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An Additional 2′-Ribofuranose Residue at a Specific Position of the DNA Primer Prevents Its Elongation by HIV-1 Reverse Transcriptase

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Abstract—Oligodeoxynucleotides containing 2′-*O*-β-D-ribofuranosyladenosine were prepared and used as modified primers in RNA-templated DNA synthesis catalyzed by HIV reverse transcriptase. It was shown that the additional 2′-ribofuranose residue in specific position of primer prevents its elongation.

Introduction

Template-directed DNA-dependent and RNA-dependent DNA polymerases play a fundamental role in all living cells. Particularly, the reverse transcriptase (RT) of human immunodeficiency virus (HIV) carries out viral replication and converts viral RNA genome into a double-stranded DNA copy. Since RT is the only DNA polymerase that operates in the cytoplasm of HIV-infected cells, it is the main target for the compounds that block viral replication.[1] Numerous highly specific nucleoside (operating as terminators of growing DNA chain) and non-nucleoside inhibitors (operating in non-competitive mode) of RT have been described.[2,3] Examples of RT inhibition by oligodeoxynucleotides (ONs) are limited mainly to primers carrying a 'terminating' nucleotide on their 3′-terminus.[4–6]

Recently, a general and useful method for the preparation of 2′-*O*-D-ribofuranosylnucleosides[7] and their suitably protected derivatives for ON synthesis[8–10] were developed. Several sets of modified ONs were prepared and their thermal stability was evaluated. Melting point determinations have demonstrated that modified ONs form stable duplexes with RNA ($\Delta T_m = 0\,^{\circ}C$).[10] The incorporation of 2′-*O*-β-D-ribofuranosylnucleosides into ONs slightly destabilizes DNA duplex formation ($\Delta T_m = 2–3\,^{\circ}C$).[10] In order to determine the influence of this modification the solution structure of the self-complementary decaribonucleotide 5′-r(GCGXAUUCGC)-3′ (X-2′-*O*-β-D-ribofuranosyl-adenosine residue) was studied using high-resolution NMR spectroscopy and restrained molecular dynamics.[11] Incorporation of such voluminous substituent into 2′-*O*-position has no profound effect on the thermal stability of the duplex. It was found that the duplex RNA maintains an A-type helical geometry with extra 2′-*O*-ribose moiety located in the minor groove.[11] It should be also noted that 2′-*O*-β-D-ribofuranosyl-adenosine was found in yeast initiator tRNA in stem region of T domain.[12]

Results and Discussion

RT catalyzes DNA synthesis, utilizing RNA or DNA as a template. The necessary condition for the elongation reaction is the presence of ON primer, containing free 3-hydroxyl group. It would be of interest to investigate substrate properties of modified primers containing bulky 2′-*O*-β-D-ribofuranosylnucleosides in DNA synthesis reactions catalyzed by HIV RT using an RNA template.

ON synthesis was performed at 1 μmol scale using commercial 2-cyanoethylphosphoroamidites and standard methodology, but for a longer coupling time (80 s)

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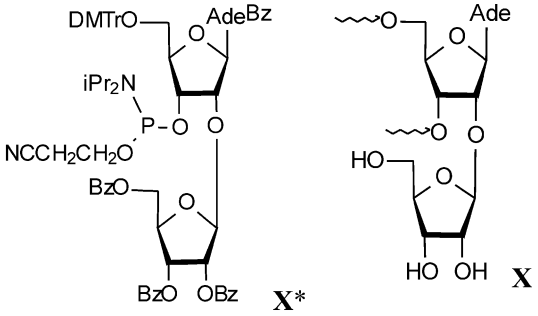
and at a higher concentration (0.15 M) for the modified synthon (**X***) to ensure high coupling yields. The ONs obtained were deprotected, removed from the solid support and purified as described.[8–10] The structure of primers **2a**–**6a** was proven by mass spectrometry.

5′-[^{32}P] end labeling of ONs **1a**–**6a** was carried out with T4 polynucleotide kinase[13] to yield corresponding **1b**–**6b**. Recombinant HIV-1 RT was purified from *Escherichia coli* strain as described.[14] A 149 nt RNA fragment obtained by T7 transcription of *Pvu*II-linearized plasmid pTZR7G[15] was used as the template. ONs **1**–**6** were used as primers. Primers were annealed with the template according to standard protocols.[13]



1a 5'-GAC-GTT-GTA-AAA-CG-3'
2a 5'-GXC-GTT-GTA-AAA-CG-3'
3a 5'-GAC-GTT-GTX-AAA-CG-3'
4a 5'-GAC-GTT-GTA-XAA-CG-3'
5a 5'-GAC-GTT-GTA-AXA-CG-3'
6a 5'-GAC-GTT-GTA-AAX-CG-3'
1b - 6b - 5'-[^{32}P] phoshorylated primers
template:
3'-(64nt)-CUG-CAA-CAU-UUU-GCU-GCC-69 nt)-5'

To analyze the RT-catalyzed primer extension reaction two assay systems were used:

(a) Assay mixture (10 μL) contained 50 mM Tris–HCl, pH 8.0; 6 mM $MgCl_2$; 10 mM dithiotreitol; 0.05% Nonidet NP-40, 3% glycerol, 200 U/mL RNasin; 20 μM of each dNTP, 0.05 μg RT; and variable concentrations of template/primers **1b**–**6b**.

