Minimal Sequence Requirements of a Functional Human Immunodeficiency Virus Type 1 Primer Binding Site

JOHN K. WAKEFIELD, HYANGSHUK RHIM, AND CASEY D. MORROW*

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

Received 27 September 1993/Accepted 9 December 1993

The initiation of human immunodeficiency virus type 1 (HIV-1) reverse transcription occurs by the extension of a tRNA₃^{Lys} 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 sequence of the HIV-1 genome which is complementary to the 3'-terminal 18 nucleotides of the tRNA₃^{Lys}. To investigate the sequence specificity of the interaction between tRNA₃^{Lys} and the PBS, we have constructed proviral genomes containing mutations in the PBS region. A mutant PBS was constructed in which the 18 nucleotides complementary to tRNA₃^{Lys} were substituted with 18 nucleotides predicted to be complementary to the 3'-terminal bases of a tRNA^{Phe} molecule [pHXB2PBS(phe)]. A second proviral genome was constructed in which the PBS complementary to tRNA^{Phe} was changed such that the first six nucleotides correspond to the wild-type PBS [pHXB2PBS(pheC)]. In all models of reverse transcription, the complementarity between the minus- and plus-strand PBS DNA facilitates the template switch and elongation of plus-strand DNA, resulting in a complete proviral genome. To test this model, we have inserted a five-nucleotide sequence 6 bp 3' of the mutant PBSs, which corresponds to the last five nucleotides of the wild-type PBSs [pHXB2PBS(phe+5) and pHXB2PBS(pheC+5)]. Transfection of plasmids containing the wild-type or mutant proviral genomes into COS-1 cells resulted in similar levels of intracellular expression of HIV-1 *gag* and *env* gene products as determined by immunoprecipitation with sera from AIDS patients and release of virus as determined by p24 assay. Transfection of pHXB2PBS(phe) or pHXB2PBS(phe+5) did not result in the production of infectious virus, while replication-competent viruses from cells transfected with pHXB2PBS(pheC) were detected very infrequently. Transfection of pHXB2PBS(pheC+5), however, consistently resulted in the production of infectious virus, although the appearance of the virus was delayed compared with those from cells transfected with pHXB2(wild type). Reinfection of SupT1 cells with equal amounts of p24 antigen resulted in similar kinetics of replication. PCR was used to amplify the PBS, and individual DNA products were subcloned into M13mp18. Sequence analysis of the PBS region of integrated proviruses derived from transfection of pHXB2PBS(pheC+5) revealed that the 18-nucleotide PBS complementary to tRNA₃^{Lys} was regenerated with a deletion of 6 bp 3' to the PBS region in all phage clones examined. These results demonstrate that a minimum of six nucleotides in the PBS are required for the initiation of reverse transcription and that complementarity between the minus-strand DNA and plus-strand DNA PBS facilitates the completion of reverse transcription of the viral genome.

The process by which the RNA genome of a retrovirus is converted to DNA is known as reverse transcription. The virally encoded reverse transcriptase initiates DNA synthesis by extension of the 3'-OH of a tRNA molecule which is bound to an 18-nucleotide sequence designated the primer binding site (PBS) (3, 29, 32, 44). The PBS, which is located near the 5' end of the viral RNA genome, is complementary to the 3'-terminal 18 nucleotides of the primer tRNA (14, 43, 46). Even though the interaction of the tRNA primer with the PBS is an essential step in the initiation of reverse transcription, the details regarding the mechanism by which the tRNA primer is positioned at the PBS are unknown. Although complementarity of the PBS with the 3'-terminal 18 nucleotides of the tRNA facilitates the interaction between the PBS and the tRNA primer, previous studies have suggested that this interaction is probably more complex. The 5' ends of several retroviral genomes encompassing the PBS have been predicted by computer modeling to possess extensive RNA secondary structure (11, 13). Stem-loop structures involving the U5, PBS, and leader sequences of the RNA genome have been proposed to

be required for efficient initiation of reverse transcription (10, 11). On the basis of recent studies, it has been suggested that interactions between the U5 region of the viral RNA genome and the T Ψ C loop of the tRNA primer (1), as well as interactions between the anticodon loop and sequences 3' to the PBS (19), may also be important in the initiation of reverse transcription.

A great deal of diversity exists among retroviruses with respect to the cellular tRNA used for initiation of reverse transcription (12, 18, 41). Human immunodeficiency virus type 1 (HIV-1) utilizes a tRNA₃^{Lys} molecule positioned at nucleotides 183 to 200 of the RNA genome to prime reverse transcription (35, 36). In a previous study focusing on the sequence requirements of the HIV-1 PBS, we demonstrated that only the first six nucleotides of the PBS (5'-UGGCGC) were sufficient to initiate reverse transcription (37). This suggested that the first six nucleotides of the PBS provided enough complementarity with the 3'-terminal nucleotides of the tRNA₃^{Lys} primer to promote initiation of reverse transcription.

Following initiation, the reverse transcriptase copies the 5' end of the viral RNA genome, generating minus-strand strong-stop cDNA. The minus-strand strong-stop cDNA translocates to the 3' end of the RNA genome via complementary binding of homologous terminal repeat sequences. This first template

* Corresponding author. Mailing address: Department of Microbiology, University of Alabama at Birmingham, 619 Lyons-Harrison Research Building, 1900 Seventh Ave. South, Birmingham, AL 35294. Phone: (205) 934-5705. Fax: (205) 934-1580.

transfer event allows synthesis of near full-length minus-strand viral cDNA, which includes a copy of the PBS at the 3' end (minus-strand PBS) (14, 15, 31). Incomplete degradation at the polypurine tract of the RNA genome by RNase H provides the RNA primer that initiates plus-strand strong-stop DNA synthesis (9, 16, 25, 34). In all models of reverse transcription, the plus-strand DNA copy of the PBS is regenerated when the reverse transcriptase copies the first 18 nucleotides of the still-attached tRNA primer during plus-strand strong-stop DNA synthesis (14, 31, 46). Complementarity between the plus- and minus-strand copies of the PBS sequence facilitates the transfer of the plus-strand strong-stop DNA to the 3' end of the minus-strand viral cDNA (depicted in Fig. 5A). Subsequent synthesis of the plus- and minus-strand DNAs by reverse transcriptase yields a double-stranded proviral DNA suitable for integration into the host cell chromosome (45, 46). In a previous study, we suggested that mutations within the PBS reduced the efficiency of the transfer of the plus-strand strong-stop DNA to the 3' end of the minus strand (37). Sequence analysis of the PBS region from infectious proviruses isolated after prolonged culture of viruses derived from transfection of proviruses containing truncated PBSs revealed that the entire 18-nucleotide PBS was regenerated and that stable deletions were found 3' to the PBS. We proposed a model in which the deletions 3' to the PBS resulted from the incomplete homology between the PBS sequences in the minus- and plus-strand DNAs (37). We suggested that the positioning in the minus-strand DNA of base pairs complementary to the plus-strand PBS might facilitate the second template transfer leading to the completion of proviral DNA.

