Comparative mutational analysis of cis-acting RNA signals for translational frameshifting in HIV-1 and HTLV-2

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) and human T cell leukemia virus type II (HTLV-2) use a similar mechanism for −1 translational frameshifting to overcome the termination codon in viral RNA at the end of the gag gene. Previous studies have identified two important RNA signals for frameshifting, the slippery sequence and a downstream stem–loop structure. However, there have been somewhat conflicting reports concerning the individual contributions of these sequences. In this study we have performed a comprehensive mutational analysis of the cis-acting RNA sequences involved in HIV-1 gag-pol and HTLV-2 gag-pro frameshifting. Using an in vitro translation system we determined frameshifting efficiencies for shuffled HIV-1/HTLV-2 RNA elements in a background of HIV-1 or HTLV-2 sequences. We show that the ability of the slippery sequence and stem–loop to promote ribosomal frameshifting is influenced by the flanking upstream sequence and the nucleotides in the spacer element. A wide range of frameshifting efficiency rates was observed for both viruses when shuffling single sequence elements. The results for HIV-1/HTLV-2 chimeric constructs represent strong evidence supporting the notion that the viral wild-type sequences are not designed for maximal frameshifting activity but are optimized to a level suited to efficient viral replication.

INTRODUCTION

Programmed ribosomal frameshifting modulates the expression of two open reading frames (ORFs) in many retrovirus, plant virus, coronavirus and protozoan genes (reviewed in 1,2). In human immunodeficiency virus 1 (HIV-1) −1 translational frameshifting of its mRNA leads to synthesis of the Gag–Pol fusion protein which gives rise to the viral protease, reverse transcriptase and integrase (3). Without frameshifting only the precursor of structural proteins, Gag, is expressed. The ratio of Gag to Gag–Pol proteins is highly regulated and critical for viral propagation (4,5). Similarly, in human T cell leukemia virus type II (HTLV-2) two −1 ribosomal frameshift events result in synthesis of the fusion proteins Gag–Pro and Gag–Pro–Pol (6,7).

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our study the region upstream of the slippery sequence as well as the spacer element that is located between the slippery site and the stem–loop. By shuffling individual or multiple elements of the HIV-1 and HTLV-2 wild-type sequences we were able to investigate the individual contributions of the RNA signals to frameshifting efficiency. Further, we show that the degree by which frameshifting efficiency is altered on exchange of the various RNA elements is strongly dependent on the nature of the particular stem–loop sequence.

**MATERIALS AND METHODS**

**Template construct for frameshifting assay**

The green fluorescent protein (GFP) gene amplified from the pEGFP-c2 vector (Clontech) by PCR was ligated to an EcoRI/ BamHI-digested pGEM-3Z vector (Promega) with a T7 promoter sequence upstream of the EcoRI site. Subsequently, the glutathione S-transferase (GST) gene, PCR-amplified from the pGEX-5X1 vector (Amersham Pharmacia), was inserted between the PstI and HindIII sites. For generation of the individual reporter constructs the BamHI and PstI sites of the resulting plasmid vector were used for cloning –1 frameshifting elements from either HIV-1 or HTLV-2. The respective sequences were obtained using annealed duplex DNA oligomers. To generate the stem–loop deletion mutants the internal BglII and PstI restriction sites were used. The accuracy of all wild-type and mutant constructs was confirmed by dideoxy DNA sequencing.

The UAG termination codon of the GFP ORF is located immediately after the inserted frameshifting region. If a –1 frameshift occurs at the slippery sequence, the termination codon is not read and translation proceeds through the GST gene, resulting in the production of a GFP–GST fusion protein.

**Frameshifting assay**

All plasmids were isolated and purified as described (11). The lyophilized DNA was dissolved in TE buffer (Tris–HCl, pH 8.0, and 1 mM EDTA). The T7/T Quick coupled T7 transcription/translation system (Promega) was used according to the manufacturer’s protocol. We compared this system with the previously used T7/T coupled T7 transcription/translation system (Promega) (11) and noticed no substantial difference in intra- and inter-assay variability except for a slightly lower product yield with the T7/T Quick coupled T7 transcription/translation system. Aliquots of 400 ng template DNAs were used in a 20 µl reaction containing 10 µl reticulocyte lysate and 0.8 µl of 10 µCi/µl 35S-labeled methionine (NEN).

