Efficient extension of a misaligned tRNA-primer during replication of the HIV-1 retrovirus

Atze T. Das and Ben Berkhout*

Department of Virology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

ABSTRACT

The human immunodeficiency virus (HIV) and other retroviruses show extensive genomic variation, which is primarily due to error-prone replication by the viral reverse transcriptase (RT) enzymes. RT errors include misincorporation with subsequent extension of the mismatched terminal base, and extension of realigned primer-template duplexes. Whereas both RT-mediated mechanisms have been extensively studied in vitro, almost no in vivo experiments have been performed. In this work, we analyzed the ability of HIV-1 RT to extend a misaligned tRNA\textsuperscript{LyS} primer in vivo. This tRNA binds with its 3'-terminal 18 nt to a complementary sequence in the viral genome, referred to as the primer-binding site (PBS). We constructed a series of mutant viral genomes with small insertions or deletions in the PBS sequence, resulting in misalignment of the tRNA primer. Extension of the misaligned primer did occur with reasonable efficiency for some of the mutants, resulting in reversion to the wild-type viral sequence. The infectivity and reversion frequency of the PBS mutants is therefore a measure of the efficiency of extending a misaligned primer in vivo. Using virion-derived primer-template complexes, we also measured the tRNA-priming efficiency in vitro. The combined results show that HIV-1 RT can elongate a misaligned primer and that the efficiency of primer extension is determined by the extent of the mismatch.

INTRODUCTION

The human immunodeficiency virus (HIV) and other retroviruses exhibit extensive genome variability, with single-base differences, short deletions and insertions making up the majority of sequence variation (1,2). The reported rate at which mutations are generated ranges from $10^{-6}$ to $10^{-4}$ mutations per nucleotide per cycle through a host cell (3,4). Three different replicases operate during the life cycle of a retrovirus. The virally encoded reverse transcriptase (RT) copies the genomic single-stranded RNA into double-stranded DNA, which integrates into the host genome. This integrated provirus is replicated by cellular DNA polymerases, and transcription of the provirus by the cellular RNA polymerase II enzyme results in the production of new viral RNA genomes. Although mutations can be introduced into the retroviral genome during each of the three replicative steps, research has focused mainly on the error-prone RT enzymes. RTs are multifunctional enzymes with RNA- and DNA-dependent DNA-polymerase and ribonuclease H activities. It is generally assumed that the high error rate of RT is caused by the lack of a 3'→5' exonuclease activity (5,6). The absence of proofreading results in the inability to remove misincorporated nucleotides from nascently synthesized DNA strands.

Several models for the introduction of sequence variation have been proposed (reviewed in 7). Two mechanisms for the introduction of base substitutions have been suggested. Such mutations can either result from misincorporation of noncomplementary nucleotides during strand synthesis (8,9) or they can be initiated by slippage of the primer strand. In the latter model, a complementary nucleotide is added to the misaligned primer, followed by realignment of the primer-template strands and subsequent extension of the mismatched 3' end of the primer (10). Two pathways have been proposed for the introduction of deletions and insertions. Such mutations can be mediated by slippage of the primer strand at repetitive template sequences, followed by extension of the terminal base of the misaligned primer (8,11,12). Alternatively, formation of these mutations is initiated by the misincorporation of a non-complementary nucleotide. Subsequent rearrangement of primer-template strands can lead to the formation of a correct terminal base pair. Extension of this realigned primer results in deletion or insertion of one or several nucleotides (13,14). All of these processes may involve both RNA templates present within a retroviral particle. Such strand-transfer reactions have been reported to occur frequently during reverse transcription (reviewed in 15).

Both the extension of mismatched 3' terminal basepairs (16–24) and the extension of misaligned primers (8,21–27) by retroviral RTs have been studied extensively in vitro. Recently, Pulsinelli and Temin (28) demonstrated that spleen necrosis virus RT efficiently extends a primer with mismatched 3' nucleotides in vivo. In this work we analyzed the ability of HIV-1 RT to extend a misaligned primer in vivo. HIV-1 uses tRNA\textsuperscript{LyS} as primer for the reverse transcription of its RNA genome into a double-stranded DNA molecule (Fig. 1). This tRNA binds with its 3'-terminal 18 nt to a complementary sequence in the viral genome, referred to as the primer-binding site (PBS; box A in Fig.

