New modified substrates for discriminating between human DNA polymerases α and ε

Maxim V. Jasko, Dmitry G. Semizarov, Lyubov S. Victorova, Dmitry Ju. Mozzherin, Alexander A. Krayevsky, Marina K. Kukhanova

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Str., Moscow 117984, Russian Federation

Received 14 September 1994; revised version received 14 November 1994

Abstract Two 2′-deoxynucleoside 5′-α-methylenephosphonyl-β,γ-diphosphates were synthesized. They were incorporated into the DNA chain by DNA polymerase α from human placenta. Meanwhile, they were not recognized by DNA polymerases ε and β of the same origin as well as by reverse transcriptases from human immunodeficiency virus and avian myeloblastosis virus.

Key words: DNA polymerase; Reverse transcriptase; Terminal deoxynucleotidyl transferase; Modified nucleoside 5′-triphosphate

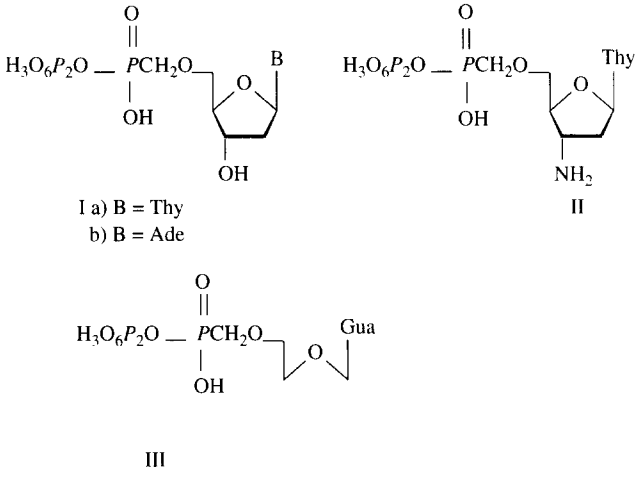
1. Introduction

Mammalian DNA polymerases α and ε display close specificity toward modified substrates. Therefore, it is rather difficult to estimate their individual contribution to DNA biosynthesis in the cell. To date, no specific substrate inhibitors discriminating between DNA polymerases α and ε have been found.

We synthesized a series of 2′-deoxynucleoside 5′-P$^{\alpha}$-methylenephosphonyl-P$^{\beta}$,P$^{\gamma}$-diphosphates (I–III) and studied their substrate properties towards mammalian DNA polymerases α, ε, β and TdT, as well as HIV and AMV RTs. A methylene group was also introduced into the acyclic nucleotide analog acyclovir to estimate the effect of this modification on both deoxyribose-related and acyclic nucleotide analogs.

2. Materials and methods

Compound Ia was synthesized as described in [1], Ib was prepared by a similar method. The procedures for synthesis of other compounds used in this work will be reported later. DNA polymerases α and ε were isolated from human placenta according to [2]. We used HIV RT [3], AMV RT (Omutninsk Chemicals, Russia), TdT and KFr (Amersham), and DNA polymerase β from calf thymus [4]. Phage M13mp10 DNA was isolated from the culture medium of the recipient *E. coli* K12XL1 strain according to [5]. The tetradecanucleotide



I a) B = Thy
b) B = Ade

II

III

primer was labeled at the 5′-terminus using [γ-^{32}P]dATP with a specific activity of 3,000 Ci/mmol (Izotop, Russia) as described in [5]. DNA polymerase assays were carried out as described in [6]. Kinetic measurements were performed within the linear region of the product formation vs. time curve according to [7], the reaction time was 2.5 min.

