Structure–function analysis of the ribosomal frameshifting signal of two human immunodeficiency virus type 1 isolates with increased resistance to viral protease inhibitors

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Expression of the pol-encoded proteins of human immunodeficiency virus type 1 (HIV-1) requires a programmed –1 ribosomal frameshift at the junction of the gag and pol coding sequences. Frameshifting takes place at a heptanucleotide slippery sequence, UUUUUUA, and is enhanced by a stimulatory RNA structure located immediately downstream. In patients undergoing viral protease (PR) inhibitor therapy, a p1/p6gag L449F cleavage site (CS) mutation is often observed in resistant isolates and frequently generates, at the nucleotide sequence level, a homopolymeric and potentially slippery sequence (UUUUCUU to UUUUUUU). The mutation is located within the stimulatory RNA downstream of the authentic slippery sequence and could act to augment levels of pol-encoded enzymes to counteract the PR deficit. Here, RNA secondary structure probing was employed to investigate the structure of a CS-containing frameshift signal, and the effect of this mutation on ribosomal frameshift efficiency in vitro and in tissue culture cells was determined. A second mutation, a GGG insertion in the loop of the stimulatory RNA that could conceivably lead to resistance by enhancing the activity of the structure, was also tested. It was found, however, that the CS and GGG mutations had only a very modest effect on the structure and activity of the HIV-1 frameshift signal. Thus the increased resistance to viral protease inhibitors seen with HIV-1 isolates containing mutations in the frameshifting signal is unlikely to be accounted for solely by enhancement of frameshift efficiency.

INTRODUCTION

Expression of the HIV-1 pol gene requires a programmed –1 ribosomal frameshift event (Jacks et al., 1988). Most ribosomes translating the viral genomic RNA terminate at the gag stop codon, generating the Gag polyprotein, encoding structural proteins. However, a shift of about 5–10 % into the overlapping pol frame in response to the frameshift signal yields the Gag–Pol fusion protein from which pol-encoded proteins viral protease (PR), reverse transcriptase (RT) and integrase are liberated by PR-mediated proteolysis. The frameshift signal of HIV-1 has two components, a slippery sequence, UUUUUUA, where the frameshift takes place, and a stimulatory RNA secondary structure immediately downstream. The precise structure of the HIV-1 stimulatory RNA has been the subject of debate (Jacks et al., 1988; Le et al., 1991; Du et al., 1996; Dinman et al., 2002; Dulude et al., 2002; Staple & Butcher, 2003; reviewed by Brierley & Dos Ramos, 2005), but from mutational analysis, secondary structure probing and nuclear magnetic resonance (NMR) analysis, the active element is most probably a stem–loop consisting of a two-stem helix capped by a tetraloop (Dulude et al., 2002; Staple & Butcher, 2005; Gaudin et al., 2005; see Fig. 1). Frameshifting is crucial to HIV replication, as expression of Gag–Pol allows targeting of replicative enzymes to the particle core during assembly. Further, it sets a precise ratio of Gag:Gag–Pol, the maintenance of which appears to be essential (Park & Morrow, 1991; Karacostas et al., 1993; Cherry et al., 1998; Shehu-Xhilaga et al., 2001; Telenti et al., 2002; Cen et al., 2004). The frameshift region of HIV-1 also encodes essential viral proteins. In Gag, PR cleavage sites almost precisely flank the frameshift signal (Fig. 1a) and yield, in addition to N-terminal proteins (MA, CA, p2, NC), the p1 and p6 (p6gag) proteins. In Gag–Pol, the equivalent

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A table of oligonucleotides used in this study is available as supplementary material in JGV Online.
overlapping coding sequences are cleaved to produce the transframe octapeptide (TFP) and p6\(^{*}\) (p6\(^{\text{pol}}\)). Changing specific residues within spacer peptide p1 affects infectivity, processing and dimer stability, probably by influencing the activity of p15-NC, the nucleocapsid-p1-p6\(^{\text{gag}}\) precursor involved in genomic RNA binding (Hill \textit{et al.}, 2002). p6\(^{\text{gag}}\), which includes a late budding domain (PTAP), is essential for virus assembly and release (Gottlinger \textit{et al.}, 1991; reviewed by Demirov & Freed, 2004; Morita & Sundquist, 2004). The transframe region of Gag–Pol has been reported to be critical for PR regulation. The TFP-p6\(^{\text{pol}}\)-PR intermediate has low dimer stability, with TFP-p6\(^{\text{pol}}\) functioning to inhibit PR until the appropriate point in maturation. Intramolecular cleavage at the p6\(^{\text{pol}}\)-PR site frees the N terminus of PR, a critical step in the formation of a stable tertiary structure of PR and enzymic activity (Louis \textit{et al.}, 1999a, b). TFP-p6\(^{\text{pol}}\) can also inhibit PR directly, allowing further regulation (Paulus \textit{et al.}, 1999).

