Structural and functional analysis of T7D promoter and its complex with *E. coli* RNA polymerase

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The DNA fragment containing D promoter of the T7 bacteriophage and its complex with DNA-dependent RNA polymerase undergo a conformational transition at 30 °C, which is accompanied by an increase in the rate of phosphodiester bond synthesis.

Key words: promoter; paranemic DNA structure; conformational transition; interaction with RNA polymerase; abortive reaction rate.

For initiation of RNA synthesis, free RNA polymerase by itself or in a complex with regulatory molecules should associate with the corresponding promoter of DNA, and then unwind two strands of DNA near the starting point of transcription. From this point of view, the process of interaction of RNA polymerase with different promoters must have the same stages. The classical two-step Scheme (1) demonstrates this generality.

Scheme 1

$$R + P \xrightarrow{K_B} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o,$$

where R and P are the RNA polymerase and the promoter, respectively; RP_c is a closed promoter complex formed by the enzyme with the double-stranded DNA, which is in an equilibrium with free RNA polymerase; and RP_o is an opened promoter complex containing *ca.* 10 unwinded pairs near the starting point.

In the present time, some promoters are studied thoroughly, and different complicated models are proposed for their complex formation.[1–9] However, it is not yet established to what extent these models reflect the individual properties of promoters; their differences may results from peculiarities of the methods used. The comparative analysis of complex formation was performed for T7D and T7A1 promoters.[10] The conformational transition induced by heating (>30 °C) was revealed for the RNA polymerase—T7D promoter complex; this transition takes place after the RP_o formation and it is not typical for T7A1. This study is devoted to investigation of the structural-functional nature of this transition.

Experimental

RNA polymerase from the *E. coli* WU-36-10-11-12 strain (leu_{am}, Tyr_{oc}, Sup E_{oc}) (*E. coli* W12) was isolated according to Burgess and Jendrisak.[11] The specific activity determined using DNA from the T7ΔD111 bacteriophage was 270 E. The content of σ-subunit according to SDS electrophoresis was 90 %.

The T7D promoter-containing fragment was restricted from DNA of T7ΔD111 bacteriophage with HinII endonuclease. The restriction products were separated by electrophoresis in 3.5% polyacrylamide gel. The corresponding DNA fragment having 341 base pairs (b.p.) was isolated as generally accepted.[12]

The rate of a synthesis of phosphodiester bonds was determined in the reaction of abortive initiation (see below) by the method from Ref. 13 using an analog of substrate labeled at the γ-position of phosphates with the fluorescent label (1-aminonaphthalene-5-sulfonate, γ-ANS-UTP). In the RNA synthesis, the elimination of pyrophosphate occurs. The significantly higher activity of fluorescence of the formed ANS-pyrophosphate makes possible to observe the kinetics of the synthesis of phosphodiester bonds.

The typical experimental curve obtained for abortive initiation is given in Fig. 1. The rate of accumulation of the abortive product was determined from the slope of the kinetic curve using the formula:

$$v = (I_{t_2} - I_{t_1})[C]U/kI_o(t_2 - t_1),$$

where I_{t_2} and I_{t_1} are the intensity of fluorescence at the t_2 and t_1 times, respectively; I_o is the intensity of fluorescence during the lag period; $k = 14$ is a coefficient of increase in the intensity of ANS fluorescence after elimination of pyrophosphate; $[C]$ is the concentration of γ-ANS-UTP in an aliquot; and $U = 2$ is a coefficient involving the relative amount of uridinemonophosphate (UMP) in the RNA product, since in the abortive synthesis in the presence of guanosinetriphosphate (GTP) and UTP, the formation of pppGpU, pppGpUpU, pppGpUpUpG, and pppGpUpUpGpG occurs.

