JOURNAL OF VIROLOGY, Feb. 1996, p. 966–975 Vol. 70, No. 2
0022-538X/96/$04.00+0

Construction of a Type 1 Human Immunodeficiency Virus That Maintains a Primer Binding Site Complementary to $tRNA^{His}$

JOHN K. WAKEFIELD, SANG-MOO KANG, AND CASEY D. MORROW*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 21 September 1995/Accepted 2 November 1995

The initiation of human immunodeficiency virus type 1 reverse transcription occurs by extension of a $tRNA_3^{Lys}$ primer bound near the 5′ end of the viral RNA genome which is designated the primer binding site (PBS). Sequences within the viral genome upstream of the PBS which are complementary to the anticodon loop (USUU) and the TΨC loop and arm ($AGGGT_mΨ$) of $tRNA_3^{Lys}$ are postulated to play a role in maintaining the selective use of $tRNA_3^{Lys}$ in reverse transcription. To investigate this possibility, proviral genomes which contain a PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{His}$ [pHXB2(His)] as well as sequences upstream of this PBS which are complementary to either the anticodon loop [CCACAA; pHXB2(His-AC)] or TΨC loop [GACCGAGG; pHXB2(His-TΨC)] of $tRNA^{His}$ were constructed. Infectious virus was recovered upon transfection into COS-1 cells of pHXB2(His), pHXB2(His-AC), or pHXB2(His-TΨC). The appearance of infectious virus after cocultivation with SupT1 cells was delayed for the proviruses containing a PBS complementary to $tRNA^{His}$ compared with that obtained by transfection of the wild-type provirus [pHXB2(WT)]. However, by several passages in SupT1 cells, the mutant viruses demonstrated replication kinetics similar to those of the wild-type virus. A DNA sequence analysis of the PBS region from integrated proviruses revealed that by day 15 of culture, the PBS of viruses derived from pHXB2(His) and pHXB2(His-TΨC) reverted back to the wild-type PBS complementary to $tRNA_3^{Lys}$. In contrast, viruses derived from pHXB2(His-AC) maintained a PBS complementary to $tRNA^{His}$ for over 4 months in culture encompassing 12 serial passages. This study, then, is the first report of a stable human immunodeficiency virus type 1 which utilizes an alternative tRNA primer and suggests that interactions between the primer tRNA anticodon loop and viral sequences upstream of the PBS contribute to the specificity of the tRNA primer used in reverse transcription.

A distinguishing feature of retrovirus replication is the conversion of a single-stranded RNA genome into a double-stranded DNA intermediate prior to integration into the host cell chromosome. This process, which is referred to as reverse transcription, is carried out by the virally encoded enzyme reverse transcriptase (RT), which utilizes a cellular tRNA bound to the primer binding site (PBS) as a primer (2, 33). The PBS is an 18-nucleotide sequence located near the 5′ end of the viral RNA genome that is complementary to the 3′-terminal 18 nucleotides of the primer tRNA. Following initiation of reverse transcription by extension of the 3′ OH of the primer tRNA, a complex process of plus- and minus-strand translocation, or "jumping," allows the RT to copy the viral RNA genome. Two copies of the PBS are generated during reverse transcription; a minus-strand copy which is the complementary sequence of the PBS originally present within the RNA genome and a plus-strand copy resulting from the copying by the RT of the 3′-terminal 18 nucleotides of the primer tRNA (9, 19, 20, 30, 31, 32, 34). As a consequence of this feature of retrovirus replication, a PBS sequence complementary to the 3′-terminal 18 nucleotides of the tRNA used to initiate reverse transcription is represented in the integrated provirus.

Within a given group of retroviruses, both the PBS and the tRNA primer used in reverse transcription are highly conserved (reviewed in reference 7). For example, avian sarcoma and leukosis viruses use $tRNA^{Trp}$ to initiate reverse transcription, whereas most of the mammalian tumor-inducing retroviruses such as murine leukemia viruses (MLVs) and the human T-cell leukemia viruses utilize $tRNA^{Pro}$. Both human immunodeficiency virus (HIV) and simian immunodeficiency virus as well as mouse mammary tumor virus utilize $tRNA_3^{Lys}$, while Mason Pfizer monkey virus, Visna virus, and spumavirus use $tRNA_{1,2}^{Lys}$ to initiate reverse transcription. The exclusive selection by retroviruses of the tRNA primers used to initiate reverse transcription has been the subject of several recent studies. Colicelli and Goff (7) isolated a replication-competent recombinant MLV that contained a PBS complementary to $tRNA^{Gln}$, suggesting that this virus replicated by means of a $tRNA^{Gln}$ primer. Lund et al. (16) provided further evidence for the utilization of a different primer tRNA in MLV replication by demonstrating the efficient transduction of an Akv MLV-based vector containing a PBS corresponding to $tRNA_1^{Gln}$, $tRNA_2^{Gln}$, or $tRNA_3^{Lys}$. Our laboratory and others have demonstrated that HIV type 1 (HIV-1) can use several different tRNAs to initiate reverse transcription if the PBS is made complementary to the respective tRNA (8, 15, 36). However, the viruses with PBSs complementary to these alternate tRNAs were not stable, and the PBS reverted back to wild-type PBS complementary to $tRNA_3^{Lys}$. Thus, it was concluded that complementarity between the primer tRNA and the PBS is not the sole determinant for the preferential use of $tRNA_3^{Lys}$ in HIV-1 reverse transcription.

The mechanism by which $tRNA_3^{Lys}$ is preferentially selected by HIV-1 for the initiation of reverse transcription is unknown. Previous studies have demonstrated by in vitro analysis that the HIV-1 RT specifically binds $tRNA_3^{Lys}$ in the presence of excess amounts of nonspecific tRNAs (3–6, 25, 27, 29, 37). The interaction between $tRNA_3^{Lys}$ and the RT, most likely in the context of the Gag-Pol polyprotein precursor, is also believed to contribute to the incorporation of $tRNA_3^{Lys}$ into the virion during particle assembly (17). However, tRNAs other than $tRNA_3^{Lys}$ have been shown to be incorporated into HIV-1 virions at levels similar to that of $tRNA_3^{Lys}$ (12, 13). Therefore, the se-

* Corresponding author. Phone: (205) 934-5705. Fax: (205) 934-1580.

lective use of tRNA$_3^{Lys}$ in reverse transcription cannot be explained solely by an exclusive presence of tRNA$_3^{Lys}$ in the virion. The interaction of tRNA$_3^{Lys}$ with sequences of the viral RNA outside the PBS might be a factor in the selective use of tRNA$_3^{Lys}$. For example, in avian sarcoma and leukosis virus, complementary base pairing between the retroviral U5 RNA and the TΨC loop and arm of the tRNA^{Trp} replication primer has been shown in vitro to be required for the efficient initiation of reverse transcription (1). Sequences complementary to the TΨC loop and arm of tRNA$_3^{Lys}$ are present in the U5 region of the HIV-1 viral RNA genome. A recent study using in vitro assays has provided evidence that a sequence in the HIV-1 U5 region just upstream of the PBS consisting of 4 adenine residues binds to the 4 uridine residues found in the anticodon loop of tRNA$_3^{Lys}$ (10, 11). These nucleotides have been predicted to be positioned within a loop structure which has been referred to as the A-rich loop (10, 11).

