BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Structural Analysis of the Hexasome, Lacking One Histone H2A/H2B Dimer from the Conventional Nucleosome

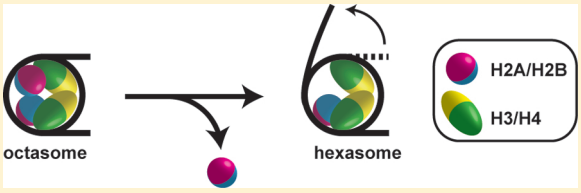
Yasuhiro Arimura,[†] Hiroaki Tachiwana,[†] Takashi Oda,[‡] Mamoru Sato,[‡,§] and Hitoshi Kurumizaka*[,†]

[†]Laboratory of Structural Biology, Graduate School of Advanced Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan

[‡]Division of Macromolecular Crystallography, Department of Supramolecular Biology, Graduate School of Nanobioscience, Yokohama City University, 1-7-29 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan

[§]RIKEN SPring-8 Center, Kouto 1-1-1, Sayo, Hyogo 679-5148, Japan

ABSTRACT: Genomic DNA is packaged into chromatin in eukaryotes, and the nucleosome is the fundamental unit of chromatin. The canonical nucleosome is the octasome, which is composed of two H2A/H2B dimers and two H3/H4 dimers. During transcription elongation, one of the H2A/H2B dimers is removed from the octasome. The depletion of the H2A/H2B dimer is also suggested to occur during DNA replication and repair. The remaining histone components are believed to maintain a nucleosomal structure called a "hexasome", which is probably important for the regulation of gene expression, DNA replication, and repair in chromatin. However, hexasomes are currently poorly understood, due to the lack of *in vivo* and *in vitro* studies. Biochemical and structural studies of hexasomes have been hampered by the difficulty of preparing purified hexasomes. In the present study, we successfully reconstituted hexasomes, using recombinant human histones. A micrococcal nuclease treatment and *in vitro* reconstitution assays revealed that the hexasome tightly wraps approximately 110 base-pairs of DNA, about 40 base-pairs shorter than the length of the DNA wrapped within the canonical nucleosome. A small-angle X-ray scattering analysis revealed that the global structure of the hexasome is similar to that of the canonical nucleosome. Our studies suggest that octasomes can be converted into hexasomes by the eviction of one of the H2A/H2B dimers, and the release of about 40 base-pairs of DNA, without involving large structural changes in the nucleosome core particle.



In eukaryotes, chromatin organizes the genomic DNA, and therefore, transcription, replication, recombination, and repair must occur within the chromatinized DNA. The nucleosome is the elemental repeating unit of chromatin and consists of 146 base-pairs of DNA and the histone octamer.[1] The histone octamer is composed of the core histones H2A, H2B, H3, and H4. Histones have a common structural motif, called the "histone-fold", within the central region of each molecule, and it is responsible for specific heterodimer formation. H2A forms a heterodimer with H2B (H2A/H2B dimer), and H3 forms a heterodimer with H4 (H3/H4 dimer) through the histone-fold domain.[2,3] Accumulating evidence indicates that the nucleosome is assembled from the H2A/H2B and H3/H4 dimers in a stepwise manner. Two H3/H4 dimers are first loaded on the DNA to form a tetrasome, in which the DNA is partially wrapped around the H3/H4 tetramer. Two H2A/H2B dimers are then incorporated, and the resulting histone octamer wraps 146 base-pairs of DNA to form the nucleosome (octasome). In addition to the canonical nucleosome containing two H2A/H2B dimers and two H3/H4 dimers, the existence of a hexasome, in which one of the two H2A/H2B dimers is depleted from the octasome, has been suggested. Previous studies have shown that the hexasome is an important intermediate structure during the disassembly, remodeling, and reassembly of nucleosomes in replication, transcription, recombination, and DNA repair.[4−6]

The importance of the hexasome has been demonstrated in transcription. The hexasome is reportedly formed when RNA polymerase II passes through the nucleosome.[7] This finding is consistent with previous observations that the nucleosome structure may be altered by transcription with RNA polymerase II, which probably involves the disassembly of an H2A/H2B dimer.[8,9] Consistently, fluorescence recovery after photobleaching (FRAP) experiments revealed that a fast-exchange H2B fraction, which may reflect the exchanged H2A/H2B dimer during transcription, was eliminated by inhibiting transcription elongation.[10] Rapid exchange of the H2A/H2B dimer also occurs at transcriptionally active loci in cells.[11] These findings support the formation of the hexasome during transcription elongation.

The RNA polymerase II-dependent hexasome formation is stimulated by the histone chaperone FACT (facilitates chromatin transcription) complex,[12] which is required to facilitate transcription elongation by RNA polymerase II on the nucleosomal DNA.[13] Interestingly, the FACT-like

Received: January 28, 2012
Revised: March 22, 2012
Published: March 26, 2012

   dx.doi.org/10.1021/bi300129b | *Biochemistry* 2012, 51, 3302−3309

hexasome formation activity during the RNA polymerase passage was also found with nucleolin, which is an abundant nucleolus protein.[14] The major histone chaperone Nap1 also promotes the eviction of a single H2A/H2B dimer from the octasome.[15−17] Studies indicated that the H2A/H2B eviction by FACT may also occur in the double-strand break repair of DNA. In this DNA repair process, FACT promotes the replacement of H2A by the phosphorylated form of H2AX (γ-H2AX),[18] which accumulates at damaged sites within DNA.[19] Similar H2A/H2B eviction was also reported with SWR1, a chromatin remodeling protein, which catalyzes the exchange of the H2A/H2B dimer with the H2A.Z/H2B dimer.[20,21] The hexasome is also suggested to be an intermediate in this H2A-H2A.Z exchange process.[22]

Despite these significant biological phenomena described for the hexasome, only limited structural information is available for it because the *in vitro* preparation of purified hexasomes has not been accomplished. In the present study, we established a method for hexasome reconstitution and purification and prepared highly purified hexasomes composed of recombinant human histones. Biochemical and structural analyses revealed that the hexasome structure is similar to the canonical nucleosome structure. We also showed that the hexasome contains a histone hexamer that stably wraps 112 base-pairs of DNA.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Human Histones. The human histones were produced as the N-terminally His_6-tagged proteins in *Escherichia coli* cells, as described previously.[23] The His_6-tagged histones were recovered from the insoluble fraction and were resuspended in 50 mL of 50 mM Tris-HCl buffer (pH 7.5), containing 7 M guanidine hydrochloride, 500 mM NaCl, and 5% glycerol. The His_6-tagged histones were then purified by a three-step procedure, including nickel−nitrilotriacetic acid (Ni-NTA) agarose chromatography (Qiagen), thrombin protease (1 unit/mg of histones; GE Healthcare) treatment, and Mono S column chromatography (GE Healthcare), as described previously.[24,25] The purified histones were dialyzed against water, freeze-dried, and stored at 4 °C.

