ISSN 0006-3509, Biophysics, 2012, Vol. 57, No. 4, pp. 428–441. © Pleiades Publishing, Inc., 2012.
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MOLECULAR BIOPHYSICS

Study of Elongation Complexes for T7 RNA Polymerase

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Received December 4, 2007; in final form, June 1, 2011

Abstract—Complexes of bacteriophage T7 RNA polymerase with a DNA template for transcription elongation were visualized by atomic force microscopy. Images for complexes of T7 RNA polymerase with terminal fragments of DNA template were obtained for single molecules. Complexes of a single DNA template molecule with several T7 RNA polymerase molecules corresponding to stages of initiation, elongation and termination of transcription were visualized under the elimination of unspecific DNA-protein binding. Immobilized on the amino mica RNA transcripts form rod-like condensed structures. Detailes of specific and unspecific complex formation for the T7 RNA polymerase–DNA system during initiation and transcription elongation are discussed.

Keywords: atomic force microscopy, transcription, T7 RNA polymerase, RNA transcript

DOI: 10.1134/S0006350912040112

INTRODUCTION

Transcription of genetic information coded by DNA is considered as a sequence of three steps—initiation, elongation and termination. In prokaryotes and eukaryotes RNA is synthesized with the aid of DNA-dependent RNA polymerases. RNA polymerase (RNAP), presenting in itself a complicated molecular machine, is responsible for binding with promoter, melting of DNA, initiation of transcription, elongation of the RNA strand and termination of transcription. In the process of transcription RNAP forms a series of complexes during scanning the DNA template. First there forms an unstable initiation complex, in consequence of which short products are synthesized before promoter clearance, and then there forms a stable elongation complex, which consists of RNAP, DNA template and growing RNA transcript [1]. With the aid of biochemical analysis and studying the kinetics of interaction of mutant RNAP with DNA template it has been shown that upon transition of the initiation complex to the elongation complex there form at least three types of transition complexes. The first complex encompasses about three–five nucleotides (nt), the second ~6–8 nt, the third ~9–14 nt [2, 3].

RNAPs can be divided into two classes: multisubunit (bacteria, eukaryotes) and monosubunit (some bacteriophages, mitochondria, chloroplasts). And although they are not characterized by structural homology and sequence similarity, for RNAPs of both classes the main steps of transcription proceed identically [4]. Monosubunit RNAPs recognize promoters without accessory molecules, while multisubunit RNAPs for binding with the promoter need additional proteins.

Transcription is controlled by the polymerase during of elongation immediately to pause or termination. Upon reaching specific positions on the DNA template the stable elongation complex dissociates in the process of transcription termination. It has been supposed that dissociation is not caused immediately by termination, but is preceded by inactivation of the elongation complex [5]. For T7 RNAP, the most studied polymerase among the class of RNAP consisting of single subunits, two types of termination or pause signals were found. Signals of I class consist of a U-rich element immediately below the sequence forming a GC-rich hairpin. Termination at sites of I class depends on the possibility of formation by the RNA molecule of secondary structure and takes place even on condition of removal of the non-template chain of DNA [3]. The other terminator of T7 RNAP, related to class II, consists of a conserved sequence of 8 nt in length. As a rule, pause or termination occur at a site that is localized lower by 7–8 nt from the indicated element. However the details of both initiation of transcription and its termination are quite difficult to clarify using traditional methods for solutions, inasmuch

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as, firstly, initiation consists of several short-term intermediate steps between promoter binding and elongation. Secondly, at each concrete time moment only a small part of RNAP molecules from the set of RNAP molecules actively participating in transcription is linked with initiation [6]. In the majority of biochemical investigations of transcription initiation they use such experimental conditions that allow studying only a fixed position of the enzyme, at which RNAP stops on a known position of the DNA template in view of the absence of a complementary nucleotide. Attempts with the aim of synchronization of transcription initiation for a fraction of RNAP molecules proved unsuccessful, inasmuch as the synchronization quickly disappeared in view of that transitions between intermediate steps bore a probabilistic character.

Relatively recently another approach has been developed to studying the transcription initiation, based on investigation of the binding with promoter, initiation and elongation for a single RNAP molecule in real time. In this method they used a DNA molecule connected by both ends with two balls that resided in optical traps. At the same time the DNA molecule resided near the surface on which there were immobilized molecules of T7 RNAP. Controlling the optical trap by means of oscillation of the ball to which by one end the DNA molecule was attached, in work [6] they observed association and dissociation of the complex of T7 RNAP with DNA promoter with a constant $K = 2.9\ s^{-1}$, transition to elongation with $K = 0.36\ s^{-1}$, synthesis with a rate of 43 nt per second and yield of an RNA transcript of length ~ 1200 nt. The authors have shown that the transition from initiation to elongation appears more prolonged as compared with the time of existence of the binary complex T7 RNAP–promoter of DNA.

Numerous investigations with the aid of X-ray analysis, DNA footprinting, fluorescence resonance spectroscopy have essentially improved our notions relative to the elongation complex of RNAP with DNA and RNA transcript [7, 8]. It is known that the growing RNA transcript is squeezed out during elongation through the outlet channel of RNAP, while contacts between the RNA transcript and RNAP are realized for 14–16 nt from the 3'-end. Obtaining new data touching on the structure of promoter, elongation complex, has become possible with the development of the microscopic technique of high resolution.

X-ray analysis, electron microscopy, scanning probe microscopy (in particular, atomic force microscopy, AFM) have allowed obtaining important information about the spatial connection between DNA, RNA and RNAP during transcription elongation, namely about the global topology of the open promoter and elongation complex. Proceeding from experimental investigations of transcription of eukaryotes and prokaryotes, for the elongation complex a model of DNA reeling has been proposed [9, 10]. In the elongation complex the reeling of DNA onto RNAP and formation of an RNA–DNA hybrid cause opening of the DNA double helix and formation of a transcription loop. However there remain quite a number of unsolved problems touching on the issue in what way RNAP transcribes long DNA fragments. Especially many suppositions have been put forth about the movement of RNAP along DNA in vivo, namely: whether RNAP rotates about DNA or the enzyme resides in such a rotationally stressed state that DNA must rotate about the helical axis so that the DNA strand passes through the protein. Also an intriguing problem remains the visualization of growing RNA molecules in the process of elongation.

Let us note that AFM visualization with high resoling power permits immediately studying biological structures in conditions approaching physiological ones, and also in the absence of crystallization, as distinct from X-ray analysis. Apart of that, in distinction from traditional microscopic methods, which provide averaged information for a set of molecules, with the aid of AFM it is possible to obtain data on the structure and functioning of single molecules. An additional advantage of AFM as compared with classical electron microscopy presents as the absence of the procedure of contrasting the specimen. For studying the topography of the surface of biomacromolecules with the aid of AFM it is only necessary that the molecule be adsorbed on a substrate. Therefore quite a number of works were devoted to AFM visualization of complexes of proteins, among them RNAPs, with DNA molecules [9, 11, 12].

In the given work conditions are optimized for conducting transcription in vitro for subsequent AFM visualization of a complex that is formed by monosubunit RNAP of bacteriophage T7 with linear DNA containing a promoter and region of termination of T7 RNAP transcription. Visualized are both specific (formed by T7 RNAP molecule with terminal fragments of DNA template) and highly specific complexes (first of all with promoter) that are formed by T7 RNAP with single molecules of DNA template. Apart of that, presented are the results of immediate visualization of RNA transcripts having formed after elongation of transcription: RNA transcripts on the surface of mica (substrate for AFM) form bunch-like condensed structures.

