Nano-scale analyses of the chromatin decompaction induced by histone acetylation*

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Summary. The acetylation of histone tails is a key factor in the maintenance of chromatin dynamics and cellular homeostasis. The hallmark of active chromatin is the hyper-acetylation of histones, which appears to result in a more open chromatin structure. Although short nucleosomal arrays have been studied, the structural dynamics of relatively long acetylated chromatin remain unclear. We have analyzed in detail the structure of

long hyper-acetylated chromatin fibers using atomic force microscopy (AFM). Hyper-acetylated chromatin fibers isolated from nuclei that had been treated with Trichostatin A (TSA), an inhibitor of histone deacetylase, were found to be thinner than those from untreated nuclei. The acetylated chromatin fibers were more easily spread out of nuclei by high-salt treatment, implying that hyper-acetylation facilitates the release of chromatin fibers from compact heterochromatin regions. Chromatin fibers reconstituted *in vitro* from core histones and linker histone H1 became thinner upon acetylation. AFM imaging indicated that the gyration radius of the nucleosomal fiber increased after acetylation and that the hyper-acetylated nucleosomes did not aggregate at high salt concentrations, in contrast to the behavior of non-acetylated nucleosomal arrays, suggesting that acetylation increases long-range repulsions between nucleosomes. Based on these data, we considered a simple coarse grained model, which underlines the effect of remaining electric charges inside the chromatin fiber.

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Introduction

In eukaryotic cells, genomic DNA is packed into a highly organized nucleoprotein complex (called chromatin). This complex is composed of nucleosomes, each of which consist of 147 bp of DNA wrapped around a protein core made up of two copies each of the four core histone proteins (H3, H2B, H2A and H4) (Kornberg, 1974; Luger *et al.*, 1997; Hizume *et al.*, 2004). Nucleosomes give

rise to a series of structures of increasing complexity. The simplest of these structures consists of an array of nucleosomes positioned in tandem on DNA in a “beads-on-a-string” fiber that has a diameter of 11 nm. This 11 nm fiber further condenses into the canonical “30 nm fiber” with the help of linker histones (Thoma and Koller, 1977; Bustamante *et al.*, 1997; Adkins *et al.*, 2004; Hizume *et al.*, 2005; Robinson and Rhodes, 2006). The 30 nm fiber forms loops and attaches to the nuclear matrix at semi-regular intervals along its length, most likely at every several tens- or hundreds-kbp (Hancock, 2000). This structure also further condenses into ~100 nm beaded fibers (Belmont and Bruce, 1994; Horn and Peterson, 2002).

Chromosome packing levels in the nucleus are believed to be closely linked to the degree of transcription. Genome regions with high transcriptional activity can be easily digested by DNase treatment (Weintraub and Groudine, 1976; Stalder *et al.*, 1980). Electron microscopy studies show that RNA labeled with ³H-urysin is predominantly located in the less condensed region of the nuclei (Belyaeva and Zhimulev, 1976). These facts suggest that loosely packed genomic regions are active in transcription. It has also been reported that transcription is suppressed by histone H1 (Weintraub, 1984; O'Neill *et al.*, 1995), which causes a compaction of the nucleosomal array into the 30 nm fiber. Taken together, these observations suggest that highly condensed “heterochromatin” regions lead to low transcriptional activities, while activated genes are located in the loosely packed “euchromatin” regions.

The chromatin state (heterochromatin or euchromatin) is dynamic and can be modulated by factors such as histone-tail modifications. Methylation of histone H3 lysine 9 induces interaction between the chromatin and heterochromatin protein 1 (HP1), which is involved in heterochromatin formation and gene silencing. Conversely, a positive correlation between histone acetylation and transcriptional activity was reported in 1964 (Allfrey *et al.*, 1964). Since then, much research has supported the idea that histone acetylation leads to euchromatin formation (Kadonaga, 1998; Tse *et al.*, 1998a; Zhang *et al.*, 1998; Strahl and Allis, 2000), i. e., that the acetylation of histones leads to chromatin decondensation and thereby a looser chromatin structure. However, we are still far from completely understanding the effects that histone acetylation has on the chromatin structure from a global stance. Here, we have attempted to address the intriguing question of chromatin structure-function using atomic force microscopy (AFM). We have carried out a nano-scale examination of chromatin dynamics *in situ* and *in vitro* and analyzed its dependence

on the hyperacetylation of histones. Since we adopted a relatively large DNA chain of 26 kbp for this study, it was possible to recognize the compacted chromatin structure as a three-dimensional fiber. The experimental system we used to reconstitute uniform fibers permitted consideration of chromatin fibers as a polymer. Since the components of chromatin are charged in solution, electrostatic interactions may govern chromatin structure. The acetylation of histone-tails alters the electrostatic charge of the histone, resulting in changes in the structural properties of the chromatin fiber. Measurements of the width of the chromatin fibers and nucleosome-nucleosome distances led us to consider a model of chromatin decondensation. These data suggested that changes in the total electrical charge of individual nucleosomes plays an important role in H1-induced 20-30 nm fiber formation and that the acetylation of histones significantly contributes to this alteration in charge.

