PROCESSIVE PYROPHOSPHOROLYSIS OF RNA BY *ESCHERICHIA COLI* RNA POLYMERASE

T. A. ROZOVSKAYA, A. A. CHENCHIK and R. Sh. BEABEALASHVILLI
Institute of Molecular Biology, USSR Academy of Sciences, Moscow 177984, USSR

Received 20 November 1981

1. Introduction

DNA-dependent RNA polymerase catalyzes polymerisation of the RNA chain from NTPs as well as the pyrophosphate exchange reaction in NTPs [1]. The enzyme does not catalyze pyrophosphorolysis of free RNA [2,3]. In contrast, small but detectable pyrophosphorolysis of RNA from the RNA–DNA hybrid has been demonstrated [3].

In our study of pause distribution along the T7 RNA chain [4,5] a model of the RNA elongation reaction has been proposed to account for the nucleotide sequence dependence of the RNA chain elongation rate. As a necessary part, occurrence of the processive pyrophosphorolysis of the nascent RNA chain is stipulated in the model. Therefore, the predicted property has been tested.

Here we show that *E. coli* RNA polymerase is capable of catalyzing the consecutive DNA-dependent RNA pyrophosphorolysis in the presence of inorganic pyrophosphate. An active ternary complex of the enzyme with DNA and nascent RNA, Mg^{2+} and inorganic pyrophosphate are needed for the reaction. NTPs are low-molecular-mass (M_r) products of the reaction. The rate of pyrophosphorolysis for particular nucleotides in different regions of RNA can differ by several orders of magnitude depending on the primary structure of the RNA region that undergoes pyrophosphorolysis. The rate of the reaction ranges from 1 nucleotide for 100 h per chain to 1 nucleotide for 1 min per chain.

Dissociation of the ternary complexes has also been studied. The ternary complex of RNA polymerase with DNA and nascent RNA has been shown to undergo site specific dissociation. The rate of dissociation is shown to be a function of the primary structure of RNA and the direction of the reaction. Details of these experiments are described in [6,7].

2. Materials and methods

Kinetics of RNA degradation from the native ternary complex with T7 DNA and RNA polymerase under the action of inorganic pyrophosphate were analyzed by electrophoresis of the RNA in polyacrylamide gel [6,8] and chromatography of the low-M_r product on PEI-cellulose. [^{32}P]RNA was synthesized from A1 promoter of T7 DNA for 8 min at 23°C in the presence of 100 μM CpA and 2.5 μM of each of the 4 NTPs, one of them being labeled with the specific activity of 100–400 Ci/mmol. The active ternary complex of RNA polymerase, T7 DNA and nascent RNA was prepared, purified from NTPs after RNA synthesis (NTP-free ternary complex) and from PP_i after pyrophosphorolysis (PP_i-free ternary complex) using A 1.5 m agarose gel-filtration. To pyrophosphorolyze the RNA, the NTP-free ternary complex was incubated with 1 mM PP_i in the presence of Mg^{2+} (details in [6]). The primary structure of RNA in the ternary complex was determined by means of specific termination of the RNA synthesis by 3′-OCH_3-NTPs [8]. Purification and properties of RNA polymerase, DNA and other materials have been described in [4–8].

3. Results and discussion

In this study we have used the ternary complex of RNA polymerase with DNA of T7 phage deletion mutant DIII and nascent RNA of 20–120 nucleotides long synthesized from AI promoter [6,8] (fig.1).

Published by Elsevier/North-Holland Biomedical Press
00145793/82/0000–0000/$02.75

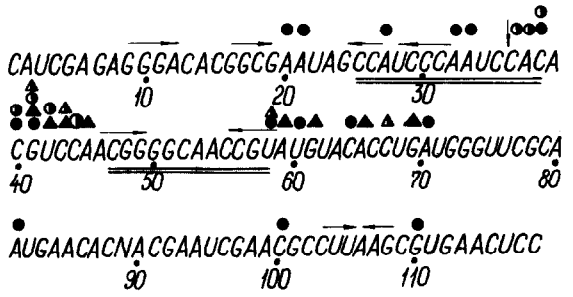

Fig.1. Nucleotide sequence of DIII T7 RNA. Horizontal arrows indicate the RNA self-complementarity region; (▲,●) nucleotides of the RNA pyrophosphorolyzed most slowly and location of strong pauses along the primary structure of RNA in the forward reaction; underlined regions are pyrophosphorolyzed with the highest rates; (◑,▲) 3′-termini of the RNAs that are relatively fast in dissociation from the ternary complexes produced in forward and backward reactions, respectively (explanations in text).

