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Human Immunodeficiency Virus Type 1 Can Use Different tRNAs as Primers for Reverse Transcription but Selectively Maintains a Primer Binding Site Complementary to $tRNA_3^{Lys}$

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The initiation of human immunodeficiency virus type 1 (HIV-1) reverse transcription occurs at a site in the viral RNA genome which is designated the primer-binding site (PBS). The HIV-1 PBS is an 18-nucleotide sequence that is complementary to the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$, which is used as the primer for reverse transcription. All HIV-1 isolates sequenced to date contain a PBS complementary to $tRNA_3^{Lys}$, suggesting that other cellular tRNAs might not function as primers for reverse transcription. To investigate this possibility, we have substituted the HIV-1 PBS with sequences predicted to be complementary to the 3′-terminal nucleotides of $tRNA_{1,2}^{Lys}$, $tRNA^{Ile}$, and $tRNA^{His}$, which previous studies have identified to be packaged into HIV-1 virions along with $tRNA_3^{Lys}$. We demonstrate that infectious viruses which utilized $tRNA_{1,2}^{Lys}$, $tRNA^{Ile}$, and $tRNA^{His}$ in reverse transcription can be recovered. However, the appearances of viruses with PBSs complementary to these alternate tRNAs were delayed compared with the wild type. After extended in vitro culture, viruses containing the PBSs complementary to these different tRNAs reverted back to the wild-type PBS complementary to $tRNA_3^{Lys}$. Furthermore, only the first 9 nucleotides of the 18 nucleotide PBSs were sufficient for HIV-1 to utilize the alternate tRNA primers in reverse transcription, demonstrating that HIV-1 does not require the complete 18-nucleotide PBS to utilize these tRNA primers for reverse transcription. These results suggest that factors other than complementarity between the PBS and the primer tRNA contribute to the selectivity of $tRNA_3^{Lys}$ to initiate HIV-1 reverse transcription.

A hallmark of retrovirus replication is the conversion of the single-stranded RNA genome into a double-stranded DNA proviral intermediate. This process, which is referred to as reverse transcription, is carried out by an RNA-dependent DNA polymerase termed reverse transcriptase (RT) which is encoded by the viral *pol* gene (4, 41). Retroviruses use a cellular tRNA as the primer for the initiation of reverse transcription. A sequence in the viral RNA genome designated the primer-binding site (PBS) is complementary to the 3′-terminal 18 nucleotides of the primer tRNA molecule. The RT initiates DNA synthesis by extension of the 3′-OH of the tRNA molecule which is bound to the PBS (38, 42).

Retroviral virions contain all of the necessary viral proteins (e.g., RT) as well as the cellular tRNA primer used for reverse transcription of the viral genome. The tRNA primer used for reverse transcription is believed to be incorporated into the virion during assembly and positioned on the PBS. Although retroviruses differ with respect to the tRNA primer used to initiate reverse transcription, the primer tRNA utilized by a given group of retroviruses is highly conserved. For example, all human immunodeficiency viruses (HIV) use $tRNA_3^{Lys}$ to initiate reverse transcription. However, the type and relative abundance of tRNA species found in retroviral virions have been shown to vary greatly, depending on the cell type from which the virion was obtained (14, 15, 17, 19, 20, 27, 28, 34). Analysis of HIV type 1 (HIV-1) virions obtained from transfection of proviral genomes into COS cells revealed that $tRNA_{1,2}^{Lys}$ and $tRNA^{Ile}$ were present at levels comparable to those of $tRNA_3^{Lys}$ (19). The same study also indicated that $tRNA^{His}$ was present within HIV-1 virions expressed from chronically infected Vero cells.

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During reverse transcription, the PBS is regenerated when the RT copies the first 18 nucleotides of the still-attached tRNA primer during plus-strand strong-stop DNA synthesis (16, 39, 40). The sequence of the PBS, then, reflects what tRNA was used to initiate reverse transcription. Since a distinguishing feature of all HIV-1 proviruses sequenced to date is that the PBSs are complementary to $tRNA_3^{Lys}$, the initiation of HIV-1 reverse transcription would be predicted to selectively use only $tRNA_3^{Lys}$, even though other tRNAs are present in the virions at relatively high levels such as $tRNA_{1,2}^{Lys}$, which also closely resembles $tRNA_3^{Lys}$ in primary structure (29). To directly test the possibility that various tRNAs present in HIV-1 virions can be used in reverse transcription, we have constructed proviral genomes in which the wild-type PBS complementary to $tRNA_3^{Lys}$ was mutated to be complementary to the 3′-terminal nucleotides of $tRNA_{1,2}^{Lys}$, $tRNA^{Ile}$, and $tRNA^{His}$. The proviral genomes containing the mutant PBSs were transfected into COS-1 cells to generate mutant viruses, which were used to infect SupT1 cells. The results provide new insights into HIV-1 reverse transcription by demonstrating that although these alternative tRNAs can be used in initiation, HIV-1 has evolved to selectively use $tRNA_3^{Lys}$ to initiate reverse transcription and maintain a PBS complementary to $tRNA_3^{Lys}$.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. Restriction endonucleases and DNA modification enzymes were obtained from New England Biolabs. The *Taq* polymerase was purchased from BRL-Gibco, and the reagents for PCR were obtained from Perkin-Elmer Cetus. Radioactive nucleotides and the reagents for enhanced chemiluminescence were purchased from Amersham Corp. [^{35}S]methionine (Translabel) was obtained from ICN. Tissue culture media and reagents were obtained from BRL-Gibco. The synthetic DNA oligonucleotides used for mutagenesis and PCR

were prepared by the Cancer Center Oligonucleotide Synthesis Facility at the University of Alabama at Birmingham.

Tissue culture. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1× G-MSG (sodium selenite [0.0067 mg/liter], insulin [10 mg/liter], and transferrin [5.5 mg/liter]) at 37°C in a 5% CO_2 atmosphere. SupT1 cells were grown in RPMI supplemented with 10% fetal bovine serum.

DNA manipulation and mutagenesis. General laboratory procedures were used for DNA manipulation, plasmid preparation, and subcloning essentially as described elsewhere (25). Single-stranded DNA of M13mp18PBS used for oligonucleotide site-directed mutagenesis was prepared as described elsewhere (43). M13mp18PBS contains HIV-1 proviral sequences between the *Hpa*I and *Pst*I sites of pHXB2*gpt* including the PBS region (32). The DNA oligonucleotides used to create the desired mutations in the HIV-1 PBS region were as follows: (i) pHXB(Ile), CGCTTTCAATCCCCGTACGGGCCACCA CTTCTA; (ii) pHXB($Lys_{1,2}$), CGCTTTCAAGCCCCACGTTGGGCGCCACTT; (iii) pHXB (Ile+5), CCCTTTCGCTCCCCTTTCAAAGGACAGCAGGGCCACCA; (iv) pHXB($Lys_{1,2}$+5), CCTTTCGCGCCCCTTTCAAAGGACAGCATGGGCGC CA; (v) pHXB(His+5), CCTTTCGCATCCGTTTCAAAGGACAGCAACGG CACCACTTCTAGAG; (vi) pHXB(Gln_2+5), CCTTTCGCATCCTTTTCAAA GGACAGCACCCCAGCCACTTCTAGAG. The PBS mutations pHXB(Ile+5) and pHXB($Lys_{1,2}$+5) were constructed by using the appropriate mutagenic oligomers on single-stranded M13mp18 phage DNA containing the Ile [M13mp18 (Ile)] or $Lys_{1,2}$ [M13mp18($Lys_{1,2}$)] mutations, respectively. Screening and subsequent identification of the M13 plaques containing the desired mutations were performed by Sanger dideoxy DNA sequencing (36) with Sequenase (U.S. Biochemicals). Each mutant PBS was subcloned into pHXB2*gpt*, a plasmid encoding the entire HIV-1 provirus flanked by unique *Hpa*I (5′) and *Xba*I (3′) restriction sites (30, 31). Briefly, replicative-form DNA containing the PBS mutation was isolated and digested with *Hpa*I and *Bss*HII, resulting in an 868-bp fragment encompassing the PBS region. The 868-bp fragment was subcloned between the *Hpa*I and *Bss*HII sites of pHXB2*gpt*, and the resulting mutant proviral plasmids were screened by restriction digests and confirmed by direct dideoxy DNA sequencing (36).