Materials and Methods

Cell culture, HDAC inhibitor treatment, and cell fractionation

HeLa S3 cells were cultured under 5% CO₂ at 37°C in complete Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA). To inhibit histone deacetylase, Trichostatin A (TSA; Wako, Osaka) or Sodium Butyrate (NaBu; Sigma) was added into the medium.

HeLa cells cultured on coverslips were washed twice with phosphate-buffered saline (PBS). The cells were treated with a nuclei isolation (NI) buffer [100 mM NaCl, 0.5% Triton X-100, 3 mM MgCl₂, 1 mM EGTA, 10 mM PIPES (pH 6.8)] at 4°C for 10 min, and then with a high salt (HS) buffer [250 mM (NH₄)₂SO₄, 0.5% Triton X-100, 3 mM MgCl₂, 1 mM EGTA, 10 mM PIPES (pH 6.8)] at 25°C for 5 min. Following fixation with PBS containing 4% paraformaldehyde at 25°C for 15 min, each specimen was mounted on slides with Vectorshield containing DAPI.

For evaluation of the amount of fibers released from the nucleus, the intensity of DAPI staining outside the nucleus was measured using the Image J program. This intensity was then divided by the number of nuclei in the image, and the amount of released fibers was presented as the DAPI intensity of released fibers per nucleus (Fig. 11).

Fig. 1. Treatment with an HDAC inhibitor induces chromatin fiber spread from nuclei. (A–H) DAPI stained nuclei isolated from non-treated cultured cells (A, B) or from cells treated with 100 ng/ml (C, D), 200 ng/ml (E, F) or 600 ng/ml (G, H) of trichostatin A for 24 h. For each condition, the same field was captured using a short (A, C, E, G) and a long (B, D, F, H) exposure time. The longer time allowed the careful detection of spread fibers although nuclear DAPI signals were saturated. **I:** DAPI intensity in the area outside the nucleus was measured to estimate the degree of fiber spread and was compiled as a histogram. **J–L:** Detection of the level of acetylation of histone H3 by Western blotting. Cells treated with the indicated concentrations of TSA were harvested and cell extracts were loaded onto an SDS-poly-acrylamide gel, transferred to a membrane, and analyzed by Western blotting using an antibody against acetylated H3 (**K**). The same extracts were analyzed by a second SDS-poly-acrylamide gel electrophoresis followed by staining of the proteins with Coomassie Brilliant Blue (CBB). Panel **L** shows subunits of core histones stained with CBB that were used as loading controls. The intensities of the bands in the Western blotting shown in panel **K** were measured and compiled into a histogram (**J**). The Y axis represents the value of the intensity relative to that of non-treated cells that was assigned a value of one.

Isolation and lysis of nuclei and fluorescence observation

HeLa cells, collected using a cell scraper, were washed twice with phosphate-buffered saline (PBS). The cells were treated with a NI buffer at 4°C for 10 min. The

nuclei were collected after centrifugation (1,000 × g for 5 min) and resuspended in a NI buffer without Triton X-100.

The nuclear suspension was dropped onto a coverslip that had been pre-treated with 0.02% poly-L-lysine at 25°C for 15 min. The surface of the coverslip was dried

once to absorb the nuclei, treated with a HS buffer at 25°C for 5 min, and then fixed with PBS containing 0.5 µg/ml DAPI and 4% paraformaldehyde at 25°C for 15 min.

To stain lipid membranes, the nuclei mounted on glass coverslips were treated with DiOC₃ (1:5,000 dilution; Invitrogen, Carlsbad, CA, USA). To stain the nuclear pore complex (NPC) or lamin B, the nuclei specimens were treated with specific primary antibodies (anti-NPC monoclonal antibody; Berkeley Antibody Company, Richmond, CA, USA, or anti-lamin B antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and fluorescent secondary antibodies (anti-mouse IgG antibody conjugated with FITC; Invitrogen, or anti-goat IgG antibody conjugated with Alexa Fluor 488; MP Biomedicals, Irvine, CA, USA). Fluorescently stained nuclei were observed using a confocal fluorescence microscope (LSM5 Pascal, Zeiss, Germany) in Figure 2.

Plasmid DNA

The plasmid used for chromatin reconstitution was a kind gift from Dr. W. De Laat (Erasmus University Medical Center, Department of Cell Biology, the Netherlands). The locus control region (LCR) of the human β -globin gene (21.5 kb) was isolated by Sal I/Cla I digestion and subcloned into pBR322, yielding the 26 kb plasmid.

