Effect of guanosine tetraphosphate (ppGpp) on the conformational state of *E. coli* RNA polymerase and transcription directed by tyrT, T7D, and T7A1 promoters

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Guanosine tetraphosphate (ppGpp) is an allosteric regulator of DNA-dependent RNA polymerase. It selectively inhibits transcription from some promoters containing the GC-rich sequence near the starting point of transcription. Using the method of the fluorescent label it was shown that interacting with the β-subunit of the enzyme ppGpp induces essential conformational transitions of the RNA polymerase. The mutation in the enzyme β-subunit that increases the polymerase affinity for T7D promoter affects ppGpp-induced conformational changes. The rate of the abortive RNA synthesis directed by the mutant enzyme from this promoter, which contains no GC-rich sequences near the starting point appeared to be ppGpp-dependent. ppGpp has no effect on T7D activity in the system containing the native enzyme. Thus, the regulatory function of ppGpp depends on the structure of the RNA polymerase and could be realized by the enzyme not only by recognition of the GC-rich block in the promoter.

Key words: RNA polymerase, promoter, ppGpp, transcription regulation, conformational transitions.

DNA-dependent RNA polymerase of *E. coli* is a main enzyme in the transcription mechanism and performs a set of complex reactions; a search and the selective activation of promoter sites of DNA, which essentially differ in structure, are among them. The capability of the initiating complex to respond to regulatory signals in a cell is the important function of RNA polymerase along with catalysis of phosphodiester bond formation. The biochemical and genetic data indicate that the promoter-selective properties of RNA polymerase depend on the structure of all its subunits. In relation to this key problem, to understand the molecular mechanisms of regulation of transcription, a hypothesis on the structural-functional organization of the promoter-recognizing enzyme center is put forward.

To explain the capability of the enzyme to interact efficiently with different promoters having various nucleotide sequences,[1–3] three models were proposed: 1) the unified promoter-recognizing center of RNA polymerase exists as a series of conformers capable of interacting significantly with promoters of various types[1]; 2) in the RNA polymerase molecule several independent promoter-recognizing centers exist[2]; 3) the unified promoter-recognizing center of RNA polymerase is formed from blocks arranged apparently at different subunits of the enzyme, which act subsequently at different stages of the complex formation with the promoter, every block containing several alternative structural elements allowing different signals in the promoter to be recognized; using one or another element at the every stage of the process depends on the structure of the complex formed (the model of alternative pathways of complex formation).[3] At the present time, there in no experimental data to ultimately support or exclude some of these models. One of the possible approaches to the choice between them is a determination of the dependence of promoter-recognizing elements in RNA polymerase, functioning at the activation of certain types of promoters, from the action of regulatory signals intended for regulation of other promoters. To solve this problem for the ppGpp regulator, pTyr, T7D, and T7A1 promoters, and two forms of RNA polymerase, which differ in promoter-selective properties, this work was performed.

Experimental

RNA polymerase from *E. coli* WU-36-10-11-12 (leu_{am}, Tyr_{oc}, Sup E_{oc}) (*E. coli* W12) and rpoB409 strains was isolated by the Burgess—Jendrisak method.[4] The specific activities determined using DNA of T7ΔD111 bacteriophage were 220—270 and 230 E for the native and mutant enzymes, respectively.

The T7D promoter containing 341 base pairs (b.p.) was prepared from T7ΔD111 bacteriophage DNA using HinII endonuclease and amplified using thermophile TtH DNA polymerase and the corresponding oligonucleotide primers complementary to 3′-ends of both strands of the fragment,

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i.e., by the polymerase chain reaction (PCR). The T7A1 promoter (1639 b.p.) was prepared from T7ΔD111 bacteriophage DNA using HinII and HaeIII restrictases. The restriction products were separated by electrophoresis in 1.2% gel of low gelling temperature agarose. The corresponding fragment of DNA was isolated from gel and purified by direct phenolic extraction.[5] The pTyr2-98 plasmid containing tyrT promoter[6] (courteously supplied by A. Travers) was transformed in *E. coli* W12. The strain was cultivated in M9 medium. The plasmid was isolated and purified by alkaline lysis.

