3′-Hydroxymethyl 2′-deoxynucleoside 5′-triphosphates are inhibitors highly specific for reverse transcriptase

Tamara V. Kutateladze, Anatoli M. Kritzyn*, Vladimir L. Florentjev*, Vadim M. Kavsan+, Zurab G. Chidgeavadze° and Robert Sh. Beabealashvilli°

*Institute of Physiology, Georgian SSR Academy of Sciences, Tbilisi 380060, *Institute of Molecular Biology, USSR Academy of Sciences, Moscow 117984, +Institute of Molecular Biology and Genetics, Ukrainian SSR Academy of Sciences, Kiev 252627* and °*National Cardiology Research Center, USSR Academy of Medical Sciences, Moscow 121552, USSR*

Received 3 August 1986

dNTP(3′-OCH_3), a 3′-*O*-methyl derivative of dNTP, is a chain terminator substrate for DNA synthesis catalyzed by AMV reverse transriptase. The enzyme seems to be the only DNA polymerase susceptible to the inhibitor while all the other DNA polymerases tested are fully resistant to the nucleotide analog. The resistant polymerases are: *E. coli* DNA polymerase I, Klenow's fragment of DNA polymerase I, phage T_4 DNA polymerase, calf thymus DNA polymerase α, rat liver DNA polymerase β and calf thymus terminal deoxyribonucleotidyl transferase.

DNA polymerase *Terminal transferase* *Chain terminator* *Nucleotide analog*

1. INTRODUCTION

A wide variety of nucleoside 5′-triphosphates modified at the sugar moiety was tested as chain terminators for a number of polynucleotide synthesizing enzymes [1–14]. Among these we have found dNTP(3′-NH_2) which is equally active against most of the tested DNA polymerases [7–12], araNTP(3′-NH_2) which is active against all but *E. coli* DNA polymerase I [9,10], dNTP(3′-*N*-fluoroscaminyl) which is not inhibitory only for the terminal deoxyribonucleotidyl transferase [10], and dNTP(3′-N_3) and araNTP(3′-N_3) active against AMV reverse transcriptase [10,12].

Here, we tested dNTP(3′-OCH_3) and found that this analog was capable of base-specific DNA chain termination with RNA-dependent DNA polymerase (reverse transcriptase) only. This finding provides a putative way to discriminate the activity of the enzyme in the cell from that of DNA polymerases α and β as well as from the activity of terminal transferase.

2. MATERIALS AND METHODS

2.1. *DNA synthesis*

DNA was synthesized by different DNA polymerases (reverse transcriptase inclusive) using a single-stranded DNA of either M13 mp11 or recombinant M13 mp9 phages [15] with a [^{32}P]d(GTAAAACGACGGCCACT) primer kindly provided by Drs Chumakov and Chernov (Institute of Molecular Biology, Moscow). The reactions were run under optimal buffer conditions for each of the enzymes. For details, see [10,11]. DNA was synthesized with terminal transferase as in [10] using the same primer. The products of DNA synthesis were analyzed by electrophoresis in 12–30% polyacrylamide gel under denaturing conditions.

2.2. *Enzymes*

DNA polymerase I was purified from *E. coli* NM182 as described [16]. Klenow enzyme was produced from DNA polymerase I according to [17].

