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Nucleotide Substitutions within U5 Are Critical for Efficient Reverse Transcription of Human Immunodeficiency Virus Type 1 with a Primer Binding Site Complementary to $tRNA^{His}$

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Sequence analysis of integrated proviruses of human immunodeficiency virus type 1 (HIV-1) which utilize $tRNA^{His}$ to initiate reverse transcription [virus derived from pHXB2(His-AC-TGT)] revealed five additional nucleotide substitutions in the U5 and primer binding site (PBS) regions (ATGAC for CCTGT at nucleotides 152, 160, 174, 181, and 200, respectively) (Z. Zhang et al., Virology 226:306–317, 1996). We constructed a mutant proviral genome [pHXB2(His-AC-GAC)] which contained the ATGAC substitutions to test if they represented a necessary adaptation by the virus for use of $tRNA^{His}$ to initiate reverse transcription. Viruses from pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) were infectious. Sequence analysis of the U5 and PBS regions of integrated provirus from a cell culture infected with virus derived from pHXB2(His-AC-TGT) revealed a G-to-A change in CCTGT at nucleotide 181 after limited in vitro culture, suggesting that this nucleotide change represented an adaptation by the virus to efficiently utilize $tRNA^{His}$ to initiate reverse transcription. To further address this possibility, we used a specific mutation in reverse transcriptase (RT), a methionine-to-valine change in the highly conserved YMDD amino acid motif of HIV-1 RT (M184V), which has been shown in previous studies to influence the fidelity and activity of the enzyme. The M184V RT mutation was cloned into pHXB2(His-AC-GAC) and pHXB2(His-AC-TGT). Virus derived from pHXB2(His-AC-GAC) with M184V RT had slightly delayed replication compared to the virus from pHXB2(His-AC-GAC) with wild-type RT; in contrast, virus from pHXB2(His-AC-TGT) with M184V RT was severely compromised in replication. Using an endogenous reverse transcription-PCR assay to analyze the reverse transcription of viruses obtained after transfection, we found that viruses derived from pHXB2(His-AC-GAC) with the wild-type RT were slightly faster in the initiation of reverse transcription than viruses with M184V RT. The initiation of reverse transcription was delayed in viruses derived from pHXB2(His-AC-TGT) with wild-type RT and M184V RT compared to viruses derived from pHXB2(His-AC-GAC). Finally, sequence analysis of U5 and PBS regions of proviruses from pHXB2(His-AC-GAC) with wild-type RT revealed considerably more nucleotide substitutions than in viruses derived from pHXB2(His-AC-GAC) containing the M184V mutation in RT after extended in vitro culture. Our studies point to a role for these additional nucleotide substitutions in U5 as an adaptation by the virus to utilize an alternative tRNA to initiate reverse transcription.

The hallmark of retroviral replication is the process of reverse transcription, in which the viral RNA genome is converted to a DNA form prior to integration into the host cell chromosome. Reverse transcription is catalyzed by a virally encoded enzyme, reverse transcriptase (RT) (4, 33). The initiation of reverse transcription occurs near the 5′ end of the viral RNA genome at a region which has been designated the primer binding site (PBS) (21, 22, 32). The PBS is an 18-nucleotide sequence which is complementary to the 3′ end of a cellular tRNA used as a primer to initiate reverse transcription (12, 22, 23, 30, 32, 34). The RT extends the bound tRNA primer from the free 3′ OH. In a series of complex steps, the RT copies the viral RNA genome, resulting in a complete DNA copy (3). The PBS is regenerated during plus-strand DNA synthesis when the RT copies the still attached tRNA primer (12, 31, 34). Thus, the proviral PBS sequence reflects the tRNA used to initiate reverse transcription.

Although all retroviruses use a cellular tRNA as the primer for RT to initiate reverse transcription, within a given group of retroviruses, the cellular tRNA selected to initiate reverse transcription is conserved (6, 10, 19). For human immunodeficiency virus (HIV) and simian immunodeficiency virus, $tRNA_3^{Lys}$ is preferentially used to initiate reverse transcription (19). Early studies found that substitution of the PBS with a region complementary to alternative tRNAs resulted in viruses which used the alternative tRNAs to initiate reverse transcription, although after extended in vitro culture, the viruses reverted to a wild-type genome with a PBS complementary to $tRNA_3^{Lys}$ (11, 17, 38, 39). New insights into the complexity of the interaction between the PBS and tRNA primer in reverse transcription have come from studies which identified regions of the HIV type 1 (HIV-1) viral genome in U5 interacting with $tRNA_3^{Lys}$ (14, 15, 37).

Recent studies from this laboratory have identified a region within U5 called the A loop which, in conjunction with mutations in the PBS, resulted in the production of stable HIV-1 isolates which utilize $tRNA^{His}$ or $tRNA^{Met}$ to initiate reverse transcription (16, 37). Analysis of the U5 and PBS regions (U5-PBS) of viruses derived after long-term in vitro culture which used $tRNA^{His}$ to initiate reverse transcription revealed that in addition to the original mutations of the A loop in U5 and the PBS, five additional mutations had occurred, resulting in the substitution of the sequence ATGAC for CCTGT (Fig. 1A) (37, 41). We postulated that the five additional mutations seen after extended culture represented a selection of viral

