

## Characteristics of $\sigma$ -Dependent Pausing by RNA Polymerases from *Escherichia coli* and *Thermus aquaticus*

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**Abstract**—The  $\sigma^{70}$  subunit of RNA polymerase (RNAP) is the major transcription initiation factor in *Escherichia coli*. During transcription initiation, conserved region 2 of the  $\sigma^{70}$  subunit interacts with the  $-10$  promoter element and plays a key role in DNA melting around the starting point of transcription. During transcription elongation, the  $\sigma^{70}$  subunit can induce pauses in RNA synthesis owing to interactions of region 2 with DNA regions similar to the  $-10$  promoter element. We demonstrated that the major  $\sigma$  subunit from *Thermus aquaticus* ( $\sigma^A$ ) is also able to induce transcription pausing by *T. aquaticus* RNAP. However, hybrid RNAP containing the  $\sigma^A$  subunit and *E. coli* core RNAP is unable to form pauses during elongation, while being able to recognize promoters and initiate transcription. Inability of the  $\sigma^A$  subunit to induce pausing by *E. coli* RNAP is explained by the substitutions of non-conserved amino acids in region 2, in the subregions interacting with the RNAP core enzyme. Thus, changes in the structure of region 2 of the  $\sigma^{70}$  subunit have stronger effects on transcription pausing than on promoter recognition, likely by weakening the interactions of the  $\sigma$  subunit with the core RNAP during transcription elongation.

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**Key words:** RNA polymerase,  $\sigma$ -dependent pausing, transcription regulation

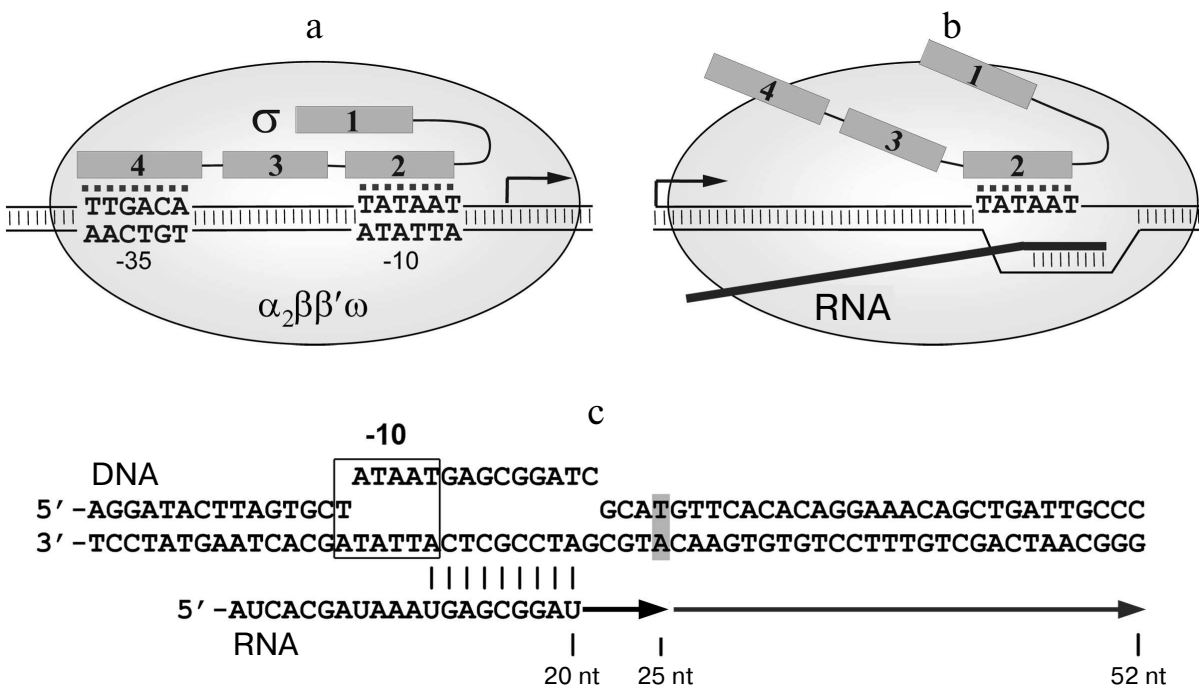
Specific recognition of promoters during transcription initiation by bacterial RNA polymerase (RNAP) occurs with the participation of the initiation factor — the  $\sigma$  subunit. Interaction of the  $\sigma$  subunit with core enzyme of RNAP (subunit composition:  $\alpha_2\beta\beta'\omega$ ) results in formation of RNAP holoenzyme, which is able to initiate RNA synthesis without participation of additional factors. During initiation the holoenzyme recognizes promoters, causes local melting of DNA around the transcription start point, binds initiating nucleotides, and starts RNA synthesis. When RNA reaches a length of about 10 nt, contacts of RNAP with the promoter are disrupted and the transition to transcription elongation occurs [1-3].

