3′-Deoxy-3′-aminonucleoside 5′-triphosphates — Terminators of RNA synthesis, catalyzed by DNA-dependent RNA polymerase from *Escherichia coli*

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1. INTRODUCTION

The NTP(3′NH_2) are of interest for the study of various biochemical processes: ATP(3′NH_2) appeared to be the substrate of ATP/CTP:tRNA-nucleotidyltransferase [EC 2.7.7.21 and EC 2.7.7.25]; the tRNAs thus obtained bore the 3′-deoxy-3′-aminoadenosine residue at the 3′-end [1,2]. The modified tRNAs of this type allowed us to reveal some details of the molecular mechanism of the aminoacyl-tRNA enzymatic synthesis [1,2] and ribosomal synthesis of proteins [2,3]. ATP(3′NH_2) also effectively inhibited the DNA-dependent RNA polymerase from the Ehrlich ascites tumour cells [4] and *E. coli* [5,6]. Indirect data were obtained indicating that the 3′-deoxy-3′-aminoadenosine residue, being incorporated in the 3′-end of the growing RNA chain terminated further RNA synthesis [4,6]. In addition, ATP(3′NH_2) was a substrate in a one-step ApC + ATP(3′NH_2) → ApCpA(3′NH_2) reaction during the abortive initiation of RNA synthesis on the T7 phage DNA as template, although unlike ATP it did not initiate RNA synthesis [7].

We have synthesised 4 NTP(3′N_3) and 4 NTP(3′NH_2) with adenine, cytosine, uracil and guanine bases and show that all these compounds are effective terminators of RNA synthesis, catalyzed by DNA-dependent RNA polymerase from *E. coli*.

Abbreviations: NTP(3′NH_2), 3′-deoxy-3′-aminonucleoside 5′-triphosphates with adenine [ATP(3′NH_2)], guanine [GTP(3′NH_2)], cytosine [CTP(3′NH_2)] and uracil [UTP(3′NH_2)] bases; NTP(3′N_3) and NTP(3′Me), 3′-deoxy-3′-azidonucleoside 5′-triphosphates and 3′-*O*-methylnucleoside 5′-triphosphates, respectively; NMP(3′N_3), 3′-deoxy-3′-aminonucleoside 5′-monophosphates with adenine, guanine, uracil and cytosine bases; CpA, cytidilyl-(3′→5′)-adenosine; C(3′NH)pA, 3′-deoxy-3′-aminocytidilyl-(3′→5′)-adenosine

2. MATERIALS AND METHODS

RNA polymerase from *E. coli* MRE-600 was isolated as in [8]. Specific activity of the enzyme was 1500 units/mg protein. Nucleoside 5′-triphosphates ATP, GTP, CTP and UTP were from Sigma (USA); [α-^{32}P]ATP and [α-^{32}P]CTP, spec. act. 100–400 Ci/mmol from Amersham (England). DNA of the deletion DIII mutant of the T7 phage initiated from A1 promotor [9] served as

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Table 1

Yield and some characteristics of the obtained compounds

Compound	Yield (%)	R_F in system 1	2	$E_{NTP}^{pH\ 7.5}$ [a]
ATP(3'N_3)	37	0.33	0.04	1.00
ATP(3'NH_2)	83	0.56	0.05	0.89
ATP	–	0.34	0.05	–
CTP(3'N_3)	41	0.22	0.08	1.00
CTP(3'NH_2)	92	0.47	0.09	0.90
CTP	–	0.28	0.06	–
UTP(3'N_3)	52	0.24	0.03	1.00
UTP(3'NH_2)	93	0.44	0.08	0.87
UTP	–	0.23	0.03	–
GTP(3'N_3)	69	0.09	0.05	1.00
GTP(3'NH_2)	82	0.15	0.05	0.96
GTP	–	0.10	0.05	–

[a] The $E_f^{pH\ 7.5}$ values for NTP were taken as 1.00
The TLC systems: N_1, 2 M HCOOH–1.6 M LiCl (1:2); N_2, isopropanol–1 M LiCl in 1 M AcOH (1:2); TLC was run on the PEI-cellulose (Polygram cell 300 PEI, Machery-Nagel)

template. NMP(3'N_3) were obtained as in [10]. *N,N'*-Carbonyldiimidazole was from Merck (FRG). C(3'NH)pA was prepared as in [11].