In the present study, we have tested whether mutations of sequences in the HIV-1 RNA genome that are complementary to the anticodon and TΨC loop of tRNA$_3^{Lys}$ affect the utilization of an alternative primer tRNA in HIV-1 reverse transcription. Since we previously demonstrated that a provirus with a PBS complementary to tRNA^{His} was infectious (36), we constructed additional mutant proviral genomes in which sequences complementary to the anticodon or TΨC loop of a tRNA^{His} molecule were also present upstream of the PBS complementary to tRNA^{His}. The PBSs of the viruses were analyzed after extended culture. Only viruses containing the sequences complementary to the anticodon loop of tRNA^{His} maintained a PBS complementary to tRNA^{His}. The results of this study point to a role for the interaction between a region in the HIV-1 genome and the anticodon loop of the primer tRNA, as well as the tRNA-PBS interaction, in maintaining the selectivity of the primer tRNA used in reverse transcription.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. Restriction endonucleases were obtained from New England Biolabs. The *Taq* DNA polymerase was purchased from BRL-Gibco, and all of the reagents used for PCR were acquired from Perkin-Elmer Cetus. Tissue culture media and reagents were obtained from BRL-Gibco. The synthetic oligonucleotides used for mutagenesis and PCR were prepared by the Cancer Center Oligonucleotide Synthesis Facility at the University of Alabama at Birmingham. The enzyme-linked immunosorbent assays (ELISAs) for p24 antigen were obtained from Coulter Laboratories.

Tissue culture. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. SupT1 cells were grown in RPMI medium supplemented with 15% fetal calf serum.

Construction of mutant proviral genomes. General laboratory procedures were followed for DNA manipulation, plasmid preparation, and subcloning essentially as previously described (18). Single-stranded DNA of M13mp18PBS used for oligonucleotide site-directed mutagenesis was prepared as previously described (35). M13mp18PBS contains HIV-1 proviral sequences between the *Hpa*I and *Pst*I sites of pHXB2*gpt*, which encompasses the PBS region (24). The oligonucleotide used to substitute the wild-type HIV-1 PBS complementary to the 3′-terminal 18 nucleotides of tRNA$_3^{Lys}$ with sequences complementary to the 3′-terminal 18 nucleotides of tRNA^{His} [pHXB2(His)] was 5′-CGCTTTCAAAT CCGAGTCACGGCACCACTGCTA-3′. The conditions used for mutagenesis were as previously described (35). Single-stranded M13mp18PBS phage DNA containing the PBS complementary to tRNA^{His} was used as the template for the construction of the mutations that changed the sequences upstream of the PBS. These mutations substituted sequences complementary to the anticodon and TΨC loops of tRNA$_3^{Lys}$ with sequences complementary to the corresponding anticodon and TΨC loops of tRNA^{His}. The oligonucleotides for each respective mutant are as follows: pHXB2(His-AC), 5′-CTGCTAGAGTTGTGGCACACT GAC-3′; pHXB2(His-TΨC), 5′-CACTGACTACCTCGGTCTGAGG-3′. Confirmation of the desired mutations was carried out by the dideoxy DNA sequencing method of Sanger et al. (28) with Sequenase (U.S. Biochemicals). The replicative form M13mp18PBS DNA containing the mutations was isolated and digested with *Hpa*I and *Bss*HII, resulting in an 868-bp fragment encompassing the PBS. The 868-bp fragment was subcloned between the *Hpa*I and *Bss*HII sites of pHXB2*gpt* (22, 23), and the resulting mutant proviral plasmids were screened by restriction digests and confirmed by DNA sequencing (28).

DNA transfections and analysis of proviral gene expression. Transfection of proviral plasmid DNA into COS-1 cells with DEAE-dextran was carried out as previously described (35). Forty-eight hours posttransfection, the supernatant was collected and filtered through a 0.45-μm-pore-size syringe filter (Nalgene). The levels of p24 antigen were determined from five independent transfections by an ELISA (Coulter Laboratories).

Analysis of virus infectivity. To test for virus infectivity, SupT1 cells, which express CD4 and support high-level replication of HIV-1, were cocultured with COS-1 cells transfected with the various proviral constructs for 3 days. Following cocultivation, the SupT1 cells were harvested by low-speed centrifugation (1,000 × *g*) and further cultured with fresh media and additional SupT1 cells. For cell-free infections, SupT1 cells (10^6 cells per ml) were infected with 100 ng of virus per ml as measured by p24 antigen. After allowing the virus to adsorb for 24 h, SupT1 cells were pelleted (1,000 × *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 intervals, were refed with fresh SupT1 cells. At the designated time intervals, samples of the culture supernatant were collected and analyzed for p24 antigen by ELISA (Coulter Laboratories).

PCR amplification and DNA sequencing of PBS-containing proviral DNA. Analysis of the proviral PBS sequences from infected SupT1 cells was carried out as previously described (35). Briefly, proviral DNA encompassing the PBS was amplified by PCR from approximately 10 μg of total cellular DNA isolated from the infected SupT1 cultures. PCR-amplified DNA was digested with *Hin*dIII and *Eco*RI and subcloned between the *Eco*RI and *Hin*dIII sites present in the polylinker region of M13mp18 replicative form DNA. Following ligation, the DNA was transformed into competent *Escherichia coli* DH5αF′ and plated on a lawn of *E. coli* DH5αF′ containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal [U.S. Biochemicals]) (35). Following identification of colorless phage plaques, the single-stranded DNA prepared from individual recombinant phage clones was sequenced (28).

RESULTS

Construction of mutant HIV-1 proviral genomes containing sequences complementary to the anticodon and TΨC loops of tRNA^{His}. In a previous study, we demonstrated that HIV-1 could use a tRNA^{His} molecule to initiate reverse transcription if the PBS was complementary to the 3′-terminal nucleotides of tRNA^{His} (36). Simply having a PBS complementary to tRNA^{His}, though, was not sufficient to produce a virus capable of maintaining the use of tRNA^{His} over multiple rounds of replication, as evidenced by the eventual reversion of the PBS back to that of a wild-type virus. Previous studies have suggested that regions outside of the PBS might be involved in the interaction of the primer tRNA with the PBS (8, 14, 15, 36). Two viral RNA sequences located upstream of the HIV-1 PBS which are complementary to the anticodon and TΨC loops of tRNA$_3^{Lys}$ have been identified. The 4 uridine residues (^{33}USUU^{36}) of the tRNA$_3^{Lys}$ anticodon loop (Fig. 1A) are complementary to the 4 adenine residues located at nucleotides 169 to 172 of the HIV-1 viral RNA (derived from molecular clone HXB2*gpt*; Fig. 1B). These 4 adenines correspond to the 4 adenines found at nucleotides 164 to 167 of the HIV-1 (Mal isolate) RNA which was shown to be positioned within a loop structure (A-loop) (10, 11). Further upstream of the A loop is the sequence GACCCU, located at nucleotides 149 to 154 (Fig. 1B), which shares complementarity with the ^{50}AGGGT$_m$Ψ^{55} sequence of the tRNA$_3^{Lys}$ TΨC loop and arm (Fig. 1A). We wanted to determine if these viral RNA sequences play a role in the selection of tRNA$_3^{Lys}$ for the initiation of reverse transcription in viruses that contained a PBS complementary to tRNA^{His}. Site-directed mutagenesis was used to substitute the wild-type PBS with sequences complementary to the 3′-terminal 18 nucleotides of tRNA^{His} [pHXB2(His), Fig. 2B]. Two additional mutations in pHXB2(His) in which each of the viral sequences which are complementary to the anticodon and TΨC loops of tRNA$_3^{Lys}$ were substituted with nucleotides complementary to the corresponding anticodon loop and TΨC loop and arm of tRNA^{His}

