLa cells as described in 'Materials and Methods' section. *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for human β -globin 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (B) About 0.125 pmol of 5'-UTR-*Renilla* RNAs (see cartoon on the graph)

(continued)

Inefficient translation driven by the HIV-2 5'-UTR

Results presented above suggest that inefficient translation of HIV-2 gag mRNA could be attributed to a negative effect of the 5'-UTR rather than any effect of the Gag coding region. To further confirm these observations, we have compared translation driven by the 5'-UTR of both human viruses in the absence of any further viral sequences (see cartoon, Figure 4A). To place our comparison in a more physiological context, we also included *Renilla* mRNAs bearing the 5'-UTR from different cellular transcripts. These cellular 5'-UTRs were selected on the basis of their length and complexity and included human β -globin, GAPDH, NADH, cyclin D2 and Line-1. As such, while the human β -globin 5'-UTR is short (52 nt long) and relatively unstructured, the length and complexity increased through GAPDH, NADH, cyclin D2 to Line-1 5'-UTR, which is longer (909 nt long), GC-rich and highly structured (see table on the left of Figure 4A). To our surprise, translation driven by the HIV-1 5'-UTR was very efficient and even higher than that of classical cellular 5'-UTR (Figure 4A). This contrasts with translation driven by the HIV-2 5'-UTR which was extremely low and only comparable to that of a *Renilla* transcript containing a 48 nt-long synthetic 5'-UTR forming an inhibitory stem-loop structure of -35 kcal/mol (SL-35 in Figure 4A). Similar to results obtained with gag mRNAs in Figure 2A, lower translation driven by the HIV-2 5'-UTR in HeLa cells (Figure 3B, left graph) may not reflect differences in transfected mRNA levels (Figure 4B, right graph) and were independent of the cell type (Figure 4C) and the HIV-2 strain used (Figure 4D) confirming that the HIV-2 5'-UTR is less efficient than that of HIV-1 to drive protein synthesis. However, it could be argued that high translation conferred by the HIV-1 5'-UTR was a peculiar feature of the NL4-3 molecular clone. Thus, 5'-UTRs from different HIV-1 clinical isolates obtained from a cohort of patients from the Croix Rousse Hospital in Lyon (France) were analyzed and compared to HIV-2 (Figure 4E). Interestingly, translation driven by the 5'-UTR of HIV-1 primary isolates was comparable to that of β -globin and NL4-3 and always higher than protein synthesis driven by the HIV-2 5'-UTR.

Taken together, these results confirm that the 5'-UTR present within the HIV-2 gRNA is very inefficient in support translation.

The TAR RNA structure interferes with HIV-2 translation

We then wanted to determine whether lower translation of HIV-2 could be attributed to any specific RNA determinant present within the 5'-UTR. For this, we have introduced 5' and 3' deletions within both viral 5'-UTRs and analyzed expression of the resulting *Renilla* transcripts (see cartoon in Figure 5). It should be mentioned that all these transcripts contains the same context surrounding the AUG start codon to avoid any bias due to differences in start site recognition. As observed in Figure 5, a mutant in which a 3' deletion was introduced from the end of PBS to the AUG start codon (called 3' del) did not significantly alter expression driven by the 5'-UTR of HIV-1 or HIV-2 indicating that the 3' end of the HIV-2 5'-UTR is not responsible for inefficient translation. However, when the TAR RNA element was deleted from the HIV-2 5'-UTR, translation was massively increased rendering *Renilla* expression as efficient as that driven by the HIV-1 5'-UTR indicating that HIV-2 TAR interferes with translation (compare HIV-1 5'-UTR and HIV-2 Δ TAR in Figure 5A). Several studies have determined the conformation of the HIV-1 and HIV-2 TAR structures (13,16–23). As such, while the HIV-1 TAR structure forms a single stem loop, the HIV-2 TAR structure can adopt two different conformations that are longer and more complex than that of HIV-1 (Figure 5B). Nevertheless, the stability of such structures could be sufficient to interfere with translation (55–57). Indeed, HIV-1 TAR was shown to be a potent inhibitor of translation in cell-free systems and *Xenopus* oocytes (24,26,27). However, such an effect has been shown to be much smaller in intact cells (58–60). Interestingly, we observed that deletion of HIV-1 TAR structure in cells did not have a strong effect on translation, thus pointing out for a specific role of HIV-2 TAR in inhibiting translation (Figure 5A). The poor inhibitory effect of HIV-1 TAR that we observed in cells compared to previous studies could be mostly attributed to the context on which its effects were analyzed. Most of the previous studies were based on the addition of a TAR structure-containing region (usually nucleotides +1 to +81, +111 or +282 from the HIV-1 5'-UTR) in front of a reporter gene (CAT for instance). Then, translational efficiency of these TAR-containing mRNAs was compared with translation of CAT mRNAs that are devoid of any RNA secondary structure (24,26,27). In this present study,