RNA synthesis was done following two methods:

Method A: The dinucleoside phosphate CpA served as initiator. The incubation mixture contained 0.1 M Tris–HCl (pH 8.0), 0.15 M KCl, 10 mM $MgCl_2$, 150 μM CpA, DIII T7 DNA (100 μg/ml), RNA polymerase (30 μg/ml) and 2 μM of each NTP, including [^{32}P]ATP (2×10^5 cpm) in 20 μl total vol. The reaction proceeded for 10 min at 22°C, then up to 500 μM inhibitor was added and after 15–20 min at 22°C excess NTP (100 μM) was added. After another 10 min incubation the reaction was quenched by EDTA, and the transcripts electrophoresed as in [12].

Method B: The mixture contained 0.15 M Tris–HCl (pH 8.0), 0.1 M KCl, 10 mM $MgCl_2$, 4 NTP (0.5 mM each), T2 phage DNA (100 μg/ml) and RNA polymerase (30 μg/ml) in μl total 20 vol. The incubation conditions were as in [8]. Electrophoresis was performed according to [12].

Table 2

Chemical shifts (δ, ppm) and spin–spin coupling constants (J, Hz) of the protons of the synthesised compounds

Compound	Proton type						
	H-8	H-2	H-6 ($J_{6,5}$)	H-5 ($J_{5,6}$)	H-1' ($J_{1',2'}$)	H-2' ($J_{2',3'}$)	Other protons
ATP(3'N_3)	8.55s	8.30s	–	–	6.16d (6.0)	5.11t (6.0)	4.84–4.13m
CTP(3'N_3)	–	–	7.95d (8.0)	6.15d (8.0)	5.98d (5.0)		4.60–4.01m
UTP(3'N_3)	–	–	7.90d (8.0)	5.97d (8.0)	5.96d (6.0)		4.84–3.80m
GTP(3'N_3)	8.08s	–	–	–	5.87d (5.5)		5.23–3.98m
ATP(3'NH_2)	8.47s	8.27s	–	–	6.23d (3.0)	5.01t	4.91–4.13m
CTP(3'NH_2)	–	–	7.90d (7.5)	6.10d (7.5)	5.91d (2.0)		5.33–3.75m
UTP(3'NH_2)	–	–	7.95d (8.0)	5.97d (8.0)	5.97d (2.5)		4.90–4.00m
GTP(3'NH_2)	7.98s	–	–	–	5.97d (3.0)		5.02–3.77m

Solvent, D_2O; internal standard, *tert*-BuOH; s, singlet; d, doublet; t, triplet; m, multiplet; spectra were taken with the Varian XL-100 spectrometer at ambient temperature

Synthesis of ATP(3'N_3), GTP(3'N_3) and UTP(3'N_3) started from the corresponding NMP(3'N_3) [10] using the route in [13]. The yield and some properties of the compounds obtained are shown in tables 1 and 2; CTP(3'N_3) was obtained from CMP(3'N_3) [10], 0.5 mmol of which was pre-treated with Ac_2O (2.5 ml) in dry pyridine (5 ml) (20°C overnight). The mixture was then evaporated, co-evaporated with pyridine (3 × 10 ml) and residue was left in 50% aqueous pyridine (10 ml). After 2 h at 20°C *n*-Bu_3N (0.12 ml) was added, the mixture was evaporated to dryness, the residue co-evaporated with dry pyridine (3 × 10 ml) and dimethylformamide (10 ml). Pyrophosphorolysis was done as in [13]. The azidonucleoside triphosphate was deacetylated (conc.NH_4OH, 20°C, 6 h) and purified as in [13]. The NTP(3'N_3)s were reduced to NTP(3'NH_2)s with Ph_3P in the NH_4OH–pyridine mixture [10]. Some properties and PMR data are given in tables 1 and 2.

