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Properties of 2′,3′-dideoxy-2′,3′-dehydrothymidine 5′-triphosphate in terminating DNA synthesis catalyzed by several different DNA polymerases

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2′-3′-Dideoxy-2′,3′-dehydrothymidine 5′-triphosphate (dddTTP) shows termination substrate properties in the DNA synthesis catalyzed by *E. coli* DNA polymerase I KF, rat liver DNA polymerase β, reverse transcriptases of avian myeloblastosis virus and Raus sarcoma virus and calf thymus terminal deoxynucleotidyl transferase. This implies that the mononucleotide residue of dddTTP incorporates into 3′-termini of newly synthesized DNA chains. However, dddTTP has no influence on the DNA synthesis catalyzed by calf thymus DNA polymerase α. In the case of some DNA polymerases dddTTP was one order of magnitude more effective in comparison with the other known termination substrates.

DNA polymerase; 2′,3′-Dideoxy-2′,3′-dehydrothymidine 5′-triphosphate; Termination substrate

1. INTRODUCTION

In 1985–1986 it was shown by NMR that dNTP substrates in [DNA polymerase I KF + dNTP] and [DNA polymerase I KF + dNTP + template analog] complexes assumed a unique conformation. Deoxyribose residues of dNTP in these complexes have the O1′-*endo* conformation, and the C1′, C2′, C3′ and C4′ atoms are practically coplanar [1,2]. For further investigation of this problem we synthesized dddTTP and investigated its termination substrate properties in the DNA synthesis reaction catalyzed by six different DNA polymerases. dddTTP is known to block DNA synthesis catalyzed by *E. coli* DNA polymerase I, however, the molecular mechanism of this action has not been examined [3].

All four carbon atoms in the 2′,3′-dehydroribose moiety of dddTTP are coplanar (owing to the presence of the double bond), excepting the endocyclic oxygen only. Therefore, dddTTP can model conformationally the substrate in the DNA synthesizing complex.

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Abbreviations: dNTP, 2′-deoxynucleoside 5′-triphosphates with natural bases; dNTP(3′-F), dNTP(3′-NH_2), dNTP(3′-N_3) and dNTP(3′-NHacet), 5′-triphosphates of 3′-fluoro-, 3′-amino-, 3′-azido- and 3′-acetamido-2′,3′-dideoxynucleosides; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphates; dNTP(2′-OCH_3), 3′-*O*-methyl-dNTP; araNTP(3′-N_3) and araNTP(3′-NH_2), 5′-triphosphates of 3′-azido- and 3′-amino-3′-deoxyarabinonucleosides

2. MATERIALS AND METHODS

dddTTP was synthesized as in [4] with some modifications [5]. DNA polymerase I KF was isolated from *E. coli* NM 182 as in [6], calf thymus

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DNA polymerase α according to [7], rat liver DNA polymerase β as described [8], and calf thymus terminal deoxynucleotidyl transferase using the method in [9]; AMV and RSV reverse transcriptases were from Amersham. The 17-member oligonucleotide d(GTAAAACCACGGCCAGT) and the 14-member d(CCCAGTCACGACGT) were labeled with [^{32}P] at the 5′-position using polynucleotide kinase and [γ-^{32}P]ATP. dNTP(3′-NH_2) and dNTP(3′-N_3) [10], dNTP(3′-F) [11], dNTP(3′-OCH_3) [12], araNTP(3′-N_3) and araNTP(3′-NH_2) [13] were synthesized as described.

All tests with DNA polymerases were made on phage M13mp10 DNA as a template and the 17-member or 14-member deoxyoligonucleotide as a primer; all experimental procedures were conducted as in [6,14]. Concentrations of substrates, inhibitors, enzymes are given in the figure legends.

3. RESULTS

The termination properties of dddTTP in DNA synthesis catalyzed by six DNA polymerases were investigated. After every incubation the reaction products were resolved by gel electrophoresis, and the termination properties of dddTTP studied using sets of bands of newly synthesized DNA chains terminated by the thymidine residue.

