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The Importance of the A-Rich Loop in Human Immunodeficiency Virus Type 1 Reverse Transcription and Infectivity

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Nucleotide segment (+169)AAAA(+172) constitutes an A-rich loop within human immunodeficiency virus type 1 (HIV-1) (HXB2D) RNA and is able to interact with the anticodon loop (33)/USUU(36) of primer $tRNA_3^{Lys}$. We have shown that the deletion of this A-rich loop resulted in diminished levels of infectivity and reduced synthesis of viral DNA in MT-2 cells and cord blood mononuclear cells. Endogenous reverse transcriptase (RT) assays revealed that the mutated viruses, termed HIV/del-A, generated fewer cDNA products than did wild-type virus, designated HIV/WT. We also employed in vitro RT assays with in vitro-synthesized viral RNA templates, recombinant HIV-1 RT(p66/51), and natural $tRNA_3^{Lys}$ as primers to show that the mutated RNA templates, designated PBS/del-A, generated less minus-strand strong-stop DNA product than did the wild-type RNA template, designated PBS/WT. The initiation efficiency of reverse transcription from the mutated RNA template was significantly impaired compared with that from the wild-type RNA template when a single-base extension assay from the $tRNA_3^{Lys}$ primer was employed. However, RT reactions performed with DNA oligonucleotides complementary to the primer binding site (PBS) as primers did not yield differences between the mutated PBS/del-A and wild-type RNA templates. Long-term culture of HIV/del-A in MT-2 cells resulted in the replacement of two G's at nucleotide positions 167 and 168 by two A's that possessed the same relationship to the 5′ end of the PBS as did the wild-type A's at positions 171 and 172. In vitro RT assays performed with recombinant enzyme with $tRNA_3^{Lys}$ as the primer showed that the RNA template thus generated, termed PBS/A2, yielded levels of minus-strand strong-stop DNA product similar to those yielded by the wild-type RNA template. Coincidentally, viruses containing A's at positions 167 and 168 were able to replicate with efficiencies similar to those of the wild-type viruses. Thus, the (+169)AAAA(+172) A-rich loop plays a key role in the synthesis of viral DNA.

Retroviral reverse transcription is initiated from a cellular tRNA primer that anneals to a complementary primer binding sequence (PBS) located downstream of the 5′ unique (U5) sequence within the long terminal repeat of the viral RNA genome (6, 20, 24). Specific tRNAs are used to prime the synthesis of viral DNA for different viruses, e.g., $tRNA^{Trp}$ for avian leukosis sarcoma viruses (ALSV) (9), $tRNA^{Pro}$ for murine leukemia virus (8), and $tRNA_3^{Lys}$ for human immunodeficiency virus type 1 (HIV-1) (22). Although viruses with PBS sequences that have been replaced may sometimes be able to utilize alternative tRNAs as primers, the efficiencies of reverse transcription and viral infectivity are low, with long-term culture resulting in the reversion of the mutated PBS to the wild type (5, 16, 29, 32). This suggests that other features of the tRNA primer, in addition to the 3′-terminal 18 nucleotides (nt) complementary to the PBS, are required for the efficient synthesis of viral DNA. Consistently, various studies have shown that viral RNA template sequences in addition to the PBS region can interact with the tRNA primer and that such interactions are important for the efficient synthesis of viral DNA (1, 2, 4, 7, 10, 12, 15, 25, 30, 31, 33, 35).

In the case of ALSV, the PBS is bound to the 3′-terminal 18 nt of primer $tRNA^{Trp}$. In addition, however, the U5-inverted-repeat stem of the ALSV genome pairs with a TΨC loop within primer $tRNA^{Trp}$ to further regulate the synthesis of minus-strand strong-stop DNA [(−)ssDNA] (1, 2, 4). Similarly, chemical and enzymatic analyses with HIV-1 have shown that certain sequences outside the PBS can interact with $tRNA_3^{Lys}$ (10, 12). The result is a specific $tRNA_3^{Lys}$–viral-RNA complex that can be efficiently employed as a template by the reverse transcriptase (RT) of HIV-1 but not by other retroviral RTs, e.g., those of HIV-2 and feline immunodeficiency virus, which also use $tRNA_3^{Lys}$ as a primer (3). In particular, an A-rich loop, (+169)AAAA(+172), upstream of the PBS interacts with the anticodon loop (33)USUU(36) of $tRNA_3^{Lys}$ in a manner dependent on a posttranscriptionally modified nucleoside 5-methoxycarbonylmethyl-2-thiouridine at position 34 of the tRNA (11, 26). The function of this interaction in viral DNA synthesis and virus replication is still unclear. While the A-rich loop has been shown to be important for the specific transition from initiation to elongation in cell-free reverse transcription assays (11), and the replacement of the A-rich loop with the appropriate sequences can generate viruses employing other tRNAs as primers (14, 28), others have reported that this region may not be essential for the generation of (−)ssDNA in studies with recombinant RT (17). Furthermore, viruses containing deletions that included the A-rich loop possessed infectivity similar to that of wild-type viruses (27).

To further investigate this subject, we deleted the A-rich loop, (+169)AAAA(+172), and assessed the effects of this change with both virus replication and cell-free reverse tran-

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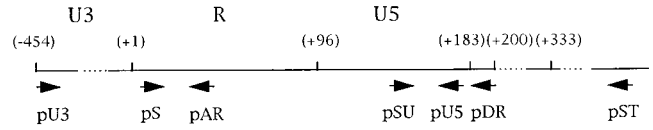

pU3: -454-TGGAAGGGCTAATTCACTCCC- -434

pS: +14-AGACCAGATCTGAGCCTGGGAG-+35

pAR: +78-TTATTGAGGCTTAAGCAGTGG-+57

pSU: +119-GTGTGACTCTGGTAACTAG-+137

PU5: +168-CCACACTGACTAAAAGGG-+150

pDR: +200-GTCCCTGTTCGGGCGCCA-+183

pST: +968-CATTCTGCAGCTTCCTCATTGATGG-+944

FIG. 1. Schematic illustration of the location of primers utilized in these experiments.

scription assays. We now show that the deletion of the A-rich loop resulted in diminished viral infectivity, which was due to the reduced synthesis of viral DNA. Cell-free experiments revealed that the A-rich loop was required for the efficient initiation of the synthesis of (−)ssDNA. In addition, long-term culture of the deletion-containing viruses, termed HIV/del-A, in MT-2 cells resulted in the partial restoration of the A-rich loop and the efficient synthesis of (−)ssDNA.

