ADENOSINE, NAD AND FAD CAN INITIATE TEMPLATE-DEPENDENT RNA SYNTHESIS CATALYZED BY *ESCHERICHIA COLI* RNA POLYMERASE

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1. Introduction

It was established [1,2] that the first nucleotide, mainly a purine, that is incorporated into the RNA chain in a RNA polymerase reaction, retains its 5′-triphosphate group; enzymes were also found that destroy this group [3,4]. It has been shown [5,6] that RNA chains can be initiated not only by nucleotide-5′-triphosphates, but also by short oligonucleotides whose 3′-OH ends are not phosphorylated. The fact that the RNA polymerase-catalyzed exchange of labeled pyrophosphate with UTP on d(A–T) copolymer is effectively stimulated not only by ATP, but also by ADP or AMP [7], suggests that the last two nucleotides can initiate RNA synthesis.

Conditions have been found [8] for the so-called abortive initiation in which the product of a RNA polymerase reaction is freed, after formation of the first phosphodiester bond, from the enzyme–template complex to accumulate in the reaction medium. We have shown [9] that abortive initiation on the d(A–T) copolymer can involve not only a dinucleotide monophosphate complementary to the template, but also certain other dinucleotide monophosphates whose 5′-nucleoside part is not complementary to the template (e.g., CpA or ApA). Accordingly, we decided to check whether other adenosine derivatives can also participate in abortive synthesis. Here it is shown that adenosine and nucleotide coenzymes such as NAD and FAD can participate in reactions with UTP that occur by an abortive mechanism on d(A–T) copolymer or on T4 DNA.

2. Methods

2.1. *Materials*

E. coli RNA polymerase holoenzyme was isolated by the method in [9]. T4 DNA was a gift from Yu. N. Zograf. ATP, UTP, poly-d(A–T) : poly-d(A–T), NAD^+ and *E. coli* alkaline phosphatase (no. 0598) were from P. L. Biochemicals; AMP was from Reanal, NADH from Boehringer Mannheim GmbH, FAD from Koch-Light, [α-^{32}P]UTP from Amersham and Norit from Serva.

2.2. *Conditions of abortive initiation*

The reaction mixture (30 μl) contained 0.02 M Tris–HCl (pH 7.9); 0.05 M NaCl; 10 mM $MgCl_2$ (or 2 mM $MnCl_2$); 0.1 mM EDTA; 0.1 mM dithiothreitol; 0.4 mM AMP or other initiating substrate; 0.2 mM [α-^{32}P]UTP (20 mCi/mmol); 30 μM poly-d(A–T) : poly-d(A–T) or 0.2 mM T4 DNA; and 30 μg/ml RNA polymerase holoenzyme. After 10 min incubation at 37°C, free phosphate groups were destroyed by alkaline phosphatase and the radioactivity adsorbed on Norit was counted as in [9].

2.3. *Isolation of abortive initiation products*

After incubation, the reaction mixtures were chromatographed on PEI-cellulose plates. To ensure better separation of the product from contaminants contained in the [α-^{32}P]UTP preparation, the FAD-containing mixture was treated with alkaline phosphatase before being applied onto a plate. For chromatography the following systems were employed:

1 M LiCl in the NAD^+ and AMP experiments, 0 2 M LiCl in the adenosine experiments, and 1 M ammonium phosphate (pH 3 4) in the FAD experiments To improve the quality of separation, the excess salts were washed out with aqueous methanol (1/1, v/v) from the applied spots onto a paper filter fixed to the plate edge, the direction of washing being opposite to that of chromatography The spot of the product of the reaction with adenosine was not preliminary desalted because of its high mobility in aqueous methanol The position of spots after chromatography was determined by radioautography, and the spots were cut out and eluted with 0 3–1 0 ml 0 7 M $MgCl_2$ plus 0 01 M Tris–HCl (pH 7 5) The eluate was desalted by gel filtration on a Sephadex G-10 column and evaporated to a small volume in a rotary evaporator