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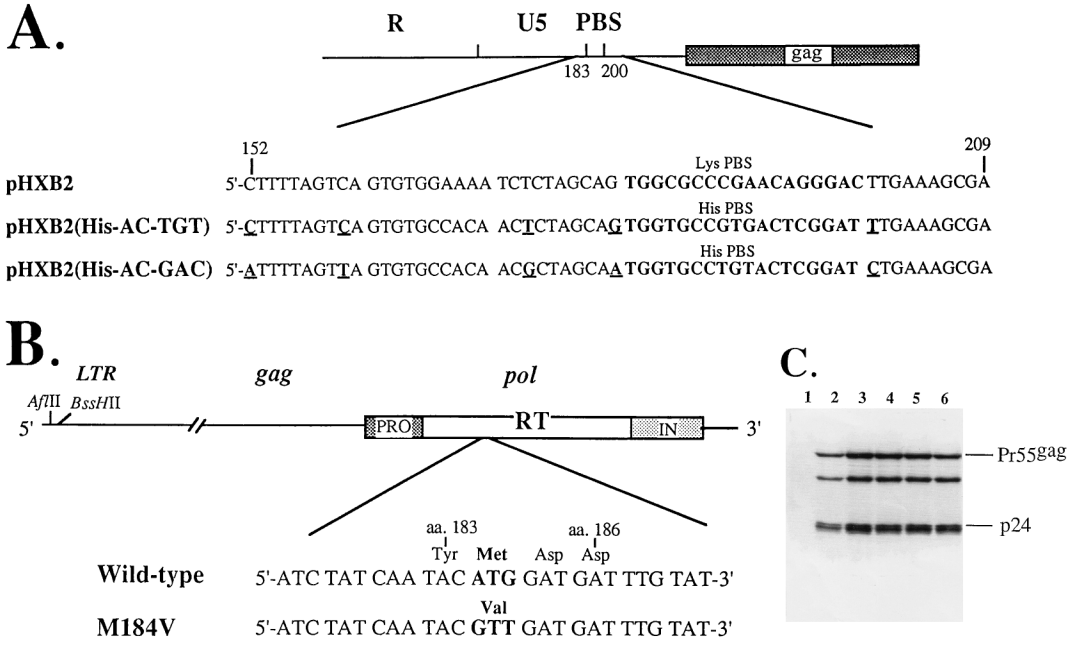

FIG. 1. (A) Diagram of the U5 and PBS regions of wild-type and mutant proviruses. The mutants pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) contain mutated U5 regions and a PBS complementary to $tRNA^{His}$ instead of $tRNA_3^{Lys}$ in the wild-type clone pHXB2. The sequence GAAAAT in the A loop of the wild type has been changed to CCACAA, which is complementary to the anticodon loop of $tRNA^{His}$ in the clones of pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) (37, 41). The nucleotides showing the CCTGT configuration in pHXB2(His-AC-TGT) and the ATGAC configuration in pHXB2(His-AC-GAC) are underlined. (B) Diagram of the RT mutations in the proviral clones. The methionine amino acid in the most conserved region of the RT (YMDD, from amino acids [aa.] 183 to 186) was replaced by a valine. The *Afl*II site at nucleotide 64 and *Bss*HII site at nucleotide 258 were used in the construction of mutants used in this study (see Materials and Methods). LTR, long terminal repeat; PRO, protease; IN, integrase. (C) Expression of viral proteins from 293T cells transfected with wild-type and mutant proviral genomes. 293T cells were transfected with various plasmids and metabolically labeled with ^{35}S-Translabel for 3 h, followed by immunoprecipitation with rabbit anti-p24 antibody. The immunoprecipitated material was separated on an SDS–10% polyacrylamide gel and then subjected to autoradiography. Lanes: 1, mock transfected; 2, wild-type pHXB2; 3, pHXB2(His-AC-TGT) with wild-type RT; 4, pHXB2(His-AC-TGT) with M184V RT; 5, pHXB2(His-AC-GAC) with wild-type RT; 6, pHXB2 (His-AC-GAC) with M184V RT. The relevant HIV-1 viral proteins (right) are marked.

genomes for the efficient use of $tRNA^{His}$ as the primer in reverse transcription. These additional nucleotide changes undoubtedly occurred during reverse transcription as a result of the inherent error-prone nature of the HIV-1 RT (37). Further support for the role of U5-PBS in interaction with $tRNA^{His}$ comes from our recent study in which we found that the virus containing the AUGAC mutations which used $tRNA^{His}$ to initiate reverse transcription had no differences in virion tRNA content or in the NC and RT genes from the wild-type virus which utilizes $tRNA_3^{Lys}$ to initiate reverse transcription (41).

In this study, we sought to establish whether the CCTGT-to-ATGAC mutations in U5-PBS play a role in the virus adaptation to utilize $tRNA^{His}$ in reverse transcription. To do this, two proviral genomes with the A loop in U5 and the PBS complementary to $tRNA^{His}$ were constructed; one proviral genome contained the CCTGT configuration, while the second contained the ATGAC substitutions. Based on our previous studies, we predicted that the proviral genome with the CCTGT configuration would mutate after in vitro culture to the ATGAC configuration as a result of, most probably, the error-prone nature of RT. Several studies found that the replacement of methionine (at amino acid 184) with valine (M184V) resulted in RT which has properties different (i.e., enzyme kinetics and fidelity) from those of the wild-type RT (20, 35). To determine if this M184V mutation in RT influences viruses utilizing $tRNA^{His}$ to initiate reverse transcription, we constructed proviruses which contained either the wild-type or M184V RT in combination with different U5-PBS regions (CCTGT and ATGAC). The results of our study provide additional support for the conclusion that nucleotide changes in U5 generated as a result of the error-prone nature of RT affect the capacity of the virus to utilize an alternative tRNA to initiate reverse transcription.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from New England Biolabs. The *Taq* DNA polymerase was purchased from Gibco BRL, and the reagents used for PCR were acquired from Perkin-Elmer Cetus. Tissue culture media and reagents were obtained from Gibco BRL. Unless otherwise specified, other chemicals used in all the experiments were purchased from Sigma Chemical Co. The enzyme-linked immunosorbent assay (ELISA) kits for p24 antigen were obtained from Coulter Laboratories. The synthetic oligonucleotides used for PCR and DNA sequencing were prepared by Gibco BRL.

Tissue culture. 293T and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at 37°C and 5% CO_2. SupT1 cells were grown in RPMI medium supplemented with 15% fetal calf serum. The proviral DNAs were transfected into COS-1 or 293T cells by using 300 μg of DEAE-dextran per ml as a facilitator. The cells were incubated in DEAE-dextran-DNA for 3 h and then in complete medium (DMEM supplemented with 10% fetal calf serum) containing chloroquine (20 μg/ml) for an additional 2 h. Cells were washed twice with DMEM and then cultured in complete medium overnight. The transfected cells were cocultured with SupT1 cells, which support high-level replication of HIV-1, for 2 days. SupT1 cells were isolated by centrifugation, washed, and further cultured with fresh RPMI at 37°C.

