Linker Histone H1 per se Can Induce Three-Dimensional Folding of Chromatin Fiber†

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Received April 5, 2005; Revised Manuscript Received July 6, 2005

ABSTRACT: Higher-order architectures of chromosomes play important roles in the regulation of genome functions. To understand the molecular mechanism of genome packing, an in vitro chromatin reconstitution method and a single-molecule imaging technique (atomic force microscopy) were combined. In 50 mM NaCl, well-stretched beads-on-a-string chromatin fiber was observed. However, in 100 mM NaCl, salt-induced interaction between nucleosomes caused partial aggregation. Addition of histone H1 promoted a further folding of the fiber into thicker fibers 20−30 nm in width. Micrococcal nuclease digestion of these thicker fibers produced an ∼170 bp fragment of nucleosomal DNA, which was ∼20 bp longer than in the absence of histone H1 (∼150 bp), indicating that H1 is correctly placed at the linker region. The width of the fiber depended on the ionic strength. Widths of 20 nm in 50 mM NaCl became 30 nm as the ionic strength was changed to 100 mM. On the basis of these results, a flexible model of chromatin fiber formation was proposed, where the mode of the fiber compaction changes depending both on salt environment and linker histone H1. The biological significance of this property of the chromatin architecture will be apparent in the closed segments (∼100 kb) between SAR/MAR regions.

The most fundamental structural unit of the eukaryotic chromosome is the nucleosome (*1*, *2*). A nucleosome is composed of 146 bp DNA wrapping around a core histone octamer for ∼1.75 turns. The higher-order arrangements of nucleosomes play important roles in a number of genome functions; transcriptional regulation is known to be tightly coupled with dynamic structural changes in nucleosomal arrays (*3*−*5*). Thicker fibers with widths of ∼30 nm were isolated from the interphase nucleus and observed by electron microscopy (EM)[1] (*6*−*10*), indicating that the nucleosome with a width of 11 nm is further folded into thicker fibers.

Linker histones have been known to play important roles in chromatin folding. Histone H1 (composed of 210−220 amino acid residues) has a globular domain in the middle part (*11*), through which it binds to linker DNA, especially, the region where DNA enters into and exits from the nucleosome core particle (*2*, *12*). The complex of a nucleosome and histone H1 is named a "chromatosome" and contains 168 bp of DNA (*13*). Removal of histone H1 resulted in unfolding of the thicker fiber into the "beads on a string" fiber (*6*, *7*). However, this property of H1 is still controversial since some studies claimed that histone H1 was not essential for chromosome condensation. The contribution of histone H1 to mitotic chromosome condensation has been examined with the use of a cell-free extract from *Xenopus* eggs, which transforms condensed sperm nuclei into metaphase chromosomes. When H1 was removed from the extract, the resultant metaphase chromosomes were indistinguishable from those formed in complete extract (*14*). Elimination of all H1 genes in *Tetrahymena* (*15*) had no phenotypic effect.

In vitro chromatin reconstitution revealed structural and functional properties of the nucleosome and chromatin. The simplest method of reconstitution is the salt dialysis method, which requires core histones and naked DNA (*16*) but no other factors. Because of such simple components, salt dialysis is the most useful method for investigating the physical properties of the chromatin fiber. The nucleosome core particle reconstituted by this method has the same properties as native particles, such as sedimentation velocity, histone content, and nuclease digestion pattern (*17*). When histone H1 was added to the reconstituted nucleosome, the transcriptional activity of the nucleosome template was repressed (*18*). Another study demonstrated that histone H1 prevented the sliding of core histones along DNA (*19*). However, despite a number of studies, the direct involvement of histone H1 in the higher-order folding of the chromatin fiber could not be demonstrated by the reconstitution system, and therefore, the molecular mechanism of chromatin folding is not yet understood.

In this study, we examined the effect of linker histone H1 on the higher-order folding of in vitro-reconstituted nucleosome fibers, and found that H1 induced formation of thick

† This study was supported by the Special Co-ordination Funds (104041500002), COE Research Grant 13CE2006, and Basic Research Grant B 15370077 from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also thank the Japan Science Society (Sasakawa Scientific Research Grant) and the Sumitomo Foundation for their strong support of this work.

[1] Abbreviations: SAR, scaffold attachment region; MAR, matrix attachment region; EM, electron microscopy; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NP-40, Nonidet P-40; AFM, atomic force microscopy; SD, standard deviation.

10.1021/bi050623v CCC: $30.25
Published on Web 09/03/2005

chromatin fibers (widths of 20−30 nm) in the physiological salt environment.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The 100 kb plasmid (pBAC7/8-α2) employed in the chromatin reconstitution was a kind gift from M. Ikeno at Fujita Health University (*20*) and used in the previous study (*21*). This plasmid was constructed as follows. First, the 25 kb alphoid fragment [tandem repeats of a 171 bp unit of alphoid DNA from the centromeric region of the 21st human chromosome (*22*)] was isolated from the cosmid clone, Q25F12, obtained from the LL21NC02 library (Lawrence Livermore Laboratory, Livermore, CA) by *Sfi*I digestion and cloned into the *Sfi*I site of pBAC-TAN that had been created by the insertion of a *Mlu*I−*Sfi*I−*Sac*II linker into the *Xho*I site of pBeloBAC11. Then, the resultant alphoid BAC, which contained 100 kb of tandem alphoid insert (four repeats of the 25 kb fragment), was digested with *Mlu*I and *Sac*II, and the *Mul*I−*Sac*II fragment was subcloned into the *Sal*I site of pBAC-108L (*23*), resulting in the 100 kb plasmid, pBAC7/8-α2.

Purification of the Histone Octamer. Core histones were purified from HeLa cells according to the method developed by O'Neill et al. (*24*) with slight modifications. The cells were harvested, washed with PBS, and then lysed with L-buffer [140 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.5% Triton X-100]. Nuclei were isolated by low-speed centrifugation and washed with W-buffer [350 mM NaCl and 10 mM Tris-HCl (pH 7.5)] three times. The nuclei were then treated with micrococcal nuclease (40 units/mg of DNA) in D-buffer [10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 M sucrose, and 0.1 mM PMSF] at 37 °C for 15 min. The reaction was stopped by 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 resuspended in N-buffer [10 mM Tris-HCl (pH 6.8), 5 mM EDTA, and 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 redialyzed against HA-buffer [0.1 M $NaPO_4$ (pH 6.7) and 0.63 M NaCl] and mixed with hydroxyapatite resin (Bio-Rad). After batch binding at 4 °C for 1 h, the resin was packed into a column and washed with 5 volumes of HA-buffer. The core histones were eluted with E-buffer [0.1 M $NaPO_4$ (pH 6.7) and 2 M NaCl]. The eluate was applied to a gel filtration column (HiPrep 16/60 S-200, Amersham Biosciences) to separate the octamer from the H3−H4 tetramer, H2A−H2B dimer, and other contaminants.

