Deletions in the tRNA^{Lys} Primer-Binding Site of Human Immunodeficiency Virus Type 1 Identify Essential Regions for Reverse Transcription

HYANGSHUK RHIM, JINSEU PARK, AND CASEY D. MORROW*

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

Received 19 February 1991/Accepted 20 May 1991

The initiation of human immunodeficiency virus type 1 (HIV-1) reverse transcription occurs by the extension of a tRNA primer bound near the 5' end of the genomic RNA at a position termed the primer-binding site (PBS). The PBS is an 18-nucleotide region of the HIV-1 genome complementary to cellular tRNA^{Lys}. To further investigate the sequence requirements for the PBS in reverse transcription, deletions in the PBS were created and subcloned into a plasmid containing the infectious HIV-1 proviral genome. The mutations deleted the entire PBS (Δ PBS) or the first 9 (Δ 1-9), the second 9 (Δ 10-18), or 12 (Δ 7-18) nucleotides of the PBS. An additional mutation in the PBS was created in which the second nine nucleotides were deleted and nine additional nucleotides were substituted [Lys(1-9)]. The transfection of plasmids containing the wild-type or mutant proviral genomes into tissue culture cells resulted in expression of the HIV-1 *gag* and *env* gene products, as determined by immunoprecipitation using sera from AIDS patients. HIV-1 virus was released from the transfected cells, as determined by analysis of the supernatants for reverse transcriptase activity. The infectivity of the viruses derived from the transfection was examined by coculture experiments with SupT1 cells, which support high-level replication of HIV-1. The transfection of plasmids containing HIV-1 proviral genomes with the Δ PBS and PBS (Δ 1-9) mutations did not produce infectious virus. In contrast, the HIV-1 proviral genomes with the Δ 10-18, Δ 7-18, and Lys(1-9) mutations in the PBS produced infectious virus upon transfection, although the kinetics of appearance was significantly delayed for the mutant viruses compared with the wild type. To further explore the nature of this defect, the PBS region from integrated proviral genomes was amplified by polymerase chain reaction and individual DNA products were subcloned into M13mp19, followed by a sequence analysis of the PBS region from individual M13 phage clones. In each of the PBS regions examined, the 18-nucleotide PBS complementary to tRNA^{Lys} was present. However, nucleotide deletions and insertions were found 3' to the PBS from the samples derived from the transfection of plasmids containing mutant proviral genomes. Upon reinfection, the revertant viruses maintained the deletions 3' to the PBS and had kinetics of replication similar to that of the wild-type virus. These results demonstrate a minimum requirement for the PBS sequence as well as the important role of cellular tRNA^{Lys} in the regeneration of the PBS during HIV-1 reverse transcription.

One of the early steps in the replication of human immunodeficiency virus type 1 (HIV-1) is the reverse transcription of the viral RNA genome into a cDNA copy. HIV-1, like all retroviruses, utilizes an RNA-dependent DNA polymerase (reverse transcriptase) to carry out this process (3, 31). Several models detailing the mechanism of retrovirus reverse transcription have been put forth and have numerous common features (8, 10, 30, 33). First, reverse transcription initiates with a cellular tRNA primer at the primer-binding site (PBS), approximately 180 nucleotides from the 5' end of the viral genome for HIV-1. The reverse transcriptase copies the short stretch of viral RNA genome to generate a cDNA called minus-strand strong-stop DNA. Second, a short terminal sequence at the 3' end of the strong-stop DNA, which is complementary to the 3' region of the viral genomic RNA, facilitates the jump of the reverse transcriptase to complete the synthesis of minus-strand DNA. Third, the initiation of plus-strand DNA synthesis occurs at the polypurine tract near the 5' end of the minus-strand DNA (21, 34). The reverse transcriptase then copies the minus-strand cDNA as well as the still attached cellular tRNA primer, which regenerates the PBS region in the viral genome (plus-strand

strong-stop DNA). The 3' region of the plus-strand strong-stop DNA, which is complementary to the end of the minus-strand cDNA, promotes a second jump by the reverse transcriptase. Subsequent elongation of the minus- and plus-strand DNAs completes the process and provides the complete proviral genome suitable for integration into cellular DNA.

The initiation of reverse transcription in all retroviruses examined to date occurs via the addition of a deoxynucleotide to the 3' end of the cellular tRNA primer which is complementary to a specific site on the genomic RNA designated the primer-binding site (PBS) (34). The PBS of HIV-1 consists of 18 nucleotides positioned at nucleotides 183 to 200 of the HIV-1 genome and is complementary to the 3' end of a cellular tRNA which is used as the primer for initiation of reverse transcription (23). A great deal of diversity exists with regard to the tRNA primer used by different retroviruses. HIV-1 uses as a primer cellular tRNA^{Lys}, as do visna virus, Mason-Pfizer monkey virus, and mouse mammary tumor virus (see reference 7 for a summary; 29). In contrast, murine leukemia virus and human T-cell leukemia virus types I and II utilize tRNA^{Pro} as a primer for reverse transcription (see reference 7 for a summary; 29). The interaction between the PBS region and the cellular tRNA is a critical step in the reverse transcription of

* Corresponding author.

the viral genome. To further understand the requirement for the interaction between the tRNA primer and the PBS in reverse transcription, we have generated mutant proviral HIV-1 genomes which contain deletions in the PBS. We report that infectious HIV-1 virus was generated upon transfection of plasmids containing HIV-1 genomes with at least the first six nucleotides of the PBS. Amplification of the PBS region from integrated proviruses by polymerase chain reaction (PCR) and subsequent DNA sequence analysis revealed that the complete 18-nucleotide PBS was regenerated, although specific nucleic acid deletions and insertions in the proviral genome 3' to the PBS were present in these viruses. The revertant viruses demonstrated replication kinetics similar to that for the wild-type virus. These results support aspects of the proposed model as well as provide new insights into the details of the mechanism of reverse transcription.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma Chemical Co. unless otherwise noted. Restriction enzymes and DNA modification enzymes were obtained from New England Biolabs. The *Taq* polymerase and reagents for PCR were purchased from Perkin-Elmer Cetus. Sequenase was purchased from U.S. Biochemical. Radioactive nucleotides were purchased from Amersham Corp., while [³⁵S] methionine (Translabel) was obtained from ICN. Tissue culture media and reagents were obtained from BRL-GIBCO. The synthetic DNA oligonucleotides used for mutagenesis were prepared by the Cancer Center Oligonucleotide Synthesis Facility at the University of Alabama at Birmingham.