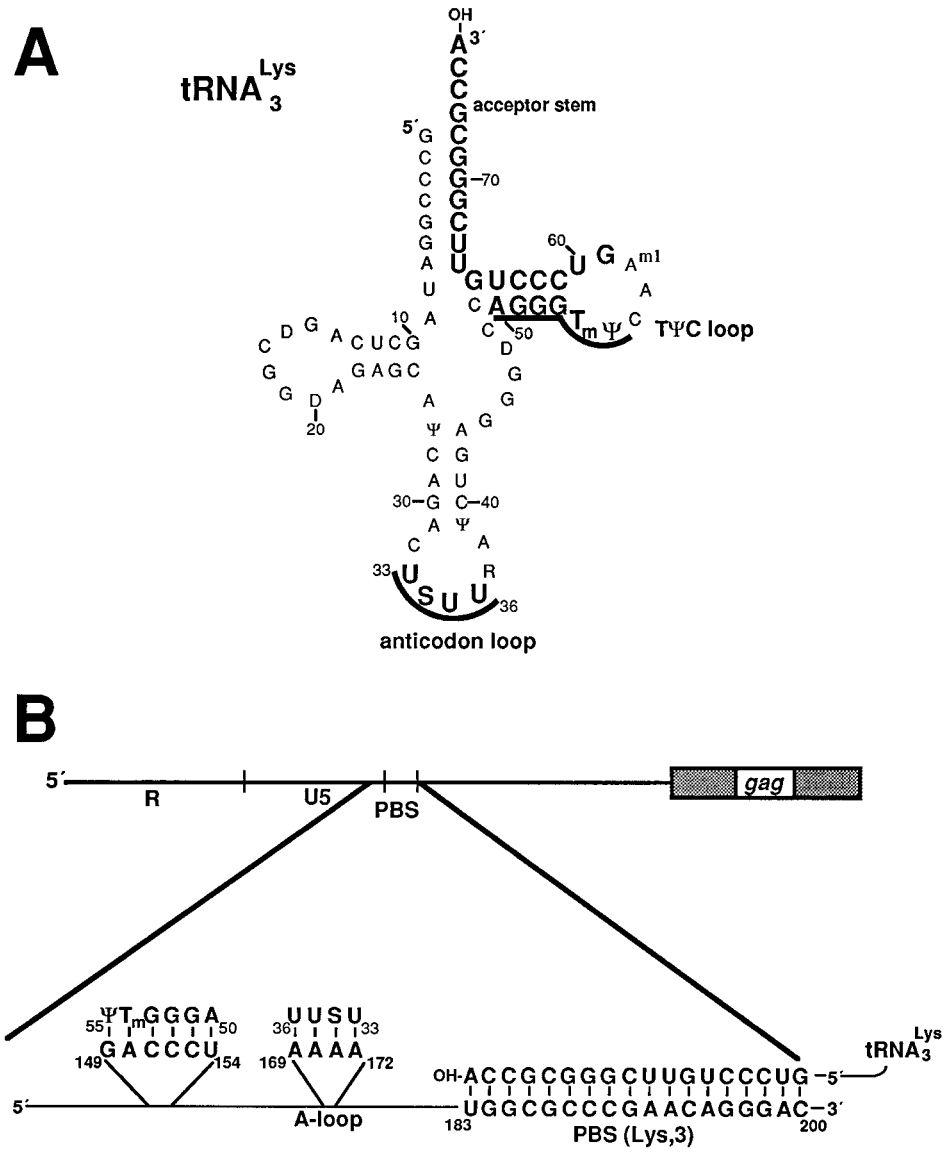

FIG. 1. Diagram of mammalian $tRNA_3^{Lys}$ and depiction of sequences found at the 5′ end of the HIV-1 viral RNA genome that are complementary to nucleotides of $tRNA_3^{Lys}$. (A) The cloverleaf diagram of rabbit liver $tRNA_3^{Lys}$ is illustrated as described by Raba et al. (21). Those nucleotides complementary to sequences in the HIV-1 viral RNA are shown in boldface type, with the nucleotides of the anticodon and TΨC loops complementary to the sequences upstream of the PBS also underlined. (B) An expanded view of the 5′ end of the HIV-1 viral RNA genome depicting the location of the sequences sharing complementarity with $tRNA_3^{Lys}$ as well as the predicted base pairing. The PBS, located between nucleotides 183 and 200, is predicted to base pair with the 3′-terminal 18 nucleotides that make up the acceptor stem of $tRNA_3^{Lys}$. By use of in vitro studies, the 4 adenine residues located upstream of the PBS (at nucleotides 169 to 172) have been shown to bind to the 4 uridine residues of the $tRNA_3^{Lys}$ anticodon loop (11). These adenines are predicted to be positioned within a loop structure, termed the A-rich loop. The sequences complementary to the TΨC loop are positioned at nucleotides 149 to 154. S, 5-methoxycarbonylmethyl-2-thiouridine; Ψ, pseudouridine; D, dihydrouridine; T_m, 2′-*O*-methyl-5-methyluridine; A^{ml}, 1-methyladenosine.

were constructed (Fig. 2A). These mutant constructs are referred to as pHXB2(His-AC) and pHXB2(His-TΨC), respectively, and are depicted in Fig. 2B. In pHXB2(His-AC), a CCACAA sequence, which is complementary to the ^{33}ΨUGU GG^{38} nucleotides found in the anticodon loop of the $tRNA^{His}$, was positioned at nucleotides 168 to 173 of the viral RNA (^{168}CCACAA^{173}). pHXB2(His-TΨC) contained the sequence GACCGAGG at nucleotides 149 to 156 (^{149}GACCGAGG^{156}), which is complementary to the ^{48}CCUCGGUΨ^{55} sequence of the $tRNA^{His}$ TΨC loop and arm.