Purification of Histone H1. Histone H1 was purified from HeLa cells according to the method developed by Mirzabekov et al. (*25*) with slight modifications. The cells were harvested, washed with PBS, and then lysed in 140 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.5% Triton X-100, followed by three washes with the same buffer without detergent. The nuclei were collected, washed with 0.35 M NaCl, and 10 mM Tris-HCl (pH 7.5), and then resuspended in 5% CCl_3COOH and rotated at 4 °C for 80 min. After centrifugation at 4000*g* for 15 min, the soluble histone H1 supernatant was dialyzed against 10 mM HCl and 2 mM 2-mercaptoethanol. The dialyzed sample was lyophilized and stored at −80 °C. For chromatin reconstitution, the lyophilized protein was resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl, 0.05% NP-40, and 20% glycerol.

Nucleosome and Chromatin Reconstitution. Equal amounts (0.5 μg) of purified DNA and the histone octamer were mixed in Hi-buffer [10 mM Tris-HCl (pH 7.5), 2 M NaCl, 1 mM EDTA, 0.05% NP-40, and 5 mM 2-mercaptoethanol] and placed in a dialysis tube (total volume, 50 μL). The dialysis was started with 150 mL of Hi-buffer with stirring at 4 °C. Lo-buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.05% NP-40, and 5 mM 2-mercaptoethanol] was added to the dialysis buffer at a rate of 0.46 mL/min, and simultaneously, the dialysis buffer was pumped out at the same speed with a peristaltic pump so that the dialysis buffer contained 50 mM NaCl after 20 h. To obtain 100 mM NaCl at the end, the pump speed was changed to 0.33 mL/min after 6.5 h, and the dialysis was continued for an additional 13.5 h. The sample was collected from the dialysis tube and stored at 4 °C.

In the case of the reconstitution with histone H1, histone H1 was added in the middle of the dialysis (at a NaCl concentration of 600 mM) or after the salt dialysis was completed (at a NaCl concentration of 50 or 100 mM). The molar ratio of histone H1 to the histone octamer is 1:1 except for the experiments described in Figure 3. The addition of histone H1 during the dialysis was performed as described previously (*26*, *27*). After the addition of histone H1, the dialysis was resumed and continued until the NaCl concentration reached 50 or 100 mM.

AFM Analysis. After fixation with 0.3% glutaraldehyde for 30 min at room temperature, the reconstituted chromatin solution was dropped onto a freshly cleaved mica substrate, which was pretreated with 10 mM spermidine. After 10 min at room temperature, the mica was washed with water and dried under nitrogen. AFM observation was performed with a Nanoscope IIIa or IV (Digital Instruments) in air under the tapping mode. The cantilever (OMCL-AC160TS-W2, Olympus) 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 accompanying the imaging module.

In the geometrical analyses of AFM images (Table 1), the "tip effect" was removed by using the apparent size of nucleosomes as a reference (*28*). The apparent dimensions of the molecules obtained by AFM are dependent upon the radius of the tip curvature and are apparently larger than the real dimensions. The relationship among 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 $W = 4(R_cR_m)^{1/2}$ (*29*). When two different molecules with R_{m1} and R_{m2} radii are imaged with the same tip, the relationship between the measured widths (W_1 and W_2) can be given by $W_1 = W_2(R_{m1}/R_{m2})^{1/2}$. Since the diameter of the nucleosome is known to be 11 nm (*30*), 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).

Table 1: Summary of the AFM Images of the Reconstituted Chromatin with or without Histone H1

hitone H1	final [NaCl] of 50 mM	final [NaCl] of 100 mM
without	beads on a string (Figure 1a−c)	beads on a string with short linker some aggregates (Figure 1d−f)
added after the salt dialysis was completed	20 nm fiber (Figure 2a,c,d)	mainly 20 nm fiber some 30 nm fiber some aggregates (Figure 4i,j)
added during the salt dialysis (at 600 mM)	20 nm fiber 30 nm fiber (Figure 5a−c)	aggregates (Figure 5f−h)
added after the salt dialysis (final NaCl concentration of 50 mM), and redialysis against the buffer containing 100 mM NaCl		mainly 30 nm fiber (Figure 4a−c)

RESULTS

Chromatin Reconstitution and Its Salt-Dependent Conformational Change. In the previous studies, we have established a highly efficient in vitro procedure for the salt dialysis-based chromatin reconstitution (*31*). Quantitative structural analyses of the reconstituted chromatin using a single-molecule imaging technique, atomic force microscopy (AFM), demonstrated that a negative supercoiling of the template DNA is critical for the efficient nucleosome formation. Namely, the more negative supercoils the template DNA carried, the more efficiently the nucleosome was reconstituted (*21*). With this reconstitution procedure, a 100 kb circular plasmid carefully isolated from *Escherichia coli* harbored 371 ± 47 nucleosomes [mean ± SD (n = 6)]; one nucleosome was formed per 270 bp (Figure 1b). In this study, this reconstitution procedure with 100 kb circular plasmids was used under different experimental conditions.

The higher-order structure of the nucleosome array is affected by the salt environment (*7*). The previous studies reported that a clear beads-on-a-string structure could be seen in 50 mM NaCl (Figure 1a,b; for high-magnification images, see Figure 1c). Under this condition, every nucleosome formed on the 100 kb circular plasmid can be seen as a bead in the beads-on-a-string (Figure 1b). When the dialysis was stopped at a NaCl concentration of 100 mM (Figure 1d), the distance between nucleosomes (Figure 1i) became much shorter than that in 50 mM NaCl (Figure 1h). In addition, partial aggregation of nucleosomes was observed (Figure 1f, right panels). This salt-dependent aggregation of nucleosomes can be explained by interaction between nucleosomes induced by a high salt concentration (*32*).

Histone H1 per se Induces Higher-Order Folding of Chromatin Fiber. Linker histone H1 was purified from HeLa cells and added to the reconstituted nucleosomes formed on the 100 kb supercoiled plasmid after salt dialysis was completed at 50 mM NaCl (Figure 2a). The micrococcal nuclease treatment of this H1-containing chromatin produced an ∼170 bp band (Figure 2b, lanes 4−6), whereas that of the chromatin fiber not containing H1 produced an ∼150 bp fragment (Figure 2b, lanes 1−3), indicating that histone H1 was incorporated into linker DNA.

When the H1-containing chromatin fiber was observed by AFM, some portions formed thick fibers (Figure 2c). The average length and width of this thick fiber were 395 ± 261 nm [mean ± SD (n = 79)] and 21.2 ± 5.13 nm [mean ± SD (n = 164)] (Figure 2f), respectively. High-magnification images (Figure 2d) revealed that this fiber was made up of the solenoid-like repetitive turns along the fiber axis. The peak-to-peak distance between adjacent solenoid turns was 23.8 ± 7.77 nm [mean ± SD (n = 104)]. The height of the fiber measured 10.1 ± 2.25 nm [mean ± SD (n = 164)] (Figure 2g), which is ∼4 times greater than that of a single nucleosome [2.71 ± 0.280 nm, mean ± SD (n = 86) (Figure 1g)], indicating that these thick chromatin fibers resulted from a three-dimensional arrangement of a nucleosomal array, and are not from a mere two-dimensional association of neighboring nucleosomes.