Preparation of the H2A/H2B, H2A.Z/H2B, H3/H4, and CENP-A/H4 Complexes. The reconstitution of the H2A/H2B, H3/H4, and CENP-A/H4 complexes was performed according to a previously published method,[26−28] with modifications. The H2A.Z/H2B complex was reconstituted by the same method used for the H2A/H2B complex. Briefly, purified histones H2A and H2B (1 mg/mL), or histones H3 and H4 (1 mg/mL), were dissolved at 1:1 stoichiometry in 50 mM Tris-HCl buffer (pH 7.5), containing 2 mM EDTA, 7 M guanidine-HCl, and 10 mM DTT and were gently mixed by rotation at 4 °C for 1 h. The mixtures were then dialyzed for 4 h against 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 2 M NaCl (500 mL). This dialysis step was repeated four times with freshly prepared dialysis buffer. For the CENP-A/H4 complex, CENP-A and H4 (0.5 mg/mL) were dissolved at 1:1 stoichiometry in 50 mM Tris-HCl buffer (pH 7.5), containing 2 mM EDTA, 7 M guanidine-HCl, and 10 mM DTT, and were gently mixed by rotation at 4 °C for 1 h. The mixtures were then dialyzed for 4 h against 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 2 M NaCl (500 mL), and the NaCl concentration was gradually decreased to 0.1 M in a stepwise manner. The reconstituted H2A/H2B, H2A.Z/H2B, H3/H4, and CENP-A/H4 complexes were fractionated by Superdex 200 (GE Healthcare) gel filtration chromatography.

Preparation of DNA Fragments for Hexasome Reconstitution. A 193-base-pair DNA fragment containing the Widom 601 sequence[29] was amplified by polymerase chain reaction (PCR), using the following primers: FW 5′- AGGTA CCAAG ATCTG ATATC GGACC CTATC GCGAG CCAGG CCTGA GAATC CGGTG CCGAG GCCGC TCAAT-3′ and REV 5′-AGGAT CCGAT ATCTA TGAAT TTCGC GACAC AAGGC CTGGA TGTAT ATATC TGACA CGTGC C-3′. The resulting 193-base-pair DNA fragment was ligated into the pGEM-T easy vector (Promega), and the 601(193)-4 plasmid, which contained four tandem copies of the 193-base-pair 601 sequence, was obtained. The 193-base-pair DNA fragment was prepared by *Eco*RV digestion.

A 112-base-pair DNA fragment containing the α-satellite sequence was prepared by self-ligation of the 54-base-pair DNA fragment containing a four-base overhang. The 58-base-pair DNA fragment (54 + 4 base-pairs) was amplified by PCR, with the α-satellite DNA[1] as the template, using the following primers: FW 5′-GGTAC CAAGA TCTGA TATCT CTACC AAAAG TGTAT TTGGA AACTG C-3′ and REV 5′-GGATC CGATA TCGAA TTCAG CTGAA CATGC CTTTT G-3′. The resulting 58-base-pair DNA fragment was ligated into the pGEM-T easy vector (Promega), and the Satellite112 plasmid, which contained 16 tandem copies of the 58-base-pair sequence, was obtained. The 54-base-pair DNA fragment containing a four-base overhang was prepared by *Eco*RV and *Eco*RI digestions. The 112-base-pair DNA was obtained by self-ligation of the 54-base-pair DNA containing a four-base overhang (5′ AATT 3′) at this cohesive site.

Preparation of the Hexasome. The purified H2A/H2B complex (54−74 μg) and H3/H4 complex (143 μg) were mixed with a 193-base-pair DNA fragment (240 μg), in a solution containing 2 M KCl. For the CENP-A nucleosome, the CENP-A/H4 complex (94−104 μg) was used instead of the H3/H4 complex. For the H2A.Z nucleosome, the H2A.Z/H2B complex (53 μg) was used instead of the H2A/H2B complex. For the hexasome reconstitution with a 112-base-pair DNA fragment (1000 μg), the purified H2A/H2B complex (680 μg) and H3/H4 complex (1100 μg) were used. The mixtures were dialyzed against 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA, 1 mM dithiothreitol, and 2 M KCl, at 4 °C. The hexasome reconstitution was then performed by gradually decreasing the KCl concentration to 250 mM with a peristaltic pump (0.8 mL/min flow rate). To eliminate the nonspecific histone−DNA binding, the reconstituted hexasomes and/or octasomes were incubated at 55 °C (for the experiments with the 193-base-pair DNA) for 1 h or 65 °C (for the experiments with the 112-base-pair DNA) for 2 h. The hexasomes and/or octasomes were separated from the free DNA and histones by nondenaturing polyacrylamide gel electrophoresis, using a Prepcell apparatus (Bio-Rad). The purified hexasomes or octasomes were concentrated and were dialyzed against 20 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA and 1 mM dithiothreitol.

Micrococcal Nuclease (MNase) Treatment Assay. The purified hexasomes (10 μg/mL) or octasomes (10 μg/mL) were treated with 0, 0.5, 1.0, and 2.0 units of MNase (Takara), in 20 μL of 57.5 mM Tris-HCl (pH 7.5), 12.5 mM NaCl, 12.5 mM $CaCl_2$, 0.75 mM DTT, and 0.75 mM EDTA. After a 5 min incubation at 23 °C, the reaction was stopped by the addition

dx.doi.org/10.1021/bi300129b | *Biochemistry* 2012, 51, 3302−3309

of 15 μL of proteinase K solution (20 mM Tris-HCl (pH 7.5), 80 mM EDTA, 0.25% SDS, and 0.5 mg/mL proteinase K (Roche)). After a 30 min incubation at 23 °C, the DNA was precipitated with ethanol and then analyzed by 10% PAGE in 0.5 × TBE buffer (9 V/cm for 2 h).

ExoIII Treatment Assay. The ExoIII treatment assay was performed according to a previously published method.[28] Briefly, the purified hexasomes (40 μg/mL) or octasomes (40 μg/mL) were treated with ExoIII (5 units, Takara), in 10 μL of 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 2 mM DTT. The reactions were incubated for 2.5, 5, and 10 min at 37 °C and were stopped by the addition of 55 μL of proteinase K solution (20 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.25% SDS, and 0.5 mg/mL proteinase K (Roche)). After a 10 min incubation at 23 °C, the DNA was extracted by phenol/chloroform and was precipitated with ethanol. The DNAs were then analyzed by denaturing 10% PAGE containing 7 M urea in 0.5 × TBE buffer (18 V/cm for 1 h).

Dynamic Light Scattering Measurements. Dynamic light scattering analyses of the hexasomes (1.0 mg/mL) and octasomes (1.0 mg/mL) were performed with Zetasizer Nano μV (Malvern Instruments) in 20 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA and 1 mM dithiothreitol, at 25 °C.