DNA transfections and analysis of viral replication. Transfection of proviral plasmid DNA into COS-1 cells by using DEAE-dextran was carried out as previously described (43). To test for viral replication and infectivity, SupT1 cells, which express CD4 and support high-level replication of HIV-1, were either cocultured with transfected COS-1 cells or infected with cell-free virus pelleted from transfected COS-1 supernatants as previously described (43). Following coculture, the SupT1 cells were removed by low-speed centrifugation (1,000 × *g*) and further cultured with fresh medium and additional SupT1 cells. For cell-free infections, SupT1 cells (10^6 cells per ml) were infected with known amounts of virus as measured by p24 antigen (50 ng/ml). After the virus was allowed to adsorb for 24 h, SupT1 cells were pelleted (1,000 × *g*), washed with phosphate-buffered saline (pH 7.0), and further cultured for as long as 3 weeks. SupT1 cultures were monitored visually for the formation of multicell syncytia, and, at various intervals postcoculture or postinfection, supernatants were collected, and cultures were split and refed with fresh SupT1 cells. Supernatant samples were filtered through a 0.45-μm-pore-size nitrocellulose syringe filter (Nalgene) and analyzed for p24 antigen by enzyme-linked immunosorbent assay (ELISA [Coulter Laboratories]).

Metabolic labeling of transfected cultures and immunoprecipitation. The procedures for metabolic labeling of COS-1 cells were as previously described (43). Briefly, the cells were washed once with Dulbecco's modified Eagle's medium and incubated in methionine-cysteine-free Dulbecco's modified Eagle's medium for 1 h, and then 100 μCi of [^{35}S]Translabel was added for 2 h. HIV-1 proteins were detected by incubating labeled cellular extracts with pooled sera from AIDS patients for 24 h, with constant rocking at 4°C. Immunoprecipitates were processed essentially as described elsewhere (43), and the proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE). Following fluorography with sodium salicylate, the dried gels were autoradiographed with Kodak X-AR film.

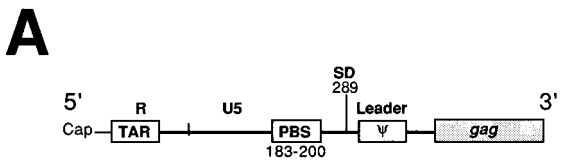
Western blot (immunoblot) analysis. Supernatants of COS-1 cells transfected with either wild-type or mutant proviral genomes were collected 60 h posttransfection and filtered through a 0.45-μm-pore-size syringe filter. Viral particles were pelleted through a 20% sucrose cushion by centrifugation for 2 h at 28,000 rpm in an SW41 rotor. Pelleted virions were resuspended in gel sample buffer and were subjected to SDS-PAGE with SDS-10% polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membrane for 2 h at 100 V. Blotted nitrocellulose filters were blocked for 1 h at room temperature with phosphate-buffered saline (pH 7.0)–0.1% Tween containing 5% nonfat dry milk and then incubated overnight at 4°C with pooled sera from AIDS patients. Following incubation with primary antibody, the blots were extensively washed with phosphate-buffered saline (pH 7.0) for 30 min and incubated with a goat-anti-human secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. The blots were again extensively washed with phosphate-buffered saline (pH 7.0) and subjected to enhanced chemiluminescence (Amersham) as described by the manufacturer.

PCR amplification and sequencing of PBS-containing proviral DNA. Sequence analysis of the proviral PBS sequences resulting from infection of SupT1 cells with wild-type and mutant viruses was carried out by a previously described protocol (43). Briefly, approximately 10 μg of total cellular DNA from infected SupT1 cultures was subjected to PCR under reaction conditions previously described (43) to amplify integrated proviral DNA encompassing the PBS. The HIV-1-specific oligonucleotide primers used to PCR amplify the PBS region were as follows: primer 1, 5′-GGCTAACTAGGGAACCCACTGC-3′ (nucleotides 42 to 63); primer 2, 5′-AGTGAATTCCTCCTTCTAGCCTC CGCTAG TC-3′ (nucleotides 309 to 330). (Note that the numbers indicating the nucleotide sequences which hybridize to the PCR primers are based on the nucleotide numbering of the pHXB2*gpt* proviral genome. In addition, the underlined nucleotides in primer 2 indicate the additional sequences at the 5′ end which correspond to an *Eco*RI restriction endonuclease site.) PCR-amplified DNA was digested with *Hin*dIII and *Eco*RI; the *Hin*dIII site is found at nucleotide 77 of the HIV-1 proviral genome, and the *Eco*RI site was engineered into primer 2. Digested PCR products were subcloned between the *Eco*RI and *Hin*dIII sites present in the polylinker region of M13mp18 replicative-form DNA. Ligation products were transformed into competent *Escherichia coli* (DH5αF′) and plated on a lawn of *E. coli* (DH5αF′) containing isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal [U.S. Biochemicals]) as previously described (43). Single-stranded DNAs prepared from individual recombinant phage clones were sequenced by Sanger dideoxy DNA sequencing (36).

RESULTS

Construction of HIV-1 proviral genomes with PBSs complementary to $tRNA^{Ile}$ and $tRNA^{Lys}_{1,2}$. In a previous study, we constructed a mutant proviral genome containing a PBS complementary to the 3′-terminal 18 nucleotides of a yeast $tRNA^{Phe}$ molecule (43). Although transfection of this provirus [pHXB2(Phe)] into COS-1 cells resulted in the production of wild-type levels of virus particles, the virus was noninfectious in SupT1 cells. Sequence differences in the 3′ acceptor stems of yeast and mammalian $tRNA^{Phe}$ species may explain why HIV-1 was unable to use a PBS complementary to yeast $tRNA^{Phe}$. Therefore, we wanted to determine if HIV-1 reverse transcription could be primed by tRNAs known to be present within the virion. In recent studies, Li et al. demonstrated that infectious viruses could be recovered with PBSs complementary to human $tRNA^{Phe}$ and $tRNA^{Lys}_{1,2}$ (23). Since $tRNA^{Ile}$ has been found in HIV-1 virions (19), we wanted to determine if viruses with a PBS complementary to $tRNA^{Ile}$ would also be infectious. To address this possibility, site-directed mutagenesis was used to substitute the wild-type PBS with the 18 nucleotides predicted to be complementary to the 3′-terminal nucleotides of human $tRNA^{Ile}$. As a positive control, a proviral genome containing a PBS complementary to $tRNA^{Lys}_{1,2}$ was also constructed. The mutant constructs are referred to as pHXB2(Ile) and pHXB2($Lys_{1,2}$), respectively, and are presented in Fig. 1B as they would appear in the viral RNA genome.