MATERIALS AND METHODS

Cells and virus infections. MT-2 and COS-7 cells were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, each medium being supplemented with 10% fetal calf serum. Cord blood mononuclear cells (CBMC) were purified by Ficoll-Hypaque density gradient centrifugation and grown in supplemented RPMI 1640 medium. CBMC were stimulated with 0.1% phytohemagglutinin (Gibco Products Inc., Mississauga, Ontario, Canada) for 7 days before being infected with HIV-1.

Virus stocks were prepared by transfecting COS-7 cells with HIV wild-type DNA (HIV/WT) or with HIV DNA with the A-rich loop deleted (HIV/del-A) (17), in the presence of calcium phosphate (23). Production of progeny virus was assessed by measuring levels of p24 (CA) antigen released into culture fluids 48 h after transfection. Similar amounts of virus, based on p24 and RT levels (i.e., 3 ng of p24 antigen/10^6 cells, equivalent to approximately 10^5 cpm of RT activity), were treated with RNase-free DNase I in the presence of 10 mM $MgCl_2$ and were then used to infect MT-2 cells or CBMC. After 2 h, cells were washed twice with serum-free RPMI 1640 and were then maintained in serum-supplemented medium. Culture fluids were collected at various times for determinations of RT activity.

Use of PCR to verify synthesis of viral DNA in infected cells. Six million MT-2 cells or CBMC were infected with viruses containing 20 ng of p24 that had been pretreated with 200 U of RNase-free DNase I in the presence of 10 mM $MgCl_2$ for 30 min at 37°C to eliminate potentially contaminating plasmid DNA. At the same time, cells were exposed to heat-inactivated viruses as a negative control. After 2 h, the cells were washed twice with serum-free RPMI 1640 and were divided equally into two tubes containing supplemented RPMI 1640 medium. Cells were collected 3 and 8 h later, i.e., 5 and 10 h after infection, and suspended in lysis buffer containing 0.5% sodium dodecyl sulfate and 1 mg of protease K per ml. After 6 h at 37°C, the lysed suspensions were extracted twice with phenol-chloroform (1:1) and precipitated with 2.5 volumes of ethanol. The recovered total cellular DNA was resuspended in 20 μl of double-distilled water, and 2 μl was used for 20-cycle PCR studies under the following conditions: 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s. The primers employed were 5′-end-labelled pS [(+14)AGACCAGATCTGAGCCTGGGAG(+35)] and pU5 [(+168)CCA CACTGACTAAAAGGG(+150)] (Fig. 1), which generated a 155-bp (−)ssDNA product that was analyzed on 6% nondenaturing acrylamide gels.

One limitation of this analysis is that PCR methodology cannot monitor full-length copies of viral DNA. For this reason, we have focused our efforts on the synthesis of early-stage (−)ssDNA product, which is made prior to the first template switch event.

Primer extension assays. Culture fluids (30 ml) from transfected COS-7 cells were clarified at 3,000 rpm for 30 min at 4°C in a Beckman GS-6R centrifuge, following which the viruses were pelleted through a 20% sucrose cushion at 40,000 rpm for 1 h at 4°C with an SW41 rotor in a Beckman L8-M ultracentrifuge. Virus pellets were suspended in TN buffer (50 mM Tris HCl [pH 7.5], 10 mM NaCl). Viral RNA was extracted from virus containing either 20, 100, or 500 ng of p24 antigen and treated with RNase-free DNase I, which was removed by phenol-chloroform extraction. Viral RNA was precipitated with 1 volume of isopropanol, after which 5′-end-labelled primer pAR [(+78)TTATTGAGGCT-TAAGCAGTGG(+57)] (Fig. 1) was annealed onto viral RNA and extended in a reaction mixture (20 μl) containing 50 mM Tris HCl, 60 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.2 mM deoxynucleoside triphosphate (dNTP), 10 U of avian myeloblastosis virus RT, and 20 U of RNA guard for 30 min at 37°C. Products were analyzed on 5% denaturing acrylamide gels.

Endogenous RT assays. Viruses collected from transfected COS-7 cells were treated with DNase I to eliminate potentially contaminating plasmid DNA and used to infect MT-2 cells. When significant numbers of MT-2 cells presented with cytopathology, viruses were harvested by ultracentrifugation as described above. Viral preparations containing 30 ng of p24 were used in endogenous assays in 50 μl containing 50 mM Tris HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 0.2 mM dNTP, and 0.05% Nonidet P-40 at 37°C for 4 h (21). As a negative control, heat-inactivated viruses were studied at the same time. Reaction mixtures were extracted with phenol-chloroform and precipitated with 2.5 volumes of 95% ethanol.

DNA products were amplified by PCR for 15 cycles as follows: 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min. pU5 and 5′-end-labelled pS were employed as primers to generate a 155-bp (−)ssDNA fragment; pU3 (−454)TG GAAGGGCTAATTCACTCCC(−434) (Fig. 1) and 5′-end-labelled pAR were used to generate a 532-bp DNA product. PCR products were analyzed on 6% nondenaturing acrylamide gels.