3. RESULTS

3.1. *Inhibition of RNA synthesis*

RNA synthesis, catalyzed by *E. coli* DNA-dependent RNA polymerase, was performed in these experiments according to [8] using T2 phage DNA as a template. The reaction was run under the conditions, providing the reinitiation of the transcription and using the excess of the RNA polymerase. The measurement of kinetics of the RNA synthesis showed that similarly to NTP(3'Me) [8], the inhibition of the reaction by NTP(3'N_3) and NTP(3'NH_2) was not reversible within the scale of incubation time. The inhibition was of the competitive type towards the natural substrate of the same nature (not shown). Since the K_i values for this type of inhibitors are meaningless, the characteristics of the relative efficiency of the inhibitors are given in fig. 1: 50% inhibition (1 h incubation at 25°C) by ATP(3'NH_2), CTP(3'NH_2) and UTP(3'NH_2) was achieved at 1:100 inhibitor:substrate (in moles). Unexpectedly, the inhibiting effect of GTP(3'NH_2) was much higher. The 50% inhibition was observed when the inhibitor:substrate ratio was 1:1000. In contrast, the ATP(3'N_3) was inhibited, the synthesis much less (50% inhibition was observed at a 1:1 ratio).

Such high efficiency of NTP(3'NH_2) and the irreversible character of the inhibition indicate that these compounds either terminate the chains of the RNAs synthesised or they are able to join the next molecule as a true substrate thus forming the NH→P bonds, but with a very low efficiency.

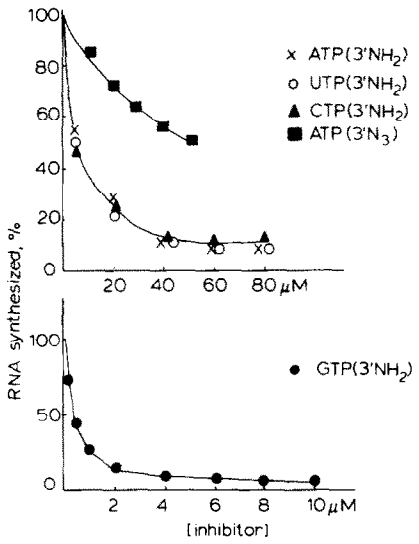

Fig. 1. The dependence of the inhibition of the RNA synthesis on the T2 phage DNA with ATP(3'NH_2), UTP(3'NH_2), CTP(3'NH_2), ATP(3'N_3) (a) and GTP(3'NH_2) (b). Experiments were according to method B; 100% of the [α-^{32}P]ATP incorporation corresponds to the 7.5×10^3 cpm or 16 pmol.

3.2. *Terminating properties of NTP(3'NH_2)*

Resulting gel pattern of the sequence analysis of ΔDIII T7 DNA transcript for the region from 30–80 nucleotides, besides the starting point of the A promoter, is shown in fig. 2. The synthesis was carried out in the presence of all 4 substrates and one NTP(3'NH_2) or NTP(3'N_3) in each experiment in the conditions of method B. The RNA synthesis in the presence of ATP(3'Me) was run as control to the experiment with ATP(3'NH_2) (fig. 2a) [8,12]. It is clear, that each track contains a discrete number of bands, which makes it possible to read fairly well the structure of the newly synthesised RNA. The sequence of about 40 nucleotide residues was determined with the use of NTP(3'NH_2), which corresponds to a similar experiment with NTP(3'Me) [9,12,14]. A direct comparison of the 1 and 2 tracks in fig. 2a also confirms that ATP(3'NH_2) are terminators of the syn-

