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Reduced Replication of Human Immunodeficiency Virus Type 1 Mutants That Use Reverse Transcription Primers other than the Natural $tRNA_3^{Lys}$

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Replication of the human immunodeficiency virus type 1 (HIV-1) and other retroviruses involves reverse transcription of the viral RNA genome into a double-stranded DNA. This reaction is primed by the cellular $tRNA_3^{Lys}$ molecule, which binds to a complementary sequence in the viral genome, referred to as the primer-binding site (PBS). In order to study the specificity of primer usage, we constructed a set of HIV-1 mutants with altered PBS sites corresponding to other tRNA species ($tRNA^{Ile}$, $tRNA_{1,2}^{Lys}$, $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Trp}$). These mutant viruses were able to replicate, although with delayed replication kinetics compared with wild-type HIV-1. Identification of the tRNA species associated with the genomic RNA demonstrated binding of tRNAs complementary to the new PBS sites. However, the occupancy of the mutant PBS sites by these new primers was reduced and correlated well with the replication potential of the mutant viruses. These results suggest that the PBS sequence is not sufficient for annealing of the tRNA primer. Upon prolonged culturing, all mutants reverted to the wild-type PBS_3^{Lys} sequence. Minor sequence changes in the nucleotides flanking the PBS site indicate that these reversions resulted from annealing of the wild-type $tRNA_3^{Lys}$ primer onto the mutant PBS sites, followed by copying of part of the $tRNA_3^{Lys}$ sequence during reverse transcription. Furthermore, the reversion efficiency of the different PBS mutants was found to correlate with their $tRNA_3^{Lys}$ binding capacity. A remarkable reversion pathway was observed for the PBS^{Pro} variant ($PBS^{Pro} \rightarrow PBS^{Ile} \rightarrow PBS^{wt}$). This pathway can be explained by efficient base pairing of $tRNA^{Ile}$ to PBS^{Pro}, followed by annealing of $tRNA_3^{Lys}$ onto the PBS^{Ile} intermediate. These results demonstrate that HIV-1 is dedicated to the $tRNA_3^{Lys}$ primer and that factors other than the PBS sequence determine the selective primer usage of this retrovirus.

The replication cycle of retroviruses involves reverse transcription of the viral RNA (vRNA) genome into a double-stranded DNA, which then becomes integrated into the host cell genome (reviewed in reference 29). This process is mediated by the virion-associated enzyme reverse transcriptase (RT), and a cellular tRNA is used as primer (Fig. 1). This tRNA binds with its 3′-terminal 18 nucleotides (nt) to a complementary sequence in the viral genome, referred to as the primer-binding site (PBS). Although retroviral particles contain a subset of cellular tRNAs, only the tRNA primer is found in tight association with the vRNA (16, 22). Apart from the complementarity between the PBS and tRNA sequences, additional base pairing interactions between the tRNA primer and vRNA template may support this binding (1, 2, 6, 14, 17). Furthermore, in vitro studies showed that both the RT and nucleocapsid protein can activate tRNA-vRNA binding (3, 4, 25). The RT protein, or the precursor Gag-Pol protein, was also suggested to be involved in selective encapsidation of the tRNA primer. First, in vitro studies demonstrated a specific interaction of the priming tRNA species with the RT protein (4, 13, 27), although nonspecific tRNA-RT binding has been reported by others (11, 17, 28). Second, selective packaging of tRNAs was shown to be affected in virions lacking a functional RT domain (19, 22, 24). For instance, virion particles containing the Gag-Pol and Gag precursor proteins contained the wild-type (wt) tRNA subset, whereas particles containing only the Gag precursor protein did not (22). Protease-deficient virions also showed a wt tRNA content, demonstrating that processing of the viral precursor proteins is not necessary for correct tRNA packaging (22). These combined results suggest that the RT domain of the Gag-Pol precursor protein mediates the selection of tRNAs that are packaged in virion particles.

The various retroviruses utilize different tRNA primers (reviewed in reference 9). The avian retroviruses utilize $tRNA^{Trp}$, whereas the majority of mammalian retroviruses utilize $tRNA^{Pro}$ (e.g., human T-cell leukemia viruses types 1 and 2 and murine leukemia viruses). However, the human immunodeficiency viruses (human immunodeficiency virus type 1 [HIV-1] and HIV-2), all simian immunodeficiency viruses, and the mouse mammary tumor virus utilize $tRNA_3^{Lys}$, while $tRNA_{1,2}^{Lys}$ is used by Mason-Pfizer monkey virus, visna virus, and spuma retroviruses. As discussed above, it is generally believed that the RT protein plays a critical role in the selective encapsidation of the tRNA primer and the binding of this tRNA to the vRNA. Therefore, retroviral RT enzymes may have evolved to selectively bind the priming tRNA species. However, recent evidence indicates that retroviral reverse transcription can be initiated with primers other than the natural tRNA primer. First, Lund et al. (21) demonstrated efficient transduction of an Akv murine leukemia virus-based vector with mutant PBS sites corresponding to $tRNA_1^{Gln}$, $tRNA_2^{Gln}$, or $tRNA_3^{Lys}$, instead of the wt PBS corresponding to $tRNA^{Pro}$. This transduction assay, however, scores for only one round of reverse transcription. Second, Colicelli and Goff (9) isolated a replication-competent revertant with an altered PBS identity from a replication-deficient Moloney murine leukemia virus. This revertant was formed by recombination with endo-

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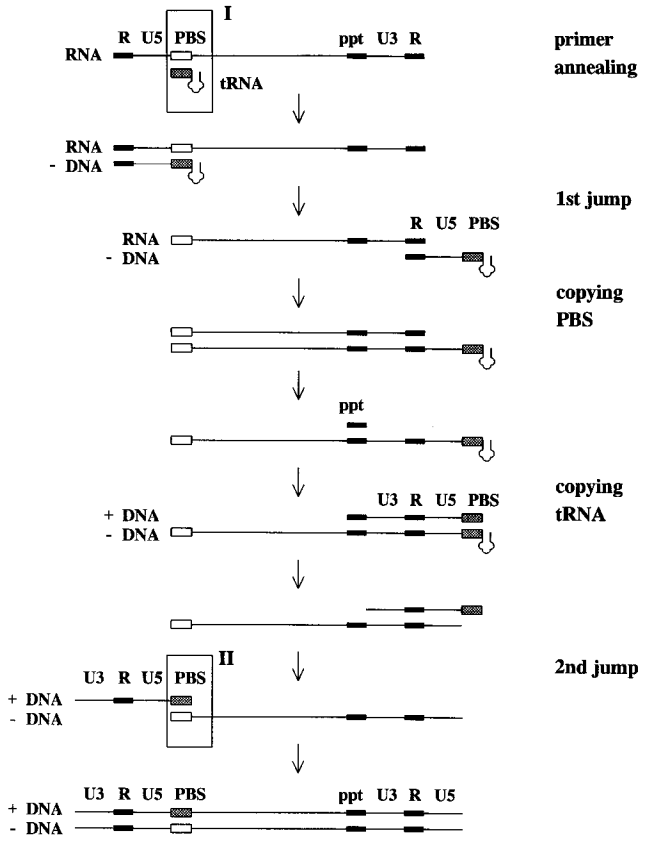


