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The presence of the TAR RNA structure alters the programmed -1 ribosomal frameshift efficiency of the human immunodeficiency virus type 1 (HIV-1) by modifying the rate of translation initiation

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ABSTRACT

HIV-1 uses a programmed -1 ribosomal frameshift to synthesize the precursor of its enzymes, Gag-Pol. The frameshift efficiency that is critical for the virus replication, is controlled by an interaction between the ribosome and a specific structure on the viral mRNA, the frameshift stimulatory signal. The rate of cap-dependent translation initiation is known to be altered by the TAR RNA structure, present at the 5′ and 3′ end of all HIV-1 mRNAs. Depending upon its concentration, TAR activates or inhibits the double-stranded RNA-dependent protein kinase (PKR). We investigated here whether changes in translation initiation caused by TAR affect HIV-1 frameshift efficiency. CD4+ T cells and 293T cells were transfected with a dual-luciferase construct where the firefly luciferase expression depends upon the HIV-1 frameshift. Translation initiation was altered by adding TAR in *cis* or *trans* of the reporter mRNA. We show that HIV-1 frameshift efficiency correlates negatively with changes in the rate of translation initiation caused by TAR and mediated by PKR. A model is presented where changes in the rate of initiation affect the probability of frameshifting by altering the distance between elongating ribosomes on the mRNA, which influences the frequency of encounter between these ribosomes and the frameshift stimulatory signal.

INTRODUCTION

The precursor of HIV-1 structural proteins, Gag, and the precursor of the viral enzymes, Pol, are translated from the full-length viral messenger RNA (mRNA). Gag is produced by conventional translation whereas Pol requires a programmed -1 ribosomal frameshift during the elongation step of translation, which generates the fusion protein Gag-Pol (1, reviewed in 2,3). Previous studies showed that a 2- to 20-fold increase in the Gag-Pol to Gag ratio prevents viral infectivity (4–7) and our group showed that a decrease in the frameshift efficiency as low as 30% severely impairs the replication of the virus in cultured cells (8). The Gag-Pol to Gag ratio is therefore critical for viral infectivity and the programmed –1 frameshift that determines this ratio represents an interesting target for the development of novel antiretroviral agents against HIV-1.

The HIV-1 frameshift event requires two *cis*-acting elements in the viral mRNA: a slippery sequence, UUUUUUA, where the frameshift occurs (1, reviewed in 2,3), followed by an irregular stem-loop (9–11), the frameshift stimulatory signal, that makes the ribosomes pause over the slippery sequence and controls the frameshift efficiency. Only a fraction of the ribosomes that encounter the stimulatory signal make a frameshift. After the pause, the ribosomes unfold the signal, which can reform after their passage.

HIV-1 can use a cap-dependent mechanism to initiate translation of its mRNAs, like most eukaryotic mRNAs (for a review on translation initiation, see 12–15). There are two major control steps in eukaryotic cap-dependent translation initiation (see details in Figure 1A). One is the binding of the initiator tRNA, Met-tRNA$_i^{Met}$, to the 40S ribosomal subunit, which requires the participation of the initiation factor 2 (eIF2) associated to GTP. The other one is the binding of the 40S subunit bearing the ternary complex to the 5′ cap structure of the mRNA, which is controlled by the eIF4F complex. Double-stranded RNA (dsRNA), such as the TAR RNA structure, can modify

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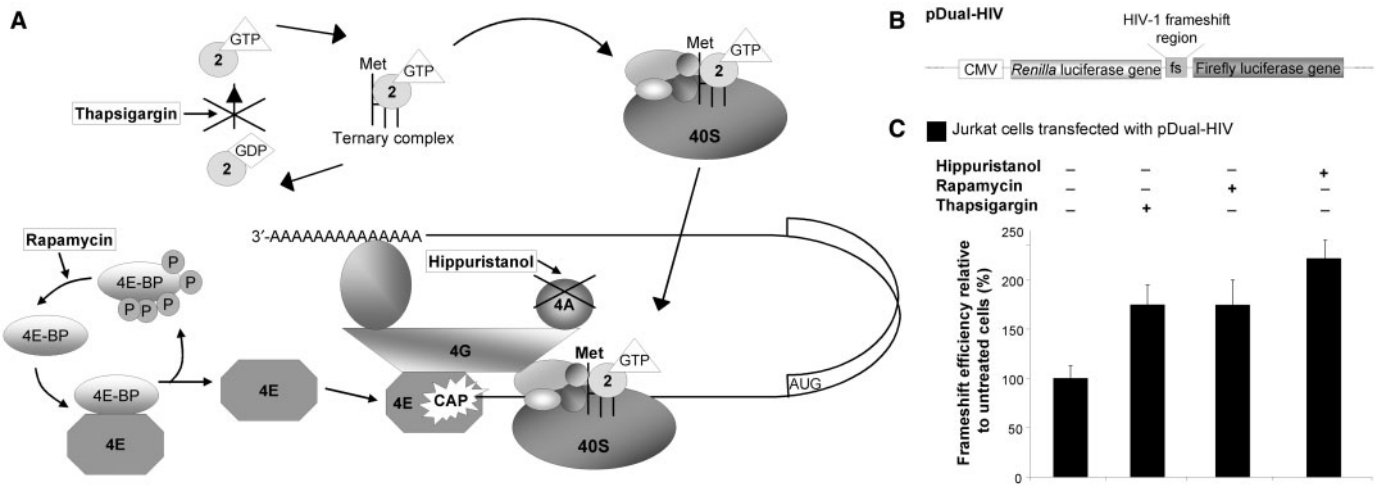