In the present study, we have further defined the minimal sequences required of the PBS to function in HIV-1 reverse transcription. A mutant PBS was constructed in which the 18 nucleotides complementary to tRNA_{Lys} were substituted with 18 nucleotides predicted to be complementary to the 3'-terminal bases of a tRNA^{Phe} molecule. Transfection of this construct did not result in the production of an infectious virus, reflecting the inability of HIV-1 to utilize a PBS complementary to tRNA^{Phe}. Additional mutations were constructed to convert this nonfunctional PBS complementary to tRNA^{Phe} into a functional PBS. On the basis of these results, we suggest that the first six nucleotides of the HIV-1 PBS are sufficient for initiation of reverse transcription, and, consistent with our previous proposition, positioning of complementary nucleotides at the 3' end of the PBS facilitates the second template transfer step, resulting in synthesis of a complete infectious proviral DNA.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma Chemical Company 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. Biochemicals. The radioactive nucleotides were purchased from Amersham, and [³⁵S]Translabel was obtained from ICN. Tissue culture media and reagents were obtained from GIBCO BRL. The synthetic DNA oligonucleotides used for mutagenesis were prepared by the Comprehensive Cancer Center Oligonucleotide Synthesis Facility at the University of Alabama at Birmingham.

Tissue culture. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and G-MSG (GIBCO) (1×) at 37°C in a 5% CO₂

atmosphere. SupT1 cells were grown in RPMI medium supplemented with 10% fetal bovine serum.

DNA manipulation and mutagenesis. General laboratory procedures were followed for DNA manipulation, plasmid preparation, and subcloning essentially as described previously (26). Single-stranded DNA of M13mp18PBS used for oligonucleotide site-directed mutagenesis was prepared as described previously (37). M13mp18PBS contains HIV-1 proviral sequences between the *Hpa*I and *Pst*I sites of pHXB2gpt, including the PBS region (37). DNA oligomers used to create the desired mutations in the HIV-1 PBS region were as follows: (i) pHXB2PBS(phe), TTTCGCTTTTCGATCCACAG AATTCGCACCACTTCTA; (ii) pHXB2PBS(phe+5), CCCC TTTCGCGTCCCTTTTCGATCCACAG; (iii) pHXB2PBS (pheC) AGAATTCGCGCCACTTCTA. Note that the PBS mutation pHXB2PBS(pheC+5) was constructed by using the mutagenic primer for pHXB2PBS(phe+5) on single-stranded M13mp18 phage DNA containing the *pheC* mutation. Screening and subsequent identification of the M13 plaques containing the desired mutations were performed with Sanger dideoxy DNA sequencing (40).

Each mutant PBS was subcloned into pHXB2gpt, a plasmid encoding the entire HIV-1 provirus flanked by unique *Hpa*I (5') and *Xba*I (3') restriction sites (37). For the purpose of clarity, this plasmid will be referred to as pHXB2(wild type). Briefly, M13mp18PBS replicative-form DNA containing the PBS mutations was isolated and digested with *Hpa*I and *Bss*HI, resulting in an 868-bp fragment encompassing the PBS region. The 868-bp fragment was subcloned between the *Hpa*I and *Bss*HI sites of pHXB2gpt, and the resulting mutant plasmids were screened by restriction digests and confirmed by direct dideoxy DNA sequencing (40).

DNA transfections and analysis of viral replication. The procedure for transfection of plasmid DNA into COS-1 cells was performed as previously described (24, 48). To test for viral replication and infectivity, transfected COS-1 cells were cocultured with SupT1 cells which express CD4 on their surface and support high-level replication of HIV-1. SupT1 cells (10⁶ cells per ml) were cocultured for 3 days essentially as described previously (48). Following coculture, the SupT1 cells were removed by low-speed centrifugation (1,000 × *g*) and were further cultured with fresh media and additional SupT1 cells. The SupT1 cultures were monitored visually for the formation of multicell syncytia, and at specified times, supernatants were collected, and cultures were split and refed with fresh SupT1 cells. Supernatant samples were filtered through a 0.45-μm-pore-size nitrocellulose syringe filter (Nalgene) and analyzed for p24 antigen with an enzyme-linked immunosorbent assay (ELISA [Coulter Laboratories]). For reinfections, SupT1 cells (10⁵ cells per ml) were infected with known amounts of virus as measured by p24 antigen (100 ng/ml) and were cultured for up to 2 weeks. Culture supernatants were collected and analyzed for p24 by ELISA (Coulter Laboratories).

Metabolic labeling of transfected cultures and immunoprecipitation. The procedures for metabolic labeling of COS-1 cells were performed as previously described (37). Briefly, the cells were washed once with Dulbecco's modified Eagle's medium and incubated in methionine and cysteine-free Dulbecco's modified Eagle's medium for 1 h, followed by the addition of 100 μCi of [³⁵S]Translabel for 6 h. To detect HIV-1-related proteins, labeled cellular extracts were incubated with pooled serum samples from five AIDS patients for 24 h at 4°C with constant rocking. The immunoprecipitates were processed essentially as described previously (37), and the proteins were resolved on sodium dodecyl sulfate (SDS)-

10% polyacrylamide gels. Following fluorography with sodium salicylate, the dried gels were autoradiographed with Kodak X-AR film.

PCR amplification and subcloning of PBS-containing proviral DNA. Total cellular DNA was extracted from infected SupT1 cells by using a modification of a previously described protocol (37). PCR was used to amplify the HIV-1 proviral DNA encompassing the PBS region. Two HIV-1-specific primers were used: 5'-GGCTAACTAGGGAACCCACTGC-3' (primer 1) and 5'-AGTGAATTCCTCTTCTAGCCTC CG CTAGTC-3' (primer 2).