FIG. 1. Reverse transcription of the retroviral genome. The tRNA primer anneals to the PBS (duplex I) and is extended by RT with the genomic RNA as template. Upon copying of the repeat (R) sequence, the minus-strand strong-stop DNA translocates to the 3′-R sequence at the 3′ end of the vRNA. After this first strand transfer, a nearly full-length minus-strand cDNA is synthesized, which contains a copy of the PBS sequence. The template RNA is subsequently degraded by the RNase H activity of the RT enzyme. Incomplete degradation at the polypurine tract of the vRNA provides a primer for plus-strand strong-stop DNA synthesis. Plus-strand cDNA synthesis copies the 3′ end of the tRNA primer. Complementarity between the minus-strand and plus-strand copies of the PBS sequence mediates the translocation of the plus-strand strong-stop DNA to the 3′ end of the minus-strand cDNA (duplex II). Upon this second strand transfer, both cDNA strands are completed, and the resulting proviral DNA is integrated into the host cell genome.

genous retroviral sequences in the host genome, which included a PBS sequence complementary to $tRNA_1^{Gln}$ instead of $tRNA^{Pro}$. These results suggest that murine retroviruses can efficiently replicate with other tRNA primers. In order to test the ability of HIV-1 to replicate with tRNA primers other than the natural $tRNA_3^{Lys}$, we replaced the PBS by sequences complementary to other tRNAs, including primers used by other retroviruses ($tRNA_{1,2}^{Lys}$, $tRNA^{Pro}$, and $tRNA^{Trp}$) but also $tRNA^{Ile}$ and $tRNA^{Phe}$. All mutant viruses showed low levels of viral replication. Upon prolonged culturing, however, all mutants reverted to the wt PBS sequence. These results suggest that HIV-1 prefers to use the $tRNA_3^{Lys}$ primer and that factors other than the PBS sequence specify this selective primer usage.

MATERIALS AND METHODS

Cells and viruses. SupT1 T cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37°C and 5% CO_2. These cells were transfected by means of electroporation. Briefly, 5×10^6 cells were washed in RPMI medium with 20% FCS, resuspended in 250 μl of RPMI medium with 20% FCS, mixed with 5 μg of DNA in 0.4-cm cuvettes, electroporated at 250 V and 960 μF, and resuspended in RPMI medium with 10% FCS. Cells were split 1 to 10 every 4 days. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 5% FCS at 37°C and 5% CO_2. These cells were transfected with 30 μg of DNA per 75-cm^2 flask (approximately 60% confluency) by the DEAE-dextran method as previously described (7).

DNA constructs. The full-length molecular HIV-1 clone pLAI (a generous gift of Keith Peden [23]) was used to produce wt and PBS-mutated viruses. Nucleotide numbers refer to the positions on the genomic RNA transcript, with +1 being the capped G residue. For mutation of the PBS sequence, an *Xba*I-*Cla*I fragment encompassing the 5′ long terminal repeat, PBS, and 5′ end of the *gag* gene (nt −454 to +376) was cloned in pBluescript (Stratagene) as previously described (Blue-5′LTR [5]). The PBS sequence in this plasmid was modified by oligonucleotide-directed in vitro mutagenesis (18) with a Muta-Gene In Vitro Mutagenesis Kit (Bio-Rad). Oligonucleotides used are PBS^{Ile}, CTCTAGCAG**T GGTGGCCCGTACGGGGA**TTGAAAGCGAAAGGG; $PBS_{1,2}^{Lys}$, GCAG**TGGC GCCCAACGTGGGGC**TTGAAAGCGAAAGGG; PBS^{Phe}, CTCTAGCAG**TG GTGCCGAAACCCGGGA**TTGAAAGCGAAAGGG; PBS^{Pro}, CTCTAGCAG**T GGGGGCTCGTCCGGGAT**TTGAAAGCGAAAGGG; PBS^{Trp}, CTCTAGCA G**TGGTGACCCCGACGTGAT**TTGAAAGCGAAAGGG; and PBS^{-}, GGAA AATCTCTAGCAG▲TTGAAAGCGAAAGGG. PBS-mutated *Xba*I-*Cla*I fragments were subsequently introduced back into the pLAI molecular clone. All mutations were verified by sequence analysis.

Isolation of HIV vRNA. Four days after transfection of HeLa cells, the culture medium (20 ml) was centrifuged at 4,000 rpm for 30 min to remove cells. The virus-containing supernatant was subsequently centrifuged through 5 ml of 15% (wt/vol) sucrose in Dulbecco's modified Eagle's medium onto 5 ml of 65% (wt/vol) sucrose in a Beckman SW28 rotor at 27,000 rpm for 3 h at 4°C. The virus-containing fraction (approximately 5 ml) was diluted with 3 volumes of Dulbecco's modified Eagle's medium, and viruses were pelleted by centrifugation through 10 ml of 20% sucrose in a Beckman SW28 rotor at 27,000 rpm and 4°C for 3 h. Virions were resuspended in 500 μl of 10 mM Tris-HCl (pH 8.0)–100 mM NaCl–1 mM EDTA. vRNA was isolated by incubation of viruses with 100 μg of proteinase K per ml in the presence of 0.5% sodium dodecyl sulfate (SDS) at 37°C for 30 min, followed by extraction with phenol-chloroform-isoamyl alcohol (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 of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and stored at −20°C.

