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Mutation in the primer binding site of the type 1 human immunodeficiency virus genome affects virus production and infectivity

T. NAGASHUNMUGAM*, A. VELPANDI*, C. S. GOLDSMITH†, S. R. ZAKI†, V. S. KALYANARAMAN‡, AND A. SRINIVASAN*§

*The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, PA 19104; †Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333; and ‡Advanced Bioscience Laboratories, Inc., 5510 Nicholson Lane, Kensington, MD 20805

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ABSTRACT **In an effort to understand the contribution of the primer-binding site (PBS) region to human immunodeficiency virus (HIV) replication, we have constructed a mutant HIV proviral DNA with an alteration in the 5′ end of the PBS. The PBS mutant proviral DNA was characterized by transfection of the viral DNA into CD4$^+$ and non-CD4$^+$ target cells. The results indicate that mutation in the PBS reduced the level of viral particles released into the medium of transfected cells in comparison to wild-type proviral DNA. The viral particles were noninfectious upon transmission to established CD4$^+$ cell lines and phytohemagglutinin-stimulated peripheral blood lymphocytes. Electron microscopic analysis of the transfected cells revealed no abnormalities in the structure of the virion directed by the mutant proviral DNA. Also, the protein and RNA contents of the mutant virions were similar to the wild type. The quantitation of intracellular viral structural protein in the transfected cells, however, indicated that the PBS mutation may have an effect on the assembly of viral particles in addition to completely abolishing reverse transcription of viral RNA into DNA. These results provide evidence that the PBS region of the viral genome has multiple functions in HIV-1 replication.**

The genome of human immunodeficiency virus type 1 (HIV-1), like other members of the lentivirus family, encodes not only the virion proteins gag, pol, and env common to all replication-competent retroviruses but also eight accessory genes, some of which are involved in the gene regulation and morphogenesis of HIV (1–3). Biochemical and structural studies have revealed that HIV is about 80–120 nm in diameter and consists of an inner core (nucleoid) surrounded by an outer envelope. The core corresponds to a ribonucleoprotein surrounded by the capsid protein. The viral RNA genome is in the ribonucleoprotein, and it is composed of two identical positive-sense RNAs with a 5′ cap and a 3′ poly(A) structure (4).

The replication cycle of HIV-1 and other retroviruses involves the reverse transcription of virion RNA to viral DNA, which then becomes incorporated into the host cellular genome. The reverse transcription is mediated by the virion-associated enzyme reverse transcriptase (RT) (5, 6). As with all DNA polymerases, RT needs a primer covering a 3′ hydroxyl (OH) group to initiate cDNA synthesis. The *in vivo* primer for the viral first-strand cDNA synthesis from an RNA template has been shown to be tRNA (6). A region near the 5′ end of the viral RNA genome designated as the primer-binding site (PBS) is complementary to the 18 nucleotides of the 3′ CCA end of the specific tRNA primer. Predominantly, many mammalian retroviruses use proline tRNA, and avian retroviruses make use of tryptophan tRNA. Sequence analysis of HIV-1 revealed PBS corresponding to the lysine tRNA as in other lentiviruses (7). It has also been recently demonstrated that HIV RT forms a stable complex with isoacceptor 3 of lysine tRNA (Lys3 tRNA) (8), as has been demonstrated for the RT enzyme of avian viruses.

Initiation of viral DNA synthesis is a crucial step in the replication of retroviruses, and the essential PBS is embedded in the complex structure of RNA (9). A number of studies have reported the importance of the secondary structure of the genome at the 5′ end in the assembly and infectivity of the virus (10–12). In an effort to understand the structural requirement of the PBS in reverse transcription and virus morphogenesis, we have introduced an insertional mutation in the PBS of HIV-1. The results obtained with the PBS mutant imply that the PBS region, in addition to its participation in reverse transcription, is also involved in the assembly of viral particles.

