a Primer Binding Site Complementary to tRNA[His]

ZHIJUN ZHANG,* SANG-MOO KANG,* ALLEN LeBLANC,† STEPHEN L. HAJDUK,† and CASEY D. MORROW*,[1]

*Department of Microbiology and †Department of Biochemistry and Molecular Genetics,
University of Alabama at Birmingham, Birmingham, Alabama 35294

The initiation of reverse transcription of the human immunodeficiency virus type 1 (HIV-1) genome requires cellular tRNA[Lys,3] as a primer and occurs at a site in the viral RNA genome, designated as the primer binding site (PBS), which is complementary to the 3′-terminal 18 nucleotides of tRNA[Lys,3]. We previously described an HIV-1 virus [designated as HXB2(His-AC)], which contained a sequence within the U5 region complementary to the anticodon region of tRNA[His] in addition to a PBS complementary to the 3′-terminal 18 nucleotides of the tRNA[His]. That virus maintained a PBS complementary to tRNA[His] after extended *in vitro* culture (Wakefield *et al., J. Virol.* 70, 966–975, 1996). In the present study, we report that subcloning a 200-base-pair DNA fragment encompassing the U5 and PBS regions from an integrated provirus of HXB2(His-AC) back into the wild-type genome (pHXB2) resulted in an infectious virus, designated as HXB2(His-AC-gac), which again stably maintained a PBS complementary to tRNA[His]. DNA sequence analysis of the 200-base-pair region revealed only three nucleotide changes from HXB2(His-AC): a T-to-G change at nucleotide 174, a G-to-A change at nucleotide 181, and a T-to-C change at nucleotide 200. The new mutant virus replicated in CD4^{+} Sup T1 cells similarly to the wild-type virus. Comparison of the nucleotide sequence of nucleocapsid gene of the wild-type and HXB2 (His-AC-gac) virus revealed no differences. Although we found numerous mutations in the reverse transcriptase gene in proviral clones derived from HXB2 (His-AC-gac), no common mutations were found among the 13 clones examined. Comparison of the virion-associated tRNAs of HXB2(His-AC-gac) with those of the wild type revealed that both viruses incorporated a similar subset of cellular tRNAs, with tRNA[Lys,3] being the predominant tRNA found within virions. There was no selective enrichment for tRNA[His] within virions of HXB2(His-AC-gac) virus which selectively use tRNA[His] to initiate reverse transcription. The results of these studies suggest that the U5 and PBS regions in the viral RNA genome are important determinants for HXB2(His-AC) viruses in the selective use of tRNA[His] to initiate reverse transcription.   © 1996 Academic Press, Inc.

INTRODUCTION

A hallmark of retrovirus replication is the process by which the RNA genome is converted to a DNA intermediate prior to integration into the host cell chromosome. This process is referred to as reverse transcription and is carried out by a virally encoded enzyme, reverse transcriptase (RT) (Baltimore, 1970; Temin and Mizutani, 1970). The initiation of reverse transcription occurs at a site, designated as the primer binding site (PBS), which is located near the 5′ end of the viral RNA genome. The PBS consists of an 18-nucleotide sequence which is complementary to the 3′ end of a cellular tRNA used as a primer for the initiation of reverse transcription. Following initiation, the RT copies the viral RNA genome into double-stranded DNA, referred to as the provirus. During this process, the PBS plays an important role in facilitating the completion of the plus-strand DNA synthesis (Gilboa *et al.,* 1979; Peters and Dahlberg, 1979; Peters and Glover, 1980; Taylor, 1977). One of the features of

reverse transcription is that the PBS is regenerated when the RT copies the still attached tRNA primer that is used to initiate reverse transcription (Taylor and Hsu, 1980). Thus, the nucleotide sequence of the PBS found in the integrated provirus is complementary to the tRNA primer used to initiate reverse transcription.

The selection of the tRNA primer utilized to initiate reverse transcription is a critical step in the virus life cycle. Within a given group of retroviruses, the tRNA primer used to initiate reverse transcription is highly conserved (Colicelli and Goff, 1987). For example, the 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 and human T-cell leukemia viruses utilize tRNA[Pro]. The human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) utilize tRNA[Lys,3] to initiate reverse transcription. In recent years, there have been considerable efforts to define the essential features involved in the selection of the tRNA[Lys,3] as primer for HIV-1 reverse transcription. In early studies, it was found that the deletion of up to 12 of the 18 nucleotides at the 3′ end of the PBS of HIV-1 did not affect the subsequent utiliza-

tion of tRNA^{Lys,3} as the primer for reverse transcription (Rhim *et al.*, 1991; Wakefield *et al.*, 1994). Further studies in which the wild-type PBS were substituted with sequences complementary to other tRNAs revealed that even though HIV-1 could utilize each alternate tRNA to initiate reverse transcription, the PBS of the recovered virus reverted back after limited *in vitro* culture to be complementary to tRNA^{Lys,3} (Das *et al.*, 1995; Li *et al.*, 1994; Wakefield *et al.*, 1995, 1996). The results of these studies suggested that factors other than the PBS are involved in the selection of tRNA^{Lys,3} as the primer for HIV-1 reverse transcription.

Analysis of the virion tRNA content of HIV-1 revealed that a subset of cellular tRNAs were selectively incorporated into HIV-1 virions; not surprisingly, tRNA^{Lys,3} was one of the major tRNA species found in the HIV-1 virions (Jiang *et al.*, 1993). The viral nucleocapsid protein (NC) and RT, were found to specifically interact *in vitro* with tRNA^{Lys,3}, leading to the suggestion that either these proteins facilitate the incorporation of tRNA^{Lys,3} into the virions or they are involved in the selective use of tRNA^{Lys,3} as the primer for minus-strand DNA synthesis (Barat *et al.*, 1989, 1991, 1993; Li *et al.*, 1996; Prats *et al.*, 1989; Robert *et al.*, 1990; Sarih-Cottin *et al.*, 1993). Further support of the idea that viral proteins are involved in the incorporation of select cellular tRNAs into virions came from a study which demonstrated that immature HIV-1 particles without the Gag-Pol polyprotein incorporated tRNAs with no selective enrichment for tRNA^{Lys,3}, while immature particles which contained Gag-Pol contained a similar composition of tRNAs as the wild-type virus (Mak *et al.*, 1994). Although it is clear that HIV-1 proteins can interact with cellular tRNAs, there is a controversy as to whether the *in vitro* interactions between them are biologically relevant since the other studies demonstrated that the *in vitro* interactions between the NC or RT and tRNA^{Lys,3} were nonspecific (Mely *et al.*, 1995; Delahunty *et al.*, 1994).

