JOURNAL OF VIROLOGY, Dec. 1996, p. 8348–8354 Vol. 70, No. 12
0022-538X/96/$04.00+0

A Dual Role of the Putative RNA Dimerization Initiation Site of Human Immunodeficiency Virus Type 1 in Genomic RNA Packaging and Proviral DNA Synthesis

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Received 4 April 1996/Accepted 21 August 1996

In retroviruses, the genomic RNA is in the form of a 60S-70S complex composed of two identical genome-length RNA molecules tightly associated through numerous interactions. A major interaction, called the dimer linkage structure, has been found near the RNA 5′ end and is probably involved in the control of translation, packaging, and recombination during proviral DNA synthesis. Recently, a small sequence corresponding to a stem-loop structure located in the 5′ leader of human immunodeficiency virus type 1 (HIV-1) RNA was found to be required for the initiation of HIV-1 RNA dimerization in vitro and named the dimerization initiation site (E. Skripkin, J.-C. Paillart, R. Marquet, B. Ehresmann, and C. Ehresmann, Proc. Natl. Acad. Sci. USA 91: 4945–4949, 1994). To investigate the possible role of this 5′ stem-loop in HIV-1 virion formation and infectivity, four mutant viruses were generated and analyzed in vivo. Results show that deletion of the stem-loop structure reduces infectivity by a factor of 10^3 whereas loop substitutions cause a decrease of 10- to 100-fold. The level of genomic RNA packaging was found to be decreased fivefold in mutants virions containing the stem-loop deletion and only twofold in the loop-substituted virions. Surprisingly, the second DNA strand transfer during reverse transcription was found to be severely impaired upon stem-loop deletion. Taken together, these results indicate that the stem-loop structure called the dimerization initiation site is a *cis* element acting on both genomic RNA packaging and synthesis of proviral DNA.

The genome of retroviruses is formed of two homologous RNA molecules that are tightly associated through numerous interactions (7, 38, 54). Sedimentation studies and electron microscopy of RNA extracted from virions revealed that these RNA molecules are joined noncovalently at a discrete site near the 5′ end of each strand named the dimer linkage structure (DLS) (7, 38). This physical linkage was considered important for the control of several steps in the retroviral life cycle. It was first suggested that genomic RNA dimerization negatively controls *gag* and *gag-pol* expression (5, 10). Indeed, monomeric RNAs from Rous sarcoma virus (RSV) (10) and human immunodeficiency virus type 1 (HIV-1) (5) were translated in vitro more efficiently than dimeric RNAs. Second, dimerization was postulated to act as a positive signal for encapsidation of two genomic RNA molecules, since *cis* elements required for in vitro dimerization of avian, murine, bovine, and human retroviruses (10, 19, 20, 35, 47, 60) were located within an essential region containing the major element required for RNA packaging (E or Psi) (2, 16, 31, 42, 45, 46, 74). In HIV-1, as in other retroviruses, the genomic RNA encapsidation signal was shown to be multipartite (8, 49), with a major element located between the 5′ splice donor (SD) site and the AUG *gag* initiation codon (2, 16, 31, 42) and two other elements located upstream of the SD site (37, 49, 62, 72) and in the 5′ end of *gag* (11, 44, 59). In addition to the *cis* packaging elements, nucleocapsid protein (NC) was shown to act as a positive *trans*-acting factor in genomic RNA packaging in virions (3, 9, 22, 28, 50, 61, 75). Third, the presence of a dimeric genome was found to be important for recombination events that take place during reverse transcription (34, 58, 71). Indeed, the apparent orientation of the two RNA molecules as observed by electron microscopy (7, 38) may facilitate DNA strand switching and thus recombination. This is thought to contribute to the genetic variability of retroviruses and to bypass breaks in genomic RNA via DNA strand switching during reverse transcription (18, 34, 69).

Extensive in vitro experiments showed that in vitro-generated RNAs corresponding to the 5′ end of the retroviral genome can efficiently dimerize (10, 20, 60). Dimerization of RNA fragments containing a region immediately downstream of the SD site could be promoted either by nucleocapsid protein (20, 63) or by cations (4, 47, 70, 73). This region, initially designated the DLS (20, 47), contains purine-rich motifs which were proposed to be involved in the dimerization process through the formation of purine quartets (4, 47, 70). Recently, by chemical modification interference and site-directed mutagenesis, we identified a region of HIV-1 (Mal strain) genomic RNA that promotes initiation of RNA dimerization in vitro (48, 56, 65). This sequence, named the dimerization initiation site (DIS), is located between the primer binding site (PBS) and the 5′ SD site (Fig. 1A) and can adopt a stem-loop structure in vitro (Fig. 1B) (30, 56, 65). It was then proposed that RNA dimer formation is initiated by annealing of the self-complementary 6-base sequence located in the loop of the stem-loop structure (56, 57, 65). This mechanism was shown to hold true for the Lai strain of HIV-1 in vitro (40, 52). However, whereas the in vitro RNA dimerization mechanism has been

