Stereoisomers of Deoxynucleoside 5’-Triphosphates as Substrates for Template-dependent and -independent DNA Polymerases*

(Received for publication, November 5, 1996)

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All four possible stereoisomers of dNTP with regard to deoxyribofuranose C-1’ and C-4’ carbon atoms were studied as substrates for several template-dependent DNA polymerases and template-independent terminal deoxynucleotidyl transferase. It was shown that DNA polymerases α, β, and ε from human placenta and reverse transcriptases of human immunodeficiency virus and avian myeloblastosis virus incorporate into the DNA chain only natural β-D-dNTPs, whereas calf thymus terminal deoxynucleotidyl transferase incorporates two nucleotide residues of α-D-dNTP and extends the resulting oligonucleotide in the presence of β-D-dNTPs. The latter enzyme also extended α-anomeric β-oligodeoxynucleotide primers in the presence of β-D-dNTPs. None of the studied enzymes utilized γ-dNTPs. These data indicate that template-dependent DNA polymerases are highly stereospecific with regard to dNTPs, whereas template-independent terminal deoxynucleotidyl transferase shows less stereodifferentiation. It is likely that the active center of the latter enzyme forms no specific contacts with the nucleic bases of both nucleotide substrate and oligonucleotide primer.

The substrate activity of the stereoisomers of DNA polymerase natural substrates and their analogs is of significant current interest, because these compounds can help to ascertain the mechanism of substrate binding by DNA polymerases and identify the parts of the dNTP molecule that specifically bind to the active center of the enzymes. Indeed, the stereoisomers of natural β-D-dNTPs differ only in the mutual orientation of the reaction center (triphosphate fragment), the genetic recognition element (nucleic base), and the sugar residue.

Recently β-l-dTTP has been shown to be an inhibitor of HIV reverse transcriptase (1). It inhibited elongation of oligo(dT)12–18 complexed with poly(rA), by 50% at a [β-l-dTTP]/[dTTP] concentration ratio of 0.1. Although it was not demonstrated by direct experiments, such a strong inhibitory effect is most likely to result from chain termination. The inhibitory effect of β-l-dTTP on mammalian mitochondrial DNA polymerase γ was weaker than that observed for HIV reverse transcriptase. DNA polymerases α and β did not utilize β-l-dTTP as a substrate (1). Focher et al. (2) have studied the substrate properties of β-l-dTTP toward human DNA polymerases α, β, γ, δ, and ε, as well as herpes simplex virus type 1 DNA polymerase, Escherichia coli DNA polymerase I, HIV reverse transcriptase, and TDT using poly(dA)-oligo(dT)20 as a template-primer for template-dependent enzymes and oligo(dT)20 as a primer for TDT. DNA polymerases β, γ, δ, and ε did not incorporate nucleotide residues of this compound into the DNA chain, whereas the other enzymes extended the primer by one or more β-l-dTMP residues. Specifically, DNA polymerases α and Klenow fragment incorporated two β-l-dTMP residues, and HIV reverse transcriptase elongated the primer by up to 3 or 4 β-l-dTMP residues.

Furthermore, it has been shown (3) that 2’,3’-dideoxy-β-l-thymidine 5’-phosphate and 2’,3’-dideoxy-2’,3’-didehydro-β-l-thymidine 5’-phosphate are incorporated into DNA chains by HIV reverse transcriptase, E. coli DNA polymerase I, and T7 DNA polymerase, but their affinity to the HIV enzyme is 10–50 times lower than that of their β-D-isomers. The β-l-stereoisomers of 2’,3’-dideoxy-2’,3’-didehydrocyclopentanetane-adenine 5’-α-methylene phosphonyl-β-g-diphasphate (4) and its guanine counterpart (5), as well as several (6) β-l-oxathiolanucleoside 5’-triphosphates (6–9) and (7) β-l-dioxolanenucleoside 5’-triphosphates (10), have been shown to be terminating substrates for a number of DNA polymerases.

To date, no compounds have been found that would specifically inhibit TDT in cell cultures. One of the reasons for that is the similarity in substrate specificity between TDT and some other DNA polymerases, especially DNA polymerase β (11, 12) and endogeneous reverse transcriptases (13).

