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Identification of the HIV-1 packaging RNA sequence (Ψ) as a major determinant for the translation inhibition conferred by the HIV-1 5′ UTR

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ABSTRACT

The HIV-1 5′ untranslated region (UTR) contains conserved sequences and unique structural motifs associated with many steps in virus replication. Because unspliced HIV mRNA containing the full-length UTR serves as a template for replication and transcription as well as packaging genomic RNA into virion, it has been postulated that the UTR may play a role in translational regulation. However, the effect and the region(s) responsible for translation control remain controversial. We used deletion mutations of the 5′ UTR region in both cell-based and *in vitro* assays to determine if secondary structural elements within the 5′ UTR confer translation inhibition, and to identify which of these elements are involved. The results indicate clearly that the entire HIV-1 5′ UTR confers translation inhibition *in vitro* and in cells; the Psi (Ψ) region specifically has the most translation inhibitory activity among the highly-structured elements in the HIV-1 5′ UTR. Moreover, it was found that the SL4 structure in the Psi (Ψ) region is the major determinant of translation inhibition, and that elimination of the SL4 RNA sequence led to increased translation. The results suggest a functional role for the Psi element and the SL4 structure in the translational control of HIV-1 full-length mRNA.

1. Introduction

The 5′ untranslated region (UTR) RNA of the Human Immunodeficiency Virus-1 (HIV-1) contains multiple *cis*-acting and conserved regulatory sequences. These include a *trans*-acting responsive element (TAR), poly(A) hairpins, a primer binding site (PBS), and dimerization (DIS) and packaging signal sequence (Psi or Ψ), in that order, from the 5′-end of the 360-base-pair-long UTR [1–3]. The TAR sequence, which is a recognition site for the HIV-1 Tat protein for initiation of transcriptional activation, is present in all HIV-1 derived mRNAs and harbors a unique secondary structure as determined in a number of studies including those using RNA nuclease mapping *in vitro* [4,5]. The PBS is a binding site of 18 nucleotides complementary to the sequence of the 3′-acceptor region of the lysine tRNA, which serves as a primer for HIV-1 reverse transcription [6,7]. The DIS and Psi are unique sequence motifs required for specific viral genome RNA dimerization and selective encapsidation of genomic RNA into virions [8–11]. In addition, the Psi element is composed of four stem-loop (SL) structures with a major splicing donor site embedded in the second SL of the Psi element [12]. These RNA sequence motifs have been shown to play many functionally important roles in various steps from the early to late stages of the virus life cycle such as reverse transcription, transcription, mRNA splicing, and genomic RNA encapsidation [13–16].

One striking feature of these elements is that they all harbor unique, highly structured, and stable secondary SL structures that have been extensively analyzed biochemically, biophysically, and genetically [3,17,18]. The presence of these highly stable secondary structures within the HIV-1 5′ UTR RNA may have the potential to affect viral mRNA translation and utilization, especially the viral mRNA encoding the Gag and Pol proteins, which should contain all of the SL structures. However, there have been conflicting observations regarding whether the HIV-1 5′ UTR itself is involved in either positive or negative translation control. A previous report showed that RNA molecules containing the TAR sequence could inhibit the translation of other mRNAs *in trans* by activating double-stranded RNA-dependent kinase (dsl, also known as p68 kinase), which in turn phosphorylates the translation initiation factor 2 (eIF-2) [19]. However, this mode of action was later challenged [20]. Several other studies, using cell-free translation extracts *in vitro* or Xenopus oocytes, have shown that the HIV-1 TAR sequence confers a strong inhibitory effect on translation [21,22]. Similar translation inhibitory activity was also observed when an extended HIV-1 5′ UTR RNA sequence, up to 282 nucleotides from the 5′-end of the HIV-1 UTR, was employed [21]. These observations, however, were determined mostly via *in vitro* cell-free

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translation systems in which the magnitude of the inhibitory effect was shown to be highly variable and dependent on the preparations of the cell-free extracts [22]. More recently, other report has also suggested that the HIV-1 leader RNA sequence may not regulate its mRNA translation [23].