From the results of several recent studies, it has become apparent that, in addition to the PBS, sequences of the retroviral RNA genomes outside of the PBS might be involved in the selective use of a specific tRNA to initiate reverse transcription (Aiyar *et al.*, 1994; Isel *et al.*, 1995). Previous studies from Isel *et al.* have delineated regions within the U5 region of an HIV-1 RNA genome that interact with regions other than the 3′ end of the tRNA^{Lys,3} (Isel *et al.*, 1995). One of these regions, designated as the A-loop region within the U5 of the HIV-1 genome, has been shown to interact with the anticodon sequences of the tRNA^{Lys,3} molecule. Recent studies from our laboratory demonstrated that a mutant HIV-1 virus [designated as HXB2(His-AC)] in which the A-loop region and the PBS were replaced with sequences complementary to the anticodon and 3′-terminal 18 nucleotides of tRNA^{His}, respectively, maintained the PBS complementary to tRNA^{His} after extended *in vitro* culturing in Sup T1 cells (Wakefield *et al.*, 1996). In the present study, we

have further characterized features of this virus. We report that substitution of a 200-base-pair DNA fragment encompassing the U5 and the PBS of this virus for the corresponding region in the wild-type genome resulted in a virus which again stably maintained a PBS complementary to tRNA^{His}. DNA sequence analysis revealed no changes in NC protein gene between this virus and the wild-type virus and no similar changes in the RT gene among proviral clones of this virus; correspondingly, analysis of the tRNA composition within virions of the virus which utilizes tRNA^{His} to initiate reverse transcription revealed no differences when compared to that from the wild-type virus which utilizes tRNA^{Lys,3}. The results of our studies are discussed with respect to the mechanism used by HIV-1 to select a tRNA to initiate reverse transcription.

MATERIALS AND METHODS

Tissue culture

COS-1 cells used for transfection were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37° and 5% CO_2. Sup T1 cells used for virus infection were cultured in RPMI medium supplemented with 15% fetal calf serum.

Construction of mutant HIV-1 proviral genome

The construction of the original mutant HIV-1 molecular clone pHXB2(His-AC) has been previously described (Wakefield *et al.*, 1996). To construct the HIV-1 proviral clone used in this study, a DNA fragment encompassing the U5 and PBS regions was amplified by PCR from approximately 2.0 μg of total cellular DNA isolated from passage 12 HXB2(His-AC) virus-infected Sup T1 cells using the following DNA primer pair: 5′-GGCTAACTA-GGGAACCCACTGC-3′ (nts 42 to 63) (nucleotides in HIV-1 genome are numbered as in Ratner *et al.*, 1987); 5′-CTCCTTCTAGCCTCCGCTAGTC-3′ (nts 331 to 310).

The amplified DNA was digested with *Afl*II and *Bss*HII releasing a 200-base-pair fragment. This DNA fragment was first cloned into a transfer vector, pUC119 PBS, which contains a *Hpa*I to *Pst*I DNA fragment from pHXB2 encompassing the 5′ LTR and PBS regions. The cloned DNA fragment was confirmed by sequencing (Sanger *et al.*, 1977). To reconstruct this proviral DNA fragment into an infectious provirus, the *Hpa*I to *Bss*HII DNA fragment from pUC119 PBS was substituted for the same DNA fragment in pHXB2 (Fig. 1); the resulting HIV-1 proviral clone is referred to as pHXB2 (His-AC-gac).

DNA transfection and virus production

COS-1 cells were transfected with 10 μg of pHXB2, pHXB2(His-AC), or pHXB2(His-AC-gac) plasmid DNA using the DEAE–dextran method as previously described (Wakefield *et al.*, 1994). To determine if the viruses produced from transfected COS-1 cells were infectious, Sup T1 cells, which
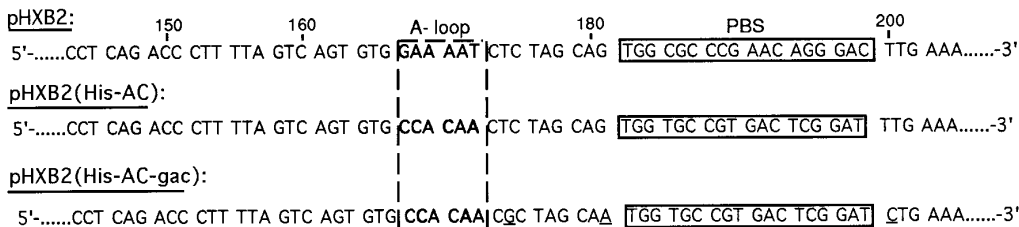
A
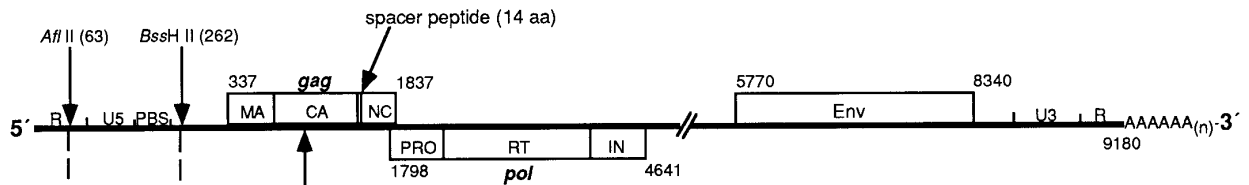
B

FIG. 1. HIV-1 (HXB2) RNA genome structure and partial nucleotide sequences in U5 and PBS regions in three HIV-1 proviral clones. (A) Schematic drawing of a wild-type HIV-1 (HXB2) RNA genome structure. The nucleotides are numbered as in Ratner (Ratner *et al.,* 1987). The coding regions on the genome are labeled with their encoded proteins (Myers *et al.,* 1994). For clarity, accessory protein genes are not depicted. *Afl*II and *Bss*HII restriction sites were used to clone a 200-bp fragment from a pHXB2(His-AC)-derived provirus to pHXB2. This resulted in a proviral clone of pHXB2(His-AC-gac). R, direct repeat sequences at the 5′ and 3′ ends of viral RNA genome; U5, 5′ end unique sequences; U3, 3′ end unique sequences; PBS, primer binding site. (B) Partial nucleotide sequences (143 to 205) in U5 and PBS regions in three HIV-1 proviral clones: pHXB2 (W.T.), pHXB2(His-AC), and pHXB2(His-AC-gac). The sequences that are located in the A-loop region and that are complementary to the anticodon loops of tRNA^{Lys,3} or tRNA^{His} are boxed and shown in boldface type. The PBS sequences that are complementary to the 3′ end 18 nucleotides of tRNA^{Lys,3} or tRNA^{His} are boxed. The 3 nucleotides (G_{174}, A_{181}, and C_{200}) present in pHXB2(His-AC-gac) but not in the original construct of pHXB2(His-AC) and wild-type pHXB2 are underlined.

express CD4 receptor and support high-level HIV-1 replication, were cocultivated with transfected COS-1 cells 20 hr after transfection (Wakefield *et al.,* 1996). Following 2 days of cocultivation, the Sup T1 cells were removed, centrifuged at 1000 *g* for 3 min, and further cultured in RPMI medium with additional uninfected Sup T1 cells. Virus productions were monitored by measuring the levels of p24 antigen in the culture medium using a commercial ELISA kit (Coulter Laboratories).

At Day 61 post-cocultivation, cell culture supernatant from HXB2(His-AC-gac) or wild-type virus infected Sup T1 cells containing 200 ng of p24 antigen (as measured by ELISA) was used to infect 2 ml of Sup T1 cells (2 × 10⁶ cells). After an overnight absorption at 37°, the infected Sup T1 cells were pelleted, washed once with RPMI medium, and resuspended in 6 ml of RPMI medium for further culturing. At 2-day intervals, 2 ml of cell culture was removed and 2 ml of fresh medium and Sup T1 cells were added to the cultures. The amounts of p24 antigen at each time point were determined by ELISA.