However, the independence of TDT from the template is a factor that could simplify the design of selective inhibitors of this enzyme. Indeed, we have recently found (4) that dNTP analogs with trans-like orientation of the nucleic base and triphosphate residue efficiently and selectively inhibit DNA synthesis catalyzed by TDT.

In this paper we synthesized all four possible stereoisomers of dNTPs with respect to the C-1’ and C-4’ carbon atoms and evaluated them as substrates for template-dependent DNA polymerases α, β, and ε from human placenta, reverse transcriptases from HIV and avian myeloblastosis virus, and TDT from calf thymus. We also synthesized two anomeric α-D-oligodeoxynucleotides and studied them as primers for TDT.

* This work was supported by Russian Foundation for Basic Research Grants N93-04-20542 and N95-03-08142a and the French CNRS (project “Cooperation Franco-russe” 1752). The costs of publication of this article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: β-D-DNTP, natural 2’-deoxy-2’-anhydroxane 5’-triphosphate; α-D-DNTP with N being C, T, or A, α-anomer at C-1’ carbon of dNTP; β-l-DNTP with N being C or T, β-l-DNTP enantiomer; α-l-DNTP with N being C, T, or A, α-anomer at C-1’ carbon of β-l-DNTP; β-l-DNMP, natural 2’-deoxyxane 5’-monophosphate; α-D-DNMP, α-anomer at C-1’ carbon of dNMP; β-l-DtMP, β-l-DTMP enantiomer; TDT, terminal deoxynucleotidyl transferase; HIV, human immunodeficiency virus; HPLC, high pressure liquid chromatography.
Stereoisomers of Deoxyribonucleoside 5'-Triphosphates

MATERIALS AND METHODS

The starting compounds, L-3-D, and L-3-L-dNTPs, and L-3-D-dNTPs, were synthesized according to Ludwig (18) using POCl₃ and pyrophosphate. For purification of dNTP stereromers, DEAE-Toyopearl 650 M (Toyosoda) and LiChroprep RP-18 (40–63 μm, Merck) were used. Their UV characteristics are listed in Table I. All stereomers were separated from possible DNA contaminants by HPLC; the retention times are given in Table I.

**Enzymes and DNA**—HIV reverse transcriptase was isolated according to Ref. 19. DNA polymerases α and δ were isolated from human placenta as described in Ref. 20. DNA polymerase β was purified according to Ref. 21. Avian myeloblastosis virus reverse transcriptase and calf thymus TDT were from Omuttnick Chemicals (Russia) and Amersham Corp., respectively.

Single-stranded M13mp10 DNA was isolated from the culture medium of the recipient E. coli K12XL1 strain as described in Ref. 22. Tetradecanucleotide primers α and β (Fig. 2) were synthesized according to Ref. 19. Labeled primers were added to a final concentration of 0.2 mM dATP, 0.1 mM dCTP, and 1 mM dGTP. The reaction products were separated by electrophoresis in 20% polyacrylamide gel, and the gels obtained were autoradiographed.

For TDT, the assay mixture contained 0.1 μM 5'-32P-labeled tetradecanucleotide primer (Fig. 2) or α-3-deoxy-1-3-3-labeled oligonucleotide, comprised of three different oligonucleotides, which were used in a molar ratio of 1:1:1. The reaction was carried out for 30 min at 37 °C and terminated by adding 3 μl of deionized formamide containing 0.5 mM EDTA and 2% bromphenol blue and xylene cyanol. The reaction products were separated by electrophoresis in 20% polyacrylamide gel, and the gels obtained were autoradiographed.

**Table I**

| Compound | UV spectrum, λ<sub>max</sub> | Retention time |
|----------|-----------------|----------------|
|          | nm              | min            |
| α-3-DTP  | (pH 7)          | 268            | 10.5           |
| β-3-DTP  | (pH 7)          | 275            | 6.5            |
| α-3-ATP  | (pH 7)          | 261            | 12.5           |
| β-3-ATP  | (pH 7)          | 275            | 7              |
| α-3-DTP  | (pH 7)          | 268            | 11.5           |
| β-3-DTP  | (pH 7)          | 281            | 12.5           |
| α-3-ATP  | (pH 7)          | 261            | 7              |
| β-3-ATP  | (pH 7)          | 275            | 7              |

**RESULTS**

The structure of the dNTP stereoisomers studied is shown in Table I. For TD assays, 2 units of the enzyme, 100 μM sodium cacodylate (pH 7.2), 10 mM MgCl₂, 1 mM CoCl₂, and 1 mM 2-mercaptoethanol were used. The fidelity of DNA synthesis catalyzed by HIV reverse transcriptase is rather low (24–26), and the probability of incorrect NMP incorporation is higher for the template positions remote from the template positions remote from the primer 3' terminus by more than two nucleotide residues. Therefore, we used different template-primers for the dCTP, dTTP, and dATP stereoisomers (type of template-primer used is specified in figure caption).