Therefore, it is necessary to first determine in further detail if the HIV-1 5′ UTR RNA by itself could actually confer translation inhibition not only *in vitro* but also in living cells. Secondly, if so, the region(s) within the HIV-l 5′ UTR that confer translation inhibition in intact cells must be clearly defined. In this study, we have investigated these issues and determined that the secondary structural elements of the HIV-1 5′ UTR can confer translation inhibition *in cis*, both *in vitro* and in intact cells. Furthermore, we found that there are differences in the degree of translation inhibition conferred by each SL of the HIV-1 5′ UTR and that, most strikingly, the Psi element is the major determinant of the translation inhibitory activity conferred by the HIV-1 5′ UTR.

2. Materials and methods

2.1. Cell culture and transfection

The tetracycline-inducible mammalian expression cell line T-Rex 293 (Invitrogen) was cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO, Invitrogen) in the absence of tetracycline in 5% CO_2 at 37 °C. Cells were transfected with reporter vectors to be tested using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.2. In vivo and In vitro translation reporter vector and other methods

All transfection reporter vectors used in this study were constructed such that reporter genes would be under the control of the CMV promoter, followed by two tandem repeats of the tetracycline operator (TetO) sequence of the pcDNA4/TO vector (Invitrogen). Reporter constructs used for the *in vitro* transcription/translation-coupled assay were constructed based on the Luciferase T7 control DNA vector (Promega) that contains the T7 RNA polymerase promoter linked upstream of the Fluc gene. Detailed descriptions of these vector constructs as well as all other assay methods used in this study are provided in the Supplementary section.

3. Results and discussion

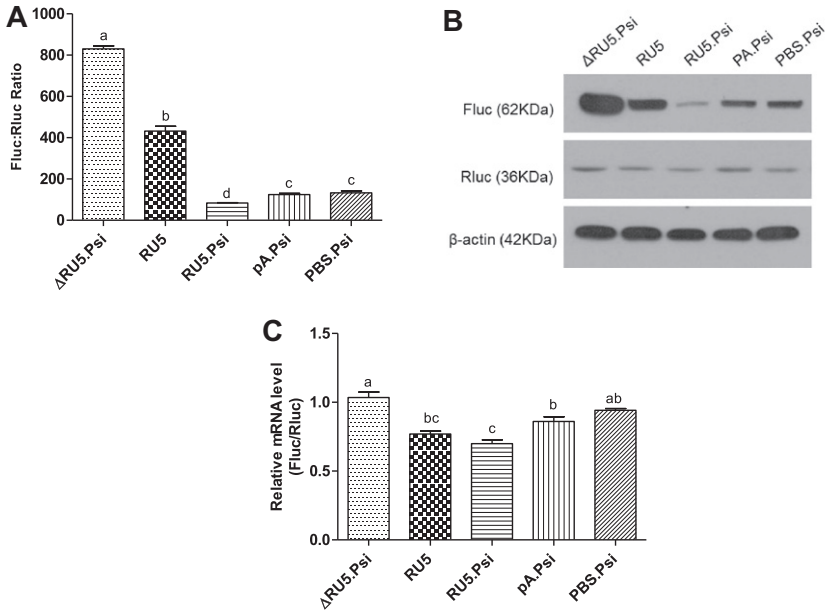
3.1. The HIV-1 5′ UTR confers translation inhibition; the Psi region is the major determinant of translation inhibition