Figure 4. Continued

were transfected in HeLa cells. *Renilla* activity and transfected RNA levels were determined at 3 hpt as described in 'Materials and Methods' section. Results were normalized to values obtained for HIV-1 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (C) About 0.125 pmol of 5'-UTR-*Renilla* RNAs (see cartoon on the graph) were transfected in Cos7 and 3T3 cells or human macrophages as described in 'Materials and Methods' section. *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for HIV-1 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (D) About 0.125 pmol of *Renilla* mRNAs containing the 5'-UTR derived from different HIV-2 strains were transfected in HeLa cells and *Renilla* expression was compared to HIV-1. *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for HIV-1 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (E) About 0.125 pmol of *Renilla* RNAs in which translation was driven by the 5'-UTR of different naturally occurring HIV-1 isolates (indicated as Pat#4, 8, 15, 18, 21) together with β -globin, HIV-1 NL4-3 and HIV-2 Rod10 (used as control references) were transfected in HeLa cells as described in 'Materials and Methods' section. *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for human β -globin 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments.

we have compared the effect of TAR deletion from the viral 5'-UTR. As such, TAR deleted constructs do still contain an important region of the viral 5'-UTR. These differences in experimental design may explain some of the functional differences that we observed.

However, we were still surprised by this result and we wanted to analyze it further. Thus, we used the *Renilla* mRNAs bearing the wild type and the Δ TAR 5'-UTRs of HIV-1 and HIV-2 to program *in vitro* translation reactions in the untreated rabbit reticulocytes lysate (40). In agreement with previous observations, the TAR structures of both HIV-1 and HIV-2 were indeed potent inhibitors of translation *in vitro* (Figure 5C). The discrepancies between results observed in cells and *in vitro* could be attributed to the absence or limiting concentration of cellular factors such as Lupus autoantigen, Staufeni or TRBP, which are able to stimulate HIV-1 translation by acting on TAR (61–63). Thus, to determine whether the lack of a strong inhibitory effect observed with HIV-1 TAR in cells could be attributed to the action of cellular factors, we complemented *in vitro* translation reactions with cell extracts derived from HeLa cells. Strikingly, addition of HeLa cell extracts to the rabbit reticulocyte lysate nicely recapitulates what was observed in cells. Whereas translation driven by the HIV-1 5'-UTR was stimulated by 5-fold by the addition of HeLa cells extract, HIV-2 5'-UTR driven translation was rather inhibited by 2-fold (Figure 5D). As such, while the inhibitory effect of HIV-1 TAR *in vitro* was almost completely lost in the presence of HeLa cells extracts, HIV-2 TAR still exerted a strong repression of translation.

Taken together, these results suggest that HIV-1 may have evolved the use of host factors to overcome the inhibitory effect of TAR on translation while HIV-2 TAR did not.

HIV-2 TAR stability impairs ribosome recruitment

Results presented above prompted us to investigate the mechanism by which HIV-2 TAR could affect translation. One possibility would be that inhibition was exerted through the induction of eIF2 α phosphorylation due to TAR-dependent activation of PKR (64,65). Another possibility is that the higher stability of the HIV-2 TAR structure interferes with entry of initiation complexes at the 5'-end.