Analysis of virus infectivity. To test for virus infectivity, samples of the culture supernatant of infected SupT1 cells were collected at 2-day intervals and assayed for the presence of p24 antigen by ELISAs as well as inspected for multinucleated cells (syncytia). For cell-free infection, the supernatants of cultures were collected and filtered through a 0.45-μm-pore-size syringe filter (Nalgene). The concentrations of the virus in the supernatants were determined based on the p24 value measured by ELISA. SupT1 cells (10^6 per ml) were infected with 100 ng of virus per ml as measured by p24 antigen. After absorbing for 24 h, SupT1 cells were pelleted by low-speed centrifugation (1,000 × *g*), washed with fresh RPMI supplemented with 15% fetal calf serum, and further cultured.

Construction of HIV-1 proviral genomes containing a modified PBS region. General laboratory procedures were followed for DNA manipulation, plasmid preparation, and subcloning as previously described (18). The full-length molecular HIV-1 clone pHXB2 (24, 25) was used to construct mutant HIV-1 proviral genomes. pHXB2(His-AC-TGT) contains the A loop and PBS regions complementary to the anticodon region and the 3′-terminal 18 nucleotides of $tRNA^{His}$, respectively, constructed previously by Wakefield et al. (37); pHXB2(His-AC-GAC) contains the U5 and PBS regions as in pHXB2(His-AC-TGT) except for five additional mutations at nucleotides 152 (C to A), 160 (C to T), 174 (T to G), 181 (G to A), and 200 (T to C); these mutations changed the sequence configuration from CCTGT to ATGAC (41).

DNA fragments containing the U5 and PBS regions with mutations were synthesized by PCR with the *Afl*II and *Bss*HII primers from clones containing the corresponding mutations. The sequences of the *Afl*II and *Bss*HII primers are 5′-ACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCA-3′ (nucleotides 64 to 98) and 5′-GGGGCGCGCTTCAGCAAGCCG-3′ (nucleotides 241 to 258), respectively. The *Afl*II-*Bss*HII DNA fragments (nucleotides 64 to 258) were first subcloned into a shuttle vector, pUC119PBS, which contains an *Hpa*I-to-*Pst*I DNA fragment including the 5′ long terminal repeat, PBS, and leader region of *gag* from pHXB2 (26); the resultant clones were named pUC119PBS (His-AC-TGT) and pUC119PBS(His-AC-GAC). The sequences of the *Afl*II-*Bss*HII DNA fragments were verified by DNA sequencing (28).

The 868-bp *Hpa*I-*Bss*HII DNA fragments from pUC119PBS(His-AC-TGT) and pUC119PBS(His-AC-GAC) were subcloned between the *Hpa*I and *Bss*HII sites of pHXB2 containing the wild-type RT and the M184V RT (36). The desired mutant plasmids were screened by restriction digestion and verified by DNA sequencing.

PCR amplification and DNA sequence analysis of PBS-containing proviral DNA. At designated days postcocultivation, genomic DNAs were isolated from infected SupT1 cells by using a Wizard DNA purification kit as instructed by the manufacturer (Promega). About 0.5 μg of cellular DNA was used to amplify the proviral DNA sequences encompassing the U5 and PBS regions of the virus, using primers 5′-GAGGTTTGACAGCCGC-3′ (nucleotides −191 to −175) and 5′-ACGCTCTCGCACCCAT-3′ (nucleotides 334 to 350).

PCR-amplified DNA fragments were gel purified and ligated into PCR II vector (Invitrogen). The ligation mixtures were transformed into *Escherichia coli* DH5α. Following identification of colorless colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; U.S. Biochemical) plates, plasmid DNA from individual *E. coli* colonies was prepared and sequenced by using primer 5′-CTTAAGCCTCAATAAAGC-3′ (nucleotides 62 to 80).

Detection of integrated proviruses. To detect integrated proviruses, 30-μl aliquots of PCR products from high-molecular-weight DNA preparations were loaded on a 0.8% agarose gel. Alternatively, to detect endogenous reverse transcription (endogenous RT)-PCR products, 30 μl of the reaction mixture was added to a 1.3% agarose gel. After electrophoresis, DNA on the gel was transferred to a 0.2-μm-pore-size nitrocellulose membrane (Protran), using a routine Southern transfer technique, in 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) (18). The membrane was baked at 80°C with vacuum for 2 h and soaked in 6× SSPE for 5 min. The DNA oligomer used for the probe was the same as that used for DNA sequencing. To radiolabel the oligomer, an aliquot of 1.5 pmol of oligomer was resuspended in 50 μl of labeling mixture containing 150 μCi of [γ-^{32}P]ATP, 1× kinase buffer (70 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 100 mM KCl, 1 mM β-mercaptoethanol), and 20 U of T4 polynucleotide kinase. Following incubation at 37°C for 45 min, unincorporated [γ-^{32}P]ATP was removed by a Sephadex G-50 column (NICK spin columns; Pharmacia Biotech). Prehybridization was carried out for 3 h at room temperature in 25% (vol/vol) formamide–6× SSPE–0.1% (wt/vol) sodium dodecyl sulfate (SDS)–5× Denhardt's solution–100 μg of single-stranded salmon sperm DNA per ml. Hybridization was initiated by adding the labeled probe to the prehybridization mixture; hybridization was continued overnight at room temperature. The membrane was then washed four times for 5 min each in washing buffer (2× SSPE, 0.1% SDS) at room temperature. Autoradiography was at −70°C with an intensifying screen.