The rate of abortive synthesis and the time of formation of the "open" promoter complexes (RP_o) ready for initiation of RNA synthesis were determined by the method from Ref. 7 using the UTP substrate analog labeled at the γ-position of the phosphate by a fluorescent label (1-aminonaphthalene-5-sulfonate, γ-ANS-UTP). In the process of RNA synthesis, the elimination of pyrophosphate occurs; the latter is accompanied by significant increase in the intensity of fluorescence of ANS-pyrophosphate formed. This makes possible monitoring of transcription process. The rate of accumulation of the abortive product was determined using the slope of the kinetic curve.[8]

An aliquot (150 μL) containing Tris-HCl (pH 7.9, 10 mmol L^{-1}), $MgCl_2$ (10 mmol L^{-1}) NaCl (100 mmol L^{-1}), dithiothreitol (0.07 mmol L^{-1}), EDTA (0.07 mmol L^{-1}), GTP (0.3 mmol L^{-1}) (for T7D and tyrT) or ATP (for T7A1), CTP (0.3 mmol L^{-1}) (for tyrT), γ-ANS-UTP (0.126 mmol L^{-1}), RNA polymerase (11—100 nmol L^{-1}), and ppGpp (50 μmol L^{-1}) (if noted) was prepared at 4 °C. The concentration of RNA polymerase was varied in different experiments in relation to the concentration of the matrix. To maintain the equilibrium conditions of complex formation, the enzyme : DNA ratio in all of the cases was ≥10. The mixture was placed in a thermostatted spectrofluorimetric cell and kept at 30 °C for 4 min; a solution of the DNA fragment (50 μL, 1—10 nmol L^{-1}) containing the T7D (T7A1) promoter or plasmid (3—10 nmol L^{-1}) was added to the same buffer. In independent experiments, the activities of T7D and T7A1 promoters were also determined using native T7ΔD111-DNA.[9] The reference probe (200 μL) did not contain the matrix. The synthesis of oligonucleotides was monitored by increase of emission of ANS at 430 nm (excitation at 360 nm).* The measurements were carried out every 6 s with a Perkin-Elmer MPF-44B spectrofluorimeter for 20 min. The time of formation of open promoter complex (τ) was determined using the point of intersection of the straight line representing the kinetics of oligonucleotide synthesis with the baseline of fluorescence. The fluorescence curve was approximated by the computational treatment of the initial data by the least-square-root method.

Conformational studies were carried out by the fluorescent label method[9] in a Tris-HCl buffer (pH 7.9) (10 mmol L^{-1}) containing $MgCl_2$ (10 mmol L^{-1}) and NaCl (100 mmol L^{-1}). The concentration of RNA polymerase was 200 nmol L^{-1}; the concentration of ppGpp was 50 μmol L^{-1}. The spectral parameters (the intensity of fluorescence at all positions of the polaroids) and the emission and excitation spectra were registered with a Perkin-Elmer MPF-44B spectrofluorimeter. The polarization coefficient was calculated using the conventional formula.[8] The fluorescence of fluorescein monomercuroacetate (FMMA) covalently bonded with RNA polymerase and excited at λ 436 nm was registered at λ_{max} of the emission spectrum (520—524 nm). The intrinsic fluorescence was excited at λ 290 nm, and emission was registered at 326 nm.

The SH-groups of the RNA polymerase were titrated at the RNA polymerase constant concentration after 2 h of modification by varying the concentration of FMMA and by measuring the intensity of fluorescence (I) and the polarization coefficient (P) at the maximum of the emission spectrum.

For subunit localization of the fluorescent label, FMMA-RNA polymerase was denatured in 0.3 % sodium dodecyl sulfate (SDS) free of mercaptoethanol at 55 °C for 30 min. The subunits were separated using SDS-electrophoresis in 15% polyacrylamide gel scanned with an SD-1 densitometric spectrofluorimeter (SCB, Pushchino) in transmitted light of a mercury lamp and using a VB-7 filter for excitation and a JGB-19 filter for registered light, respectively. The presence of a protein in bands was controlled by staining of this gel with Coomassie Blue R-250.

Results and Discussion

1. Modification of rpoB409 RNA polymerase by the fluorescent label. According to the local surroundings, the SH-groups of cysteine residues located on the enzyme surface have rather different affinities for FMMA. The data on spectrofluorimetric titration of native RNA polymerase with increasing concentration of FMMA indicates a possibility of specific interaction with one and, to smaller extent, with two molecules of the label.[9] The changes in spectral parameters of FMMA upon interaction with two most reactive SH-groups differ significantly. The SH-groups located at the α-subunit of the enzyme have the strongest affinities for FMMA.

