PHOTO-MODIFICATION STUDIES OF THE CONTACTS OF THE 5′-TERMINUS OF GROWING RNA WITH THE SUBUNITS OF RNA-POLYMERASE

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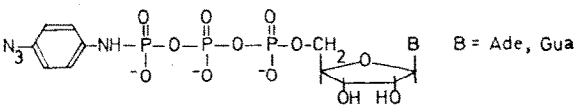
1. Introduction

Due to the substrate activity of NTP γ-amidates towards RNA-polymerase [1,2] it is possible to introduce various reactive groupings into the 5′-terminus of RNA synthesized by RNA-polymerase to study the contacts of this terminus with the enzyme in the course of transcription.

We had shown [3,4] that ATP and GTPγ-azidoanilidates:



may be used for photo-affinity modification of RNA-polymerase. UV-irradiation of reaction mixtures containing RNA-polymerase, promoter templates, one of the above non-radioactive photo-reactive substrates and different combinations of radioactive NTPs results in covalent binding of newly-synthesized radioactive oligonucleotides to the enzyme. However, these data did not give information on the functional topography of the transcribing complex, because the lengths of the oligonucleotides bound by different subunits have not been determined. One could not rely upon synchronous termination of all irradiated RNA-polymerase molecules due to lack of given substrate(s), because at the early stages of transcription RNA-polymerase easily looses the product and re-initiates [5,6].

This paper describes the determination of the lengths of radioactive oligonucleotides bound covalently by the β- and the σ-subunits of *E. coli* RNA-polymerase in the system described above. The major contacts of the 5′-terminus of growing RNA, as deduced by this technique, are the case at transcript lengths 2, 6 and 12 nucleotide residues for the β-subunit, and 2 and 6 residues for the σ-subunit.

2. Materials and methods

The sources and methods of preparation of *Escherichia coli* RNA-polymerase, of the promoter-containing *Bsp*I-fragment of T7 DNA, of ATP and GTP γ-azidianilidates have been described in [1–4,6,7].

2.1. *Photo-affinity modification*

The reaction mixture which contained 100 μg promoter fragment/ml, 100 μg RNA-polymerase/ml, 25 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT was kept for 2 min at 37°C and supplemented with the mixture of substrates to concentrations given in the legend to fig.2. In 10–20 s it was irradiated in a 1 mm silica cell during 2 min by a high-pressure mercury lamp through a filter transparent in the region 300–440 nm. After this, 1/10 vol. 0.2 M EDTA, 10% SDS, 10% mercaptoethanol was added, the solution heated for 2 min at 90°C and applied onto a 5% polyacrylamide slab (30 × 10 × 0.2 cm) prepared in 0.1 M sodium phosphate (pH 7.2)–0.1% SDS. Electrophoresis was run at 100 V until bromophenol blue migrated to the end of the gel. In preparative experiments, the gel was directly radioautographed. In analytical experiments, the gel was at first stained by Coomassie R-250, and radioautographed after drying. Counting of the radioactivities of the β- and σ-subunits revealed that the total yield of modification was ~1% on the molar basis; this figure is in accord with [3,4].

2.2. *Determination of the lengths of oligonucleotides covalently bound*

The modified subunits were electro-eluted from the gel [8] in 10 mM Tris–borate (pH 8.2), 0.4 mM EDTA in the presence of 100 μg carrier tRNA. The electro-eluate (500 μl) was supplemented with HCOOH to 10%, and kept for 2 h at 37°C. Acid was removed by evaporation, and the oligonucleotides were precipitated by ethanol. It was found in control experiments, that:

(i) Such treatment leads to complete transformation of ATP γ-azidoanilidate to ATP;
(ii) Radioactivity is quantitatively cleaved off protein;
(iii) No degradation of phosphodiester bonds in oligonucleotides takes place. The oligonucleotides thus obtained were analyzed by gel-electrophoresis in 25% polyacrylamide gel as detailed in [6]. Quantitative treatment of the radioautographs was performed by V. A. Zabelin and V. N. Dementyev in the Computer Center of the Siberian Division using a computerized system of photographic image processing.

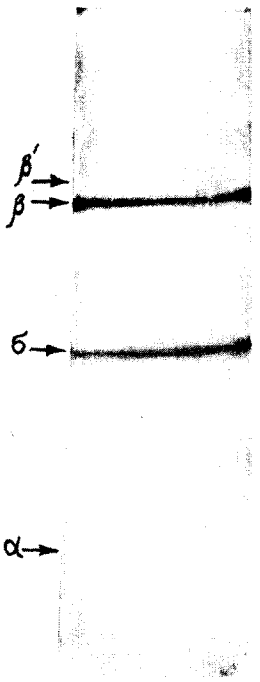


Fig.1. Separation of the photo-modified subunits of RNA-polymerase (radioautograph). Photo-modification and gel-electrophoresis were done as in section 2.1 with 10^{-4} M GTP γ-azidoanilidate, 10^{-4} M CTP and 10^{-6} M [α-^{32}P]UTP as substrates. Positions of the RNA-polymerase subunits as revealed by staining are indicated by arrows.