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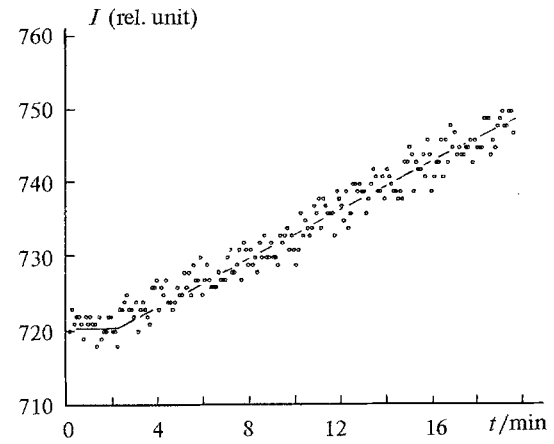

Fig. 1. Time dependence of the synthesis of oligonucleotides with T7D promoter at 20 °C (*I* is the intensity of fluorescence of ANS-pyrophosphate).

An aliquot (170 μL) containing Tris-HCl (10 mmol L^{-1}, pH 7.9), $MgCl_2$ (10 mmol L^{-1}), NaCl (100 mmol L^{-1}), dithiothreitol (0.01 mmol L^{-1}), EDTA (0.01 mmol L^{-1}), GTP (0.3 mmol L^{-1}), γ-ANS-UTP (47 μmol L^{-1}), and active molecules of RNA polymerase (0.82 nmol L^{-1}) was prepared at 4 °C. The mixture was placed in a thermostatted spectrofluorimetric cell, and after 5 min, the mixture (30 μL) containing Tris-HCl (10 mmol L^{-1}, pH 7.9), $MgCl_2$ (10 mmol L^{-1}), NaCl (100 mmol L^{-1}), and the DNA fragment containing the T7D promoter (1 nmol L^{-1}) was added. The synthesis of oligonucleotides was monitored by increase in the intensity of fluorescence of ANS at 430 nm (excitation at 360 nm). The measurements were performed with a Perkin-Elmer MPF-44B spectrofluorimeter every 6 s for 20 min. The corresponding fit of the fluorescence curve was obtained by the computational treatment of the initial data by the least-square-root method.

The determination of electrophoretic mobility of the DNA fragment in 3.5% polyacrylamide gel was performed in a thermostatted camera. The temperature was maintained within *ca.* ±2 °C intervals. The product of DNA restriction of Pvu II of the λ bacteriophage and Hin 1I of the T7 bacteriophage containing 343, 440, and 265 and 143 n.p., respectively, were used as standards. To visualize bands, gel plates were stained with ethidium bromide (0.5 μg mL^{-1}) and photographed.

The 3′-end of the matrix strands of the D promoter was labeled by α^{32}-P-deoxycytidinetriphosphate (α^{32}P-dCTP). The reaction mixture (100 μL) contained Tris-HCl (pH 8.5, 10 mmol L^{-1}), NaCl (25 mmol L^{-1}), $MgCl_2$ (5 mmol L^{-1}), bovine serum albumine (100 μg mL^{-1}), T7-DNA (130 μg), α^{32}P-dCTP (0.125 mCi, specific activity 2×10^6 Ci mol^{-1}), dGTP (0.3 mmol L^{-1}), HinII endonuclease (10 E), and TtH DNA polymerase (2.5 E). The mixture was incubated for 2 h at 37 °C and for 30 min at 70 °C. The labeled fragments were separated in 3.5% polyacrylamide gel and identified by autoradiography. The fragment containing the T7D promoter was isolated from gel using the standard procedure.[12] The specific activity of the labeled fragment was $2\cdot10^5$ imp. μg^{-1}.

Localization of the promoter protected by RNA polymerase from DNAase I action (footprinting). The reaction mixture (10 or 20 μL) contained Tris-HCl (pH 7.9, 10 mmol L^{-1}), $MgCl_2$ (10 mmol L^{-1}), KCl (25 mmol L^{-1}), T7D promoter (112 nmol L^{-1}), and RNA polymerase (570 nmol L^{-1}). The mixture was incubated for 10 min at 35 °C and pancreatic DNAase (up to 1 μg mL^{-1}) dissolved in the same buffer was added. The mixture was incubated for 20 s at 35 °C. The hydrolysis was stopped by addition of the equivalent volume of 8 *M* NH_4OAc and RNA (250 μg mL^{-1}). Nucleic acids were precipitated with 2.5 volumes of cold distilled ethanol. The sample was kept at −20 °C for 1 h, precipitated, washed with 70% ethanol, dried *in vacuo* and dissolved in a mixture (10 μL) of 90 % deionized formamide, 0.001 % bromophenol blue, and NaOH (8 mmol L^{-1}); the mixture was heated for 5 min at 95 °C and electrophoresis in 12.5% polyacrylamide gel in 8 *M* urea was performed. The same DNA fragment hydrolyzed by guanine-specific reaction[12] was used as the marker for identification of bands.

