View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

ELSEVIER
Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virology 345 (2006) 127 – 136
VIROLOGY
www.elsevier.com/locate/yviro

Decreasing the frameshift efficiency translates into an equivalent reduction of the replication of the human immunodeficiency virus type 1

Dominic Dulude [a], Yamina A. Berchiche [a,b], Karine Gendron [a], Léa Brakier-Gingras [a,*], Nikolaus Heveker [a,b,*]

[a] *Département de Biochimie, Université de Montréal, 2900 Boul. Édouard-Montpetit, Montréal, Québec, Canada H3T1J4*
[b] *Centre de Recherche, Hôpital Sainte-Justine, 3175 Chemin de la Côte Sainte-Catherine, Montréal, Québec, Canada H3T1C5*

Received 16 June 2005; returned to author for revision 18 August 2005; accepted 31 August 2005
Available online 25 October 2005

Abstract

The Gag–Pol polyprotein of the human immunodeficiency virus type 1 (HIV-1) is the precursor of the virus enzymatic activities and is produced via a programmed −1 translational frameshift. In this study, we altered the frameshift efficiency by introducing mutations within the slippery sequence and the frameshift stimulatory signal, the two elements that control the frameshift. These mutations decreased the frameshift efficiency to different degrees, ranging from ~0.3% to 70% of the wild-type efficiency. These values were mirrored by a reduced incorporation of Gag–Pol into virus-like particles, as assessed by a decrease in the reverse transcriptase activity associated to these particles. Analysis of Gag processing in infectious mutant virions revealed processing defects to various extents, with no clear correlation with frameshift decrease. Nevertheless, the observed frameshift reductions translated into equivalently reduced viral infectivity and replication kinetics. Our results show that even moderate variations in frameshift efficiency, as obtained with mutations in the frameshift stimulatory signal, reduce viral replication. Therapeutic targeting of this structure may therefore result in the attenuation of virus replication and in clinical benefit.
© 2005 Elsevier Inc. All rights reserved.

Keywords: HIV-1; −1 ribosomal frameshifting; Gag–Pol

Introduction

The human immunodeficiency virus type 1 (HIV-1) encodes its structural and enzymatic components from the overlapping *gag* and *pol* open reading frames, respectively (reviewed in Frankel and Young, 1998; Freed and Martin, 2001; Tang et al., 1999). When ribosomes of infected cells translate the full-length viral messenger RNA, Gag is synthesized according to conventional rules of decoding, whereas Pol is produced from the same messenger as a Gag–Pol precursor via a programmed −1 ribosomal frameshift event. The frequency of this event was assessed at 2–10%, depending upon the system used to measure it (Biswas et al., 2004; Cassan et al., 1994; Dulude et al., 2002; Grentzmann et al., 1998; Harger and Dinman, 2003; Jacks et al., 1988; Reil et al., 1993). The programmed −1 ribosomal frameshift is widely used by viruses to produce Gag–Pol (reviewed in Brierley, 1995). It allows a precise control of the Gag–Pol to Gag ratio and also ensures the incorporation of the viral enzymes upon assembly of the viruses due to the fusion of the Pol sequence to Gag. The HIV-1 Gag polyprotein, p55, is the precursor of various endproducts that result from proteolytic breakdown by the HIV-1 protease. These are the matrix (MA or p17), the capsid (CA or p24), the p2 spacer peptide (SP1), the nucleocapsid (NC or p7), the p1 spacer peptide (SP2), and p6 (reviewed in Frankel and Young, 1998; Freed and Martin, 2001; Tang et al., 1999). Gag–Pol shares proteins MA to NC with Gag, but not p1 and p6 because of the frameshift event that overlaps with the p1 reading frame. These two proteins are replaced in Gag–Pol by a transframe (TF) octapeptide and p6*, the roles of which are still

* Corresponding authors. L. Brakier-Gingras is to be contacted at fax: +1 514 343 2210. N. Heveker, Centre de Recherche, Hôpital Sainte-Justine, 3175 Chemin de la Côte Sainte-Catherine, Montréal, Québec, Canada H3T1C5, fax: +1 514 345 4801.

E-mail addresses: lea.brakier.gingras@umontreal.ca (L. Brakier-Gingras), nikolaus.heveker@recherche-ste-justine.qc.ca (N. Heveker).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.08.048

controversial (see Hill et al., 2002; Paulus et al., 2004 and references therein). The Pol portion of Gag–Pol contains the three enzymes that are essential for the replication of the virus: the protease (PR), which cleaves the Gag and Gag–Pol polyproteins, producing mature proteins, the reverse transcriptase (RT), which converts the viral RNA genome into a proviral DNA duplex, and the integrase (IN), which inserts the proviral DNA into the host chromosome (Frankel and Young, 1998; Freed and Martin, 2001; Tang et al., 1999). The Gag polyprotein directs assembly and release of new virions from infected cells (reviewed in Freed, 1998). During or after the release, the viral protease cleaves the Gag and Gag–Pol polyproteins, producing mature, infectious viral particles.

Previous studies of the −1 programmed frameshift events demonstrated the requirement of two mRNA *cis*-acting elements for efficient frameshifting (reviewed in Brierley and Pennell, 2001) (see Figs. 1A and B). In HIV-1, the first element is a heptamer sequence UUUUUUA, called the slippery sequence, where the −1 frameshift occurs. The second element is a long helix structure located immediately downstream of the slippery sequence, which stimulates the frameshift. This structure, which contains two stems separated by a purine-rich bulge, was first characterized by probing with enzymatic attack and mutagenesis (Dulude et al., 2002) and recently confirmed by NMR (Gaudin et al., 2005; Staple and Butcher, 2005).