For comparative analysis of conformational transformations caused by ppGpp in the molecules of parent and mutant enzymes, it was necessary to determine that the mutation does not influence on the interaction of the enzyme with the fluorescent label.

The changes in intensity of emission during titration of both enzymes with FMMA (Fig. 1) reveal two prominent bends at close label : enzyme ratios for the parent (0.9±0.02 and 1.7±0.08) and mutant (0.9±0.02 and 1.6±0.1) RNA polymerases. For both enzymes, when the ratio label : protein > 1 increases, a characteristic decrease in intensity of fluorescence (I) is observed, while the polarization coefficient (P) remains constant. Evidently, the rpoB409 mutation changed the conditions of interaction of the label with less reactive cysteine residues. Hence, comparative analysis of conformational transitions of the parent and mutant enzymes induced by ppGpp was carried out only under conditions of equimolar modification.

In Fig. 2, data on the analysis of the subunit localization of the first two labels for the mutant enzyme are presented. It was shown that with equimolar modification (label : protein = 0.8) the label was practically only in the band related to the α-subunit. An increase in the label : protein ratio up to 2 results in appearance of fluorescence in the band related to the β,β′-subunits. Simultaneously, the amount of the label in the band of the α-subunit doubles. Analogous results were obtained

* Synthesis and purification of the fluorescent analog of γ-ANS-UTP was performed by N. Bulychev at the Institute of Bioorganic Chemistry, SB RAS.

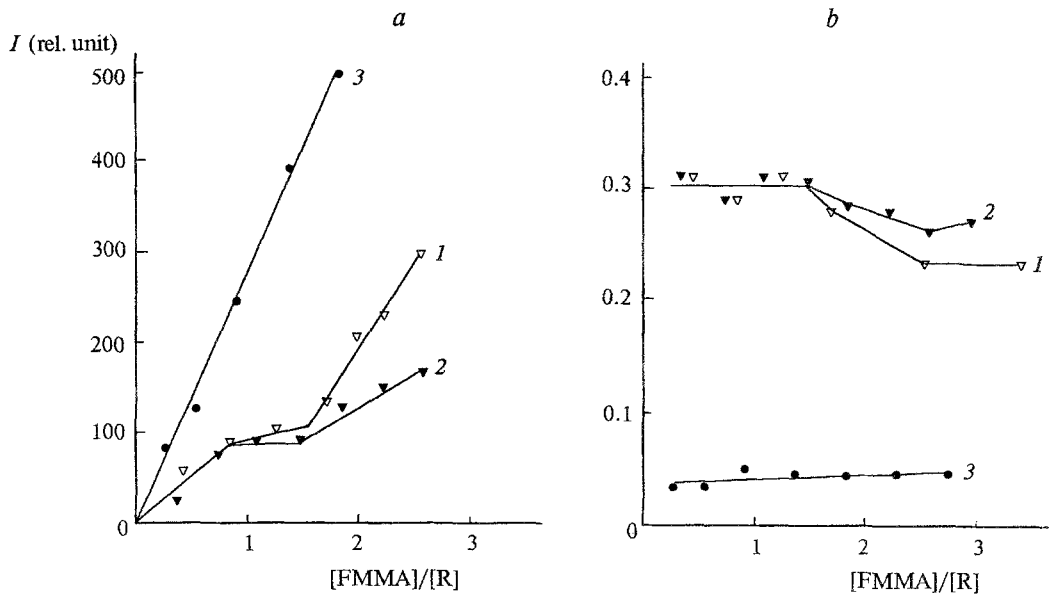

Fig. 1. Titration of the parent (*1*) and mutant (*2*) RNA polymerases with FMMA from data on intensity (*a*) and polarization (*b*) of fluorescence. Straight lines (*3*) indicate the change in spectral parameters for the free label. The concentration of the parent *E. coli* W12 RNA polymerase is 41.8 nmol L^{-1}, and the concentration of mutant enzyme rpoB409 is 40 nmol L^{-1}.

previously for the RNA polymerase of the native strain.[9] Therefore, one can conclude that the mutation in the β-subunit of the RNA polymerase does not change the character of its modification by the first two FMMA molecules.