Primer 1 hybridizes at nucleotides 42 to 63 of the HIV-1 proviral genome; primer 2 hybridizes at nucleotides 309 to 330 of the HIV-1 proviral genome and contains additional sequences at the 5' end corresponding to an *Eco*RI restriction endonuclease site. The reaction conditions were as described by the manufacturer with 0.5 U of *Taq* DNA polymerase per reaction mixture (Perkin-Elmer) (38, 39). Approximately 10 μ g of high-molecular-weight DNA was subjected to 30 cycles of amplification, with each cycle consisting of a 94°C denaturation step (1 min), a 42°C annealing step (2 min), and a 72°C polymerization step (3 min). Following the 30 cycles, a 7-min polymerization step was performed at 72°C. One-tenth of each PCR-amplified product was analyzed on a 1% agarose gel, while the remainder of each was digested with *Eco*RI and *Hind*III (*Hind*III site at nucleotide 77 of the HIV-1 proviral genome). Digested PCR products were subcloned between the *Eco*RI and *Hind*III sites present in the polylinker region of M13mp18 replicative-form DNA. Ligation products were transformed into competent *Escherichia coli* (DH5 α F') and plated on a lawn of *E. coli* (DH5 α F') containing isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as described previously (27, 49). Colorless phage plaques, indicating recombinants, were grown, and single-stranded DNA was prepared for DNA sequencing. At least 10 independent white plaques were grown and sequenced by Sanger dideoxy DNA sequencing for each sample (40).

RESULTS

Construction of HIV-1 proviral genomes containing defined mutations in the PBS. The HIV-1 PBS is positioned within the 5' noncoding region (nucleotides 183 to 200) between the transactivation response region, splice-donor site, and the putative RNA encapsidation signal (ψ) (2, 20, 35, 36) (Fig. 1A). The PBS consists of 18 nucleotides which are complementary to the 3'-terminal 18 nucleotides of a cellular tRNA_{Lys} molecule. To further investigate the nucleotide sequence requirements of a functional HIV-1 PBS, oligonucleotide site-directed mutagenesis was used to create mutations within the PBS region of the HIV-1 proviral genome. These modifications are presented as they would appear in the viral RNA genome in Fig. 1B. Initially, a mutation in the PBS was constructed in which the 18 nucleotides complementary to tRNA_{Lys} were substituted with 18 nucleotides predicted to be complementary to the 3'-terminal bases of a tRNA^{Phe} molecule; this construct was designated pHXB2PBS(phe). tRNA^{Phe} has not been shown to act as a tRNA primer for HIV-1 reverse transcription and has not been detected in HIV-1 virions (17). However, the PBS of pHXB2PBS(phe) contains five of the first six nucleotides present in the wild-type PBS. Since our previous studies have shown that the first six nucleotides are sufficient for the function of the PBS (37), a mutant PBS of pHXB2PBS(phe) was constructed in which the first six nucleotides were identical to the wild-type HIV-1 PBS, while the remaining 12 nucle-

FIG. 1. The HIV-1 5' nontranslated region and description of the mutations within the PBS region. (A) Expanded view of the 5' nontranslated region and location of the relevant RNA structures. The PBS is positioned between the transactivation response region (TAR) and the splice-donor site (SD). A putative encapsidation signal (ψ) is located at nucleotides 300 to 319 (2, 20). The translational initiation for the *gag* gene is located at nucleotide 334. (B) Expanded view of the PBS region of HIV-1. PBS is located between nucleotides 183 and 200 in the HIV-1 genome. The nucleotide sequence corresponding to the wild-type PBS which is complementary to the cellular tRNA_{Lys} is depicted in pHXB2(wild type). A mutation in the PBS was constructed such that the resultant mutant PBS is now complementary to tRNA^{Phe} [pHXB2PBS(phe)]. The nucleotide differences from the wild-type PBS region are denoted in boxes. The PBS corresponding to tRNA^{Phe} was modified by the change of a guanosine to a cytosine at position 4. The resulting PBS [pHXB2PBS(pheC)] now contains the first six nucleotides identical to the wild-type PBS. An additional mutation was constructed outside of the PBS region in which the five nucleotides GGGAC were inserted 6 bp downstream from the last nucleotide of the PBS. The 5-bp insertion is noted in plasmids pHXB2PBS(phe+5) and pHXB2PBS(pheC+5).

tides corresponded to tRNA^{Phe}. Oligonucleotide site-directed mutagenesis was used to construct this mutant, called pHXB2PBS(pheC), which substituted the fourth nucleotide (guanosine) with a cytosine.

In our previous study, we suggested that mutations in the PBS might affect the strand transfer reaction that occurs between minus- and plus-strand DNA and that these effects might be overcome by positioning base pairs at the PBS region

complementary to the plus-strand strong-stop DNA (see Fig. 5A). These complementary bases might facilitate the completion of the plus strand to generate an infectious provirus (37). In order to test this, we have constructed in the HIV-1 genome 3' of the mutant PBS a five-nucleotide insertion (GGGAC) which is identical to the last five nucleotides of the wild-type PBS. Upon copying of this sequence during reverse transcription, the sequence generated (CCCTG) would be complementary to the plus-strand PBS DNA. We have constructed this five-nucleotide insertion into the plasmids pHXB2PBS(phe) and pHXB2PBS(pheC) to make the corresponding HIV-1 proviral genomes designated pHXB2PBS(phe+5) and pHXB2PBS(pheC+5).

Expression of HIV-1 proteins from mutant PBS proviral genomes. A previous study suggested that mutations within the PBS affect the release of virus particles from transfected cells (28). In contrast, studies from this laboratory have demonstrated no significant differences in the intracellular processing and release of viral particles between wild-type and proviral genomes containing mutations within the PBS region when transfected into COS-1 cells (37). To determine whether the mutation introduced into the PBS region of the new proviral genomes constructed for this study might affect the expression of viral proteins and subsequent release of virus, the intracellular expression of viral proteins from transfected COS-1 cells was analyzed by immunoprecipitation (Fig. 2A). No significant differences in the intracellular expression of viral proteins, particularly that of the *gag* gene products (Pr55, p41, p24, and p25), as well as the *env* gene proteins (gp160, gp120), were evident within COS-1 cells transfected with the various proviral constructs. To determine whether the PBS mutations had any effect on the release of virus, supernatants of COS-1 cells transfected with either wild-type or mutant proviral genomes were analyzed for p24 antigen with a commercially available ELISA. Again, no significant differences in the levels of p24 antigen released from the transfected cells were noted (Fig. 2B). Any minor variations observed are believed to be due to differences in transfection efficiencies and were not consistently observed during repeat transfections (data not shown).