Histones

Core histones were purified by a previously described procedure (Hizume *et al.*, 2005, 2007). Briefly, nuclei were isolated by low-speed centrifugation from HeLa cells, which had been treated with a buffer containing 0.5% Triton X-100. The nuclei were then treated with micrococcal nuclease (40 units/mg of DNA) at 37°C for 15 min, and solubilized fractions containing mono- or oligo-nucleosomes were collected. Core histones were separated from DNA by hydroxyapatite column chromatography (Simon and Felsenfeld, 1979), and the purified core histones were applied to a gel filtration column (HiPrep 16/60 S-200, GE Healthcare UK Ltd., Buckinghamshire, England) to separate the octamer from the H3-H4 tetramer, the H2A-H2B dimer, and other contaminants. Histone H1 was obtained from Calbiochem.

The p300 acetyltransferase (p300), which was used for histone-tail acetylation, was purified from recombinant baculovirus-infected Sf21 cells as a His6-tagged protein using a nickel-nitrilotriacetic acid affinity column (Qiagen, Hilden, Germany), as described previously (Balasubramanyam *et al.*, 2003).

To obtain acetylated histones, purified core histones were dialyzed against a buffer containing 50 mM Tris-

HCl (pH 8.0), 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, 0.1 mM EDTA (pH 8.0), and 10 mM NaBu at 4°C for 24 h. The dialyzed core histones (16 µg) were treated with p300 (60 ng) and acetyl-CoA (56 µM) (Sigma) at 36°C for 7 h. As a non-acetylated control, the dialyzed core histones were treated with acetyl-CoA only.

Acetylation levels of histones were analyzed by gel electrophoresis using an AUT gel, as described elsewhere (Zweidler, 1978). The acetylated histones were incubated with an equivalent volume of a loading buffer [8 M urea, 0.2% Pyronin Y, 1.4 mM 2-mercaptoethanol] for 5 min. The mixture was applied onto a gel containing 5% acetic acid, 8 M urea, 12% acrylamide, and 0.4% Triton X-100 and electrophoresed in 0.9 N acetic acid. Proteins were stained using a silver staining kit (Wako).

Chromatin reconstitution

Equal amounts (0.5 µg) of purified DNA and the histone octamer were mixed in a Hi-buffer [10 mM Tris-Cl (pH 7.5), 2 M NaCl, 1 mM EDTA, 0.05% NP-40, 5 mM β -mercaptoethanol, and 0.1 mM PMSF] and placed in a dialysis tube (total volume, 50 µl). Dialysis was begun by stirring the tube in 150 ml of the Hi-buffer at 4°C. A Lo-buffer [10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.05% NP-40, 5 mM 2-mercaptoethanol, and 0.1 mM PMSF] was then added to the dialysis buffer at a rate of 0.46 ml/min, and, simultaneously, the existing dialysis buffer was pumped out at the same speed with a peristaltic pump so that the final dialysis buffer contained 50 mM NaCl after 20 h. The samples were collected from the dialysis tube and stored at 4°C until further use.

For reconstitution with histone H1, this was added after the salt dialysis was completed (at a NaCl concentration of 50 mM). The molar ratio of histone H1 to the histone octamer was 1:1 unless otherwise stated.

For AFM imaging, the reconstituted chromatin solution was fixed with 0.3% glutaraldehyde for 30 min at room temperature. The fixed sample was dropped onto a freshly cleaved mica surface that had been pretreated with 10 mM spermidine. After 10 min incubation at room temperature, the mica was washed with water and dried under nitrogen gas.

AFM observation

AFM observations of the reconstituted chromatin and isolated nuclei were performed in air with both a Multi Mode and Bioscope II (Digital Instruments, Santa Barbara, CA, USA), using the tapping mode. The cantilever (OMCL-AC160TS-W2, Olympus, Tokyo) used was 129 µm in length with a spring constant of

33–62 N/m. The scanning frequency was 2–3 Hz, and images were captured with the height mode in a 512×512 pixel format. The obtained images were plane-fitted and flattened by the computer program supplied in the imaging module to remove the background curvature of the mica surface.

The widths of chromatin fibers in AFM images were affected by the size of the tips used, and the apparent dimensions of the molecules obtained by AFM were dependent upon the radius of the tip curvature and appeared larger than the actual dimensions. To prevent this broadening effect caused by the tip (“tip effect”) for the reconstituted chromatin fiber shown in Figure 4, the apparent size of the nucleosomes was used as a reference. This method is the same as that used in our previous reports concerning reconstituted chromatin (Hizume *et al.*, 2004, 2005). The relationship between the width of the globular molecule in the AFM image (W), the radius of the tip curvature (R_c), and the radius of the molecule (R_m), is given by $W=4(R_c R_m)^{1/2}$. When two different molecules with R_{m1} and R_{m2} radii were imaged with the same tip, the relationship between the measured widths (W_1 and W_2) was given by $W_1=W_2(R_{m1}/R_{m2})^{1/2}$. Since the diameter of the nucleosome is known to be 11 nm (Luger *et al.*, 1997), we used the diameter of nucleosomes in our AFM images as W_1 , and calculated the radius of the fiber (R_{m2}) from the apparent width of the fiber (W_2).

