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The virion-associated Gag–Pol is decreased in chimeric Moloney murine leukemia viruses in which the readthrough region is replaced by the frameshift region of the human immunodeficiency virus type 1

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Abstract

The human immunodeficiency virus type 1 (HIV-1) requires a programmed −1 translational frameshift event to synthesize the precursor of its enzymes, Gag–Pol, when ribosomes from the infected cells translate the full-length viral messenger RNA. Translation of the same RNA according to conventional translational rules produces Gag, the precursor of the structural proteins of the virus. The efficiency of the frameshift controls the ratio of Gag–Pol to Gag, which is critical for viral infectivity. The Moloney murine leukemia virus (MoMuLV) uses a different strategy, the programmed readthrough of a stop codon, to synthesize Gag–Pol. In this study, we investigated whether different forms of the HIV-1 frameshift region can functionally replace the readthrough signal in MoMuLV. Chimeric proviral DNAs were obtained by inserting into the MoMuLV genome the HIV-1 frameshift region encompassing the slippery sequence where the frameshift occurs, followed by the frameshift stimulatory signal. The inserted signal was either a simple stem-loop, previously considered as the stimulatory signal, or a longer bulged helix, now shown to be the complete stimulatory signal, or a mutated version of the complete signal with a three-nucleotide deletion. Although the three chimeric viruses can propagate essentially as the wild-type virus in NIH 3T3 cells, single-round infectivity assays revealed that the infectivity of the chimeric virions is about three to fivefold lower than that of the wild-type virions, depending upon the nature of the frameshift signal. It was also observed that the Gag–Pol to Gag ratio was decreased about two to threefold in chimeric virions. Comparison of the readthrough efficiency of MoMuLV to the HIV-1 frameshift efficiency, by monitoring the expression of a luciferase reporter in cultured cells, revealed that the frameshift efficiencies were only 30–60% of the readthrough efficiency. Altogether, these observations indicate that replacement of the readthrough region of MoMuLV with the frameshift region of HIV-1 results in virions that are replication competent, although less infectious than wild-type MoMuLV. This type of chimera could provide an interesting tool for in vivo studies of novel drugs targeted against the HIV-1 frameshift event.

Keywords: HIV-1; Murine leukemia virus; Ribosomal frameshift; Gag–Pol/Gag ratio; Readthrough

Introduction

The acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus type 1 (HIV-1), has killed over 21 million people since its appearance (Los Alamos National Laboratory, 2002). The current therapy, based on the use of inhibitors of viral enzymes, is hampered by the emergence of resistant variants to these drugs (Coakley et al., 2000; Isel et al., 2001; LaBonte et al., 2003; Prabu-Jeyabalan et al., 2003). Novel antiviral drugs that target other steps of the viral replication cycle, such as viral entry, were recently developed (Chantry, 2004; Clapham and McKnight, 2002; Lee and Rossi, 2004; Matthews et al., 2004). However, the virus also develops a resistance to these drugs (Miller and Hazuda, 2004). Promising results were obtained in cultured cells with the

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RNA interference strategy (Berkhout, 2004; Lee and Rossi, 2004), but escape mutants were also isolated with this approach (Boden et al., 2003). It is therefore imperative to investigate other potential viral targets.

The programmed translational frameshift event used by HIV-1 to produce Gag–Pol, the precursor of its enzymes (Jacks et al., 1988), is one potential target worth investigating. Gag, the precursor of the viral structural proteins, and Gag–Pol are both translated from the full-length viral mRNA but are encoded in two different reading frames. Gag is synthesized according to conventional translation rules whereas Gag–Pol synthesis requires a −1 ribosomal frameshift during the translation of the messenger. This strategy allows the virus to maintain a specific Gag–Pol to Gag ratio, which is critical for particle assembly, RNA dimerization, viral replication and infectivity (Hung et al., 1998; Karacostas et al., 1993; Park and Morrow, 1992; Shehu-Xhilaga et al., 2001). The synthesis of Gag–Pol depends upon two *cis*-acting elements, the slippery sequence, where the shift occurs and a specific downstream secondary structure, which acts as a frameshift stimulatory signal (reviewed in Brierley, 1995; Brierley and Pennell, 2001). This secondary structure was long assumed to be a simple 11-bp stem-loop, the classical stimulatory signal (Jacks et al., 1988; Kang, 1998). However, Dulude et al. (2002) showed that the stimulatory signal is more complex and consists in a longer stem-loop where the upper part corresponds to the classical or short stem-loop signal and the lower part results from pairing the spacer region following the slippery sequence and preceding the short stem-loop with a complementary segment downstream of this stem-loop. A three-purine bulge separates the two parts of the helix. This bulged helix, which we name the long frameshift stimulatory signal, stimulates the frameshift efficiency twice as much as the short signal.

It will be interesting to develop novel anti-HIV-1 agents that perturb the frameshift efficiency and, consequently, the Gag–Pol to Gag ratio in HIV-1. However, assays with primates that are traditionally used for investigating anti-HIV drugs in vivo are very expensive and, most often, only small numbers of animals can be used, which limits the number of compounds that can be tested. It is therefore important to develop models using small mammals to assess in vivo the effects of potential anti-frameshift agents. We thus decided to construct a chimera of a murine retrovirus, the Moloney murine leukemia virus (MoMuLV), in which the frameshift region of HIV-1 directs the synthesis of the Gag–Pol polyprotein from MoMuLV. MoMuLV is a simple C-type murine retrovirus that induces T-cell lymphomas in susceptible mice after a latency of several months (reviewed in Gardner, 1978). In this well-characterized virus (Goff, 1984; Jones et al., 1989; Shinnick et al., 1981; Tsichlis, 1987), the *gag* and *pol* genes are in the same reading frame and the Gag–Pol polyprotein is expressed by a translational readthrough of the UAG stop codon at the end of the *gag* coding sequence. The UAG stop codon is followed at a distance of 8 nucleotides by a pseudoknot structure that acts as a readthrough stimulatory signal (Alam et al., 1999; Felsenstein and Goff, 1988, 1992; Feng et al., 1992; Wills et al., 1991, 1994; Yoshinaka et al., 1985). In a previous study, we constructed a chimeric MoMuLV in which we inserted the short frameshift region of HIV-1. The construct was such that the synthesis of Gag–Pol depends upon a −1 frameshift rather than the readthrough signal normally used by MoMuLV (Brunelle et al., 2003). When NIH 3T3 cells were transfected with this chimeric proviral DNA, the mutated virus propagated about as well as the wild-type virus. However, the conditions of propagation in transfected NIH 3T3 cells could fail to detect small changes in infectivity that can affect viral propagation in vivo. In the present study, we constructed a chimeric MoMuLV containing the long frameshift region of HIV-1 and another chimera containing a mutant of this long signal with a three-nucleotide deletion. We then assessed the function of the HIV-1 frameshift signal in the three chimeras by measuring the capacity of the viral particles produced with the chimeric proviral DNA to propagate in cultured cells and by monitoring their infectivity with a one-cycle infectivity assay. We also determined the ratio of Gag–Pol to Gag in the chimeric virions. In parallel, we compared the readthrough efficiency of the MoMuLV to the frameshift efficiency of HIV-1 using a reporter gene, the firefly luciferase, whose expression depended upon the readthrough region of MoMuLV or the short, the long and the long mutated frameshift region of HIV-1. Altogether, the results obtained show that all the chimeric virions are replication competent, but less infectious than the wild-type virus, a defect that can be directly correlated to a decrease in the Gag–Pol to Gag ratio, consistent with the lower efficiency of the frameshift compared to the readthrough.