Tissue culture cells. COS-1 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 5% newborn calf serum and 5% fetal bovine serum (complete medium). The SupT1 cells were obtained from B. Hahn (University of Alabama at Birmingham) and maintained in DMEM supplemented with 10% fetal calf serum.

DNA manipulation and mutagenesis. Routine procedures for DNA manipulation were carried out as previously described (15). For these studies, the replicative form (RF) of M13mp18 was modified by the addition of a synthetic DNA oligomer to create an *HpaI* restriction site at the *SmaI* site in the polylinker region (36). To do this, M13mp18 RF DNA was digested with *SmaI* and then ligated to a phosphorylated DNA linker corresponding to a *HpaI* restriction site. The RF DNA was digested with *HpaI*, religated, and transformed into *Escherichia coli* TG-1. Individual plaques were picked and used to infect TG-1; the RF DNA was screened for the presence of a unique *HpaI* site and was designated M13mp18 *HpaI*.

A plasmid containing the infectious HIV-1 provirus, pHXB2gpt, encodes the entire HIV-1 provirus flanked by unique *HpaI* (5') and *XbaI* (3') restriction sites (22). For these studies, pHXB2gpt was digested with *HpaI* and *PstI* restriction enzymes, and a 1,552-bp fragment was isolated and ligated into an *HpaI*-*PstI*-digested RF of M13mp18 *HpaI*. Following transformation into competent TG-1, the resultant phage were plaque purified and the RFs were screened by restriction digestion for the presence of the *HpaI*-*PstI* DNA fragment of pHXB2gpt. The recombinant phage, designated M13mp18 PBS, was used to prepare single-stranded DNA for mutagenesis (16).

Oligonucleotide site-directed mutagenesis was done by

previously described methods (37, 38). The DNA oligomers used to create the desired mutations in the PBS region are as follows:

```

5' GCG CCA CTT CTA GAG AT 3' (XbaI)
5' AGA GAG CTC TGG ATC CCC TTT CGC TTT 3' (BamHI)
5' GCT CTG GAT CCC CTT TCG CTT TCA AGT CCC TGT
   TCT TCT AGA GAT 3' ( $\Delta$ 1-9)
5' TCG CTT TCA ACG GGC GCC AC 3' ( $\Delta$ 10-18)
5' CCC TTT CGC TTT CAA GCG CCA CTT CTA GAG 3' ( $\Delta$ 7-18)
5' CGC TTT CAA TTC GCA CCA CGG GCG CCA 3' Lys(1-9)

```

The bacteriophage M13 plaques were screened by hybridization to the [γ -³²P]ATP kinased mutagenic oligonucleotide (5×10^5 cpm/ml) in $6 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and $10 \times$ Denhardt's solution ($1 \times$ Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) at a temperature that was 20 to 25°C below the theoretical dissociation temperature. The filters were washed with prewarmed $6 \times$ SSC at increasing temperatures until 5°C below the dissociation temperature. The filters were then exposed to X-ray film at -70°C with an intensifying screen to identify positive plaques.

Mutant DNAs identified by plaque hybridization were confirmed by DNA sequencing of plaque-purified phage DNA, using the dideoxy sequencing technique (27). A sequencing DNA oligonucleotide was designed to hybridize 78 bp 3' from the PBS region (nucleotides 279 to 295).

To reconstruct the mutant PBS back into pHXB2gpt, the RF DNA was isolated and digested with *HpaI* and *BssHII*, which gives a 868-bp fragment. For subcloning, pHXB2gpt, also digested with *HpaI* and *BssHII*, and the mutant PBS contained on the *HpaI* and *BssHII* DNA fragments were ligated and transformed into competent *E. coli* HB101. The resultant colonies were screened by restriction digestion, with confirmation of the desired clone by direct sequencing of the plasmid DNA by dideoxy sequencing using Sequenase enzyme (U.S. Biochemical) (27).

DNA transfections and generation of HIV-1. The CsCl-purified plasmids were transfected into COS-1 cells with use of DEAE-dextran (500,000 Da) as a facilitator (14). The DNA (10 μ g) was mixed with DEAE-dextran (500 μ g/ml) in 4 ml of DMEM. The COS-1 cells were washed two times with DMEM and incubated with the DEAE-dextran DNA mixture for 3 h. The medium was then removed and replaced with complete medium and chloroquine (20 μ g/ml) for an additional 3 h. The cells were then shocked with 10% dimethyl sulfoxide-DMEM for 2 to 3 min and rinsed two times before addition of complete medium.

To test for virus infectivity, the transfected cells were cocultured with SupT1 cells (10^6 cells per ml) for 4 days. At the end of this time, the nonadherent SupT1 cells were removed and cultured in a separate flask with fresh SupT1 cells (10^5 cells per ml). For reinfection, the SupT1 cells (0.5×10^6) were infected with known amounts of virus as measured by supernatant reverse transcriptase (10^6 cpm of reverse transcriptase). After 24 h, the virus was removed, and fresh medium with 0.5×10^6 SupT1 cells was added for further culture. The cells were monitored visually for the formation of multicell syncytia; at specified times, the supernatants were analyzed for reverse transcriptase activity.

Reverse transcriptase assays. Virus in the supernatant of transfected cells was pelleted by centrifugation at 40,000 rpm for 2 h in an SW41 rotor. The pellet was resuspended in 100 μ l of TEN buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA). For the reverse transcription analysis, 5 μ l of the virus pellet or 25 μ l of the infected SupT1 cell cultures

was placed in an Eppendorf tube with 15 μ l (or 75 μ l for SupT1 cultures) of a reaction cocktail containing 50 mM Tris-HCl [pH 8.0], 5 mM dithiothreitol, 5 mM MgCl₂, 150 mM KCl, 0.1% Triton X-100, 1 μ g of poly(A)-oligo(dT), and 2 μ Ci of [α -³²P]TTP. The reaction was allowed to proceed for 60 min before the addition of 100 μ l of tRNA (0.5 mg/ml), followed by precipitation with an equal volume of 20% trichloroacetic acid. The precipitate was collected on 0.45- μ m-pore-size Gelman filters and dried; radioactivity was determined in a Beckman scintillation counter, or the filters were exposed to X-ray film at -70°C with an intensifier.

