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Citation	Biochemical and Biophysical Research Communications, 400(1), 181-186 https://doi.org/10.1016/j.bbrc.2010.08.041
Issue Date	2010-09-10
Doc URL	http://hdl.handle.net/2115/44031
Type	article (author version)
File Information	BBRC400-1_181-186.pdf

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**Intermediate structure between chromatin fibers and chromosome revealed by
mechanical stretching and SPM measurement**

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Abstract

The morphology of chromosomes (certain rod-shaped structures) is highly reproducible despite the high condensation of chromatin fibers ($\sim 1 \text{ mm}$) into chromosomes ($\sim 1 \text{ }\mu\text{m}$). However, the mechanism underlying the condensation of chromatin fibers into chromosomes is unclear. We assume that investigation of the internal structure of chromosomes will aid in elucidating the condensation process. In order to observe the detailed structure of a chromosome, we stretched a human chromosome by using a micromanipulator and observed its morphology along the stretched region by scanning probe microscopy (SPM). We found that the chromosome consisted of some fibers that were thicker than chromatin fibers. The found fiber was composed of approximately 90-nm-wide beads that were linked linearly. To explore the components of the fiber, we performed immunofluorescence staining of the stretched chromosome. Fluorescence signals of topoisomerase (Topo) II α , which is known to interact with and support chromatin fibers, and DNA were detected both on the found fiber and beads. Furthermore, after micrococcal nuclease and trypsin treatments, the fibers were found to be mechanically supported by proteins. These results suggest that chromosome comprises an intermediate structure between chromatin fibers and chromosomes.

Keywords

chromosome; scanning probe microscopy; intermediate structure; chromatin fiber

Introduction

The DNA of eukaryotic cells essentially condenses into chromosomes at the M phase of the cell cycle. The DNA is in the uncondensed form in the nucleus at interphase to synthesize proteins by transcription of the encoded genes. At the M phase, DNA condenses to form chromosomes; this plays an essential role in the equal segregation of the chromosomes. Thus, it is important to understand the structure of a chromosome and the process of DNA condensation leading to chromosome formation.

A chromosome consists of DNA and many kinds of proteins and has hierarchical architecture. DNA that is 2 nm in diameter is spooled around histone octamers to form nucleosome cores that are 11 nm in diameter, resulting in the beads-on-a-string morphology [1, 2]. The cores are further packed to form a chromatin fiber that is 30 nm in diameter [3-5]. Although the kind of substance that connects and condenses the chromatin fibers is unknown, they condense into a chromosome that is 700 nm in diameter.

In a chromosome, chromatin fibers are connected by scaffold proteins. When histone proteins are removed from a human chromosome by using a low concentration of dextran sulfate, the network-like structure of non-histone proteins remains intact and anchors with the loop-shaped DNA [6, 7]. These non-histone proteins are called scaffold

proteins. Topoisomerase (Topo) II α is proposed as a candidate scaffold protein [8]. These proteins are considered to be responsible for constituting and maintaining the chromosome morphology. However, whether scaffold proteins connect with each other and form a mechanically stable fibrous network continues to be a subject of discussion.

In newt chromosomes, scaffold proteins are not mechanically stable fibriform structures; instead, they are piecemeal structures. To decide whether scaffold proteins are mechanically stable fibrous structures, Poirier et al. applied a tensile force of approximately 100 pN to a newt chromosome and digested the DNA by using a micromanipulator [9]. The morphology of newt chromosomes is attributable to not only to the non-histone scaffold proteins but also to the short DNA chains with several thousands of base pairs; this is because treatment with both, micrococcal nuclease and restriction enzyme completely reduced the elastic contraction force of the stretched chromosomes. This result shows that there is no contiguous scaffold protein structure to which chromatin “loops” are merely tethered in a newt chromosome. However, it is not clear whether human scaffold proteins connect with each other and form a mechanically stable fibrous network.

Our aim is to clarify whether and how the stable structure of a chromosome is formed by chromatin fibers and scaffold proteins. Therefore, we unraveled a part of a

single human chromosome into fibers by external stretching with a micromanipulator and observed the morphology of the fiber by scanning probe microscopy (SPM). We found that the fiber was thicker than chromatin fiber. Furthermore, we confirmed that the found fibers were comprised of DNA and scaffold proteins by immunofluorescence microscopy. These results suggest that the found fibers consist of chromatin fibers and scaffold proteins, and the found fibers will be a hierarchical intermediate structure between chromatin fibers and chromosomes.