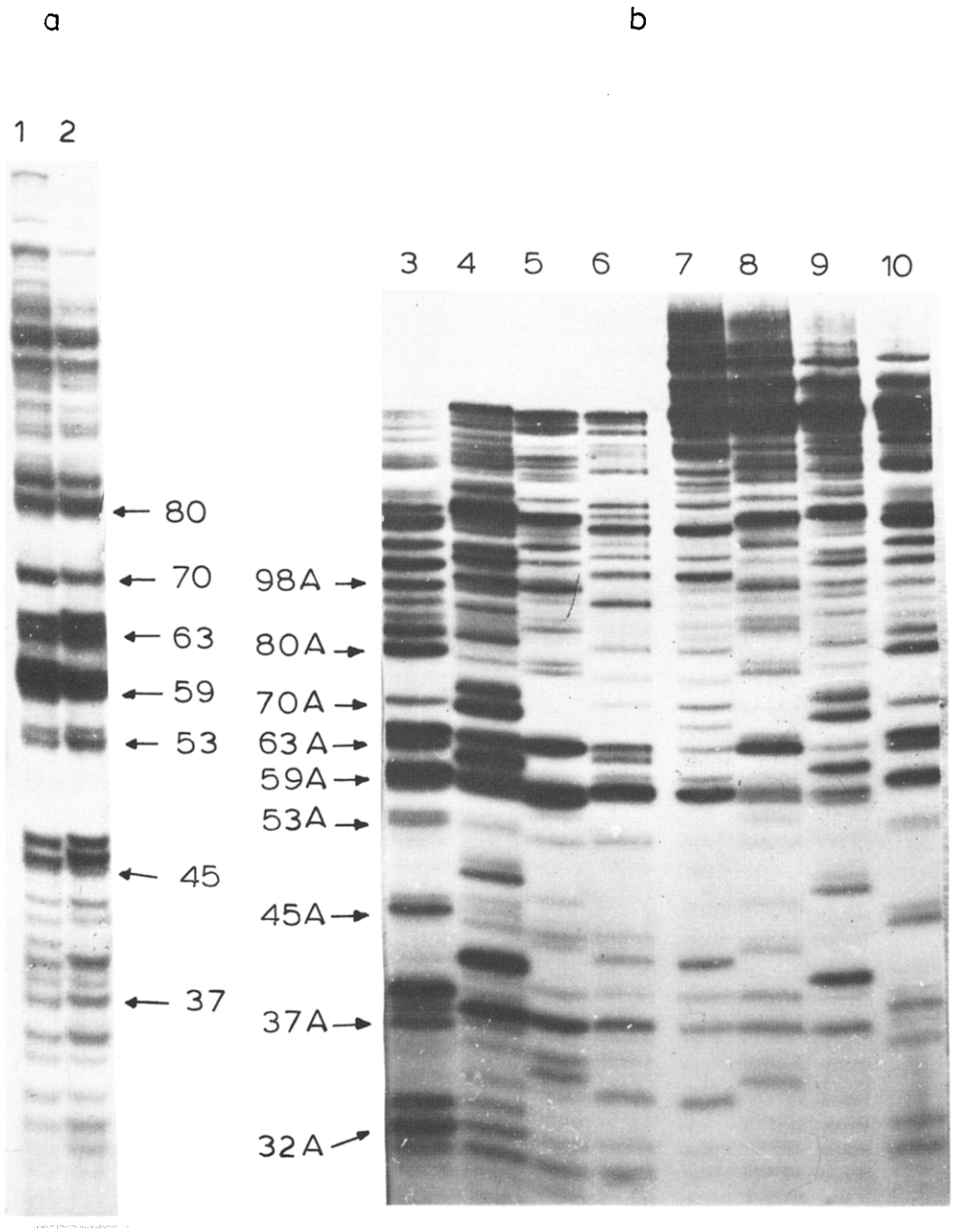

Fig. 2. The gel pattern of the sequence analysis of the T7 phage DNA DIII transcripts, terminated by NTP(3′NH_2) and by NTP(3′N_3): (a) 1, control ATP(3′Me); 2, ATP(3′NH_2); (b) 3, ATP(3′NH_2); 4, GTP(3′NH_2); 5, CTP(3′NH_2); 6, UTP(3′NH_2); 7, UTP(3′N_3); 8, CTP(3′N_3); 9, GTP(3′N_3); 10, ATP(3′N_3).

thesis. NTP(3′N_3) like NTP(3′NH_2) can also serve as terminators, although they are far less effective (fig. 2, tracks 7–10).