Bacterial cells generally contain several different  $\sigma$  subunits responsible for transcription of different gene groups. Transcription of most genes is mediated by a so-called major  $\sigma$  subunit ( $\sigma^{70}$  in *Escherichia coli*,  $\sigma^A$  in other bacteria). Promoters recognized by the  $\sigma^{70}$  subunit con-

tain several conservative elements, the main being the  $-10$  and the  $-35$  elements (consensus sequences TATAAT and TTGACA, respectively) (Fig. 1a) [1]. The  $\sigma$  subunit in the holoenzyme directly recognizes promoter elements and participates in DNA melting in the starting point of transcription. All major  $\sigma$  subunits include several conserved regions, which are divided into subregions (1.1-1.2, 2.1-2.4, 3.0-3.2, 4.1-4.2) and perform different functions during transcription initiation. In particular, the 2.3/2.4 and the 4.2 regions participate in the recognition of the  $-10$  and  $-35$  promoter elements, respectively, and the 2.1 and the 2.2 regions play a major role in interactions with the core RNAP enzyme. It should be noted that the remaining regions of the  $\sigma$  subunit are also involved in interactions with the core enzyme (Fig. 1a) [1, 4-7]. The melting of DNA is initiated by the 2.3 region of the  $\sigma^{70}$  subunit, which contains several aromatic and positively charged residues and interacts with the non-template DNA chain at the  $-10$  promoter element. Mutations of these residues lead to an increase in the temperature of promoter DNA melting during transcription initiation, which confirms their important role in separation of DNA strands [8-12].

Abbreviations: nt, nucleotides; RNAP, RNA polymerase.

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**Fig. 1.** Participation of the RNAP  $\sigma$ -subunit in promoter recognition and in formation of transcription pauses. a) Scheme of promoter recognition by the  $\sigma$ -subunit. Conserved regions of the  $\sigma$ -subunit are numbered. The sequences of  $-10$  and  $-35$  promoter elements are shown; the transcription start point is indicated by an arrow. b) Scheme of pause signal recognition by the  $\sigma$ -subunit of RNAP. c) Scheme of the synthetic template used in this study for analysis of  $\sigma$ -dependent transcription pause. Pause signal, corresponding to the  $-10$  promoter element, is outlined by a frame. DNA nucleotides corresponding to the RNA 3'-end in the pause site are shown in gray. Arrows indicate the transcripts corresponding to transcription pause (25 nt) and full-sized RNA product (52 nt).

It was initially thought that during the transition to elongation the  $\sigma$  subunit dissociates from the transcription complex. However, the  $\sigma^{70}$  subunit in *E. coli* was found to interact with the elongation complex (EC) and to induce pauses during transcription. Such pauses were initially found in the initially-transcribed promoter regions of lambdoid bacteriophages (phages  $\lambda$ , 82, and 21) [13, 14]. Later the  $\sigma^{70}$ -dependent pauses were found in the promoter-proximal region of the *lac* gene *in vitro* [15-17] and also in a number of *E. coli* genes *in vivo* [18]. In the case of lambdoid phage promoters, the  $\sigma$ -dependent pausing plays a regulatory role and is required for interaction of the EC with the Q protein, which acts as a transcription antiterminator [14]. However, the functional role of  $\sigma$ -dependent pauses in the transcription of other genes remains unknown.

Pausing requires the presence in the transcribed DNA of a region similar to the  $-10$  promoter element. This region interacts with region 2 in the  $\sigma^{70}$  subunit (Fig. 1b) [13, 15, 17]. The binding of the  $\sigma$  subunit to EC is likely to occur through interactions of region 2  $\sigma$  with the  $\beta'$  subunit of the RNAP core enzyme; mutations in the 2.2 region of the  $\sigma^{70}$  subunit disrupt pausing [14, 19-21]. It should be noted that the presence of RNA transcript in EC prevents the interaction of the core enzyme with regions 3 and 4 of the  $\sigma$  subunit, which in the holoenzyme

are located in the RNA exit channel [2, 6, 22]. Binding of  $\sigma^{70}$  to the  $-10$ -like element leads to "anchoring" of the EC at the upstream edge of the transcription bubble. Continuation of the RNA synthesis leads to the formation of unstable stressed EC, which can then backtrack on the DNA template; as a result, the RNAP active site loses contact with the 3'-end of RNA, leading to stop of transcription [14, 15, 17, 23, 24].

Many details of  $\sigma^{70}$ -dependent pausing as well as the EC structure during pausing remain unknown. In particular, the molecular mechanisms of interactions of the  $\sigma$ -subunit with the RNAP core enzyme and with the non-template DNA chain in the EC are not sufficiently studied. Additionally, it is not known whether  $\sigma$ -dependent pausing occurs during transcription in other bacteria and if so, whether there are any differences of this process in comparison with *E. coli* RNAP.