Oligonucleotide-primer and tRNA-primer extension assays. In the oligonucleotide-primer extension assay, 1 μl of vRNA was incubated with 20 ng of oligonucleotide primer in 12 μl of 83 mM Tris-HCl (pH 7.5)–125 mM KCl at 85°C for 2 min and 65°C for 10 min and then cooled to room temperature in approximately 30 min. The annealed primer was extended by addition of 6 μl of 3× RT buffer (9 mM $MgCl_2$, 30 mM dithiothreitol, 150 μg of actinomycin D per ml, 30 μM dATP, 30 μM dGTP, 30 μM dTTP, 1.5 μM dCTP), 0.5 μl of [α-^{32}P]dCTP (800 Ci/mmol, 10 mCi/ml), and 5 U of recombinant HIV-1 RT (kindly provided by D. Stammers, Wellcome Research Laboratories, Beckenham, Kent, United Kingdom) and incubation at 42°C for 3 min. After addition of 1 μl of deoxynucleoside triphosphate (dNTP) mix (10 mM 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)–70% ethanol at −20°C. The pellet was resuspended in formamide loading buffer, heated at 85°C for 3 min, and analyzed on a denaturing 6% polyacrylamide–urea sequencing gel. The primer C(N1) is complementary to nt +123 to +151. Assays were performed in the linear range, and products were quantitated with a PhosphorImager (Molecular Dynamics).

A similar protocol was used in the tRNA-primer extension assay, except that 2 μl of vRNA was used, no oligonucleotide primer was added, and the heat-denaturation step was omitted. For the degradation of the tRNA part of the extended product, samples were incubated with 0.45 N NaOH for 20 min at 55°C and then the cDNA product was precipitated in 25 mM EDTA–0.3 M Na-acetate (pH 5.2)–70% ethanol at −20°C.

Western blot (immunoblot) 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 an SDS–10% polyacrylamide gel, transferred to Immobilon-P (16 h, 60 V), and subsequently blocked with phosphate-buffered saline buffer containing 5% nonfat dry milk, 3% bovine serum albumin, 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 immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad), and developed with the BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium) protocol (Sigma).

Gag-p24 and RT assay. Gag-p24 levels were determined by enzyme-linked immunosorbent assay (ELISA) (Abbott). RT assays were performed as previously described (31). Each reaction contained 10 μl of virus sample and 50 μl of RT buffer (60 mM Tris-HCl [pH 8.0], 75 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM EDTA, 4 mM dithiothreitol) supplemented with 0.25 μg of poly(A) and 8 ng of oligo(dT)18 primer and 0.1 μl of [α-^{32}P]dTTP (3,000 Ci/mmol, 10 mCi/ml). After 2 h at 37°C, 10 μl was spotted onto DE-81 paper, which was washed three times in 5% Na_2HPO_4 and 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.

TABLE 1. PBS region of wt and mutated vRNA

vRNA	PBS (in capital letters)	Used by[a]:
Lys-3	ucucuagcagUGGCGCCCGAACAGGGACuugaaagcga	HIV, SIV, MMTV
Ile	ucucuagcagUGGUGGCCCGUACGGGGAuugaaagcga	
Lys-1,2	ucucuagcagUGGCGCCCAACGUGGGGCuugaaagcga	MPMV
Phe	ucucuagcagUGGUGCCGAAACCCGGGAuugaaagcga	
Pro	ucucuagcagUGGGGGCUCGUCCGGGAUuugaaagcga	HTLV, MLV
Trp	ucucuagcagUGGUGACCCCGACGUGAUuugaaagcga	AMV
PBS$^-$	ucucuagcag----------uugaaagcga	

[a] HIV, human immunodeficiency viruses; SIV, simian immunodeficiency viruses; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; HTLV, human T-cell leukemia viruses; MLV, murine leukemia viruses; AMV, avian myeloblastosis virus.

Proviral DNA analysis. Proviral DNA sequences were PCR amplified from total cellular DNA with the 5′ U3 region primer 5′CE (positions −111 to −91) and the 3′ Gag primer SK39 (positions +1177 to +1204). A *Hin*dIII-*Cla*I fragment (nt +77 to +376) was cloned into pSP73 (Promega). Sequence analysis was performed with the Taq DyeDeoxy Terminator cycle sequencing protocol (Applied Biosystems) and an Applied Biosystems 370A DNA sequencer.

RESULTS

Replication potential of PBS-mutated HIV-1 viruses. To test whether HIV-1 can replicate with primers other than $tRNA_3^{Lys}$, we replaced the wt PBS site of the infectious plasmid pLAI by sequences complementary to the 3′ end of $tRNA^{Ile}$, $tRNA_{1,2}^{Lys}$, $tRNA^{Phe}$, $tRNA^{Pro}$, or $tRNA^{Trp}$ (Table 1). One additional HIV-1 construct with a deletion of the 18-nt PBS was constructed (PBS$^-$). To study the replication potential of the mutant viruses, these proviral genomes were transfected into SupT1 cells. SupT1 cells 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 (Fig. 2A). Some Gag-p24 production was observed for all mutants in the first 3 days posttransfection, reflecting the ability to express virus from the transfected plasmids. Upon prolonged culture of cells transfected with wt HIV-1 plasmid, the Gag-p24 concentration rapidly increased, resulting from the infection of other cells and subsequent rounds of viral replication. In contrast, the deletion mutant PBS$^-$ showed no further increase in virus production, and Gag-p24 levels gradually decreased in the second week posttransfection. This result demonstrates that the transiently produced PBS$^-$ virions are not replication competent. The PBS-replacement mutants showed intermediate phenotypes, with a further increase in virus production after a lag period of variable length. The observed order for the replication potential was $PBS^{wt} > PBS^{Ile} > PBS_{1,2}^{Lys}$, $PBS^{Pro} > PBS^{Phe}$, $PBS^{Trp} > PBS^-$.

At 5 days posttransfection, all PBS variants except the PBS$^-$ deletion mutant had replicated to some extent (Fig. 2A). Equal amounts of these virions, as based on Gag-p24 levels, were used to infect fresh SupT1 cells, and virus was allowed to replicate for several weeks (Fig. 2B). Upon infection, the wt virus showed rapid virus production, whereas the $PBS_{1,2}^{Lys}$, PBS^{Pro}, PBS^{Phe}, and PBS^{Trp} mutants showed an increase in virus level only after a lag period of varying extent. Thus, the replication rate of these mutants was significantly reduced compared with the wt virus. Furthermore, the order of replication potential was similar to that initially observed upon

