Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution *in vitro*

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Centromere protein A (CENP-A) is a variant of histone H3 with more than 60% sequence identity at the C-terminal histone fold domain. CENP-A specifically locates to active centromeres of animal chromosomes and therefore is believed to be a component of the specialized centromeric nucleosomes on which the kinetochores are assembled. Here we report that CENP-A, highly purified from HeLa cells, can indeed replace histone H3 in a nucleosome reconstitution system mediated by nucleosome assembly protein-1 (NAP-1). The structure of the nucleosomes reconstituted with recombinant CENP-A, histones H2A, H2B, and H4, and closed circular DNAs had the following properties. By atomic force microscopy, ''beads on a string'' images were obtained that were similar to those obtained with nucleosomes reconstituted with four standard histones. DNA ladders with repeats of approximately 10 bp were produced by DNase I digestion, indicating that the DNA was wrapped round the protein complex. Mononucleosomes isolated by glycerol gradient sedimentation had a relative molecular mass of ≈200 kDa and were composed of 120–150 bp of DNA and equimolar amounts of CENP-A, and histones H4, H2A, and H2B. Thus, we conclude that CENP-A forms an octameric complex with histones H4, H2A, and H2B in the presence of DNA.

Human centromere protein A (CENP-A) is one of the proteins recognized by anti-centromere autoantibodies from calcinosis/Raynaud's phenomenon/esophageal dysmotility/sclerodactyly/telangiectasia variant (CREST) serum (1). The C-terminal two-thirds of CENP-A is highly homologous to histone H3, but the remaining N-terminal one-third is unique (2, 3). The putative histone-fold domain located in the C-terminal region is essential for targeting CENP-A to the centromeric region (3). Recently, the mouse CENP-A gene was shown to be essential by gene targeting (4). In *Saccharomyces cerevisiae* the CENP-A homologue, CSE4, was isolated and shown to be essential for chromosome segregation (5). By mutational analysis, the functional domains of Cse4p were shown to be distributed across the entire histone-fold domain and the N-terminal domain (6). CENP-A homologues also have been identified in *Caenorhabditis elegans* and *Drosophila melanogaster* and shown to be essential for chromosome segregation (7, 8). CENP-A has been detected among mononucleosome fractions (9) and is located only at active centromeres in mammalian cells (10). Therefore, it has been speculated that formation of chromatin containing CENP-A on centromeric DNA may be essential for establishing active centromeres.

The centromere of *S. cerevisiae* is genetically defined within a 125-bp sequence (centromere; CEN sequence) (11), and at least seven centromere proteins, including Cse4p, are bound to this CEN region (12–14). In *Schizosaccharomyces pombe*, a much longer DNA sequence (40–100 kb), consisting of a 4- to 7-kb unique sequence flanked by tens of kilobases of inverted repeat sequence, is located at the centromeres (15, 16). In mammalian cells, centromeric regions contain a few megabases of highly repetitive DNA, whose sequences differ between species (17). In humans, α-satellite (alphoid) arrays varying from 500 kb to 5 Mb are found at the centromeres. Alphoid arrays consist of a repeated 171-bp monomer that shows chromosome-specific variation in sequence and in higher-order repeat arrangement (18, 19). On rare occasions, however, stable neocentromeres have been found in euchromatic regions lacking alphoid sequences (20). It has been suggested that the DNAs and centromeric proteins are organized into a special type of heterochromatin (prekinetochore) in S phase and form a trilaminar kinetochore in M phase.

A critical question is what triggers formation of the functional centromere in a long repetitive DNA region. Three centromere-associated proteins, CENP-A (17 kDa), CENP-B (80 kDa), and CENP-C (140 kDa) (21) have been located at the centromeres of human chromosomes throughout the cell cycle, and therefore these proteins are candidates for components of prekinetochores. The CENP-B dimer binds two CENP-B boxes to fold α-satellite DNA (22–24) and promote nucleosome positioning around the CENP-B box regions (25). Gene knockout analysis of CENP-B in mice indicates that this protein is not essential (26). CENP-C is located at the inner kinetochore plate (27) and is essential for chromosome segregation (28, 29), but its molecular role has yet to be elucidated. As CENP-A is a histone H3 variant widely conserved from human to *S. cerevisiae* and is an essential protein for centromere function, it seems very likely that nucleosomes containing CENP-A may be what distinguish the centromeric chromatin from other euchromatic or heterochromatic regions, and that they may promote the formation of functional kinetochores. At present there is very little information about the biochemical properties of CENP-A and no direct evidence that CENP-A can actually replace histone H3 in nucleosome formation.