EXPERIMENTAL

Conduction of transcription. In the quality of template for transcription we use an amplicon of 1414 base pairs (bp) in length, containing promoter A1 (one of the 17 known promoters, which is contained by the genome of bacteriophage T7) and the region of termination of T7 RNAP transcription (Fig. 1). The DNA template was obtained by means of enzymatic treatment with restriction endonuclease *ScaI* (New England Biolabs, England) of supercoiled DNA pGE-

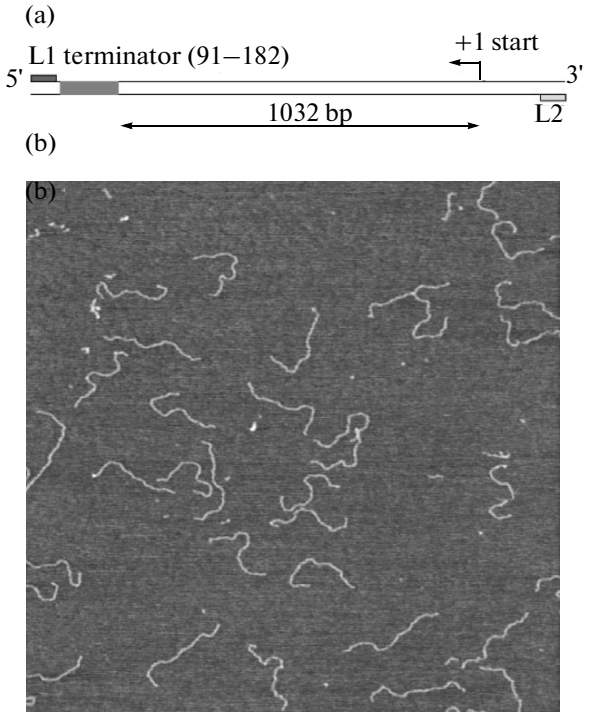

Fig. 1. Schematic representation of the template of linear DNA that was used for conducting transcription (a) and its AFM image (b). Primers L1 and L2, shown by rectangles, flank a fragment of pGEMEX DNA 1414 bp in length, which contains a promoter and a terminator of T7 RNAP transcription. For visualization the pGEMEX DNA was immobilized on freshly cleaved mica in HEPES buffer containing 2.5 mM $MgCl_2$. Frame size – 2.23 μm × 2.23 μm.

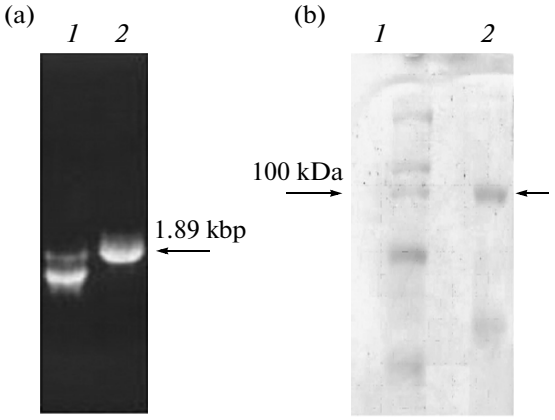

Fig. 2. (a) Analysis of RNA transcripts after conducting transcription with T7 RNAP on a template of a fragment of linear DNA pGEMEX with length of 1414 bp in 1.8% agarose gel with formaldehyde and control plasmid pTRI-Xef with length of 1890 bp. Expected length of transcripts – 1122 nt. (b) Analysis of T7 RNAP with the aid of electrophoresis in 12.5% PAG-SDS: *1*—molecular mass marker; *2*—T7 RNAP (Ambion, USA). Arrow shows the band that corresponds to the main fraction of protein with molecular mass ~99 kDa. Gel stained with Coomassie blue.

MEX DNA (Promega, USA) of 3993 bp in length, with subsequent amplification and purification of the amplicon. The reaction of transcription was conducted according to the protocols presented below with the use of kits for transcription with the aid of T7 RNAP (Promega, USA), MegaScript T7 (Ambion, USA) and a kit from New England Biolabs (England) at different temperature and temporal parameters.

At that we used three buffers for transcription—buffer A (Promega, USA), buffer B (Ambion, USA) and buffer C [9]. Buffer A contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 10 mM dithiotritol, 2 mM spermidine, 0.05% Tween 20, 40 un. RNasin, 20 un. RNAP in a total volume of 20 μL. The reaction of transcription was initiated by addition of nucleotides (CTP, GTP, UTP and ATP) to a final concentration of 200 μM.

Buffer B contained the reaction mix for transcription (Ambion, USA), 40 un. RNasin, 20 un. RNAP, 20 μM CTP, GTP, UTP and ATP in a total volume of 20 μL. After 20–65 min incubation at a temperature of 31°C the reaction was stopped by heating for 15 min at a temperature of 70°C.

Buffer C contained 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiotritol, 40 un. RNasin, 20 un. RNAP in a total volume of 20 μL. The reaction of transcription was initiated by addition of nucleotides to a final concentration of 100 μM CTP, GTP, UTP and ATP. After 65 min incubation at a temperature of 31°C the reaction was stopped by rapid cooling to 0°C.

For removal of DNA template and degradation of DNA that may contaminate the RNA preparation, after conducting the transcription to the reaction mix we added 1 μL of DNase I free of RNases (Ambion, USA) and incubated for 15 min at a temperature of 37°C. For inactivation of DNase the reaction mix was incubated at a temperature of 70°C for 10 min. For control of transcription effectiveness we conducted electrophoresis in 1.2% agarose gel containing 1.8% formaldehyde (Fig. 2a). Transcription was conducted in a special room destined for work with RNA, with the use of ultrapure water, to which we added DEPC for inhibition of RNases. For conducting electrophoresis in denaturing conditions to the reaction mix we added an equal volume of buffer for sample layering, which contained 90% formamide, 0.01% xylene cyanole, 0.01% bromphenol blue, 10 mM EDTA and 0.01% sodium dodecylsulfate. For estimation of the size of RNA transcripts forming after conducting transcription on the DNA template pGEMEX, we used a control DNA template pTRIXef from the kit for conducting transcription MegaScript T7 (Ambion, USA). During conducting transcription in accordance to the conditions of the manufacturer there formed only full-sized RNA transcripts with length of 1890 nt, for estimation of the size of which, in turn, we used RNA markers G 319A (Promega, USA).

Preparations of T7 RNAP were controlled with the aid of conducting electrophoresis in 12.5% PAG in the presence of sodium dodecylsulfate (Fig. 2b) and used without additional purification. For enzymes T7 RNAP (Promega) and T7 RNAP (New England Biolabs), characteristic was the presence of one band on the electrophoregram, while for T7 RNAP (Ambion) we found two bands. Therefore in the work we prevalently used T7 RNAP (Promega).

Atomic force microscopy. In the study we used an atomic force microscope Nanoscope IV MultiMode System (Veeco Instruments Inc., USA) with an E-scanner, which provides a maximal scanning range up to 12 μm. The AFM images of DNA were recorded with the aid of a vibrating variant of AFM in air in the "height" regime with the use of OMCLAC160TS cantilevers (Olympus Optical Co., Japan) with resonance frequency 340–360 kHz and stiffness constant 42 N/m. Images were obtained in the format of 512 × 512 pixels, smoothed and analyzed with the aid of Nanoscope software (version 5.12r3) (Veeco Instruments Inc., USA).

Preparation of DNA samples for PCR and T7 RNAP–DNA complexes for AFM. For conducting a polymerase chain reaction (PCR) we have constructed primers L1 and L2 delimiting the pGEMEX DNA fragment that contained the promoter and termination region of T7 RNAP transcription. Primers L1 and L2, the sequences of which with corresponding positions on circular DNA of pGEMEX are presented below, were obtained from Sigma (Japan):

5'-cgc tta caa ttt cca ttc gcc att c-3'– direct primer L1 (3748–3772)

5'-ctg att ctg tgg ata acc gta tta ccg-3'– reverse primer L2 (1168–1142).

PCR with a hot start was conducted in a reaction mix volume of 50 μL on an amplifier GeneAmp 9700 (Perkin Elmer, USA) at the following temperature and temporal parameters: initial incubation – 95°C, 2 min; denaturation – 95°C, 1 min; annealing – 71°C, 1 min; synthesis – 74°C, 1 min; number of cycles – 35. The annealing temperature was determined theoretically with the aid of program Oligo [13].

For visualization of amplicons 15 μL of PCR product was resolved with the aid of electrophoresis in 2% agarose gel with subsequent staining with ethidium bromide.

For purification of the amplified DNA fragment we use the following procedure. After conducting electrophoresis we excised the gel stripe containing the amplicon, irradiating the gel with a long-wavelength UV source of radiation of low intensity (BioRad, USA). For further purification of the amplicon from nucleotides, primers, DNA polymerase and ethidium bromide we used the QIAquick PCR purification kit (QIAgen, Japan) according to manufacturer's recommendations, and also extraction with phenol/chloroform with subsequent reprecipitation with ethanol.