The region of the genome which includes the frameshift region is likely to be of considerable importance in the development of resistance to antiviral drugs, particularly PR inhibitors. There are several potential mechanisms that can be envisaged, including greater PR and RT production through frameshift regulation, enhanced packaging of viral enzymes via changes in p6\(^{\text{gag}}\) and control of activation of the viral PR, via p6\(^{\text{pol}}\) (Peters \textit{et al.}, 2001). In support of the first mechanism, a Gag cleavage site mutation (p1/p6\(^{\text{gag}}\) L449F)
has been reported in HIV-1 variants resistant to PR inhibitors both in vitro (Doyon et al., 1996) and in clinical isolates (Maguire et al., 2002). In these viruses, the p1/p6RNA L449F cleavage site mutation frequently generates an additional homopolymeric and potentially slippery sequence [UUUUCUU to UUUUUUU (CS mutation)] that has been reported to increase levels of pol-encoded enzymes to counteract the PR deficit (Doyon et al., 1998). This change is located within the lower stem of the stimulatory RNA, yet the C to U transition is not predicted to destabilize the helix greatly (GU replaces GC pair). (Fig. 1b). Despite lacking an obvious stimulatory RNA downstream, the novel sequence has been shown to be functional in frameshifting in vitro (Doyon et al., 1998). It also supports Gag–Pol synthesis and PR activity in HIV molecular clones in which the authentic slippery sequence is inactivated, although no productive infection of T cells could be demonstrated (Doyon et al., 1998). Another mutation at the HIV-1 frameshift site that has been described in HIV-1-infected individuals undergoing therapy with protease inhibitors is a three nucleotide insertion (GGG) in the loop of the stimulatory RNA (Robinson et al., 2002). This change does not generate a novel slippery sequence according to accepted dogma (Atkins et al., 2001), so would conceivably act by a different mechanism, perhaps by influencing stability of the stimulatory RNA.

In this study, we have used chemical and enzymic structure probing to assess the impact of the CS and GGG changes on the structure of the stimulatory RNA and have measured the frameshift efficiency of the HIV-1 signal containing these, and certain related, mutations. We found that the CS and GGG mutations led to a modest stimulation of frameshifting in vitro, but had no effect on frameshifting in tissue culture cells. The mutations also had little effect on the structure of the stimulatory RNA. Together, these observations suggest that the increased resistance to viral protease inhibitors seen with HIV-1 isolates containing mutations in the ribosomal frameshifting signal is unlikely to be mediated by modulation of frameshift efficiency.

**METHODS**

**Site-directed mutagenesis.** Site-specific mutagenesis was carried out by a procedure based on that of Kunkel (1985) as described previously (Brierley et al., 1989). Mutations were introduced by deoxy sequencing of single-stranded templates (Sanger et al., 1977). Sequencing through G + C-rich regions was facilitated by replacing dGTP with deaza-GTP in the sequencing mixes.

