

## Identification of a Sequence within U5 Required for Human Immunodeficiency Virus Type 1 To Stably Maintain a Primer Binding Site Complementary to tRNA<sup>Met</sup>

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**Initiation of reverse transcription of human immunodeficiency virus type 1 (HIV-1) occurs by extension from the 3' end of a cellular tRNA complexed to the primer binding site (PBS) located near the 5' end of the viral RNA genome. Although the PBSs for all naturally occurring HIV-1 viruses are complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup>, we identified an HIV-1 virus which contained a PBS complementary to the 3' nucleotides of tRNA<sup>Met</sup>; the PBS of this virus was not stable upon extended culture and reverted back to the wild type (S.-M. Kang, J. K. Wakefield, and C. D. Morrow, *Virology* 222:401–414, 1996). To further characterize the virus with a PBS complementary to tRNA<sup>Met</sup>, a DNA fragment encompassing the PBS and U5 region from this proviral genome was substituted for the same region in the infectious HIV-1 proviral clone [named pHXB2(AC-Met)]. Three additional proviral genomes were also created: pHXB2(Met), which is isogenic with pHXB2 except for the PBS complementary to tRNA<sup>Met</sup>; pHXB2(Met-AC-Met), which contains the PBS sequence complementary to the 3'-terminal nucleotides and the sequence upstream of this PBS in U5 complementary to the anticodon region of tRNA<sup>Met</sup>; and pHXB2(Met-C-Met), which contains two G-to-C changes predicted to disrupt complementarity within the tRNA<sup>Met</sup> anticodon region. Viruses derived from the transfection of these proviral genomes were infectious, although the appearance of the viruses was delayed compared to that of the wild-type virus. PCR amplification and DNA sequence analysis of the PBS regions from proviral genomes revealed that the PBSs from viruses derived from pHXB2(Met) and pHXB2(AC-Met) reverted back to the wild type by days 16 and 44 postcoculture, respectively. Two new, novel mutant viruses were identified among viruses derived from pHXB2(Met-C-Met) at day 35 postcoculture: one contained a PBS complementary to tRNA<sup>Lys</sup>, while the second maintained a PBS complementary to tRNA<sup>Met</sup> but contained a 26-nucleotide deletion in U5 upstream of the anticodon-complementary region. By day 125 postcoculture, the PBS in the virus from this culture had reverted back to the wild type, complementary to tRNA<sup>Lys</sup>. In contrast, the viruses derived from pHXB2(Met-AC-Met) stably maintained a PBS complementary to tRNA<sup>Met</sup> during the 125-day culture period examined. The results of these studies support the idea that HIV-1 can maintain a PBS complementary to alternative tRNAs provided that the appropriate complementarity exists between the U5-PBS region of the viral RNA genome and the tRNA molecule used to initiate reverse transcription.**

An essential step in retrovirus replication is the reverse transcription of the viral RNA genome into a DNA intermediate prior to integration into the host cell chromosome. The initiation of reverse transcription occurs near the 5' end of the viral genome, designated the primer binding site (PBS), which is complementary to the 3'-terminal 18 nucleotides of a cellular tRNA used as a primer. The reverse transcription is carried out by a virally encoded enzyme, reverse transcriptase (RT), which extends from the 3'OH of the tRNA primer bound to the PBS and copies the viral RNA genome to generate a double-stranded DNA copy of the viral genome (3, 5, 20, 21, 29, 31). During the synthesis of plus-strand DNA, the PBS is generated when RT copies the 3'-terminal 18 nucleotides of the tRNA primer (30). As a consequence, the PBS in the integrated provirus genome reflects the 3'-terminal 18 nucleotides of the tRNA used to initiate reverse transcription.

For a given retrovirus, the tRNA used to initiate reverse transcription is highly conserved. For example, human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus uses tRNA<sup>Lys</sup>, Mason-Pfizer monkey virus, visna virus, and spumavirus use tRNA<sup>Lys</sup>, murine leukemia virus uses tRNA<sup>Pro</sup>, and avian leukosis virus (ALV) uses tRNA<sup>Trp</sup> (19).

The mechanism by which the tRNA is selected from the cellular milieu is unknown. Previous results suggest that the RT of the Gag-Pol precursor protein in HIV-1 mediates the selection of tRNAs that are packaged into virus particles (14, 17). However, the selective incorporation of the tRNA which is used as the primer does not completely explain the use of a single tRNA to initiate reverse transcription. For example, HIV-1 incorporates into virions tRNA<sup>Lys</sup> and tRNA<sup>Lys</sup> as major tRNA species, but only tRNA<sup>Lys</sup> is preferentially used to initiate reverse transcription (12). Early on, it was thought that complementarity between the PBS and the 3'-terminal nucleotides of a tRNA might determine the use of the tRNA to initiate reverse transcription. HIV-1 and ALV viruses containing mutant PBSs corresponding to the 3'-terminal 18 nucleotides of alternative tRNAs could utilize different tRNAs to initiate reverse transcription (4, 16, 34, 35). However, a common feature of those studies was that viruses with PBSs complementary to alternate tRNAs were not stable, and the PBSs of these viruses reverted back to the wild-type PBS upon extended culture. Thus, it was suggested that regions of the viral genome outside the PBS sequence are involved in the preferential use of the wild-type primer for the initiation of reverse transcription in retroviruses (1, 2).

Using enzyme and chemical probes, Isel et al. defined additional regions in U5 of the HIV-1 viral RNA genome, which

