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MOLECULAR BIOPHYSICS

Visualization of Amplicons after Polymerase Chain Reaction

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Abstract—Linear DNA molecules amplified by the polymerase chain reaction (PCR) were visualized by atomic force microscopy. The measured contour length of the PCR product of the 1414-bp sequence was 435 ± 215 nm. Taking into account that the calculated value of the distance between the nucleotides along the duplex axis is 0.31 nm, it was assumed that linear DNA molecules on the surface of mica, which serves as a support in the atomic force microscopy technique, are in the A form. The influence of surface properties of the mica and the sample drying procedure on the conformation of adsorbed DNA molecules is discussed. Possible causes of the Gaussian distribution of the contour length of the synthesized amplicon are considered.

Key words: amplicons, atomic force microscopy, polymerase chain reaction

Modern molecular-genetic methods allow manipulations with genes and their fragments with a high (to one nucleotide) accuracy. Polymerase chain reaction (PCR) is used for solving both applied and fundamental problems in nearly every modern laboratory dealing with molecular biology. Using modern PCR kits and primers flanking the genomic fragment of interest, researchers amplify the fragment specified. After visualization of the amplified DNA fragment by electrophoresis and subsequent staining with ethidium bromide (provided that the gel contains only one band, which corresponds to the DNA fragment analyzed), as well as after purification from primers, nucleotides, and DNA polymerase, it can be assumed that the sample contains the sought DNA fragment of the specified length with an accuracy to one nucleotide. But is this true in reality? Does the contour length of DNA fragments amplified in PCR correspond to the predicted length of the expected DNA fragment?

An answer to these questions can be obtained using atomic force microscopy (AFM), a modern method of nanobiology. Earlier it was shown that the contour length of entire DNA molecules and their restriction fragments can be easily measured using AFM and the corresponding software [1, 2]. In this study, the 1414-bp-long pGEMEX DNA fragment was amplified by PCR. The purified PCR product was visualized using AFM. The contour length of the amplicon was measured, and statistical analysis of the distribution of the contour length of amplified DNA fragments was performed.

EXPERIMENTAL

PCR was performed with linear DNA obtained by treating pGEMEx coiled-coil DNA 3993 bp in length (Promega, United States) with the restriction endonuclease *Sca*I (New England Biolabs, United Kingdom). Primers L1 and L2, manufactured by us, limited the DNA fragment containing the T7 RNA polymerase promoter and the transcription termination region. The scheme of the amplified DNA fragment 1414 bp in length is shown in Fig. 1. Primers L1 and L2, whose sequences with the corresponding positions on the pGEMEX DNA are shown below, were obtained from Sigma (Japan):

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

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

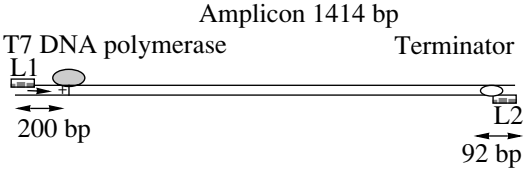

Fig. 1. Scheme of the DNA template used in this study. The primers shown with rectangles flank the pGEMEX DNA fragment 1414 bp in length, which contains the promoter and transcription terminator of T7 RNA polymerase.

