A STUDY OF THE CONDITIONS OF THE FIRST PHOSPHODIESTER BOND FORMATION BY *E. COLI* RNA POLYMERASE

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1. Introduction

The RNA polymerase-catalyzed template-dependent synthesis of RNA is a multistage process [1]. Of particular importance in this process is the initiation stage leading to the formation of the first phosphodiester bond of RNA. The mechanism of initiation was difficult to study because of the complexity of this stage as well as the absence of reliable methods for uncoupling it from other transcription stages.

Recently Johnston and McClure [2] have found that a special limitation of the nucleoside triphosphate set in the RNA polymerase-λ DNA system results in specific synthesis of initial dinucleotides on λ promoters. They called this synthesis 'abortive initation' and showed that not only nucleoside triphosphates but also diphosphates or monophosphates can be incorporated into the 5'-half of the product. This has made possible direct study of the mechanism by which the first phosphodiester bond is formed.

The purpose of this paper is to examine the conditions of abortive synthesis by the holoenzyme *E. coli* RNA polymerase on the (dA–dT) copolymer template. It was found that the dependence on Mg^{2+} concentration of pApU synthesis from AMP and UTP did not differ from the analogous dependence of polymer RNA synthesis. In contrast, these two reactions differed significantly in their dependence on Mn^{2+} and NaCl concentrations. The substitution of ppA for the initiating pA resulted in a 2-fold decrease of dinucleotide synthesis, and the substitution of UpA for pA caused UpApU synthesis to increase 5-fold. No synthesis of pUpA from UMP and ATP was in evidence while ApUpA synthesis from ApU and ATP proceeded stoichiometrically.

2. Methods

2.1. *Isolation of RNA polymerase holoenzyme*

RNA polymerase was isolated from *E. coli* MRE600 by the method in [3] except that denaturated DNA agarose (3.5%, containing 3 mg/ml DNA; elution with a linear 0.2–1.2 M NaCl gradient) was used instead of native DNA cellulose. This made possible recovery of the σ-saturated holoenzyme which emerged just after the core. Resulting holoenzyme was > 98% purity and 2000 U/mg spec. act. [3].

2.2. *Conditions of abortive synthesis*

A standard reaction mixture (50 μl) contained 0.02 M Tris–HCl, pH 7.9, 0.05 M NaCl, 10 mM $MgCl_2$ 0.1 mM ethylenediamine tetra-acetate (EDTA), 0.1 mM dithiothreitol, 0.4 mM AMP, 0.2 mM [α-^{32}P]-UTP (1–20 mCi/mmol, Amersham), 10–20 μM poly(dA–dT):poly(dA–dT) (P-L Biochemicals) and 10–20 μg/ml RNA polymerase holoenzyme. Incubation was for 10 min at 37°C. Unlabelled nucleotides were purified in acid and in neutral 7 M urea on DE-23 (Whatman) columns [4] to remove not only nucleotide contaminants but also an unidentified inhibitor in the UTP (P-L Biochemicals) that caused a 4–5-times reduction of both abortive and normal RNA synthesis.

2.3. *Registration of abortive synthesis products*

This was achieved by two methods:

1. The reaction was stopped with a 30% excess (to

Me^{2+}) of EDTA, and 10 μl aliquots were applied onto a polymer foil with a thin layer of PEI cellulose (Machery-Nagel); the plates were subjected to ascending chromatography in 1 M NaCOOH, pH 3.4 [5], and the product spots were cut out and counted in a NAG β M gasflow counter.

2. After the product synthesis, 0.05 ml of *E. coli* alkaline phosphatase in 0.1 M Tris-HCl, pH 7.9 (5 U/ml, P-L Biochemicals, 0598), was added to each tube which was incubated for 90 min at 55°C and chilled to 0°C, then adding to each 1 ml 1 M HCl–0.02 M NaH_2PO_4 and 0.05 ml of 40% Norit (Serva) suspension in 0.25 M HCl–0.05 M NaH_2PO_4; after stirring for 10–15 min the mixtures were filtered through GF/C filters (24 MM, Whatman) which were then washed with 50 ml 10 mM HCl–2 mM NaH_2PO_4, dried and counted.