The inhibitory effects of NTP(3′NH_2) on the RNA synthesis at pH 7, 8, 8.5, 9 and 9.5 were about the same (not shown). The gel pattern of transcripts obtained at pH 7 and 9.5 is also similar (fig. 3). These facts indicate that NTP(3′NH_2), be-

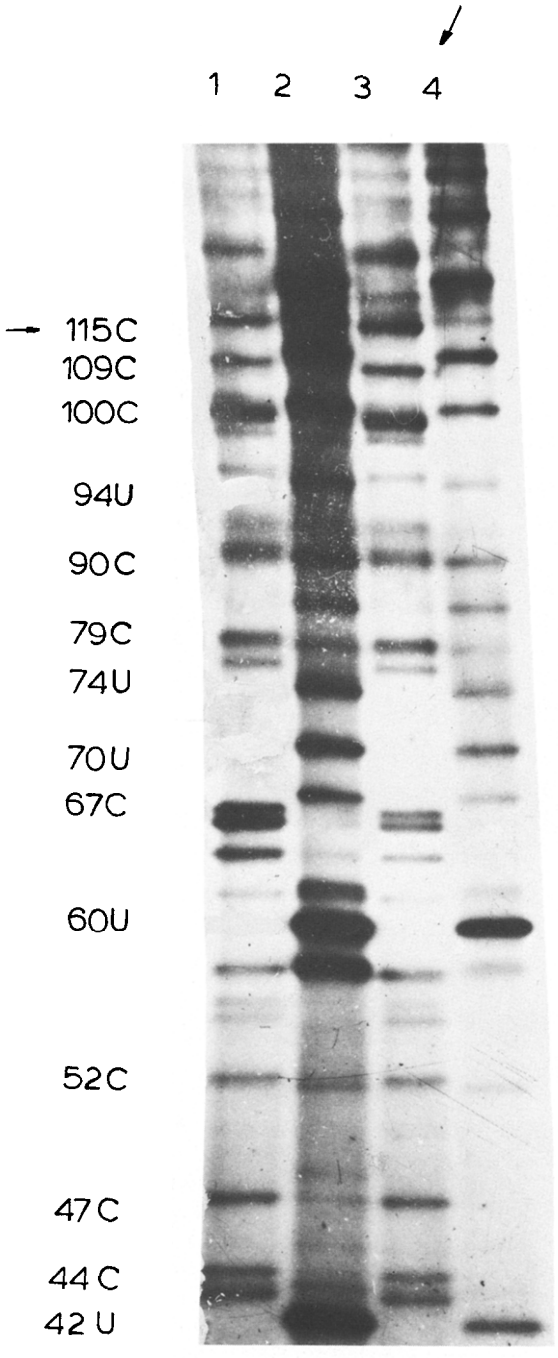

Fig. 3. The gel pattern of the sequence analysis of T7 phage DNA DIII transcripts, terminated by NTP(3′NH_2) at different pH: 1, CTP(3′NH_2) pH 8.0; 2, UTP(3′NH_2) pH 8.0; 3, CTP(3′NH_2) pH 9.5; 4, UTP(3′NH_2) pH 9.5.

ing incorporated in the 3′-end of the growing chain, cannot serve as acceptors of the following nucleotide residues and thus form the phosphamide bonds. These experiments were carried out at the pH values at which the aliphatic amino-group of NTP(3′NH_2) was either protonated or deprotonated. The measurements of the chemical shifts of the H_3' proton (PMR) of AMP(3′NH_2) at different pD values [titration of AMP(3′NH_2) solution in D_2O with DCl or NaOD] allowed to determine the pK_a value of the sugar NH_2-group ($pK_a \sim 8.5$). All these data lead to conclusion that the NTP(3′NH_2) over pH 7–9.5 are terminators of the RNA synthesis. Even if they are capable of polymerisation, the velocity of this process is very low.

