Specificity of Priming Reaction of HIV-1 Reverse Transcriptase, 2'-OH or 3'-OH*  

(Received for publication, November 19, 1993)

Masamitsu Shimada, Hideo Hosaka, Hiroshi Takaku, Jeffrey S. Smith, Monica J. Roditi, Sumiko Inouye, and Masayori Inouye*  

From the Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854 and the Department of Industrial Chemistry, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275, Japan

It has not been unambiguously demonstrated whether the priming reaction of human immunodeficiency virus, type 1 (HIV-1) cDNA synthesis initiates with either the 2'-OH or 3'-OH group of the 3'-terminal adenosine residue of tRNA. In this report, we synthesized tRNA of which the 3'-terminal adenosine residue lacks either a 2'-OH or 3'-OH. These tRNA molecules were used for the HIV-1 cDNA priming reaction in a cell-free system consisting of a 141-base RNA template and purified HIV-1 reverse transcriptase. It was found that under the conditions used, the tRNA containing the 2'-deoxyadenosine was able to initiate the cDNA synthesis, whereas the tRNA with the 3'-deoxyadenosine was not. The results show that retroviral reverse transcriptase specifically primes cDNA synthesis from the 3'-OH group. This is in contrast to bacterial reverse transcriptase, which initiates cDNA synthesis from the 2'-OH group of an internal guanosine residue of a template RNA.

For the priming reaction of retroviral minus-strand DNA synthesis, tRNA molecules are known to be utilized (see Ref. 1 for a review). It is believed that the 3'-terminal adenosine residue of the tRNA molecules is used for the priming reaction forming a 3',5'-phosphodiester linkage between tRNA and the first nucleotide of cDNA. However, this has not been unambiguously demonstrated. In particular, two recent findings raised a serious question of whether retroviral reverse transcriptases may initiate cDNA synthesis from the 2'-OH rather than the 3'-OH group of their own individual primer tRNA molecules. The first finding is that seemingly primitive reverse transcriptases from bacterial retroelements exclusively prime cDNA synthesis from the 2'-OH group of an internal guanosine residue of the template RNA molecules forming a 2',5'-phosphodiester linkage (see Ref. 2 for a review). The second finding is that yeast retrotransposon Ty-1 requires a debranching enzyme for efficient transposition (3), raising the possibility that a 2',5'-phosphodiester linkage is formed during the cDNA syn-

* This work was supported in part by a grant from the United States Public Health Service (to M. I. and S. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of the New Jersey Commission on Cancer research fellowship.

‡ Scholar of the Leukemia Society of America, Inc.

thesis by its reverse transcriptase. In this report, we examined if HIV-1 reverse transcriptase initiates its cDNA synthesis from either the 2'-OH or the 3'-OH group of the primer tRNA.

EXPERIMENTAL PROCEDURES

Synthesis of the Trinucleotides, pCC (2'-Deoxy)Ap—The cytidine and deoxyadenosine 2'-phosphoramide unit was prepared from the reaction of 5'-dimethoxytrityl-N'-protected-2'-O-(1-3)-chloroethoxyethyl cytidine or 5'-dimethoxytrityl-N'-protected-2'-deoxyadenosine with 2-cyanomethyl-N,N-diisopropylchlorophosphoramide (8). The oligonucleotides having 5',2'-terminal phosphates were synthesized by means of the phosphoramidite approach using the 2'-phosphates modifier (DMTOCH$_2$SO$_2$CH$_2$OCHOCH$_3$cPG, where cPG is controlled pore glass) and the 5'-terminal modifier (DMTOCH$_2$SO$_2$CH$_2$OPNiPr$_2$OCH$_2$CH$_3$), where iPr is isopropyl) (8). The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.

Synthesis of tRNA$_{syn-3}$ (2'-OH) and tRNA$_{syn-3}$ (3'-OH)—tRNA$_{syn-3}$ was synthesized in vitro by T7 RNA polymerase using pTL9, which contains the entire tRNA$_{syn-3}$ gene under a T7 promoter (6). To amplify the tRNA$_{syn-3}$ gene lacking the CCA sequence at the 3'-end, the following two PCR primers were used: primer 1, 5'-CGCCGAGTTACA; and primer 2, 5'-CGCCGAGCTAG. To amplify the full-size tRNA$_{syn-3}$ gene, primer 1 and primer 3 (5'TGGGCGCGAACACG) were used. Primer 1 was designed to initiate PCR reactions immediately upstream of the T7 promoter. DNA fragments amplified were subsequently used to synthesize the tRNA in a cell-free system with T7 RNA polymerase (7).