In the present work we show that CENP-A purified from HeLa cells can replace histone H3 in nucleosome reconstitution *in vitro*. Using recombinant CENP-A, the structure of reconstituted nucleosomes containing CENP-A has been extensively analyzed and compared with that of nucleosomes formed with standard histones, and we demonstrate that the CENP-A nucleosomes are composed of an octameric complex of CENP-A and histones H4, H2A, and H2B with 120–150 bp of DNA wrapped around it.

Methods

Cell Growth and Acid Extraction of Chromatin Proteins. HeLa S3 cells were grown in RPMI 1640 medium (Nissui Seiyaku, Tokyo)

Abbreviations: CENP, centromere protein; NAP-1, nucleosome assembly protein-1; TCA, trichloroacetic acid; EK, enterokinase; AFM, atomic force microscopy; Topo I, topoisomerase I.

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containing 5% calf serum (ICN) in spinner flasks (total 2×10^{11} cells). The nuclei were isolated as described (23). The nuclear pellet (1×10^{10} nuclei equivalent) was dissolved in 100 ml of ice-cold 1 M NaCl-EB [EB: 20 mM Hepes, pH 8.0/0.5 mM EDTA/0.5 mM DTT/15% (vol/vol) glycerol/0.5 mM PMSF/0.5 mg/ml pepstatin/2 mg/ml leupeptin] with sonication. Proteins soluble in HCl were extracted and recovered by precipitation with 5% trichloroacetic acid (TCA) (2).

Purification of CENP-A and Core Histones. CENP-A in the acid-extracted proteins (1×10^{10} nuclei equivalent) was purified twice by reversed-phase HPLC (Waters) (2). The CENP-A fractions then were separated by SDS/PAGE. CENP-A in the gel was electro-eluted in Tris/glycine/SDS buffer, dialyzed against 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, 0.1% SDS, 1 mM DTT, 0.1 mM PMSF, and precipitated with acetone. To prepare a CENP-A/H4 complex, histone H4 was added to CENP-A in the gel before electro-elution. The eluted CENP-A/H4 mixture was dissolved in 10 mM HCl. Each histone was purified from the first reversed-phase-HPLC fractions by TAU/PAGE (histone H2B, H3, and H4) (30) or SDS/PAGE (histone H2A) (31). Triton X-100 or SDS was removed as described (32, 33). Purified H2B, H3, and H4 were precipitated with acetone and dissolved in 10 mM HCl. Purified H2A was dialyzed against 0.5 M NaCl-EB.

CENP-A Gene and the Baculovirus System. CENP-A and histone H4 genes were amplified by PCR using human cDNA and cloned into pUC 119 vector. The primers were 5′-ATTGAATTCATGGGCCCGCGCCGCCGGAG-3′ and 5′-ATTCTGCAGTCAGCCGAGTCCCTCCTCAAG-3′ for CENP-A and 5′-ATTCTCGAGATGCATCACCATCACCATCACTCTGGGCGCGGCAAAG-3′ and 5′-ATTTCTAGACTCAGCCGCCAAAGCCATACAG-3′ for H4. The CENP-A gene with a 6× histidine tag at its N terminus was cloned into pFastBac vector (GIBCO/BRL). Using pFastBac DUAL vector (GIBCO/BRL), the CENP-A gene and the histone H4 gene with 6× histidine tag at its N terminus were cloned into a polyhedrin promoter site and a p10 promoter site, respectively.

Purification of the Recombinant CENP-A. The cells (5×10^8 in PBS-1 M NaCl) infected with recombinant CENP-A baculovirus were lysed by sonication in the presence of 0.1% Triton X-100. Acid-soluble proteins were extracted with HCl and precipitated with TCA as described above. The pellet was suspended in 20 ml of 5 mM imidazole, 0.5 M NaCl, 20 mM Tris·HCl (pH 7.9), 0.25 mM PMSF, 0.25 μg/ml pepstatin, 1 μg/ml leupeptin, and 6 M urea. The sample was applied to an Ni column and eluted with a 0.01–0.5 M imidazole gradient. His6CENP-A fractions were pooled and dialyzed against 10 mM HCl, 0.5 mM DTT, at 4°C. A yield of 2 mg of His6CENP-A was obtained from 5×10^8 cells. To purify CENP-A/His6H4 complexes in the native state, nuclei were isolated as described (23). The CENP-A/His6H4 complexes were solubilized in 2 M NaCl-EB and purified on an Ni column.