Figure 1. HIV-1 frameshift efficiency increases in the presence of inhibitors of cap-dependent translation initiation. (**A**) Major control steps of cap-dependent translation initiation in eukaryotes (15). The figure is adapted from Gebauer and Hentze (14). Only the factors we refer to in the text are named. The 40S ribosomal subunit associates with the ternary complex [initiation factor 2 (eIF2) plus GTP plus the initiator tRNA, Met-tRNA$_i^{Met}$] and with other factors, and binds to the 5′ cap structure of the mRNA. This binding requires the eIF4F complex formed by three initiation factors: eIF4E, the cap-binding protein, eIF4G, a scaffold protein and eIF4A, a RNA helicase that unfolds secondary structures. After each round of initiation, eIF2 is released from the ribosome in association with GDP. Phosphorylation of the α subunit of eIF2 (eIF2-α) prevents the recycling of eIF2-GDP in eIF2-GTP, blocking translation initiation. Thapsigargin induces endoplasmic reticulum stress, which stimulates the PERK kinase that phosphorylates eIF2-α, reducing the level of functional eIF2 (55–57). Rapamycin shuts down the mammalian target of rapamycin (mTOR) pathway, which blocks the phosphorylation of the translation repressor 4E-BP, and hypophosphorylated 4E-BP sequesters the initiation factor eIF4E (58,59). Hippuristanol is a selective inhibitor of eIF4A (60), which interferes with the binding of the 40S subunit to the mRNA. (**B**) Plasmid pDual-HIV contains the *R*luc and the Fluc coding sequences under the control of a CMV promoter and separated by the HIV-1 frameshift region. (**C**) The frameshift efficiency was assessed in lysates from Jurkat cells transfected with 2 μg of pDual-HIV(-1) or (0) and, subsequently, treated with thapsigargin, rapamycin or hippuristanol or left untreated (see 'Materials and Methods' section for details). The frameshift efficiency with untreated cells transfected with pDual-HIV was arbitrarily set at 100%. Results are the means ± SD of at least four independent experiments.

the rate of translation initiation. TAR, the transactivation response element, is a 59-nt stem-bulge-loop structure present at the 5′ and 3′ end of all HIV-1 mRNAs in the nucleus and the cytoplasm (reviewed in 16). It is also present under a free form of 58-66 nt in the cytoplasm of cells infected with the virus (17,18). In the nucleus, TAR mediates transcription activation by binding to the viral Tat protein and the cellular cyclinT protein (19,20). In the cytoplasm, a low concentration of TAR activates PKR, the dsRNA-dependent protein kinase, whereas a higher concentration of TAR inhibits this kinase by blocking its dimerization, which is essential for its activity (reviewed in 21). When PKR is activated, it phosphorylates the α subunit of eIF2, interfering with translation initiation, whereas, when it is inhibited, the amount of eIF2 phosphorylated decreases and the rate of translation initiation increases.

In this study, we investigated whether the presence of TAR affects HIV-1 frameshift efficiency in relationship with the changes it causes in the rate of cap-dependent translation initiation. To this end, we used a dual-luciferase construct (8) which expresses the *Renilla* luciferase (*R*luc) and the firefly luciferase (Fluc) separated by HIV-1 frameshift region as a fusion protein. *R*luc is expressed following conventional rules of translation whereas Fluc expression requires a -1 frameshift in the HIV-1 frameshift region. This type of construct is adapted from Grentzmann *et al*. (22), who pioneered the use of a dual-luciferase reporter for studying recoding signals. CD4+ T cells (Jurkat) or 293T cells were transfected with the dual-luciferase plasmid and TAR was added either in *cis* or in *trans* of the reporter mRNA. Several conditions were assayed to characterize the effect of TAR on frameshift efficiency and the involvement of PKR in this effect, such as the introduction of a small or a large amount of TAR in the cells, the use of mutants of TAR that cannot perturb PKR activity and the silencing of PKR expression with short interfering RNA (siRNA).

Our results show that HIV-1 frameshift efficiency increases at a low concentration of TAR, when cap-dependent translation initiation is slowed down, whereas it decreases at a high concentration of TAR, when translation initiation is stimulated. These effects were shown to be dependent on PKR. A model is presented which relates the effects of TAR on frameshift efficiency to changes in the spacing between the elongating ribosomes on the mRNA caused by changes in the rate of translation initiation. Such changes affect the frequency of encounter between the ribosomes and the frameshift stimulatory signal.

MATERIALS AND METHODS

Plasmids

To measure HIV-1 frameshift efficiency, we used the dual-luciferase reporters pDual-HIV(-1) and (0) (8). These plasmids are derived from pcDNA3.1Hygro+ (Invitrogen) and contain the HIV-1 frameshift region inserted between the coding sequences of the *Renilla*

luciferase (*R*luc) and the firefly luciferase (Fluc). Expression of these genes is under control of a CMV promoter, which is followed by a T7 promoter. Plasmid pDual-HIV(0) differs from pDual-HIV(-1) by the addition of an adenine after the slippery sequence in the frameshift region. Derivatives of pDual-HIV(-1) and (0) were constructed where the TAR sequence was inserted after the CMV and T7 promoters. A TAR-containing fragment flanked with HindIII sites obtained from pcDNA3-RSV-TAR-*R*luc plasmid (23), a kind gift from L. DesGroseillers (Université de Montréal), was cloned in the HindIII site of pDual-HIV to produce pDual-HIV-TAR(-1) and (0), where the TAR sequence is located at a distance of about 40 nt from the 5′ end of the reporter mRNA. To produce pDual-HIV-50TAR(-1) and (0), where the TAR sequence is at a larger distance from the 5′ end of the reporter mRNA, a cassette of a 50-nt non-coding sequence was inserted in the AflII site of pDual-HIV, followed by the insertion of TAR immediately after these 50 nt, in the HindIII site. The oligonucleotides for the cassette were cass50nt-fwd and cass50nt-rev (see the sequence of all the oligonucleotides used in this study in Table 1 of the Supplementary Data). Plasmid pTAR, which expresses the free TAR sequence in *trans* from the reporter mRNA, was made by inserting the TAR-containing fragment flanked with HindIII sites into the HindIII restriction site of pcDNA3.1Hygro+. Derivatives of pTAR, pTARuucg* and pTAR△bulge*, which express mutants of TAR, were constructed by cloning oligonucleotide cassettes (cass_TAR-uucg* fwd and cass_TAR-uucg* rev or cass_TAR-bulge* fwd and cass_TAR-bulge* rev) between the two NheI restriction sites present in the TAR sequence of pTAR. In the first mutant, the upper loop, CUGGGA, is replaced with UUCG and, in the second mutant, the bulge UCU preceding the upper loop is deleted. Plasmid pCGN△C [a generous gift from N. Hernandez, Cold Spring Harbor Laboratory (24)] expresses a mutant of the TAR-binding protein Tat (Tat*), named TatC30,31A.