* To whom correspondence should be addressed
between other domains of the tRNA primer and the viral RNA templates. The combined results show that HIV-1 RT can elongate a misaligned primer, although the efficiency of primer extension was found to depend on the extent of the mismatch.

**MATERIALS AND METHODS**

**Cells and viruses**

SupT1 and C8166 T cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37°C and 5% CO2. SupT1 cells were transfected by means of electroporation. Briefly, 5 x 10^6 cells were washed in RPMI with 20% FCS, resuspended in 250 µl RPMI with 20% FCS, mixed with 5 µg DNA in 0.4 cm cuvettes and electroporated at 250 V and 960 µF, followed by resuspension of the cells in RPMI with 10% FCS. Cells were split 1 to 10 every 4 days. HeLa cells and HeLa-CD4 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 5% FCS at 37°C and 5% CO2. These cells were transfected with 30 µg DNA per 75 cm² flask (~ 60% confluency) by the DEAE-dextran method as previously described (37). The infectivity of the viruses was determined by the endpoint titration method (38) using C8166 cells.

**DNA constructs**

The full-length molecular HIV-1 clone pLAI (39) was used to produce wild-type and PBS-mutated viruses. Nucleotide numbers refer to the positions on the genomic RNA transcript, with +1 being the capped G residue. The PBS site (position +182/+199) was deleted. All constructs were verified by sequence analysis.

**Isolation of HIV viral RNA**

Four days after transfection of HeLa cells, the culture medium (20 ml) was centrifuged at 4000 r.p.m. for 30 min to remove cells. The virus-containing supernatant was subsequently centrifuged through 5 ml 15% (w/v) sucrose in DMEM onto 5 ml 65% (w/v) sucrose in a Beckmann SW28 rotor at 27000 r.p.m. for 3 h at 4°C. The virus-containing fraction (~ 5 ml) was diluted with 3 vol. of DMEM and viruses were pelleted by centrifugation through 10 ml 20% sucrose in a Beckmann SW28 rotor at 27000 r.p.m. and 4°C for 3 h. Virions were resuspended in 500 µl 10 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA. Viral RNA was isolated by incubation of viruses with 100 µg/ml proteinase K in the presence of 0.5% SDS at 37°C for 30 min, followed by extraction with phenol–chloroform–isoamylalcohol (25:24:1) and precipitation in 0.3 M Na-acetate pH 5.2 and 70% ethanol at -20°C. The RNA was dissolved in 10 µl 10 mM Tris–HCl pH 8.0, 1 mM EDTA and stored at -20°C.

**Oligo-primer and tRNA-primer extension assays**

In the oligo-primer extension assay 1 µl viral RNA was incubated with 2 pmol oligo primer in 12 µl 83 mM Tris–HCl pH 7.5, 125 mM KCl at 85°C for 2 min and 65°C for 10 min, followed by
cooling to room temperature in ~30 min. The annealed primer was extended by addition of 6 nM 3× RT buffer (9 mM MgCl₂, 30 mM DTT, 150 μg/ml actinomycin D, 30 μM dATP, 30 μM dGTP, 30 μM dTTP and 1.5 μM dCTP), 0.5 μCi [α-32P]dCTP (800 Ci/mmol, 10 μCi/ml) and 5 U recombinant HIV-1 RT (kindly provided by Dr D. Stammers, Wellcome Research Labs, Beckenham, Kent, UK) and incubation at 42 °C for 3 min. After addition of 1 μl dNTP mix (10 mM of each dNTP) incubation was continued at 42°C for 30 min. The cDNA product was precipitated in 25 mM EDTA, 0.3 M Na-acetate pH 5.2 and 70% ethanol at -20°C.