DNA sequencing of the A-rich loop region of long-term-cultured mutated viruses. MT-2 cells infected by mutated HIV/del-A, which had been treated with RNase-free DNase I, were maintained in supplemented RPMI 1640 medium. When about 30% of cells presented with cytopathology, culture fluids were collected in order to initiate another round of infection. At the same time, cellular DNA was extracted from infected MT-2 cells as described above. PCR was performed to amplify the region containing the A-rich loop with primers pS and pST [(+968)CATTCTGCAGCTTCCTCATTGATGG(+944)] (Fig. 1). PCR products were digested with *Bgl*II and *Pst*I and cloned into a pSP72 vector (Promega, Madison, Wis.). At least 10 different clones containing the PCR product were sequenced to verify the presence of the A-rich loop.

Construction of RNA expression plasmids and preparation of HIV-1 RNA. Construction of PBS/WT and PBS/del-A were as described previously (17). Plasmid PBS/A2, which contains two A's at positions +167 and +168, was derived from the above-mentioned PCR product by cloning. To prepare RNA transcripts, plasmids were linearized with *Acc*I and used as templates in a Mega-Scripts kit (Ambion, Austin, Tex.) according to the manufacturer's instructions.

RT with in vitro-synthesized viral RNA templates. One picomole of natural $tRNA_3^{Lys}$ from human placenta (13) was placed onto one picomole of HIV RNA template by denaturing a mixture of the two at 85°C for 5 min and annealing at 55°C for 10 min in a volume of 12 μl containing 83 mM Tris HCl (pH 7.5) and 125 mM KCl. After this solution was cooled to 37°C, other reagents were added to constitute a mixture containing 50 mM Tris HCl (pH 7.5), 75 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 200 μM dATP, 20 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1 μl of [α-^{32}P]dCTP (specific activity, 4,500 Ci/mmol), 20 U of RNA guard, and 50 ng of HIV-1 wild-type RT (p66/51) (34). Reactions were terminated at different times (i.e., 15 and 30 s and 1, 2, 4, and 16 min) by adding EDTA at a final concentration of 50 mM. After extraction with phenol-chloroform, reaction mixtures were precipitated with 2.5 volumes of ethanol. Recovered products were boiled in formamide denaturing buffer for 5 min and fractionated in 5% denaturing polyacrylamide gels containing 7 M urea.

The same reactions were also performed with a DNA oligonucleotide, pDR [(+200)GTCCCTGTTCGGGCGCCA(+183)] (Fig. 1), complementary to the PBS as a primer instead of $tRNA_3^{Lys}$.

To specifically study the initiation of (−)ssDNA synthesis with natural $tRNA_3^{Lys}$ as a primer, reactions were performed as described above except that only [α-^{32}P]dCTP (specific activity, 4,500 Ci/mmol) was utilized. Under these conditions, only one nucleotide, C, could be extended from annealed $tRNA_3^{Lys}$.

PCR to verify production of (−)ssDNA product. Reverse transcription reactions were performed as described above with natural $tRNA_3^{Lys}$ as a primer except that nonlabelled dNTPs were employed. PCR amplifica tions were carried out with 2 μl of reaction product, with primers pSU[(+119)GTGTGACTCTGGTAACTAG(+137)] (Fig. 1) and 5′-end-labelled pU5 for 10 cycles as follows: 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s. The 50-bp DNA products generated were analyzed on 6% nondenaturing acrylamide gels.

Placement of $tRNA_3^{Lys}$ onto the RNA template. One picomole of 5′-end-labelled $tRNA_3^{Lys}$ was incubated with one picomole of HIV RNA template in 12 μl containing 83 mM Tris HCl (pH 7.5) and 125 mM KCl for 5 min at 85°C and then for 10 min at 55°C. Five microliters of reaction mixture was analyzed on 6% nondenaturing acrylamide gels.

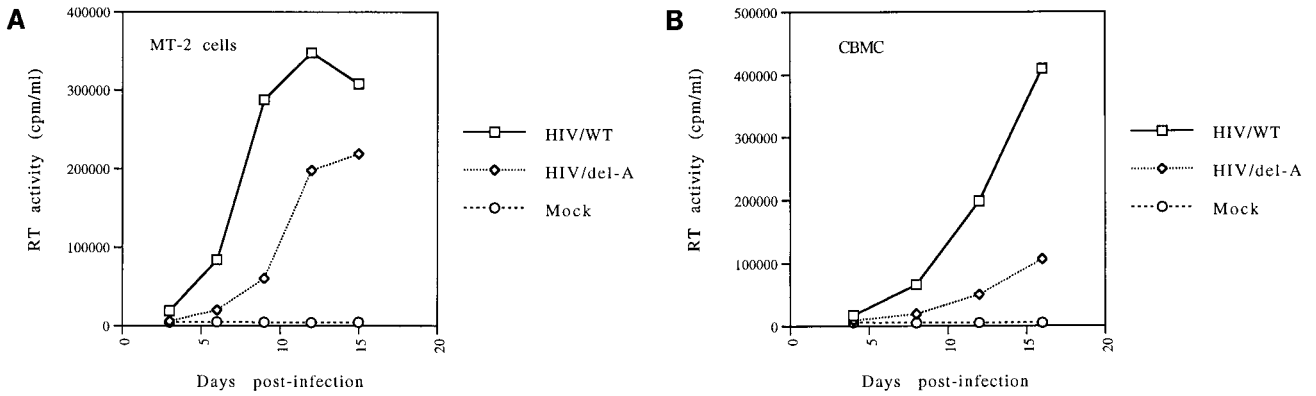

FIG. 2. Infection of MT-2 cells (A) or of CBMC (B) with viruses harvested from transfected COS-7 cells. One million cells were infected with HIV/del-A or HIV/WT, equivalent to 3 ng of p24 antigen, and virus production was monitored by RT assay.