To measure the width of the fiber spread from nuclei (Fig. 3), the fiber widths were estimated at the half-maximum height (FWHM: full width at half-maximum). This method of measuring fiber width was previously used in our studies of genome fiber spread from cells (Kim *et al.*, 2004; Kobori *et al.*, 2006, 2007). This measurement is expected to subtract the broadening effect of the tip as described previously (Schneider *et al.*, 1998) without the need for any size references.

Gyration radius

The positions of the nucleosomes were digitized as $\vec{r}_i$. The radius of gyration (R_g^2) was calculated as follows:

$$R_g^2 = \frac{\sum_{i=1}^N (\vec{r}_i - \vec{r}_G)^2}{N},$$

where $\vec{r}_G$ is the center of mass.

Results

TSA treatment affects chromatin packing

To analyze the role of histone acetylation on chromatin fiber structure, we first assayed the effect of the inhibition of histone deacetylase on chromatin fiber spread from the cell nuclei. HeLa cells, cultured on coverglasses, were therefore treated with various concentrations of the histone deacetylase inhibitor TSA, and chromatin fibers were microscopically analyzed. For this purpose, the cells were treated with detergent to remove the plasma membrane as well as with cytosol (0.5% Triton X-100, 10 min) followed by a high-salt (250 mM $(\text{NH}_4)_2\text{SO}_4$) treatment to remove nucleoplasmic proteins. Nucleic acids were then stained by DAPI (Fig. 1). During this lysis procedure, there was no spread of chromatin fiber out of nuclei which had not been treated with TSA (Fig. 1A, B). Therefore, the genome structures of control cells were tightly held inside the nucleus and remained inside the nuclei even after $(\text{NH}_4)_2\text{SO}_4$ treatment to remove the nucleoplasm. Treatment with high concentrations of TSA induced the appearance of more fibrous structures that did spread out of the nuclei (Fig. 1C–H). The degree of fiber release from the isolated nuclei was estimated based on the intensity of DAPI staining outside the nucleus (see Materials and Methods for details). As the concentration of TSA increased, there was a corresponding increase in the number of chromatin fibers that spread out of the nuclei. Western blotting analysis of acetylated histone H3 (Fig. 1K) indicated that the level of histone acetylation increased in tandem with the TSA concentration used and also correlated with the increased DAPI-staining of the spread-out fibers (Fig. 1I). Presumably, upon hyperacetylation due to the inhibition of histone deacetylases, the chromatin fibers decondensed, detached from the nuclear membrane scaffold, and were therefore more easily released from the nucleus.

Nuclear isolation and AFM observation of TSA treated cells

To analyze the structure of the chromatin fibers that spread from the nucleus following TSA treatment, AFM analysis was performed on isolated nuclei. HeLa nuclei were isolated by gentle centrifugation ($1000 \times g$, 5 min) following detergent treatment (0.5% Triton X-100, 10 min), and were then deposited on a glass surface (Fig. 2A). At this stage, the nuclear envelope was still intact, as shown by staining with DiOC6—which recognizes lipid membranes (Fig. 2B)—and by staining with anti-NPC (nuclear pore complex) (Fig. 2C) and anti-nuclear lamina

Fig. 2. Fluorescent analysis of nuclei isolated from HeLa cells by high salt-lysis on a glass surface. **A:** A brief outline of the procedure for nuclei isolation is shown. Cultured HeLa cells were harvested and treated with a buffer containing 0.5% Triton X-100. Nuclei were isolated by low speed centrifugation, mounted on a glass surface, and lysed with a buffer containing 250 mM $(\text{NH}_4)_2\text{SO}_4$. **B–G:** Isolated nuclei before (**B–D**), and after (**E–G**), $(\text{NH}_4)_2\text{SO}_4$ treatment were observed by fluorescent microscopy following staining with DiOC6—which selectively labels lipid membranes (**B** and **E**)—or following immunostaining with the anti-NPC (**C** and **F**), or anti-laminB (**D** and **G**) antibodies. Prior to high salt lysis, the DiOC6 fluorescent signal was clearly detected both on the edge of and outside the nuclei, indicating that both the nuclear membrane and the ER were intact. The isolated nuclei were also observed to contain both NPC (**C**) and lamin (**D**) prior to lysis. **H, J:** DAPI-stained images of an isolated nucleus before (**H**), and after (**J**), high salt-lysis. **I, K:** AFM images of the same nuclei shown in **H** and **J** respectively. The dotted lines in (**I**) and (**K**) represent the sites at which the height profiles shown in panels **L** and **M** were respectively taken. Bars = 10 μm