Endogenous RT-PCR. The procedure for endogenous RT was adapted from that of Masuda et al. (18a). Supernatants from cell cultures transfected with designated proviruses were treated with RNase-free DNase (Boehringer Mannheim) at a final concentration of 20 U/ml for 1 h at 37°C in the presence of 10 mM $MgCl_2$. DNase-treated supernatant were overlaid onto 5-ml cushions containing 20% diatrizoate–80% TEN buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and centrifuged at 27,000 rpm for 2 h in an SW28 rotor at 4°C. The pellet was resuspended in 50 μl of ice-cold TEN buffer, and the amount of virus was determined by p24 ELISA assay. Approximately 1 ng of virus p24 was added to the endogenous RT mixture (0.01% Triton X-100, 50 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol, 5 mM $MgCl_2$, 250 μM each dATP, dCTP, dGTP, and TTP) to a final volume of 30 μl and incubated at 37°C. At each time point (0, 5, 10, 15, 20, 30, and 60 min), reactions were terminated by adding 30 μl of cold termination buffer (50 μg of proteinase K per ml, 1.5 mM EDTA [pH 8.0]). After incubation at 62°C for 1 h, the complete mixture was boiled for 10 min. For each PCR, 16.7 pg of p24 (1-μl aliquot of the reaction mixture) was used. One of the primers (5′-GTGATCGTATAGTGGTTAGTACTC-3′) used for PCR has the sequence of $tRNA^{His}$ from nucleotides 5 to 27 (29). The other primer (5′-GTAGTGTGTGCCCGTCTG-3′) used for PCR has the sequence of pHXB2 proviral DNA from nucleotides 99 to 116 in U5. PCR was performed for 40 cycles of amplification, each consisting of a denaturing step at 94°C for 1 min, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min. Amplified products were resolved on a 1.3% agarose gel. Southern hybridization was used to verify the signals from endogenous RT-PCR. The ^{32}P-end-labeled oligomer (5′-GCGTTGTGGCCGCAGCAA-3′) used for Southern hybridization corresponds to nucleotides 29 to 46 of $tRNA^{His}$.

RESULTS

Construction of mutant HIV-1 proviruses. In a recent report, we described two modifications of HIV-1 that contain a PBS complementary to the 3′-terminal 18 nucleotides of $tRNA^{His}$ and a sequence in U5 (called the A-loop region in pHXB2) which is complementary to the anticodon loop of $tRNA^{His}$ (37). Transfection of this proviral clone, designated originally pHXB2(His-AC), into cells resulted in the production of an infectious virus. DNA sequence analysis of the PBS region from recovered virus revealed additional nucleotide substitutions around the PBS region (T to G at nucleotide 174, G to A at nucleotide 181, and T to C at nucleotide 200 [collectively referred to as GAC]). Additional substitutions upstream of the GAC mutations in U5 were found at nucleotides 152 (C to A) and 160 (C to T); the total substitutions in the U5-PBS analyzed resulted in the CCTGT in the input viral genome changed to ATGAC after culture (37, 41). To determine the significance of this change in reverse transcription in this study, the DNA sequence (nucleotides 64 to 255) of the wild-type pHXB2 containing U5-PBS was replaced with the mutated sequences encompassing the A loop and the PBS complementary to $tRNA^{His}$ with either the CCTGT [same as in pHXB2(His-AC) (37)] or ATGAC [the mutation in U5-PBS recovered from a culture of pHXB2(His-AC)] configuration. The resultant proviral DNA was named pHXB2(His-AC-TGT) for the CCTGT configuration or pHXB2(His-AC-GAC) for the ATGAC configuration (Fig. 1A).

Previous studies suggested that an HIV-1 RT with a methionine-to-valine change in the highly conserved YMDD (amino acids 183 to 186) amino acid motif at the enzyme catalytic site affected both the activity and apparent fidelity of the enzyme (20, 35). To determine the effect of the RT mutations on the virus which utilizes $tRNA^{His}$ to initiate reverse transcription, proviruses were constructed to replace the wild-type RT in the original pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) with the M184V RT (Fig. 1B).

Expression of viral proteins and analysis of infectivity. To determine if mutations in both U5-PBS and RT [i.e., pHXB2 (His-AC-TGT) with M184V RT and pHXB2(His-AC-GAC) with M184V RT] affected viral protein expression, we introduced each of the proviral clones into 293T cells by transfection followed by metabolic labeling and immunoprecipitation with rabbit anti-p24 antibodies. No difference was observed in the expression of viral proteins in cells transfected with the mutant proviral genomes or the wild-type genome (Fig. 1C). To determine if the mutations affect virus release, we compared the levels of p24 antigen in cultures transfected with the mutant proviruses with that in the culture transfected with the wild-type provirus. Again, we detected no significant difference with respect to the levels of p24 antigen released from the cells transfected with the mutant or wild-type proviral genome (data not shown).

We next examined the infectivity of the viruses derived from transfection of the proviral genomes. Since COS-1 cells do not express CD4 and are not infected by HIV-1, we used coculture with SupT1 cells. This procedure has been previously used by our laboratory for analysis of the PBS and RT mutants (36, 37). Following 2 days of coculture, the nonadherent SupT1 cells

from the coculture were isolated and recultured with additional SupT1 cells. Viral replication was monitored by assaying the p24 antigen level in the culture supernatant and visual inspection for large, giant cell syncytia. We readily detected the presence of virus from cultures transfected with pHXB2(His-AC-GAC) and pHXB2(His-AC-TGT) with the wild-type RT. The time frames for the appearance of virus were similar, with the virus derived from pHXB2(His-AC-TGT) slightly delayed compared to that from pHXB2(His-AC-GAC). In contrast, the appearance of virus derived from the transfection of pHXB2 (His-AC-GAC) encoding the M184V RT was slower than that of the virus with the wild-type RT (Fig. 2A). Surprisingly, we did not detect a similar level of p24 production from cocultures derived from transfection of pHXB2(His-AC-TGT) containing M184V RT. To further explore this result, we reanalyzed the supernatant from the culture transfected with pHXB2(His-AC-TGT) with M184V RT for p24 antigen at a lower dilution (Fig. 2B). In this case, we detected a very low level of p24 from the culture derived from pHXB2(His-AC-TGT) containing M184V RT by day 28 postcoculture. Visual inspection of the cultures transfected with pHXB2(His-AC-TGT) containing M184V RT revealed no large, multicell syncytia after 110 days of culture. To further verify these results, we used PCR and Southern blot analysis to detect integrated proviruses in these cultures (Fig. 2C and D). As expected, samples from the cultures which contained high levels of p24 were clearly positive with this method of detection (Fig. 2C). Consistent with the results in Fig. 2B, we found that a sample from the culture transfected with pHXB2(His-AC-TGT) with M184V RT was also positive on day 35 of coculture (Fig. 2D), although no signal was detected on day 15 postcoculture in this assay.