To determine whether the HIV-1 5′ UTR RNA sequence itself could affect translation efficiency within cells, we generated a number of reporter vectors that contained either the entire 360-base-pair 5′ UTR of HIV-1, or a series of deletions, placed upstream of either the Fluc or EGFP reporter genes, as depicted in Supplementary Fig. S1. All reporter vectors constructed were under the control of the same CMV promoter and tetracycline operators, so that translation efficiency could be measured precisely in a tightly regulated manner upon tetracycline induction. For all transient transfection assays, individual reporter vectors were transfected into T-Rex293 cells along with a control vector, pRL-SV40, which expressed *Renilla* luciferase, for transfection efficiency determination. Using this cell-based dual luciferase reporter gene assay, we found that the RU5.Psi vector harboring the full-length HIV-1 5′ UTR strongly inhibited luciferase activity compared to the positive control vector that did not contain the HIV-1 5′ UTR sequence (ΔRU5.Psi), resulting in more than 90% inhibition, while a little less than 50% inhibition was obtained with the RU5 region only (Fig. 1A). The result was verified further by western blot analysis of the reporter gene assay where the RU5.Psi vector showed the greatest suppression of Fluc gene production (compare lanes 1 and 3 of Fig. 1B). Even though all the reporter vectors tested in this study were driven by the same CMV promoter, we further examined the possibility that the translation inhibition effect observed might be derived from differences at the transcriptional level. To this end, quantitative real time-PCR (qRT-PCR) analysis was performed, revealing no gross difference in Fluc reporter gene mRNA levels between the reporter vectors tested, as shown in Fig. 1C. Although mRNA levels generated from the RU5.Psi reporter vector were slightly lower than from the other vectors, this difference is unlikely to account for the huge decrease in the Fluc reporter gene product as observed in the western blot (Fig. 1B). Moreover, another independent *in vitro* transcription/translation-coupled assay also yielded similar results (Fig. 1D), indicating strongly that the HIV-1 5′ UTR does indeed confer translation inhibitory activity.

To further map out which region(s) of the HIV-1 5′ UTR cause translation inhibition, we tested a series of reporter vectors containing serial deletions of major SL structures in the HIV-1 5′ UTR, such the TAR, poly(A), and Psi element regions. When the TAR and poly(A) regions were deleted successively, as in the case of the pA.Psi and PBS.Psi vectors, translation suppression was slightly and incrementally relieved (Fig. 1A and B). However, these mutants were not able to recover the level of translation efficiency seen with the control ΔRU5.Psi vector; a substantial degree of translation inhibition remained, indicating that neither TAR nor poly(A) were the major sites that conferred translation inhibition. On the other hand, when we deleted the Psi region only, which is equivalent to the RU5 reporter vector, but preserved the TAR, Poly(A), and PBS regions, more than 50% of the translation efficiency seen in the case of the ΔRU5.Psi vector was recovered (compare lanes1 and 2 of Fig. 1A and B). Taken together, these results suggest that among the elements of the HIV-1 5′ UTR, the Psi region is the major determinant of the translation inhibitory activity of the HIV-1 5′ UTR sequence. The retention of some degree of translation inhibition activity with the RU5 region in this assay is in good agreement with observations of translation inhibition from a previous report [21].

3.2. The SL4 stem-loop of Psi region is responsible for the translation inhibitory activity

Next, we set out to identify the sequence or motif in the Psi region that is responsible for the observed translational inhibition. To do this, we generated a series of reporter vectors containing the full sequence of Psi (SL1234) and serial deletion constructs from the 5′-end of the Psi region, namely SL234, SL34, and SL4; the SL4 region contains the AUG initiation codon for the Gag–Pol protein and thus was maintained for all reporter vectors that were examined for translation efficiency. From our analysis of these constructs, we found that the SL1234 (Psi) reporter vector yielded only about 20% of the luciferase activity of the ΔRU5.Psi control vector (lanes 1 and 2 in Fig. 2A), indicating more than 80% translation inhibition with the Psi sequence. This result confirms again that the Psi region is the most significant and major determinant of translational inhibition in the HIV-1 5′ UTR. The other deletion constructs (SL234, SL34, and SL4) exhibited nearly equal levels of translation inhibition (lanes 3–5 in Fig. 2A and B). Similar results were obtained by the *in vitro* transcription/translation-coupled assay and the corresponding western blot analysis (Fig. 2D and E). The efficiency of mRNA synthesis was nearly equal in all the reporter vectors examined by qRT-PCR analysis (Fig. 2C). These data indicate that the presence of the SL4 sequence resulted in the greatest translation inhibition, as evidenced from both the transient transfection reporter gene assay and the *in vitro* transcription/translation-coupled