**Construction of plasmids.** Plasmid pHXB2/SM was constructed by inserting four pairs of complementary synthetic oligonucleotides (RG1–RG8; supplementary Table S1, available in JGV Online Table 1), including the frameshift region of HIV-1 strain HXB2, into the Xbal and Psfl sites of plasmid pUC119 δV (Arnvig et al., 2004), a derivative of pUC18T7PstV (Jovine et al., 2000; gift of Dr Sandra Searles) (Fig. 1b). The cloned HIV-1 sequences contained the two-stem hairpin flanked by 25 and 45 nt at the 5’ and 3’ ends, respectively. A derivative of this plasmid, pHXB2/SM/A, was prepared by introducing a unique AflII restriction site, using site-directed mutagenesis, between the 3’ end of the HIV-1 sequences and the region encoding the hepatitis delta virus ribozyme. Derivatives of pHXB2/SM/A containing the CS or GGG mutations were prepared by site-directed mutagenesis. Plasmid p2luc/HXB2 was constructed by inserting two pairs of complementary synthetic oligonucleotides (RG9–RG12; supplementary Table S1, available in JGV Online Table 1) encoding the HIV-1 HXB2 frameshift region between the renilla and firefly luciferase genes of the dual luciferase reporter plasmid p2luc (Grentzmann et al., 1998), using the Sall and BamHI restriction sites (Fig. 3). A ‘100 % frameshift’ in-frame control plasmid, p2luc/HXB2/100 % was prepared by inserting an adenine residue immediately downstream of the slippery sequence to align the luciferase frames (T TT T T TA AG G G). To perform mutagenesis of the HIV-1 frameshift region, a T989 bp HindIII–EcoRI fragment from p2luc/HXB2 or p2luc/HXB2/100 % was subcloned into plasmid pKT0 (Tibbles et al., 1995) to generate pKT0/HXB2 and pKT0/HXB2/100 %, respectively. Following mutagenesis, the HindIII–EcoRI fragments were sequenced in their entirety and reintroduced into p2luc for subsequent frameshift assays.

**RNA structure mapping.** RNA for structure mapping was prepared by in vitro transcription of AflII-digested pHXB2/SM/A, pHXB2/SM/A/GGG and pHXB2/SM/A/CS using T7 RNA polymerase. Transcription reactions were on a 200 μl scale and contained 20 μg plasmid DNA, 2.5 mM each rNTP and 500 units T7 RNA polymerase (NEB) in a buffer containing 40 mM Tris (pH 8), 15 mM MgCl2 and 5 mM DTT. After 3 h at 37 °C the RNA was heated at 65 °C for 5 min, slow-cooled to 50 °C and held at this temperature for a further 30 min. RNA was recovered by single extraction with phenol/chloroform/isomyl alcohol (49:49:2, by vol.) followed by ethanol precipitation in 5 M ammonium acetate. The RNA pellet was dissolved in water, and the 115 nt HIV transcript was purified from a 6 % polyacrylamide/7 M urea sequencing gel and dissolved in water. RNA structure mapping was carried out using a 5’-end-labelling procedure as described by Manktelow et al. (2005) and followed the general principles outlined by others (van Belkum et al., 1988; Wyatt et al., 1990; Polson & Bass, 1994). All reactions (50 μl final vol.) contained 10 000–50 000 c.p.m. 5’-32P-end-labelled RNA transcript, 10 μg Escherichia coli rRNA and the relevant enzymic or chemical probe (detailed in the figure legends). Each RNA was probed between two and four times and yielded highly reproducible data.

**In vitro transcription and translation.** Plasmids were prepared using a commercial kit (WizardPlus SV Miniprep; Promega). In vitro transcription reactions employing the bacteriophage T7 RNA polymerase were carried out with EcoRI-linearized p2luc/HXB2 templates essentially as described by Melton et al. (1984) and included the synthetic cap structure 7meGpppG (New England Biolabs) to generate capped mRNA. Product RNA was recovered by a single extraction with phenol/chloroform/isomyl alcohol (49:49:2, by vol.) and remaining unincorporated NTPs were removed by Sephadex G-50 chromatography. RNA was recovered by ethanol precipitation, dissolved in water and checked for integrity by electrophoresis on a 1.5 % agarose gel before use for in vitro translation. mRNAs were translated in rabbit reticulocyte lysates (RRL) as described previously (Brierley et al., 1987). Translation products were analysed on SDS-15 % (w/v) polyacrylamide gels. The relative abundance of non-frameshifted and frameshifted products on the gels (marked by arrows in Fig. 3) was determined by direct measurement of [35S]methionine incorporation using a Packard Instant Imager 2024 and adjusted to take into account the differential methionine content of the products. The frameshift efficiencies quoted are the mean values of at least three independent measurements which varied by less than 10 %, i.e. a measurement of 30 % frameshift efficiency was between 27 and 33 %. The calculations of frameshift efficiency take into account the differential methionine content of the various products.
Frameshift assays in tissue culture. Cos-7 and 293T cells were maintained in Dulbecco’s modification of Eagle’s medium supplemented with 10% (v/v) fetal calf serum. Plasmids for transfection were cultured in E. coli DH5α cells, purified using a commercial kit (Qiagen) and transfected using a commercial liposome method (FuGene 6; Roche). Cells were seeded in 60 mm dishes and grown for 18–24 h until 80% confluency was reached. Transfection mixtures [containing plasmid DNA, serum-free medium (Optimem; Gibco-BRL) and FuGene] were set up as recommended by the manufacturers and added directly (dropwise) to the tissue culture cell growth medium. The cells were harvested 24 h post-transfection and reporter gene expression was determined using a dual luciferase assay system kit (Promega). Each data point represents the mean value from six separate transfections.