Expression of viral proteins from proviral genomes containing mutant PBSs. The 5′ nontranslated region of the HIV-1 genome has many *cis*-acting elements such as the transactivation response region (nucleotides 1 to 59) and the splice-donor site (nucleotide 289), as well as the putative Ψ encapsidation signal (nucleotides 300 to 319) (3, 10, 22) (Fig. 1A). Since the HIV-1 PBS is also located at nucleotides 183 to 200 within the 5′ nontranslated region, the substitutions in the PBS may affect viral gene expression (26). To determine if substitution of the wild-type PBS complementary to $tRNA^{Lys}_3$ with sequences complementary to $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$ affects the expression of viral proteins, the intracellular expression of viral proteins in COS-1 cells transfected with these mutant proviral genomes was analyzed by immunoprecipitation (Fig. 2A). Similar levels of *gag* gene products ($Pr55^{gag}$ and p24) and *env* gene product (gp160) were detected in COS-1 cell lysates transfected with the various proviral constructs. To determine whether a substituted PBS affects levels of extracellular particles, supernatants of transfected COS-1 cells were analyzed by Western blotting by using pooled sera from AIDS patients to identify viral proteins. The virion-associated viral proteins detected in



B **Primer Binding Site Substitutions**

	PBS Sequence
pHXB2(WT)	UGG CGC CCG AAC AGG GAC
pHXB2(Ile)	UGG UGG CCC GUA CGG GGA
pHXB2(Lys$_{1,2}$)	UGG CGC CCA ACG UGG GGC

FIG. 1. The 5′ nontranslated region of the HIV-1 genome and PBS mutants. (A) Schematic diagram of the 5′ nontranslated region in the HIV-1 genome showing the locations of relevant *cis*-acting elements. The PBS is positioned at nucleotides 183 to 200 within the 5′ nontranslated region between the viral U5 and the leader RNA sequences (30, 31). Also located within the 5′ nontranslated region is the transactivation response region (TAR), the splice-donor site (SD), and one of the putative encapsidation signals (Ψ). (B) Nucleotide sequence of the wild-type and substituted PBSs. The wild-type HIV-1 PBS, which is complementary to the 3′-terminal 18 nucleotides of a cellular tRNA$_3^{Lys}$ molecule, is designated pHXB2(WT). Two mutant proviral genomes, designated pHXB2(Ile) and pHXB2(Lys$_{1,2}$), were constructed such that the wild-type PBS was substituted with a sequence predicted to be complementary to the 3′-terminal 18 nucleotides of either tRNA^{Ile} or tRNA$_{1,2}^{Lys}$, respectively. The nucleotide differences between the wild-type and mutant PBSs are denoted in the boxes.

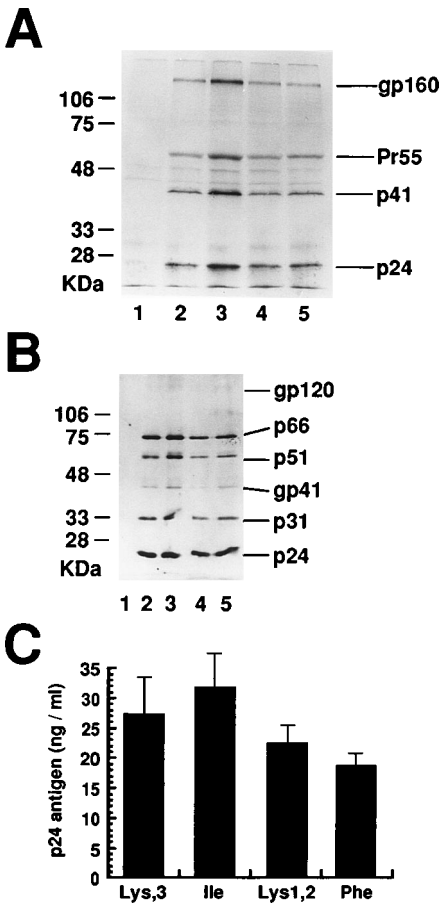


FIG. 2. Intracellular expression of viral proteins and analysis of released virion particles. (A) COS-1 cells were transfected with either the wild type or the various mutant proviral genomes and were metabolically labeled 24 h posttransfection with [^{35}S]Translabel for 2 h, and then HIV-1-specific proteins were immunoprecipitated with pooled sera from AIDS patients. The immunoprecipitated proteins were analyzed by separation on an SDS-10% polyacrylamide gel, which was followed by fluorography and autoradiography. Lanes: 1, mock transfected; 2, pHXB2(WT); 3, pHXB2(Ile); 4, pHXB2(Lys$_{1,2}$); 5, pHXB2PBS(phe). The molecular mass markers and the relevant HIV-1 proteins are noted. (B) Western blot analysis of virions released from transfected COS-1 cells. Supernatants of COS-1 cells transfected with the indicated proviral DNAs were filtered through a 0.45-μm-pore-size syringe filter, and the virus particles were pelleted through a 20% sucrose cushion by centrifugation in a SW41 rotor at 28,000 rpm for 2 h. The viral pellets were resuspended in gel sample buffer, electrophoresed through an SDS-10% polyacrylamide gel, and electrophoretically transferred to nitrocellulose membrane for Western blotting. Lanes: 1, mock transfected; 2, pHXB2(WT); 3, pHXB2(Ile); 4, pHXB2(Lys$_{1,2}$); 5, pHXB2PBS(phe). The molecular mass markers and the relevant HIV-1 proteins are noted. (C) Supernatants of COS-1 cells transfected with wild-type (Lys,3) or proviral genomes containing PBSs complementary to tRNA^{Ile} (Ile), tRNA^{Lys1,2} (Lys$_{1,2}$), or tRNA^{Phe} (Phe). At 48 h posttransfection, the culture supernatants were analyzed for released virus particles by quantitation of p24 antigen (Coulter). The bar graph illustrates the mean values of p24 antigen detected from six independent transfections, with standard deviations represented by error bars.

supernatants from cells transfected with the wild type were similar to those from cells transfected with proviruses containing the mutant PBSs (Fig. 2B). Finally, we assayed the supernatants for soluble p24 antigen by an ELISA. Similar amounts of p24 antigen were detected in the supernatants from the cells transfected with the different proviral constructs, indicating that mutations in the PBS did not affect release of virus particles (Fig. 2C).

Replication potential of proviral genomes containing substituted PBS. To determine whether HIV-1 can utilize PBSs complementary to the 3′-terminal 18 nucleotides of tRNA species different from that of tRNA$_3^{Lys}$, SupT1 cells were either cocultured with COS-1 cells transfected with the mutant or wild type proviral constructs or were infected with equal amounts of cell-free virus (50 ng of p24 per ml) obtained from transfected COS-1 supernatants. Samples of culture supernatants were collected at various times (postcoculture or postinfection), and replication of both the wild-type and mutant viruses was measured by quantitating the levels of p24 antigen. Cell-free virus derived from COS-1 cells transfected with proviral genomes possessing PBSs complementary to either tRNA^{Ile} (Ile) or tRNA$_{1,2}^{Lys}$ (Lys$_{1,2}$) replicated less efficiently in SupT1 cells than the wild-type virus [Lys,3(WT)], with a 3-day delay in the appearance of wild-type virus in the culture supernatants (Fig. 3). In agreement with previous results, viruses derived from proviral genomes containing a PBS complementary to yeast tRNA^{Phe} (Phe) failed to replicate (43). The overall patterns for the replication kinetics were similar when coculture of COS-1 and SupT1 cells was used to initiate virus infection (data not shown).

Sequence analysis of the PBS from proviruses after extended in vitro culture. The model of retroviral reverse transcription predicts that the PBS is regenerated during plus-strand strong-stop viral DNA synthesis. The RT copies the first 18 nucleotides of the tRNA used to initiate reverse transcription which is still attached to the 5′ end of the minus-strand viral cDNA (16, 39, 40, 42). Therefore, the PBS present in the proviral form will be complementary to the tRNA species used to initiate minus-strand synthesis. To determine which tRNA species was used to initiate reverse transcription, PCR was used to amplify the PBS region from proviruses derived from

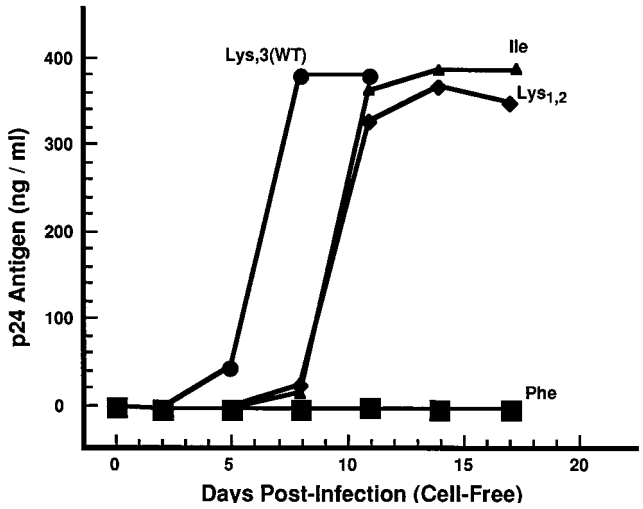


FIG. 3. Kinetics of replication of viruses containing substituted PBSs. Equal amounts of virus (approximately 50 ng/ml) isolated from COS-1 cells transfected with either pHXB2(WT) [Lys,3(WT)], pHXB2(Ile) (Ile), pHXB2($Lys_{1,2}$) ($Lys_{1,2}$), or pHXB2PBS(phe) (Phe) were used to infect 10^6 SupT1 cells in RPMI medium containing 10% fetal bovine serum. At 24 h postinfection, the virus-containing medium was removed by low-speed centrifugation (1,000 × *g*) and the SupT1 cells were washed once in phosphate-buffered salne (pH 7.0), which was followed by further culturing in fresh medium and new SupT1 cells. Samples of culture supernatants were removed at specified times postinfection and assayed for soluble p24 antigen. The origins of the samples obtained for infection of SupT1 cells with wild-type and mutant viruses are as marked.