Metabolic labeling of transfected cultures and immunoprecipitation. To analyze the expressed proteins from the proviral genomes, COS-1 cells were metabolically labeled at 48 h posttransfection. The cells were washed once with DMEM and then incubated in DMEM without methionine for 1 h, followed by addition of [³⁵S]methionine (100 μ Ci) at 1 mCi/ml for 2×10^5 cells for 6 h. The cells were processed for immunoprecipitation by removal of the labeling media and addition of 1 ml of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 0.025 M Tris-HCl, pH 7.0). The cellular DNA and debris were removed by centrifugation ($13,000 \times g$) for 5 min. To detect HIV-1-related proteins, the supernatants were incubated with pooled sera from five AIDS patients for 24 h at 4°C with constant rocking. The immunoprecipitates were collected by incubation with protein A-Sepharose (20 μ l of 10% [wt/vol] in RIPA) for 1 h at room temperature. The protein A-Sepharose beads were washed three times with RIPA, and the bound material was eluted from the beads by boiling in sample buffer (50 mM Tris-HCl [pH 6.8], 5% SDS, 5% β -mercaptoethanol, 0.023% bromophenol blue). The eluted proteins were separated on 10% SDS-polyacrylamide gels and then subjected to fluorography according to standard techniques. The dried gels were autoradiographed with use of Kodak X-AR film and an intensifier at -70°C.

PCR amplification and subcloning of PBS DNA. Total DNA was extracted from infected SupT1 cells by using modifications of previously described protocols (25, 26). Briefly, infected cells were pelleted by low-speed centrifugation and washed once in phosphate-buffered saline. The cells were lysed in 400 μ l of a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% SDS, and 100 μ g of proteinase K per ml. After incubation at 56°C for 3 h, the samples were transferred to microcentrifuge tubes. The samples were extracted with phenol-chloroform (1:1, vol/vol) and centrifuged, and the aqueous phase was extracted again with chloroform. Following centrifugation, the aqueous phase was adjusted to 0.3 M sodium acetate and 2.5 volumes of ethanol was added. A sealed Pasteur pipette was placed at the interface between the aqueous and ethanol phases, and the DNA was gently spooled onto the pipette until the phases were completely mixed. The pipette with the spooled DNA was gently washed with 70% ethanol, air dried, incubated in 200 μ l of TE buffer (10 mM Tris-HCl [pH 7.8], 1 mM EDTA), and incubated with RNase for 5 min at 37°C. The solution was then reextracted with phenol-chloroform and precipitated with ethanol. Following centrifugation, the pellet was resuspended in TE buffer, and the amount of nucleic acid was quantitated by the UV A₂₆₀.

Amplification of the specific DNA by PCR was performed with 10 μ g of DNA (25, 26). Two HIV-1-specific primers were used for these studies: 5'-CAAGGCTACTTCCCTG ATTGGCAGAACTAC-3' (primer 1) and 5'-CTTTGGTCCT TGTCTTATGTCTAGAATGC-3' (primer 2). Primer 1 hybridizes to the HIV-1 region at nucleotides -391 to -362 (in

the 5' long terminal repeat region), while primer 2 is specific for nucleotides 1176 to 1204. The reaction conditions were as described by the manufacturer, using 2 U of *Taq* polymerase. The thermocycling temperatures used were 94°C for 1 min, 50°C for 2 min, and 60°C for 3 min, for a total of 30 cycles. To analyze the amplified products, 1/10 of the reaction product (approximately 500 ng) was analyzed on 0.8% neutral agarose gels. The remaining sample was extracted with phenol-chloroform (1:1), and the aqueous phase was made 0.3 M sodium acetate and precipitated with 3 volumes of ethanol at -70°C. The precipitated nucleic acids were collected by centrifugation ($13,000 \times g$ for 30 min at 4°C), washed once with 70% ethanol, and dried. The pellet was resuspended in a total volume of 50 μ l, and the DNA was digested with *Xba*I and *Bam*HI for 1 h at 37°C. The restriction enzymes were inactivated by phenol-chloroform, and the nucleic acids were precipitated with 0.3 M sodium acetate and ethanol at -70°C. The DNA was pelleted, dried, and ligated into RF M13mp19 previously digested with *Bam*HI and *Xba*I, using T4 DNA ligase (1 U) at 15°C overnight. The sample was used to transform competent TG-1, and the cells were plated on LB plates with a lawn of TG-1. The identification of phage containing the inserts was facilitated by including 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 200 μ g/ml) in the agar overlay. White phage plaques, indicating putative recombinants, were grown, and single-stranded DNA was prepared for sequencing. For these studies, we used the universal M13 forward sequencing primer (New England Biolabs) with the dideoxynucleotide sequencing protocol (27). At least 10 independent white plaques were grown and sequenced from the PCR products obtained from each infected cell culture.

RESULTS

Construction of HIV-1 proviral genomes containing mutations in the PBS. Oligonucleotide site-directed DNA mutagenesis was used to create deletions in the PBS region of the HIV-1 proviral genome. The PBS of the HIV-1 genome is positioned at nucleotides 183 to 200 between the transactivation response region, splice donor, and putative RNA encapsidation sequence (reviewed in references 1, 5, and 13) (Fig. 1). Plasmid, pHXB2gpt, containing the complete infectious HIV-1 proviral genome, was used for these experiments (22). The initial mutations that were constructed resulted in one- and two-nucleotide changes to create *Xba*I and *Bam*HI restriction sites, respectively, in pHXB2gpt. In preliminary experiments, we found that these nucleotide changes did not affect the infectivity of the proviral genome (data not shown). Because of the convenient *Xba*I and *Bam*HI restriction sites flanking the PBS, we have made all subsequent mutant PBS with the *Xba*I-*Bam*HI mutant as the template DNA. For convenience, this plasmid is referred to as pHXB2PBS (wild type).