DNA sequence analysis of the U5 and PBS region of proviruses

At the designated days post-cocultivation, genomic DNAs were isolated from wild-type HXB2 and mutant

HXB2(His-AC-gac) virus-infected Sup T1 cells using the Wizard genomic DNA purification kit according to the manufacturer's instructions (Promega). About 2.0 μg of cellular DNA was used to amplify the proviral DNA sequences encompassing the U5 and PBS regions of the virus by PCR using the following primer pair: 5′- TTGACA-GCCGCCTAGC-3′ (nts 8895 to 8910); 5′-GCGCGCTTC-AGCAAGCCG-3′ (nts 262 to 245).

PCR-amplified DNA fragments were gel purified and ligated into pCR II vector (Invitrogen). The ligation mixtures were transformed into *Escherichia coli* (strain DH5α); putative recombinants were identified as previously described (Sambrook *et al.,* 1989). Plasmid DNA from individual *E. coli* colonies were prepared and sequenced using the primer: 5′-GGCTAACTAGGGAAC-CCACTGC-3′ (nts 42 to 63).

DNA sequence analysis of the *gag* and *pol* genes of proviruses

At Day 61 post-cocultivation, genomic DNAs were isolated from HXB2 and HXB2(His-AC-gac) virus-infected Sup T1 cells. PCR was used to amplify a DNA fragment from the integrated proviral genomes spanning nucleotides from −191 (in the U3 region of the 5′ LTR) to 3774

(the 3′ end of the reverse transcriptase gene) using the following primers: 5′-TTGACAGCCGCCTAGC-3′ (nts 8895 to 8910); 5′-TAGTACTTTCCTGAT-3′ (nts 3774 to 3760).

The PCR-amplified DNAs were ligated into pCR II vector followed by transformation into *E. coli* (strain DH5α). Recombinants were identified by restriction enzyme screening of plasmid minipreparations. Six individual clones of HXB2(His-AC-gac) provirus were used to determine the DNA sequences encoding for the viral NC protein and 13 HXB2(His-AC-gac) proviral clones and 5 wild-type HXB2 proviral clones were sequenced to determine the DNA sequences encoding for the RT. The following primers were used for DNA sequencing: 5′-GGGACC-AGCGGCTACACTAG-3′ (nts 1345 to 1364); 5′-GATTGT-ACTGAGAGACAGGC-3′ (nts 1607 to 1626); 5′-TAGTAG-GACCTACACCTGTC-3′ (nts 2024 to 2043); 5′-GGAATA-CCACATCCCGCAGG-3′ (nts 2071 to 2390); 5′-ATC-AATACATGGATGATTTGTATGTA-3′ (nts 2636 to 2661); 5′-GACAGTACAGCCTATAGTGC-3′ (nts 2811 to 2830); 5′-AGAATGAGGGGTGCCCACAC-3′ (nts 3159 to 3179); 5′-GGATATGTTACTAATAGAGG-3′ (nts 3459 to 3479).

Analysis of tRNAs from HIV-1 virions

Wild-type and mutant viruses were isolated from 25-ml infected Sup T1 cell cultures at Day 61 post-cocultivation. The media were first subjected to low-speed centrifugation to pellet the cellular debris (1000 g for 10 min). The supernatants were layered onto a 20% sucrose solution and centrifuged at 25,000 rpm (82,700 g) for 16 hr at 4° in a SW28 rotor. Total viral RNA was isolated from the viruses using the guanidinium isothiocyanate method described by Chomczynski and Sacchi (1987). About 2.5 μg total viral RNA was obtained and 0.6 μg of this RNA was labeled at the 3′ end with [^{32}P]pCp under the following reaction conditions (50 μl in total): 36 μl of RNA sample in DEPC–H_2O, 5 μl of 10× T4 RNA ligase buffer [50 mM Tris–HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 1 mM ATP], 5 μl of DMSO, 2 μl of [^{32}P]pCp (10 mCi/ml, Amersham), and 2 μl (40 units) of T4 RNA ligase (Bruce and Uhlenbeck, 1978). The reaction was incubated at 4° overnight. Approximately 1 μg of yeast tRNAs (GIBCO, BRL) was labeled under the same conditions. After labeling, 2 μg of λ DNA was added to the labeled RNA samples followed by precipitation with 0.1 vol of 3 M NaOAc (pH 5.4) and 2.5 vol of 100% ethanol. After an incubation at −70° for 1 hr, the nucleic acids were recovered by centrifugation at 4° for 20 min in a microfuge. The RNA pellets were washed once with 70% ethanol, dried in a speed vac, and resuspended in 10 μl of DEPC-H_2O.

The RNA samples were heated at 90° for 2 min prior to mixing with 10 μl of 2× glycerol loading buffer [10% glycerol, 0.2 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol FF] (Sambrook *et al.*, 1989) and electrophoresed at 200 V in a 7% polyacrylamide/4 M urea gel with 1× TBE running buffer [0.089 M Tris base, 0.089 M boric acid, and 0.002 M EDTA] at 4° for 6 hr. After autoradiography, the gel was stained with ethidium bromide and the nucleic acids visualized under UV light. The regions of the gel corresponding to the tRNAs (as determined by migration of the yeast tRNA controls) were excised from the gel and embedded on the top of a second gel at an orientation of 90° to the first dimension. The second dimension gel contained 17.6% polyacrylamide/8 M urea. The samples were electrophorezed at 300 V in 1× TBE running buffer at room temperature for 40 hr. The gel was then subjected to autoradiography without drying (Jiang *et al.*, 1992).

Direct sequencing of isolated virion-tRNAs

Sequencing of tRNAs were carried out using the procedures described by LeBlanc and Hajduk (1996). Briefly, following autoradiography, regions of the gel that contained tRNAs were excised and the tRNAs eluted into 300 μl of 0.01× TAE [1× TAE contains 0.089 M Tris base, 0.04 M acetic acid, and 0.001 M EDTA] using an Isco's electroelutor (3 W, 1 hr at room temperature). The tRNAs were precipitated in ethanol overnight at −70° and then recovered by centrifugation in a microfuge. The tRNA pellets were dried in a speed vac and resuspended in 10 μl of DEPC–H_2O. Some tRNAs were not completely resolved into single species by two-dimensional gel electrophoresis. These tRNAs were further purified on a sequencing gel containing 8% acrylamide and 8 M urea (30 W for 2 hr). After autoradiography, tRNAs were excised from the gel and eluted into 200 μl of elution solution (0.5 M NH$_4$OAc, 1.0 mM EDTA, and 0.1% of SDS) by shaking overnight at 37°. The tRNAs were precipitated in ethanol overnight at −70° and recovered by centrifugation. The sequences of the isolated tRNAs were determined by enzyme digestion using Pharmacia's RNA sequencing kit. The 3′-terminal nucleotide sequences were resolved by electrophoresis on a gel containing 17.6% acrylamide and 8 M urea.