Transfection of Jurkat and HEK 293T cells

Jurkat cells (CD4+ T cells) were maintained in RPMI 1640 medium (Wisent) supplemented with 10% (v/v) FBS (Wisent) and HEK 293T cells (human embryonic kidney cells transformed with adenovirus and simian virus 40 large-T) were maintained in DMEM (Gibco) supplemented with 10% (v/v) FBS. Transfections were performed with polyethylenimine (PEI) (Polysciences, Inc.) in six-well plates containing Jurkat cells (1.2×10^6), 293T cells (4.0×10^5) or 293T stable transfectants (6.0×10^5 cells) expressing a dual-luciferase HIV reporter (see subsequently). PEI was added drop-wise to serum-free medium and incubated 10 min at room temperature. In parallel, serum-free medium was added to DNA. The diluted PEI was added to the DNA solution (PEI to DNA ratio of 2:1) and incubated at least 15 min at room temperature. An empty plasmid, pcDNA3.1Hygro+, was added, when required, to maintain an equivalent DNA input.

Effect of translation inhibitors

Translation inhibitors were added as follows: rapamycin (Fisher), 16 h post-transfection (final concentration: 25 nM), hippuristanol (a generous gift from J. Pelletier, McGill University), 24 h before harvest (final concentration: 400 nM) and thapsigargin (Sigma), 4 h before harvest (final concentration: 300 nM). Transfected cells were harvested 48 h post-transfection. Non-adherent cells were centrifuged at 3000 *g* for 5 min, washed with PBS and lysed in 100 µl of Cell Passive Lysis Buffer (Promega). Adherent cells were washed with PBS and lysed in 400 µl of Cell Passive Lysis Buffer. Cell lysates were centrifuged 2 min at 13 000 *g* at 4°C to remove cell debris, before luciferase assays.

Selection of stable 293T transfectants expressing a dual-luciferase HIV reporter

Plasmids pcDNA5-Dual-HIV(-1) and (0) were made by inserting the HindIII–ApaI fragment from pDual-HIV(-1) or (0), respectively, into pcDNA5-FRT (Invitrogen), which contains a resistance gene to hygromycin B. An in-frame construct without the HIV-1 frameshift region was generated by cloning an oligonucleotide cassette (inframe-fwd and inframe-rev) into the KpnI and BamHI restriction sites of linearized pDual-HIV. In pDual-in-frame, the luciferase coding sequences are in the same reading frame and separated by a short linker. The HindIII–ApaI fragment from pDual-in-frame was cloned into pcDNA5-FRT.

Cell lines stably expressing the (-1) or (0) dual-luciferase HIV reporter, or the in-frame construct, were generated following the manufacturer's instructions, using 293T Flp-in™ cells (Invitrogen). Individual clones that stably incorporated the plasmids were selected on the basis of their resistance to hygromycin B (Wisent) (250 µg/ml) and maintained in hygromycin B.

Silencing of PKR with siRNA

293T transfectants (6.0×10^5 cells) stably expressing the (-1) and (0) dual-luciferase HIV reporter were transfected with 150 ng of the PKR ShortCut®siRNA Mix or the eGFP ShortCut®siRNA Mix (New England BioLabs), using PEI. The TAR-expressing plasmids were transfected 24 h after the transfection with a siRNA mix. Cells were harvested 48 h after this second transfection and luciferase assays were performed.

Control of PKR silencing by western blotting

293T transfectants, transfected with a siRNA mix, as described above, were harvested 48 h after the transfection, washed in PBS and lysed in 100 µl of Ripa-Doc (final concentration: 140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.05% sodium dodecyl sulphate), containing a cocktail of protease and phosphatase inhibitors. Equal amounts of proteins (15 µg) were separated on a 10% SDS–PAGE gel, transferred on a nitrocellulose membrane and immunoblotted with a mouse anti-PKR hybridoma supernatant (clone F9)

(a generous gift from A. Koromilas, McGill University) and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham) diluted 1/1500. After detection of the antigen–antibody complexes, the membrane was washed with 25 ml of stripping buffer (final concentration: 0.08 M β-mercaptoethanol, 2% sodium dodecyl sulphate and 0.06 M Tris–HCl, pH 6.9) for 30 min at 50°C, and immunoblotted with a mouse anti-α-tubulin monoclonal antibody (clone B-5-1-2 Sigma) diluted 1/5000 and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody diluted 1/1500. Antigen–antibody complexes were detected with an enhanced chemiluminescence (ECL) system.

Luciferase assays

The Fluc versus the *R*luc activities of the (-1) and (0) constructs were measured as relative light units with a Berthold Lumat LB 9507 luminometer, as previously described (8). A Dual-Luciferase Reporter Assay System kit (Promega) was used for Jurkat cells and home-made reagents (25) were used for 293T cells. The *R*luc activity is used to normalize the Fluc activity (Fluc/*R*luc). The frameshift efficiency is equal to:

$$[\mathrm{Fluc}(-1)/R\mathrm{luc}(-1)]/[\mathrm{Fluc}(0)/R\mathrm{luc}(0) + \mathrm{Fluc}(-1)/R\mathrm{luc}(-1)].$$

RESULTS

Inhibition of cap-dependent translation initiation with specific inhibitors increases HIV-1 frameshift efficiency

Our aim was to investigate whether the presence of TAR affects HIV-1 frameshift efficiency in relationship with its effect on cap-dependent translation initiation. To this end, we used a dual-luciferase construct, pDual-HIV(–1), which contains the *R*luc and the Fluc reporter genes separated by the HIV-1 frameshift region (Figure 1B). In this construct, the Fluc is produced only by ribosomes that make a –1 frameshift when translating the HIV-1 frameshift region. To assess the frameshift efficiency, we used a control construct, pDual-HIV(0), in which an adenine is added after the slippery sequence in the frameshift region, so that the Fluc coding sequence is in-frame with the *R*luc coding sequence. The *R*luc is synthesized by conventional translation in both (-1) and (0) constructs. Before investigating the effect of TAR, we verified that changes in cap-dependent translation initiation affect HIV-1 frameshift efficiency. Jurkat cells, a CD4+ T-cell line, were transfected with pDual-HIV(-1) or (0) plasmids and treated with thapsigargin, rapamycin or hippuristanol, three inhibitors perturbing a different step of cap-dependent translation initiation (Figure 1A). The frameshift efficiency, which is 5.1 ± 0.4% in the absence of inhibitors, was increased about twofold in the presence of either one of these three inhibitors (Figure 1C).

