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Effects of Alterations of Primer-Binding Site Sequences on Human Immunodeficiency Virus Type 1 Replication

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Received 31 March 1994/Accepted 29 June 1994

The human immunodeficiency virus type 1 genomic RNA primer-binding site (PBS) sequence comprises 18 nucleotides which are complementary to those at the 3’ end of the replication initiation primer tRNA<sub>Lys</sub>. To investigate the role of the PBS in viral replication, we either deleted the original wild-type PBS (complementary to tRNA<sub>Lys</sub>) or replaced it with DNA sequences complementary to either tRNA<sub>Phe</sub> or tRNA<sub>Phe</sub>. Transfection of COS cells with such molecular constructs yielded similar levels of viral progeny that were indistinguishable with regard to viral proteins and tRNA content. Virus particles derived from PBS-deleted molecular clones were noninfectious for MT-4, Jurkat, and CEM-T4 cells. However, infectious viruses were derived from constructs in which the PBS had been altered to sequences complementary to either tRNA<sub>Phe</sub>, although mutated forms showed significant lags in replication efficiency in comparison with wild types. Molecular analysis of reverse-transcribed DNA in cells infected by the mutated viruses indicated that both tRNA<sub>Phe</sub> and tRNA<sub>Phe</sub> could function as primers for reverse transcription during the early stages of infection. Sequencing of full-length proviral DNA, obtained 6 days after infection, revealed the mutated PBS, indicating that a complete cycle of reverse transcription had occurred. During subsequent rounds of infection, reversion of the mutated PBS to wild-type sequences was observed, accompanied by increased production of viral gene products. Reversion to wild-type PBS sequences was confirmed both by specific PCR analysis, using distinct primer pairs, and by direct sequencing of amplified segments. We also performed endogenous in vitro reverse transcription experiments in which synthesis of minus-strand strong-stop viral DNA was primed from a synthetic RNA template containing a PBS complementary to various tRNA isoacceptors. These results showed that tRNA<sub>Phe</sub> was a much more efficient primer of such reactions than either tRNA<sub>Lys</sub> or tRNA<sub>Phe</sub>.

An early, critical step in the human immunodeficiency virus type (HIV-1) life cycle is reverse transcription of viral RNA into proviral DNA, which can then be integrated into the infected host cell genome. This process is carried out by the multifunctional viral enzyme reverse transcriptase (RT) and requires a primer annealed to a single-stranded template to initiate DNA synthesis (9, 14, 18, 45, 47, 51). All retroviruses use a cell-derived specific tRNA as primer, which is packaged into mature virions (10, 11, 17, 22, 25, 34). Eighteen nucleotides (nt) at the 3’ end of tRNA<sub>Lys</sub> is complementary to an 18-nt sequence of HIV-1 genomic RNA, termed the primer-binding site (PBS). The PBS is found approximately 180 nt from the 5’ end of the viral genome (39). Preferential packaging of tRNA<sub>Lys</sub> and its tight association with viral RNA in the virion suggests that it may function as a primer of reverse transcription from the PBS. In addition, HIV-1 RT has been shown to bind specifically to tRNA<sub>Lys</sub> through interactions with the anticodon loop, TWC loop, and D loop of tRNA<sub>Lys</sub> (1, 2, 4, 27). However, the question of specific binding between HIV-1 RT and complexes of tRNA<sub>Lys</sub> annealed to a PBS template has not been studied.

The viral genomic PBS is believed both to provide a site for binding of primer tRNA, thereby allowing initiation of reverse transcription, and to facilitate the second template switch (36, 46, 48, 50). Neither of these functions is well understood. In addition, the PBS may play a role in the specific selection and packaging of primer tRNA<sub>Lys</sub>. Primer tRNA<sub>Lys</sub> may also play a dual role in HIV reverse transcription, by initiating RNA-dependent DNA polymerization from the PBS and by acting as the PBS template during synthesis of plus-strand DNA (9, 13).

To study these multiple functions, the wild-type PBS sequence (complementary to the 3’ end of tRNA<sub>Lys</sub>) in an infectious molecular clone of HIV-1 was either deleted or replaced with sequences complementary to either tRNA<sub>Phe</sub> or tRNA<sub>Phe</sub>. The rationale for choosing these particular tRNA species was that (i) tRNA<sub>Phe</sub> is utilized as primer for reverse transcription in other retroviruses, e.g., Mason-Pfizer monkey virus (27, 50), whereas tRNA<sub>Phe</sub> has never been identified as a reverse transcription initiation primer, and (ii) while tRNA<sub>Phe</sub> is packaged into wild-type HIV-1 in amounts even greater than those of the wild-type primer tRNA<sub>Lys</sub>, tRNA<sub>Phe</sub> is present at much lower levels, i.e., around 1% (22, 49).