Experimental procedures were described above except that pCC (2'-deoxy)Ap was used instead of pCC (2'-deoxy)A2'-OH. The 3'-phosphate was removed by alkaline phosphatase after ligation. The product was analyzed by 7.5% polyacrylamide gel electrophoresis in 8 M urea.

HIV-1 cDNA Synthesis—The template was synthesized in vitro by T7 RNA polymerase using a 165-base pair DNA fragment. The DNA fragment produced by primers 1 and 2, tRNA$_{syn-3}$ (ACCA) (37.5 pmol) was ligated with a trinucleotide, 5'-pCC (3'-deoxy)A2'-OH (A$_{300}$ = 0.1; 375 pmol), by T4 RNA ligase (Pharmacia LKB Biotechnology Inc.) in 25 mM Hepes buffer pH 7.5, containing 15 mM MgCl$_2$, 3.5 mM dithiothreitol, 10 µg/ml bovine serum albumin, 15% dimethyl sulfoxide, and 50 µM ATP. The reaction was carried out in 20 µl at 16 °C for 16 h. tRNA$_{syn-3}$ (3'-OH) was synthesized in the same manner as described above except that pCC (2'-deoxy)Ap was used instead of pCC (2'-deoxy)A2'-OH. The 3'-phosphate was removed by alkaline phosphatase after ligation. The product was analyzed by 7.5% polyacrylamide gel electrophoresis in 8 M urea.

The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; pCC (2'-deoxy)Ap, 5'-monophosphate cytidylic acid 2'-deoxyadenosine 3'-monophosphate; PCR, polymerase chain reaction; DMTO, O-(4,4'-dimethoxytrityl); T7 RNA polymerase, and the 3'DMTO, O-(4,4'-dimethoxytrityl) cytidylic acid 2'-deoxyadenosine 3'-monophosphate; PCR, polymerase chain reaction; DMTO, O-(4,4'-dimethoxytrityl); T7 RNA polymerase, and the 3'-terminal modifier (DMTOCH$_2$SO$_2$CH$_2$OPNiPr$_2$OCH$_2$CH$_3$), where iPr is isopropyl) (8). The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.

The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.

The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.

The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.

The unblocked oligonucleotides were purified by use of reverse phase and ion-exchange HPLC. The product was used for determination of base composition by enzymatic degradation to nucleotides followed by HPLC analysis.
for 30 min in 20 μl of 50 mM Tris-HCl, pH 8.0, containing 8 mM MgCl₂, 50 mM KCl, and 2 mM dithiothreitol. The reaction was stopped by adding 50 μl of the stop solution (20 mM EDTA, 0.5% SDS). The products were treated with ribonuclease A and analyzed by 7.5% polyacrylamide gel electrophoresis in 8 M urea, and the products were detected by autoradiography.

RESULTS AND DISCUSSION

Two kinds of tRNA<sup>lys</sup>-3 were synthesized; one tRNA<sup>lys</sup>-3 contained only the 2'-OH group (3'-deoxy), designated tRNA<sup>lys</sup>-3 (2'-OH), and the other only a 3'-OH group (2'-deoxy), designated tRNA<sup>lys</sup>-3 (3'-OH). First, tRNA<sup>lys</sup>-3 lacking the 3'-terminal CCA sequence was synthesized in vitro using a T7 expression system. To this 73-base tRNA<sup>lys</sup>-3, synthetic pCC (3'-deoxy)A<sub>2</sub>'-OH or pCC (2'-deoxy)Ap was ligated with RNA ligase. In the latter case, the 3'-phosphate was removed by alkaline phosphatase after ligation. The analysis of the resultant tRNA<sup>lys</sup>-3 is shown in Fig. 1. As a result of ligation, a new band appeared at the 76 nucleotides corresponding to the full-sized tRNA<sup>lys</sup>-3 for both tRNA preparations (compare lanes 3 and 4 with lane 2). Approximately 70% of tRNA<sup>lys</sup>-3 (ACCA) was ligated with the trinucleotide. These tRNA preparations were then used for the priming reaction using a 141-base template RNA synthesized in vitro in a T7 system. This template RNA contains the 18-base PBS and a 48-base sequence including a 43-base leader sequence downstream of the PBS derived from HIV-I NY5 (4). This U5-leader stem sequence is reported to enhance the retroviral cDNA initiation by reverse transcriptase (5). It also contains 74 nucleotides upstream of the PBS en-
Priming Reaction of HIV-1 Reverse Transcriptase

