INITIATION BY *ESCHERICHIA COLI* RNA-POLYMERASE: TRANSFORMATION OF ABORTIVE TO PRODUCTIVE COMPLEX

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1. Introduction

Initiation of RNA synthesis by *E. coli* RNA-polymerase is a complicated sequence of events. It is believed that during elongation RNA-polymerase forms a stable complex with bihelical DNA and with the RNA product which is capable of processive synthesis of RNA as suggested by experiments with rifampicin [1]. Contrary to this, at the first steps of transcription, di- and trinucleotides of the general formulae pppNpN and pppNpNpN do not form long-living complexes, and easily dissociate into solution. The so-called abortive initiation takes place with appropriate combinations of two substrates – dissociation of products and re-initiation are many times repeated [2–4]. This process is not inhibited by rifampicin.

In this study we tried to find out at which length of newly-synthesized RNA the ternary complex becomes long-living. It appeared that this transformation occurs at product length of 10 nucleotide residues, i.e. approximately at the same time as the dissociation of the σ subunit [5].

2. Materials and methods

RNA-polymerase of *E. coli* isolated according to [6] and purified additionally according to [7] was prepared by Mr V. V. Khomov. The enzyme contained an equimolar amount of the σ-subunit and had spec. act. 15 000 units/mg as measured according to [7].

The promoter-containing fragment of T7 DNA, which involves its left-hand terminus, the promoters A_0, A_1, A_2, A_3 and gene 0.3, was isolated according to [8] using the restriction nuclease *Bsu*I. The length of the fragment according to revised data [9] is 1400 base pairs. Synthesis of RNA on this promoter-containing template afforded 4 RNA species corresponding to startpoints on the promoters (cf. [10]).

[α-^{32}P]UTP and [α-^{32}P]CTP (400 Ci/mmol) were purchased from Amersham (England). The purity of these radioactive compounds was checked by chromatography on PEI-cellulose. Unlabelled NTPs from Reanal (Hungary) were purified by chromatography on Dowex 1 in a LiCl gradient containing 3×10^{-3} M HCl to be sure that they are not cross-contaminated.

Gel-electrophoresis was run on slabs (20 × 20 × 0.04 cm) with 25% polyacrylamide gel in 7 M urea–0.05 M Tris.borate according to [11]. The products were visualized by radioautography.

2.1. *Synthesis and elongation of initiating oligonucleotides*

Reaction mixtures contained: promoter fragment, 100 μg/ml (1.2×10^{-7} M); RNA-polymerase, 100 μg/ml (2×10^{-7} M); 25 mM Tris . HCl (pH 7.9); 50 mM NaCl; 0.5 mM dithiothreitol; 10 mM $MgCl_2$. This mixture was preincubated for 2 min at 37°C, and 1/10 vol. of one of the following two mixtures was added: (i) 10^{-3} M each ATP, GTP, CTP and 4×10^{-7} M [α-^{32}P]UTP; (ii) 10^{-3} M each ATP, GTP, UTP and 4×10^{-7} M [α-^{32}P]CTP. After 20 or 60 s the reactions were stopped by adding EDTA to 20 mM. Alternatively, isotopic dilution of the labelled NTP was effected by adding the same non-labelled compound to 10^{-4} M, the mixture was incubated for 2 min at 37°C, and the reaction stopped by adding EDTA to 20 mM. The 8 mixtures obtained were analyzed by gel-electrophoresis (fig.1).

2.2. *Determining the lengths of labelled oligonucleotides*

The radioactive products IX and XI (fig.1A, lane 3)

obtained from [α-^{32}P]CTP, were eluted from the gel according to [11] in the presence of 100 μg tRNA as carrier, desalted on DEAE-cellulose and dephosphorylated by *E. coli* phosphomonoesterase under the conditions of complete transformation of ATP to adenosine. The products obtained were subjected to microcolumn chromatography on anionite Lichrosorb . NH_2 in a gradient of potassium phosphate pH 7 (0–0.15 M). Length markers were oligonucleotides of the general formula $A(pA)_n$.

2.3. *Sequencing oligonucleotides*

The dephosphorylated radioactive oligonucleotides obtained as in section 2.2 were subjected to partial digestion with snake venom phosphodiesterase followed by two-dimensional separation (first direction, electrophoresis on cellulose acetate at pH 3.5; second direction, homochromatography [12]). The sequences were read as in [12]. Obviously, using this technique it is only possible to read the sequence to the right of the cytidine residue nearest to the 5′-terminus, because the oligonucleotide is obtained from [α-^{32}P]CTP.

3. Results

The use of such short promoter-containing template was a necessary pre-requisite of the experiments, discussed below, because it enabled initiation of RNA synthesis at a molar concentration of promoters not too different from that of the substrates.

The promoter-containing fragment, RNA-polymerase, 3 NTPs at high concentrations and one highly-radioactive pyrimidine NTP at a very low concentration were incubated for a short time as in section 2. Gel-electrophoresis showed, that radioactive oligonucleotides were synthesized (fig.1A, lanes 1,3; fig.1B, lanes 1,3) The synthesis of RNA in these experiments stopped presumably because the radioactive NTP was consumed to a large extent.