The presence of a high amount of TAR decreases HIV-1 frameshift efficiency

We next assessed the effect of TAR on the frameshift efficiency. TAR (Figure 2A) was inserted at about 40 nt from the 5′ end of the mRNA in pDual-HIV, generating pDual-HIV-TAR(-1) and (0) (Figure 2B). We avoided placing TAR at the very end of the mRNA, since such a position could interfere with the binding of the 40S subunit to the messenger (23,26,27 and references therein). We first examined the effect of a high amount of TAR that inhibits PKR and stimulates translation initiation (21). The frameshift efficiency was assessed in Jurkat and 293T cells. When 2 μg of pDual-HIV-TAR were delivered into the cells, the frameshift efficiency was decreased to 70% of its value in absence of TAR in either Jurkat or 293T cells (Figure 2C and D). Under the conditions of these assays, the frameshift efficiency in absence of TAR was 6.1 ± 0.2% in Jurkat cells and 11.3 ± 0.9% in 293T cells. These values, and the value of 5.1 ± 0.4% observed in the experiment described in the preceding section with Jurkat cells that were transfected under slightly different conditions (see details in 'Materials and Methods' section), are comparable to the values obtained with different heterologous systems containing the HIV-1 frameshift region, which were shown to range between 2 and 10% in mammalian cultured cells (8,22,28,29). It can be recalled here that several groups observed that the absolute value of the frameshift efficiencies changes, depending upon various parameters such as the conditions used for the assay and the type of cultured cells (30).

We then investigated whether the decrease in frameshift efficiency observed with pDual-HIV-TAR was influenced by the position of TAR in *cis* or in *trans* from the reporter mRNA. Two other constructs were used, pDual-HIV-50TAR, where the distance between TAR and the 5′ end of the reporter mRNA was increased by 50 nt compared to pDual-HIV-TAR, and pTAR, that provides TAR in *trans* from the reporter mRNA expressed from pDual-HIV (Figure 2B). The frameshift efficiency was decreased to 75 and 60%, respectively, in Jurkat cells and 293T cells transfected with pDual-HIV-50TAR compared to the value in absence of TAR. When Jurkat and 293T cells were co-transfected with 2 μg of pDual-HIV and 2 μg of pTAR, the frameshift efficiency was reduced to 70% of its value in absence of TAR, a decrease similar to that observed when TAR was present in *cis* of the reporter mRNA (Figure 2C and D). These results indicate that it is the presence of TAR in the cells and not its presence in the reporter mRNA that decreases HIV-1 frameshift efficiency. The effect of TAR on the frameshift efficiency was confirmed when using an infection system to deliver the reporters into the cells (see Figure 1 in the Supplementary Data).

Inhibiting PKR decreases HIV-1 frameshift efficiency

To verify that PKR was involved in the changes in HIV-1 frameshift efficiency observed with a high amount of TAR, we created two constructs, pTAR△bulge* and pTARuucg*, expressing mutants of TAR that cannot bind PKR (31) (Figure 3A). When Jurkat cells were co-transfected with pDual-HIV and plasmids generating these TAR mutants, the frameshift efficiencies were similar to that obtained in absence of TAR and significantly higher than the value obtained in the presence