3. Results

Fig.1 shows the electrophoretic pattern obtained with RNA-polymerase after its irradiation by UV light in a reaction mixture which contained the *Bsp*I promoter fragment of T7 DNA [7], non-radioactive GTP γ-azidoanilidate, CTP and [^{32}P]UTP. It is seen, that the radioactive label is bound by the β- and σ-subunits. A similar pattern was obtained when ATP γ-azidoanilidate, GTP γ-azidoanilidate, UTP, and a small amount of [α-^{32}P]CTP were taken as substrates.

Fig.2 shows the gel-electrophoretic patterns of radioactive oligonucleotides cleaved off the modified RNA-polymerase subunits. The lengths of these oligonucleotides were determined by comparison of their

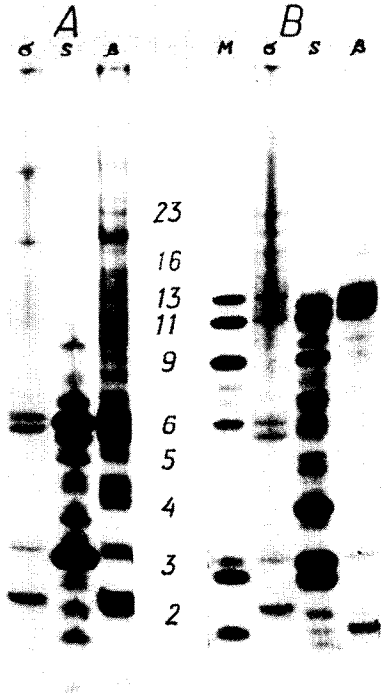


Fig.2. Analysis of the oligonucleotides cleaved off the modified subunits of RNA-polymerase. Figures correspond to the lengths of the oligonucleotides in lane 'M'. (A) Photo-modification with 10^{-4} M GTP γ-azidoanilidate, 10^{-4} M CTP, 10^{-6} M [α-^{32}P]UTP; (σ) oligonucleotides cleaved off the σ-subunit; (s) oligonucleotides synthesized in the non-irradiated reaction mixture; (β) oligonucleotides cleaved off the β-subunit. (B) Photo-modification run with 10^{-4} M GTP–azidoanilidate, 10^{-4} M ATP γ-azidoanilidate, 10^{-4} M UTP and 4×10^{-8} M [α-^{32}P]CTP; (σ,β,s) as in 'A'; (M) markers (see text).

electrophoretic mobilities with those of the products synthesized by RNA-polymerase on the *Bsp*I promoter fragment of T7 DNA in the presence of ATP, GTP, UTP and a small amount of [α-^{32}P]CTP (lane M, fig.2B); these lengths have been found out by direct sequencing as described in [6].

It is seen in fig.2 that:

(i) The patterns of the oligonucleotides cleaved off the subunits are specific;

(ii) The distribution of the intensities differs drastically from that characteristic of the non-bound oligonucleotides present in the reaction mixtures;

(iii) In the 3-substrate experiment (fig.2A), synthesized and bound covalently are oligonucleotides some of which are longer than those that should have been obtained with the template taken (for the primary structure of the promoter fragment, see [9]).

The latter fact may be due to either small contamination of the substrates by ATP, or non-fidelity of the transcription at the early stages.

Fig.3 shows the results of the quantitative treatment (two-dimensional photometry, digitizing and background subtraction) of the radioautographs presented in fig.2. It is seen, that prevalent on the molar basis are di-, hexa- and dodecanucleotides among the products cleaved off the β-subunit, and di- and hexanucleotides among those cleaved off the σ-subunit.

4. Discussion

The arguments in favour of the conclusion that it is affinity modification by intra-complex, photo-