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## B

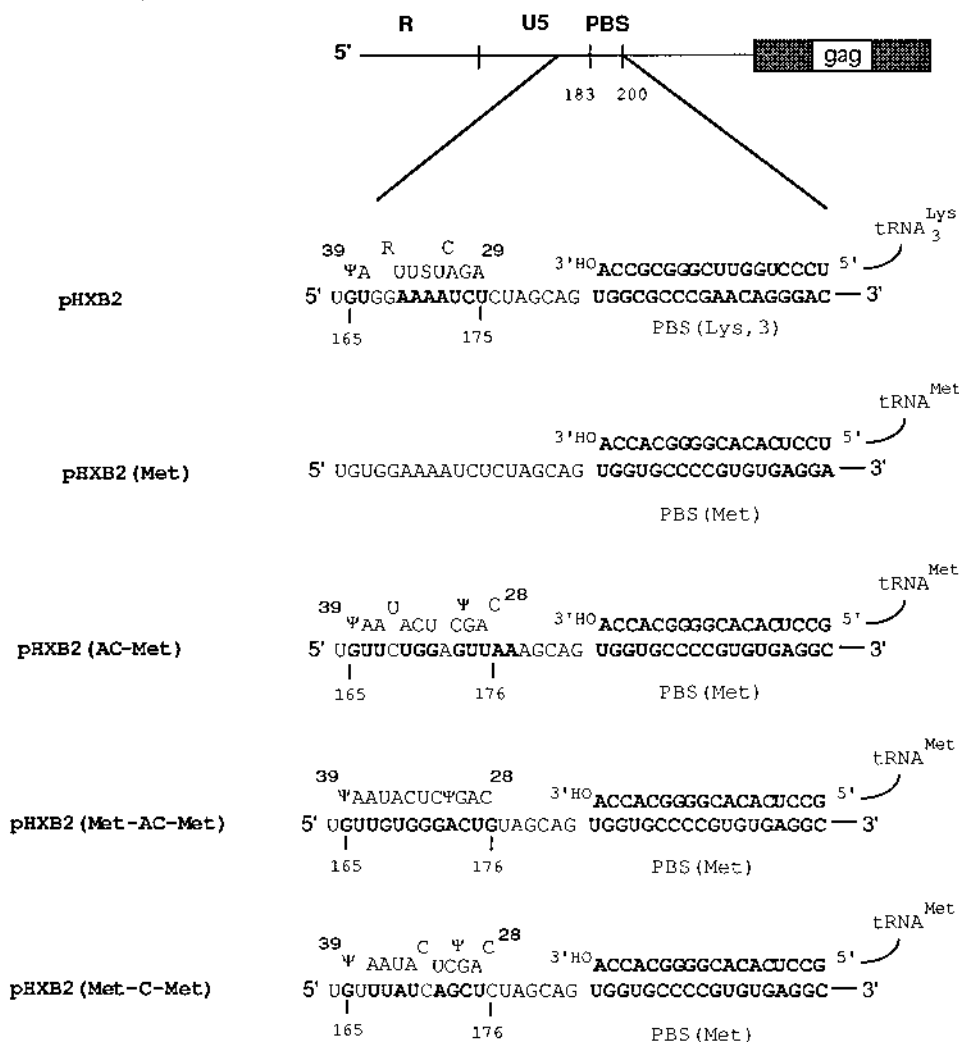


FIG. 1—Continued.

pHXB2 (23, 24) was used to make mutant HIV-1 proviral genomes. To construct pHXB2(AC-Met), a DNA fragment encompassing the U5 and PBS regions was PCR amplified from an integrated provirus derived from the transfection of a proviral genome containing a PBS and the sequences complementary to the 3'-terminal nucleotides and anticodon loop of tRNA<sup>Lys</sup>, respectively (13). For subcloning, an *AflIII*-*Bss*HII DNA fragment (nucleotides 64 to 255) was first cloned into a shuttle vector, pUC119(PBS), which contains an *HpaI*-to-*PstI* DNA fragment including the 5' long terminal repeat, PBS, and leader region of the *gag* gene from pHXB2 (25); the resulting clone was named pUC119(AC-Met). The complete DNA sequence of the *AflIII*-*Bss*HII fragment was determined by sequencing (26). pUC119(AC-Met) was used for the subsequent preparation of pUC119(Met-AC-Met) and pUC119(Met-C-Met); pUC119(Met) was prepared from pUC119(PBS) (25). Site-directed mutagenesis was carried out by two consecutive PCRs (27). Megaprimers containing mutant sequences were produced by the first PCR with a primer in the U3 region of the 5' long terminal repeat (5'-TTGACAGCCGCTAGC [nucleotides 8895 to 8910]) and a mutagenic oligonucleotide. The oligonucleotides for the mutants are as follows: pHXB2(Met), 5'-TCGCTTTCAATCCTCACACGGGGACCACTGCTAG-3'; pHXB2(Met-AC-Met), 5'-ACCACTGCTACAGTCCCACAACACTGACTAA A-3'; and pHXB2(Met-C-Met), 5'-ACCACTGCTAGAGCTGATAAACAACACTG ACTAAAA-3'. The second PCR was performed to generate restriction enzyme recognition sites for cloning into pUC119(PBS) by using a megaprimer containing mutant sequence and a primer downstream of the PBS (5'-GCGCGCTC AGCAAGCCG-3' [nucleotides 262 to 245]). These mutagenic DNA fragments were digested with *Bgl*II and *Bss*HII and were cloned into pUC119(PBS). All constructs were sequenced to verify that only the desired mutations in U5 and

the PBS were present. Each of the pUC119 plasmid constructs containing the mutations was digested with *HpaI* and *Bss*HII, resulting in an 868-bp fragment encompassing the U5 and PBS. The 868-bp fragment was subcloned between the *HpaI* and *Bss*HII sites of pHXB2, and the resulting mutant proviral plasmids were screened by restriction digestions and again verified by DNA sequencing (26).

**DNA transfections and analysis of proviral gene expression.** Transfection of proviral plasmid DNA into COS-1 cells by using DEAE-dextran was carried out as previously described (34). After 48 h posttransfection, the supernatant was collected and filtered through a 0.45- $\mu$ m-pore-size syringe filter (Nalgene). The levels of p24 antigen were determined from two independent transfections by enzyme-linked immunosorbent assay (ELISA) (Coulter Laboratories).

**Analysis of virus infectivity.** To test for virus replication and infectivity, the various proviral constructs were transfected into COS-1 cells, which was followed 24 h later by coculture with SupT1 cells ( $5 \times 10^5$ ), which support high-level replication of HIV-1. After 48 h, the SupT1 cells were harvested by low-speed centrifugation ( $1,000 \times g$ ) and further cultured with fresh medium and additional SupT1 cells. The infected SupT1 cells were maintained by splitting 1:6 every 3 days. For cell-free infections at days 27, 35, 44, and 61 postcoculture, SupT1 cells ( $10^6$  cells per ml) were infected with equal amount of virus as measured by p24 antigen. After the virus was allowed to adsorb for 24 h, SupT1 cells were pelleted ( $1,000 \times g$ ), washed with phosphate-buffered saline (pH 7.0), and further cultured. SupT1 cultures were monitored visually for the formation of multicell syncytia and at various time intervals were refed with SupT1 cells and medium. At the designated time intervals, samples of the culture supernatant were collected and analyzed for p24 antigen by ELISA (Coulter Laboratories).