Infectivity of proviral genomes containing mutations in the PBS. To test the effects of the mutations in the PBS on virus infectivity, the proviral genomes were transfected into COS-1 cells; since COS-1 cells do not support HIV-1 replication, the cells were cocultured with SupT1 cells for 3 days. Following coculture, SupT1 cells were isolated by low-speed centrifugation and further cultured with new media and fresh SupT1 cells for up to 3 weeks. Samples of the culture supernatants were removed and analyzed for the presence of soluble p24 antigen (Fig. 3). Transfection of the wild-type proviral genome, pHXB2(wild type), into COS-1 cells and subsequent coculture with SupT1 cells resulted in the production of infectious virus as measured by p24 antigen in the culture supernatant. The production of p24 antigen and the formation of multinucleated cells (syncytia) occurred rapidly in these cultures and could be detected after approximately 3 to 6 days. Transfection of pHXB2PBS(phe) did not result in the production of an infectious virus, as judged by the absence of p24 in the culture supernatant, reflecting the inability of HIV-1 to utilize a PBS complementary to tRNA^{Phe}. Infectious virus was produced following transfection of pHXB2PBS(pheC) in only 1 of 20 independent transfections, indicating that the restoration of the first six nucleotides of the PBS was sufficient, in rare instances, for the reverse transcription of the viral genome.

The model for reverse transcription predicts that the second template transfer is facilitated by complementarity between the minus- and plus-strand viral DNA genomes (14, 31, 46).

FIG. 2. Expression of viral proteins and release of virus from COS-1 cells transfected with the wild-type and mutant proviral genomes. (A) COS-1 cells were transfected with the designated plasmids and metabolically labeled with [³⁵S]Translabel 24 h following transfection. The HIV-specific proteins were immunoprecipitated with AIDS patients' sera, and the immunoprecipitates were run on an SDS-10% polyacrylamide gel, followed by fluorography and autoradiography. The molecular mass standards and the relevant HIV viral proteins are noted. Lanes: 1, mock transfected; 2, pHXB2(wild type); 3, pHXB2PBS(phe); 4, pHXB2PBS(phe+5); 5, pHXB2PBS(pheC+5); 6, pHXB2PBS(pheC). (B) COS-1 cells were transfected with the designated plasmids, and, at 48 h posttransfection, the supernatant was analyzed for released p24 antigen. The levels of p24 antigen were determined from five independent transfections. The data are presented as the percentage of p24 relative to the levels of p24 detected in the supernatants from COS-1 cells transfected with the wild-type genome, with standard deviations represented by error bars.

From a preliminary examination, we found that little, if any, complementarity would exist between the minus- and plus-strand PBSS from viruses derived from transfection of pHXB2PBS(pheC). It was possible that the low levels of complementarity accounted for the infrequent recovery of infectious virus from independent transfections of pHXB2PBS(pheC). To determine if this was the case, we constructed an additional mutation in pHXB2PBS(pheC) to include five nucleotides corresponding to the last five nucleotides found at the 3' end of the wild-type (tRNA₃^{Lys}) PBS. These five nucleotides were inserted 6 bp 3' of the mutant PBS and should facilitate the second-strand transfer in reverse transcription [pHXB2PBS(pheC+5)]. Transfection of pHXB2PBS(pheC+5) resulted in the consistent production of infectious virus in all transfections (20 of 20 independent transfections). To determine whether the five-nucleotide insertion 3' to the PBS would obviate the necessity for the first six nucleotides, we constructed a proviral genome with a PBS complementary to tRNA^{Phe} with the five-nucleotide insertion [pHXB2PBS(phe+5)]. Twenty independent transfections of pHXB2PBS(phe+5) did not result in the production of infectious virus. Taken together, these results highlight the importance of the first six nucleotides of the PBS for initiation of

FIG. 3. Kinetics of the appearance of infectious virus derived from transfection of wild-type and mutant proviral genomes. The plasmids containing the wild-type and mutant proviral genomes were transfected into COS-1 cells and then cocultured with SupT1 cells for an additional 3 days. After this time, the SupT1 cells were isolated by centrifugation, washed three times, and further cultured with fresh SupT1 cells (day 0). At subsequent times post-coculture, supernatant was removed and analyzed for p24 antigen. The samples are as marked in the figure.

reverse transcription and suggest that the complementarity 3' to the PBS in the pHXB2PBS(pheC+5) mutant facilitated the completion of plus-strand DNA and generation of an infectious provirus.

Analysis of the PBS region from integrated proviruses. One of the common features of the models for reverse transcription is that the PBS is regenerated by copying of the still-attached tRNA primer during plus-strand DNA synthesis (14, 31, 42). In a previous study, we validated this aspect of the model by demonstrating the presence of the complete PBS complementary to tRNA₃^{Lys} in proviruses derived initially from transfection of proviral genomes containing deletions of the last 9 or 12 nucleotides of the PBS. However, we noted that the infectious provirus with a complete PBS region contained deletions 3' to the PBS of up to 11 nucleotides (37). On the basis of these studies, we proposed a model in which complementarity between the minus- and plus-strand PBSs would facilitate the completion of reverse transcription. To test the model, we constructed a proviral genome with defined mutations in the PBS to allow initiation and the second template transfer in reverse transcription [pHXB2PBS(pheC+5)]. If the model was correct, the viruses recovered from transfection of pHXB2PBS(pheC+5) would contain a wild-type PBS complementary to tRNA₃^{Lys}. However, it could be argued that a recombination event between the transfected proviral DNA and retrovirus-like sequences might result in regeneration of a wild-type PBS complementary to tRNA₃^{Lys}. To distinguish between a recombination event that generates the wild-type PBS from a PBS regenerated during reverse transcription, we designed the five-nucleotide insertion such that viruses undergoing successful reverse transcription would contain a deletion of six nucleotides 3' to the PBS. Previous studies from this laboratory have demonstrated that viruses with a six-nucleotide deletion 3' to the PBS replicate with kinetics similar to that of the wild-type virus (37). Transfection of pHXB2PBS(pheC+5) into COS-1 cells initially resulted in the appearance of an infectious virus more slowly than that derived from transfection of wild-type pHXB2PBS. The virus recovered [vHXB2PBS(pheC+5)] demonstrated kinetics of replication similar to that of wild-type virus under conditions in which equal amounts of p24 antigen were used to initiate the

FIG. 4. Kinetics of replication of the infectious virus isolated from transfection of cells with pHXB2(wild type) [vHXB2(wild type)] and pHXB2PBS(pheC+5) [vHXB2PBS(pheC+5)]. SupT1 cells were infected with equal amounts (approximately 100 ng/ml) of virus isolated from cocultures of cells initially transfected with either pHXB2(wild type) or pHXB2PBS(pheC+5). 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 p24 antigen in the medium at the designated times postinfection. The samples are as marked.

