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Visualization of RNA Transcripts by Atomic Force Microscopy

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Abstract—RNA transcripts following transcription with T7 RNA polymerase in vitro are visualized by atomic force microscopy. A fragment of linearized pGEMEX DNA 1414 nucleotide pairs in length containing a promoter and the transcription termination region of the bacteriophage T7 is used as template for the transcription. RNA transcripts immobilized on mica (the substrate for atomic force microscopy) form condensed, rod-like structures 122 ± 10 nm in length with characteristic length-to-width ratio 4.5–5. Features of immobilization of RNA molecules on mica for subsequent visualization by atomic force microscopy are discussed.

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INTRODUCTION

RNA is a multifunctional molecule for which DNA functions as well as functions of different proteins are typical. The principal functions of RNA is complementary replication, encoding and decoding of proteins, molecular recognition through the formation of a unique spatial surface globular structure (created by RNA molecules), and a catalytic function (catalytic RNA are called ribosomes) [1].

In prokaryotes and eukaryotes RNA is synthesized by DNA-dependent RNA polymerase. The latter is responsible for binding with a promoter, DNA fusion, initiation of transcription, elongation of RNA chain, and termination of transcription. Three different RNA polymerases (RNAP) are known for eukaryotes, RNAP I, RNAP II, and RNAP III, each of which transcribes different classes of genes. Many studies by X-ray crystallographic analysis, DNA foot printing, and fluorescent resonance spectroscopy have improved our conceptions of the RNAP elongation complex with DNA and RNA transcript [2, 3]. It is known that RNA transcript is extruded in the course of elongation through the RNAP exit channel, while the contacts between RNA transcript and RNAP are maintained for 14–16 nucleotides from the 3'-end. It became possible to obtain new data on the structure of the promoter and the elongation complex as a result of the development of high-resolution microscopy equipment. Electron microscopy and atomic force microscopy have made it possible to obtain important information on the spatial association between DNA, RNA, and RNAP in the course of transcription elongation, and even on the global topology of an open promoter and the elongation complex. A model of DNA winding has been proposed for the elongation complex based on experimental studies on transcription of eukaryotes and prokaryotes [4]. In the elongation complex wrapping of DNA on RNAP and the creation of RNA–DNA hybrid induces unwinding of the double DNA helix and the appearance of the transcription loop. However, there remain quite a number of unanswered questions concerning the way in which RNA polymerase transcribes lengthy DNA fragments. There have been especially numerous suggestions that attempt to account for the movement of RNAP along DNA in vivo, specifically, whether RNAP wraps around DNA or whether the fragment exists in a rotationally taut state such that DNA is forced to wrap around the helix axis in such a way as ti enable the DNA strand to pass through the protein. Visualization of RNA molecules that grow in the course of elongation also remains a problem.

Since RNA molecules have been studied by atomic force microscopy to a significantly lesser extent than DNA molecules, in the present article results will be presented from direct visualization of RNA transcripts that were synthesized following transcription with bacterial T7 RNA polymerase. Visualization will be carried out by atomic force microscopy. Our studies have shown that RNA transcripts on the surface of a substrate form condensed, rod-like structures that are characterized by a length-to-width ratio of 4.5–5.

MATERIALS AND METHODS

Preparation of DNA samples for polymerase chain reaction and atomic force microscopy. To conduct the polymerase chain reaction linear DNA was obtained by restriction of superhelical pGEMEX DNA (Promega, United States) 3993 nucleotide pairs in length using *ScaI* endonuclease (New England Biolabs, Great Britain). The L1 and L2 primers that we created flank the DNA fragment, which contains a promoter and transcription termination region of T7 RNA polymerase. Primers L1 and L2, the sequences of which together with corresponding positions on the pGEMEX RNA presented below, were obtained from Sigma (Japan): 5'-cgc tta caa ttt cca ttc gcc att c-3', the sense primer L1 (3748–3772); and 5'-ctg att ctg tgg ata acc gta tta ccg-3', the antisense primer L2 (1168–1142).

Hot-start PCR was performed in a 50 μl reaction mixture on GeneAmp 9700 amplifiers (Perkin Elmer, United States), The PCR parameters were as follows: initial incubation, 95°C, 2 min; denaturation, 95°C, 1 min; annealing, 69–73°C, 1 min; synthesis, 74°C, 1 min; number of cycles, 35. The annealing temperature was determined theoretically by the Oligo program. Several PCR procedures at different annealing temperatures (69, 71, and 73°C) were performed in order to minimize amplification of nonspecific fragments.