Western blot analysis

Cells were washed once with phosphate-buffered saline and resuspended in reducing SDS sample buffer (50 mM Tris–HCl pH 7.0, 2% SDS, 10% β-mercaptoethanol, 5% glycerol). Proteins were resolved in a 10% SDS–polyacrylamide gel, transferred to Immobilon-P (16 h, 60 V), and subsequently blocked with PBS buffer containing 5% non-fat dry milk, 3% BSA and 0.05% Tween-20. Filters were subsequently incubated with serum of an HIV-1 infected individual (patient H) for 1 h at room temperature, washed, incubated with goat anti-human IgG–alkaline phosphatase conjugate (BioRad) and developed using the BCTP-NBT protocol (Sigma).

Gag-p24 and reverse transcriptase assay

Gag-p24 levels were determined by ELISA (Abbott). RT assays were performed as previously described (40). Each reaction contained 10 μl of virus-sample and 50 μl RT buffer (60 mM Tris–HCl pH 8.0, 75 mM KCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM EDTA, 4 mM DTT) supplemented with 0.25 μg polyA and 8 ng oligo(dT)18 primer and 0.1 μl [α-32P]dCTP (3000 Ci/mmol, 10 μCi/ml). After 2 h at 37°C, 10 μl was spotted onto DE-81 paper, which was washed three times in 5% Na₂HPO₄, once in ethanol and air-dried. RT activity was measured in the linear range of the
assay and radioactive spots were quantitated on a Molecular Dynamics PhosphorImager.

Proviral DNA analysis

Proviral DNA sequences were PCR-amplified from total cellular DNA using the 5' U3 region primer 5'CE (position -111/-91) and the 3' Gag primer SK39 (position +1177/+1204). A HindIII-ClaI fragment (nucleotides +77/+376) was cloned into pSP73 (Promega). Sequence analysis was performed using the Taq DyeDeoxy Terminator cycle sequencing protocol (Applied Biosystems) and an Applied Biosystems 370A DNA sequencer.

RESULTS

Replication potential of PBS-mutated HIV-1

To produce misaligned primer–template structures, the primer binding site (PBS) of the infectious proviral HIV-1 clone pLAI was mutated to varying extent by insertion or deletion of 2 or 4 nt. The possible basepair interactions between the tRNA \( ^{15} \) primer and wild-type (wt) and mutant viral RNA templates (mutants NK, NS, KK and KS) are shown in Fig. 2A. The number of basepairs that can be formed between primer and template, and the size of the gap that has to be introduced for proper annealing of the 3'-end of the tRNA, is indicated for the different viruses. Whereas wild-type HIV-1 RNA can form a perfect 18 bp duplex, the most severe mutant KS can form only 2 + 12 bp, including a 4 nt loop in the primer tRNA. For some of the mutants, alternative basepairing schemes are possible, which are shown on the right hand side (Fig. 2A). For instance, the NK mutant can form either 2 + 16 bp or 6 + 12 bp by changing the position of the two looped-out nucleotides in the vRNA template.

The wild-type and mutant proviral genomes were transfected into SupT1 cells and HeLa-CD4 cells (Fig. 3). Both cell types express the CD4 receptor and are fully susceptible for virus replication. Virus production was monitored by measuring the Gag-p24 level in the culture medium. All constructs produced low levels of Gag-p24 in the 4 days post-transfection, reflecting the ability to produce virus from the transfected HIV plasmids. Upon prolonged culture of cells transfected with the wild-type HIV-1 DNA, the Gag-p24 concentration rapidly increased, resulting from the infection of other cells and subsequent rounds of viral replication. In contrast, the most severe KS mutant showed no further increase in Gag-p24 levels. These results demonstrate that the transiently produced KS-viruses are not replication-competent. The other three mutant viruses showed intermediate phenotypes, with a further increase in virus production after a lag period of varying length. We consistently observed the following order of virus growth in multiple transfection experiments in both SupT1 and HeLa-CD4 cells: wt > NK > NS > KK > KS.