Results and Discussion

1. Effect of temperature on the rate of the abortive reaction performed by RNA polymerase on T7D promoter. In the presence of initiating and one or two elongating substrates, *i.e.*, under conditions of an incomplete set of nucleosidetriphosphates, so-called abortive initiation of RNA synthesis takes place. This reaction exhibits promoter-specific synthesis of short oligonucleotides complementary to a matrix. This reaction involves all stages of interaction of the RNA polymerase with the promoter except the latter stage, *viz.*, transformation of the complex in the productive one, and this may be used to verify whether the conformational transition at 30 °C revealed by the method of fluorescent labeling is of the functional significance and manifests itself at any preceding stages.

Fig. 2 indicates that the drastic increase in the rate of the abortive reaction begins from 28 °C. This means that the conformational transition is really functionally significant.

2. Analysis of the structure of T7D promoter and its complex with RNA polymerase. The nucleotide sequence of the T7D promoter is given in Fig. 3. It contains 2 series of the CTTTAGG segments repeated with a 13 b.p. period and arranged symmetrically in 6 blocks around the "−35" area. In addition, in the T7D promoter longer

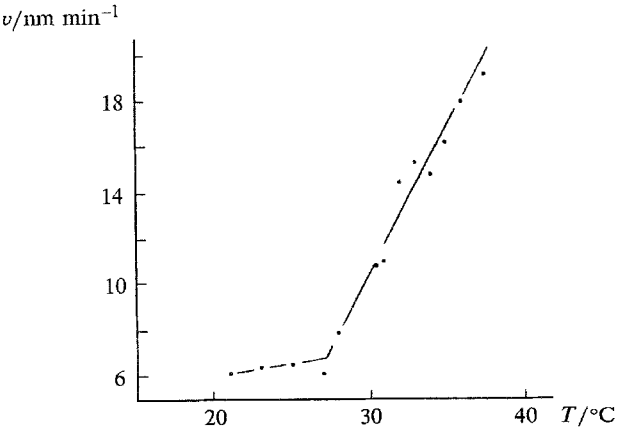

Fig. 2. Temperature dependence of the rate of abortive synthesis of RNA (υ) performed by RNA polymerase on T7D promoter.