To visualize the amplicons 15 μl PCR-product was separated by electrophoresis in 2% agarose gel in a buffer with subsequent staining with ethidium bromide.

To purify the amplified DNA fragment, a strip of gel containing amplicon was cut off following electrophoresis after the gel had been irradiated by a short-wave ultraviolet source, of low-intensity radiation (Bio-Rad, United States). The QIAquick PCR purification kit (QIAgen, Japan) was used for further purification of the amplicon from nucleotide and primers, DNA polymerase, and ethidium bromide in accordance with the manufacturer's recommendations. Extraction of phenol/chloroform with subsequent precipitation by ethanol was also performed.

Two types of high-precision, thermally stable DNA polymerase were used: Pyrobest DNA polymerase (TaKaRa Co., Japan) and Invitrogen Platinum DNA polymerase (Invitrogen, Japan).

10 mM HEPES buffer containing 2.5 mM $MgCl_2$ was used to deposit the DNA on freshly cleaved mica. A drop of DNA solution with concentration 0.1 μg/ml in a 10-μl TE buffer (10 mM tris-HCl, pH 7.9, 1 mM EDTA) was deposited on a mica strip 1 cm^2 in area, washed following 2-h exposure to deionized water, and blown dried with an argon flow; the sample was then held under a pressure of 100 mm Hg for 20 min.

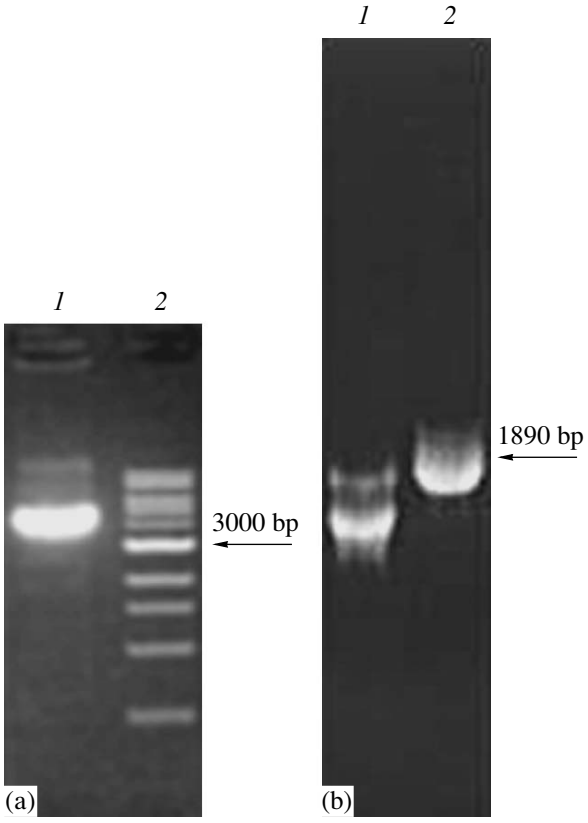

Fig. 1. (a) Analysis of linear pGEMEX DNA following restriction of superhelical pGEMEX DNA with the endonuclease *ScaI* in 1.5 % agarose gel: *1*—linear pGEMEX DNA 3993 bp long; *2*—100 bp marker of molecular weight; the strip of elevated intensity corresponds to a fragment of length 3000 bp; (b) Analysis of RNA transcripts following transcription with T7 RNA polymerase on a template of a fragment of linear pGEMEX DNA of length 1414 bp in 1.8% agarose gel with formaldehyde and pTRI-Xef control plasmid of length 1890 bp. Expected length of transcripts 1122 nucleotide.

Transcription and visualization of transcripts. An amplicon 1414 bp in length containing a promoter and the transcription termination region of T7 RNA polymerase was used as the template for the transcription. The DNA template was obtained by restriction of the endonuclease *ScaI* (Fig. 1a) of superhelical pGEMEX DNA with subsequent amplification and purification of the amplicon. The transcription reaction was performed according to the following protocol using a kit for transcription by T7 RNA polymerase (Promega, United States). The reaction mixture contained a DNA template (2 mM), transcription buffer (40 mM tris-HCL, pH 7.9, 6 mM $MgCl_2$, 10 mM NaCl), 10 mM dithiothreitol, 2 mm spermidine, 0.05% Twin 20, 40 units RNasine, and 20 units RNA polymerase, with a total volume of 20 μl. The transcription reaction was initiated by the addition of the nucleotides CTP, GTP, UTP, and ATP to a final concentration of 2.5 mM. After 60 min incubation the reaction was halted by heating