The deletions created in the PBS are summarized in Table 1. The PBS of the HIV-1 genome consists of at least 18 nucleotides which are complementary to the 3' 18 nucleotides of cellular tRNA^{Lys} (23). A proviral genome was created in which the PBS was deleted by *Xba*I and *Bam*HI digestion, fill-in, and blunt-end ligation. The resultant plasmid, pHXB2 (Δ PBS) has a deletion of nucleotides 180 to 215 encompassing the PBS region. Since the Δ PBS mutation deletes nucleotides outside of the PBS, oligonucleotide site-directed mutagenesis was used to construct exact deletions of the first 9 nucleotides [pHXB2PBS (Δ 1-9)], the second 9 nucleotides [pHXB2PBS (Δ 10-18)], and the second

FIG. 1. HIV-1 proviral genome organization and location of the PBS. (A) Organization of the HIV-1 proviral genome and locations of the various open reading frames (5). The position of the 5' nontranslated region (NTR) is noted. A restriction site for *BssHII* (nucleotide position 255) is noted; a second restriction site used in these studies for subcloning, *HpaI*, is found in the flanking cellular sequences near the 5' long terminal repeat (LTR) and is not shown. Both the *HpaI* and *BssHII* restriction sites are unique in plasmid pHXB2gpt (22, 23). The DNA fragment contained between the *HpaI*-*BssHII* sites was subcloned into RF M13mp18. (B) Expanded view of the 5' nontranslated region and locations of relevant RNA structures. The PBS is positioned at nucleotides 183 to 200, between the identified regions designated transactivation response region (TAR), splice donor (SD), and the putative encapsidation signal (Ψ) (1, 13). The translational start for the *gag* gene at nucleotide position 334 is also noted.

12 nucleotides [pHXB2PBS (Δ7-18)] of the PBS. Since previous studies have suggested the length and the positioning of the PBS region are important for reverse transcription (6), a mutant PBS, in which the first 9 nucleotides were conserved with the authentic HIV-1 PBS and the second 9 were substituted with unrelated nucleotides which conserve the 18-nucleotide spacing [pHXB2PBS Lys(1-9)], was created.

Expression of HIV-1 proteins from mutant PBS proviral genomes. To determine the effects of the mutated PBS on the expression of HIV-1 proteins, the plasmids containing the mutant proviral clones were transfected into COS-1 cells and the expression of viral proteins was analyzed by immunoprecipitation with AIDS patient sera (Fig. 2A). No significant difference was observed in the expression of *gag*-

FIG. 2. Expression of viral proteins and release of virus from COS-1 cells transfected with wild-type and mutant proviral genomes. (A) COS-1 cells were transfected with various plasmids and metabolically labeled with ³⁵S-Translabel for 6 h, followed by immunoprecipitation with sera from AIDS patients. The immunoprecipitated material was run on a 10% SDS-polyacrylamide gel and then subjected to autoradiography. The molecular size standards (left) and the relevant HIV-1 viral proteins (right) are marked. Lanes: 1, mock transfected; 2, pHXB2PBS (wild type); 3, pHXB2 (ΔPBS); 4, pHXB2PBS (Δ1-9); 5, pHXB2PBS (Δ10-18); 6, pHXB2PBS (Δ7-18); 7, pHXB2PBS Lys(1-9). (B) COS-1 cells were transfected with various plasmids; and 48 h later, the supernatant was harvested and then ultracentrifuged to pellet the virus. Reverse transcriptase activity was analyzed as described in Materials and Methods. Following the reactions, the radioactive filters were counted and exposed to X-ray film for autoradiography. Lanes: 1, mock transfected; 2, pHXB2 (Δpol) (a plasmid containing a proviral genome lacking a complete *pol* gene); 3, pHXB2PBS (wild type); 4, pHXB2 (ΔPBS); 5, pHXB2PBS (Δ1-9); 6, pHXB2PBS (Δ10-18); 7, pHXB2PBS (Δ7-18); 8, pHXB2PBS Lys(1-9). The numbers reflect the individual radioactivity of the filters in counts per minute.

related proteins (p55, p41, and p24/25) and *env* (gp160) from that of the proteins detected from COS-1 cells transfected with pHXB2PBS (wild type).

The supernatants of transfected COS-1 cells were analyzed for release of virus by using ultracentrifugation, followed by analysis of reverse transcriptase activity (Fig. 2B). For controls, supernatants from mock-transfected cells as well as cells transfected with a plasmid containing the proviral HIV-1 genome in which regions of the *pol* gene (nucleotides 2165 to 4097) had been deleted were analyzed; preliminary studies demonstrated this deletion did not sig-

TABLE 1. Mutations in the PBS region of the HIV-1 genome

Nucleotide location ^a	PBS DNA sequence	Resultant PBS
181-201	AG TGG CGC CCG AAC AGG GAC ---TTG AAA ^b	Wild-type PBS (PBS underlined)
180-215	AG (delete 180-215) GAT CC	Delete PBS (ΔPBS)
183-191	AG (delete 183-191) AAC AGG GAC ---TTG AAA	Delete first 9 nucleotides of PBS (Δ1-9)
192-200	AG TGG CGC CCG (delete 192-200) ---TTG AAA	Delete second 9 nucleotides of PBS (Δ10-18)
189-200	AG TGG CGC (delete 189-200) -----TTG AAA	Delete second 12 nucleotides of PBS (Δ7-18)
183-200	AG TGG CGC CCG /TGG TGC GAA---TTG AAA	PBS of tRNA ^{Lys} (1-9)

^a Numbering as in reference 23.

^b Dashes are used for spacing only and do not reflect nucleotide additions or deletions.

nificantly affect the release of viruslike structures (data not shown). The levels of reverse transcriptase detected were similar between the cells transfected with pHXB2PBS (wild type) and those transfected with plasmids containing the mutant PBS in the HIV-1 proviral genome. The minor variations observed were due to differences in transfection efficiencies and were not consistently observed during repeat independent transfections (data not shown).

Infectivity of proviral genomes containing mutant PBS. To determine viral infectivity, COS-1 cells were transfected with plasmids containing the complete proviral genomes with wild-type or mutant PBS, followed by coculture with SupT1 cells. In this system, the transfection of the COS-1 cells with plasmids containing the wild-type or mutant proviral genomes results in the production of virus. Since the COS-1 cells cannot be infected with HIV-1, this assay requires the spread of virus to the SupT1 cells which have the CD4 receptor and support high level of HIV-1 replication. Following coculture for 4 days, the SupT1 cells, which grow as suspension cells, were removed and cultured for up to 3 weeks with addition of medium every 4 to 5 days. The viral infectivity was monitored by visual inspection for syncytium formation and quantitated by reverse transcriptase activity released into the supernatant (Fig. 3A). The production of infectious virus from the transfection of pHXB2PBS (wild type) and subsequent spread of the virus to SupT1 cells during the coculture period accounts for the initial (day 0) high level of supernatant reverse transcriptase activity. The replication of the wild-type virus proceeds at a rapid rate such that after 5 days of culture (i.e., 5 days after separation of the SupT1 cells from the transfected COS-1 cells), the entire culture of SupT1 cells consists mainly of large multinucleated giant cell (syncytia) with high levels of reverse transcriptase in the supernatant. In contrast, no infectious virus was detected from transfection of pHXB2 (Δ PBS) or pHXB2PBS (Δ 1-9). Infectious virus was produced following transfection of pHXB2PBS (Δ 10-18) and pHXB2PBS (Δ 7-18), although there was a substantial delay in the appearance of infectious virus. The transfection of pHXB2PBS Lys(1-9) also resulted in the production of infectious virus, with delayed kinetics of appearance compared with that obtained from pHXB2PBS (wild type) (Fig. 3B). These results are representative of three independent transfections.