MATERIALS AND METHODS

Cell Lines. A human rhabdomyosarcoma (RD) cell line (American Type Culture Collection) was maintained as a monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and L-glutamine (540 μg/ml) at 37°C with 5% CO_2. CEM × 174 and HUT78 cells were maintained as suspension cultures in RPMI 1640 medium; phytohemagglutinin-stimulated (10 μg/ml) peripheral blood lymphocytes were grown in RPMI 1640 medium containing T-cell growth factor (10%).

Construction of the PBS Mutant. The HIV proviral DNA plasmid designated pARV was derived from cells infected with HIV_{SF2} (13). The pARV plasmid was cleaved with the restriction enzyme *Nar* I, and the resultant linear molecule was purified by agarose gel electrophoresis. The *Nar* I overhang sequence in the DNA was filled in by using Klenow polymerase and ligated by using T4 ligase (14). The recombinant plasmid obtained from the transformed colonies was checked for the presence of the *Nar* I cleavage site. The plasmid (pARV-NAR) lacking the *Nar* I site was further confirmed by sequence analysis with a Sequenase kit (United States Biochemical).

Transfection. RD cells (1×10^6 cells per plate) were split 24 hr before transfection, and growth medium was replaced 1–2 hr before the addition of calcium phosphate-precipitated DNA (15). Cells were exposed to the precipitate for 8 hr followed by a 90-sec glycerol shock (16). For DEAE-dextran transfection, CEM × 174 cells (1×10^7) were washed and

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Abbreviations: RT, reverse transcriptase; PBS, primer-binding site; RD, rhabdomyosarcoma; HIV-1, human immunodeficiency virus type 1; RIPA, radioimmunoprecipitation analysis.

§To whom reprint requests should be addressed.

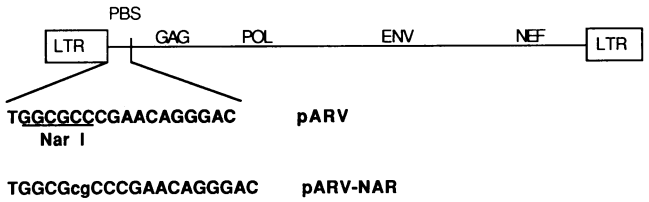

FIG. 1. Structure of wild-type and mutated proviral DNAs used in this study. The wild-type PBS sequence is shown. The insertion mutation in the PBS region of the mutant proviral DNA (pARV-NAR) is indicated in lowercase letters. LTR, long terminal repeat.

suspended in 1 ml of serum-free medium containing 100 μg of DEAE-dextran and 10 μg of plasmid DNA for 1–2 hr in a Petri dish. The cells were washed once with serum-free medium and were grown in RPMI with 10% serum (17).

HIV Antigen and RT Assay. The HIV antigen assay (Coulter) was performed according to the manufacturer's guidelines to quantitate the amount of virus (18). The RT assay procedure was essentially similar to that described (19). The endogenous RT reaction was carried out in the absence of the exogenous template poly(A)·$(dT)_{12-18}$ (20).

Immunoprecipitation Analysis. RD cells (2×10^6) transfected with 10 μg of pARV or 10 μg of mutant pARV-NAR proviral DNA were labeled 60 hr after glycerol shock. The labeling continued for 12 hr with 8 ml of methionine- and cysteine-deficient serum-free medium containing 50 μCi (1 Ci = 37 GBq) of Tran^{35}S-label per ml. The cell-free medium and cells were analyzed by using HIV-positive patient sera and normal human sera (21).

EM. After transfection, cells were fixed for EM with 2.5% glutaraldehyde, postfixed with osmium tetroxide, and embedded in Epon/Araldite. Sections were stained with lead citrate and uranyl acetate (22).

Nucleic Acid Analysis. The virus particles that sedimented from the medium were lysed and immobilized on a nitrocellulose paper and hybridized to the probe representing full-length viral DNA (23, 24). For localizing lysine tRNA present in the virion, the specific oligonucleotide 5′-TCTGATGC-TCTACCGACTGAGCT-3′ derived from Lys3 tRNA was used as a probe (8).