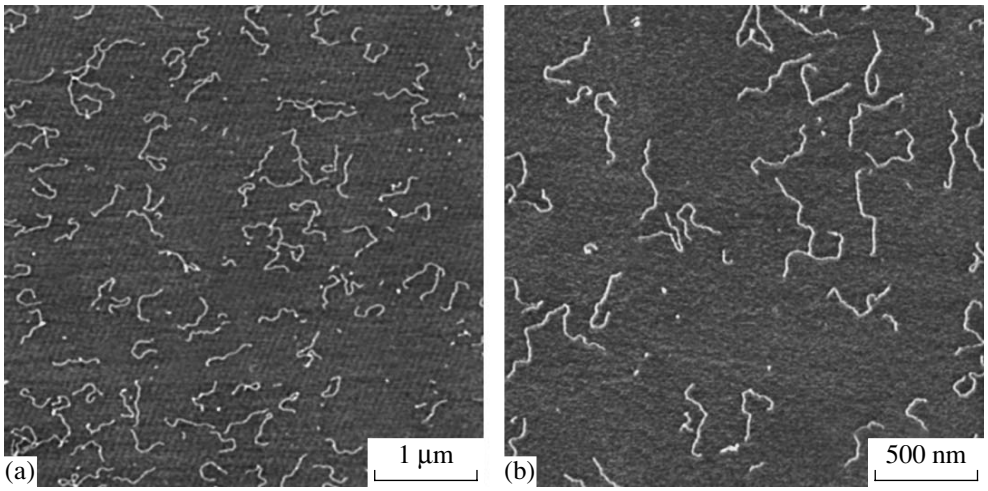

Fig. 2. Image of fragment of linear pGEMEX DNA obtained by atomic force microscopy 1414 bp in length used as template for transcription: (a) scan size, 4 μm × 4 μm; (b) scan size, 2.1 μm × 2.1 μm.

for 15 min at a temperature of 70°C. 1 μl DNAase I free of RNAase (Ambion, United States) was added to the reaction mixture after transcription had been performed for the purpose of expelling the DNA template and degradation of the DNA, which could have led to contamination of the RNA sample, and the mixture was incubated for 15 min at a temperature of 37°C. In order to inactive the DNAase the reaction mixture was incubated at a temperature of 70°C for 10 min. To monitor the efficiency of transcription, electrophoresis was performed in 1.2% agarose gel containing 1.8% formaldehyde (Fig. 1b). In order to carry out electrophoresis under denatured conditions, an equal volume of buffer was added to the reaction mixture to precipitate a sample containing 90% formaldehyde, 0.01% xylene cyanol, 0.01% bromphenol blue, 10 mM EDTA, and 0.01% SDS.

Atomic force microscopy. The Nanoscope IV Multi-Mode System atomic force microscope (Veeco Instruments, Inc., United States) with E-scanner was used in the study. An AFM image of the DNA was taken by tapping mode atomic force microscopy in open air in a "height" regime using OMCL-AC160TS cantilevers (Olympus Optical Co., Japan) with resonance frequency 340–360 kHz and spring constant 42 N/m. The images were obtained in a 512 × 512 pixel format, flattened, and analyzed by the Nanoscope software package (v. 5.12r3) (Veeco Instruments Inc.).

RESULTS AND DISCUSSION

An important feature of the elongation complexes that are utilized in transcription (DNA–RNA-polymerase—RNA transcript) is the fact that the elongation complex rapidly dissociates after the stop of transcription. Moreover, the rate of dissociation for bacterial T7 RNA polymerase is significantly higher than that of eukaryotic polymerase. This well-known fact produces an essential complication in visualization of the entire elongation complex as well as in visualization of individual RNA transcripts. For the purpose of atomic force microscopy-based visualization of the elongation complex, therefore, we used DNA templates where the nucleotide sequence of the templates was synthesized so that transcription would halt at certain positions. As an example, three such templates were used in [6]. One of these templates, 24 bp in length, was characterized by the fact that the DNA contained only three nucleotides (dTTP, dATP, dCTP) from the position at the start of transcription initiation to position 24 above the start of transcription initiation. Moreover, only three of the four ribonucleotides (with the exception of CTP) were added to the reaction mixture, which resulted in termination of transcription at position 25, the location of dGTP.

In the present study a different approach to prevent rapid dissociation of the elongation complex was used. That is, we used a DNA template that contained a T7 RNA polymerase transcription termination region at a distance of 1122 nucleotides from the transcription initiation site. The efficiency of transcription termination could be regulated, in that it depends on the concentration of the components of the reaction mixture, the ionic strength, and pH of the buffer used for the transcription and may reach 70–80%.