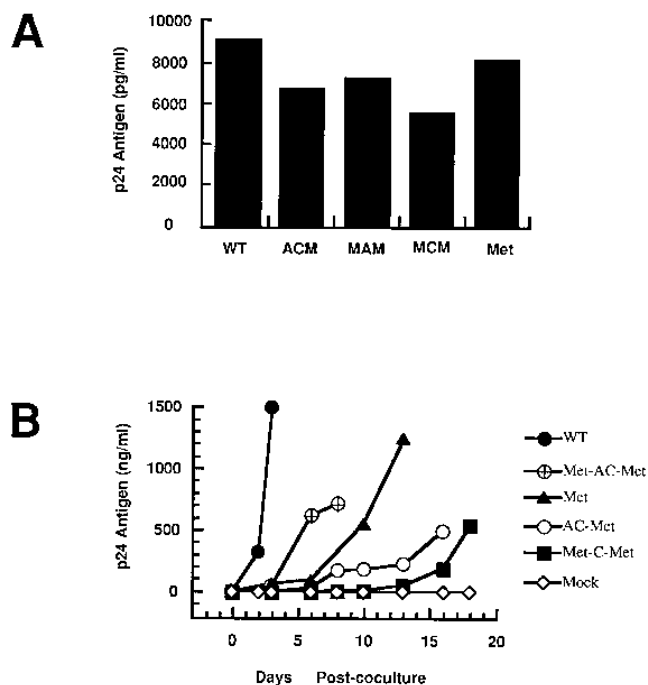


FIG. 2. Protein expression and infectivities of proviruses with mutations in U5 and the PBS. (A) Release of virus from COS-1 cells transfected with the wild-type and mutant proviral genomes. COS-1 cells were transfected with the designated plasmids. At 48 h posttransfection, the culture supernatants were analyzed for released virus particles by quantitation of p24 antigen by ELISA (Coulter). The bar graph illustrates the mean values of p24 antigen detected from two independent transfections. WT, wild-type pHXB2; ACM, pHXB2(AC-Met); MAM, pHXB2(Met-AC-Met); MCM, pHXB2(Met-C-Met); Met, pHXB2(Met). (B) Kinetics of the appearance of infectious virus derived from transfection of wild-type and mutant proviral genomes. The plasmids containing the wild-type and mutant proviral genomes were transfected into COS-1 cells; this was followed 24 h later by coculture with SupT1 cells ( $5 \times 10^5$ ). After 48 h of coculturing, the SupT1 cells were then isolated by centrifugation, washed once, and further cultured with more uninfected SupT1 cells and fresh medium (day 0). At various intervals postcoculture, culture supernatants were collected and the p24 antigen was quantitated by ELISA (Coulter).

**PCR amplification and DNA sequencing of PBS-containing proviral DNA.** Analysis of the proviral PBS sequences from infected SupT1 cells was carried out by using minor modifications of protocols previously described (33). Briefly, at the designated days postcoculture, genomic DNA was isolated from infected SupT1 cells by using the Wizard genomic DNA purification kit according to the manufacturer's instruction manual (Promega). Approximately 2  $\mu$ g of cellular DNA was used to amplify integrated proviral DNA sequences comprising the U5 and PBS regions of the provirus genome by using two HIV-1-specific oligonucleotide primers, as follows: primer 1, 5'-GCTCTAGACCAGATCTGAGCCTGGGAGCTC-3' (nucleotides 17 to 38); and primer 2, 5'-CGGAATTCCTCC TTCTAGCCTCCGCTAGTC-3' (nucleotides 309 to 330) (the underlined nucleotides indicate the additional sequences at the 5' end which correspond to *Xba*I and *Eco*RI restriction endonuclease sites for subcloning of PCR products). PCR-amplified DNA was digested with *Xba*I and *Eco*RI and then subcloned between the *Xba*I and *Eco*RI sites present in the polylinker region of pUC119 DNA or was directly ligated into the pCRII vector (Invitrogen). Following ligation, the DNA was transformed into competent *Escherichia coli* (DH5 $\alpha$ ) and plated on 1.5% Luria-Bertani agar medium containing isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (U.S. Biochemical). Following identification of colorless clones, the DNAs prepared from individual recombinant clones were sequenced by using the primer 5'-GGCTAACTAGGGAACCCACTGC-3' (nucleotides 42 to 63) (26).

## RESULTS

**Construction of HIV-1 proviral genomes with U5 and PBS mutations.** In a previous study, we described the construction of a mutant proviral genome, pHXB2(Trp-AC), which contains a PBS complementary to the 3'-terminal 18 nucleotides

of tRNA<sup>Trp</sup> and an 8-nucleotide substitution at nucleotides 167 to 175 of the HIV-1 genome (<sup>167</sup>UCUGGAGU<sup>175</sup>) complementary to the anticodon loop of tRNA<sup>Trp</sup> (13). Analysis of the PBS sequences from proviral genomes derived from the transfection of pHXB2(Trp-AC) revealed that the majority, but not all, were complementary to a completely unexpected tRNA, tRNA<sup>Met</sup>, at 27 days following infection of SupT1 cells. After further in vitro culture, though, we found that the PBSs of the viruses derived from pHXB2(Trp-AC) reverted back to be complementary to tRNA<sup>Lys</sup>. It was not possible to discern the sequence of the reversion back to the wild-type PBS; that is, it was possible that the PBS complementary to tRNA<sup>Trp</sup> first mutated to be complementary to tRNA<sup>Met</sup> and then subsequently reverted to a PBS complementary to tRNA<sup>Lys</sup>. If this was the case, we would expect the proviruses containing a PBS complementary to tRNA<sup>Met</sup> to be unstable. Alternatively, the PBS complementary to tRNA<sup>Trp</sup> might revert so as to be complementary to either tRNA<sup>Met</sup> or tRNA<sup>Lys</sup>; if this was the case, we would expect that proviruses with a PBS complementary to tRNA<sup>Met</sup> might be stable. To address this question, a DNA fragment encompassing nucleotides 64 to 255 from the provirus with a PBS complementary to tRNA<sup>Met</sup> was cloned into an infectious clone of HIV-1, pHXB2; this new construct, referred to as pHXB2(AC-Met), contains a complete HIV-1 provirus which has a PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Met</sup>, an 8-nucleotide sequence (UCUGGAGU) (nucleotides 167 to 174) complementary to the anticodon loop of tRNA<sup>Trp</sup>, and two additional nucleotide mutations (<sup>176</sup>AA<sup>177</sup>) between the PBS and the anticodon-complementary sequence (Fig. 1B).

Previous studies have shown that mutating a PBS complementary to alternate tRNAs was not sufficient to produce a virus capable of maintaining the use of alternate tRNAs over multiple rounds of retrovirus replication (4, 16, 34, 35). To determine if this was also the case for a PBS complementary to tRNA<sup>Met</sup>, site-directed mutagenesis was used to replace the wild-type PBS with sequences complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Met</sup> [pHXB2(Met) (Fig. 1B)]. Since the anticodon-complementary sequence in pHXB2(AC-Met) does not have base complementarity with the anticodon region of tRNA<sup>Met</sup>, an additional mutation in pHXB2(AC-Met) was made so that the corresponding sequence complementary to the anticodon region of tRNA<sup>Met</sup> was present; the resulting construct was named pHXB2(Met-AC-Met) (Fig. 1B). Note that to avoid the insertion of a new AUG codon within the HIV-1 genome, the mutation was made to substitute a guanine for the adenine (GUG instead of AUG); complementarity with the anticodon region of tRNA<sup>Met</sup> would still be facilitated by virtue of a G · U base pair (28). Finally, to investigate the effect that a disruption of the complementarity between the sequence in U5 and the anticodon region of tRNA might have on the maintenance of a substituted PBS, pHXB2(Met-C-Met) was constructed, in which nucleotides cytosines 171 and 176 within this region (<sup>167</sup>UUAUCAGCUC<sup>176</sup>) were substituted for guanines; this mutation would disrupt the potential base pair with cytosines in the anticodon loop (3'-<sup>38</sup>AAUACUC $\Psi$ GAC<sup>28</sup>-5') of tRNA<sup>Met</sup> (underlined nucleotides) (Fig. 1B).