To test the first hypothesis, phosphorylated eIF2 α was visualized by western blot in extracts derived from cells previously transfected with a control *Renilla* mRNA (β -globin-*Renilla*) or with the *Renilla* mRNAs carrying the HIV-1 5'-UTR, HIV-2 5'-UTR or HIV-2 Δ TAR (Figure 6A, lanes 2–5). As controls mock transfected cells untreated or treated with sodium arsenite were also included (Figure 6A, lanes 1 and 6, respectively). Although phosphorylated eIF2 α was detected after transfection of all the mRNA tested, these levels were similar to the basal levels of phosphorylated eIF2 α observed in mock transfected cells and lower to that observed in cells treated with sodium arsenite suggesting that induction of eIF2 α phosphorylation was not dependent on HIV-2 TAR (Figure 6A, compare lanes 1–5 with line 6). Moreover,

it should be mentioned that treating the cells with sodium arsenite virtually killed translation driven by the β -globin or the HIVs 5'-UTRs indicating that phosphorylation of eIF2 α could not benefit translation of any of the mRNAs used in this study (data not shown).

As showed in Figure 5B, and references herein, HIV-2 and HIV-1 5'-UTRs strongly differ in the length and predicted folding of their TAR structures. Although both motifs are expected to be in close proximity to the cap structure, the much stronger stability of HIV-2 TAR could be responsible for reduced translational efficiency. However, the HIV-2 5'-UTR contains a 140 nt intron that is removed in multiple spliced transcripts (66). Interestingly, Lodmell and colleagues (67) recently showed that this intron could also be removed from the gRNA 5'-UTR resulting in an mRNA with enhanced translation. Removal of this intron results in the destabilization of the TAR RNA structure as at least half of its sequence is contained in the intron (see cartoon in Figure 6B). Given the results obtained so far by our group and these new data from Lodmell's lab, we created a *Renilla* mRNA carrying the spliced version of the 5'-UTR present within the HIV-2 gRNA as recently described (67). This HIV-2 5'-UTR spliced mRNA was transfected in HeLa cells and translational efficiency was compared to that of *Renilla* mRNAs carrying the wild-type HIV-2 and HIV-1 5'-UTRs (Figure 6B, left panel). Similar to results obtained with the HIV-2 Δ TAR *Renilla* RNA and in agreement with previous results (67), destabilization of HIV-2 TAR resulted in an enhanced translation that reached the high levels of the HIV-1 5'-UTR, confirming that the higher stability of the HIV-2 TAR structure strongly interferes with ribosome recruitment at the 5'-end.

Then, we wanted to determine the effects that such a splicing event could have in the context of an HIV-2 gag mRNA (Figure 6B, right panel). Interestingly, and in agreement with data obtained with the HIV-1/HIV-2 Gag chimera (Figure 3), removal of the 5'-UTR intron resulted in 5-fold stimulation of Gag expression. However, this enhancement was not sufficient to reach the levels of HIV-1 Gag production further indicating that ribosome recruitment at the 5'-end may interfere with the IRES located within the Gag coding region.

DISCUSSION

The molecular mechanism controlling HIV-1 translation has been a matter of debate for many years (68). While some people have found that cap-dependent ribosomal scanning is the major mechanism taking place (25,69,70), others have demonstrated the presence of a functional IRES element within the 5'-UTR (28–30,71,72) and the Gag coding region (31). In the case of HIV-2, IRES elements have only been described within the Gag coding region (32–35) and the role of the 5'-UTR remained largely unknown. Although a large number of these studies have focused on the molecular mechanism of ribosome recruitment (i.e. IRES-driven versus cap-dependent) none of them have investigated how overall