Results

Description of the chimeras used in the study

In this study, we assessed the infectivity of a chimeric murine retrovirus derived from MoMuLV for which the synthesis of Gag–Pol depends upon the HIV-1 frameshift region instead of the region responsible for the readthrough of a stop codon. We had previously constructed such a chimeric provirus, pGMofs-short, which contains the complete proviral DNA of MoMuLV with the short frameshift region of HIV-1 group M subtype B inserted between the *gag* and *pol* genes (Brunelle et al., 2003). Here, we introduced the long frameshift region of HIV-1 between the MoMuLV *gag* and *pol* genes, generating pGMofs-long. We also investigated a third chimera, pGMofs-B*, which contains the frameshift region of a natural isolate of HIV group M subtype B, B*, where the frameshift stimulatory signal is mutated into a helix interrupted by an internal loop, consequently to a three-

nucleotide deletion (Fig. 1). In all the chimeras, the HIV-1 frameshift region is inserted between two cleavage sites of the viral protease so that the insertion is eliminated during the maturation of the viral proteins (Fig. 2). The Gag stop codon (UAG) where the readthrough occurs in wild-type MoMuLV was replaced with a glutamine codon (CAG), thus minimizing the probability of reversion of the chimeric viruses if the inserted frameshift region were eliminated during viral replication. It can be noted that readthrough of the Gag stop codon in the chimeric construct would not have resulted in the expression of the *pol* gene that is now in a −1 frame relative to the *gag* gene. To allow the production of the Gag precursor, other stop codons were introduced in the (0) frame, as described in Materials and methods (see Fig. 1).

The chimeric viruses can replicate in NIH 3T3 cells

To examine the replication of the chimeric viruses, we transfected cultured mouse fibroblasts permissive for MoMuLV replication (NIH 3T3 cells) with 5 μg of the proviral DNA of either wild-type or chimeric MoMuLV. We examined the propagation of the virus in cultured cells by measuring the reverse transcriptase activity in the supernatants every third day during 15 days. Under these conditions, the propagation of the chimeric MoMuLV derivatives was similar to that of the wild-type MoMuLV (data not shown). These assays show that the chimeric murine viruses expressing *pol* via a frameshift event can replicate in cultured cells. An amplification of viral RNAs by RT-PCR confirmed that the HIV-1 insertion in the chimeric virions was stable and unaltered for periods of time up to 30 days (data not shown). Although the replicative potential of the chimeric viruses appears to be normal in the propagation assays, minor differences which could affect the propagation of the virus in infected mice could be missed in these assays with which the virions are produced in large amounts. Also, the measurement of the reverse transcriptase activity could be misleading if the number of virion-associated reverse transcriptase molecules is altered in the chimeric virions.

In order to further quantify the replicative potential of the chimeric viruses, an alternative approach was considered. Culture media from cells chronically infected with either one of plasmids pGMo, pGMofs-short, pGMofs-long and pGMofs-B* were first harvested. The amount of viruses released was assessed by a quantitative protein dot blot assay, using an anti-p30 (CA) antiserum, which recognizes

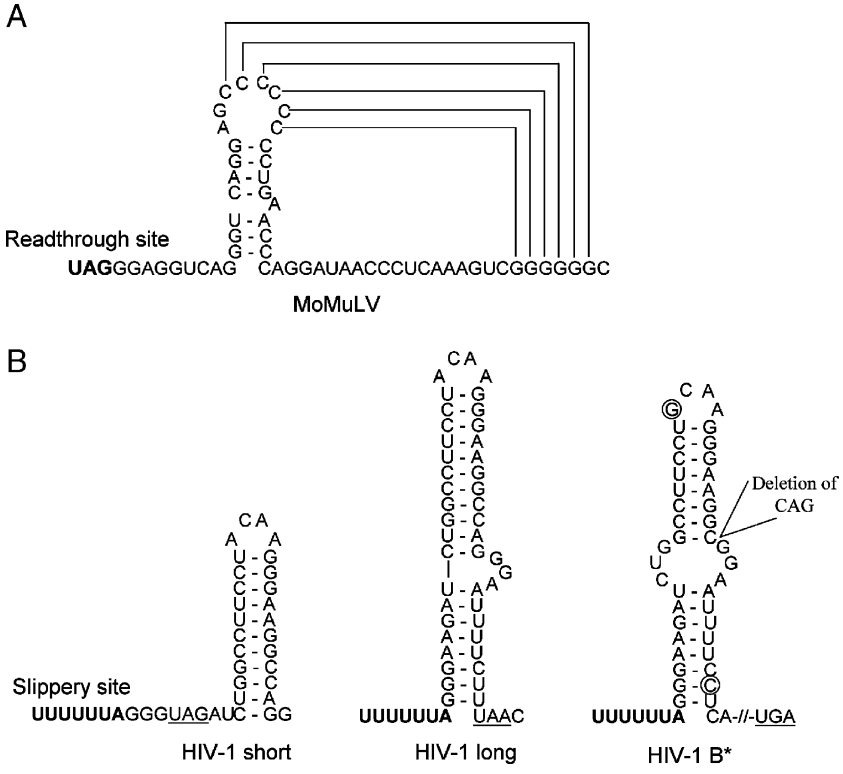


Fig. 1. Structure of the HIV-1 frameshift region inserted in the genome of Moloney murine leukemia virus (MoMuLV) at the *gag–pol* junction. (A) Readthrough region of MoMuLV containing the site of readthrough in bold and the stimulatory pseudoknot. (B) Frameshift region of the human immunodeficiency virus type 1 (HIV-1) containing the short (HIV-1 short), the long (HIV-1 long) or a mutated (HIV-1 B*) frameshift stimulatory signal with a three-nucleotide deletion. Circles in the mutated signal indicate the bases that differ from the long stimulatory signal (group M subtype B). The slippery site is in bold. Stop codons that were introduced in the (0) frame to terminate the synthesis of the Gag precursor are underlined. Note that for HIV-1 B* the stop codon does not directly follow the frameshift signal but is located at the junction of the frameshift region and the inactivated readthrough region, at a cleavage site for the MoMuLV protease (see details in Materials and methods).