For conducting PCR we used a thermostable DNA polymerase of high fidelity of two kinds—Pyrobest DNA polymerase (TaKaRa Co., Japan) and Invitrogen Platinum DNA polymerase (Invitrogen, Japan).

For immobilization of T7 RNAP–DNA complexes onto freshly cleaved mica we used 10 mM HEPES buffer, which contained 2.5 mM $MgCl_2$, or buffer in which we conducted transcription, inasmuch as it also contained magnesium cations necessary for binding negatively charged DNA molecules with the negatively charged surface of freshly cleaved mica. Onto a strip of mica sized 1 cm^2 we applied a drop of solution of complex of volume 10 μL with a molar relationship of concentrations of T7 RNAP and DNA varying in the range 1–100, in TE buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA), washed after a 2-min exposure with ultrapure water free of RNases, blew with a flow of nitrogen and at once conducted the visualization.

The concentration of T7 RNAP in the initial solution was determined by a spectrophotometric method with the use of a control set of reagents for determination of the concentration of bovine serum albumin (BCA Protein Assay Reagent, Pierce, USA). From the constructed calibration plot of the dependence of optical density on the known concentration of albumin for three values of albcumin concentration we determined the concentration of an intermediate solution of T7 RNAP, proceeding from the measured value of absorption. The concentration of the initial solution of T7 RNAP (Promega) constituted $6.6 \cdot 10^{-6}$ M, while the concentration of linear DNA pGEMEX, which was used in the quality of template for transcription, – 200–800 pM (amplicon) in AFM visualization. For its determination we conducted measurements of optical density and, consequently, concentration of the initial solution of purified PCR product. Complexes were prepared by addition of T7 RNAP solution to an equal-volume solution of DNA template, which was prepared by the method of consecutive dilutions of the initial solution of the amplicon. For visualization of the T7 RNAP–DNA complex the ratio of molar concentrations of polymerase and DNA was varied from 1 to 100. The corresponding solutions of T7 RNAP were prepared by the method of consecutive dilutions of the initial solution.

RESULTS AND DISCUSSION

The effectiveness of transcription is influenced by several factors—temperature and temporal parameters of reaction, concentration of components, ionic strength of solution. Therefore in the given work we visualized the T7 RNAP–DNA complexes after conducting transcription at different temperature and temporal parameters, and also with the use of several buffers for transcription, distinguished by composition and concentration of components.

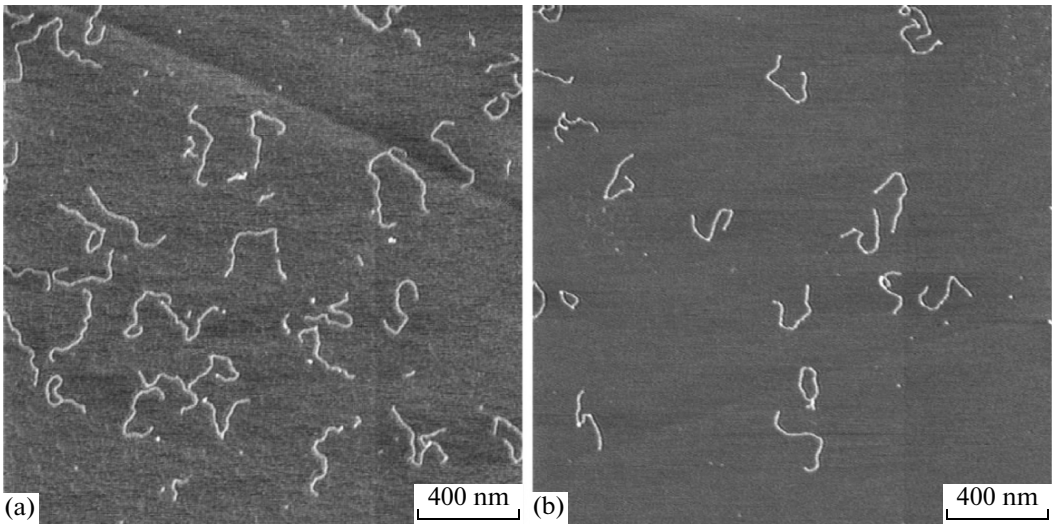

Fig. 3. AFM image: (a) of molecules of linear pGEMEX DNA with length of 1414 bp (after conducting PCR with DNA polymerase Invitrogen Platinum), which was used in the quality of template for transcription, immobilized on freshly cleaved mica in HEPES buffer containing 2.5 mM $MgCl_2$; (b) of complex T7 RNAP–DNA in buffer A for transcription (without conducting transcription). Frame size: (a) 2.08 μm × 2.08 μm; (b) 2 μm × 2 μm.

It is known that lowering the temperature of incubation during conducting transcription from $T = 37$ to $T = 30$°C leads to an increase in the quantity of full-sized RNA transcripts. At the same time a rather high concentration of NaCl (>30 mM) may lead to a decline in the number of RNA transcripts in view of the possible precipitation of the DNA template [14]. Let us note that the ionic strength (I) of the used buffer solutions for transcription was equal to 70–80 mM Na^+.

For investigation of the structure of T7 RNAP–DNA complexes by means of AFM we used immobilization of biomolecules onto freshly cleaved mica with addition of magnesium ions. That as a result of transcription the RNA transcripts of expected length are synthesized is evidenced by the presence of the corresponding band on the electrophoregram after conducting electrophoresis in denaturing conditions of the products of transcription with T7 RNAP (Fig. 2). Controls were AFM images of the molecules of DNA template adsorbed on mica (Fig. 1b, Fig. 3a), which had nonextended shape, smoothed contours, their fragments uniformly immobilized on the substrate surface. The presence on the electrophoregram of two bands testifies, in our opinion, to synthesis of RNA transcripts of length 1122 and 1032 nt (band of higher intensity), corresponding to transcription products with the termination region and without the transcription termination region. Despite that the reaction mix was not a synchronized one, it is seen that after transcription there form only full-sized RNA transcripts and shorter transcripts after stoppage at the termination site.

After addition of T7 RNAP to the transcription mix with DNA template in the process of transcription there form elongation complexes, which are characterized by typical bends for DNA–protein complexes [9, 15, 16]. In Fig. 4 and Fig. 5 we present AFM images of a fragment of linearized DNA pGEMEX after transcription with T7 RNAP at different temperature and temporal conditions at a significant excess of T7 RNAP molecules. Attracting attention is that for molecules of DNA in complex with T7 RNAP inherent are the presence of the noted bends, which are widely discussed in literature, and formation of peculiar loops after conducting transcription at room temperature (Fig. 4a, Fig. 5a). At the same time in the absence of T7 RNAP molecules the bends and loops in linear molecules of DNA pGEMEX on AFM images do not reveal themselves (Fig. 1a).

In general, formation of bends of a DNA molecule, on the one hand, is caused by complex formation with proteins, and on the other one, bends reveal themselves during visualization of DNA–protein complexes adsorbed on mica surface. This effect may be explained by a change in the density of the surface charge of the DNA molecule upon interaction with protein. Adsorption of DNA molecule onto the surface of uniformly charged mica takes place mainly at the expense of electrostatic interaction of uniformly negatively charged sites of DNA with positively charged surface groups of mica. As a result of such interaction of two uniformly charged surfaces (DNA and substrate) the DNA molecules uniformly immobilize on the substrate surface. This signifies that there exists a definite (empirically established) relationship between the surface properties of DNA and the surface properties of the substrate on which the DNA molecules are immobilized.