The reason why no infectious virus was obtained following the transfection of certain proviral genomes may be that the PBS mutation affected the encapsidation of the genomic RNA. Although the putative encapsidation signal has been identified (1, 13), and the PBS mutations do not overlap this region, the actual mechanism of RNA encapsidation is unknown. Therefore, we analyzed the virus pellets obtained from ultracentrifugation of the supernatants of COS-1 cells transfected with the appropriate plasmids for virion RNA. Following DNase treatment to remove any contaminating plasmid DNA, a dot blot analysis was used with radiolabeled probes specific for virion RNA. Comparable levels of virion RNA were detected in the virus pellets derived from transfection of COS-1 cells with pHXB2PBS (wild type), pHXB2 (Δ PBS), and pHXB2PBS (Δ 1-9) (data not shown). Thus, the encapsidation of the genomic RNA was not affected by mutations in the PBS region.

Analysis of the PBS region of infectious viruses. All of the viruses generated from transfection of pHXB2PBS (Δ 10-18), pHXB2PBS (Δ 7-18), and pHXB2PBS Lys(1-9) exhibited delayed kinetics of appearance compared with virus derived from transfection of pHXB2PBS (wild type). However, the

FIG. 3. Kinetics of appearance and replication kinetics of infectious virus derived from transfection of wild-type and mutant proviral genomes. The plasmids containing the wild-type and mutant proviral genomes were transfected into COS-1 cells and then by cocultured with SupT1 cells for an additional 4 days. The nonadherent SupT1 cells were removed and cultured with additional fresh medium and uninfected SupT1 cells (day 0). The supernatants were assayed for reverse transcriptase as described in Materials and Methods at sequential time periods of culture. (A) Kinetics of appearance of virus derived from cells transfected with the proviral genomes containing wild-type or deleted PBS. The cultures were assayed until the supernatant reverse transcriptase peaked at approximately 100,000 cpm, at which time the infected cell samples were used for PCR amplification of the PBS of integrated proviral genomes. (B) Kinetics of appearance of viruses derived from transfection of HIV-1 proviral genomes containing the Lys(1-9) PBS region. (C) Replication kinetics of viruses derived from transfection of wild-type and mutant proviral genomes containing the Δ 10-18 and Δ 7-18 mutant PBS. SupT1 cells were infected with equal amounts of cell free virus as described in Materials and Methods. After 24 h, the initial virus-containing medium was removed, and new medium with SupT1 cells was added to the cultures. Supernatants were removed and assayed for reverse transcriptase activity. The values for reverse transcriptase activity are for 25 μ l of culture supernatant.

TABLE 2. Sequence analysis of the PBS of HIV-1 proviruses

SAMPLE	PBS SEQUENCE	FREQUENCY ^a
vHXB2PBS (wild type)^b		
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----TTG AAA GCG AAA G ^c (WILD TYPE)	9/11
	AAG <u>TGG CGC CCG AAC GGG GAC</u> -----TTG AAA GCG AAA G	1/11
	AAG <u>TGG TGC CCG AAC AGG GAC</u> -----TTG AAA GCG AAA G	1/11
Mutant Sequence^d		
AAG TGG CGC CCG (delete 192-200) TTG AAA GCG AAA G		
vHXB2PBS (Δ 10-18)		
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----GCG AAA G (Δ TTG AAA) ^e	8/12
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----ACG AAA G (Δ TTG AAA)	1/12
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----A G (Δ TTG AAA GCG AA)	1/12
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----G AAA G (Δ TTG AAA GC)	1/12
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----GTG AAA GCG AAA G	1/12
Mutant Sequence		
AAG TGG CGC (delete 189-200)-----TTG AAA GCG AAA G		
vHXB2PBS (Δ 7-18)		
	AAG <u>TGG CGC CCG AAC AGG GAC</u> -----A G (Δ TTG AAA GCG AA)	10/10
Mutant Sequence		
AAG <u>TGG CGC CCG</u> TGG TGC GAA TTG AAA GCG AAA G (Lys)		
vHXB2PBS Lys(1-9)		
	AAG <u>TGG CGC CCG AAC AGG GAC</u> TTG AAA GCG AAA G (WILD TYPE)	2/10
	AAG <u>TGG CGC CCG AAC AGG GAC</u> GAA TTG AAA GCG AAA G (GAA INSERTION)	5/10
	AA A TGG CGC CCG AAC AGG GAC TTG AAA GCG AAA G	1/10
	AAG <u>TGG CGT</u> CAC AAC AGG GAC GAA TTG AAA GCG AAA G (GAA INSERTION)	1/10
	AAG <u>TGG CGC CC- AAC</u> GT-GG GAC GAA TTG AAA GCG AAA G (G and A deletion; GT and GAA INSERTION)	1/10

^a DNA sequences of the PBS region obtained from independent M13 phage clones.^b Designates the PBS regions PCR amplified and sequenced from genomic DNA isolated from SupT1 cells infected with viruses derived from the transfection of the corresponding plasmids (e.g., pHXB2PBS (wild type)).^c Dashes are used for spacing only and do not reflect nucleotide deletions or insertions.^d The mutant sequence refers to the initial mutation in the PBS region.^e Δ designates deletion of the following nucleotides from the wild-type sequence.

final amounts of virus, as measured by supernatant reverse transcriptase and visual inspection of the cultures for syncytia, were similar. Thus, it was possible that reversions or second-site mutations had occurred during the culture period to compensate for the deletions in the PBS region. To further explore this possibility, we utilized PCR to amplify the regions of the PBS from integrated HIV-1 proviral DNA obtained from infected SupT1 cultures described in the previous section. Our rationale for analyzing the high-molecular-weight DNA from the infected SupT1 cells reflects the fact that only those viruses which have undergone a successful reverse transcription will be integrated into the chromosome. The amplified DNA was isolated and subcloned into the similarly digested RF M13mp19 as described in Materials and Methods. Individual phage plaques were identified, and the DNA sequence in the region correspond-

ing to the PBS was determined (Table 2). Since the PBS regions of integrated proviruses obtained from highly infected cultures were analyzed, we believe that the DNA sequence of each PBS obtained in the M13 phage plaques accurately reflects the PBS population present in the infectious viruses. In support of this notion, we found that 9 of 11 PBS sequenced from the proviruses of cultures infected with vHXB2PBS (wild type) (i.e., cultures infected with viruses derived originally from the transfection the corresponding plasmid, pHXB2PBS [wild type]), were the wild-type sequence, and 2 of the 11 had sequence changes consisting of an A-to-G or C-to-T conversion. At present, we are uncertain of the significance of these mutations with relation to the viability of these proviruses.