It should be noted that most data about RNAP structure was obtained in the study of *T. aquaticus* RNAP, as well as RNAP of the closely related bacterium *Thermus thermophilus*, which makes functional analysis of this RNAP during different stages of transcription very important. *Escherichia coli* and *T. aquaticus* are not evolutionarily related, and their RNAP differ greatly in their sequence, structure [25-28], and transcriptional properties [29, 30]. The major  $\sigma$ -subunits in *E. coli* and *T.*

*aquaticus* ( $\sigma^{70}$  and  $\sigma^A$ , respectively) were found to exhibit significant differences at the stage of transcription initiation. In particular, the  $\sigma^A$ -subunit is not able to melt promoter DNA at the transcription start point at temperatures below 45°C, unlike the  $\sigma^{70}$ -subunit [7, 30, 31]. Thus, comparison of the mechanisms of pause formation by *E. coli* and *T. aquaticus* RNAPs can give important information about the features of transcription regulation mechanisms in different groups of bacteria.

The purpose of this study was to examine the process of *T. aquaticus* RNAP pausing compared with *E. coli* RNAP and to determine how the differences in the structures of the  $\sigma$ -subunits from these bacteria affect the species-specific characteristics of transcription pausing.

## MATERIALS AND METHODS

**Enzymes and DNA.** Core enzymes and RNAP  $\sigma$ -subunits of *E. coli* and *T. aquaticus* were isolated from *E. coli* cells as described previously [30]. Preparation of mosaic  $\sigma$ -subunits is described in [7]. DNA and RNA oligonucleotides were synthesized by Syntol (Moscow). Promoter DNA fragment containing the *lacUV5*-promoter (from position -57 to +58) was obtained by PCR as described previously [16].

**RNAP activity measurement.** The transcription reaction was performed in buffer containing 40 mM Tris-HCl, pH 7.9, 40 mM KCl, and 10 mM MgCl<sub>2</sub>. To obtain the holoenzyme, RNAP core enzyme of *E. coli* (in final concentration of 100 nM) was mixed with a fivefold excess of  $\sigma$ -subunit (500 nM), incubated for 5 min at 37°C, then promoter DNA fragment (10 nM) was added, the mixture was incubated for another 5 min, and the samples were placed on ice for 3 min. The sample was then transferred to the desired temperature, incubated for 5 min, and a mixture of nucleotides (100  $\mu$ M ATP, GTP, CTP, 10  $\mu$ M UTP with addition of  $\alpha$ -[<sup>32</sup>P]UTP) was added. The reaction was stopped after 5 min by addition of an equal volume of stop buffer containing 8 M urea and 50 mM EDTA. RNA products were analyzed by electrophoresis in 15% (29 : 1.5) polyacrylamide gel.