infection (Fig. 4). To determine whether the viruses derived from pHXB2PBS(pheC+5) contained a wild-type PBS with a six-nucleotide deletion 3' to the PBS, PCR was used to amplify the PBS region from integrated proviruses by using primers that flank the PBS. A similar approach was previously used to analyze integrated proviruses following infection of SupT1 cells with viruses containing deletions in the PBS (37). Amplifying the PBS region from high-molecular-weight DNA from these long-term cultures, then, allows analysis of only those viruses which have undergone successful reverse transcription. Amplified DNA was isolated and subcloned into replicative-form M13mp18, single-stranded DNA from individual recombinant phage plaques was isolated, and the DNA sequence of the PBS region was determined (Table 1). The DNA sequences of the PBS region from at least 10 recombinant phage plaques were determined. As a control for our analysis, proviruses from SupT1 cultures infected with viruses obtained from transfection of pHXB2(wild type) into COS-1 cells were also sequenced. We found that 10 of 10 PBSs sequenced from proviruses derived from transfection of pHXB2(wild type) were of the predicted nucleotide sequence, including the entire region surrounding the PBS. Sequence analysis of the PBS region from 11 of 11 proviruses derived from pHXB2PBS(pheC+5) revealed the conversion of the mutant PBS (complementary to tRNA^{Phe}) to an intact 18-nucleotide wild-type sequence complementary to tRNA₃^{Lys}. In addition to the regeneration of the wild-type PBS, DNAs isolated from proviruses of cultures infected with pHXB2PBS(pheC+5) contained a deletion of the six nucleotides (ΔTTG AAA) adjacent 3' to the PBS. Taken together, these results provide strong support for minus- and plus-strand complementarity of the PBS as a facilitator for the successful completion of reverse transcription.

DISCUSSION

In this study, we have investigated the minimal PBS sequences required for HIV-1 reverse transcription by constructing and characterizing proviral genomes containing defined mutations in the PBS. Initially, a mutant HIV-1 proviral genome was constructed by substituting the wild-type PBS,

TABLE 1. Sequence analysis of the PBS of HIV-1 proviruses derived from pHXB2PBS(wild type) and pHXB2PBS(pheC+5)

Provirus sample	PBS sequence	Frequency ^a
vHXB2PBS(wild type) ^b	AAG TGG CGC CCG AAC AGG GAC TTG AAA GCG AAA G (Wild type)	10/10
Mutant sequence ^c	AAG TGG CGC GAA TTC TGT GGA TTG AAA GGGAC GCG AAA G	
vHXB2PBS(pheC+5)	AAG TGG CGC CCG AAC AGG GAC GCG AAA G ^d (Δ TTG AAA) ^e	11/11

^a DNA sequences of the PBS region obtained from independent M13 phage clones.

^b 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 The mutant sequence refers to the initial mutation in the PBS region from pHXB2PBS(pheC+5). Note the fourth nucleotide (boldface C) and the five additional base pairs (boldface GGGAC).

^d Dots reflect nucleotide deletions.

^e Δ designates deletion of the following nucleotides from the wild-type sequence.

which is complementary to the 3'-terminal 18 nucleotides of a tRNA^{Lys} molecule, with a sequence complementary to the 3'-terminal 18 nucleotides of tRNA^{Phe}. Transfection of this proviral genome [pHXB2PBS(phe)] into COS-1 cells resulted in the production of a noninfectious virus. Analysis of the mutations which produced a functional HIV-1 PBS demonstrated that only the first six nucleotides found at the 5' end of the PBS were sufficient to allow initiation of reverse transcription [pHXB2PBS(pheC)]. Furthermore, our results suggest that sequences found at the 3' end of the PBS provide the complementary nucleotides necessary to interact with the 3' end of the plus-strand strong-stop DNA during the second template transfer of reverse transcription [pHXB2PBS(pheC+5)]. The appearance of infectious virus in SupT1 cells cocultured with COS-1 cells transfected with pHXB2PBS(pheC+5) was slower than that from cultures transfected with pHXB2(wild type). DNA sequence analysis of the PCR-amplified PBS region revealed a regeneration of the wild-type PBS sequence complementary to the 3'-terminal 18 nucleotides of tRNA^{Lys}. A stable 6-bp deletion 3' to the PBS was also present, providing evidence that the infectious viruses recovered were not the result of a recombination event during reverse transcription.

The PBS of HIV-1 is located at nucleotides 183 to 200 in the viral genome between the transactivation response region, the splice-donor sequence (nucleotide 289), and one of the viral genome encapsidation signals (ψ; nucleotides 300 to 319) (12, 20, 35, 36). We were concerned that, given the complexity of this region of the viral RNA genome, mutations in the PBS might have additional, pleiotropic effects on the expression of viral proteins and the assembly and release of virus. Indeed, an earlier study found that mutations in the PBS site affected virus assembly and subsequent release from the host cell (28). Those results, though, were in contrast to results from previous studies from this laboratory in which we found that mutations in the PBS did not result in significant differences in the levels of intracellular expression of viral proteins or release of virus (37). We have confirmed our previous results in finding that, under our experimental conditions, no differences in the intracellular expression of viral protein or amounts of released p24 antigen were evident in COS-1 cultures transfected with the wild-type and mutant proviral genomes. The reason for this discrepancy between the two studies is unclear, but may be due to differences in the COS-1 expression system used in our studies and the human rhabdoid sarcoma cell line used by Nagashunmugam et al. (28).

It is generally believed that, during virion assembly, retroviruses selectively incorporate the tRNA primer used in the initiation of reverse transcription. Our initial intention in

choosing to mutate the PBS region to be complementary to tRNA^{Phe} was to determine whether the specificity for the tRNA primer used in reverse transcription was determined solely by complementarity to the PBS. Previous studies have described the isolation and characterization of a recombinant murine leukemia virus with a PBS complementary to tRNA^{Gln} rather than the usual tRNA^{Pro}. The virus was replication competent and now used tRNA^{Gln} as the primer for reverse transcription (12). We believed that changing the PBS of HIV-1 to be complementary to tRNA^{Phe} would test the possibility that tRNA complementarity to the PBS dictates which tRNA would be used in reverse transcription, since tRNA^{Phe} is not utilized by other retroviruses as a primer for reverse transcription. It was clear from our results with the transfection of pHXB2PBS(phe) that the complementarity of the PBS region with a particular tRNA alone is not the sole determinant for which tRNA primer will be used in reverse transcription. The inability of the HIV-1 proviral genome pHXB2PBS(phe) to utilize a PBS complementary to tRNA^{Phe} and produce an infectious virus may be due to the absence of tRNA^{Phe} in the virion. A recent report demonstrated that HIV-1 virions produced from COS cells incorporate four major tRNA species, including tRNA^{Lys}, which is found tightly associated with the RNA genome (17); tRNA^{Phe} was not identified in the HIV-1 virions. The mechanism by which the cellular tRNA is incorporated into the virion is largely unknown. The results of previous studies with murine (21) and avian (33) retroviruses, as well as those with yeast retrotransposons (8), have suggested that the tRNAs are incorporated into virions independent of the PBS region. Instead, packaging of the primer tRNAs may be due to an interaction of the reverse transcriptase enzyme with the specific tRNA (4–7, 22, 30, 33, 47). The nucleocapsid protein, which is required for initiation of reverse transcription, probably plays a role in tRNA primer selection and positioning of the primer on the PBS (6). It is also possible that recognition of certain tRNAs might involve unique features of individual tRNAs, possibly the anticodon regions (5). Future studies will be required to answer this question.