Small-Angle X-ray Scattering (SAXS). SAXS measurements of the reconstituted hexasomes and octasomes, in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT, were performed at the RIKEN structural biology beamline I (BL45XU) of SPring-8 (Hyogo, Japan).[30] The scattering intensities of the hexasome and octasome solutions were measured with a PILATUS300 K-W detector at 20 °C with a sample-to-detector distance of 3509 mm, which was calibrated by the powder diffraction from silver docosanoate. Circular averaging of the scattering intensities was then performed, to obtain the one-dimensional scattering data $I(q)$ as a function of q ($q = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle, and the X-ray wavelength λ = 1.0 Å). Nine successive measurements were made for each solution, with an exposure time of 20 s. The resultant nine data sets were combined after inspections for X-ray radiation damage to the solution and the existence of instrumental artifacts. SAXS measurements of the buffer solution for background subtraction were performed after each measurement of the nucleosome solutions, using the same conditions and procedure as those for the nucleosome solutions. To correct the interparticle interference effect, the $I(q)$ data were collected at three protein concentrations (0.7, 1.0, and 1.3 mg/mL) and extrapolated to zero concentration. The data were processed and analyzed using the software applications embedded in the ATSAS package. The radius of gyration, R_g, was estimated by fitting the $I(q)$ data using the Guinier approximation $I(q) = I(0) \exp(-q^2R_g^2/3)$, where $I(0)$ is the forward scattering at the zero scattering angle, in a smaller angle region of $qR_g < 1.3$. The error of R_g was estimated from the least-squares fitting. The distance distribution function $P(r)$ and its error were calculated by the program GNOM.[31] The maximum dimension, D_{max}, was estimated from the $P(r)$ function as the distance r, where $P(r) = 0$,[32] and its error was estimated from the errors of the $P(r)$ values around $P(r) = 0$.

RESULTS

Reconstitution of the Hexasome. We purified the human histones H2A, H2B, H3 (H3.1), and H4 as bacterially expressed recombinant proteins, and the H2A/H2B and H3/H4 complexes were prepared. We then performed the nucleosome reconstitution by the salt-dialysis method. A 193-base-pair DNA fragment, containing the 601 nucleosome positioning sequence,[29] was used as the DNA substrate. The nucleosome reconstitution was performed with a reduced amount of the H2A/H2B complex (73.8 μg), relative to the amount of the H3/H4 complex (143 μg). Under these conditions, two nucleosomal bands were detected upon an electrophoretic mobility shift assay (EMSA) (Figure 1A). The nucleosomes reconstituted on the 193-base-pair DNA fragment are very stable and are maintained after an incubation at 55 °C, while nonspecific histone-DNA binding is disrupted by this

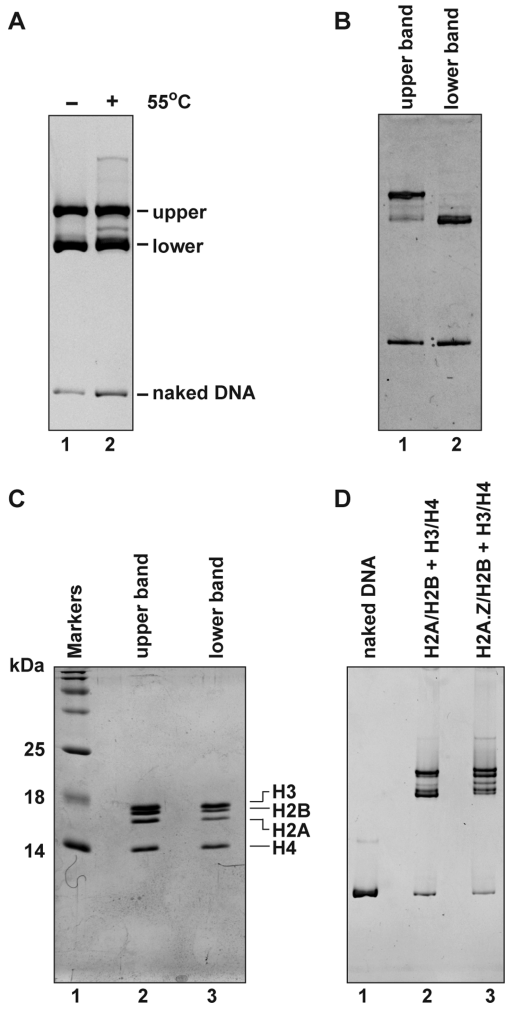


Figure 1. Reconstitution of hexasomes and octasomes with a 193-base-pair DNA fragment. (A) The hexasomes and octasomes were reconstituted by the salt-dialysis method and were analyzed by nondenaturing 6% PAGE. Lanes 1 and 2 indicate the samples before and after a 55 °C incubation, respectively. DNA was visualized by ethidium bromide staining. (B) The octasomes (upper band, lane 1) and the hexasomes (lower band, lane 2) were purified using a Prepcell apparatus and were analyzed by nondenaturing 6% PAGE with ethidium bromide staining. (C) Histone compositions of the purified octasomes (upper band, lane 2) and hexasomes (lower band, lane 3) were analyzed by 18% SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1 indicates molecular mass markers. (D) The hexasomes and octasomes were reconstituted by the salt-dialysis method with histone H2A.Z. Lane 1 indicates the naked DNA. Lane 2 represents a positive control experiment with canonical H2A. Lane 3 indicates the experiment with H2A.Z. The samples were analyzed by nondenaturing 6% PAGE with ethidium bromide staining.

dx.doi.org/10.1021/bi300129b | Biochemistry 2012, 51, 3302–3309

heat treatment.[24] As shown in Figure 1A (lane 2), both the upper and lower bands were still detected after a 1 h incubation at 55 °C, suggesting that both bands correspond to nucleosomes. Therefore, two types of nucleosomes were reconstituted under the conditions with a reduced amount of the H2A/H2B complex by the salt-dialysis method.

We then purified these two nucleosomes by fractionation on a nondenaturing polyacrylamide gel, using a Prepcell apparatus (Figure 1B). The upper band contained histones H2A, H2B, H3, and H4 with a 1:1:1:1 stoichiometry (Figure 1C, lane 2), indicating that it is the canonical nucleosome (octasome). On the other hand, the lower band contained all four histones, but the band intensities of H2A and H2B were half of those of H3 and H4 (the band intensity of H2A was 51.9%, relative to the H4 band intensity) (Figure 1C, lane 3). This suggests that the lower band corresponds to the hexasome, consisting of one H2A/H2B dimer and two H3/H4 dimers that form the histone hexamer, and the DNA fragment that wraps around the histone complex.

Multiple bands, probably corresponding to multiple nucleosome positions, were observed when H2A.Z (H2A.Z1) was used instead of H2A under the experimental conditions for the hexasome formation (Figure 1D). Therefore, the H2A.Z hexasome may be more mobile than the hexasome containing canonical H2A.