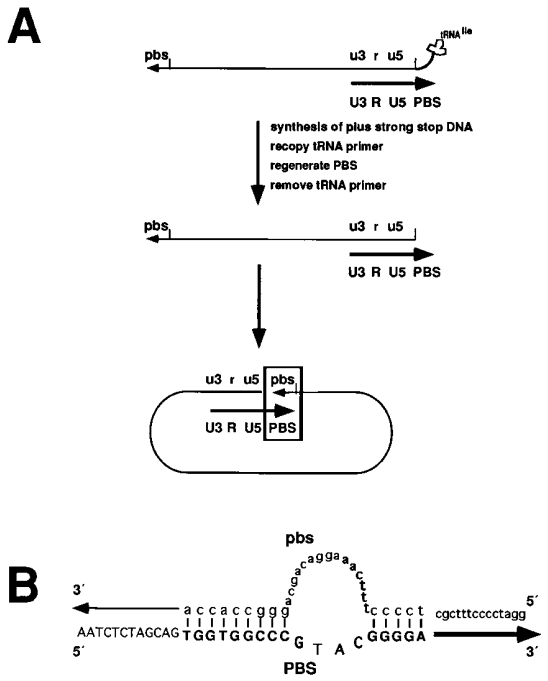
infection of viruses containing PBS substitutions. The proviral PCR products were subcloned into M13 replicative-form DNA, and the PBS region contained in the individual recombinant M13 phage clones was sequenced. Table 1 shows the PBS sequences of proviruses derived from infected SupT1 cells at two different time points: early (day 4) and late (day 8) postinfection. At day 4 postinfection, all of the proviruses sequenced maintained PBSs that were complementary either to $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$, but not the wild-type sequence. However, at day 8 postinfection, all but one of the proviruses sequenced possessed a wild-type PBS complementary to $tRNA^{Lys}_3$. These results suggest that HIV-1 can utilize alternate tRNA primers to initiate reverse transcription if a PBS complementary to that particular tRNA species is present in the viral RNA genome.

Utilization of alternate tRNA primers for HIV-1 reverse transcription does not require complete PBS. In previous studies, we and others have demonstrated that HIV-1 does not require a complete 18-nucleotide PBS to initiate reverse transcription (13, 23, 32, 43). We have exploited this feature to demonstrate that only the first six nucleotides of the PBS are required to initiate reverse transcription (32). In the present study, we have constructed proviral genomes in which the first nine nucleotides correspond to alternate tRNAs. To distinguish between the PBS sequence present in the input genomes from the PBS sequences of proviruses derived from reverse transcription, mutant proviral genomes were constructed in which the PBS region was altered so that proviruses which were generated as a result of reverse transcription would possess a 6-bp deletion 3′ to the PBS as depicted in Fig. 4. These constructs are based on our previous results which demonstrated that an insertion downstream of a mutated PBS of five nucleotides identical to the last five of the wild-type PBS could be used to facilitate the second template transfer event of reverse transcription (43). As a consequence, complementary binding of these five nucleotides, which are deleted in the mutated form of the PBS, with the last five nucleotides of the plus strand PBS sequence would result in a deletion of the intervening six nucleotides. For the present studies, five nucleotides corresponding to the last five of either the Ile or $Lys_{1,2}$ PBS were inserted 3′ of a mutated form of these PBSs, respectively. The first 9 nucleotides of the mutated form of the PBSs are complementary to either $tRNA^{Lys}_{1,2}$ or $tRNA^{Ile}$, while the last 9 of the 18 nucleotides have been substituted with noncomplementary nucleotides (Fig. 5). Therefore, if either a $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$ initiates reverse transcription, in addition to regenerating the authentic 18-nucleotide Ile or $Lys_{1,2}$ PBS, a 6-bp deletion 3′ of this PBS would be present in the integrated proviruses. The advantage of this strategy is that it

TABLE 1. Sequence analysis of the proviral PBSs derived from pHXB2(Ile) and pHXB2($Lys_{1,2}$)

Provirus sample[a]	PBS sequence	Frequency[b]
Input sequence[c]	TGG TGG CCC GTA CGG GGA TTG AAA (Ile)	
vHXB(Ile) (day 4 postinfection)	TGG TGG CCC GTA CGG GGA TTG AAA (Ile)	8/8
vHXB(Ile) (day 8 postinfection)	TGG CGC CCG AAC AGG GAC -[d]TG AAA (Lys,3)	5/5
Input sequence[c]	TGG CGC CCA ACG TGG GGC TTG AAA ($Lys_{1,2}$)	
vHXB2($Lys_{1,2}$) (day 4 postinfection)	TGG CGC CCA ACG TGG GGC TTG AAA ($Lys_{1,2}$)	9/9
vHXB2($Lys_{1,2}$) (day 8 postinfection)	TGG CGC CCG AAC AGG GAC TTG AAA (Lys,3)	8/10
	TGG CGC CCG AAC AGG GAC C[e]TG AAA (Lys,3)	1/10
	TGG CGC CCA ACG TGG GGC TTG AAA ($Lys_{1,2}$)	1/10

[a] PBS regions PCR amplified and sequenced from genomic DNAs isolated from SupT1 cells infected with viruses derived from transfection of the corresponding plasmids.
[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones.
[c] The input sequence refers to the initial mutation in the PBS region.
[d] Nucleotide deletion.
[e] Nucleotide difference from original proviral sequence.



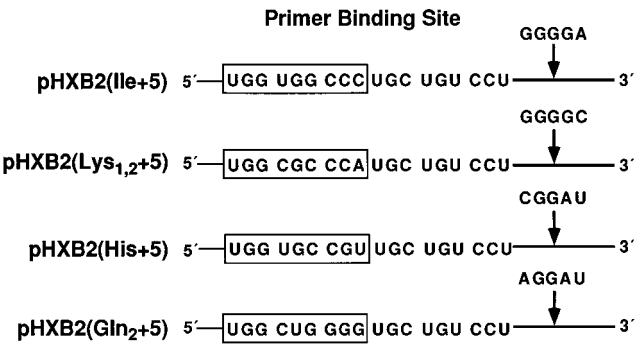


FIG. 5. Depiction of the PBS sequences with 3′ nucleotide insertions. The four mutated PBS sequences found in the proviral constructs are depicted. The first 9 nucleotides of the PBSs are complementary to 3′-terminal 9 nucleotides of the corresponding primer tRNAs (shown in boxes). The second 9 nucleotides are designed such that they are not complementary to the primer tRNAs. The arrows indicate the insertion of the 5 nucleotides, which correspond to the last 5 nucleotides of the authentic substituted PBS, that are positioned 6 bp downstream from the end of the PBS (CCU). Upon successful reverse transcription, the entire PBS will be complementary to the 3′-terminal 18 nucleotides of the initiating tRNA as well as the 6-nucleotide deletion. PBS sequences complementary to the 3′-terminal 18 nucleotides of $tRNA^{His}$ and $tRNA_2^{Gln}$ are as follows: 5′-UGG UGC CGU GAC UCG GAU (His) and 5′-UGG CUG GGG UAC GAG GAU (Gln_2), respectively.