2.4. *Demonstrating the structure of abortive synthesis products*

The product of the reaction of AMP with [α-^{32}P]-UTP was obtained in a standard mixture of 0.2 ml and treated with alkaline phosphatase. The dephosphorylated product was purified on a Sephadex G-15 column in H_2O, the product emerging after the P_i. The process was checked by chromatography on PEI cellulose plates in 4 M LiCl:1 M CH_3COOH (1:1). The product fraction was evaporated to 12 μl. To 6 μl of that, 1 μl snake venom phosphodiesterase I (40 U/ml in 5 mM Tris–HCl, pH 8.9–0.5 mM $MgCl_2$) was added and the mixture was incubated for 1 h at 37°C. To the other 6 μl, 0.5 μl 2 mM cold ApU and 0.5 μl 1 M KOH were added; the mixture was sealed in a glass capillary, heated for 1 h at 100°C and neutralized with 25 mM HCl. After PEI cellulose chromatography in 1 M HCOOH [5] the radioactive product of phosphodiesterase I action coincided with the cold 5′-UMP spot; the product of alkaline hydrolysis, with the cold 3′-AMP spot; and the initial product, with the ApU spot. The structure of the UpApU resulting from the reaction of UpA with [α-^{32}P]UTP was demonstrated in the same manner but without the removal of $^{32}PP_i$.

3. Results

Figure 1 shows the kinetics of pApU synthesis from AMP and [α-^{32}P]UTP under three types of ionic conditions. The synthesis can proceed linearly for a long time (curve 3), its slowing down (curves 1 and 2) being associated with [α-^{32}P]UTP exhaustion.

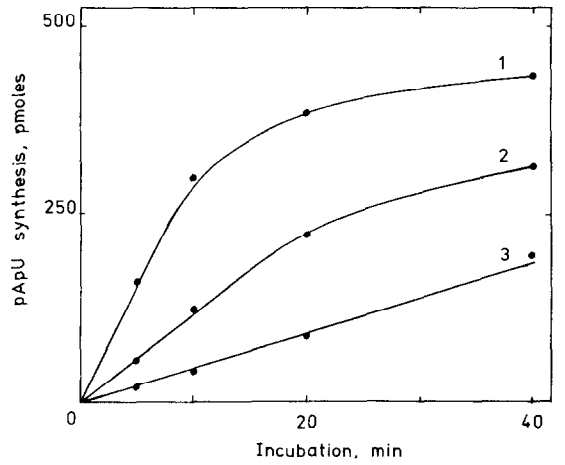

Fig.1. Abortive synthesis of pApU. Standard conditions but AMP = 0.2 mM; [α-^{32}P]UTP = 10 μM; incubation, 20 min at 37°C; and ionic compositions: (1) 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM NaCl; (2) 10 mM $MgCl_2$ and 50 mM NaCl; (3) 10 mM $MgCl_2$ and 200 mM NaCl.

The observed large differences in the initial velocity of pApU synthesis under the different conditions (fig.1) stimulated us to study in more detail the dependence of the reaction on Mg^{2+} and Mn^{2+}

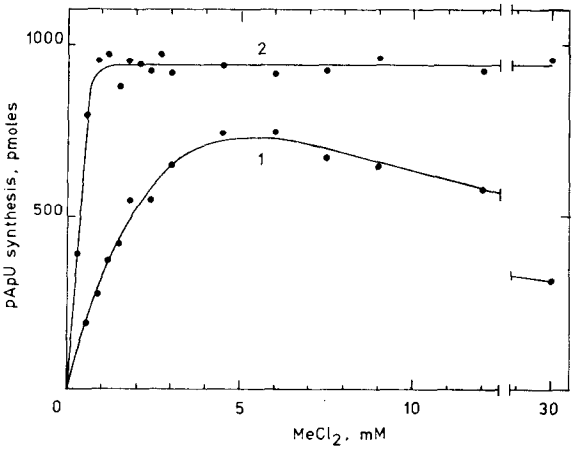

Fig.2. Effect of $MgCl_2$ (1) and $MnCl_2$ (2) concentration on pApU synthesis. Standard conditions except Me^{2+}.