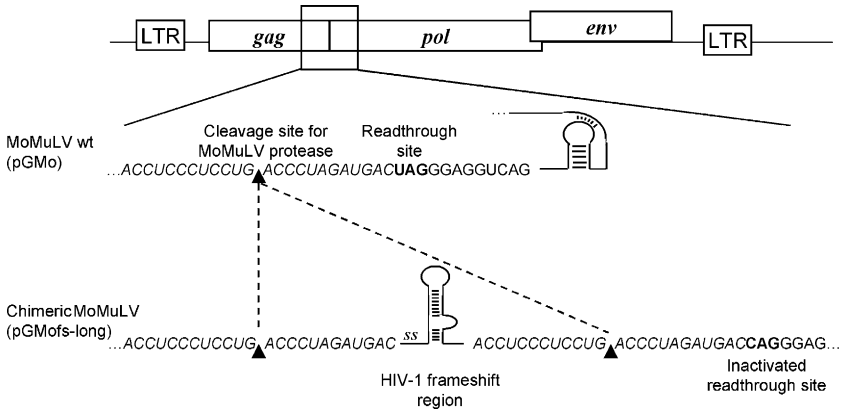


Fig. 2. Details on the sequence of the *gag–pol* junction in the proviral DNA of wild-type and chimeric MoMuLV. Plasmid pGMo contains the wild-type MoMuLV DNA. In the enlarged *gag–pol* junction, the ▲ indicates a cleavage site for MoMuLV protease, and the readthrough site, in bold, is followed by the stimulatory pseudoknot. Plasmid pGMofs-long contains the chimeric proviral DNA, with the long frameshift region of HIV-1. The insertion is bracketed between two cleavage sites. The *ss* represents the slippery site that is followed by the long stimulatory signal and by the readthrough region, where the original site of readthrough is replaced with CAG (in bold). The same type of chimera was obtained with the short frameshift stimulatory signal (pGMofs-short) and the mutated stimulatory signal (pGMofs-B*).

the Gag precursor and one of its cleavage products, the capsid protein (CA). Equivalent small amounts of viruses were then used to infect NIH 3T3 cells for 8 h in presence of polybrene. Culture medium was harvested every day during 15 days and the amount of viruses released post-infection was assessed by the dot blot assay monitoring the presence of Gag. It was observed that virus production in all three chimeras was reduced compared to the wild-type virus during this period of time, the decrease being about two- to threefold for pGMofs-long and pGMofs-short and fourfold for pGMofs-B* (Fig. 3A), suggesting a decrease in the infectivity of the chimeric virions. The released virions were also analyzed for their content of Gag–Pol and for the processing of Gag (see later on). After a longer period of time, the differences in the virus production disappeared (data not shown). Analysis of viral RNAs by RT-PCR for periods of time up to 15 days confirmed that the HIV-1 insertion was stable and unaltered in the chimeric virions (data not shown).

The infectivity of the chimeric virions is decreased in single-round infectivity assays

To further investigate the decrease of infectivity of the chimeras suggested by the replication assays described above, single-round infectivity assays were used (Wang and Goff, 2003). Proviral DNA (pGMo, pGMofs-short, pGMofs-long or pGMofs-B*) was cotransfected in 293FT cells with a murine retrovirus vector (SRαLluc) carrying firefly luciferase as a reporter gene (An et al., 1999). The chimeric or the wild-type MoMuLV acts as a helper virus that encapsidates the RNA corresponding to the luciferase gene. Equivalent volumes (normalized for the efficiency of transfection) of culture media of either the wild-type or the mutant virus were used to infect NIH 3T3 cells during 1 h in presence of polybrene and the infectivity corresponding to the helper viruses was monitored by measuring the luciferase activity in lysates of the infected cells 36 h post-infection. It was found that, under these conditions, cells infected with the wild-type MoMuLV helper virus produce high levels of luciferase activity whereas luciferase activity was reduced by about three to fivefold in the cells infected with either one of the chimeric viruses (Fig. 3B). These assays therefore confirm that the chimeric viruses, in which the synthesis of Gag–Pol depends on a −1 frameshift, are infectious but show that their infectivity is decreased compared to the wild-type virus.

The processing of the Gag polyprotein is not affected but the Gag–Pol to Gag ratio is decreased in the chimeric virions

The processing of the Gag polyprotein and the amount of Gag–Pol incorporated relative to the amount of Gag was investigated in the chimeric virions released from the infected NIH 3T3 cells. The total amount of Gag contained in these virions had been quantified with a protein dot blot, as described above. To investigate whether there were changes in the processing of the Gag polyprotein, aliquots containing the virions released from NIH 3T3, as described above in the standard infectivity assays, were analyzed by SDS–PAGE and by immunoblotting with the anti-p30 (CA) antiserum that detects Gag and the capsid protein. It can be seen (Fig. 4A) that the protein profiles obtained with all the chimeric virions are identical to that obtained with the wild-type virus, the CA/Gag ratio being about 10:1 for each type of viruses, indicating that the insertion of the HIV-1 frameshift region and the inactivation of the readthrough process do not affect the maturation of the Gag polyprotein (Fig. 4A). The amount of Gag–Pol incorporated in the released virions was assessed by measuring their associated reverse transcriptase activity, which indicates the total amount of Gag–Pol and one of its cleavage products, the reverse transcriptase. It was observed that the amount of Gag–Pol relative to Gag decreased by two

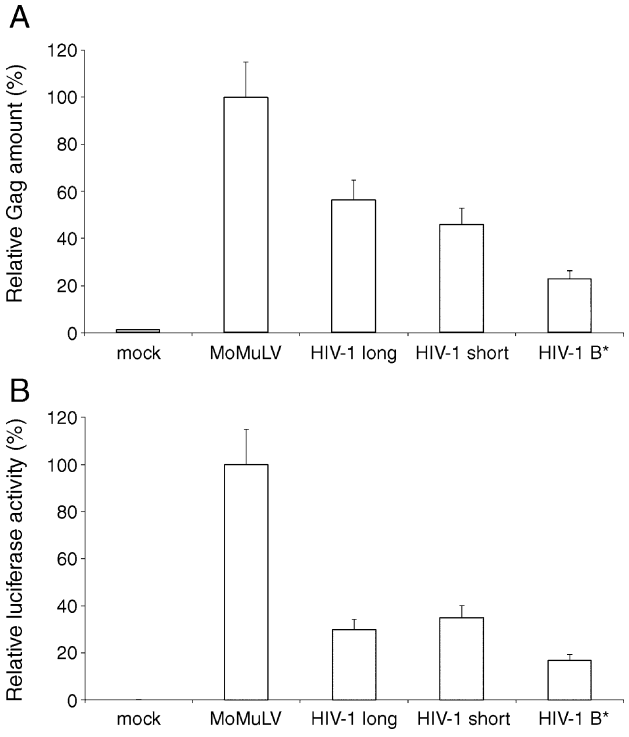


Fig. 3. Analysis of the infectivity of the chimeric viruses by replication and single-round infection assays. (A) Replication assays. Gag dot blotting was used to monitor the amount of virions produced in the supernatant following the infection of NIH 3T3 cells with either wild-type or chimeric virions obtained from cells chronically infected with pGMofs-long (HIV long), pGMofs-short (HIV short) or pGMofs-B* (HIV B*). Supernatants were harvested post-infection every third day and treated as described in Materials and methods to quantify the amount of Gag released from the infected cells. The figure is representative of three independent experiments and shows the amount of viruses obtained after 9 days. A value of 100% was arbitrarily ascribed to the mean amount of Gag released from wild-type MoMuLV virions. Standard errors of the means are shown as error bars. (B) Single-round infectivity assays. 293FT cells were co-transfected with pGMo (MoMuLV), pGMofs-long (HIV-1 long), pGMofs-short (HIV-1 short) or pGMofs-B* (HIV-1 B*), plus a murine retrovirus vector (SRαLluc) coding for the firefly luciferase and pcDNA3.1/hygro(+)/lacZ, coding for β-galactosidase, as described in Materials and methods. The infectivity of the viruses was assayed by measuring 36 h post-infection the luciferase activity of lysates of NIH 3T3 cells infected with equal amounts of luciferase reporter virus packaged by mutant viruses serving as helpers. The figure presents the means of four independent experiments. A value of 100% was arbitrarily ascribed to the mean luciferase activity with wild-type MoMuLV virions. Standard errors of the means are shown as error bars.

to threefold in the chimeric viruses compared to the wild type (Fig. 4B), suggesting that less Gag–Pol is synthesized during infection by the chimeric viruses.