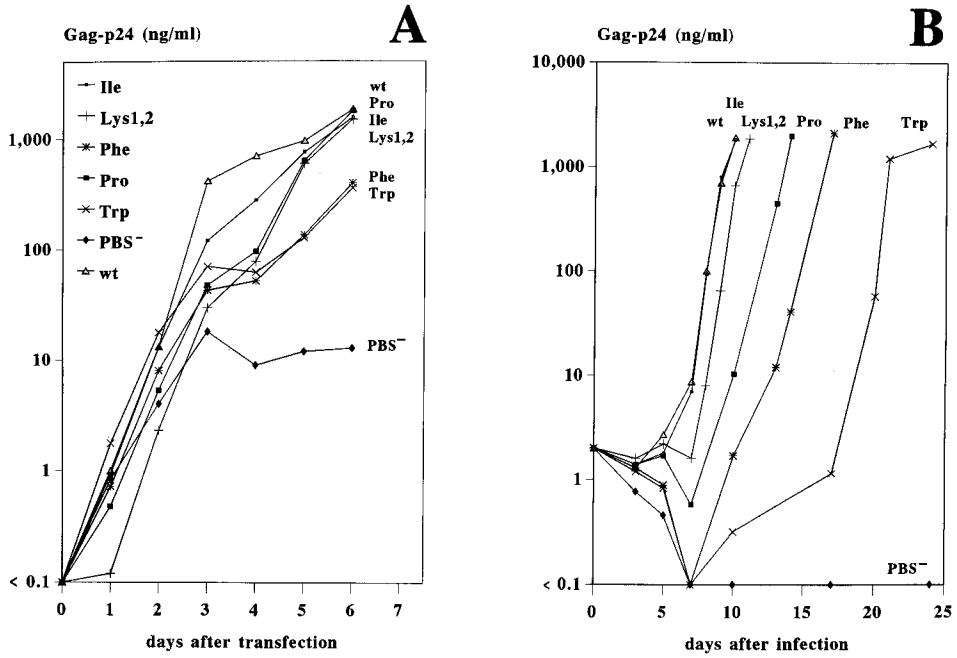


FIG. 2. Replication of wt and PBS-mutated HIV-1 viruses. SupT1 cells were transfected with wt and PBS-mutated proviral constructs (A). Virions were harvested at day 5 posttransfection, and equal amounts (corresponding to 10 ng of Gag-p24) were used to infect fresh SupT1 cells (B). Viruses were allowed to replicate for several weeks, and virus-associated Gag-p24 production was measured in the culture supernatant at several time points.

TABLE 2. Proviral PBS sequences upon replication of wt and mutant viruses

PBS input	Day[a]	PBS progeny	PBS[b]	Frequency
Lys-3	9	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	2/2
Ile	9	Lys-3	tctctagcagTGGCGCCCGAACAGGGAC▲tgaaagcga	3/5
		Lys-3a	tctctagcagTGGCGCC**T**GAACAGGGAC▲tgaaagcga	1/5
		Lys-3	tctctagcagTGGCGCCCGAACAGGGAC**a**tg▲aagcga	1/5
Lys-1,2	10	Lys-1,2	tctctagcagTGGCGCCCAACGTGGGGCttgaaagcga	5/5
	17	Lys-1,2	tctctagcagTGGCGCCCAACGTGGGGCttgaaagcga	3/5
		Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	2/5
	31	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	3/4
		Lys-3a	tctctagcagTGGCGCC**T**GAACAGGGACttgaaagcga	1/4
	49	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	5/5
Phe	14	Lys-3	tctctagcagTGGCGCCCGAACAGGGAC▲tgaaagcga	4/5
		Lys-3a	tctctagcagTGGCGCC**T**GAACAGGGAC▲tgaaagcga	1/5
Pro	13	Pro	tctctagcagTGGGGGCTCGTCCGGGATttgaaagcga	3/5
		Ile	tctctagcagTGGTGGCCCGTACGGGGA**t**ttgaaagcg	2/5
	24	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	5/5
	28	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	5/5
	49	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	5/5
Trp	21	Lys-3	tctctagcagTGGCGCCCGAACAGGGACttgaaagcga	4/4

[a] Days postinfection (see Fig. 2B).

[b] Nucleotide sequences from positions +1 to +381 were determined. The PBS sequence (positions +182 to +199) is shown in uppercase letters; the surrounding sequences are shown in lowercase letters. Nucleotides differing from the input or wt sequence are indicated in boldface and underlined (▲ = deletion). Some additional nucleotide substitutions were observed in the sequenced region of individual clones, which may reflect RT or PCR errors.

transfection. These results suggest that at 5 days posttransfection these virions still contained their input PBS sequence. It therefore seems likely that HIV-1 is able to replicate by using $tRNA^{Lys}_{1,2}$, $tRNA^{Pro}$, $tRNA^{Phe}$, or $tRNA^{Trp}$ as reverse transcription primer, although at a reduced rate compared with wt HIV-1. Only the PBS^{Ile} progeny showed replication kinetics similar to that of wt upon infection (Fig. 2B). This result was somewhat unexpected because we observed a delayed replication phenotype upon transfection (Fig. 2A). Thus, the PBS^{Ile} progeny had either reverted to a wt PBS sequence or adapted by other means such that the replication potential was increased to the wt level (see below).

Reversion of the PBS region upon prolonged replication. A PBS mutant can revert to the wt sequence in a single round of reverse transcription by annealing of the natural $tRNA^{Lys}_3$ primer onto the mutant PBS sequence (duplex I in Fig. 1). Upon the second strand transfer, a duplex molecule is formed between a copy of the $tRNA^{Lys}_3$ primer and a copy of the viral PBS (plus strand and minus strand, respectively, in duplex II). Upon completion of reverse transcription, integration in the host genome, and one round of DNA replication, these strands are separated and both wt and PBS-mutated proviruses are produced (8, 10, 26). Poor replication of the mutant virus will eventually result in outgrowth of the wt virus. We therefore determined the genotype of the progeny virions. At the peak of SupT1 infection (shown in Fig. 2B), total cellular DNA was isolated and HIV-1 DNA was PCR amplified and cloned. The nucleotide sequence of the PBS was determined for several clones. As shown in Table 2, the progeny of the PBS^{Ile}, PBS^{Phe}, and PBS^{Trp} mutants had reverted to the wt PBS sequence. Apparent outgrowth of wt virus indicates that reversion had occurred and confirms that HIV-1 replication is more efficient with the natural $tRNA^{Lys}_3$ primer than with $tRNA^{Ile}$, $tRNA^{Phe}$, or $tRNA^{Trp}$.

Interestingly, the wt revertants of the $tRNA^{Ile}$ and $tRNA^{Phe}$ variants contained minor sequence alterations immediately downstream of the PBS site (Table 2). Indeed, $tRNA^{Lys}_3$-mediated reversion of the PBS^{Ile} and PBS^{Phe} mutants is expected to result in deletion of 1 nt directly downstream of the PBS due to realignment of nucleotides in duplex II (Fig. 3A and 3C, respectively). The wt progeny of the PBS^{Trp} virus did not show any additional modifications (Table 2), which is also consistent with the proposed reversion mechanism (Fig. 3D). One of the PBS^{Ile}-derived clones contained a ttga- to -atg mutation directly downstream of the PBS. Although we cannot explain how this mutation was generated, its position suggests that it also resulted from misalignment in duplex II. Since the PBS^{Ile} progeny all showed the wt sequence, it now seems likely that the efficiently replicating virus observed upon infection (Fig. 2B) was in fact the wt revertant. The extremely rapid reversion of this mutant can be explained by the efficient binding of the $tRNA^{Lys}_3$ primer to the PBS^{Ile} site (Fig. 3A, 17 bp in duplex I; see below).