The Hexasome Wraps 112 Base-Pairs of DNA. We next examined the DNA length that is wrapped within the hexasome. To do so, we treated the fractions containing the reconstituted octasomes and hexasomes with micrococcal nuclease (MNase), which preferentially digests the DNA region detached from the histone surface in the nucleosomes. In the octasome, about 150 base-pair DNA fragments were protected from MNase digestion (Figure 2A, lanes 2−5). On the other hand, in the hexasome, only about 115 base-pairs of DNA were protected from MNase digestion (Figure 2A, lanes 6−9). These results suggest that about 115 base-pairs of DNA were tightly wrapped within the hexasome, and about 30−40 base-pairs from the DNA ends are detached from the histone surface. To confirm this, we performed a nucleosome reconstitution experiment with a 112-base-pair DNA. We found that only one type of nucleosome was formed using this DNA (Figure 2B). The reconstituted nucleosome contained all four histones, but the H2A/H2B band intensities were about half of those of H3/H4 (the band intensity of H2A was 50.3%, relative to the H4 band intensity) (Figure 2C). Thus, we found that the reconstitution of nucleosomes on a DNA fragment that is about 30 base-pairs shorter than the canonical size (146 base-pairs) results in the formation of a hexasome, instead of an octasome.

The Hexasome Containing CENP-A. We previously reported that CENP-A, the centromere-specific histone H3 variant, forms the octasome, and the DNA segments at the entry/exit sites are detached from the histone surface.[28,33−35] We performed the MNase assay with the CENP-A hexasome. The CENP-A octasome and hexasome were reconstituted by the same method used for the canonical H3 octasome and hexasome (Figure 3A, lane 1). The upper and lower bands, which corresponded to the CENP-A octasome and hexasome, respectively, were purified on a nondenaturing polyacrylamide gel, using a Prepcell apparatus (Figure 3A, lanes 2 and 3). Consistent with the previous results, the DNA segments at the entry/exit sites of the CENP-A ocatsome were more susceptible to nuclease (MNase) than those of the H3

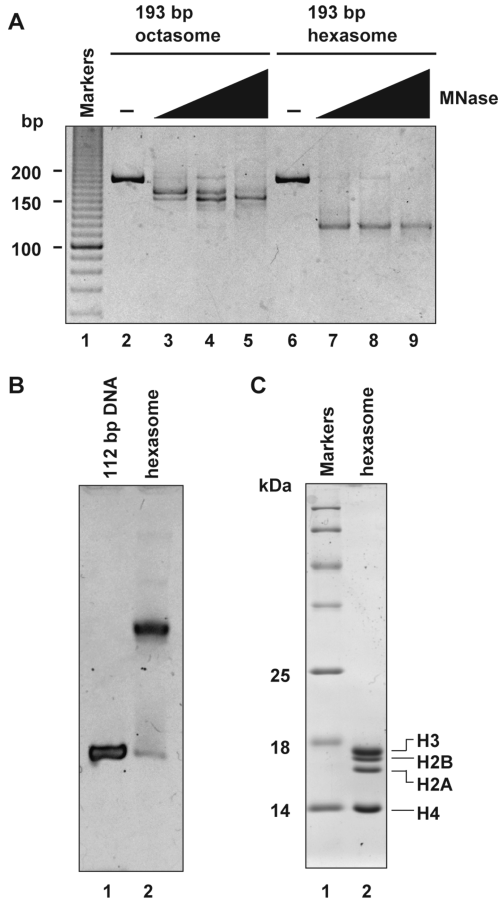


Figure 2. Formation of the hexasome with a 112-base-pair DNA fragment. (A) MNase treatment assay. The purified octasomes (Figure 1B, lane 1) and hexasomes (Figure 1B, lane 2) were treated with 0 units (lanes 2 and 6), 0.5 units (lanes 3 and 7), 1.0 units (lanes 4 and 8), and 2.0 units (lanes 5 and 9) of MNase. The resulting DNAs were analyzed by 10% PAGE. Lane 1 indicates 10-base-pair DNA ladder markers. (B) The hexasomes reconstituted with a 112-base-pair DNA fragment were purified using a Prepcell apparatus and were analyzed by nondenaturing 6% PAGE with ethidium bromide staining. (C) Histone compositions of the purified hexasomes were analyzed by 18% SDS-PAGE with Coomassie Brilliant Blue staining (lane 2). Lane 1 indicates molecular mass markers.

octasome (Figure 3B, lane 2 and lanes 4−7). In the CENP-A hexasome, the DNA segments at the entry/exit sites were quite susceptible to the MNase digestion (Figure 3B, lanes 8−11), as compared to those of the H3 octasome (Figure 3B, lane 3). Therefore, the flexible nature of the DNA within the CENP-A octasome at the entry/exit sites may also be conserved in the CENP-A hexasome.

Asymmetric DNA Wrapping in the Hexasome. To identify the boundaries of the entry/exit sites of the DNAs in the octasome and the hexasome with histones H2A, H2B, H3, and H4, we performed an ExoIII assay. In this assay, the nucleosomal DNA was treated with ExoIII, which exonucleolytically digests a single strand only from the 3′ end, and consequently generates a 5′ single-stranded region. The DNA region directly bound to the histone surface was protected from the ExoIII digestion. In the octasome with the 601 sequence, the 145-base-pair DNA region is tightly wrapped around the histone octamer.[36,37] Therefore, a single-stranded DNA (ssDNA) fragment containing about 165−170 bases would be detected by a denaturing PAGE analysis, if the DNA was

dx.doi.org/10.1021/bi300129b | Biochemistry 2012, 51, 3302−3309

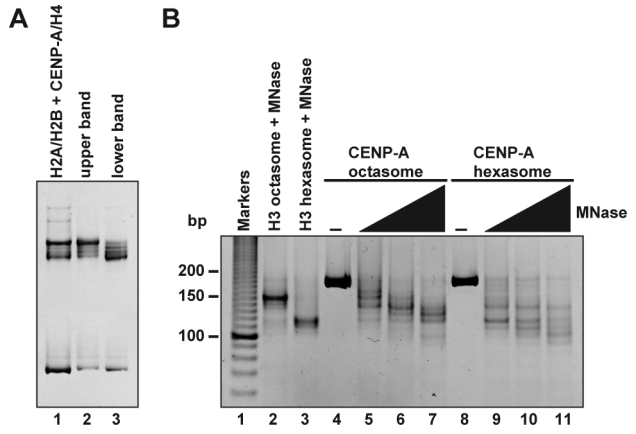


Figure 3. Reconstitution of hexasomes and octasomes with CENP-A and a 193-base-pair DNA fragment. (A) The CENP-A hexasomes and octasomes were reconstituted by the salt-dialysis method and were analyzed by nondenaturing 6% PAGE. DNA was visualized by ethidium bromide staining. Lane 1 indicates the sample before fractionation. Lanes 2 and 3 represent the octasomes (upper band, lane 2) and the hexasomes (lower band, lane 3), purified using a Prepcell apparatus. (B) MNase treatment of the CENP-A nucleosomes. The purified CENP-A octasomes (Figure 3A, lane 2) and the CENP-A hexasomes (Figure 3A, lane 3) were treated with 0 units (lanes 4 and 8), 0.5 units (lanes 5 and 9), 1.0 units (lanes 6 and 10), and 2.0 units (lanes 7 and 11) of MNase. Lanes 2 and 3 indicate control experiments with the H3 octasome and the H3 hexasome in the presence of 2.0 units of MNase. The resulting DNAs were analyzed by 10% PAGE. Lane 1 indicates 10-base-pair DNA ladder markers.