Nucleosome Assembly *in Vitro*. pUC-αdimer DNA (human α-satellite dimer DNA cloned into pUC119), pUC-α11-mer DNA, and nucleosome assembly protein-1 (NAP-1) were prepared as described (25, 34). Mixtures of purified CENP-A and histones were dissolved in 10 mM HCl. The mixture of His6CENP-A/H4 was denatured by dialysis against 7 M guanidine-HCl, 50 mM Tris·HCl (pH 8.0), 10 mM DTT, and 2 mM EDTA for 1 h at room temperature and overnight at 4°C. The mixture then was renatured by stepwise dialysis against 2 M NaCl-EB followed by 0.5 M NaCl-EB for several h at 4°C. CENP-A/core histones (a mixture of CENP-A, H4, H2A, and H2B) were reconstituted by mixing the renatured His6CENP-A/H4 or the native CENP-A/His6H4 with the native H2A/H2B complex, which had been purified on a hydroxyapatite column (35). Nucleosome assembly *in vitro* was carried out as described (25). The histidine tag of His6CENP-A was removed during the nucleosome formation step by digestion with 0.2 units of enterokinase (EK, Novagen) in the presence of 3 mM $CaCl_2$.

Atomic Force Microscopy (AFM). The nucleosome complexes were isolated by gel filtration using Sepharose CL4B (Amersham Pharmacia) in 50 mM NaCl, 20 mM Hepes-NaOH (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mM DTT. Samples were fixed with 0.05% glutaraldehyde for 60 min at room temperature and imaged by using AFM in the tapping mode (36).

DNase I Digestion. The nucleosome complexes were digested with pancreatic DNase I (GIBCO/BRL) at 37°C. The reaction was terminated by the addition of EDTA. The DNAs, extracted with phenol, were labeled with ^{32}P by using [γ-^{32}P]ATP and T4 PNKase after dephosphorylation with calf intestinal phosphatase. The DNAs were separated by using an 8% polyacrylamide-urea gel.

MNase Digestion and Glycerol Density Gradient Sedimentation. The conditions of MNase digestion of the nucleosome complexes and glycerol density gradient sedimentation were as described (25). The isolated nuclei (2×10^8 nuclei/ml) from HeLa cells (25) were digested with 40 units/ml MNase for 20 min at 37°C and centrifuged at 10,000 *g* for 10 min at 4°C. Nucleosomes in the soluble fraction (100 μl) were subjected to glycerol density gradient sedimentation. Nucleosomes were assembled *in vitro* with 4 μg of DNA (pUC119-α11mer relaxed form) and 4.8 μg of native core histones or 15.6 μg of His6CENP-A/core histones, in 120-μl reaction mixtures. The samples then were digested with 0.1–0.4 unit of MNase for 10 min at 37°C and centrifuged through glycerol gradients.

Results

Purification of CENP-A and Core Histones from HeLa Cells. To analyze CENP-A function, we purified CENP-A from HeLa cells to near homogeneity (Fig. 1*A*, lane 4) with two stages of reversed-phase HPLC and SDS/PAGE as described in *Methods* (Fig. 1*A*). CENP-A (17 kDa) formed a homodimer and gave a 34-kDa band (Fig. 1*A*, lane 5). Results of each purification step are shown in Table 1. Approximately 5 μg of CENP-A was recovered from 1×10^{10} HeLa cells. The core histones were purified from the first HPLC fractions as described in *Methods*.