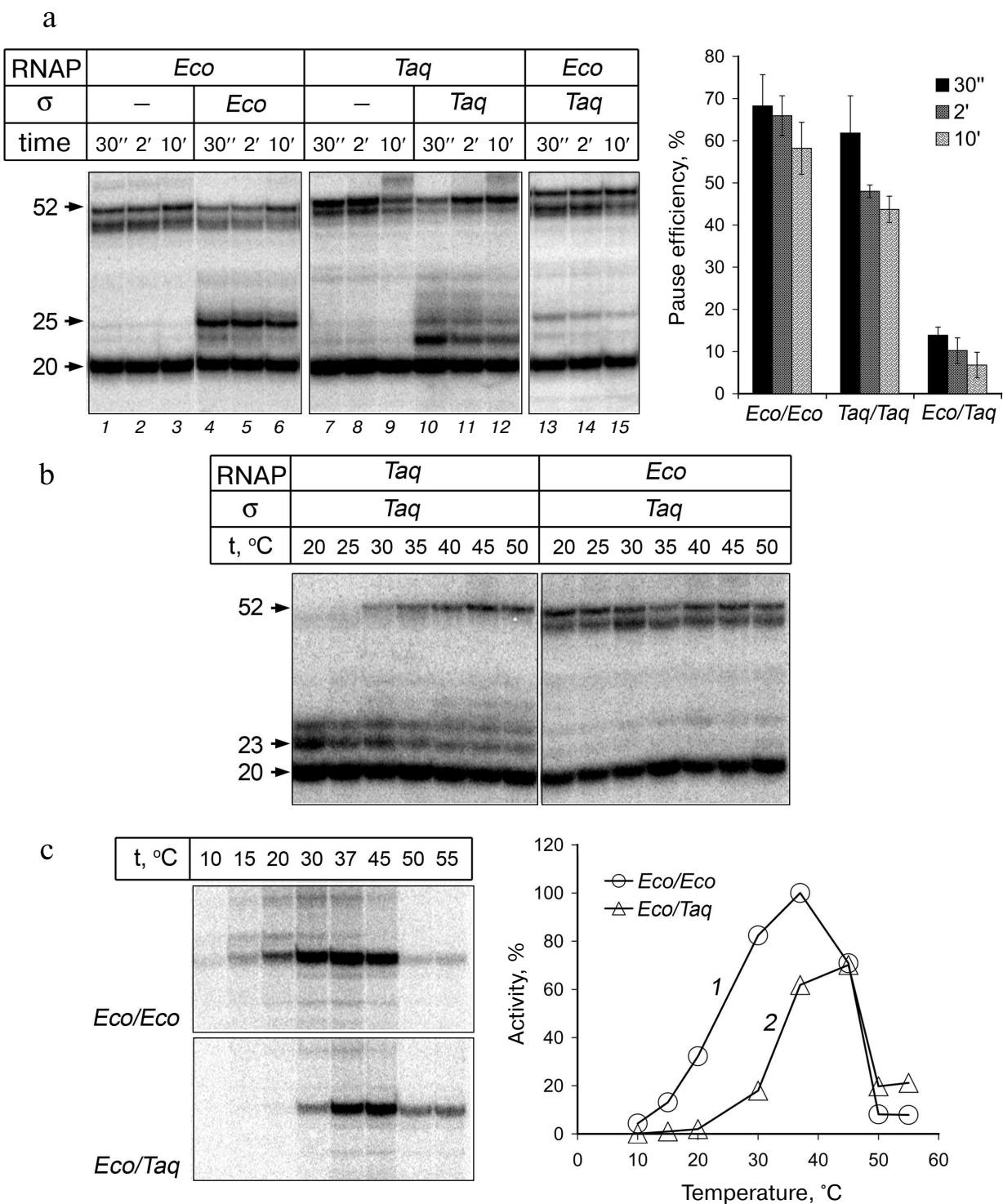
**Analysis of pausing.** Artificial EC was obtained using a modified method [32, 33]. Oligonucleotide sequences used for the complex assembly are shown in Fig. 1c. RNA oligonucleotide was labeled at the 5'-end with T4 polynucleotide-kinase (New England Biolabs, USA) and  $\gamma$ -[<sup>32</sup>P]ATP. Assembly was carried out in buffer containing 40 mM Tris-HCl, pH 7.9, and 40 mM KCl. The oligonucleotide corresponding to the template DNA strand was incubated with RNA oligonucleotide (final concentrations of 2.5  $\mu$ M and 250 nM, respectively) for 3 min at 65°C and cooled for 30 min to 20°C. The mixture was diluted three times with buffer, RNAP core enzyme was added (250 nM), and the mixture was incubated for 20 min at 40°C. The oligonucleotide corresponding to the

non-template DNA strand (1  $\mu$ M) was added to the sample, and the mixture was incubated for 20 min at 40°C and transferred to 20°C. The resulting EC was diluted 10 times with buffer containing 40 mM Tris-HCl, pH 7.9, and 40 mM KCl,  $\sigma$ -subunit was added (final concentration 500 nM), and the mixture was incubated for 5 min at 37°C. Samples were transferred to the desired temperature, incubated for 3 min, then NTP substrates were added to the reaction mixture to the final concentration of 100  $\mu$ M or 1 mM, and MgCl<sub>2</sub> solution was added (10 mM). The reaction was stopped after the required periods of time by addition of an equal volume of stop buffer. RNA products were separated by electrophoresis in 15% polyacrylamide gel and analyzed with a phosphorimager (GE Healthcare). Three or four independent experiments were conducted for each of the studied RNAPs.

## RESULTS

**Formation of  $\sigma$ -dependent transcription pauses by RNAPs from *E. coli* and *T. aquaticus*.** To study the formation of  $\sigma$ -dependent RNAP pauses, we used the method of *in vitro* EC assembly. This approach allows to assemble fully functional EC using synthetic DNA and RNA oligonucleotides and RNAP core enzyme, bypassing the stage of transcription initiation [32, 33]. The scheme of oligonucleotide template used in the experiments is shown in Fig. 1c. Two complementary DNA oligonucleotides (template and non-template) with a length of 60 nt and RNA oligonucleotide with length of 20 nt, complementary to the template DNA oligonucleotide, were used for the EC assembly (see "Materials and Methods"). A -10 element of the promoter was introduced into the template sequence; its position relative to the 3'-end of the RNA corresponded to its position relative to the transcription starting point in the promoter complex (Fig. 1c). We then added to the EC assembled on this template the  $\sigma$ -subunit and nucleotide substrates. The transcription reaction was stopped after different time intervals, RNA products were analyzed by gel electrophoresis. The results of measurements of RNAP transcriptional activity in this experiment are shown in Fig. 2a.