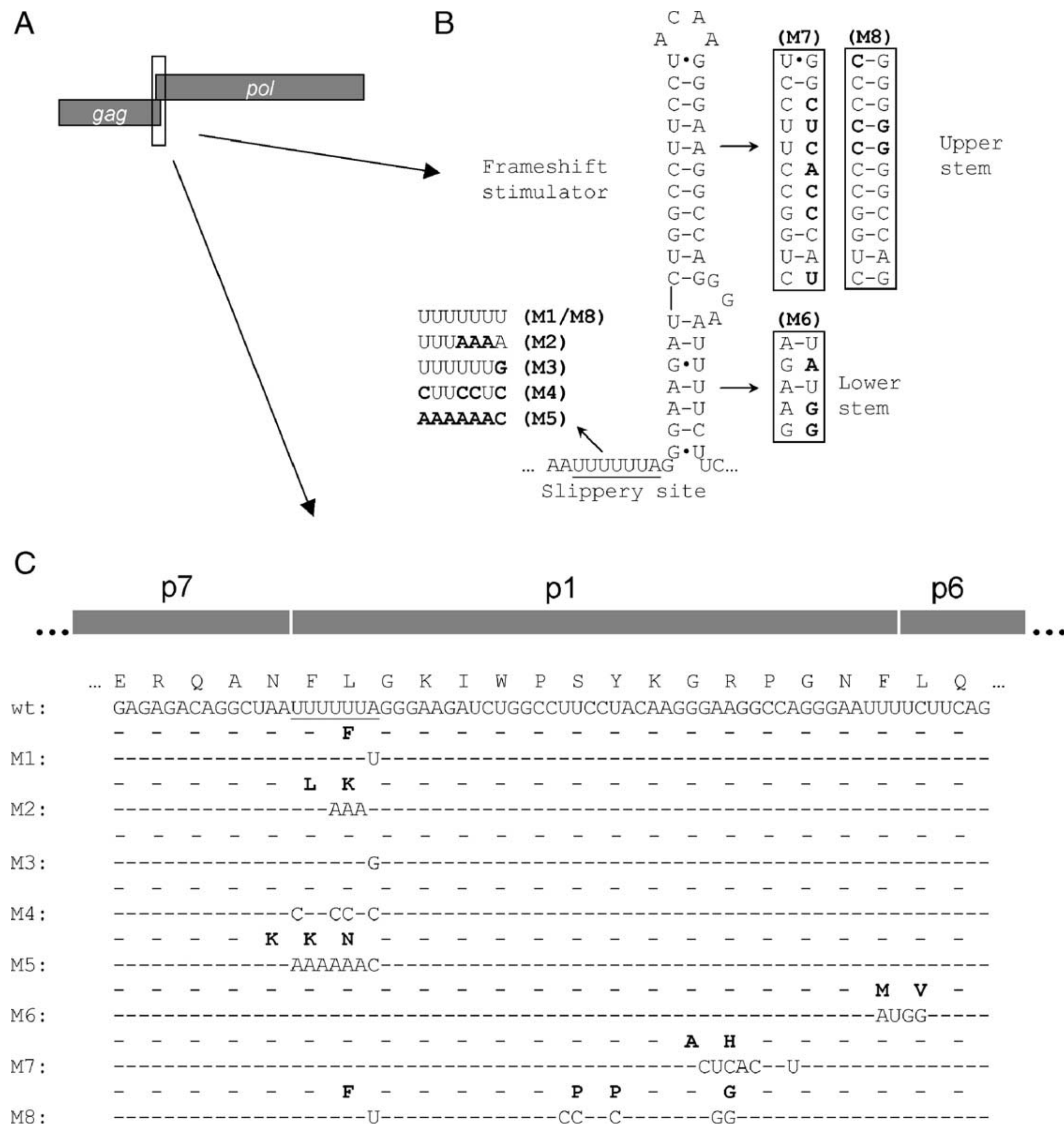

Fig. 1. Frameshift region of wild-type HIV-1 and its mutants. (A) HIV-1 *gag* and *pol* open reading frames. The overlapping segment contains the programmed −1 ribosomal frameshift region. The *pol* gene is in the −1 frame relative to *gag* and it is expressed as a Gag–Pol polyprotein via a −1 frameshift. (B) Structure of the frameshift stimulatory signal of HIV-1 and summary of the mutations introduced in the frameshift region. The underlined slippery site is followed by a frameshift stimulator consisting of a long irregular stem-loop, where the upper and the lower stems are separated by a three-purine bulge. (C) Amino acid and nucleotide sequences of wild-type HIV-1 and its derivatives mutated in the frameshift region. The elements required for the frameshift are located in the p1 spacer peptide, between the end of p7 (nucleocapsid) and the beginning of p6. The slippery sequence is underlined in the wild-type nucleotide sequence (wt). Amino acid and nucleotide residues that are not altered in the Gag mutants (M1 to M8) are represented by dashed lines.

Although the importance of the frameshift is clearly established, several questions remain to be answered in order to exploit the frameshift event as a potential drug target. In particular, it remains to determine the extent to which variations in frameshifting efficiently translate into reduced viral replication and therefore into potential clinical benefit. Biswas et al. (2004) found that replacing the slippery sequence of HIV-1 with other slippery sequences severely decreases frameshifting and viral infectivity. However, the slippery sequence most likely does not form a defined structure, making specific interactions with candidate compounds difficult. In contrast, targeting of the frameshift stimulator structure by small molecules may be envisioned. A report by Telenti et al. (2002) analyzed natural and laboratory HIV-1 isolates with sequence variations in the upper stem of the stimulatory signal and found reduced viral replication only for two variants with more than 60% reduction in frameshift efficiency, whereas variants with a 16–42% reduction did not show any detectable replication deficit. The interpretation of these data suggested the existence of a threshold of tolerance for frameshift variations under which they would have no effect on viral replication. This in turn implied that attempts to modulate frameshift would be of no therapeutic value unless efficiently overcoming this threshold.

The present study was designed to elucidate the impact of mutations in the two *cis*-acting elements of the HIV-1 frameshift region on both frameshifting efficiency and viral replication. We introduced mutations into the slippery site, the upper stem and the lower stem of the stimulatory signal structure. The effects of these mutants cover a wide range of frameshift efficiencies that were paralleled by a decreased infectivity of mutant virions and a reduced replication in long-term cultures. Our results show that moderate reductions of frameshift efficiency are able to slow down viral replication, which suggests that compounds causing such reductions should provide clinical benefit.

Results

Description of HIV-1 mutants altered in the frameshift region

In order to study the influence of changes in frameshift efficiency on HIV-1 replication, we investigated eight mutants containing mutations within the slippery sequence and/or the stimulatory frameshift signal. Fig. 1 shows the HIV-1 wild-type frameshift region and describes the mutants M1 to M8. Mutants M1 to M5 contain mutations that alter the slippery site and influence the frameshift efficiency by changing the dynamics of the codon–anticodon interaction at the A and/or P site. Mutants M1 (UUUUUUU), M2 (UUUAAAA), and M3 (UUUUUUG) have a mutated A-site codon. M5 is mutated in both the A- and P-site codons of the slippery site, the sequence that replaces the HIV-1 slippery sequence being AAAAAAC, which is used as a frameshift slippery site by several viruses (see below in the Discussion section). Mutant M4, for which the UUUUUUA slippery sequence was substituted with CUUCCUC, served as a negative frameshift control since it does not allow slippage of the two tRNAs and re-pairing in the −1 frame.

The other mutants are altered in the frameshift stimulatory signal. Mutant M6 is destabilized in the lower stem and M7 in the upper stem of the signal. M8 has mutations that increase the thermodynamic stability of this upper stem by replacing two U–A pairs with two C–G pairs, in addition to having a C–G instead of a U·G pair on top of this stem and having the M1 mutation. This mutant was initially designed to increase frameshifting.

Frameshift efficiency of the HIV-1 mutants altered in the frameshift region

The frameshift efficiency of wild-type and mutant constructs was measured with a dual-luciferase system (Fig. 2). The wild-type HIV-1 frameshift region caused a frameshift with an efficiency of 9 ± 1% in this system, which is in agreement with previous studies that measured HIV-1 frameshifting with similar reporter systems (Biswas et al., 2004; Harger and Dinman, 2003). The frameshift efficiency of the negative control (M4) was reduced to a background level, as expected. For mutant M1, the frameshift efficiency was 48% of the wild-type whereas for M2 and M3 the frameshift was 8% and 60% of the wild-type, respectively. When the slippery site of HIV-1 was changed to the AAAAAAC sequence (M5) used by other viruses, the frameshift efficiency decreased to 35% of the wild-type construct. Destabilization of the lower stem (M6) led to a frameshift level of 70% and destabilization of the upper stem (M7) to 23%. Finally, with mutant M8, designed to stabilize the upper stem, the frameshift efficiency decreased to 42% of the wild-type value. Therefore, all the eight mutations decreased the frameshift efficiency to variable degrees.