Fig. 3. AFM analysis of chromatin fibers spread out from nuclei. Nuclei were isolated from HeLa cells grown in the absence of treatment (**A–E**), or following treatment with 600 ng/ml TSA and 10 mM NaBu for 24 h (**F–J**). The nuclei were mounted on a glass surface, lysed with high-salt, and then the same region of the slide was observed under a fluorescence microscope following DAPI staining (**A, F**) and by AFM (**B–D, G–I**). Rescanning of the area within the dotted square in panels **B** and **C** is shown in panels **C** and **D**, respectively. Rescanning of the dotted square in panels **G** and **H** is shown in panels **H** and **I**, respectively. The width of the fibers shown in panels **D** and **I** were measured and compiled into a histogram (**E** and **J** respectively). Cross-sections of the fibers (as shown in the inset of panel **I**) were analyzed to measure FWHM (see materials and methods). Cross-sections of 53 areas of 13 fibers in panel **D**, and of 147 areas of 21 fibers in panel **I**, were analyzed. Bars = 2 μ m

Fig. 4. Nucleosome reconstitution using acetylated core histones. **A:** Core histones were treated with or without acetyl-CoA and p300 and were then fractionated by Triton- acrylamide -urea gel electrophoresis. The histones were detected by silver-staining. The mobility of the core histone subunits was retarded with acetylation (arrows). **B, D, F:** An AFM image of nucleosomes reconstituted on a 26-kb plasmid using non-acetylated core histones (Mock). Nucleosomes were fixed in a buffer containing 50 (**B**), 100 (**D**) or 200 mM (**F**) NaCl. **C, E, G:** An AFM image of nucleosomes reconstituted on a 26-kb plasmid using acetylated core histones. Nucleosomes were fixed in a buffer containing 50 (**C**), 100 (**E**) or 200 mM (**G**) NaCl. **H:** The average minimum radius required for a circle that would enclose an entire chromatin molecule was measured in AFM images such as those shown in panels **B–G**. In the 200 mM NaCl buffer, the minimum radius of the non-acetylated nucleosomes was significantly smaller (* $p = 0.0014$) than that of the acetylated nucleosomes.

Fig. 5. An H1 induced fiber reconstituted on a 26 kb plasmid using non-acetylated or acetylated core histones. **A–H:** AFM images of H1-induced chromatin fibers reconstituted on a 26-kb plasmid using non-acetylated core histones (**A**, **C**, **E**, **G**) or acetylated core histones (**B**, **D**, **F**, **H**). The samples were fixed in a buffer containing 50 mM (**A** and **B**) or 100 mM (**C–H**) NaCl. Histone H1 and the core histone octamer were added at a molar ratio of 1:1 (**A–D**), 1:2 (**E** and **F**) or 1:4 (**G** and **H**). At a molar ratio of 1:1 of core histone octamer to linker histone H1, the lengths of the fibers were 207 nm and 224 nm in acetylated and non-acetylated chromatin, respectively. **I–L:** The widths of the fibers shown in panels **A**, **B**, **C** and **D** were measured and compiled into a histogram. Cross-sections of 644 areas of 20 fibers (**E**), 568 areas of 25 fibers (**G**), 444 areas of 10 fibers (**I**) and 439 areas of 21 fibers (**K**) were analyzed to measure the bottom-to-bottom distance and to remove the ‘tip effect’ as described in Materials and Methods.

(Fig. 2D) antibodies. Subsequently, the nuclei were treated with 250 mM $(\text{NH}_4)_2\text{SO}_4$ to remove nucleoplasmic proteins. The DiOC6 and NPC-fluorescence signals could not be detected after this treatment (Figs. 2E and F, respectively). Although the nuclear lamina-fluorescence signal could still be detected (Fig. 2G), it was much weaker than before the treatment. Examination of the nucleus by DAPI staining revealed a distinct round-shaped nucleus prior to $(\text{NH}_4)_2\text{SO}_4$ treatment (Fig. 2H). However, the outline of the nucleus was difficult to identify by AFM imaging (Fig. 2I), and AFM images showed a smooth nuclear surface (Fig. 2L), possibly due to the remaining ER surrounding the nucleus. Following $(\text{NH}_4)_2\text{SO}_4$ treatment, the outline of the nucleus was clearly identified by both DAPI-staining (Fig. 2J) and AFM imaging (Fig. 2K). It is likely that this treatment removed the nuclear envelope (Fig. 2E), resulting in a decrease in the height of the AFM image, and the appearance of a rough surface (Fig. 2M).

Few chromatin fibers were detected outside of the nuclei in the absence of TSA treatment, indicating that chromatin fibers were still tightly packed even after removal of the nuclear envelope (Fig. 3A–D). The fibers that were observed by AFM imaging were calculated to have a width of 43.0 ± 15.6 nm (mean $\pm$ S.D., $n=53$; Fig. 3E), which is in agreement with our previous work (Kobori *et al.*, 2006). In contrast, the AFM images of TSA-treated-nuclei revealed many fiber bundles of various widths (Fig. 3E–J). The thinnest fibers appeared to co-exist in bundles. Interestingly, the width of the thinnest fibers was measured as 23.1 ± 5.9 nm (left peak of Fig. 3J), which is thinner than the fibers of the non-TSA-treated nuclei.