**Replication potential of mutant proviral genomes.** Since the 5' region of the HIV-1 genome contains many *cis*-acting elements, we wanted to determine whether the mutations in U5 and the PBS would have any effect on expression of HIV-1 proteins and on release of virions into medium upon transfection. Plasmids containing the HIV-1 wild-type and mutant proviral genomes were transfected into COS-1 cells, followed by analysis of the culture supernatants for p24 antigen (Fig. 2A). Taking into consideration the variability inherent with trans-

TABLE 1. Partial sequences of the U5 and PBS regions of pHXB2(Met) mutant proviruses isolated after coculture

Day post-coculture	Sequence <sup>a</sup>													Frequency <sup>b</sup>	
	ACS						PBS								
0 <sup>c</sup>	5'-AGATCCCT	CAGACCCTTT	TAGTCAGT	GTGG <b>AAAA</b>	TCTCTA	GCAG	<b>TGG</b>	<b>TGC</b>	<b>CCC</b>	<b>GTG</b>	<b>TGA</b>	<b>GGA</b>	TTGAAAGCG-3'		
16	5'-*****	*****	*****	*****	*****	****	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***<sup>d</sup></b>	*****-3'	1/8	
	5'-*****	*****	*****	*****	*****	****	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***<sup>e</sup></b>	*****-3'	2/8	
	5'-*****	*****	*****	*****	*****	****	<b>TGG</b>	<b>CGCCCCA</b>	<b>ACG</b>	<b>TGC</b>	<b>GGC<sup>f</sup></b>	*****-3'	1/8		
	5'-*****	*****	*****	*****	*****	****	<b>*T**</b>	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC<sup>f</sup></b>	*****-3'	1/8
	5'-*****	*****	*****	*****	*****	****	<b>*A</b>	<b>TGG</b>	<b>CGCCCCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC<sup>g</sup></b>	*****-3'	1/8	
	<b>TGG CGC CCG AAC AGG GAC ACAAATCTCTAGCAA<sup>h</sup></b>														
	5'-*****	*****	*****	*****	*****	****	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***</b>	<b>***<sup>i</sup></b>	*****-3'	2/8	
35	5'-*****	*****	*****	*****	*****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC<sup>j</sup></b>	*****-3'	6/7	
	5'-*****	*****	*****	*****	*****	****	<b>*T**</b>	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	1/7

<sup>a</sup> Asterisks indicate identity with the input sequence (day 0); boldface indicates nucleotides in ACS or PBS; dots indicate deletions. ACS, nucleotides located in the A-rich loop region in the HXB2 HIV-1 clone.

<sup>b</sup> Frequency of the DNA sequence of the PBS region obtained from independent clones.

<sup>c</sup> The sequence is the input sequence with the initial mutations in the PBS region (PBS complementary to tRNA<sup>Met</sup>).

<sup>d</sup> One nucleotide difference from the input PBS complementary to tRNA<sup>Met</sup> (implying the presence of isoacceptor for tRNA<sup>Met</sup>).

<sup>e</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup> (with one nucleotide insertion).

<sup>f</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup>.

<sup>g</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup> (with one nucleotide insertion).

<sup>h</sup> Insertion of PBS complementary to tRNA<sup>Lys</sup> and duplicated nucleotides (upstream sequence of PBS) before the PBS complementary to tRNA<sup>Met</sup>.

<sup>i</sup> One nucleotide deletion during reversion from PBS complementary to tRNA<sup>Lys</sup> to PBS complementary to tRNA<sup>Lys</sup>.

fection efficiencies, no significant difference between the amounts of p24 antigen released from cultures transfected with different viral genomes and the wild type was observed.

To determine the infectious potential of each of the mutant proviral genomes, transfected COS-1 cells were cocultured with SupT1 cells, which are susceptible to HIV-1 infection. At various times postcoculture, replication of both the wild-type and mutant viruses was monitored by visual inspection for syncytium formation and quantitated by levels of viral capsid (p24) antigen in the supernatants from the cultures (Fig. 2B). All of the viruses derived from mutant proviral genomes demonstrated a delay in virus production compared with that of the wild-type virus. The levels of supernatant p24 from viruses derived from pHXB2(Met) rapidly increased from day 10 post-coculture, reaching a level similar to that from wild-type virus at approximately 13 days postcoculture. The results of these studies demonstrate that the proviruses with the mutations in U5 and the PBS all gave infectious virus upon transfection, although the appearance of the viruses was delayed compared to that of the wild-type virus.

**Analysis of the PBSs from viruses derived from pHXB2(Met) and pHXB2(AC-Met).** Since the PBS is generated when the first 18 nucleotides of the primer tRNA are copied during plus-strand strong-stop viral DNA synthesis of reverse transcription, analysis of the proviral PBS sequences will determine which tRNA species was used to initiate reverse transcription. We first analyzed the proviruses which were isogenic with HXB2 except for the PBS which was complementary to tRNA<sup>Met</sup> [pHXB2(Met)]. The viruses derived from pHXB2(Met) at day 10 postcoculture maintained a PBS complementary to the 3' nucleotides of tRNA<sup>Met</sup>, although additional single-nucleotide substitutions were found around the PBS (data not shown). By 13 days postcoculture, the level of p24 antigen increased greatly (Fig. 2B). Analysis of PBS sequences from proviruses at day 16 revealed that the viruses were heterogeneous (Table 1); two clones in which the PBS reverted back to be complementary to tRNA<sup>Lys</sup> were identified. Two

other clones contained a PBS complementary to tRNA<sup>Lys</sup> inserted upstream of a PBS complementary to tRNA<sup>Met</sup>, with 15 nucleotides of additional intervening sequence between the dual PBSs. We previously found proviruses with dual PBSs from viruses derived from a mutant proviral genome where the 3' regions of U5 and the PBS were mutated to be complementary to the anticodon region and the 3'-terminal 18 nucleotides of tRNA<sup>Pro</sup>, respectively (13); it is possible that this insertion occurred as a result of aberrant template switching during reverse transcription (8). Additional analysis revealed a PBS complementary to tRNA<sup>Lys</sup>. By 35 days postcoculture, though, all of the PBS clones isolated contained a wild-type PBS complementary to tRNA<sup>Lys</sup>.