The sequence analysis of the PBS region from the proviruses derived from vHXB2PBS (Δ10-18), vHXB2PBS (Δ7-

18), and vHXB2PBS Lys(1-9) were different in several respects. First, all of the regions PCR amplified and sequenced contained the complete intact 18-nucleotide wild-type PBS. Second, most of the regions analyzed contained significant nucleotide changes 3' to the PBS. For example, 11 of the 12 DNAs from the proviruses of cultures infected with vHXB2PBS (Δ 10-18) contained nucleotide deletions 3' to the PBS ranging from 6 nucleotides (8 of 12) to 11 nucleotides. This clustering of nucleotide deletions was also evident from the analysis of proviruses from the cultures infected with vHXB2PBS (Δ 7-18), in which the only 3' PBS region contained deletions of 11 nucleotides.

The sequence of the PBS regions from the PCR-amplified DNA from the proviruses derived from vHXB2PBS Lys(1-9) presented a different pattern. Two of the ten regions sequenced contained PBS regions identical to the wild-type sequence. More interestingly, 7 of 10 contained a 3-nucleotide GAA insertion immediately after the 18-nucleotide PBS. One of the DNAs contained a G-to-A change 5' to the start of the PBS. In addition, 2 of the 10 PBS had significant nucleotide deletions and insertions within the PBS; in the first, a C-to-T change and C-to-A change were found, while the second had two deletions (G and A) with a GT insertion in the predicted wild-type PBS sequence.

Although viruses vHXB2PBS (Δ 10-18), vHXB2PBS (Δ 7-18), and vHXB2PBS Lys(1-9) demonstrated delayed kinetics of appearance compared with the wild type, the final levels of virus as determined by reverse transcriptase were similar. To further characterize the revertant viruses, we compared the replication kinetics with that of the wild-type virus. For these studies, SupT1 cells were infected with equal amounts of virus as measured by reverse transcriptase, and the subsequent virus replication was monitored by the appearance of supernatant reverse transcriptase (Fig. 3C). No difference was apparent between the replication kinetics of vHXB2PBS (Δ 10-18), vHXB2PBS (Δ 7-18), and wild-type virus as measured by supernatant reverse transcriptase and by visual inspection for multinucleate giant cell syncytia. In separate experiments, no difference in replication kinetics was observed between vHXB2PBS Lys(1-9) and wild-type virus (data not shown).

To further characterize the revertant viruses, we analyzed the PBS regions from the integrated proviruses obtained after reinfection by using PCR followed by subcloning into M13mp19. The DNA sequence from vHXB2PBS (Δ 10-18) contained one the 6-bp deletion (Δ TTG AAA) found in 8 of the 12 PBS analyzed in the original sample (Table 2). The PBS region from proviruses obtained from reinfection with vHXB2PBS (Δ 7-18) all contained the 11-bp deletion (Δ TTG AAA GCG AA). In total, six PBS for each preparation were analyzed (data not shown). Taken together, this further characterization demonstrates the revertant viruses with the deletions 3' to the PBS are stable and have replication kinetics similar to that of the wild-type virus.

DISCUSSION

In this report, we have described the construction and characterization of HIV-1 proviral genomes with defined mutations in the PBS. Transfection of the plasmids containing these proviral genomes into COS-1 cells resulted in production of virus, as determined by immunoprecipitation of intracellular viral proteins and virus-associated reverse transcriptase. Infectious virus was generated from transfection with plasmids containing HIV-1 proviral genomes which had at least the first six nucleotides of the PBS. To further

characterize the nature of the PBS of the infectious viruses, this region was amplified from integrated proviruses by PCR and subcloned into M13mp19, and the DNA sequence was determined for individual M13 phage clones. This analysis revealed that the entire 18-nucleotide PBS was regenerated and that significant nucleotide deletions and insertions were found 3' to the PBS. Furthermore, the revertant viruses demonstrated replication kinetics similar to that of the wild type upon reinfection and had stable deletions 3' to the PBS.

The results of this study provide several insights into the initiation of HIV-1 reverse transcription. Recent studies by Whitcomb et al. have suggested that HIV-1 is typical of most retroviruses in that reverse transcription initiates with a dC residue, which is the first nucleotide following the CCA triplet at the end of the tRNA (35). These studies are entirely consistent with our results in which no infectious virus was recovered upon transfection of the plasmid containing a proviral genome with the deletion of the first nine nucleotides of the PBS [i.e., PBS (Δ 1-9)]. As demonstrated by our experiments, this mutation in the PBS does not affect the expression of viral proteins or encapsidation of genomic RNA. We believe that the PBS (Δ 1-9) mutant is blocked at the initiation step of reverse transcription. That is, during reverse transcription, the 3' nine nucleotides of tRNA^{Lys} would not base pair with the mutant PBS lacking the first nine nucleotides. The complementarity between the second nine nucleotides of the tRNA and the PBS might be enough to facilitate the interaction between the tRNA and the PBS, but there appears to be no mechanism by which the virus can remove the non-base-paired tRNA (i.e., the 3' nine nucleotides) to initiate reverse transcription. In contrast, the copia retroviruslike particles of *Drosophila melanogaster* uses nucleotides 25 to 39 of tRNA^{Met} as a primer rather than the 3'-terminal nucleotides (11). The cleavage of the remaining nucleotides of tRNA^{Met} is postulated to be accomplished by a 3'-5' exoribonuclease, possibly by RNase activities mediated by a cellular RNase P-like enzyme (12).