The GFP–GST fusion product yields a protein of 58 kDa that contains 18 methionine residues, whereas the non-frameshifting GFP protein product is 30 kDa (HIV-1) or 28 kDa (HTLV-2) with six methionines. The high number of methionines in GST enhances the sensitivity for measuring frameshifting rates since the levels of frameshifting efficiency in HIV-1 and HTLV-2 are low compared to other viral systems such as BWYV and PLRV (11,12). In order to separate the GFP–GST fusion protein from the non-frameshifting product (GFP) the samples were separated through 12% SDS–polyacrylamide gels (Fig. 2B). After electrophoresis, gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics). Quantitation of signal intensities was done using PhosphorImager software (Molecular Dynamics). Frameshifting efficiencies were calculated using the formula [\(I_{FS}/I_{NFS}\)], where \(I_{FS}\) is the signal intensity of the frameshifting product and \(I_{NFS}\) is the signal intensity of the non-frameshifting product. All individual in vitro assays were accompanied by HIV-1 wild-type controls and repeated three times or more to determine average frameshifting efficiencies. The mean ± standard deviation frameshifting efficiency of the HIV-1 wild-type reactions in this study was 5.6 ± 0.4%.

**RESULTS**

**Experimental strategy**

Figure 1A compares the sequence regions of HIV-1 and HTLV-2 involved in translational frameshifting of the viral gag–pol or gag–pro genes, respectively. The predicted secondary structure of the stem–loop motif is shown in Figure 1B. For quantitative in vitro analysis of frameshifting activity promoted by wild-type and mutant HIV-1 or HTLV-2 RNA elements we inserted them between the ORFs of GFP and GST (Fig. 2). The non-frameshifting product yields a protein of 30 kDa (HIV-1 constructs) or 28 kDa (HTLV-2 constructs) with six internal methionines. Upon –1 frameshifting a fusion protein of 58 kDa containing 18 methionines is produced in reticulocyte extracts. To determine frameshifting efficiencies of HIV-1 and HTLV-2 constructs the ratios of frameshifting to non-frameshifting product were determined from at least three independent reactions each. Figure 3 shows an example of an autoradiogram obtained from electrophoretically separated in vitro translation products from incubations with HIV-1 and HTLV-2 wild-type reporter constructs and selected mutants. The HIV-1 wild-type construct yielded a frameshifting activity of ~5.6%, whereas the HTLV-2 wild-type sequence yielded ~9.3%. Deleting the stem–loop of the HIV-1 or HTLV-2 wild-types drastically reduced frameshifting (0.8% for HIV-1, 1.3% for HTLV-2) without eliminating it completely (Fig. 3), in accordance...
Contribution of slippery sites to frameshifting in HIV-1 and HTLV-2 is context dependent

The slippery sequences in HIV-1 and HTLV-2 have been shown to be essential for frameshifting (9,10), with an optimal repetition of A₆ or U₆ within the consensus X₆Y. If the HIV-1 slippery site U₆A is replaced by G₃A₃C, frameshifting is almost eliminated (Table 1). In the case of HTLV-2 the same slippery sequence (G₃A₃C) leaves a residual frameshifting activity of 2.7%. However, when in addition the stem–loop is replaced by the HIV-1 stem–loop frameshifting is nearly abolished.

The seemingly conflicting results of Kollmus et al. (9) and Honda et al. (10) concerning the influence of the slippery sequence on frameshifting efficiencies of HIV-1 and HTLV-2 prompted us to investigate the contribution of the slippery sequence on frameshifting within the different sequence backgrounds of the HIV-1 and HTLV-2 mRNAs. As shown in Figure 3, frameshifting increases by 63% (from 5.6 to 9.1%) when the wild-type slippery sequence UUUUUUA is replaced by the slippery site AAAAAAC of HTLV-2. Furthermore, an HIV-1 construct lacking the stem–loop but with the HTLV-2 slippery site still had higher frameshifting activity than the complete HIV-1 wild-type sequence (7.8% compared to 5.6%; Fig. 3).

Intriguingly, changing the wild-type slippery sequence of HTLV-2 from A₆C₄ to the U₆A sequence of HIV-1 also greatly increased frameshifting efficiency, from 9.3 to 15.3%. Taken together, these results strongly argue for a modulatory role of the upstream sequences and/or the spacer element on frameshifting.