(b) To determine the kinetic parameters (K_m and relative V_{max}) of primer extension on one nucleotide the same assay mixture containing 2×10^6 cpm [α-^{32}P]dATP and variable concentrations (1–10 μM) of unlabeled template/primers **1a**–**6a**. To determine K_i values of **2a**–**6a** the reaction was monitored at variable concentrations of template/primer **1b** and several fixed concentrations of template/**2a**–**6a**.

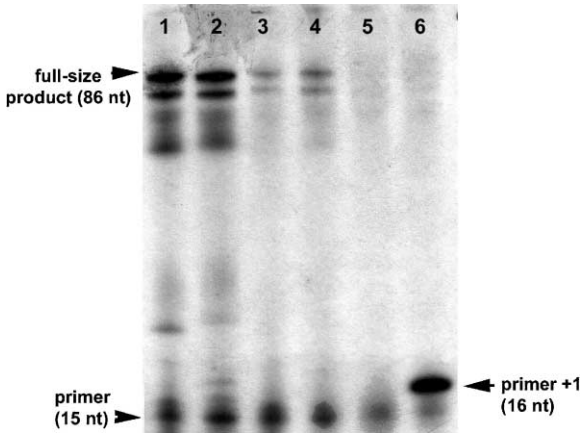


Figure 1. RT-catalyzed elongation of 5′-[^{32}P]-end labeled modified primers 1, **1b** (control); 2, **2b**; 3, **3b**; 4, **4b**; 5, **5b**; 6, **6b**. After completing, the reactions were stopped by addition of 5 μL of sample buffer (10 mL of formamide containing 1 mg/mL each of bromphenol blue and xylene cyanol, 1mM EDTA), heated for 5 min at 95 °C and subjected to electrophoresis in 15% polyacrylamide-8 M urea.[13] After electrophoresis the gels were subjected to autoradiography for 12 h at −70 °C. For quantitative determination of primer extension and for the determination of apparent kinetic parameters of the reaction the respective autoradiography patterns were scanned with FAST SCAN 300A densitometer (Molecular Dynamics) using the software Image Quant.

The samples were incubated for 45 min at 37 °C and subjected to polyacrylamide gel electrophoresis according to ref 13 (further details see in the legend to Fig. 1).

Figure 1 and Table 1 demonstrate the extension of ON primers containing 2′-*O*-β-D-ribofuranosyladenosine. As can be seen, primer **2b** with modification in position-13 from 3′-end (reaction center) is extended with the same efficiency as unmodified ON **1b**. As the modification shifts to 3′-end of the primer the efficiency of its elongation decreases, though direct relation is not observed: primer **4b**, modified at position (-5) is extended more effectively, than **3b** [modification in a position-(6)]. Primer **5b** with modification in position -4 is not elongated practically by RT. At the same time primer **6b**, containing modification even closer to 3′-end (-3) is extended. However elongation happens only on one nucleotide unit. Thus formally, with **6b**, dNTP acts like a terminator of the reaction. In this connection, it is possible to assume, that for the efficient elongation of a primer it is important not only relative remoteness of the modified nucleotide from 3′-end, but also the specific position of the latter within nucleotide chain,

Table 1. Modified ONs as primers in RT-catalyzed reaction

Primer	Product	Elongation (%)	K_m, (μM)	V_{max} (rel units)	V_{max}/K_m
1	DNA	100	0.45±0.11	100	222
2	DNA	90	0.62±0.13	100	161
3	DNA	10	1.47±0.24	86	58
4	DNA	15–20	0.19±0.05	18	93
5		No elongation, $K_i = 1.05\pm0.33$			
6	Short product, +1 nucleotide	90	0.56±0.07	100	178

determined by the topography of the primer binding site of the enzyme. It should be mentioned that ONs containing 1-β-D-glucopyranosylthymine were used as primers in DNA synthesis reaction catalyzed by *E. coli* DNA polymerase (Klenow fragment).[16] No elongation was observed when the modification is located close to 3′-end of the primer.[16]

Table 1 demonstrates the kinetic parameters (K_m and relative V_{max}) of primer extension on one nucleotide (these parameters, unlike 'overall' K_m and V_{max}, characterize the elementary act of the reaction more adequately). As is seen, the K_m and V_{max} values vary rather insignificantly. It is known, that the parameter V_{max}/K_m is a measure of the efficiency of enzymatic reaction, and correlates with a total product yield observed in reaction with all four dNTPs. Both parameters K_m and V_{max} decrease in the case of primer **4b** [modification in position (-5)]. The decrease of the former might be attributed to formation by the additional ribofuranosyl moiety of new hydrogen bond(s) with protein; decrease of V_{max} can be explained by formation of incorrect enzyme–substrate (ES) complex. However, the overlapping of these two effects leads to rather considerable V_{max}/K_m value and, therefore, to efficient primer extension. It is interesting that the kinetic parameters of single nucleotide primer extension for **6** are close to those for the unmodified ON **1**.

