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MOLECULAR BIOLOGY

Study of RNA Polymerase Transcription Through Nucleosome Using the Cryo-Electron Microscopy Approach

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Abstract— A promising and reliable method for the analysis of macromolecule structures is capturing images using cryo-electron transmission microscopy and consequently performing three-dimensional reconstructions. In this study, using cryo-electron microscopy, we analyzed the structure of the complex, formed by RNA polymerase stalled at position +42 during its transcription through the nucleosome. We obtained both projection images and a three-dimensional structure of the EC-42 complex at 2.5 nm resolution. This allowed us to confirm the conformational integrity of the nucleosome during the passage of the RNA polymerase.

Keywords: RNA polymerase, transcription, cryo-electron microscopy, nucleosome.

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Recent studies have shown that transcription elongation plays an important role in the regulation of the expression of many genes of higher eukaryotes. Regulation at the stage of elongation is most often performed when the RNA polymerase II (RNAP II) leaves the promoter and transcribes the DNA of the first nucleosome (at +1). This regulatory process involves enzymes that modify histones and remodel the chromatin, as well as various elongation factors. Current research in this field is focused on defining the nature of the nucleosome barrier for the RNAP II transcription, and determining the mechanisms that will allow to overcome it. *In vitro* studies have shown that a nucleosome structure becomes a serious obstacle for RNAP II—most RNAP II complexes stop at the first encounter with a nucleosome [1–4]. Higher nucleosome barriers occur at positions +15 and +45 of the nucleosomal DNA [4]; their height determines the efficiency of the chromatin transcription [5].

One of the more informative approaches for the study of mechanisms of chromatin transcription by RNA polymerase II is to stall the movement of a functioning polymerase complex at a certain position at the nucleosomal DNA during the transcription and to thoroughly study the properties of the stalled complexes (intermediates). Previously, studies of such complexes were performed using molecular genetic approaches [4]. Currently, the crystal structures of the nucleosome [6] and RNAP II [7] have been revealed. However, structural basis for the formation of the nucleosome barrier has not been sufficiently understood, and the complete RNAP-nucleosome complex has not been crystallized yet.

It is known that changes in protein conformation reflect onto its functional activity [8]. Thus, knowledge of spatial structures of macromolecular complexes allows us to interpret conformational changes in molecules during activation, inhibition, and ligand binding. This is necessary both for understanding mechanisms (and, thus, identifying domains of the protein as targets to fight a disease), and for design of novel drugs.

Widely recognized methods for the study of the structure and the conformational changes in protein molecules are: X-ray analysis, NMR, method of spin labels, luminescence detection, as well as other spectroscopic methods. Each of these methods has its advantages and limitations. Most of these approaches can detect only minor changes in a polypeptide chain, and only a few can pinpoint which part of the protein has undergone a conformational change and to what extent.

The advantage of transmission electron microscopy (TEM) over other structural biology methods is that this technology allows not only to visualize the three-dimensional structure, but can also reveal the dynamics (conformational rearrangements) of a variety of nanoobjects with resolutions ranging from 2–5 nm to atomic size (1.8 nm). In addition, the TEM method lacks many flaws of other structural methods:

there are no limitations in particle size, there is no need for crystallization; the amount and concentration of the sample are quite small. In addition, modifications of this method (cryo-TEM) allow one to examine macromolecules in their native hydrous environment, in a close to physiological state [9]. Combined with a single particle reconstruction method, cryo-TEM allows to obtain three-dimensional structures of macromolecular complexes [10].

In this study, we used a cryo-TEM method to obtain the three-dimensional structure of the complex, formed by *E. coli* RNA polymerase, stalled at position +42 during its transcription through the nucleosome.