The nucleotide sequence of the viruses present at the peak of Gag-p24 production was determined. For this reason, culture medium was used to infect fresh cells (either SupT1 or HeLa-CD4), cellular DNA was isolated after 3 days, followed by PCR-amplification of HIV-1 proviral DNA sequences. PCR fragments were cloned and the nucleotide sequence of the PBS site was determined for several clones. It appeared that the PBS of the NK, NS and KK progeny had reverted to the wild-type sequence. The PBS mutants can revert to the wild-type sequence in a single round of reverse transcription by annealing of the natural tRNA \( ^{15} \) primer onto the mutant PBS site and proper extension (41,42) (see also Fig. 1). Upon the second strand-transfer, a duplex molecule is formed between a copy of the tRNA \( ^{15} \) primer and a copy of the viral PBS (plus-strand and minus-strand, respectively; box B in Fig. 1). Upon completion of reverse transcription, integration in the host genome and one round of DNA replication, these strands are separated and both wild-type and mutant proviruses are produced (43). Poor
replication of the mutant virus will result in outgrowth of the wild-type virus. The variable lag time, observed before replication of the PBS mutants was evident, indicates that the various mutants differ in their ability to revert. Since reversion is mediated by successful priming with tRNA_Lys^3, the order of reversion reflects the priming potential of the tRNA primer onto the different vRNA templates.

Characterization of transiently produced virions

To further characterize the defect resulting from the altered PBS site, mutant virions were produced by transient transfection of HeLa cells (not expressing the CD4 receptor). Viruses were harvested at day four post-transfection and purified by ultracentrifugation. These viruses contain genomic RNA molecules corresponding to the input proviral genome, i.e. having a wild-type or mutated PBS sequence. The Gag-p24 level and reverse transcriptase (RT) activity of both culture medium and purified virus samples, as well as the observed RT:Gag-p24 ratio, did not vary significantly for the different viruses (Table 1). Small differences in the Gag-p24 and RT levels reflect experimental variation in transfection efficiency. Transient expression of viral proteins was also assayed by Western blot analysis of total HeLa cellular extracts. As shown in Figure 4, transfection with the different constructs resulted in the production of similar levels of viral proteins. These combined data show that there are no differences in virus production, indicating that the PBS mutants are not affected in transcription, translation and virion assembly. To quantify the replication defect of the PBS-mutated virions, we determined the infectivity of the transiently produced viruses by the endpoint titration method. As shown in Table 2, the infectivity of the NK mutant was ~32% of that of the wild-type virus, that of the NS mutant was only ~1% of the wild-type level, whereas the infectivity of the KK and KS mutants was below the level of detection. As expected, the infectivity of the different viruses corresponds well with the order of reversion as observed in the previous experiment (Fig. 3).

Table 1. Virus production upon transfection of wild-type and mutated HIV-1 constructs into HeLa cells

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>NK</th>
<th>NS</th>
<th>KK</th>
<th>KS</th>
<th>mock</th>
<th>RT^-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag-p24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ng/ml</td>
<td>43</td>
<td>18</td>
<td>39</td>
<td>6</td>
<td>19</td>
<td>&lt;</td>
<td>34</td>
</tr>
<tr>
<td>RT (x10^5/ml)</td>
<td>770</td>
<td>391</td>
<td>774</td>
<td>101</td>
<td>395</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>RT/Gag-p24 (ng/ml)</td>
<td>18</td>
<td>22</td>
<td>20</td>
<td>17</td>
<td>21</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Pelleted virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gag-p24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ng/ml</td>
<td>233</td>
<td>91</td>
<td>222</td>
<td>40</td>
<td>111</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>RT (x10^5/ml)</td>
<td>13500</td>
<td>4855</td>
<td>12780</td>
<td>1365</td>
<td>6925</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>RT/Gag-p24 (ng/ml)</td>
<td>58</td>
<td>53</td>
<td>58</td>
<td>34</td>
<td>62</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

*Samples were taken at 4 days post-transfection.
|    | Virions were isolated from the culture supernatant by ultracentrifugation.
|    | <c> not detectable.