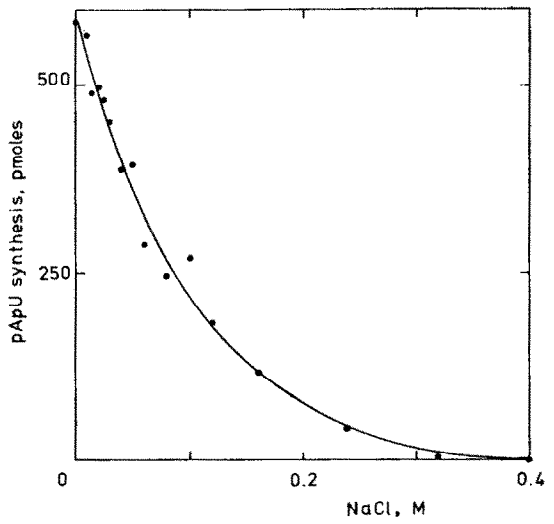

Fig.3. Effect of ionic strength on pApU synthesis. Standard conditions except NaCl.

concentrations and on ionic strength. Raising the Mg^{2+} concentration (fig.2, curve 1) was found to result in enhanced abortive synthesis of pApU (to a maximum at 5–6 mM $MgCl_2$) followed by its gradual decline. pApU synthesis attained a maximal rate at ~1 mM $MnCl_2$ (fig.2, curve 2) remained at a constant level up to 30 mM $MnCl_2$. pApU production was appreciably depressed even at 0.01–0.02 M NaCl and about 50% inhibited at 0.06 M NaCl (fig.3).

Table 1
Dependence of abortive synthesis on the first substrate structure

First substrate	Incorporation of [α-^{32}P]UTP (pmol)[a]
pA	177
ppA	93
UpG	5
ApC	14
ApU	3
GpA	12
ApA	26
CpA	96
UpA	898

[a] Control without incubation = 20 pmol was subtracted

Standard conditions but RNA polymerase = 0.5 μg/tube, first substrate = 0.2 mM, and [α-^{32}P]UTP = 0.1 mM

Next, we considered abortive synthesis in relation to the nature of the substrate incorporated into the 5′-end of the product (table 1). The substitution of ADP for AMP decreased the abortive synthesis about 50% while the substitution of UpA for AMP increased it 5-fold. Also, there was a pronounced reaction with UTP when CpA was used instead of UpA and a noticeable reaction with it when UpA was replaced by ApA.

We checked the structure of the products of pA or UpA reactions with [α-^{32}P]UTP and became convinced that it was really pApU which formed in the former case and UpApU in the latter case (see section 2).

4. Discussion

Study of the effects of Me^{2+} and NaCl concentrations has revealed an interesting feature of pApU abortive synthesis, namely that its dependence on Mg^{2+} is essentially the same as, while its dependences on Mn^{2+} and ionic strength are very different from, the corresponding dependences of RNA synthesis and elongation (cf. [6–8]). The inhibitory effect of NaCl on abortive synthesis may be accounted for by a substantial contribution of ion action to the binding of the first two nucleotides with the enzyme, unless this effect results from inhibition of the enzyme–template complex formation. More difficult to explain is the same effect of Mg^{2+} on the normal and abortive RNA syntheses and the different effects of Mn^{2+} on these two kinds of synthesis. It may be assumed that high Mn^{2+} concentrations prevent translocation of the product or that the enzyme–template complex contains several nucleoside triphosphate-binding sites which respond to Mg^{2+} differently than to Mn^{2+}.