Dose Dependency of Linker Histone H1 for Formation of Thicker Fibers. In the experiments shown in Figure 2, the molar ratio of histone H1 to the core histone octamer was 1. When this ratio was reduced to 0.25, the nucleosome particles could still be seen all over the plasmid, but thick chromatin fibers were hardly detected (Figure 3a). Although only some portions formed 20 nm fibers (Figure 3b,d), the length was significantly shorter than that in Figure 2 (Figure 3c). This result suggests that the 20 nm fibers are the first and thinnest unit above nucleosomes for constructing higher-order architectures.

On the other hand, the addition of an increased amount of H1 tended to induce thicker chromatin structures. When the H1:core histone ratio equaled 2, thick bead-like structures were formed in addition to the 20 nm fibers (Figure 3e). When the histone H1−core histone octamer was added at a ratio of 4, the 20 nm fiber was no longer observed (Figure 3g), and the entire chromatin was compacted into a single large structure composed of many beads which had a diameter of 93.8 ± 27.3 nm [mean ± SD (n = 176)] (Figure 3f). Thus, the amount of H1 in the reconstitution system was found to be critical for the formation of the thicker fibers.

Physiological Ionic Strength Is Necessary for the Formation of 30 nm Fibers. We observed three-dimensionally folded thick chromatin fibers by just adding the linker histone to the reconstituted chromatin fiber. However, most of the fiber had a width of ∼20 nm. The 30 nm fiber was previously observed by EM and AFM after being isolated from the nucleus. To elucidate the difference between these fibers and our fibers, the H1-containing chromatin fiber was exposed to the solution with different ionic strengths.

Histone H1 was added to the reconstituted chromatin fiber at a NaCl concentration of 50 mM, and then, the NaCl concentration was slowly increased to 100 mM (Figure 4a). The AFM observation (Figure 4b,c) revealed that the width of the fiber was relatively uniform and measured around 30 nm (Figure 4d). The height was 12.5 nm (Figure 4e), which is larger than that of the 20 nm fiber (10.1 nm, Figure 2g). The appearance of the 30 nm fiber was similar to that of the 20 nm fiber, i.e., solenoid-like repetitive turns. However, the peak-to-peak distance of the solenoid turn was 21.6 ± 6.28

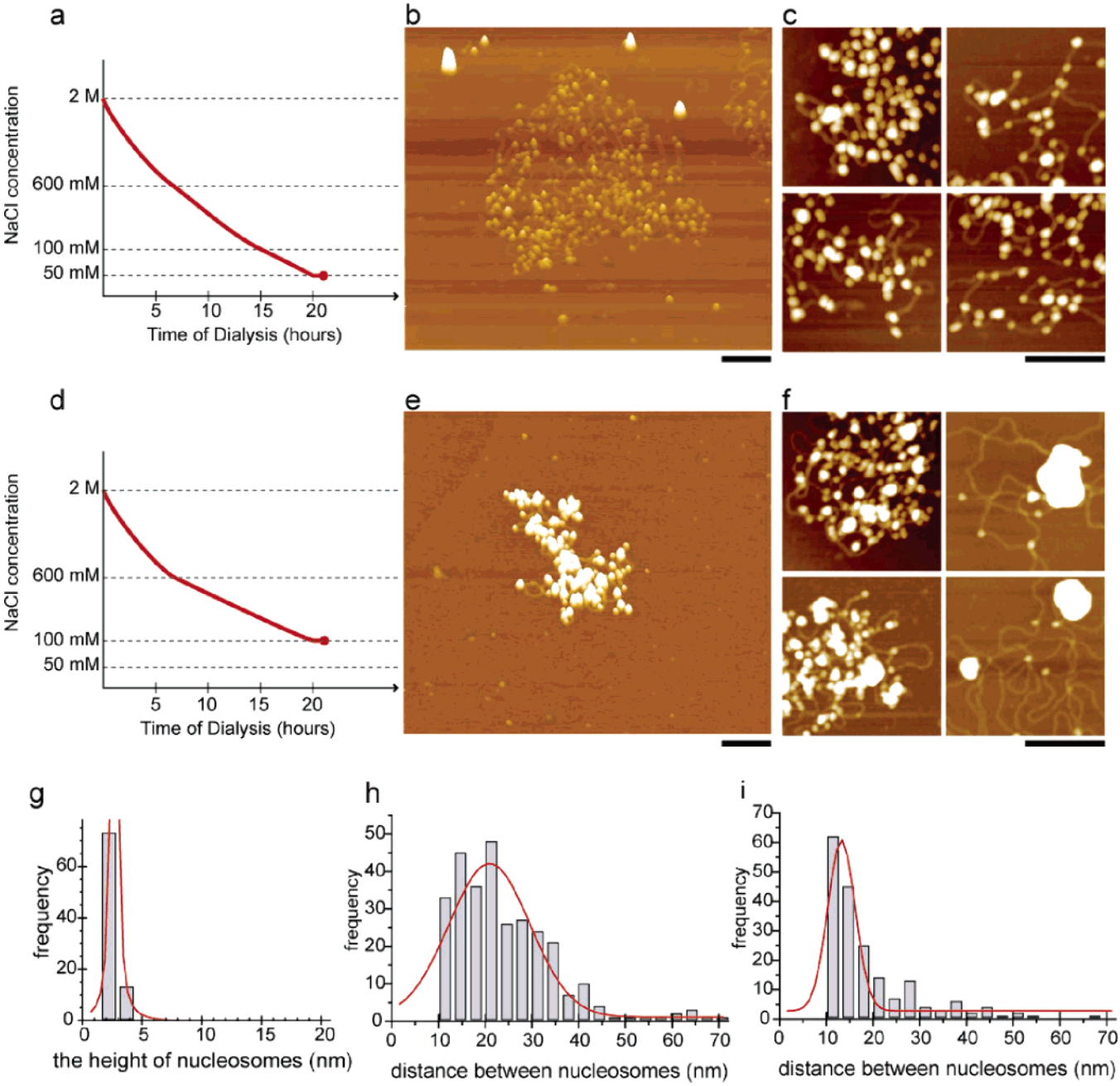

FIGURE 1: Chromatin reconstitution on the 100 kb plasmid in the absence of histone H1. The chromatin was reconstituted by the salt dialysis procedure and observed by AFM. The final concentration of the dialysis was either 50 mM (a−c, g, and h) or 100 mM (d−f and i). (a and d) Diagrams of the salt dialysis procedure. (b, c, e, and f) Representative AFM images of the reconstituted chromatin. Bars are 200 nm. The height of the nucleosome (g; $n = 86$) and the nucleosome−nucleosome distance (h and i; $n = 190$ and 292, respectively) were measured and summarized. Due to the imaging in air, the height of the nucleosome in our AFM image was approximately half of that from the X-ray crystallography data (5.6 nm) (*30*).

nm [mean ± SD ($n = 89$)] and was significantly ($p = 0.028$) shorter than that of the 20 nm fiber. When the salt concentration was reduced to 50 mM, the 20 nm fibers became dominant (Figure 4f−h), indicating that the salt-induced conformational change between 20 and 30 nm fibers is a reversible process.