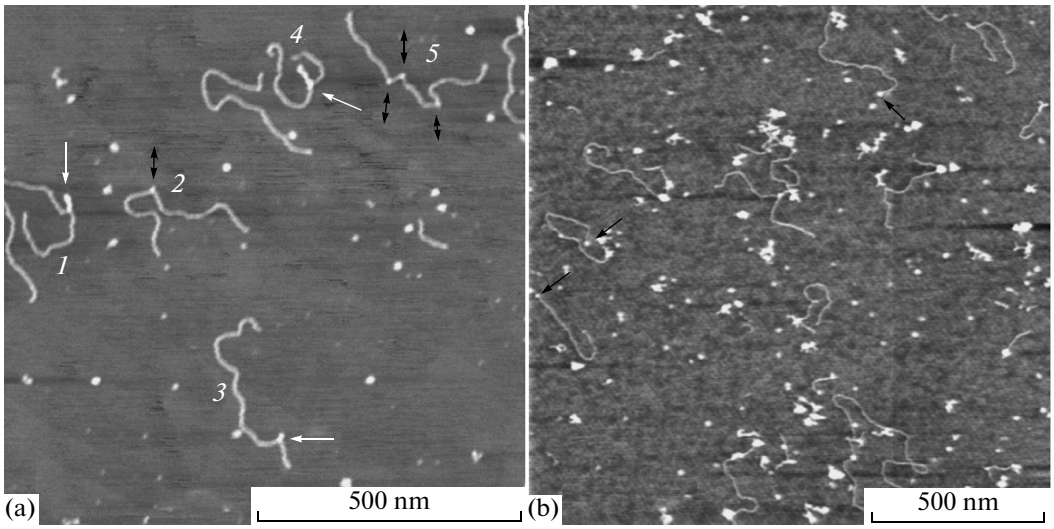

Fig. 4. AFM image a fragment of linear pGEMEX DNA with length of 1414 bp used in the quality of template for transcription, and T7 RNAP (which appear as white dots) after transcription in the course of 60 min in buffer A for transcription at room temperature (analogously to conditions of [8]) (a) and in the course of 65 min at $T = 31°C$ in buffer C for transcription (b). (a) Shown with white arrows are involute fragments of DNA template that have arisen as a result of complex formation with molecules of T7 RNAP, while with black – molecules of T7 RNAP having formed complexes in the middle of DNA template. (b) Shown with back arrows are T7 RNAP molecules that have formed a complex with the ends of DNA template. Frame size: (a) 1 μm × 1 μm; (b) 1.48 × 1.48 μm. Relationship of molecules of T7 RNAP and DNA constitutes 20.

An important peculiarity of elongation complexes forming in transcription (DNA–RNAP–RNA transcript) appears that after ending the transcription the elongation complex quickly dissociates. At that the rate of dissociation for bacterial T7 RNAP is significantly higher as compared with eukaryotic polymerases. The indicated fact substantially complicates visualization of both the whole elongation complex and of separate RNA transcripts. Therefore for AFM visualization of the elongation complex use is often made of DNA templates the sequence of nucleotides of which is synthesized in such a way that transcription stops in definite positions [9].

In the given work we applied a different approach for preventing rapid dissociation of the elongation complex, namely: we used a DNA template containing at a distance of 1122 nt from the transcription initiation site a region of termination of T7 RNAP transcription. The effectiveness of transcription termination can be regulated: it depends on the concentration of reaction mix components, ionic strength and pH of buffer for transcription and may reach 70–80%. The DNA template for transcription is constructed in such a way that it contained a promoter and termination region of T7 RNAP transcription, which are asymmetrically localized at the ends of an amplicon of 1414 bp in length. Thus the promoter of transcription is situated in position 1212–1231 bp (i.e. at a distance of 200 bp from the 3'-end of the template strand of DNA), while an internal terminator of T7 RNAP transcription – in position 91–182 bp (i.e. at a distance of 90 bp from the 5'-end of the template strand of DNA) (Fig. 1). Inasmuch as in the accessible literature we have not found data relative to the indicated termination region, which is cloned in pGEMEX vector, a question arose of detecting termination signals for T7 RNAP by analysis of the sequence of the transcription terminator. With the aid of the package of applied programs GeneBee [17] we have revealed a hairpin structure with a stem length (with one unpaired nucleotide) 13 bp, loop sized 3 nt and free energy of hairpin formation $\Delta G = -16.4$ kcal/mol.

In the quality of additional control we used an AFM image of the molecule of DNA template, or amplicon, immobilized on freshly cleaved mica in HEPES buffer, and also an AFM image of DNA–T7 RNAP complex with conducting transcription (Fig. 3b). An important peculiarity of AFM is that from AFM images with the aid of software one can immediately measure the contour length of DNA molecules with a sufficiently high resolution. By the way, one of the first applications of AFM was just the measurement of DNA contour length [18]. From the plotted graph of Gaussian distribution of the contour length of amplicons [19], measured immediately from the AFM image, we have determined the contour length of the DNA template. The obtained value of contour length of the investigated amplicons – 435 ± 15 nm – is smaller than the theoretical value of the length of DNA molecules in B-form approximately by 10%. For the DNA template having formed a complex with T7 RNAP in buffer A for transcription (without conducting transcription, Fig. 3b), the contour length that we determined in the same way from the Gaussian

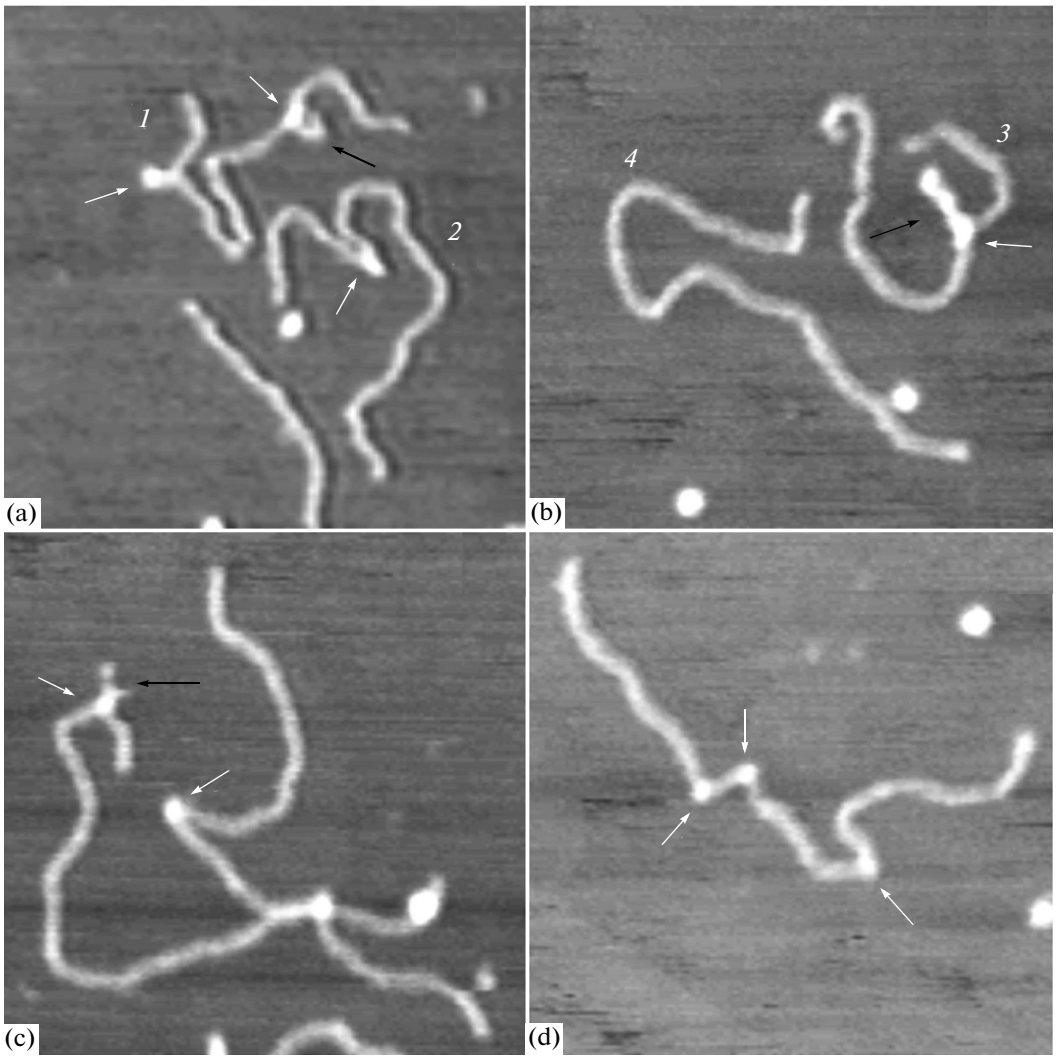

Fig. 5. AFM image with high resolution of complexes of T7 RNAP and DNA. Molecules T7 RNAP appear as spheres of diameter about 8 nm. White arrows point to complexes formed by molecules of T7 RNAP and DNA, while black ones – to RNA transcript (a, c) and involute fragment of DNA template (b). Transcription was conducted in buffer A at room temperature for 60 min, after which the reaction mix was instantly used for AFM visualization. Frame size: (a) 348 nm × 348 nm; (b) 306 nm × 306 nm; (c) 298 nm × 298 nm; (d) 299 nm × 299 nm. The contour length of molecules constitutes: (a) 445–450 nm, (b) 454–457 nm.