Influence of frameshift mutations on virus-like particles (VLPs)

We then investigated how changes in frameshift efficiency might affect the release of VLPs and the incorporation of Gag–Pol in these particles. As shown in Fig. 3, no mutation of the frameshift region altered the quantity of released p24. However, we observed changes in the amount of RT activity present in the VLPs and consequently in the ratio of Gag–Pol to Gag incorporated. These variations correlated perfectly with the changes in frameshift efficiency. Those mutations that most dramatically decrease the frameshift efficiency cause the largest decrease in the amount of Gag–Pol incorporated (see the inset of Fig. 3).

Influence of changes in frameshift efficiency on Gag processing

The processing of the Gag polyprotein is mediated by the viral protease contained within Gag–Pol. We therefore investigated the effect of frameshift changes on the maturation of Gag within virions produced from cells transfected with the mutant derivatives of HIV-1_{LAI} altered in the frameshift region. The assay consisted in assessing the amount of the capsid protein, p24, produced upon Gag processing. As shown in Fig.

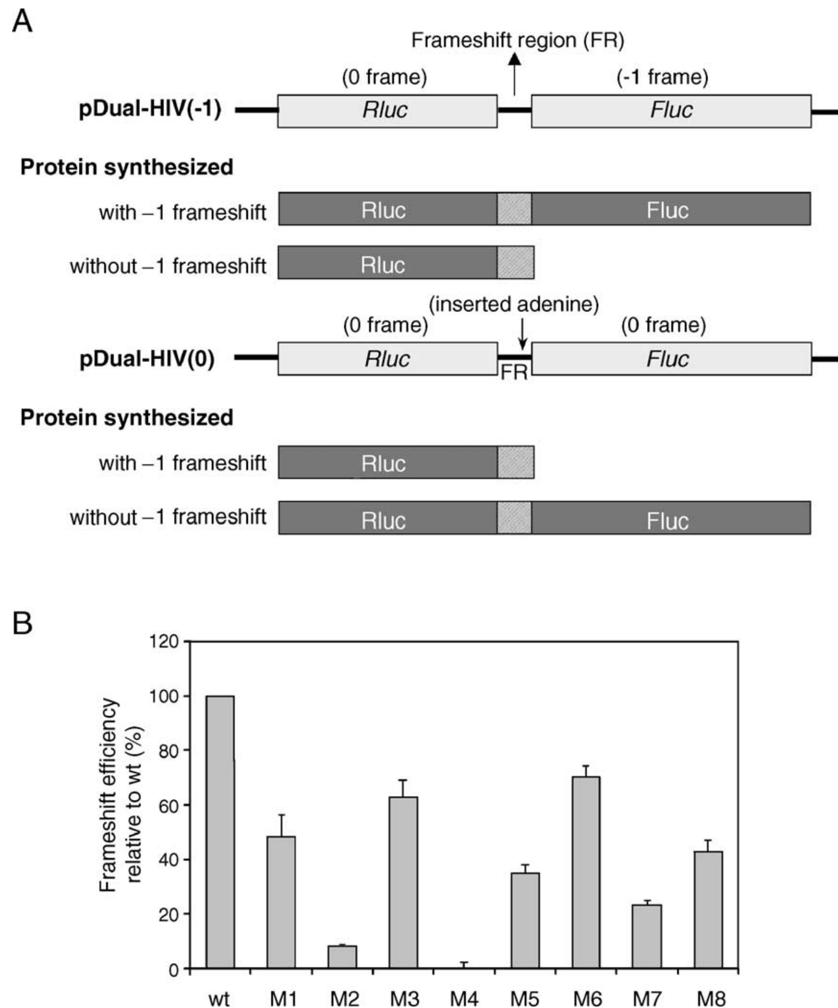

Fig. 2. Measurement of the frameshift efficiency of HIV-1 derivatives with a dual-luciferase system. (A) Scheme of the vectors used to measure the frameshift efficiency. In the pDual-HIV construct, the HIV-1 wild-type or mutant frameshift region (FR) is inserted between the *Renilla* luciferase (*Rluc*) gene and the firefly luciferase (*Fluc*) gene. With the pDual-HIV(−1) construct, the firefly luciferase (Fluc) is synthesized as a fusion to the *Renilla* luciferase (Rluc) only by ribosomes that make a −1 frameshift in the inserted frameshift region. Plasmid pDual-HIV(0) is an in-frame control, in which an additional adenine is inserted immediately after the slippery site. With this construct, Fluc is synthesized as a fusion to Rluc only by ribosomes that translate the messenger according to conventional rules. Frameshift efficiencies were determined by dividing the ratio of the firefly to *Renilla* luciferase activities from the wild-type or mutant constructs by the firefly to *Renilla* luciferase ratio from the in-frame pDual-HIV(0) control construct. (B) Frameshift efficiency of the frameshift mutants. 293FT cells were transfected with the different dual-luciferase constructs and the activities of *Renilla* and firefly luciferase were measured 48 h later. The frameshift efficiencies are indicated relative to the wild-type construct, which is arbitrarily set at 100% (the absolute value is 9 ± 1%). Each value represents the mean ± standard deviation of at least four independent experiments.

4, the Gag polyprotein was highly processed in the wild-type virions but there was no detectable Gag processing for mutants M2 and M4, which have a very low frameshift efficiency. The Gag processing was decreased about twofold compared to the wild-type virions for mutants M1, M3, M6, M7, and M8, which have frameshift efficiencies ranging between about 23% and 70% of the wild-type value, and mutant M5, with a frameshift efficiency of 35%, had a decrease of about fivefold in Gag processing. Similar profiles were obtained when analyzing Gag processing within VLPs produced from COS-7 cells transfected with pGAGPOL-xm-wt and its mutant derivatives (data not shown). These Gag processing profiles suggest a lack of proportionality between the decrease in frameshift efficiency and processing. Indeed, mutants M6 and M7, with, respectively, a frameshift efficiency of 70% and 23% of the wild-type value, had a reduction of 50% in the production of the p24 breakdown product.

Influence of changes in frameshift efficiency on viral infectivity

We also investigated the effect of changes in frameshift efficiency on the infectivity of HIV-1 by performing single-round infectivity assays. Infectivity is the relative number of infectious units per standardized p24 content in the inoculum. As shown in Fig. 5, the infectivity of virions produced from cells transfected with the mutant derivatives of HIV-1_{LAI} with an altered frameshift region was inferior to that of the wild-type construct. The decrease in infectivity was proportional to the decrease observed in frameshifting, the effect on viral infectivity being slightly amplified. One apparent exception