It was shown that the EC of *E. coli* RNAP obtained on this template is transcriptionally active. After addition of nucleotides to the EC obtained in the absence of  $\sigma$ -subunit, RNAP synthesizes an RNA product of 52 nt that corresponds to transcription up to the end of the template (Fig. 2a, lanes 1-3). A fraction of the starting RNA oligonucleotide (50-70% in our experiments) is not extended, likely because the efficiency of EC assembly does not reach 100%, and part of the RNA oligonucleotide is not included in the EC and is not involved in transcription (data not shown). When the reaction was performed in the presence of  $\sigma^{70}$ -subunit we observed a transcription pause that occurs when the RNA reaches a



**Fig. 2.** Formation of transcription pauses by RNAPs from *E. coli* and *T. aquaticus*. a) Electrophoretic analysis of transcription products synthesized on the synthetic template by RNAPs from *E. coli* and *T. aquaticus* in the absence or in the presence of *E. coli* (*Eco*) or *T. aquaticus* (*Taq*)  $\sigma$ -subunits. The position of RNA products of different length is indicated on the left of the figure. The efficiencies of pause formation against time are shown in the plot on the right for RNAPs from *E. coli* (*Eco/Eco*), *T. aquaticus* (*Taq/Taq*), and for the hybrid RNAP consisting of *E. coli* core enzyme and the  $\sigma^A$ -subunit of *T. aquaticus* (*Eco/Taq*). The experiment was performed at 37°C in the presence of 100  $\mu$ M NTP. b) Pausing of RNAPs from *T. aquaticus* and *E. coli* in the presence of the  $\sigma^A$ -subunit at different temperatures. The experiment was performed at 1 mM NTP concentration. c) Activities of *E. coli* RNAP (1) and the hybrid RNAP (2) on the template containing the *lacUV5*-promoter at different temperatures. The plot shows the efficiency of synthesis of full-length RNA product as a percentage of maximum activity of *E. coli* RNAP.

length of 25 nt (lanes 4-6). The effectiveness of pausing is about 70% and gradually decreases over time, as a result of RNAP transition to productive elongation (Fig. 2a, plot on right). High efficiency of  $\sigma$ -dependent pause formation on this template allows using this approach in further studies of the mechanism of pausing.

RNAP of *T. aquaticus* also possesses transcription activity on the synthetic template and is able to synthesize full-length RNA product (Fig. 2a, lanes 7-9). It is worth noting that RNAP from *T. aquaticus* has a higher temperature optimum of activity than RNAP from *E. coli* [29, 30]. However, under the conditions of our experiments, RNAP from *T. aquaticus* can synthesize full-length RNA at 37°C with the same efficiency as the RNAP from *E. coli* (Fig. 2a). When the reaction was performed in the presence of  $\sigma^A$ -subunit, we observed a transcription pause and the efficiency of pausing was comparable to that of RNAP from *E. coli* (Fig. 2a, lanes 10-12 and plot). Thus, RNAP of *T. aquaticus* is also capable of  $\sigma$ -dependent pausing during transcription elongation. It is worth noting that, in contrast to RNAP from *E. coli*, the pause is mainly formed when the length of the RNA is 23 nt and, to a lesser extent, 25 nt. Analysis of the temperature dependence of the reaction showed that the efficiency of the RNAP pause formation in *T. aquaticus* is not changed when the temperature is increased to 50°C, but it rises significantly with temperature decrease and reaches almost 100% at 20°C (Fig. 2b).

**The  $\sigma^A$ -subunit of *T. aquaticus* cannot induce transcription pausing of RNAP from *E. coli*.** It was shown previously that the  $\sigma^A$ -subunit of *T. aquaticus* forms an active holoenzyme in complex with core enzyme of *E. coli* [30, 31, 34]. This allows to directly compare the properties of  $\sigma$ -subunits of *E. coli* and *T. aquaticus* in complex with *E. coli* core enzyme and also to investigate to what extent the characteristics of RNAP pause formation in *T. aquaticus* are associated with structural features of the  $\sigma^A$ -subunit. It is worth noting that the  $\sigma^{70}$ -subunit of *E. coli* is not capable of forming the holoenzyme with the core enzyme of *T. aquaticus*, which makes it impossible to compare properties of these two subunits in the complex with the core enzyme of *T. aquaticus* [29, 34].

