SUBUNITS OF *E. COLI* RNA POLYMERASE FORMING 3′-OH BINDING SITE AT INITIAL STAGES OF TRANSCRIPTION PROCESS

Possible role of σ-subunit

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1. Introduction

During RNA synthesis, subunits of *E. coli* RNA polymerase form, apparently, several functional sites responsible for binding with the 3′-OH end of the growing RNA chain, with entering nucleoside triphosphate and with a template [1].

Here, to establish the enzyme sites in contact with 3′-OH end of the growing RNA at the first steps of the synthesis, we used photoaffinity modification by oligonucleotides synthesizing in situ, containing photoreactive 5-halogenopyrimidine residues [2] in the vicinity of 3′-OH end.

After comparison of the obtained and known data concerning the phenomena observed at the first stages of transcription the hypothesis has been advanced that:

(1) σ-Subunit participates directly in the formation of 3′-OH binding site of RNA polymerase;
(2) After σ-subunit dissociation the structure of this site changes.

2. Materials and methods

RNA polymerase was isolated by the method [4]. 5′–3′ Guanosyluridine was purchased from 'Sigma'. Uridine triphosphate from Reanal (Hungary) was purified by column chromatography on Whatman DE-52 DEAE-cellulose using gradient elution by triethylammonium bicarbonate buffer (TEAB) (pH 7.5) [α-^{32}P]GTP with spec. radioact. 400 Ci/mmol was purchased from Amersham (England).

DNA from phage λ and DNA fragment from phage λ *imm*434, containing p_O and p_R promoters were isolated as in [5].

Synthesis of 5-iodouridine triphosphate (5-IU) and bromouridinetriphosphate (5-BrU) was done as in [6]. The products were characterised by UV spectra and chromatographic mobilities on the PEI-cellulose plates with eluent 0.75 M KH_2PO_4, (pH 3.5) [6]. The obtained compounds after irradiation by UV light ($\lambda \geqslant 260$ nm) change their spectra and in both cases the final spectra are identical to that of uridine triphosphate.

Photoaffinity modification of RNA polymerase was done as in [2,3].

The reaction mixture (20 μl), containing phage λ DNA (400 μg) or *Eco*RI-G fragment of phage λ *imm*434 (1 μg), bovine serum albumin (BSA) as the inner control (4 μg), 20 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM DTT, 0.2 mM GpU, 0.2 mM either 5-IU or 5-BrU and 6 μM [α-^{32}P]-GTP (350 Ci/mmol), was incubated for 5 min at 37°C. Then the mixture was irradiated for 5 min using the light of a SVD-12 A mercury lamp focussed with a quartz lens and transmitted through a BS-4 ($\lambda > 290$ nm) filter. The distance from the light source to the irradiated solution was 10 cm. The solution (20 μl) containing 10% glycerol, 3% SDS and 5% 2-mercaptoethanol was added to the irradiated mixture and heated for 2 min at 90°C; RNA polymerase subunits were separated in 20 × 20 × 0.15 cm slabs of 6% polyacrylamide gel, containing 0.1% SDS, 0.1 M sodium phosphate and Tris-borate buffer (0.05 M) with 1 mM EDTA (pH 7.5). The electrode buffer consisted of Tris-borate (0.05 M, pH 8.3) with 0.1% SDS. Electrophoresis was at 200 V for 17 h up to the

removal of bromophenol blue from the gel. The radioactive subunits were located by autoradiography on RT-1 film at room temperature for 48 h.

3. Results

As has been mentioned [7] 5-BrU and 5-IU are substrates of RNA polymerase. We used these analogs for labeling of the enzyme active centre. The modification was done according to the scheme developed in [2,3]. *Eco*RI-G fragment of phage λ *imm*434 DNA contains 2 active in vitro promoters (p_o and p_R) programming RNA synthesis with 5′-terminal sequences GpUpUpG and GpUpUpUpG, respectively [8]. Consequently, using this fragment as template, GpU as a primer, 5-IU or 5-BrU (5HalU) and [α-^{32}P]GTP as substrates the synthesis of short oligonucleotides of GpUpHalU^{32}pG structure for p_o promoter and of GpUpHalUpHalU^{32}pG structure for p_R promoter can be carried out. Synthesized oligonucleotides unlike the original nucleoside triphosphates combine the properties of photoreactivity and radioactivity depending on HalU and [^{32}p]G units, respectively. Therefore the discovery of radioactivity covalently bound with subunits of RNA polymerase should unequivocally indicate the modification of the subunits by the reaction products. It is also correct when using the whole DNA of phage λ as template. However in this latter case the described synthesis will take place only on p_o promoter. Besides, for the whole DNA one should expect an increase of the level of non-specific synthesis.