When histone H1 was added to the reconstituted chromatin at 100 mM NaCl (Figure 4i), the 30 nm fiber was also observed (Figure 4j). However, 80% of the thick fiber had a width of 20 nm (Figure 4k), suggesting that the higher-order folding of the chromatin fiber depends not only on the final salt concentration but also on the ionic environment when histone H1 is added. The incorporation of H1 into the chromatin fiber and/or the intermolecular interaction of histone H1 might be affected by the salt concentration.

To further investigate the effect of salt, histone H1 was added during the reconstitution (Figure 5a,f). In this procedure, histone H1 was added to the reconstitution reaction mixture at a NaCl concentration of 600 mM, where the nucleosome structure has already been formed (*17*). After the addition of H1, the dialysis of the reaction mixture was continued to a final NaCl concentration of 50 or 100 mM. When the dialysis was continued to 50 mM, only a small fraction of the chromatin formed the 30 nm fiber (Figure 5b−d). However, the average length of the fiber was 179 ± 94 nm [mean ± SD ($n = 44$)], approximately one-half of

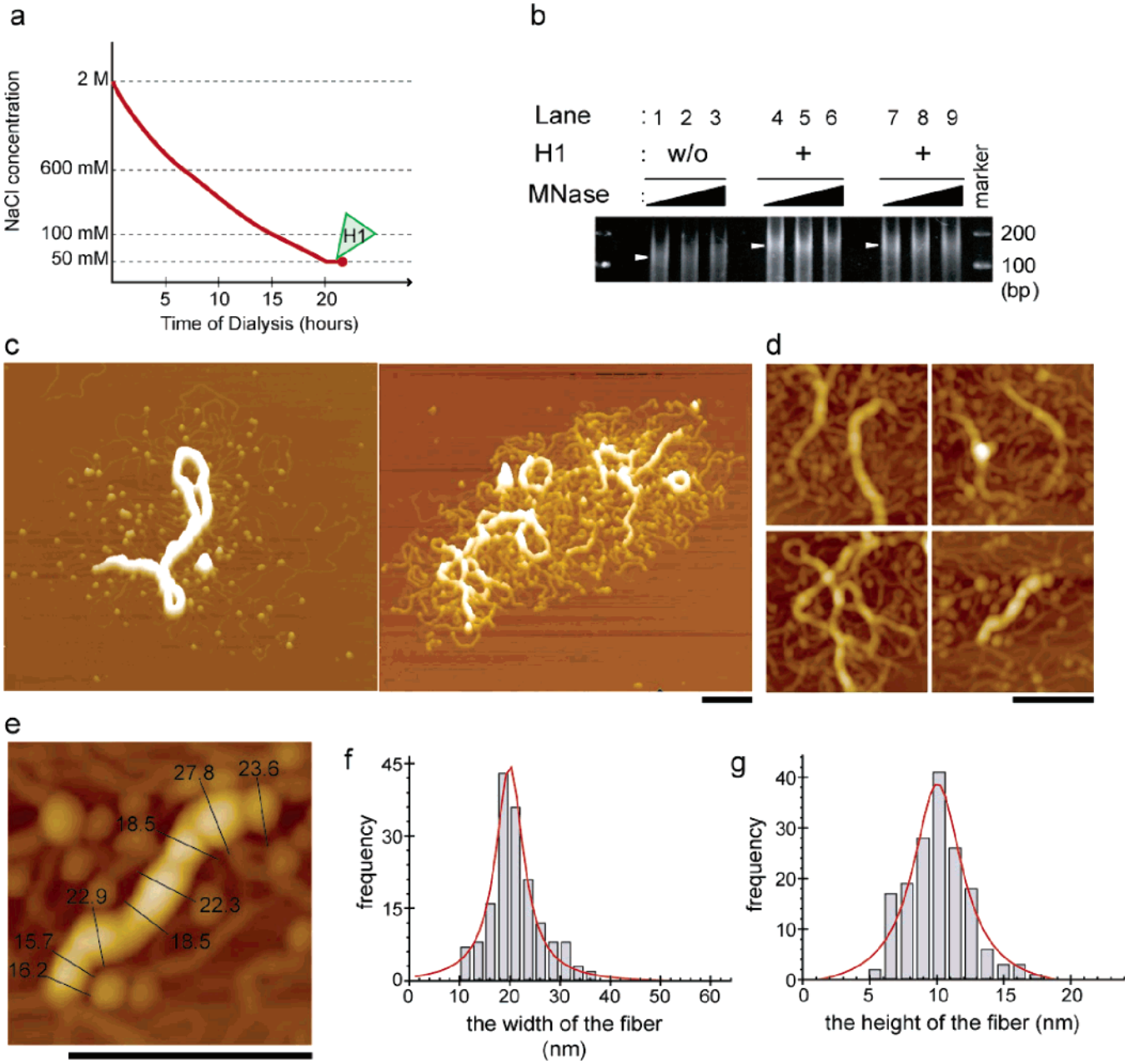

FIGURE 2: Reconstituted chromatin with linker histone H1 in 50 mM NaCl. Histone H1 was added to the reconstituted chromatin after the salt dialysis was completed at 50 mM NaCl. (a) Diagram of the salt dialysis procedure. (c and d) Representative AFM images of the thick chromatin fiber with large *x* and *y* and small *z* scales (c) and small *x* and *y* and large *z* scales (d). The fiber shown in the left part of panel c was composed of 78 helical turns. The chromatin image in the right part of panel c was supposed to contain two chromatin fibers, because of the total number of nucleosomes (712). Bars are 200 nm. (e) Enlarged image of the thick chromatin fiber seen in panel d. The widths of the fiber were indicated. The width (f; $n = 164$) and the height (g; $n = 164$) of the thick fiber were measured on AFM images and summarized. (b) Micrococcal nuclease digestion assay of reconstituted chromatin fibers. The chromatin without histone H1 (lanes 1−3), with histone H1 added at 50 mM NaCl (lanes 4−6), or with H1 added in the middle of the dialysis (600 mM) (lanes 7−9) was subjected to micrococcal nuclease treatment and analyzed by polyacrylamide gel electrophoresis.

the length of that formed after salt dialysis (Figures 2a−c and 4a−c). When the dialysis was stopped at 100 mM NaCl (Figure 5f), the thick fibers could hardly be seen, but large aggregations of the nucleosomes were found (Figure 5g,h). The incorporation of histone H1 into linker DNA could be seen in the micrococcus nuclease assay (Figure 2b, lanes 7−9), but the signal was slightly weaker than when the 20 nm fiber was formed (Figure 2b, lanes 4−6). All of these results indicate that not only the final salt concentration but also the timing of the H1 addition largely affects the higher-order folding of the chromatin fiber.