It is evident from Fig. 3 that β-3-DTP is not incorporated into the DNA chain by HIV reverse transcriptase (Fig. 3A, lanes 4–6). In the control assays, dATP (Fig. 3A, lane 2), dCTP + dCTP (Fig. 3A, lane 3) were used. Similar results were obtained for avian myeloblastosis virus reverse transcriptase (data not shown). We also evaluated β-3-DCTP and β-3-DTTP as substrates for human DNA polymerases α, β, and δ. It can be seen in Fig. 4 that β-3-DCTP is also not incorporated into the DNA chain by these enzymes (Fig. 4A and B, lanes 4–6). It was not a substrate for dopamine polymerase β (data not shown).

**Fig. 2. Structure of the template-primers.**

**Fig. 3. Series A, primer extension catalyzed by HIV reverse transcriptase.** Lane 1, template-primer α + enzyme; lane 2, as in lane 1 + 2 μM dATP; lane 3, as in lane 1 + 2 μM dATP + 2 μM dCTP; lanes 4 and 5, as in lane 1 + 2 μM dATP + 2 μM (lanes 4 and 5) 1-3-DTP; lane 6, as in lane 1 + 2 μM dATP + 20 μM β-3-DCTP + 20 μM dATP. Series B, primer extension catalyzed by TDT. Lane 1, primer α + enzyme; lane 2, as in lane 1 + 0.2 μM (lane 2) and 2 μM (lanes 3) dCTP; lanes 4–6, as in lane 1 + 0.2 μM (lane 4) and 2 μM (lanes 5 and 6) 1-3-DCTP.
Similar results were obtained for efficient primer extension depended on the dNTP concentration. The efficiency of primer extension depended on the dNTP concentration. The products being dependent on the dNTP concentration.

It can be seen in Fig. 5 that α-δ-dNTPs (lanes 3, 4, 6, and 7) and α-δ-dNTPs (lanes 8, 9, 11, and 12) are not substrates for HIV reverse transcriptase. We assume that α-δ-dATP practically does not interact with the DNA-synthesizing complex, because it did not inhibit primer extension by dTMP and dGMP residues even at a high concentration (Fig. 5, lanes 6–7), whereas α-δ-dATP completely inhibited primer extension at a [α-δ-dATP]/[dNTP] concentration ratio of 10:1 (Fig. 5, lane 12). We attribute the presence of a weak heptadecanucleotide band in lane 2 of Fig. 5 to the error-prone properties of HIV reverse transcriptase. Both α-δ-dNTPs and α-δ-dNTPs were not utilized as substrates by human DNA polymerases (data not shown).

It is evident from Fig. 6 that two α-δ-dTMP (lanes 10–12) and α-δ-dAMP (lanes 13–15) residues are incorporated into the primer by TDT, and one more residue is incorporated less efficiently. The efficiency of primer extension depended on the substrate concentration. Similar results were obtained for α-δ-dCTP (data not shown). The α-δ-dTTP (Fig. 6, lanes 4–6) and α-δ-dATP were not utilized by the enzyme. In the control assays (Fig. 6, lanes 2 and 3), dTTP was used as a substrate. We ascribe the presence of weak pentadecanucleotide bands on lanes 1, 5, and 6 of Fig. 6 to contamination of the TDT preparation with trace amounts of dNTPs.

Then, we examined the ability of TDT to elongate oligonucleotides terminated by α-δ-dNMP residues in the presence of different concentrations of dNTPs. It can be seen in Fig. 7 that oligodeoxy nucleotides containing α-δ-dTMP and α-δ-dAMP residues at the 3’ end are elongated by TDT in the presence of 0.2, 2, and 20 μM dNTPs (lanes 6–8 and 11–13), the length of the products being dependent on the dNTP concentration.