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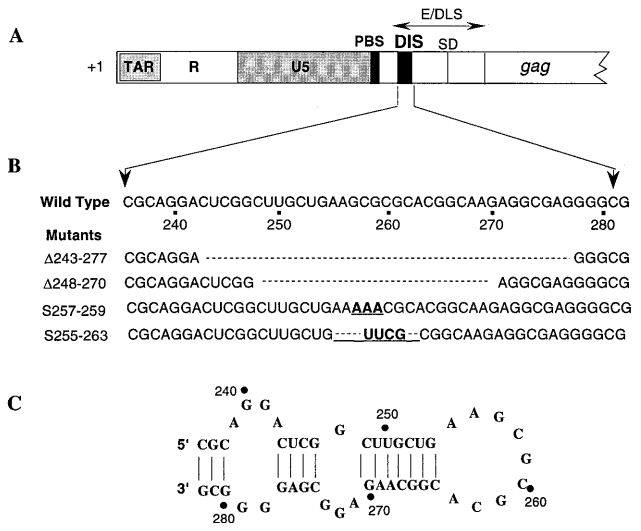

FIG. 1. Wild-type and mutated DIS of HIV-1. (A) Schematic representation of the 5′ end of HIV-1 genomic RNA. R, repeat sequence with TAR (*trans*-acting responsive element); E/DLS, encapsidation-dimerization sequence of the genomic RNA; *gag*, 5′ end of the structural proteins coding sequence. (B) Sequences of wild-type and mutant DIS RNAs. Deletions are indicated by dashed lines; substitutions are in boldface and underlined. Numbers are from +1 of genomic RNA. (C) Secondary structure of the wild-type DIS (30, 65).

well studied, little is known about genomic RNA dimerization and its role in vivo.

To investigate the possible function of this 5′ stem-loop structure, we introduced several mutations into the DIS stem-loop structure of an infectious molecular clone of HIV-1. Effects of these mutations on virus infectivity, RNA encapsidation, and proviral DNA synthesis were investigated. Our data strongly suggest that this stem-loop is part of the encapsidation signal and appears to be required for efficient proviral DNA synthesis.

MATERIALS AND METHODS

HIV-1 DNA mutagenesis. The *Aat*II-*Sph*I fragment of the pNL4.3 HIV-1 molecular clone (1) was introduced in M13mp19, and in vitro site-directed mutagenesis was carried out on the single-stranded DNA by the procedure of Kunkel (39). The mutated oligonucleotides were Δ243.277 (5′ GTCGCCGCCC TCCTGCGTCG 3′), Δ248.270 (5′ CCCCTCCGCCTCCGAGTCCTGC 3′), S257.259, (5′ GCCGTGCG**TTT**TTCAGCAAGC 3′), and S255.263 (5′ CCTCT TGCCC**GCGAAC**AGCAAGCCG 3′). The underlined nucleotides represent the borders of the deletions, and the boldface nucleotides correspond to substitutions. All mutations were checked by sequencing (64), and fragments were reinserted into pNL4.3 DNA. Positions of deletions and substitutions are shown in Fig. 1B, and numbers correspond to the position on HIV-1 genomic RNA.

Cell cultures and HIV-1 DNA transfection. Cos7 cells used for transfection experiments were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), and 2 mM glutamine. DNA transfection was performed by a modification of the calcium phosphate procedure (13), using 20 μg of plasmid DNA for 10^6 cells in 10 ml. Three days after transfection, 24-h supernatants were harvested and clarified by low-speed centrifugation (15,000 × *g* for 10 min). Infectivity and viral titers were determined as previously described (55). Viruses harvested were used to infect SupT1 lymphocytic T cells (66) which were grown in RPMI 1640 supplemented as for Cos7 cells.

ELISA and RT assays. To normalize supernatants to an equal number of viral particles prior to infection of SupT1 cells and to compare individual clones, the amount of CA p24 core antigen was determined by enzyme-linked immunosorbent assay (ELISA) (kindly provided by V. Cheney and B. Mandrand, Biomérieux, Lyon, France). Determination of reverse transcriptase (RT) activity was performed by using a modification of the protocol initially described by Goff et al. (27).