RESULTS

Construction of an HIV Proviral DNA with Alteration in the PBS. To analyze the requirement of the PBS sequences for viral replication, we initially compared the sequences of a number of HIV-1 isolates already available (7). Such a comparison revealed few changes in the PBS, and the mutations noted are all present in the 3′ end of the PBS. Inspection of the PBS sequences also indicated the presence of recognition sequences for the restriction enzyme *Nar* I. The unique *Nar* I cleavage site was utilized to introduce the changes in the PBS sequences. HIV-1 proviral DNA was cleaved with *Nar* I and treated with Klenow polymerase to fill in the overhanging sequences. Blunt-end ligation of the DNA fragment generated a PBS containing two additional bases (Fig. 1). Proviral DNA with this mutation (pARV-NAR) in the PBS was confirmed by restriction enzyme and sequence analyses.

Biological Characterization of HIV-1 Proviral DNA with an Altered PBS. To test the mutant for replication competence, wild-type and mutant proviral DNAs were directly transfected into a $CD4^+$ cell line designated CEM × 174. A positive result is indicative of a spreading HIV-1 infection in this $CD4^+$ cell line. Transfected CEM × 174 cells with wild-type proviral DNA showed high levels of HIV-1 p24 antigen in the medium, in comparison to the PBS mutant (Table 1). These results indicate that virus particles directed by the mutant proviral DNA are unable to initiate a spreading infection.

Effect of the PBS Mutation on Virus Production. To distinguish the effect of the mutation on virus production (synthesis and assembly of viral particles), we have carried out transfection experiments using a transient expression system involving monolayer cells. Human RD cells are ideal for these studies, because they have been shown to release high levels of viral particles upon transfection as monitored by RT and a viral antigen assay (24–26). Transfection of the mutant proviral DNA consistently showed a 40–60% reduction in the release of viral particles in comparison to wild type. The differences observed in the extracellular viral p24 prompted us to quantitate the intracellular p24 in cells transfected with mutant and wild-type proviral DNA (Table 2). Unlike extracellular p24 levels, intracellular p24 levels were similar in both wild-type and mutant proviral DNA-transfected cells (Table 3).

The viral particles released from the transfected cultures were also tested for infectivity potential. Since our previous studies (27) and others (28) have shown that the proviral clone pARV-derived virus is not able to infect CEM × 174 cells as a cell-free virus, we have used the cocultivation method to assess the infectivity of the virus. CEM × 174 cells were directly added to the proviral DNA-transfected RD cells. The cells cocultivated with mutant proviral DNA-transfected RD cells failed to show an increase in virus, which indicates the noninfectious nature of the virus derived from the mutant proviral DNA (data not shown).

Biochemical Analysis of Virions. The lack of infectivity of the virus derived from the mutant proviral DNA could be due to any of a number of factors, including the absence of virion RNA (29) in the virus particle or the presence of an inappropriate primer complex that is unable to initiate reverse transcription following infection. To determine the presence of virion RNA, virus particles released from wild-type and mutant DNA-transfected cells were pelleted by ultracentrifugation. Based on the p24 antigen values, 25, 50, 75, and 100 ng of p24 antigen equivalent virus particles were lysed and immobilized on a nitrocellulose filter and hybridized to a probe representing the whole viral DNA (Fig. 2). Hybridization data showed equal intensities in both wild-type and mutant virions. Further, hybridization studies with a probe specific for lysine tRNA also revealed the presence of lysine tRNA packaged in the viral particles. Mutant virus particles were evaluated for the functional RT enzyme molecule. When the exogenous template primer poly(A)·$(dT)_{12-18}$ was used, similar levels of RT activity were noted (Fig. 3*A*).

Table 1. Kinetics of p24 antigen released from transfected CEM × 174 cells

Proviral DNA	p24 antigen released into the medium, pg/ml								
	3 days	6 days	9 days	13 days	16 days	19 days	23 days	28 days	32 days
pARV	15	21	162	11,500	112,809	119,404	498,019	690,257	563,613
pARV-NAR	15	15	23	33	25	42	11	12	11

CEM × 174 cells (10^7) were transfected with 10 μg of wild-type pARV or mutant pARV-NAR by the DEAE-dextran method as described in the text. The level of p24 antigen released into the medium was monitored at different intervals (every 3–5 days) after transfection. The cut-off value for this experiment was 7 pg/ml.