Table 2. Characterization of wild-type and mutant virions

<table>
<thead>
<tr>
<th>Order of reversion</th>
<th>Infectivity</th>
<th>Viral RNA level</th>
<th>tRNA priming efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID50 (pg Gag-p24)</td>
<td>Inf. units (pg Gag-p24)</td>
<td>vRNA/Gag-p24 (%)</td>
</tr>
<tr>
<td>wild-type</td>
<td>-</td>
<td>10^5</td>
<td>100</td>
</tr>
<tr>
<td>NK</td>
<td>1</td>
<td>10^3</td>
<td>1651 (100)</td>
</tr>
<tr>
<td>NS</td>
<td>2</td>
<td>10^4</td>
<td>521 (32)</td>
</tr>
<tr>
<td>KK</td>
<td>3</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>KS</td>
<td>4</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>mock</td>
<td>-</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>RT^-</td>
<td>-</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

*As determined in Figure 3.

aWild-type and mutant virions were produced by transfection of HIV-1 constructs into HeLa cells (see Table 1). Serial dilutions of the culture supernatant were used to infect C8166 cells and the ID50 and number of infectious units was determined according to Reed and Muench (38).

bViral RNA content and tRNA priming efficiency were determined by quantification of the cDNA products in Figure 5.

cThe Gag-p55 precursor protein of the RT^- virus is only partially processed (not shown), resulting in a relatively low level of Gag-p24 protein and a relatively high vRNA/Gag-p24 ratio.
Next, we analyzed vRNA–tRNA complexes in the PBS-mutated virions. First, vRNA was isolated from the purified virions by phenol–chloroform extraction and quantitated by primer extension using an oligonucleotide complementary to position 123–151 (Fig. 5A). The extended cDNA product (151 nt) was quantitated using a PhosphorImager and compared to the amount of input virus (Gag-p24 level or RT activity). Table 2 lists these relative vRNA levels for wild-type and mutant virions. This analysis demonstrates that the genomic RNA content of the wild-type and PBS-mutated viruses does not vary significantly, indicating that the PBS mutations do not affect viral RNA packaging. Secondly, we determined the priming efficiency of the natural tRNA<sup>lys</sup> primer on the wild-type and mutant vRNA templates. This primer remains bound to the genomic RNA upon extraction from virions, and extension of this primer can be measured upon addition of reverse transcriptase and dNTPs (Fig. 5B). The size of the extended product was as expected for a tRNA-primed cDNA (76 nt tRNA<sup>lys</sup> plus 181 nt cDNA = 257 nt). The identity of this band was confirmed by NaOH-mediated degradation of the RNA part, leaving a cDNA of 181 nt (Fig. 5B, compare lanes 1 and 2). The products of this tRNA-primer extension assay were quantitated using a PhosphorImager and compared to the amount of input viral RNA (Table 2). We measured reduced tRNA-extension efficiency for the NK-mutant (19%) compared to the wild-type template, whereas no extended products were detected for all other PBS mutants. This efficiency of cDNA synthesis correlates well with the observed infectivity/reversion of the different viruses.

Reduced cDNA production as observed for the PBS mutants may result from decreased binding of the tRNA primer onto the PBS. Alternatively, normal levels of tRNA are bound, but these primers are not efficiently extended due to incomplete basepairing of the 3' end of the tRNA with the mutant vRNAs. To discriminate between these two possibilities, we determined the tRNA-primer occupancy of the wild-type and mutant PBS sites. As a control, we used a virus lacking any tRNA primer. For this reason, we constructed one additional mutant with an almost complete deletion of the RT domain of the Gag–Pol fusion protein (RT<sup>−</sup> mutant). Transiently produced RT<sup>−</sup> virions contain normal levels of vRNA (Fig. 5A, lane 7), but when this vRNA template was used in the tRNA-primer extension assay, hardly any cDNA product was detected (Fig. 5B, lane 9; 2% of the wild-type production, only visible upon prolonged exposure of the autoradiograph). This result shows that the tRNA-primer occupancy of the PBS is strongly reduced in RT-lacking virions, in concordance with the proposed role of RT in the selective packaging of tRNA<sup>lys</sup> into virions (44).