Analysis of initiation of reverse transcription. To determine if the differences which we observed in the replication of the viruses containing mutations in U5 were due to reverse transcription, we used a new method for endogenous RT-PCR. We designed for PCR a DNA oligonucleotide which had the 24-nucleotide sequence of $tRNA^{His}$ at the 5′ end; a second DNA primer which had a sequence in a region within U5 in the HIV-1 proviral genome was designed. Using equal amounts of virus derived from transfection of proviral clones, we performed endogenous RT reactions followed by PCR with the specific oligonucleotide primers. Another primer was designed for Southern hybridization to verify the signal received from RT-PCR which had the $tRNA^{His}$ sequence downstream of that used for PCR. The results of endogenous RT-PCR–Southern hybridization are shown in Fig. 3A and B. Analysis of the kinetics of the in vitro endogenous RT reaction revealed that both viruses containing the wild-type or M184V RT derived from pHXB2(His-AC-GAC) underwent reverse transcription faster than those derived from pHXB2(His-AC-TGT) (Fig. 3C and D). This finding is consistent with our finding from analyzing the replication of viruses that those viruses with the M184V mutation in RT demonstrated the greatest difference in the endogenous RT reaction.

Analysis of U5-PBS of viruses after in vitro culture. The foregoing results demonstrate a difference in infectivity between the viral genomes derived from pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC). Since the virus derived from pHXB2(His-AC-TGT) with the wild-type RT was replication competent, we first wanted to determine if the TGT nucleotides were present after in vitro culture of the virus. For this analysis, we used PCR to amplify the U5-PBS (from nucleotides −191 to 350) from integrated proviruses of pHXB2(His-AC-TGT) with wild-type RT, and the DNA sequences from individual clones were determined (Table 1). Three of the five clones contained the input (i.e., CCTGT) sequence (day 15

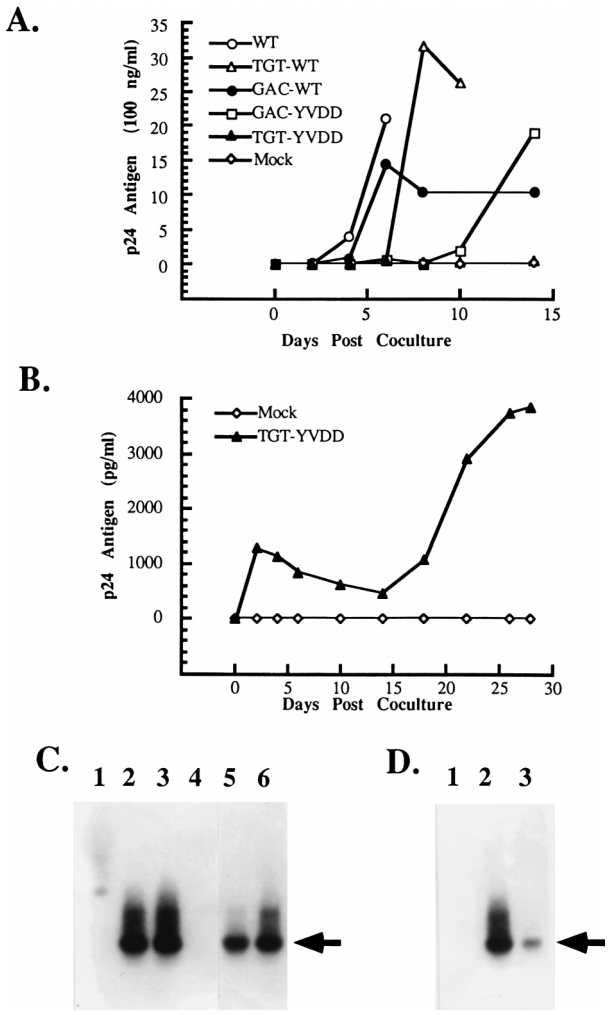

FIG. 2. Kinetics of the appearance of infectious virus derived from transfection of wild-type and mutant proviral genomes. (A) The plasmids containing the wild-type and mutant proviral genomes were transfected into COS-1 cells, which were cocultured 24 h later with 5×10^5 SupT1 cells. After 48 h of coculture, the SupT1 cells were isolated by centrifugation, washed once, and further cultured with additional uninfected SupT1 cells and medium (day 0). At 2-day intervals postcoculture, culture supernatants were collected and the p24 antigen was determined by ELISA. (Note that the values in the figure should be multiplied by 100 ng/ml to obtain the final culture concentrations.) WT refers to the wild-type genome of pHXB2; TGT-WT and GAC-WT refer to the genomes of pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) with the wild-type RT, respectively; TGT-YVDD and GAC-YVDD refer to the genomes of pHXB2 (His-AC-TGT) and pHXB2(His-AC-GAC) with M184V RT. (B) Expanded curve of panel A indicating the low level of p24 antigen in the culture transfected with proviral DNA pHXB2(His-AC-TGT) with M184V RT (TGT-YVDD). Note that the concentration of p24 antigen is in picograms per milliliter. By day 22 postcoculture, virus from pHXB2(His-AC-TGT) with M184V RT contained an increased level of p24 antigen in the supernatant of the culture, although the level was not comparable to that of the wild-type or other mutant genomes [pHXB2(His-AC-TGT), pHXB2(His-AC-GAC), or pHXB2(His-AC-GAC) with M184V RT]. (C) Detection of integrated proviral genomes by PCR and Southern hybridization at day 15 postcoculture. The high-molecular-weight DNA used for PCR was isolated at day 15 postcoculture. The PCR products were analyzed by Southern blot procedures (Materials and Methods). The arrow denotes the 541-bp PCR product. Lanes: 1, mock; 2, wild-type pHXB2; 3, pHXB2(His-AC-TGT); 4, pHXB2(His-AC-TGT) with M184V RT; 5, pHXB2(His-AC-GAC); 6, pHXB2(His-AC-GAC) with M184V RT. (D) Detection of integrated proviral genomes by PCR and Southern hybridization at day 35 postcoculture. The high-molecular-weight DNA used for PCR was isolated at day 35 postcoculture. The arrow denotes the 541-bp PCR product. Lanes: 1, mock; 2, wild-type pHXB2; 3, pHXB2(His-AC-TGT) with M184V RT.