Table 1. Frameshifting efficiencies of HIV-1 (I) and HTLV-2 (T) wild-type constructs and chimeras

| UP       | SL       | SP       | STL                          | Composition | Efficiency (%) |
|----------|----------|----------|------------------------------|-------------|----------------|
| HIV-1   | CAGGCUAA | UUUUUUA  | GGGAAGA                      | I-STL       | 5.6 ± 0.4 (WT) |
| HTLV-2  | CCUGAGGA | AAAAAAC  | UCCUUA                       | I-STL       | 4.5 ± 0.3      |
|          |          |          | UCCUUA                       | I-STL       | 9.1 ± 0.6      |
|          |          |          | UCCUUA                       | I-STL       | 3.2 ± 0.2      |
|          |          |          | UCCUUA                       | I-STL       | 2.3 ± 0.7      |
|          |          |          | UCCUUA                       | I-STL       | 2.2 ± 0.1      |
|          |          |          | UCCUUA                       | I-STL       | 10.9 ± 0.4     |
|          |          |          | UCCUUA                       | I-STL       | 1.9 ± 0.3      |
|          |          |          | UCCUUA                       | T-STL       | 9.3 ± 0.9 (WT) |
| HIV-1   | CAGGCUAA | AAAAAAC  | GGGAA                        | I-STL       | 14.3 ± 0.5     |
|          | CCUGAGGA | UUUUUUA  | GGGAAGA                      | T-STL       | 17.0 ± 0.6     |
|          | CCUGAGGA | AAAAAAC  | GGGAA                        | T-STL       | 11.0 ± 1.0     |
|          | CCUGAGGA | UUUUUUA  | GGGAAGA                      | T-STL       | 17.9 ± 1.1     |
|          | CCUGAGGA | AAAAAAC  | GGGAA                        | T-STL       | 18.9 ± 0.8     |
|          | CCUGAGGA | UUUUUUA  | GGGAA                        | T-STL       | 15.5 ± 0.5     |
|          | CCUGAGGA | UUUUUUA  | GGGAAGA                      | T-STL       | 12.8 ± 0.7     |
|          | CCUGAGGA | UUUUUUA  | GGGAAGA                      | I-STL       | 0.5 ± 0.1      |
|          | CCUGAGGA | GGGAAAC  | UCCUU                        | I-STL       | 0.2 ± 0.1      |
|          | CCUGAGGA | GGGAAAC  | UCCUU                        | T-STL       | 2.7 ± 0.1      |

UP, upstream sequence; SL, slippery site; SP, spacer element; STL, stem–loop (underlined).

Figure 2. Schematic representation of reporter constructs and resulting protein products used for in vitro ribosomal frameshifting measurements. Boxed regions indicate ORFs for GFP (shaded light gray) and GST (dark gray).

Figure 3. Schematic representation of reporter constructs and resulting protein products used for in vitro ribosomal frameshifting measurements. Boxed regions indicate ORFs for GFP (shaded light gray) and GST (dark gray).

with earlier in vitro and in vivo results from other groups (10,13,14).

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In this case the HTLV-2 A 6C slippery sequence was extended to an A 4C motif because of the HIV-1 upstream sequence. A stimulatory effect on frameshifting through an increasing number of adenines in the slippery sequence is consistent with the earlier results of Honda et al. (10).

In HTLV-2 double replacement of the upstream and slippery sequences or the upstream sequence and the spacer both increased frameshifting rates (17.9 and 18.9%, respectively; Table 1). Individual exchange of the slippery sites boosted frameshifting to 17%, while spacer replacement yielded 11% (Table 1). However, exchanging all three elements resulted in 12.8% frameshifting.

**Frameshiftings mediated by the HIV-1 or HTLV-2 stem–loops show different sensitivities to changes in surrounding sequences**

Of the several RNA elements examined, the stem–loop sequence, in concert with a slippery site, is certainly the most important component of the frameshifting region, as has been demonstrated by several groups (9,10,13). This was confirmed in our *in vitro* experiments (see Fig. 3). Further, it has been shown for HIV-1 that frameshifting rate correlates with the thermodynamic stability of the stem–loop (15).