3. Results

The substrate properties of Ia,b were evaluated using M13mp10 DNA and a synthetic tetradecanucleotide as the template-primer (Scheme 1). Fig. 1 presents the results of a primer extension assay employing DNA polymerase α. Clearly, Ia was incorporated into the DNA chain (lane 3), the efficiency of incorporation being only slightly lower than that for dTTP (lane 2). The primer containing a residue of Ia was efficiently extended to give a modified heptadecamer (lane 5). In the control assay (lane 4), dTMP and dGMP residues were incorporated into the primer. The heptadecamer containing two residues of Ia was elongated, but its extension in the presence of dATP (lane 7) was markedly less efficient than elongation of the control oligonucleotide (lane 6). Lanes 7 and 9 are similar, implying that the enzyme can incorporate no more than two nucleotide residues of Ia into the DNA chain; the control reaction with four natural dNTPs is shown in lane 8. Compound Ib was incorporated three times, the insertion of the third

```
            10        20
3′-GGGTCAGTGCTGCAACATTTTGCTCCGGTCA.....
5′-CCCAGTCACGACGT ---->
```

Scheme 1. Structure of the template–primer complex used in this work.

*Corresponding author. Fax: (7) (095) 135 14 05.

Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; AMV, avian myeloblastosis virus; TdT, terminal deoxynucleotidyl transferase; KFr, DNA polymerase I Klenow fragment from *E. coli*; dNTP, 2′-deoxynucleoside 5′-triphosphate; dNMP, 2′-deoxynucleoside 5′-monophosphate; d_2NTP, 2′,3′-dideoxynucleoside 5′-triphosphate.

0014-5793/95/$9.50 © 1995 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)01319-5

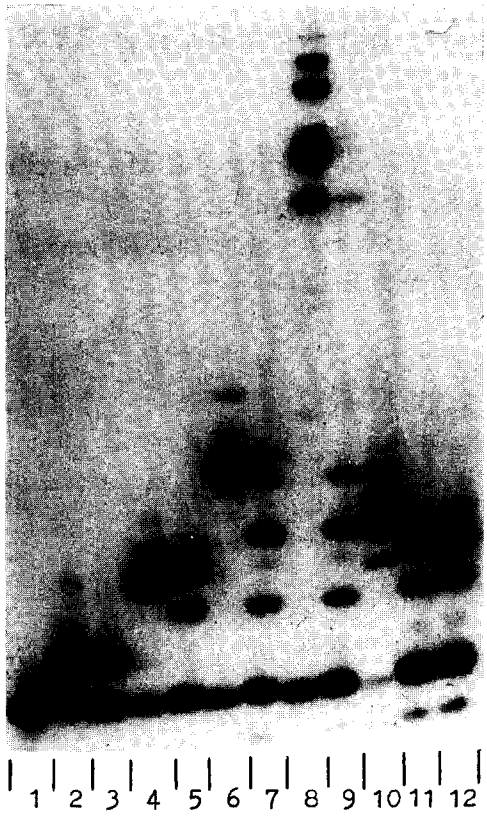


Fig. 1. Primer extension catalyzed by DNA polymerase α. (1) Primer-template + enzyme; (2) as (1) + 10 μM dTTP; (3) as (1) + 10 μM Ia; (4) as (1) + 10 μM dTTP + 10 μM dGTP; (5) as (1) + 10 μM Ia + 10 μM dGTP; (6) as (1) + 10 μM dTTP + 10 μM dGTP + 10 μM dATP; (7) as (1) + 10 μM Ia + 10 μM dGTP + 10 μM dATP; (8) as (1) + 10 μM dTTP + 10 μM dGTP + 10 μM dATP + 10 μM dCTP; (9) as (1) + 10 μM Ia + 10 μM dGTP + 10 μM dATP + 10 μM dCTP; (10) as (1) + 10 μM Ib + 10 μM dGTP + 10 μM dTTP; (11) as (1) + 10 μM Ia + 10 μM Ib + 10 μM dGTP + 10 μM dCTP.

residue being markedly less efficient (lane 10). When both Ia and Ib were added to the assay mixture, incorporation of two residues of Ia and one residue of Ib (lane 8) was observed.

It can be seen in Fig. 2 that AMV RT (series A), HIV RT (B), and DNA polymerase ε (D) did not utilize Ia. DNA polymerase β also did not recognize the compound (data not shown). At the same time, the nucleotide residue of Ia was incorporated into DNA chain twice by DNA polymerase α (series C) and once by TdT (series E). In the TdT assay, chasing with dTTP resulted in extension of the primer containing a residue of Ia by only two dTMP residues (lane 5).