Table 2. Quantitation of the virus particles produced from the RD cells transfected with wild-type or mutant proviral DNA

Proviral DNA	p24 antigen released into the medium, pg/ml		
	Exp. 1	Exp. 2	Exp. 3
pARV	47,950	31,441	24,887
pARV-NAR	15,881	16,464	13,840

RD cells (10^6) were transfected with 5 μg of wild-type (pARV) or mutant (pARV-NAR) proviral DNA. The supernatant was assayed for p24 antigen after 72 hr by an ELISA. The cut-off values were 4 pg/ml for Exp. 1, 5 pg/ml for Exp. 2, and 4 pg/ml for Exp. 3. The values are from three independent experiments.

However, when RT activity was monitored in the absence of an exogenous template primer, only the wild-type virus showed considerable activity (Fig. 3*B*). These results clearly indicate that the mutant virus is unable to initiate reverse transcription in an endogenous assay.

Analysis of Viral Proteins. The differences in the extracellular and intracellular p24 antigen levels prompted us to analyze the viral proteins in the transfected cells. The low level of virus production observed in the mutant DNA-transfected cells could result from a defect in the gag and envelope protein synthesis and/or processing. The intracellular synthesis of viral proteins was analyzed by using radioimmunoprecipitation analysis (RIPA). Cells transfected with the proviral DNA were labeled with [^{35}S]cysteine and [^{35}S]methionine, and RIPA was carried out with both the cell extract and the medium. The intracellular and extracellular protein banding patterns in both the wild-type and mutant proviral DNA-transfected cells were indistinguishable (Fig. 4 and data not shown).

Analysis of Virions by EM. Transfected RD cells were fixed 48 hr after glycerol shock to analyze the effect of the PBS mutation on virion morphology by transmission EM. HIV particles produced in wild-type and mutant viral DNA-transfected cells averaged about 100 nm in diameter and showed identical morphology (Fig. 5).

DISCUSSION

Although the vital role played by the tRNA bound to the genomic RNA as primer has been analyzed (30, 31), studies on the minimal requirement of PBS sequences for binding of primer tRNA and consequences of alteration in the PBS have been limited (32). The 5′ noncoding region of retroviral RNA can be subdivided into R, U5, primer-binding, and leader regions. Sequences in these regions are important for reverse transcription, translation, and packaging of viral RNA and for integration of viral DNA. These regions contain numerous inverted repeat sequences, which can potentially form RNA secondary structures. Analysis of virion RNA by EM revealed a stable structure, including U5, a PBS, and leader sequences of Rous sarcoma virus and Moloney murine leukemia virus. In addition, computer modeling of Rous sarcoma virus, feline leukemia virus, mouse mammary tumor virus, murine leukemia virus, spleen necrosis virus, and HIV has indicated that the tRNA primer may, in each case, be positioned within an extensive RNA structure (11, 12). Cobrinik *et al.* (10) provided genetic evidence that the secondary structures in the 5′ end of the genome of avian retroviruses are critical for replication and efficient growth. Sequence analyses of the PBS in a number of HIV-1 isolates indicate that the PBS is highly conserved, and only minimal changes were noted in the 3′ end of the PBS.

In this report, we present evidence that the PBS has multiple functions; it provides a target for anchoring tRNA primer and also participates in virion morphogenesis. For assessing the effect of the PBS mutation, both wild-type and

Table 3. Quantitation of intracellular p24 antigen in RD cells transfected with wild-type or mutant proviral DNA

Proviral DNA	Intracellular p24 antigen, pg/ml				
	24 hr	48 hr	72 hr	96 hr	120 hr
pARV	27,467	246,605	614,225	252,100	221,070
pARV-NAR	22,950	259,427	757,122	540,945	228,895

RD cells (10^6) were transfected with 10 μg of wild-type (pARV) or mutant proviral DNA (pARV-NAR). The cells were collected from the plates at different days after transfection, and the cell pellet was lysed in 250 μl of distilled water by freeze-thawing. The cut-off value in the assay was 5 pg/ml.