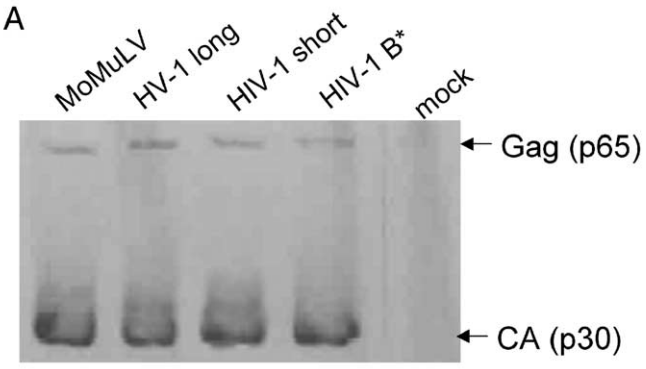
The readthrough efficiency of MoMuLV is higher than the frameshift efficiency of HIV-1

The decreased Gag–Pol to Gag ratio observed in the chimeric virions suggests that the frameshift event is less efficient than the readthrough event. We therefore decided to use constructs with a heterologous reporter gene to compare the readthrough efficiency promoted by the readthrough region of MoMuLV to the frameshift efficiency corresponding to the frameshift regions of HIV-1 inserted in the chimeric MoMuLV. We introduced the readthrough region of MoMuLV in a vector containing the firefly luciferase gene under control of a CMV promoter (Baril et al., 2003), generating pLUC/MoMuLV (readthrough), for which the insertion is such that the synthesis of luciferase depends upon the readthrough of the stop codon. A control construct, pLUC/MoMuLV (control), was made with an identical insertion except that the stop codon was replaced with a glutamine codon. The ratio of luciferase produced with pLUC/MoMuLV (readthrough) to that produced with the pLUC/MoMuLV (control) construct was used to assess the readthrough efficiency. For measuring the frameshift efficiency, the frameshift region of HIV-1, with the short, long or mutated stimulatory signal, was inserted in the beginning of the luciferase coding sequence such that only ribosomes that make a −1 frameshift synthesize luciferase (see Fig. 5A). This insertion was followed by the inactivated readthrough region of MoMuLV so as to reproduce the situation encountered by the ribosomes when translating the viral mRNA in cells infected with the chimeric viruses. This generated pLUC/HIVshort-MoMuLV (−1), pLUC/HIV-long-MoMuLV (−1) and pLUC/HIVB*-MoMuLV (−1). A control in-frame construct was derived from each (−1) construct by inserting one base immediately downstream the slippery site, so that only ribosomes that do not make a frameshift synthesize luciferase. The readthrough efficiency and the frameshift efficiency were assessed in cultured 293FT cells by measuring the luciferase activity in lysates from cells transfected with the appropriate vectors (Fig. 5B). The readthrough efficiency of MoMuLV is 5.2%, a value comparable to those obtained previously with an appropriate heterologous reporter in cultured cells, which were 3.7% and 4.2%. (Grentzmann et al., 1998; Orlova et al., 2003). We found that the frameshift efficiency of the long frameshift region of HIV-1, the short frameshift region and the frameshift region of subtype B* are, respectively, 2.9%, 1.5% and 1.3%, corresponding to about one-half to one-fourth of the readthrough efficiency. This lower efficiency of the frameshift event compared to the readthrough event can directly account for the lower Gag–Pol to Gag ratio in the chimeric virions.

Discussion

As indicated in the Introduction, the Gag–Pol to Gag ratio is critical for virus replication and infectivity. Baril et al. (2003), who examined the frameshift efficiencies in all the subtypes of HIV-1 group M, found that these values fall within a narrow window, the maximal deviation being only 35% of the mean value. Increasing the Gag–Pol to Gag ratio in HIV-1 by as little as two to threefold was sufficient to inhibit HIV-1 replication (Hung et al., 1998; Shehu-Xhilaga et al., 2001) and it was recently shown that a fourfold

A



B

	MoMuLV	HIV-long	HIV-1 short	HIV-1 B*	mock
RT activity (cpm)	90696	39241	30941	26139	1138
Relative Gag-Pol/Gag ratio	100%	51%	44%	30%	

Fig. 4. Analysis of the processing of the Gag polyprotein by Western blot and assessment of the Gag–Pol to Gag ratio in the chimeric virions. (A) Total amount of Gag (Gag + p30 bands) in the virions, as revealed by immunoblotting. The viral proteins were fractionated by SDS–PAGE and Gag and p30 were revealed with an anti-p30 (CA) antiserum. Equal total amounts of Gag were used in these assays. The CA/Gag ratio was about 10:1 for the wild-type virus and for the chimeras. This figure is a representative example of four independent experiments. (B) Reverse transcriptase activity associated to the virions. The reverse transcriptase activity reflects the amount of Gag–Pol and of one of its cleavage products, the reverse transcriptase, incorporated into the virions. The Gag–Pol to Gag ratio was calculated from the reverse transcriptase activities and the amount of Gag determined as in Fig. 3A value of 100% was arbitrarily ascribed to the Gag–Pol to Gag ratio of the wild-type MoMuLV. Results are the means of three independent experiments and correspond to virions released after 9 days. The standard error of the means was inferior or equal to 15%.

decrease of frameshift efficiency decreased HIV-1 infectivity a hundred-fold (Biswas et al., 2004). Also, Telenti et al. (2002) analyzed HIV-1 mutants, altered in the upper stem-loop structure of the frameshift stimulatory signal, and found that viruses with more than 60% reduction in frameshift efficiency presented a profound defect in viral replication and infectivity. In the case of MoMuLV, Felsenstein and Goff (1988) showed that, with a mutant of MoMuLV expressing only Gag–Pol but not Gag, there was no assembly and no release of viral particles. However, to our knowledge, there are no other data on the effect of a decrease or an increase in the Gag–Pol to Gag ratio on MoMuLV infectivity. Studies with another retrovirus using a −1 ribosomal frameshift to synthesize Gag–Pol, the equine infectious anemia virus indicated that mutations that reduced frameshift efficiency by as much as 50% continued to sustain viral replication, but that greater reductions in frameshifting efficiency led to replication defects (Chen and Montelaro, 2003).