Fig.2K shows a typical electrophoreogram of RNA in the ternary complex with RNA polymerase and DIII T7 DNA. Each band in the electrophoreogram corresponds to RNA molecules of a certain length. The intensity of a band is proportional to the amount of RNA of a particular length. The relative intensity of a band is inversely proportional to the time period which the particular nucleotide remains terminal in the growing RNA chain (details in [4,5]).

To establish the mutually unambiguous correlation between the position of each band in the electrophoreogram and the length of RNA corresponding to this band, the primary structure of RNA in the ternary complex has been determined by means of the method in [8] using 3′-OMe-ATP (fig.2J), 3′-OMe-GTP (fig.2I), 3′-OMe-CTP (fig.2H) and 3′-OMe-UTP (fig.2G).

The ternary complexes used in our experiments are stable. The results presented in fig.2A–F indicate that ≤10–20% of all the ternary complexes dissociate at 20–23°C within 22–44 h. RNA dissociates relatively fast from the ternary complex which comprises RNA molecules of 36,37,38,40,41,42 and 44 nucleotides in length. The ternary complexes containing RNA molecules of the above-mentioned length dissociate faster than by 30–50% within 44 h.

Addition of the high concentration of unlabeled NTPs to the ternary complex (fig.3B) reveals that the NTP-free ternary complex is active. Most of the radioactivity is moved from the RNA bands in the region of 20–150 nucleotides (fig.3D) to the bands corresponding to the RNAs having over 150 nucleotides in length (fig.3B).

Fig.3D–I and 4A–J show electrophoreograms of RNA in the NTP-free ternary complex before and after incubation with inorganic pyrophosphate for different time intervals. One can see that long-term incubation of the ternary complex free of NTPs in the presence of Mg^{2+} (fig.4A–F) and in the absence

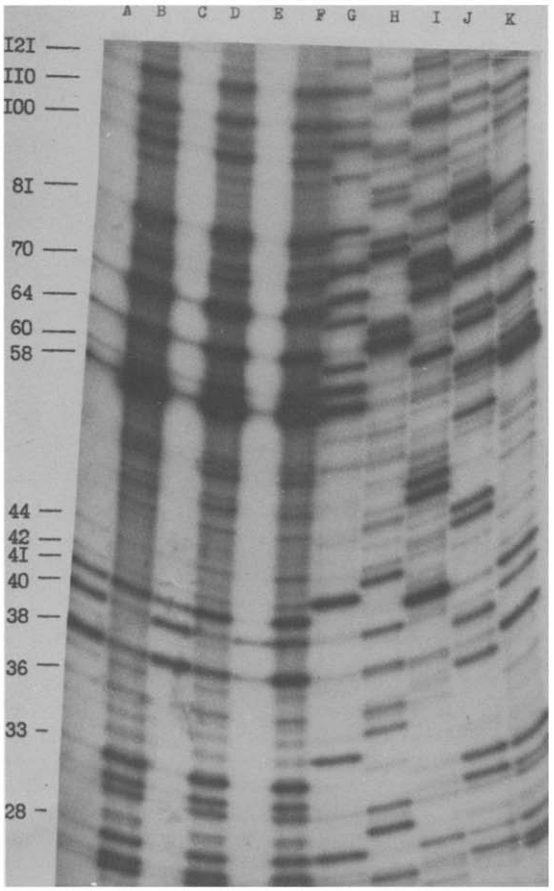

Fig.2. Dissociation of the ternary complex at 23°C. Distribution of [^{32}P]RNA according to its length: (K) original ternary complex (RNA was synthesized for 8 min at 23°C); (J–G) RNA synthesis was terminated with 3′-OMe-ATP (J), 3′-OMe-GTP (I), 3′-OMe-CTP (H), 3′-OMe-UTP (G); (A–F) the ternary complex (K) was incubated 0 h (E,F), 22 h (C,D), 44 h (A,B), and chromatographed on Bio-Gel A 1.5 m; (B,D,F) high-M_r fractions after the chromatography (ternary complex); (A,C,E) low-M_r fractions (the dissociated RNA). All the reactions were stopped by adding EDTA to 50 mM. Nucleotides are numbered starting from the initiating dinucleotide CpA.