In contrast to viruses derived from pHXB2(Met), viruses derived from pHXB2(AC-Met) contained the PBS complementary to tRNA<sup>Met</sup> at 16 days postcoculture. Most of the PBSs analyzed maintained an original input sequence upstream of the PBS; two clones had mutations just upstream or downstream of the PBS (data not shown). By day 35 postcoculture, 6 of the 11 clones analyzed maintained a PBS complementary to tRNA<sup>Met</sup> (Table 2). A point mutation was found at nucleotide 201 (G to A) downstream of the PBS in all clones. Four of six clones with a PBS complementary to tRNA<sup>Met</sup> had an additional mutation at nucleotide 171 (G to A) within the anticodon region-complementary sequence. Interestingly, the other clones (5 of 11) had a PBS complementary to tRNA<sup>Lys</sup> rather than tRNA<sup>Lys</sup>. Since we did not detect a PBS complementary to tRNA<sup>Lys</sup> by day 35, we wanted to compare the replication of the virus with that of the wild type. Culture supernatants at day 35 were collected, and equal amounts of virus as measured by the level of p24 antigen were used to infect SupT1 cells (Fig. 3A). The viruses still grew slowly compared to the wild-type virus, although the delay was not as long as that observed at earlier times postcoculture (Fig. 2B). Since tRNA<sup>Lys</sup> species were reported to be encapsidated into HIV-1 virion particles (12, 14), we wanted to determine if the revertant viruses with a PBS complementary to tRNA<sup>Lys</sup> were sta-

TABLE 2. Partial sequences of the U5 and PBS regions of pHXB2(AC-Met) mutant proviruses isolated after coculture

Day postcoculture	Sequence <sup>a</sup>														Frequency <sup>b</sup>
	ACS							PBS							
0 <sup>c</sup>	5'-AGAGATCCCT	CAGACCCTTT	TAGTCAGTGT	<b>TCTGGAGT</b>	<b>TAAA</b>	GCAG	<b>TGG</b>	<b>TGC</b>	<b>CCC</b>	<b>GTG</b>	<b>TGA</b>	<b>GGC</b>	GAAAGCG-3'		
35	5'-*****	*****	*****	*****	****	****	***	***	***	***	***	***	A*****-3'	2/11	
	5'-*****	*****	*****	***** <b>A</b> ***	****	****	***	***	***	***	***	***	A*****-3'	3/11	
	5'-*****	*****	*****	***** <b>A</b> ***	****	****	***	***	***	***	***	***	A*****-3'	1/11	
	5'-*****	*****	*****	*****	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b> <sup>c</sup>	A*****-3'	5/11	
44	5'-*****	*****	*****	*****	****	****	***	***	***	***	***	***	*****-3'	1/11	
	5'-*****	*****	***** <b>Δ</b> *	*****	****	****	***	***	***	***	***	***	A*****-3'	1/11	
	5'-*****	*****	*****	*****	****	****	<b>A</b> **	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11
	5'-*****	*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	***** <b>A</b> *	***** <b>A</b> ***	****	<b>A</b> ***	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	*****	***** <b>Δ</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	*****	***** <b>A</b> ***	****	<b>A</b> ***	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	***** <b>Δ</b> *	***** <b>A</b> ***	****	<b>A</b> **T	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCA</b>	<b>ACG</b>	<b>TGG</b>	<b>GGC</b>	A*****-3'	1/11	
	5'-*****	*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b> <sup>d</sup>	A*****-3'	1/11	
	5'-*****	*****	***** <b>G</b> **	*****	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	A*****-3'	1/11	
	61	5'-*****	*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	8/15
5'-*****		*****	*****	***** <b>A</b> ***	<b>A</b> ***	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	2/15	
5'-*****		*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	A*****-3'	1/15	
5'-*****		*****	*****	***** <b>A</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	1/15	
5'-*****		*****	*****	***** <b>AA</b> ***	****	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	1/15	
5'-*** <b>A</b> *****		*****	*****	***** <b>A</b> ***	<b>A</b> ***	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	1/15	
5'-*****		*****	<b>A</b> *****	***** <b>A</b> ***	<b>A</b> ***	****	<b>TGG</b>	<b>CGC</b>	<b>CCG</b>	<b>AAC</b>	<b>AGG</b>	<b>GAC</b>	*****-3'	1/11	

<sup>a</sup> Asterisks indicate identity with the input sequence (day 0); boldface indicates nucleotides in ACS or PBS; the dot indicates a deletion. ACS, anticodon-complementary sequences located in the 3' region of U5 which were made complementary to the anticodon loop of tRNA<sup>Met</sup>.

<sup>b</sup> Frequency of the DNA sequence of the PBS region obtained from independent clones.

<sup>c</sup> The sequence is the input sequence with the initial mutations in the PBS region (PBS complementary to tRNA<sup>Met</sup>).

<sup>d</sup> One nucleotide difference from the input PBS complementary to tRNA<sup>Met</sup> (implying the presence of isoacceptor tRNA<sup>Met</sup>).

<sup>e</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup><sub>35</sub>.

<sup>f</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sup>Lys</sup><sub>5</sub>.

ble. By day 44 postcoculture, we identified PBSs in 2 of 11 clones complementary to tRNA<sup>Lys</sup><sub>35</sub>; a variety of mutations upstream of PBSs in most clones were also observed. Only two clones which maintained a PBS complementary to tRNA<sup>Met</sup> were found. The remaining clones contained a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub>, accompanying adenine substitution mutations within the anticodon loop-complementary sequence (<sup>169</sup>T to A and <sup>171</sup>G to A). By day 61 postcoculture, all of the clones analyzed contained the wild-type PBS and also acquired the G-to-A substitution at nucleotide 171. As might be expected, viruses analyzed at this time replicated similarly to the wild-type virus (Fig. 3B).

**Analysis of PBSs of viruses derived from pHXB2(Met-AC-Met) and pHXB2(Met-C-Met).** The delay in the reversion of the PBSs of viruses derived from pHXB2(AC-Met) to wild type convinced us to construct proviruses with modified U5 regions that might enhance the stability of the PBS. The most logical choice would be to construct a provirus with both the anticodon-complementary sequence in U5 and a PBS complementary to the corresponding region of tRNA<sup>Met</sup> [pHXB2(Met-AC-Met)]. The appearance of viruses derived from transfection of pHXB2(Met-AC-Met) was faster than that of the other mutant viruses (Fig. 2B). In all of the clones analyzed from day 16 postcoculture, the anticodon-complementary region and PBS were complementary to tRNA<sup>Met</sup> (Table 3). A single clone was found to have a T-to-A change at nucleotide 169 within the anticodon region-complementary sequence. Analysis at day 35 postcoculture revealed that all clones still