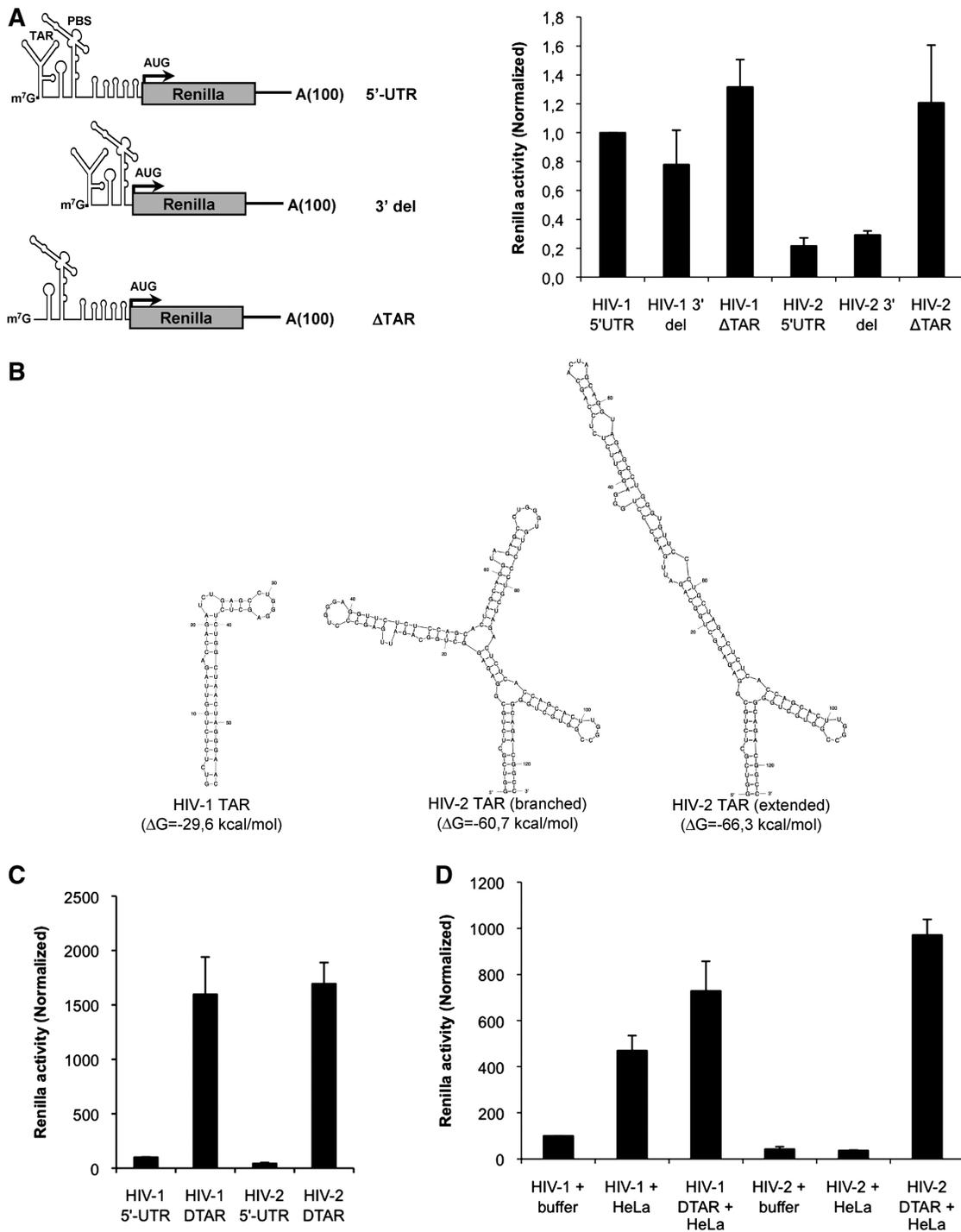


Figure 5. TAR interferes with ribosome recruitment onto the HIV-2 gRNA. (A) About 0.125 pmol of *Renilla* mRNAs containing the wild-type viral 5'-UTR or 5' and 3' deletions (see cartoon on the graph) were transfected in HeLa as described in 'Materials and Methods' section. *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for HIV-1 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained in three independent duplicate experiments. (B) Secondary structure prediction and stability of HIV-1 and HIV-2 TAR RNA structure using the mfold software as indicated in 'Materials and Methods' section. (C) The untreated rabbit reticulocyte lysate was programmed with 0.125 pmol of wild-type or Δ TAR *Renilla* mRNAs and *Renilla* activity was measured after 30 min. Results were normalized to values obtained for HIV-1 5'-UTR RNA (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained at least in three independent experiments. (D) The untreated rabbit reticulocyte lysate was programmed with 0.125 pmol of wild-type or Δ TAR *Renilla* mRNAs in the presence of buffer or HeLa cells extracts as described in 'Materials and Methods' section. *Renilla* activity was measured after 30 min. Results were normalized to values obtained for HIV-1 5'-UTR RNA in the presence of buffer (arbitrary set to 1) and expressed as mean \pm SD corresponding to values obtained at least in three independent experiments.

