Chromatin Reconstitution: Development of a Salt-dialysis Method Monitored by Nano-technology

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Summary. The regulation of DNA replication and transcription is achieved by dynamic structural changes of chromatin in which a series of proteins will acquire accessibility to specific regions of the DNA strand. A combination of biochemistry and nano-technology is essential to address questions regarding the structural basis for such macromolecular mechanisms. In the present study, we established an efficient salt-dialysis method of chromatin reconstitution and employed atomic force microscopy (AFM) as a single-molecule-imaging technique, to monitor the efficiency of the reconstitution. At first, the reconstitution efficiency with short DNA molecules of several kilo-base pairs was low, although the salt dialysis yielded a “beads-on-a-string” structure of oligonucleosomes with each nucleosome trapping 158 ± 27 bp DNA. However, the efficiency for nucleosome formation became higher when longer DNA molecules with a super-helical constraint were used. A statistical analysis of the obtained AFM images identified a first-order relationship between the efficiency of the reconstitution and the length of the super-coiled DNA used. A high efficiency

of ~ 290 bp/nucleosome that is close to the *in vivo* situation was obtained with a ~ 100 kbp template DNA. This enabled the structure-function studies of long chromatin molecules under well-defined conditions.

Since the proposal of the double helix model of DNA in 1953, a large number of structural studies that utilize techniques including NMR and X-ray crystallography have developed the molecular account of the interactions between DNA and DNA-binding proteins. Although a combination of techniques in conventional biochemistry and structural biology has been very useful for analyzing the interactions between the regulatory proteins and naked short DNA, the elucidation of the higher-order structures of several thousands of base-pairs or longer DNA associated with nuclear proteins has yet to be realized due to difficulties in crystallizing the complexes.

On the other hand, atomic force microscopy (AFM) has become a standard technique visualizing the structures of DNA and DNA/protein assemblies. Structural analyses of nucleic acids and specific binding proteins by AFM have revealed the distinct higher-order structures of DNA, including supercoiling, stem-loop structures and enhancer-promoted DNA loops (REES et al., 1993; OHTA et al., 1996; CARY et al., 1997; YOSHIDA et al., 1999; SHLYAKHTENKO et al., 2000; YOSHIMURA et al., 2000a, b).

A number of proteins play crucial roles in the maintenance of well-organized higher-order structures of DNA in cells. In bacteria, the genome DNA is packed in a cell ($\sim 1 \mu\text{m}$ in diameter) as a form of a “nucleoid” (PETTIJOHN, 1996). In eukaryotic cells, DNA exists in chromatin form and is packed in a nucleus (KORNBERG, 1974; MCGHEE and FELSENFELD, 1980). In either case, a set of distinct structural

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DNA-binding proteins, such as histones in eukaryotic cells, constitutively play major roles in organizing DNA into higher-order structures (FELSENFIELD and MCGHEE, 1986; WOODCOCK and DIMITROV, 2001). The gene regulation by a set of proteins must be achieved via a series of structural changes of the higher-order DNA/protein complexes (OWEN-HUGHES and WORKMAN, 1994; JACKSON and COOK, 1995; HORN and PETERSON, 2002), and thus the molecular mechanisms of gene regulation must be understood at the chromatin level. The structures of nucleosomes and chromosomes have also become the subject of AFM study (ZLATANOVA et al., 1994; SATO et al., 1999; HOSHI and USHIKI, 2001). However, due to a lack of reliable methods for the specimen preparation, little progress has been made in the structural analyses of large chromatin complexes. In addition, although it is now widely accepted that the chromatin structure plays important roles in gene regulation and other genomic events, little is known about the physical characteristics of the chromatin fiber itself.

The aim of this study to develop a biochemical specimen-preparation procedures led us to establish a high-efficiency *in vitro* reconstitution method, and applied AFM technology to the characterization of the physical properties of long chromatin fibers. Since chromatin is a fundamental structure of the chromosome, the understanding of the chromatin fiber is indispensable for a total understanding of the genome function.

MATERIALS AND METHODS

Purification of histone octamers

Core histones were purified from HeLa cells according to the method developed by O'NEILL et al. (1992), with slight modifications. The cells were harvested, washed with PBS, and then lysed with L-buffer (140 mM NaCl, 10 mM Tris-Cl [pH 7.5], 0.5% Triton-X100). Nuclei were isolated by low speed centrifugation and washed by W-buffer (350 mM NaCl, 10 mM Tris-Cl [pH 7.5]) for three times. The nuclei were then treated with micrococcal nuclease (40 units per mg of DNA) in D-buffer (10 mM Tris-Cl [pH 7.5], 1.5 mM MgCl₂, 1 mM CaCl₂, 0.25 M sucrose, 0.1 mM PMSF) at 37°C for 15 min. The reaction was stopped by an addition of EGTA to a final concentration of 2 mM, and the nuclei were pelleted by centrifugation at 10000 g for 5 min. The pellet was re-suspended in N-buffer (10 mM Tris-Cl [pH 6.8], 5 mM EDTA, 0.1 mM PMSF), and dialyzed against N-buffer overnight at 4°C. The sample was centrifuged at 10000 × g for 10 min and the soluble chromatin supernatant was

