Mechanical Elongation of the Centromere in the Barley Metaphase Chromosome

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Summary. The present study investigated the mechanical elongation of the centromere in the barley chromosomes by a microneedle manipulation method for the structural analysis of the chromosomes. Chromosomes were extracted from barley root cells, affixed on a cover slip by a standard preparation method, and elongated in either distilled water, phosphate buffered saline (PBS), or 2× sodium saline citrate (SSC). The mechanical property of the chromosome elongation was assessed by the measurement of the force required for the elongation of chromosomes. This assessment has shown that the chromosomes in distilled water were much firmer than those in the PBS or 2× SSC. To confirm the elongation of the centromere, the elongated chromosomes were investigated by fluorescence in situ hybridization with a centromere probe. The fluorescence information indicated that the extent of the loosening of the centromere during elongation differed depending on the buffers used; the centromere elongated in 2× SSC was more loosened than that in the PBS. Atomic force microscopy also revealed the structure of the unelongated centromere after the mechanical elongation, when rows of fibrous structures about 30 to 50 nm thick were clearly observed in the centromere elongated in 2× SSC. The investigation of elongated chromosomes should prove useful for an understanding of the structural analysis of chromosomes.

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Metaphase chromosomes are produced by the packing of chromatin fibers, which consist of DNA and its associated proteins. Several investigators have recently applied atomic force microscopy (AFM) to studies of the structure of extended or unraveled chromatin fibers and their manner of packing (Fritzsche et al., 1994; Leuba et al., 1994; Satô et al., 1999). These studies have suggested that the unpacking of chromosomes by micromanipulation could prove valuable in studies on the high order structure of chromosomes in relation to their functions. Such an approach to chromosome research has been conducted for the analyses of chromosomal movement in mitosis (Nicklas, 1983; Skibbens et al., 1997), G-bands (HLHCS et al., 1997), and the folding structure of chromosomes (Houchmandzadeh et al., 1997). More recently, Poirier et al. (2000, 2001, 2002) have further established an advanced method of analysis for unfixed native chromosomes of the newt by micromanipulation, and revealed dynamic mechanical properties of the chromosomes.

In regard to chromosomal structure, it is well known that the centromere is more specific and different than other regions of the chromosome (Rieder et al., 1998). The question of why the chromosome has such a specific structure raises interest from the viewpoint of genetic and cytological aspects because its structure is considered to be closely involved in chromosomal movement and separation in mitosis. However, chromatin fibers in this region are densely packed, and the structure hidden inside chromosomes present difficulties even for AFM observation. The present study therefore aimed at introducing an elongation method for the centromere regions of barley chromosomes for the analysis of their structures. It has succeeded in elongating the centromere by micromanipulation and subsequently...
investigated the elongated centromere by fluorescence in situ hybridization (FISH) with a centromere probe and by atomic force microscopy (AFM).

**MATERIALS AND METHODS**

**Chromosome preparation**

Barley (*Hordeum vulgare*, cv. Minorimugi) chromosomes were prepared by a modified dillé's method (1990) as follows. After the seeds were germinated on moist filter paper in Petri dishes, the roots were cut and placed in colchicine solution (5 μg/ml) at 0°C for 24 h. They were then fixed with a methanol-acetic acid (1:1) fixative for 10 min under an aspirated condition and further immersed in a fresh fixative for 60 min. After washing for 25 min in distilled water, the root tips (1 mm in length) were cut off and digested with an enzyme solution (2% cellulase Onozuka RS, 1% pectolyase Y-23, 7.5 mM KCl and 7.5 mM EDTA, pH 4.0) at 37°C for 60 min. The resultant suspension (consisting mainly of meristem cells) was centrifuged and resuspended in distilled water for 30 min for hypotonic treatment. For storage, the cells were fixed with Carnoy's fixative (methanol: acetic acid = 2:1). A drop of this sample suspension was spread on a cover slip, which had been pretreated in 2N NaOH for 90 min to acquire hydrophilicity. The cover slip was washed with a few drops of Carnoy's fixative to remove indigested fragments or cytoplasm, and air-dried. Unless otherwise noted, all preparations were conducted at room temperature.