RESULTS

Construction of HIV-1 proviruses containing mutant PBS

In a previous study, our laboratory constructed an HIV-1 virus, HXB2(His-AC), which contained a PBS sequence complementary to the 3′-terminal 18 nucleotide sequences of tRNA^{His} and 6 nucleotide sequences (CCA-CAA) upstream of the PBS in U5 of the viral RNA genome complementary to the anticodon loop of tRNA^{His} (Wakefield *et al.*, 1996). This virus maintained a PBS complementary to tRNA^{His} after extended *in vitro* culture in Sup T1 cells. DNA sequence analysis of the PBS regions recovered from the integrated proviruses of HXB2(His-AC) revealed that a number of nucleotide substitutions were present around the 18-nucleotide PBS. Three nucle-
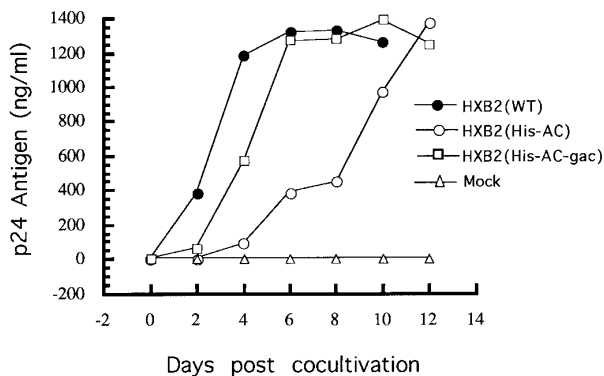
FIG. 2. Kinetics of appearance of viruses produced from cells transfected with pHXB2, pHXB2(His-AC), or pHXB2(His-AC-gac). COS-1 cells were transfected with the designated plasmids and then cocultured with Sup T1 cells. After 2 days of coculture, the Sup T1 cells were collected by centrifugation, washed once with RPMI medium, and further cultured in fresh medium with additional fresh Sup T1 cells. At 2-day intervals, the culture supernatants were collected and assayed for p24 antigen by ELISA.

otide substitutions, however, were found in most of the clones of the PBS region that were recovered: a T-to-G change at nucleotide 174, a G-to-A change at nucleotide 181, and a T-to-C change at nucleotide 200 (Wakefield *et al.,* 1996). All of these changes were found within the *Afl*II to *Bss*HII DNA restriction fragment (200 base pairs) encompassing the U5 and PBS regions of the viral genome (Fig. 1). To determine if the sequence changes in the U5 and PBS regions alone were sufficient for the HXB2(His-AC) virus to maintain a PBS complementary to tRNA^{His}, we cloned this 200-base-pair DNA fragment into an infectious HIV-1 clone, pHXB2. The resulting clone, referred to as pHXB2(His-AC-gac), contains a complete HIV-1 provirus which has a PBS complementary to the 3′-terminal 18 nucleotides of tRNA^{His}, a 6-nucleotide sequence (CCACAA) complementary to the anticodon loop of tRNA^{His}, and the 3 additional nucleotide substitutions (G_{174}, A_{181}, and C_{200}) around the PBS and the A-loop region (Fig. 1).

Infectivities of mutant HIV-1 viruses derived from pHXB2(His-AC) and pHXB2(His-AC-gac)

In preliminary experiments, we determined that transfection of pHXB2(His-AC-gac) or pHXB2(His-AC) into COS-1 cells resulted in production of similar levels of p24 antigen in the culture supernatant as that from COS-1 cells transfected with pHXB2 (data not shown). To compare the infectivities of the mutant and wild-type viruses produced in the transfected cells, we cocultured transfected COS-1 cells with Sup T1 cells; the production of viruses was detected by the release of p24 in the Sup T1 culture supernatant (Fig. 2). The appearance of infectious virus derived from pHXB2(His-AC-gac)-transfected cells was approximately 1–2 days slower than that for virus derived from pHXB2. Even with the slight delay, both viruses reached similar levels of p24 antigen in the cul-

ture supernatants by Day 6 post-cocultivation (Fig. 2). In contrast to the pHXB2(His-AC-gac)-derived virus, as previously reported (Wakefield *et al.,* 1996), virus produced from the parent pHXB2(His-AC)-transfected cells had a 4-day delay in appearance in Sup T1 cells compared with that of the wild-type virus; approximately 12 days of culture was required for HXB2(His-AC)-derived viruses to reach a similar level of p24 antigen as that for the wild-type virus (Fig. 2).

DNA sequence analysis of the U5 and PBS regions

Since the kinetics of appearance for viruses derived from transfection of pHXB2(His-AC), which contained the A-loop and a PBS complementary to tRNA^{His}, was considerably slower (4- to 6-day delay) than that for the wild-type virus derived from transfection of pHXB2 (Fig. 2 and Wakefield *et al.,* 1996), the fact that the viruses derived from pHXB2(His-AC-gac) had similar kinetics of appearance as the wild-type virus (Fig. 2) suggested that either the 200-nucleotide fragment transferred from HXB2(His-AC) proviral DNA was sufficient to support the replication of the HXB2(His-AC-gac) viruses or the original PBS in HXB2(His-AC-gac) complementary to tRNA^{His} had quickly reverted back to wild-type PBS complementary to tRNA^{Lys,3}. To investigate this possibility, we analyzed the proviral DNA from the viruses derived from pHXB2(His-AC-gac) at Days 10, 29, 45, and 61 post coculture. PCR was used to amplify a DNA fragment which contained the U5 and PBS regions of integrated proviruses; the amplified DNA was subcloned into the pCRII vector and individual clones analyzed by DNA sequencing. We found that, after an extended cell culture (2 months), the viruses derived from pHXB2(His-AC-gac) still maintained a PBS complementary to the 3′-terminal 18 nucleotides of tRNA^{His}, and a CCACAA sequence that is complementary to the anticodon loop sequence of tRNA^{His} along with the original 3 nucleotide substitutions (G_{174}, A_{181}, C_{200}) (Table 1). We did find a number of additional nucleotide substitutions and insertions within the U5 region. Of particular note were two substitutions upstream of the A-loop region found in most proviral DNA clones analyzed starting at Day 29 post-cocultivation: a C-to-A change at position 152 and a C-to-T change at position 160 (Table 1).

Comparison of replication of HXB2(His-AC-gac) virus obtained from extended culture with that of the wild-type virus

Since the pHXB2(His-AC-gac)-derived viruses exhibited slightly delayed kinetics of appearance as compared to the wild-type virus following the initial cocultivation (Fig. 2), it was possible that, after 2 months of *in vitro* culture, the viruses containing the additional C-to-A change at position 152 and C-to-T change at position 160 in U5 region would now have different growth characteristics (Table 1). To determine if this was the case,

TABLE 1

Partial DNA Sequences in U5 and PBS Regions of Proviruses Derived from pHXB2(His-AC-gac) at Days 29 and 61 Post-cocultivation

Sequence[a]	Frequency[b]
5'-CCT CAG ACC CTT TTA GTC AGT GTG **CCA CAA** CGC TAG CAA TGG TGC CGT GAC TCG GAT CTG AAA-3'	Input[c]
Day 29 post-coculture	
5'-*** *** *** A** *** **T *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***-3'	4/11
5'-*** *** *** A** *** **T *** **A *** *** *** *** *** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** A** *** **T *** *** *** T** *** *** *** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** A** *** **T *** *** *** A** *** *** *** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** A** *** **T *** *** *** *T* *** *** *** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** A** *** **T *** *** *** *** *** *** T** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** *** *** *** *** *** *** *T* *** *** *** *** *** *** *** *** *** *** ***-3'	1/11
5'-*** *** *** *** *** *** *** *** *** *** **T *** *** *** *** *** *** *** *** *** ***-3'	1/11
Day 61 post-coculture	
5'-*** *** *** A** *** **T *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***-3'	8/15
5'-*** *** *** A** *** **T *** *** A** *** *** *** *** *** *** *** *** *** *** *** ***-3'	2/15
5'-*** *** *** A** *** **T *** *** *** A** *** *** *** *** *** *** *** *** *** *** ***-3'	1/15
5'-*** *** *** A** *** **T *** *** *** *** *** *** A** *** *** *** *** *** *** *** ***-3'	1/15
5'-*** *** C** A** *** **T *** *** *** *** *** **A *** *** *** *** *** *** *** *** ***-3'	1/15
5'-*** *** *** A** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***-3'	1/15
5'-*** *** *** A*C *** **T *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***-3'	1/15