The concentrations of radioactive NTPs were optimized to obtain a mixture of short oligonucleotides. Surprizingly, these optimal concentrations appeared to be an order of magnitude smaller than those of RNA-polymerase and promoters; at higher concentrations, much longer products predominated. Presumably this was because RNA-polymerase contained a large proportion of transcriptionally inactive molecules. Another possible explanation is that

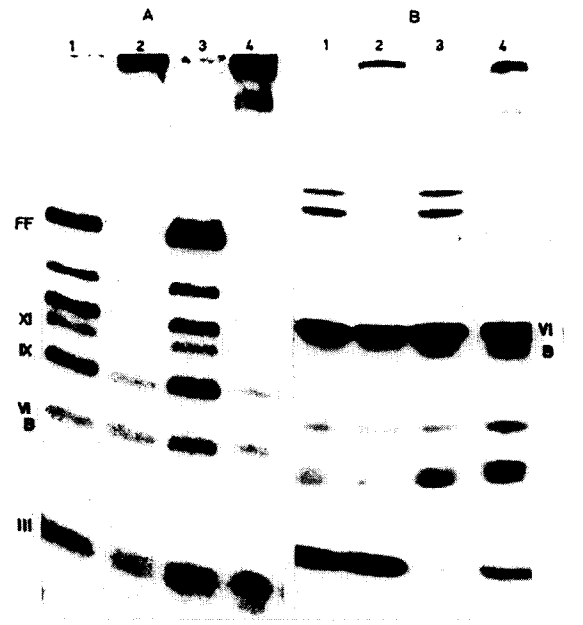

Fig.1. Gel-electrophoretic patterns. (A) Experiment with [α-^{32}P]CTP; (B) experiment with [α-^{32}P]UTP. (1,3) Before isotopic dilution, synthesis during 20 s and 60 s. (2,4) Isotopic dilution after 20 s and 60 s of synthesis with a limiting amount of the radioactive NTP, 2 min synthesis after isotopic dilution. Roman figures correspond to lengths of oligonucleotides which are tentative except for zones IX and XI in the experiment with [α-^{32}P]CTP. FF, xylene cyanol FF; B, bromocresol blue.

initiation is a rare event compared with the binding to promoter.

In parallel experiments, after 20 or 60 s of synthesis in the presence of a low concentration of a labelled pyrimidine NTP, chase-up was effected by adding the same non-labelled NTP at a high concentration. Electrophoretic patterns of such chase-up reaction mixtures incubated for 2 min after the isotopic dilution are shown in fig.1 (A, lanes 2,4; B, lanes 2,4). It is seen that after the chase with non-labelled NTP the longer oligonucleotides disappear to give radioactive RNA which stays at the start, whereas the shorter oligonucleotides remain in their places. Obviously, this is because the shorter oligonucleotides are synthesized abortively, whereas the longer ones form productive ternary complexes.

The borderline which separates abortive and productive oligonucleotides lies between zones IX and XI of the experiment with radioactive CTP (fig.1A). The lengths of the products in these zones are, respectively,

9 and 11 nucleotide residues, as suggested by the following evidence:

(i) Dephosphorylation of the radioactive products IX gave radioactive oligonucleotides which moved together with the marker $A(pA)_8$ in chromatography on Lichrosorb. Na_2;

(ii) As found by sequencing, zone IX contained two radioactive products, the 3′-termini of which were read as . . . ApGpGpUpApA and . . . CpGpA. The 5′-terminal sequences of RNAs synthesized on T7 promoters are presented below:

A_0 pppGUUGGCUUUAGGAUGGAC. . .
(Pletnyov and Zaychikov)

A_1 pppAUCGAGAGGGACAC. . . [13]

A_2 pppGCUAGGUAACAC. . . [14]

A_3 pppAUGAAACGACAGAGUGAG. . . [14]

Hence, the two radioactive nonanucleotides of zone IX are most probably the products synthesized on promoters A_2 and A_3;

(iii) The 3′-terminal sequence of one of the products of zone XI was found to be . . . GpApGpGpGpA. The position of this zone in gel-electrophoresis and this sequence indicate that the product is the undecanucleotide synthesized on promoter A_1.

The conclusion is that the longest poorly-elongated products are nonanucleotides, whereas undecanucleotides and longer products are elongated efficiently.

4. Discussion

It follows from the above data that productive ternary complexes, enzyme–template–RNA, become long-living only after RNA-polymerase reads some 10 base residues of the template, i.e., one turn of the double helix. It is known that at this stage the transcribing complex undergoes a profound conformational rearrangement, e.g., loses the σ-subunit [5].

Evidently, the reason for the instability of the full transcription complex is the weak binding of the 3′-terminus of elongated RNA by the catalytic center.

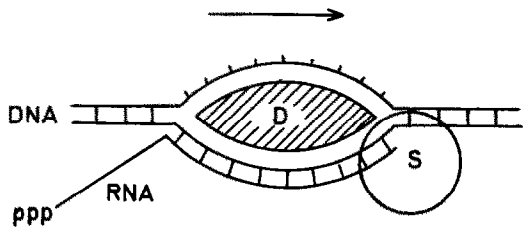

Fig.2. Partial model of the transcription complex: S, catalytic center; D, denaturating center.

One can imagine (fig.2) that in the course of elongation the strength of this binding gradually increases because newly-synthesized RNA fills the denatured part of the template. The length of the denatured part is known to be equal to some 10 nucleotide residues [15]. After the product passes the distal end of the denatured part, it is presumably displaced into solution by the non-codogenic strand.

Maizels [16] has done chase-up experiments with the lactose promoter, an initiating dinucleoside monophosphate and all 4 NTPs at low concentrations. She observed a pause giving rise to a heptanucleotide and claimed that this heptanucleotide was efficiently elongated after chase-up with non-radioactive NTPs.

It has long been known that some dinucleoside monophosphates are efficient initiators of the RNA-polymerase reaction [17,18] and that they serve as primers for RNA occupying a position immediately to the left of the normal start-point. Remarkably, there is space in the model shown in fig.2 for such initiating oligonucleotides.

The data presented above give only a qualitative picture. Actually, the ability of oligonucleotides to be elongated is time-dependent. A more detailed account of this kinetics will be published elsewhere.

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