DISCUSSION

In this study, we successfully demonstrated that histone H1 per se is able to induce a three-dimensional folding of the chromatin fiber into 30 nm fibers at the physiological salt concentration. The effect of histone H1 on the higher-order folding of the chromatin fiber has previously been investigated by using di- or oligonucleosome (*27*, *33*). However, although preceding EM studies have demonstrated that the in vivo chromatin fiber forms a thick fiber with a width of ~30 nm (*7*, *8*, *34*), how the linker histone is involved in the formation of this 30 nm fiber despite the

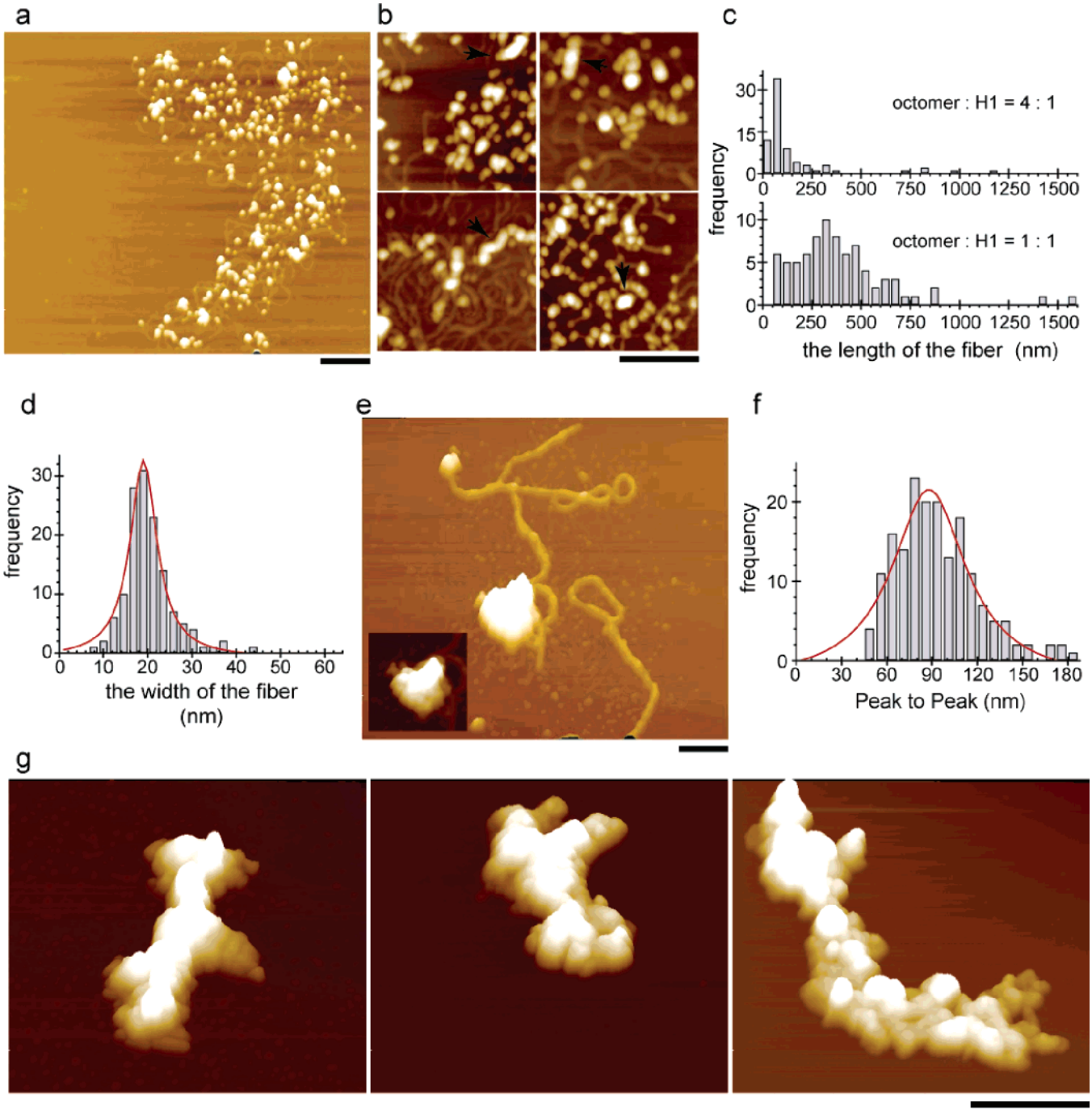

FIGURE 3: Reconstitution of chromatin with various amounts of linker histone H1. Various amounts of H1 were added to the reconstituted chromatin after the salt dialysis was stopped at 50 mM NaCl. Histone H1 and histone octamer were added at a molar ratio of 1:4 (a−d), 2 (e), or 4 (f and g). Scale bars in the AFM images are 200 nm (a, b, and e) and 1 μm (g). Arrows in panel b indicate the 20 nm fibers, the lengths [c, top (n = 79)] and the widths [d (n = 136)] of which were measured and histogrammed. The lengths of the fibers reconstituted with an equal molar ratio of H1 and histone octamer (Figure 2) were also measured (n = 72) and histogrammed (c, bottom). At a molar ratio of 4, large complexes were observed (g), which consisted of beads. The bead sizes were measured (n = 176) as the peak-to-peak distance, and histogrammed (f).

fact that linker histone H1 binds to the linker DNA still remains unknown.

Principles of Beads-on-a-String Nucleosome Formation. Our results demonstrate that the higher-order folding of the chromatin fiber depends on both the presence of linker histone and the interaction between nucleosomes. From these results, together with other results from other studies (*21*, *31*, *33*, *35*), an unfolding−refolding model of the chromatin fiber was proposed (Figure 6a). In this model, "highly supercoiled" and "long circular" DNA is necessary as a template (*21*). At the very low salt concentration (0−50 mM), chromatin fiber without histone H1 is well-extended and exhibits a beads-on-a-string configuration (Figures 1 and 6a). As the salt concentration is increased to ~500 mM, the interaction between nucleosomes becomes stronger, which then induces nucleosome aggregation (Figures 1 and 6a) (*32*). When the salt concentration becomes higher than 600 mM, core histones dissociate from DNA (*17*). These steps can be reversed when the salt concentration is slowly reduced, which was seen in our salt dialysis procedure (Figures 1 and 6a).