Our aim was to construct a chimeric murine retrovirus whose replication depends upon the ribosomal frameshift event of HIV-1, with the expectation that such a chimera could be used to study the effect of agents targeted against HIV-1 frameshift region in mice. Chimeric retroviruses in which regions of the genome of MoMuLV are replaced by the corresponding regions of HIV-1 genome (Kondo et al., 1995; Yuan et al., 2000) and in which regions of the genome of HIV-1 are replaced by the corresponding regions of MoMuLV (Chen et al., 2001; Reed et al., 2002) have been described. These chimeras were replication competent in cultured cells but, in general, less infectious than the corresponding wild-type virus. Our results show that chimeric derivatives of MoMuLV producing Gag–Pol under the control of the frameshift region of HIV-1 are also less infectious than the wild type. When NIH 3T3 cells are transfected with chimeric proviral DNA, enough infectious virions are formed under the conditions of transfection to sustain productive spread through the culture. However, infectivity assays show that, although the frameshift region of HIV-1 allows the production of Gag–Pol, the resulting viral particles are less infectious than the wild-type particles. Assessment of the frameshift efficiency for the chimeric viruses showed that it was decreased to one-fourth to one-half of the readthrough efficiency, accounting for the decrease in the Gag–Pol to Gag ratio observed in the chimeric virions and roughly correlating with the decreased virus infectivity. However, we did not observe any difference between chimeric and wild-type virions when comparing the efficiency of Gag processing. Gag is processed by the viral protease, one of the mature products of Gag–Pol. Since there is less Gag–Pol synthesized in chimeric virions, there is less protease produced. To account for the lack of difference in Gag processing, we suggest that the viral protease is produced in excess over the amount required for efficient Gag cleavage in wild-type virions.

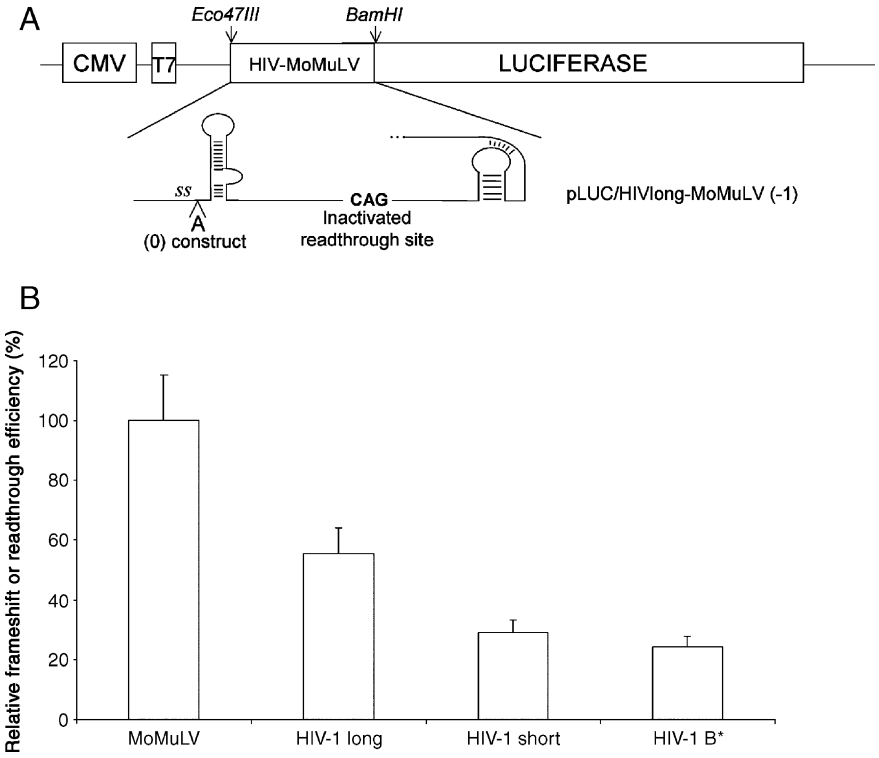


Fig. 5. Comparison of the frameshift efficiency of HIV-1 to the readthrough efficiency of MoMuLV. (A) Example of a vector expressing a luciferase reporter used to compare HIV-1 frameshift efficiency to MoMuLV readthrough efficiency in cultured cells. The HIV-1 frameshift region (short, long or B*), followed by the inactivated readthrough region of MoMuLV, is inserted in the beginning of the luciferase coding sequence, between the *Eco*47III and *Bam*HI sites. This generated the (−1) constructs, in which the *luc* coding sequence is in a −1 reading frame relative to the initiator codon so that a −1 frameshift is required to synthesize luciferase. For each (−1) construct, a corresponding in-frame (0) construct was made by inserting an adenine immediately downstream the slippery site so that luciferase is produced by conventional translation. The figure presents pLUC/HIVlong-MoMuLV (−1) with the slippery site (*ss*), the long stimulatory signal and the inactivated readthrough region of MoMuLV, so as to reproduce the context of the frameshift region in the chimeric virus. When the readthrough efficiency is measured, the readthrough region of MoMuLV is inserted between the *Eco*47III and *Bam*HI sites such that the luciferase production is dependent on a readthrough event, generating pLUC/MoMuLV (readthrough) (not shown). (B) Relative frameshift or readthrough efficiency monitored by the luciferase activity in lysates from 293FT cells transfected with the vectors containing the HIV-1 short, long or mutated frameshift region, compared to the readthrough efficiency of MoMuLV. The mean readthrough efficiency of MoMuLV is arbitrarily set at 100%, its experimental value being 5.2 ± 0.5%. The luciferase activity of the cell lysates was measured as described in Materials and methods. Results are the means of at least four independent experiments. Standard errors of the means are shown as error bars.

How a small change in the Gag–Pol to Gag ratio decreases the virus infectivity remains mysterious. We observed that the processing of Gag is not perturbed and it is known that the placement of the primer tRNA on the primer binding site does not require *pol* gene expression in murine retroviruses (Fu et al., 1997). An attractive hypothesis is that the decreased infectivity of the chimeric virions results from an interference with the maturation of genomic RNA dimerization. Indeed, although the Gag protein is the major mediator of genomic RNA encapsidation and dimerization in HIV-1 and MoMuLV (see Evans et al., 2004; Feng et al., 1999, and references therein), the maturation of RNA dimerization is known to be sensitive to the changes in the amount of Gag–Pol (Shehu-Xhilaga et al., 2002). In addition to this possible maturation problem, it must also be added that Orlova et al. (2003) recently demonstrated that MoMuLV reverse transcriptase is involved in the regulation of its own expression, by sequestering eRF1, the eukaryotic translation release factor 1 that recognizes stop codons, and, in association with the release factor eRF3, causes termination and polypeptide release from the ribosome. This interaction thus enhances the efficiency of readthrough (see also Goff, 2004). Such *trans*-acting additional regulation mechanism for the control the Gag–Pol to Gag ratio is obviously missing in the chimeric viruses. The lack of this control could also contribute to affect the Gag–Pol to Gag ratio and the infectivity of the chimeras, for which the synthesis of Gag–Pol is not up-regulated by the reverse transcriptase.

In conclusion, our results demonstrate that chimeras derived from MoMuLV, where the synthesis of Gag–Pol depends upon the −1 ribosomal frameshift from HIV-1, are replication competent but less infectious than the wild-type virus. Among these chimeras, the one with the long frameshift region of HIV-1 could represent an interesting tool to investigate agents targeted against the frameshift stimulatory signal. Although it is premature to predict the effect of the decreased infectivity when mice are infected

with such a chimera, it is likely that this defect will delay the propagation of the chimeric virions. However, this should not prevent a comparison of virus propagation in mice in the presence or the absence of anti-frameshift drugs and an assessment of the efficiency of these drugs.