To estimate the relative affinity of Ia to the (DNA polymerase α–template–primer) complex, we determined the kinetic parameters for this compound and dTTP in the one-step primer extension reaction. The K_m values for Ia and dTTP were 8.9 ± 0.3 and 3.8 ± 0.2 μM, the V_{max}(Ia)/V_{max}(dTTP) ratio was equal to 0.48.

Compound II was not utilized by all the enzymes examined (data not shown).

As can be seen in Fig. 3, III was a substrate for AMV RT (series A), and HIV RT (B), comparatively poor substrate for KFr (C), and DNA polymerase α (E), but was not recognized by DNA polymerase β (D). For AMV RT, nonspecific incorporation of one nucleotide residue was observed (lane 2); this effect is well known [6]. We also evaluated it as a chain terminator in several sequencing assays using different DNA polymerases. Termination of HIV RT-catalyzed DNA synthesis by III is shown in Fig. 4. The band intensity depended on the III concentration, the minimal concentration producing the termination picture being 5 μM (III/dGTP concentration ratio 2.5).

4. Discussion

Among all examined DNA polymerases, DNA polymerase α is one of the most substrate-specific enzymes [8,9]. To date, no nucleotide substrates specific to DNA polymerase α have been found. We showed compounds Ia,b to be specific substrates for template-dependent DNA polymerase α and template-independent TdT. Meanwhile, they were not recognized by such low-specific enzymes as retroviral RTs, *E. coli* KFr, and mammalian repair DNA polymerase β. Besides, they did not display substrate properties toward human replicative DNA polymerase ε . We successfully capitalized on this selectivity during simultaneous isolation of DNA polymerases α and ε , which often co-purify upon chromatography on different resins. The purity of DNA polymerase ε preparations was monitored by primer extension tests in the presence of Ia. This method proved to be very sensitive, and its results were con-

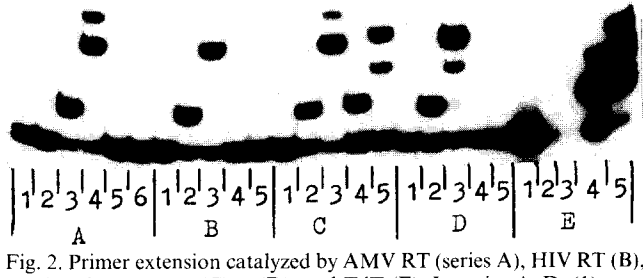


Fig. 2. Primer extension catalyzed by AMV RT (series A), HIV RT (B), DNA polymerases a (C), e (D), and TdT (E). In series A–D: (1) template–primer + enzyme; (2) as (1) + 10 μM dTTP; (3) as (1) + 10 μM dTTP + 10 μM dGTP; (4) as (1) + 10 μM Ia; (5) as (1) + 10 μM Ia + 10 μM dGTP. In series E: (1) primer + enzyme; (2) and (3) as (1) + 10 μM dTTP; (4) and (5) as (1) + 10 μM Ia; assays (3) and (5) were chased with 300 μM dTTP.

firmed by tests with monoclonal antibodies specific to DNA polymerase α.

In all experiments, compounds Ia,b were efficiently incorporated into the DNA chain only twice, implying that the oligonucleotide containing two modified residues cannot serve as a primer, presumably, because of the conformational distortion of the extended template–primer complex. This effect has been earlier observed with arabinonucleoside 5′-triphosphates [10].