2 4 *Alkaline hydrolysis of abortive initiation products*

To 18 μl of a solution of the purified reaction product (5–20 μM), 2 μl 1 0 M KOH was added, and the mixture incubated in sealed glass capillaries for 1 h at 100°C The hydrolyzates obtained were neutralized with 1 M HCl, tested with a strip of universal pH indicator paper, and chromatographed in 1 M LiCl or in 0 8 M CH_3COOH–0 8 M LiCl after preliminary desalting as described above

3 Results

As can be seen from table 1, adenosine, NAD^+, NADH, and FAD are all capable of reacting with [α-^{32}P]UTP to transform ^{32}P into a form inaccessible to alkaline phosphatase action This reaction is catalyzed by RNA polymerase not only on a synthetic, but also on a natural template It occurs in the presence of both Mg^{2+} and Mn^{2+} Its rate depends on the nature of the first substrate (the one incorporating into the 5′-end of the product) These differences are more conspicuous in the Mg-containing system, but depend only slightly on whether a natural or a syn-

Table 1
Participation of adenyl derivatives in the reaction with [α-^{32}P]UTP catalyzed by *E coli* RNA polymerase on T4 DNA or d(A–T) copolymer

First substrate	Incorporation of [α-^{32}P]UTP (nmol)[a]			
	T4 DNA		d(A–T) copolymer	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
Adenosine	0 11	0 18	0 23	0 62
AMP	0 39	0 94	0 67	1 83
UpA	1 25	0 69	3 20	2 34
NAD^+	0 07	0 34	0 16	0 91
NADH	0 10	0 44	0 20	1 01
FAD	0 14	0 37	0 20	0 74

[a] Controls without first substrate have been subtracted column 1 and column 2, 0 03 nmol, column 3 and column 4, 0 04 nmol See section 2 for description of conditions of product synthesis and identification

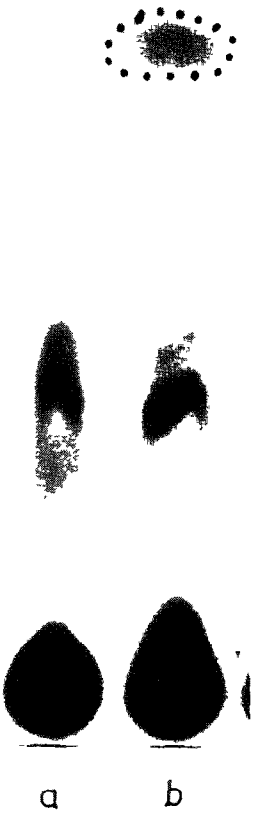

Fig 1 Chromatography in 0 2 M LiCl of standard reaction mixtures (a) incubated without adenosine, (b) incubated in presence of adenosine Dots mark contours of the ultraviolet-visible ApU spot applied to the starting point as marker

thetic template is used. It is remarkable that in nearly all cases the reaction rate in the Mn-containing system was higher, except for the reaction between UpA and UTP whose rate was higher in the presence of Mg^{2+}. It is also of interest that NAD and FAD resemble AMP but differ from UpA in this respect.

Inasmuch as the [α-^{32}P]phosphate of UTP was converted to a form resistant to alkaline phosphatase in the reactions considered, we indeed observed an abortive initiation of RNA synthesis. An additional confirmation that the product of the adenosine reaction with [α-^{32}P]UTP was ApU, was its chromatographic identity to unlabeled ApU (fig.1).

The products of the AMP, FAD, and NAD^+ reactions differed in their chromatographic mobility in a neutral medium (fig.2a–c) but were all converted to the same compound, pA*p, after alkaline hydrolysis (fig.2d–f). A similar situation was seen on chromatography in an acid medium (0.8 M CH_3COOH–0.8 M LiCl). These results enable one to ascribe the structure $N^+(5')pp(5')A*pU$(I) to the product of the NAD^+ reaction with [α-^{32}P]UTP and the structure