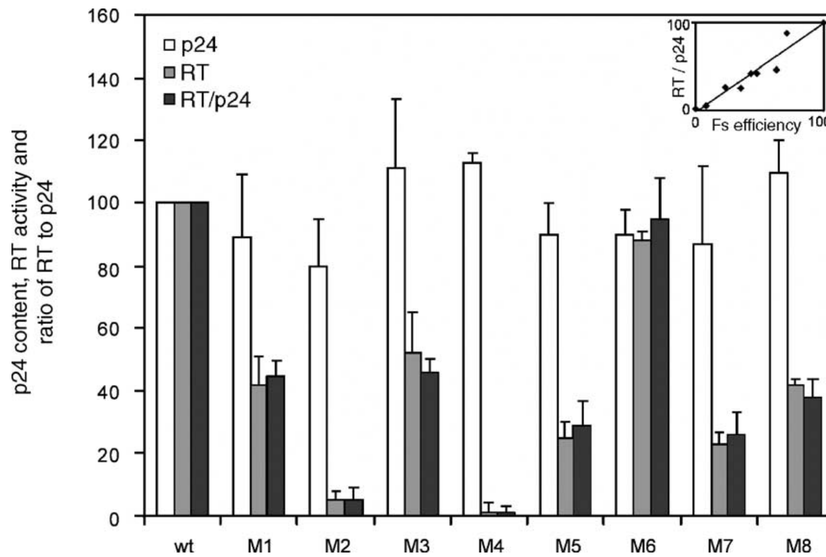

Fig. 3. Effect of changes in frameshift efficiency on the release of VLPs, on the RT content of the released VLPs, and on the ratio of Gag–Pol to Gag in these VLPs. Four micrograms of pGAGPOL-xm-wt or pGAGPOL-M1 to -M8 and 2.5 μg of pRev1 were introduced into COS-7 cells by transfection. The p24 and the RT content of the VLPs in the culture supernatant were measured 64 h posttransfection (see details in Materials and methods). White and grey bars represent, respectively, the p24 and the RT level of the VLPs released in the supernatant. Black bars represent the ratio of RT to p24 for the released VLPs. Changes in the amount of released p24, RT and in the RT to p24 ratio for the mutants are indicated relative to the wild-type construct, which is arbitrarily set at 100%. The inset presents the correlation (R^2 = 0.923) between the RT to p24 ratio and the frameshift efficiency (Fs) of the wild-type construct and frameshift mutants. Each value represents the mean ± standard deviation of three to four experiments.

to this proportionality was mutant M5, which appeared almost non-infectious, despite an appreciable frameshift efficiency and RT incorporation in VLPs.

Influence of changes in frameshift efficiency on viral replication

In order to investigate whether the observed decrease in frameshift efficiency and the reduction in infectivity would cause cumulative effects in long-term cultures, we analyzed the replication kinetics of mutants M1 to M8 in CEM T-cells (see Fig. 6). With mutants M2, M4, and M5, there was no detectable virus production during the period of observation and the replication of mutants M1, M7, and M8 was severely attenuated as compared to the wild-type control HIV-1_{LAI}.

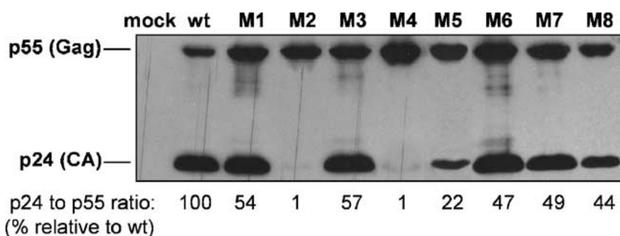

Fig. 4. Analysis of the processing of Gag within virions mutated in the frameshift region. Virion samples produced from provirus transfection in HeLa-P4 cells were resolved by a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed by Western blot with an anti-p24 antibody. A representative blot from three separate experiments is shown. P55 (Gag) corresponds to the non-processed polyprotein, whereas p24 (CA) represents a mature component of the processed Gag. The wt lane corresponds to the wild-type protein profile, and lanes M1 to M8 correspond to the frameshift mutants. The ratio of p24 to p55 in the wild-type and mutant virions is indicated under each lane, expressed as the percentage of processing in wild-type virions.

Interestingly, the p24 production of mutant M6 was less than 50% of that of the wild-type virus at all time points, indicating reduced viral replication in spite of a moderate effect on frameshift efficiency. Finally, mutant M3 also produced less p24, and the peak of production was delayed by more than 3 days as compared to the control. These results demonstrate that all mutants with altered frameshift efficiency were appreciably attenuated in long-term virus replication.

Discussion

In the present work, we introduced mutations in the frameshift slippery site and in the downstream frameshift stimulatory signal of HIV-1. Our study shows that even relatively mild effects on frameshift efficiency, such as those resulting from mutations in the frameshift stimulatory signal, lead to a reduced replication of the mutant virions. The frameshift stimulatory signal has a defined structure with which antiviral compounds could interact, unlike the slippery site, which may be a more difficult target. Indeed, even the replication of mutants for which no dramatic changes in Gag processing were detected by Western blot analysis was affected in comparison to the wild-type virus. Therefore, and in contrast to the view expressed by Telenti et al. (2002), our results suggest that any change in frameshift efficiency results in reduced viral replication and that no threshold of frameshift alteration must be reached to become phenotypically manifest.

In addition to showing a strong correlation between frameshift and virus replication, our results yield new information on the different elements of the frameshift region. Destabilization of the lower stem of the HIV-1 frameshift stimulatory signal (M6) results in a mild but clearly detectable

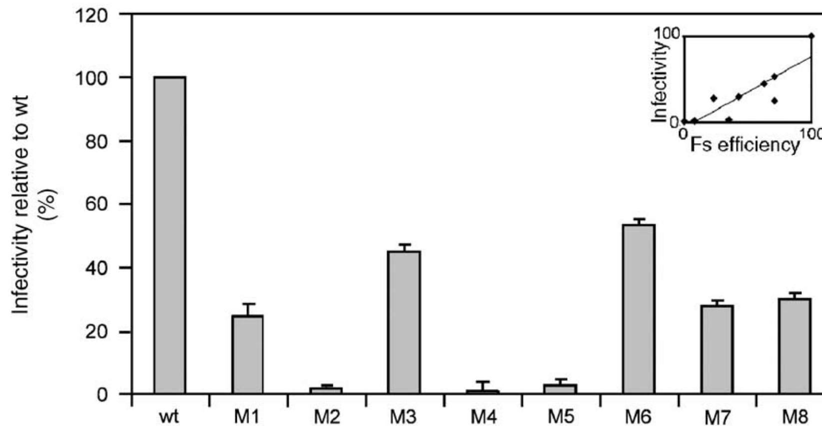

Fig. 5. Single-round infectivity assays with HIV-1 and its derivatives mutated in the frameshift region. HeLa-P4 cells were infected with p24-normalized amounts of wild-type HIV-1_{LAI} or mutant viruses. Changes in the infectivity of the mutant viruses (M1 to M8) are indicated relative to the wild-type HIV-1 (wt), which is arbitrarily set at 100%. The inset presents the correlation (R^2 = 0.752) between the infectivity and the frameshift efficiency (Fs) of the wild-type virus and the frameshift mutants. Each value represents the mean ± standard deviation of a triplicate analysis.

replication phenotype. Destabilization of the upper stem (M7) drastically reduces the replicative capacity of the mutant virus. In contrast to mutants M6 and M7, mutant M8 stabilizes the upper stem but it nevertheless results in a decreased frameshift efficiency and in a dramatic decrease in the replicative capacity of the corresponding mutant virus. Interestingly, Bidou et al. (1997) also reported that increasing the stability of the upper stem decreases the frameshift efficiency of HIV-1 and concluded that increasing the pause of the ribosome on the slippery site disfavors the frameshift. Our results illustrate the importance of maintaining the structural integrity and stability of the frameshift stimulatory signal for the infectivity of the virus but they contrast with the results of Telenti et al. (2002). Indeed, these researchers failed to detect reduced infectivity or replication for variants containing substitutions that destabilize the upper stem of the frameshift stimulatory signal and reduce frameshift efficiency to a value of about 60% compared to wild-type. However, they observed a reduced infectivity for two mutants with a frameshift efficiency decreased at and below 40% of the wild-type. Telenti et al. concluded that this 40% value constitutes a threshold efficacy, above which variations in frameshifting have no effect on viral replication. The reason for this discrepancy between our results and those of Telenti et al. is unclear and may lie in differences between the respective experimental systems.