The transfection of pHXB2PBS (Δ 10-18), pHXB2PBS (Δ 7-18), and pHXB2PBS Lys(1-9) resulted in production of infectious viruses. We then used PCR to amplify the PBS region of the integrated proviruses from the highly infected cultures. In a similar experimental approach, several recent studies have used PCR to amplify and analyze the DNA sequence of the long terminal repeat circle junction sequences (9, 28, 35). In several of the DNA sequences examined in these studies, nucleotide substitutions, deletions, and insertions were described and attributed to errors in HIV-1 replication. Given the low error rate observed for *Taq* polymerase (32), coupled with the significantly higher error rate of reverse transcription (24), it is reasonable to conclude that most of the single-base changes observed in the PBS analyzed in our studies are also the result of errors in replication. This hypothesis is supported by recent studies which have fully characterized mutations, including deletions and insertions, that occurred during a single replication cycle of spleen necrosis virus (18, 19). Although we do not know whether all of the PBS regions amplified are actually derived from infectious proviruses or defective genomes, we believe that the viruses must have gone through at least one, and possibly multiple, rounds of viral replication. In support of this view, further analysis of vHXB2PBS (Δ 10-18) and vHXB2PBS (Δ 7-18) by reinfection revealed that these viruses maintained deletions of 6 and 11 nucleotides, respectively, 3' to the PBS and demonstrated replication kinetics similar to that of the wild-type virus. Thus, even with the 3'

FIG. 4. Predicted base pairings at the elongation step of plus-strand DNA synthesis to account for the observed DNA sequences 3' to the PBS. (A) Proposed step in reverse transcription at which the nucleotide deletions and insertions occur in the proviral genomes containing the mutated PBS. The convention used for labeling the steps in reverse transcription were as described by Gilboa et al. (8). Lowercase letters indicate the minus-strand cDNA; the tRNA^{Lys} primer attached to the initial minus-strand cDNA is the starting point corresponding to step V. The subsequent synthesis of plus-strand strong-stop DNA (uppercase letters) and regeneration of the PBS by copying of the tRNA primer are depicted. The region referred to in panels B to E (c and C) is boxed. (B) Proposed base pairings required for successful reverse transcription by the wild-type genome. c, minus-strand cDNA; C, the result of synthesis of plus-strand strong-stop DNA. It is important to note that in the first reverse transcription of the viral genome during the initial infection of SupT1 cells, the minus-strand cDNA (c) will contain the PBS with the mutations, while the plus-strand strong-stop DNA (C) will consist of the complete PBS derived from copying of the cellular tRNA primer. (C) Proposed base pairings during the first reverse transcription from the genome derived from transfection of pHXB2PBS (Δ10-18) to account for the deletions observed in 8 of 12 of the regions 3' to the PBS. The boxed nucleotides below the arrow designate the starting point to complete the plus-strand DNA during reverse transcription. The completed bottom strand (C) will have the 18-nucleotide PBS followed by boxed nucleotides, which account for the DNA sequence of the PBS region in Table 2. (D) Proposed base pairings during the first reverse transcription to account for deletions observed in the 3' region to the PBS in all of the viruses obtained from transfection of pHXB2PBS (Δ7-18). (E) Proposed base pairings to account for GAA insertion observed in 7 of the 10 PBS regions from viruses derived from transfection of pHXB2PBS Lys(1-9).

deletions, the revertant viruses undergo efficient reverse transcription and replication.

An important feature of nearly all of the PBS regions analyzed was the presence of the intact 18-nucleotide PBS. In all models for reverse transcription, the PBS is regenerated when the reverse transcriptase copies the cellular tRNA primer during the synthesis of strong-stop plus-strand DNA (8, 10, 17, 30, 33) (Fig. 4A). Our results are significant because they support this aspect of the model. Furthermore, our results support the idea that the inclusion of the tRNA primer into the HIV-1 virion most probably occurs by specific interaction with the viral *gag* or *pol* gene product, as suggested by previous studies which demonstrated that the reverse transcriptase of HIV-1 specifically interacts with tRNA^{Lys} (2, 4). The reverse transcriptase has also been shown to be the major determinant for packaging of the cellular tRNA primer used in the replication of avian myeloblastosis virus (20).

We believe that the deletions in the region 3' to the PBS in the proviruses derived from infection with vHXB2PBS (Δ10-18) and vHXB2PBS (Δ7-18) occurred at the synthesis of the complementary plus-strand DNA step in reverse transcription during the initial spread of the infection from the COS-1 to the SupT1 cells. This can be visualized from inspection of the proposed model for the second jump in reverse transcription. This step is facilitated by the complementarity of the 3' end of the minus-strand viral cDNA with the PBS region as shown for the wild type HIV-1 genome (Fig. 4B). A series of hypothetical base pairs required for this step of reverse transcription is presented to explain the deletions and insertions observed in the PBS regions. The base pair homologies predicted between the proviral genomes containing the PBS with deletions of nucleotides 10 to 18 at this step in reverse transcription are depicted in Fig. 4C. The first nine nucleotides of the c-C region will match perfectly between the plus-strand strong-stop DNA and the 3' cDNA. However,

the plus-strand strong-stop DNA (C) will contain the second nine nucleotides corresponding to those complementary to tRNA^{Lys}, while the cDNA strands will contain nucleotides complementary to the original PBS with the deleted nucleotides from 10 to 18 (c). Completion of the reverse transcription reaction would be accomplished if partial regions of base pair homology could be established; we believe that *gag* (nucleocapsid) and possibly the reverse transcriptase, which have been demonstrated to be required for annealing of the tRNA to the viral genome (4), facilitate this step. The reverse transcriptase then continues the replication process to complete the synthesis of double-stranded HIV-1 proviral DNA. As depicted in the model, the bottom-strand DNA (C) would contain the complete 18-nucleotide PBS followed by nucleotides GCG AAA G, (and subsequent deletion of the AACTTT nucleotides), which was observed in our sequence analysis 8 out of 12 times. Following DNA repair by the host cell, a complete integrated proviral genome in the SupT1 cells would have the 18 nucleotides of the PBS containing the observed 3' deletions. A similar model is presented to account for the deletions observed for the PBS sequence from the analysis of viruses obtained from transfection of pHXB2PBS (Δ 7-18) (Fig. 4D). In this case, the degree of homology extending to 7 of 9 nucleotides can be predicted to account for the 11-nucleotide deletion observed in 10 of 10 of the PBS regions sequenced. The majority of the PBS region amplified and sequenced from the proviruses obtained from infection with vHXB2PBS Lys(1-9) contained a three-nucleotide insertion, GAA, observed immediately 3' to the PBS. A possible mechanism by which this insertion is generated involves three G · C base pair homologies, which includes a G · C pair immediately prior to the start of the reverse transcriptase (Fig. 4E). The elongation of the plus-strand strong-stop (C) DNA would proceed with GAA TTG AAA . . . to account for the observed GAA insertion. For generation of the wild-type PBS region found in 2 of 10 cases, two G · C and one A · U homology can also be predicted in this region (not shown). Finally, this model would predict that positioning of the complementary base pairs at the site immediately before extension of the plus-strand strong-stop (C) DNA might facilitate the completion of the reverse transcription reaction. Experiments are under way to test this hypothesis by generating further mutations in the PBS of HIV-1.