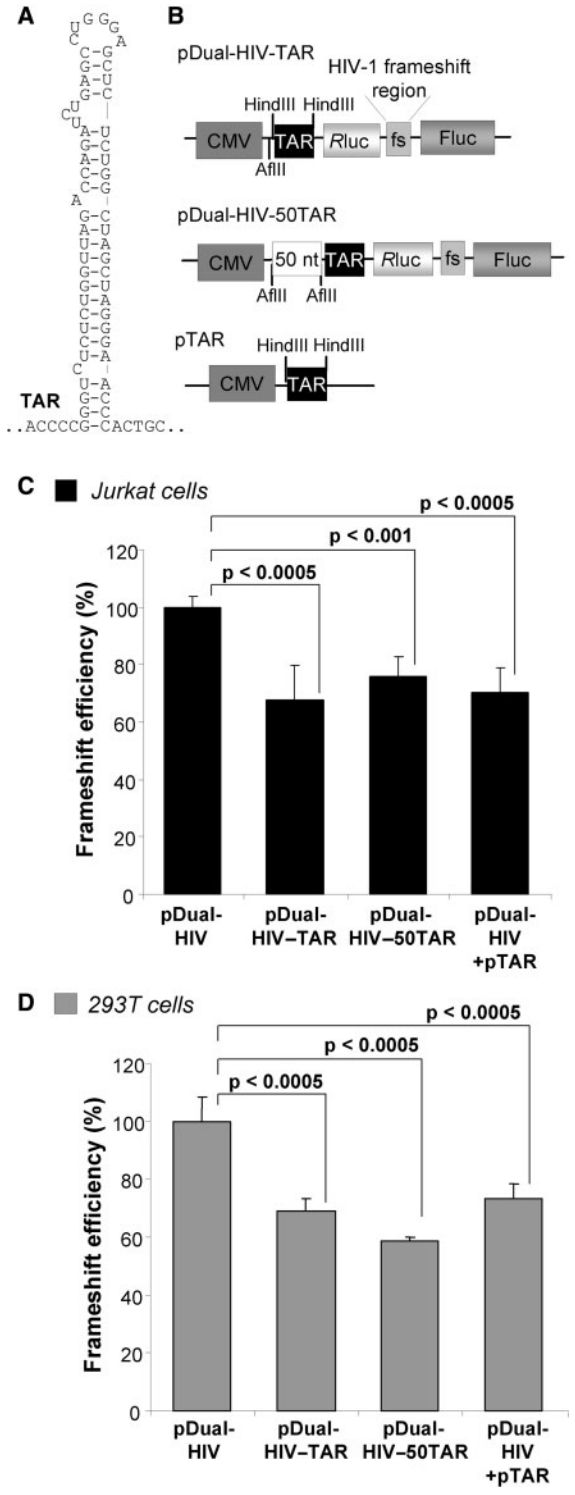

Figure 2. HIV-1 frameshift efficiency decreases when a high amount of TAR is present. (**A**) Sequence and structure of wild-type TAR RNA. (**B**) Plasmids pDual-HIV-TAR and pDual-HIV-50TAR are derivatives of pDual-HIV with the TAR-coding sequence inserted, respectively, about 40 nt downstream from the CMV promoter or at an additional distance of 50 nt from this promoter. Plasmid pTAR generates the free TAR sequence in *trans* from the reporter mRNA expressed from pDual-HIV. The frameshift efficiency was assessed in lysates from Jurkat cells (**C**) and 293T cells (**D**) transfected with 2 μg of pDual-HIV or pDual-HIV-TAR or pDual-HIV-50TAR or co-transfected with 2 μg of pDual-HIV and 2 μg of pTAR. The frameshift efficiency with Jurkat cells and 293T cells transfected with pDual-HIV was arbitrarily set at 100% in (C) and (D), respectively. Results are the means ± SD of at least four independent experiments. The *P*-values, calculated according to the Student's *t*-test, are indicated.

of wild-type TAR (Figure 3B). This result supports that PKR is involved in the changes of frameshift efficiency observed in the presence of TAR.

To further confirm that inhibiting PKR decreases HIV-1 frameshift efficiency, a plasmid expressing Tat, a HIV-1 viral protein, was co-transfected with the dual-luciferase plasmids. In addition to its well-characterized transactivation effect on transcription of the viral mRNAs by binding to TAR, Tat influences translation by inhibiting PKR, either directly by binding this kinase or indirectly by blocking the binding of TAR to PKR (32,33). We used a Tat mutant (Tat*) that can bind TAR and inhibit PKR but cannot transactivate transcription, and, thereby, that does not affect mRNA levels (24). Jurkat cells were co-transfected with the plasmid coding for this Tat mutant and with pDual-HIV, pDual-HIV-TAR or pDual-HIV-50TAR. In the presence of Tat*, the frameshift efficiency was decreased to approximately 60% of its value in absence of Tat* (Figure 3C). The decrease with Tat* was the same, whether TAR was present or not, which suggests that Tat* and TAR both act via the same mechanism, the inhibition of PKR.

TAR increases or decreases HIV-1 frameshift efficiency depending upon its concentration and this dose-dependent effect is mediated by PKR

Next, we investigated the effect of a small amount of TAR, which activates PKR and thus interferes with translation initiation (21). We used stable 293T transfectants expressing a dual-luciferase HIV reporter. Stable transfectants expressing a (-1) or (0) dual-luciferase HIV reporter were transfected with pTAR, pTAR△bulge* or pTARuucg* in amounts ranging from 0 to 2.3 μg. Figure 4A shows the effect of wild-type TAR. In the presence of a small quantity of TAR, the frameshift efficiency increases to about 140% of its value in absence of TAR but with a larger quantity of TAR, the frameshift efficiency decreases to about 80%, a decrease comparable to that observed with a transient transfection of pDual-HIV (Figure 2). As a control, we used stable 293T transfectants expressing *R*luc and Fluc in-frame, separated by a linker instead of the HIV-1 frameshift region. The ratio of Fluc activity to *R*luc activity in lysates from these transfectants was unchanged in the presence of pTAR (data not shown), confirming that changes in the Fluc to *R*luc ratio observed with stable transfectants expressing the dual-luciferase HIV reporter are due to variations in the frameshift efficiency. When the stable 293T transfectants expressing the dual-luciferase HIV reporter were transfected with plasmids producing TAR mutants that cannot bind PKR, the frameshift efficiency was unaltered (Figure 4B). The effect of a low amount of TAR was also assessed by transient co-transfection of Jurkat cells with pDual-HIV and different quantities of pTAR, ranging from 0 to 2 μg, the ratio of pTAR to pDual-HIV being equal or inferior to 1:1. The frameshift efficiency also

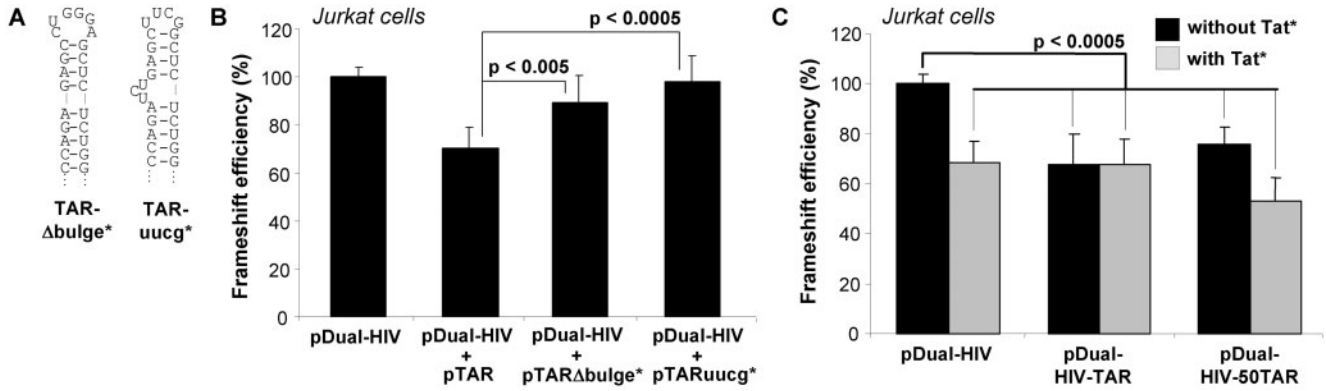

Figure 3. PKR is involved in the decrease in HIV-1 frameshift efficiency observed when a high amount of TAR is present. (**A**) Sequence and structure of TAR mutants that cannot bind PKR (31) used in this study. (**B**) The frameshift efficiency is not affected by the TAR mutants. The frameshift efficiency was assessed in lysates from Jurkat cells co-transfected with pDual-HIV and plasmids expressing wild-type TAR or mutants of TAR. The frameshift efficiency with pDual-HIV was arbitrarily set at 100%. Results are the means ± SD of at least five independent experiments. The *P* values are indicated. The values with pDual-HIV and pTARΔbulge* or pDual-HIV and pTARuucg* were not significantly different from the value with pDual-HIV, but were significantly higher than the value with pDual-HIV and pTAR. (**C**) The presence of a Tat mutant (Tat*) that inhibits PKR decreases the frameshift efficiency. The frameshift efficiency was assessed in lysates from Jurkat cells co-transfected with pDual-HIV, pDual-HIV-TAR or pDual-HIV-50TAR and a plasmid coding for Tat* or an empty vector in a 1:1 ratio. The frameshift efficiency with pDual-HIV without Tat* was arbitrarily set at 100%. Results are the means ± SD of at least four independent experiments. The *P*-values are indicated. The values with pDual-HIV-TAR and pDual-HIV-50TAR, with or without Tat*, were not significantly different from the value with pDual-HIV with Tat* but significantly lower than the value with pDual-HIV without Tat*.