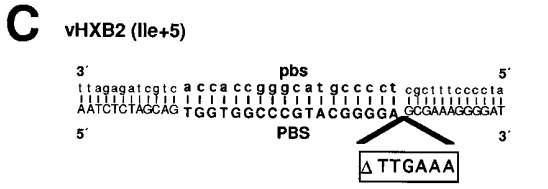


FIG. 4. Model for the second template transfer of Ile+5 reverse transcription and the predicted sequence of the proviral PBS region. (A) Steps involved in the second template transfer event of reverse transcription (16, 40, 42). Plus-strand strong-stop viral DNA synthesis (thick line) initiates from an RNA primer that remains following incomplete digestion by the viral RNase H activity at the polypurine tract located just upstream of the U3 region in the viral genome. DNA synthesis continues until the RT copies the first 18 nucleotides of the still-attached primer tRNA used to initiate minus-strand cDNA synthesis, regenerating the plus-strand copy of the PBS (denoted by the uppercase PBS). Removal of the primer tRNA by the RNase H activity allows the plus-strand PBS to bind with the minus-strand copy of the viral PBS (lowercase pbs). Binding of the plus- and minus-strand PBSs (illustrated in box) facilitates the template transfer of the plus-strand strong-stop DNA to the 3′ end of the minus-strand cDNA (thin line), which is required for completion of full-length proviral DNA. U3, R, and U5, long terminal repeat. (B) Predicted base pairings of the plus- and minus-strand PBSs during the initial reverse transcription reaction of viral genomes containing an Ile+5 PBS. When $tRNA^{Ile}$ is used to initiate HIV-1 reverse transcription, the plus-strand copy of the PBS will be complementary to the 3′-terminal 18 nucleotides of $tRNA^{Ile}$. It is important to note that during the initial reverse transcription reaction, the minus-strand PBS will contain the complementary sequences of the mutated Ile+5 PBS as well as the 5-nucleotide insertion corresponding to the last 5 nucleotides of the full-length PBS complementary to $tRNA^{Ile}$, which are located downstream of this PBS (see Fig. 5). Although the first 9 nucleotides of the plus- and minus-strand PBSs form base pairs in a complementary manner, similar complementarity does not exist for the last 9. However, insertion of the 5 nucleotides (which are a cccct in the minus strand) provides the complementary sequences which can base pair with the last 5 nucleotides of the plus-strand PBS, allowing elongation of the plus strand by the RT to continue. Utilization of the 5 nucleotides to facilitate the second template transfer will cause a looping out of the intervening nucleotides including the 6 nucleotides (aacttt) between the mutated Ile+5 PBS and the 5-nucleotide insertion. Previous studies from this laboratory have validated this aspect of the model (43). The non-base paired nucleotides will be removed by the host cell DNA strand repair machinery after the full-length proviral DNA integrates into the host chromosome. (C) Predicted sequence of the Ile+5 proviral PBS region following reverse transcription host cell DNA strand repair. Repair of the minus strand will result in a provirus containing an authentic 18-nucleotide Ile PBS along with a deletion of the adjacent 6 nucleotides (TTGAAA) 3′ of the regenerated PBS.

allows identification of integrated proviral genomes which have been generated as a result of reverse transcription (Fig. 5). To extend our analysis, two additional mutant HIV-1 proviral genomes were constructed which contained PBSs predicted to be complementary to mammalian $tRNA^{His}$ and an *E. coli* $tRNA_2^{Gln}$, which are designated pHXB2(His+5) and pHXB2(Gln_2+5), respectively (Fig. 5). We were especially interested in the viral genomes with PBS complementary to $tRNA^{His}$, since this tRNA has been identified in viruses from chronically infected Vero cells (19). *E. coli* $tRNA_2^{Gln}$ has been shown in vitro to be used by the HIV-1 RT to prime DNA synthesis (21). However, we anticipate, that a proviral genome with a PBS complementary to *E. coli* $tRNA_2^{Gln}$ would be noninfectious because of the sequence differences in the 3′ acceptor stem between mammalian and *E. coli* $tRNA^{Gln}$.

In preliminary experiments, we again found that these mutations in the PBS did not affect the intracellular expression of the viral proteins. The amounts of p24 antigen in the supernatants of COS-1 cells transfected with the proviruses containing the mutant PBSs were similar to amounts found in COS-1 cells transfected with the wild-type genome (data not shown). Virus infection was assayed following coculture with SupT1 cells by measuring soluble p24 antigen levels in the supernatant. Although there was a delay compared with the wild type, infectious virus was recovered following transfection of pHXB2 (Ile+5) and pHXB2($Lys_{1,2}$+5). Interestingly, infectious virus was seen from the transfection of pHXB2(His+5), although its appearance was considerably delayed compared with the others. Infectious virus was not recovered from transfection of pHXB2(Gln_2+5), even after prolonged in vitro culture of as long as 3 months (Fig. 6).

To determine the status of the PBSs of the viruses derived from transfection of pHXB2(Ile+5), pHXB2($Lys_{1,2}$+5), and pHXB2(His+5), PCR was used to amplify the PBS regions from integrated proviruses. Sequence analysis of the PBS regions derived from Ile+5 viruses early in postcoculture (day 5) revealed that in 50% (8 of 16) of the recombinant M13 clones sequenced, a PBS complementary to $tRNA^{Ile}$ was regenerated with a 6-bp deletion 3′ of the PBS (Table 2). When proviral

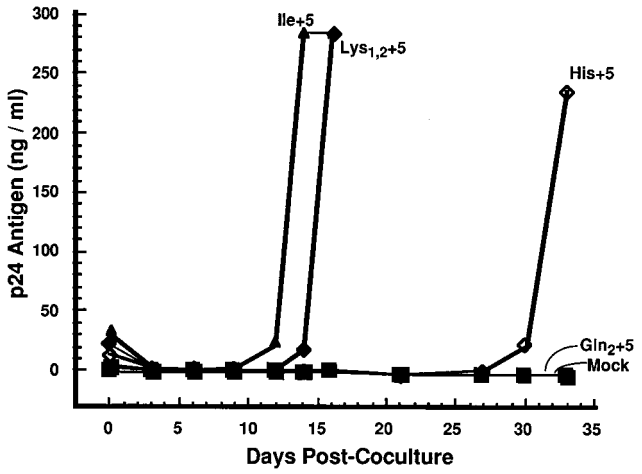


FIG. 6. Kinetics of the appearance of infectious virus. Proviral genomes containing mutant PBSs were transfected into COS-1 cells, which was followed 24 h later by coculture with SupT1 cells (5×10^5). After 48 h of coculturing, the SupT1 cells were isolated by low-speed centrifugation and were further cultured with fresh SupT1 and media (day 0). At designated times postcoculture, samples of supernatants were collected and analyzed for p24 antigen. The origins of the samples are as marked and refer to the initial mutations in the PBS.

PBSs that resulted from the reverse transcription of proviral genomes containing a $Lys_{1,2}$+5 PBS were sequenced, 8 of 14 proviruses had a PBS complementary to $tRNA^{Lys}_{1,2}$ along with a 6-bp deletion 3′ of the PBS (Table 3). Taken together, these results demonstrate that both $tRNA^{Ile}$ and $tRNA^{Lys}_{1,2}$ were used as primers to initiate HIV-1 reverse transcription, since regeneration of a complete 18-nucleotide PBS complementary to $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$ requires elongation from these alternate species. Even with the original PBS complementary to $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$, the virus was able to utilize $tRNA^{Lys}_3$ for initiation of reverse transcription, as evidenced by the isolation of PBS complementary to $tRNA^{Lys}_3$, which also contained the 6-bp deletion. Analysis of the proviruses after 10 and 12 days of in vitro virus culture revealed that the viruses containing the PBS complementary to $tRNA^{Lys}_3$ were predominant, indicating that the viruses containing the PBS complementary to $tRNA^{Lys}_3$ had an advantage in replication over viruses containing the PBS complementary to $tRNA^{Lys}_{1,2}$ or $tRNA^{Ile}$.