maintained a PBS complementary to tRNA<sup>Met</sup>. Two additional mutations were observed most commonly: the T-to-A change at nucleotide 169 and a G-to-A change at nucleotide 201 just downstream of the PBS. We also isolated a single clone containing a substitution mutation of C to T at nucleotide 145, which is 38 nucleotides upstream of the PBS. The appearance of this virus was a little slower than that of the wild type when analyzed with samples from day 35 postcocultures (Fig. 3A). Surprisingly, it was found that all of the clones isolated at days 44 and 61 still maintained a PBS complementary to tRNA<sup>Met</sup>. A substitution of C to T at nucleotide 145 was predominant at days 44 (7 of 12 clones) and 61 (15 of 18 clones). Two other prevalent point mutations were clear at day 61: a G-to-A change at nucleotide 171 (12 of 18 clones) within the anticodon region-complementary sequence and a G-to-A change at nucleotide 201 (15 of 18 clones) just downstream of the PBS. Viruses isolated at this time replicated similarly to the wild-type virus (Fig. 3B). Finally, analysis of the PBSs from the viruses derived from pHXB2(Met-AC-Met) at 125 days postcoculture revealed that the PBS was still complementary to tRNA<sup>Met</sup> (Table 3). Interestingly, no single dominant clone was recovered; all of the clones had mutations in U5. Several adenine substitution mutations were found at the positions further upstream of the PBS (<sup>140</sup>G, <sup>161</sup>C, and <sup>179</sup>G to A). Furthermore, clones which contained a 9-nucleotide deletion (<sup>135</sup>AACTAGAGA<sup>141</sup>) at the 42-nucleotide upstream position of the PBS were recovered (5 of 12 clones). Taken together, the results of these experiments demonstrate that mutation in

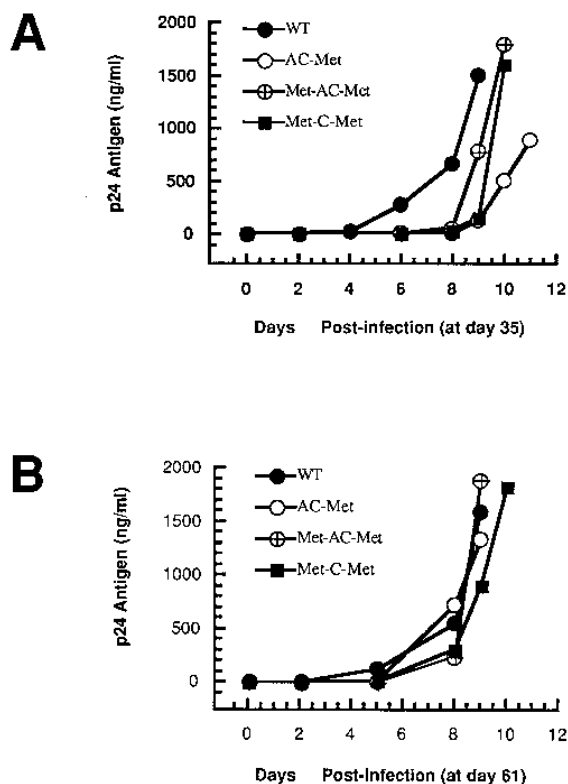


FIG. 3. Replication of the infectious virus. SupT1 cells were infected with equal amounts (20 ng/ml) of virus isolated after 35 (A) and 61 (B) days of coculture. At the designated times postinfection, supernatants were removed and assayed for p24 antigen. The initial mutations in the PBS region are indicated.

nucleotides in U5 to be complementary to the anticodon region of tRNA<sup>Met</sup> resulted in a virus which stably maintained a PBS complementary to tRNA<sup>Met</sup>.

To further understand the role that the U5 sequences played in the virus maintaining a PBS complementary to tRNA<sup>Met</sup>, we analyzed the effects that a change within the anticodon-complementary sequence would have on maintenance of the PBS. We altered nucleotides from G to C, which would disrupt base pairing with the anticodon region of tRNA<sup>Met</sup> [pHXB2(Met-C-Met)] (Fig. 1B). The appearance of viruses derived from transfection of pHXB2(Met-C-Met) was slower than that of the other mutant viruses. By day 16 postcoculture, all clones analyzed maintained a PBS complementary to tRNA<sup>Met</sup>, although none of the clones contained the exact input sequence. Additional mutations were found around the 3' end of U5; a single substitution mutation at nucleotide 180 (C to T or A) was prominent. Two striking changes were observed in the clones isolated at 35 days postcoculture (Table 4). One was a 26-nucleotide deletion just upstream of the anticodon-complementary region in U5. All clones having this deletion (4 of 11) maintained a PBS complementary to tRNA<sup>Met</sup> and also had the same point mutation as at day 16 at nucleotide 180 (C to T or A). The other change was that in 4 of 11 clones the PBS was complementary to tRNA<sup>Lys</sup>. Additional mutations of one or two nucleotides (C to A at nucleotide 180 or GC to AA at nucleotides 179 and 180) in the clones with the PBS complementary to tRNA<sup>Lys</sup> were found. Three clones analyzed at this time maintained a PBS complementary to tRNA<sup>Met</sup> without deletion of nucleotides in U5 and contained the same 2-nucleotide substitutions (GC to AA) as those in clones with a PBS

complementary to tRNA<sup>Lys</sup>. Analysis of the virus replication at day 35 revealed that the production of p24 antigen in the culture supernatant from virus derived from pHXB2(Met-C-Met) was delayed compared to that from the wild-type virus (Fig. 3A).

To further examine the stabilities of the PBSs of these viruses derived from pHXB2(Met-C-Met), we analyzed the PBS sequences at days 44 and 61 (Table 4). No reversion to a PBS complementary to tRNA<sup>Lys</sup> was detected. Most of the clones with the deletions in U5 and the PBS complementary to tRNA<sup>Met</sup> contained mutations of C to A at nucleotide 180 and G to A at nucleotide 201. In contrast, the clones with a PBS complementary to tRNA<sup>Lys</sup> were heterogeneous within U5, containing several substitution mutations within the anticodon region-complementary sequence and around the PBS at day 61. Interestingly, we did find that a GC-to-AA mutation at nucleotides 179 and 180 occurred in the viruses with a PBS complementary to tRNA<sup>Lys</sup>, which resulted in a stretch of four adenine nucleotides (nucleotides 178 to 181); one of these mutations (C to A at nucleotide 180) was within the integration motif (CA, nucleotides 180 and 181), which is known to be required for integration of proviral DNA into the chromosome (15). Since the nucleotides within the anticodon-complementary sequence in pHXB2(Met-C-Met) are not complementary to the anticodon loops of both tRNA<sup>Lys</sup> and tRNA<sup>Met</sup>, we wanted to determine the stability of the PBS after further extended *in vitro* culture. As might be expected, then, by 125 days postcoculture all of the PBSs recovered were wild-type PBSs complementary to tRNA<sup>Lys</sup> (Table 4). Additional mutations within the anticodon-complementary sequence in U5 (a G-to-A change at nucleotide 173) and a G-to-C change at the 3' end of the PBS were also found. The results of these studies demonstrate that mutations within the anticodon-complementary region in U5 influence the stability of the maintenance of the PBS complementary to tRNA<sup>Met</sup>.