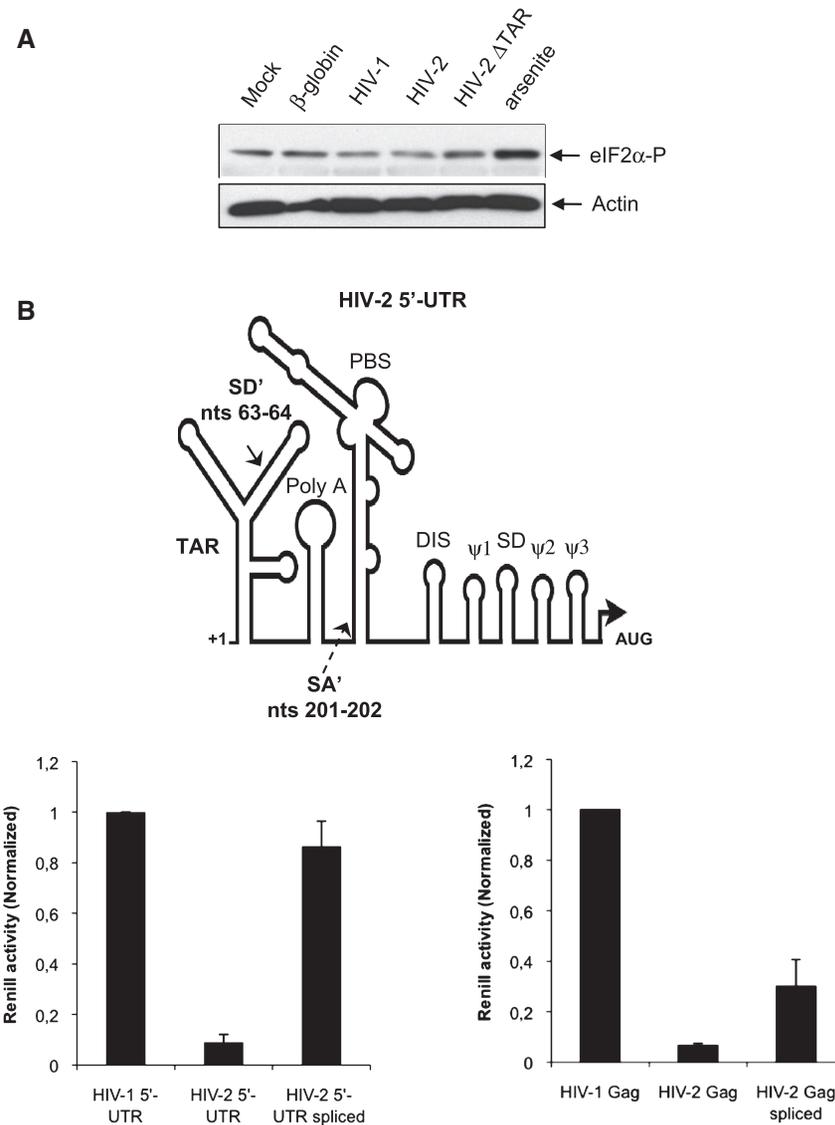


Figure 6. HIV-2 TAR stability impairs ribosome recruitment. (A) HeLa cells were transfected with equal molar quantities of *Renilla* mRNAs as indicated in the figure and cells extracts were prepared at 3 hpt for western blot analyses. In parallel, cells extracts from mock transfected cells or mock transfected cells treated with sodium arsenite during 30 min were also prepared and used as controls. (B) HeLa cells were transfected with 0.125 pmol of a control RNA or with HIV-1 5'-UTR, HIV-2 5'-UTR or HIV-2 ΔTAR-*Renilla* RNAs and whole cellular extracts were prepared at 3 hpt and probed against eIF2α, eIF2α phosphorylated and PABP by western blot as described in 'Materials and Methods' section. (C) Schematic representation not at scale of the HIV-2 5'-UTR indicating the position of the alternative splice donor (SD') and splice acceptor (SA') that results in the removal of the 5'-UTR intron described by Lodmell *et al.* (67). About 0.125 pmol of wild-type or 5'-UTR spliced HIV-2 *Renilla* mRNA (left graph) or 5'-UTR spliced HIV-2 Gag-*Renilla* mRNA (right graph) were transfected in HeLa cells together with the corresponding HIV-1 *Renilla* mRNA and *Renilla* activity was measured at 3 hpt. Results were normalized to values obtained for HIV-1 mRNAs (arbitrary set to 1) and expressed as mean ± SD corresponding to values obtained in three independent duplicate experiments.

translational efficiency of these two related viruses would be compared. This is probably due to the fact that a side-by-side comparison is technically difficult to perform in the context of viral infection as the viral life cycle and the nature of the proteins produced by each virus are very different. To circumvent some of these problems, we have used a very efficient mRNA transfection strategy of different synthetic constructs carrying the different RNA elements that have been shown to be involved in gRNA translation (i.e. the 5'-UTR and the Gag coding region).