To determine the tRNA-primer occupancy for the PBS-mutated viruses, the viral RNA–tRNA complexes were used as template for extension of oligonucleotide primers complementary to sequences 3' of the PBS (Fig. 6B and C). The cDNA products were quantitated using a PhosphorImager and corrected for the amount of input viral RNA as determined in a primer extension assay using an oligonucleotide complementary to sequences 5' of the PBS (Fig. 6A). The primer-extension efficiencies of the wild-type and PBS-mutated viruses were then compared with the corresponding efficiencies of the tRNA-lacking RT<sup>−</sup> mutant (Table 3). Extension of the downstream primers is reduced to ~33% for the wild-type vRNA–tRNA complex compared to the tRNA-less vRNA of the RT<sup>−</sup> mutant, suggesting that ~67% of the wt PBS sites are occupied by tRNA primer. However, we cannot exclude that associated tRNA primers are partially lost during RNA isolation or displaced in the primer extension assay, resulting in an underestimation of the tRNA occupancy. Most importantly, we observed a similar reduction in the extension efficiency of downstream primers for the PBS-mutated vRNAs, although the extent of this reduction was smaller for some of the mutants. These results indicate that the PBS site of the wt and PBS-mutated vRNAs is occupied by tRNA primer, although the level of occupancy varies from ~67%
We therefore produced viral genomes with small in vivo. The downstream quantified using a PhosphorImager. The primer upstream of the PBS, C(N1), was used to determine the input viral RNA level, which varied due to variation in transfection efficiency and viral RNA recovery (Fig. 6A). The additional bands observed in (B), lane 4 and 5 (NK and wild-type, respectively), represent the 257 nt tRNA-extended products, as were already seen in the tRNA-primer extension (Fig. 5B, lane 6 and 7).

![Figure 6](image)

<table>
<thead>
<tr>
<th>Relative cDNA production*</th>
<th>AUG primer (%)</th>
<th>SD primer (%)</th>
<th>mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>31</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>NK</td>
<td>25</td>
<td>42</td>
<td>34</td>
</tr>
<tr>
<td>NS</td>
<td>55</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td>KK</td>
<td>38</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>KS</td>
<td>54</td>
<td>69</td>
<td>62</td>
</tr>
<tr>
<td>mock</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>RT&lt;sup&gt;-&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* cDNA products from the primer extension assays shown in Figure 6 were quantified using a PhosphorImager. The primer upstream of the PBS, C(N1), was used to determine the input viral RNA level, which varied due to variation in transfection efficiency and viral RNA recovery (Fig. 6A).

**DISCUSSION**

In this study we analyzed the ability of HIV-1 RT to extend the natural tRNA<sup>18s</sup> primer when misaligned on a mutated HIV-1 genome in vivo. We therefore produced viral genomes with small insertions or deletions in the primer-binding site (PBS; Fig. 2). These mutations affected neither the synthesis of viral RNA or proteins, nor the production of virions, as was observed upon transfection of mutant HIV-1 proviral genomes into cells. The replication potential of the PBS-mutated viruses, however, was significantly reduced. We measured decreased infectivity and delayed replication of these viruses.

Upon prolonged replication, the progeny of the mutant viruses had reverted to the wild-type PBS sequence. This reversion is thought to be mediated by a tRNA<sup>Lys</sup> molecule that successfully primed onto the mutant PBS sequence. The model of reverse transcription predicts that the 18 nt at the 3' end of the tRNA primer will be copied into the cDNA, resulting in a wild-type PBS sequence in the proviral genome (Fig. 1). The time it took for the appearance of revertant viruses correlated well with the infectivity of the mutant viruses, showing that reversion is dependent on replication, in accordance with the proposed reversion mechanism.