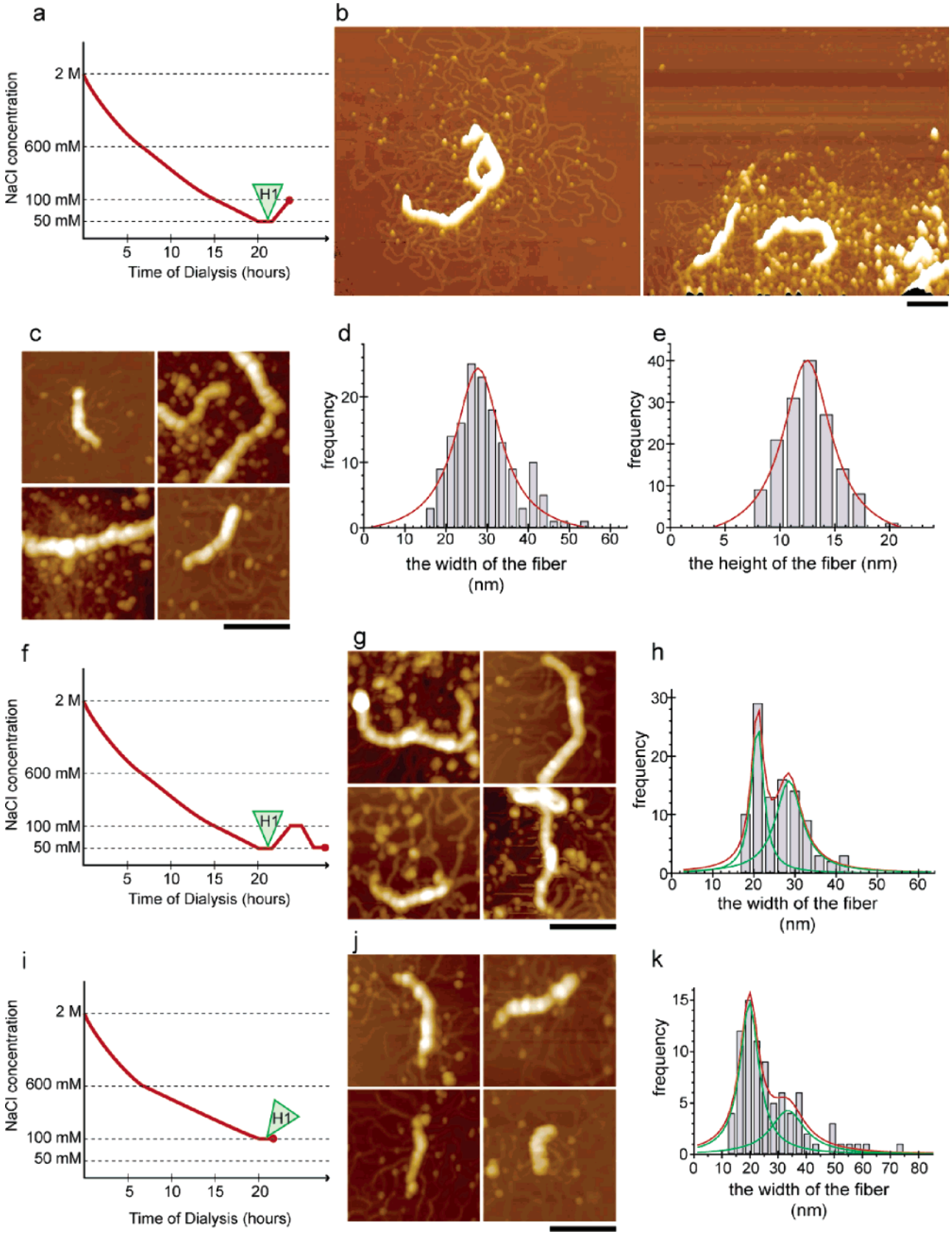

FIGURE 4: Reconstituted chromatin with linker histone H1 in 100 mM NaCl. (a−e) Nucleosomes were reconstituted by dialysis from 2 M to 50 mM. Histone H1 was added as shown in Figure 2, and then the mixture was dialyzed against 100 mM NaCl. (f−h) The reconstituted mixture was prepared as shown in panel a, and then dialyzed against 50 mM NaCl again. (i−k) The dialysis was stopped at a NaCl concentration of 100 mM, and then histone H1 was added. (a, f, and i) Diagrams of the dialysis procedure. (b, c, g, and j) AFM images of the thick chromatin fibers. Bars are 200 nm. The width [d ($n = 151$), h ($n = 99$), and k ($n = 84$)] and the height [e ($n = 151$)] of the thick fiber were measured and summarized.

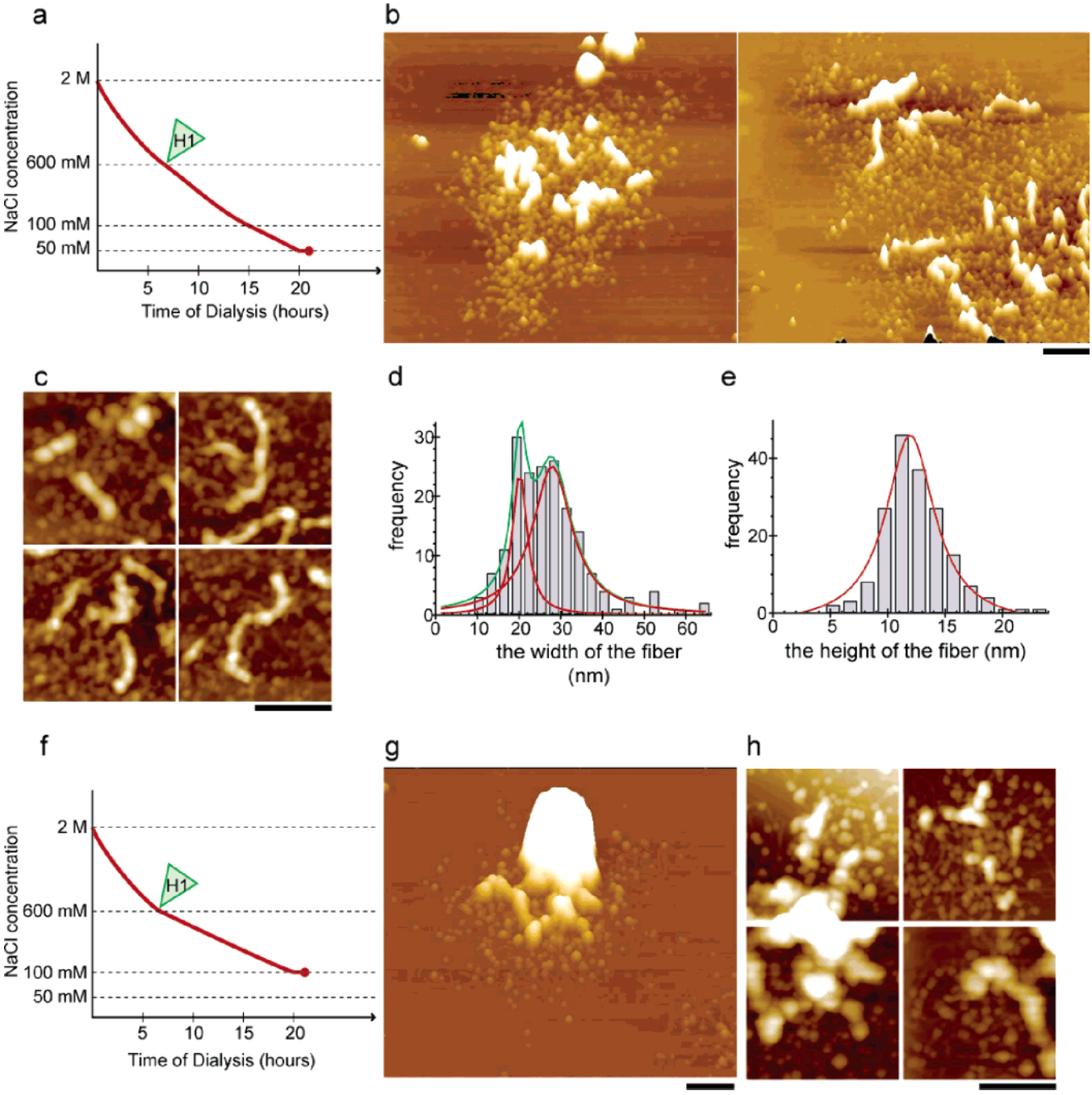

FIGURE 5: Histone H1 was added in the middle of the dialysis. Histone H1 was added to the reconstitution reaction mixture at a NaCl concentration of 600 mM, and then the dialysis was continued to the final NaCl concentration of 50 (a−e) or 100 mM (f−h). (a and f) Diagrams of the salt dialysis procedure. (b, c, g, and h) Representative AFM images of the thick chromatin fibers. Bars are 200 nm. The width [d (n = 180)] and the height [e (n = 179)] of the thick fiber were measured and summarized.