The AFM image of a fragment of linear pGEMEX DNA 1414 bp in length which was used for the transcription is shown in Fig. 2. The contour length of the amplicons determined directly from the AFM image was 435 nm ±15 nm, which corresponds to the distance between the nucelotides along the axis of the helix, or 3.07 Å, which is within the range of the expected

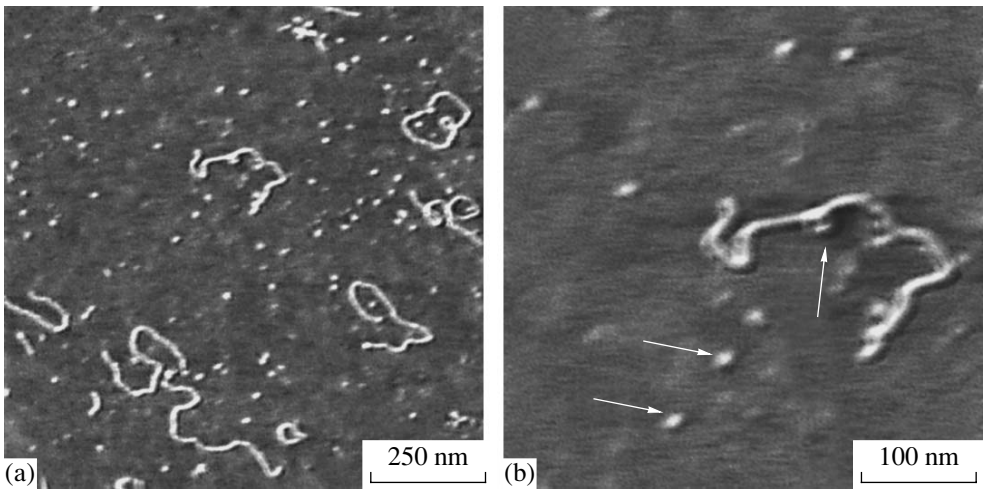

Fig. 3. Atomic force microscopy image of fragment of linear pGEMEX DNA following transcription with T7 RNA polymerase without treatment with DNAase I: (a) scan size, 1 μm × 1 μm; (b) scan size 400 nm × 400 nm. Arrows point to T7 RNA polymerase molecule.

length under the assumption of B-form DNA. Note that the promoter and transcription terminator for T7 RNA polymerase are localized asymmetrically with respect to the 5'- and 3'-ends of the fragments, while the RNAP T7 transcription promoter (with length of 19 nucleotides) is found at position 91–182 bp. Thus, the expected length of the transcripts is equal to 1122 nucleotides (without the transcription terminator) and 1214 nucleotides (including the termination region).

The Promega transcription buffer (Fig. 3) was used to achieve atomic force microscopy-generated visualization of the T7 RNA polymerase complexes with linear RNA without conducting transcription. The T7 RNAP molecules, the molecular weight of which was 98.8 kDa, appear in the AFM images to be spheres with diameter 18 nm. It was noted that with this high ratio between the molar concentrations of T7 RNAP and DNA (for the molecules shown in Fig. 3, this ratio is equal 20) it is apparent that binding of RNA polymerase to the DNA fragment containing the promoter (that is, the high-specific binding site) is not specific. A high-resolution image (Fig. 3b) shows that a complex of 4 or 5 T7 RNAP molecules are formed with this amplicon.

Our results are in agreement with previous data [5]. In this study it was shown that, by comparison with the plasmid pBR322, the sequence of which does not contain any promoter, binding of T7 RNA polymerase to the plasmid pAR11, which carries the T7 RNA polymerase promoter, is nonspecific in the range of ionic force (I) 40–100 mM Na^+. In addition, binding of DNA molecules to other sites occurs together with binding of T7 RNA polymerase with promoter (through electrostatic interaction of positively charged T7 RNAP sites with negatively charged DNA phosphate groups). Only with a value I = 0 binding occurs exclusively to the T7 RNAP promoter.

To analyze RNA transcripts directly, we removed the DNA-template and primers by incubation of the reaction mixture with DNAse after transcription. (The fact that this DNA fragment is transcribed successfully is confirmed by the results of analysis of transcription products by electrophoresis in agarose gel with formaldehyde under denaturing conditions (Fig. 1b).)