Results presented herein show that expression from the HIV-1 gag mRNA was higher than that of the HIV-2 gag

mRNA in different cell lines including T-lymphocytes and PBMC-derived human macrophages (Figures 1 and 2). Importantly, no evident differences in the levels of transfected mRNAs or the specific activity and stability of both Gag-*Renilla* fusion proteins were observed indicating that differences in expression between HIV-1 and HIV-2 gag mRNAs were exclusively at the level of translation. Switching the 5'-UTR of both gag mRNAs and the use of reporter constructs bearing the 5'-UTR in the absence of any further viral coding region showed that lower translational efficiency of the HIV-2 gag mRNA was merely due to the presence of its highly structured 5'-UTR

(Figures 3 and 4). This was confirmed by deletion experiments pointing out a specific role of the HIV-2 TAR RNA structure (Figure 5). We observed that complementation of *in vitro* translation reactions with HeLa cells extracts was rather inhibitory for translation driven by the HIV-2 5'-UTR (Figure 5), indicating that host factors may contribute to the low translation observed from the HIV-2 gRNA in intact cells. Moreover, we observed that swapping the 5'-UTR (Figure 3) or destabilizing TAR (Figure 6) did not result in HIV-2 Gag levels resembling those observed with HIV-1 despite similar properties of both Gag–*Renilla* fusions. Interestingly, the activity of the HIV-2 gag IRES was increased by several folds when the entire 5'-UTR was removed from the mRNA (32–34). These results suggest that ribosomal entry at the 5' end could potentially interfere with ribosome recruitment from the Gag IRES and both mechanisms may be tightly regulated during viral replication.

Although a strong inhibitory effect of TAR was seen for both viral mRNAs *in vitro*, such a strong effect of TAR was only seen with HIV-2 in intact cells in a mechanism that was dependent on its intrinsic stability rather than PKR activation (Figures 5 and 6). As such, protein synthesis driven by the HIV-1 5'-UTR was very efficient and even higher than translation driven by short and unstructured cellular 5'-UTR such as β -globin, GAPDH or NADH (Figure 4). Several host RNA-binding proteins including Staufen1, La autoantigen and TRBP were shown to stimulate HIV-1 translation by acting at the level of the TAR RNA structure, and thus may contribute to alleviate TAR interference (61–63). In fact, complementation of the rabbit reticulocyte lysate *in vitro* system with HeLa cells extracts resulted in an important loss of inhibition by TAR and the concomitant stimulation of HIV-1 translation (Figure 5). Interestingly, addition of HeLa cells extracts to *in vitro* translation reactions had no the same effect on HIV-2 translation. To date, it is unknown whether cellular factors participate in the regulation of HIV-2 translation through TAR. However, destabilization of the TAR structure by removal of an intron present within the 5'-UTR (66,67) and the use IRES elements within the Gag coding region (32–35) confers two alternative ways to overcome TAR constraints and allow Gag and Gag-Pol production. Thus, the direct binding of eIF3 and the 40S ribosomal subunit to the Gag IRES (35) may allow the full-length unspliced HIV-2 gRNA to be associated with polysomes. Nevertheless, the poor translation of the HIV-2 genomic RNA provides an explanation for limited Gag production observed during viral replication (Figure 1) and may be required for *cis* co-translational packaging of the gRNA (73,74). Interestingly, the lack of splicing event in the HIV-2 5'-UTR or mutations in TAR from the closely related virus SIVmac were shown to not impact viral replication despite its ability to increase translation of a reporter gene (67,75). Moreover, the HIV-2 5'-UTR spliced gRNA was shown to be excluded from the viral particles despite the presence of all packaging signals (67) (and our unpublished data), further supporting a physiological role for slowed translation and gRNA packaging during the HIV-2 replication cycle.

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