RESULTS

Impact of deletion of the A-rich loop on viral infectivity. We deleted the A-rich loop, (+169)AAAA(+172), upstream of the PBS from HIV-1 proviral DNA (17). Constructs containing either HIV/WT or HIV/del-A were transfected into COS-7 cells. After 48 h, virus in culture fluids was standardized on the basis of p24 levels and viruses containing 12 ng of p24 antigen were used to infect 4×10^6 MT-2 cells as described in Materials and Methods. The results shown in Fig. 2A indicate that MT-2 cells infected by HIV/del-A generated less progeny virus, as measured by RT activity in culture fluids, than did those infected by wild-type viruses, e.g., a fivefold difference was present after 9 days. In similar studies, when 30 ng of viral p24 antigen was used to infect 10^7 phytohemagglutinin-prestimulated CBMC, HIV/del-A replicated more slowly than wild-type viruses (Fig. 2B). Therefore, deletion of the A-rich loop impacted virus replication negatively.

Diminished production of viral DNA in cells infected by mutated viruses. To understand how the A-rich loop might influence viral replication, we used PCR analysis to study the synthesis of viral DNA at early stages of infection.

Either MT-2 or CBMC were infected with HIV/del-A or wild-type viruses. Total cellular DNA was extracted after 5 or 10 h, and PCR was used to amplify a viral DNA fragment from +14 to +168. Mock controls with heat-inactivated viruses yielded no PCR product (Fig. 3A and B), indicating that potentially contaminating plasmid DNA, eliminated by treatment with DNase I, had not interfered with the assay. The results in Fig. 3A show that wild-type viruses generated three- to fourfold more such DNA than did mutated HIV/del-A in MT-2 cells. In CBMC, the mutated viruses yielded six- to eightfold less viral DNA than did wild-type viruses (Fig. 3B).

We next assessed whether the deletion of the A-rich loop might result in deficient packaging of genomic RNA, since this could have caused the impaired synthesis of viral DNA described above. Viral RNA was extracted from viral suspensions containing either 20, 100, or 500 ng of p24 antigen and quantified by primer extension assay. The results shown in Fig. 3C indicate that both the wild-type and the mutated viruses contained similar levels of RNA for each preparation evaluated. Therefore, the diminished synthesis of viral DNA in cells infected with HIV/del-A must have resulted from deficient reverse transcription.

Use of endogenous RT assays to study production of viral DNA. To further study the role of the A-rich loop in the synthesis of viral DNA, endogenous RT assays were performed with wild-type and mutated viruses collected from infected MT-2 cells. Viruses produced by transfected COS-7 cells were not studied because of potential contamination with plasmid DNA. To ensure that the viruses harvested from the infected MT-2 cells still contained the A-rich loop deletion, total cellular DNA was extracted from the infected MT-2 cells. Se-

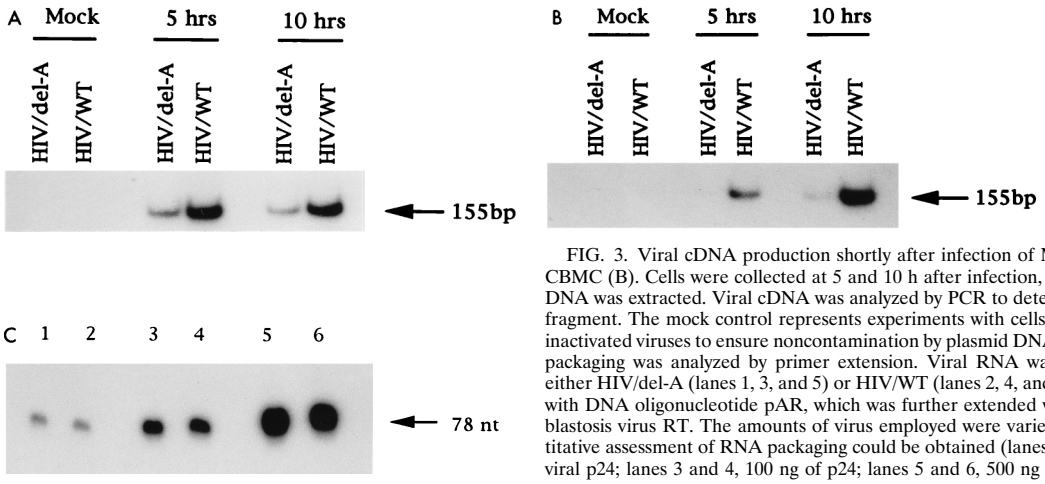

FIG. 3. Viral cDNA production shortly after infection of MT-2 cells (A) or CBMC (B). Cells were collected at 5 and 10 h after infection, and total cellular DNA was extracted. Viral cDNA was analyzed by PCR to detect a 155-bp DNA fragment. The mock control represents experiments with cells exposed to heat-inactivated viruses to ensure noncontamination by plasmid DNA. (C) Viral RNA packaging was analyzed by primer extension. Viral RNA was extracted from either HIV/del-A (lanes 1, 3, and 5) or HIV/WT (lanes 2, 4, and 6) and annealed with DNA oligonucleotide pAR, which was further extended with avian myeloblastosis virus RT. The amounts of virus employed were varied so that a quantitative assessment of RNA packaging could be obtained (lanes 1 and 2, 20 ng of viral p24; lanes 3 and 4, 100 ng of p24; lanes 5 and 6, 500 ng of p24).