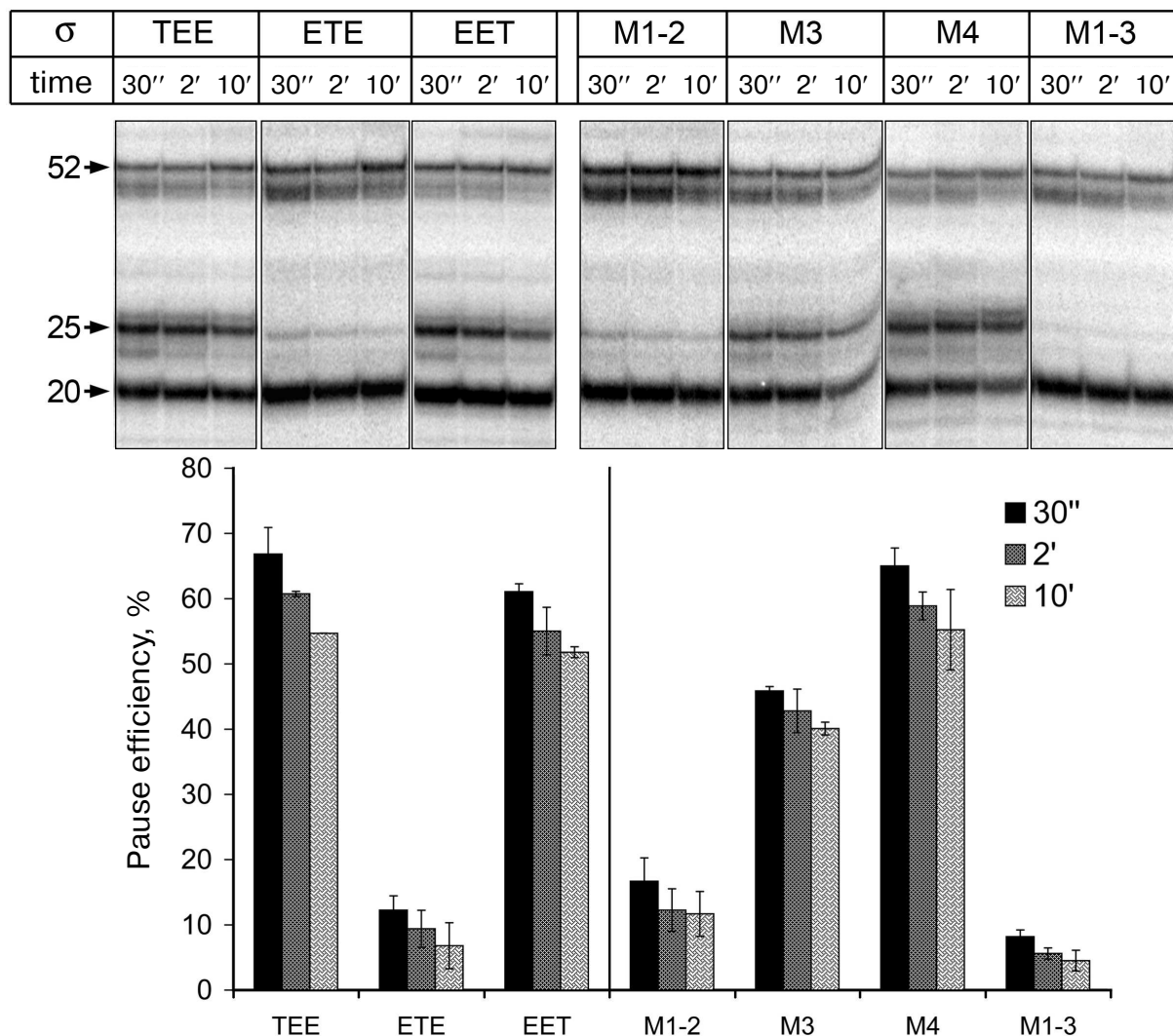
We investigated whether the  $\sigma^A$ -subunit of *T. aquaticus* can interact with EC of *E. coli* RNAP and induce a transcription pause. It was found that pause formation occurs with much lower efficiency ( $\leq 10\%$ ) in the presence of the  $\sigma^A$ -subunit than in the presence of the  $\sigma^{70}$ -subunit (Fig. 2a, lanes 13-15, and plot on right). The efficiency of pause formation does not increase when changing the reaction temperature (in the range from 20 to 50°C; Fig. 2b). The pause occurs mainly in EC containing RNA with length of 25 nt, i.e. in the same template position as in the case of pausing in the EC of *E. coli* RNAP in the presence of the  $\sigma^{70}$ -subunit (Fig. 2a). Thus, the differences in the pause position between RNAPs from *E. coli* and *T. aquaticus* are apparently determined by structural features of the RNAP core enzyme.

To confirm that the  $\sigma^A$ -subunit of *T. aquaticus* is able to stimulate promoter-dependent transcription of *E. coli* RNAP under the conditions of our experiments, we compared the activities of *E. coli* RNAP and the hybrid RNAP containing the *E. coli* core enzyme and the  $\sigma^A$ -subunit in an *in vitro* transcription test using a DNA fragment containing the *lacUV5*-promoter as a template. This promoter is one of the well-studied classical promoters and contains -10 and -35 elements [15]. It was shown that the hybrid RNAP is capable of transcription initiation at the *lacUV5*-promoter. The temperature optimum of promoter-dependent activity of the hybrid RNAP is slightly shifted towards higher temperatures, but its activity at 37-45°C is comparable to the activity of *E. coli* RNAP (Fig. 2c). Thus, the  $\sigma^A$ -subunit of *T. aquaticus* is able to interact with the core enzyme of *E. coli* and to recognize the -10 and -35 promoter elements.