AFM images of the RNA transcripts obtained are shown in Fig. 4. It is apparent that the DNA molecules have been completely extruded, which is in agreement with the results of electrophoresis, while the white formations correspond to RNA polymerase molecules in the complex in which the RNA transcripts were found. Because of the high-resolution AFM image, it was determined that the RNA transcripts immobilized on the mica formed condensed, rod-like structures 122 ± 10 nm in length with ratio of length to width of 4.5–5. Since the length of these structures is much less than the expected length of the transcripts, or 336 nm (1122 nm × 0.3 nm = 336 nm), it may be concluded that single-stranded RNA molecules form condensed multichain structures. It has previously also been shown [8] using atomic force microscopy that the length of RNA molecules which are transcribed from a linear DNA template is one-half the length of the DNA template and that these molecules form similar condensed structures.

The morphological features thus found for RNA molecules that have been visualized by atomic force microscopy may possibly be explained by the significant influence of the surface properties of mica on which the RNA transcripts were immobilized. The sur-

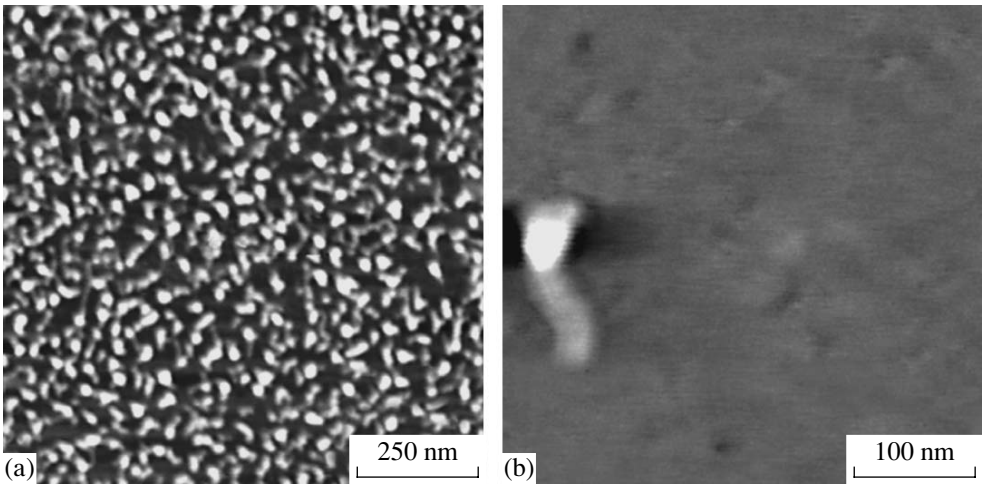

Fig. 4. AFM image of RNA transcripts following transcription with T7 RNA polymerase on template of linear pGEMEX DNA of length 1414 bp and treatment with DNAase I. Expected length of transcripts 1100 nucleotides: (a) scan size, 1 μm × 1 μm; (b) scan size, 315 nm × 315 nm.

face properties of the substrate are, in turn, determined by the hydrophobicity and density of the cations that are localized on the mica surface. In fact, the same mica that was used to visualize the DNA molecules was also used for visualization of the RNA molecules—that is, mica with degree of hydrophobicity and surface density of cations for which linear and superhelical double-chain DNA molecules do not form condensed structures upon immobilization on the mica surface, but are instead characterized by a uniform distribution of DNA fragments. We have previously demonstrated that even a slight change in the degree of hydrophobicity and density of cations on a mica surface will lead to a significant change in the morphology of immobilized DNA molecules [8, 9]. For example, a decrease in degree of hydrophobicity leads to substantial lengthening of superhelical DNA molecules, a process which is characterized by an increase in the helical rise, that is, distance between nucleotides along the duplex axis, from 0.34 nm (typical of B-form DNA) to 4.8–5.3 nm. At the same time, with an increase in degree of hydrophobicity and density of cations on the mica surface, the molecules of superhelical DNA become more compact, turning into S-DNA or compressed DNA molecules with distance between nucleotides along the helical axis 0.2 nm. These remarks lead us to conclude that a change in hydrophobicity and surface density of cations on mica leads to marked compaction or stretching of molecules of linear and superhelical DNA.

An analysis of the results that have been obtained from visualization of RNA transcripts as well as superhelical DNA molecules immobilized on mica with different degrees of hydrophobicity and surface charge density [8, 9] shows that hydrophobicity and the surface density of cations on mica used to immobilize RNA transcripts is too high to achieve visualization of linear single-stranded RNA molecules, but not the condensed structures formed by them. Therefore, to visualize isolated RNA molecules it is necessary to optimize the surface density of the mica cations so that once RNA molecules are immobilized on the mica, stem-loop structures or stretched molecules are formed rather than the condensed structures visualized here.

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