retained. This supernatant was re-dialyzed against HA-buffer (0.1 M NaPO₄ [pH 6.7], 0.63 M NaCl), and mixed with hydroxyapatite resin (Bio-Rad, USA). After batch binding at 4°C for one hour, the resin was packed into a column, and washed in 5 volumes of HA-buffer. The core histones were eluted by E-buffer (0.1 M NaPO₄ [pH 6.7], 2 M NaCl). The eluate was applied to gel-filtration column (HiPrep 16/60 S-200, Amersham Biosciences, USA) to separate the octamer from H3/H4-tetramer, H2A/H2B-dimer and other contaminants.

DNA templates and nucleosome reconstitution

The 437 bp DNA template contains a two 197 bp tandem repeat of a *Xenopus borealis* somatic 5S RNA gene and associated upstream sequences (−64 to +122 relative to the start site of transcription, +1) (O'NEILL et al., 1992). For longer DNA preparations, human centromere-specific repetitive sequences (alphoid DNA) of various lengths (25-kbp, 50-kbp, 100-kbp fragments) were subcloned into either pUC119 (2-kbp fragment) or a bacterial artificial chromosome (BAC) (SHIZUYA et al., 1992) to stably maintain the long DNA fragment. (The 50-kbp and 100-kbp plasmids were kindly given by Dr. IKENO of the Fujita Health Science University.) The long DNA were prevented from freezing and kept at 4°C until use.

Nucleosomes were reconstituted by the salt dialysis method (URA et al., 1995). Purified core histones (0.6 µg) were mixed with template DNA molecules (0.5 µg) varying lengths in 50 µl of a buffer containing 2.0 M NaCl, 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM PMSF and 1 mM 2-mercaptoethanol at 4°C. The samples were dialyzed at 4°C against a buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 1 mM 2-mercaptoethanol and varying concentrations of NaCl as follows: 2.0 M NaCl, 1 h; 1.5 M NaCl, 4 h; 1.0 M NaCl, 4 h; 0.75 M NaCl, 4 h. The final dialysis was performed in 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 mM PMSF and 1 mM 2-mercaptoethanol at 4°C overnight.

Sample preparation for AFM imaging

Unless otherwise indicated, reconstituted nucleosomes were fixed with 0.1% glutaraldehyde at 4°C overnight. After overnight fixation, nucleosomes were applied onto a spermidine (1 mM)-treated mica surface. After 3 min, the mica was gently washed with distilled water and dried under nitrogen gas. Imaging was performed using a Nanoscope IIIa with type E scanner (Digital Instrument Inc., USA) in air under Tapping Mode™ at room temperature. Probes used were made of a single silicon crystal with cantilever length of 129 µm and spring constant of 33–62

N/m (Digital Instrument Inc.). Imaging was performed in a height mode with a scanning rate of 3–4 Hz and a driving amplitude of 40–80 mV. The images were captured in a 512×512 pixel format and the captured images were flattened and plain fitted before analysis.

RESULTS

When a short DNA (~450 bp) containing two 197 bp tandem repeats of the 5S RNA gene was used as a template in the salt-dialysis reconstitution, a variety of nucleosomes were formed; i.e., mono-nucleosome, di-nucleosomes, and tri-nucleosomes (Fig. 1a, b, c). Among them, the major population was the mono-nucleosomes. The statistical analysis of the nucleosome position along the template DNA revealed that most of the nucleosomes were located at the end of the DNA strand (Fig. 1d, e) (see DISCUSSION).

When chromatin fibers were reconstituted on the linearized 3-kbp plasmid, a well-known 'beads-on-a-string structure' was observed under the AFM (Fig. 2a). Detailed analyses of the AFM images revealed that ~150-bp DNA was incorporated in a nucleosome (Fig. 2b). This salt-dialysis method, however, has a limitation in that the efficiency of the reconstitution was very low.

To evaluate the effect of DNA length on the reconstitution, we compared the efficiency of nucleosome formation on the DNA fragments with different lengths (3 kbp, and 50 kbp) (Fig. 3a). In these image analyses, the number of nucleosomes on each DNA molecule was counted and taken into account to the calculation of a frequency of nucleosome formation as [length of DNA (bp) used]/[number of nucleosomes formed]. In the case of linear 50 kbp DNA, the efficiency was 650 bp/nucleosome, while that of the 3 kbp DNA was 640 bp/nucleosome (Fig. 3e). Thus, longer DNA did not improve the reconstitution efficiency when linearized.