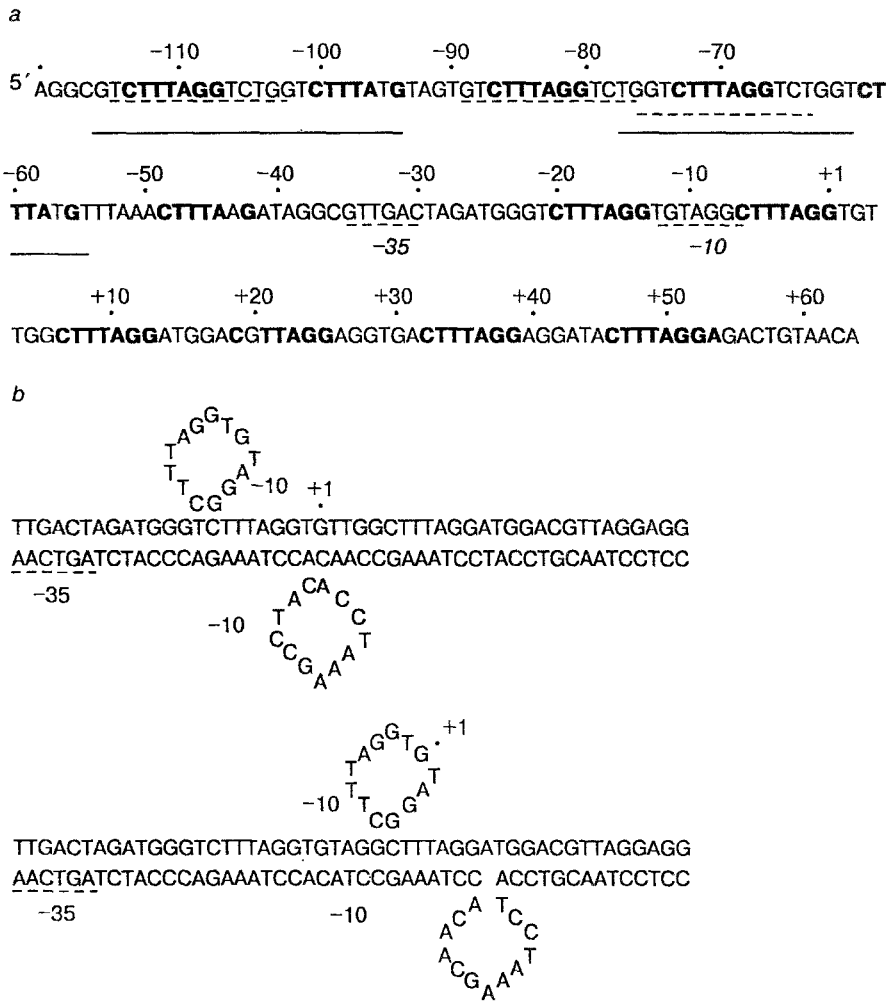

Fig. 3. *a*. Sequence of bases of T7D promoter. The repeating structures are printed in bold type and underlined by dashed and solid lines; *b*. Probable ways of formation of slippage structures near the initiation site of transcription.

periods with 13 and 22 b.p. were revealed. Directly repeated oligonucleotide sites are also found in some other promoters.[14,15] It is known that in the DNA fragments containing direct repeats are capable to exist in a complex configuration, and some their structural elements may be significant for activation of the respective promoter with RNA polymerase. To elucidate how these specific fragments of nucleotide sequence are realized in the spatial structure of the T7D promoter, the temperature dependence of its electrophoretic mobility and the accessibility of different bases of the promoter for the action of DNAase I were studied.

Fig. 4, *a* indicates that at 10—30 °C the DNA fragment containing the D promoter (341 n.p.) moves slightly slower than longer marker (343 n.p.). Since in the range of correct resolution the mobility of polynucleotides in the electric field depends linearly from logarithm of their length, one can estimate the expected mobility of the fragment studied using the mobilities of the neighboring bands. The expected position of the band related to T7D promoter is indicated by an arrow; the real mobility was found to be lower than the expected one. The change in the relative mobility of the fragments studied was observed at temperatures higher than 30 °C. The data in Fig. 4, *b* indicate that increase in temperature causes an increase in mobility of the DNA fragment containing T7D promoter; simultaneously, the relative mobilities of other fragments were unchanged. This means that some features of spatial structure of the T7D promoter impeding its motion in gel at a low temperature disappears at a high temperature. The similar effect can be caused by the formation of the stable bend in the promoter DNA. However, the primary structure of the fragment studied is free from sequences assisting in formation of the stable DNA bend. The presence of direct repeats may cause the formation of the slippage pin-structures, analogous to those presented in Fig. 3, *b*.[16] In fact, the preliminary data on the action of the S1 nuclease indicate the presence of stable one-strand fragments in the structure of T7D promoter.