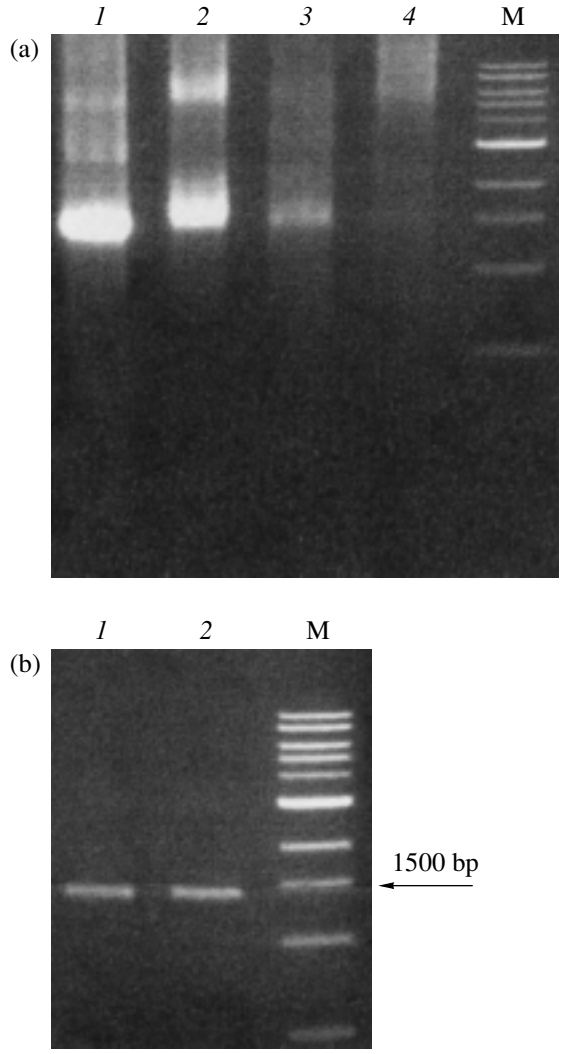

Fig. 2. (a) Amplification of the fragment of linear pGEMEX DNA under different conditions of PCR. Notations: lanes *1*, *2*, annealing temperature T_{ann} = 71°C; lanes *3*, *4*, T_{ann} = 73°C; lanes *1*, *3*, Pyrobest TaKaRa DNA polymerase; lanes *2*, *4*, Invitrogen Platinum DNA polymerase; *M*, molecular-weight marker. (b) Purification of amplicons, after gel electrophoresis and subsequent cutting off the band of the PCR product, by extraction with the QIAquick gel extraction kit and with phenol/chloroform and reprecipitation with ethanol. Amplification with (*1*) Pyrobest TaKaRa and (*2*) Invitrogen Platinum DNA polymerase. *M* denotes the molecular-weight markers (the 3000-bp fragment is marked).

Hot-start PCR was performed in 50 μl of reaction medium in a GeneAmp 9700 amplifier (Perkin-Elmer, United States) under the following temperature and temporal conditions: initial incubation (95°C, 2 min), denaturation (95°C, 1 min), annealing (69–73°C, 1 min), and synthesis (74°C, 1 min). In total, 35 cycles were performed. The annealing temperature was determined theoretically using Oligo software. To minimize the amplification of nonspecific fragments, we performed several PCR sets at different annealing temperatures (69, 71, and 73°C).

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

The amplified DNA fragment was purified as follows. After electrophoresis, the band of gel containing the amplicon was excised using a low-intensity long-wavelength UV lamp (BioRad, United States) as the detector. Further purification of the amplicon from nucleotides, primers, and DNA polymerase was performed using a QIAquick PCR purification kit (QIAgen, Japan) in accordance with the recommendations provided by the manufacturer, as well as extraction by phenol–chloroform with subsequent reprecipitation with ethanol.

PCR was conducted with a thermostable high-fidelity DNA polymerase of two types—Pyrobest DNA polymerase (TaKaRa Co., Japan) and Invitrogen Platinum DNA polymerase (Invitrogen, Japan).

Freshly cleaved mica was used as a support for AFM. DNA was applied on the mica using a buffer containing 10 mM HEPES and 2.5 mM $MgCl_2$. A drop of DNA solution (10 μl; concentration, 01–1 μg) in a TE buffer containing 10 mM Tris–HCl (pH 7.9) and 1 mM EDTA was applied onto a piece of mica (1 cm^2) and allowed to absorb for 2 min. Then, the sample was washed with deionized water, dried under argon flow, and incubated under pressure of 100 mmHg for 20 min. Buffer solutions and DNA samples were prepared in ultrapure water with a specific resistance of ~17 MΩ cm, which was obtained using the Milli Q device (Millipore, United States).

AFM images of DNA were recorded using a Nanoscope IV MultiMode System atomic force microscope (Veeco Instruments Inc., United States) in the tapping mode. Images were obtained in the "height" mode with the use of OMCL-TR cantilevers (Olympus Optical Co., Japan) at a resonance frequency of 340–360 kHz and a rigidity constant of 42 N/m. The images with a resolution of 512 × 512 pixels were smoothed and analyzed using Nanoscope 5.12r3 software (Veeco Instruments Inc., United States).