Materials and methods

Construction of plasmids

Plasmid pGMo is a derivative of pGEM7Zf (Promega) that contains the proviral DNA of wild-type MoMuLV. Plasmid pGMofs-short is a derivative of pGMo in which the short frameshift region of HIV-1 was inserted at the junction of the *gag* and *pol* genes, such that the *pol* gene is in a −1 frame relative to the *gag* gene. In the readthrough region, the Gag stop codon (UAG) where the readthrough occurs was mutated to a glutamine codon (CAG). Both plasmids were described previously (Brunelle et al., 2003). In this study, we constructed plasmid pGMofs-long, which contains the long frameshift region of HIV-1 group M subtype B (GenBank KO2007), at the junction of the *gag* and *pol* genes and pGMofs-B*, which contains the long HIV-1 frameshift region with a mutated stimulatory signal, corresponding to a natural variant of HIV-1 (GenBank M17449), which we name subtype B*. Both pGMofs-long and pGMofs-B* were obtained from a plasmid derived from pGMofs-short, pGEMt-XhoMfe-short, by a standard PCR with four primers (Ho et al., 1989) using the Vent DNA polymerase (New England Biolabs). Plasmid pGEMt-XhoMfe-short that had been derived from pGMofs-short to facilitate molecular cloning contains the portion of MoMuLV genome between the *Xho*I and *Mfe*I restriction sites. The PCR products obtained from pGEMt-XhoMfe-short were first introduced between the *Nru*I and *Age*I sites of pGEMt-XhoMfe-short to produce pGEMt-XhoMfe-long and pGEMt-XhoMfe-B* and the *Xho*I–*Mfe*I fragment of these plasmids was subsequently cloned in pGMofs-short to produce pGMofs-long and pGMofs-B*. The four primers were, respectively, extern-fwd: 5′-GAAGGAGGTCCC-AACTCGATCGCGACCAGTGTGCCTACTG-3′; extern-rev: 5′-GGTCCCATAACCTGAGCTCCTGATCCCT-CAAAGTGGATTTGGGC-3′; long-fwd: 5′-CAATTTTT-TAGGGAAGATCTGGCCTTCCTACAAGGGAAGGC-CAGGGAATTTTCTTTAACCTCCCTCCTGACCCTAG-3′; long-rev: 5′-CTAGGGTCAGGAGGGAGGTTAAAG-AAAATTCCCTGGCCTTCCCTTGTAGGAAGGCCA-GATCTTCCCTAAAAAATTG-3′ for the cloning of pGMofs-long and extern-fwd; extern-rev; B*-fwd: 5′-GGC CTTCCTGCAAGGGAAGGCGGAATTTTCCTCAA-CCTCC-3′; B*-rev: 5′-GGAGGTTGAGGAAAATTCC-GCCTTCCCTTGCAGGAAGGCC-3′ for the cloning of pGMofs-B*. In all these chimeric constructs, the inserted frameshift region is bracketed between two sites of cleavage for the viral protease. The readthrough codon is also replaced with a glutamine codon (CAG). In all the chimeric constructs, there is a stop codon between the frameshift region and the readthrough region, at the downstream site of cleavage by the MoMuLV protease, which allows the production of the Gag precursor. For the pGMofs-short and pGMofs-long, as a precautionary measure, an additional stop codon (UAG or UAA) had been created by mutagenesis in the spacer between the slippery sequence and the short stem-loop and immediately after the frameshift region, respectively.

To compare the frameshift efficiency of each of the chimeric viruses to the readthrough efficiency of MoMuLV, we used a vector derived from pcDNA3.1/hygro(+) (Invitrogen) containing the firefly luciferase coding sequence under control of a CMV promoter (described in Baril et al., 2003). The readthrough region of MoMuLV was inserted between the *Eco*47III and the *Bam*HI sites of the vector, at the beginning of the luciferase coding region, such that luciferase production is dependent on a readthrough event, generating pLUC/MoMuLV (readthrough). The pLUC/MoMuLV (control) construct was made by replacing the readthrough stop codon by a glutamine codon so that luciferase is synthesized following conventional translation rules. The readthrough efficiency was calculated as the ratio of the luciferase activity (in %) with pLUC/MoMuLV (readthrough) to that with pLUC/MoMuLV (control). The luciferase activity was measured as described below. To determine the frameshift efficiency, we inserted the short frameshift region of HIV-1 subtype B, the long frameshift region of HIV-1 subtype B and the frameshift region of HIV-1 subtype B* followed by the readthrough region where the stop codon was replaced with a glutamine. This generated pLUC/HIVshort-MoMuLV (−1), pLUC/HIV-long-MoMuLV (−1) and pLUC/HIVB*-MoMuLV (−1), respectively. For these (−1) constructs, the *luc* coding sequence is in a −1 reading frame relative to the initiator codon so that a −1 frameshift is required to synthesize luciferase. For each (−1) construct, a corresponding in-frame (0) construct was made by inserting an adenine immediately downstream the slippery site so that luciferase is produced by conventional translation. The frameshift efficiency was calculated by dividing the level of luciferase activity produced with pLUC/HIV-MoMuLV (−1) constructs by the sum of the luciferase activity of the (−1) and the (0) control constructs (in %).

Quantitative analysis of viral replication

NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 15% (v/v) fetal bovine serum. Transient transfections were made in cells at 40% confluence in 100-mm plates as described above, with 5 μg of pGMo, pGMofs-short, pGMofs-long or pGMofs-B* that contain the complete proviral DNA of either wild-type or chimeric MoMuLV. The cells were passaged every third day until they became chronically infected. To investigate viral replication and propagation, the culture media were

harvested every third day during 2 weeks and centrifuged 10 min at 3000 rpm to remove cell debris. Viruses were pelleted by a 1-h centrifugation at 40 000 rpm in 50 Ti rotor (Beckman) and resuspended overnight at 4 °C in 100 μl of Tris–EDTA, pH 8.0. The virion-associated reverse transcriptase (RT) activity of the harvest was measured with a standard reverse transcriptase activity assay described below. Alternatively, chronically infected NIH 3T3 cells (100-mm dishes) at confluence were covered with a minimal volume (3 ml) of culture medium. Supernatants were harvested and replaced with fresh medium every 2 h for 8 h and kept on ice until the last harvest. The harvests of each type of virus (pGMo, pGMofs-short, pGMofs-long or pGMofs-B*) were pooled and frozen at −80 °C. Fresh NIH 3T3 cells at 20% confluence were infected for 8 h with an equal small amount of viruses diluted in 3 ml of culture medium, as measured by dot blotting with goat antiserum against MoMuLV p30, which detects the Gag polyprotein and one of its cleavage product, the capsid protein (CA or p30). Infection was done in the presence of polybrene (Sigma) at 8 μg/ml. Cells were passaged every third day and culture medium was harvested every day during 15 days as described above in the analysis of viral replication and propagation section. The amount of viruses produced was detected by dot blotting with MoMuLV p30 and the reverse transcriptase activity of these virions was also measured to assess the relative Gag–Pol to Gag ratio associated to the virions.