However, when a supercoiled circular plasmid of 50 kbp was used, the efficiency was drastically increased (Fig. 3b). In this case, the efficiency was 310 bp/nucleosome, a half of that of the linearized case (Fig. 3e). We then prepared a series of circular plasmid DNA with various lengths (3, 5, 30, 50, and 100 kbp) and analyzed the efficiency of the reconstitution. In the case of the supercoiled 3 kbp, 5 kbp (Fig. 3c), 30 kbp, and 50 kbp (Fig. 3b) plasmid DNAs, the efficiencies were calculated to be one nucleosome per 830 bp (i.e., 830 bp/nucleosome), 770 bp, 410 bp, and 310 bp, respectively (Fig. 3e). For the supercoiled 100-kbp plasmid (Fig. 3d), it was 290 bp/nucleosome.

Fig. 1. Typical AFM images of chromatin reconstituted on a 437-bp DNA fragment by the salt dialysis method. The imaging was done by Digital Instrument Nanoscope IIIa with a type E scanner under tapping mode in air at room temperature. They contain one (a), two (b), or three (c) nucleosomes. d. AFM image containing many chromatins. Many of them had a nucleosome at the termini. e. Probability distribution of the position of histone on the DNA chain. R_p is measured from the AFM image as shown schematically in the figure.

Fig. 2. An AFM image of reconstituted chromatin and an analysis of the relationship between the chromatin lengths and the number of nucleosomes. **a.** AFM image of the reconstituted chromatin with core histones purified from HeLa cells and linearized pBluescript DNA. Reconstituted sample was put on spermidine-treated mica washed with distilled water, and dried under nitrogen gas. **b.** Linear relationship between the apparent length of the reconstituted chromatin and the number of nucleosomes formed on the DNA. The length of chromatin strands with different numbers of nucleosomes formed was measured from one end to the other. The data were collected from 50 individual images and used for a calculation of the length of DNA.

Namely, as the length of the supercoiled plasmid became longer, the length of DNA necessary for a nucleosome formation became shorter. When we used the circular plasmid DNA relaxed by topoisomerase I, the efficiency was very low (3500 bp/nucleosome) (data not shown). Thus the tension of the supercoiled template DNA must be critical for this cooperative nucleosome formation.

A close look at the nucleosome formation found that the nucleosome formation was biased for the DNA molecule (Fig. 4). Namely, an apparent random distribution of nucleosomes seen in a several kbp chromatin could not be observed in a several ten-kbp chromatin. There were always the scattered regions containing a few far-isolated nucleosomes (Fig 4b, arrowhead) and compacted regions with many nucleosomes gathering and sometimes touching each other. These results suggest that the assembly of nucleosome on a supercoiled DNA is a cooperative process.

DISCUSSION

In this report, we developed an efficient protocol for *in vitro* chromatin reconstitution by salt dialysis, and investigated the characteristics of the nucleosome

formation with an aid of AFM. Compared with the chromatin reconstitution procedure by using cell extract (BECKER and WU, 1992) that has a specific merit for examining chromatin functions, the advantages of the salt-dialysis method are in its simplicity and flexibility that allow one to change experimentally the environment and examine the changes in the physical properties of chromatin.

Dynamics of histone-DNA interaction

The simplest structure of the chromatin is the mononucleosome. From the structural observation of the nucleosome reconstituted on the short DNA of ~450 bp, we found that most of the nucleosomes were located at the end of the DNA strand (Fig. 1). The molecular mechanism of this phenomenon was clearly explained by the result of the theoretical simulation of histone-DNA interaction (SAKAUE et al., 2001). When the interaction between DNA and histone was strong, nucleosome-like structure was not formed because DNA randomly binds to the protein surface. However, when the attracting force between DNA and histone was moderate, the nucleosomal structure was stably formed and, intriguingly, histone could slide along the DNA strand with the nucleosome structure being maintained. After a random slide

Fig. 3. AFM images of chromatin reconstituted on linearized 56-kbp plasmid (a), circular and supercoiled 56-kbp plasmid (b), circular and supercoiled 5-kbp plasmid (c), and circular and supercoiled linear 106-kbp plasmid (d). The 100-kbp and 50-kbp fragments of the human centromeric region were inserted into a BAC (bacterial artificial chromosome) vector (6 kbp). The 5-kbp plasmid was constructed with a pUC119 vector and a 2-kbp centromeric insertion fragment. The linear 56-kbp plasmid DNA was made by a single-site cutting with a restriction enzyme, Sal I. e. Relationship between the plasmid length and the frequency of nucleosome formation in the reconstitution process. The frequency was plotted against the length of the template DNA. The frequency was characterized by nucleosome formation per unit length of DNA.