The virus derived from transfection of cells with pHXB2 (His+5) exhibited a strikingly different infection profile. Early in culture (prior to 25 days), the levels of virus were undetectable by the p24 antigen capture assay (Fig. 6). At day 30 of in vitro culture, the level of p24 antigen in the culture was higher than the background (uninfected SupT1 cells). Surprisingly, PCR amplification and DNA sequencing of the PBS revealed that a complete 18-nucleotide PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{His}$, along with a 6-nucleotide deletion adjacent 3′ to the PBS, was present at a higher frequency than the PBS complementary to $tRNA^{Lys}_3$ (Table 4). The results of this analysis clearly demonstrate that HIV-1 can utilize $tRNA^{His}$ in reverse transcription. By day 37 of culture, the levels of p24 antigen had increased substantially; PCR amplification and sequence analysis revealed that the PBSs of the viruses were now complementary to $tRNA^{Lys}_3$. The initial delayed appearance and slow virus growth appeared to be the result of the PBS being complementary to $tRNA^{His}$. Once $tRNA^{Lys}_3$ was utilized to initiate reverse transcription from the PBS complementary to $tRNA^{His}$, the revertant would contain a PBS complementary to $tRNA^{Lys}_3$ (generated from reverse transcription) resulting in a virus with wild-type replication kinetics.

DISCUSSION

In this study, we have determined whether changing the PBS of HIV-1 can result in viruses utilizing different tRNAs in reverse transcription. Proviral genomes were initially constructed which substituted the wild-type PBS with sequences complementary to the 3′-terminal 18 nucleotides of either $tRNA^{Ile}$ or $tRNA^{Lys}_{1,2}$, which are known to be present within virions expressed from COS cells (19). Transfection into COS-1 cells of these proviral genomes [pHXB(Ile) and pHXB($Lys_{1,2}$), respectively] resulted in the production of infectious virus competent to replicate in SupT1 cells. DNA sequence analysis of the PBS region PCR amplified from integrated proviruses revealed that the mutant PBSs were maintained early in infection, suggesting the use of $tRNA^{Ile}$ and $tRNA^{Lys}_{1,2}$ in HIV-1 reverse transcription. To confirm that alternate tRNAs could substitute for $tRNA^{Lys}_3$, proviral genomes were constructed which contained a mutated form of PBSs complementary to $tRNA^{Ile}$, $tRNA^{Lys}_{1,2}$, $tRNA^{His}$, or a $tRNA^{Gln}_2$ molecule. These proviral genomes were constructed such that if the specific tRNA had been used to prime initiation from these PBSs, in addition to regenerating the entire 18-nucleotide PBS complementary to the initiating tRNA, a 6-nucleotide deletion 3′ of the regenerated PBS would occur. Full-length PBSs com-

TABLE 2. Sequence analysis of the PBSs of HIV-1 proviruses derived from pHXB2(Ile+5)

Provirus sample	PBS sequence[a]	Frequency[b]
Input sequence (Ile+5)[c]	TGG TGG CCC TGC TGT CCT TTG AAA GGGGA GCG AAA GGG	
vHXB(Ile+5) (day 6 postcoculture)	TGG CGC CCG AAC AGG GAC ... ... .CG AAA GGG GAT CCA (Lys,3)	8/16
	TGG TGG CCC GTA CGG GGA ... ... GCG AAA GGG GAT CCA (Ile)	8/16
vHXB(Ile+5) (day 10 postcoculture)	TGG CGC CCG AAC AGG GAC ... ... .CG AAA GGG GAT CCA (Lys,3)	12/14
	TGG TGG CCC GTA CGG GGA ... ... GCG AAA GGG GAT CCA (Ile)	2/14

[a] Sequences of the PBS regions PCR amplified from genomic DNAs isolated from SupT1 cells infected with viruses derived from pHXB2(Ile+5). Note that the PBS sequence located in the square represents the first nine nucleotides that are complementary to the 3′-terminal nine nucleotides of $tRNA^{Ile}$. The five nucleotides inserted 3′ of the mutated PBS are underlined. Dots reflect the numbers and positions of the deleted nucleotides.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones.

[c] The input sequence refers to the initial mutation in the PBS region of pHXB2(Ile+5).

TABLE 3. Sequence analysis of the PBSs of HIV-1 proviruses derived from pHXB2($Lys_{1,2}$+5)

Provirus sample	PBS sequence[a]	Frequency[b]
Input sequence ($Lys_{1,2}$+5)[c]	TGG CGC CCA TGC TGT CCT TTG AAA GGGGC GCG AAA GGG	
vHXB($Lys_{1,2}$+5) (day 6 postcoculture)	TGG CGC CCG AAC AGG GAC ... ... GCG AAA GGG GAT CCA (Lys,3)	6/14
	TGG CGC CCA ACG TGG GGC ... ... GCG AAA GGG GAT CCA ($Lys_{1,2}$)	8/14
vHXB($Lys_{1,2}$+5) (day 12 postcoculture)	TGG CGC CCG AAC AGG GAC ... ... GCG AAA GGG GAT CCA (Lys,3)	14/16
	TGG CGC CCA ACG TGG GGC ... ... GCG AAA GGG GAT CCA ($Lys_{1,2}$)	2/16

[a] Sequences of the PBS regions PCR amplified from genomic DNAs isolated from SupT1 cells infected with viruses derived from pHXB2($Lys_{1,2}$+5). Note that the PBS sequence located in the square represents the first nine nucleotides that are complementary to the 3′-terminal nine nucleotides of $tRNA^{Lys}_{1,2}$. The five nucleotides inserted 3′ of the mutated PBS are underlined. Dots reflect the numbers and positions of the deleted nucleotides.
[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones.
[c] The input sequence refers to the initial mutation in the PBS region of pHXB2($Lys_{1,2}$+5).

plementary to either $tRNA^{Ile}$, $tRNA^{Lys}_{1,2}$, or $tRNA^{His}$ accompanied by the 6-bp deletion were detected in the proviral genomes, confirming the use of these alternate tRNAs in the initiation of HIV-1 reverse transcription. After extended culture, viruses were detected with wild-type PBS sequences complementary to $tRNA^{Lys}_3$.

The results of this study clearly demonstrate that HIV-1 can utilize tRNA primers other than $tRNA^{Lys}_3$ in reverse transcription. In one recent study, Li et al. mutated the PBS of HIV-1 such that it was complementary to that of human $tRNA^{Lys}_{1,2}$ or $tRNA^{Phe}$ (23). Transfection of the proviral genomes with these mutant PBSs gave rise to infectious virus with delayed kinetics of appearance compared with those of the wild type. We have extended their results by analyzing PBSs complementary to additional tRNAs, $tRNA^{Ile}$, and $tRNA^{His}$, which have been found to be present within HIV-1 virions (19). Viruses with PBSs complementary to $tRNA^{Lys}_{1,2}$, $tRNA^{Ile}$, and $tRNA^{His}$ were infectious. At early times postinfection, low levels of p24 antigen in the culture supernatant for the cultures infected with viruses with the substituted PBSs were seen. The cultures did not have the giant, multinucleated cells (syncytia) characteristic of wild-type infection of SupT1 cells. Sequence analysis of the proviral PBS region from high-molecular-weight DNA at this time revealed that the PBSs were complementary to the substitute primer (i.e., $tRNA^{Lys}_{1,2}$, $tRNA^{Ile}$, or $tRNA^{His}$). In our experimental design, we also made use of the fact that complementarity between the plus- and minus-strand copies of the PBS sequence is required for transfer of plus-strand strong-stop DNA to complete the synthesis of the full-length double-stranded provirus. By placing a 5-nucleotide sequence complementary to the last five nucleotides of the plus-strand PBS, we can facilitate this step in reverse transcription (43) (Fig. 4). Following reverse transcription and integration, the proviruses will contain a complete PBS complementary to the 3′-terminal sequences of the tRNA used to initiate minus-strand synthesis. The six nucleotides located 3′ of the minus-strand PBS, which are not base paired with the plus-strand PBS, would be removed by the host DNA repair mechanisms. Deletion of the 6 bp 3′ to the PBS, then, serves as a genetic marker to identify only PBSs generated as a result of reverse transcription. One of the important points of this study was that our results unequivocally demonstrate that the alternate tRNAs were used to initiate HIV-1 reverse transcription, since regeneration of the entire 18-nucleotide PBS sequences complementary to $tRNA^{Lys}_{1,2}$, $tRNA^{Ile}$, and $tRNA^{His}$, as well as the accompanying 6-nucleotide deletion, could be explained only by utilization of these respective tRNAs as primers for reverse transcription.