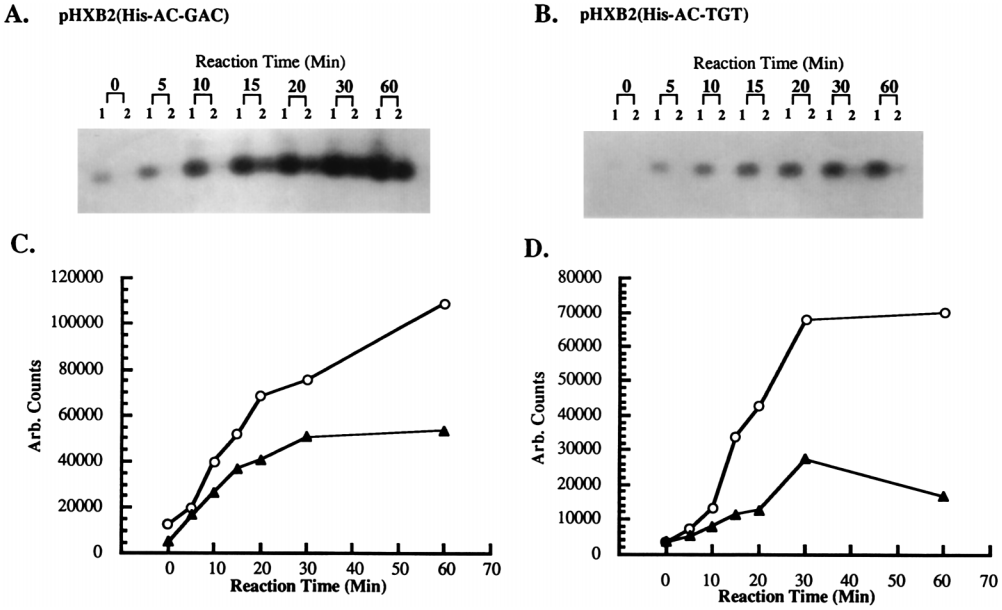

FIG. 3. Analysis of reverse transcription from viruses derived from HXB2(His-AC-GAC) or HXB2(His-AC-TGT). (A and B) For each endogenous RT reaction, virus equal to 1 ng of p24 antigen was used in a 30-μl reaction volume. At each time point (0, 5, 10, 15, 20, 30, and 60 min), reactions were terminated by adding 30 μl of termination buffer. For each PCR, a 1-μl aliquot of the reaction mixture was used. Following transfer to nitrocellulose, the blot was probed with a radioactive oligomer which had the sequence of nucleotides 29 to 46 of $tRNA^{His}$. Results of endogenous RT-PCR–Southern hybridization from viruses isolated from transfection of proviruses pHXB2(His-AC-GAC) (A) and pHXB2(His-AC-TGT) (B) are shown. Lanes: 1, virus with the wild-type RT; 2, virus with M184V mutated RT. (C and D) The radioactivity on the Southern blot was quantitated with a phosphorimager. The arbitrary (Arb.) counts were plotted for the signals from HXB2(His-AC-GAC) (circles) and HXB2(His-AC-TGT) (triangles). (C) Viruses with wild-type RT; (D) viruses with M184V RT.

postculture). It is possible, though, that some of these clones were actually amplified from the input plasmid DNA (37). Analysis of the clones recovered from proviruses obtained at 44 days postculture revealed mutations in the U5-PBS which contained a G-to-A change (CCTGT to CCTAT) at nucleotide 181. We observed considerable heterogeneity with respect to the clones recovered; that is, we did not recover a single clone which contained the input sequence.

Presumably, the mutations which we detected in U5-PBS during replication were made by the wild-type HIV-1 RT because of the error-prone nature of the enzyme (5, 7–9, 27). If this is the case, the virus derived from pHXB2(His-AC-TGT) containing the HIV-1 RT with the M184V mutation might have a lower infectivity due to the inability to generate the mutations which are required for the efficient use of $tRNA^{His}$ to initiate reverse transcription. Furthermore, it is possible that pHXB2(His-AC-GAC) with M184V mutant RT grew more slowly than virus derived from pHXB2(His-AC-GAC) with wild-type RT (Fig. 1A) because additional nucleotide substitutions within U5-PBS may be necessary for optimal reverse transcription using $tRNA^{His}$ as a primer (Table 2). To explore this possibility, we analyzed the U5-PBS sequences of integrated proviruses from pHXB2(His-AC-GAC) with the wild-type and M184V RT after in vitro culture. The sequences of the majority of the U5-PBS clones from both cultures at day 15 postculture were the same as the input sequence (data not shown). In contrast, by day 44 postculture, when we noted considerable virus growth in all of the cultures as measured by the concentration of p24 antigen and the formation of syncytia, we found striking differences with respect to the heterogeneity

TABLE 1. DNA sequences of U5 and PBS regions of proviruses derived from pHXB2(His-AC-TGT)[a]

Day[b]	Frequency[c]	Input DNA sequence[d] (5′–3′)
		TCCCTCAGACCcTTTTAGTcAGTGTGCCACAACtCTAGCAgTGGTGCCGTGACTCGGATtTGAAAGCGAAAGGGAAACC
15	3/5	**********
	1/5	**********T**********
	1/5	**********T**********A**********
44	2/11	**********A*A***T**********A**********
	4/11	**********A*A**********A**********
	3/11	**********T*A**********A**********
	1/11	**********A*******A**********
	1/11	**********A**********A**********

[a] pHXB2(His-AC-TAT) refers to the mutant clone containing an A loop and a PBS complementary to $tRNA^{His}$ and the configuration of CCTGT in the U5-PBS region (Fig. 1). The RT encoded in this clone is wild type.
[b] Day postculture when the high-molecular-weight DNA was isolated.
[c] Frequency of the DNA sequence surrounding the PBS region obtained from independent PCR clones.
[d] Sequence of the original clone pHXB2(His-AC-TGT). Lowercase letters show the configuration of CCTGT at nucleotides 152, 160, 174, 181, and 200, respectively. Asterisks denote identity with the input sequence.