Acetylated nucleosomes form fiber structures in vitro in the presence of the linker histone H1 that are thinner than those formed in non-acetylated nucleosomes

In order to confirm the effect of histone acetylation on chromatin fiber structure, we performed AFM studies using an *in vitro* reconstituted chromatin fiber. For this purpose, highly purified HeLa core histones were acetylated with histone-acetyltransferase (p300). The results of the Acid-Urea-Triton (AUT) gel electrophoresis shown in Figure 4A confirm that subunits of core histones treated with p300 and acetyl-CoA exhibit decreased in mobility (lane 3) compared with non-treated core histones (lane1) or with core histones treated with only acetyl-CoA (lane 2). This suggests that the positive charges of the core histones subunits were decreased by acetylation; it is consistent with the expectation that the acetylation of

lysine residues will neutralize some of the positive charge of histones. Excess treatment with p300 and acetyl-CoA did not further change the gel mobility of histones (lane 4), suggesting that the core histones in lane 3 were acetylated at all p300 targets within the protein.

AFM images of reconstituted chromatin, formed by the assembly of a 26-kb plasmid DNA and core histones treated with only acetyl-CoA (non-acetylated core histones, Mock) or with acetyl-CoA and p300 (acetylated core histones), are shown in Figures 4B and C respectively. The chromatin reconstituted with non-acetylated core histones contained 55 ± 5.0 ($n=8$) nucleosomes, and that reconstituted with acetylated core histones contained 52 ± 7.9 nucleosomes per molecule of 26-kb plasmid, indicating that histone-tail acetylation did not affect the efficiency of nucleosome formation as such. Interestingly, the distance between nucleosomes was affected by acetylation of the histones. Measurement of the gyration radius of the reconstituted nucleosomes in the AFM images using the method described in the Materials and Methods section indicated a gyration radius of 41 ± 11 nm (mean $\pm$ SD) for the non-acetylated nucleosome, but a radius of 57 ± 13 nm for the acetylated nucleosome ($p<0.001$). The degree of salt-induced compaction of the acetylated and non-acetylated nucleosomes was compared in a buffer containing 100 or 200 mM NaCl by AFM imaging (Fig. 4D–G). Non-acetylated nucleosomes bound to each other and formed aggregates in the higher salt buffer whereas the acetylated chromatin exhibited an open, beads-on-a-string structure at this salt concentration. The minimum radius of a circle required to encompass an entire chromatin molecule was measured from these AFM images (Fig. 4H). As the salt concentration increased, the average minimum radius of non-acetylated chromatin—but not that of acetylated chromatin—decreased. This difference may be caused by the increased electrical repulsion between nucleosomes contributed by the histone acetylation (see Discussion).

The addition of linker histone H1 into reconstituted chromatin is known to induce the formation of a fiber structure (Hizume *et al.*, 2005). The width of the fiber is dynamic and changes depending on the buffer conditions. Thus, in 50 mM NaCl, the fiber width is 20 nm, but in 100 mM NaCl it is 30 nm. As shown in Figures 5A–H, the addition of histone H1 to either the non-acetylated or acetylated chromatin led to fiber formation. The efficiency of fiber formation was similar at different molar ratios of core histones:histone H1. However, the width of the fibers was dramatically decreased in the acetylated sample. In a buffer containing 100 mM salt, the width of the non-acetylated fibers (26.0 ± 4.96 nm, $n=444$; Fig. 5K) was significantly greater than that of the acetylated fibers

Fig. 6. Schematic models of the structural dynamics of the chromatin induced by histone acetylation. **A:** Potential effect of electric charge on the interaction between nucleosomes. The gyration radius of acetylated nucleosome was higher than that of non-acetylated nucleosomes, suggesting that the decrease in positive charge due to acetylation gives the nucleosome a more negative charge and produces a higher repulsive force between nucleosomes. **B:** The width of the H1-induced 30 nm fiber decreases depending on the degree of histone acetylation. **C:** Variation of fiber width with different charge densities.

(17.7 ± 5.14 nm, $n=439$; Fig. 5L) although this difference was hard to detect under conditions of 50 mM salt (Fig. 5I, J).

Discussion

Histone-acetylation has been studied using genetic, biochemical, and cell biological analyses with a view towards understanding the mechanism by which histone-acetylation controls interactions between nuclear proteins and nucleosomes/chromatin. Proteins that contain a bromodomain, such as chromatin remodeling factors, histone acetyltransferases, and many transcription factors, have been shown to specifically interact with acetylated histones (Ornaghi *et al.*, 1999; Jacobson *et al.*, 2000; Shen *et al.*, 2007). These studies suggest that specific proteins open the chromatin structure and lead to higher transcriptional activity. In this report, we have investigated the mechanism by which histone acetylation affects linker histone H1-induced fiber formation using both an *in vitro* reconstitution system (mediated by acetylated core histones) and native chromatin that is released from nuclei treated with TSA. Acetylation

resulted in: 1) easy release of chromatin fibers out of nuclei, 2) a loss of salt-induced compaction *in vitro*, and 3) a decrease in the width of reconstituted H1-induced fibers.