Viral protein analysis. Viral particles were pelleted after transfection by ultracentrifugation through a 20% sucrose cushion at 30,000 rpm for 1 h at 4°C and dissolved in 25 mM Tris-HCl (pH 7.5)–50 mM NaCl–1 mM EDTA–1% sodium dodecyl sulfate (SDS). For analysis of the viral proteins in transfected cells, cells were washed twice in cold phosphate-buffered saline solution and scraped. The pellet was suspended in 80 μl of 20 mM Tris-HCl (pH 8.8)–2 mM $CaCl_2$. Cells were lysed by adding 9 μl of a solution containing 10% β-mercaptoethanol and 3% SDS, heated 2 min at 95°C, and immediately thawed on ice. Samples were treated with 5 μl of DNase solution (0.5 M Tris-HCl [pH 7], 50 mM $MgCl_2$, 0.67 mg of DNase per ml) and stored at −20°C before use.

Proteins were fractionated through a 10% polyacrylamide gel (acrylamide/bisacrylamide, 40:1.3) containing 2% SDS and electroblotted onto nitrocellulose membrane (Hybond C+; Amersham) in Tris-glycine (pH 6.5) buffer with 40% methanol. Proteins were detected by immunoblotting with a chemiluminescence system (ECL kit; Amersham), using anti-CA p24, anti-MA p17, and anti-gp120 monoclonal mouse antibodies.

HIV-1 genomic RNA analysis. (i) Viral RNA preparation from infected cells. Viral pellets were prepared from 60 ml of supernatants of SupT1 cells 2 weeks after infection and centrifuged as described above. Viral pellets were lysed and RNA was extracted as already described (55). Samples were serially diluted and immobilized onto a nitrocellulose membrane by using a slot blot manifold (Schleicher & Schuell, Inc.). HIV-1 RNA was fixed by UV irradiation and probed with a ^{32}P-labeled DNA made by random-primer labeling of a 5.3-kb *Sac*I-*Sal*I fragment of plasmid pNL4.3 (corresponding to *gag* and *pol* sequences), using the conditions specified by the manufacturer (Appligene-Oncor). Membranes were washed as previously described (64). The virus-specific RNA in both cases was quantified by integrating intensity of the bands with a Bio-Imager apparatus (Fuji).

(ii) Viral RNA preparation from transfected cells. The protocol described above was used except that the viral pellet was prepared from 10 ml of supernatant of Cos7 cells 72 h after transfection.

Preparation of total cellular DNA. Viruses recovered upon DNA transfection of Cos7 cells were used to infect SupT1 cells (10^6 cells in 5 ml of RPMI 1640 in the presence of Promega RQ1 DNase [5,000 U/ml] to remove contaminating pNL4.3 DNA). Five hours after infection, cells were centrifuged for 5 min at 1,000 rpm at 20°C and washed with 1 ml of phosphate-buffered saline solution to remove extracellular virions. Twenty hours after infection, cells were washed and lysed in the presence of proteinase K (100 μg/ml). After RNase treatment, phenol-chloroform extraction, and ethanol precipitation, total DNA was resuspended in H_2O, and an aliquot was analyzed by electrophoresis on a 0.8% agarose gel to examine the integrity of the preparation. Prior to analysis, DNA was digested with *Dpn*I to cleave residual contaminating plasmid DNA.

PCR amplification and analysis of newly synthesized viral DNA. Total DNA from 5×10^4 cells at 20 h postinfection was amplified by 30 cycles of PCR with different primer pairs designed to permit the detection of DNA at different stages of reverse transcription. PCR primer pairs were as follows: U3.18 (sense; GGACTTTCCAGGGAGGTG) and MA.20 (antisense; TGATGCACACAAT AGAGGAC); U3.18 (sense) and U5.20 (antisense; CTGCTAGAGATTTTCC ACA); and pPBS (sense; ACTTGAAAGCGAAAGTAAAGC) and MA.20 (antisense). The thermocycling temperatures used for all sets of primers were 94°C for 30 s, 59°C for 30 s and 72°C for 60 s. Semiquantitative PCR was performed simultaneously with standards containing known amounts of pNL4.3. PCR products were electrophoresed on a native 1.2% agarose gel (supplemented with ethidium bromide [0.5 μg/ml]). Viral DNA bands were detected by UV, and quantification of each band was performed with an Image Analyzer (Bio-Rad).