MATERIALS AND METHODS

Nucleosome assembly. The matrix for the creation of the stalled complex (EC-42) was obtained using the polymerase chain reaction (PCR) method. To obtain the RNA polymerase complex with nucleosome, the nucleosomes were assembled from purified histones in the presence of T7A1-603/+42 DNA, containing a strong T7A1 promoter of the *E. coli* RNA polymerase, a 603 DNA sequence which supports the assembly of an accurately positioned nucleosome (located 50 bp from the point of the initiation of the transcription) and a DNA sequence which is responsible for stalling the RNA polymerase at 42 bp from the promoter-proximal border of the nucleosome [4]. For this, a mixture was prepared from the following components: T7A1-603/+42 DNA, 5M NaCl, 1M Tris, pH 8, 2% NP-40, 4mM EDTA, H2A/2B, H3/4, and water distilled to 50 μl. The NaCl concentration was gradually decreased from 2M to 10 mM by use of dialysis.

To obtain the transcription complex assembled nucleosomes with a concentration of 400 ng/μl (T7A1-603/+42, 269 n.b.p.) were used. A TB40 buffer was added to a mixture of nucleosomes with RNAP *E. coli* (nucleosomes (400 ng/μl), *E. coli* holoenzyme). The mixture was incubated at 37°C for 10 min. To form the EC-39 complex (containing 11 bp long RNA), 400 μm [ApUpC], 1 mM ATP, and 1 mM GTP were added to the mixture and incubated at 37°C for 10 min. Next, to form the EC-45 complex (containing 45 bp long RNA), Rifampicin, CH_3OH, 50 μM CTP and 150 μM KCl were added to the mixture, incubated at room temperature for 10 min, then followed by dialysis against TB300 for 2-3 h. To form the EC-42 complex, 1 μM mixture of CTP, UTP and GTP, and 25 μM 3'-dATP were added to the mixture and incubated at room temperature for 4 min. The RNAP stalling occurs at this precise position due to limiting the transcription reaction by one ribonucleotide. Due to lack of substrate, as the enzyme reaches a site on the matrix, wherein the corresponding ribonucleotide would be included to the generated transcript, it stops, due to lack of corresponding ribonucleotides in the solution.

As a result, a RNAP *E. coli* complex with a nucleosome stalled at position +42 (EC-42) with a concentration of 86 ng/μl was obtained. The purity of the complex was evaluated by electrophoresis.

Preparation of samples for cryo-TEM studies. The prepared samples in the amount of 3 μl were immediately applied to the grids using the Vitrobot Mark IV apparatus (FEI, United States). Quantifoil copper grids (Germany), with a diameter of 3 mm and 400 mesh/2.54 cm^2, coated with a plastic film containing 1.2 μm diameter holes, were used. Inside the Vitrobot Mark IV apparatus chamber a constant temperature (22°C) and humidity (95%) were maintained. The grids were clamped with tweezers and automatically, within 2 sec, blotted using paper filters to remove any excess liquid. Immediately thereafter, the tweezers holding the grid were instantaneously dipped in liquid ethane to generate amorphous ice, containing protein particles. Grids with applied samples were stored in containers, submerged in liquid nitrogen.

Cryo-electron microscopy and image processing. Grids were placed into a 626 Gatan cryo-holder (Gatan), and viewed in a Tecnai G2 Spirit TEM cryo-electron microscope (FEI, United States) at an acceleration voltage of 120 kV under low dose conditions (10 e/$Å^2$). Image capture was performed using CCD Eagle (FEI, United States) at a resolution of 4000 × 4000 pixels. Complex particles were collected from images in a semiautomated mode using the Boxer program [11] and were copied into a single file for further analysis and classification. 3500 complex particles have been collected in total. Image processing was performed using the Imagic software [12].

RESULTS AND DISCUSSION

A nucleosome complex with RNAP stalled at + 42 (EC-42) was obtained in an amount of 100 μl (86 ng/μl). Presence of correctly assembled complexes was confirmed by electrophoretic analysis. Samples containing complexes were frozen in vitrified ice. Since the process of freezing in cryo-microscopy happens instantly (in less than 1 μs [13]), we have reason to believe that the structure of the complex, corresponding to one of the phases of the RNA polymerase passing through the nucleosome, has been preserved in all the complexes to an equal extent. An identical structure of single complexes is a necessary prerequisite for the successful single particle three-dimensional reconstructions [14].