Materials and methods

Preparation of human chromosomes

Human cell line, BALL-1 (RCB0256), was obtained from RIKEN Cell Bank (Riken; Tsukuba, Japan). BALL-1 cells were grown in a 40-ml flask in the RPMI 1640 medium (Invitrogen; Tokyo, Japan) at 37 °C under an atmosphere containing 5% CO₂ and 95% air. After the cells were arrested with 0.06 mg/ml colcemide for 12 h, they were suspended and centrifuged at $190 \times g$ (1000 rpm) for 10 min at 4 °C. Thereafter, the cells were resuspended as a pellet in 10 ml of the culture medium for 20 min, centrifuged again at $190 \times g$ for 10 min, and then exposed to 75 mM KCl for 15 min. The cells were collected after centrifuging and isolated using the hexylene glycol method as described previously [10]. Briefly, the cells were gently resuspended in a hexylene glycol buffer composed of 1.0 M hexylene glycol (2-methyl-2,4-pentanediol), 0.5 mM CaCl₂, and 0.1 mM PIPES (pH 6.5) and incubated at 37 °C for 10 min. The cells were further broken using a Dounce homogenizer (15 ml capacity) with 5 gentle strokes [11] and centrifuged at $1680 \times g$ (5000 rpm) at 4 °C for 15 min in order to concentrate the crude chromosome suspension. It should be noted that the preparation process does not include any fixatives, which may strongly affect the mechanical properties of the chromosome.

Mechanical micromanipulation

The chromosome was spread on poly-L-lysine (PLL)-coated glass; PLL has positive electric charge, and hence, it binds to chromosomes. For confirmation, the chromosomes were stained with 1 g/ml 4',6-diamidino-2-phenylindole (DAPI) and observed by fluorescence microscopy. Micromechanical experiments were performed using an inverted microscope (IX70, 100 $\times$, 1.4 numerical aperture (NA); Nikon Instech Co.; Kawasaki, Japan) equipped with 2 micromanipulators (MP-285; Sutter Instruments; Novato, CA, USA) and a charge-coupled device (CCD) camera (KP-F120, Hitachi Kokusai Electric; Tokyo, Japan). The CCD camera and software can acquire phase-contrast images at 15 frames/s. A force-measuring micropipette was pulled and cut to have a deflection force constant of approximately 0.1 nN/ μ m and then mounted directly on the microscope stage to minimize mechanical noise. One glass micropipette was used to hold the chromosome and another to hitch and pull it in a hexylene glycol buffer. Force-extension experiments were performed by moving a stiff micropipette at a rate of 2 μ m/s while observing the deflection of the force-measuring micropipette. After each experiment, the force-measuring micropipette was calibrated according to the elastic contact with a standard specimen with a known spring constant. The standard

specimen was the cantilever of the scanning probe microscope with a spring constant of 0.18 N/m. The extension of the chromosome and the applied external force were estimated based on the distance between the glass capillaries and the deflection of the glass capillary.

Scanning probe microscopy

For the SPM measurements, we used a commercial instrument equipped with a piezoscanner with a maximum xy-scan range of 20 μm in each direction and a z range of 1.2 μm (SPA400 and SPI4000; Seiko Instruments Inc.; Chiba, Japan). For imaging, an atomic force microscope (AFM) was operated in a dynamic force mode (i.e., intermittent contact mode) in hexylene glycol buffer solution. It should be noted that the spring constant of the cantilevers was chosen to be comparable to that of the samples, because this reduces sample damage and retains maximum sensitivity for elasticity measurements. We used commercially available single crystal silicon cantilever with a tip height of approximately 7 μm and a tapered cone tip with a radius of curvature of approximately 10 nm (Au-ISC; Team Nanotec GmbH; Villingen-Schwenningen, Germany). The typical spring constant of this cantilever was 3 N/m; resonance frequency was approximately 25 kHz in liquid; scanning frequency was 0.25–0.5 Hz;

and the images were captured with the height mode in a 512×256 pixel format. The obtained images were plane-fitted and flattened by the computer program accompanying the imaging module.