Our data raise the question of what properties of compounds Ia,b determine their selectivity. In view of the fact that 3′-amino-2′,3′-dideoxythymidine 5′-triphosphate is a substrate for DNA polymerase α [11], we synthesized compound II containing an amino group at the 3′ position. This modification, however, inactivated the compound. The presence of the 3′ hydroxyl is not necessary to maintain the substrate properties of the nucleotide analog to DNA polymerase α: compound III, as well as acyclovir 5′-triphosphate [12] and acyclo-2′,3′-dideoxy-2′,3′-didehydronucleoside 5′-triphosphates [13] were shown to be substrates for DNA polymerase α. Thus, it remains unclear which structural elements of Ia,b impart them with the unique property of being specific substrates for mammalian DNA polymerases and, specifically DNA polymerase α.

Our results and the earlier reported data [14] indicate that introduction of a methylene group into the molecule of a deoxyribose-related nucleotide markedly alters its substrate properties, whereas the same modification of an acyclic nucleotide analog, acyclovir, has no marked effect on its substrate properties toward retroviral RTs and human DNA polymerase α. It has been assumed [15] that upon binding to the DNA-synthesizing complex, the glycone dNTP functions only as a framework maintaining the correct distance between the nucleic base and triphosphate residue, whereas the latter directly binds specifically to the enzyme's active center. We suppose that for acyclic nucleotide analogs, the change of this distance, caused by introduction of an additional group is compensated by a higher flexibility of the open pseudosugar residue, while for deoxyribose-related compounds, the modification markedly changes the structure and thus precludes binding to the DNA polymerase active center.

We are planning to evaluate compounds I as substrates and inhibitors of mammalian DNA polymerases δ and γ. Regardless of the results obtained with these enzymes, our data on DNA polymerase α and TdT can be used in designing anticancer agents.

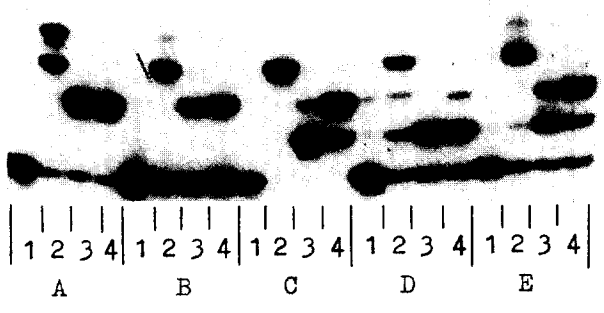


Fig. 3. Primer extension catalyzed by AMV RT (series A), HIV RT (B), KFr (C), DNA polymerase β (D), and DNA polymerase α (E). (1) template–primer + enzyme; (2) as (1) + 5 μM dTTP + 5 m M dGTP; (3) as (1) + 5 μM dTTP + 5 μM III; (4) as (1) + 5 μM dTTP + 10 μM III.