Expression of HIV-1 proteins from mutant proviral genomes. To determine if the mutations in the 5′ region of the HIV-1 genome affected viral gene expression, we analyzed COS-1 cells transfected with the mutant and wild-type proviral genomes for intracellular viral proteins and the supernatants for released virus particles. No differences in the intracellular viral proteins between the cultures transfected with the plasmid containing wild-type or mutant proviruses were evident, as determined by metabolic labeling and immunoprecipitation with sera from AIDS patients (data not shown). To determine if the mutations had any effect on the release of virus, the culture supernatants were measured for p24 antigen by using an ELISA. No significant differences between the amounts of p24 released from cultures transfected with the wild-type or

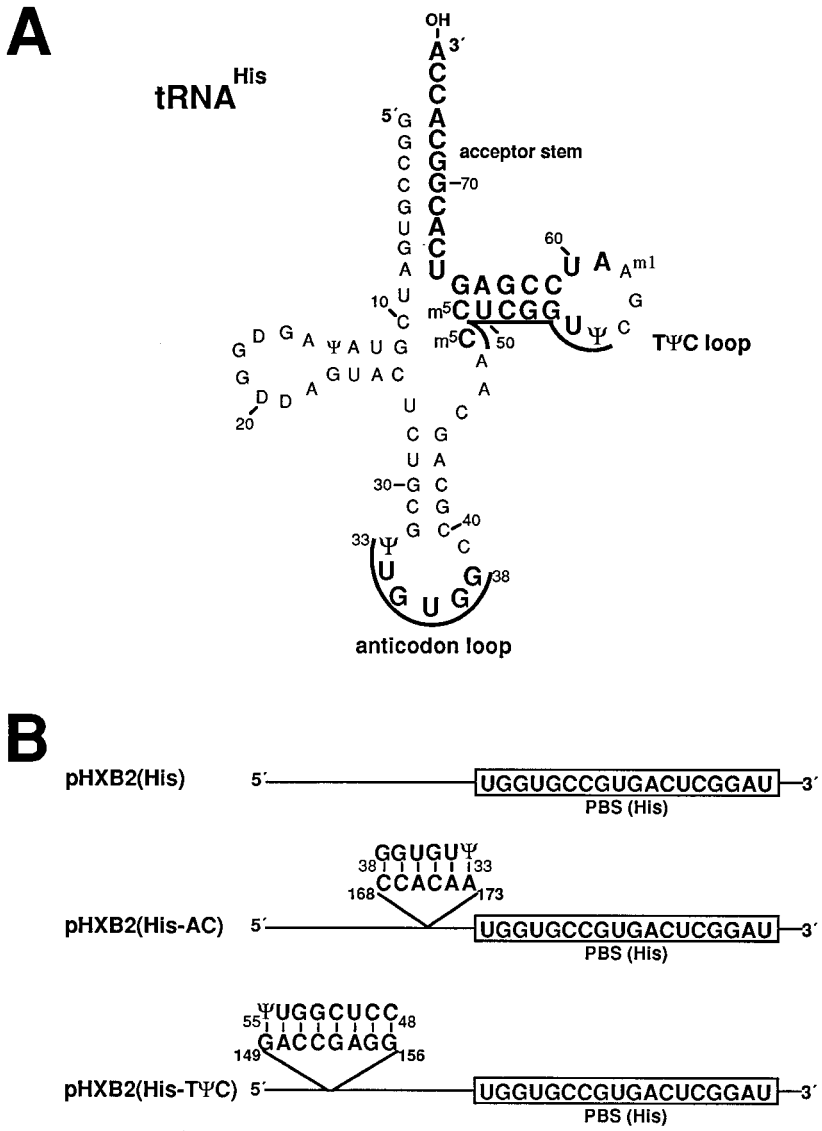

FIG. 2. Diagram of tRNA^{His} and description of the mutations within the PBS region. (A) A diagram of HeLa cell tRNA^{His} is depicted as described by Rosa et al. (26). The nucleotides of tRNA^{His} which were used to model the mutations within the PBS region are depicted in boldface type, with the anticodon and TΨC loop sequences also underlined. (B) A mutation in the PBS was constructed such that the PBS (box) was complementary to the 3′-terminal 18 nucleotides of tRNA^{His} [pHXB2(His)]. Two additional mutant proviral genomes, which contained sequences complementary to the anticodon loop [pHXB2(His-AC)] and TΨC loop [pHXB2(His-TΨC)] of tRNA^{His} in place of those sequences complementary to the corresponding anticodon and TΨC loops, respectively, of tRNA$_3^{Lys}$, were constructed. The predicted base pairing between the mutant viral RNA sequences and the anticodon and TΨC sequences are also depicted. The numbers indicate the nucleotide positions of the mutant residues within the RNA genome. m^{5}C, 5-methylcytidine.

mutant proviruses were seen (Fig. 3). The minor variations seen were due to differences in the transfection efficiencies from the five independent experiments.

Replication potential of mutant HIV-1 viruses. To determine the replication potential of the mutant viruses, SupT1 cells were cocultured with the transfected COS-1 cells. At various times postcoculture, replication of both the wild-type and mutant viruses was monitored by quantitating the levels of the viral capsid antigen (p24) in the supernatant. The increasing levels of p24 antigen in the culture supernatants indicated that viruses derived from COS-1 cells transfected with either pHXB2(His), pHXB2(His-AC), or pHXB2(His-TΨC) were infectious in SupT1 cells (Fig. 4). The increase in the level of p24 antigen in the cultures also correlated with the increase in the number of syncytia. Although the appearance of virus from cells transfected with proviruses containing the mutant PBS was delayed compared with the case for the wild type, by day 15 postcoculture peak levels of p24 antigen in all of the culture supernatants were similar to that found in the culture of the wild-type virus (Fig. 4).

DNA sequence analysis of the PBS and surrounding region. To determine the DNA sequence of the PBS region, high-molecular-weight DNA was isolated from each of the infected SupT1 cultures at days 9 and 15 postcoculture. The PBS regions of the integrated proviruses were amplified by PCR. Since the first 18 nucleotides of the primer tRNA are copied during plus-strand strong-stop viral DNA synthesis, analysis of the proviral PBS sequence will determine which tRNA species was used to initiate reverse transcription. At day 9 postcoculture, the viruses derived from pHXB2(His), pHXB2(His-AC),

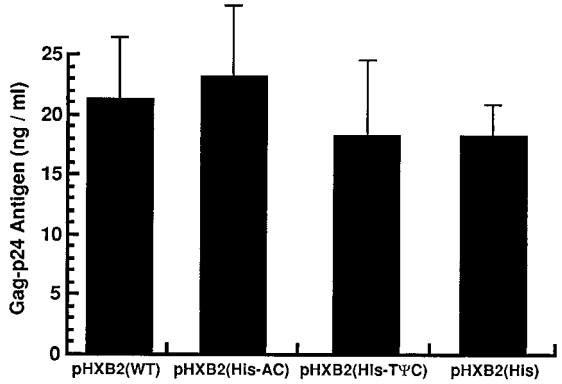

FIG. 3. Release of virus from COS-1 cells transfected with the wild-type and mutant proviral genomes. COS-1 cells were transfected with the designated plasmids, and at 48 h posttransfection, the supernatant was analyzed for released p24 antigen by ELISA (Coulter). The levels of p24 antigen (in nanograms per milliliter of supernatant) were determined for five independent transfections. Standard deviations are represented by error bars.

and pHXB2(His-TΨC) maintained a PBS complementary to $tRNA^{His}$ (Table 1). The viruses derived from pHXB2(His-AC) also contained several nucleotide substitutions upstream of the mutant PBS. Similar substitutions were not present in the proviral sequences derived from pHXB2(His) or pHXB2(His-TΨC).