We asked whether frameshifting mediated by the HIV-1 and HTLV-2 stem–loops would be similarly influenced by changes in the other *cis*-acting RNA elements, a behavior that would indicate a purely additive contribution from each element to frameshifting function. As shown in Figure 4A, the changes in the extent of frameshifting brought about by changes in the slippery sequence were different for the HIV-1 and HTLV-2 stem–loops. The range of frameshifting rates in HIV-1 was much smaller (1–9%) than the range for HTLV-2 stem–loop constructs (2–18%). This behavior might be a direct consequence of the inherently different thermodynamic stabilities of the two stem–loop structures at 37°C, which for HTLV-2 was calculated to be $\Delta G = –18.3$ kJ/mol, while for HIV-1 $\Delta G = –20.9$ kJ/mol, as estimated using the Mulfold program (16). In order to examine this property more directly we introduced mutations into the HTLV-2 stem–loop that only changed the orientation of base interactions but did not substantially influence the calculated thermodynamic stability of the stem–loop structures. As documented in Figure 4B, only minor changes in frameshifting efficiency were observed with mutants M1–M4 in combination with the HTLV-2 wild-type slippery sequence. However, when introducing the HIV-1 slippery site, 2-fold differences in frameshifting rates were measured between mutants (Fig. 4B). The most drastic change was apparent after inversion of the terminal three G-C base pairs of the stem, which might alter base stacking within the helix. Similarly, inverting the medial three G-C base pairs decreased frameshifting substantially, whereas inversion of all six G-C base pairs yielded frameshifting activities close to wild-type levels (Fig. 4B). In line with the results described above, an analogous mutational analysis with the HIV-1 stem–loop resulted in only minor differences in frameshifting efficiencies (data not shown).

**DISCUSSION**

The frameshifting regions of HIV-1 and HTLV-2 mRNA contain several RNA elements, of which the slippery sequence
and the stem–loop have been studied extensively with respect to their importance for frameshifting function. Here we have extended the analysis of cis-acting RNA signals to the neighboring upstream region and the spacer sequence. We have examined their functional interrelationships by shuffling HIV-1 and HTLV-2-derived elements. Our results from interchanging the slippery sites provide an explanation for the apparently contradictory results obtained by other groups (9,10). As reported by Honda et al. (10) and confirmed in our experiments, the combination of the HIV-1 stem–loop and the HTLV-2 slippery sequence results in higher frameshifting activity than the HIV-1 stem–loop combined with the HIV-1 slippery site. However, if the surrounding RNA elements, the upstream sequence and the spacer, are also exchanged for the respective HTLV-2 sequences, then we obtain the opposite result. In fact, an HTLV-2-based construct with the HIV-1 stem–loop was used in the study by Kollmus et al. (9) and this explains how they came to the conclusion that the HIV-1 slippery sequence is more efficient in mediating –1 frameshifting.

If we compare all possible combinations of HIV-1/HTLV-2 chimeras that differ only in the slippery sequence (mutant nos 1/3, 2/5, 4/7, 6/8 and 11/9), then three out of five combinations give higher frameshifting with the HIV-1 slippery site. Clearly, the frameshifting activity induced by a given combination of RNA elements is not predictable from the individual contributions of single components, but rather is the result of a complex interplay between each sequence region within the mRNA and probably their interaction with the translational machinery.

Interestingly, the construct with the highest frameshifting activity (Table 1, mutant no. 14) was a mosaic of alternating RNA elements from HIV-1 and HTLV-2. Also, the second best frameshifting chimera (mutant no. 13) contained two RNA elements from each virus. The question arises, why did the frameshifting region evolve to harbor at least four cis-acting RNA signals even though one or two of these elements, optimally combined, can yield the same level of frameshifting? It is widely known that the efficiency of translational frameshifting is regulated during the viral life cycle (for reviews see 17–19). Such mechanisms require additional sequences that interact with viral and/or host factors. A number of trans-acting factors that positively or negatively influence the efficiency of frameshifting have already been genetically identified (20–23). Within viral RNA the candidate region most likely to be subject to regulatory control is the stem–loop. It is well known that during translation of the frameshift region the secondary structure has to be unfolded for the ribosome to proceed along the mRNA. The stem–loop is believed to collide with the moving ribosomal machinery causing it to stall (24,25). This pausing is a prerequisite for efficient frameshifting and it has recently been shown that the average ribosomal pause time is greater for that fraction of ribosomes that proceed in the –1 frame (26). Cellular or viral trans-acting factors that interact with the stem–loop region could thus positively or negatively influence frameshifting by stabilizing or destabilizing the secondary structure. In fact, Kollmus et al. (27) have demonstrated that when replacing the HIV-1 stem–loop by the iron-responsive element, frameshifting rates increase under conditions that allow binding of iron regulatory proteins.