Two lines of evidence support the induction of chromatin decompaction by histone acetylation: inhibition of salt-dependent precipitation and reduction of the width of H1-induced fibers

The “higher-order” folding of a reconstituted nucleosome array has previously been detected as a salt-dependent precipitation of nucleosomal arrays (Garcia-Ramirez *et al.*, 1995; Tse *et al.*, 1998b; Shogren-Knaak *et al.*, 2006). These studies have also shown that histone-acetylation inhibits the salt-dependent compaction of nucleosomes. Salt-dependent precipitation studies have raised the idea that the acetylation of specific lysine residues of histone tails is critical for chromatin decompaction. Indeed, using a native chemical ligation strategy to generate histone H4 acetylated at specific lysine residues, it has been shown that the acetylation of lysine 16 of histone H4 is sufficient to inhibit any salt-dependent compaction (Shogren-Knaak *et al.*, 2006). The analysis of nucleosomal arrays,

reconstituted from mutant core histones with lysine-to-glutamine substitutions to mimic acetylation, showed that acetylation-mimics of H2B and H4 inhibit the salt-dependent precipitation of nucleosomes (Wang and Hayes, 2008). In contrast to these previous studies, our study focused on the effect of hyper-acetylation on structural changes such as changes in the width of the H1-induced fiber rather than on the sedimentation properties (or solubility) of the nucleosome array. Unexpectedly, our results showed that histone-acetylation did not prevent the formation of the H1-induced fiber. However, the fiber formed from hyper-acetylated nucleosomes was thinner than that formed from non-acetylated nucleosomes. It has been reported that histone H1 can bind to both hyper-acetylated and non-acetylated chromatin but that it cannot induce 0.15 M NaCl-dependent precipitation of an hyper-acetylated nucleosome array (Ridsdale *et al.*, 1990). This indicates that histone H1 induces “less folded state” chromatin with hyper-acetylated chromatin. The thinner fibers that we detected in this study could be fibers that exist in this lower folded state.

Coarse-grained model of the decrease in the width of chromatin fibers due to acetylation

To understand the mechanism by which acetylation induces a reduction in the width of the H1-induced fiber, it may be necessary to consider the physical properties of the fiber as a polymer. Since we did not examine the acetylation of specific lysine residues, the contribution of specific acetylation sites to the effects we observed is not clear. However, it is still possible to discuss the mechanism of structural change induced by acetylation in relation to the electrostatic properties of chromatin from a global point of view. DNA is a highly charged polymer that carries two negative elementary charges per bp. Core histones and H1 proteins are also charged polymers, carrying 147 and 54 positive elementary charges, respectively. One nucleosome forms from 147 bp of nucleosomal DNA, resulting in a total of 147 negative elementary charges per nucleosome. If this negatively charged nucleosome is acetylated on 22 lysine residues by p300, the number of charges per nucleosome increases to -169 e because acetylation of the histone tail neutralizes the positive charges of the lysines. This increase in negative charge should, in turn, increase the repulsing force between nucleosomes, so that the distance between nucleosomes increases, giving rise to the “beads-on-a-string” structure that was detected even under conditions of 200 mM salt (Fig. 4G, 6A). This phenomenon that we observed is consistent with previous AFM studies that showed “cooperativity” (cooperative

nucleosome occupation of a DNA fragment) of nucleosomes reconstituted on an ~2 kb fragment as well as that interaction between neighboring nucleosomes was decreased by hyperacetylation (Yodh *et al.*, 2002; Solis *et al.*, 2007).

This electrostatic feature is important not only for nucleosome-nucleosome interaction but also for decompaction of the 30 nm fiber. EM observation of chromatin fibers purified from NaBu-treated HeLa cells indicated that acetylated chromatin fibers were thinner than non-acetylated fibers (Annunziato *et al.*, 1988). In our study, we also detected thinner fibers in both hyper-acetylated nuclei and in reconstituted H1-induced fibers. Our *in vitro* experiments suggested that acetylation per se is directly responsible for making the fiber thinner, without the need for protein addition.