ACKNOWLEDGMENTS

We thank Etty Benveniste, Eric Hunter, Sandra Jablonski, Weon Sang Choi, and John Wakefield for reading the manuscript and for helpful suggestions. We thank Etty Benveniste and Il Yup Chung for assistance with PCR. We thank B. Hahn for plasmids and some of the PCR primers used in this study and Debbie Morrison for preparation of the manuscript.

The HIV virus culture was carried out in the UAB Center for AIDS Research Central Virus Core Facility, supported by center core grant AI-27767. This research was supported by Public Health Service grant AI27290 from the National Institutes of Health (C.D.M.).

REFERENCES

1. Aldovini, A., and R. A. Young. 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging results in production of noninfectious virus. *J. Virol.* **64**:1920-1926.
2. Araya, A., L. Sarih, and S. Litvak. 1979. Reverse transcriptase mediated binding of primer tRNA to the viral genome. *Nucleic Acids Res.* **6**:3831-3843.
3. Baltimore, D. 1970. RNA-dependent DNA polymerase in viruses of RNA tumor viruses. *Nature (London)* **226**:1209-1211.
4. Barat, C., V. Lullien, O. Schatz, G. Keith, M. T. Nugeyre, F. Gruninger-Leitch, F. Barre-Sinoussi, S. F. J. LeGrice, and J. L. Darlix. 1989. HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *EMBO J.* **8**:3279-3285.
5. Cann, A. J., and J. Karn. 1989. Molecular biology of HIV: new insights into the virus life-cycle. *AIDS* **3**(Suppl):S19-S34.
6. Cobrinik, D., L. Soskey, and J. Leis. 1988. A retroviral RNA secondary structure required for efficient initiation of reverse transcription. *J. Virol.* **62**:3622-3630.
7. Colicelli, J., and S. P. Goff. 1986. Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. *J. Virol.* **57**:37-45.
8. Gilboa, E., S. W. Mitra, S. Goff, and D. Baltimore. 1979. A detailed model of reverse transcription and tests of crucial aspects. *Cell* **18**:93-100.
9. Hong, T., K. Drlica, A. Pinter, and E. Murphy. 1990. Circular DNA of human immunodeficiency virus: analysis of circle junction nucleotide sequences. *J. Virol.* **65**:551-555.
10. Hu, W.-S., and H. M. Temin. 1990. Retroviral recombination and reverse transcription. *Science* **250**:1227-1233.
11. Kikuchi, Y., Y. Ando, and T. Shiba. 1986. Unusual priming mechanism of RNA-directed DNA synthesis in *copia* retrovirus-like particles in *Drosophila*. *Nature (London)* **323**:824-826.
12. Kikuchi, Y., N. Sasaki, and Y. Ando-Yamagami. 1990. Cleavage of tRNA within the mature tRNA sequence by the catalytic RNA of RNase P: implication for the formation of the primer tRNA fragment for reverse transcription in *copia* retrovirus-like particles. *Proc. Natl. Acad. Sci. USA* **87**:8105-8109.
13. Lever, A., H. Gottlinger, W. Haseltine, and J. Sodrosky. 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63**:4085-4087.
14. Lopata, M., D. Cleveland, and B. Sollner-Webb. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**:5707-5717.
15. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20-78.
17. Panganiban, A. T., and D. Fiore. 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. *Science* **241**:1064-1069.
18. Pathak, V. K., and H. M. Temin. 1990. Broad spectrum of *in vivo* forward mutations, hypermutations and mutational hot-spots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. *Proc. Natl. Acad. Sci. USA* **87**:6019-6023.
19. Pathak, V. K., and H. M. Temin. 1990. Broad spectrum of *in vivo* forward mutations, hypermutations and mutational hot-spots in a retroviral shuttle vector after a single replication cycle: deletions with insertions. *Proc. Natl. Acad. Sci. USA* **87**:6024-6028.
20. Peters, G. G., and J. Hu. 1980. Reverse transcriptase as the major determinant for selective packaging of tRNA's into avian sarcoma virus particles. *J. Virol.* **36**:692-700.
21. Pullen, K. A., and J. J. Champoux. 1990. Plus-strand origin for human immunodeficiency virus type 1: implications for integration. *J. Virol.* **64**:6274-6277.
22. Ratner, L., A. Fisher, L. Linda, J. Agodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal. 1987. Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**:57-69.
23. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**:277-284.

24. Roberts, J. D., K. Bebenek, and T. A. Kunkel. 1988. The accuracy of reverse transcription from HIV-1. *Science* **242**: 1171–1173.
25. Saiki, R., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–491.
26. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350–1354.
27. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
28. Smith, J. S., S. Kim, and M. J. Roth. 1990. Analysis of long terminal repeat circle junctions of human immunodeficiency virus type 1. *J. Virol.* **64**:6286–6290.
29. Taylor, J. M. 1977. An analysis of the role of tRNA species as primers for transcription into DNA of RNA tumor virus genomes. *Biochim. Biophys. Acta* **473**:57–71.
30. Temin, H. 1981. Structure, variation and synthesis of retrovirus long terminal repeat. *Cell* **27**:1–3.
31. Temin, H. M., and S. Mizutani. 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. *Nature (London)* **226**:1211–1213.
32. Tindall, K. R., and T. A. Kunkel. 1981. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27**:6008–6013.
33. Varmus, H. 1982. Form and function of retroviral proviruses. *Science* **216**:812–820.
34. Varmus, H., and R. Swanstrom. 1982. Replication of retroviruses, p. 369–512. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *Molecular biology of tumor viruses. RNA tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. Whitcomb, J. M., R. Kumar, and S. H. Hughes. 1990. Sequence of the circle junction of human immunodeficiency virus type 1: implications for reverse transcription and integration. *J. Virol.* **64**:4903–4906.
36. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
37. Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* **100**:468–500.
38. Zoller, M. J., and M. Smith. 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA* **3**:479–488.