## DISCUSSION

In this study, we have investigated the effect that mutations in the U5 region have on the selection of the tRNA primer used to initiate reverse transcription. For these studies, we reconstructed a series of HIV-1 proviral genomes in which the PBS was complementary to tRNA<sup>Met</sup>. The choice of tRNA<sup>Met</sup> was based on a previous study in which we found HIV-1 viruses which contained a PBS complementary to tRNA<sup>Met</sup> (13). Our starting construct contained a DNA fragment (nucleotides 64 to 255) from a mutant provirus containing a PBS complementary to tRNA<sup>Met</sup>, which was found in a virus culture derived from transfection of a plasmid containing a provirus with a PBS and an anticodon-complementary region complementary to tRNA<sup>Trp</sup> (13). HIV-1 proviral genomes containing a PBS complementary to tRNA<sup>Met</sup> [pHXB2(Met)] as well as proviral genomes with mutant sequences located in U5 which are complementary to the anticodon loop of tRNA<sup>Met</sup> [pHXB2(Met-AC-Met) and pHXB2(Met-C-Met)] were also created. Transfection of each of these constructs into cells resulted in the production of infectious virus. A DNA sequence analysis of the U5 and PBS regions from integrated provirus genomes revealed that some viruses derived from pHXB2(Met) reverted to have a wild-type PBS by day 16 postcoculture. In contrast, the viruses derived from pHXB2(AC-Met), pHXB2(Met-AC-Met), and pHXB2(Met-C-Met) still maintained a PBS complementary to tRNA<sup>Met</sup> at this time. Upon extended culture, though, only the virus derived from pHXB2(Met-AC-Met) maintained a PBS complementary to tRNA<sup>Met</sup>.

The results of our study are unique for several reasons. First,





TABLE 4. Partial sequences of the U5 and PBS regions of pHXB2(Met-C-Met) mutant proviruses isolated after coculture

Day postco-culture	Sequence <sup>a</sup>	ACS	PBS	Frequency <sup>b</sup>	
0 <sup>c</sup>	5'-TGTGACTCTGGTAACTAGAGATCCCTCAGACCC <sup>d</sup> TTT <sup>e</sup> TAGTCAGTGT	<b>TTATCAG</b>	CTCTAGCAG <b>TGGTGCCCCGTGTGAGGC</b>	GAA-3'	
16	5'.....	<b>*****</b>	<b>*****T**</b>	<b>*****</b> ***-3'	7/11
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> ***-3'	2/11
	5'.....	<b>*****</b>	<b>T***AT**</b>	<b>*****</b> ***-3'	2/11
35	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>*****</b> ***-3'	1/11
	5'.....	<b>*****</b>	<b>**A*AA**</b>	<b>*****</b> A*-3'	1/11
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>*****</b> A*-3'	1/11
	5'.....	<b>*****</b>	<b>*****T**</b>	<b>*****</b> ***-3'	1/11
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> A*-3'	3/11
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC<sup>f</sup></b> ***-3'	1/11
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	2/11
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/11
44	5'.....	<b>***A***</b>	<b>T***AA**</b>	<b>*****</b> ***-3'	1/10
	5'.....	<b>*****</b>	<b>*****T**</b>	<b>*****A*</b> ***-3'	1/10
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> ***-3'	1/10
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> A*-3'	1/10
	5'.....	<b>***T**</b>	<b>***AT**</b>	<b>*****</b> ***-3'	1/10
			TAGAAG <sup>g</sup> ↓		
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> ***-3'	1/10
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	3/10
	5'.....	<b>*****</b>	<b>***GA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/10
61	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****</b> A*-3'	3/13
	5'.....	<b>*****</b>	<b>*****A**</b>	<b>*****A</b> A*-3'	3/13
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> ***-3'	1/13
	5'.....	<b>*****A</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> ***-3'	1/13
	5'.....	<b>A*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/13
	5'.....	<b>A*****A</b>	<b>A***AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/13
	5'.....	<b>A**G***A</b>	<b>A***AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> ***-3'	1/13
	5'.....	<b>A**G**G*A</b>	<b>A***AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/13
		↓			
	5'.....	<b>*****</b>	<b>*****AA**</b>	<b>TGGCGCCCAACGTGGGGC</b> A*-3'	1/13
125	5'..... <b>A</b> .....	<b>*****A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC<sup>h</sup>C</b> ***-3'	1/11
	5'.....	<b>*****A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> ***-3'	1/11
	5'.....	<b>*****A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>*A***A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> ***-3'	1/11
	5'.....	<b>*****A</b>	<b>T***AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>*****A</b>	<b>T***AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>*****A</b>	<b>T***AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>A***A*A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>A***T**</b>	<b>T***AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'.....	<b>AA**T*A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> C*-3'	1/11
	5'..... <b>A</b> .....	<b>*****A</b>	<b>*****AA**</b>	<b>TGGCGCCCGAACAGGGAC</b> ***-3'	1/11

<sup>a</sup> Asterisks indicate identity with the input sequence (day 0); boldface indicates nucleotides in ACS or PBS; dots indicate deletions. ACS, anticodon-complementary sequence located in the 3' region of U5 which would be predicted to disrupt the complementarity with the anticodon loop of tRNA<sup>Met</sup>.  
<sup>b</sup> Frequency of the DNA sequence of the PBS region obtained from independent clones.  
<sup>c</sup> The sequence is the input sequence with the initial mutations in the PBS region (PBS complementary to tRNA<sup>Met</sup>).  
<sup>d</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sub>3</sub><sup>Met</sup>.  
<sup>e</sup> One nucleotide difference from the input PBS complementary to tRNA<sup>Met</sup> (implying the presence of isoacceptor for tRNA<sup>Met</sup>).  
<sup>f</sup> Nucleotides inserted (duplicated nucleotides upstream of PBS).  
<sup>g</sup> PBS complementary to the 3'-terminal 18 nucleotides of tRNA<sub>3</sub><sup>Met</sup>.