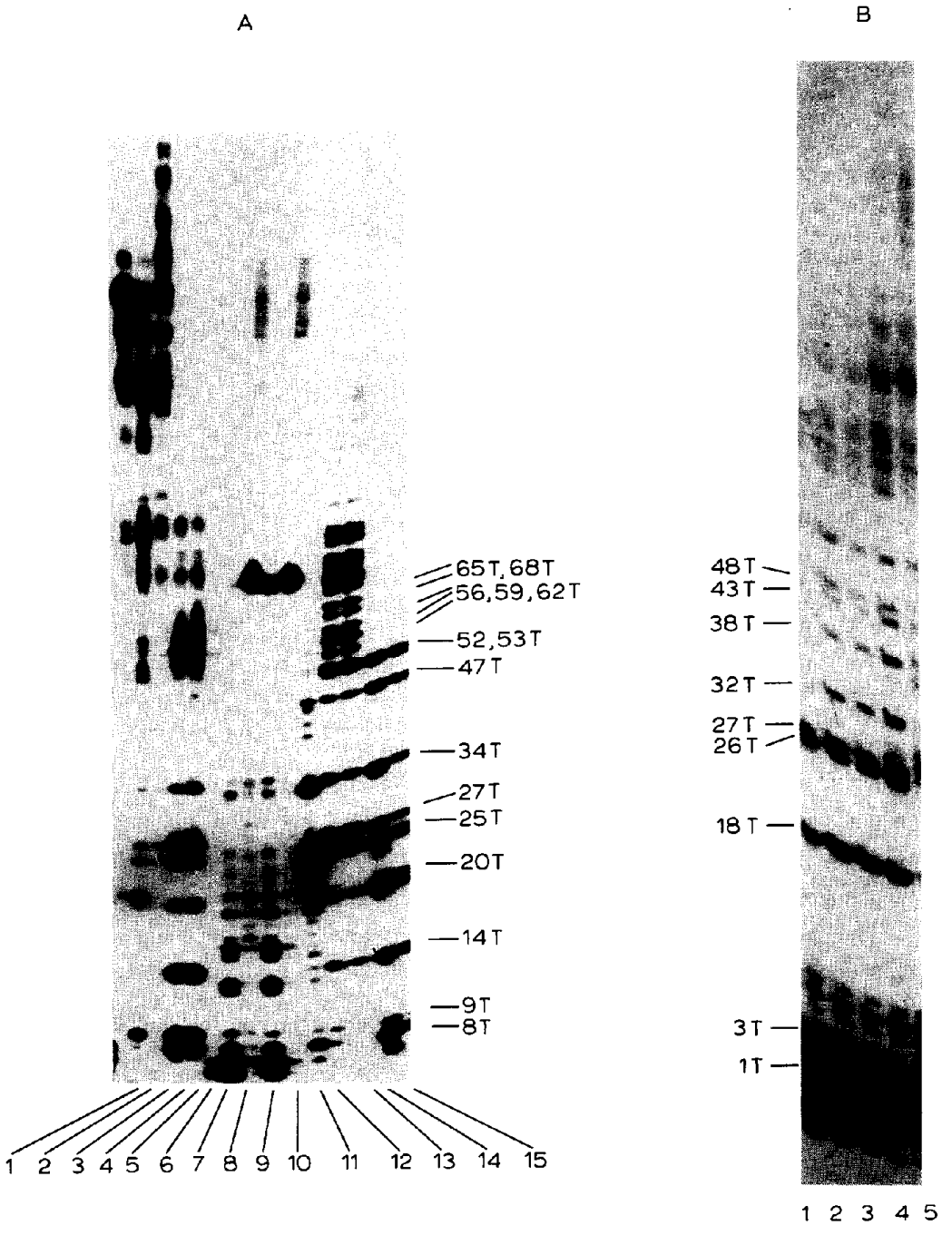
3.1. *DNA polymerase I KF from* E. coli, *AMV and RSV reverse transcriptases and DNA polymerase α from calf thymus*

Fig.1A presents the results of dddTTP action on primer chain elongation catalyzed by three DNA polymerases. The structure of the newly synthesized DNA fragment is shown in scheme 1a. From tracks 4–5 it can be seen that dddTTP terminates DNA chain elongation catalyzed by AMV reverse transcriptase, the effectiveness of this process being significantly greater than that in control tracks. For these control tracks, termination was achieved with dNTP(3′-OCH_3) (tracks 2,3) [12]. Strong termination of DNA synthesis in the presence of dddTTP is observed in a set of bands with shorter DNA fragments (the fragments that terminated on 8T, 9T, 14T, 20T, 25T, 27T, 34T) in comparison with DNA fragments in tracks 2 and 3. However, neither dddTTP (tracks 9,10) nor dNTP(3′-OCH_3) (tracks 7,8) terminated the DNA synthesis catalyzed by DNA polymerase α, which can be seen from the analysis of the sets of bands corresponding to normal pauses (cf. track 6). In the case of DNA polymerase I KF, the effect of dddTTP was compared with that of dTTP(3′-F) [11]; dTTP(3′-F) is known to be one of the strongest termination substrates for the system with DNA polymerase I KF [12]. From tracks 12 and 14,15 it