Immunofluorescence

Mouse monoclonal antibody (catalogue number NA 14-100UG) against human Topo II α was purchased from Oncogene. This antibody shows specificity for Topo II α . For immunofluorescence, the stock was diluted to 1:100 in phosphate-buffered saline (PBS). The chromosomes were incubated with the antibody solution for approximately 17 h at room temperature and then washed with 0.01 M PBS for 3 times. Next, the preparations were incubated for approximately 6 h with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG Alexa Fluor 488 (Sigma; St, Louis, MO, USA) diluted at 1:200 in PBS. Thereafter, the slides were washed again in PBS for 3 times and the preparations reincubated for approximately 10 min with YOYO-3 (Invitrogen; catalogue number Y-3606) diluted to 1:2500 in PBS at room temperature. Finally, the slides were washed in PBS again.

Micrococcal nuclease and trypsin digestion assay

A tensile force of approximately 100 nN was applied to the human chromosome before exposing it to DNA and protein digestion enzymes. The enzymes were added to a sample buffer before monitoring the tensile force. Phase-contrast images were acquired at 1 frame/s or 10 frames/min after exposure to each enzyme; pipette positions (force and extension) and chromosome morphology were simultaneously recorded. For DNA digestion, micrococcal nuclease was obtained from TaKaRa (Bio Inc., Shiga, Japan); it is an endonuclease that digests the 5'-phosphodiester bonds of DNA, yielding 3'-phosphate mononucleotides and oligonucleotides. The final concentration of this enzyme in the sample buffer was decided such that it conformed to the manufactory's protocol for producing a DNA fragment of about 150 bp in 1 min at room temperature with a human chromosome as the substrate (data not shown). Trypsin was obtained from Gibco; it is a serine protease that catalyses the hydrolysis of peptide bonds. It was prepared at a final concentration of 0.25 % in appropriate sample buffers.

Results

We observed changes in the morphology of a single human chromosome after external stretching (Figure 1.A, Supplementary movie S1). External stretching transformed a part of the chromosome into a thin structure, (indicated by a red square in Figure 1.A) with abrupt extension of the chromosome. Because each area of the separated chromosome followed the movement of the micropipette (indicated by yellow arrows in Figure 1.A), we could confirm that the 2 separated parts were connected. The extension of the chromosome and the external force due to a moving glass capillary were estimated by measuring the distance between the glass capillaries and deflection of the glass capillary. The mechanical properties of the chromosome were divided into 2 stages: 1st, elastic deformation (Figures 1.B,C a–c) and 2nd, plastic deformation (Figures 1.B,C d,e). The 1st deformation involved a slow time-dependent increase in the extension, and the force acting on the glass capillary was directly proportional to the extension. The 2nd deformation involved a rapid increase in the extension, and the contraction force was inversely proportional to the extension. This abrupt increase in the extension and the decrease in force were due to the transformation of the chromosome into thin structure, as shown by the time-lapsed optical images (indicated by red arrows in Figures 1.B,C). The force which was required to transform chromosome was $1.6 \times$

$10^{-7} \pm 0.5 \times 10^{-7}$ N (mean $\pm$ standard deviation [SD], [n = 4]). These results suggest that chromosomes were transformed to a thin structure.

In order to clarify the thin structure, its morphology was observed in detail by SPM (Figure 2). Figure 2A and 2B show that the thin structure resembled a “string of beads” (indicated by a green arrow in Figure 2.B). Next, the chromosome that was stretched to approximately 2–3 times from its original length was observed by SPM (Figure 2.C). The stretched chromosome was composed of some fibers which were oriented parallel to the stretch axis, and exhibited the same morphology as that of the thin structure. To confirm the mechanical stability of the string of beads structure, we stretched some chromosomes on different-magnitude of force conditions. The widths of the beads were independent of the applied force. The averaged width was 89.2 ± 24.56 nm (mean $\pm$ SD, [n = 74]) (Figure 2.D). This width was considerably larger than that of a single chromatin fiber (ca. 30 nm). These results indicate that the string of beads structure was not a single chromatin fiber but a different mechanically stable structure.

Next, we investigated the molecular components of the string of beads structure by fluorescence staining. Figure 3 shows the fluorescence images of the structure when the stretched chromosomes were stained for DNA and Topo II α . Topo II α is one of the non-histone proteins and is considered as a scaffold protein involved in

chromosome condensation [7, 12]. Both DNA and Topo II α localized on the string of beads structure. These results suggest that the string of beads structure consists of chromatin fibers and scaffold proteins.