By 15 days postcoculture, there were substantial amounts of virus in all of the cultures, as determined by the level of p24 antigen (Fig. 4). Analysis of the PBS sequences from proviruses derived from pHXB2(His) and pHXB2(His-TΨC) revealed the presence of sequences complementary to the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$ as well as $tRNA^{His}$ (Table 2). All the viruses derived from the pHXB2(His-TΨC)-infected cultures contained the original sequences upstream of the PBS which were made complementary to the TΨC loop of $tRNA^{His}$. Therefore, the TΨC-complementary sequences upstream of the PBS did not stabilize the use of $tRNA^{His}$ in reverse transcription. Four of 13 revertant viruses derived from

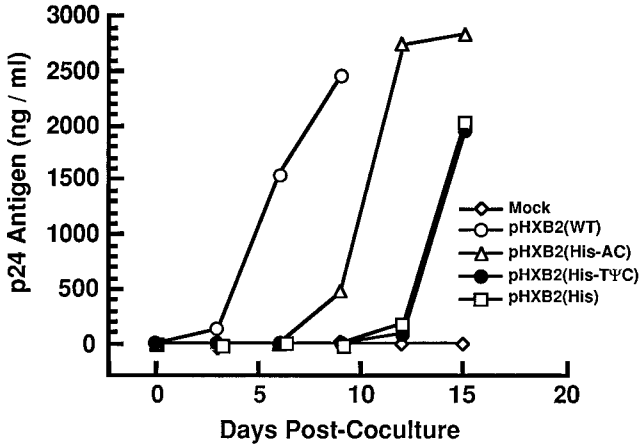

FIG. 4. Kinetics of the appearance of infectious virus derived from transfection of wild-type and mutant proviral genomes. The plasmids containing the wild-type and mutant proviral genomes were transfected into COS-1 cells and then cocultured with SupT1 cells for an additional 3 days. The SupT1 cells 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).

pHXB2(His) contained an insertion of 12 nucleotides downstream of the wild-type PBS which were identical to the last 12 nucleotides of the mutant PBS. This insertion could have occurred during the second template transfer of reverse transcription through inappropriate base pairing between the plus-strand PBS copy, which corresponds to the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$, and the minus-strand copy of the PBS, which is complementary to the original mutant PBS (24, 35). A completely different result was found upon analysis of the PBS region from viruses derived from pHXB2(His-AC). The PBS remained complementary to the 3′-terminal 18 nucleotides of $tRNA^{His}$ in all of the sequences analyzed (13 of 13). No PBS sequences complementary to $tRNA_3^{Lys}$ were found. The A-loop sequences of these viruses also remained complementary to the anticodon loop of $tRNA^{His}$; however, more single-nucleotide changes were found surrounding these mutant sequences.

Stability of a PBS complementary to $tRNA^{His}$ in viruses derived from pHXB2(His-AC). To determine the stability of the PBS in the mutant viruses, culture supernatants were collected at day 15 postcoculture, and equal amounts of virus as measured by the level of p24 antigen (100 ng/ml) were used to infect fresh SupT1 cells. The viruses were allowed to replicate for 10 days, after which the viruses were again collected and used to infect fresh SupT1 cells as before. The viruses were passaged in SupT1 cells following this protocol for 12 serial passages. At passages 5 and 10, the replication kinetics of the viruses were analyzed. The viruses derived from pHXB2(His) and pHXB2(His-TΨC) exhibited replication kinetics similar to those of the wild type at passages 5 and 10 (Fig. 5). At passage 5, the viruses derived from pHXB2(His-AC) replicated more slowly than the wild type; however, by passage 10 the virus had replication kinetics similar to those of the wild type.

After five serial passages, the PBS sequences of all viruses isolated from pHXB2(His) and pHXB2(His-TΨC) had reverted to a wild-type PBS sequence complementary to the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$ (Table 3). Again, the sequence complementary to the $tRNA^{His}$ TΨC loop was present in viruses derived from pHXB2(His-TΨC). The surprising result was that viruses derived from pHXB2(His-AC) contained PBS sequences complementary to $tRNA^{His}$ after 5, 10, and 12 serial passages, representing a time of over 4 months in continuous culture. Sequence analysis of 48 independent M13mp18 phage clones (17 at passage 5; 21 at passage 10; and 10 at passage 12) revealed PBS sequences complementary only to $tRNA^{His}$. Taken together, these results point to a stable virus containing a PBS that is complementary to $tRNA^{His}$.

Although the PBS sequence of the virus derived from pHXB2(His-AC) did not change, the number of point mutations found upstream of the PBS increased over time in culture (Table 4). In the majority of clones sequenced, two changes were observed in the PBS region: a G-to-A change at position 182 and a T-to-G change at nucleotide 175. We did find several changes within the ^{168}CCACAA^{173} sequence. A C-to-A change for C-168 was found most frequently; C-171 was replaced with an A in a single clone found at passages 5 and 10. A single clone with a C-to-A change at position 169 resulting in a ^{168}CAACAA^{173} sequence was found from analysis of passage 10 viruses. Both of these viruses, though, were unstable, since a PBS with these mutations were not recovered at passage 12 (Table 5). In contrast, an A-to-T change at position 172 resulting in a ^{168}CCACTA^{173} sequence was found in a single clone at both passage 10 and passage 12. No nucleotide substitutions for A-170 or A-173 were found in any of the 48 clones exam-

TABLE 1. Sequences of the PBS region of proviruses isolated at day 9 postculture

Provirus sample	Sequence[a]	Frequency[b]
pHSB2(His-AC)	5′-CCT CAG ACC CTT TTA GTC AGT GTG **CCA CAA** CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
	5′-*** *** *** *** *** *** *** *** *** *** *** *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	5/9
	5′-*** *** *** *** *** *** **A *** *** *** *** *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	2/9
	5′-*** *** *** *** *** *** *** *** *** *** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/9
	5′-*** *** *** *** C** *** *** *** *** *** *** *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	1/9
pHXB2(His-TΨC)	5′-CCT CA GACCGAGG TA GTC AGT GTG GAA AAT CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
	5′-CCT CA GACCGAGG TA GTC AGT GTG GAA AAT CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	8/8
pHXB2(His)	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAT TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	3/3

[a] The sequences located in the A-rich loop region which were made complementary to the anticodon loop of tRNA^{His} are shown in boldface type. Asterisks denote identity with the input sequences. The sequences complementary to the TΨC loop of tRNA^{His} are indicated by underlining. The PBS sequence, which is complementary to tRNA^{His} in all cases, is boxed.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones. The input sequence refers to the initial mutations in the PBS region.

ined, encompassing the samples obtained from passages 5, 10, or 12.