In fig.1,2 are shown the results of the experiments with 5-BrU and 5-IU on the *Eco*RI-G fragment of phage λ *imm*434 DNA and on whole DNA of phage λ. One can see that when using as a template the *Eco*RI-G fragment, σ-subunit appears to be labeled. In fig.1 the results of a number of control experiments are shown. They indicate that this modification is specific and occurs inside the transcriptional complex.

(1) The labeling of subunits takes place only in the presence of template, primer and photoreactive analog.
(2) The considerable amount of albumin which was in the mixture as an inner control as well as β′, β, and α-subunits of the enzyme are not labeled.
(3) If after the synthesis the complex is destroyed and then irradiated, none of the subunits are

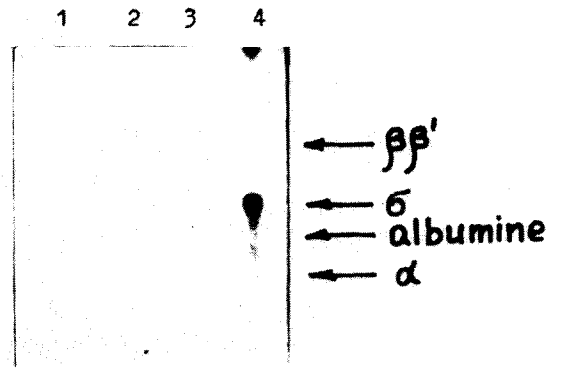

Fig.1. Autoradiogram of RNA polymerase subunits, covalently bound with oligonucleotides after irradiation of transcriptional complex, formed on *Eco*RI-G fragment of phage λ *imm*434 DNA in the presence of GpU, 5-IU and [α-^{32}P]GTP. Synthesis, electrophoresis and autoradiography were done as in section 2. (1) Reaction mixture did not contain 5-IU. (2) Reaction mixture contained UTP instead of 5-IU. (3) The reaction mixture did not contain the template. (4) The standard reaction mixture.

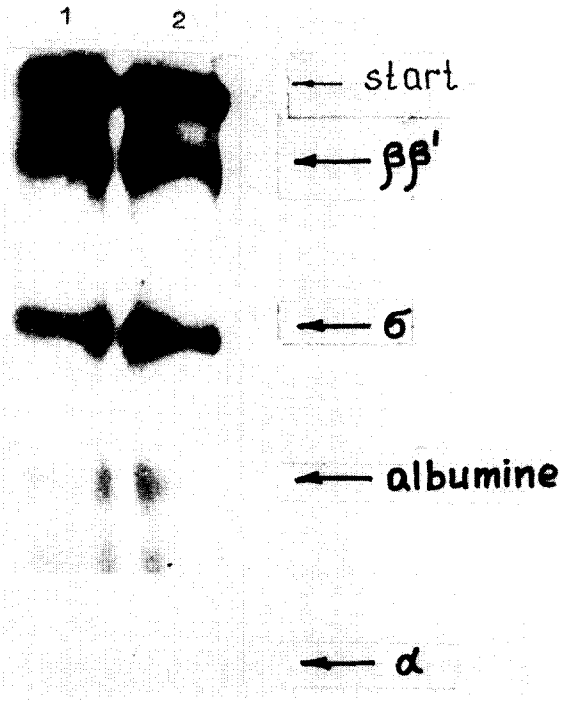

Fig.2. Autoradiogram of RNA polymerase subunits, covalently bound with oligonucleotides after irradiation of the transcriptional complex, formed on λ phage DNA in the presence of GpU, 5-BrU or 5-IU and [α-^{32}P]GTP. Synthesis, electrophoresis, and autoradiography were done as in section 2. (1) Reaction mixture contained 5-IU. (2) Reaction mixture contained 5-BrU.

bound with the radioactive product (data not shown).

(4) The subunits remain unlabeled if in the reaction mixture UTP is used instead of HalUTP.

When whole phage λ DNA is used as a template σ-subunit also appears to be labeled. Besides in this case the labeling of β and/or β′ subunit is observed. Control experiments analogous to these described above show that this labeling is also specific.

Disagreements between the experiments with whole DNA and with the fragment can depend on considerable non-specificity of the transcription processes in the former case.