In order to clear the contribution of scaffold proteins and DNA to the mechanical stability of the string of beads structure, chromosome was digested with micrococcal nuclease or trypsin during stretching, and the force acting on the chromosome was monitored (Figure 4). The force was estimated from the deflection of the glass capillary and normalized to the initial force. Under short-term treatment of trypsin or micrococcal nuclease, trypsin reduced the force required to stretch chromosomes, on the other hand, micrococcal nuclease rarely reduced the force (Figure 4.A). Long-term treatment of micrococcal nuclease reduced the force to approximately 75% of the initial value (Figure 4.B). These results indicate that the morphology of unraveled fibers can be mainly attributed to scaffold proteins and to some extent to DNA.

Discussion

The unraveled fibers are a hierarchical intermediate structure between chromatin fibers and chromosomes.

In the present study, we unraveled the human chromosome into a fiber by mechanical stretching (Figure 1) and observed the structure of the unraveled fibers by SPM (Figure 2.A). This observation showed that the structure of the unraveled fibers resembled a string of beads with widths of approximately 90 nm. The widths were stable and not affected by the magnitude of the applied force (Figure 2.D). In addition, we found that the unraveled fibers comprised scaffold proteins and DNA (Figure 3). These results strongly indicate that the unraveled fibers are mechanically stable and composed of DNA and scaffold proteins.

Next, we discuss the morphology of the unraveled fibers based on the experiment results and the size of the chromatin fibers. Since the unraveled fibers were observed without dehydration or fixation, their morphology is thought to be natural. The apparent dimensions of the unraveled fibers obtained by SPM depended on the radius of the tip curvature and were apparently larger than the actual dimensions [13]. Since we used a cantilever in which the radius of the tip curvature was either less or approximately equal to 10 nm, the actual widths of the beads were considered to be a

little less than the value estimated by SPM (cantilever tip effect). The widths of the beads were approximately 90 nm; this width was considerably larger than that of a chromatin fiber (30 nm). Based on these facts, we elucidate that the unraveled fibers are definitely thicker than chromatin fibers and their morphology differs from that of the chromatin fibers. Moreover, we conclude that the unraveled fibers are a hierarchical intermediate structure between chromatin fibers and chromosomes.

The unraveled fibers have a mechanically stable fibrous structure of scaffold proteins.

We now discuss how chromatin fibers and scaffold proteins compose the unraveled fibers. We confirmed that the unraveled fibers contain scaffold proteins by fluorescence staining. Poirier et al. suggested that the scaffold proteins in newt chromosomes were not mechanically stable fibriform structures but piecemeal structures [9]. It was considered that the morphology of newt chromosomes was attributed to not only the non-histone scaffold proteins but also to short DNA chains several thousands of base pairs in length. However, we demonstrated that the force required to unravel a human chromosome was 1000 times larger (160 nN) than that required for disassembling a chromatin fiber to a nucleosome (10–60 pN) [14-16], a nucleosome to a histone core and DNA (~20 pN) [17], or the breakdown force of a

DNA filament (476 pN) [18]. This result indicates that the mechanical properties of the unraveled fibers, including the yielding force, are not attributable to the mechanical properties of chromatin fibers or DNA alone. In order to decide whether the complexes of scaffold proteins are mechanically stable fibrous structures in human chromosomes, we applied a tensile force of approximately 100 nN to a human chromosome and digested the DNA and proteins by micrococcal nuclease and trypsin, respectively. Trypsin digestion completely reduced the force required to stretch the human chromosome, while micrococcal nuclease digestion rarely reduced the force (Figure 4.A). These results indicate that the complexes of scaffold proteins are mechanically stable fibrous structures in the unraveled fibers.

The chromatin fibers regularly connect the axis of scaffold proteins in the unraveled fibers.

Here, we discuss the formation of chromatin fibers in the unraveled fibers. The observation of unraveled fibers revealed that the beads were linearly linked. Chromatin fibers regularly connect the axis of the scaffold proteins and condense to form the unraveled fibers. These fibers are considered to have a simple regular structure and not a disordered complex structure, since chromatin fibers of 30-nm diameter form beads

that are approximately 90-nm wide.