[a] The nucleotides (CCACAA) that are located in the A-loop region and that are complementary to the anticodon loop of tRNA^{His} are shown in boldface type. The PBS sequence complementary to the 3'-terminal 18 nucleotides of tRNA^{His} is boxed. The nucleotides (G_{174}, A_{181}, and C_{200}) present in input pHXB2(His-AC-gac) that are different from those in pHXB2(His-AC) are underlined. Asterisks indicate identity with the input sequences.
[b] Frequencies of DNA sequences in U5 and PBS regions among proviral PCR clones analyzed.
[c] Partial DNA sequence (143–205) in U5 and PBS regions in the input pHXB2(His-AC-gac).

equal amounts of cell-free mutant and wild-type virus (as measured by p24 antigen) were collected from the infected Sup T1 cultures at Day 61 post-cocultivation and used to infect Sup T1 cells. The virus replication was monitored by measuring their p24 antigen levels in the culture medium (Fig. 3). After 2 months of culture, the HXB2(His-AC-gac) virus with the 2 additional nucleotide changes in U5 region from the starting virus (A at nucleotide 152 and T at nucleotide 160) had a nearly identical replication profile as that of the wild-type virus, in contrast to the slight delay observed for the kinetics of appearance of the early HXB2(His-AC-gac) virus (Figs. 2 and 3).

DNA sequence analysis of proviral NC and RT genes

Previous studies have suggested that the NC protein of the *gag* gene and the RT protein of the *pol* gene might be involved in the interaction with, and possible selection of, the tRNA primer used for reverse transcription (Barat *et al.*, 1989, 1991, 1993; Robert *et al.*, 1990; Sarih-Cottin *et al.*, 1993). Thus, there was a possibility that the NC and/or RT was mutated in the viruses derived from pHXB2(His-AC-gac) which were responsible for the selective use of tRNA^{His}. One way to address this possibility is to compare the sequence of the NC and RT genes of the mutant and wild-type virus. PCR was used to amplify proviral DNA encompassing nucleotides from −191 (in U3 region) to 3774 (the end of the RT gene) at the 61-day post-coculture time point. The PCR-amplified DNA was ligated into the pCRII vector; six individual PCR clones from pHXB2(His-AC-gac)-derived proviruses were sequenced to determine the nucleotide sequence encoding for the NC protein. Thirteen clones from pHXB2(His-AC-gac)-derived proviruses and 5 clones from wild-type pHXB2-derived provirus were analyzed for RT gene sequences. The U5 and PBS regions in these proviral
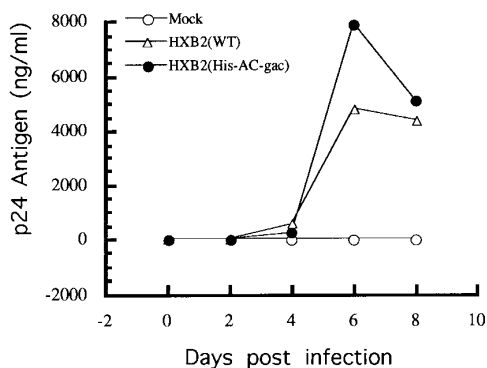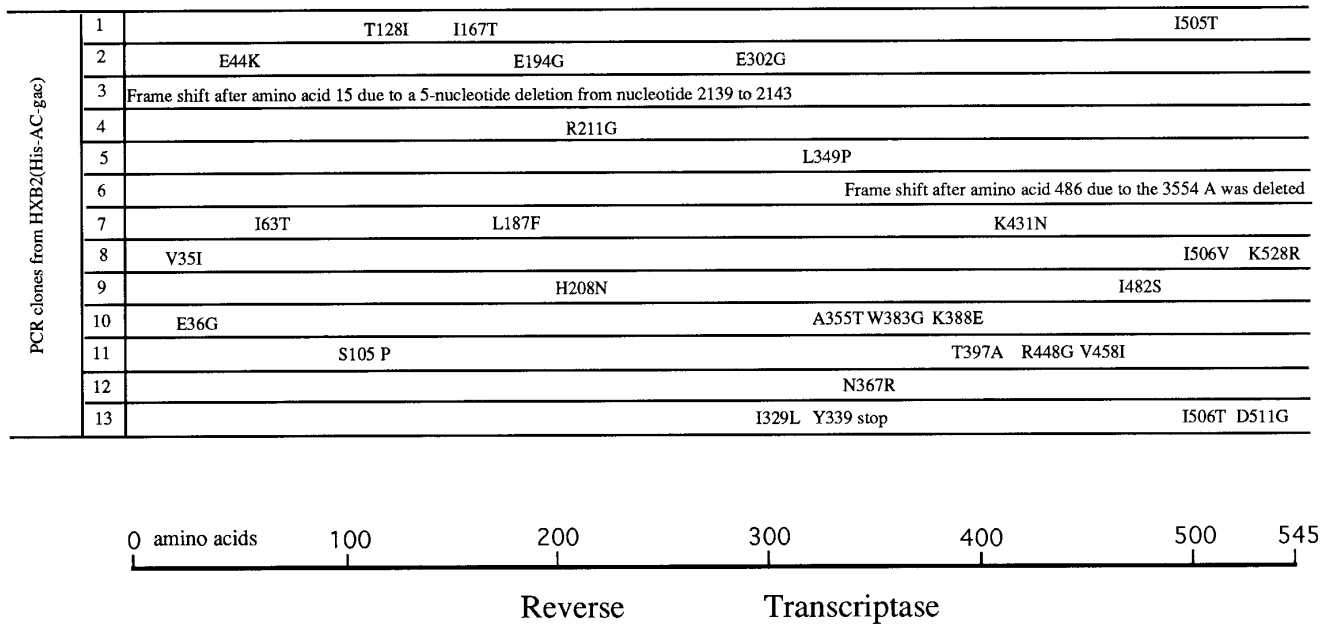

FIG. 3. Replication kinetics of the viruses after 61 days of *in vitro* culture. Supernatants from infected Sup T1 cells containing mutant HXB2(His-AC-gac) or wild-type HXB2 viruses were collected at Day 61 post-cocultivation and the p24 levels were determined by ELISA. Equal amounts of mutant and wild-type viruses (200 ng of p24) were used to infect fresh Sup T1 cells (2×10^6 cells in 2 ml of RPMI medium). After an overnight incubation, the medium was removed and the infected Sup T1 cells were washed once with medium and further cultured in 6 ml of fresh medium with additional uninfected Sup T1 cells. At 2-day intervals, the culture supernatants were collected and assayed for p24 antigen concentration by ELISA.