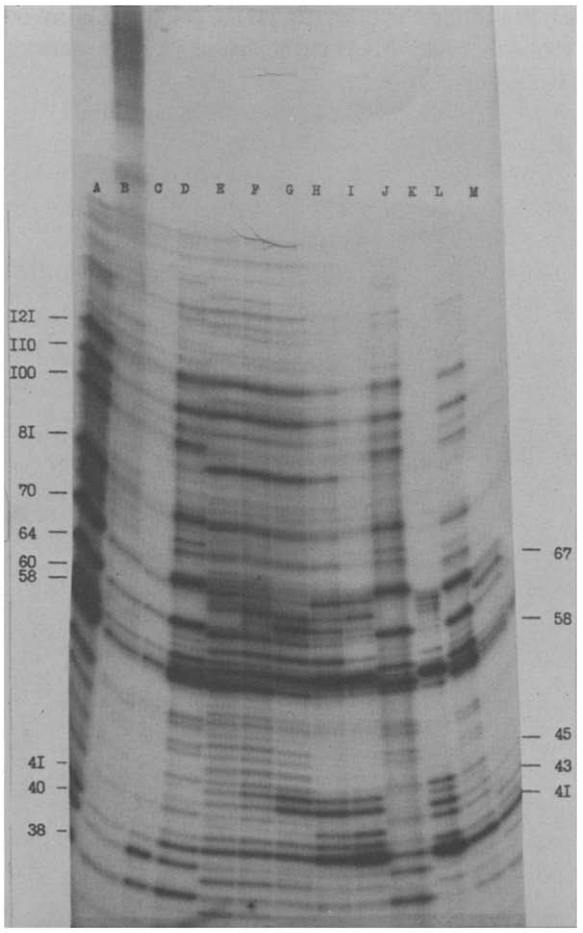

Fig.3. Pyrophosphorolysis of RNA in the ternary complex with RNA polymerase and DIII T7 DNA at 23°C. Electrophoresis of [^{32}P]RNA: (A) original ternary complex, RNA was synthesized for 8 min at 23°C; (D) ternary complex free of NTPs (the first peak after chromatography of A on Bio-Gel A 1.5 m); (C) RNA released from the ternary complex as a result of its dissociation (the second peak after chromatography of A on Bio-Gel A 1.5 m); (B) unlabeled NTPs (100 μM) were added to the NTP-free ternary complex (D), and the mixture was incubated for 60 min at 23°C; (E–I) PP_i (1 mM) was added to the NTP-free ternary complex (D), and the mixture was incubated for 1,2,5,25 and 90 min, respectively; (J) ternary complex free of NTPs (D) was heated at 90°C for 10 min, then cooled to 23°C and 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.9), 50 μg RNA polymerase/ml and 1 mM PP_i were added to the complex, and the mixture was incubated for 90 min at 23°C; (K,M) ternary complex after pyrophosphorolysis for 90 min (I) was chromatographed on Bio-Gel A 1.5 m ((K) PP_i-free ternary complex after pyrophosphorolysis; (M) RNA dissociated in the course of pyrophosphorolysis); (L) unlabeled NTPs (2 μM) were added to the PP_i-free ternary complex after pyrophosphorolysis (K), and the mixture was incubated for 4 min at 23°C. All the reactions were terminated by adding EDTA to 50 mM.

of PP_i causes minor redistribution of the band intensities in electrophoreograms within the region of RNA lengths from 57–60 nucleotides. Here, the intensity of bands 58 and 60 decreases while that of bands 57 and 59 increases. No other alterations occur. No change in the RNA chain distribution has been registered for the ternary complex incubated in the absence of Mg^{2+} (not shown). If PP_i is added to the ternary complex, intensities of the bands in electrophoreograms are considerably redistributed (fig.3E–I and 4G–J): intensities of the bands corresponding to the RNAs having >67 nucleotides in length decrease steadily, those for RNAs from 40–46 and 58–67 nucleotides long increase for 2 h but