At the second stage of this research we evaluated α-d[(T)p(9)T] and α-d[(T)p(9)T] as primers for TDT in the presence of natural dNTPs and α-dNTP (Fig. 8, B and D). Oligonucleotides β-d[(T)p(9)T] (Fig. 8A) and β-d[(T)p(9)T] (Fig. 8C) were used as reference primers. All four oligothymidylates were extended in the presence of dGTP, and the length of the products depended on the dGTP concentration (Fig. 8, lanes 2–4). Similar results were obtained with dCTP as substrate.

**DISCUSSION**

Our results indicate that template-dependent DNA polymerases utilize as substrates only β-δ-dNTPs, whereas for template-independent TDT α-δ-dNTPs but not β-δ-dNTPs or α-δ-dNTPs are substrates. It is noteworthy that α-δ-dNTPs but not β-δ-dNTPs or α-δ-dNTPs inhibited primer extension catalyzed by HIV and avian myeloblastosis virus reverse transcriptases. It seems likely that the 3’ hydroxyl of l-dNTPs hinders formation of the productive [DNA polymerase + template-primer + dNTP] complex by creating steric obstacles for dNTP binding to the enzyme.

The inconsistency between our results and the data of Focher et al. (2) may be attributed to the following differences in the experimental conditions. First, these authors used a homopolymeric template-primer, whereas in our experiments random-
sequence tetradecanucleotides and M13mp10 DNA were employed. Thus, our system better models the natural conditions of DNA biosynthesis. Second, Focher et al. used groundlessly high concentrations of β-L-dTTP (up to 0.5–1 mM). Obviously, this may initiate various side processes. When we repeated our experiments using these high concentrations of β-L-dNTPs, many uninterpretable bands were observed. Finally, formation of oligo(dT)₉₁9 observed by Focher et al. (2) upon primer extension by DNA polymerase α, HIV reverse transcriptase, and TDT (Fig. 5 in Ref. 2) may result only from pyrophosphorylation of oligo(dT)₂₀, because these enzymes do not possess 3'-TDT (Fig. 5 in Ref. 2). It is likely that many uninterpretable bands were observed. Thus, our system better models the natural conditions of DNA biosynthesis. Second, Focher et al. used groundlessly high concentrations of β-L-dTTP (up to 0.5–1 mM). Obviously, this may initiate various side processes. When we repeated our experiments using these high concentrations of β-L-dNTPs, many uninterpretable bands were observed. Finally, formation of oligo(dT)₉₁9 observed by Focher et al. (2) upon primer extension by DNA polymerase α, HIV reverse transcriptase, and TDT (Fig. 5 in Ref. 2) may result only from pyrophosphorylation of oligo(dT)₂₀, because these enzymes do not possess 3'-TDT (Fig. 5 in Ref. 2). It is likely that many uninterpretable bands were observed.

The results obtained may imply that the nucleic bases of dNTP and oligonucleotide primers do not bind in a specific manner to the substrate- and primer-binding sites, respectively, of the TDT active center. Indeed, we have found that phenylphosphonyldiphosphate (II) in Fig. 9 was not a substrate for TDT (data not shown), but inhibited TDT-catalyzed primer extension by 50% at a [III]/[dTTP] molar concentration ratio of 1:1.

Acknowledgments—We are grateful to Dr. L. Victorova (Engelhardt Institute of Molecular Biology, Moscow, Russia) for providing DNA polymerase β and to Drs S. Spadari and F. Focher (Istituto di Genetica Biochimica ed Evoluzionistica, Pavia, Italy) for useful discussion.

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Fig. 8. Extension of β-d[(Tp)₉] (A), α-d[(Tp)₉] (B), β-d[(Tp)₉] (C), and α-d[(Tp)₉] (D) catalyzed by TDT. Lanes 1, oligodeoxythymidylate + enzyme; lanes 2–4, as in lanes 1 + 0.2 μM lanes 2, 2 μM (lanes 3), and 20 μM (lanes 4) dGTP.

Fig. 9. Structure of the TDT substrate carbocyclic α-δ- and l-dNTP analogs I and II and the TDT inhibitor phenylphosphonyldiphosphate III.
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J. Biol. Chem. 1997, 272:9556-9560.
doi: 10.1074/jbc.272.14.9556

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