We introduced five mutations that alter the slippery sequence, M1 to M5. For mutant M1 (with a stretch of 7U), we detect a 50% reduced frameshift efficiency. With the 7U sequence (M1 and M8), frameshifting occurs with a $tRNA^{Phe}$ in the A-site rather than a $tRNA^{Leu}$ as it is the case for the wild-type. It is possible that $tRNA^{Phe}$ is less prone to slip than $tRNA^{Leu}$. Similarly, for UUUAAAA (M2), it is also possible that $tRNA^{Lys}$ in the A-site is less prone to slip than $tRNA^{Leu}$. The importance of the identity of the tRNA for the frameshift efficiency is also supported by previous observations from Chamorro et al. (1992). A change in the identity of the A-site $tRNA^{Leu}$ (from $tRNA^{Leu}$ with an IAG anticodon to a $tRNA^{Leu}$ with a CAG anticodon) also likely accounts for the decreased

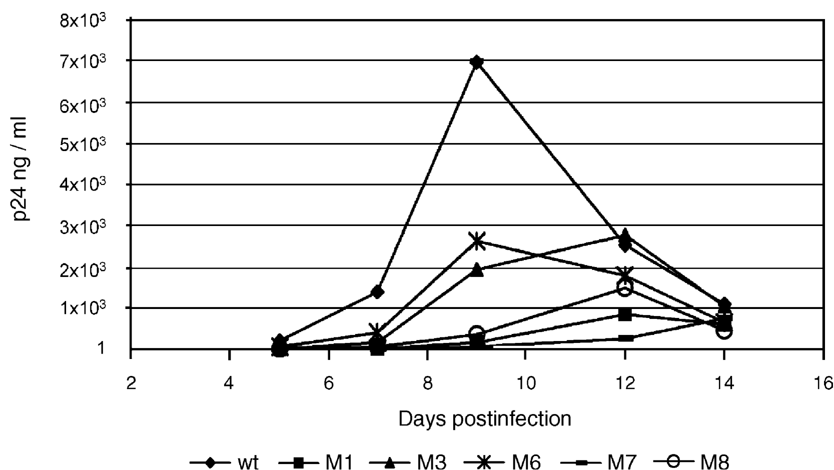

Fig. 6. Replication kinetics of HIV-1 and mutants with an altered frameshift efficiency. CEM T-cells were infected with p24-normalized amounts of wild-type HIV-1_{LAI} (wt) or mutant viruses. Viral replication was monitored at regular intervals by quantification of the p24 concentration in the culture supernatant with a p24 ELISA assay. Mutants M2, M4, and M5 replicate below the level of detection of the assay. A representative analysis from three separate experiments is shown.

frameshift efficiency with the UUUUUUG sequence (M3) (see Brierley et al., 1992). The AAAAAAC sequence (M5) contains a slippery sequence found in astroviruses, in human T-cell leukemia virus type 2, in mouse mammary tumor virus (MMTV), and in equine anemia infectious virus (EIAV) (reviewed in Brierley, 1995). The frameshift efficiency of M5 is 35% of the wild-type, in agreement with Biswas et al. (2004), who also replaced the slippery sequence of HIV-1 with AAAAAAC. Interestingly, it has been reported that replacing the AAAAAAC sequence of EIAV with the slippery sequence of HIV-1 decreased the frameshift efficiency to 50% in the presence of the pseudoknot that acts as a frameshift stimulator in EIAV (Chen and Montelaro, 2003). Therefore, some degree of functional interdependence seems to exist between the slippery sites and a matching stimulatory signal since both elements cannot be arbitrarily interchanged between viruses. All the mutations that were introduced in the slippery site severely decreased the capacity of the virus to replicate, the severity of this effect correlating with the decrease in the frameshift efficiency, with the exception of M5 (see below).

The mechanisms accounting for the observed effects of the frameshift mutations on HIV-1 replication may be multiple. The frameshift changes result in reduced Gag–Pol production and consequently reduced incorporation of the viral enzymes into the virions. The amount of RT activity within VLPs directly mirrors reduced frameshift efficiency, and it is likely that the decreased RT amount largely contributes to the decreased viral infectivity, knowing that viral infectivity is very sensitive to changes in RT activity (see Ambrose et al., 2004; Julias et al., 2001). The reduction in Gag–Pol incorporation can result in altered protease activity leading to altered Gag processing, but it appears that protease activity must be reduced by more than fourfold to significantly impair viral infectivity (Rosé et al., 1995). We therefore do not believe that maturation defects play a major part in the reduced infectivity observed for the mutant viruses, except when the amount of protease is dramatically decreased (mutants M2 and M4) and in the case of mutant M5 (see below). It is also possible that the decreased integrase incorporation contributes to reduce the infectivity of mutant viruses. In addition, mechanisms other than just the reduction of incorporated viral enzymes may be at work. It has been shown that over- or underexpressing Gag–Pol impairs the maturation of the virion genomic RNA dimers (Shehu-Xhilaga et al., 2001, 2002), and it can be hypothesized that such an impairment could contribute to the decreased infectivity of mutant viruses. Also, the incorporation of $tRNA^{Lys}$ into viruses is a crucial event in the viral replication cycle, which is driven by Gag–Pol (Mak et al., 1994) and could be affected by a decrease in Gag–Pol.