DISCUSSION

HIV-1 proviral genomes which contained not only a PBS complementary to the 3′-terminal 18 nucleotides of tRNA^{His} [pHXB2(His)] but also one of two sequences positioned upstream of the PBS which are complementary to the anticodon loop [pHXB2(His-AC)] and TΨC loop and arm [pHXB2 (HisTΨC)] of tRNA^{His} were constructed. Transfection into COS-1 cells of each of these constructs resulted in the production of infectious virus. PCR amplification of the PBS region and DNA sequence analysis revealed that at day 9 following infection, the PBSs were complementary to tRNA^{His}. By day 15 in culture, viruses derived from pHXB2(His) and pHXB2(His-TΨC) contained PBS sequences complementary to tRNA$_3^{Lys}$. In contrast, viruses derived from pHXB2(His-AC) maintained a PBS complementary to tRNA^{His}. The virus was stable and maintained a PBS complementary to tRNA^{His} for over 4 months in culture, encompassing 12 serial passages in SupT1 cells.

The generation of a stable HIV-1 virus which maintains a PBS complementary to a tRNA other than tRNA$_3^{Lys}$ is a significant step towards understanding the complexities of the interaction between the tRNA primer and the viral RNA genome, which is a prerequisite first step in the initiation of

TABLE 2. Sequences of the PBS region of proviruses isolated at day 15 postculture

Provirus sample	Sequence[a]	Frequency[b]
pHXB2(His-AC)	5′-CCT CAG ACC CTT TTA GTC AGT GTG **CAA CAA** CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
	5′-*** *** *** *** *** *** *** *** *** *** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	5/13
	5′-*** *** *** *** *** *** *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	4/13
	5′-*** *** *** *** *** *** *** *** *** *** *** *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	2/13
	5′-*** *** *** *** *** *** *** *** *** *** **T *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/13
	5′-*** *** *** *** *** *** *** **A *** *** *** *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	1/13
pHXB2(His-TΨC)	5′-CCT CA GACCGAGG TA GTC AGT GTG GAA AAT CTC TAG CAG *TGG CGC CCG AAC AGG GAC* TTG AAA-3′	12/13
	5′-CCT CA GACCGAGG TA GTC AGT GTG GAA AAT CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	1/13
pHXB2(His)	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG *TGG CGC CCG AAC AGG GAC* TTG AAA-3′	5/13
	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG *TGG CGC CCG AAC AGG GAC* ▼TTG AAA-3′	4/13
	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	4/13

[a] The sequences located in the A-rich loop region which were made complementary to the anticodon loop of tRNA^{His} are shown in boldface type. Asterisks denote identity with the input sequences. The sequences complementary to the TΨC loop of tRNA^{His} are indicated by underlining. The PBS sequence is boxed; roman letters represent a PBS which is complementary to tRNA^{His}, and italic letters indicate reversion back to a PBS complementary to the 3′-termini 18 nucleotides of tRNA$_3^{Lys}$. ▼, insertion site of sequence CGTGACTCGGAT, which corresponds to the last 12 nucleotides of the PBS complementary to the 3′-terminal nucleotides of tRNA^{His}.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones. The input sequence refers to the initial mutations in the PBS region.

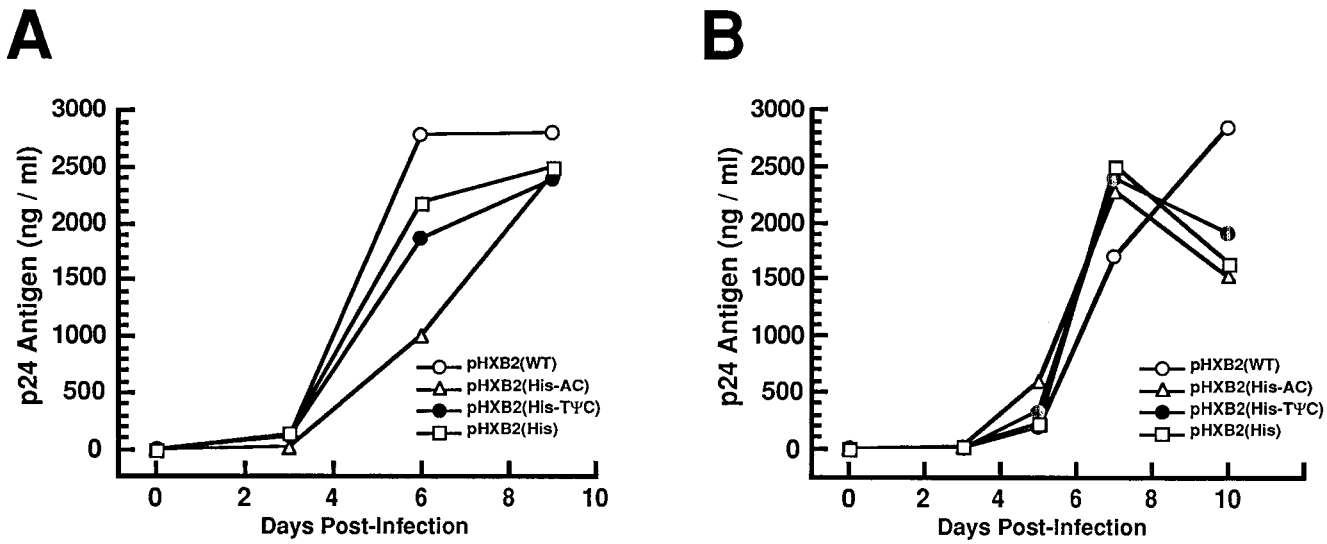

FIG. 5. Kinetics of replication of the infectious virus. SupT1 cells were infected with equal amounts (approximately 100 ng/ml) of virus isolated after 5 or 10 serial passages. After 24 h, the initial virus-containing medium was removed and new medium and SupT1 cells were added to the cultures. Supernatants were removed and assayed for p24 antigen. (A) Kinetics of virus replication after 5 serial passages. (B) Kinetics of replication of viruses obtained after 10 serial passages.

reverse transcription. Using in vitro systems, previous studies have identified several regions in the 5′ end of the RNA genome outside of the PBS which might interact with the $tRNA_3^{Lys}$ primer used for reverse transcription (10, 11, 14). Whether the interactions between $tRNA_3^{Lys}$ and these viral RNA sequences had any significance with respect to replication could only be addressed in the context of the complete viral genome and generation of infectious virus. Our approach used HIV-1 proviral cDNAs containing mutations in the PBS region. Since the PBS is regenerated during plus-strand synthesis of reverse transcription when the RT copies the 3′-terminal 18 nucleotides of the initiating tRNA, PCR amplification of the integrated proviral PBS region followed by DNA sequence analysis allows the determination of which tRNA was used as the primer for reverse transcription. Analysis of the PBS sequences of viruses derived from pHXB2(His),