**Force measurement during chromosome elongation**

To investigate the mechanical properties of the elongation, the force required for stretching the chromosomes at their centromeric regions was measured in three different solutions: distilled water, phosphate buffered saline (PBS), and 2×sodium saline citrate (SSC). For the force measurement, two different microneedles, one stiff and the other pliant, were controlled independently by different micromanipulators (OUN-31P, Olympus, Tokyo). The stiff microneedle was used to peel the chromosome from the substrates, and the pliant needle (tip diameter of 1.3 μm) was utilized for the force measurement. The latter needle has an elasticity of 165 nN/μm, which was calibrated by using an AFM cantilever with a spring constant of 0.08 N/m (OMCL-TR400PSA-H1W, Olympus) against the perpendicular force to deflect the needle tip; this calibration was conducted by the analysis of the force-displacement ratio of the needle and cantilever observed under an inverted light microscope. In the measurement of the stretching force, the procedure consisted of three steps (Fig. 1). First, a centromere of the target chromosome affixed on a cover slip by the chromosome preparation was partially scraped and peeled from the cover slip using the stiff needle (Fig. 1a, b). Secondly, the tip of the pliant needle was hooked at the peeled region, and the microscope stage was driven in a direction perpendicular to the long axis of the chromosome with a motion rate of 1 μm/sec (Fig. 1c). Consequently, the centromere and the other regions were dragged and stretched together by the needle (Fig. 1d) until the distortion of the chromosome or its unhooking occurred. Lastly, the stretching force and resultant elongated length of the chromosome were estimated from the video images of the stretching process, based on the spring constant and deflection of the needle tip.

**Mechanical elongation of chromosomes**

The chromosomes prepared were elongated in the PBS or 2×SSC. One milliliter of the buffer was deposited on the area of the cover slip, where locations of the chromosomes had been preliminarily confirmed in the bright field by light microscopy. The elongation process was as follows (Fig. 2). By means of a stiff microneedle (less than 1 μm in a tip diameter), a centromere region of a target chromosome was dragged slowly and perpendicularly to the long axis of the chromosome (Fig. 2b). One arm of the chromosome was subsequently peeled from the cover slip with the arm adhering to the needle tip (Fig. 2c) and elongated to approximately twice that of its original state (Fig. 2d, e). The elongated chromosome spontaneously adhered to the cover slip, owing to its hydrophilicity. After removing the buffer deposited on the cover slip by suction through a pipette, the area was rinsed twice by 2 ml distilled water to avoid the crystallization of salt in the buffer. The deposition and removal of the distilled water were also made by using a pipette. These unpacking processes described above were conducted at 25°C.

**Fluorescence in situ hybridization (FISH) with a centromere probe**

The barley GC-rich centromeric sequence was amplified by PCR (Hudakova et al., 2001) and used as a FISH probe. Tetrameres of the AGGGAG motif and its complementary sequence were used as primers for PCR in the absence of template DNA under the following conditions: 10 cycles consisting of 60 sec at 95°C, 30 sec at 58°C, and 60 sec at 72°C; the next 30 cycles of 60 see at 95°C, 30 sec at 60°C, and 90 sec at 72°C, and final extension for 5 min at 72°C. The primer-multimer products were labeled with
**Fig. 1.** Time-serial images (light microscopy) of the force measurement based on the microneedle deflection caused by the stretching of chromosomes fixed. a and b. A centromere region of the target chromosome was moderately scraped and peeled from the cover slip using a stiff microneedle. c and d. The tip of a pliant microneedle was hooked at the region peeled and fixed. Then the microscope stage was driven in the direction perpendicular to the long axis of the chromosome. Consequently, the centromere region and also the other regions were dragged and stretched by the needle.