Reverse transcriptase activity assays

The standard reverse transcriptase activity assay was adapted from Smith et al. (1990). An aliquot of the harvest (35 μl) was mixed with a cocktail reaction in a final volume of 100 μl (final concentration: 10 μM dTTP, 50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml bovine serum albumin, 0.05% Nonidet P-40, 20 μg/ml poly (rA)•$dT_{12–18}$ (Roche) and 4 μCi [^{3}H]dTTP (Perkin-Elmer, 88 Ci/mmol)). The reaction was incubated for 1 h at 37 °C, followed by the addition of a freshly made solution of $MnCl_2$ at a final concentration of 2 mM for 15 min. Precipitates were formed by adding trichloroacetic acid (12.5% w/v), sodium pyrophosphate (50 mM) and baker's yeast tRNA (0.5 mg) in a final volume of 1 ml. The mixture was kept on ice for 2 h and filtered through glass microfibre filters 934-AH (Whatman). The radioactivity was counted in a Liquid Scintillation Counter Rack-beta1217 (Perkin Elmer).

Analysis of viral RNA by RT-PCR

Viral RNA was extracted from viral suspensions with Trizol LS reagent (Invitrogen) following the instructions of the manufacturer. The first-strand cDNA synthesis was made using MoMuLV reverse transcriptase according to a standard protocol (Invitrogen), with a primer named oligo B (5′-AGGAGGTCCCAACTCGATCGCGACC-3′), which is complementary to a *gag* sequence upstream the readthrough region of MoMuLV. The PCR reactions were made with primers that are complementary to *gag* or *pol* sequences flanking the HIV-1 insertion. Ten percent of the first-strand reaction was used for the first PCR amplification reaction performed with oligo A (5′-CCCATAACCTGAGCTCCT-GATCCCTC-3′) and oligo B. One tenth of this PCR reaction was used for the second PCR reaction that was performed with oligo B and an internally located primer called oligo C (5′-GGTCCAGCGATACCGCTTTCCTCC-3′). This second PCR reaction was sequenced by automated DNA sequencing, using oligo C as a primer.

Analysis of the viral proteins by dot blot and Western blot

Virions were harvested from infected cells, resuspended in Tris–EDTA and lysed in a 2× RIPA buffer (final concentration: 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.2, 140 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate) containing a cocktail of protease inhibitors. Cell debris was removed by a 10-min centrifugation at 3000 rpm in an Allegra Centrifuge (Beckman Coulter). Samples were either analyzed by a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Western blot) or directly spotted on a nitrocellulose film (dot blot). Samples were then immunoblotted, using a goat anti-p30 antiserum diluted 1/3000 (National Cancer Institute) and a horseradish peroxidase-conjugated swine anti-goat secondary antibody (Cedarlane). Antigen-antibody complexes were detected by enhanced chemiluminescence and autoradiography. Dots and bands from lightly exposed films (Biomax, MR) were scanned and quantified with Quantity One (Bio-Rad), using serial dilutions.

Single-round infectivity assays

Single-round infectivity assays were performed as described by Wang and Goff (2003). 293FT cells (Invitrogen) were transiently co-transfected with 8 μg proviral DNA (pGMo, pGMofs-long, pGMofs-short or pGMofs-B*), 2 μg of a murine retrovirus vector (SRαLluc) carrying a reporter gene coding for the firefly luciferase (An et al., 1999) and 1 μg of pcDNA3.1/hygro(+)/lacZ. The culture media were collected 48 h post-transfection. The β-galactosidase activity of the cell lysates was measured to normalize for variations in transfection efficiency, as described above in the luciferase assays section. Equivalent volumes (1 or 3 ml) of culture medium containing either the wild-type or the mutant reporter virus were used to infect NIH 3T3 cells during 1 h in presence of polybrene at 8 μg/ml. Cells were trypsinized 36 h post-infection, pelleted and washed with PBS, lysed with 200 μl of the Culture Cell Lysis Reagent (Promega) and the infectivity of the viruses was assayed by measuring the luciferase activity in lysates (5 μl) of the infected cells, as described above.

Luciferase assays in cultured cells

293FT cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum. About 2×10^6 cells were seeded in 6-well plates 1 day prior to transiently transfecting, with a standard calcium phosphate precipitation method, 2 μg of the different pLUC vectors and 1 μg of pcDNA3.1/hygro(+)/lacZ (Invitrogen), as an internal reporter to normalize the transfection efficiency. Cells were harvested 48 h post-transfection and lysed with 600 μl of the Cell Culture Lysis Reagent (Promega). The luciferase activity in 2 μl of this cell lysate was determined with a standard luciferase assay reagent made according to Dyer et al. (2000) or with a Luciferase Assay Reagent (Promega) for the single-round infectivity assays and measured in relative light units in a Berthold Lumat LB 9507 luminometer. The β-galactosidase activity of the cell lysates was measured with the chlorophenol-red-β-D-galactopyranoside substrate (Calbiochem), using a standard procedure (Eustice et al., 1991).

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References

Alam, S.L., Wills, N.M., Ingram, J.A., Atkins, J.F., Gesteland, R.F., 1999. Structural studies of the RNA pseudoknot required for readthrough of the gag-termination codon of murine leukemia virus. J. Mol. Biol. 288 (5), 837–852.

An, D.S., Morizono, K., Li, Q.X., Mao, S.H., Lu, S., Chen, I.S., 1999. An inducible human immunodeficiency virus type 1 (HIV-1) vector which effectively suppresses HIV-1 replication. J. Virol. 73 (9), 7671–7677.

Baril, M., Dulude, D., Gendron, K., Lemay, G., Brakier-Gingras, L., 2003. Efficiency of a programmed −1 ribosomal frameshift in the different subtypes of the human immunodeficiency virus type 1 group M. RNA 9 (10), 1246–1253.

Berkhout, B., 2004. RNA interference as an antiviral approach: targeting HIV-1. Curr. Opin. Mol. Ther. 6 (2), 141–145.

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.

Boden, D., Pusch, O., Lee, F., Tucker, L., Ramratnam, B., 2003. Human immunodeficiency virus type 1 escape from RNA interference. J. Virol. 77 (21), 11531–11535.

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

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

Brunelle, M.N., Brakier-Gingras, L., Lemay, G., 2003. Replacement of murine leukemia virus readthrough mechanism by human immunodeficiency virus frameshift allows synthesis of viral proteins and virus replication. J. Virol. 77 (5), 3345–3350.

Chantry, D., 2004. HIV entry and fusion inhibitors. Expert Opin. Emerg. Drugs 9 (1), 1–7.

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.

Chen, B.K., Rousso, I., Shim, S., Kim, P.S., 2001. Efficient assembly of an HIV-1/MLV Gag-chimeric virus in murine cells. Proc. Natl. Acad. Sci. U.S.A. 98 (26), 15239–15244.

Clapham, P.R., McKnight, A., 2002. Cell surface receptors, virus entry and tropism of primate lentiviruses. J. Gen. Virol. 83 (Pt. 8), 1809–1829.

Coakley, E.P., Gillis, J.M., Hammer, S.M., 2000. Phenotypic and genotypic resistance patterns of HIV-1 isolates derived from individuals treated with didanosine and stavudine. AIDS 14 (2), F9–F15.

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.