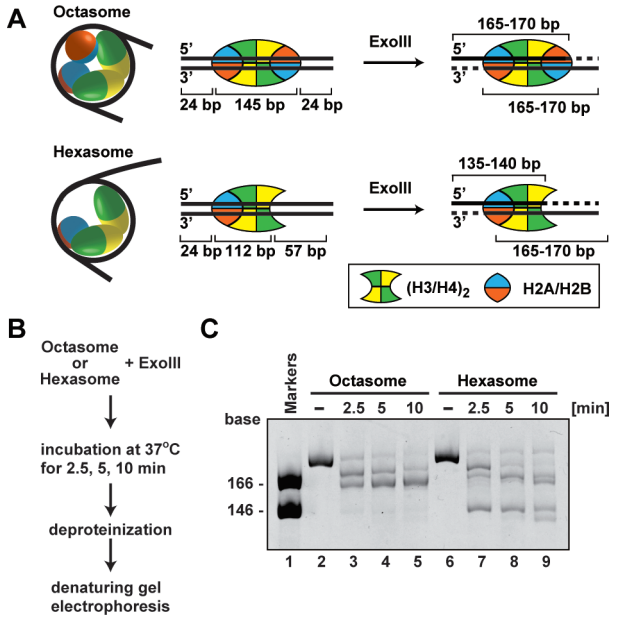


Figure 4. The ExoIII treatment assay. (A) Schematic representations of the hexasome and octasome used in the ExoIII treatment assay. In this assay, the canonical histones H2A, H2B, H3, and H4 are used for the octasome and hexasome reconstitutions. The octasome and the hexasome are represented in the upper and lower panels, respectively. The ssDNA fragments detected by this assay are represented by solid lines. Dashed lines indicate the ssDNA regions, which may be exonucleolytically digested in the hexasome and octasome. (B) Schematic representation of the ExoIII treatment assay. (C) The purified octasomes and hexasomes were treated with 5 units of ExoIII. The resulting DNAs were analyzed by denaturing 10% PAGE. Lane 1 indicates molecular mass markers, including 146- and 166-base ssDNAs. Lanes 2–5 and 6–9 indicate experiments with the octasome and the hexasome, respectively. Lanes 2 and 6 indicate negative control experiments in the absence of ExoIII. Reaction times were 2.5 min (lanes 3 and 7), 5 min (lanes 4 and 8), and 10 min (lanes 5 and 9).

symmetrically detached at both sides of the entry/exit sites in the nucleosome (Figure 4A, upper panel). On the other hand, two ssDNA fragments, encompassing about 135−140 bases and 165−170 bases, would be detected if the DNA was asymmetrically detached (Figure 4A, lower panel). As anticipated, in the Exo III digestion experiments with the octasome, an ∼165-base ssDNA was detected by denaturing PAGE analysis (Figure 4C, lanes 2−5). In addition, two ssDNA fragments of approximately 145 and 165 bases were detected, when the hexasome was treated with ExoIII (Figure 4C, lanes 6−9). These results strongly suggested that the DNA was asymmetrically detached at either the entry or exit site (probably at the site lacking an H2A/H2B dimer) in the hexasome.

Dynamic Light Scattering Analysis of the Hexasome. The MNase and ExoIII treatment assays revealed that the MNase- or ExoIII-susceptible DNA region of the hexasome is larger than that of the octasome, suggesting that the DNA segments located at the entry/exit site of the nucleosome may be detached from the histone surface in the hexasome. If this is the case, then the hydrodynamic radius of the hexasome may be larger than that of the octasome, when both nucleosomes are formed with the same length of DNA (Figure 5A). To test this possibility, we analyzed hexasomes and octasomes that were reconstituted on the 193-base-pair DNA fragment by dynamic light scattering (DLS). The measurements revealed that the mean particle size of the octasome with the 193-base-pair DNA was about 13.5 nm in diameter (Figure 5B). By contrast, the mean particle size of the hexasome with the 193-base-pair DNA was about 16 nm in diameter, which was clearly larger than that of the octasome (Figure 5B). These DLS data are consistent with the MNase data and indicate that the DNA segment is unwrapped at the entry/exit of the hexasome.

Small-Angle X-ray Scattering Analysis of the Hexasome. We next performed a small-angle X-ray scattering (SAXS) analysis of the hexasome. In the SAXS measurements, we used the hexasome containing a 112-base-pair DNA fragment (Figure 2B). For comparison, the octasome containing a 146-base-pair DNA fragment was reconstituted and subjected to the SAXS analysis, as previously reported.[38] Interestingly, despite the difference in the DNA lengths in the hexasome and the octasome, the hexasome generated nearly identical SAXS curves to those of the octasome (Figure 6A). The distance distribution function ($P(r)$) profiles of the hexasome and the octasome were also quite similar (Figure 6B). These results indicated that the overall structures of the hexasome and the octasome are similar. On the other hand, the radius of gyration (R_g = 38.5 Å) and the maximum diameter (D_{max} =115 Å) of the hexasome were substantially smaller than those of the octasome (R_g = 42.0 Å, D_{max} = 135 Å). This may reflect local structural differences in the nucleosomes due to the absence of one H2A/H2B dimer and the shorter DNA in the hexasome.

We then modeled the hexasome structure (hexasome^{cryst}) from the octasome crystal structure (PDB ID 3AFA)[24] by removing one H2A/H2B dimer and the DNA segment located near the H2A/H2B dimer (Figure 7A). This hexasome^{cryst} model contained 112 base-pairs of DNA. The SAXS curve