TABLE 2. DNA sequences of U5 and PBS regions of proviruses pHXB2(His-AC-GAC) isolated at day 44 postculture

Proviral DNA	Frequency[a]	Input DNA sequence[b] (5′–3′)
		TCCCTCAGACCATTTTAGTTAGTGTGCCACAACGCTAGCAATGGTGCCGTGACTCGGATCTGAAAGCGAAAGGGAAACC
GAC-WT[c]	3/12	**********
	1/12	**********A*********A**********
	4/12	**********A**********
	2/12	**********A**********
	1/12	**********A**********
	1/12	*****A**********T***AA**C**********
		↑
		G[d]
GAC-YVDD[e]	12/12	**********

[a] Frequency of the DNA sequence surrounding the PBS region obtained from independent PCR clones.
[b] Sequence of the original clone of pHXB2(His-AC-GAC). Asterisks denote identity with the input sequence.
[c] pHXB2(His-AC-GAC), which contains a PBS complementary to $tRNA^{His}$ and the ATGAC configuration in the U5-PBS region (Fig. 1A). This provirus encodes wild-type (WT) RT.
[d] Nucleotide insertion in the PBS region.
[e] pHXB2(His-AC-GAC) with the M184V RT, which contains a PBS complementary to $tRNA^{His}$ and the ATGAC configuration in the U5-PBS region. The RT gene encoded by this provirus has a mutation which replaced the methionine amino acid with a valine in the highly conserved YMDD motif.

of the U5-PBS sequences. In the case of the viruses from pHXB2(His-AC-GAC) with the wild-type RT, we found that only 3 of the 12 clones recovered contained the starting nucleotide sequence, while all 12 of the U5-PBS sequences recovered from the virus with the M184V mutation were identical to the starting sequence (Table 2). Sequence analysis of the RT genes at this time revealed that the original starting mutations in the M184V sequence were still present (data not shown). To extend these results, the culture was analyzed at day 60 postcoculture. As before, we found considerable heterogeneity in the U5-PBS sequences from the proviral DNA pHXB2(His-AC-GAC) with the wild-type RT. Only 4 of the 11 clones analyzed contained the starting sequence; numerous changes (mostly adenine substitutions) were found. In contrast, the proviral DNA from pHXB2(His-AC-GAC) with the M184V RT contained the starting sequence in 10 of 12 clones analyzed. Two clones with changes from the starting sequence contained a cytosine rather than the adenine substitutions found in the case of the wild-type RT (data not shown). At day 68, we recovered only a single input sequence from the wild-type cultures; the remaining 14 of the 15 clones analyzed contained numerous substitutions, mostly adenines, in the U5 region. In contrast, over 50% of the clones recovered from the integrated proviral DNA with the M184V RT had the starting sequence; the majority of the remaining clones had only a single nucleotide (adenine) change (data not shown). Finally, after 90 days of coculture, no starting U5-PBS sequence was detected from pHXB2(His-AC-GAC) with the wild-type RT (Table 3). We found numerous adenine substitutions within U5-PBS. In contrast, 2 of the 10 clones recovered from the viruses with the M184V mutation in RT still had the starting sequence; the remaining 8 clones contained only one nucleotide change, the majority being the same adenine substitution as seen at day 68. By day 90, all subclones of the RT gene recovered from pHXB2(His-AC-GAC) with M184V RT still contained the YVDD motif (data not shown). Taken together, the results of these studies demonstrate that the viruses with the M184V RT have fewer nucleotide substitutions in the U5 after extended in vitro culture.

DISCUSSION

In this study, we used novel HIV-1 proviral genomes with mutations in the U5 and PBS which allow the virus to stably maintain a PBS complementary to $tRNA^{His}$ (37) to further identify the functions of the U5 region in the initiation of reverse transcription. The mutated U5 region containing a five-nucleotide change (CCTGT to ATGAC), acquired from extended

TABLE 3. DNA sequences of U5 and PBS regions of proviruses isolated at day 90 postculture

Proviral DNA	Frequency[a]	Sequence[b] (5′–3′)
		TCCCTCAGACCATTTTAGTTAGTGTGCCACAACGCTAGCAATGGTGCCGTGACTCGGATCTGAAAGCGAAAGGGAAACC
GAC-WT[c]	4/10	**********A**********
	3/10	**********A**********A**********
	1/10	****A**********A**********A**********A**********
	1/10	**T**********A**********A**********
	1/10	**********A***A*********A**********
GAC-YVDD[d]	2/10	**********
	6/10	**********A**********
	1/10	**********A**********
	1/10	**********T**********

[a] Frequency of the DNA sequence surrounding the PBS region obtained from independent PCR clones.
[b] Sequence of the original clone of pHXB2(His-AC-GAC). Asterisks denote identity with the input sequence.
[c] pHXB2(His-AC-GAC), which contains a PBS complementary to $tRNA^{His}$ and the ATGAC configuration in the U5 region (Fig. 1A). This provirus encodes a wild-type (WT) RT.
[d] pHXB2(His-AC-GAC) with the M184V RT, which contains a PBS complementary to $tRNA^{His}$ and the ATGAC configuration in the U5 region. The RT gene encoded by this provirus has a mutation which replaced the methionine amino acid with a valine in the highly conserved YMDD amino acid motif.

culture of virus which used $tRNA^{His}$ as a primer for reverse transcription, was subcloned into pHXB2. The infectivities of the resultant provirus, pHXB2(His-AC-GAC), and the starting provirus, pHXB2(His-AC-TGT), with either wild-type or M184V mutant RT were compared. The virus derived from pHXB2(His-AC-TGT) containing both wild-type and M184V RT replicated more slowly than the pHXB2(His-AC-GAC) counterparts, and virus from pHXB2(His-AC-TGT) with M184V mutant RT was severely delayed in the initiation of reverse transcription compared to virus derived from pHXB2 (His-AC-GAC) with the same RT. Sequence analysis of integrated proviral DNA revealed several nucleotide substitutions in the U5 region of the viruses derived from pHXB2(His-AC-GAC) and pHXB2 (His-AC-TGT) containing the wild-type RT, but not the proviruses containing the M184V RT, after extended in vitro culture.