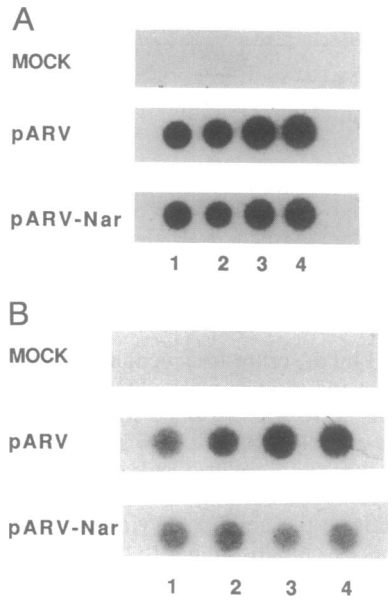

FIG. 2. Analysis of virion RNA. The viral supernatants obtained from RD cells transfected with proviral DNA were quantitated for p24 antigen. Twenty-five (lane 1), 50 (lane 2), 75 (lane 3), and 100 (lane 4) ng of p24 antigen equivalent virus particles were lysed and immobilized on nitrocellulose. The filters were hybridized with the full-length proviral pZ6 probe (*A*) (23) or with an oligonucleotide probe specific for lysine tRNA (*B*).

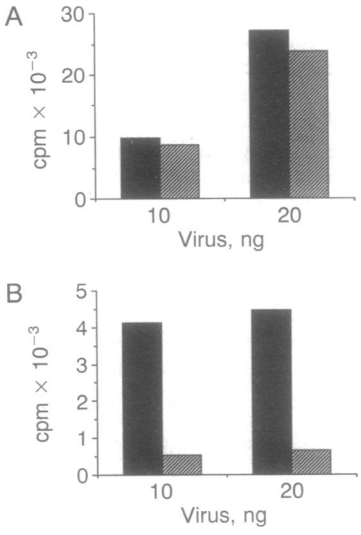

FIG. 3. RT assay. Ten and 20 ng of p24 antigen equivalent virus particles were analyzed for RT activity in the presence of exogenous template poly(A)·$(dT)_{12-18}$ (*A*) or in the absence of exogenous template (*B*) in the reaction. Solid bars, pARV; hatched bars, pARV-NAR.

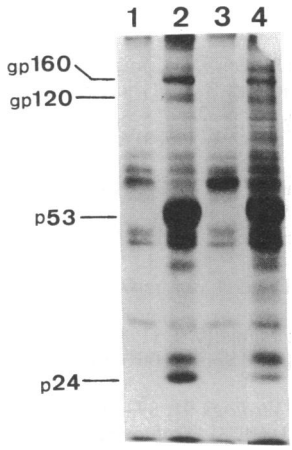

FIG. 4. RIPA. RD cells were transfected with 10 μg of pARV (lanes 1 and 2) or 10 μg of pARV-NAR (lanes 3 and 4) proviral DNA, and cells were labeled with Tran^{35}S-label (50 μCi/ml), which contains methionine and cysteine, 60 hr after glycerol shock. After a 12-hr incubation, the cells were washed with phosphate-buffered saline and lysed in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS). Immunoprecipitation was done with normal sera (lanes 1 and 3) or with HIV-positive sera (lanes 2 and 4).

mutant proviral DNA were transfected into CD4$^+$ CEM × 174 cells. This experimental system relies on the ability of the virus to initiate a new infection upon release from the transfected cells. Generally, a high amount of RT activity or viral antigen is observed after 10–15 days. The results generated by using this system clearly indicated that the virus released by mutant proviral DNA is noninfectious because there was no spreading infection.