RESULTS

Construction of HIV-1 5′ stem-loop mutants. To examine the role of the 5′ stem-loop structure called DIS in virion formation and infectivity, mutations were created in DIS of the HIV-1 pNL4.3 molecular clone (Fig. 1A and B). Mutations were designed to minimize structural perturbations in this region. The two deletions (Δ243–277 and Δ248–270) removed part of the stem-loop structure (Fig. 1C). Three nucleotides in the loop (S257–259) were substituted by AAA, or the nine-nucleotide loop (S255–263) was replaced by a UNCG loop. Such tetraloops were shown to be very stable in several RNA structures (14). These mutations were investigated in vitro and found to impair HIV-1 RNA dimerization (56, 65).

Expression and processing of HIV-1 proteins. Cos7 cells were transfected with wild-type or mutant pNL4.3 clones. Synthesis and processing of the Gag polyprotein precursor were analyzed by immunoblotting. Similar amounts of Pr55^{gag} precursor were expressed in Cos7 cells transfected with wild-type and HIV-1 mutants (Fig. 2A). To characterize the Gag proteins present in the viral particles, Western blot (immunoblot) analyses were performed on virions pelleted from the supernatant of transfected cells, using antibodies directed against CA p24 and MA p17 (Fig. 2B). A wild-type pattern of viral

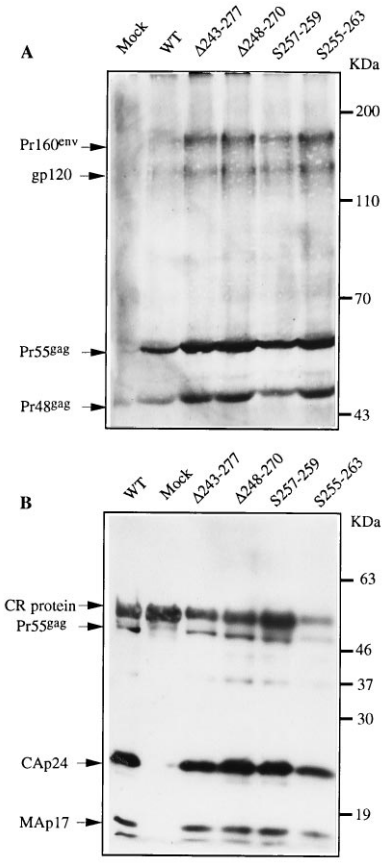

FIG. 2. Analysis of viral proteins. (A) Analysis of viral proteins synthesized in Cos7 cells upon transfection with wild-type and mutants DIS clones. Viral proteins were extracted as described in Materials and Methods, separated on an SDS–10% polyacrylamide gel, and electrotransferred. The membrane was probed first with anti-CA p24 and subsequently with antibodies against SU gp120. (B) Analysis of the structural Gag proteins in viral particles produced by cells transfected with HIV-1 wild type and DIS mutants. Cells were lysed; proteins were separated as described above and detected by immunoblotting with anti-CA p24 and anti-MA p17 monoclonal antibodies. In all lanes, these antisera cross-reacted with a ≈60-kDa protein (CR protein) that probably corresponds to albumin. Lanes represent the wild type (WT), negative control (Mock), and HIV-1 mutants (Δ243–277, Δ248–270, S257–259, and S255–263). Positions of HIV-1 proteins (left) and molecular weight markers (right) are indicated. The differences observed between the wild type and mutants (A) and between mutant S255–263 and other mutants or the wild type (B) are due to the lower amount of sample loaded on the gel.

Gag proteins was found in all stem-loop mutant viruses. Similarly, the presence and integrity of the Env precursor $Pr160^{env}$ and the $gp120^{env}$ protein were examined in transfected cells (Fig. 2A) and pelleted viruses (data not shown). With respect to the Gag proteins, no differences were detected between wild-type and mutant viruses. RT levels were determined by monitoring RT activity in the supernatant of transfected cells and found to be identical for all viruses. Thus, mutations in the 5′ stem-loop structure did not significantly affect assembly and maturation of viruses.

Infectivity of the HIV-1 stem-loop mutants. To assess the viral titers of wild-type and mutant viruses, human SupT1 cells were infected with serial dilutions of cell-free medium harvested from Cos7 culture (normalized to identical levels of CA p24). The viral titer was defined as the highest dilution able to infect SupT1 cells as measured by cytopathic effects or syncytium formation (Table 1). The Δ243–277 and Δ248–270 mutant viruses were poorly infectious, having titers of approximately 10 IU/ml; i.e., they were 10^3-fold less infectious than the wild-type virus. The S257–259 and S255–263 mutants had titers of 10^2 and 10^3 IU/ml, respectively; i.e., they were 10- to 100-fold less infectious than the wild-type (Table 1). These results were confirmed by measuring RT activity in SupT1 cell supernatant (data not shown). The genotypes of all four mutant viruses were investigated 4 weeks after infection by PCR amplification of the viral DNA on a fragment encompassing the 5′ stem-loop. Sequencing of the amplified products did not reveal any changes or reversion of the mutated sequences. These data indicate that the four HIV-1 mutant viruses displayed decreased infectivity, the more drastic effect being observed with the stem-loop deletion mutants.