We now describe the effects of alterations in the HIV-1 PBS on viral replication and on virion tRNA content. We found that the quantities and patterns of tRNA species incorporated into virions were unaffected either by the absence of a PBS or by the presence of altered PBS sequences, indicating that the PBS does not play a significant role in the selection and incorporation of primer tRNA during HIV-1 assembly. However, deletion of the 18-nt wild-type PBS completely abolished viral infectivity, whereas its replacement with sequences complementary to either tRNA<sub>Phe</sub> or tRNA<sub>Phe</sub> impaired but did not arrest viral infectivity. Interestingly, the mutant PBS sequences reverted to wild type during infection. The ability of these various viruses to replicate was closely related to the status of the PBS. During early stages of infection, the two PBS mutants, in conjunction with tRNA<sub>Phe</sub> and tRNA<sub>Phe</sub>, appar-
ently functioned as primers for reverse transcription, although less efficiently than wild-type primer tRNA\textsubscript{lys}\textsuperscript{3\textprime}.

This research was largely performed by X.L., under the joint supervision of M.A.W. and M.A.P., in partial fulfillment of the requirements for the Ph.D. degree, Faculty of Graduate Studies and Research, McGill University, Montreal, Quebec, Canada.

**MATERIALS AND METHODS**

**Cells, viruses, and plasmids.** The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: the pBH10 noninfected plasmid used as a nick translation probe in Northern (RNA) blots (donated by R. C. Gallo); the HXB2D infectious molecular clone of HIV-III\textsubscript{B}, containing the full-length HIV-1 proviral genome (provided by G. Shaw and B. Hahn); and the CD4\textsuperscript{+} MT-4 cell line (contributed by D. Richman). Other cells, including the CD4\textsuperscript{+} RD (rhabdomyosarcoma), simian-derived COS-7, CD4\textsuperscript{+} CEM, and Jurkat cell lines, were purchased from the American Type Culture Collection, Rockville, Md. Each of the CD4\textsuperscript{+} cell lines was susceptible to acute cytopathic HIV-1 infection, while the CD4\textsuperscript{-} lines were not. Cells were routinely maintained in RPMI 1640 medium (Gibco-BRL Laboratories, Toronto, Ontario, Canada) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 \mu g of streptomycin per ml. pSCV21, a eukaryotic expression vector containing the full-length HIV-1 genome with a simian virus 40 origin of replication (15), was a gift of E. Cohen, Université de Montréal, Montreal, Quebec, Canada. pSVK3 and pSP72 were purchased from Pharmacia Inc. (Montreal, Quebec, Canada) and Promega (Nepean, Ontario, Canada), respectively. Recombinant HIV-1 RT (p66/p51 heterodimer) was kindly provided by Casey Morrow, University of Alabama at Birmingham. Restriction enzymes and other modified enzymes were obtained from Pharmacia.

**Construction of HIV-1 proviral plasmids with altered PBS sequences.** The PBSs of HIV-1 molecular clones were altered by using a combination of linker replacement and site-directed mutagenesis (44). Briefly, HXB2D was cut with \textit{SacI} and \textit{ApaI} to generate a 3.7-kb fragment comprising the 5\textprime{} region of the HIV-1 proviral genome and cellular flanking sequences. This fragment was subcloned into \textit{SacI}-\textit{ApaI}-digested pSVK3 to give pSVPBS, which was used for the subsequent construction of PBS mutants. PBS-PBS was digested with \textit{NotI} and treated with a mung bean nuclease to generate blunt ends. The plasmid was then cut with \textit{BstHI} to remove 70 HIV nucleotides including the PBS and downstream sequences. We then ligated various 70-nt oligonucleotide sequences into the gap created by the \textit{NotI} and \textit{BstHII} digestions described above. These insertions contained PBS-like sequences complementary to tRNA\textsubscript{lys}\textsuperscript{3\textprime} and tRNA\textsubscript{Phe} to yield pSVPBS-Lys1,2 and pSVPBS-Phe, respectively. Standard site-directed mutagenesis was used to delete the entire 18-nt region of pSVPBS to yield pSVPBS(−).