RESULTS

Structure probing of the HIV-1 stimulatory RNA and mutant variants (CS, GGG)

The structure of the stimulatory RNA present at the HIV-1 frameshift site has been the subject of considerable debate. Originally thought to be a simple stem–loop (Jacks et al., 1988), more elaborate models have been proposed, including typical (Le et al., 1991; Du et al., 1996) and atypical (Dinman et al., 2002) RNA pseudoknot structures. Recent NMR analysis, however, supports the proposal of Dulude et al. (2002) that the structure is in fact a two-stem helix (Fig. 1b). Here, we assessed the potential effects of the CS and GGG mutations on the HIV-1 stimulatory RNA by enzymic and chemical structure probing. The region probed included the proposed two-stem helix flanked by 25 and 45 nt at the 5’ and 3’ ends, respectively. The HIV sequences were cloned into a plasmid vector in such a way that following in vitro transcription, they were flanked by hammerhead and hepatitis delta virus ribozymes to allow unit-length RNAs containing solely HIV-1 sequences to be purified (Fig. 1b; Methods). Such transcripts also possess a hammerhead and hepatitis delta virus ribozymes to allow the synthesis of run-off transcripts. Thus, following hammerhead cleavage, the probe RNA (~115 nt) in fact retained non-HIV bases at the 3’ end, albeit only four templated bases (UUUA). After end-labelling, the HIV RNAs were subjected to limited chemical and enzymic digestion prior to analysis on denaturing polyacrylamide gels. The chemical probes were imidazole (I) and lead acetate (Pb), specific for cleavage of single-stranded regions. Enzymic probes were RNases T1 and U2, which preferentially cleave single-stranded G and A residues respectively, and RNase CV1, which targets regions that are in a double-stranded or stacked conformation.

Fig. 2 shows representative structure probing gels of 32P-end-labelled RNAs containing the wild-type HIV-1 (HXB2) framesshifting signal (a), or RNAs containing the CS (b) or GGG (c) mutations. The cleavage patterns seen were strongly supportive of the two-stem helix model and revealed differences in the relative stabilities of the upper and lower stems. In the wild-type molecule, the presence of the classical stem–loop (Jacks et al., 1988; the upper stem of Fig. 2) was evident from RNase CV1 cuts in either arm and resistance to cleavage from single-stranded enzymic and chemical probes. The stability of the upper stem was also emphasized by its protection from alkaline hydrolysis (lane OH). The imidazole cleavage pattern evident in the loop was consistent with a four-membered loop with occasional breathing of the U:G pair at the top of the stem. NMR data support this conclusion, indicating that the upper stem consists of a stable A-form helix capped by an ACAA tetraloop (Staple & Butcher, 2003). Only limited accessibility of the loop to enzymic probes was seen, with only the first loop base (A44) being cleaved noticeably. The region of RNA corresponding to the lower stem of the structure, as expected from its reduced thermodynamic stability (Staple & Butcher, 2005), was more accessible to the chemical probing reagents, particularly imidazole. The lower stem, however, was resistant to enzymic probing reagents and partially resistant to lead probing, especially in the first arm (bases 24–32 in Fig. 2). The three purines (GGA) that separate the upper and lower arms (internal loop centred around position 60) were susceptible to chemical cleavage and showed some weak accessibility to single-stranded specific RNases U2 and T1, supporting the view that the two stems are interrupted by an unpaired region. RNase T1 cleaved predominantly at G60 at the apex of the internal loop, yet unexpectedly, RNase U2 was most active on A62 just 3’ of the loop, possibly indicating that the U12:A62 base pair can breath or adopts an unusual conformation.