Genomic RNA content of the HIV-1 5′ stem-loop mutant virions. The impaired infectivity observed for the various mutants might reflect a defect in the viral RNA content such as inefficient encapsidation. Since virus formation and genomic RNA packaging probably depend on the cell types used and transfection versus infection, virion genomic RNA content was determined in supernatants of transfected Cos7 and infected SupT1 cells. Virus RNAs were extracted from wild-type and mutant virions and immobilized onto nitrocellulose membranes, and viral RNAs were detected by hybridizing a labeled 5.3-kb *Sac*I-*Sal*I fragment corresponding to *gag-pol* sequences. The level of genomic RNA extracted from virions obtained upon cell transfection failed to reveal any significant differences between wild-type and mutant viruses (data not shown). However, HIV-1 mutant virions produced by infected T cells contained substantially less viral RNA than the wild-type virions (Fig. 3A). The average RNA content in Δ243–277 and Δ248–270 viruses, determined in three independent experiments, was about fivefold lower than in wild-type virus (Fig. 3B). In substitution mutant particles S257–259 and S255–263, the decrease was only twofold. These results indicate that the 5′ stem-loop structure contributes to the genomic RNA packaging signal. Integrity of the virion genomic RNA was investigated by Northern (RNA) blotting experiments, and results indicate that genomic RNA was mostly intact in wild-type and mutant virions (results not shown).

Analysis of HIV-1 reverse transcription products. To further investigate the nature of the defect of the DIS mutant viruses, we analyzed viral DNA synthesized following infection of SupT1 cells, using equal amounts of wild-type and mutant viruses obtained from transfected Cos7 cells. It should be noted that wild-type and mutant viruses produced by transfected Cos7 cells contain similar levels of genomic RNAs (see above). Total DNA harvested 20 h after infection was subjected to PCR amplification using primer pairs designed to detect viral DNA at different stages of reverse transcription (see Materials and Methods; locations of the primer pairs are shown in Fig. 4).

HIV-1 DNA corresponding to the 5′ long terminal repeat

TABLE 1. Virus titers of HIV-1 DIS mutants[a]

Virus	Titer (no. of infectious particles/ml)
Wild type	10^4
Δ243–277	10
Δ248–270	10
S257–259	10^2
S255–263	10^3

[a] SupT1 cells were cultured with serial 1:10 dilutions of supernatant from transfected Cos7 cells. Virus titer was defined as the last infecting dilution leading to syncytium formation.

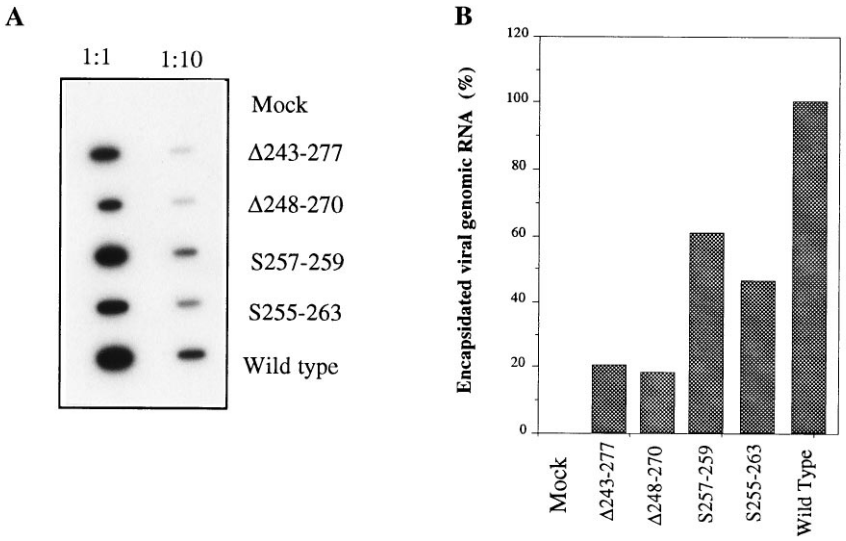

FIG. 3. Slot blot analysis of viral RNA packaged in wild-type and mutants virions produced by infected cells. (A) Equivalent amounts of viral RNA (according to p24) extracted from pelleted virions were bound onto a nitrocellulose membrane and detected with an HIV-1-specific *gag-pol* probe. Sample dilutions were loaded as indicated above the lanes. (B) The amounts of virion RNA were quantified with a Bio-Imager (Fuji). All values were normalized relative to the wild type. Wild-type, negative control, and mutant virions are indicated.