When comparing HIV-1 and HTLV-2 constructs, mutants of the frameshifting region had a more profound effect on frameshifting in the context of the HTLV-2 stem–loop than with the HIV-1 sequence, indicating a higher sensitivity of this element to changes in the surrounding sequences. These results are in line with earlier work demonstrating that the HTLV-2 stem–loop is much more sensitive to changes in the length of the spacer element than the HIV-1 stem–loop (9,28). The differences might be solely dependent on the thermodynamic stability of the stem–loop structure. However, when we tested mutants of the HTLV-2 stem–loop that do not change the overall thermodynamic stability of the secondary structure but only alter base pair orientation, the frameshifting efficiencies of individual mutants still varied depending on the nature of the slippery sequence. The HIV-1 slippery site proved much more sensitive to mutations in the stem–loop than that derived from HTLV-2. Taken together, these findings also argue for the involvement of additional cis- and/or trans-acting factors.

It has recently been shown that the introduction of an upstream or downstream termination codon relative to the slippery site also influences frameshifting efficiency (29,30).
An upstream termination codon in the –1 frame located at various positions impairs frameshifting via an unknown mechanism (29). However, a downstream termination codon in the –1 frame (30) enhances frameshifting, probably because it represents another pausing element for the translating ribosomes acting in concert with the RNA secondary structure. The translational termination signal probably leads to sequestration of protein factors to the frameshifting region either positively or negatively interfering with the frameshifting process.

Although the mechanics of ribosomal frameshifting are largely unknown, we should not be surprised to find that frameshifting efficiency is affected by the upstream sequences and spacer sequences, as well as the more thoroughly studied slippery sequence and downstream structural motif, in this case a stem–loop. The spacer element usually has 6 or 7 nt. It is likely that they are normally in a stacked configuration with ~3.4 Å per base. A number of nuclease digestion experiments have been done and they reveal that the number of nucleotides found between the coding site and the outside of the ribosome where the nuclease acts is 12–15 nt in prokaryotes (31,32) and 20 nt in eukaryotes (33). This clearly suggests that the spacer segment undergoes considerable elongation before ribosomal frameshifting occurs. The power behind this extension is the translational mechanism of the ribosome, which moves the mRNA–tRNA complex one codon (probably ~10 Å) associated with tRNA translocation from the A site to the P site. This translocation moves in the upstream direction and presumably elongation of the spacer segment is due to the fact that the downstream secondary structure, in this case the stem–loop, cannot enter the ribosomal mRNA channel. We do not as yet know the mechanism behind the translocation process and mRNA movement, but it is likely that a significant component consists of pressures to move the tRNA itself. It is this movement in the upstream direction which, when faced with an extended mRNA and a secondary structural element that does not unravel, leads to sliding of the tRNA by ~1 nt in the upstream direction. The detailed sequence of bases in the spacer segment will determine both its initial stacking energy and its gradual loss through unstacking and extension, as well as the extent to which it interacts with other ribosomal components forming the mRNA channel. In the same way, the upstream sequence of the message must continue to move through the ribosome during the translational process. Thus, it too may have an opportunity to be influenced by contacts with those elements that make the ribosomal mRNA channel. However, it has recently been shown that specific inhibition of EF-2-mediated translocation by pokeweed antiviral protein does not change the efficiency of –1 ribosomal frameshifting. Therefore, the frameshift must occur before completion of the peptidyl-transferase reaction (34).

An important element is, of course, the interaction of the stem–loop structure with the ribosome, which helps to determine whether it will unravel, and therefore not frameshift, or maintain its structure, leading to frameshifting. Here it is not a matter of the stabilizing energy of the isolated stem–loop. Instead, it is the energy of the stem–loop as it abuts the ribosome. As seen by the variation in frameshifting associated with changes in the stem–loop structure (Fig. 4B), it seems clear that a string of CG base pairs are important for stability near the end of the stem–loop structure. However, there is a curious destabilization associated with mutants M2 and M3 in which the six CG base pairs have been changed to blocks of three with the adjacent block inverted. These lose considerable frameshifting ability with the HIV-1 slippery sequence, but much less so with HTLV-2. This may reflect the fact that interaction of the stem–loop with the ribosome is a much more important component of frameshifting in HIV-1 than in HTLV-2.

Discussions of the mechanics of ribosomal frameshifting will shortly undergo an abrupt change due to the recent publication of high resolution X-ray crystallographic studies of both the large and small ribosomal subunits (35–37). At present it is possible to locate the position of the two tRNAs in the A and T sites and give a precise description of that environment. In the near future, with further developments in this area, we will be able to transform the discussion of ribosomal frameshifting from generalities to highly specific suggestions about which structural elements and interactions may be important in understanding this process.

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