Thus, in the absence of histone H1 and at varying salt concentrations, unfolding and refolding processes of a beads-on-a-string fiber are reversible without formation of well-organized thicker fibers.

The nucleosome−nucleosome interaction is largely affected by salt concentration and composition. The EM study of oligonucleosomes (*32*) and the measurement of the sedimentation coefficient of chicken erythrocyte chromatin depleted of H1 (*36*) have demonstrated that a high salt concentration induces nucleosome compaction. The amino-terminal tails of the core histones have been thought to become more extended under high-salt conditions, which then strengthen the interaction between nucleosomes (*37*, *38*). The amino-terminal tails of core histones are relatively unstructured and undergo a variety of post-translational modifications such as phosphorylation, acetylation, and methylation (*39*). Histone tails are not necessary for the formation of the nucleosome core (*40*) but are involved in the regulation of chromatin structure through (i) the interaction with nucleosomal and linker DNA, (ii) the interaction with non-nucleosomal proteins, and (iii) nucleosome−nucleosome interaction (*41*). Thus, the salt may affect the interactions of histone tails, resulting in folding or unfolding of the beads-on-a-string fiber.

A Model of 30 nm Chromatin Fiber Folding. When linker histone H1 was added to the chromatin fiber at 50 mM NaCl,

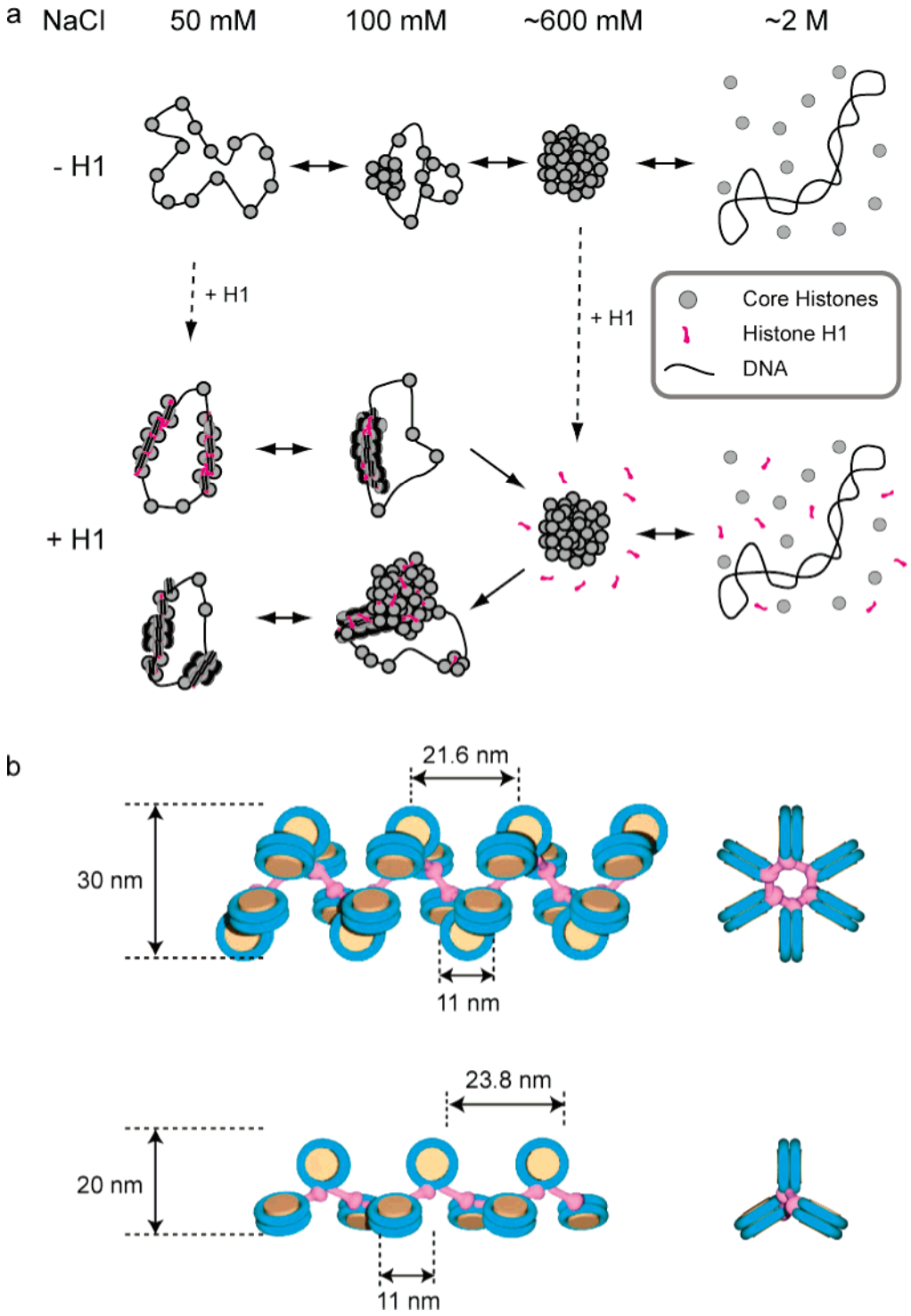

FIGURE 6: Structure and dynamics of thick chromatin fibers. (a) Dynamic structural changes in the chromatin fiber in the absence (top) or presence (bottom) of linker histone H1 (magenta) with a change in the NaCl concentration. The vertical dashed arrows indicate the addition of H1. See the text for details. (b) Models of nucleosome arrangement in the 30 nm fiber (top) and the 20 nm fiber (bottom). The position of the linker histone (magenta) was based on the previous studies (*12*). For simplicity, nucleosomes were represented without linker DNA. Side views of these fibers are given at the right.

H1 can properly bind to linker DNA and mediates the formation of the 20 nm fiber (Figures 2 and 6a). When the salt concentration was then increased to 100 mM, the chromatin fiber became thicker (~30 nm) (Figures 4a−e and 6a). Geometrical analyses of the thick chromatin fibers on AFM images predict that the 20 nm fiber is reasonably made up by triad positioning of nucleosomes, as shown in Figure 6b (left), whereas the 30 nm fiber is supposed to have a hexagonal nucleosome positioning along the fiber axis (Figure 6b, right). In the chromatin fiber shown in Figure 2c (left), the 20 nm thick fiber contains ~251 nucleosomes [this number was deduced by subtracting the number of nucleosomes in the beads-on-a-string conformation (119) from the total number of nucleosomes (supposed to be ~370 from the result in Figure 1)]. Since the thick fiber had 78 solenoidal turns (Figure 2), each turn should contain 3.2 nucleosomes on average, implying that the nucleosomes are arranged in a triadic pattern as shown in Figure 6b. Similarly,

the 30 nm fiber (Figure 4b) should contain 5.6 nucleosomes per turn (Figure 6b), which is close to the previously proposed "hexagonal arrangement" (*7*, *10*, *42*).