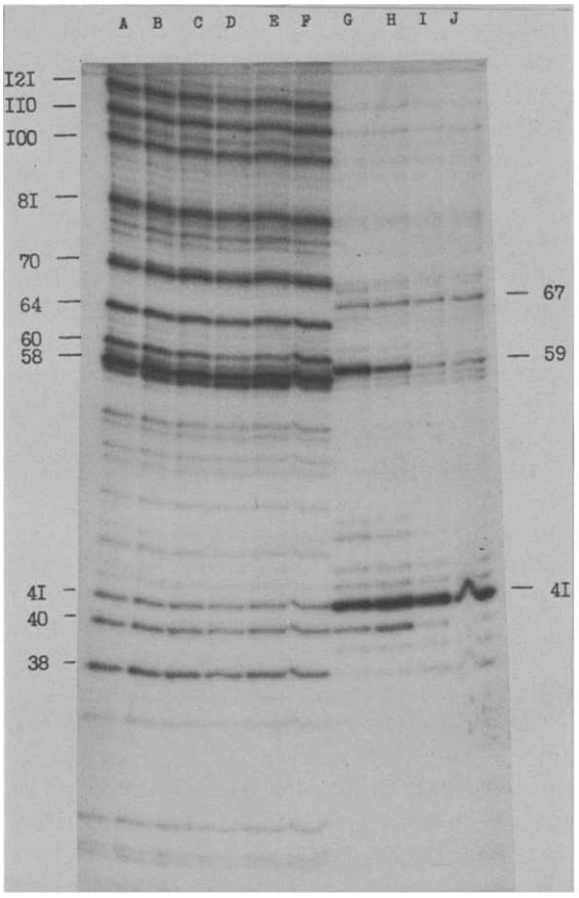

Fig.4. Pyrophosphorolysis of RNA in the ternary complex with RNA polymerase and DIII T7 DNA at 23°C. Electrophoresis of [^{32}P]RNA: (A–F) ternary complex free of NTPs was incubated for 0 h (A,F), 1.5 h (B), 3 h (C), 16 h (D), 25 h (E) without PP_i; (G–J) PP_i (1 mM) was added to the NTP-free ternary complex (A) and incubated for 1.5 h (G), 3 h (H), 16 h (I) and 25 h (J). All the reactions were stopped by adding EDTA to 50 mM.

then slowly decrease, and those for RNAs shorter than 40 nucleotides decrease fast. The intensive bands corresponding to RNAs of 38,40,58,60,64,70, 81,100,110 and 121 nucleotides in length virtually disappear after 90 min incubation at 23°C, whereas intensities of the bands 41,42,43,45,46,59,67 and 69 increase significantly within the same time. Such changes in electrophoreograms may occur only if the length of RNA in the ternary complex decreases continuously, i.e., if degradation of RNA takes place.

In the course of RNA degradation a low-M_r radioactive reaction product was accumulated. The product was analyzed by chromatography on PEI-cellulose plates (fig.5). It should be noted that the chromatographic system we used makes it possible to separate nucleoside mono-, di- and triphosphates, as well as all of the 4 nucleoside triphosphates. Under these conditions, the low-M_r labeled product of RNA degradation does not differ in its chromatographic behaviour from ATP (fig.5B–D). If this product of RNA degradation is treated with alkaline phosphatase, the final resultant labeled product is [^{32}P]P_i (inorganic orthophosphate) (fig.5E,F). Therefore, the products of this RNA degradation reaction are nucleoside triphosphates ([α-^{32}P]ATP in this case).

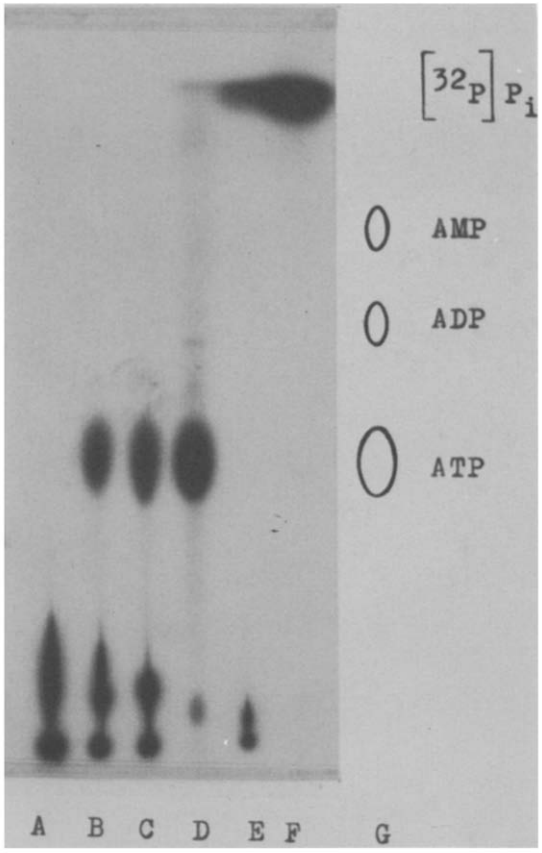

Fig.5. Chromatography of the pyrophosphorolysis reaction products on the PEI-cellulose plates: (A) ternary complex free of NTPs; (B,C) PP_i (1 mM) was added to (A) and incubated for 1.5 h (B) and 3 h (C); (D) [α-^{32}P]ATP; (E) alkaline phosphatase ('Worthington', MA) (2 μl) was added to the ternary complex (4 μl) after pyrophosphorolysis and incubated for 30 min at 65°C; (F) alkaline phosphatase (2 μl) was added to 2 μM [α-^{32}P]ATP (4 μl) and incubated for 30 min at 65°C; (G) a mixture of ATP, ADP and AMP.