Dyer, B.W., Ferrer, F.A., Klinedinst, D.K., Rodriguez, R., 2000. A noncommercial dual luciferase enzyme assay system for reporter gene analysis. Anal. Biochem. 282 (1), 158–161.

Eustice, D.C., Feldman, P.A., Colberg-Poley, A.M., Buckery, R.M., Neubauer, R.H., 1991. A sensitive method for the detection of beta-galactosidase in transfected mammalian cells. Biotechniques 11 (6), 739–740, 742–743.

Evans, M.J., Bacharach, E., Goff, S.P., 2004. RNA sequences in the Moloney murine leukemia virus genome bound by the Gag precursor protein in the yeast three-hybrid system. J. Virol. 78 (14), 7677–7684.

Felsenstein, K.M., Goff, S.P., 1988. Expression of the gag–pol fusion protein of Moloney murine leukemia virus without gag protein does not induce virion formation or proteolytic processing. J. Virol. 62 (6), 2179–2182.

Felsenstein, K.M., Goff, S.P., 1992. Mutational analysis of the gag–pol junction of Moloney murine leukemia virus: requirements for expression of the gag–pol fusion protein. J. Virol. 66 (11), 6601–6608.

Feng, Y.X., Yuan, H., Rein, A., Levin, J.G., 1992. Bipartite signal for readthrough suppression in murine leukemia virus mRNA: an eight-nucleotide purine-rich sequence immediately downstream of the gag termination codon followed by an RNA pseudoknot. J. Virol. 66 (8), 5127–5132.

Feng, Y.X., Campbell, S., Harvin, D., Ehresmann, B., Ehresmann, C., Rein, A., 1999. The human immunodeficiency virus type 1 Gag polyprotein has nucleic acid chaperone activity: possible role in dimerization of genomic RNA and placement of tRNA on the primer binding site. J. Virol. 73 (5), 4251–4256.

Fu, W., Ortiz-Conde, B.A., Gorelick, R.J., Hughes, S.H., Rein, A., 1997. Placement of tRNA primer on the primer-binding site requires pol gene expression in avian but not murine retroviruses. J. Virol. 71 (9), 6940–6946.

Gardner, M.B., 1978. Type C viruses of wild mice: characterization and natural history of amphotropic, ecotropic, and xenotropic MuLv. Curr. Top. Microbiol. Immunol. 79, 215–259.

Goff, S.P., 1984. The genetics of murine leukemia viruses. Curr. Top. Microbiol. Immunol. 112, 45–71.

Goff, S.P., 2004. Genetic reprogramming by retroviruses: enhanced suppression of translational termination. Cell Cycle 3 (2), 123–125.

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.

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.

Hung, M., Patel, P., Davis, S., Green, S.R., 1998. Importance of ribosomal frameshifting for human immunodeficiency virus type 1 particle assembly and replication. J. Virol. 72 (6), 4819–4824.

Isel, C., Ehresmann, C., Walter, P., Ehresmann, B., Marquet, R., 2001. The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase. J. Biol. Chem. 276 (52), 48725–48732.

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.

Jones, D.S., Nemoto, F., Kuchino, Y., Masuda, M., Yoshikura, H., Nishimura, S., 1989. The effect of specific mutations at and around the gag–pol gene junction of Moloney murine leukaemia virus. Nucleic Acids Res. 17 (15), 5933–5945.

Kang, H., 1998. Direct structural evidence for formation of a stem-loop structure involved in ribosomal frameshifting in human immunodeficiency virus type 1. Biochim. Biophys. Acta 1397 (1), 73–78.

Karacostas, V., Wolffe, E.J., Nagashima, K., Gonda, M.A., Moss, B., 1993. Overexpression of the HIV-1 gag–pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. Virology 193 (2), 661–671.

Kondo, E., Mammano, F., Cohen, E.A., Gottlinger, H.G., 1995. The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J. Virol. 69 (5), 2759–2764.

LaBonte, J., Lebbos, J., Kirkpatrick, P., 2003. Enfuvirtide. Nat. Rev. Drug Discov. 2 (5), 345–346.

Lee, N.S., Rossi, J.J., 2004. Control of HIV-1 replication by RNA interference. Virus Res. 102 (1), 53–58.

Los Alamos National Laboratory, 2002. In: Human Retroviruses and AIDS (Eds.), A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Los Alamos National Laboratory.

Matthews, T., Salgo, M., Greenberg, M., Chung, J., DeMasi, R., Bolognesi, D., 2004. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat. Rev., Drug Discov. 3 (3), 215–225.

Miller, M.D., Hazuda, D.J., 2004. HIV resistance to the fusion inhibitor enfuvirtide: mechanisms and clinical implications. Drug Resist. Updat. 7 (2), 89–95.

Orlova, M., Yueh, A., Leung, J., Goff, S.P., 2003. Reverse transcriptase of Moloney murine leukemia virus binds to eukaryotic release factor 1 to modulate suppression of translational termination. Cell 115 (3), 319–331.

Park, J., Morrow, C.D., 1992. The nonmyristylated Pr160gag–pol polyprotein of human immunodeficiency virus type 1 interacts with Pr55gag and is incorporated into viruslike particles. J. Virol. 66 (11), 6304–6313.

Prabu-Jeyabalan, M., Nalivaika, E.A., King, N.M., Schiffer, C.A., 2003. Viability of a drug-resistant human immunodeficiency virus type 1 protease variant: structural insights for better antiviral therapy. J. Virol. 77 (2), 1306–1315.

Reed, M., Mariani, R., Sheppard, L., Pekrun, K., Landau, N.R., Soong, N.W., 2002. Chimeric human immunodeficiency virus type 1 containing murine leukemia virus matrix assembles in murine cells. J. Virol. 76 (1), 436–443.

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.

Shinnick, T.M., Lerner, R.A., Sutcliffe, J.G., 1981. Nucleotide sequence of Moloney murine leukaemia virus. Nature 293 (5833), 543–548.

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.

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.

Tsichlis, P.N., 1987. Oncogenesis by Moloney murine leukemia virus. Anticancer Res. 7 (2), 171–180.

Wang, M.Q., Goff, S.P., 2003. Defects in virion production caused by mutations affecting the C-terminal portion of the Moloney murine leukemia virus capsid protein. J. Virol. 77 (5), 3339–3344.

Wills, N.M., Gesteland, R.F., Atkins, J.F., 1991. Evidence that a downstream pseudoknot is required for translational read-through of the Moloney murine leukemia virus gag stop codon. Proc. Natl. Acad. Sci. U.S.A. 88 (16), 6991–6995.

Wills, N.M., Gesteland, R.F., Atkins, J.F., 1994. Pseudoknot-dependent read-through of retroviral gag termination codons: importance of sequences in the spacer and loop 2. EMBO J. 13 (17), 4137–4144.

Yoshinaka, Y., Katoh, I., Copeland, T.D., Oroszlan, S., 1985. Murine leukemia virus protease is encoded by the gag–pol gene and is synthesized through suppression of an amber termination codon. Proc. Natl. Acad. Sci. U.S.A. 82 (6), 1618–1622.

Yuan, B., Campbell, S., Bacharach, E., Rein, A., Goff, S.P., 2000. Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. J. Virol. 74 (16), 7250–7260.