(a)	1	11	21	31	
	GCCAAGCTTG	GGCTGCAGGT	CGACTCTAGA	GGATCCCCGG	
	41	51	61	71	
	GCGAGCTCGA	ATTCGTAATC	ATGGTCATAG	CTGTTTCGTG	
(b)	1	11	21	31	41
	TGTAAAACGA	CGGCCAGTGC	CAAGCTTGGG	CTGCAGGTCG	ACTCTAGAGG

Scheme 1. Nucleotide sequence in a newly synthesized DNA chain

Fig.1. (A) Gel pattern of DNA synthesis in the presence of a ^{32}P-labeled 17-member primer catalyzed by AMV reverse transcriptase (tracks 1–5), DNA polymerase α (tracks 6–10) and DNA polymerase I KF (tracks 11–15). Experimental conditions for tests with reverse transcriptases: assay mixture contained 6 units enzyme, 100 μM dNTP; incubation time 2 min, 42°C (track 1). Termination substrates at 500 μM were then added to the reaction mixtures, and incubation continued for a further 60 min (tracks 2,4). Reaction mixtures were incubated for the third time with 250 μM dNTP and 6 units of an additional enzyme portions (tracks 3,5). Experimental conditions for DNA polymerase α: each test tube contained 2 units enzyme and 10 μM each of dNTP; incubation time 1 min, 37°C (track 6). Termination substrates at 500 μM were then added to other test tubes and the incubation continued for another 60 min (tracks 7,9). Mixtures were incubated (tracks 8,10) for the third time with 250 μM dNTP and a further 2 enzyme units for 60 min at 37°C. Experimental conditions for DNA polymerase I KF: each test tube contained 3 units enzyme, 1 μM dNTP; incubation time 10 s, 37°C (track 11). Termination substrates at 500 μM were added to other test tubes and the incubation



continued for 20 min at 37°C (tracks 12,14). Mixtures (tracks 13,15) were incubated for the third time with 250 μM dNTP for 20 min at 37°C. Termination substrates: dUTP(3'-OCH_3), tracks 2,3,7,8; dTTP(3'-F), tracks 12,13; dddTTP, tracks 4,5,9,10,14,15. (B) Gel pattern of DNA synthesis in the presence of RSV reverse transcriptase and the ^{32}P-labeled 14-member oligonucleotide. Other experimental conditions as in A, tracks 1–5. After the first incubation dddTTP was added to the reaction mixtures: 1, 500 μM; 2, 250 μM; 3, 100 μM; 4, 50 μM. Track 5, control without dddTTP.

is clear that both compounds terminate DNA synthesis but the effect of dddTTP is stronger. This is obvious from the set of strong bands corresponding to fragments terminated with 8T, 9T, 14T, 20T, 25T, 27T, 34T, 52–53T, whereas longer fragments are practically absent (tracks 14,15). In the case of dTTP(3′-F) (at concentrations equal to those of dddTTP) one can observe only prolonged fragments starting from 25T and 27T.

A more careful investigation of the termination reaction vs dddTTP concentration when the reaction was catalyzed by DNA polymerase I KF showed that dddTTP is 10–30-times more effective than other termination substrates (not shown). Similar results were also obtained for RSV reverse transcriptase (fig.1B). A clear picture of termination was observed at such dddTTP/dNTP ratios as 1:2 (mol/mol), which is 10–50-times less than for other termination substrates (not shown).

3.2. *DNA polymerase β from rat liver*

The action of dddTTP on DNA synthesis catalyzed by DNA polymerase β is illustrated in fig.2. A 14-member primer was used, and the structure of the newly synthesized DNA is pictured in scheme 1b. In all cases (tracks 2–6) a clear termination is observed (bands correspond to 1T, 3T, 18T, 26T, 27T, 32T, 38T). Even at a 1:1 ratio of dddTTP/dNTP concentration (track 4) the sequence pattern is readable. The same quality sequence pattern was obtained from a concentration ratio of 2.5:1 for dTTP(3′-NH_2)/dTTP (not shown).

3.3. *Terminal deoxynucleotidyltransferase from calf thymus*

Fig.3 presents the data on termination of the 17-member oligonucleotide by this enzyme in the presence of dddTTP. The initial oligonucleotide (tracks 1,2) was elongated by dTTP (track 3) and terminated by dTTP(3′-F) (tracks 4–6), nearly 50% of the termination being observed at 200 μM dTTP(3′-F) (track 6). Tracks 8–10 demonstrate the pattern of termination for the same oligonucleotide by dddTTP, 60–70% termination being observed at a dddTTP concentration of 10 μM. This implies that in the case of terminal deoxynucleotidyltransferase the activity of dddTTP is 20-times greater than that of dTTP(3′-F).