Correct priming of tRNA<sup>Lys</sup> on the mutant PBS sites is possible by loop out of nucleotides in either the primer or template strand (Fig. 2). Alternatively, when the tRNA primer is not realigned with the vRNA template, the mismatched 3' end of the primer can be extended. The latter scenario will result in deletion or insertion of nucleotides directly upstream of the PBS site, which we did not observe in the progeny viruses. It is possible, however, that viruses with minor sequence alterations upstream of the PBS site are (partially) replication incompetent, explaining their absence in the progeny viruses. In vitro studies, however, demonstrated a similar preference of RT enzymes to extend a realigned primer with a basepaired 3' end (26).

**In vitro** experiments using virion derived vRNA-tRNA complexes, showed that the reduced replication potential correlated with a decreased priming efficiency of the PBS mutants. We showed that this resulted from a less efficient extension of the misaligned tRNA primer on the mutant viral RNA template, rather than from the absence of this primer. We therefore believe that both the viral infectivity and the reversion rate of the PBS-mutants reflect the in vivo extension efficiency of a misaligned tRNA primer-vRNA template complex.

The PBS mutations may affect not only the initiation of reverse transcription, but also other stages of reverse transcription. In particular, a basepairing defect is predicted to occur during the second jump, in which a DNA copy of the wild-type tRNA primer hybridizes to a DNA copy of the PBS site (Fig. 1, duplex B). This situation is schematically depicted in Figure 2B for the NK mutant. The major difference with the vRNA-tRNA initiation complex (duplex A in Fig. 1) is that the second-jump product will be extended in both directions. Because the duplex will have ≥12 consecutive basepairs at the 3' end of the tRNA-derived cDNA, this end will be extended efficiently (plus strand synthesis). As a result, this duplex will be stabilized considerably, which may improve the extension of the minus strand. Therefore, we believe that the predominant effect of the PBS mutations is at the level of initiation of reverse transcription, which is consistent with the in vitro primer extension assays.

Characteristics of the tRNA-vRNA complex that may affect the efficiency of productive tRNA-priming are: (i) the total number of basepairs in the duplex, (ii) the number of non-basepaired nucleotides and (iii) the number of basepairs at the 3' end of the tRNA primer (Fig. 2). The total number of basepairs and the size of the bulge are expected to affect the amount of tRNA primer bound to the PBS site. Indeed, a reasonable correlation
between these parameters (bp total/bulge size: wild-type 18/0, NK 18/2, KK 18/4, NS 16/2, KS 14/4) and tRNA-primer occupancy of the PBS sites (Table 3: wild-type > NK > KK > NS > KS) was observed. The extension efficiency of a misaligned primer may be particularly sensitive to the number of 3' terminal basepairs and the number of non-basepaired nucleotides. Indeed, priming efficiency and replication of the viruses (wild-type > NK > NS > KK > KS) is correlated with decreased bulge size (NK 2, NS 4, KK 4, KS 4 nt) and increased number of 3' terminal basepairs (NK 6, NS 4, KK 6, KS 2 bp).

Finally we measured rather efficient extension of the tRNA-ys3 primer on the least severe mutant, NK. Our in vitro and in vivo data indicate that this mutant PBS site is utilized with an efficiency of 20–30% compared to the wild-type PBS site. Several in vitro studies have also demonstrated the ability of RT enzymes to accept misaligned primers (8,21-27). These combined results strongly suggest that this mechanism can play an important role in the generation of genomic variation by the error-prone RT replicases of retroviruses.

ACKNOWLEDGEMENTS

We thank Albert van Wijk for help in the Gag-p24 ELISA, Wilman Est for photography work and Dr D. Stammers (Wellcome Research Labs, UK) for making the HIV-1 RT enzyme available to us (MRC AIDS Reagent Project ADP631.1). This work was supported by the Dutch Cancer Society (KWF).

REFERENCES