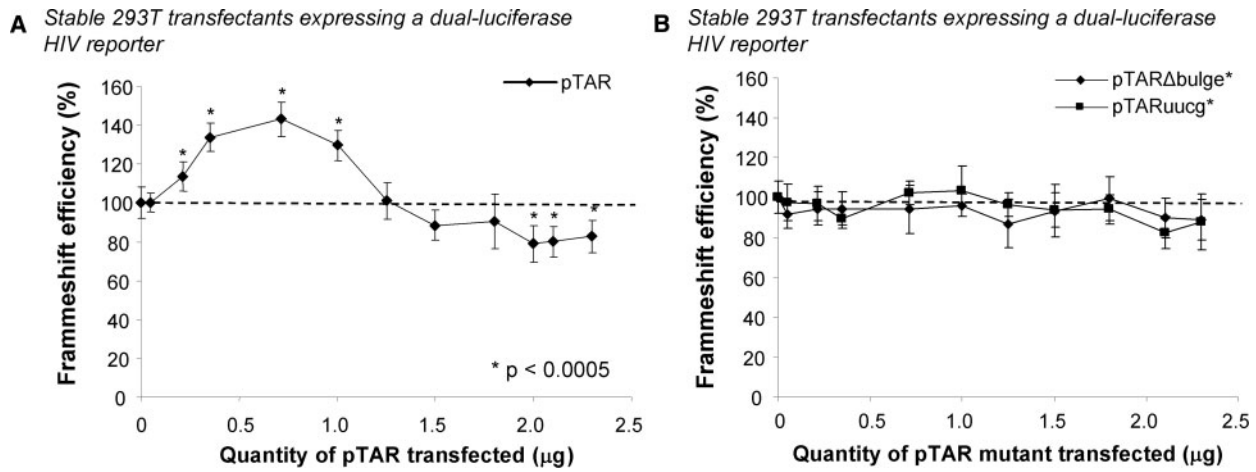

Figure 4. Wild-type TAR, but not the TAR mutants, increases or decreases HIV-1 frameshift efficiency in a dose-dependent manner. The frameshift efficiency was assessed in lysates from stable 293T transfectants expressing the (-1) or (0) dual-luciferase HIV reporter transfected with pTAR (**A**), pTAR△bulge* (**B**) or pTARuucg* (B) in different amounts ranging from 0 to 2.3 μg. The asterisks indicate the frameshift efficiencies that significantly differ from the frameshift efficiency without pTAR ($P < 0.0005$). Results are the means ± SD of at least six independent experiments.

increases under the conditions corresponding to low amounts of TAR, the highest increase being ~140% of the frameshift efficiency without TAR (data not shown).

We investigated the involvement of PKR in the changes in frameshift efficiency observed with a low amount of TAR. To this end, PKR expression was silenced by transfecting a PKR siRNA mix into stable 293T transfectants expressing a dual-luciferase HIV reporter. After 24 h, cells were transfected with pTAR in different amounts and harvested 48 h later. As a negative control, an eGFP siRNA mix targeting GFP was used. In the presence of the eGFP siRNA, the frameshift efficiency increases when TAR is present. However, when PKR expression is silenced, this effect disappears, supporting that it is related to PKR activation (Figure 5A). Effective silencing of PKR is achieved under the conditions of the assay as shown in Figure 5B. It can be noted that the response of the cells to the increase in the amount of TAR appears to differ from that in Figure 4. This is due to a difference in the experimental protocol resulting in a lower ratio of the quantity of transfected pTAR to the number of cells (see 'Materials and Methods' section).

DISCUSSION

Using a dual-luciferase reporter system in Jurkat and 293T cells, we showed that the presence of TAR alters

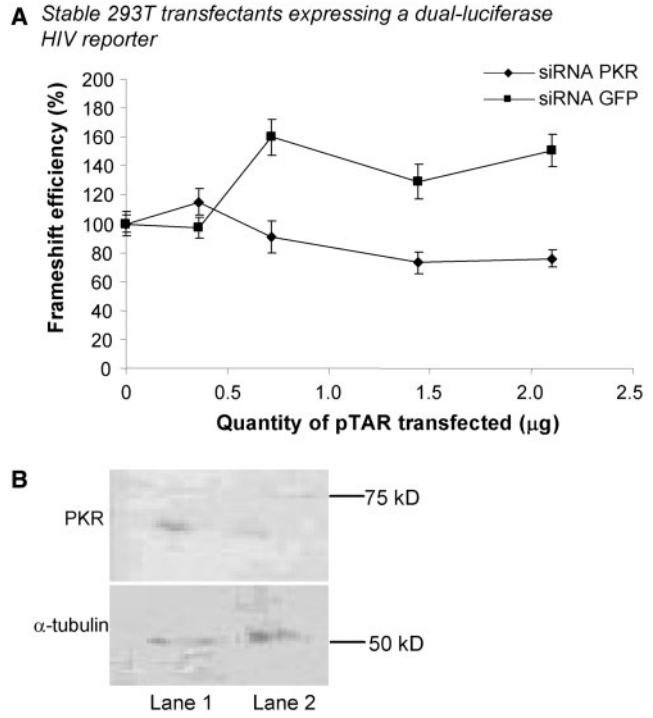

Figure 5. The effect of TAR on HIV-1 frameshift efficiency disappears when PKR expression is silenced. (**A**) The frameshift efficiency was assessed in lysates from stable 293T transfectants expressing the (-1) or (0) dual-luciferase HIV reporter and transfected first with a eGFP siRNA mix (negative control) or a PKR siRNA mix, and, after 24 h, with pTAR in different amounts ranging from 0 to 2.3 μg. Results are the means ± SD of at least three independent experiments. (**B**) Control of the silencing of PKR expression. Equal amounts of proteins from lysates of stable 293T transfectants expressing a dual-luciferase HIV reporter and transfected with either the eGFP siRNA mix (lane 1) or PKR siRNA mix (lane 2) were separated by SDS–PAGE, transferred on a nitrocellulose membrane and immunoblotted with a mouse anti-PKR monoclonal antibody. Anti-α-tubulin blotting was used as an internal control for loading.