The strong correlation that we observe between the decrease in frameshift efficiency and the decrease in viral infectivity supports our interpretation that the changes in the amount of Gag–Pol produced in the mutants investigated account for the decrease in viral infectivity. However, other changes besides the decrease in frameshift efficiency and the resulting reduction in Gag–Pol production could also contribute to lower the virus infectivity. For example, it could be hypothesized that the mutations that we introduced in the frameshift region influence the secondary and tertiary structure of the viral RNA. These structural changes could perturb long-range interactions such as those involved in the stabilization of the association between the two copies of genomic viral RNA forming a dimer (Hibbert and Rein, 2005; Paillart et al., 2002, 2004). Mutants M1 and M3 have a comparable frameshift efficiency, a comparable level of RT activity associated to the VLPs and do not differ in Gag processing. However, M1 appears to be less infectious than M3 by about twofold. Although purely speculative, an effect of the mutation in M1 on the viral RNA secondary and tertiary structure could account for its lower infectivity compared to M3. Also, it is difficult to mutate the frameshift signal of HIV-1 without simultaneously changing the amino acid sequence of the corresponding Gag p1 spacer peptide and that of the p7/p1 and p1/p6 cleavage sites, which might also affect viral replication. For example, two proline residues (positions 7 and 13) of the 16-amino acid p1 have been shown to be required for efficient HIV-1 infectivity (Hill et al., 2002). Interestingly, the only frameshift mutants found to have reduced replication by Telenti et al. (2002) contain a proline to lysine substitution at position 7. It could therefore be envisioned that the experimental conditions used by Telenti et al. were able to detect defects due to p1 mutations but were not sensitive enough to identify more subtle effects due to frameshift variations. The impact of some p1 mutations on the viral replication is also illustrated by the drastic phenotype of our mutant M5. This mutant contains three amino acid substitutions at the p7/p1 protease cleavage site, among which the introduction of a lysine residue at the P1 position of this cleavage site, a substitution that was previously shown to abolish the cleavage reaction (Pettit et al., 2002). It could be suggested that these substitutions at the p7/p1 cleavage site affect the overall protease activity. The observed decrease of protease activity of mutant M5 could indeed contribute to lower its infectivity, which is also disproportional to the effect of the mutation on frameshifting. We should, however, point out that this observation applies only to mutant M5, and it is unlikely that p1 mutations have a major influence on the phenotypes of all our mutants. For example, the mutation of M3 does not affect the amino acid sequence of p1, yet affects viral replication.

Altogether, our study provides evidence that variations in the frameshift stimulatory signal lead to moderate reductions of frameshift efficiency but nevertheless reduce the replicative capacity of the virus. Even a partial reduction of virus replication can translate into clinical benefit. This seems indeed to be the case for many drug-resistant HIV-1 strains that bear mutations reducing their replicative fitness (Berkhout, 1999). Reduction of viral replication by compounds targeting the stimulatory signal may thus be of significant therapeutic interest.

Materials and methods

Construction of plasmids

pGAGPOL vectors

The HIV-1 *gag–pol* expression vector used in this study for the synthesis of virus-like particles (VLPs) was derived from

pGAGPOL-RRE-r. This vector contains, under the control of the SV40 promoter, the *gag–pol* gene and the *rev*-responsive element (RRE) from the HIV-1 proviral clone BH10-HXB2 (Smith et al., 1990). Cotransfection in COS-7 cells (see details below) of this plasmid together with pRev1 (Smith et al., 1990), which codes for HIV-1 Rev, results in the efficient release of VLPs in the culture supernatant of the transfected cells. To facilitate the cloning of mutants of pGAGPOL-RRE-r altered in the frameshift region, we created a cloning derivative of this plasmid, named pGAGPOL-xm. In this plasmid, the frameshift region was deleted and replaced with a short cassette containing a *Xho*I restriction site at one end and a *Mlu*I restriction site at the other end. This was made by PCR amplification with a standard overlap extension procedure (Ho et al., 1989). The wild-type construct and the frameshift mutants were made by inserting, between the digested *Xho*I and *Mlu*I sites of pGAGPOL-xm, the appropriate annealed and phosphorylated 74-bp oligonucleotide cassette, purchased from Sigma Genosys. This generated pGAGPOL-xm-wt and pGAGPOL-M1 to pGAGPOL-M8, respectively (Fig. 1C). The insertion of a *Xho*I and a *Mlu*I restriction site in pGAGPOL-xm-wt and its derivatives substituted a tyrosine with a lysine residue and a proline with a valine residue at the amino acid position 427 and 453 of Gag, respectively. These modifications did not change the reverse transcriptase (RT)-associated content of the released VLPs compared to the parental construct (data not shown).

Dual-luciferase reporter plasmids

A dual-luciferase reporter plasmid was created, based on previously published systems (Grentzmann et al., 1998; Harger and Dinman, 2003). Briefly, the HIV-1 frameshift region was inserted between the coding sequence of the *Renilla* luciferase (*Rluc*) and the firefly luciferase (*Fluc*) gene in an appropriate vector. In this construct, only the ribosomes that initiated translation at the initiator codon for the *Renilla* luciferase but changed the reading frame by one base in the 5′ direction in the frameshift region synthesize the firefly luciferase, which is thus fused to *Renilla* luciferase (see Fig. 2A). To create this vector, the *Renilla* luciferase coding sequence and part of its 5′ untranslated region were amplified by PCR from pRluc-C1, a *Renilla* luciferase fusion protein expression vector (a generous gift from Dr. M. Aubry, Université de Montréal). The forward and reverse mutagenic primers: 5′GCTGGTTTAGTGAAGCTTCAGATCCGCTAGAGCCACC-3′ and 3′AACGAGCAGGGGGTACCCGGCGAGCTCTC′5 used for this amplification introduced a *Hin*dIII and a *Kpn*I restriction site at the 5′ and 3′ end of the gene, respectively. The amplified *Rluc* gene was then inserted upstream of the *Fluc* gene of *Hin*dIII–*Kpn*I-digested pcDNA3.1-luc (Dulude et al., 2002), generating the parental pDual-luc construct. The wild-type and the mutated frameshift regions (nucleotides 2037–2139 according to HXB2 genome nomenclature) were amplified by PCR from pGAGPOL-xm-wt or pGAGPOL-M1 to pGAGPOL-M8 and inserted between the *Rluc* and *Fluc* genes of pDual-luc, generating pDual-HIV(-1) and the pDual-HIV-M1 to pDual-HIV-M8 constructs, respectively. The amplification used the following primers: Fwd-*Kpn*I (5′-GGGCTGTTGGTACCTGGAAAGGAAGG-3′) and Rev-*Bam*HI (5′-GGGCTGTTGGATCCCACGCGTGACTG-3′), and the digested PCR products were then inserted between the *Kpn*I and *Bam*HI restriction sites of pDual-luc. All these constructs were used to measure the level of firefly luciferase expressed relative to the *Renilla* luciferase. An in-frame plasmid control, pDual-HIV(0), in which the *Rluc* gene is in the same frame as the *Fluc* gene, was created by PCR amplification. This was made by inserting an adenine immediately after the slippery site, which was mutated from UUUUUUA to CUUCCUC to prevent −1 frameshifting, as described by Grentzmann et al. (1998). This plasmid was used to measure the translation level of the in-frame *Fluc* coding sequence relative to that of the *Rluc* gene (see Fig. 2A).

Proviral clones

For infectivity assays, we used the LAI proviral clone, which contains the complete genome of HIV-1_{LAI} (Peden et al., 1991). The wild-type construct, pLAI-wt, and the frameshift mutants, pLAI-M1 to pLAI-M8, were LAI-BH10 chimeras made by substituting a 1.2-kb *Age*I-*Bst*1107I fragment from the pGAGPOL-xm-wt and pGAGPOL-M1 to pGAGPOL-M8 plasmids, respectively, into the *Age*I- and *Bst*1107I-digested LAI proviral clone. With the inserted *Age*I–*Bst*1107I BH10 fragment, amino acid residues 389, 401, 427, 453, and 473 of Gag and residues 19–22 and 53 of Pol, were changed, compared to wild-type HIV-1_{LAI}.