Acetylation of the lysine residues in core histones can locally adjust the charge of a chromatin component, a H1-containing nucleosome. A detailed model has been published (Arya and Schlick, 2009), in which the positional distribution of histone tails under different conditions such as the absence or presence of monovalent salts or of linker histones was analyzed and in which the effect of tail distribution on chromatin higher-order structure was simulated. In our present study, we found that the width of the chromatin fiber changed from 26.0 nm to 17.7 nm depending on the degree of acetylation in the histone tails. We considered this effect of hyper-acetylation to correspond to a decrease in the remaining charge inside the fiber. By focusing on this decrease in fiber width caused by hyper-acetylation, we quantified the correlation between the change in the width of the fibers and the level of the charge that remains inside the fibers as follows: Since the H1-containing acetylated nucleosome contains more negative charges than the H1-containing non-acetylated nucleosome we considered a coarse-grained model to express the width of the chromatin fiber. Let us consider the fiber as a cylinder of radius R and length l , with a total charge per unit volume of $-\rho e$, and the solution conditions with ions as characterized by a Bjerrum length $l_B \equiv e^2 / \epsilon k_B T$ (ϵ : dielectric constant of the solvent) and a Debye screening length $\kappa^{-1} = (8\pi c_s l_B)^{-1/2}$ (c_s : salt concentration with 1:1 valences). The volume energy in the fiber per unit volume (U_1) excluding the Coulombic contribution would then be given as:

$$U_1 = -a$$

where a is a constant that represents the degree of attractive energy between nucleosomes. Consider that the destabilization energy, or surface energy, of the nucleosomes is located on the surface of the fiber since

the nucleosomes on the surface of the fiber are not entirely surrounded by other nucleosomes. Accordingly, the surface energy of the cylinder per unit volume (U_2) is:

$$U_2 = 2\gamma / R.$$

In this calculation the effect of the cylinder edge was neglected. When the fiber has no electronic charge, an infinite radius is possible. Because of the remaining negative charges, we consider the electrostatic energy (U_3) using the following equation:

$$U_3 = \frac{e^2 \rho^2}{4\epsilon} R^2 \left[\frac{1}{4} + \ln \left(\frac{R + l_D}{R} \right) \right].$$

For the sake of simplicity the exact distribution of salts was neglected in the above equation.

The total energy of the fiber per unit volume (U) can then be approximated by the following equation:

$$U = U_1 + U_2 + U_3.$$

By minimizing the total energy U with R , the width of the fiber is given as a function of the remaining charges (Fig. 6B, C). The more charges that remain, the wider the fiber becomes. This tendency is consistent with our experimental observations and suggests that, when a large amount of net charge remains, the fiber becomes unstable and tends to unfold into separate nucleosomal fibers without considering the role of small ions. The pair interaction between mononucleosomes has been shown to be complex; it has a minimum repulsive interaction at a concentration of 160 mM sodium (Mangenot *et al.*, 2002). It was also found that acetylated mononucleosomes induced a longer end-to-end distance of the linker DNA than non-acetylated mononucleosomes, indicating that acetylation causes an opening of the nucleosome structure (Toth *et al.*, 2006). Unlike nucleosome core particles, the salt-concentration dependence of the pair potential of nucleosomes in a nucleosomal array (i.e. nucleosomes linked with each other by linker DNA) has been unclear although the dependence of the pair potential on the histone/DNA ratio has been reported (Nakai *et al.*, 2005). This constitutes a highly interesting topic for future study. However, in the absence of a detailed model, this particular trend regarding the width of the fiber can be roughly explained by a simple coarse-grained model. For a more detailed understanding of the structural effect of hyper-acetylation on chromatin fibers, a mesoscale structural simulation as previously described (Arya and Schlick, 2009) would be required.

“Chromatin release” due to histone acetylation

It is believed that chromosomes are partitioned in a well-organized manner in the nucleus in well defined three-dimensional territories (Cremer *et al.*, 2006). One proposed model suggests that inactivated genes are located in condensed nuclear compartments. Conversely, activated genes are believed to be released into open areas called interchromatin compartments where they become accessible to the transcription machineries. Fluorescent microscopy studies have shown that HP1 dissociates from chromatin upon histone acetylation (Taddei *et al.*, 2001; Maison *et al.*, 2002) and that condensed chromatin regions are subsequently decondensed (Toth *et al.*, 2004). Although this demonstrates that histone-acetylation affects chromatin organization in the nucleus, the detailed structural dynamics of chromatin fibers cannot be determined by fluorescence microscopy.

Chromatin fibers are more easily released from the nucleus after treatment with an HDAC inhibitor (Fig. 1). This result suggests that acetylated chromatin fibers are not “locked into” compact heterochromatin compartments. However, further experiments are required to conclude definitively that chromatin release is caused by histone-acetylation and not by a general weakening of nuclear integrity by HDAC inhibition. The resulting “detachment” of chromatin could facilitate gene relocation from the heterochromatin regions to the euchromatin regions. Our present observations of isolated nuclear chromatin suggest that this “detachment” is the most drastic structural change induced by histone acetylation. A combination of chromatin release from the condensed region, and specific binding between acetylated histones and proteins such as transcription factors, chromatin remodeling factors, and RNA polymerase complexes would therefore determine the status of “euchromatin.”

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