The use of proviruses containing different mutations in the PBS has provided insights into the interaction at the nucleotide level between the tRNA primer and the PBS. Although tRNA^{Phe} is not utilized in the initiation of HIV-1 reverse transcription, it does contain some sequence homology at the 3'-terminal end with tRNA^{Lys}, with five of the first six bases being identical. Thus, tRNA^{Lys} may still prime reverse transcription, provided there is enough complementarity with the mutant (phe) PBS. Previous studies from this laboratory have indicated that a deletion of the last 12 nucleotides of the

FIG. 5. Predicted base pairings at the elongation step of plus-strand DNA synthesis. (A) Proposed step at which the interactions between the PBSs generated during minus-strand and plus-strand DNA synthesis occur. In the first step, the initiation of plus-strand synthesis is depicted on the minus-strand DNA template. It is important to note that the PBS identified in lowercase (pbs) represents the minus-strand DNA copy of the PBS region found in the virion RNA. Following the initiation of plus-strand DNA synthesis to generate plus-strand strong-stop DNA, the reverse transcriptase recopies the still-attached tRNA^{Lys} primer. The tRNA primer is removed by the action of RNase H activity of reverse transcriptase. Thus, the plus-strand copy of the PBS (denoted by uppercase PBS) is generated by copying of the tRNA primer. The complementarity between the PBS on the minus strand (lowercase pbs) and the plus strand (uppercase PBS) results in the reverse transcriptase extending from the plus-strand PBS to complete the synthesis of the proviral genome. The region of complementarity between the minus- and plus-strand PBSs is boxed in this panel. (B) Base pair interactions between the minus- and plus-strand PBSs in the reverse transcription of the wild-type genome. The 18-nucleotide complementarity that exists between the PBSs of the minus- and plus-strand DNAs in the wild-type virus is depicted in this panel. This complementarity facilitates the extension reaction for both minus- and plus-strand DNA synthesis, resulting in the completion of an infectious proviral DNA. The six nucleotides boxed on the minus-strand DNA template (AAGTTT) represent those nucleotides which would be predicted to be deleted in proviral genomes containing the PBS found on the plasmid pHXB2PBS(pheC+5). (C) Predicted base pairings between the minus- and plus-strand PBS regions during this step in reverse transcription from viral genomes generated by transfection of pHXB2PBS(pheC). The viruses generated from transfection of COS cells with pHXB2PBS(pheC) will contain the PBS region corresponding to tRNA^{Phe} with the single uridine-to-cytosine change. These viruses will undergo initiation of reverse transcription upon infection of susceptible SupT1 cells. The PBS region present on the minus-strand DNA template would be generated from copying of the viral RNA genome by reverse transcriptase. The minus-strand DNA would thus contain a PBS complementary to tRNA^{Phe} with the guanosine-to-cytosine mutation. However, the PBS on the plus-strand DNA would be synthesized upon recopying of the cellular tRNA^{Lys} used as the primer for reverse transcription. In this instance, there would be very little complementarity between the minus- and plus-strand PBS regions. In fact, we would predict that only three potential base pair interactions might occur, which would be insufficient to allow the reverse transcriptase to complete the plus-strand DNA extension (denoted by the X), resulting in a noninfectious virus. (D) Potential interactions between the minus- and plus-strand PBS regions in viruses generated from transfection of pHXB2PBS(pheC+5). In this case, the PBS region denoted in the minus-strand DNA would again contain a copy of the original mutated PBS region corresponding to tRNA^{Phe} with the guanosine-to-cytosine change. In addition, we have inserted in the original provirus a five-nucleotide insertion, GGGAC, which in the minus-strand DNA sequence would be read as CCCTG. As depicted in this figure, this five-nucleotide insertion would then be complementary to the last five nucleotides of the plus-strand DNA PBS generated by recopying of the cellular tRNA^{Lys}. It is important to note that we have constructed the mutation pHXB2PBS(pheC+5) such that the five-nucleotide insertion, if utilized during reverse transcription, would delete the nucleotides AAGTTT (represented on the minus strand and boxed in panel B). In 11 of the 11 PBS regions which were PCR amplified and sequenced, we found that the 6-bp deletion had occurred, thus providing strong support for this model. vHB2(wild type), vHB2PBS(pheC), and vHB2PBS(pheC+5) denote viruses derived from pHXB2(wild type), pHXB2PBS(pheC), and pHXB2PBS(pheC+5), respectively.

18-nucleotide HIV-1 PBS resulted in the production of an infectious virus (37). These results suggested that, for initiation of reverse transcription to occur, only the 3'-terminal six nucleotides of tRNA₃^{Lys} are needed to base pair with the 5'-terminal six nucleotides of the HIV-1 PBS. By changing the fourth nucleotide of the *phe* PBS from a guanine to a cytosine, a PBS mutant was created in which the first six nucleotides of the PBS were complementary to the 3'-terminal six nucleotides of tRNA₃^{Lys}. As demonstrated by our experiments, this mutant proviral genome, designated pHXB2PBS(*pheC*), produced infectious virus following transfection into COS-1 cells. It appears, then, that a minimal complementarity between the RNA template and the 3' end of the primer tRNA is required for initiation. As depicted in Fig. 1B, the presence of a non-base-paired nucleotide at position 4 between tRNA₃^{Lys} and the PBS complementary to tRNA^{Phe} creates a 1-bp mismatch which prevents the initiation of reverse transcription. Results from earlier studies suggested that the nucleocapsid protein promotes complementary base pairing between the PBS and the 3'-terminal nucleotides of the tRNA primer by binding to the tRNA and unwinding the RNA secondary structures of both the tRNA and the 5' end of the viral RNA genome (6). It is possible that the 6-bp complementarity represents the minimum number required by nucleocapsid protein or reverse transcriptase to promote unwinding of the base pairing between tRNA₃^{Lys} and the PBS. Alternatively, the nucleotide at position 4 could be an essential component of an RNA recognition sequence for the nucleocapsid protein or reverse transcriptase. Studies are ongoing with additional mutants to distinguish between these possibilities.