was amplified by using primer pairs U3.18 and U5.20. Similar amounts of amplified DNA were detected for wild-type and mutant viruses (Fig. 5a). The differences observed for the Δ243–277 and S255–263 mutants did not appear to be significant. These data indicate that the first-strand transfer was not significantly altered by the stem-loop mutations. Next, HIV-1 DNA corresponding to 5′ *gag* sequences was amplified by using primers MA.20 and pPBS (see Materials and Methods and Fig. 4). These primers amplify only minus-strand DNA containing *gag*, *pol*, *env*, and the 3′ leader sequences. As shown in Fig. 5b, similar amounts of DNA were detected for the wild-type and mutant viruses except for mutant Δ243–277, in which case a twofold decrease in newly synthesized viral DNA was observed (53% versus 100%) (Fig. 5b, lane 2). Finally, primers U3.18 and MA.20 were used to amplify proviral DNA once the second-strand transfer had occurred (Fig. 5c). A 10-fold decrease was observed for the large-deletion mutant (Fig. 5c, lane 2), and a 3-fold reduction was seen for deletion mutant Δ248–270. Substitutions (S257–259 and S255–263) did not significantly alter full-length DNA synthesis (1.5 and 1.25 times less than for the wild type). These quantifications result from an average of three independent experiments. Identical analyses were done with Hirt supernatants (33), and the results confirmed the data for wild-type and mutant viruses (results not shown).

Thus, these findings favor the notion that deletion of the 5′ stem-loop structure corresponding to the putative DIS interfered with proviral DNA synthesis as a result of a lower level of plus-strand DNA initiation or/and of second-strand transfer.

DISCUSSION

A key feature of the retroviral genome is its dimeric nature, which is thought to promote a high level of genetic recombination, ensuring virus viability and variability (18, 21, 26, 34). The DLS represents a major interaction between the two-genome-length RNA and is located in the 5′ leader within the major packaging signal (10, 19, 20, 47, 54, 60, 74). In HIV-1, a stem-loop structure located between the PBS and SD site was shown to initiate dimerization of genomic RNA fragments in vitro and thus named DIS (40, 52, 56, 65). To examine the possible role of this 5′ stem-loop in HIV-1 replication, nucleotide substitutions and deletions in the stem-loop were introduced into an infectious HIV-1 molecular clone. The four mutations were found to alter virus infectivity, the most extensive effect being with stem-loop deletions (a 10^3-fold decrease of infectivity was observed [Table 1]), while loop substitutions decrease viral infectivity by 10- to 100-fold. These findings show that the 5′ stem-loop DIS is functionally important in the course of HIV-1 replication.

Genomic RNA packaging. To determine the origin of the replication defect of the HIV-1 stem-loop mutants, viral particles and proviral DNA synthesis were analyzed. Immunoblots did not detect differences in the virion protein content between wild-type and DIS mutants. Genomic RNA was packaged at similar levels in wild-type and mutant virions produced by transfected Cos7 cells. However, upon T-cell infection, genomic RNA packaging was found to be decreased from twofold (substitution mutants) to fivefold (deletion mutants) (Fig. 3). Therefore, the 5′ stem-loop does not appear to be required per se for encapsidation but is likely to optimize the process. This view is consistent with previous data indicating that the packaging signal of HIV-1 is multipartite (8, 49). A major packaging element, which can adopt a stem-loop structure, is located between the major SD site and the *gag* initiation codon (17, 30, 32). Additional *cis*-acting sequences, located upstream of the SD in the U5 region (62, 72) or in a region including the putative DIS (37, 49) and in *gag* (2, 42, 44, 59), were also found to contribute to HIV-1 genomic RNA packaging. Similar observations were made with RSV (36, 67), murine leukemia virus (MuLV) (6, 53) and bovine leukemia virus (46). The DIS deletion mutants exhibited an RNA encapsidation defect similar to that resulting from a deletion of nucleotides 240 to 252 in HIV-1 genomic RNA (37). Several lines of evidence suggest that genomic RNA dimerization and encapsidation are linked events and that RNA dimerization precedes packaging in HIV-1 (23) and MuLV (24, 43). In contrast, monomeric RNA was found to be encapsidated in RSV, and dimerization was proposed to proceed later (12, 15, 41). However, Stoltzfus and Snyder (68) observed that an unstable RSV RNA dimer was encapsidated and was disrupted during phenol extraction. Re-