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00145793/86/$3.50

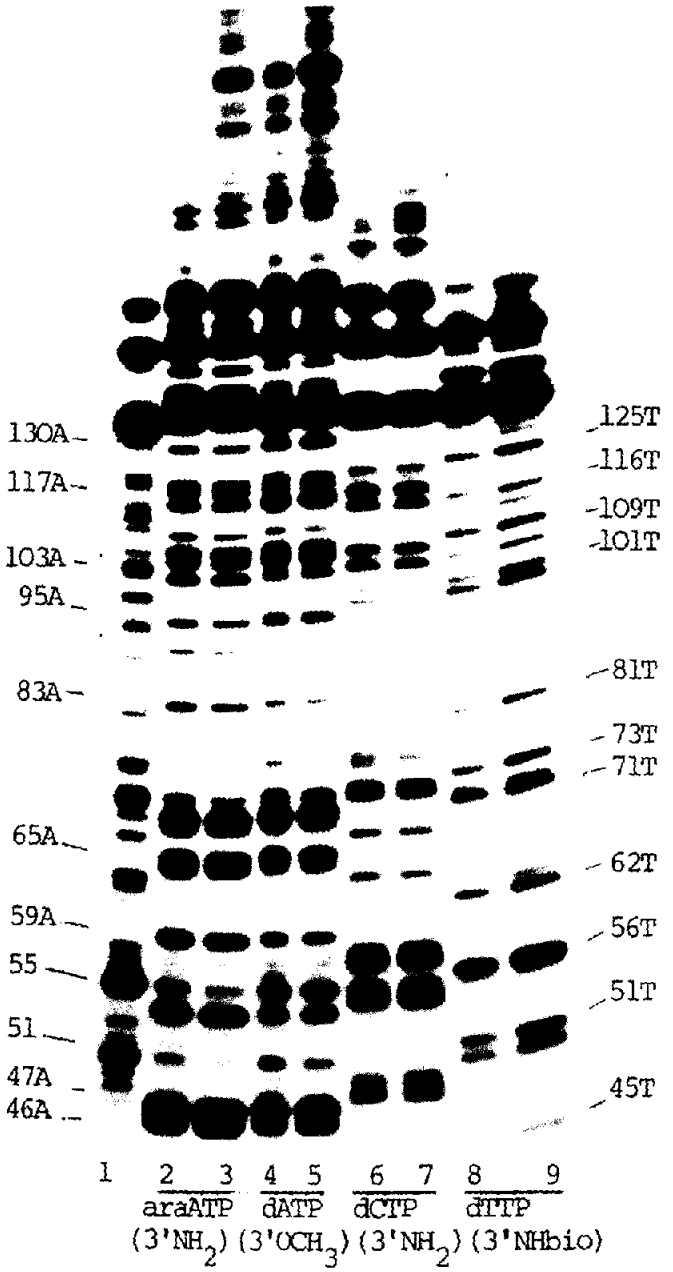

Fig.1. DNA synthesized from M13 mp9 recombinant phage [15] by reverse transcriptase. The nucleotide sequence of the product is:

41. ACTGTAACCT TGACCTCCAG GTTCAAGCAA TCCTCCCGCC TCAGCCCCCC 90

91. AGGTAGCCTC TGACTACATG AGTGCTACCA CACCTGCCTA TTTCTTCCTA 140

Nucleotides are numbered starting from the 5'-end of the primer. Nucleotide analogs used (in the tracks): (1) control, incubation without an analog, synthesis for 3 min; (2,3) araATP(3'-NH_2); (4,5) dATP(3'-OCH_3); (6,7) dCTP(3'-NH_2);

DNA polymerase α (7 S) was purified as in [18]. Terminal transferase was prepared according to the modified procedure of Chang and Bollum [19]. These enzymes were nearly homogeneous as revealed by electrophoresis in polyacrylamide gel, and contained no endonucleases as demonstrated by the resistance of supercoiled pBR322 DNA to overnight incubation with the enzyme preparation at 37°C under the conditions optimal for each enzyme. Reverse transcriptase was purified according to [20]. The specific activity and concentrations of stock enzyme solutions were as follows: DNA polymerase I, 60 U/μg, 20 U/μl; Klenow enzyme, 20 U/μg, 2 U/μl; DNA polymerase α, 5 U/μg, 0.4 U/μl; terminal transferase, 20 U/μg, 2 U/μl; reverse transcriptase, 7 U/μl. T_4 DNA polymerase was from PL Biochemicals (USA); DNA polymerase β (with an activity of 3 U/μg, 0.1 U/μl) was kindly provided by Dr Atrazhev (Institute of Molecular Biology, Moscow).

2.3. *Nucleotide analogs*

dATP(3′-OCH_3), dGTP(3′-OCH_3), dUTP(3′-OCH_3) and dCTP(3′-OCH_3) were synthesized as described in [21].

The nucleotide analogs contained about 1% of unmodified nucleoside triphosphates. Besides dCTP(3′-OCH_3) preparations contained an unidentified admixture (≈5%) of nucleoside triphosphates more lipophilic than dCTP(3′-OCH_3). Most probably it is the 4-*N*-methyl derivative of dCTP.

dNTP(3′-F), araNTP(3′-NH_2), dNTP(3′-NH_2) and dTTP(NHbio) were kindly donated by Dr Mikhailopulo (Institute of Bioorganic Chemistry, Minsk), Dr Papchikhin (Kuybyshev State University) and Dr Krayevsky (Institute of Molecular Biology, Moscow). The properties of these compounds, their nomenclature and ability to inhibit DNA polymerases were described earlier [7–12]. dNTPs were from Sigma (USA); [α-^{32}P]dNTP and [γ-^{32}P]ATP were from Isotop (USSR); [α-^{35}S]thio-dATP was from Amersham (England).