It is noteworthy that not only a dinucleotide but a trinucleotide as well can be synthesized by the abortive mechanism. Moreover the reaction of UpA with UTP resulting in UpApU formation was much faster than the synthesis of pApU from AMP and UTP. This suggests higher affinity of the enzyme RNA-binding site for UpA substrate than for AMP substrate and/or its lower affinity for UpApU product than for pApU product. The question arises whether or not the 5′-nucleoside part of a dinucleotide primer makes

a contribution to the abortive synthesis. We have concluded that it does since the reactions of GpA or ApA with UTP are very weak while, at the same time, CpA is capable of effective participation in the reaction with UTP. This allows one to extend the Crick 'wobble' hypothesis [9] to the C–A interaction which is quite likely to occur [10].

In the system we studied, no abortive synthesis of the U–p–A bond was found. The UMP–[α-^{32}P]ATP reaction did not occur at all (we could notice the formation of 1 UpA molecule/100 molecules of the enzyme) while ApU reacted with [α-^{32}P]ATP stoichiometrically rather than catalytically. This result is in good agreement both with the predominant initiation of RNA from purine nucleotides [1] and with the results in [11].

Analysis of the conditions of abortive synthesis suggests that it may be a consequence of combining some elements of the initiation and the termination mechanisms. Basic to it is the high affinity of the RNA binding site of RNA polymerase for purine nucleotides which is important for initiation and its low affinity for pyrimidine nucleotides which appears to be important for termination. It will be recalled that, in all the cases reported, the terminated RNAs have an oligouridyl sequence at the 3′-end [12]. The present results are consistent with the opinion that for the normal template synthesis to take place, the RNA binding site should interact with several 3′-end nucleotides of the growing RNA chain. Also, more than one nucleoside triphosphate are possibly bound by the binding site. In view of this the concept of 'initiation' should not probably be restricted to include only the formation of the first phosphodiester bond but should be extended to include several subsequent bonds, perhaps all those formed before the σ subunit leaves the ternary complex.

References

[1] Chamberlin, M. J. (1976) in: RNA Polymerase (Losick, R. and Chamberlin, M. eds) pp. 17–67, Cold Spring Harbor Lab., Cold Spring Harbor, NY.
[2] Johnston, D. E. and McClure, W. R. (1976) in: RNA Polymerase (Losick, R. and Chamberlin, M. eds) pp. 413–428, Cold Spring Harbor Lab., Cold Spring Harbor, NY.
[3] Burgess, R. R. and Jendrisak, J. J. (1975) Biochemistry 14, 4634–4638.
[4] Tener, G. M. (1967) Methods Enzymol. 12/A, 398–404.
[5] Randerat, K. and Randerat, E. (1967) Methods Enzymol. 12/A, 323–347.
[6] Chamberlin, M. J. and Berg, P. (1962) Proc. Natl. Acad. Sci. USA 48, 81–94.
[7] Rhodes, G. and Chamberlin, M. J. (1974) J. Biol. Chem. 249, 6675–6683.
[8] Khesin, R. B., Astaurova, O. B., Shemyakin, M. F., Kamzolova, S. G. and Manyakov, V. F. (1967) Molek. Biol. 1, 736–753.
[9] Crick, F. H. C. (1966) J. Mol. Biol. 19, 548–555.
[10] Topal, M. D. and Fresko, J. R. (1976) Nature 263, 285–289.
[11] So, A. G. and Downey, K. M. (1970) Biochemistry 9, 4788–4793.
[12] Gilbert, W. (1976) in: RNA Polymerase (Losick, R. and Chamberlin, M. eds) pp. 193–225, Cold Spring Harbor Lab., Cold Spring Harbor, NY.