Our model shown in Figure 6b is based on a simple solenoid model, although more complicated models involving different nucleosome arrangements (*42*) are now available. A recent report favors a zigzag model which can be explained by the theoretical prediction and biochemical/EM data after internucleosome cross-linking (*43*). However, since we did not detect any "zigzag" configurations of the beads-on-a-string nucleosome array in our reconstituted chromatin, a simple solenoidal model seems to be sufficient to explain the dynamic changes of the fiber widths as expected from the earlier EM observation on the fibers isolated from the cell nucleus (*7*). A calculation of the energy of nucleosomes with linker DNA of various lengths and linker entry−exit angles has predicted that there are two stable structures in the nucleosome arrays independent of the entry−exit angle (*44*). These data together with our results suggest that the chromatin fibers have several different conformational states depending on a subtle environmental change.

Salt-Induced Conformational Change between 20 and 30 nm Fibers. Under our experimental conditions, approximately half of the total nucleosomes in a chromatin fiber (67% in the case of Figure 2c) were incorporated into the 20 nm fiber and the rest of the nucleosomes are still in a beads-on-a-string configuration. The nucleosome−nucleosome distance in the beads-on-a-string fiber in Figure 2c is larger than that of the fiber in the absence of histone H1 (Figure 1). On the basis of the fact that H1 reduces the nucleosome−nucleosome distance when it binds to linker DNA (*33*), and the fact that generally nucleosome can freely slide along the DNA strand (*45*), histone H1 likely assembles the nucleosomes along the DNA strand and contributes to the formation of a well-organized solenoid structure. On the other hand, the transition from 20 to 30 nm fibers is possibly caused by salt-induced nucleosome−nucleosome interaction. Although an increasing salt concentration results in a random aggregation of nucleosomes in the absence of histone H1 (Figure 1), it induces a coordinated compaction of H1-containing thick chromatin fiber from 20 to 30 nm (Figures 4a−e and 6a). This could be supported by the fact that the pitch of the solenoid in the thick chromatin fiber was slightly smaller in the 30 nm fiber (21.6 nm) than in the 20 nm fiber (23.8 nm).

When histone H1 was added at 100 mM NaCl, the width of the thick fiber was distributed between 20 and 30 nm (Figure 4i−k). This is probably because at higher salt concentrations, the chromatin fiber is already somehow compacted and it is more difficult for histone H1 to access to linker DNA. When the salt concentration increases further to 500 mM, histone H1 starts to dissociate from the native chromatin (*34*). On the basis of this result, it was once suggested that if histone H1 was added in the middle of the salt dialysis procedure (at ∼500 mM NaCl), H1 would be properly incorporated into the chromatin fiber and mediate the higher-order chromatin folding (*26*, *34*, *46*). However, under our experimental conditions, the addition of histone H1 in the middle of the reconstitution step could not dramatically increase the extent of formation of the 30 nm fiber (Figure 5); when the dialysis was continued to 100 mM NaCl after the addition of H1, a large aggregation of nucleosomes which is different from the thick chromatin fiber was formed (Figures 5 and 6a). Considering the fact that in a high-salt environment (∼600 mM NaCl) the nucleosomes are tightly compacted and aggregated, the histone H1 may not be able to access linker DNA properly and may cause a random aggregation, although it can still dissociate from the chromatin. Thus, the chromatin folding process with histone H1 by salt dialysis does not seem to be a simple reverse process of salt-mediated unfolding (Figure 6a).

As discussed above, histone H1 promotes the coordinated formation of 30 nm solenoid fibers. This process would produce additional superhelical tensions. Just after the formation of beads-on-a-string structures with core histones, most of the tensions given by the negative supercoiling of the 100 kb plasmid are expected to be absorbed by nucleosome formation (*21*). If we assume that the all the beads-on-a-string structures on the 100 kb plasmid turned into 30 nm fibers upon addition of histone H1, ∼61 solenoidal turns would be created to form a single entire fiber. This much change in the superhelical tensions will easily be absorbed by the system, considering the entire length (100 kb) of the DNA used in the reconstitution. It is interesting that, in reality, ∼67% of the beads-on-a-string structures usually turn into 30 nm solenoid fibers.

Understanding of the Higher-Order Chromatin Folding in Vivo. The 30 nm solenoid fibers are thought to be packed into much higher-order structures in vivo. The chromatin fibers are highly packed into condensed chromosomes and heterochromatin in the mitotic and interphase cells, respectively. Lines of evidence suggest that the 70−80 nm fiber or bead structures exist in the interphase (*47*) and mitotic cells (*48*−*51*). The molecular mechanisms of such higher-order structure formation remain to be solved. However, other lines of evidence imply the important role of physical properties of a chromatin fiber in such a structural transition (*52*, *53*). In this context, chromatin dynamics in response to ionic conditions analyzed in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (*54*) is intriguing; a dynamic change of the chromatin structure from 30−40 nm to ∼100 nm fibers was detected just with a change in the NaCl concentration. Namely, the construction of higher-order architectures is highly dependent on subtle changes in the environmental conditions, and is governed by the physical nature (i.e., phase transition) of the chromatin fibers (*35*). It is of interest that the ∼90 nm beaded structure was reconstituted by addition of a larger amount of H1 (Figure 3f).

All the available biological models include a series of regulatory proteins that lead to chromatin remodeling (*3*, *55*−*57*). On the other hand, our model relies mostly on the physical properties of chromatin and its behavior in different environments. It seems critical for gene regulation that the biological significance of the chromatin architecture is determined to some extent by the physical nature of both the length of DNA (i.e., ∼100 kb between SAR/MAR regions) and the nucleosome−nucleosome interaction. The condensin complex and topoisomerase II are expected to hold the two DNA strands at the remote regions to give rise to a tension to the trapped DNA (*58*).

NOTE ADDED IN PROOF

During the course of the preparation of this paper, a paper on the thicker fiber reconstitution (*59*) was published. Since this investigation was done by EM and from a very different point of view, the information provided will well complement the results reported herein. As shown in panels f and g of Figure 3, we observed an ~90 nm beaded structure in the sample reconstituted at a 4:1 ratio of histone H1 to core histones. After 20 μL of the reconstituted chromatin with 20 ng of DNA was put on the mica surface, only one or two complexes harboring the ~90 nm beads were detected in the 100 μm × 100 μm scannning area, whereas ~500 chromatins in the same scannning area existed in the case of other samples shown in this study. The volume calculated from the AFM image of the ~90 nm beaded complex was $0.89-6.63 \times 10^8$ nm^3, which was 1000 times bigger than that of the chromatin without H1. These results suggest that the ~90 nm beaded complex consisted of hundreds of thousands of 100 kb chromatin fibers. The mechanism of the formation of the 90 nm beads is unclear and beyond the scope of this paper, but it should be one more step higher-order structure than the 30 nm fiber. As described in the last section of Discussion, the 80−100 nm structures have been observed in the different areas of the nuclear chromatin.

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BI050623V