The sequences of our various 18-nt PBS constructs are as follows: 5\textprime{}-TGG CGC CCG AAC AGG GAC-3\textprime{} (pPBS-WT), 5\textprime{}-TGG CGC CCA ACG TGG GGC-3\textprime{} (pPBS-Lys1,2), and 5\textprime{}-TGG TGC CGA AAC CCG GGA-3\textprime{} (pPBS-Phe).

The \textit{SacI}-\textit{ApaI} fragments from pSVPBS, pSVPBS(−), pSVPBS-Lys1,2, and pSVPBS-Phe were then cloned into the appropriately digested pSCV21 molecular clone of HIV-1 to yield expression plasmids containing full-length HIV-1 proviral DNA with the wild-type PBS (pPBS-WT) and mutated PBSSs [pPBS(−), pPBS-Lys1,2 and pPBS-Phe]. All constructs were sequenced to verify that the correct modifications in PBS sequence had been achieved.

Each of the plasmids described above, containing altered PBS sequences, was cut with \textit{BglII} and \textit{PstI} to generate a fragment of 947 bp (473 to 1420), comprising the PBS/USR region of HIV-1 proviral sequences (3). Such fragments were then ligated into the RNA expression vector pSP72, at the gap created by \textit{PstI} and \textit{BglII}, to generate various HIV-1 RNA expression plasmids containing altered PBS sequences.

**Synthesis of minus-strand strong-stop DNA in an endogenous in vitro RT assay.** The HIV-1 RNA expression vectors described above were transfected into a mung bean RNAse A (cont 850) (39) and used in the Promega Riboprobe Gemini Core System to generate runoff transcripts of 483 ribonucleotides with alterations in the PBS region. In vitro RT assays were carried out in a volume of 20 \mu l containing 10 mM dithiothreitol, 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM MgCl\textsubscript{2}, and 0.2 mM each of the four deoxynucleoside triphosphates as described previously (3). tRNA\textsubscript{lys}\textsuperscript{3\textprime}, tRNA\textsubscript{Phe}\textsuperscript{3\textprime}, and tRNA\textsubscript{lys}\textsuperscript{3\textprime} were purified from human placenta (22, 42). Reaction mixtures were supplemented with 0.3 \mu g of recombinant HIV-1 RT together with 200 U of RNasin (RNase inhibitor; Gibco-BRL, Toronto, Ontario, Canada) and incubated at 37°C for up to 15 min. Reactions were terminated at various times by addition of EDTA to 100 mM. The terminated mixtures were extracted with phenol-chloroform and chloroform and passed through a Sephadex G-25 (Pharmacia) column to remove unincorporated free radioactive nucleotides. The products of these reactions were boiled for 4 min in formamide gel loading buffer (44) and chilled on ice for 5 min before being loaded onto a 5% denaturing polyacrylamide gel. The full-length minus-strand strong-stop DNA synthesized in these reactions is 249 nt long.

**Infection of target cells.** COS-7 cells were transfected with the pPBS constructs by electroporation. After 60 h of incubation, cell-free virus stocks were prepared by centrifugation of culture supernatants at 3,000 rpm at 4°C for 30 min in a Beckman bench-top centrifuge and filtration through a 0.2-\mu m-pore-size sterile membrane (Becton Dickinson, Oxnard, Calif.). To remove possible contaminating plasmid DNA, which could interfere with the infection of the target cells, the virus stock was treated at 37°C for 30 min with excess DNase I at a final concentration of 100 U/ml in the presence of 10 mM MgCl\textsubscript{2} (35). The virus-containing clarified culture fluids were stored at −70°C until use.

Infectiousness of virus particles produced by transfection of COS-7 cells was determined by using MT-4, Jurkat, or CEM cells as targets. Briefly, 5 × 10\textsuperscript{5} cells were harvested during exponential growth, washed once by centrifugation, and incubated in medium containing 5 ng of viral p24, supplemented with 10 \mu g of Polybrene per ml, at 37°C for 3 h with occasional gentle shaking. Unbound viruses were removed by four separate centrifugation washings in serum-free medium, and the cells were resuspended in fresh medium. To ensure complete removal of contaminating plasmid DNA, medium from the fourth wash was checked by PCR using primer pairs specific for the HIV-1 p24 gene (16). Cell culture medium was changed at 3- to 4-day intervals. Samples of cells and cell-free culture supernatants were collected at regular intervals and assayed for virus content by HIV-1 p24 antigen expression and RT assay (5). Samples from MT-4 cells infected by heat-inactivated pPBS-WT (60°C, 30 min) served as negative controls (52).