RESULTS AND DISCUSSION

The specificity and accuracy of PCR are determined by several parameters, including the degree of primer homology, primer annealing temperature, DNA polymerase fidelity, Mg^{2+} concentration, and composition of the reaction medium for PCR. The accuracy of PCR can be increased using the hot-start variant of PCR and an amplifier at high rates of heating and cooling. For this reason, we conducted PCR using a high-speed amplifier and two high-fidelity DNA polymerases (Invitrogen Platinum and Pyrobest TaKaRa), which ensure minimal error of synthesis. In addition, increased annealing temperatures made it possible to minimize the formation of nonspecific amplification products. The electrophoretogram of amplicons

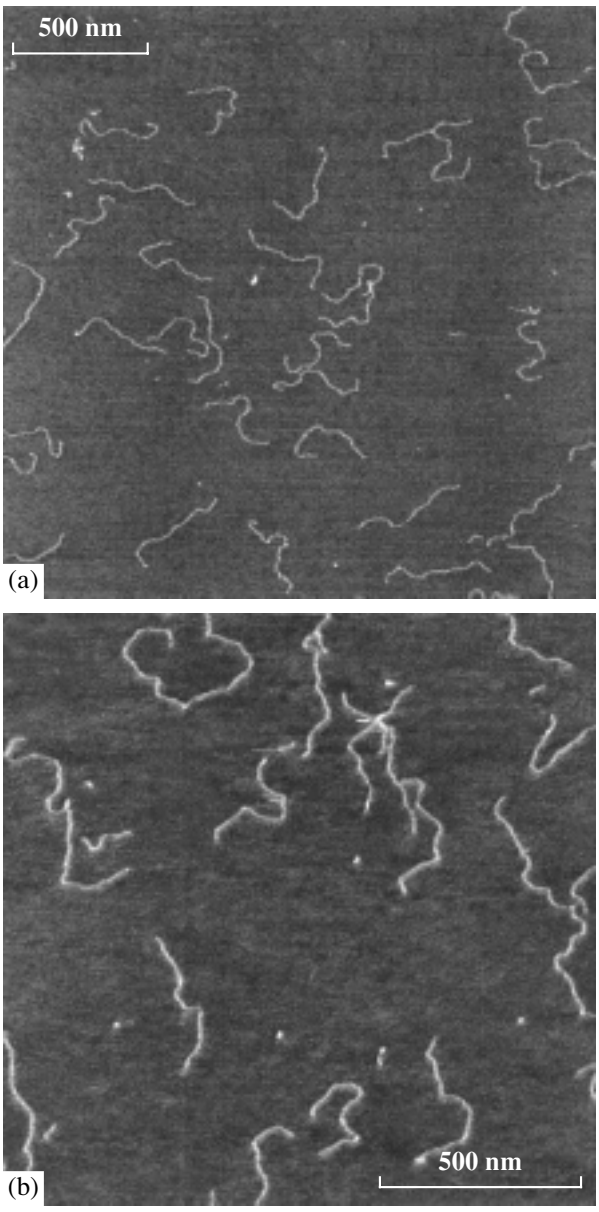

Fig. 3.

obtained at different T_{ann} values is shown in Fig. 2a. It can be seen that, at T_{ann} = 71°C (lanes *1* and *2*), sufficiently high quantities of the amplicon are produced by both DNA polymerases and nonspecific low-molecular-weight amplification products are absent; however, nonspecific high-molecular-weight amplification products are present in trace amounts, as judged by the tail above the intensive band of amplicons. An increase in temperature to 73°C resulted in a considerable decrease in the amount of amplicon synthesized by the Pyrobest TaKaRa DNA polymerase (lane *3*) and disappearance of the band corresponding to the amplicon synthesized by the Invitrogen Platinum DNA polymerase (lane *4*), which was indicative of the absence of synthesis of the PCR product under these conditions. After cutting off