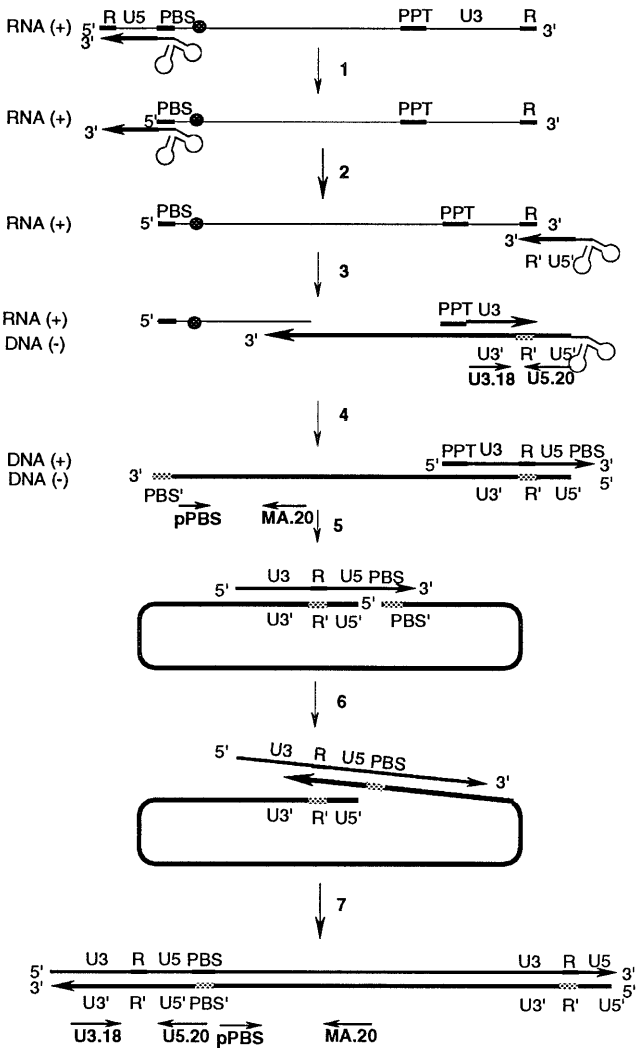

FIG. 4. Models for retroviral reverse transcription. The thin lines represent RNA, thick lines represent DNA, $tRNA_3^{Lys}$ is represented by two hairpins, and the DIS is defined by a full circle. PPT, polypurine tract. Step 1, synthesis of minus-strand strong-stop DNA and RNase H degradation of the RNA template strand. Step 2, first-template switching by hybridization of the repeat (R) region from the minus-strand strong-stop DNA to the same or the second RNA molecule. Step 3, extension of the minus-strand DNA up to the PBS, degradation of the RNA template by RNase H, and initiation of the plus-strand strong-stop DNA from the conventional PPT. Step 4, synthesis of the plus-strand strong-stop and minus-strand DNAs. Step 5, second-template transfer. The minus-strand DNA base pairs with the plus-strand DNA by using PBS complementary sequences transcribed from the tRNA primer by forming a circular intermediate. Step 6, extension of the plus-strand strong-stop DNA and of the minus-strand DNA by displacement along its plus-strand template. Step 7, completion of double-stranded DNA. Horizontal small arrows indicate DNA primers that are used for the detection of intermediate proviral DNA in SupT1 cells (see Materials and Methods).

cently, Haddrick et al. have shown that the HIV-1 dimeric RNA genome with 5′ stem-loop DIS mutations was destabilized, as evidenced by an increased monomer-to-dimer ratio (29).

Proviral DNA synthesis. The slight reduction of genomic RNA packaging (fivefold less than the wild-type level) caused by the two deletions in the putative DIS cannot explain the strong defect in HIV-1 replication. This prompted us to examine viral DNA synthesis in newly infected cells. Synthesis of minus- and plus-strand DNA was analyzed by PCR amplification using appropriate primer pairs (Fig. 4). Results show that none of the mutations impaired minus-strand DNA synthesis corresponding to the 5′ long terminal repeat domain. Synthesis of complete minus-strand DNA was not significantly altered except for the large stem-loop deletion causing a twofold decrease (Fig. 5). The most unexpected result was that plus-strand DNA synthesis was reduced by a factor of 10 for the large stem-loop deletion and by a factor of 3 for the short one. This DNA synthesis defect probably resides at the second-strand transfer level. Since reverse transcription is a complex multistep process probably requiring a spacial architecture of the two nucleic acid templates within the virion nucleocapsid structure, any element bringing in a close proximity the redundant ends of the viral nucleic acids would favor strand transfer. How a defect in this organization, induced by deleting the putative DIS, might alter late events of the process remains a matter of speculation. Nevertheless, the 5′ stem-loop DIS is one of the last RNA regions to be reverse transcribed, and this may not be fortuitous. Accordingly, possible specific nucleic acid structures (within the nucleocapsid), reminiscent of the initial RNA dimer linkage structure, may influence the second-strand transfer. In agreement with this view, it has been shown that the region corresponding to the DLS of MuLV genomic RNA (60) is a hot spot for recombination (51). The other possibility excludes any relationship with RNA dimerization and subsequent events but would implicate the minus-strand