D

Reporter vectors	Luciferase activity(RLU)	Translation efficiency (%)
ΔRU5.Psi	4594117 (±410652.8)	100
RU5	2218607 (±327826.7)	48.19(±4.6)
RU5.Psi	378370 (±27617.3)	8.24(±0.2)
PA.Psi	527509 (±75863.8)	11.44(±0.8)
PBS.Psi	571653 (±93830.9)	12.39(±0.9)

Fig. 1. Translation efficiencies of the wild type HIV-1 5′ UTR and its deletion mutants. (A) Cell-based dual luciferase assay. T-Rex293 cells were co-transfected with 1 μg of the indicated firefly luciferase reporter gene constructs along with 0.1 μg of *Renilla* luciferase reporter vector. To normalize translation efficiency, relative luciferase activity was expressed as a ratio (Fluc/Rluc). Values are represented as mean ± SD ($n = 5$); bars with different letters are significantly different ($P < 0.01$). (B) Western analysis of cell lysates obtained from the transfection assay in (A). The antibodies (dilutions) used were anti-firefly luciferase (1:1000), anti-*Renilla* (1:500), and anti-β-actin (1:10,000), and bands were detected with goat anti-mouse IgG conjugated with HRP. The band intensities of β-actin indicate that equal amounts of cell lysate were loaded for the analysis. (C) Total RNA from T-Rex293, extracted and reverse transcribed, as described in Section 2. The levels of Fluc, Rluc and GAPDH mRNA were measured by qRT-PCR. Data are represented as mean ± SD ($n = 4$); bars with different letters are significantly different ($P < 0.05$). Relative expression levels of each gene in T-Rex293 cells were normalized to that of the ΔRU5.Psi reporter vector. (D) *In vitro* transcription/translation-coupled luciferase assay. Firefly luciferase activities of 1 μg of each of the constructs driven by the T7 promoter were determined by an *in vitro* transcription/translation assay as described in Section 2. Raw values of firefly luciferase activity in relative light units (RLU), and translation efficiency, are shown. The data are shown as mean values with standard deviations from three independent experiments.

assay, suggesting that the SL4 stem-loop is the major determinant of translational inhibition within the Psi sequence.

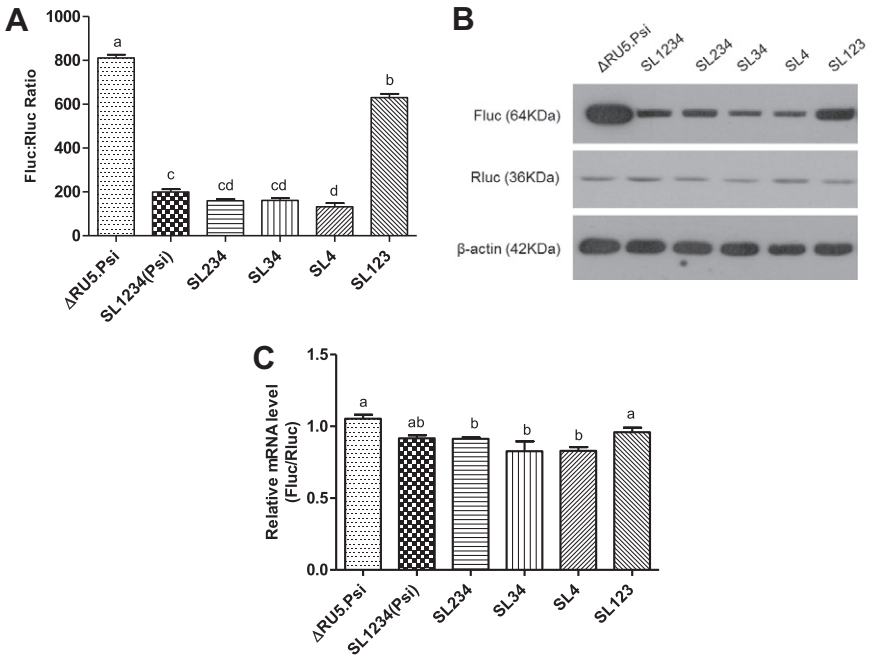
We also constructed and examined another construct, SL123, containing a deletion of the SL4 sequence. Up to 80% of the translation efficiency was observed with this SL123 reporter construct when compared with the ΔRU5.Psi construct. The result was further confirmed by concomitant Western blot analysis and *in vitro* transcription/translation-coupled assay (Fig. 2A–E).