3927
coded by a U5 sequence and one extra G residue at the 5'-end, which was added for efficient transcription from the T7 promoter. When the full-size tRNA^{ly3} is used as a primer for cDNA synthesis by HIV-1 reverse transcriptase, 75 deoxyribonucleotides are added onto the tRNA. Digestion of this product with ribonuclease A yields a 76-base product (75-base DNA plus one adenosine at the 5'-end; see reaction 1 in Fig. 2A). On the other hand, the reaction with tRNA^{ly3} lacking CCA (ΔCCA) yields a 79-base product (78-base DNA plus one guanosine; see reaction 2 in Fig. 2A). These products are clearly observed in Fig. 2B, lanes 1 and 2, for reactions 1 and 2, respectively.

When the tRNA^{ly3} (2'-OH) was used for the priming reaction, only a faint band at the 79-base position, but not at all at the 76-base position, was observed (Fig. 2B, lane 3). This indicates that the full-length tRNA^{ly3} (2'-OH) was not used as a primer, which would yield the 76-base band as illustrated in Fig. 2A, reaction 3. The 79-base band in lane 3 is derived from the parental tRNA^{ly3} (ΔCCA), which remained in the tRNA^{ly3} (2'-OH) preparation (see Fig. 1, lane 3). In contrast to tRNA^{ly3} (2'-OH), tRNA^{ly3} (3'-OH) was able to produce the 76-base band (Fig. 2B, lane 4) as in the case of tRNA^{ly3} (lane 1). The 79-base band observed in lane 4 is again due to the unligated tRNA^{ly3} (ΔCCA) present in the reaction.

The present results now demonstrate that HIV-1 reverse transcriptase is able to prime cDNA synthesis from the 3'-terminal adenosine residue of tRNA^{ly3} but not from the 2'-OH group under the conditions used. However, in the present cell-free system, no other proteins other than reverse transcriptase were added, the tRNA^{ly3} was not modified, and a shortened template RNA was used. Therefore, one may not completely rule out the possibility that HIV-1 reverse transcriptase in vivo is capable of initiating cDNA synthesis from the 2'-OH group of the 3'-end adenosine residue of tRNA^{ly3}.

Acknowledgments—HIV-1 reverse transcriptase heterodimer purified from *Escherichia coli* was provided by Jeffrey Culp and Christine DeBouck, Department of Protein Biochemistry, Smith Kline Beecham Pharmaceuticals. We thank Dr. Stuart F. J. Le Grice for plasmid pTL9 and Dr. Arnold B. Robson for plasmid pNL4-3.

REFERENCES

1. Weiss, R., Teich, N., Varmus, H., and Coffin, J. (eds) (1985) *RNA Tumor Virus*, Ed. 2, pp. 76-82, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Inouye, M., and Inouye, S. (1991) *Annu. Rev. Microbiol.* 45, 163-186
3. Chapman, K. B., and Boeke, J. D. (1991) *Cell* 65, 483-492
4. Beno, S., Rutledge, R., Folks, T., Gold, J., Baker, L., McCormick, J., Fearinio, P., Piot, P., Quinn, T., and Martin, M. (1985) *Science* 229, 549-551
5. Cobrini, D., Sokey, L., and Leis, J. (1988) *J. Virol.* 62, 3622-3630
6. Barat, C., Le Grice, S. F. J., and Darlix, J.-L. (1991) *Nucleic Acids Res.* 19, 751-757
7. Richter-Cook, N. J., Howard, K. J., Cirino, N. M., Wöbli, B. M., and Le Grice, S. F. J. (1992) *J. Biol. Chem.* 267, 15952-15957
8. Sakatsume, O., Yamaguchi, T., Ishikawa, M., Hirao, I., Miyazaki, K., and Takaku, H. (1991) *Tetrahedron* 47, 8717-8728
9. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabaon, A., and Martin, M. A. (1986) *J. Virol.* 59, 284-291