TABLE 3. Sequences of the PBS region of proviruses isolated at passage 5

Provirus sample	Sequence[a]	Frequency[b]
pHXB2(His-AC)	5′-CCT CAG ACC CTT TTA GTC AGT GTG **CAA CAA** CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
	5′-*** *** *** *** *** *** *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	8/17
	5′-*** *** *** *** *** *** *** *** *** *** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	2/17
	5′-*** *** *** *** *** *** *** *** A** *** *A* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *** *** *** *** *** A** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *** *** *** *** *** *** *** *** *A* *** *** TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *** *** *** *** *** *** *** A** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *** *** *** *** *** *** *** *** *G* *G* **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *** *** *** *** *A* *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
	5′-*** *** *A* *** *** *** *** *** *** *** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/17
pHXB2(His-TΨC)	5′-CCT CA GACCGAGG TA GTC AGT GTG GAA AAT CTC TAG CAT *TGG CGC CCG AAC AGG GAC* TTG AAA-3′	12/12
pHXB2(His)	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG *TGG CGC CCG AAC AGG GAC* TTG AAA-3′	9/12
	5′-CCT CAG ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAG *TGG CGC CCG AAC AGG GAC*▼ TTG AAA-3′	3/12

[a] The sequences located in the A-rich loop region which were made complementary to the anticodon loop of $tRNA^{His}$ are shown in boldface type. Asterisks denote identity with the input sequences. Single nucleotides in boxes denote nucleotide changes found in the mutant A-rich loop sequences (CCA CAA). The sequences complementary to the TΨC loop of $tRNA^{His}$ are indicated by underlining. The PBS sequence is boxed; roman letters represent a PBS which is complementary to $tRNA^{His}$, and italic letters indicate reversion back to a PBS complementary to the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$. ▼, insertion site of sequence CGTGACTCGGAT, which corresponds to the last 12 nucleotides of the PBS complementary to the 3′-terminal nucleotides of $tRNA^{His}$.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones. The input sequence refers to the initial mutations in the PBS region.

TABLE 4. Sequences of the PBS region of proviruses derived from pHXB2(His-AC) viruses at passage 10

Sequence[a]	Frequency[b]
5′-CCT CAG ACC CTT TTA GTC AGT GTG **CCA CAA** CTC TAG CAG TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
5′-*** *** *** *** *** *** *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	8/21
5′-*** *** *** *** *** *** *A* *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	3/21
5′-*** *** *** *** *** A** *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	2/21
5′-*** *** *** *** *** *** *** A*A *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *A* *** *** *** *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *** *** *** **T *** *** *[A]* *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *** *** *** *** *A* *** *** [A]** *** *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *** *** *** *** *** *** [A]** *** *G* *** **a TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *** *** *** *** *GA *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21
5′-*** *** *** *** *** *** *** A** [A]** *** *GA *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	1/21
5′-*** *** *** *** *** **T *** *** *** *[T]* *G* *** **A TGG TGC CGT GAC TCG GAT *** ***-3′	1/21

[a] The sequences located in the A-rich loop region which were made complementary to the anticodon loop of tRNA^{His} are shown in boldface type. Asterisks denote identity with the input sequences. Single nucleotides in boxes denote nucleotide changes found in the mutant A-rich loop sequences (CCA CAA). The PBS sequences, which are complementary to tRNA^{His} in all cases, are boxed.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones. The input sequence refers to the initial mutations in the PBS region.

pHXB2(His-TΨC), and pHXB2(His-AC) at early passages revealed that tRNA^{His} was used to prime reverse transcription. This result is consistent with previous studies from our laboratory as well as others which have established that multiple tRNA species can function to initiate reverse transcription of HIV-1 (8, 15, 36). In these studies, even though the viral genomes contained a PBS complementary to the 3′-terminal 18 nucleotides of the alternate tRNAs, the PBS sequences of all of the mutant viral genomes reverted back to a wild-type sequence, complementary to tRNA$_3^{Lys}$. The period of time before these revertant viruses appeared in culture varied from 8 to 30 days. In the present study, the PBS sequences of viruses derived from transfection of pHXB2(His) and pHXB2(His-TΨC) also reverted back to wild-type by 15 days in culture. However, viruses in which the A-rich loop was made complementary to the anticodon loop of tRNA^{His} maintained a PBS complementary to tRNA^{His} for over 4 months. The results of our studies, then, demonstrated that the presence of a sequence upstream of the PBS that is complementary to the anticodon loop of tRNA^{His} was critical for the maintenance of a PBS complementary to tRNA^{His}.

The generation of a virus which stably maintains a PBS complementary to a tRNA other than tRNA$_3^{Lys}$ provides insights into how the virus selects the tRNA used for reverse transcription. It was previously demonstrated that incorporation of tRNAs into HIV-1 virions is independent of the PBS (17). Thus, similar levels of tRNA$_3^{Lys}$ probably existed in viruses derived from pHXB2(His-AC), pHXB2(His), and pHXB2 (His-TΨC). The reversion back to the wild type of viruses derived from pHXB2(His) and pHXB2(His-TΨC) clearly demonstrates the ability of tRNA$_3^{Lys}$ to prime initiation of reverse transcription from a PBS complementary to tRNA^{His}. The fact that tRNA$_3^{Lys}$ can initiate reverse transcription from a PBS which is complementary to an alternate tRNA is consistent with previous studies from our laboratory which demonstrated that only the first 6 nucleotides of the HIV-1 PBS are

TABLE 5. Sequences of the PBS region of proviruses derived from pHXB2(His-AC) viruses at passage 12

Sequence[a]	Frequency[b]
5′-CCT CAG ACC CTT TTA GTC AGT GTG **CCA CAA** CTC TAG CAT TGG TGC CGT GAC TCG GAT TTG AAA-3′	Input
5′-*** *** *A* *** *** *** *** *** [A]** *** *G* *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	3/10
5′-*** *** *** *** *** **T *** *** *** *** *G* *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	2/10
5′-*** *** *** *** *** *** *** *** *** *[T]* *G* *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	2/10
5′-*** *** *A* *** *** A** *** *** [A]** *** *** *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	1/10
5′-*** *** *A* *** *** A** *** *** [A]** *** *G* *** **A TGG TGC CGT GAC TCG GAT C** ***-3′	1/10
5′-*** *** *** *** *** *** *** *** *** *** *G* *** **T TGG TGC CGT GAC TCG GAT C** ***-3′	1/10

[a] The sequences located in the A-rich loop region which were made complementary to the anticodon loop of tRNA^{His} are shown in boldface type. Asterisks denote identity with the input sequences. Single nucleotides in boxes denote nucleotide changes found in the mutant A-rich loop sequences (CCA CAA). The PBS sequences, which are complementary to tRNA^{His} in all cases, are boxed.