Another significant point is that it is clear that complete complementarity between the entire 18-nucleotide HIV-1 PBS and the 3′-terminal nucleotides of $tRNA^{Lys}_3$ was not necessary to initiate reverse transcription. Even with our initial constructions of the pHXB2($Lys_{1,2}$+5), pHXB2(Ile+5), and pHXB2(His+5) proviral clones, only the first 9 nucleotides of the PBSs were complementary to $tRNA^{Lys}_{1,2}$, $tRNA^{Ile}$, and $tRNA^{His}$, respectively. Since the first 3 nucleotides of each PBS are the same (TGG), our results show that the remaining 6 nucle-

TABLE 4. Sequence analysis of the PBSs of HIV-1 proviruses derived from pHXB2(His+5)

Provirus sample	PBS sequence[a]	Frequency[b]
Input sequence (His+5)[c]	TGG TGC CGT TGC TGT CCT TTG AAA CGGAT GCG AAA GGG	
vHXB(His+5) (day 30 postcoculture)	TGG TGC CGT GAC TCG GAT ... ... GCG AAA GGG GAT CCA (His)	19/24
	TGG CGC CCG AAC AGG GAC ... ... GCG AAA GGG GAT CCA (Lys,3)	5/24
vHXB(His+5) (day 37 postcoculture)	TGG CGC CCG AAC AGG GAC ... ... GCG AAA GGG GAT CCA (Lys,3)	10/10

[a] Sequences of the PBS regions PCR amplified from genomic DNA isolated from SupT1 cells infected with viruses derived from pHXB2(His+5). Note that the PBS sequence located in the square represents the first nine nucleotides that are complementary to the 3′-terminal nine nucleotides of $tRNA^{His}$. The five nucleotide inserted 3′ of the mutated PBS are underlined. Dots reflect the numbers and positions of the nucleotides deleted.
[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones.
[c] The input sequence refers to the initial mutation in the PBS region of pHXB2(His+5).

otides were sufficient for the utilization of these alternate tRNAs in the initiation of reverse transcription. Studies are ongoing to define the minimum number of complementary nucleotides required for the initiation event.

After extended culture times, we noted a rapid rise in the levels of p24 in the culture supernatant which correlated with an increase in syncytia and cytopathicity. Analysis of the PBS regions from proviruses which started with PBSs complementary to $tRNA^{Lys}_{1,2}$, $tRNA^{Ile}$, or $tRNA^{His}$ revealed the presence of the PBS complementary to $tRNA^{Lys}_3$. This means that for reversion to wild-type PBS, a $tRNA^{Lys}_3$ must have been used to prime reverse transcription from the PBSs complementary to either $tRNA^{Ile}$, $tRNA^{Lys}_{1,2}$, or $tRNA^{His}$. In order for this to happen, the 3′-terminal 18 nucleotides of $tRNA^{Lys}_3$ must bind to the different PBSs to initiate reverse transcription. This is in agreement with earlier studies which have demonstrated that $tRNA^{Lys}_3$ could prime reverse transcription from PBSs containing either deletions or nucleotide substitutions (13, 32, 43). It is possible that infectious proviruses could not be recovered from proviruses with a PBS complementary to $tRNA^{Gln}_2$ because there was not sufficient complementarity with $tRNA^{Lys}_3$. That is, including guanine-uridine base pairing (G-U), only 67% (12 of 18) of the nucleotides of the PBS complementary to $tRNA^{Gln}_2$ are complementary to the 3′-terminal nucleotides of $tRNA^{Lys}_3$. However, the interaction between tRNA and the PBS is undoubtedly complex, because the number of complementary nucleotides between $tRNA^{Lys}_3$ and the PBSs complementary to $tRNA^{Ile}$ and $tRNA^{His}$ is only slightly higher (14 of 18 [77%]). It is possible, then, that there are specific base pair interactions between the $tRNA^{Lys}_3$ and PBS that are required for the initiation of reverse transcription. Future studies will be required to identify which nucleotides in the HIV-1 PBS are important for the interaction with $tRNA^{Lys}_3$.

Finally, reversion of the PBSs back to wild-type PBS provides an explanation for why only PBSs complementary to $tRNA^{Lys}_3$ have been found in all of the HIV-1 proviruses identified to date. It appears that HIV-1 has evolved such that it preferentially selects and utilizes $tRNA^{Lys}_3$ in the initiation of reverse transcription. The reversion to a PBS complementary to $tRNA^{Lys}_3$ cannot be explained solely by the availability of the tRNA in the virion. Previous studies have shown that the levels of $tRNA^{Lys}_{1,2}$ and $tRNA^{Ile}$ are relatively high in HIV-1 virions expressed from COS cells; in fact, the levels of $tRNA^{Lys}_{1,2}$ are comparable to, if not greater than, those of $tRNA^{Lys}_3$ (19). It is possible, then, that regions of the HIV-1 viral RNA genome outside the PBS are important for efficient utilization of alternate tRNAs in reverse transcription. Previous studies of avian retroviruses suggested that maintenance of stem-loop structures involving the U5, PBS, and leader sequences was critical for efficient initiation of reverse transcription (1, 2, 8, 11, 12). It is also possible that sequences within the tRNA such as the TΨC (1) and the anticodon loops (21) are important for the proper positioning of the primer tRNA at the PBS. In support of this idea, a recent study by Isel et al., demonstrated in vitro that a region located upstream of the PBS, termed the A-rich loop, interacts with the $tRNA^{Lys}_3$ used in reverse transcription (18). Selectivity may also be mediated by viral proteins such as the HIV-1 RT, which has been shown in vitro to specifically bind $tRNA^{Lys}_3$ even in the presence of higher levels of competitor tRNAs (5–7, 9, 33, 35, 37, 44). Recent studies have suggested that primer tRNA selection may actually occur in the context of the Gag-Pol precursor protein (24). Thus, antiviral agents targeted to prevent interaction of $tRNA^{Lys}_3$ with virion proteins or disruption of the positioning of $tRNA^{Lys}_3$ at the PBS will likely significantly impair replication. Although it is clear that the implementation of these strategies will require further development, the results of the present studies provide a clear rationale to consider the tRNA-PBS interaction a target of strategies to inhibit HIV-1 replication.