4. Discussion

In the experiments described the σ-subunit of RNA polymerase is specifically labeled by a photoreactive group located in the vicinity of the 3′-terminus of the synthesized ribooligonucleotide. Earlier the photoaffinity labeling of σ-subunit of RNA polymerase with 5′-terminal group of the growing RNA chain has been demonstrated [2,3]. Thus, at the first stages of transcription before dissociation of σ-subunit from the transcriptional complex, both 5′- and 3′-terminal groups of synthesized oligonucleotides are in contact with this subunit. It is noticeable that for 3′-terminal group this contact is the main one. From the data obtained it appears reasonable to assume that σ-subunit directly participates in the forming of the product binding site and 3′-OH binding site at the initial stages of RNA synthesis*. If this conclusion is correct then it is clear that the structure of this site changes after dissociation of σ-subunit from the transcriptional complex. Are there any data in favour of this hypothesis? Stability of binding of rather long synthesized RNA with the transcriptional complex is well known [1]. At the same time in our experiments on affinity labeling of RNA polymerase by short oligonucleotides synthesized by this enzyme it was shown that binding of these nucleotides with the complex is very unstable. It has been shown [11] that the synthesized oligonucleotides easily dissociate from the transcriptional complex until their lengths are equal to 8–12 residues. From this moment the stability of the association of oligonucleotide with the complex sharply increases. This can be connected with the changes in the structure of the complex after the oligonucleotide attains the corresponding length. In particular these changes may be due to the removal of σ-subunit from the product binding site. The conclusion on the instability of the bonds of short oligonucleotides with the transcriptional complex follows also from the numerous data on the abortive synthesis [9], occuring in case of the incomplete set of nucleosidetriphosphates. It was shown that abortive synthesis is realized by the holoenzyme with active σ-subunit and not realized by core enzyme [10]. These data again give an idea that σ-subunit is somehow connected with the product being synthesized and defines its unstable binding with the transcriptional complex. Moreover, the transcription inhibitor rifamicin, which attacks the β-subunit [13] does not affect the first phosphodiester bond formation [10] and blocks the elongation step. This fact is again in accordance with the idea of the difference between the structures of enzyme active centres at the moment of RNA synthesis initiation and the structures during the following steps of the synthesis. Additional evidence supporting idea of the direct participation of σ-subunit in initiation of the RNA synthesis comes from the data in [12], on crosslinking of the RNA polymerase with *lac* UV-5 promoter. The σ-subunit has been shown to be in direct contact with DNA in the vicinity of the initiation point.

One of the reasons for the changes in the active site of RNA polymerase can be the following: RNA polymerase at the first stages of RNA syntheses remains in interaction with the specific sequences of the promoter. This causes, seemingly, a definite conformation of the enzyme depending on these sequences. At this stage the σ-subunit which, possibly, participates in the interactions both with the promoter and product is necessary. When the length of the synthesizing product increases RNA polymerase stops interacting with the promoter. The template devoid of specific promoter sequences no longer has a necessity for the σ-subunit. The conformation of the RNA binding site changes, and at the same time changes the structure of the product binding site.

Naturally, this schematic working hypothesis needs to be supported by the additional experimental data, however it explains the data now available.

* This hypothesis has been earlier made on the basis of photoaffinity labeling of σ-subunit with 5′-terminal groups of the growing RNA [2,3]

References

[1] Krakow, J. S., Rhodes, J. and Jovin, T. M. (1976) in: RNA polymerase (Losic, R. and Chamberlin, M. eds) pp. 127–157, Cold Spring Harbor Lab., NY.
[2] Sverdlov, E. D., Tsarev, S. A., Levitan, T. L., Lipkin, V. M., Modyanov, N. N., Grachev, M. A., Zaychikov, E. F., Pletnev, A. G. and Ovchinnikov, Yu. A. (1979) in: Macromolecules in the functioning cell (Salvatore, F. and Marino, G. eds) pp. 149–158, Plenum, London, New York.
[3] Sverdlov, E. D., Tsarev, S. A., Modyanov, N. N., Lipkin, V. M., Grachev, M. A., Zaychikov, E. F. and Pletnev, A. G. (1978) Bioorg. Chem. USSR 4, 1278–1280.
[4] Burgess, R. R. and Yendrisak, J. (1975) Biochemistry 14, 4634–4638.
[5] Sverdlov, E. D., Monastyrskaya, G. S. and Rostapshov, V. M. (1978) Bioorg. Chem. USSR 4, 894–900.
[6] Dale, R. M. K., Ward, D. C., Livingston, D. C. and Martin, E. (1975) Nucleic Acid Res. 2, 915–930.
[7] Kahan, F. M. and Hurwitz, J. (1962) J. Biol. Chem. 237, 3778–3785.
[8] Ovchinnikov, Yu. A., Guryev, S. O., Krayev, A. S., Monastyrskaya, G. S., Skryabin, K. G., Sverdlov, E. D., Zacharyev, V. M. and Bayev, A. A. (1979) Gene 6, 235–249.
[9] Jonston, D. E. and McClure, W. R. (1976) in: RNA polymerase (Losic, R. and Chamberlin, M. eds) pp. 413–428, Cold Spring Harbor Lab., NY.
[10] Hansen, U. M. and McClure, W. R. (1979) J. Biol. Chem. 254, 5713–5717.
[11] Grachev, M. A. and Zaychikov, E. F. (1980) submitted.
[12] Simpson, R. B. (1979) Cell 18, 277–285.
[13] Zillig, W., Palm, P. and Heil, A. (1976) in: RNA polymerase (Losic, R. and Chamberlin, M. eds) pp. 101–125, Cold Spring Harbor Lab., NY.