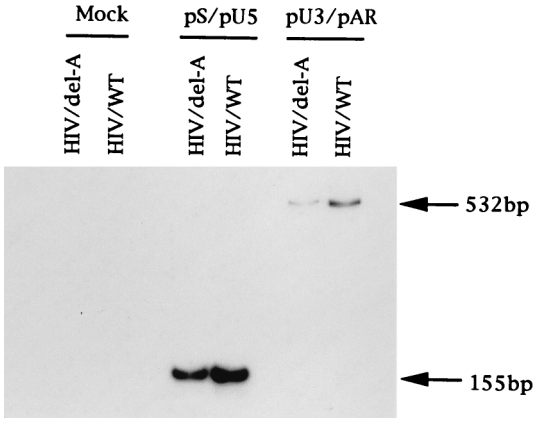

FIG. 4. Viral cDNA production in endogenous RT assays, as analyzed by 15-cycle PCR with primer pairs pS/pU5 and pU3/pAR. These primer pairs amplify DNA products prior to and after the first template switch, respectively. The mock control represents reactions performed with heat-inactivated viruses.

quence analysis showed that the A-rich loop was absent from DNA in cells infected by HIV/del-A.

The cDNA products generated in endogenous reactions were amplified by PCR with primer pair pS and pU5 to amplify (−)ssDNA of 155 bp or with primer pair pU3 and pAR to amplify viral cDNA of 532 bp that was made after the first template switch. Heat-inactivated viruses were utilized as a mock control and yielded no detectable products in our 15-cycle PCR. PCR with both sets of primers showed that HIV/del-A produced two- to threefold less viral DNA than did the wild type (Fig. 4). Therefore, these endogenous RT assays further support the notion that deletion of the A-rich loop can negatively modulate viral reverse transcription.

Cell-free RT assays for generation of (−)ss viral DNA. We also wished to study the efficiency of the synthesis of viral (−)ssDNA in cell-free reactions performed with wild-type recombinant HIV-1 RT (p66/51), natural $tRNA_3^{Lys}$ primer, and in vitro-synthesized RNA template with the A-rich loop deleted. While reactions performed with PBS/WT and PBS/del-A RNA templates yielded similar levels of full-length (−)ssDNA product over 16-min incubations, more partially extended DNA products were observed with PBS/WT than with PBS/del-A (Fig. 5A, lanes 6 and 12). In addition, reactions for shorter times, i.e., 15 and 30 s and 1, 2, and 4 min, showed that wild-type template produced more DNA product than did mutated RNA (Fig. 5A, see especially the 4-min time point, lanes 5 and 11). In contrast, when an 18-nt DNA oligonucleotide (pDR) complementary to the PBS was employed as a primer, similar patterns of viral DNA production were observed with both wild-type and mutated RNA templates at all time points (Fig. 5B).

PCRs were also performed to amplify a DNA segment at positions +119 to +168 in order to quantify both partially and fully extended viral DNA products generated with $tRNA_3^{Lys}$. The results shown in Fig. 5C indicate that reverse transcription reactions involving mutated PBS/del-A RNA template produced less total DNA than did wild-type template, consistent with the data shown above on the generation of (−)ssDNA.

Figure 5A shows that significantly less extended DNA product was generated with PBS/del-A than with PBS/WT, which implied that the defective initiation of reverse transcription might have resulted from deletion of the A-rich loop. To further investigate whether the A-rich loop might influence the initiation of (−)ssDNA synthesis, single-nucleotide extension reactions were performed, with [α-^{32}P]dCTP as the only nucleotide available to be extended from the annealed $tRNA_3^{Lys}$ primer. The results shown in Fig. 5D indicate that reactions performed with PBS/del-A RNA template generated less extended product than those with wild-type RNA at each time point studied.

Conceivably, the deletion of the A-rich loop might have caused less $tRNA_3^{Lys}$ to be placed onto the mutated RNA template PBS/del-A after heating, resulting in fewer initiation products. Therefore, heat-denatured 5′-end-labelled $tRNA_3^{Lys}$ was incubated with a mutated or wild-type RNA template as described in Materials and Methods, and complex formation was analyzed on nondenaturing gels. The results shown in Fig. 5E indicate that comparable levels of $tRNA_3^{Lys}$ were associated with the mutated and wild-type RNA templates. Thus, the deletion of the A-rich loop impeded the initiation of the synthesis of (−)ssDNA and not the placement of $tRNA_3^{Lys}$ onto the viral RNA template.

Partial restoration of deleted A's after long-term culture of mutated viruses in MT-2 cells. Since HIV/del-A replicated less efficiently than did wild type viruses, HIV/del-A was passaged in MT-2 cells, as described in Materials and Methods, to select for potential revertants with a higher proliferation capacity.

After various numbers of passages, cellular DNA was extracted for PCR amplification of viral DNA containing the A-rich loop region, and this DNA was sequenced. The results shown in Table 1 indicate that five of eight clones of viral DNA that were sequenced after eight passages possessed (+167)AA(+168) in place of (+167)GG(+168), while three other clones were unchanged. By 11 passages, all 11 viral DNA clones studied contained this change. After 20 passages, a further substitution, of +166T with +166A, was observed in 1 of 12 clones, and 2 of 12 clones sequenced contained the latter substitution after passage 26. Thus, the virus population became relatively stabilized after two G's mutated to A's at positions 167 and 168.

The first two A's to reappear possessed the same relationship to the PBS as did the original A-rich loop, i.e., the 3′ terminal A was 10 nt upstream of the PBS in each case. We therefore sought to determine whether an RNA template containing the two A's at positions 167 and 168 (PBS/A2) would be active in our in vitro RT assays. The results shown in Fig. 6A indicate that template PBS/A2 generated levels of (−)ssDNA product similar to those of wild-type RNA at each time point evaluated. This finding was confirmed by PCR results regarding the amplification of the viral DNA segment from +119 to +168 and the quantification of total (−)ssDNA product (Fig. 6B). In addition, the viruses containing these restored A's (HIV/A2) were able to replicate with an efficiency similar to that of HIV/WT in MT-2 cells and CBMC in experiments in which cells were infected under the same conditions as those employed for experiments whose results are shown in Fig. 2 (Fig. 6C and D).