**PCR analysis and DNA sequencing.** Infected cells (5 × 10\textsuperscript{5}) were suspended in 0.5 ml of TE buffer (50 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg of pronase per ml and incubated at 37°C for
FIG. 1. Primer location and strategy for detection of viral DNA by PCR. (A) Sequences of primers used in PCR. (B) PCR strategy. Primer pairs were chosen to distinguish the three types of PBS studied (PS/Lys1,2, PS/Phe, and PS/Lys3) or to detect full-length proviral DNA (PS/PA). The latter primer pair was used to amplify a region flanking the PBS. Amplified fragments were subsequently analyzed by using the PS primer to sequence minus-strand DNA and the PA primer to sequence plus-strand DNA, using a dsDNA cycling sequencing system (Gibco-BRL).

5 to 8 h with gentle shaking. The samples were then extracted with TE-saturated phenol and chloroform-isoamyl alcohol. Both total DNA and high-molecular-weight (HMW) DNA were isolated by standard methods (44); the former was used for PBS sequence analysis, while the latter served for determinations of integrated HIV-1 proviral DNA. Samples were then analyzed by PCR.

Selected primer pairs were used in PCR analysis of the PBS sequences of various viral DNA species ( unintegrated intermediates or integrated forms). The sequences, locations, and orientations of the primer pairs, designed to detect DNA species with contiguous R and PBS sequences, as well as a description of the products formed, are illustrated schematically in Fig. 1. Primer pair PS/PA amplifies full-length proviral DNA and therefore detects the completion of reverse transcription (13, 52). Three sets of primer pairs, PS/Lys1,2, PS/Phe, and PS/Lys3, were used to distinguish the three types of PBS studied. To distinguish PBS forms, highly stringent PCRs were performed with 50 ng of sample DNA, 50 mM Tris-Cl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 5 pmoles of 32P-end-labeled sense primer, and 20 pmol of unlabeled antisense primer. Reactions were run for 25 cycles of 94°C (2 min) and 65°C (2 min). Other PCRs were performed with 50 pM of unlabeled primers (sense and antisense) for 30 cycles of 94°C (2 min), 60°C (2 min), and 72°C (2 min). Reactions were standardized by simultaneous amplification of β-globin DNA (52) (primer pair, 5′-ACACCACTGTGTTCACTAG C-3′ [sense] and 5′-CAACTTCTACCTGGTACCAT-3′ [antisense]). For direct sequencing of the R/US/PBS region (LTR/Gag), PS/PA amplified fragments (367 bp) were resolved by electrophoresis, purified by electrolutions, and sequenced by using a PCR-based double-stranded DNA (dsDNA) cycling sequencing system (Gibco-BRL).

Identification of tRNA species in virus particles. A dot blot assay using DNA oligonucleotides complementary to the 3′ end of tRNA<sub>Lys</sub> (probe sequence, 5′-TGGCGCAGCAACG GAC-3′), tRNA<sub>Phe</sub> (probe sequence, 5′-TGGCGCACGAC GTGGGC), or tRNA<sub>Phel</sub> (probe sequence, 5′-TGGTGGCAG AAACCCGGA-3′) was used to identify specific tRNA species. Positive controls, including tRNA<sub>Lys</sub>, tRNA<sub>Phe</sub>, and tRNA<sub>Phel</sub>, were purified from human placenta (22, 42). The specificities of these probes and hybridization conditions have been described elsewhere (22, 30). Total RNA was purified from viruses as described previously (6), and the amount of viral RNA was normalized according to copy numbers of HIV-1 genomic RNA. Total RNA corresponding to 4 × 10<sup>8</sup> copies of viral genomic RNA was used in each analysis. RNA samples were blotted onto Hybond N filters (Amer sham) and hybridized separately with each of the three probes. Following high-stringency washing (22, 30), the filters were air dried and exposed to X-ray film at -70°C.

RESULTS

Alteration of PBS sequences does not affect expression of the proviral genome. The infectious HIV-1 clone pPBS-WT, which possesses a wild-type PBS complementary to tRNA<sub>Lys</sub> was altered (i) by deleting the 18-nt PBS to give pPBS(-) or (ii) by replacing the wild-type PBS with sequences complementary to tRNA<sub>Lys</sub> or tRNA<sub>Phe</sub> to give pPBS-Lys1,2 or pPBS-Phe, respectively. These mutant constructs were transfected into COS-7 cells, and virus particles were harvested after 60 h. Northern blotting and Western blotting (immunoblotting) were performed to study expression of the proviral genome. All of our wild-type (pPBS-WT) and mutated molecular clones produced the usual three major HIV RNA transcripts; no differences were noted among the various clones, nor were differences found in the patterns of proteins in the viral
progeny produced after transfection with each of the constructs.