3. RESULTS AND DISCUSSION

Figs 1 and 2 show that dNTP(3′-OCH_3) is capable of terminating DNA synthesis catalyzed by reverse transcriptase with a correct termination of the growing DNA chain at sites directed by the nature of a nucleotide base. The correct sequence pattern does not change when an excess of natural dNTPs is added after the analog has been incorporated into the 3′-end of the DNA. It follows therefore that: (i) incorporation of the analog into the DNA chain is irreversible and (ii) prolongation of the terminated chain is impossible. dAMP(3′-OCH_3), dGMP(3′-OCH_3) and dUMP(3′-OCH_3) are incorporated in place of dAMP, dGMP and dTMP as one can see from the comparison with data for the corresponding dNTP(3′-F) [8] or araNTP(3′-NH_2) [9] and from the ability to read the correct DNA sequence from the electropherogram (figs 1 and 2). dCTP(3′-OCH_3) is also highly inhibitory. It terminates the DNA chain correctly but the sequencing pattern on the gel (fig.2, track 8) is shifted a little with respect to other nucleotide terminated patterns. Moreover, dCTP(3′-OCH_3) produces a rather smeared pattern.

It seems that dCTP(3′-OCH_3) preparations contain an admixture of a nucleotide substrate which, being incorporated into the DNA chain instead of dCMP, makes the DNA electrophoretic mobility differ from that in the case of natural dCMP. Most probably, the nucleotide is a dCTP analog modified somewhere in the base.

The presence of an admixture is confirmed by the results of experiments with the terminal transferase shown in fig.3. The enzyme cannot use dNTP(3′-OCH_3) as a substrate since the analog does not prevent oligonucleotide elongation in the presence of dNTP (fig.3a, tracks 5,6). When ^{32}P-

(8,9) dTTP(3′-NHbio). The reaction is initiated by adding 3.5 U reverse transcriptase to 20 μl of the medium containing 0.1 μg single-stranded DNA, preannealed with the ^{32}P-primer oligonucleotide and purified by A15 m agarose chromatography. The incubation medium contains: 50 mM Tris-HCl (pH 8.4), 10 mM $MgCl_2$, 50 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA and 50 μM each of the four natural dNTPs. After 3 min at 42°C an NTP analog is added to 1 mM concentration and the mixture incubated for 30 min. Then the reaction is either terminated by cooling to −20°C (tracks 2,4,6,8) or prolonged for another 30 min interval after adding a mixture of all the four dNTPs to a concentration of 1 mM (tracks 3,5,7,9).