DISCUSSION

We have studied the role of the A-rich loop in HIV-1 DNA synthesis in both in vivo and in vitro systems. Deletion of the A-rich loop resulted in the diminished synthesis of viral DNA in both MT-2 cells and CBMC as well as reduced levels of viral infectivity. In addition, in vitro RT assays showed that the mutated RNA template, designated PBS/del-A, generated less (−)ssDNA than did the wild-type RNA template. Long-term culture in MT-2 cells of the mutated virus, HIV/del-A, resulted in the reappearance of two to three A's at or near A-rich loop positions. This restored the efficient synthesis of (−)ssDNA.

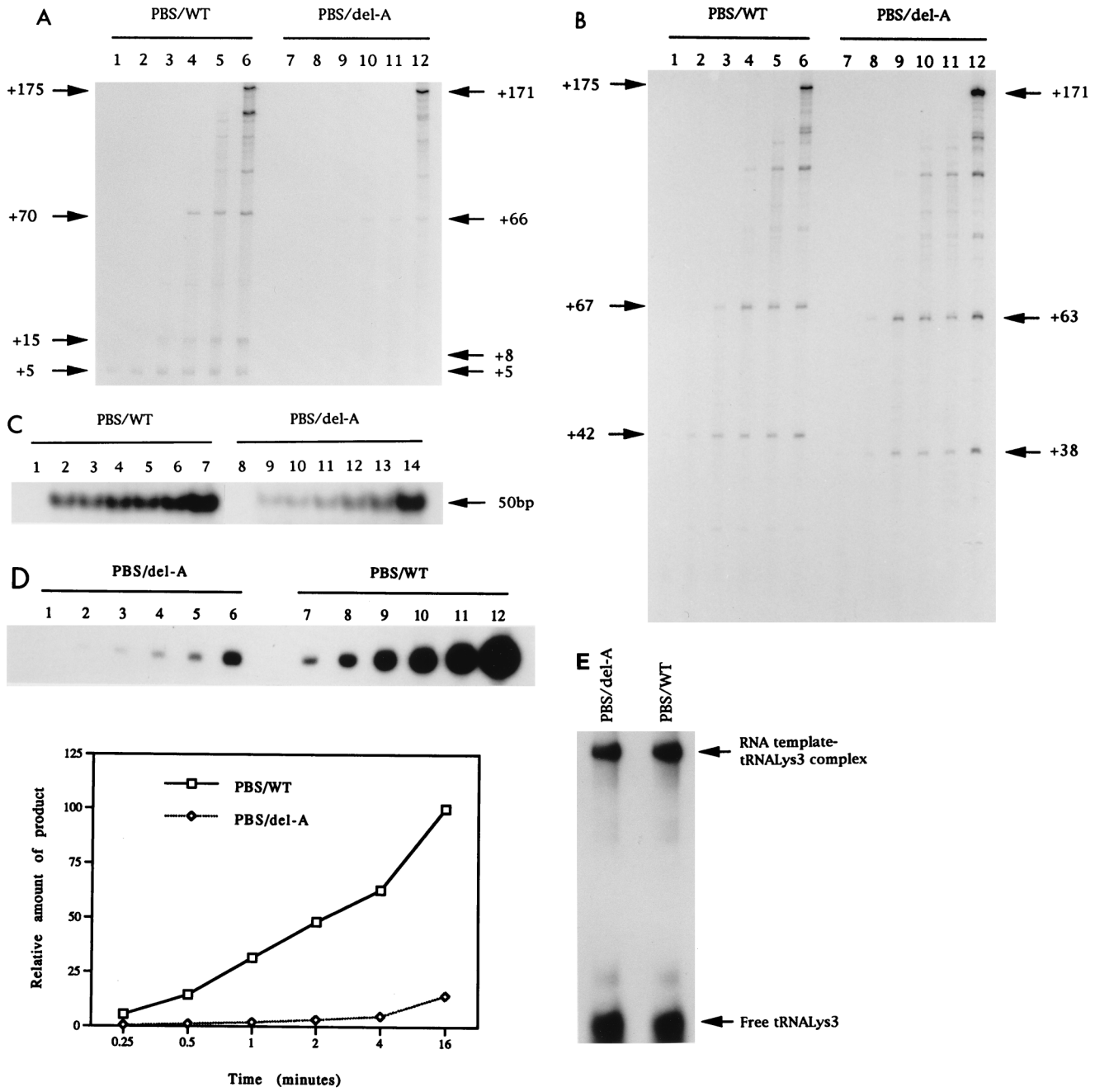

FIG. 5. (A) (−)ssDNA synthesis in an in vitro RT assay with natural $tRNA_3^{Lys}$ as a primer. Lanes 1 to 6, reactions stopped at 15 and 30 s and 1, 2, 4, and 16 min after the addition of RT. Lanes 7 to 12 are in the same order as lanes 1 to 6. (B) (−)ssDNA synthesis in an in vitro RT assay with DNA oligonucleotide pDR as a primer. (C) PCR to detect cDNA synthesis in the in vitro RT assay with natural $tRNA_3^{Lys}$ as a primer. Lanes 1 and 8, negative controls of reverse transcription reactions without HIV-1 RT; lanes 2 to 7, reactions stopped at 15 and 30 s and at 1, 2, 4, and 16 min after the addition of RT. Lanes 9 to 14 are in the same order as lanes 2 to 7. (D) One-base extension from the annealed $tRNA_3^{Lys}$ on PBS/WT or PBS/del-A RNA template. Reaction times for lanes 1 to 6 and 7 to 12 were the same as described for panel A. Products were quantified by molecular imaging and are shown in the graph; the product from PBS/WT at 16 min (lane 12) was arbitrarily set at 100. (E) Placement of 5′-end-labelled $tRNA_3^{Lys}$ onto PBS/WT or PBS/del-A RNA template by heating.