dx.doi.org/10.1021/bi300129b | Biochemistry 2012, 51, 3302−3309

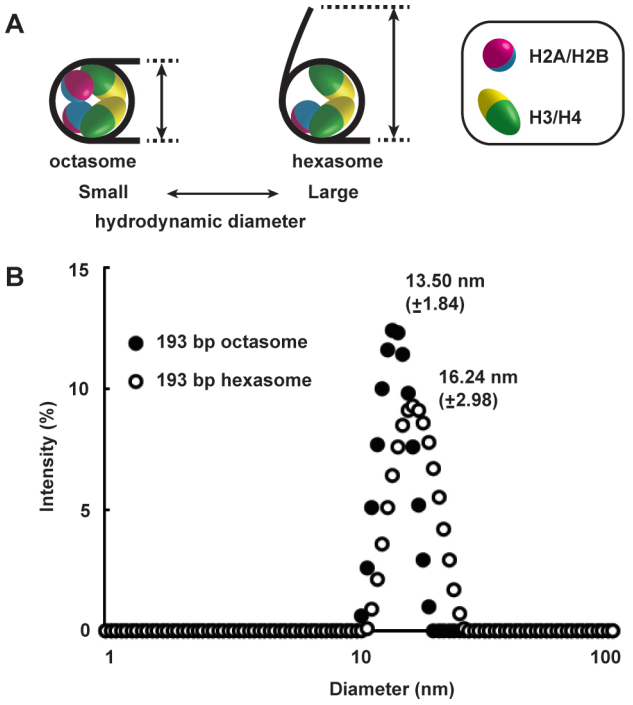


Figure 5. Dynamic light scattering (DLS) analysis of the hexasome. (A) Schematic representations of the octasome and the hexasome. DNAs are depicted by bold black lines. (B) The DLS profiles of the octasome and the hexasome with a 193-base-pair DNA fragment. The distributions of the particle sizes of the octasomes (closed circles) and the hexasomes (open circles) are plotted. The mean particle sizes of the octasomes (13.50 nm) and the hexasomes (16.24 nm) are indicated with errors.

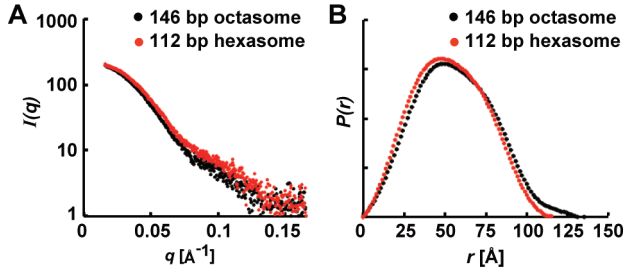


Figure 6. Small-angle X-ray scattering (SAXS) measurements of the hexasome. (A) Normalized SAXS curves of the hexasome (with a 112-base-pair DNA) and the octasome (with a 146-base-pair DNA). Red and black lines show the SAXS curves as a function of q ($q = 4\pi \sin \theta/\lambda$) for the hexasome and the octasome, respectively. (B) Distance distribution functions $P(r)$ of the hexasome (red dots) and the octasome (black dots). The maximum dimensions D_{max} of the hexasome and the octasome, estimated from the $P(r)$ function as the distance r, where $P(r) = 0$.

obtained for the hexasome^{cryst} model fit very well with the SAXS curve of the actual hexasome (Figure 7B). The distance distribution function ($P(r)$) profiles (curve shapes) of the hexasome^{cryst} and the hexasome were also very similar (Figure 7C), although the D_{max} value of hexasome^{cryst} was slightly smaller, which probably reflects the absence of the flexible DNA ends in the static crystal structure and the higher flexibility of the overall structure in solution, as compared to that in the crystal. Therefore, we concluded that the structure of the hexasome core particle containing a 112-base-pair DNA fragment is similar to that of the hexasome^{cryst} structure, which

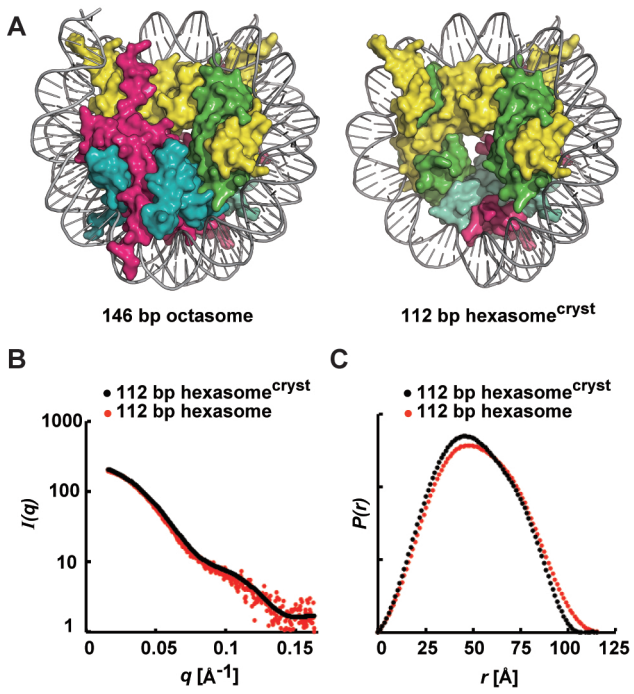


Figure 7. Comparison of the hexasome with its model. (A) The hexasome model (right panel, hexasome^{cryst}), created by removing one H2A/H2B dimer and the DNA segment located near the H2A/H2B dimer from the octasome crystal structure (PDB ID 3AFA), shown in the left panel. This hexasome^{cryst} model contained 112 base-pairs of DNA. (B) The normalized SAXS curve of the hexasome^{cryst} model (black line). For comparison, the SAXS data of the hexasome presented in Figure 6A are also presented (red dots). (C) The distance distribution function $P(r)$ of the hexasome^{cryst} model (black dots). For comparison, the $P(r)$ distribution profile of the hexasome, shown in Figure 6B, is also presented (red dots).

was generated by removing one H2A/H2B dimer from the canonical nucleosome.

DISCUSSION

The hexasome is considered to be an intermediate structure formed during the assembly and disassembly processes of mature nucleosomes.[4−6,22] Previous studies suggested that the hexasome has a specific function during transcription elongation.[7,12] In addition, the hexasome appears to be important during DNA replication and repair processes.[4] Based on these findings, the biological significance of the hexasome has been extensively discussed. However, the hexasome structure remains to be elucidated.

In the present study, we reconstituted the hexasome with human histones H2A, H2B, H3, and H4 and successfully purified the hexasome to near-homogeneity. The reconstituted hexasome was not disrupted by an incubation for 1 h at 55 °C. This heat-stable nature of the hexasome is the same as that of the octasome containing two each of the conventional histones.[24,25] The SAXS analysis revealed that the structure of the reconstituted hexasome was similar to the modeled hexasome, in which one H2A/H2B dimer is removed from the crystal structure of the human octasome.

Importantly, we found that the hexasome efficiently forms with a 112-base-pair DNA fragment. Consistently, in the hexasome reconstituted with a 193-base-pair DNA fragment, about 115 base-pairs of DNA are protected from the MNase

dx.doi.org/10.1021/bi300129b | Biochemistry 2012, 51, 3302−3309

digestion, indicating that this protected DNA region is tightly bound to the histone hexamer. In our reconstitution experiments, the octasome was not stably formed with a 112-base-pair DNA, suggesting that the 112-base-pair fragment may be too short to stably form the octasome, but is sufficient for the hexasome. In agreement with this possibility, octasomes and hexasomes were simultaneously formed, when a 193-base-pair DNA fragment was used in the nucleosome reconstitution. Approximately 145−147 base-pairs of DNA are required for stable octasome formation.[1,36,37,39,40] Therefore, about 35 base-pairs of DNA become detached from the histone surface, upon H2A/H2B eviction from the octamer. In the octasome structure, each H2A/H2B dimer symmetrically binds to the DNA region about 10 base-pairs away from the proximal DNA end and covers about 30−40-base-pair regions.[1] The absence of one H2A/H2B dimer in the hexasome may asymmetrically detach this DNA region from the histone surface. Consistent with this idea, our ExoIII treatment assay revealed the asymmetric detachment of the DNA region at the entry/exit sites in the hexasome, but not in the octasome.