We also transfected the various PBS constructs into the CD4+ RD cell line, which permits only one round of viral replication (32). No significant differences were observed with regard to levels of RT activity in culture fluids after various times following transfection with pPBS-WT, pPBS(−), pPBS-Lys1,2, or pPBS-Phe (data not shown). The transient nature of these infections is apparent by the peak in RT activity after 10 to 15 days. These results are not surprising, considering that neither the PBS nor reverse transcription is involved in viral replication following transfection with proviral DNA.

Effects of alterations in the PBS on viral infectivity. Viral particles were harvested from COS-7 transfection cultures after 60 h, normalized according to p24 content (approximately 5 ng), and used to infect a variety of HIV-susceptible cell lines, including MT-4, Jurkat, and CEM. Culture supernatants were regularly monitored both for HIV-1 p24 antigen expression and RT activity over 4 weeks.

Infection of MT-4 cells with pPBS-WT resulted in the rapid emergence of RT activity, syncyta, p24 Ag+ cells as measured by indirect immunofluorescence assay, and other cytopathic effects within 2 days. Virtually, all cells were p24 Ag+ after 1 week, at which time RT activity had peaked. RT activity gradually declined with death of infected cells. In contrast, only 5 to 10% of cells transfected by pPBS-Lys1,2 or pPBS-Phe were p24+ after this period, and RT activity in culture fluids was low (Fig. 2A). Virus particles produced from COS cells transfected with pPBS(−) were unable to infect MT-4 cells. No cytopathic effect, p24 antigen, or RT was detected even after 30 days. We noted that virus production by cells infected with the two PBS mutants reached normal levels by 15 to 18 days. Similar virus production kinetics were noted when either Jurkat or CEM cells were used as targets (data not shown), suggesting that cell type differences were not responsible for the observed results with mutated PBS-containing viruses.

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FIG. 2. (A) Infection of MT-4 cells with viruses harvested from COS-7 transfections. Viral inocula were equalized on the basis of either p24 content (5 ng) or RT activity (800,000 cpm) to infect 5 × 10⁵ cells. Cultures were regularly monitored for HIV-1 production by RT assay following infection by PBS(−) (O), PBS-Lys1,2 (●), PBS-Phe (Δ), or PBS-WT (▲). A, infection by pPBS-WT (heat-inactivated virus). No cells were added during the 24-day study period, in order to observe viral particle accumulation in culture supernatants. (B) Second round of infection of MT-4 cells by viruses obtained from initially infected cultures (9 days following infection by pPBS-WT and 18 days following infection by pPBS-Lys1,2 or pPBS-Phe). For symbols, see above.

The progeny of these MT-4 infections (9 days from pPBS-WT infection and 18 days from pPBS-Lys1,2 and pPBS-Phe infection) were then used in a second round of replication in MT-4 cells. Figure 2B shows that high rates of replication, equivalent to those obtained with wild-type viruses, were observed when the progeny of pPBS-Lys1,2 or pPBS-Phe, obtained at 18 days, were studied for the ability to infect MT-4 cells. In contrast, when the progeny of MT-4 infections, obtained after 6 days, were compared for infectiousness in a second-round infection, infection kinetics similar to those of Fig. 2A were observed (data not shown). This result suggests that wild-type PBS forms had preferentially emerged during the 24-day period of study; this subject will be considered in Discussion.

PCR analysis of the PBS sequence of proviral DNA in infected cells. The specificity of our PCR assay was monitored by mixing 0.1 ng of cloned HIV plasmid (wild type or mutated) with 50 µg of MT-4 DNA from uninfected cells. The R/U5/PBS region of each type of HIV genomic DNA was amplified by using the three sets of primer pairs, PS/Lys1,2, PS/Phe, and PS/Lys 3, described in Fig. 1. Each HIV clone could be amplified only by its own specific primer pair, e.g., pPBS-Lys1,2 by PS/Lys1,2 and not PS/Lys3 or PS/Phe (Fig. 3A, lane 2). Also, primer pair PS/Lys1,2 could not amplify any of the R/U5/PBS region of PBS(−) (Fig. 3A, lane 1), pPBS-Phe (lane 3), or pPBS-WT (lane 4).