distribution constituted 386 ± 13 nm. Such a substantial reduction of DNA contour length may be explained by several causes. Firstly, the availability in the composition of the transcription buffer along with magnesium ions of spermidine ions essentially changes the surface properties of mica on which DNA molecules are immobilized, as compared with immobilization from HEPES buffer. An increase in the density of cations on mica surface may lead to transition of DNA molecules from the canonical B-form into compressed S-form of DNA. Let us note that earlier it has been shown by us that linear and supercoiled molecules of S-DNA form on the surface of mica with high surface charge density and are characterized by a significant decrease of contour length at the expense of the decrease of the distance between nucleotides along the helix axis [20]. Secondly, the decrease in the contour length of DNA having formed a complex with T7 RNAP may be explained by reeling of the DNA molecule onto the RNAP molecule. A corresponding model was proposed in work [21]. Analogous results on DNA compactization were obtained for *E. coli* RNAP in work [9], the authors of which the aid of AFM visualized the elongation complexes of RNAP with DNA template with the use of the method of stopping transcription on definite sites and came to a conclusion that the DNA molecule indeed reels onto the RNAP molecule.

From the AFM image presented in Fig. 4a it is seen that DNA molecules with numbers from 1 to 4 have formed complexes only with one T7 RNAP molecule, while with molecule 5 three polymerase molecules have bound. At the same time the DNA–T7 RNAP

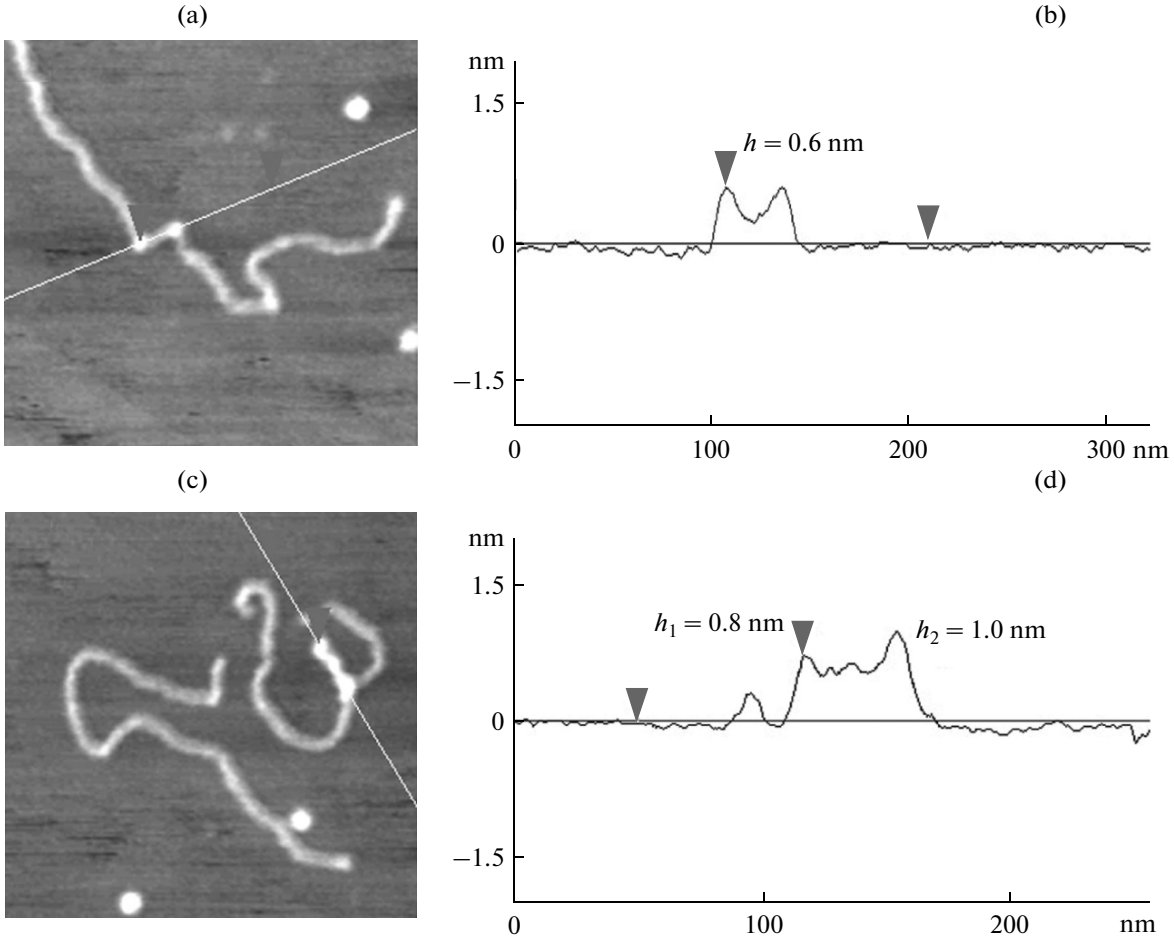

Fig. 6. AFM images of complexes of pGEMEX DNA with T7 RNAP (a, c) and the corresponding longitudinal sections (b, d). Shown is the line along which the section plane is drawn (a, c) perpendicular to the plane of the figure. Peaks on the sections correspond to the maximal height of complexes or molecules. (b) the height h of both complexes pGEMEX DNA–T7 RNAP equals 0.6 nm; (d) h_1 corresponds to the height of pGEMEX DNA–T7 RNAP complex; h_2 – maximal height of the involute fragment of pGEMEX DNA.

complexes for molecules 1, 3, 4 qualitatively differ from the complexes for molecules 2 and 5, binding with which is accompanied by formation of characteristic bends. With white arrows for molecules 1, 3, 4 shown are fragments that may be interpreted as RNA transcripts (let us note that in work [9] upon thorough analysis of the therein presented AFM images of like size of the frame we have not found a single DNA–RNAP complex that would have contained a growing RNA transcript). However, thorough analysis of AFM images of these (and other) molecules with higher resolution (Fig. 5), construction of cross sections of molecule fragments (Fig. 6), and also their reconstructed 3D images (Fig. 7b) permit supposing that the indicated fragments of molecules relate to the DNA template.

Let us once again turn attention to that the contour length of the DNA template measured by us after amplification was equal to 435 ± 15 nm. The determined contour length of DNA molecules having formed a complex with T7 RNAP also resides in the limits of this statistically reliable determined value – 445–457 nm (Fig. 5). Let us note that the height of a DNA molecule immobilized on the mica surface measured with the aid of AFM equals 0.3–0.5 nm. On the AFM image the T7 RNAP molecules, the molecular mass of which constitutes 98.8 kDa, look like spheres the diameter of which equals 8 nm. The height of the DNA–T7 RNAP complex constitutes from 0.6 nm (Fig. 6a,b) to 1 nm (Fig. 6c,d). The length of molecule 3 with an involute fragment (which is shown by black arrow in Fig. 5b) constitutes 457 nm, which points to that the visualized fragment presents a part of the DNA template rather than an RNA transcript. Apart of that, the height of knots having formed upon twisting of double-stranded DNA equals 0.8 nm (Fig. 6d), which also coincides with the height of knots of twisted DNA immobilized on mica [20].