In the presence of unmodified primer **1b,** primers **3a**–**6a** were also effective competitive inhibitors of the reaction. Their inhibition constants as a whole (and, particularly, **5a** which is not elongated by RT) were close to appropriate K_m values which specified its similarity with true dissociation constants of an enzyme-template/primer complex.

In order to get insight in the different behavior of primers, we have carried out the simulation of modified primer binding in the RT active site on the basis of three-dimensional structure of its complex with the template and primer.[17] The simulations of primer binding within the RT active site was carried out using the program WebLabViewerPro 3.7 (Molecular Simulations Inc) according to the instructions of the manufacturer. As a model the structure of RT complex with substrates was utilized.[17] (Brookhaven Protein Data Bank, ID 1RTD). Close inter-atomic contacts between additional ribose and protein that are within 70% of the sum of the covalent radii were chosen to estimate the steric hindrance.

The arrangement and conformation of the additional ribose residue was modeled according to solution structure of a RNA decamer duplex, containing 2′-*O*-β-D-ribofuranosyladenosine.[11] The applicability of the RNA

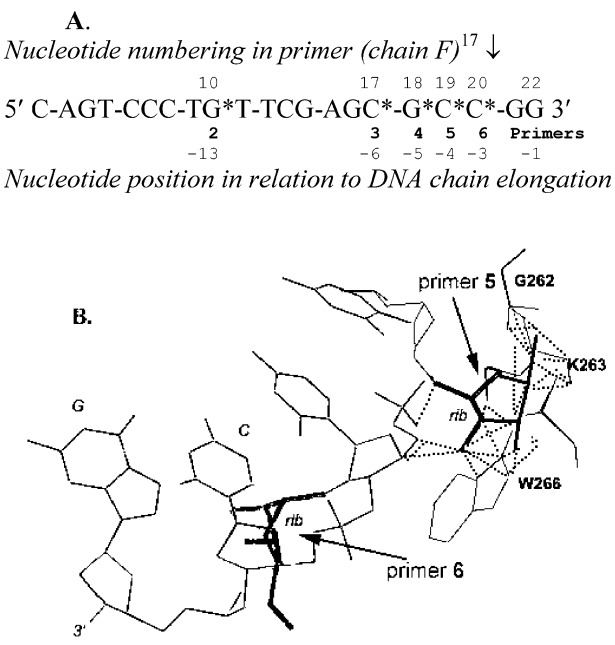


Figure 2. Simulation of modified primer binding within RT active site. (A). Structure of ON primer from paper.[17] This structure was used for introduction of additional ribose residues that topologically correspond to modifications in primers **2**–**6.** Positions of modified nucleotides are marked with asterisks. (B). Model of the binding of primers **5** and **6** within the RT active site. Only primer DNA chain is shown. Close inter-atomic contacts between additional ribose (***rib***) and protein that are within 70% of the sum of the covalent radii are shown with dotted lines.

duplex structure (A-form) to the given model implies from the fact, that DNA template-primer conforms more closely in structure to classical A-form of DNA near the RT polymerase active site.[18] According to NMR data[11] the extra ribose moiety has a fixed orientation in the minor groove and adopts a C3′-*endo* conformation. The position of this moiety relative to the parent nucleic acid chain is characterized by an O2′–C1″–C2″–O2″ torsion angle of −134°. Introduction of additional ribose moieties in nucleotides 19 and 20 of the RT/template/primer complex[17] which topologically corresponds to the modifications as in primers **5** and **6** is shown in Figure 2. It is seen that in the case of **5** considerable close inter-atomic contacts between additional ribose and protein (α-helical segment 260–270, see Table 2) are present, while for **6** no such interactions are detectable. Moreover, some contacts with neighboring phoshate are visible in **5**. The importance of this phosphate group between (-3) and (-4) nucleosides was mentioned during the studies of phosphorothioate containing primer elongation by terminal deoxyribonucleotidyl transferase.[19,20] Alterations of the O2′–C1″–C2″–O2″ torsion angle do not result in essential differences of the observed pattern: for primer **5** interactions with protein are still preserved, and for **6** they remain

Table 2. Close inter-atomic contacts between additional ribose moiety in primers **5** and **6** and protein upon binding within RT active site

Torsion angle	Primers **2**–**4**	Primer **5**	Primer **6**
$\chi = -134^{o}$	No close contacts	Lys263, Trp266	No close contacts
Other		Gly262, Lys263, Leu264, Trp266	

absent (data not shown). Thus, **6** retains a considerable conformational freedom within the complex, which gives it the possibility to interact correctly within the RT active site and to react with the incoming dNTP. After such elongation and subsequent isomerization of the ES complex the latter becomes identical to that formed with primer **5**, and the reaction stops.