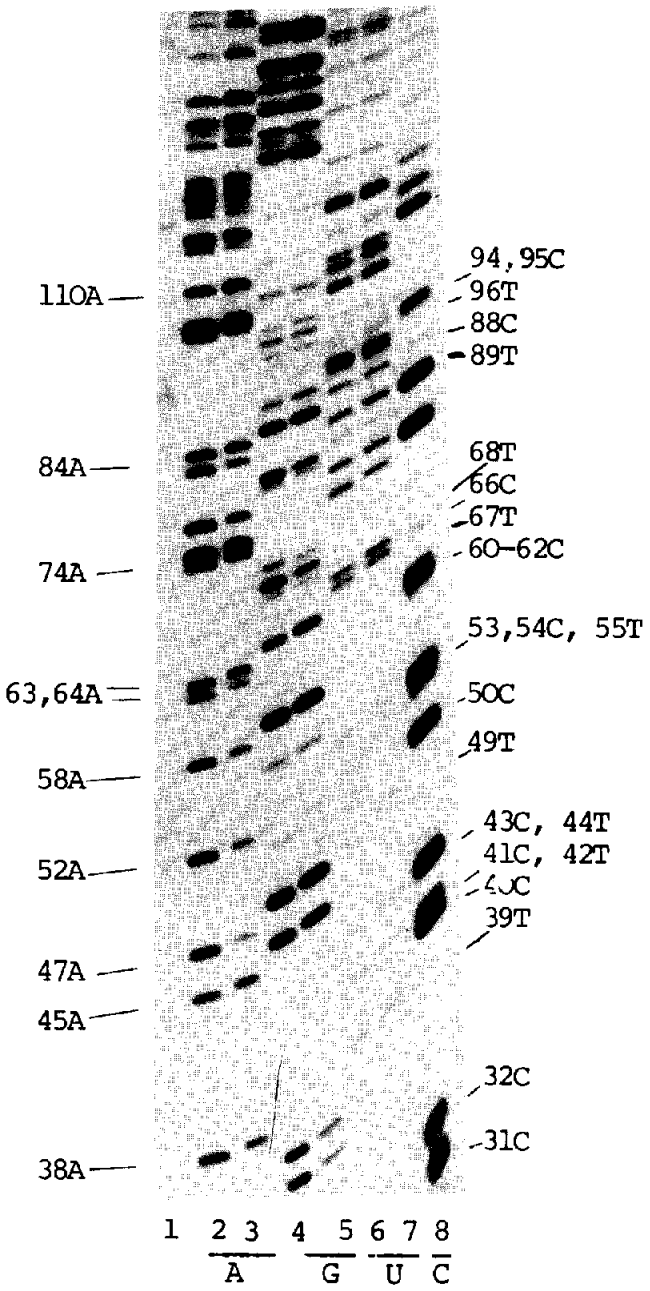

Fig.2. M13 mp11 DNA sequence with dNTP(3′-OCH_3). The reaction conditions are as in fig.1 except the concentrations: DNA, 0.5 μg/ml; reverse transcriptase, 0.1 U/μl; dNTP, 50 μM; nucleotide analogs, 1 mM. The reaction time is 30 min everywhere but track 7. Here after the incubation with dUTP(3′-OCH_3) for 30 min, a mixture of dNTPs is added to a concentration of 0.5 mM for an additional 30 min. dNTP analogs in the tracks: (1) no analog; (2) araATP(3′-NH_2); (3) dATP(3′-OCH_3); (4) araGTP(3′-NH_2); (5) dGTP(3′-OCH_3); (6,7) dUTP(3′-OCH_3); (8) dCTP(3′-OCH_3). The DNA sequence is:

31. CCCGGGGATC CTCTAGAGTC GACCTGCAGC CCAAGCTTGG CGTAATCATG 80

81. GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA 130

Nucleotide numbering starts from the 5′-end of the primer.