To characterize the effect of the PBS mutation on virus production, we have used monolayer cells as target cells for transfection of proviral DNA. We and others (24, 25, 27, 28) have extensively used human RD cells, as a transient system,

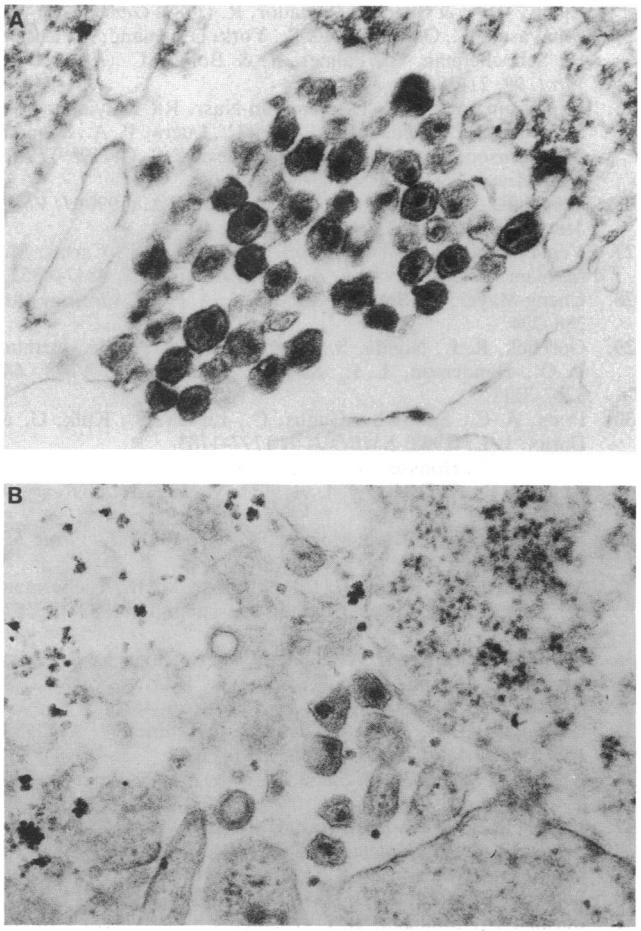

FIG. 5. EM of transfected RD cells. Cells transfected with proviral DNA (*A*, pARV; *B*, pARV-NAR) were collected 72 hr after glycerol shock. The cells were fixed and analyzed by transmission electron microscopy. (×15,500.)

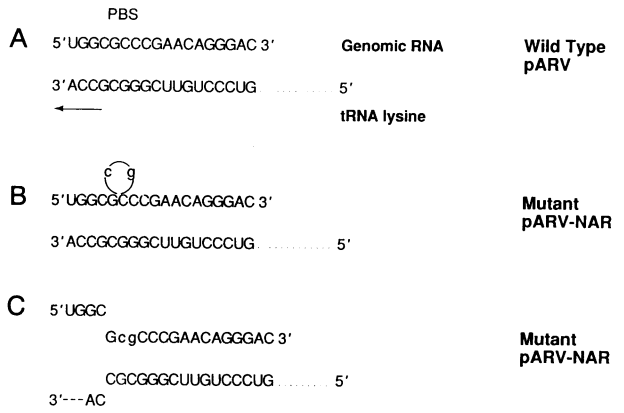

FIG. 6. Interaction of lysine tRNA with wild-type and PBS mutant genomic RNA. (*A*) Sequence homology between the 3′ end of the tRNA and the genomic RNA PBS enables successful reverse transcription in wild-type virions. (*B* and *C*) Possible models for the interaction of lysine tRNA with the PBS mutant genomic RNA. Mutant sequences in the PBS region are indicated by lowercase letters. The arrow indicates the direction of reverse transcription.