**Effect of substitutions in conserved regions of the  $\sigma^{70}$ -subunit on pause formation.** Reduced efficiency of pause formation by the hybrid RNAP containing core enzyme of *E. coli* and the  $\sigma^A$ -subunit of *T. aquaticus* allowed us to search for regions of the  $\sigma$ -subunit that play a functionally important role in pausing. To locate these regions, we used an approach based on analysis of mosaic  $\sigma$ -subunits comprised of different parts of the *E. coli* and *T. aquaticus*  $\sigma$ -subunits. Since the recognition of the pause signal (the -10 element) involves conserved region 2 of the  $\sigma$ -subunit, the *E. coli* and *T. aquaticus* sequences were connected at the boundaries of this region. In total, we prepared and studied three mosaic  $\sigma$ -subunits based on the *E. coli*  $\sigma^{70}$ -subunit, in which some parts were replaced with the corresponding sequences of the  $\sigma^A$ -subunit of *T. aquaticus* (Fig. 3a): 1)  $\sigma$ TEE,  $\sigma$ -subunit with the replacement of conserved region 1 and a nonconserved segment between regions 1 and 2 (residues 1-386 of *E. coli* replaced by residues 1-209 of *T. aquaticus*; the difference in the lengths of fragments is explained by the different length of the nonconserved segments in these  $\sigma$ -subunits); 2)  $\sigma$ ETE,  $\sigma$ -subunit with the replacement of conserved region 2 (residues 387-455 of *E. coli* replaced by residues 210-278 of *T. aquaticus*); 3)  $\sigma$ EET,  $\sigma$ -subunit with the replacement of conserved regions 3 and 4 (residues 456-613 replaced by residues 279-438).

It was shown previously that all three mosaic  $\sigma$ -subunits form active holoenzymes with core enzyme of *E. coli* [7]. Mosaic subunits were expressed in *E. coli* cells and obtained in purified form. Analysis of *E. coli* RNAP transcription on a synthetic template containing the pause signal in the presence of these subunits showed that the replacement of regions 1, 3 and 4 in the  $\sigma$ -subunits  $\sigma$ TEE and  $\sigma$ EET had practically no effect on the pause formation. However, the replacement of region 2 in the  $\sigma$ -subunit  $\sigma$ ETE reduced the efficiency of the pause formation to the same level as observed in the case of the  $\sigma^A$ -subunit of *T. aquaticus* (Fig. 4). Thus, the low efficiency of *E. coli* RNAP transcription pausing with  $\sigma^A$ -subunit of





**Fig. 4.** Transcription pausing by *E. coli* RNAP in the presence of mosaic  $\sigma$ -subunits. The experiment was performed at 37°C in the presence of 100  $\mu$ M NTP substrates.

and by RNAP interaction with additional regulatory factors [35-37]. The  $\sigma^{70}$ -dependent pauses were detected in initially-transcribed regions of lambdoid bacteriophage late genes, as well as in many genes of *E. coli* [14, 18, 38]. Although the functional role of these pauses is largely unknown, it is assumed that they can change the properties of EC and affect its interaction with transcription factors [38]. In this study we compared the formation of  $\sigma$ -dependent RNAP pauses of *E. coli* and *T. aquaticus* and investigated how substitutions of evolutionary nonconserved amino acids in different parts of the  $\sigma$ -subunit, including the regions involved in interactions with DNA and RNAP in the elongation complex, influence pausing.

It is worth noting that the majority of studies of  $\sigma$ -dependent pauses have been devoted to analysis of *E. coli* RNAP. At the same time, RNAP of *T. aquaticus* is one of the main models for structural studies of molecular mechanisms of transcription, since for this RNAP the

high-resolution three-dimensional structure was first deciphered [22, 39]. This allows using the results of studies of transcription pausing by *T. aquaticus* RNAP for structural modeling of the pausing process. Our studies have shown that RNAP of *T. aquaticus* is capable of forming  $\sigma^A$ -dependent transcription pauses, and the pause formation efficiency is comparable with that of *E. coli* RNAP at temperatures  $\geq 37^\circ\text{C}$ . As the temperature decreases, the efficiency of *T. aquaticus* RNAP pausing increases dramatically. This is likely to be explained by a significantly lower rate of transcription elongation by RNAP of *T. aquaticus* at low temperatures [30, 40], which stimulates transcription stop at the pause site.