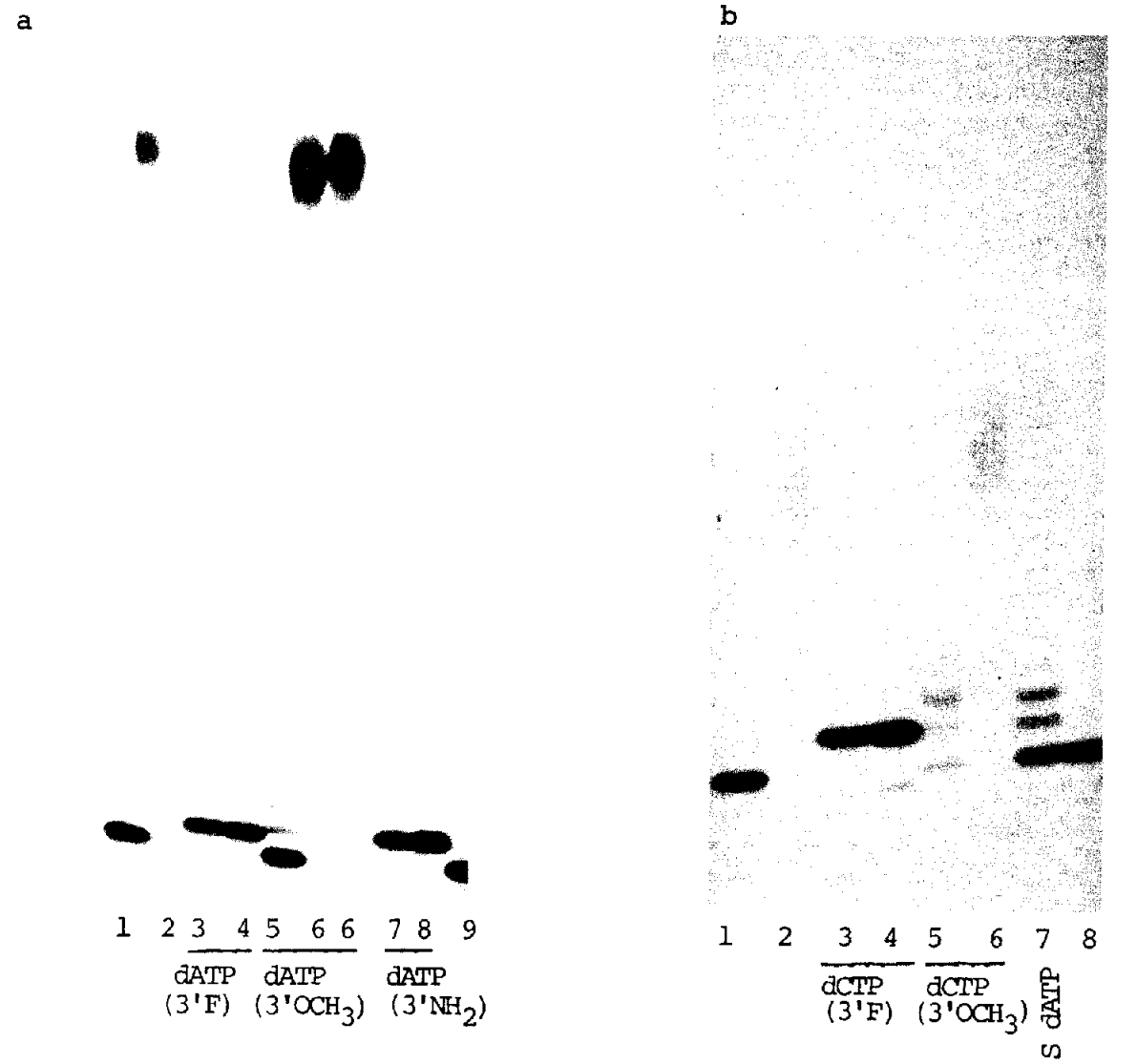

Fig.3. Incorporation of nucleotide analogs into the 3′-end of the primer. [^{32}P]d(GTAAAACGACGGCCAGT) is incubated for 30 min at 37°C with terminal transferase in 5 μl medium containing: 100 mM K cacodylate buffer (pH 6.8), 50 mM CH_3COOK, 5 mM $MgCl_2$, 0.5 mM $CoCl_2$, 2 mM 2-mercaptoethanol, 200 μM dNTP analog. Terminal transferase, 0.1 U/μl; primer, 0.2 μM (a) or 1 μM (b). After preincubation, the probes are diluted 10-fold with a fresh medium containing neither the primer nor a dNTP analog, and either frozen at −20°C (tracks 1,3,5,7) or incubated for an additional 30 min at 37°C after adding 1 mM dATP (a: 2,4,6,8) or 1 mM dCTP (b: 2,4,6). Tracks correspond to: (a) 1,2, no inhibitor; 3,4, dATP(3′-F); 5,6, dATP(3′-OCH_3); 7,8, dATP(3′-NH_2); 9, original primer; (b) 1,2, no inhibitor; 3,4, dCTP(3′-F); 5,6, dCTP(3′-OCH_3); 7, 2 μM dATP + 10 μM [α-^{35}S]thio-dATP; 8, original primer.

oligonucleotide is preincubated with terminal transferase in the presence of either dATP(3′-OCH_3) (fig.3a, track 5) or dCTP(3′-OCH_3) (fig.3b, track 5), the oligonucleotide slowly elongates. The elongation is due to the presence of about 1% of the original dNTP in the analog preparations. With dCTP(3′-OCH) in contrast to dATP(3′-OCH_3), the oligonucleotide elongated by one or two nucleotides displays duplicated lines (fig.3, track 5). Further elongation yields a