It is now clear that mutations in U5 can influence the selection of the tRNA used to initiate reverse transcription. Previous studies from our laboratory have described proviral genomes containing mutations in both the U5 and PBS regions which allow the virus to stably utilize alternative tRNAs to initiate reverse transcription (16, 37, 41). These mutations were positioned in a region within U5 which had been postulated to interact with the anticodon loop of the $tRNA_3^{Lys}$ used to initiate wild-type HIV-1 reverse transcription (14). One common feature in all of these studies is that analysis of the U5 region of viruses which utilize alternative tRNAs to initiate reverse transcription revealed a considerable number of nucleotide substitutions. For example, the U5 region of the virus which used $tRNA^{His}$ contained numerous substitutions, the most predominant being a CCTGT-to-ATGAC change. We speculated that these mutations were an adaptation by the virus for the efficient use of the alternative $tRNA^{His}$ to initiate reverse transcription.

Several results from our study establish that these mutations in U5 are critical for the virus to use the alternative $tRNA^{His}$ to initiate wild-type HIV-1 reverse transcription. First, viruses with the wild-type RT derived from pHXB2(His-AC-TGT) replicated more slowly than viruses derived from pHXB2(His-AC-GAC) (Fig. 2A). The difference in replication between viruses derived from pHXB2(His-AC-TGT) and pHXB2(His-AC-GAC) was magnified when they contained the M184V RT (Fig. 2A and B). The differences in replication could have been due to defects in one or multiple steps in reverse transcription. To address this question, we used an endogenous RT-PCR method to compare the early events in reverse transcription. From this analysis, we conclude that the initiation of reverse transcription for viruses derived from pHXB2(His-AC-TGT) was compromised compared to that of viruses derived from pHXB2(His-AC-GAC). Again, this difference was clearly magnified with viruses which contained the M184V RT. This result would appear to conflict with those of previous studies from our laboratory and others, which have demonstrated that viruses with the M184V RT mutation had replication profiles similar to that of the wild-type virus (20, 36, 40). It is possible that under certain conditions, such as using an alternative tRNA primer to initiate reverse transcription, the differences between the wild-type and M184V RT are magnified. In support of this idea, previous studies by Arts et al. clearly demonstrated that efficient initiation of HIV-1 reverse transcription is dependent on the RT to disrupt the U5-tRNA interaction (1, 2). Thus, it is possible that the M184V mutation in RT affects the capacity of the enzyme to disrupt these interactions when $tRNA^{His}$ is used to initiate reverse transcription. Further studies are required with the M184V RT, using mutant HIV-1 viruses which utilize alternative tRNAs to initiate reverse transcription, to resolve this point.

A second series of results which support our contention that mutations in U5 are critical for the virus to use $tRNA^{His}$ comes from our sequence analysis of integrated proviral genomes. The proviral genomes analyzed after limited in vitro culture of pHXB2(His-AC-TGT) containing the wild-type RT all revealed a G-to-A mutation. Consistent with this result is the fact that this virus, which contained the G-to-A mutation, replicated similarly to the virus derived from pHXB2(His-AC-GAC) (17a). Analysis of the U5-PBS sequences from these viruses with the wild-type RT revealed numerous adenine substitutions within the U5 region after extended culture. In fact, by day 68 postcoculture, we were able to detect only 1 in 15 clones of PCR fragments which still had the input sequence from the cultures of pHXB2(His-AC-GAC) containing the wild-type RT gene. In contrast, even after extended culture representing 90 days postcoculture, we were able to identify the input sequence from the cultures of pHXB2(His-AC-GAC) containing the M184V RT gene. This result, when considered together with the finding that viral genomes derived from pHXB2(His-AC-TGT) with the M184V RT had severely reduced infectivity, supports the idea that the M184V RT has a defect in the capacity to generate nucleotide substitutions within U5-PBS which are essential for the virus to adapt to the use of $tRNA^{His}$ to initiate reverse transcription.

The details of the role that these critical nucleotides in U5 play in the initiation of reverse transcription require further study. Most probably, the specific changes are essential for U5-PBS to form an RNA structure which facilitates reverse transcription. An extensive interaction between U5-PBS of the genome and $tRNA_3^{Lys}$ has been found by using chemical and enzymatic probing (14). This analysis revealed the interaction of a region in U5 described as an A loop with the anticodon of $tRNA_3^{Lys}$, as well as additional interactions of other regions in U5 with $tRNA_3^{Lys}$. The overall importance of these interactions was put in question when proviruses with deletions in U5 were found to be replication competent (13). This led to the interesting suggestion by Huang et al. that interactions of U5 and alternative tRNAs might be important only to exclude $tRNA_3^{Lys}$ from the PBS (13). If this were the case, though, we would have expected that the viruses derived from pHXB2(His-AC-TGT) would have reverted to use $tRNA_3^{Lys}$ to initiate reverse transcription, rather than change a single nucleotide and continue using $tRNA^{His}$ to initiate reverse transcription. It is possible, then, that the combination of the U5-PBS complementary to $tRNA^{His}$ is necessary for both selection of the alternative tRNA and exclusion of $tRNA_3^{Lys}$. The finding that the M184V RT reduces replication and generation of nucleotide substitutions provides us with an important tool for future studies designed to dissect the interaction of the tRNA with U5-PBS required for initiation of reverse transcription. Finally, the appreciation that the interaction between the tRNA and U5-PBS represents an important step in reverse transcription opens the possibility for the design of therapeutics to disrupt this RNA-RNA interaction.

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