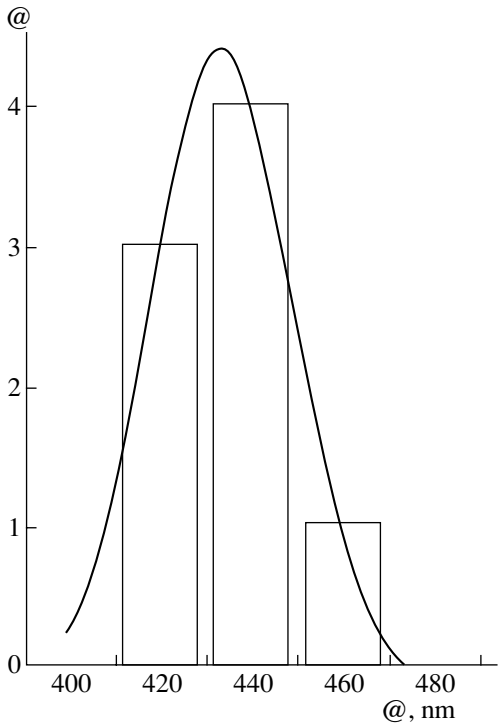

Fig. 4.

the bands of amplicons from agarose gel (lanes *1* and *2*, Fig. 2a) and purification (see EXPERIMENTAL), the preparations of PCR products, which contained only the expected DNA fragment 1414 bp in length (as evidenced from Fig. 2b), were visualized by AFM. The AFM images of the PCR product obtained with the use of DNA polymerases Invitrogen Platinum and Pyrobest TaKaRa are shown in Figs. 3a and 3b, respectively.

DNA samples were absorbed on the surface of freshly cleaved mica in HEPES buffer in the presence of Mg^{2+}. In this case, the surface of mica has a lower absorbing capacity compared to other known methods of mica treatment, such as aminosilication of mica surface in liquid [3, 4] and vapors of aminosilane derivatives [5]. For this reason, if DNA samples are placed on mica surface in the presence of Mg^{2+}, the surface properties of mica have a weaker effect on the conformation of amplicons. The authors of [7, 8] believe that this way of sample preparation for AFM shifts the equilibrium from the three-dimensional structure of DNA in solution to the two-dimensional structure on a mica surface. The loss of one degree of freedom allows DNA molecules to move in the two remaining directions. The plot of Gaussian distribution of the contour length of DNA amplicons is shown in Fig. 4. The obtained value of the contour length of DNA amplicons, (435 ± 15) nm, is lower than that predicted for B-form DNA by ~10%. Earlier, the authors of [7] reported that the contour length of DNA molecules measured on AFM images of molecules in air is always smaller than the theoretical length of B-form DNA molecules, assuming that the

distance between the nucleotide pairs is 0.34 nm. In the opinion of the authors of [7], possible causes of this effect may include insufficient resolution of the atomic force microscope, inaccuracy of the algorithm for calculating the contour length of DNA, and drying of the DNA sample before visualization with AFM. However, we think that the first two factors cannot lead to a statistically significant decrease in the contour length of DNA. Such a decrease in the length of a linear molecule by ~45 nm for an amplicon of 1414 bp in length (or 480 nm, assuming that DNA is in the B form) is significantly greater than the equipment error and considerably greater that the resolution capacity of AFM for macromolecules. Drying of a DNA sample after its application onto a mica surface may lead to the transition of molecules from the B form, characteristic of DNA molecules in solution, into a conformation distinct from the B form; this, in turn, may decrease the contour length of DNA molecules.

Knowing the contour length of the amplicon (L = 435 nm) with the 1414-bp sequence length, we determined the distance between the nucleotide residues of the amplicon and obtained h = 0.31 nm. Among the most widespread A, B, and Z families of double-stranded DNA, the A form of DNA is characterized by the greatest fluctuations in the distance h between nucleotides—from 0.26 to 0.33 nm. It is also known that DNA molecules pass to the A form if relative humidity drops below 76% [8, 9]. Note that the published data mentioned above were obtained for the crystalline form of DNA in fibers. However, if these data are extrapolated to DNA molecules in solutions or to genomic DNA in vivo, several important moments should be taken into consideration.

In addition to the procedure of sample drying, which can induce the B $\longrightarrow$ A transition of DNA molecules, the surface properties of mica (the support on which DNA molecules are adsorbed) may also significantly affect DNA conformation. It is known that, in an aqueous electrolyte solution, the surface properties of mica (primarily the surface charge density) are determined by the superposition of surface charges in accordance with their multiple isoelectric points. Because silanol groups are exposed on a mica surface at a ratio significantly exceeding the amount of imino and amino groups, the mica surface appears negatively charged within a broad pH range [10], including neutral pH values, at which DNA molecules are adsorbed for AFM visualization.