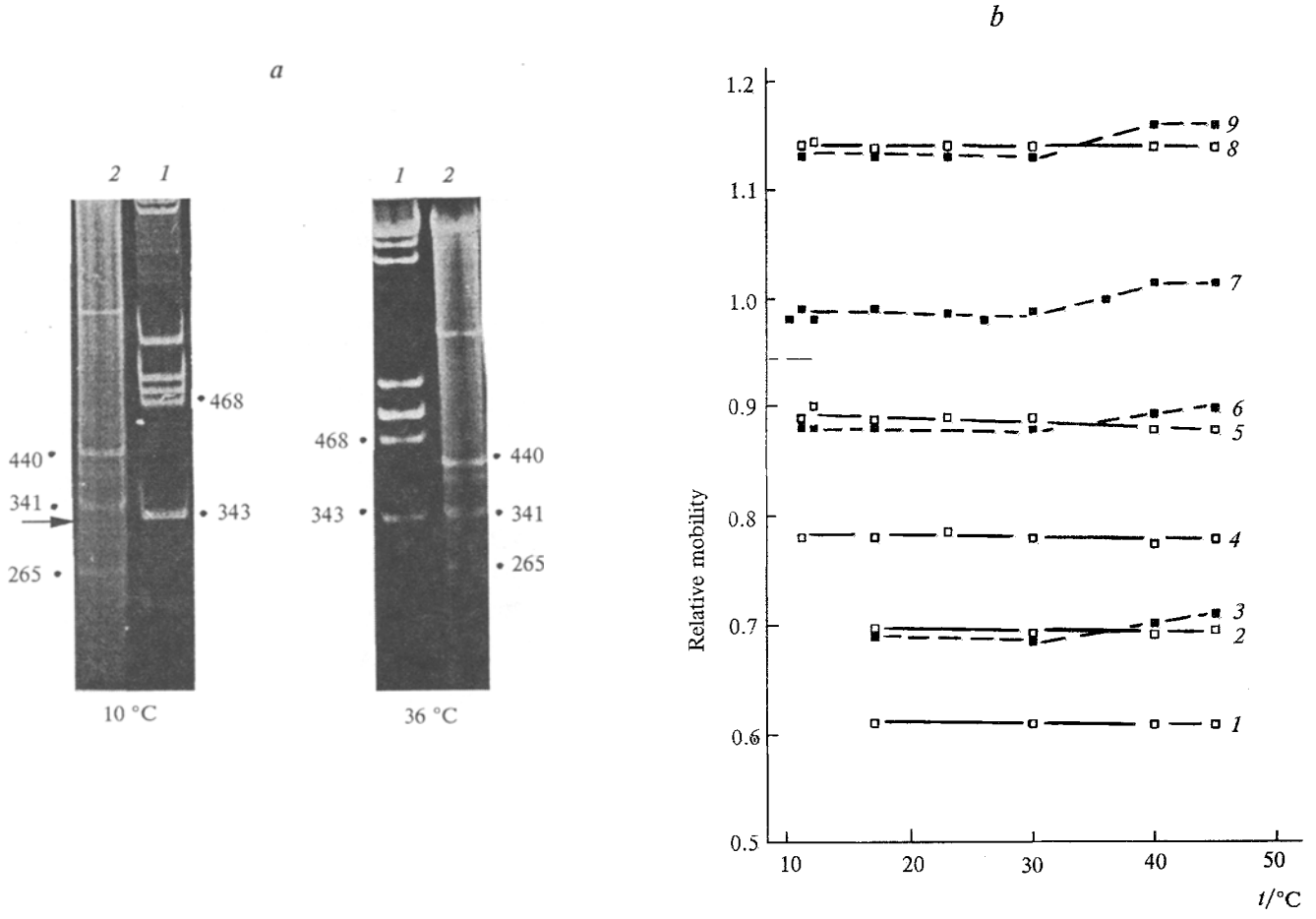

Fig. 4. Temperature dependence of the relative mobilities of different fragments of DNA in an electric field. *a*. Photographs of gels after electrophoretic separation of the restrictive fragments and staining with ethidium bromide. The theoretically expected position of the DNA fragment containing the T7D promoter is marked by an arrow. The length of fragments restriction of DNA of λ bacteriophage by Pvu II endonuclease (*1*) and DNA of T7 bacteriophage by Hin II endonuclease (*2*) are given numerically. *b*. Relative mobilities of different fragments calculated by the formula R_f^i/R_f^j, where R_f^i is the mobilities of 440, 343, and 341 b.p. fragments, and R_f^j is the mobilities of the 143, 265, 343, and 440 b.p. fragments, respectively: *1*, 440/143; *2*, 343/143; *3*, 341/143; *4*, 440/265; *5*, 343/265; *6*, 341/265; *7*, 341/343; *8*, 343/440, and *9*, 341/440.