Conclusion

The additional 2′-ribofuranose residue in -4 position of a DNA primer prevents its elongation by HIV-1 RT due to the steric hindrances. This finding might be of general value and such primers with bulky groups might be used for specific inhibition of different polymerases.

Acknowledgements

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References and Notes

1. Richman, D. D. *Nature* **2001**, *410*, 995.
2. Kaufmann, G. R.; Cooper, D. A. *Curr. Opin. Microbiol.* **2000**, *3*, 508.
3. Rando, R. F.; Nguyen-Ba, N. *Drug Discov. Today* **2000**, *5*, 465.
4. Heidenreich, O.; Kruhoeffer, M.; Grosse, F.; Eckstein, F. *Eur. J. Biochem.* **1990**, *192*, 621.
5. Austermann, S.; Kruhoffer, M.; Grosse, F. *Biochem. Pharmacol.* **1992**, *43*, 2581.
6. Canard, B.; Sarfati, S. R.; Richardson, C. C. *J. Biol. Chem.* **1998**, *273*, 14596.
7. Mikhailov, S. N.; Efimtseva, E. V.; Gurskaya, G. V.; Zavodnik, V. E.; De Bruyn, A.; Rozenski, J.; Herdewijn, P. *J. Carbohydr. Chem.* **1997**, *16*, 75.
8. Tunitskaya, V. L.; Rusakova, E. E.; Memelova, L. V.; Kochetkov, S. N.; Van Aerschot, A.; Herdewijn, P.; Efimtseva, E. V.; Ermolinsky, B. S.; Mikhailov, S. N. *FEBS Lett.* **1999**, *442*, 20.
9. Gritsenko, O. M.; Mikhailov, S. N.; Efimtseva, E. V.; Van Aerschot, A.; Herdewijn, P.; Gromova, E. S. *Nucleosides, Nucleotides Nucleic Acids* **2000**, *19*, 1805.
10. Efimtseva, E. V.; Bobkov, G. V.; Mikhailov, S. N.; Van Aerschot, A.; Schepers, G.; Busson, R.; Rozenski, J.; Herdewijn, P. *Helvetica Chim. Acta* **2001**, *84*, 2387.
11. Luyten, I.; Esnouf, R. M.; Mikhailov, S. N.; Efimtseva, E. V.; Michiels, P.; Heus, H. A.; Hilbers, C. W.; Herdewijn, P. *Helvetica Chim. Acta* **2000**, *83*, 1278.
12. Basavappa, R.; Sigler, P. B. *EMBO J.* **1991**, *10*, 3105.
13. Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor University Press: Cold Spring Harbor, NY, 1982.
14. Pokholok, D. K.; Gudima, S. O.; Esipov, D. S.; Dobrynin, V. N.; Rechinsky, V. O.; Kochetkov, S. N. *FEBS Lett.* **1993**, *325*, 237.
15. Gudima, S. O.; Kostyuk, D. A.; Grishchenko, O. I.; Tunitskaya, V. L.; Memelova, L. V.; Kochetkov, S. N. *FEBS Lett.* **1998**, *439*, 302.
16. Ermolinsky, B. S.; Fomitcheva, M. V.; Efimtseva, E. V.; Meshkov, S. V.; Mikhailov, S. N.; Esipov, D. S.; Boldyreva, E. F.; Korobko, V. G. *Nucleosides Nucleotides* **1996**, *15*, 1619.
17. Huang, H.; Chopra, R.; Verdine, G. L.; Harrison, S. C. *Science* **1998**, *282*, 1669.
18. Jacobo-Molina, A.; Ding, J.; Nanni, R. G.; Clark, A. D., Jr.; Lu, X.; Tantillo, C.; Williams, R. L.; Kamer, G.; Ferris, A. L.; Clark, P.; Hizi, A.; Hughes, S. H.; Arnold, E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6320.
19. Koziolkiewicz, M.; Maciaszek, A.; Stec, W. J.; Semizarov, D.; Victorova, L.; Krayevsky, A. *FEBS Lett.* **1998**, *434*, 77.
20. The authors are grateful to a referee for the indication of the importance of phosphoryl group of the third nucleotide from 3′-end of the primer in the case of terminal deoxyribonucleotidyl transferase.