It is worth noting that upon interaction of a DNA molecule with T7 RNAP there form specific and

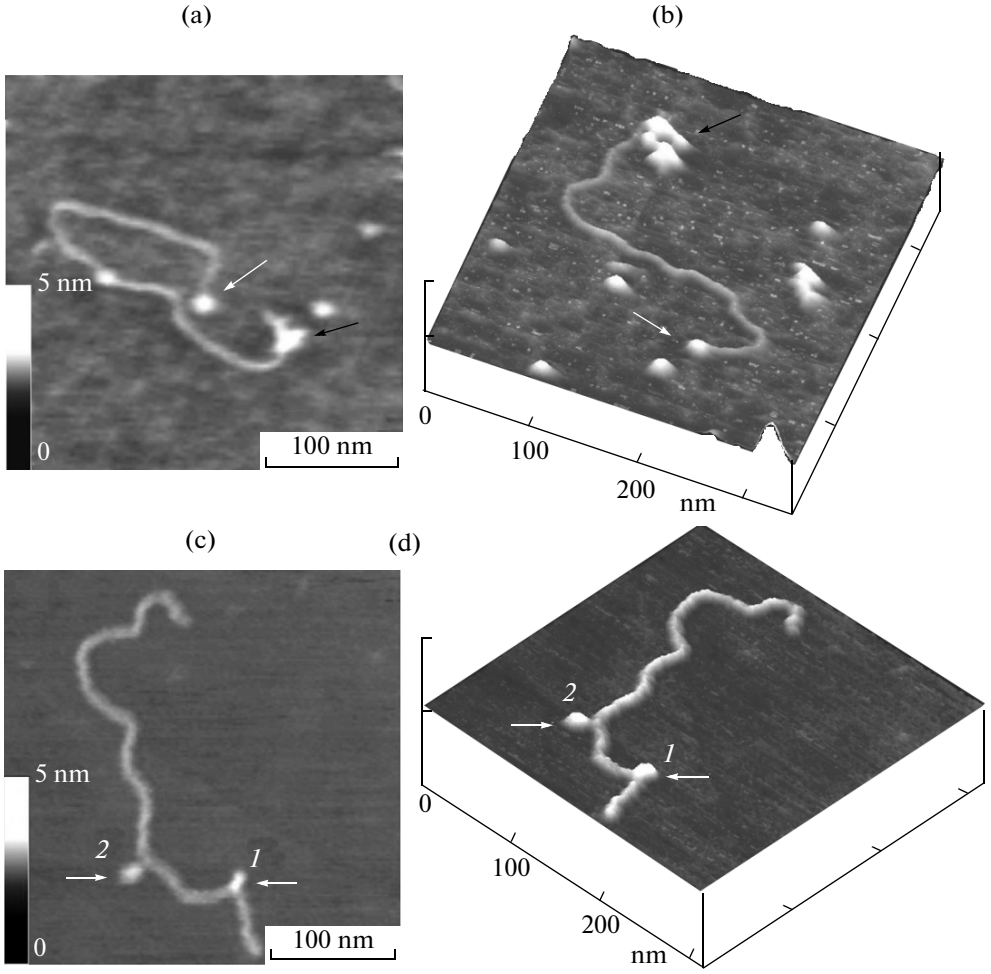

Fig. 7. AFM image (a, c) and 3D image (b, d) of complexes formed by molecules of T7 RNAP with fragments of DNA template. (a) White arrow points to a complex of transcription initiation (frame size – 297 nm × 297 nm), while black one (b) to a complex in the region of transcription termination. (c) White arrows point to a complex T7 RNAP–DNA in the promoter region (*1*) and to a complex T7 RNAP–DNA in which a fragment of DNA molecule is reeled onto a RNAP molecule (*2*). Frame size – 314 nm × 314 nm. Presented is a scale of grades of gray that corresponds to a range of height from 0 to 5 nm. Transcription was conducted in the course of 65 min at $T = 31°C$ (a); (c) transcription was not conducted.

unspecific complexes. Specific binding (interaction with promoter) appears relative insensitive to a change of the ionic strength of solution, but it depends on the conformation of the DNA fragment. Unspecific DNA–T7 RNAP complexes form at the expense of electrostatic interaction of positively charged residues of the polymerase with negatively charged phosphate groups of the DNA. Unspecific binding appears extraordinarily sensitive to a change of the ionic strength of the reaction mix, but insensitive to the degree of DNA supercoiling. From an analysis of the properties of specific and unspecific DNA–RNAP complexes in work [22] a conclusion is made that the main component parts of the mechanism of transcription regulation of bacteriophage T7 appear to be the effectiveness and the rate of formation of an open complex of RNAP with promoter rather than binding with different promoters that enter into the composition of the bacteriophage genome.

In the given work we have used the promoter that is localized in position 1212–1231 bp of the DNA template, i.e. at a distance about 200 nt from the end of the amplicon, which corresponds to a distance of ~70 nm (200 nt × 0.34 nm). Let us note that the possibilities of AFM allow reliably visualizing a complex formed at such distance from the end of the amplicon. At the same time among quite a large set of analyzed DNA–T7 RNAP complexes (over 200) we have visualized complexes formed by polymerase exactly with the terminal fragments of DNA (about 30 complexes). Typical AFM- and 3D images of such complexes are presented in Fig. 7 and Fig. 8.

Let us consider also in more detail the complexes visualized by us that are formed by T7 RNAP at both

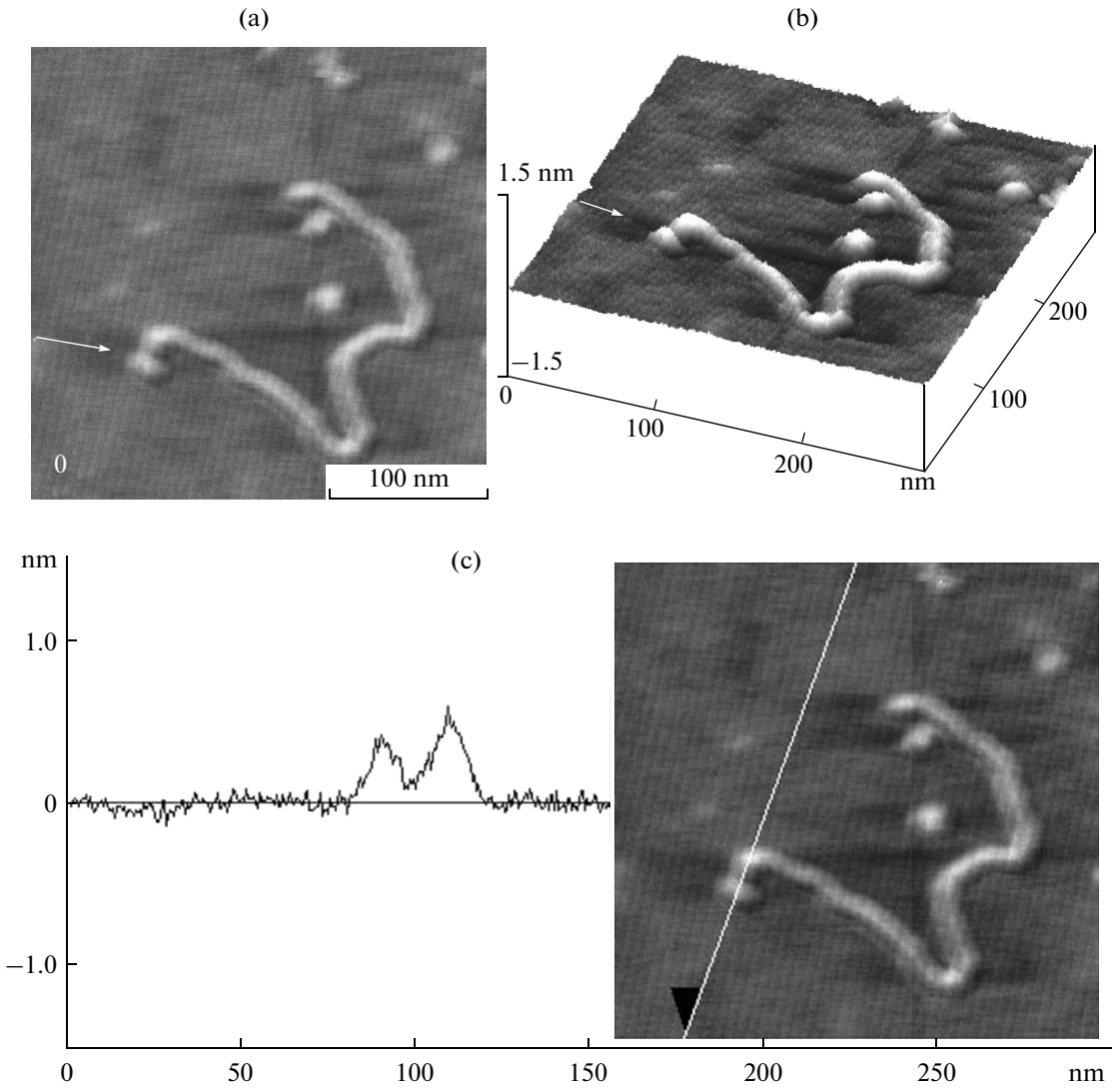

Fig. 8. Image of a single linear DNA with length of 1414 bp having formed a complex with T7 RNAP (shown by arrow) after conducting transcription in buffer A for 4 min at $T = 37°C$. Molecules of T7 RNAP appear as spheres of diameter about 8 nm. (a) AFM image. Frame size – 280 nm × 280 nm. (b) 3D image. (c) Cross-section of a complex of T7 RNAP with DNA from which we determined the height of molecules of DNA and RNAP. In the inset, shown is the line along which the section plane is drawn perpendicular to the plane of the figure. The height of T7 RNAP molecule (H_1) in the T7 RNAP–DNA complex constitutes 0.3 nm, while of DNA molecule (H_2) – 0.5 nm.