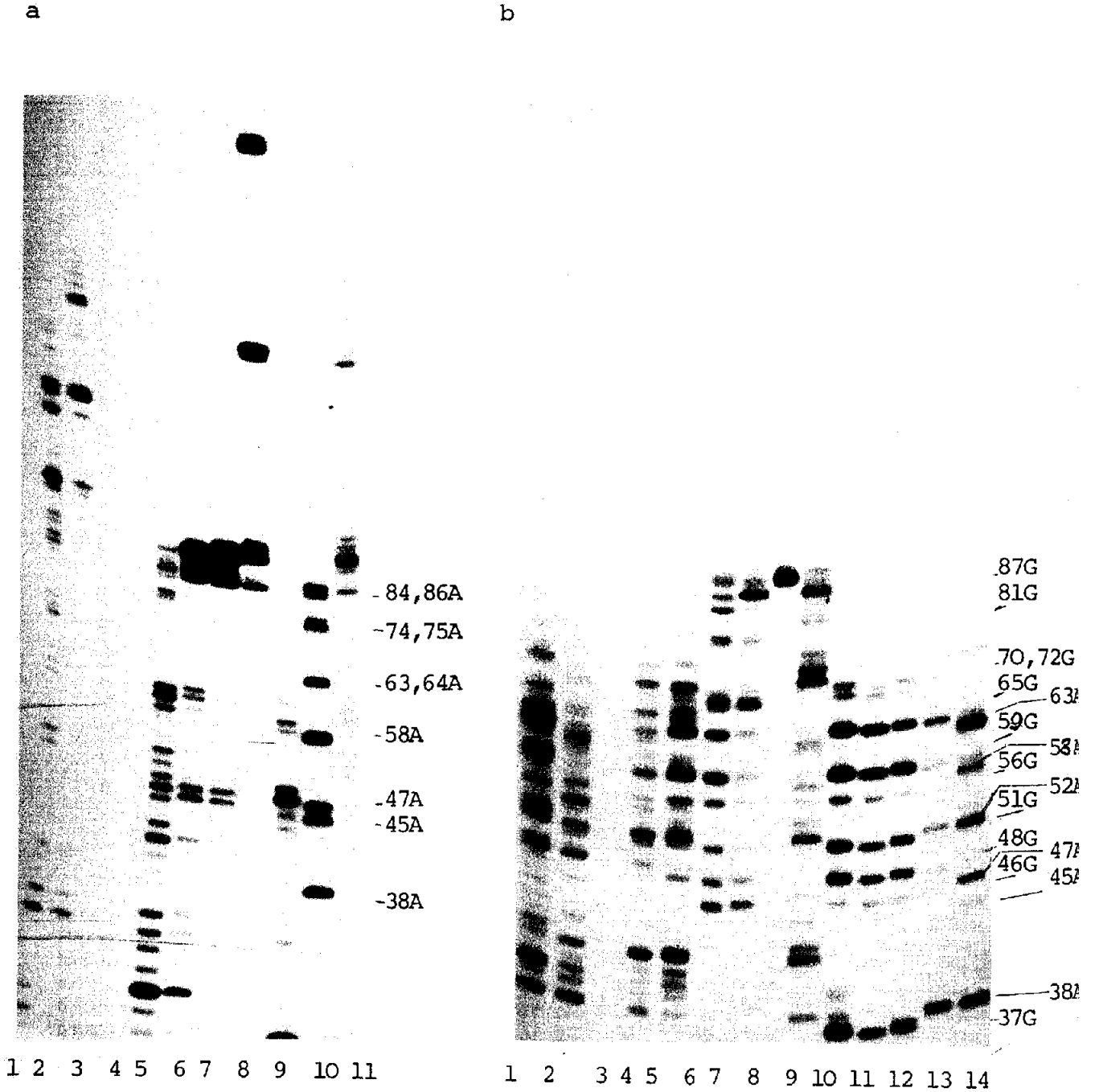

Fig.4. Influence of dNTP(3′-OCH_3) on M13 mp11 DNA elongation and hydrolysis by DNA polymerases α, I and T_4. Experimental conditions are as described in fig.2. The enzyme and dNTP concentrations and the reaction time are different. (a) Tracks 1,2,3,4, DNA polymerase I, 0.5 U/μl, 1 μM dNTPs; 1, without inhibitor, 15 s; 2,3,4, 1 mM dATP(3′-OCH_3), 30 s, 1 min and 3 min, respectively. Tracks 5–8, T_4 DNA polymerase, 0.5 U/μl; 1 μM dNTPs; (5) without inhibitor, 15 s; (6,7,8) 1 mM dATP(3′-OCH_3), 30 s, 1 min and 3 min respectively. Tracks 9–11 DNA polymerase α, 2 U/probe; 10 μM dNTPs; (9) without inhibitor, 2 min; (10) 1 mM araATP(3′-NH_2), 60 min; (11) 2 mM dATP(3′-OCH_3), 60 min. (b) [^{32}P]DNA for the experiment is prepared by elongation of the ^{32}P-primer on M13 DNA