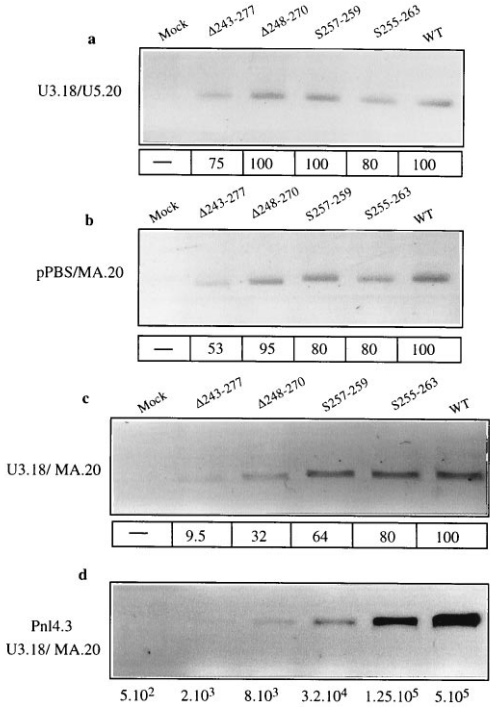

FIG. 5. Detection of viral DNA synthesized in newly infected cells. Sup T1 cells were infected with equal amounts of wild-type and mutant viruses and maintained for 20 h at 37°C. Total HIV-1 cellular DNA was extracted, and PCR was performed with different primer pairs (indicated at the left; see Fig. 4 for their locations). Viral DNAs detected after the first-strand transfer (a), just before the second-strand transfer (b), and after the second-strand transfer (c) were run on a 1.2% agarose gel containing ethidium bromide (0.5 μg/ml). Bands detected were quantified with the Bio-Rad Image Analyzer, and the percentage of each band compared with the wild type is indicated below the gel. Lanes represent the wild type (WT), negative control (Mock), and mutants. (d) PCR amplification using primers U3.18 and MA.20 and known amounts of the pNL4.3 molecular clone. The copy number is indicated under each lane. Linearity of the curve was verified after quantification of each band as described above.

DNA itself, through a *cis*-acting sequence specifying a specific DNA structure or/and a recognition site for a viral factor (e.g., NC protein) required for efficient strand transfer.

Contribution of the 5′ stem-loop forming the putative DIS. An unexpected result was that the stem-loop deletions had more severe consequences on virus infectivity than loop substitutions. However, loop substitutions decreased virus infectivity by a factor of 10 to 100, indicating that the loop has a biological function. In support of this observation, Haddrick et al. reported that the loop-loop interaction is important for genomic RNA dimer formation and/or stability (29). Deletion of another putative candidate for RNA dimerization was found to have no effect (29). Obviously, other elements in the RNA genome or *trans*-acting factors are able to compensate the loop substitutions. Since the deletions of the stem-loop have more dramatic effects, a possible explanation is that melting of the stem and formation of an extended intermolecular interaction can occur in vivo (23, 24). However, this hypothesis was not confirmed by our in vitro physicochemical experiments (57) and by using a *trans*-complementing mutant in vivo (49). Most likely the DIS stem-loop is important not only to provide the loop sequence but also as an intrinsic hairpin structure. In this view, the DIS hairpin was shown to bind NC protein with high affinity (17). Therefore, the ex vivo situation appears to be more complex than in vitro experiments had suggested, and the involvement of the stem in the dimerization or any other process remains an open question that is under investigation.

In conclusion, our results provide evidence for a dual role of the DIS, since it functions as a positive *cis* element allowing efficient encapsidation of the genomic RNA and extensive proviral DNA synthesis. Since the DIS element controls both early and late events of the retroviral cycle, it represents a well-adapted target for the design of an antiviral drug.

ACKNOWLEDGMENTS

This work was supported by grants from the Agence Nationale de Recherches sur le SIDA (ANRS) and the Mutuelle Générale de l'Education Nationale. J.-C.P. is a fellow of the ANRS.

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