The structure of the CS variant was remarkably similar to that of the wild-type RNA, except for changes consistent with a decrease in the stability of the lower stem, as would be predicted from the mutation involved (a change from a G:C pair to G:U). As can be seen in Fig. 2, the bases in the lower stem were more sensitive to lead acetate (especially evident for bases in the first arm) and showed reduced reactivity to CV1 cleavage. Consistent with a more labile lower stem, the GGA bulge between the two stems showed slightly increased accessibility to single-stranded enzymic probes. No obvious changes in global conformation were observed. The patterns of hydrolysis seen with the GGG variant were also very similar to those seen with the wild-type molecule. The most noticeable difference was that the GGG insertion opened up the loop, allowing increased accessibility of RNases T1 and U2, the enzymic probes specific for single-stranded G and A residues. In this RNA, five of the six reactive nucleotides present in the loop were cleaved (all of the As and two of the three inserted G bases). The patterns of imidazole and lead acetate (data not shown) cleavage were also very similar to that of the wild-type molecule. In the GGG panel of Fig. 2, the lead acetate probing reactions shown (lanes X) were inadvertently contaminated with RNase A (cleaves single-stranded UpA and Cpa), but the cleavage pattern seen was highly consistent with the two-helix model with an expanded loop.
Efficiency of ribosomal frameshifting promoted by the HIV-1 stimulatory RNA and mutant variants

The structure mapping data indicated that the CS and GGG mutations did not significantly alter the global conformation of the HIV-1 frameshift-stimulatory RNA. To test whether this was reflected in an unchanged frameshift-promoting activity, frameshift assays were performed in vitro in the rabbit reticulocyte lysate system (RRL), and in vivo in cultured cells (cos 7 and 293T). The HIV-1 frameshift signal, either wild-type or modified (Fig. 3a) was inserted between the Renilla (R-luc) and firefly luciferase (F-luc) genes of the dual reporter plasmid p2luc (Grentzmann et al., 1998; see Methods) in such a way that expression of the R-luc : F-luc fusion protein (analogous to Gag : Pol) was dependent upon frameshifting at the inserted HIV signal. In these constructs, the inserted HIV-1 sequence was shorter than that used in the structure probing, lacking some 30 nt from the 3’ end, (but retaining 15 nt downstream of the stimulatory two-stem helix). However, frameshift efficiency was essentially unaffected by inclusion of the ‘missing’ 30 nt (construct SM; Fig. 3b).

In vitro assays were carried out by translating mRNAs derived from EcoRI-digested p2luc/HXB2, or a mutant derivative, in RRL and the non-frameshifted (39 kDa) and frameshifted (60 kDa) products were quantified by densitometry. In in vivo assays, the p2luc/HXB2 plasmid, or a mutant derivative, was transfected into mammalian cells and luciferase activities were measured 24 h post-transfection. To quantify frameshift efficiency in vivo, a ‘100 % frameshift’ control plasmid was prepared in which the R-luc and F-luc sequences were aligned in-frame by insertion of an A residue immediately after the slippery sequence. Such 100 % frameshift control constructs were prepared for each test construct to ensure that any effects of the primary sequence changes in the frameshift region on the activity of the R-luc : F-luc fusion were normalized. The in vitro assays are shown in Fig. 3(b) and the frameshiffficiencies engendered in vitro and in vivo are summarized in Fig. 3(c).

For the wild-type HIV-1 (HXB2) signal, the frameshift efficiencies in RRL and cultured cells were 7 and 5 %, respectively, values similar to those seen in previous studies (Bidou et al., 1997; Dinman et al., 2002; Dulude et al., 2002). The majority of the introduced mutations led to a moderate, but reproducible, stimulation of frameshifting in RRL, albeit less than 1.5-fold in any case. The CS and GGG mutations gave frameshift efficiencies of 10 and 8 %, respectively, with the frameshift efficiency of a GGG/CS double mutant also 10 %. The moderate stimulation by the GGG insertion was not restricted to this triplet, as a CCC insertion at the same site promoted about 10 % frameshifting. The only change that did not stimulate frameshifting in RRL was one in which the top 5 base pairs of the upper stem were flipped (FLIP; 6 %). As expected, in a control construct in which the slippery sequence was inactivated (KO), frameshift efficiencies were at baseline levels. As observed by Doyon et al. (1998), the CS mutation was active in RRL when in combination with the KO mutation (KO/CS, 4 %). However, the CS itself was poorly functional in vivo (KO 0.3 %; KO/CS 0.5 %). It is known that U-rich stretches can prove to be effective as slippery sequences in the absence of a stimulatory RNA in RRL (Brierley et al., 1992), but clearly the U7 stretch that results from the CS mutation has greatly reduced activity in vivo. In contrast to the pattern seen in RRL, all functional constructs (with > 0.5 % frameshifting) displayed a frameshift efficiency in vivo almost indistinguishable from that of the wild-type signal. These experiments were repeated in another cell line (293T) with essentially identical results (Fig. 3c). Why subtle differences in frameshift efficiency were seen in RRL but less so in vivo is considered in the Discussion. Whatever the explanation, it is clear that in the cellular environment, the CS and GGG mutations have little effect on frameshift efficiency and would not be expected to promote increased synthesis of replicative enzymes in the context of the virus itself.