Frameshift assays

The effect of the mutations on the −1 frameshift efficiency was measured using the dual-luciferase reporter plasmids described above. Frameshifting was monitored by transient transfection of the dual-luciferase vectors into 293FT cells (Invitrogen). The day before transfection, 2×10^4 cells/well were seeded in 24-well plates and maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Wisent). Briefly, 0.5 μg of each dual-luciferase reporter construct was transfected into cells, using a standard calcium phosphate precipitation method (Jordan et al., 1996), and cells were grown for 48 h before being harvested. Cells were washed twice with PBS and lysed with 100 μl of the Cell Passive Lysis Buffer (Promega). The firefly versus the *Renilla* luciferase activities of each construct were measured as relative light units with a Berthold Lumat LB 9507 luminometer, using a Dual-Luciferase Reporter Assay System kit from Promega.

Production of VLPs

COS-7 cells (3×10^5 per plate) were plated in 60-mm dishes and cultured in 4 ml of DMEM supplemented with 10% FBS for 24 h prior to transfection. Four micrograms of pGAGPOL-xm-wt or its mutant derivatives and 2.5 μg of pRev1 were introduced in cells by transfection, using a standard calcium phosphate precipitation method. The culture medium was removed and replaced with fresh medium 16

h posttransfection. Cells and supernatants were harvested 64 h posttransfection. The culture medium was centrifuged at 3000 rpm for 20 min at 4 °C to remove cellular debris. The clarified medium was centrifuged again for 1 h at 40,000 rpm (Beckman 50Ti rotor) to pellet the VLPs. Pelleted VLPs were resuspended in 200 μl of 10 mM Tris–HCl (pH 7.5) and frozen at −80 °C or used immediately. In parallel, cells were washed twice with 2 ml of PBS and lysed with 1 ml of 1% Triton X-100/PBS. Cell lysates were collected and stored at −80 °C or used immediately.

RT assays

VLP-associated RT levels were assayed by a standard procedure (Smith et al., 1990). In a 100-μl reaction volume, 65 μl of VLPs resuspended in the Tris buffer were incubated for 90 min at 37 °C with 4 μCi of H^3-TTP (Perkin-Elmer) and 2.5 μg of poly$A_{dT(12-18)}$ (Pharmacia) in an RT buffer at a final concentration of 50 mM Tris–HCl (pH 7.4), 60 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 0.1% Triton X-100, and 1 mM EDTA. The reaction products were precipitated for 1 h on ice with 1 ml of cold 10% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate, collected by filtration on glass-fiber filters and counted for radioactivity.

p24 ELISA assays

p24 ELISA assays were performed to measure the amount of Gag inside the cells and in the VLPs released in the supernatant following the transfection of COS-7 cells with the pGAGPOL vectors. Quantification of p24 was also used to normalize the input of viruses in the infectivity assays and to determine the amount of viruses produced in the replication kinetics studies. These assays were performed using a commercially available p24 antigen detection kit (Beckman-Coulter), according to the instructions provided by the manufacturer. In each experiment, a standard curve was generated, using serial dilutions of a p24 standard. Samples were diluted so that the experimental values were within the linear range of the assay.

Virus production

HeLa-P4($CD4^+$/LTR-*lac*Z^+) cells (Clavel and Charneau, 1994), which contain the *lac*Z gene under the control of the HIV-1 LTR promoter, were used for virus production and single-round infectivity experiments (see below). HeLa-P4 cells (8×10^5 cells/well) were seeded in 6-well plates and cultured in DMEM supplemented with 10% FBS for 24 h before transfection with 5 μg of proviral DNA, using the Polyfect Transfection Reagent (Qiagen). The medium was changed 24 h after transfection. Virus-containing supernatants were collected 48 h posttransfection, clarified of cells debris by a 5-min centrifugation (13,000 rpm), and stored at −80 °C. The p24 content in the supernatants was determined by a p24 ELISA assay (see above) prior to the single-round infectivity assays and to the long-term replication assays.

Gag processing in virions

To examine the level of processing of Gag incorporated into virions, 6 ml of supernatant from HeLa-P4 cells transfected with proviral DNA were centrifuged at 25,000 rpm for 3 h at 4 °C through a 20% sucrose cushion with a Beckman Coulter Avanti J25 centrifuge, using a JA.25.50 rotor. The viral pellets were resuspended in 200 μl of PBS and 15 μl was resolved by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. Samples were immunoblotted, using a mouse anti-HIV-1 p24 monoclonal antibody (no 24-2), diluted 1/2000 (reagent obtained from Dr. Michael Malim of the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). Horseradish peroxidase-conjugated sheep anti-mouse was used as the secondary antibody. Antigen–antibody complexes were detected by enhanced chemiluminescence and bands were obtained with films (Biomax, XAR) exposed for a short period. Bands were scanned and quantified with Quantity one (Bio-Rad).

Single-round infectivity assays

HeLa-P4 cells were plated in 96-well plates (10^4 cells/well) in DMEM supplemented with 10% FBS and infected in triplicate with a normalized amount of HIV-1 or mutant viruses (180 ng/ml of p24 of virus per well) for 24 h. The supernatant was then removed, cells were lysed with lysis buffer (0.2% Triton X-100/PBS), and β-galactosidase activity was determined, using the chromogenic substrate chlorophenolred-β-D-galactopyranoside, as described (Brelot et al., 2000).

Replication kinetics of mutant virus

CEM T-cells were used for long-term replication experiments. CEM T-cells (2×10^6) in 1 ml of RPMI 1640 medium were infected with 360 ng/ml of p24 of wild-type or mutant virus for 2 h, then washed and cultured in T-25 flasks with 5 ml of fresh medium. The cultures were split every second day by replacing 50% of the culture with the same volume of fresh medium. Samples of culture supernatant were withdrawn from the medium at regular intervals and p24 quantified as a measure of ongoing virus replication.

Acknowledgments

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) to L.B-G. and N.H. Y.B. holds a studentship from the Fondation de l'Hôpital Sainte-Justine. N.H. holds a Chercheur-Boursier award from the Fonds de la Recherche en Santé du Québec (FRSQ). We gratefully acknowledge Gerardo Ferbeyre and Jim Omichinski for helpful discussions and critical reading of this manuscript. We thank Peter Kreuzaler for his participation in this study as an exchange student from the Freie Universität of Berlin, Germany.

References

Ambrose, Z., Boltz, V., Palmer, S., Coffin, J.M., Hughes, S.H., Kewalramani, V.N., 2004. In vitro characterization of a simian immunodeficiency virus-human immunodeficiency virus (HIV) chimera expressing HIV type 1 reverse transcriptase to study antiviral resistance in pigtail macaques. J. Virol. 78 (24), 13553–13561.

Berkhout, B., 1999. HIV-1 evolution under pressure of protease inhibitors: climbing the stairs of viral fitness. J. Biomed. Sci. 6 (5), 298–305.

Bidou, L., Stahl, G., Grima, B., Liu, H., Cassan, M., Rousset, J.P., 1997. In vivo HIV-1 frameshifting efficiency is directly related to the stability of the stem-loop stimulatory signal. RNA 3 (10), 1153–1158.

Biswas, P., Jiang, X., Pacchia, A.L., Dougherty, J.P., Peltz, S.W., 2004. The human immunodeficiency virus type 1 ribosomal frameshifting site is an invariant sequence determinant and an important target for antiviral therapy. J. Virol. 78 (4), 2082–2087.