The PBS also plays an important role in the extension step of plus-strand synthesis in reverse transcription (Fig. 5A). The complementarity between the minus- and plus-strand DNA PBSs facilitates the template transfer required to synthesize the complete proviral genome (Fig. 5B) (14, 23, 31, 46). In our previous study, we found that proviruses with 9- to 12-bp deletions in the PBS gave rise to viruses with a full-length (18-nucleotide) wild-type PBS complementary to tRNA₃^{Lys} (37). Thus, as predicted by the model for reverse transcription, the PBS is regenerated by copying of the tRNA primer during plus-strand synthesis (14, 42). However, upon analysis of the PBSs from integrated proviruses, we found numerous nucleotide deletions, some up to 11 bp, 3' of the PBS. We suggested that an insertion of nucleotides complementary to the 3' end of the plus-strand PBS DNA might facilitate the template transfer (37) (Fig. 5B). To test this possibility, we positioned the five-nucleotide insertion 3' to the PBS such that the resulting virus would delete six nucleotides 3' from the PBS if the inserted five nucleotides complementary to tRNA₃^{Lys} were used to promote the extension of plus-strand DNA. We found that the PBSs of proviruses generated from transfection of pHXB2PBS(*pheC*+5) had the predicted 6-bp deletion in 11 of 11 PBS regions analyzed. Taken together, these results provide strong support for our previous explanation as to the origin of the deletions 3' to the PBS found in viruses derived from mutant proviruses in which the last 9 or 12 nucleotides of the PBS had been deleted (37). Our results also suggest a reason why infectious virus was isolated only sporadically from transfection of pHXB2PBS(*pheC*). We believe that transfection of pHXB2PBS(*pheC*) or pHXB2PBS(*pheC*+5) into COS-1 cells results in the production of viruses that, upon initial infection, undergo initiation of reverse transcription at similar efficiencies. However, during the second template transfer, the viruses generated from pHXB2PBS(*pheC*) undergo abortive reverse transcription because of the unfavorable complementarity between the minus- and plus-strand DNA containing the PBS

(Fig. 5C). It is important to note here that, in the initial infection during the first reverse transcription reaction, the minus-strand PBS DNA will contain the mutated PBS region (complementary to tRNA^{Phe}), while the plus-strand DNA PBS region will contain the wild-type PBS complementary to tRNA₃^{Lys} since this is generated by copying of the tRNA₃^{Lys} used as the primer. Only viruses derived from transfection of pHXB2PBS(*pheC*+5) would have sufficient complementarity in the minus- and plus-strand DNA PBS to complete reverse transcription (Fig. 5D). In completing reverse transcription, the viruses derived from transfection of pHXB2PBS(*pheC*+5) delete six nucleotides from the region 3' of the PBS. Although the inclusion of the five-nucleotide insertion facilitates the reverse transcription of the viral genomes derived from pHXB2PBS(*pheC*+5), it is clear from the delayed appearance of the virus compared with that of the wild type that the overall reverse transcription process is less efficient than that of the wild-type virus. In support of this notion, we were unable to reproducibly detect the reverse-transcribed genomes at early times (days 3 to 6) in cultures infected with viruses derived from transfection of pHXB2PBS(*pheC*+5). There are several possible reasons for the delay in appearance of the virus from transfection of pHXB2PBS(*pheC*+5) compared with that from transfection of pHXB2(wild type). First, even though the 3'-terminal nucleotides of tRNA₃^{Lys} can presumably anneal to the 5' nucleotides of the PBS of pHXB2PBS(*pheC*), the remaining non-base-paired nucleotides of the tRNA₃^{Lys} and the PBS may result in an unstable interaction between the reverse transcriptase (or reverse transcriptase-nucleocapsid protein) and the tRNA bound at the PBS. If the interaction between tRNA-PBS-reverse transcriptase-nucleocapsid protein is reduced, this might result in a sporadic initiation of reverse transcription. Alternatively, the insertion of the PBS complementary to tRNA^{Phe} may alter the RNA secondary structures between the U5, PBS, and leader sequences, which have been shown to play a role in the efficient initiation of reverse transcription of avian sarcoma leukosis viruses (1, 10). A second possibility for the delay in the recovery of infectious virus from transfection of pHXB2PBS(*pheC*+5) is that the putative DNA structures presented in Fig. 5D would be a target for cellular DNA repair enzymes after integration into the host cell chromosome. As a result of DNA repair, the wild-type PBS (derived from plus-strand DNA) might be altered, resulting in a noninfectious provirus. If this were the case, upon subsequent culture of the SupT1 cells, the resulting noninfectious viruses produced might be diluted out during passage. Future studies will be needed to distinguish between these possibilities.

ACKNOWLEDGMENTS

We thank David Ansardi and Sandra Jablonski for helpful comments and Nancy Vaida for preparation of the manuscript. C.D.M. expresses thanks to M.A.R. for invaluable help and discussion.

Culture of HIV was carried out at the University of Alabama at Birmingham AIDS Research Central Virus Core Facility (AI-27767). Some of the oligonucleotides were prepared by the DNA Oligonucleotide Cancer Center Core Facility, University of Alabama at Birmingham (NCI contract CA 13148). The University of Alabama at Birmingham AIDS Molecular Biology Core Facility provided help with PCR techniques (AI-27767). J.K.W. was supported by training grant GM08111. This study was supported by Public Health Services grant AI34749 from the National Institutes of Health (C.D.M.).