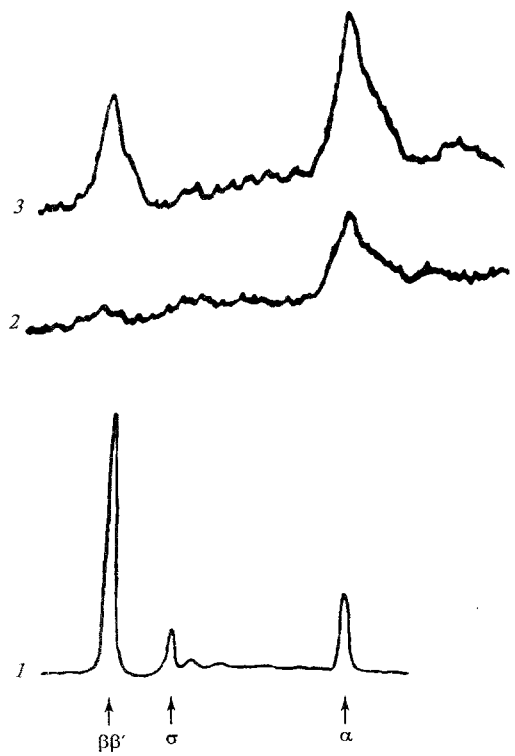

Fig. 2. Densitograms of FMMA-modified RNA polymerase of the mutant strain after separation of the subunit by electrophoresis in 15% polyacrylamide gel in the presence of SDS. *1*) subunits of rpoB409 RNA polymerase after staining of gel by Coomassie Blue; *2*) and *3*) fluorogram of gel for FMMA-modified RNA polymerase at label : protein ratios of 0.8 (*2*) and 2.0 (*3*), respectively.

2. Conformational transformations induced by ppGpp in the molecules of the parent and mutant RNA polymerases. It was assumed that the addition of ppGpp to the β-subunit of RNA polymerase induces the conformational changes in the enzyme, which cause changes of its promoter-selective properties. The existence of conformational changes in the RNA polymerase caused by interaction with ppGpp was observed previously with change in the molar ellipticity of the enzyme[10] and the rate of its sedimentation in a glycerol gradient.[11] One can assume that the use of the fluorescent label covalently and specifically bonded with the α-subunit makes possible to approximate the range of these changes and, probably, the dependence of the conformational transformations of the enzyme from the conformation of its β-subunit.

In all of the experiments, the parameters of the intrinsic fluorescence of the RNA polymerase were registered along with the spectral parameters of the fluorescent label (Fig. 3). Statistically treated data on six independent experiments are presented in Table 1. The change in spectral parameters of the label, 15% quenching of the intrinsic fluorescence of the RNA polymerase, and the bathochromic shift in the excitation spectrum of tryptophan residues indicate the presence of ppGpp-induced conformational transformations of the RNA polymerase. Evidently, these changes modify a significant part of the enzyme surface, since the interaction of guanosine tetraphosphate with the β-subunit[12,13] affects the conformation of α-subunit of the RNA polymerase and strongly changes the parameters of intrinsic fluores-