PCR clones from HXB2(His-AC-gac)

#	Amino acid changes
1	T128I I167T I505T
2	E44K E194G E302G
3	Frame shift after amino acid 15 due to a 5-nucleotide deletion from nucleotide 2139 to 2143
4	R211G
5	L349P
6	Frame shift after amino acid 486 due to the 3554 A was deleted
7	I63T L187F K431N
8	V35I I506V K528R
9	H208N I482S
10	E36G A355T W383G K388E
11	S105 P T397A R448G V458I
12	N367R
13	I329L Y339 stop I506T D511G

```
0  amino acids   100        200        300        400        500   545
|--------------|----------|----------|----------|----------|-----|
           Reverse       Transcriptase
```

PCR clones from HXB2 (W.T.)

#	Amino acid changes
1	I202L V317M E404G G541E
2	W153 stop
3	D113N P225S
4	I495L
5	I2T N54D S162N D192N R199G D471G

FIG. 4. Amino acid changes in RTs caused by nucleotide mutations in proviruses derived from wild-type pHXB2 and mutant pHXB2(His-AC-gac) at Day 61 post-coculture. Amino acids are represented by single letters. The letters before the numbers refer to the amino acids of the original wild-type RT of HIV-1 (HXB2) and the letters after the numbers denote the mutated amino acids derived from the mutated nucleotide sequences of PCR clones (Myers *et al.*, 1994; Ratner *et al.*, 1987).

clones were sequenced at the same time to ensure that all of the clones contained a PBS complementary to tRNA^{His} for the mutant viruses or tRNA^{Lys,3} for the wild-type virus.

Our initial sequencing efforts were focused on a 420-base-pair region of DNA (nucleotides 1424 to 1837) encoding for the NC and the spacer peptide located between the capsid protein (CA) and the NC (Krausslich *et al.*, 1995; Myers *et al.*, 1994) (Fig. 1). Sequence analysis of six individual PCR subclones from proviruses derived from pHXB2(His-AC-gac) revealed no nucleotide changes which would have resulted in the use of different amino acids in any of these clones (data not shown). We next focused our sequence analysis on the RT genes from proviruses derived from pHXB2(His-AC-gac) (Fig. 4). Three of 13 clones analyzed contained mutations that would disrupt the translational reading frame and thus would encode a nonfunctional RT (clones 3, 6, and 13). The remaining 10 clones all contained nucleotide changes which would result in the substitutions of different amino acids from the starting clone. However, we did not find a single mutation which was common in any of the 13 clones analyzed. This is a striking contrast to

the consistent nucleotide changes in the U5 region of these same proviral clones (Table 1). Analysis of the RT gene from the PCR clones derived from the wild-type proviral clone pHXB2 also revealed numerous nucleotide changes resulting in amino acid changes (Fig. 4); we also found a single clone (of five analyzed) which contained a stop codon in the early part of the RT gene. Again, there was no identical amino acid change among these clones. The mutations that were identified in proviruses derived from pHXB2(His-AC-gac) and pHXB2 were not located at any of the sites in the RT which correspond to the reported catalytic site (nucleotides 2641 to 2652) (Argos, 1988), nucleotide binding site (nucleotides 2881 to 2892) (Johnson *et al.*, 1986), or the site postulated to bind the primer tRNA (nucleotides 2959 to 2982) (Delahunty *et al.*, 1994).

Comparison of tRNAs in virions derived from HXB2(His-AC-gac) and the wild-type viruses

We next wanted to address the question of whether viruses derived from pHXB2(His-AC-gac) incorporated a different set of cellular tRNAs than that present within

the wild-type virions. At Day 61 post-cocultivation, viruses were isolated from supernatants of Sup T1 cells infected with either HXB2(His-AC-gac) or HXB2. The virion-associated RNAs were 3′-end labeled with [^{32}P]pCp and separated sequentially by one-dimensional and then by two-dimensional gel electrophoresis (Figs. 5A and 5B). No significant differences with respect to the composition of tRNAs were evident between the HXB2 (His-AC-gac) viruses and the wild-type virus. Although there were many spots on the autoradiogram, we could readily identify four major tRNA species (spots) from each preparation which had similar labeling intensities; we designated these four tRNA species with numbers for sequence analysis (Fig. 5B; Spots 1, 2, 3, and 4).

The sequences of the four predominant tRNA species present in HXB2(His-AC-gac) and the wild-type viruses were determined. Before sequencing, spots 2 and 4 were further resolved using a polyacrylamide gel to separate cross-contaminating tRNA species (Fig. 5C). Each tRNA was eluted from individual gel spot and sequenced using standard enzymatical RNA sequencing methods (Fig. 6). Comparing the tRNA sequences from each spot with the published tRNA sequences allowed the determination that the tRNA in spot 1 is tRNA^{Lys,2}; tRNA in spot 2 (the most predominant tRNA species in both mutant and wild-type viruses) is tRNA^{Lys,3}; tRNA in 3 is tRNA^{Asn} and tRNA in 4 is tRNA^{Lys,1} (Table 2) (Sprintzl *et al.*, 1989). These results confirm a previous study which found that tRNA^{Lys,3} and tRNA^{Lys1,2} were the major tRNA species present in HIV-1 virions isolated from transfected COS-7 cells (Jiang *et al.*, 1993). Interestingly, using Sup T1 cells as virus producing cells, we found that tRNA^{Asn} is also a major tRNA species present in HIV-1 virions. Most importantly, the major tRNA species found in HXB2(His-AC-gac) viruses were the same tRNA species present in the wild-type virus. Furthermore, tRNA^{His} is not among the major tRNA species found in HXB2(His-AC-gac) virus which uses it as replication primer.