The three sets of primer pairs were used to analyze PBS sequences in DNA harvested from MT-4 cells at various times after infection by the various viral clones. Total cellular DNA was used to simultaneously detect PBS sequences in intermediate viral DNA species as well as in full-length integrated proviral DNA. At all times, we detected only wild-type PBS sequences in DNA extracted from MT-4 cells exposed to pPBS-WT virions, starting 3 days after infection (Fig. 3B). At 24 days after infection, the intensity of this band had decreased, apparently as a result of virus-induced cytopathicity.
In contrast, only mutated PBS forms were present in cells infected with pPBS-Lys1,2 or pPBS-Phe at 6 days after infection (Fig. 3C and D). By 9 days after infection, the wild-type PBS had started to emerge, while mutated PBS forms had begun to disappear, concomitant with high levels of release of infectious progeny by these cells (Fig. 2A). To rule out possible PCR artifacts, we used primer pair PS/PA (Fig. 1) to amplify a 367-bp fragment flanking the PBS region in DNA isolated from MT-4 cells infected with each of the wild-type and mutated viral forms. Sequencing confirmed that the PBS which was detected early (i.e., up to 6 days) in cells infected by mutated viruses contained the original mutations. However, at later times, only wild-type PBS sequences were found, while mixtures were apparently present at intermediate time points, as suggested by the presence of multiple bands at such times. A summary of DNA sequencing results with respect to the PBS is shown in Table 1.

**Determination of minus-strand strong-stop DNA synthesis as measured by endogenous in vitro reverse transcription.** To determine the efficiency of reverse transcription initiated by different tRNA primers, we used an endogenous in vitro reverse transcription assay (3) that employed synthetic RNA templates containing mutated PBS sequences together with their respective tRNA primers. When synthetic pPBS-WT RNA template was primed with tRNA\textsuperscript{T}\textsuperscript{\textsubscript{c}}, full-length minus-strand strong-stop DNA products were detectable within 1 min of addition of RT to the reaction mixture (Fig. 4, lane 1). However, when reactions involving synthetic pPBS-Lys1,2 and pPBS-Phe RNA templates were primed with tRNA\textsuperscript{T}\textsuperscript{\textsubscript{l}}, tRNA\textsuperscript{T}\textsuperscript{\textsubscript{e}}, and tRNA\textsuperscript{T}\textsuperscript{\textsubscript{m}}, respectively, full-length minus-strand strong-stop DNA (249 nt) was not detectable until after 15 min (Fig. 4, lanes 7 and 11). None of the tRNAs were able to prime minus-strand strong-stop DNA synthesis from a synthetic PBS(−) RNA template (Fig. 4, lanes 4, 8, and 12), indicating the specificity of the 249-nt products generated. The fast-moving bands (molecular size, <249 nt) in Fig. 4 are probably due to pausing of DNA synthesis by RT during DNA synthesis (19, 24).

**Alteration of HIV-1 PBS does not abolish the reverse transcription cycle.** The retrovirus PBS is thought to facilitate the second template switch which occurs during reverse transcription. To detect full-length integrated proviral DNA by PCR, we studied HMW DNA of infected MT-4 cells by using primer pair PS/PA (Fig. 1), which amplifies the last region of reverse-transcribed proviral DNA (13). Figure 5 shows that full-length proviral DNA was detected throughout the course of infection by wild-type and mutated viruses. This was true even at early time points of infections (6 days) involving mutated viruses, when only mutated PBS forms were present (Fig. 5).

The identity of the PBS sequences of integrated proviral DNA was next confirmed by sequencing 367-bp fragments amplified by primer pair PS/PA (Fig. 1). The PBS sequences in

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HMW DNA after 6 days were all of the mutated variety in cells infected with the mutant virus PBS-Lys1,2 or PBS-Phe. However, only wild-type PBS sequences were detected after 24 days. The PBS sequences at intermediate times (e.g., day 12 in PBS-Lys1,2-infected cells and day 9 in PBS-Phe-infected cells) were a mixture of both mutants and wild types.