3.3. *The priming of the RNA synthesis with the C(3′NH)pA*

A chemical synthesis of a number of dinucleoside phosphate analogues with the NH→P bonds was reported in [11]. We show here the ability of the C(3′NH)pA to initiate the RNA synthesis on the AI promoter at low concentration of substrate. Fig. 4 points out that C(3′NH)pA initiates the RNA synthesis nearly as effectively as CpA. This indicates that the C(3′NH)pA as well as CpA complementarily binds to the corresponding region of DNA.

4. DISCUSSION

In the introduction we have already mentioned the activity of ATP(3′NH_2) in a cell-free system of

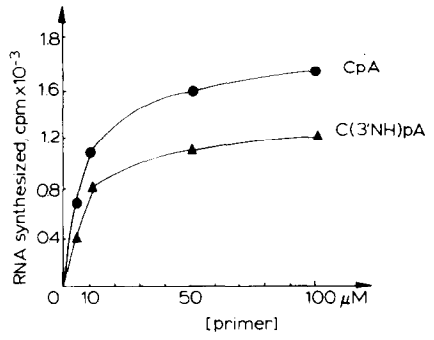

Fig. 4. The initiation of the RNA synthesis on the T2 phage DNA with C(3′NH)pA and CpA. The experiment was according to method A.

RNA synthesis. It is indirectly shown that the ATP(3'NH_2) may serve as RNA synthesis terminator. This was one of the factors which stimulated this investigation.

A probable terminating activity of NTP(3'NH_2) was also promoted by general speculations. The substitution of the hydroxyl for the NH_2-group in the 3'-position of the nucleoside 5'-triphosphates should not cause the significant conformational distortion in molecules as follows from the analysis of the PMR spectra [10,15]. Therefore, one could assume that this modification should not considerably change the affinity of these analogues for the enzyme during their binding to the transcription complex and joining the primer.

Here, 4 NTP(3'N_3) and 4 NTP(3'NH_2) were obtained and some of their properties studied: all NTP(3'N_3) and especially NTP(3'NH_2) were effective terminators of the RNA synthesis. Two types of RNA synthesis terminators, namely 3'-deoxynucleoside 5'-triphosphates [16] and NTP(3'Me) were reported [8,12]. The NTP(3'NH_2) possess the same properties when used as terminators. Furthermore, they have one significant advantage. The transcripts obtained by termination with NTP(3'NH_2) a chemically reactive group in the 3'-position. This amino-group can be easily used for specific introduction of the fluorescent or spin labels, or the residues for affinity labelling [6].

It should also be mentioned that GTP(3'NH_2) proved to be a much more effective terminator than other NTP(3'NH_2). We are not yet able to explain this.

The independence of the inhibitory properties of NTP(3'NH_2) over pH 7–9.5 and the same position of bonds on the electrophoretograms of transcripts obtained at different pH values are in favour of the conclusion that the aminonucleotide residues are not inserted in the middle of the chain.

The transcripts, synthesized in the presence of NTP(3'NH_2), were stable in 1 M AcOH, 20°C, 15 h. It was expected that N→P bonds would be hydrolysed in this condition, as we suggested using model dinucleoside monophosphates. However, no reaction was noticed. This fact also demonstrated that the aminonucleotide residues were not incorporated in the middle of the chain.

Finally, the presence of the priming ability of C(3'NH)pA provides a possibility to create a new type of primers for RNA and probably DNA synthesis, which can be of use for the investigation of the initiator complexes of transcription, reverse transcription and, probably, replication. One of the obvious advantages of these modified dinucleoside phosphates in comparison to the natural dinucleoside phosphates is their stability towards pyrophosphorolysis under the action of the RNA polymerase, thus excluding an uncontrolled accumulation of the NTP from the primer, especially in the system of abortive synthesis.

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