The PBS sequence of the $PBS^{Lys}_{1,2}$ progeny had not reverted at up to 10 days postinfection (Table 2). However, upon prolonged culturing, this progeny also reverted to the wt PBS sequence. At 17 days postinfection, a mixed progeny was found (three $PBS^{Lys}_{1,2}$ and two wt clones) and rapid outgrowth of the wt virus was observed in the samples of days 31 and 49. The delayed reversion of the $PBS^{Lys}_{1,2}$ variant suggests that HIV-1 replication proceeds more efficiently with $tRNA^{Lys}_{1,2}$ compared with either $tRNA^{Ile}$, $tRNA^{Phe}$, or $tRNA^{Trp}$ as primer. As shown in Fig. 2A, the replication potential of the $PBS^{Lys}_{1,2}$ mutant was indeed higher compared with that of the PBS^{Phe} and PBS^{Trp} mutants (rapid reversion of the PBS^{Ile} mutant makes it impossible to accurately assess its replication potential). Another possibility is that $tRNA^{Lys}_3$-mediated reversion is particularly difficult for the $PBS^{Lys}_{1,2}$ mutant. However, inspection of the duplex structures I and II (Fig. 3B) suggests this explanation to be unlikely. For example, the slowly reverting $PBS^{Lys}_{1,2}$ mutant can form 14 bp in both duplexes I and II, whereas the more rapidly reverting PBS^{Trp} mutant forms duplexes of maximally 15 and 13 bp.

An interesting reversion pathway was observed for the PBS^{Pro} variant. At 13 days postinfection, three clones still contained the PBS^{Pro} sequence, while two clones had changed to PBS^{Ile}. Generation of the PBS^{Ile} virus suggests an efficient priming of $tRNA^{Ile}$ onto the PBS^{Pro} sequence, and outgrowth of the PBS^{Ile} variant indicates that HIV-1 can replicate more efficiently with $tRNA^{Ile}$ compared with $tRNA^{Pro}$. Upon pro-

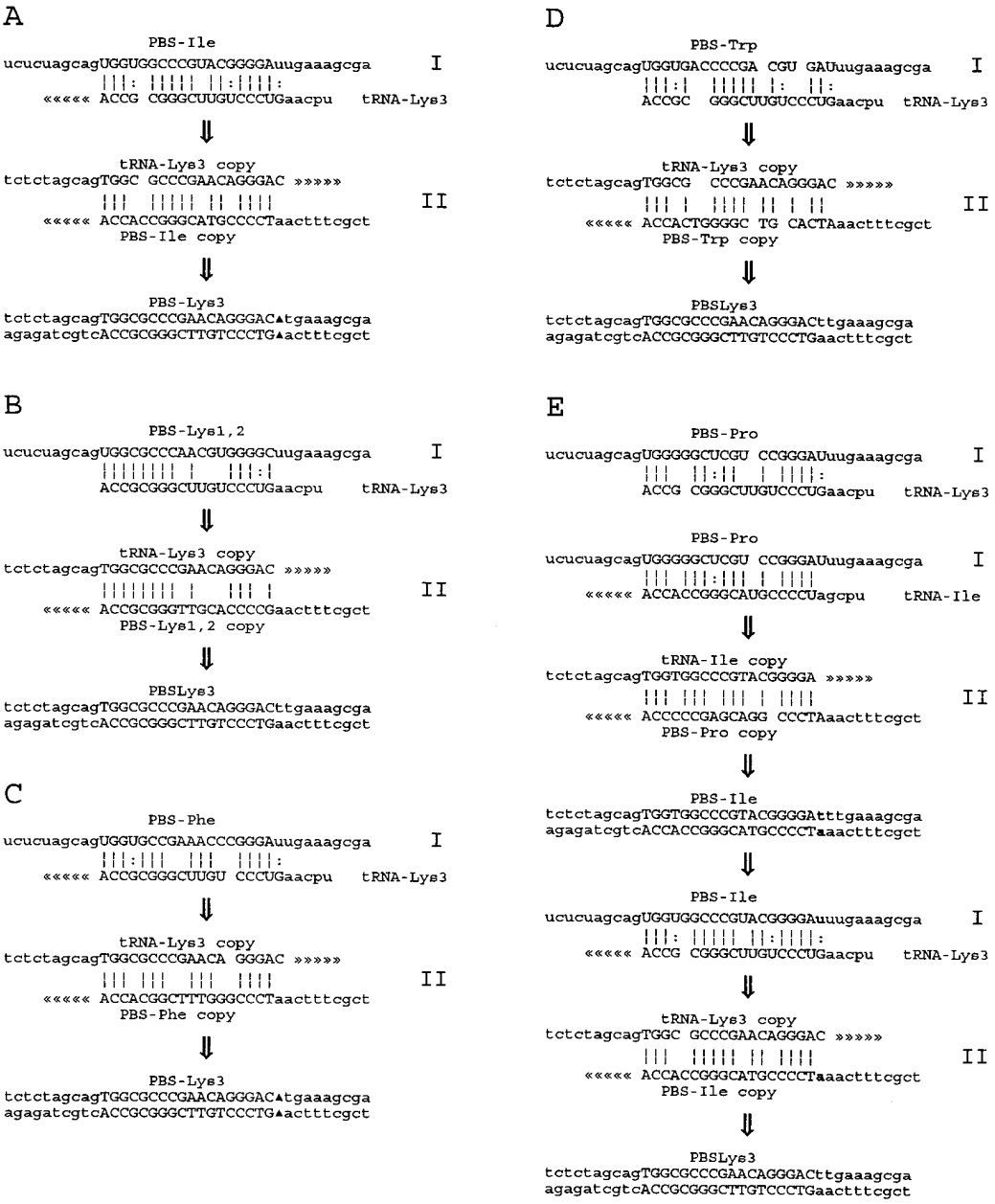


FIG. 3. Reversion of PBS mutants. Reversion is mediated by the annealing of the $tRNA_3^{Lys}$ primer to the mutant vRNA template, as is shown for the PBS^{Ile} (A), $PBS_{1,2}^{Lys}$ (B), PBS^{Phe} (C), and PBS^{Trp} (D) viruses. Reversion of the PBS^{Pro} mutant (E) is initiated by a $tRNA^{Ile}$-priming event, resulting in the production of PBS^{Ile} virions, followed by a $tRNA_3^{Lys}$-priming event, which results in the production of wt virions. Duplex I, possible base pair interactions between the 3′-terminal 18 nt of the tRNA primer (lower strand) and the mutant PBS region (upper strand, capital letters). Duplex II, possible base pairing between the tRNA primer and vRNA copies of the PBS sequence (upper and lower strands, respectively). E, base pair interaction of PBS^{Pro} with either $tRNA_3^{Lys}$ or $tRNA^{Ile}$ is shown to demonstrate that the latter complex is more stable. « and », direction of strand elongation by RT. ▲, deletion.