for a role for this region in the selection of the tRNA to initiate reverse transcription. Viruses derived from pHXB2(AC-Met) and pHXB2(Met-C-Met), which had nucleotides in the 3' region of U5 that would disrupt base pairing with the anticodon region of tRNA<sup>Met</sup>, did not stably maintain a PBS complementary to tRNA<sup>Met</sup>. Third, although the PBS in the virus derived from pHXB2(Met-AC-Met) was stable, it appears that the

virus was still evolving after 125 days postcoculture. Additional mutations in and around the anticodon region-complementary sequence were observed in proviral clones derived from pHXB2(Met-AC-Met) after extended culture days. For example, a point mutation (T to A at nucleotide 169) in the region complementary to the anticodon region of tRNA<sup>Met</sup> was identified in a single clone at day 16. The number of clones with this

mutation increased at day 35 (6 of 13 clones); however, this mutation was found only in a single clone at day 61. Instead, point mutations at nucleotide 145 (C to T) 38 nucleotides further upstream of PBS at day 35 and at nucleotide 171 (G to A) within the anticodon-complementary sequence at day 44 became predominant by 61 days in culture. Coincidentally, at this time we found that viruses with a PBS complementary to tRNA<sup>Met</sup> replicated similarly to the wild-type virus as measured by p24 antigen in the culture (Fig. 3B). It is not clear if these additional nucleotide changes in U5 provided an advantage for viruses to use tRNA<sup>Met</sup> as a primer. Previous studies with ALV and HIV-1 reported that the genomic RNA from PBS mutant virions with slower growth early in culture was associated with less occupancy of the corresponding tRNA primer on the mutant PBS compared with that of the wild type (4, 35). In our case, it is possible that the nucleotide changes result in a virus which could utilize tRNA<sup>Met</sup> more effectively to initiate reverse transcription. In support of this idea, previous studies by Isel et al. have demonstrated a complex structure between the U5-PBS and tRNA<sup>Lys</sup> primer, extending the interactions of tRNA<sup>Lys</sup> with viral sequences 39 nucleotides upstream of the PBS (10); interestingly, the C-to-T mutation at nucleotide 145 found predominantly at the later culture times is located at this position. Additional studies will be required to delineate the interaction between the U5-PBS and tRNA<sup>Met</sup>.

Further evidence for a complex RNA structure at the 5' end of the RNA genome required for selection of tRNA<sup>Met</sup> and maintenance of the PBS complementary to tRNA<sup>Met</sup> comes from the fact that the viruses derived from transfection of pHXB2(AC-Met) reverted back to a wild-type PBS. The presence of a sequence in U5 complementary to the anticodon loop of tRNA<sup>Trp</sup>, then, did not stabilize the maintenance of a PBS complementary to tRNA<sup>Met</sup>. Thus, the reversion to a PBS complementary to tRNA<sup>Lys</sup> by the virus with the anticodon-complementary sequence in U5 and a PBS complementary to tRNA<sup>Trp</sup> was most probably through a virus which contained a PBS complementary to tRNA<sup>Met</sup>. The fact that the viruses with the sequence in U5 complementary to the anticodon loop of tRNA<sup>Trp</sup> and a PBS complementary to tRNA<sup>Met</sup> had additional mutations resulting in a stretch of three adenine nucleotides [nucleotides 176 to 178 in pHXB2(AC-Met)] upstream of the PBS might have facilitated the reversion to wild type. That is, even subtle mutations within the anticodon-complementary region influence the maintenance of an alternate PBS, as evidenced from the analysis of the viruses derived from pHXB2(Met-C-Met). In this case, the virus reverted to contain a PBS complementary to tRNA<sup>Lys</sup> or, even more dramatically, resulted in a provirus which contained a deletion of 26 nucleotides upstream of the anticodon-complementary region. The fact that few nucleotide changes could initiate a cascade of mutations resulting in the virus utilizing a different PBS is, to our knowledge, without precedence. Our results lend support to a growing appreciation that a complex RNA secondary structure which influences both the selection of the tRNA primer and initiation of reverse transcription exists in the 5' end of the viral genome. This region of secondary structure could, in fact, be extensive, since a recent study demonstrated that a single base pair change in the TAR region influences minus-stranded DNA synthesis (7).

One of the more interesting results of this study was the generation of a virus containing a PBS complementary to tRNA<sup>Lys</sup> as found in the day 35 cultures derived from pHXB2(Met-C-Met) and pHXB2(AC-Met). Again, the use of tRNA<sup>Lys</sup> is not without precedence, since tRNA<sup>Lys</sup> is the primer for Mason-Pfizer monkey virus, visna virus, and spumaviruses (19). More importantly, analysis of HIV-1 virions

revealed that tRNA<sup>Lys</sup><sub>1,2</sub> is packaged at levels comparable to those for tRNA<sup>Lys</sup><sub>3</sub> (12). Previous studies from this as well as other laboratories, though, have demonstrated that viruses with a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> rapidly reverted back to wild type after limited in vitro culture (16, 34). We observed that viruses derived from pHXB2(AC-Met) contained a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> at day 35, but by day 61 the viruses contained only a PBS complementary to tRNA<sup>Lys</sup><sub>3</sub>. Some of viruses derived from pHXB2(Met-C-Met) maintained a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> for over 61 days, although the PBS eventually reverted back to wild type. It is not clear why the viruses with a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> derived from pHXB2(Met-C-Met) were delayed in the reversion to a wild-type PBS. One of the major differences between the viruses with a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> derived from pHXB2(AC-Met) and pHXB2(Met-C-Met) can be found within the U5 region. In the case of viruses from pHXB2(AC-Met), we noted the substitution of adenine nucleotides within the anticodon-complementary region, in some cases, creating a substitution of three adenines similar to the wild-type sequence. In contrast, the viruses with a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> derived from pHXB2(Met-C-Met) had substitution of GC to AA at nucleotides 179 and 180, which created a stretch of four adenine nucleotides. These base changes in combination with the anticodon-complementary sequence in U5 might have delayed reversion of the PBS of viruses derived from pHXB2(Met-C-Met) to wild type. The fact that the virus derived from pHXB2(Met-C-Met) could maintain a PBS complementary to tRNA<sup>Lys</sup><sub>1,2</sub> for the extended time in culture is surprising. How the virus discriminates between tRNA<sup>Lys</sup><sub>1,2</sub> and tRNA<sup>Lys</sup><sub>3</sub>, which are known to be encapsidated into the HIV-1 virions in similar amounts (12), is a major question. The stability of additional tRNA-RNA template interactions might depend on particular base modifications found only in some tRNAs (9–11). The sequence comparison between tRNA<sup>Lys</sup><sub>1,2</sub> and tRNA<sup>Lys</sup><sub>3</sub> shows that a single base differs in the anticodon loop, two base pairs (tRNA<sup>Lys</sup><sub>1</sub>) or a single base pair (tRNA<sup>Lys</sup><sub>2</sub>) differs in the anticodon stem, and two base pairs and a single base differ in the T $\Psi$ C stem-loop; there is no difference in the D stem-loop (22). It is possible that these sequence differences between two tRNAs might allow the virus to discriminate tRNA<sup>Lys</sup><sub>1,2</sub> from tRNA<sup>Lys</sup><sub>3</sub>. Further analysis of these mutant viruses will help to delineate the complex interactions during the initiation of reverse transcription.

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