DISCUSSION

In this study we characterized the secondary structure of the HIV-1 frameshift signal derived from two isolates (CS, GGG) that displayed increased resistance to protease inhibitors. We also determined the frameshift efficiency engendered by these signals in vitro and in cultured cells. The structure mapping data were strongly supportive of the two-stem helix model of Dulude et al. (2002), but neither the CS nor GGG mutations had a dramatic effect on the secondary
structure. The lower stem of the CS stimulatory RNA showed reactivities consistent with a moderate destabilization of this helix, whereas the predominant feature of the GGG stimulatory RNA was an increased loop size, with no additional changes evident. Despite such modest effects on structure, the frameshift efficiencies were somewhat elevated in vitro. The stimulation seen with the CS change is likely to be a consequence of the resulting homopolymeric stretch (U₇) acting as a novel slippery sequence in vitro, since this change allowed frameshifting to occur in the absence of the authentic slippery sequence (KO/CS construct). The capacity of U-rich stretches to induce frameshifting in vitro in the absence of a functional stimulatory RNA has been documented previously (Brierley et al., 1992). The pattern of frameshifting seen with the other mutants, however, is harder to account for. In Fig. 3(a), the predicted
stabilities (ΔG) of the upper helix of the various mutant structures are shown, calculated according to the rules of Turner et al. (1988). The changes in base stacking that arise as a consequence of the FLIP mutation suggest a modest destabilization of the structure, consistent with the slight reduction in frameshifting efficiency. The increased stability predicted for the GGG and CCC mutations is also in line with the stimulation of frameshifting seen. However, it is known that the upper stem of the wild-type RNA is stabilized by the ACAA tetraloop (Staple & Butcher, 2005), a factor not included in the stability calculation formula. Indeed, on this basis, one would expect the GGG and CCC insertions to destabilize the upper stem. The molecular basis of the frameshift stimulation seen with these mutants remains to be determined. Whatever the explanation, it is clear that the specific formation of a tetraloop is not required for frameshifting in vitro or in vivo.

The subtle changes in frameshift efficiency seen in vitro were less evident in vivo. A molecular explanation for this is lacking, but may relate to differences in ribosomal density, rates of protein synthesis, translation error rates or the types and abundance of mRNA-associated proteins. It was significant, however, that in combination with the KO mutation, the CS change showed very low levels of frameshifting in vivo. In apparent contradiction, Doyon et al. (1998) found that proviral clones containing the CS mutation alone (with the authentic slippery sequence inactivated by sequence changes) were still able to secrete virions containing p24 (capsid antigen) and, in the presence of the protease inhibitor palinavir, detectable Gag-Pol polyprotein. However, it was noticeable that in comparison to wild-type clones, a great deal of unprocessed Gag was present in the CS virions, suggesting that very low levels of protease were produced, indicating much lower levels of frameshifting. This is consistent with the low levels of frameshifting seen here for the CS mutation in vivo.

The CS and GGG mutations present in HIV-1 isolates resistant to protease inhibitors do not appear to affect ribosomal frameshifting, as judged from our structure mapping and in vivo frameshift assays. So why do these mutations arise? Recent trials have indicated that the incidence of the GGG insertion in protease-treated and untreated populations is in fact very similar, with about 1% of isolates displaying this insertion (R. Elston and others, unpublished). In support of this, of 190 isolates from naive patients listed in HIV databases (http://hiv-web.lanl.gov/content/index), two have a GGG insertion at the same location as that studied here. Of 1256 isolates (naïve and experienced) listed in the same database, there are ten examples of a GGG insertion in the loop and seven of GGA (total 1.4%). Thus it is questionable whether this particular mutation arises as a result of drug therapy. Nevertheless, if it is involved in resistance, one mode of action could be to influence cleavage at the TFP-p6* junction. The transframe region (TFR) (see Fig. 1), expressed as part of the Gag-Pol polyprotein, consists of two domains, the 8 aa N-terminal TFP (FLREDLA F) and 48 aa of p6* separated by a protease cleavage site (F-L). Louis et al. (1998, 1999a, b) have shown that TFR acts to inhibit the mature protease by destabilizing its structure. PR is released in a two-step process, with cleavage at the TFP-p6* junction being followed by release of PR from the p6*-PR product. The GGG insertion (encoding glycine) is located close to the TFP-p6* cleavage site and could influence cleavage between TFP and p6* in such a way as to increase the rate of PR activation (Whitehurst et al., 2003).