The pause occurs earlier in the case of *T. aquaticus* RNAP than in the case of RNAP from *E. coli*: preferential position of pause is shifted two nucleotides upstream on the template DNA. Analysis of *E. coli* RNAP pausing in the presence of  $\sigma^A$ -subunit from *T. aquaticus* showed

that such a shift in the pause position is probably caused by the properties of RNAP core enzyme. We can propose that the position of the pause is affected by the differences in contacts between core enzymes of *E. coli* and *T. aquaticus* and transcribed DNA template. The stressed complex formed during transcription at the pause site is probably less stable in the case of *T. aquaticus* RNAP, which leads to an earlier stop of transcription.

We found that the  $\sigma^A$ -subunit of *T. aquaticus* is not able to induce transcriptional pausing of RNAP from *E. coli* with high efficiency, at neither low nor high temperatures. However, a hybrid holoenzyme that consists of *E. coli* core enzyme and the  $\sigma^A$ -subunit possesses normal transcriptional activity comparable to that of the holoenzyme of *E. coli*. The most likely explanation for this fact is that the  $\sigma^A$ -subunit cannot efficiently bind to the EC of *E. coli* RNAP.

We analyzed mosaic subunits containing various conserved regions from *E. coli* and *T. aquaticus* to find differences in the regions of the  $\sigma$ -subunits that affect pause formation. It was found that the inability of  $\sigma^A$ -subunit to induce a pause in the transcription by RNAP of *E. coli* is caused by structural features of subunit region 2. This region plays the main role in interactions of  $\sigma$ -subunit with core enzyme of RNAP and  $-10$  element during transcription initiation. In particular, the substitutions of amino acid residues in subregions 2.1 and 2.2 (V387A, L402F, D403A, Q406A, E407A, N409D, M413T) disrupt the interaction of  $\sigma^{70}$  with RNAP core enzyme [5], and substitutions of the conserved aromatic and positively charged residues in subregions 2.3 and 2.4 affect the interaction of RNAP holoenzyme with promoters and increase the temperature of transcription initiation (Fig. 3b) [8-10, 12, 41].

The influence of nonconserved amino acid substitutions in region 2 of the  $\sigma^{70}$ -subunit on transcription pausing could be explained both by a change in its interactions with the RNAP core enzyme and by a change in contacts with the non-template DNA chain in the transcribed region. The study of substitutions in different parts of the  $\sigma^{70}$ -subunit region 2 showed that the formation of transcription pauses is most greatly influenced by substitutions in subregions 2.1 and 2.2; the introduction of corresponding substitutions from the  $\sigma^A$ -subunit to the  $\sigma^{70}$ -subunit leads to a significant decrease in the efficiency of pause formation. The effect of substitutions in subregions 2.1 and 2.2 on pause formation is most probably directly caused by their influence on the interaction of the  $\sigma$ -subunit with the RNAP core enzyme. The amino acid substitution G424R in subregion 2.3 also leads to some decrease in pausing efficiency, and a combination of substitutions in subregions 2.1, 2.2, and 2.3 has the most substantial effect. G424R substitution is located in a protein loop that apparently does not interact with DNA and RNAP core enzyme; this substitution may affect the conformational changes of the  $\sigma$ -subunit that occur during its interactions with the core enzyme and DNA [7]. It was

shown previously that conserved amino acid substitutions in subregion 2.2 of  $\sigma^{70}$  (L402F, E407K, N409D; Fig. 3b) disrupt pausing, probably due to changes in interactions with the RNAP core enzyme [19, 21]. Our results indicate an important role of nonconserved amino acid residues in interactions with RNAP core enzyme in the process of transcription elongation.

It was shown previously that the replacement of non-conserved amino acid residues in region 2 of  $\sigma^{70}$  for corresponding residues of the  $\sigma^A$ -subunit of *T. aquaticus* increases promoter melting temperature compared with the wild-type  $\sigma^{70}$ -subunit [7]. As in the case of  $\sigma$ -dependent transcription pausing, nonconserved amino acid substitution in subregions 2.1 and 2.2 had the greatest effect on promoter recognition. Nevertheless, all the studied mutant variants of the  $\sigma^{70}$ -subunit as well as the  $\sigma^A$ -subunit of *T. aquaticus* can form active holoenzyme with core enzyme of *E. coli* and are capable of promoter-dependent transcription initiation (reference [7] and this work). Thus, the substitutions in region 2 have a stronger impact on the interaction of  $\sigma$ -subunit with transcription complex during elongation than on the recognition and melting of promoters during initiation. This is probably due to the fact that the recognition of promoters and the interaction with the RNAP core enzyme in the promoter complex involves additional regions of  $\sigma$ -subunit (including regions 3 and 4), whereas the main role in the interaction of the  $\sigma$ -subunit with EC is played by region 2.

Our studies allowed us to identify  $\sigma$ -subunit regions involved in pause formation and revealed the role of non-conserved amino acid region 2 in this process. Thus, the study of mosaic subunits is a promising approach for future studies of transcription mechanisms in different organisms. A similar approach can be used to identify functionally important regions of RNAP and to identify evolutionary changes that alter the functional properties of the protein and have adaptive value.

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