Brelot, A., Heveker, N., Montes, M., Alizon, M., 2000. Identification of residues of CXCR4 critical for human immunodeficiency virus coreceptor and chemokine receptor activities. J. Biol. Chem. 275 (31), 23736–23744.

Brierley, I., 1995. Ribosomal frameshifting viral RNAs. J. Gen. Virol. 76 (Pt 8), 1885–1892.

Brierley, I., Pennell, S., 2001. Structure and function of the stimulatory RNAs involved in programmed eukaryotic-1 ribosomal frameshifting. Cold Spring Harbor Symp. Quant. Biol. 66, 233–248.

Brierley, I., Jenner, A.J., Inglis, S.C., 1992. Mutational analysis of the "slippery-sequence" component of a coronavirus ribosomal frameshifting signal. J. Mol. Biol. 227 (2), 463–479.

Cassan, M., Delaunay, N., Vaquero, C., Rousset, J.P., 1994. Translational frameshifting at the *gag–pol* junction of human immunodeficiency virus type 1 is not increased in infected T-lymphoid cells. J. Virol. 68 (3), 1501–1508.

Chamorro, M., Parkin, N., Varmus, H.E., 1992. An RNA pseudoknot and an optimal heptameric shift site are required for highly efficient ribosomal frameshifting on a retroviral messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 89 (2), 713–717.

Chen, C., Montelaro, R.C., 2003. Characterization of RNA elements that regulate *gag–pol* ribosomal frameshifting in equine infectious anemia virus. J. Virol. 77 (19), 10280–10287.

Clavel, F., Charneau, P., 1994. Fusion from without directed by human immunodeficiency virus particles. J. Virol. 68 (2), 1179–1185.

Dulude, D., Baril, M., Brakier-Gingras, L., 2002. Characterization of the frameshift stimulatory signal controlling a programmed −1 ribosomal frameshift in the human immunodeficiency virus type 1. Nucleic Acids Res. 30 (23), 5094–5102.

Frankel, A.D., Young, J.A., 1998. HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. 67, 1–25.

Freed, E.O., 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology 251 (1), 1–15.

Freed, E.O., Martin, M.A., 2001. HIVs and their replication. In: Knipe, P.M.H.D.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, PA, pp. 1971–2041.

Gaudin, C., Mazauric, M.H., Traikia, M., Guittet, E., Yoshizawa, S., Fourmy, D., 2005. Structure of the RNA signal essential for translational frameshifting in HIV-1. J. Mol. Biol. 349 (5), 1024–1035.

Grentzmann, G., Ingram, J.A., Kelly, P.J., Gesteland, R.F., Atkins, J.F., 1998. A dual-luciferase reporter system for studying recoding signals. RNA 4 (4), 479–486.

Harger, J.W., Dinman, J.D., 2003. An in vivo dual-luciferase assay system for studying translational recoding in the yeast Saccharomyces cerevisiae. RNA 9 (8), 1019–1024.

Hibbert, C.S., Rein, A., 2005. Preliminary physical mapping of RNA-RNA linkages in the genomic RNA of Moloney murine leukemia virus. J. Virol. 79 (13), 8142–8148.

Hill, M.K., Shehu-Xhilaga, M., Crowe, S.M., Mak, J., 2002. Proline residues within spacer peptide p1 are important for human immunodeficiency virus type 1 infectivity, protein processing, and genomic RNA dimer stability. J. Virol. 76 (22), 11245–11253.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77 (1), 51–59.

Jacks, T., Power, M.D., Masiarz, F.R., Luciw, P.A., Barr, P.J., Varmus, H.E., 1988. Characterization of ribosomal frameshifting in HIV-1 *gag–pol* expression. Nature 331 (6153), 280–283.

Jordan, M., Schallhorn, A., Wurm, F.M., 1996. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res. 24 (4), 596–601.

Julias, J.G., Ferris, A.L., Boyer, P.L., Hughes, S.H., 2001. Replication of phenotypically mixed human immunodeficiency virus type 1 virions containing catalytically active and catalytically inactive reverse transcriptase. J. Virol. 75 (14), 6537–6546.

Mak, J., Jiang, M., Wainberg, M.A., Hammarskjold, M.L., Rekosh, D., Kleiman, L., 1994. Role of Pr160gag-pol in mediating the selective incorporation of tRNA (Lys) into human immunodeficiency virus type 1 particles. J. Virol. 68 (4), 2065–2072.

Paillart, J.C., Skripkin, E., Ehresmann, B., Ehresmann, C., Marquet, R., 2002. In vitro evidence for a long range pseudoknot in the 5′-untranslated and matrix coding regions of HIV-1 genomic RNA. J. Biol. Chem. 277 (8), 5995–6004.

Paillart, J.C., Shehu-Xhilaga, M., Marquet, R., Mak, J., 2004. Dimerization of retroviral RNA genomes: an inseparable pair. Nat. Rev., Microbiol. 2 (6), 461–472.

Paulus, C., Ludwig, C., Wagner, R., 2004. Contribution of the Gag–Pol transframe domain p6* and its coding sequence to morphogenesis and replication of human immunodeficiency virus type 1. Virology 330 (1), 271–283.

Peden, K., Emerman, M., Montagnier, L., 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology 185 (2), 661–672.

Pettit, S.C., Henderson, G.J., Schiffer, C.A., Swanstrom, R., 2002. Replacement of the P1 amino acid of human immunodeficiency virus type 1 Gag processing sites can inhibit or enhance the rate of cleavage by the viral protease. J. Virol. 76 (20), 10226–10233.

Reil, H., Kollmus, H., Weidle, U.H., Hauser, H., 1993. A heptanucleotide sequence mediates ribosomal frameshifting in mammalian cells. J. Virol. 67 (9), 5579–5584.

Rosé, J.R., Babe, L.M., Craik, C.S., 1995. Defining the level of human immunodeficiency virus type 1 (HIV-1) protease activity required for HIV-1 particle maturation and infectivity. J. Virol. 69 (5), 2751–2758.

Shehu-Xhilaga, M., Crowe, S.M., Mak, J., 2001. Maintenance of the Gag/Gag–Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. J. Virol. 75 (4), 1834–1841.

Shehu-Xhilaga, M., Hill, M., Marshall, J.A., Kappes, J., Crowe, S.M., Mak, J., 2002. The conformation of the mature dimeric human immunodeficiency virus type 1 RNA genome requires packaging of pol protein. J. Virol. 76 (9), 4331–4340.

Smith, A.J., Cho, M.I., Hammarskjold, M.L., Rekosh, D., 1990. Human immunodeficiency virus type 1 Pr55gag and Pr160gag-pol expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. 64 (6), 2743–2750.

Staple, D.W., Butcher, S.E., 2005. Solution structure and thermodynamic investigation of the HIV-1 frameshift Inducing element. J. Mol. Biol. 349 (5), 1011–1023.

Tang, H., Kuhen, K.L., Wong-Staal, F., 1999. Lentivirus replication and regulation. Annu. Rev. Genet. 33, 133–170.

Telenti, A., Martinez, R., Munoz, M., Bleiber, G., Greub, G., Sanglard, D., Peters, S., 2002. Analysis of natural variants of the human immunodeficiency virus type 1 *gag–pol* frameshift stem-loop structure. J. Virol. 76 (15), 7868–7873.