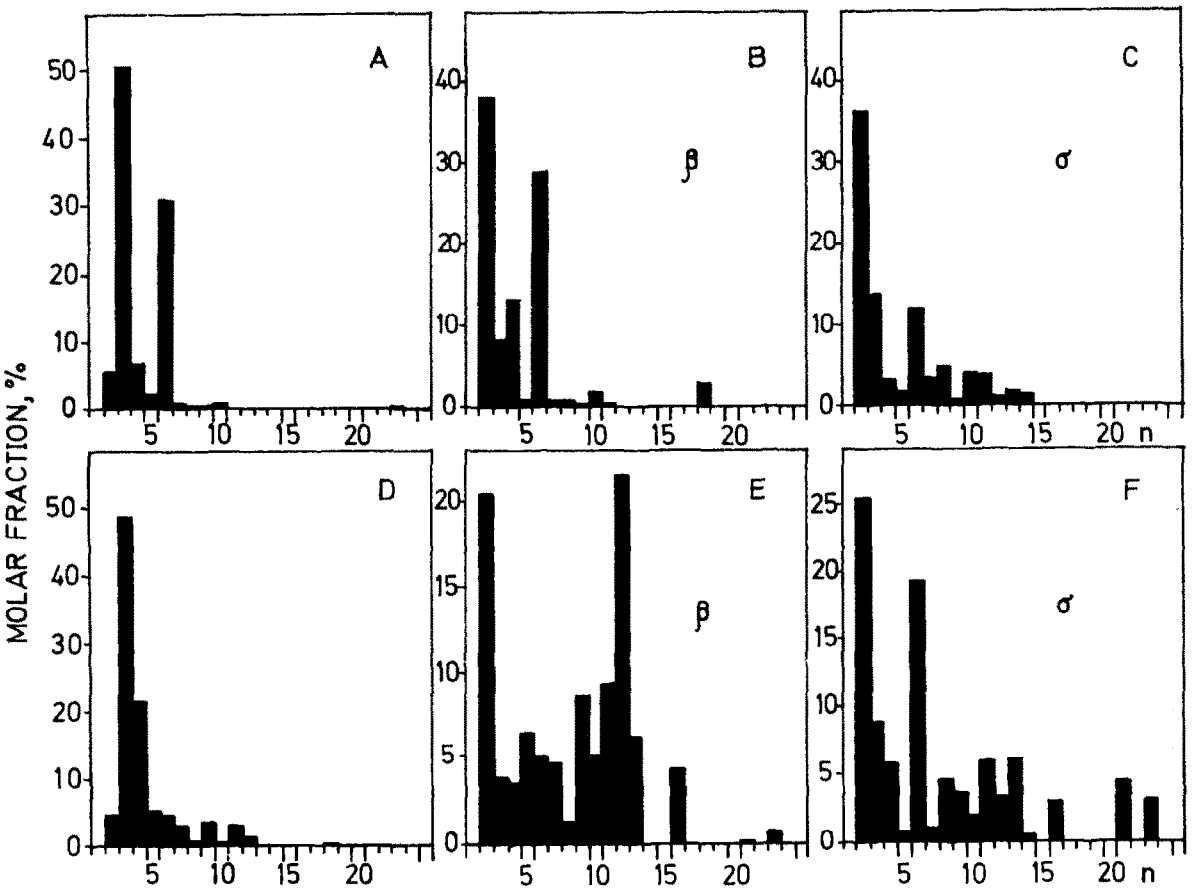


Fig.3. Quantitation of the radioautographs shown in fig.2. Radioautograph presented in fig.2 was subjected to computer treatment as in section 2.2. Ordinates, molar fraction of oligonucleotides of given length; abscissae, lengths of oligonucleotides (n), nucleotide residues. The molar fractions were calculated from the intensities of the bands relative to sum of the intensities of the bands of given lane, from the content of labelled residues as follows from the structure of the template, and on the assumption of equal efficiency of the 4 promoters A_0–A_3.

reactive, newly-synthesized oligonucleotides which takes place in the system described above have been discussed [3,4]. The strongest argument is the fact that such oligonucleotides are both photo-reactive and radioactive, whereas the radioactive substrates taken are not photo-reactive, and the photo-reactive ones are non-radioactive. Another argument is the specificity of the modification towards different subunits of the enzyme.

The results outlined above give some information on the topography of the transcribing complex. Knowing the maximum distance between the 5′-hydroxyl of the ribose of the 5′-terminal nucleotide residue and the azido-group, and, assuming, that the oligonucleotide sits firmly in the respective center, one may conclude, that at the stage of the synthesis of the third phosphodiester bond, a domain of the β-subunit, a domain of the σ-subunit, and the 5′-terminus mentioned meet together within a sphere of a 18 Å radius. These data support the hypothesis [10,11] on the possible direct participation of the σ-subunit in the formation of the active center of initiation. Furthermore, one may conclude that a similar triple contact occurs also at the stage of the synthesis of the seventh phosphodiester bond. After the synthesis of this bond, no close contacts of the 5′-triphosphate terminus with the σ-subunit occur, whereas one more close contact with the β-subunit takes place when phosphodiester bonds 11–13 are synthesized.

The data outlined above may be regarded as reflection of dramatic conformational rearrangements of the transcribing complex at the first stages of RNA synthesis. For example, the abrupt decrease of the yield of photo-modification after the synthesis of the second phosphodiester bond might be due to a dramatic change of the environment of the 5′-terminus of growing RNA. Presumably, the action of rifampicin results in inhibition of this particular rearrangement. According to [12], rifampicin added before substrates does not inhibit the abortive synthesis of di- and trinucleotides, but inhibits completely the synthesis of longer products. As for the significant decrease of the extent of photo-modification after the synthesis of the first 5 phosphodiester bonds, this might be due to the rearragement which results finally in dissociation of the σ-subunit after the synthesis of the first 7 phosphodiester bonds [13].

It will be mentioned in conclusion that the photoreagents employed have both great advantages and great disadvantages because of their non-selectivity [14]. The non-selectivity leads to a very low yield of photo-modification (~1%), and it will be very difficult to identify the modified amino acid residues. However, due to the non-selectivity one may believe, that the pattern obtained reflects the general topography of the complex, rather than the topography of particular reactive amino acid residues, which would be revealed by similar experiments with oligonucleotides carrying chemically reactive groups.

Acknowledgements

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