The (+169)AAAA(+172) A-rich loop in HIV-1 RNA has been identified by chemical and enzymatic analyses as able to bind to the anticodon loop (33)USUU(36) of $tRNA_3^{Lys}$ (10, 12). Its involvement in reverse transcription has been reported by different labs. In vitro studies suggested that the A-rich loop facilitates the specific transition from initiation to elongation of reverse transcription, since the mutation of the four A's (from GUAAAA to CUAUG) caused the accumulation of unelongated $tRNA_3^{Lys}$ primer (11). In contrast, the RTs of feline immunodeficiency virus and HIV-2, which cannot ordinarily utilize $tRNA_3^{Lys}$ when annealed to wild-type HIV-1 RNA template, were able to efficiently extend from $tRNA_3^{Lys}$ placed onto a mutated HIV-1 RNA template containing the A-rich loop deletion, i.e., the deletion of the A-rich loop freed a blockage of reverse transcription (3). At the same time, both PBS/del-A and PBS/WT HIV-1 RNA templates can yield sim-

TABLE 1. Sequencing analysis of HIV/del-A and of viruses emergent in tissue culture after passage in MT-2 cells[a]

Passage no.	Sequence	No. of clones containing conversion/no. sequenced
8	TTTTAGTCAGTGTGGTCTCTAGCAGTGG	3/8
	TTTTAGTCAGTGTAATCTCTAGCAGTGG	5/8
11	TTTTAGTCAGTGTAATCTCTAGCAGTGG	11/11
20	TTTTAGTCAGTGTAATCTCTAGCAGTGG	11/12
	TTTTAGTCAGTGAAATCTCTAGCAGTGG	1/12
26	TTTTAGTCAGTGTAATCTCTAGCAGTGG	10/12
	TTTTAGTCAGTGAAATCTCTAGCAGTGG	2/12

[a] Long-term culture of HIV/del-A in MT-2 cells, resulting in the conversion of nucleotide (+166)TGG(+168) to (+166)AAA(+168). Mutated nucleotides are underlined. HIV/WT sequence, (+154)TTTTAGTCAGTGTGGAAAATCTCTAGCAGTGG(+185); HIV/del-A sequence, (+154)TTTTAGTCAGTGTGG----TCTCTAGCAGTGG(+185).

ilar levels of full-length (−)ssDNA in cell-free RT reactions (17). In this study, we have examined earlier stages of the same reactions to show that less DNA product was generated by mutated PBS/del-A RNA template than by PBS/WT template (Fig. 5A and C). This implies that the deletion of the A-rich loop resulted in deficient initiation of (−)ssDNA, a conclusion that is further supported by the one-base extension experiments (Fig. 5D). Since 16-min incubation reactions with PBS/del-A and PBS/WT RNA templates yielded similar levels of full-length (−)ssDNA despite differences in initiation efficiency, our data support the earlier conclusion that the deletion of the A-rich loop frees a blockage of reverse transcription (3). While minor differences in results among various labs might be due to the introduction of different mutations (3, 11, 17), all in vitro studies to date support a regulational role for the A-rich loop in the synthesis of (−)ssDNA.

Although the mutated PBS/del-A RNA template generated about the same amount of full-length (−)ssDNA as did the wild-type RNA template (17), reactions with the latter generated more partially extended DNA products, particularly those