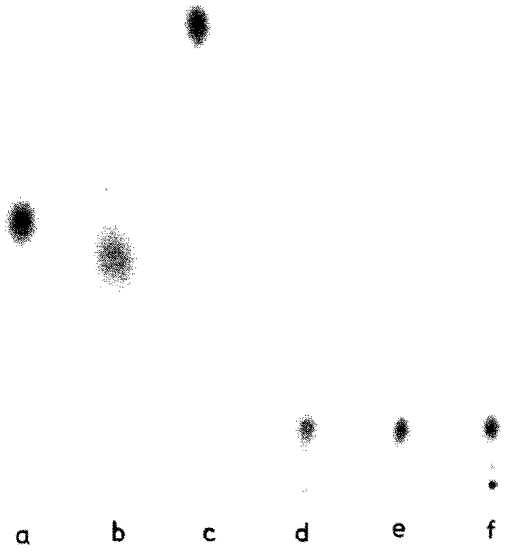

Fig.2. Chromatography in 1.0 M LiCl of isolated products of [α-^{32}P]UTP reaction with: (a) AMP; (b) FAD; (c) NAD^+, and their alkaline hydrolysates (d, e, f, respectively).

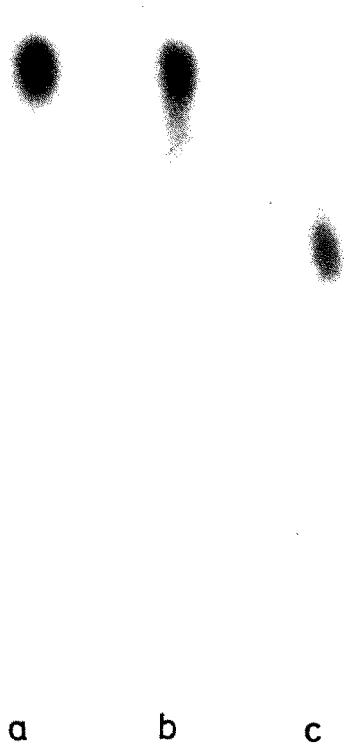

Fig.3. Chromatography in 1.0 M LiCl of isolated products of reactions of: (a) NAD^+; (b) NADH; (c) AMP, with [α-^{32}P]UTP.

F(5′)pp(5′)A*pU(II) to the product of the FAD reaction with [α-^{32}P]UTP.

Chromatography of the products of the NAD reactions with [α-^{32}P]UTP yielded two spots, one of which contained ~80% of the radioactivity, was indistinguishable chromatographically from compound I, and converted to pA*p after alkaline hydrolysis. The initial product of the NADH reaction with UTP probably corresponded to another spot which had a smaller R_F value since this product converted to compound I almost completely during elution and desalting (fig.3b), apparently as a result of spontaneous oxidation. These findings warrant the conclusion that both NAD^+ and NADH are directly involved in an abortive initiation reaction with the formation of a (3′→5′)-phosphodiester bond.

Adenosine, NAD, and FAD can be involved not only in an abortive initiation reaction, but also in initiation of polymeric RNA synthesis as is suggested

Table 2
Stimulation of poly(A–U) synthesis on d(A–T) copolymer by adenyl derivatives

Initiating substrate	Relative incorporation of [α-^{32}P]UTP into TVU-insoluble sediment
–	1 0
Adenosine (0 3 mM)	1 2
AMP (0 3 mM)	1 3
UpA (2 μM)	2 2
NAD^+ (0 3 mM)	1 6
FAD (0 3 mM)	1 9

Conditions as in section 2, but ATP and UTP were 1 μM each

by the results of an experiment presented in table 2 Indeed, when ATP and UTP were present in very low concentrations, all the adenyl derivatives studied were found to increase the amount of label incorporated into the acid-insoluble product

4. Discussion

These results serve to enlarge the list of compounds of nucleotide nature that are able to initiate RNA synthesis We have detected an abortive initiation reaction of adenosine with UTP Since no stimulation by adenosine of the labeled pyrophosphate exchange with UTP on d(A–T) copolymer was observed [7] this reaction may be thought to be less reversible than that of phosphorylated adenosine derivatives with UTP

Of particular interest is the finding that RNA synthesis can be initiated by nucleotide coenzymes In addition to NAD and FAD, the cytoplasm is known to have many other metabolites whose molecules contain nucleotide residues In many cases the functional role of these residues or of the metabolites themselves remains obscure, and it is tempting to assume that some of these metabolites can take part in the control of RNA synthesis at the initiation level In this connection it should be noted that the RNA structure with a NAD or FAD residue at the 5′-end, resembles the structure of mRNAs carrying the so-called cap at their 5′-end [10–12]

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