Identification of the tRNA species in mutant viruses. Three different oligonucleotide probes, complementary to the 3' ends of tRNA\textsubscript{12}, tRNA\textsubscript{Phe}, and tRNA\textsubscript{35}, respectively, were used to detect these tRNA species in purified virus particles, using hybridization conditions and purified human placental tRNA isoacceptor species, as described previously (22, 30). These analyses were carried out by dot blot hybridization; all patterns of reactivity were specific, and no cross-hybridization was observed among the probes used with any tRNA isoacceptor species. For example, the tRNA\textsubscript{Phe}-specific probe did not show cross-hybridization to either tRNA\textsubscript{12} or tRNA\textsubscript{35} (Fig. 6A to C), which share more than 60% homology at the 18-nt 3' end.

No significant differences in levels of tRNA\textsubscript{12}, tRNA\textsubscript{Phe}, or wild-type primer tRNA\textsubscript{35} were found in each of the three mutant compared with wild-type viruses (Fig. 6A to C). Thus, replacement of the wild-type PBS with sequences complementary to tRNA\textsubscript{12} or tRNA\textsubscript{35} did not alter the pattern of tRNA species incorporation into viral progeny (Fig. 6). Two-dimensional polyacrylamide gel electrophoresis analysis of tRNA species in these various virus particles did not reveal significant differences among the wild type and PBS(−), PBS-Lys1,2, and PBS-Phe mutants (data not shown). Thus, the PBS does not appear to be involved in the selective incorporation of tRNA species into mature virions.

DISCUSSION

Reverse transcription of retroviral genomic RNA into viral DNA is an early and essential step in the HIV life cycle. The role of the PBS in this process is to provide a complementary region for the binding of the specific tRNA isoacceptor species, which serves as a primer for RNA-dependent DNA polymerization, and to facilitate the second template switch, allowing completion of full-length double-stranded proviral DNA (12, 26, 28, 31, 33, 38, 43).

This study of PBS sequences was based on the utility of tRNA as a primer of retroviral RT and the abundance of tRNA species in HIV-1 virions. We were unable to detect significant differences among tRNA isoacceptors in terms of packaging into mature virions, consistent with results that showed deletion of either long terminal repeat or PBS sequences did not disrupt tRNA patterns in viruses (22). Viral proteins, including the Pr160\textsubscript{ gagpol } precursor, probably play an important role in the selection of tRNA isoacceptors (30).

Virus particles harvested from transfected COS-7 cells were assessed for infectivity, using MT-4, CEM-T4, and Jurkat cells as targets. Not surprisingly, virus produced from PBS(−) transfections were noninfectious, consistent with previous observations (40). A novel finding of this study is that virus produced after transfection by the two PBS replacement mutants, PBS-Lys1,2 and PBS-Phe, were infectious, although less so than wild-type viruses. However, despite a delay in production of viral p24 (CA [capsid]) and RT, the rate of
viruses by these mutants eventually reached wild-type levels. Mutant viruses derived from early and late stages of infection were used to reinitiate MT4 cells; we found that the early-stage-derived virus continued to lag in infection kinetics, while late-stage virus behaved indistinguishably from the wild type. As discussed below, this shift in phenotype corresponded to a reversion to a wild-type PBS. Other workers have also reported differential results with use of mutated PBS sequences in a different system involving only a single round of viral replication (29).

During plus-strand DNA synthesis, tRNA serves as the template for the generation of the PBS, thus enabling identification of the tRNA isoacceptor used as a primer. PCR analysis at early time points of the PBS sequences found within the HMW DNA of cells infected by pPBS-Lys1,2 or pPBS-Phe showed complementarity to tRNA\textsubscript{Lys\footnote{1,2}} and tRNA\textsubscript{Phe}, respectively. In contrast, the PBS sequences of proviral DNA from later stages of these infections were complementary to wild-type primer tRNA\textsubscript{Lys\footnote{1,2}}, indicating that synthesis and apparent selection of wild-type forms had occurred. Thus, both tRNA\textsubscript{Lys\footnote{1,2}} and tRNA\textsubscript{Phe} appear to serve as primers for reverse transcription during early stages of infection. However, at late stages, it is likely that tRNA\textsubscript{Lys\footnote{1,2}} alone can serve this role. Our data show that the replication competence of HIV-1 clones is closely related to the status of the PBS. Our use of synthetic RNA templates and mutated PBS in cell-free RT assays showed that tRNA\textsubscript{Lys\footnote{1,2}} and tRNA\textsubscript{Phe} were inefficient primers of minus-strand strong-stop DNA synthesis. This may explain the lag in virus production kinetics when the two PBS mutants were studied in a replication-competent system.