The detachment of the DNA segments at the edge of nucleosomes has been reported in nucleosomes containing C-terminally truncated H2A mutants.[41] The histone H2A.Bbd, an H2A variant that appears to be associated with active chromatin, reportedly forms the octasome with 118−130 base-pairs of DNA, indicating that the DNA segments at the entry/exit sites of the nucleosome are detached.[42,43] These results are consistent with the view that the H2A/H2B dimer plays an important function for the stable association of the DNA segment at the entry/exit sites of the nucleosome. Intriguingly, we found that the H2A.Z and CENP-A histone variants also have the potential to form hexasomes *in vitro*; however, these hexasomes may be less stable, as compared to those containing canonical H2A, H2B, H3, and H4. Therefore, the hexasome formation with histone variants may function to regulate gene expression and maintenance in chromatin.

During transcription processes, hexasome formation is actively promoted by the FACT complex, which removes one H2A/H2B dimer from the octasome.[12] This H2A/H2B eviction activity of FACT may relieve the kinetic barrier of the nucleosomal DNA during RNA polymerase passage.[44,45] When the RNA polymerase passes through the nucleosome, the DNA ahead of the polymerase detaches from the histone surface.[46] This may allow RNA polymerase to proceed with transcription elongation in the DNA region that is normally incorporated within the octamer. FACT may promote the formation of the hexasome just in front of RNA polymerase by the H2A/H2B-eviction activity and may detach the DNA from the histone surface for transcription elongation through nucleosomal DNA.

AUTHOR INFORMATION

Corresponding Author

*Tel +81-3-5369-7315; Fax +81-3-5367-2820; e-mail kurumizaka@waseda.jp.

Funding

This work was supported in part by Grants-in-Aid from the Japanese Society for the Promotion of Science (JSPS), and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. H.K. was also supported by the Waseda Research Institute for Science and Engineering.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. W. Kagawa for critical reading of this manuscript. We are also grateful to Dr. T. Hikima for his assistance with data collection at the BL45XU beamline of SPring-8.

ABBREVIATIONS

DLS, dynamic light scattering; D_{max}, maximum dimension; EMSA, electrophoretic mobility shift assay; FACT, facilitates chromatin transcription; FRAP, fluorescence recovery after photobleaching; MNase, micrococcal nuclease; PAGE, polyacrylamide gel electrophoresis; R_g, radius of gyration; SAXS, small-angle X-ray scattering; SDS, sodium dodecyl sulfate.

REFERENCES

(1) Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature 389*, 251−260.

(2) Arents, G., Burlingame, R. W., Wang, B. C., Love, W. E., and Moudrianakis, E. N. (1991) The nucleosomal core histone octamer at 3.1 Å resolution: a tripartite protein assembly and a left-handed superhelix. *Proc. Natl. Acad. Sci. U. S. A. 88*, 10148−10152.

(3) Arents, G., and Moudrianakis, E. N. (1995) The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc. Natl. Acad. Sci. U. S. A. 92*, 11170−11174.

(4) Groth, A., Rocha, W., Verreault, A., and Almouzni, G. (2007) Chromatin challenges during DNA replication and repair. *Cell 128*, 721−733.

(5) Lavelle, C., and Prunell, A. (2007) Chromatin polymorphism and the nucleosome superfamily: a genealogy. *Cell Cycle 6*, 2113−2119.

(6) Zlatanova, J., Bishop, T. C., Victor, J.-M., Jackson, V., and van Holde, K. (2009) The nucleosome family: dynamic and growing. *Structure 17*, 160−171.

(7) Kireeva, M. L., Walter, W., Tchernajenko, V., Bondarenko, V., Kashlev, M., and Studitsky, V. M. (2002) Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. *Mol. Cell 9*, 541−552.

(8) Baer, B. W., and Rhodes, D. (1983) Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes. *Nature 301*, 482−488.

(9) González, P. J., and Palacián, E. (1989) Interaction of RNA polymerase II with structurally altered nucleosomal particles. Transcription is facilitated by loss of one H2A.H2B dimer. *J. Biol. Chem. 264*, 18457−18462.

(10) Kimura, H., and Cook, P. R. (2001) Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. *J. Cell Biol. 153*, 1341−1353.

(11) Thiriet, C., and Hayes, J. J. (2005) Replication-independent core histone dynamics at transcriptionally active loci in vivo. *Genes Dev. 19*, 677−682.

(12) Belotserkovskaya, R., Oh, S., Bondarenko, V. A., Orphanides, G., Studitsky, V. M., and Reinberg, D. (2003) FACT facilitates transcription-dependent nucleosome alteration. *Science 301*, 1090−1093.

(13) Orphanides, G., LeRoy, G., Chang, C. H., Luse, D. S., and Reinberg, D. (1998) FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell 92*, 105−116.

(14) Angelov, D., Bondarenko, V. A., Almagro, S., Menoni, H., Mongélard, F., Hans, F., Mietton, F., Studitsky, V. M., Hamiche, A., Dimitrov, S., and Bouvet, P. (2006) Nucleolin is a histone chaperone with FACT-like activity and assists remodeling of nucleosomes. *EMBO J. 25*, 1669−1679.

(15) Levchenko, V., and Jackson, V. (2004) Histone release during transcription: NAP1 forms a complex with H2A and H2B and