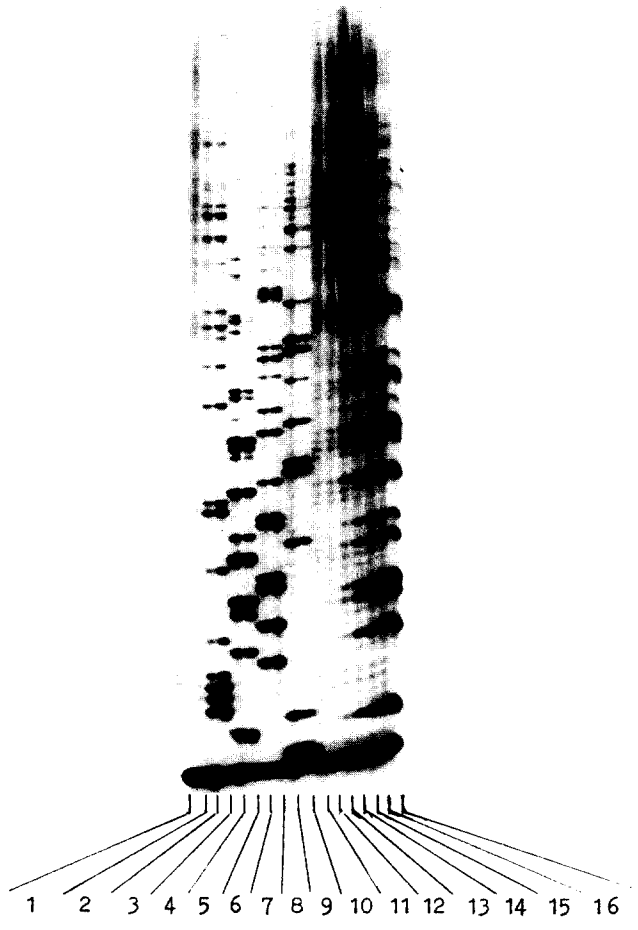


Fig. 4. Termination of DNA synthesis catalyzed by HIV RT. (1) DNA synthesis in the absence of terminators (control); (2–9) in the presence of 0.5 μM (2) and 1 μM (3) ddATP; 1 μM (4) and 2 μM (5) ddGTP; 1 μM (6) and 2 μM (7) ddCTP; 2 μM (8) and 4 μM (9) ddTTP. (10–16) in the presence of 0.5 μM (10), 1 μM (11), 2 μM (12), 5 μM (13), 10 μM (14), 20 μM (15), and 50 μM (16) III. All assays were chased with a mixture of 500 μM dNTPs.

Acknowledgements: This work was supported by the Russian State Fund of Fundamental Research (Grant N 93-04-20524) and the Program National Priorities in Medicine and Public Health. AIDS (Grants SP290 and SP306).

References

[1] Kukhanova, M.K., Kuznetsova, E.V., Jasko, M.V., Morin, J., Becker, D., O'Hara, B. and Gluzman, J. (1994) Mol. Biol. (Russian) 28, 530–541.
[2] Rozovskaya, T.A., Belogurov, A.A., Lukin, M.A., Chernov, D.N., Kukhanova, M.K. and Beabealasvilli, R.Sh. (1993) Mol. Biol. (Russian) 28, 618–630.
[3] Mozzerin, D.Yu., Atrazev, A.M. and Kukanova, M.K. (1992) Mol. Biol. (Russian) 26, 999–1010.
[4] Kolocheva, T.I. and Nevinsky, G.A. (1993) Mol. Biol. (Russian) 27, 1368–1379.
[5] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press.
[6] Victorova, L.S., Dyatkina, N.B., Mozzerin, D.Yu., Atrazhev, A.M., Krayevsky, A.A. and Kukhanova, M.K. (1992) Nucleic Acids Res. 20, 783–789.
[7] Semizarov, D.G., Victorova, L.S., Krayevsky, A.A. and Kukhanova, M.K. (1993) FEBS Lett. 327, 45–48.

[8] Krayevsky, A.A., Kukhanova, M.K., Atrazhev, A.M., Chidgeavadze, Z.G. and Beabealashvilli, R.Sh. (1987) Mol. Biol. (Russian) 21, 33–38
[9] Krayevsky, A.A. (1992) Mol. Biol. (Russian) 26, 725–744.
[10] Cozzarelli, R.N. (1977) Annu. Rev. Biochem. 46, 641–668.
[11] Chidgeavadze, Z.G., Beabealashvilli, R.S., Atrazhev, A.M., Kukhanova, M.K., Azhayev, A.V. and Krayevsky, A.A. (1984) Nucleic Acids Res. 12, 1671–1684.
[12] Germershausen, J., Bostedor, R., Field, A.K., Perry, H., Liou, R., Bull, H., Tolman, R.L. and Karkas, J.D. (1983) Biochem. Biophys. Res. Commun. 116, 360–367.
[13] Krayevsky, A.A., Victorova, L.S., Mozzherin, D.Ju. and Kukhanova, M.K. (1993) Nucleosides Nucleotides 12, 83–93.
[14] Herdewijn, P., Jie, L., Van Aershot, A., Balzarini, J., Jnssen, G., Busson, R., Hoodmartens, J. and De Clercq, E. (1990) J. Med. Chem. 33, 2481–2486.
[15] Krayevsky, A.A. and Watanabe, K.A. (1993) Nucleosides Nucleotides 12, 649–670.