Despite the ability of tRNA\textsubscript{Lys\footnote{1,2}} and tRNA\textsubscript{Phe} to serve as primers, both mutated PBS forms eventually reverted to wild-type complementary to tRNA\textsubscript{Lys\footnote{1,2}}. It is unlikely that this development was due to contamination and amplification of small quantities of wild-type forms, since our molecular proviral clones had been repeatedly subcloned and purified and were pure by sequencing. Also, specific PCR showed that only mutant PBS forms were present in proviral DNA at early stages of infection by mutated viruses. No reversion of the PBS\textsuperscript{−} mutant was noted, as might have been expected if the reversions with PBS-Lys1,2 and PBS-Phe were due to contamination.

Although the mechanisms responsible for the observed reversion are uncertain, it is known that the HIV RT binds preferentially to tRNA\textsubscript{Lys\footnote{1,2}} (4, 21, 37, 41) and that interaction between retroviral US RNA and the 3' VLP of the tRNA\textsuperscript{Phe} may be required for efficient initiation of reverse transcription (1, 7, 8, 27). Recent studies have shown that a 4-nt sequence in the anticodon loop of tRNA\textsubscript{Phe} interacts with HIV-1 genomic RNA in a region upstream from the PBS (20). The resulting loop-loop interaction between tRNA and RNA template, combined with normal PBS-tRNA binding, might give rise to significant alterations in secondary structure of the primer-template complex relative to that occurring when only the 18 nt of the viral PBS interact with tRNA (as in the case of the pPBS-Lys1,2 and pPBS-Phe mutants). The stability of additional tRNA-RNA template interactions might be dependent on particular base modifications found only in tRNA\textsubscript{Lys\footnote{1,2}}. Such interactions could play a role in formation of RT-tRNA-RNA template transcription complexes, thereby affecting transcription efficiency. Results of our endogenous, in vitro reverse transcription reaction showed that initiation with tRNA\textsubscript{Lys\footnote{1,2}} and tRNA\textsubscript{Phe} occurred less efficiently than with tRNA\textsubscript{Lys\footnote{1,2}} when minus-strand strong-stop DNA synthesis was primed from an RNA template containing a PBS complementary to the respective tRNA isoacceptors (Fig. 4).

tRNA\textsubscript{Lys\footnote{1,2}} has extensive 3'-end homology with both tRNA\textsubscript{Lys\footnote{1,2}} (71%) and tRNA\textsubscript{Phe} (62%) and was incorporated into our PBS mutant viruses at levels similar to those found with wild types. Homology among these tRNA species implies that tRNA\textsubscript{Lys\footnote{1,2}} could conceivably anneal to a mutant PBS; such annealing might be further stabilized by the tRNA-RNA template interactions discussed above. Thus, tRNA\textsubscript{Lys\footnote{1,2}} might be able to prime reverse transcription even from a mutant PBS. Since reverse transcription with tRNA\textsubscript{Lys\footnote{1,2}} is more efficient than with tRNA\textsubscript{Lys\footnote{1,2}} or tRNA\textsubscript{Phe}, it is conceivable that mutant viruses might preferentially use a wild-type primer, leading to the reverse transcription from tRNA\textsubscript{Lys\footnote{1,2}} of a wild-type PBS and a
consequent increase in viral production. Other factors that may affect reversion of the PBS to wild type include specific interactions between the HIV-1 RT and tRNA$^{\text{Lys}}$ and the preferential incorporation of tRNA$^{\text{Tyr}}$ isoacceptors into virions. Since the PBSs of both proviral plus-strand strong-stop DNA and full-length plus-strand DNA reflect the identity of the tRNA primer, the use of tRNA$^{\text{Lys}}$ as a primer for viruses containing a mutated PBS might eventually result in reversion to a wild-type sequence. It is still unclear whether cellular factors may be involved in reverse transcription (24, 52). Further insight will be possible once the factors involved in the selection, incorporation, and placement of primer tRNA onto the HIV-1 PBS in vivo are better understood (23, 25, 30, 32).

ACKNOWLEDGMENTS

We are grateful to C. Morrow, University of Alabama at Birmingham, for a gift of recombinant wild-type p66/p51 heterodimeric HIV-1 RT. We thank Francine Busschaert for assistance in preparation of the manuscript.

We thank Health and Welfare Canada, the Medical Research Council of Canada, and the American Foundation for AIDS Research for grant support to M.A.W. and M.A.P. X.L. was the recipient of a predoctoral studentship from the Medical Research Council of Canada.

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