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REFERENCES

1. **Aiyar, A., D. Cobrinik, Z. Ge, H.-J. Kung, and J. Leis.** 1992. Interaction between retroviral U5 tRNA and the TΨC loop of the $tRNA^{Trp}$ primer is required for efficient initiation of reverse transcription. J. Virol. **66:**2464–2472.
2. **Aiyar, A., Z. Ge, and J. Leis.** 1994. A specific orientation of RNA secondary structures is required for initiation of reverse transcription. J. Virol. **68:**611–618.
3. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64:**1920–1926.
4. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226:**1209–1211.
5. **Barat, C., S. F. J. Le Grice, and J.-L. Darlix.** 1991. Interaction of HIV-1 reverse transcriptase with a synthetic form of its replication primer, $tRNA^{lys,3}$. Nucleic Acids Res. **19:**751–757.
6. **Barat, C., V. Lullien, O. Schatz, G. Heith, M. T. Nugeyre, F. Gruninger-Leitch, F. Barre-Sinoussi, S. F. J. Le Grice, and J.-L. Darlix.** 1989. HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. EMBO J. **8:**3279–3285.
7. **Barat, C., O. Schatz, S. Le Grice, and J.-L. Darlix.** 1993. Analysis of the interactions of HIV1 replication primer $tRNA^{lys3}$ with nucleocapsid protein and reverse transcriptase. J. Mol. Biol. **231:**185–190.
8. **Baudin, F., R. Marquet, C. Isel, J.-L. Darlix, B. Ehresmann, and C. Ehresmann.** 1993. Functional sites in the 5′ region of human immunodeficiency virus type 1 RNA form defined structural domains. J. Mol. Biol. **229:**382–397.
9. **Bordier, B., L. Tarrago-Litvak, M.-L. Sallafranque-Andreola, D. Robert, D. Tharaud, M. Fournier, P. J. Barr, S. Litvak, and L. Sarih-Cottin.** 1990. Inhibition of the p66/p51 form of human immunodeficiency virus reverse transcriptase by $tRNA^{lys}$. Nucleic Acids Res. **18:**429–436.
10. **Cann, A. J., and J. Karn.** 1989. Molecular biology of HIV: new insights into the virus life cycle. AIDS **3(Suppl.):**S19–S34.
11. **Cobrinik, D., A. Aiyar, Z. Ge, M. Katzman, H. Huang, and J. Leis.** 1991. Overlapping retrovirus U5 sequence elements are required for efficient integration and initiation of reverse transcription. J. Virol. **65:**3864–3872.
12. **Cobrinik, D., L. Soskey, and J. Leis.** 1988. A retroviral RNA secondary structure required for efficient initiation of reverse transcription. J. Virol. **62:**3622–3630.
13. **Das, A. T., S. E. C. Koken, B. B. Oude-Essink, J. L. B. van Wamel, and B. Berkhout.** 1994. Human immunodeficiency virus uses $tRNA^{Lys,3}$ as primer for reverse transcription in HeLa-$CD4^+$ cells. FEBS Lett. **341:**49–53.
14. **Erikson, E., and R. L. Erikson.** 1971. Association of 4S RNA with oncornavirus RNA. J. Virol. **8:**254–256.
15. **Faras, A. J., A. C. Gerapin, W. E. Levinson, J. M. Bishop, and H. M. Goodman.** 1973. Characterization of the low-molecular weight RNAs associated with the 70S RNA of Rous sarcoma virus. J. Virol. **12:**334–342.
16. **Gilboa, E., S. W. Mitra, S. Goff, and D. Baltimore.** 1979. A detailed model of reverse transcription and tests of crucial aspects. Cell **18:**93–100.
17. **Harada, F., G. G. Peters, and J. E. Dahlberg.** 1979. The primer tRNA for Moloney murine leukemia virus DNA synthesis: nucleotide sequence and aminoacylation of $tRNA^{Pro}$. J. Biol. Chem. **21:**10979–10985.
18. **Isel, C., R. Marquet, G. Keith, C. Ehresmann, and B. Ehresmann.** 1993. Modified nucleotides of $tRNA^{Lys,3}$ modulate primer/template loop-loop interaction in the initiation complex of HIV-1. J. Biol. Chem. **268:**25269–25272.
19. **Jiang, M., J. Mak, A. Ladha, E. Cohen, M. Klein, B. Rovinski, and L. Kleiman.** 1993. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. J. Virol. **67:**3246–3253.
20. **Jiang, M., J. Mak, M. A. Wainberg, M. A. Parniak, E. Cohen, and L. Kleiman.** 1992. Variable tRNA content in $HIV\text{-}1_{IIIB}$. Biochem. Biophys. Res. Commun. **185:**1005–1015.

21. **Kohlstaedt, L. A., and T. A. Steitz.** 1992. Reverse transcriptase of human immunodeficiency virus can use either human $tRNA^{Lys,3}$ or *Escherichia coli* $tRNA^{Gln,2}$ as a primer in an *in vitro* primer-utilization assay. Proc. Natl. Acad. Sci. USA **89:**9652–9656.
22. **Lever, A., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. J. Virol. **63:**4085–4087.
23. **Li, X., J. Mak, E. J. Arts, Z. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak.** 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. J. Virol. **68:**6198–6206.
24. **Mak, J., M. Jiang, M. A. Wainberg, M.-L. Hammarskjöld, D. Rekosh, and L. Kleiman.** 1994. Role of $Pr160^{gag\text{-}pol}$ in mediating the selective incorporation of $tRNA^{Lys}$ into human immunodeficiency virus type 1 particles. J. Virol. **68:**2065–2072.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Nagashunmugam, T., A. Velpandi, C. S. Goldsmith, S. R. Zaki, V. S. Kalyanaraman, and A. Srinivasan.** 1992. Mutations in the primer binding site of the type 1 human immunodeficiency virus genome affects virus production and infectivity. Proc. Natl. Acad. Sci. USA **89:**4114–4118.
27. **Peters, G., F. Harada, J. E. Dahlberg, A. Panet, W. A. Haseltine, and D. Baltimore.** 1977. Low-molecular-weight RNAs of Moloney murine leukemia virus: identification of the primer for RNA-directed DNA synthesis. J. Virol. **21:**1031–1041.
28. **Peters, G. G., and C. Glover.** 1980. tRNAs and priming of RNA-directed DNA synthesis in mouse mammary tumor virus. J. Virol. **35:**31–40.
29. **Raba, M., K. Limburg, M. Burghagen, J. R. Katze, M. Simsek, J. E. Heckman, U. L. Rajbhandary, and H. J. Gross.** 1979. Nucleotide sequence of three isoaccepting lysine tRNAs from rabbit liver and SV40-transformed mouse fibroblasts. Eur. J. Biochem. **97:**305–318.
30. **Ratner, L., A. Fisher, L. Linda, J. Agodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3:**57–69.
31. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
32. **Rhim, H., J. Park, and C. D. Morrow.** 1991. Deletions in the $tRNA^{Lys}$ primer-binding site of human immunodeficiency virus type 1 identify essential regions for reverse transcription. J. Virol. **65:**4555–4564.
33. **Robert, D., M.-L. Sallafranque-Andreola, B. Bordier, L. Sarih-Cottin, L. Tarrago-Litvak, P. V. Graves, P. J. Barr, M. Fournier, and S. Litvak.** 1990. Interactions with $tRNA^{lys}$ induce important structural changes in human immunodeficiency virus reverse transcriptase. FEBS Lett. **277:**239–242.
34. **Rosenthal, L. J., and P. C. Zamecnik.** 1973. Amino acid acceptor activity of the "70S-associated" 4S RNA from avian myeloblastosis virus. Proc. Natl. Acad. Sci. USA **70:**1184–1185.
35. **Sallafranque-Andreola, M. L., D. Robert, P. J. Barr, M. Fournier, S. Litvak, L. Sarih-Cottin, and L. Tarrago-Litvak.** 1989. HIV RT expressed in transformed yeast cells. Biochemical properties and interactions with bovine $tRNA^{Lys}$. Eur. J. Biochem. **184:**367–374.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
37. **Sarih-Cottin, L., B. Bordier, K. Musier-Forsyth, M.-L. Andreola, P. J. Barr, and S. Litvak.** 1992. Preferential interaction of human immunodeficiency virus reverse transcriptase with two regions of primer $tRNA^{lys}$ as evidenced by footprinting studies and inhibition with synthetic oligoribonucleotides. J. Mol. Biol. **226:**1–6.
38. **Taylor, J. M.** 1977. An analysis of the role of tRNA species as primer for transcription into DNA of RNA tumor virus genomes. Biochim. Biophys. Acta **473:**57–71.
39. **Taylor, J. M., and T. W. Hsu.** 1980. Reverse transcription of avian sarcoma virus RNA into DNA might involve copying of the tRNA primer. J. Virol. **33:**531–534.
40. **Temin, H.** 1981. Structure, variation and synthesis of retrovirus long terminal repeat. Cell **27:**1–3.
41. **Temin, H. M., and S. Mitzutani.** 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226:**1211–1213.
42. **Varmus, H., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses: RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Wakefield, J. K., H. Rhim, and C. D. Morrow.** 1994. Minimal sequence requirements of a functional human immunodeficiency virus type 1 primer binding site. J. Virol. **68:**1605–1614.
44. **Weiss, S., B. Konig, H.-J. Muller, H. Seidel, and R. S. Goody.** 1992. Synthetic human $tRNA^{Lys,3}$ (UUU) and natural bovine $tRNA^{Lys,3}$ (SUU) interact with HIV-1 reverse transcriptase and serve as specific primers for retroviral cDNA synthesis. Gene **111:**183–197.