DISCUSSION

In this report, we have characterized a unique HIV-1 virus [HXB2(His-AC)] which maintained a PBS complementary to tRNA^{His}. We found that substitution of a 200-base-pair DNA fragment which contained the region of the U5-PBS of this virus into a plasmid containing a wild-type provirus (pHXB2) conferred the capability of the resulting virus [HXB2(His-AC-gac)] to utilize tRNA^{His} to initiate reverse transcription. The HXB2(His-AC-gac) virus replicates similar to the wild-type HXB2 virus. Sequence analysis of selective regions of the *gag* and *pol* genes of HXB2(His-AC-gac) proviruses revealed no changes in NC protein gene and no conserved changes in RT gene among the proviral clones analyzed. Analysis of the virion–tRNA contents of the virus which maintained a PBS complementary to tRNA^{His} revealed that this mutant virus contained similar tRNA species as that of the wild-type
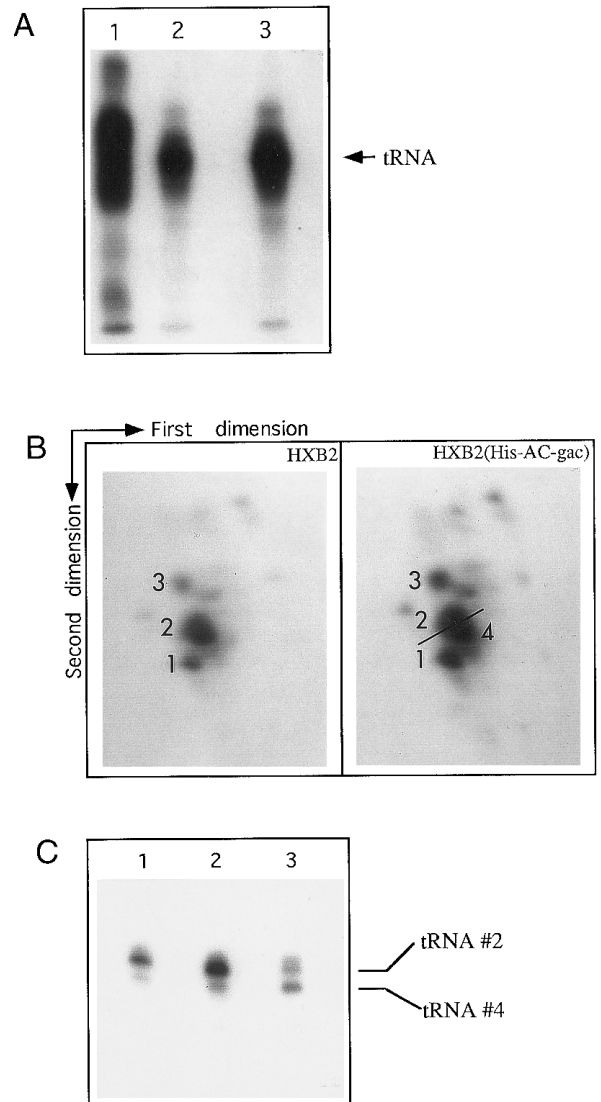
FIG. 5. Gel electrophoresis of tRNAs isolated from HIV-1 virions. Virion-associated RNAs were isolated from pHXB2 or pHXB2(His-AC-gac)-derived viruses isolated from culture media of infected Sup T1 cells at Day 61 post-coculture. (A) First-dimensional gel electrophoresis. Total viral RNAs were 3′-end labeled with [^{32}P]pCp, followed by separation on a polyacrylamide gel. Lane 1, [^{32}P]pCp-labeled total yeast tRNAs. Lanes 2 and 3, RNAs from virions of wild-type HXB2 and mutant HXB2(His-AC-gac) viruses, respectively. Autoradiography was used to identify the virion-associated tRNAs. (B) Second-dimensional gel electrophoresis. The gel slices in the first-dimensional gel that contained virion-associated tRNAs were excised and embedded on the top of the second-dimensional gel. Following electrophoresis, the gel was processed for autoradiography. The numbers refer to regions of the gel excised after autoradiography for further purification [spot 2 of HXB2; spots 2 and 4 of HXB2(His-AC-gac)] or direct sequence determination [spots 1 and 3 of both HXB2 and HXB2(His-AC-gac)] of the tRNAs. (C) Further purification of virion-associated tRNAs obtained from the second dimensional gel electrophoresis. Lane 1, Electrophoresis of tRNA species isolated from spot 2 of HXB2. After autoradiography, only the top strong band was excised and the tRNA was eluted from the gel and sequenced. Lanes 2 and 3, Electrophoresis of tRNA species isolated from spots 2 and 4 of HXB2(His-AC-gac), respectively. The tRNA bands were excised from the gel separately, and the two top bands (tRNA 2) and two bottom bands (tRNA 4) in lanes 2 and 3 were combined and the tRNAs were eluted and sequenced.

tRNAs within the HIV-1 virion does not necessarily correlate with the tRNA used to initiate reverse transcription. In other words, factors other than NC protein, RT or virion–tRNA compositions were responsible for the selection of the tRNA^{His} used to initiate the reverse transcription in HXB2(His-AC-gac) virus. Based on our results that nucleotide substitutions within the U5 region, in combination with a PBS complementary to tRNA^{His} that resulted in an HIV-1 virus which now uses tRNA^{His} to initiate the reverse transcription, we postulate that it is the HIV-1 RNA genome that selects the tRNA primer for initiation of reverse transcription. It is possible that this selection occurs via a complex RNA–tRNA secondary structure formed by the 5′ end of the HIV-1 RNA genome and the primer tRNA. There are several lines of evidence to support this hypothesis. First, after 2 months of culture, the virus derived from pHXB2(His-AC-gac) showed very similar replication kinetics to that for the wild-type virus (Fig. 3). The majority of the PBS clones recovered at this time point from this mutant virus contained two additional mutations in U5 region (a C-to-A change at nucleotide 152 and a C-to-T change at nucleotide 160) (Table 1). This result, coupled with the fact that HXB2(His-AC-gac) virus replicated more rapidly than HXB2(His-AC) virus on Sup T1 cells (Fig. 2), supports the concept that during the in vitro culturing, viruses with a PBS complementary to tRNA^{His} were still evolving. It is possible then that these single nucleotide changes in the U5 region provide a structurally competitive advantage to the selection of tRNA^{His} to initiate the reverse transcription. Studies are ongoing to address this possibility. Second, there is a precedent for a U5-PBS-tRNA interaction. Previous studies by Isel et al. (1995) have demonstrated the existence of a complex RNA secondary structure at the 5′ end of the viral RNA genome in the absence of the tRNA. Using an in vitro chemical and enzymatic analysis, the same investigators found that regions in the U5 as well as the PBS interact with the tRNA^{Lys,3} (Isel et al., 1995).

Finally, the results of our previous study also support a complex interaction between the tRNA^{Lys,3} and U5-PBS (Wakefield et al., 1996). In that study, we demonstrated that a sequence in the A-loop region in U5 complementary to the anticodon region of tRNA^{His} was critical for an HIV-1 to maintain a PBS complementary to tRNA^{His} (Wakefield et al., 1996). The result of the current study that a 200-base-pair region encompassing U5 and the PBS was all that was required for the virus to maintain a PBS complementary to tRNA^{His} favors the idea that the viral RNA genome is the major determinant in the selection of tRNA^{His} for the initiation of reverse transcription. Further studies will be required with additional mutant HIV-1 proviral genomes to determine if this is the case for other HIV-1 viruses which use alternative tRNAs to initiate reverse transcription (Kang et al., 1996).

ACKNOWLEDGMENTS

REFERENCES

Aiyar, A., Cobrinik, D., Ge, Z., Kung, H.-J., and Leis, J. (1994). Interaction between retroviral U5 RNA and TψC loop of tRNA^{Trp} is required for efficient initiation of reverse transcription. J. Virol. 66, 2461–2472.

Argos, P. (1988). A sequence motif in many polymerases. Nucleic Acids Res. 6, 3831–3843.

Baltimore, D. (1970). Viral RNA-dependent DNA polymerase. Nature 226, 1209–1211.

Barat, C., Grice, S. F. J. Le, and Darlix, J.-L. (1991). Interaction of HIV-1 reverse transcriptase with a synthetic form of its replication primer tRNA^{Lys,3}. Nucleic Acids Res. 19, 751–757.

Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre, M. T., Gruninger-Leitch, F., Barre-Sinoussi, F., LeGrice, S. F. J., and Darlix, J. L. (1989). HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. EMBO J. 8, 3279–3285.

Barat, C., Schatz, O., Grice, S., Le, and Darlix, J.-L. (1993). Analysis of the interactions of HIV-1 replication primer tRNA^{Lys,3} with nucleocapsid protein and reverse transcriptase. J. Mol. Biol. 231, 185–190.

Bruce, A. G., and Uhlenbeck, O. C. (1978). Reactions at the termini of tRNA with T4 RNA ligase. Nucleic Acids Res. 5, 3665–3677.