to generate HIV upon transfection. These target cells lack CD4 receptors on the cell surface, and this feature enables absolute quantitation of virus released from the transfected cells. PBS mutant proviral DNA showed a low level of virus production in comparison to the wild-type DNA. These results are in sharp contrast to the data recently reported by Rhim *et al.* (32) involving deletion mutational analysis of the PBS. The difference between our data and that of Rhim *et al.* (32) may be due to the difference in the cell system used for transfection studies. In RD cells, HIV has been shown to bud from the cell surface as observed in H9, MOLT-3, MOLT-4, and CEM cells (4). The reduced level of virus production observed could result from a number of events including transcription, RNA stability, translation, and posttranslational processing. The quantitation of the viral structural protein p24, in the form of viral particles released into the medium, showed low levels from mutant DNA-transfected cells. However, the levels of intracellular structural protein of the virus remained the same in both the mutant and wild-type DNA transfected cells, indicating a possible defect in any one of the steps associated with the virion assembly. It has been reported that the RT enzyme and nucleocapsid protein participate in the selection and binding of the tRNA primer to the PBS in the genomic RNA. The leader and replication primer tRNA are highly structured, and hybridization seems unlikely without disruption of secondary structures. A role for the nucleocapsid protein in the unwinding of the RNA structure, which promotes a base pairing interaction between complementary sequences, has been reported (8, 9). In the presence of RT, in addition to nucleocapsid protein, tRNA binds more efficiently to the PBS. It is possible that the PBS mutation interferes with this process, which results in diminished virion morphogenesis.

Concomitant to the data observed in CEM × 174 cells, virus released from the monolayer cells was noninfectious in an assay involving cocultivation of CEM × 174 cells with proviral DNA-transfected RD cells. The lack of infectivity of the virus could result from impaired processing of viral proteins. Studies have shown that the cleavage of the env precursor gp160 into the gp120–gp41 heterodimer is critical for infectivity (33). HIV particles containing uncleaved gp160 due to a mutation are not infectious. Similarly, the HIV-specific protease has to process the p55^{gag} precursor to render the assembled particles infectious (34, 35). To rule out this possibility, analysis of viral proteins in the transfected cells was carried out by using sera from HIV-positive individuals.

The protein profile was similar in both wild-type and PBS mutant DNA-transfected cells.

Biochemical analysis of the viral particles directed by the PBS mutant proviral DNA was carried out to understand the basis of the noninfectious nature of the virus. Hybridization analysis of the viral particles utilizing the probes specific for the HIV-1 genome and the lysine tRNA primer showed that packaging of the viral RNA and the tRNA primer is not affected. The viral particles derived from the PBS mutant were also characterized for the presence of functional RT enzyme. Although functional RT enzyme was detected by using an exogenous template primer, endogenous reverse transcription with the PBS mutant virus was negligible. These results indicated that there is a block at the initiation of reverse transcription in the mutant virus.

The PBS mutation analyzed in this study contains a 2-base insertion, which is located 5 bases downstream of the 5′ end of the PBS. Even though there is evidence for the use of truncated tRNA primers (36–38) or incorrectly processed tRNA (39), the insertion of 2 bases completely eliminated the PBS function in reverse transcription. Because of the homology, tRNA anneals efficiently to the genomic RNA PBS and is able to initiate the reverse transcription process in the wild type (Fig. 6*A*). The lack of endogenous reverse transcription in the mutant virions could be attributed to two possible mechanisms, as depicted in Fig. 6 *B* and *C*. In the first model (Fig. 6*B*), the tRNA primer binds to the genomic RNA, leaving nonhomologous mutant sequences in the PBS as a bulge, which would then lead to successful initiation of reverse transcription. The results obtained with endogenous reverse transcription, however, were in favor of the second model (Fig. 6*C*), where binding occurs only at the 3′ end of PBS with the tRNA molecule. The nonhomology at the 5′ end of PBS, because of the insertion of bases, leaves the 3′ OH termini of the tRNA primer ineffective in priming. Such an observation has also been noted with oligonucleotide primers in the polymerase chain reaction (40).

The exact mechanisms by which alteration in the PBS affects virus production are not known. The observations of increased levels of structural protein p24 inside the cells, dissimilar levels of virus released into the cultural medium of the proviral DNA-transfected cells, and the noninfectious nature of the virus produced by the mutant (pARV-NAR) suggest that the PBS mutation possibly interferes with the processes associated with the virion morphogenesis in addition to abolishing reverse transcription. These results support the notion that the PBS region of the retroviral genome has multiple functions in retrovirus replication (41, 42). Additionally, the noninfectious HIV-1 resulting from the PBS mutation provides an important alternative source of immunization of animals for evaluating vaccine strategies.

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