Fig. 4. AFM images of fully reconstituted 106-kbp chromatin (a) and its intermediates showing uneven distribution of nucleosomes (b). *Arrowhead* indicates the scattered regions containing a few far-isolated nucleosomes.

along the DNA, nucleosome came to the end of the DNA and was stably positioned. This indicated that nucleosome structure is very flexible and dynamic, and that the histone core could always slide along the DNA strand.

Elucidating of chromosome architecture

Although the molecular structure of the nucleosome has been resolved by X-ray crystallography (LUGER et al., 1997), it is still uncertain how the chromatin fiber is folded up to a higher-order fiber, and finally up to a chromosome. At present, the general consensus seem to be that the higher-order architectures of chromatin are attained by a balance between the biological nature of the nuclear proteins that interact with chromatin and the physical properties of the chromatin fiber. Therefore, making progress towards an elucidation of the chromosome structures and functions will require studies not only about the functions of the number of chromosomal proteins — especially those involved in the higher-order arrangement of chromosome structure — but also the physical properties of DNA and chromatin.

AFM has been particularly powerful in visualizing and directly analyzing the effects of protein binding

on the DNA structures. The chromatin compaction and the formation of higher-order architectures have been known to involve several nuclear proteins, including linker histones (MCGHEE and FELSENFELD, 1980; BEDNAR et al., 1998) and a complex of SMC (structural maintenance of chromosome) and non-SMC proteins (SAKA et al., 1994; SUTANI et al., 1999). AFM analyses have demonstrated that the histone H1 indeed promotes a nucleosome compaction (SATO et al., 1999) and that the condensin holo-complex has a head-and-tail structure and the non-SMC subunits are included in the head region (YOSHIMURA et al., 2002). It has been also suggested that the SMC heterodimer interacted with DNA, forming a large DNA/protein aggregate, whereas the non-SMC subunits regulate the aggregation.

Further more, as to the physical properties of DNA, AFM has provided several intriguing observations on much higher-order structural changes of DNA with several kilo-base pairs. When a replication initiator of *E. coli* F-plasmid, RepE, bound to the supercoiled replication origin (*ori2*), the superhelical strain was reduced and the entire plasmid (2.5 kbp) was completely relaxed, though RepE possesses neither a topoisomerase nor a nicking activity (YOSHIMURA et al., 2000a). Similarly, a tumor suppressor

Fig. 5. A schematic representation of the implication obtained from the present study, and a model for chromatin architectures. **a.** Linearized short DNA without supercoiled helicity is not a good template for an efficient chromatin reconstitution, and the nucleosomes formed tends to slip off from the edge. **b.** Supercoiled small plasmid DNA can be used for a more efficient reconstitution, but still is not satisfactory. **c.** Supercoiled ~100-kbp plasmid DNA is a good template for an efficient chromatin reconstitution with almost the same efficiency as *in vivo*. The existence of cooperativity is a prerequisite for the efficient nucleosome formation. **d.** A loss of super-helicity leads to an unsuccessful reconstitution. **e.** Additional chromosomal proteins are required for the higher-order architectures.

protein, p53, specifically relaxed the supercoiled DNA without changing the linking numbers (PALECEK et al., 1997; JETT et al., 2000). These results demonstrate that the local binding of a particular protein to the target supercoiled plasmid can promote the changes in the entire state of super-helical twisting of

the plasmid without changing the linking number. Though the molecular mechanism of the plasmid relaxation has not yet been well understood, a structural change from B-type to A-type or Z-type DNA might be involved in this process, as is seen in the enhancer of SV40 (GRUSKIN and RICH, 1993).

These lines of evidence are consistent with our results and the deduced model (Fig. 5) that indicate the importance of the super-helical tension for the formation of the higher-order architectures of chromatin. The strongest evidence for this is that a linearization of the template DNA did loosen the reconstitution efficiency. Additionally, our present results demonstrated that the assembly of nucleosomes on a template DNA was cooperative only in the presence of the superhelicity of the DNA (Fig. 5c, d). We expect that the higher-order structure of the genome will be attained in a similar process, and that the local binding of regulatory proteins can drastically change the chromatin structure towards much higher-order chromatin architectures (Fig. 5e).

In the post-genome era, every possible technology needs to be combined on the basis of all biological information, including genomic sequence, protein sequence, three-dimensional structure of protein and more. As shown in this study, a single-molecule-imaging technique in biology has been well developed and combined with conventional biochemical and molecular biological techniques. This new strategy 'nano-biology' should greatly promote the understanding of the structure and function of the gene.

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