longed culturing of this mixture of viruses, the PBS was shown to revert to the wt genotype. This may not be surprising, since we demonstrated rapid reversion of the PBS^{Ile} mutant (Table 2). We therefore propose a $PBS^{Pro} \rightarrow PBS^{Ile} \rightarrow PBS^{wt}$ reversion pathway. Priming of the PBS^{Pro} site with $tRNA^{Ile}$ instead of $tRNA_3^{Lys}$ can be explained by the more efficient binding of the $tRNA^{Ile}$ molecule (duplexes I in Fig. 3E: $tRNA^{Ile}$ annealing, 15 bp with 3 nt in bulges; $tRNA_3^{Lys}$ annealing: 14 bp with 5 nt in bulges). The $tRNA^{Ile}$-mediated reverse transcription will result in the addition of a T nucleotide directly downstream of the PBS, which is eventually lost in a subsequent $tRNA_3^{Lys}$-priming event (Fig. 3E). Indeed, we detected this T insertion only in the intermediate PBS^{Ile} sequences (Table 2).

In the course of this study, we frequently observed a variant PBS_3^{Lys} sequence. This variant, with a C→T substitution in the center of the PBS motif, was observed upon reversion of the PBS^{Ile}, PBS^{Phe}, and $PBS_{1,2}^{Lys}$ mutants (Table 2). These data do suggest the presence of a minor $tRNA_3^{Lys}$ species with a G→A substitution at position 69 in the acceptor stem. For the moment, we refer to this variant as $tRNA_{3a}^{Lys}$ (Table 2). A mouse gene encoding a putative $tRNA_{3a}^{Lys}$ species was previously described (12). This tRNA molecule contains a total of five nu-

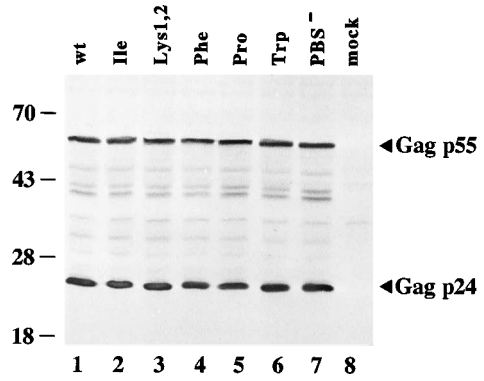


FIG. 4. Western blot analysis of transiently produced viral proteins. HeLa cells were transfected with wt and PBS-mutated proviral constructs. At 4 days posttransfection, total cellular extracts were prepared and analyzed by Western blot analysis. Viral proteins were identified by using serum from an HIV-1 infected individual. The positions of HIV-1 Gag precursor protein p55 and mature Gag protein p24 are indicated on the right. The positions of the molecular mass marker proteins (in kilodaltons) are indicated on the left. Lane 1, transfection of PBS^{wt} construct; lanes 2 to 7, transfection of PBS-mutated constructs; lane 8, mock-transfected cells.

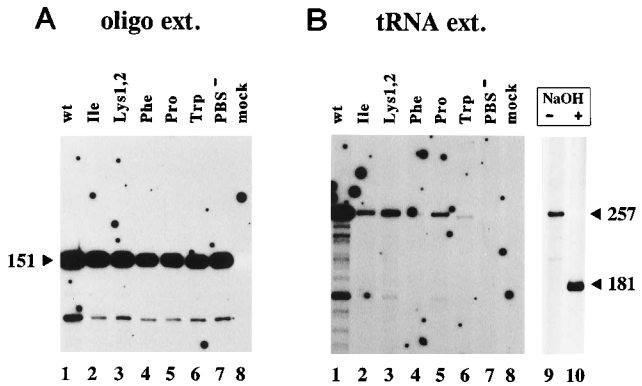


FIG. 5. Oligonucleotide-primer and tRNA-primer extension assays. HeLa cells were transfected with wt and PBS-mutated HIV-1 constructs. At 4 days posttransfection, viruses were purified from the culture supernatant and vRNA was isolated by phenol-chloroform extraction. (A) vRNA was quantitated in an oligonucleotide-primer extension assay, by using a primer complementary to positions 123 to 151 of the genomic RNA. Lane 1, PBS^{wt} vRNA; lanes 2 to 7, PBS-mutated vRNA; lane 8, no vRNA (mock-transfected cells). (B) The tRNA primer associated with and copurified with HIV-1 genomic RNA was extended by addition of RT and dNTPs (lanes 1 to 8 as described for panel A). For the wt virus, the length of this tRNA-primed cDNA is 257 nt (76-nt $tRNA_3^{Lys}$ plus 181-nt cDNA [lane 9]). Incubation of this cDNA product with NaOH resulted in the degradation of the tRNA part, leaving an 181-nt cDNA (lane 10). Products were analyzed on a 6% polyacrylamide-sequence gel.

cleotide differences compared with the human $tRNA_3^{Lys}$ sequence (positions 4, 15, 17, 48, and 69).

Characterization of transiently produced virions. All PBS-replacement viruses showed reduced replication rates and could not be stably maintained because of reversion. In order to study the mutant viruses in more detail, we transfected HeLa cells (not expressing CD4) and analyzed cells and transiently produced virions. These virions contain genomic RNA molecules corresponding to the input proviral genome, i.e., having a wt or PBS-mutated sequence. First, we assayed expression of viral proteins by Western blot analysis of total cell extracts. As shown in Fig. 4, transfection with the different constructs resulted in the production of similar levels of viral proteins. We next analyzed virus production by measuring the amount of Gag-p24 protein and RT activity in the culture supernatant. These levels did not vary significantly for the different constructs, including the PBS^- deletion mutant (Table 3). The observed small differences reflect experimental variation in the transfection assay. These combined results demonstrate that the PBS mutants are not affected in transcription, translation, and virion production, indicating that the HIV-1 PBS sequence does not overlap regulatory elements involved in one of these processes.