ends of the amplicon. For the variant of transcription at $T = 37°C$ in the course of 4 min we disclosed only one type of complex (Fig. 8) – a T7 RNAP molecule attached to one of the ends of the DNA template. The measured height of this complex (Fig. 8a, shown by arrow) immediately from the AFM image with the aid of constructing sections (Fig. 8c) was equal to ~ 0.5 nm, which corresponds to the height of a single molecule of T7 RNAP. At the same time after conducting transcription at $T = 31°C$ in the course of 65 min we visualized two types of complex localized at both ends of the DNA template (Fig. 7), – complexes of T7 RNAP both with promoter and with terminator. The height of another T7 RNAP–DNA complex shown by black arrow in Fig. 7a and Fig. 7b was equal to 0.93–1.0 nm.

It is important that the process of transcription may be regarded as a peculiar scanning of the DNA template by RNAP. The direction of scanning is set by the sequence of the promoter in the template strand of the DNA [23]. But for the search of promoter and exclusion of the possibility of missing the promoter site by such a high-precision "machine" as RNAP is, in our opinion, this molecular motor starts the scanning from one of the terminal fragments of the DNA. It is just by this circumstance that we explain the rather large number of visualized terminal complexes, or complexes that precede the formation of transcription ini-

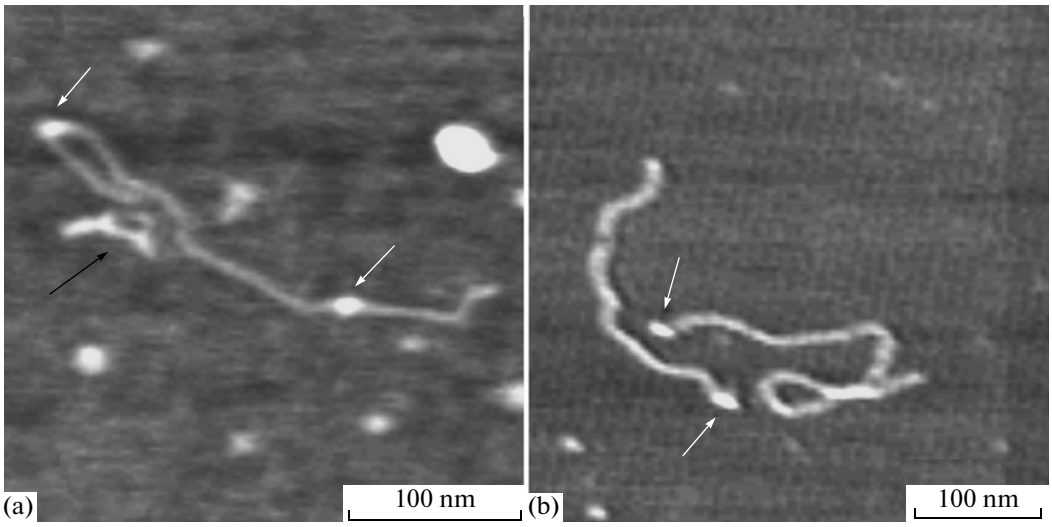

Fig. 9. AFM image of complexes of T7 RNAP with the DNA template. (a) White arrow points to a molecule of RNAP having formed a complex with a single DNA molecule in the process of transcription, while the black one – to a complex of RNAP with transcription terminator. Frame size – 323 nm × 323 nm. (b) White arrows point to complexes of single molecules of T7 RNAP with terminal fragments of single molecules of DNA template. Frame size – 418 nm × 418 nm. Transcription was conducted for 65 min at $T = 31°C$.

tiation complexes. Our suppositions are consistent with the experimental results on AFM visualization of RNAP diffusion during searching for promoter [24] and a model of migration of protein along DNA (fast transfer of protein between different DNA fragments with the aid of mechanisms of one-dimensional diffusion and weak unspecific binding) [25].

At the 5'-end of the template strand of the amplicon, located is a region of transcription termination at a distance of ~ 90 bp from the end of the amplicon. Upon reaching a terminator the elongation complex (DNA–RNAP–RNA transcript) stops and dissociates. However we have visualized several DNA molecules having formed a T7 RNAP complex at both ends of the template (Fig. 7). At that in Fig. 7a one can see three T7 RNAP molecules bound with DNA: one has formed a complex of initiation (shown by white arrow), the second – a complex in the transcription termination region (shown by black arrow), while the third molecule resides in the middle of the DNA template. The presence of several T7 RNAP molecules having formed a complex with a single DNA molecule confirms that after the beginning of transcription by one T7 RNAP molecule another enzyme molecule can bind with the terminal fragment of the DNA template localized beside a promoter of T7 RNAP, for conduction of subsequent preinitiation and elongation of transcription.

In the transcription termination region several T7 RNAP molecules may be bound with the DNA template (shown by black arrow in Fig. 7a and Fig. 7b). In the 3D image (Fig. 7b) apart of terminal T7 RNAP–DNA complexes one can see still five molecules of polymerase that have not formed a complex with the DNA template. Inasmuch as the reaction of transcription was conducted at an excess of T7 RNAP and at a rather high value of ionic strength (I = 70–80 mM Na^+), which leads to elimination of unspecific binding of polymerase with DNA, and in the given conditions there formed only one T7 RNAP–DNA complex, then the visualized terminal complex (shown by white arrow in Fig. 7a and Fig. 7b) appears specific. Analogous complexes of T7 RNAP with DNA were also visualized in work [26]. But, in our view, they were erroneously interpreted as unspecific.

The presence of several molecules of T7 RNAP having formed a complex with a DNA template in the process of initiation and elongation of transcription (Figs. 7a, 9a) show that not one but several RNAP molecules initiate transcription from one promoter. These data are consistent with the results of work [27] in which a possibility is shown of association of RNAP molecules in the process of transcription elongation. It is known that in bacteria and eukaryotic cells transcription is actualized at a high rate despite the presence of numerous blocks of transcription. In work [27] it is demonstrated that the majority of internal and external blocks of transcription (signals of pause and stop) disappear if more than one RNAP molecule initiates transcription from one and the same promoter. Conversely, upon conduction of exactly single cycle of transcription the presence of internal terminators substantially influences the rate of elongation and the yield of full-sized transcripts.