**Fig. 2.** Time-serial images (light microscopy) of the elongating process conducted in a PBS buffer. a and b. By means of a microneedle (less than 1 μm in diameter), a centromere region of a target chromosome was dragged slowly and perpendicularly to the long axis of the chromosome. c. One arm of the chromosome was peeled from the cover slip with the arm adhering to the needle tip. d and e. The chromosome was elongated to approximately twice the length of its original state.
biotine-16-dUTP (Roche Molecular Systems, Inc., NJ, USA) using a Nick Translation Kit (Roche). The standard FISH protocol employed for labeling the chromosomes is described below. The cover slip with the chromosomes was serially rinsed with RNase (100 μg/ml in 1× SSC) at 37°C for 60 min, and three times with 2× SSC for 5 min. After being dehydrated by an ethanol series (70% for 10 min twice, and 100% for 5 min) and overnight incubation at 65°C, the chromosomes were denatured at 70°C for 2 min in 70% formamide in 2× SSC and then immediately placed serially in ice-cold 70% ethanol for 5 min and 100% ethanol for 5 min. The biotinylated DNA probe was denatured at 75°C for 10 min and quenched in an ice-cold bath. The DNA probe and target chromosomes were hybridized at 37°C for 20–24 h in a humid chamber. After the hybridization, the samples were successively washed with 50% formamide in 2× SSC at 37°C for 20 min, 2× SSC for 15 min, and 1× SSC for 15 min (twice). A biotin-labeled DNA probe was detected by 25 μg/ml Alexa488-streptavidin conjugates in 4× SSC containing 10 mg/ml bovine serum albumin fraction V at 37°C for 45 min. To remove non-specifically bound Alexa488-streptavidin conjugates, the samples were rinsed serially with 4× SSC, 0.1% TritonX in 4× SSC, 4× SSC, and 2× SSC for 5 min each. They were finally mounted with 2.5 μg/ml propidium iodide (PI) in an anti-bleach mounting medium (Vectorshied, Vector Laboratories, Burlingame, CA, USA). Unless otherwise noted, all preparations were conducted at room temperature.

Atomic force microscopy of elongated chromosomes

Topographic images of chromosomes after the FISH treatment were obtained by using an AFM (NVB100, Olympus) in air. The tapping mode was used as an operation mode with a standard silicon nitride cantilever (OMCL-AC160TS-C2, spring constant 42 N/m, resonance frequency 300 kHz, Olympus). The set point voltage was adjusted to the lowest value to avoid damage to the samples.

RESULTS

Force measurement of chromosome elongation

The stretching force during chromosome elongation was investigated in distilled water, PBS, or 2× SSC. In the observation of the stretching process, fixed barley chromosomes soaked in the distilled water appeared elastic like a spring, and were much firmer than those in the buffer solutions. Because of this elastic property, unhooking of the needle tip from the hooking position of the chromosomes was readily accomplished, resulting in a shortage of the elongation. This elastic property of the chromosomes in the distilled water was correlated with elongation rates between the original length of the chromosome and its elongated length. When the elongation rates ranged from 1.5 to 2, the elongation was reversible, the chromosome behaved like a coiled spring, and the ratio of the force to the length represented an apparent spring constant approximated at 147 nN/μm. On the other hand, a rate of more than 3 resulted in the irreversible elongation of the chromosome, in that the chromosome was distorted and permanently elongated.

In contrast, the length of the elongation in the buffers (i.e., PBS or 2× SSC) was greater than that in the distilled water. The stretching forces were also considerably smaller than those in the distilled water, indicating that chromosomes in the PBS or 2× SSC were very soft. An apparent spring constant of the chromosome was unable to be defined in these buffers because an irreversible elongation immediately occurred even over a slight range of elongation, though the chromosomes in 2× SSC tended to be disorganized more easily than those in the PBS.

Process of mechanical elongation of the chromosomes

In the buffers, the chromosomes appeared soft and sticky, since the stretched chromosomes easily adhered to the hydrophilic cover slip, which resulted in the retention of the stretched state. The elongation process in 2× SSC was similar to that in the PBS, although the elongation in the 2× SSC often easily provided a distortion of the chromosome like a mass of torn cotton.

FISH analysis of mechanically elongated chromosomes

In FISH treated specimens, the centromeric sequence was successfully hybridized and the centromeric region emitted green fluorescence. In specimens stained with PI after the FISH treatment, a yellowish band corresponding to the centromeric region was clearly distinguished in the primary constriction of the chromosomes (Fig. 4).