Chomczynski, P., and Saachi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Colicelli, J., and Goff, S. P. (1987). Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. J. Virol. 57, 37–45.

Crawford, S., and Goff, S. P. (1985). A deletion mutation in the 5′ part of the pol gene of moloney murine leukemia virus blocks proteolytic processing of the Gag and Pol polyproteins. J. Virol. 53, 899–907.

Das, A. T., Klaver, B., and Berkhout, B. (1995). Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primer other than the natural tRNA^{Lys,3}. J. Virol. 69, 3090–2097.

Delahunty, M. D., Wilson, S. H., and Karpel, R. L. (1994). Studies on primer binding of HIV-1 reverse transcriptase using a fluorescent probe. J. Mol. Biol. 236, 469–479.

Gilboa, E., Mitra, S. W., Goff, S., and Baltimore, D. (1979). A detailed model of reverse transcription and tests of crucial aspects. Cell 18, 93–100.

Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., and Marquet, R. (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.

Jiang, M., Mak, J., Ladha, A., Cohen, E., Klein, M., Rovinski, B., and Kleiman, L. (1993). Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. J. Virol. 67, 3246–3253.

Jiang, M., Parniak, M. A., and Kleiman, L. (1992). Isolation and fractionation of retroviral tRNAs. J. Virol. Methods 38, 205–214.

Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J., and Doolittle, R. F. (1986). Computer analysis of retroviral pol genes: Assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. Proc. Natl. Acad. Sci. USA 83, 7648–7652.

Kang, S.-M., Wakefield, J. K., and Morrow, C. D. (1996). Mutations in both the U5 region and primer binding site influence the selection of the tRNA used for the initiation of HIV-1 reverse transcription. Virology 222, 401–444.

Krausslich, H.-G., Facke, M., Heuser, A.-M., Konvalinka, J., and Zentgraf, H. (1995). The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity. J. Virol. 69, 3407–3419.

LeBlanc, A. J., and Hajduk, S. L. (1996). Identification of a nuclear encoded and mitochondrial imported precursor tRNA[Leu] in *Trypanosoma brucei*. Submitted for publication.

Levin, J. G., and Seidman, J. G. (1981). Effect of polymerase mutations on packaging of primer tRNA[Pro] during murine leukemia virus assembly. *J. Virol.* **38**, 403–408.

Li, X., Johnson, M., Arts, E. J., Gu, Z., Kleiman, L., Wainberg, M. A., and Parniak, M. A. (1994). Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. *J. Virol.* **68**, 6198–6206.

Li, X., Quan, Y., Arts, E. J., Li, Z., Preston, B. D., de Rocquigny, H., Roques, B. P., Darlix, J.-L., Kleiman, L., Parniak, M. A., and Wainberg, M. A. (1996). Human immunodeficiency virus type 1 nucleocapsid protein (NCp7) directs specific initiation of minus-strand DNA synthesis primed by human tRNA[Lys,3] in vitro: studies of viral RNA molecules mutated in regions that flank the primer binding site. *J. Virol.* **70**, 4996–5004.

Mak, J., Jiang, M., Wainberg, M. A., Hammarskjold, M.-L., Rekosh, D., and Kleiman, L. (1994). Role of Pr160 $^{gag-pol}$ in mediating the selective incorporation of tRNA[Lys] into human immunodeficiency virus type 1 particles. *J. Virol.* **68**, 2065–2072.

Mely, Y., Rocquigy, H., Sorinas-Jimeno, M., Keith, G., Roques, B., Marquet, R., and Gerard, D. (1995). Binding of the HIV-1 nucleocapsid protein to the primer tRNA[Lys,3], *in vitro,* is essentially not specific. *J. Biol. Chem.* **270**, 1650–1656.

Myers, G., Wain-Hobson, S., Henderson E. L., Korber, B., Jeang, K.-T., and Pavslakis, N. G. (1994). Human Retroviruses and Aids. Los Alamos National Laboratory, Los Alamos, NM.

Najera, I., Holguin, A., Quinones-Mateu, M. E., Munoz-Fernandez, M. A., Najera, R., Lopez-Galindez, C., and Domingo, E. (1995). *pol* gene quasispecies of human immunodeficiency virus: Mutations associated with drug resistance in virus from patients undergoing no drug therapy. *J. Virol.* **69**, 23–31.

Peters, G., and Dahlberg, J. E. (1979). RNA-directed DNA synthesis in the Moloney murine leukemia virus: Interaction between the primer tRNA and the genome. *J. Virol.* **31**, 398–407.

Peters, G., and Glover, C. (1980). tRNAs and priming of RNA-directed DNA synthesis in mouse mammary tumor virus. *J. Virol.* **35**, 31–40.

Peters, G., and Hu, J. (1980). Reverse transcriptase as the major determinant for selective packaging of tRNAs into avian sarcoma virus particles. *J. Virol.* **36**, 692–700.

Prats, A. C., Sarih, L., Gobus, C., Litvak, S., Keith, G., and Darlix, J. L. (1988). Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA. *EMBO J.* **7**, 1777–1783.

Ratner, L., Fisher, A., Linda, L., Agodzinski, J., Mitsuya, H., Liou, R.-S., Gallo, R. C., and Wong-Staal, F. (1987). Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**, 57–69.

Rhim, H., Park, J., and Morrow, C. D. (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.

Robert, D., Sallafranque-Andreola, M.-L., Bordier, B., Sarih-Cottin, L., Tarragp-Litvak, P., Graves, P. V., Barr, P. J., Fournier, M., and Litvak, S. (1990). Interactions with tRNA[Lys] induce important structural changes in human immunodeficiency virus reverse transcriptase. *FEBS Lett.* **277**, 239–242.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Sarih-Cottin, L., Bordier, B., Musier-Forsyth, K., Andreola, M.-L., Barr, P. J., and Litvak, S. (1993). 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.

Sprintzl, M., Hartmann, T., Weber, J., Blank, J., and Zeidler, R. (1989). Complilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res. Suppl.* **17**, rl–r172.

Stewart, L., Schatz, G., and Vogt, V. M. (1990). Properties of avian retrovirus particles defective in viral protease. *J. Virol.* **64**, 5076–5092.

Taylor, J. M. (1977). An analysis of the role of tRNA species as primers for transcription into DNA of RNA tumor virus genomes. *Biochem. Biophys. Acta.* **473**, 57–71.

Taylor, J. M., and Hsu, T. W. (1980). Reverse transcription of avian sarcoma virus RNA into DNA might involve copying of the tRNA primer. *J. Virol.* **33**, 531–534.

Temin, H. M., and Mizutani, S. (1970). RNA-directed DNA polymerase in virions of Rous sarcoma virus. *Nature* **226**, 1211–1213.

Wakefield, J. K., Kang, S.-M., and Morrow, C. D. (1996). Construction of a type 1 human immunodeficiency virus that maintains a primer binding site complementary to tRNA[His]. *J. Virol.* **70**, 966–975.

Wakefield, J. K., Rhim, H., and Morrow, C. D. (1994). Minimal sequence requirements of a functional human immunodeficiency virus type 1 primer binding site. *J. Virol.* **68**, 1605–1614.

Wakefield, J. K., Wolf, A. G., and Morrow, C. D. (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[Lys,3]. *J. Virol.* **69**, 6021–6029.