[b] Frequencies of the DNA sequences of the PBS region obtained from independent M13 phage clones. The input sequence refers to the initial mutations in the PBS region.

sufficient for the initiation of reverse transcription (24). Taken together, the results of these studies again point to the fact that different tRNAs can be used to initiate reverse transcription. The results from our studies and the in vitro binding studies of Isel et al. (10, 11) clearly support the idea that $tRNA_3^{Lys}$ interacts with both the A-rich loop and the PBS. In viruses derived from pHXB2(His) and pHXB2(His-TΨC), the A-rich loop was complementary to the anticodon loop of $tRNA_3^{Lys}$, and this complementarity may have stabilized the binding between the mutant PBS and the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$, allowing $tRNA_3^{Lys}$ to be used to initiate reverse transcription. After one round of replication in which $tRNA_3^{Lys}$ was used to initiate reverse transcription, 50% of the proviruses would contain a PBS complementary to $tRNA_3^{Lys}$. The substitution of the A-rich loop with sequences complementary to the $tRNA^{His}$ anticodon loop might have stabilized the interaction between the 3′-terminal 18 nucleotides of $tRNA^{His}$ and the PBS. Alternatively, the A-rich loop complementary to $tRNA^{His}$ might have prevented the positioning of the $tRNA_3^{Lys}$, at the PBS complementary to $tRNA^{His}$. Further studies will be needed to characterize the binding between $tRNA^{His}$ and the mutant viral RNA. If indeed complementary binding between the A-rich loop region and the anticodon loop of $tRNA_3^{Lys}$ is required for selection of the $tRNA_3^{Lys}$ primer, we would predict that HIV-1 viruses maintaining PBSs complementary to different tRNAs could be generated by construction of proviruses with changes in the A-rich loop region. Studies to test this possibility are ongoing. Finally, the inability of viruses derived from pHXB2(His-TΨC) to maintain a PBS complementary to $tRNA^{His}$ is also consistent with the findings of Isel et al. (10), which demonstrated that the TΨC arm of $tRNA_3^{Lys}$ did not interact with the HIV-1 viral RNA but instead base paired with nucleotides located within the 5′ strand of the $tRNA_3^{Lys}$ acceptor stem.

The viruses derived from transfection of pHXB2(His-AC) exhibited several characteristics different from those of the wild-type virus. The viruses derived from pHXB2(His-AC) had slower replication kinetics at passage 5, but by 10 serial passages the kinetics of replication were similar to those of the wild type. A number of nucleotide substitutions were found in and around the viral ^{168}CCACAA^{173} sequence which is complementary to the anticodon loop of $tRNA^{His}$. We believe that these mutations are not random, because a similar buildup of mutations adjacent to the sequences complementary to the TΨC loop of $tRNA^{His}$ were not seen following multiple rounds of replication of viruses derived from pHXB2(His-TΨC). Several nucleotide changes were also common to all of the PBS clones analyzed from the viruses derived from pHXB2(His-AC). For example, 16 of the 17 clones analyzed at passage 5 and all (21 of 21) analyzed at passage 10 contained a G-to-A substitution at the first nucleotide 5′ of the PBS (nucleotide 182). A T-to-G substitution at position 175 was also found in a majority of the proviral sequences analyzed. Additional mutations were evident in all of the samples, such that the complete input sequence from pHXB2(His-AC) was not found by passage 10 or 12. Given the fact that the pHXB2(His-AC)-derived viruses at passage 10 had replication kinetics similar to those of the wild type, we favor the hypothesis that these additional mutations represent an evolution of the virus to stabilize use of $tRNA^{His}$ as the primer for reverse transcription. Extended serial passage of the virus followed by analysis of the PBS region will be required to determine if the number of mutations continue to increase or a virus with a predominant sequence emerges. Finally, we did find that several nucleotides within the ^{168}CCACAA^{173} mutation were maintained throughout all of the PBS regions analyzed. In particular, the ^{169}CAC^{171} mutation was maintained in all of the clones analyzed at passage 12. The ^{169}CAC^{171} mutation might be important, because these nucleotides would be predicted to interact with the anticodon of $tRNA^{His}$. Thus, the maintenance of the ^{169}CAC^{171} mutation might be critical for the continued use of $tRNA^{His}$ as a primer and maintenance of a PBS complementary to $tRNA^{His}$. Studies with additional mutants to test this possibility are ongoing.

ACKNOWLEDGMENTS

We thank Zhijun Zhang for helpful comments and Dee Martin for preparation of the manuscript. C.D.M. expresses thanks to M.A.R. for continued help and discussion.

Culture of HIV was carried out at the UAB AIDS Research Center Virus Core facility (AI-27767). Oligonucleotides were prepared by the Cancer Center Core facility (NCI contract CA13148). The UAB AIDS Molecular Biology Core facility provided help with PCR. This study was supported by PHS grant AI34749 from NIH (C.D.M.).

REFERENCES

1. **Aiyar, A., D. Cobrinik, Z. Ge, H.-J. Kung, and J. Leis.** 1992. Interaction between retroviral U5 RNA and the TΨC loop of the $tRNA^{Trp}$ primer is required for efficient initiation of reverse transcription. J. Virol. **66:**2464–2472.
2. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226:**1209–1211.
3. **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.
4. **Barat, C., V. Lullien, O. Schatz, G. Keith, 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.
5. **Barat, C., O. Schatz, S. Le Grice, and J.-L. Darlix.** 1993. Analysis of the interactions of HIV-1 replication primer $tRNA^{lys3}$ with nucleocapsid protein and reverse transcriptase. J. Mol. Biol. **231:**185–190.
6. **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.
7. **Colicelli, J., and S. P. Goff.** 1987. Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. J. Virol. **57:**37–45.
8. **Das, A. T., B. Klaver, and B. Berkhout.** 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural $tRNA_3^{Lys}$. J. Virol. **69:**3090–3097.
9. **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.
10. **Isel, C., C. Ehresmann, G. Keith, B. Ehresmann, and R. Marquet.** 1995. Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/$tRNA^{Lys,3}$ (template/primer) complex. J. Mol. Biol. **247:**236–250.
11. **Isel, C., R. Marquet, G. Keith, C. Ehresmann, and B. Ehresmann.** 1993. Modified nucleotides of $tRNA^{Lys,3}$ modulate primer/template loop-loop interactions in the initiation complex of HIV-1. J. Biol. Chem. **268:**25269–25272.
12. **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.
13. **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.
14. **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.
15. **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.
16. **Lund, A. H., M. Duch, J. Lovmand, P. Jorgensen, and F. S. Pedersen.** 1993. Mutated primer binding sites interacting with different tRNAs allow efficient murine leukemia virus replication. J. Virol. **67:**7125–7130.
17. **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.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

19. **Peters, G., and J. E. Dahlberg.** 1979. RNA-directed DNA synthesis in Moloney murine leukemia virus: interaction between the primer tRNA and the genome RNA. J. Virol. **31:**398–407.
20. **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.
21. **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.
22. **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.
23. **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.
24. **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.
25. **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.
26. **Rosa, M. D., J. P. Hendrick, Jr., M. R. Lerner, J. A. Steitz, and M. Reichlin.** 1980. A mammalian tRNA^{His}-containing antigen is recognized by the polymyositis-specific antibody anti-Jo-1. Nucleic Acids Res. **11:**853–870.
27. **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.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **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.
30. **Taylor, J. M.** 1977. An analysis of the role of tRNA species as primers for transcription into DNA of RNA tumor virus genomes. Biochim. Biophys. Acta **473:**531–534.
31. **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.
32. **Temin, H.** 1981. Structure, variation and synthesis of retrovirus long terminal repeat. Cell **27:**1–3.
33. **Temin, H. M., and S. Mizutani.** 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226:**1211–1213.
34. **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.
35. **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.
36. **Wakefield, J. K., A. G. Wolf, and C. D. Morrow.** 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA$_3^{Lys}$. J. Virol. **69:**6021–6029.
37. **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.