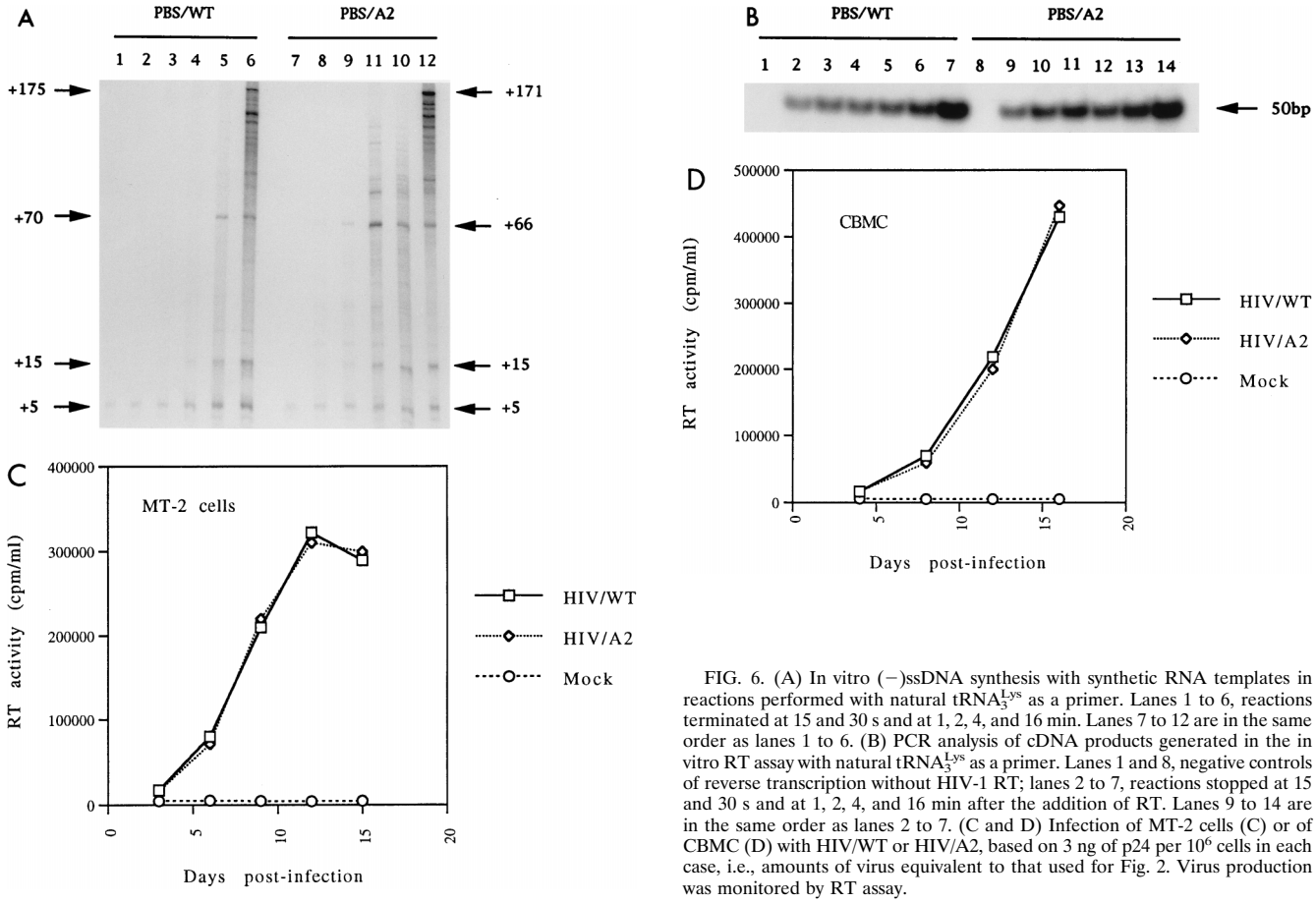

FIG. 6. (A) In vitro (−)ssDNA synthesis with synthetic RNA templates in reactions performed with natural $tRNA_3^{Lys}$ as a primer. Lanes 1 to 6, reactions terminated at 15 and 30 s and at 1, 2, 4, and 16 min. Lanes 7 to 12 are in the same order as lanes 1 to 6. (B) PCR analysis of cDNA products generated in the in vitro RT assay with natural $tRNA_3^{Lys}$ as a primer. Lanes 1 and 8, negative controls of reverse transcription without HIV-1 RT; lanes 2 to 7, reactions stopped at 15 and 30 s and at 1, 2, 4, and 16 min after the addition of RT. Lanes 9 to 14 are in the same order as lanes 2 to 7. (C and D) Infection of MT-2 cells (C) or of CBMC (D) with HIV/WT or HIV/A2, based on 3 ng of p24 per 10^6 cells in each case, i.e., amounts of virus equivalent to that used for Fig. 2. Virus production was monitored by RT assay.

of about the 140 nt that includes part of the 3′ end of the R region (Fig. 5A, lanes 6 and 12). The importance of these partially extended DNA products is that they might play a role in the initiation of the first template switch. This could result in more viral cDNA product, enabling wild-type viruses to replicate more efficiently than mutated (HIV/del-A) variants.

The importance of the A-rich loop in HIV-1 reverse transcription can also be inferred from in vivo studies. The substitution of the A-rich loop region with sequences complementary to the anticodon loop of $tRNA^{His}$ or $tRNA^{Met}$ yielded replication-competent HIV-1 that stably utilized either $tRNA^{His}$ or $tRNA^{Met}$ as primers (14, 28). At the same time, the deletion of viral DNA, containing the A-rich loop, had little effect on virus replication (27). Our studies show that the deletion of the A-rich loop caused a diminution in the levels of synthesis of viral DNA in infected cells, resulting in reduced infectiousness of the mutated viruses, HIV/del-A. This effect (reduced infectiousness) was more pronounced in cells of primary origin, i.e., CBMC, than in the MT-2 cell line. Moreover, endogenous RT assays also revealed that viruses containing HIV/del-A RNA generated less viral DNA than did wild-type viruses.

In further support of the advantage to HIV replication of A's in the region immediately upstream of the PBS, we have shown that long-term culture of HIV/del-A in MT-2 cells led to the substitution of A's for G's at nt positions 167 and 168 (Table 1). It is notable that these newly generated A's possess the same relationship to the PBS as did the A's in the original A-rich loop; in both cases, they are located 10 nt away from the 5′ end of the PBS. Cell-free assays also demonstrated that an RNA template, PBS/A2 containing a (+167)AA(+168) motif, was able to generate (−)ssDNA with an efficiency similar to that of the wild-type RNA template.

However, the A-rich loop of HIV/del-A was not completely regenerated, not even after 26 passages (130 days) of replication in MT-2 cells (Table 1). This is because the (+167)AA(+168) reversion generated viruses with infectivity comparable to that of the wild type. Based on the model of interaction between the A-rich loop, (+169)AAAA(+172), and the anticodon loop, (33)USUU(36), of $tRNA_3^{Lys}$ (10), the modified nucleotide 34S of the anticodon seemingly interacts with +171A. Furthermore, this modified 34S is essential to the maintenance of the interaction between the A-rich loop and the $tRNA_3^{Lys}$ anticodon (10–12, 26). In all likelihood, the regenerated (+167)AA(+168) in HIV/del-A was able to interact with (33)/US(34), including the modified 34S, of $tRNA_3^{Lys}$ in the same manner as nucleotides (+171)AA(+172) normally do, thus stabilizing the interaction between $tRNA_3^{Lys}$ and the viral RNA template.

The fact that the HIV/del-A studied were less infectious than the wild-type viruses suggests that the former incorporated fewer copies of the cognate $tRNA_3^{Lys}$ primer than did the latter. However, previous research by our group and by others has shown that only the $Pr160^{gag\text{-}pol}$ precursor protein is involved in specifying the incorporation of $tRNA_3^{Lys}$ into HIV-1 particles. Indeed, extensive deletions in multiple regions of the HIV-1 genome did not impact the incorporation of tRNA into virions, an event that has been shown to occur independently of genomic RNA packaging (18, 19).

In conclusion, the (+169)AAAA(+172) A-rich loop is important for both the initiation of efficient reverse transcription and viral replication. In support of this conclusion, long-term culture of HIV/del-A in MT-2 cells resulted in the reappearance of an A-rich-loop-like motif.

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