facilitates a topologically dependent release of H3 and H4 from the nucleosome. *Biochemistry 43*, 2359−2372.
(16) Levchenko, V., Jackson, B., and Jackson, V. (2005) Histone release during transcription: displacement of the two H2A−H2B dimers in the nucleosome is dependent on different levels of transcription-induced positive stress. *Biochemistry 44*, 5357−5372.
(17) Mazurkiewicz, J., Kepert, J. F., and Rippe, K. (2006) On the mechanism of nucleosome assembly by histone chaperone NAP1. *J. Biol. Chem. 281*, 16462−16472.
(18) Heo, K., Kim, H., Choi, S. H., Choi, J., Kim, K., Gu, J., Lieber, M. R., Yang, A. S., and An, W. (2008) FACT-Mediated Exchange of Histone Variant H2AX Regulated by Phosphorylation of H2AX and ADP-Ribosylation of Spt16. *Mol. Cell 30*, 86−97.
(19) Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. (1999) Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J. Cell Biol. 146*, 905−916.
(20) Mizuguchi, G., Shen, X., Landry, J., Wu, W.-H., Sen, S., and Wu, C. (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science 303*, 343−348.
(21) Luk, E., Ranjan, A., Fitzgerald, P. C., Mizuguchi, G., Huang, Y., Wei, D., and Wu, C. (2010) Stepwise histone replacement by SWR1 requires dual activation with histone H2A.Z and canonical nucleosome. *Cell 143*, 725−736.
(22) Ryan, D. P., and Owen-Hughes, T. (2011) Snf2-family proteins: chromatin remodellers for any occasion. *Curr. Opin. Chem. Biol. 15*, 649−656.
(23) Tanaka, Y., Tawaramoto-Sasanuma, M., Kawaguchi, S., Ohta, T., Yoda, K., Kurumizaka, H., and Yokoyama, S. (2004) Expression and purification of recombinant human histones. *Methods (San Diego, CA, U. S.) 33*, 3−11.
(24) Tachiwana, H., Kagawa, W., Osakabe, A., Kawaguchi, K., Shiga, T., Hayashi-Takanaka, Y., Kimura, H., and Kurumizaka, H. (2010) Structural basis of instability of the nucleosome containing a testis-specific histone variant, human H3T. *Proc. Natl. Acad. Sci. U. S. A. 107*, 10454−10459.
(25) Tachiwana, H., Osakabe, A., Shiga, T., Miya, Y., Kimura, H., Kagawa, W., and Kurumizaka, H. (2011) Structures of human nucleosomes containing major histone H3 variants. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 67*, 578−583.
(26) Tachiwana, H., Osakabe, A., Kimura, H., and Kurumizaka, H. (2008) Nucleosome formation with the testis-specific histone H3 variant, H3t, by human nucleosome assembly proteins in vitro. *Nucleic Acids Res. 36*, 2208−2218.
(27) Osakabe, A., Tachiwana, H., Matsunaga, T., Shiga, T., Nozawa, R.-S., Obuse, C., and Kurumizaka, H. (2010) Nucleosome formation activity of human somatic nuclear autoantigenic sperm protein (sNASP). *J. Biol. Chem. 285*, 11913−11921.
(28) Tachiwana, H., Kagawa, W., Shiga, T., Osakabe, A., Miya, Y., Saito, K., Hayashi-Takanaka, Y., Oda, T., Sato, M., Park, S. -Y., Kimura, H., and Kurumizaka, H. (2011) Crystal structure of the human centromeric nucleosome containing CENP-A. *Nature 476*, 232−235.
(29) Lowary, P. T., and Widom, J. (1998) New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. *J. Mol. Biol. 276*, 19−42.
(30) Fujisawa, T., Inoue, K., Oka, T., Iwamoto, H., Uruga, T., Kumasaka, T., Inoko, Y., Yagi, N., Yamamoto, M., and Ueki, T. (2000) Small-angle X-ray scattering station at the SPring-8 RIKEN beamline. *J. Appl. Crystallogr. 33*, 797−800.
(31) Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr. 25*, 495−503.
(32) Glatter, O., and Kratky, O. (1982) *Small-angle X-ray Scattering*, Academic Press, New York.
(33) Conde e Silva, N., Black, B. E., Sivolob, A., Filipski, J., Cleveland, D. W., and Prunell, A. (2007) CENP-A-containing nucleosomes: easier disassembly versus exclusive centromeric localization. *J. Mol. Biol. 370*, 555−573.
(34) Sekulic, N., Bassett, E. A., Rogers, D. J., and Black, B. E. (2010) The structure of $(CENP\text{-}A\text{-}H4)_2$ reveals physical features that mark centromeres. *Nature 467*, 347−351.
(35) Panchenko, T., Sorensen, T. C., Woodcock, C. L., Kan, Z.-Y., Wood, S., Resch, M. G., Luger, K., Englander, S. W., Hansen, J. C., and Black, B. E. (2011) Replacement of histone H3 with CENP-A directs global nucleosome array condensation and loosening of nucleosome superhelical termini. *Proc. Natl. Acad. Sci. U. S. A. 108*, 16588−16593.
(36) Makde, R. D., England, J. R., Yennawar, H. P., and Tan, S. (2010) Structure of RCC1 chromatin factor bound to the nucleosome core particle. *Nature 467*, 562−566.
(37) Vasudevan, D., Chua, E. Y. D., and Davey, C. A. (2010) Crystal Structures of Nucleosome Core Particles Containing the "601" Strong Positioning Sequence. *J. Mol. Biol. 403*, 1−10.
(38) Yang, C., van der Woerd, M. J., Muthurajan, U. M., Hansen, J. C., and Luger, K. (2011) Biophysical analysis and small-angle X-ray scattering-derived structures of MeCP2-nucleosome complexes. *Nucleic Acids Res. 39*, 4122−4135.
(39) Davey, C. A., Sargent, D. F., Luger, K., Maeder, A. W., and Richmond, T. J. (2002) Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 A resolution. *J. Mol. Biol. 319*, 1097−1113.
(40) Richmond, T. J., and Davey, C. A. (2003) The structure of DNA in the nucleosome core. *Nature 423*, 145−150.
(41) Shukla, M. S., Syed, S. H., Goutte-Gattat, D., Richard, J. L. C., Montel, F., Hamiche, A., Travers, A., Faivre-Moskalenko, C., Bednar, J., Hayes, J. J., Angelov, D., and Dimitrov, S. (2011) The docking domain of histone H2A is required for H1 binding and RSC-mediated nucleosome remodeling. *Nucleic Acids Res. 39*, 2559−2570.
(42) Bao, Y., Konesky, K., Park, Y. J., Rosu, S., Dyer, P. N., Rangasamy, D., Tremethick, D. J., Laybourn, P. J., and Luger, K. (2004) Nucleosomes containing the histone variant H2A.Bbd organize only 118 base pairs of DNA. *EMBO J. 23*, 3314−3324.
(43) Doyen, C.-M., Montel, F., Gautier, T., Menoni, H., Claudet, C., Delacour-Larose, M., Angelov, D., Hamiche, A., Bednar, J., Faivre-Moskalenko, C., Bouvet, P., and Dimitrov, S. (2006) Dissection of the unusual structural and functional properties of the variant H2A.Bbd nucleosome. *EMBO J. 25*, 4234−4244.
(44) Studitsky, V. (2004) Chromatin remodeling by RNA polymerase. *Trends Biochem. Sci. 29*, 127−135.
(45) Kireeva, M. L., Hancock, B., Cremona, G. H., Walter, W., Studitsky, V. M., and Kashlev, M. (2005) Nature of the Nucleosomal Barrier to RNA Polymerase II. *Mol. Cell 18*, 97−108.
(46) Studitsky, V. M., Clark, D. J., and Felsenfeld, G. (1994) A histone octamer can step around a transcribing polymerase without leaving the template. *Cell 76*, 371−382.