3.3. Translation inhibition caused by Psi and SL4 sequence in a different reporter gene expression vector

In order to eliminate any possibility that the translation inhibition observed thus far using the luciferase assay is due to or unique to the luciferase gene used, and to further verify the results directly within cells (without cell lysis), we evaluated translation inhibition using another reporter construct containing different regions of the HIV-1 5′ UTR upstream of the EGFP gene, similar to the luciferase reporter constructs. To do this, the Fluc reporter gene in the ΔRU5.Psi, RU5.Psi, RU5, SL1234(Psi), SL123, and SL4 reporter vectors was substituted with EGFP, and the translation efficiency was evaluated using fluorescence microscopy after co-transfection with pDS-Red as a transfection efficiency control vector (Fig. 3A). Similar to the luciferase assay, we observed efficient production of EGFP in the absence of the upstream HIV-1 5′ UTR sequence, whereas the RU5.Psi vector showed the greatest inhibition of EGFP translation efficiency, followed by the SL1234 vector. Interestingly, the signal intensity seen with the SL4-EGFP vector appeared to be equal to or slightly lower than that seen with the SL1234 construct. Conversely, the RU5 construct, containing a deletion of the Psi sequence, as well as SL123 exhibited a much greater efficiency of EGFP production than either the SL1234 or SL4 reporter vectors. Again, qRT-PCR analysis showed that the translation inhibition conferred by the Psi or SL4 sequence was not due to any deficiency in the transcription of the EGFP reporter vectors (Fig. 3B). These results were consistent with the results of the luciferase reporter gene assay and reconfirm the importance of Psi as well as SL4 in the translation inhibition activity of the HIV-1 5′ UTR sequence.

3.4. Comparison of translation inhibition efficiency of Psi to other stably structured HIV-1 RNA elements

To examine whether the strong translation inhibitory activity of the Psi region of the HIV-1 5′ UTR is unique or might be a general fea-



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Reporter vectors	Luciferase activity(RLU)	Translation efficiency (%)
ΔRU5.Psi	4594117 (±410652.8)	100
SL1234(Psi)	601861 (±89054.8)	13.07 (±0.9)
SL234	573795 (±89846.1)	12.44 (±0.9)
SL34	598829 (±151842.8)	12.91 (±2.1)
SL4	507193 (±44261.7)	11.04 (±0.4)
SL123	3381560 (±328638.1)	73.57 (±0.6)

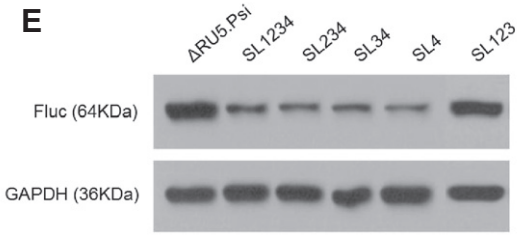

Fig. 2. The effect of the Psi region and SL4 stem-loop on translation inhibition. (A) Cell-based dual luciferase assay as described in Fig. 1. Values are represented as mean ± SD ($n = 5$); bars with different letters are significantly different ($P < 0.01$). (B) Equal amounts (25 μg) of cell lysates from the transfection assay in A were analyzed for the production of Fluc protein by western blot. (C) Total RNA was isolated from T-Rex293 cells and analyzed by qRT-PCR. Data are represented as mean ± SD ($n = 4$); bars with different letters are significantly different ($P < 0.05$). (D and E) Results of the *in vitro* transcription/translation assay and its western blot analysis as described in Fig. 1. For immunoblotting analysis, the primary antibodies anti-firefly luciferase (1:2000), and anti-GAPDH (1:10,000) were used. GAPDH was used as an internal control for each of the *in vitro* transcription/translation reactions.