Next, we discuss chromatin fiber formation based on the mechanical properties of human chromosomes. Micrococcal nuclease digestion relaxed the elastic contraction force and reduced it to approximately 75% of the initial force (Figure 4.B). This result indicates that the morphology of the unraveled fibers is mainly attributable to scaffold proteins and to some extent to DNA, suggesting that DNA not only connects scaffold proteins in the unraveled fibers but also condenses to maintain the morphology of the scaffold proteins. These discussions suggest that the chromatin fiber is coiled to form the unraveled fiber in the center of the scaffold proteins.

Conclusion

We unraveled human chromosome into thin fiber structure and observed the structure in detail by SPM. Human chromosome consists of fibers with 90-nm-wide beads. The fibers are a hierarchical intermediate structure between chromatin and chromosomes. In the near future, we will report if the intermediate structure in human chromosome is observed in other animal species.

Acknowledgments

This study was supported by Special Coordination Funds from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan. It was also supported by a Grant-in-Aid (18340122, 14GS0301 for K. K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Figure legends

Figure 1. The morphology of a single human chromosome under external stretch. (A) Time-lapse images of a human chromosome under mechanical stretch in a hexylene glycol buffer. Two glass capillaries driven by a computer-controlled micromanipulator hitch 2 points of a single chromosome and stretch it at a constant velocity of 2 $\mu\text{m/s}$ (direction indicated by blue arrows). Two glass capillaries and the chromosome are indicated by green arrows and an orange circle, respectively. Time-lapse images of the human chromosome at 8.9, 10.6, 12.2, 13.9, and 14.9 s are labeled as a, b, c, d, and e, respectively. Mechanical stretching transformed a part of a chromosome into a filiform structure with abrupt extension of the chromosome. The filiform structure and the 2 parts of the separated chromosome are indicated by a red square and yellow arrows, respectively. Bar = 2 μm . (B) Time dependence of the extension of the chromosome under external stretch. The extension was estimated based on the distance between the glass capillaries. The extension increased slowly with time when it was 2~3-fold; however, the extension increased rapidly with time when it exceeded 3-fold. This abrupt increase in extension was due to the transformation of the chromosome into the filiform structure, as shown by the time-lapse optical images (indicated by a red arrow). (C) Curve showing the relationship between the extension of the chromosome and external

force. External force due to a moving glass capillary was estimated from the deflection of the glass capillary. The external force was directly proportional to the extension when the extension was 2~3-fold; however, the external force was inversely proportional to the extension when the extension exceeded 3-fold. This decrease in force was due to the transformation of the chromosome into the filiform structure due to extension, as shown in Figure 1.B by a red arrow.

Figure 2. Topography of the filiform structure measured by SPM in hexylene glycol buffer. (A) Topography and morphology of the filiform structure was observed in detail by SPM. Bar = 250 nm. (B) Close-up of the topography and morphology of the filiform structure. The unraveled fiber resembled a string of beads, as indicated by green arrows. Bar = 100 nm. (C) Topography and morphology of the stretched chromosome observed in detail by SPM. Bar = 500 nm. (D) Widths of the beads subjected to different magnitudes of force are measured and averaged. The average width was 89.2 nm (SD = 24.56, n = 74).

Figure 3. Fluorescence images of the unraveled fibers. (A) Phase-contrast image of the unraveled fiber of the chromosome (indicated by a red arrow). (B)

Immunofluorescence image of Topo II α . (C) Fluorescence image of the sample stained with YOYO-3. Bar = 5 μ m.

Figure 4. Mechanical response of the human chromosome microdigested with micrococcal nuclease and trypsin. A tensile force of approximately 100 nN was applied to the human chromosome, and the chromosomal DNA and proteins were digested using micrococcal nuclease and trypsin, respectively. The force that was normalized to the initial force by deflecting the glass capillary was measured and averaged. (A) Force applied for 2 min and (B) force applied for 60 min. Black squares represent normalized contraction force by applied initial force ($n = 3$), red circles represent it by applied micrococcal nuclease ($n = 3$), and green triangles represent it by applied trypsin ($n = 3$). Trypsin digestion for 1 min completely reduced the elastic contraction force, while micrococcal nuclease and buffer digestion for 2 min rarely reduced it. Micrococcal nuclease digestion for 60 min relaxed the elastic contraction force and reduced it to approximately 75% of the initial force.

A

B

C

D

A **B** **C**

A

B