Fig. 3. Ribosomal frameshifting activity of the CS and GGG mutants. (a) Plasmid p2luc/HXB2 contains a 77 bp fragment encompassing the HIV-1 frameshift region cloned between the renilla (R-luc) and firefly luciferase (F-luc) genes of the reporter plasmid p2luc. Expression of firefly luciferase requires a −1 ribosomal frameshift at the HIV-1 slippery sequence (underlined). A number of mutations were introduced into the cloned HIV-1 sequences, including the GGG and CS changes. Additional mutants tested were KO, where the slippery sequence was inactivated without changing the relevant amino acids of Gag; CCC, where a C triplet was inserted into the loop of the stimulatory RNA; FLIP, where the top 5 base pairs of the upper stem were flipped, and SM, where an additional 30 nt of HIV-1 coding sequence was inserted. Two combination mutants were also tested, KO/CS and GGG/CS. At the top of the panel, the predicted ΔG values (kcal mol⁻¹) of the upper stem of the wild-type (WT), GGG, CCC and FLIP mutants are given. Also shown are the site and amino acid composition of the PR cleavage sites of Gag p7/p1, p1/p6 and Gag-Pol TFP/p6*. (b) In vitro frameshift assays in RRL. Plasmid p2luc/HXB2, or a mutant derivative, was linearized with EcoRI, transcribed with T7 RNA polymerase and transcripts translated in RRL at a final RNA concentration of approximately 15 μg ml⁻¹. Products were labelled with [35S]methionine, separated on a 15% SDS-polyacrylamide gel and detected by autoradiography. The non-frameshifted (stop; predicted 39 kDa) and frameshifted (FS; predicted 60 kDa) species are marked with arrows. M represents 14C protein markers (Ameraham Pharmacia Biotech). A control translation of an mRNA derived from EcoRI-linearized plasmid p2luc/MMTV gag/pro (Grentzmann et al., 1998), which contains the retrovirus mouse mammary tumour virus gag/pro frameshift signal, is also shown (MMTV gag–pro). The SM stop product is somewhat larger than that of other mutants due to the increased length of the HIV region in this plasmid. The frameshift efficiencies (%) measured for each mRNA are shown above the lanes. In vitro frameshift assays were performed on at least three independent occasions and the mean frameshift efficiencies are shown. (c) In vivo frameshift assays in tissue culture cells. A selection of the p2luc-based plasmids were used to measure frameshift efficiency in cos-7 (dark grey bars) and 293T (white bars) cells. These data are presented graphically and include in vitro data for comparison purposes (pale grey bars). The mean frameshift efficiencies shown for cos-7 and 293T cells were derived from in vivo experiments employing six replicates for each plasmid. Error bars represent SEM.
There is ample evidence that the CS mutation arises frequently in response to protease inhibitor therapy (Doyon et al., 1996; Zhang et al., 1997; Carrillo et al., 1998; Bally et al., 2000; Maguire et al., 2002; Feher et al., 2002). Whilst the experiments described here are not supportive of a major contribution of frameshifting to the viability of protease-resistant isolates, we cannot rule out that a minor increase in frameshifting above the level engendered by the natural site may contribute to viability. An alternative mode of action for the CS mutation could be in modulating the activity of p6* (since the CS mutation also affects the amino acid sequence of Gag–Pol) which could influence PR maturation (Louis et al., 1999a, b). However, as the L to F change generates a more effective cleavage site for both wild-type and PR-inhibitor-resistant proteases (Doyon et al., 1996), the most likely explanation is that the CS mutation acts to facilitate release of p6 to improve particle maturation. The natural p1/p6 site does not appear to be optimized for rapid hydrolysis and mutation of this rate-limiting cleavage site would compensate to some extent for the reduced catalytic activity of drug-resistant mutant HIV-1 proteases (Feher et al., 2002).

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REFERENCES


Frameshifting in HIV-1 protease-resistant mutants