smeared pattern for the oligonucleotide preincubated with dCTP(3′-OCH_3) in contrast to a sharp pattern produced after preincubation with dATP(3′-OCH_3) (cf. fig.3a and b, track 6). Each oligonucleotide preincubated with either dATP(3′-OCH_3) or dCTP(3′-OCH_3) elongated freely after adding a dNTP excess (fig.3a,b, track 6) indicating that dNTP(3′-OCH_3) was not incorporated in the chain. Otherwise elongation of the chain blocked by the 3′-OCH_3 group would hardly be possible. When a 3′-modified nucleotide such as dNTP-(3′-F) or dNTP(3′-NH_2) is incorporated, elongation does not occur (cf. fig.3a and b, tracks 3,4 and fig.3a, tracks 7,8). One can see a band doubling similar to that in the case of dCTP(3′-OCH_3) in the experiment with a mixture of dATP and α-thio-dATP (fig.3b, track 7).

Others of the tested DNA polymerases are fully resistant to dNTP(3′-OCH_3). Fig.4a,b demonstrates autoradiograms obtained for *E. coli* DNA polymerase I, phage T_4 and calf thymus DNA polymerase α. Neither of the DNA polymerases is terminated with dNTP(3′-OCH_3). The same is true for DNA polymerase β from rat liver and for Klenow enzyme (not shown). Even DNA hydrolysis, which is in general more sensitive to the terminator nucleotides [11] due to the absence of a competing natural substrate from the medium than the elongation reaction, is not inhibited by dNTP(3′-OCH_3) in a sequence-specific manner. As can be seen from comparing tracks 5 and 9 with 10 and 14 in fig.4b, dNTP(3′-NH_2) and ddNTP virtually do not terminate the synthesis in the presence of 1 mM natural dNTPs although they rather effectively terminate DNA hydrolysis in the absence of a natural substrate. The same is true of araNTP(3′-NH_2) and DNA polymerase I [11].

The results of this work suggest that dNTP(3′-OCH_3) can be used for selective inhibition of DNA synthesis catalyzed by reverse transcriptase. Preliminary experiments with a mixture of enzymes, either DNA polymerase α and reverse transcriptase or Klenow enzyme and reverse transcriptase, have shown that the presence of an enzyme unable to utilize a certain nucleotide analog does not prevent chain termination by the enzyme which is inhibited by this analog. Therefore, one may expect that the above analogs can be used in studying the role of reverse transcriptase in both normal and transformed cells.

Comparison of the data presented here with those concerning other nucleotide analogs modified at the sugar moiety [8–12] clearly demonstrates that the viral reverse transcriptase possesses lower substrate specificity than any cellular DNA polymerase tested. The conclusion, extrapolated to other DNA polymerases of viral origin, opens up a wide variety of nucleotide and nucleoside analogs for testing as putative drugs against any disease which requires reproductive retrovirus (or other virus) infection dependent on activity of the virus encoded polymerase.

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with DNA polymerase I. DNA is then purified by phenol extraction and A15 m agarose chromatography. The product is shown in track 1. The DNA is either elongated or hydrolysed in the absence of dNTPs, but in the presence of 1 mM dNTP analogs. The reaction conditions are the same as specified in a. DNA, 0.5 ng/μl; T_4 DNA polymerase, 0.5 U/μl. Tracks correspond to: (2,4) DNA hydrolysis without an inhibitor for 2–5 min; (4) for 1 min; (3) elongation in the presence of 100 μM dNTPs without inhibitor, 1 min; (5–9) DNA elongation, 1 mM dNTPs and 1 mM dNTP analog, 1 min; (10–14) DNA hydrolysis, 1 mM dNTP analog, 5 min; (5,10) dGTP(3′-NH_2); (6,11) dGTP(3′-F); (7,12) araGTP(3′-NH_2); (8,13) dGTP(3′-OCH_3); (9,14) ddATP.

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