REFERENCES

1. Aiyar, A., D. Cobrinik, Z. Ge, H.-J. Kung, and J. Leis. 1992. Interaction between retroviral U5 RNA and the T Ψ C loop of the

- tRNA^{Trp} primer is required for efficient initiation of reverse transcription. *J. Virol.* **66**:2464–2472.
2. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. *J. Virol.* **64**:1920–1926.
 3. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature (London)* **226**:1209–1211.
 4. **Barat, C., S. F. J. Le Grice, and J.-L. Darlix.** 1991. Interaction of HIV-1 reverse transcriptase with a synthetic form of its replication primer, tRNA^{Lys,3}. *Nucleic Acids Res.* **19**:751–757.
 5. **Barat, C., V. Lullien, O. Schatz, G. Keith, M. T. Nugéyre, F. Gruninger-Leitch, F. Barre-Sinoussi, S. F. J. Le Grice, and J. L. Darlix.** 1989. HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *EMBO J.* **8**:3279–3285.
 6. **Barat, C., O. Schatz, S. Le Grice, and J.-L. Darlix.** 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.
 7. **Bordier, B., L. Tarrago-Litvak, M.-L. Sallafranque-Andreola, D. Robert, D. Tharaud, M. J. Fournier, P. Barr, S. Litvak, and L. Sarih-Cottin.** 1990. Inhibition of the p66/p51 form of human immunodeficiency virus reverse transcriptase by tRNA^{Lys,3}. *Nucleic Acids Res.* **18**:429–436.
 8. **Chapman, K. B., A. S. Bystrom, and J. D. Boeke.** 1992. Initiator methionine tRNA is essential for Ty1 transposition in yeast. *Proc. Natl. Acad. Sci. USA* **89**:3236–3240.
 9. **Charneau, P., and F. Clavel.** 1991. A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract. *J. Virol.* **65**:2415–2421.
 10. **Cobrinik, D., A. Aiyar, Z. Ge, M. Katzman, H. Huang, and J. Leis.** 1991. Overlapping retrovirus U5 sequence elements are required for efficient integration and initiation of reverse transcription. *J. Virol.* **65**:3864–3872.
 11. **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.
 12. **Colicelli, J., and S. P. Goff.** 1987. Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. *J. Virol.* **57**:37–45.
 13. **Darlix, J. L., M. Zuker, and P.-F. Spahr.** 1982. Structure-function relationship of Rous sarcoma virus leader RNA. *Nucleic Acids Res.* **10**:5183–5196.
 14. **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.
 15. **Hu, W.-S., and H. M. Temin.** 1990. Retroviral recombination and reverse transcription. *Science* **250**:1227–1233.
 16. **Hungnes, O., E. Tjotta, and B. Grinde.** 1992. Mutations in the central polypurine tract of HIV-1 result in delayed replication. *Virology* **190**:440–442.
 17. **Jiang, M., J. Mak, A. Ladha, E. Cohen, M. Klein, B. Rovinski, and L. Kleiman.** 1993. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. *J. Virol.* **67**:3246–3253.
 18. **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.
 19. **Kohlstaedt, L. A., and T. A. Steitz.** 1992. Reverse transcriptase of human immunodeficiency virus can use either human tRNA^{Lys,3} or *Escherichia coli* tRNA^{Gln,2} as a primer in an *in vitro* primer-utilization assay. *Proc. Natl. Acad. Sci. USA* **89**:9652–9656.
 20. **Lever, A., H. Gottlinger, W. Haseltine, and J. Sodroski.** 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63**:4085–4087.
 21. **Levin, J. G., and J. G. Seidman.** 1979. Selective packaging of host tRNA's by murine leukemia virus particles does not require genomic RNA. *J. Virol.* **29**:328–335.
 22. **Levin, J. G., and J. G. Seidman.** 1981. Effect of polymerase mutations on packaging of primer tRNA^{Pro} during murine leukemia virus assembly. *J. Virol.* **38**:403–408.
 23. **Li, P., A. J. Stephenson, L. J. Kuiper, and C. J. Burrell.** 1993. Double-stranded strong-stop DNA and the second template switch in human immunodeficiency virus (HIV) DNA synthesis. *Virology* **194**:82–88.
 24. **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.
 25. **Luo, G., L. Sharmeen, and J. Taylor.** 1990. Specificities involved in the initiation of retroviral plus-strand DNA. *J. Virol.* **64**:592–597.
 26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 27. **Messing, J.** 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20–78.
 28. **Nagashunmugam, T., A. Velpandi, C. S. Goldsmith, S. R. Zaki, V. S. Kalyanaraman, and A. Srinivasan.** 1992. Mutation in the primer binding site of the type 1 human immunodeficiency virus genome affects virus production and infectivity. *Proc. Natl. Acad. Sci. USA* **89**:4114–4118.
 29. **Panet, A., and H. Berliner.** 1978. Binding of tRNA to reverse transcriptase of RNA tumor viruses. *J. Virol.* **26**:214–220.
 30. **Panet, A. C., W. Haseltine, D. Baltimore, G. Peters, F. Harada, and J. Dahlberg.** 1975. Specific binding of tRNA^{Trp} to AMV reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **72**:2535–2539.
 31. **Panganiban, A. T., and D. Fiore.** 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. *Science* **241**:1064–1069.
 32. **Peters, G., and J. E. Dahlberg.** 1979. RNA-directed DNA synthesis in Moloney murine leukemia virus: interaction between the primer tRNA and the genome RNA. *J. Virol.* **31**:398–407.
 33. **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.
 34. **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.
 35. **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.
 36. **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. Ghayeb, 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.
 37. **Rhim, H., J. Park, and C. D. Morrow.** 1991. Deletions in the tRNA^{Lys} primer-binding site of human immunodeficiency virus type 1 identify essential regions for reverse transcription. *J. Virol.* **65**:4555–4564.
 38. **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.
 39. **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.
 40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 41. **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.
 42. **Taylor, J. M., and T. W. Hsu.** 1980. Reverse transcription of avian sarcoma virus RNA into DNA might involve copying of the tRNA primer. *J. Virol.* **33**:531–534.
 43. **Temin, H.** 1981. Structure, variation and synthesis of retrovirus long terminal repeat. *Cell* **27**:1–3.
 44. **Temin, H. M., and S. Mizutani.** 1970. RNA-directed DNA poly-

- merase in virions of Rous sarcoma virus. *Nature (London)* **226**:1211–1213.
45. **Varmus, H.** 1982. Form and function of retroviral proviruses. *Science* **216**:812–820.
46. **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.
47. **Vzorov, A. N., M. I. Bukrinsky, V. B. Grigoriev, Y. Y. Tentsov, and A. G. Bukrinskaya.** 1991. Highly immunogenic human immunodeficiency viruslike particles are produced by recombinant vaccinia virus-infected cells. *AIDS Res. Hum. Retroviruses* **7**:29–36.
48. **Wakefield, J. K., S. A. Jablonski, and C. D. Morrow.** 1992. In vitro enzymatic activity of human immunodeficiency virus type 1 reverse transcriptase mutants in the highly conserved YMDD amino acid motif correlates with the infectious potential of the proviral genome. *J. Virol.* **66**:6806–6812.
49. **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.