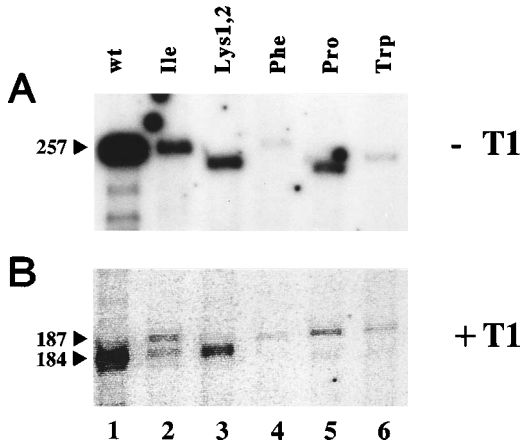
We also measured the vRNA content of the PBS-mutated viruses. Virions were purified by ultracentrifugation, and vRNA was isolated by phenol-chloroform extraction and subsequently analyzed in a primer extension assay with an oligonucleotide primer complementary to the +123-+151 region (Fig. 5A). Equal levels of cDNA were synthesized by the wt and PBS-mutated RNA genomes, reflecting similar levels of vRNA for the different virions. These data show that the PBS sequence does not contribute to packaging of genomic RNA. We next tested for the presence of a tRNA primer associated with the vRNA. The tRNA primer remains bound to the genomic RNA upon isolation and can be visualized in a tRNA-primer extension assay upon the addition of RT enzyme and dNTPs (Fig. 5B). The identity of this 257-nt-long tRNA-cDNA product was confirmed by NaOH-mediated degradation of the tRNA part, leaving an 181-nt cDNA (Fig. 5B, compare lanes 9 and 10). The extended tRNA-cDNA products were quantitated and expressed as a percentage of the cDNA production obtained for wt vRNA (Table 3). Greatest cDNA production was observed for the wt vRNA, whereas no product could be detected for the PBS-deleted mutant. Intermediate levels were observed for the $PBS_{1,2}^{Lys}$, PBS^{Pro}, and PBS^{Ile} mutants, and low levels were observed for the PBS^{Phe} and PBS^{Trp} viruses (Fig. 5B and Table 3). Similar results were obtained when either avian myeloblastosis virus or Moloney murine leukemia virus RT enzymes were used instead of HIV-1 RT (22a). These results indicate that retroviral RT enzymes do not preferentially extend their natural tRNA primer species. Thus, differences in cDNA production reflect differences in tRNA occupancy. The observed tRNA occupancies correlate reasonably well with the reduced replication kinetics of the PBS mutants.

In order to analyze the tRNA species bound to the mutant PBS sites in more detail, we separated the tRNA-primed cDNA products on a low-percentage polyacrylamide gel, thereby visualizing small size differences. All tRNA-PBS combinations will produce a cDNA of 181 nt, but small length differences exist for the various tRNA primers (Fig. 6C, 75 to 77 nt). A complicating factor is the tRNA-specific modification of nucleotides, which can lead to unpredictable migration ef-

TABLE 3. Analysis of wt and PBS-mutated virions

PBS	Gag-p24[a] (ng/ml)	RT activity[a,b] (%)	tRNA-priming efficiency[c] (%)	
wt (Lys-3)	500	100	100	
Ile	540	84	10	(5%$tRNA^{Ile}$,5%$tRNA_3^{Lys}$)
Lys-1,2	560	79	20	
Phe	400	97	5	
Pro	580	88	15	
Trp	460	95	3	
PBS^-	520	85	0	
Mock	<0.1	0	0	

[a] Measured in the culture supernatant.
[b] RT activity of the wt virus was set at 100%.
[c] tRNA-priming efficiencies were determined by quantification of the tRNA-primed cDNA products in Fig. 5B. These values were corrected for vRNA concentration (Fig. 5A), and priming activity obtained for wt vRNA was set at 100%.



C

tRNA primer		tRNA sequence	tRNA nt	cDNA-tRNA - RNase T1 nt	cDNA-tRNA + RNase T1 nt
Lys3	3′	ACCGCGGGCUUGUCCCUG... 5′	76	257	184
Ile		ACCACCGGGCAUGCCCCU...	77	258	187
Lys1,2		ACCGCGGGUUGCACCCCG...	76	257	184
Phe		ACCACGGCUUUGGGCCCU...	77	258	186
Pro		ACCCCCGAGCAGGCCCUA...	75	256	187
Trp		ACCACUGGGGCUGCACUA...	75	256	187

FIG. 6. Identification of tRNAs associated with the wt and PBS-mutated vRNAs. (A) The tRNA primer associated with the HIV-1 genomic RNA was extended by addition of RT and dNTPs. (B) The RNA of these tRNA-cDNA products was digested with RNase T_1, which specifically cleaves at G residues. (A and B) Products were analyzed on a 4.5% polyacrylamide-sequence gel. Lane 1, PBS^{wt} vRNA; lanes 2 to 6, PBS-mutated vRNA. (C) Expected fragment size of the tRNA-primed products upon RNase T_1 cleavage. Cleavage at the 3′-most G residue of the tRNA part (arrow) determines the size of the resulting fragment. The fragment sizes before and after RNase T_1 digestion are indicated.

fects. Therefore, the tRNA-cDNA products were digested with RNase T_1, which specifically cleaves at G residues in the tRNA part (Fig. 6C). RNase T_1 digestion will remove all modified tRNA nucleotides, allowing a direct size comparison of the resulting fragments. Figure 6C summarizes the expected length of the tRNA-cDNA fragments before and after RNase T_1 digestion for every putative tRNA primer. Before RNase T_1 incubation, tRNA-primed products of the $PBS^{Lys}_{1,2}$, PBS^{Phe}, PBS^{Pro}, and PBS^{Trp} mutants showed a different migration than the wt product (Fig. 6A). Upon RNase T_1 incubation, the sizes of the $PBS^{Lys}_{1,2}$ and wt products were identical and the PBS^{Phe} product was 2 nt larger than the wt product, whereas the PBS^{Pro} and PBS^{Trp} products were 3 nt larger than the wt product (Fig. 6B). These results confirm that $PBS^{Lys}_{1,2}$, PBS^{Phe}, PBS^{Pro}, and PBS^{Trp} are occupied by the corresponding tRNAs.