HIV-1 frameshift efficiency. The addition of a high amount of TAR, in *cis* or in *trans* of the reporter mRNA, decreases the frameshift efficiency. This effect is related to an inhibition of PKR. Conversely, a low amount of TAR increases the frameshift efficiency, by activating PKR.

Activation or inhibition of PKR is well-known to affect translation initiation via changes in eIF2 phosphorylation (reviewed in 21). However, it is also known that transformed cells, such as those we used in this study, tolerate a certain degree of endoplasmic reticulum stress leading to a certain level of phosphorylation of eIF2 via PERK, a kinase functionally homologous to PKR (34). Our experimental conditions do not drastically affect the expression of our reporters, implying that the changes in the translation initiation rate caused by activation or inhibition of PKR are small and that the changes in eIF2 phosphorylation should be modest. Using western blotting, we could not detect significant variations in the phosphorylation level of eIF2 in 293T or Jurkat cells transfected with different quantities of TAR (data not shown). We nevertheless suggest that the effect of PKR on HIV-1 frameshift efficiency results from changes in eIF2 phosphorylation that are too small to be detected in presence of the endogenous signal for phosphorylated eIF2 in these cells. However, we cannot exclude that PKR could also influence HIV-1 frameshift efficiency via another yet undiscovered mechanism.

Contradictory effects were seen in previous observations on the influence of the translation initiation rate on the frameshift efficiency. The frameshift efficiency of a plant virus, the beet western yellow virus (BWYV), was higher in a reticulocyte lysate than in a wheat germ extract, which has a lower rate of translation initiation (35). Also, the frameshift efficiency of the human T-cell leukemia virus type II (HTLV-2), when measured in a reticulocyte lysate, was higher with capped than with uncapped mRNAs, which have a lower rate of translation initiation (36). These observations disagree with our results that show a negative relationship between the rate of translation initiation and the frameshift efficiency. However, Paul *et al.* (37), when comparing the frameshift efficiency of the barley yellow dwarf virus (BYDV) with capped and uncapped mRNAs in a yeast extract, found that increasing the translation initiation rate decreased the frameshift efficiency. Furthermore, Lopinski *et al.* (38), who investigated *in vivo* the effect of a reduced translation initiation rate on the frameshift efficiency of the L-A virus of *S. cerevisiae*, found that this efficiency was increased under these conditions. The results of Paul *et al.* (37) and Lopinski *et al.*(38) are in perfect agreement with our findings, and, in line with them, we present the following model that explains our results (Figure 6).

When a ribosome translates the HIV-1 frameshift region, it encounters the frameshift stimulatory signal and makes a pause, its decoding center covering the slippery sequence (39–41). During the pause, the ribosome can shift or not the reading frame, and, after the pause, the ribosome unfolds the frameshift stimulatory signal and translation continues. If the upstream ribosome reaches the frameshift region before the signal has refolded, the probability that the frameshift occurs is extremely weak. The spacing between ribosomes translating the HIV-1 frameshift region, which is determined by the rate of translation initiation [basal rate estimated to about one initiation event every 6.5 s (42)], could thus affect the frameshift efficiency. Therefore, if we assume an average elongation speed of five amino acids per second per ribosome, corresponding to a displacement of 15 nt per second on the mRNA (42), the minimal distance between the decoding centers of two ribosomes translating a mRNA would be of about 100 nt. A ribosome covers about 32 nt on the mRNA and heel-printing studies showed that the first base of the P-site codon is at a distance of 12 nt from the 5′ edge of the ribosome and of 20 nt from the 3′ edge (43). From these calculations, there would be about 70 exposed nt between two elongating ribosomes. Thus, the HIV-1 frameshift region, including the 43-nt frameshift stimulatory signal, would be exposed after the passage of the first ribosome. The signal would then re-form, which takes only a few microseconds (44),