The structure of T7D promoter and its complex with the enzyme were studied by the DNAase footprinting. For this purpose, the 3′-end of the matrix strand of the promoter was labeled by radioactive α^{32}P-dCTP. Free DNA and the polymerase—promoter complex were treated with a low concentration of DNAase 1, the hydrolytic fragments after denaturation were separated using electrophoresis. Data in Fig. 5 show that in the absence of the enzyme, the accessibilities of bases of the promoter for the action of DNAase are different. The most reactive bases are marked by arrows on the autograph and in Scheme. Their positions are related to those of repeating structural fragments. In addition, the fragment from −55 to −44 enriched by AT-track appeared relatively stable to the action of endonuclease. Since the action of DNAase 1 depends on the width of the minor groove of DNA and does not depend on the type of a base.[17] One can suggest that in the T7D promoter several structural peculiarities exist, some of them correlate in position with those of repeating sequences.

In the presence of RNA polymerase, as well as in the case of the majority of other studied promoters, the area from −58 to +24 is protected from the action of the nuclease. The enzyme does not influence on the reactivity of the areas, which are remote from the startpoint. However, some positions become more sensitive to the action of the nuclease. At first, that are the positions −25/−24 and −38/−37, located inside the protected area. More than 2/3 of promoters studied by DNAase footprinting, have such "hypersensitive" fragments inside the contact area. One can assume that the interaction with the enzyme creates stressed conformation of DNA, increasing its accessibility for the DNAase. The second