Interestingly, the PBS^{Ile} mutant generated two different fragments upon RNase T_1 treatment (Fig. 6B, lane 2). The longer product is expected for a $tRNA^{Ile}$-primed cDNA (187 nt), while the other is identical in size to a $tRNA^{Lys}_3$-initiated product (184 nt). These results suggest that PBS^{Ile} sites are occupied by either $tRNA^{Ile}$ or $tRNA^{Lys}_3$. Consistent with this idea, $tRNA^{Lys}_3$ can efficiently bind to the PBS^{Ile} sequence (Fig. 3A; 18 bp, with two small bulges), and this interaction is likely to drive the rapid reversion of the PBS^{Ile} mutant.

DISCUSSION

To assay for the ability of the HIV-1 virus to use primers other than $tRNA^{Lys}_3$ for the initiation of reverse transcription, we replaced the wt PBS by sequences complementary to the 3′ end of $tRNA^{Ile}$, $tRNA^{Lys}_{1,2}$, $tRNA^{Phe}$, $tRNA^{Pro}$, and $tRNA^{Trp}$. We found that HIV-1 is able to replicate with all of these tRNA primers, although at a markedly reduced rate. Reduced replication of HIV-1 viruses with $tRNA^{Lys}_{1,2}$ or $tRNA^{Phe}$ as primer was recently reported by others. Li et al. (20) observed similar replication kinetics for both mutants, whereas Wakefield et al. (30) measured an absolute replication defect of the PBS^{Phe} mutant. With the extensive set of PBS mutants analyzed in this paper, we observed the following order of replication: $PBS^{wt} > PBS^{Ile} > PBS^{Lys}_{1,2}$, $PBS^{Pro} > PBS^{Phe}$, PBS^{Trp}. Only the virus with a deleted PBS sequence did not replicate in our system. The genomic RNA of every mutant virion was found to be in association with the corresponding tRNA primer. However, the tRNA occupancy of the mutant PBSs was strongly decreased compared with the wt PBS^{Lys}_3, varying from 3 to 20% of the wt level. This tRNA-priming efficiency correlated reasonably well with the replication rate of the wt and mutant HIV-1 viruses. These results suggest that a change in PBS identity leads to reduced annealing of the new tRNA primer, resulting in a lower rate of viral replication.

It is possible that the tRNA occupancy of a mutant PBS is a reflection of the concentration of individual tRNA species in viral particles. HIV-1 particles contain high concentrations of $tRNA^{Lys}_3$ and $tRNA^{Lys}_{1,2}$ and lower levels of other tRNAs, although the tRNA content may also depend on the host cell type (15, 16, 22). We observed highest tRNA occupancies for the wt (PBS^{Lys}_3) and $PBS^{Lys}_{1,2}$ viruses, suggesting that the tRNA occupancy indeed correlates with the viral tRNA content. However, the differences in tRNA content of HIV-1 virions cannot fully explain the observed differences in tRNA occupancy. For instance, whereas virions contain similar amounts of $tRNA^{Lys}_{1,2}$ and $tRNA^{Lys}_3$, the occupancy of $PBS^{Lys}_{1,2}$ was only 20% compared with the wt PBS^{Lys}_3. Therefore, factors other than the PBS may also play an important role in the selective binding of the natural $tRNA^{Lys}_3$ primer. RT, or its precursor Gag-Pol protein, may be such a factor, since in vitro studies not only showed that binding of the tRNA primer to the vRNA is stimulated by RT but also demonstrated specific interaction of the priming tRNA species with the RT protein (3, 4, 13, 25, 27). In addition, we recently observed a strongly reduced $tRNA^{Lys}_3$ occupancy of the wt PBS in the absence of a functional RT protein (9a). Alternatively, additional base pairing interactions between $tRNA^{Lys}_3$ and the vRNA may result in a more efficient annealing of this primer species (1, 2, 6, 14, 17).

None of the slowly replicating PBS mutants could be stably maintained because they all reverted to the wt sequence upon prolonged culturing. The reversion pathway involves the binding and extension of the wt $tRNA^{Lys}_3$ primer at the mutant PBS, resulting in the production of 50% wt progeny (Fig. 1) (8, 26). This mechanism can explain the small mutations observed directly downstream of the PBS in some of the revertants. The PBS mutants reverted in a specific order ($PBS^{Ile} > PBS^{Phe} > PBS^{Trp} > PBS^{Pro} > PBS^{Lys}_{1,2} > PBS^{-}$), which correlated with the $tRNA^{Lys}_3$-binding capacity of the mutant PBSs (Fig. 3, $PBS^{wt} > PBS^{Ile} > PBS^{Phe} > PBS^{Lys}_{1,2} > PBS^{Trp} > PBS^{Pro} >$

PBS$^-$). The one exception to this rule is the PBS$^{Lys}_{1,2}$ virus, which reverted relatively slowly. The efficient replication of this mutant is likely to have delayed outgrowth of the wt virus. A remarkable reversion pathway was observed for the PBS^{Pro} virus, which first changed to PBS^{Ile} and then rapidly reverted to the wt sequence. This route can be explained on the basis of the more efficient binding of tRNA^{Ile} than of tRNA$^{Lys}_3$ at the PBS^{Pro} site (compare duplexes I in Fig. 3E).

The reduced replication rate of HIV-1 with nonnatural tRNA primers suggests a difference in primer specificity for HIV-1 and murine leukemia virus. Lund et al. (21) demonstrated efficient transduction of an Akv murine leukemia virus-based vector with tRNA$^{Gln}_1$, tRNA$^{Gln}_2$, or tRNA$^{Lys}_3$ primer instead of the natural tRNA^{Pro} primer. Furthermore, Colicelli and Goff (9) isolated a recombinant murine leukemia virus, which successfully utilized tRNA$^{Gln}_1$ instead of tRNA^{Pro} as primer. These results suggest that tRNA primer selection is more flexible for the murine leukemia viruses than it is for HIV-1. Alternatively, the apparent differences in primer specificity result from differences in the experimental approach. For instance, the transduction assay scores for only one round of reverse transcription, whereas we measured multiple rounds of virus replication. It should also be noted that the recombinant virus described by Colicelli and Goff had replaced both the PBS and flanking regions by endogenous retroviral sequences. These flanking sequences may have provided additional tRNA$^{Gln}_1$-vRNA interactions, thereby forcing the use of this unnatural tRNA primer. Alternatively, the recombinant virus could have adapted to the use of tRNA$^{Gln}_1$ by the acquisition of mutations in, e.g., the RT protein. In fact, analysis of such second-site revertants will be extremely useful for further characterization of the viral protein(s) involved in primer selection. We should note that such studies are not possible with our set of HIV-1 mutants given the genetic instability of their PBS sequence.

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