At the same time, it is known that, as a result of silication with aminosilane derivatives, pK values of amino groups of aminosilane near the mica surface decrease by 3 to 6 pH units compared to pK values of amino groups in solutions [11, 12], which points to a direct effect of the mica surface on the molecules adsorbed on it.

Our data are consistent with the results of other studies [7, 13]. The authors of [7] used AFM to measure the contour lengths of four DNA fragments 1008–1055 bp in length. The h value for these amplicons, determined on the basis of data presented in [7], constituted 0.31–0.33 nm; for the DNA fragment 538 bp in length (according to [13]), this value was 0.32 nm, which also corresponded to the characteristic distance between nucleotide pairs along the axis of the A-form DNA helix.

Visualization and measurement of the length of single DNA molecules after amplification by PCR showed that PCR yields amplicons with distribution of the contour length rather than DNA fragments with a strictly fixed length, as could be expected from results of gel electrophoresis after PCR. The heterogeneity of the contour length of amplicons in our study is ~7%; in [7] and [13], ~11 and 6%, respectively. We believe that the presence of the Gaussian distribution of the contour length of amplicons visualized by AFM can be explained by a heterogeneous surface charge density of the mica on which DNA molecules are adsorbed in the presence of Mg^{2+}, rather than by errors produced by DNA polymerase during the synthesis of amplicons (the probability of which during the synthesis of amplicons ~1400 bp in length is extremely low). Local distribution of the charge density in the vicinity of adsorption sites of DNA molecules may cause changes in the conformation of a DNA fragment (e.g., B $\longrightarrow$ A transitions), which, in turn, results in a decrease in the contour length of DNA.

CONCLUSIONS

AFM-based determination of the contour length of linear DNA molecules amplified by PCR showed that, despite the use of high-fidelity thermostable DNA polymerases, PCR yields DNA fragments (435 ± 15) nm in length. The distance between the nucleotide residues (h = 0.31 nm) along the axis of the helical duplex is within the value characteristic of DNA in the A form. It is assumed that the main factors that induce the B $\longrightarrow$ A transition of DNA molecules absorbed on the surface of freshly cleaved mica, after drying of the sample in a buffer containing magnesium ions, are the procedure of sample drying as such and the surface properties of mica.

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REFERENCES

1. Y. Lubchenko, B. Jacobs, and S. Lindsay, Nucleic Acids Res. **20** (15), 3983–3986 (1992).

2. Y. Lyubchenko, A. Call, L. Shluakhtenko, *et al.*, J. Biomol. Struct. Dyn. **10** (3), 589–606 (1992).
3. T. Okaiuma, H. Arakawa, M. Alam, *et al.*, Biophys. Chem. **107**, 51–61 (2004).
4. T. Kodama, H. Ohtani, H. Arakawa, and A. Ikai, Chem. Phys. Lett. **385**, 507–511 (2004).
5. A. Limanskii, Biopolim. Kletka **17** (4), 292–297 (2001).
6. T. Berge, N. Jenkins, R. Hopkick, et al., Nucleic Acids Res. **30** (13), 2980–2986 (2002).
7. C. Rivetti, S. Codeluppi, G. Dieci, and C. Bustamante, J. Mol. Biol. **326**, 1413–1426 (2003).
8. A. Perez, A. Noy, F. Lanks, *et al.*, Nucleic Acids Res. **32** (20), 6144–6156 (2004).
9. Saenger, W., *Principles of Nucleic Acid Structure*, Ed. by Cantor, C.R., (Springer, New York, 1984; Mir, Moscow, 1987).
10. H. Butt, Biophys. J. **60**, 1438–1444 (1991).
11. D. Vezenov, A. Noy, L. Rozsnyai, and C. Liebdr, J. Am. Chem. Soc. **119**, 2006–2015 (1997).
12. H. Zhang, H. He, J. Wang, *et al.*, Appl. Phys. **A66** (1998).
13. M. Lysetska, A. Knoll, D. Boehringer, et al., Nucleic Acids Res. **30** (12), 2686–2691 (2002).

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