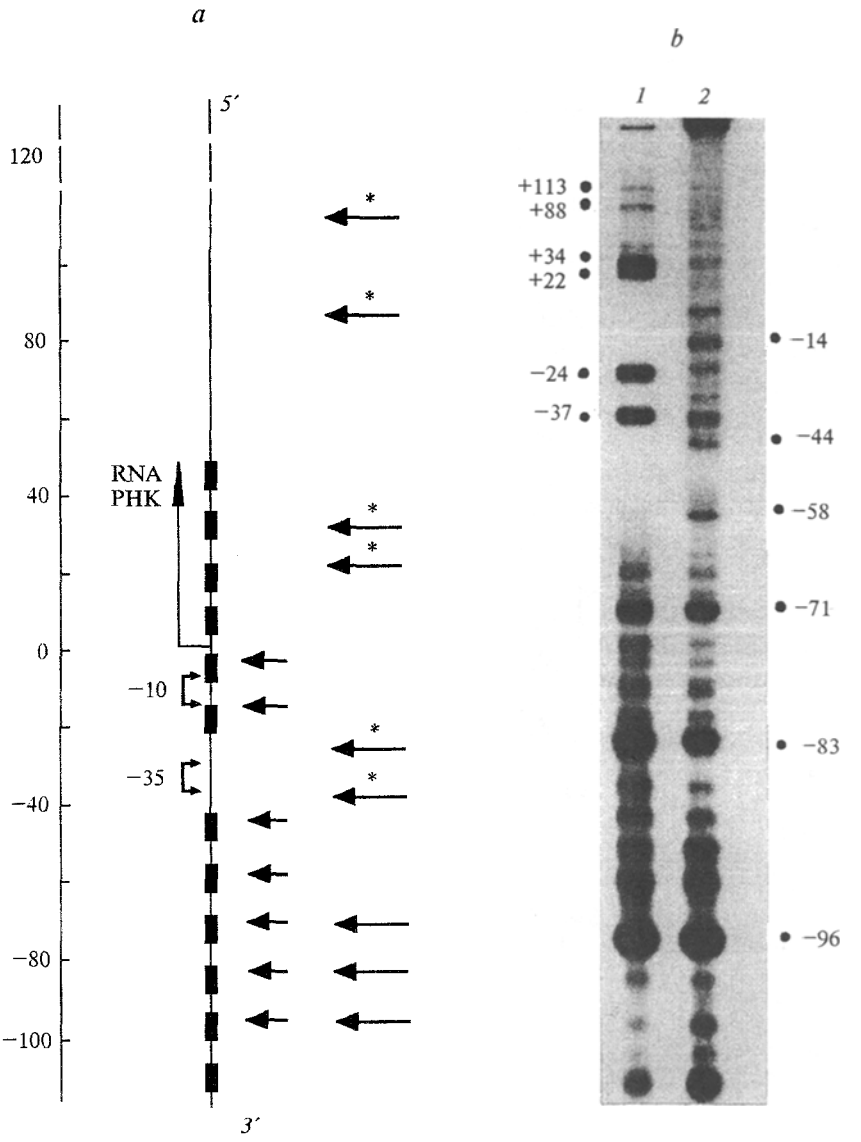

Fig. 5. *a*. Schematic presentation of the positions of the repeated oligonucleotide fragments CTTTAGG and bases of the promoter, having high sensitivity to DNAase in the free state (short arrows) and in the complex with RNA polymerase (long arrows). *b*. Accessibilities of the different bases of the T7D promoter to the action of DNAase 1 in the free state (*b*, *2*) and in the presence of RNA polymerase (*b*, *1*). Hypersensitive sites are marked with asterisks.

group of fragments hypersensitive to the DNAase is located in the primarily transcribed part of the gene (~+22/+23 and ~+35/+36). At least, five other promoters (pap,[18] rrnBP1,[19,20] lacUV5,[21] tac-UM-138,[22] deoP1[23]) are hypersensitive to DNAase action in this area. In all cases, as well for T7D promoter, the sites of increase in sensitivity to the nuclease in the transcribed part of the gene are in the area of directly repeating oligonucleotides. Thus, in the pap promoter, repeated three times the TTGA tetranucleotide (5′-ended nucleotide at −6, +6, and +13, respectively) and the sequences TAAG and TAAAG (5′-ended nucleotides at +20 and +27, respectively) is present. Hypersensitivity is demonstrated at +13/+14 and +22/+23 positions. An increase in sensitivity to the DNAase 1 action in the T7D promoter appears in ~+22/+23 and +34/+35 positions, inside the TTAGGA repeats, which begin at the +20 and +33 positions, respectively. One can suppose that the interaction in the RNA polymerase leads to the formation, or, on the contrary, to the destruction of some paranemic structures, *i.e.*, to the cooperative conformational transformation in DNA, which is capable to change sensitivity to DNAase not only in the area of the direct contact with the enzyme, but also in neighboring sites.

In the presence of RNA polymerase, the increase in sensitivity to DNAase I was registered in two sites located in the remote coding area of the T7D promoter (~+88, +113; Fig. 5). Yet the other promoters, which complexes with the enzyme possess this property, are not found. One can propose that hypersensitivity in the remote transcribed part of the gene is conditioned by addition of the second RNA polymerase molecule to the DNA fragment studied. However, the search algorithm for the promoter-sequences[24] does not reveal promoter-like sites in this part of the gene. One can assume that the formation of T7D-polymerase complex leads to the cooperative change in the structure of the promoter DNA and this change is spread on a large distance.

Thus, one can conclude that the spatial structure of the D promoter really possesses some peculiarities. The decreased electrophoretic mobility of the corresponding fragment (Fig. 4); the presence of periodically located fragments having larger sensitivity in relation to DNAase 1 (in comparison to the neighboring ones) (Fig. 5, *b*, *2*) and cooperative changes in DNA in the coding part of the gene under interaction with RNA polymerase (Fig. 5, *b*, *1*) confirm this fact. The temperature dependence (Fig. 4) of stability of the T7D-specific paranemic structure is probably the basis of the conformational transition in the polymerase—promoter complex registered by the fluorescent label method at 30 °C. It was proved that this transition is functionally important, since the increase in temperature in this region leads to significant increase in the rate of the phosphodiester bond formation (Fig. 2). This is of fundamental importance, because in conjunction with data from Ref. 10, it directly indicates the difference in the molecular mechanisms of activation of different promoters.

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