Intensity of the centromere signals of the elongated chromosomes appeared markedly different depending on the buffer conditions used for the elongation. Fluorescent bands of the centromeres were mostly distinguishable in the elongated chromosome in the PBS, though their intensities were weaker than those
Fig. 3. Force-enlongation relationship measured in distilled water (DW), phosphate buffered saline (PBS), or 2×SSC, based on the microneedle deflection caused by the stretching of chromosomes fixed.

Fig. 4. Fluorescence images of barley chromosomes containing elongated ones. After the elongation in PBS (a to c) or 2×SSC (d to f), their centrometers were FISH-labeled by the centromeric sequences with Alexa488 (green) followed by counterstaining with PI (red). Yellowish bands show that green and red fluorescence were superimposed.
Fig. 5. Topographic AFM images of elongated chromosomes containing their centromeres defined by the FISH signals shown in Figures 4b and 4e. a to b. The chromosome elongated in PBS is shown in Figure 4b. d-f. The other one elongated in 2×SSC is shown in Figure 4e. Rectangular areas in the left images, covering the centromeres defined, were magnified and shown in the right ones. Height scales are common to all the images as indicated in the left ones.

of the intact chromosomes. In such a case, the centromeres were stretched and transformed along the long axis of the chromosomes. In contrast, elongated chromosomes in the 2×SSC condition showed extremely dim bands of the centromeres, which could be difficult to distinguish in the images for reproduction (Fig. 4d, f).

AFM imaging

In the topographic images of the chromosome elongated in PBS, the height of the elongated chromosome was generally less than 50 nm. In the topographic images of the centromeric region in the chromosome elongated in 2×SSC and defined by the FISH signal, fiber-like stripes arranged in parallel along the long axis were clearly observed, suggesting that those fibrous structures are derived from the unpacked chromatin fibers in the centromere.

DISCUSSION

The present study has shown the physical properties and structural changes of barley chromosomes during or after their mechanical elongation by micro-needle manipulation. We have measured the force required for elongating the chromosomes and clarified that the chromosomes in distilled water are far firmer than those in PBS or 2×SSC, and that these buffers facilitate the elongation.

The stretching forces measured in the distilled water provided markedly higher values than those in the other solutions, while the lengths in the distilled
water were somewhat shorter. Clausensen et al. (1994) reported that there was no distinguishable difference in the stretching manner of human chromosomes immersed in distilled water, PBS, saline solution, or ethanol. However, our observation of the stretching process showed that fixed barley chromosomes soaked in the distilled water were elastic like a spring, and much firmer than those in the buffers.

It is interesting that fixed barley chromosomes soaked in distilled water were elastic like a spring. As for the force required for chromosome elongation, Poirier et al. (2000, 2001, 2002) directly extracted unfixed mitotic chromosomes from the newt nuclei and then measured the force in a suspended state; the force to stretch unfixed mitotic chromosomes of the newt was ~1 nN for their elongation by several micrometers. In contrast, the force that we have obtained in this study shows more than several hundred nanonewtons. Because the chromosomes investigated in our work were fixed on substrates by a standard method, it is probable that the fixation by standard fixatives influences mechanical properties of the chromosomes, though the species difference between the newt and barley should not be overlooked.

The FISH observation of the centromeres elongated in the buffers indicates that the homogeneous expansion of the centromere regions in the buffer was made by a loosening of their structures. Our studies have also shown that the centromeres elongated in 2×SSC might be expanded more apparently than those elongated in the PBS because their signals were faint. These findings indicate the composition of the solution for chromosome accommodations is important for the tight packing of the chromosomes.

By AFM, the height of the chromosome elongated was mostly less than 50 nm. Heights of barley chromosomes, which were fixed by a standard method and air-dried, were reported as 600–700 nm by AFM (Schaper et al., 2000). Thus, a certain influence of the FISH treatment and also the elongation to the height change is assumed.

Topographic AFM images of elongated chromosomes treated by FISH also show the presence of fibrous structures appearing in the centromere regions, which were especially enhanced in 2×SSC. These findings indicate that mechanical elongation of the chromosome holds promise for use in AFM studies of the high order structure of chromosomes.

As a future study, we are planning more precise analyses of the force-elongation relationship under various buffer conditions which can be obtained by microneedle manipulation.

REFERENCES