ture of RNAs with a stable secondary structure, we tested the effect of substituting either the HIV-1 Rev responsive element (RRE) or TAR (*Trans*-Activation Response) sequence, both of which have been shown to contain extremely stable secondary structures [5,24], in place of the RU5 sequence. Moreover, we wanted to compare side by side the translation inhibitory activity of Psi to that of the TAR sequence, as it has previously been proposed to have translation inhibitory activity [21]. The translation efficiencies of constructs containing the RRE and TAR sequences were lower than that of the ΔRU5.Psi construct. However, the levels of translation inhibitory activity were similar to that of the RU5 region only and they all showed at least two-fold or higher translation efficiency than that of SL1234 (Psi), which was verified not due to any increase in transcription (Fig. 4A–C). These results indicate that the Psi region is rather unique and exhibits a much stronger inhibitory effect on translation than the HIV-1 RRE sequence, the RU5 sequence, or the TAR sequence alone, suggesting an importance and possible role of the Psi element in HIV-1 viral Gag translation control.

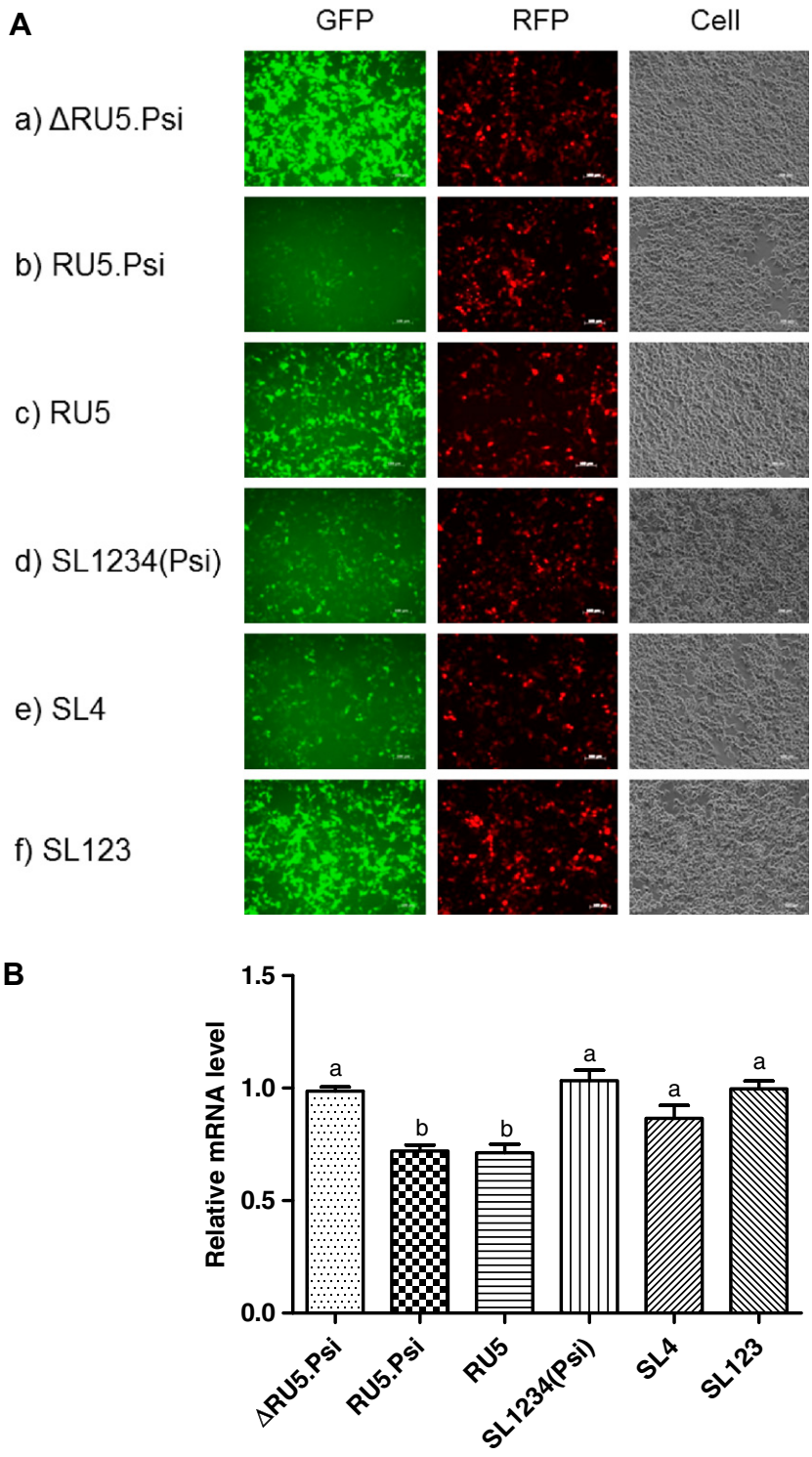

Fig. 3. Translation efficiency of the HIV-1 5′ UTR and its derivatives, determined using the EGFP reporter gene. (A) EGFP reporter vectors (1 μg of each) as indicated in the figure were used to co-transfect T-Rex293 cells along with a transfection efficiency control vector, pDS-Red (0.05 μg) that expresses a red fluorescent protein (RFP). Expression of the reporter genes in transfected cells then was examined by fluorescence microscopy as described in Section 2. (B) Total RNA was isolated from T-Rex293 cells and analyzed by qRT-PCR. RNA levels were normalized to RFP mRNA levels. Data are represented as mean ± SD ($n = 4$); bars with different letters are significantly different ($P < 0.05$).

3.5. Effect of SL4 stem-loop of Psi on the efficiency of HIV-1 Gag expression

It is well documented that the HIV-1 Gag protein is naturally kept at a low level within host cells [25,26]. To test the effect of Psi, and particularly the SL4 sequence, on the synthesis of the HIV-1 Gag protein, we constructed two different HIV-1 Gag expression vectors; one with the SL4 sequence as in the original HIV-1 Gag coding sequence, and the other without it but containing the rest of amino acid sequence in frame. As shown in Fig. 4, the latter