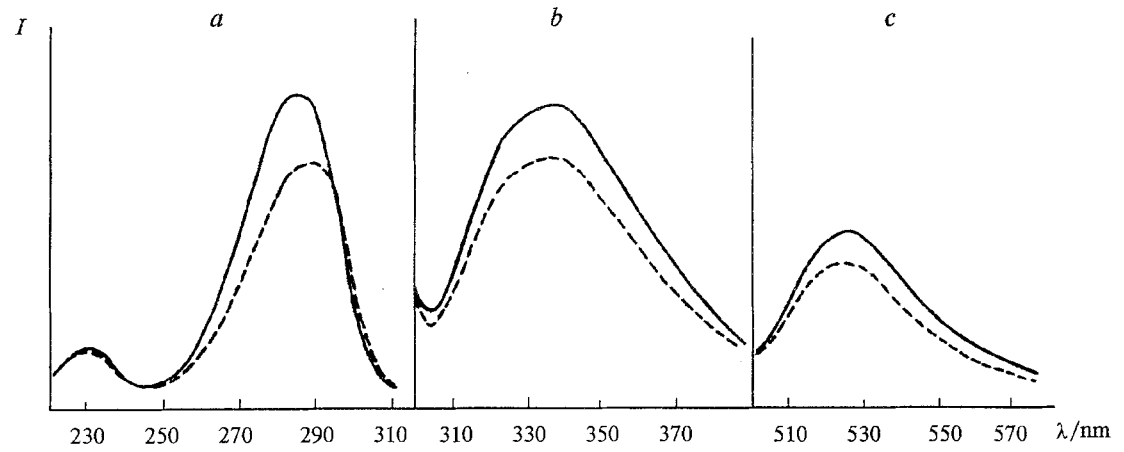

Fig. 3. *a*. Excitation spectrum of intrinsic fluorescence for FMMA-modified *E. coli* W12 RNA polymerase (emission at 326 nm). *b*. Emission spectrum of fluorescence of the modified protein (excitation at 290 nm). *c*. Fluorescence spectrum of the label covalently bonded with RNA polymerase (excitation at 436 nm). Without ppGpp (solid line), in the presence of 50 μmol L^{-1} of ppGpp (dashed line), λ is the wavelength of excitation (*a*) or registration (*b,c*), *I* is the intensity of emission.

Table 1. Effect of ppGpp on spectral parameters of FMMA-modified RNA polymerase of the parent *(E. coli* W12*)* and mutant (rpoB409) strains

Enzyme	I_{524} (% in relation to parent)	*P* presence of ppGpp		I_{326} (% in relation to parent)	λ_{max} presence of ppGpp	
		−	+		−	+
E. coli W12	81.9±5.5	0.32±0.01	0.28±0.01	84.8±2.6	285	290
rpoB409	94.5±2.7	0.31±0.01	0.31±0.01	89.0±1.7	285	290

Note: I_{524} is the intensity of fluorescence of the FMMA-modified RNA polymerase at excitation at 436 nm; *P* is a polarization coefficient of fluorescence FMMA-modified RNA polymerase; I_{326} is the intensity of intrinsic fluorescence of RNA polymerase at excitation at 290 nm; λ_{max} is a maximum of the intrinsic fluorescence of the enzyme.

cence of the enzyme. However, for the mutant RNA polymerase, local conformational transformations in the intimate surroundings of the fluorescent label are significantly smaller than for the parent enzyme. Thus, addition of ppGpp to rpoB409 RNA polymerase does not change the local mobility of FMMA (the constant *P* value indicates this); simultaneously, significant changes in parameters of intrinsic fluorescence of the RNA polymerase is observed. Probably, this fact means that the presence of the regulatory important mutation of rpoB409 causes the change in the structure of the centers, which transfer the conformational perturbation from the β-subunit of the RNA polymerase to its α-subunit. Does this effect the capability of the mutant RNA polymerase to respond to the regulatory signal of ppGpp?

3. Effect of ppGpp on the activation of tyrT promoter. Guanosine tetraphosphate inhibits the transcription of the genes of ribosomal and transfer RNA. One of the most representative members of this family is the tyrT promoter.[6,14] Its primary structure (Fig. 4) contains the GC-rich sequence between −10 and +1. One can assume that the area of the promoter is responsible for its regulation by ppGpp.[14] The effect of ppGpp on the abilities of the parent and mutant RNA polymerases to activate tyrT promoter was studied in five independent experiments on RNA polymerase from *E. coli* W12 and in eleven experiments on the mutant enzyme (Table 2). For both enzymes, the time of formation of open promoter complex (τ) increases, and the rate of abortive synthesis of oligonucleotides decreases. This means that the rpoB409 mutation does not affect the capability of RNA polymerase to respond to the regulatory signal of ppGpp on the promoter controlled by this factor.

4. Effect of ppGpp on the activation of promoters free of GC-rich sequences between −10 and +1. It is known that ppGpp may affect the activity of promoters free of GC-rich sequences near the starting point of transcription. Several genes whose activities were increased in the presence of ppGpp were revealed. Promoters of these genes frequently contain the AT-rich block instead of the GCGC sequence. It was shown for two promoters of such type (lac and his operons) that activation by ppGpp depends on the promoter structure in the −10 region. Nucleotide changes in this region increased the homology with the so-called consensus