By means of kinetic analysis of initiation and elongation of transcription for single molecules of T7 RNAP [4] it has been disclosed that the initiation step lasts 4 s, and consequently, for synthesis of an RNA

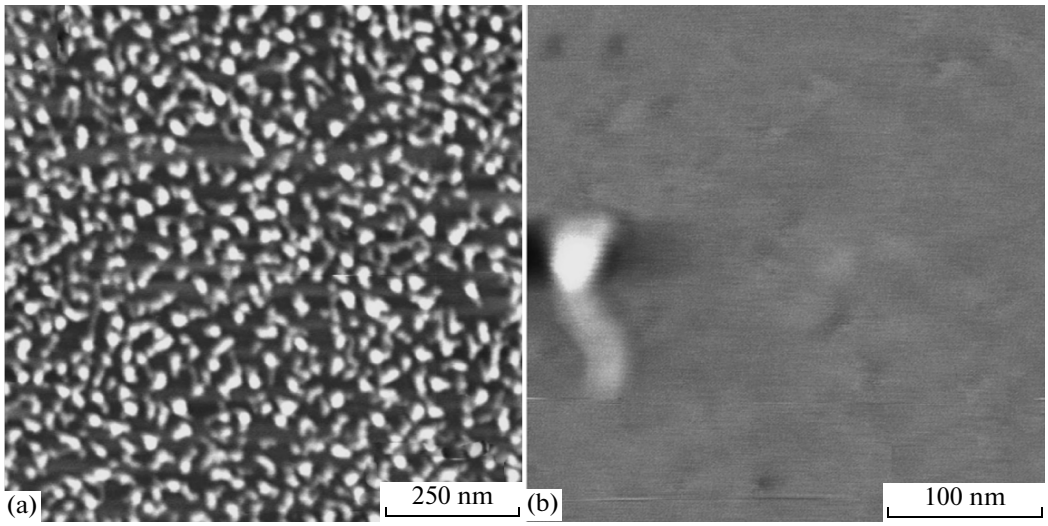

Fig. 10. AFM image of RNA transcripts after conducting transcription with T7 RNAP on a template of linear DNA pGEMEX of length 1414 bp and treatment with DNase I. The expected length of transcripts constitutes 1100 nt. Frame size: (a) 1 μm × 1 μm; (b) 315 nm × 315 nm.

transcript of 1200 nt length one needs about 30 s. Therefore in our case the time during conduction of transcription in the course of 4 min is quite enough for synthesis of RNA transcripts on a DNA template of 1414 bp length, which was used in the given work. However upon conducting transcription in the course of 4 min (in buffer A) we visualized complex inherent to which is binding with only one end of the DNA template, similar to the variant depicted in Fig. 8. At the same time upon increasing the time of transcription to 65 min we have detected complexes of T7 RNAP with both terminal fragments of the DNA template (Fig. 7). In our opinion, the complexes shown by black arrows in Fig. 7 and Fig. 9 correspond to a complex formed by T7 RNAP with the DNA template in the transcription termination region. Possibly, after elongation on the terminator there is "stoppage" one after another of several T7 RNAP molecules with corresponding RNA transcripts. This is pointed to by the rather large size of complex, and also the presence of a T7 RNAP molecule inside the DNA template given the presence of complexes at both terminal sites of the DNA template.

Visualization of a large enough quantity of T7 RNAP complexes with terminal fragments of the DNA template may be explained, in our opinion, by several circumstances. Firstly, it is exactly at the ends of the DNA template that the promoter and the transcription termination region are localized. Inasmuch as the binding constant for T7 RNAP molecule with the highly specific site of promoter significantly exceeds the constant of unspecific binding (with any site inside the DNA template) [22], then under conditions of dissociation of T7 RNAP–DNA complexes the promoter–T7 RNAP complex appears the most stable of those formed in the processes of initiation and termination of transcription. Secondly, the time of dissociation of the T7 RNAP–promoter complex significantly exceeds the time of transition to the elongation complex; therefore there exists a large probability of visualization of the complex with promoter other conditions being equal. Thirdly, pause and arrest of enzyme occur in the termination region, which also statistically elevates the probability of visualization of complexes with terminal fragments of the DNA template.

For visualization of immediately RNA transcripts the DNA template and primers were removed with the aid of incubation of the reaction mix with DNase I after conducting transcription. The obtained AFM images of RNA transcripts are presented in Fig. 10. One can see that DNA molecules are fully removed, which coincides with the results of electrophoresis (on the electrophoregram in Fig. 2 one can see only a band of RNA transcripts), while white dots correspond to molecules of RNAP in complex with which the RNA transcripts actually are remaining. From an AFM image with high resolution it was determined that RNA transcripts immobilized on mica form bunch-like condensed structures of length 122 ± 10 nm with a relationship of length and width of 4.5–5.0. Inasmuch as this value is significantly smaller than the expected length of transcripts, equaling 336 nm (1122 nt × 0.3 nm = 336 nm), one may make a conclusion that single-stranded RNA molecule form multichain condensed structures (Fig. 10b). Earlier with the aid of AFM [28] it has also been demonstrated that RNA molecules that are transcribed from a linear DNA template form similar condensed structures, while their length is two times smaller as compared with the length of the DNA template.

The noted morphological peculiarities of RNA molecules visualized with the aid of AFM re explained, in our opinion, by a significant influence of the surface properties of mica on which the RNA transcripts are immobilized. In their turn, the surface properties of substrate are determined by the hydrophobicity and density of cations localized on the mica surface. The matter is that for visualization of RNA molecules use is made of the same mica as for visualization of DNA molecules, i.e. mica with the same hydrophobicity and surface density of cations, at which linear and supercoiled double-stranded molecules of DNA do not form condensed structures upon immobilization on mica surface, but are characterized by a uniform distribution of DNA fragments. Earlier we have shown that even an insignificant change of the hydrophobicity and density of cations of the mica surface leads to a significant change in the morphology of immobilized DNA molecules [23, 27]. For example, a decrease of hydrophobicity leads to a significant stretching of supercoiled DNA molecules, which is characterized by an increase in the helical rise, i.e. distance between nucleotides along the duplex axis, from 0.34 nm (characteristic of B-form DNA) to 0.48–0.53 nm. At the same time upon an increase of hydrophobicity and density of cations of the mica surface the molecules of supercoiled DNA compactize—transit into S-DNA, or compressed molecules of DNA with a distance between nucleotides along the helix axis of 0.2 nm. All the said gives a possibility of making a conclusion that a change of hydrophobicity and surface density of mica cations leads to striking compactization or stretching of the molecules of linear and supercoiled DNA.

Comparative analysis of the obtained results on visualization of condensed molecules of RNA transcripts and single supercoiled DNA molecules immobilized on aminomica with different hydrophobicity and surface charge density [20, 29] shows that the hydrophobicity and surface density of cations of aminomica that is used for immobilization of RNA transcripts appear too high in order to visualize linear single-stranded molecules of RNA rather than the thereby formed condensed structures by them. Therefore for visualization of single RNA molecules is it necessary to decrease the surface density of cations of aminomica in such a way that upon immobilization thereon of RNA molecules there would form hairpin structures or plectonemic molecules as distinct from the visualized condensed structures.

In this way, in the work by means of AFM we visualized RNA transcripts and complexes that T7 RNAP forms with terminal fragments of the DNA template upon elongation of transcription. It is shown that on one molecules of DNA template (which contains a promoter and internal terminator of T7 RNAP transcription) in conditions of elimination of unspecific binding the T7 RNAP molecules form both complexes with terminal fragments of the amplicon and specific complexes in the region of termination and in the process of elongation of transcription.

Study of the complexes that are formed by RNAP in the process of transcription may be important for understanding the resistance of RNAP to antibacterial preparations widely discussed in the literature. Inhibition of bacterial RNAP presents a commonly accepted strategy of antituberculous therapy, inasmuch as RNAP presents a target for some antibiotics. Analysis of the crystalline structure of complexes of *T. aquaticus* RNAP with antituberculous preparations rifampicin and sorangicin has allowed establishing that binding of the given antibiotics near the RNAP active center causes blocking of elongation of RNA transcripts [30]. The resistance to rifampicin, which can eradicate nonreplicating mycobacteria of tuberculosis, includes replacement of residues within the site of rifampicin binding on RNAP, which immediately decreases the binding of rifampicin with the RNAP molecule. Therefore, from the point of view of future AFM investigations, it appears important to study the elongation complexes of clinical isolates of tuberculosis mycobacteria the RNAP of which appears more than a 1000 times sensitive to the first-line antituberculous antibiotic rifampicin as compared with the RNAP of *E. coli* [31]. Further investigations with the use of the possibilities of scanning probe microscopy (visualization in vitro, measurement of the force of intermolecular interactions for pairs of singe molecules) will allow obtaining, in the opinion of the authors, additional information of the mechanisms of intermolecular interaction of antituberculous antibiotics rifampicin, sorangicin, streptolydigin, myxopyronin with bacterial RNAP which presents a molecular target for them [30].

ACKNOWLEDGMENTS

The authors express gratitude to Prof. A.V. Sivolob (Shevchenko Kiev National University) for critical comments in article preparation.

The work was supported by the International Center of Science and Technologies (Japan) and National Academy of Medical Sciences of Ukraine (AMS grant no. 72/2007).

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