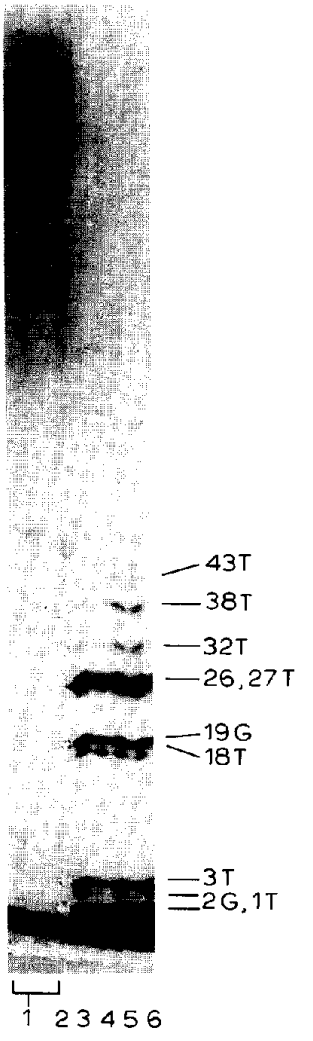


Fig.2. Gel pattern of DNA synthesis in the presence of DNA polymerase β and a ^{32}P-labeled 14-member primer. Each test tube contained 2 units enzyme, 4 mM $MgCl_2$, 10 μM dNTP and was incubated for 15 min at 37°C (track 1). dddTTP and 2 μM dNTP were then added. Incubation was continued for 15 min at 37°C. dddTTP concentrations: 10 μM (track 1), 20 μM (3), 50 μM (4), 100 μM (5), 1000 μM (6).

4. DISCUSSION

The above results demonstrate that dddTTP intensively terminates the synthesis of DNA catalyzed by DNA polymerase I KF and β, two reverse transcriptases and terminal deoxynucleotidyltransferase, but not that of DNA polymerase

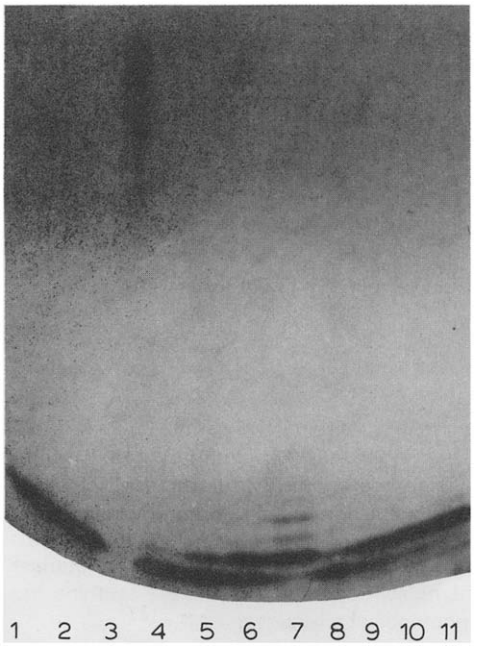


Fig.3. DNA synthesis catalyzed by terminal nucleotidyltransferase in the presence of ^{32}P-labeled 17-member oligonucleotide. Track 1, initial oligonucleotide. Each test mixture contained 1 unit enzyme; incubation time 60 min, 37°C (track 2); track 3, with 200 μM dNTP. Mixtures for tracks 4–7 were incubated as for track 2 but in the presence of dNTP(3′-F): 4, 1 μM; 5, 10 μM; 6,7, 200 μM; 200 μM dNTP added to the mixture for track 7 after the first incubation and another incubation was undertaken for 60 min, 37°C. The reaction mixtures for tracks 8–11 were made as for tracks 4–7, but instead of dTTP(3′-F), dddTTP was added: 1 μM (track 8), 10 μM (9), 200 μM (10,11).

α. Taking into account the strong termination activity of dddTTP and the coplanar structure of its C_1', C_2', C_3' and C_4' part of the sugar residue, we propose that dddTTP conformationally models the substrate in the [enzyme + template primer + dNTP] complex. Therefore, it may be supposed that compounds of this type open up perspectives for the investigation of substrate conformation at the transition state in the complete DNA synthesizing complex.

A stronger activity of dddTTP in comparison with that of dTTP(3′-F) demonstrates that template-independent DNA polymerase (terminal deoxynucleotidyltransferase) utilizes substrates after their transformation into conformations similar to that of the substrate for template-directed DNA polymerases.

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