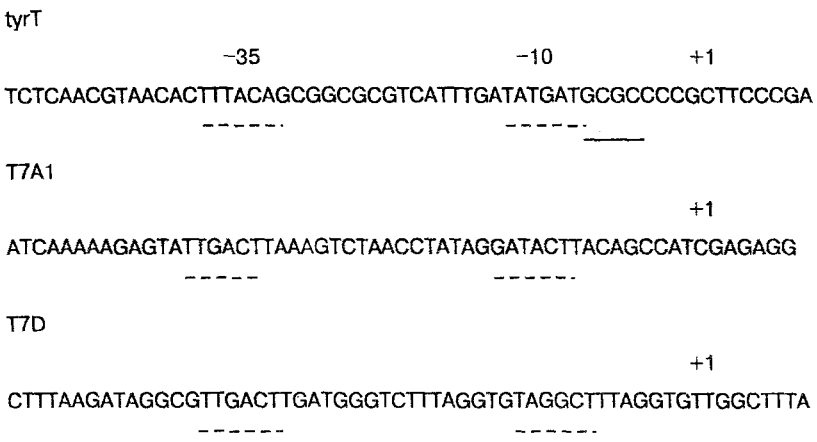

Fig. 4. Primary structure of pTyr, T7D, and T7A1 promoters.

sequence (TATAAT) resulted in increase in efficiency of such promoters and disappearance of the dependence on ppGpp.[15,16] In addition, data on ppGpp-induced inhibition of the activity of T7A1 promoter, which nucleotide sequence near the starting point of transcription is neither AT- nor GC-rich (Fig. 4), are known.[10] This promoter is one of the most studied.[3,17,18] It was shown by the fluorescent label method[3] that the molecular mechanism of its activation differs from the mechanism of activation of T7D promoter. In addition, the rpoB409 mutation increases the affinity of the RNA polymerase for T7D promoter and does not affect the activation of T7A1 promoter. The nucleotide sequence of the T7D promoter near the starting point also has no obvious dependence on its content. Hence, T7A1 and T7D were chosen for study of the influence of ppGpp on activation of similar promoters. The data from Table 3 show that the presence of 50 μmol L^{-1} of ppGpp inhibits the abortive synthesis of oligonucleotides performed by the native enzyme from T7A1 promoter. The activity of T7D promoter in the system of the native enzyme was independent of ppGpp. This is in accord with the difference in the molecular mechanisms of interactions of the RNA polymerase with T7A1 and T7D promoters.[3] In the system of the mutant enzyme, the activities of both promoters are equally inhibited by guanosine tetraphosphate. This means that the mutation-induced conformational changes in RNA polymerase, which increase its activity in relation to T7D promoter, simultaneously lead to a dependence of the corresponding polymerase-promoter complex on ppGpp. Since the rpoB409 mutation increases the affinity of the RNA polymerase for this promoter, one can assume that the analogous effect was observed in this case; this effect was opposite to that observed for promoters of lac and his operons. In another way, T7D promoter is not inhibited if its activity is low, but T7D is inhibited if it is high; the promoters of *lac* and *his* operons are activated by ppGpp if their activity is low, and they are not activated if it is high.

Table 2. Effect of ppGpp on kinetic parameters of the abortive synthesis of RNA from tyrT promoter by the parent and mutant enzymes

Enzyme	V_{+ppGpp}/V_{-ppGpp} (%)	$\tau_{+ppGpp}/\tau_{-ppGpp}$ (%)
E. coli W12	73.5±8.3	146
rpoB409	75.7±6.5	145

Table 3. Effect of ppGpp on the rate of the abortive synthesis of RNA (V) performed by the parent (*E. coli* W12) and mutant (rpoB409) enzymes from T7D and T7A1 promoters

Enzyme	V_{+ppGpp}/V_{-ppGpp} (%)	
	T7A1	T7D
E. coli W12	76.3±8.5 (n = 5)	102.2±3.9 (n = 5)
rpoB409	76.8±9.9 (n = 6)	76.8±7.9 (n = 14)

The data obtained are of principal importance, because they indicate that the regulatory action of ppGpp may be realized through recognition of some signals of the promoter by ppGpp-RNA polymerase, which differ from its enrichment with AT- or GC-pairs in the area neighboring the initiation point. The data are in accord with the supposition on the difference in molecular mechanisms used by the enzyme for activation of T7A1 and T7D. Therefore, the promoter-recognizing centers of the enzyme, which work upon interaction with promoters of different type, are not completely independent.

In addition, the obtained data imply that both the relative strengths of the promoters and the sensitivity of the RNA polymerase to regulatory signals in the cell depend on the structural and conformational state of its β-subunit.

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