Because particles generally have no preferred orientation in ice, various projections display different views of the molecule (Fig. 1a).

To enhance the contrast of the cryo-images, we applied the combined effects of spherical aberration of the electron microscope and a defocus of 1.5–1.9 μm from the true focus parameters [15]. This action induces a phase shift defined by the Contrast Transfer

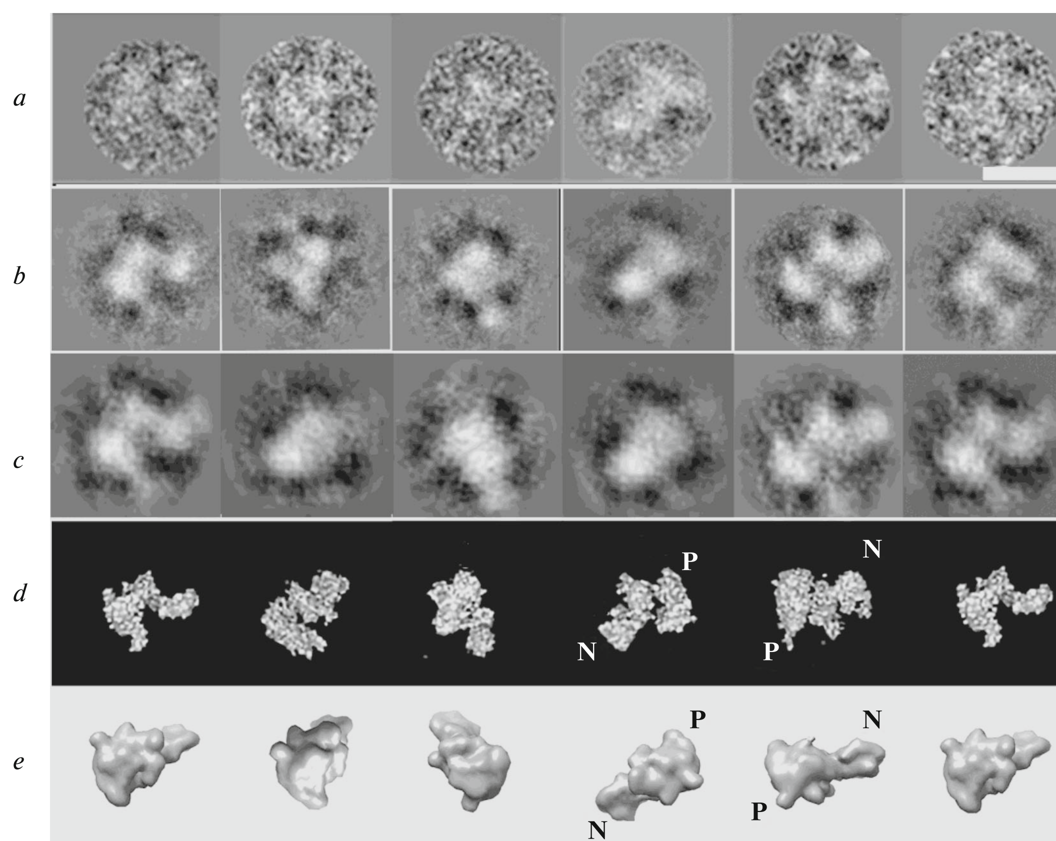

Fig. 1. Image analysis and a three-dimensional reconstruction of the EC-42 complex using cryo-data: *a*—individual EC-42 particles in ice after band-pass filtration, the contrast has been inverted (protein is white); *b*—class-sum images of complexes in different orientations; *c*—reprojections of the three-dimensional structure of the complex, calculated using images from (*b*) in corresponding orientations; *d*—a three-dimensional reconstruction of the EC-42 complex in corresponded orientations; *e*— RNAP (PDB ID: 2O5I) and nucleosome (PDB ID:1AOI) structures at 2.5 nm resolution in orientation corresponding to images in (*d*); N—nucleosome; P—RNAP. Scale bar, 20 nm.