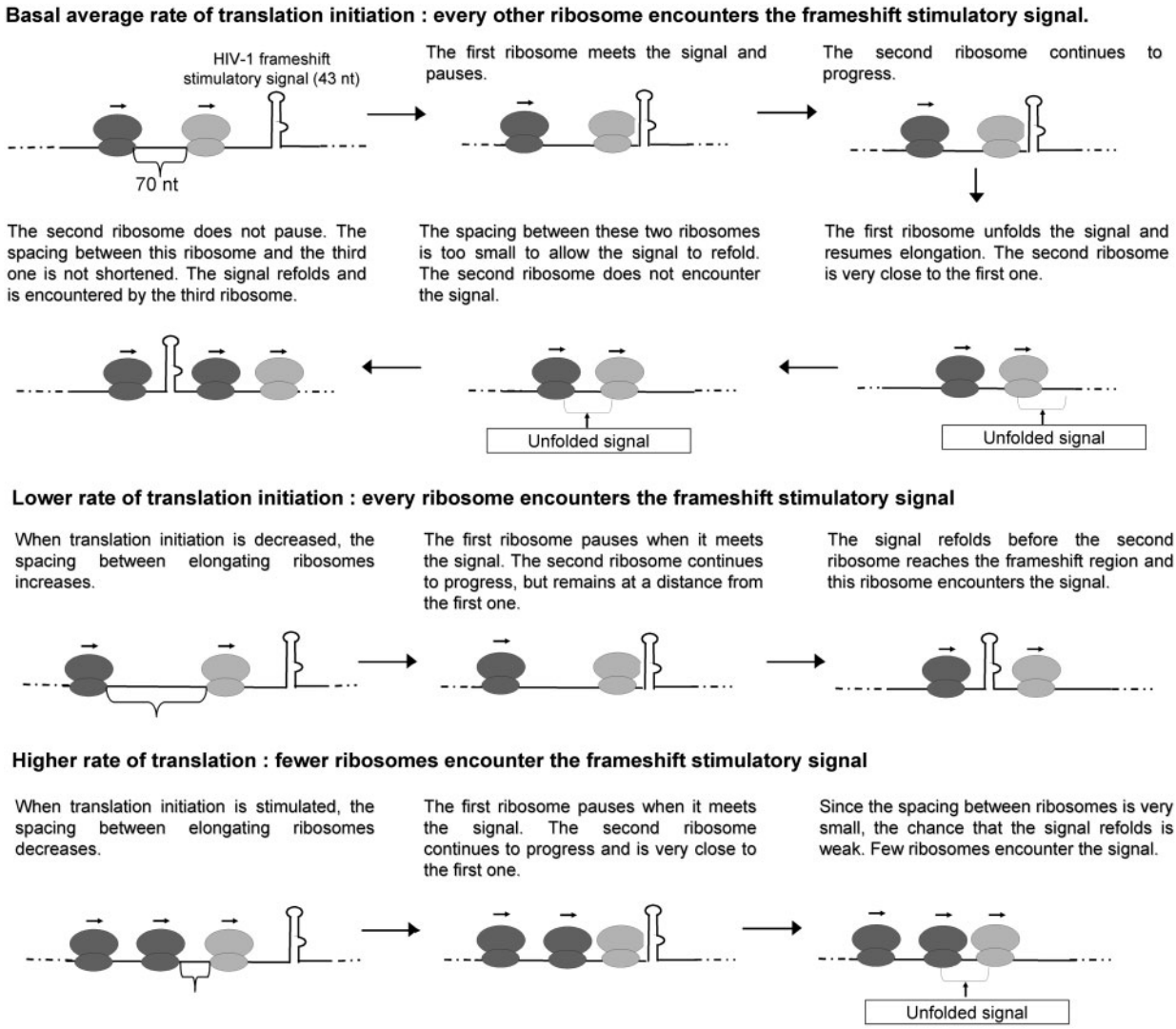

Figure 6. Changes in the rate of translation initiation influence the frameshift efficiency by modifying the spacing between elongating ribosomes. This model shows elongating ribosomes that reach the frameshift region and explains how the rate of translation initiation, which determines the spacing between these ribosomes, affects the frameshift efficiency (see the text). Note that a ribosome must encounter a folded frameshift stimulatory signal to make a frameshift, but this encounter does not ensure that frameshifting will occur.

before the upstream ribosome reaches the region of the mRNA containing the sequence of this signal. However, the pause made by the first ribosome when encountering the signal decreases the distance with the following ribosome, which has continued to progress during the pause of the first ribosome. This second ribosome could reach the region corresponding to the stimulatory signal before this signal could refold, being still partially covered by the first ribosome. A pause of about three seconds for the first ribosome is sufficient to prevent the refolding of the stimulatory signal. The second ribosome would thus avoid frameshifting and the spacing between this ribosome and the third ribosome would not be altered. As a consequence, the third ribosome would encounter the stimulatory signal and pause, and frameshifting would be possible. This analysis shows that the signal affects every other ribosome under basal conditions. According to this model, an increase in the rate of translation initiation would decrease the frameshift efficiency, since ribosomes would be closer to each other and a smaller proportion of ribosomes would encounter the folded frameshift stimulatory signal. Conversely, a decrease in translation initiation would increase the frameshift efficiency since ribosomes would be further apart and it is very likely that each ribosome would encounter the folded signal. Interestingly, Lopinski *et al.* (38), when studying the effect of a reduced translation initiation rate with the L-A virus in yeast cells, observed that the frameshift efficiency doubled, independently of the severity of the initiation defect. Their interpretation was that every other ribosome encounters the signal under basal conditions and that, with a reduced initiation rate, every ribosome encounters this signal. Our analysis fully supports this interpretation.

Although HIV-1 does not induce a rapid and dramatic global shutdown of host cell translation following infection, in contrast to other viruses such as poliovirus, cap-dependent translation initiation is decreased due to cellular stress following infection by this virus (25,45) and this decrease can be related to PKR activation (46). Our results suggest that a change in cap-dependent translation initiation could affect HIV-1 frameshift efficiency in infected cells. As mentioned in the 'Introduction' section, the virus replication appears to be exquisitely sensitive to changes in frameshift efficiency. Given the detrimental effect of such changes, the virus likely uses various strategies to counteract this effect. One strategy is inhibition of PKR (reviewed in 25,47) to stimulate translation initiation. HIV-1 uses two major ways to inhibit PKR: its Tat protein inhibits PKR and its TAR RNA structure blocks PKR dimerization when present in large quantities. TAR is located at the 5′ and 3′ end of all HIV-1 mRNAs and is also present under a free cytoplasmic form of 58-66 nt (17,18). All these forms of TAR can participate in the inhibition of PKR.

However, inhibition of cap-dependent translation initiation can occur independent of PKR activation. Indeed, the HIV-1 Vpr protein is capable of inducing G2 arrest in cultured CD4+ T cells (48,49 and references therein), and, during such arrest, cap-dependent translation initiation is severely impaired (50). Another possible strategy to circumvent the problem caused by this situation is the use of a cap-independent mechanism by HIV-1 to initiate the translation of its full-length mRNA (25,45). The virus would thus continue to express Gag and Gag-Pol and would maintain a frameshift efficiency that is optimal for its replication. An internal ribosomal entry site (IRES) was identified in the 5′UTR region of HIV-1 full-length mRNA (51) and another IRES was found in the beginning of the *gag* coding sequence (52). IRES have also been found in HIV type 2 (53) and in simian immunodeficiency virus (54), two viruses related to HIV-1. However, the use of an IRES by HIV-1 in the context of replication-competent viruses remains to be proven (45). The two strategies that are described above are not mutually exclusive. HIV-1 could first counteract changes in cap-dependent translation initiation by inhibiting PKR, until a larger stress in the cellular environment severely perturbs cap-dependent initiation. The virus would then switch to an IRES-driven mode to translate its full-length mRNA.

This scheme is deduced from studies in cultured cells and it will now be important to investigate the frameshift efficiency in the context of a viral infection. A detailed understanding of the mechanisms used by HIV-1 to control its frameshift efficiency will provide valuable information for the design of drugs targeting the frameshift event.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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