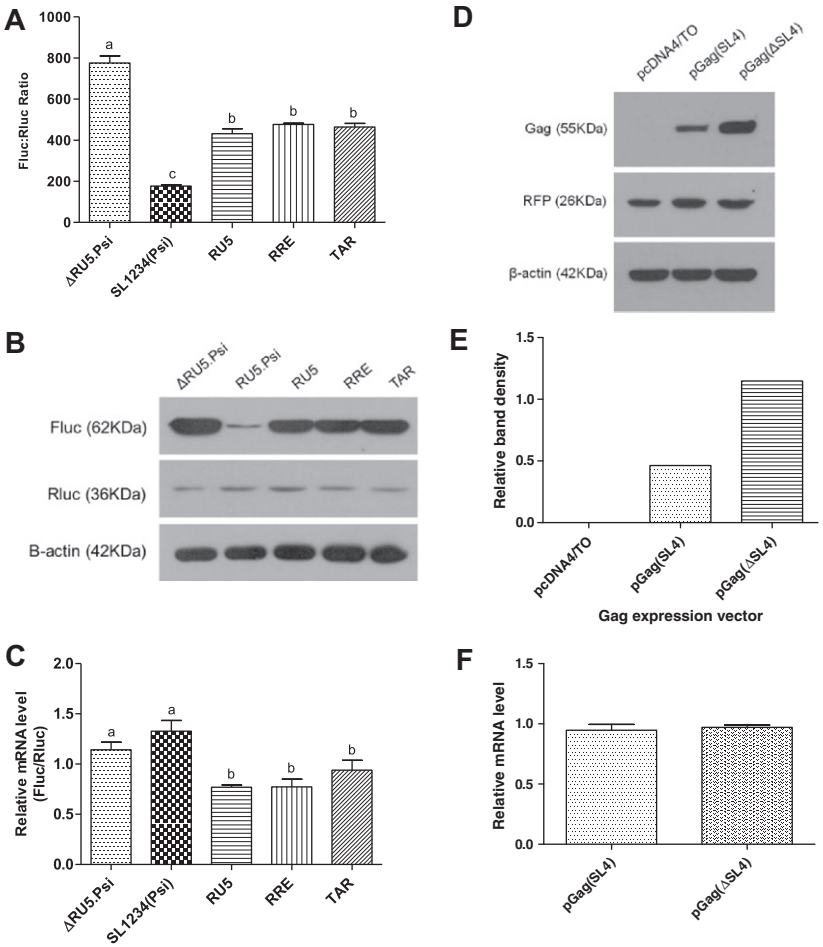

Fig. 4. Translation inhibition efficiency by the Psi element compared to other HIV-1 secondary structural elements, and the effect of the SL4 region of Psi on HIV-1 Gag expression. (A) T-Rex293 cells were co-transfected with 1 μg of each of ΔRU5.Psi, SL1234, RU5, RRE, and TAR reporter vectors, 0.1 μg *Renilla* luciferase pRL-SV40 vector an internal control. Data shown are the results of five independent experiments. Values are represented as mean ± SD ($n = 5$); bars with different letters are significantly different ($P < 0.01$). (B) Western blot analysis. Cell lysates from the transfection assay in A were analyzed for Fluc protein production by western blot analysis. (C) After total RNA extraction, expression from the ΔRU5.Psi, SL1234, RU5, RRE, and TAR vectors was analyzed using qRT-PCR and normalized to Rluc mRNA. Data are represented as mean ± SD ($n = 4$); bars with different letters are significantly different ($P < 0.05$). (D) Extracts prepared from T-Rex293 cells after transfection for 24 h with the pGag(SL4) and pGag(ΔSL4) plasmids, as indicated, were subjected to SDS–PAGE. Proteins were transferred to a nitrocellulose membrane, and Gag, RFP, and β-actin were visualized by immunoblotting. The antibodies (dilutions) used were anti-p24 (CA) (1:1000), anti-RFP(1:500), and anti-β-actin (1:10,000) dilution. RFP was used as an internal control to monitor transfection efficiency. The band intensity of β-actin indicates that equal amounts of cell lysate were loaded for the analysis. (E) Densitometer scanning of relative Gag protein production between the pGag(SL4) and pGag(ΔSL4) expression vectors. (F) Relative mRNA expression levels of the Gag gene in T-Rex293 cells transfected with pGag(SL4) and pGag(ΔSL4) were estimated by qRT-PCR. Data are represented as mean ± SD ($n = 4$); bars with different letters are significantly different ($P < 0.05$).

expression vector yielded nearly 3-fold more Gag protein than the former, which harbors the full SL4 stem-loop sequence (Fig. 4D and E). qRT-PCR analysis verified that the result was not due to transcriptional differences between the expression vectors (Fig. 4F). These results confirm clearly that the HIV-1 Psi region, specifically SL4 of it, is the major site for translational inhibition conferred by the HIV-1 5′ UTR.

The HIV-1 Gag gene harbors a so-called INS sequence (inhibitory sequence) that hampers its efficient translation, resulting in a low level production of Gag and Gag–pol proteins [27,28]. It has been suggested that this low level of Gag and Gag–pol production might be a strategy employed by HIV-1 for evading host cell immune response during HIV-1 infection [29]. Lately, a possible role of the Gag protein, as a *trans*-acting factor, to confer a translational inhibition by a possible interaction to the HIV-1 Psi of 5′ UTR has been also reported by an *in vitro* study [30].

In addition, it is intriguing that the Gag-initiation codon, AUG, is localized in the SL4 loop of the Psi sequence of HIV-1 5′ UTR. Having found in this study that the SL4 region is able to confer translation inhibition and is the major region in the HIV-1 5′ UTR responsible for this activity, it is thus possible to reason that the Psi-SL4 region harboring the AUG codon may well be a *cis*-acting translation inhibitory element which provides another level of translation control mechanisms to ensure the low level of Gag and Gag–pol production.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.149.

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