Function (CTF). To recreate actual structural information, a CTF correction on the images was performed.

All 3500 projection images of the complexes were subjected to band-pass filtration to remove any unwanted spatial frequencies. Next, particles were centered by determining peak intensity for each signal, in comparison to the background. Thereafter, using the Imagic [12] software, each image was automatically shifted in a way that would allow the amount of peak intensity near its center to be at a maximum. The centered particles were aligned using statistical analysis [16] and an iterative algorithm.

For classification, similar images of particles were composed into separate classes. 100 classes of particles were obtained altogether, some of which are shown in Fig. 1*b*. For each class Euler angles were calculated – directions that determine the position and alignment of each particle relative to a common center. To perform a three-dimensional reconstruction of the EC-42 complex, we used a search of analogous projections in the Fourier space, e.a. in a space of reverse coordinates [14]. The Fourier transformation of the experimental projections is revealed in the formation of layers around a common center; this is why the crossovers in these projections are unique, and their relative alignment can be determined using three or more projections.

As result, a three-dimensional reconstruction of the EC-42 complex was calculated (Fig. 1*d*). The selected contour level for the visualization of the reconstruction was based on the data of the molecular weight of the complex ~550 kDa and an average density of the protein of 810 Da/nm^3. The resulting three-dimensional structure is represented by two connected electron densities, the larger being ~20 nm in height and ~10 nm in width, and the smaller having a diameter of ~8 nm. The obtained resolution, calculated using the Fourier shell correlation [17, 18] at a correlation coefficient of 0.5, proved to be 2.5 nm.

To interpret the obtained reconstruction, we used the crystal structures of the nucleosome [6] and RNAP [19], filtered at resolution of 2.5 nm using the UCSF Chimera program [20] (Fig. 1*e*). At this resolu-

tion, the dimensions of the reconstructed crystal structures approach the sizes of domains determined using cryo-TEM. The RNAP structure resembles the larger domain of the three-dimensional reconstruction (marked "P" in Figs. 1*d*, 1*e*). The smaller domain resembles the nucleosome (marked "N" in Figs. 1*d*, 1*e*). This interpretation is further supported by the correspondence of the spatial volumes of all subdomains and the crystal structures of the RNAP and nucleosome, at 2.5 nm resolution (Fig. 1 *d*). Domains in this configuration are interconnected with a short stretch of DNA, along which the RNAP normally moves. However, this connector cannot be detected at the obtained resolution.

This positioning of EC-42 complex components is consistent with an earlier hypothesis that the RNAP II-type transcription mechanism of chromatin allows to preserve H3/H4 histones bearing specific covalent modifications (a so-called "histone code") in original positions to maintain the epigenetic status of the cell [4]. According to this hypothesis nucleosomes do not shift during a RNAP II-type transcription, and only an exchange of H2A/H2B histones exist. Indeed, we obtained the three-dimensional structure of the transcription intermediate, stalled at position +42, contains two domains; this indicates that the nucleosome does not disassemble completely whilst passing the RNAP.

Thus, we were able to demonstrate a prominent possibility of studying RNAP complexes with nucleosome stalled in a certain position, using cryo-electron microscopy. Based on our data we built a three-dimensional reconstruction of the RNAP complex stalled at position +42 with a nucleosome, at a resolution of 2.5 nm. This complex consists of two domains, which correspond to the connected RNAP and nucleosome. The achieved results can be used as a starting model for the analysis and identification of surfaces which stabilize interactions in RNAP complexes with nucleosome during transcription.

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