The reaction requires the presence of Mg^{2+}. EDTA added in excess inhibits the reactions. The reaction of RNA degradation requires also the existence of a native ternary complex. If the ternary complex is denatured by heating, and Mg^{2+}, Tris–HCl (pH 7.9) and a new portion of RNA polymerase are added to the initial concentration together with PP_i, the original distribution of band intensities is not changed (fig.3J). Therefore, the RNA degradation reaction, like RNA synthesis, is a template-dependent process and is not caused by any nuclease activity that might be present in the reagents or preparations of RNA polymerase used in our experiments.

Consequently; (i) the reaction is DNA-dependent; (ii) it requires the presence of Mg^{2+} and PP_i; (iii) P_i cannot replace PP_i; (iv) the reaction is catalyzed by RNA polymerase; (v) its low-M_r products are nucleoside triphosphates. Thus, the observed reaction of RNA degradation is pyrophosphorolysis.

RNA in the ternary complex subjected to pyrophosphorolysis (fig.3I) and purified from PP_i by chromatography on Bio-Gel A 1.5 m (fig.3K) can be elongated if NTPs are added to it. Original distribution of RNA molecules, typical of the ternary complex before pyrophosphorolysis, is restored (fig.3L).

The ternary complex subjected to pyrophosphorolysis dissociates differently than the ternary complex produced in the forward reaction of RNA elongation. Comparing fig.3K and 3M one can see that the overall dissociation rate is rather low, similar to that of the ternary complex produced in the forward reaction. But there are some sites where the complex subjected to pyrophosphorolysis dissociates at a relatively high rate. These are ternary complexes with DIII T7 RNAs of 41,43,58 and 67 nucleotides in length. They dissociate by 20–30% for the 41 nucleotide long RNA and by 70–80% for 43,58 and 67 nucleotide-long RNAs for a 90 min incubation. In the forward reaction, RNAs of 58 and 67 nucleotides

do not dissociate even during the 22 h incubation, while DIII T7 RNA molecules of 38,40 and 41 nucleotides dissociate within the 22 h incubation, faster by 30–50%. It appears that a rather fast dissociation occurs in the backward reaction when RNA molecules become as short as 6–8 nucleotides. In this range dissociation of ternary complexes is complete (not shown). Similar observations have been made with the other template used [5,6].

The results of this work are summarized in fig.1. Similar to the forward reaction of RNA elongation [4,5], pyrophosphorolysis and the ternary complex dissociation reaction are shown to be strongly dependent on the primary structure of the template. We hope that the quantitative analysis of these phenomena which is now in progress will provide an opportunity for evaluation of efficiency of the putative termination signals from the sequence data only.

References

[1] Krakow, J. S. and Fronk, E. (1969) J. Biol. Chem. 244, 5988–5993.
[2] Furth, J. J., Hurwitz, J. and Anders, M. (1962) J. Biol. Chem. 237, 2611–2619.
[3] Maitra, V. and Hurwitz , J. (1967) J. Biol. Chem. 242, 4897–4907.
[4] Aivasashvili, V. A., Beabealashvilli, R. Sh., Vartikyan, R. M. and Kutateladze, T. V. (1981) Molekul. Biol. 15, 653–667.
[5] Aivasashivili, V. A., Beabealashvilli, R. Sh., Vartikyan, R. M. and Kutateladze, T. V. (1981) Molekul. Biol. 15, 915–929.
[6] Rozovskaya, T. A., Chenchik, A. A. and Beabealashvilli, R. Sh. (1981) Molekul. Biol. 15, 636–652.
[7] Rozovskaya, T. A., Chenchik, A. A., Beabealashvilli, R. Sh., Tarusova, N. B. and Khomutov, R. M. (1981) Molekul. Biol. 15, 1205–1223.
[8] Axelrod, V. D., Vartikyan, R. M., Aivasashvili, V. A. and Beabealashvilli, R. Sh. (1978) Nucleic Acids Res. 5, 3549–3563.