Mixtures of CENP-A/H4 and CENP-A/H4/H2A/H2B Introduced Supercoils into Closed Circular DNA in the Presence of NAP-I. We first addressed whether the purified CENP-A from HeLa cells could replace histone H3 in nucleosome formation *in vitro*. Nucleosome reconstitution on closed circular DNA was mediated by NAP-I and monitored by the change of linking number of the DNA after topoisomerase I (Topo I) treatment (Topo I analysis). Because CENP-A is difficult to recover in soluble form, CENP-A in the gel after SDS/PAGE was electro-eluted into the buffer containing the purified histone H4, and CENP-A and H4 were copurified. Fig. 1*B* shows that the mixture of CENP-A and histone H4 (lanes 7–9), as well as the mixture of histone H3 and H4 (lanes 1–3), exhibit supercoiling activity dependent on NAP-1, but neither histone H4 (lanes 13–15) nor histone H3 alone induce supercoiling (data not shown). Fig. 1*C* shows that a mixture of histone H2A, H2B, and H4 has no supercoiling activity (lanes 11 and 12), whereas the addition of CENP-A to the mixture (CENP-A/H4/H2A/H2B, CENP-A core histones; lanes 7 and 8) as well as the addition of histone H3 (H3/H4/H2A/H2B, reconstituted core histones; lanes 1–3) restores the supercoiling activities. These results indicate that CENP-A can replace histone H3 in nucleosome assembly *in vitro*.

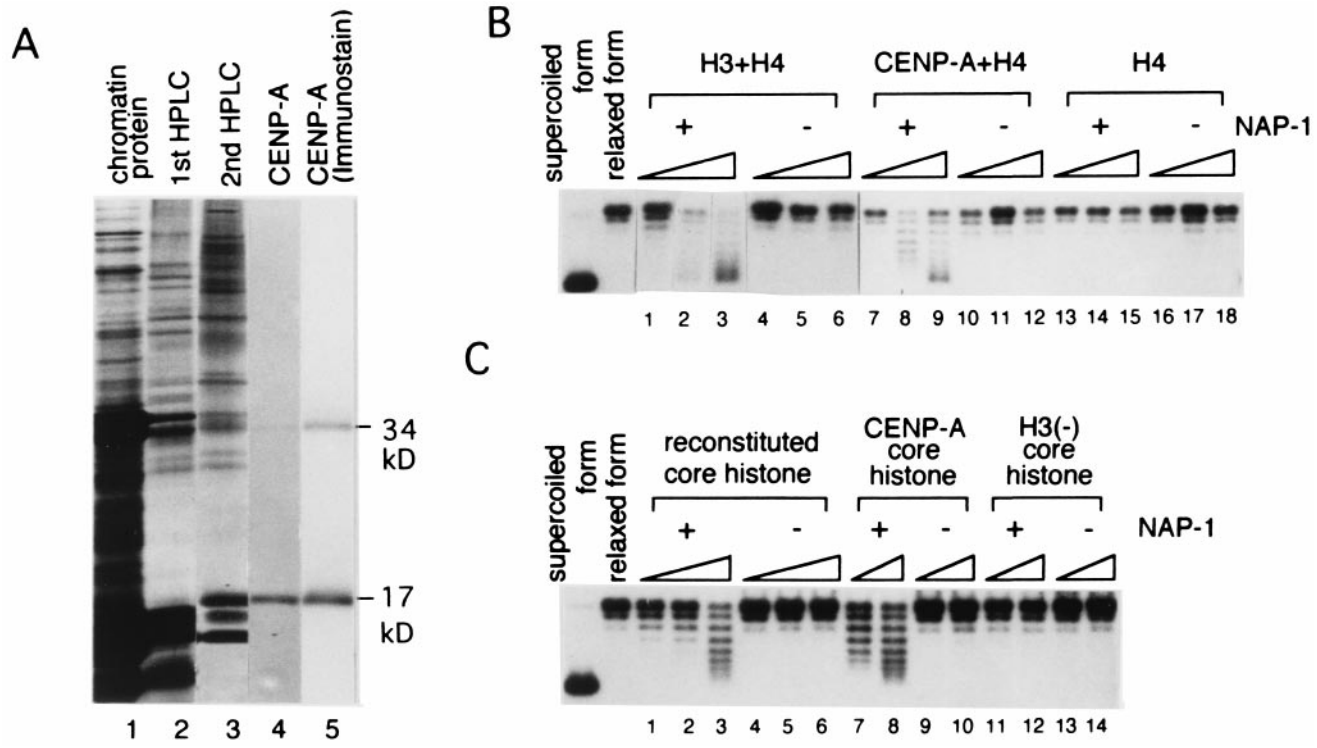

Fig. 1. Topo I analyses using the purified CENP-A, histone H4, H2A, and H2B. (*A*) Samples from each step of purification were identified by silver staining (lanes 1–4) or by immunoblotting with anti-centromere auto-antibody serum (lane 5) after 13% SDS/PAGE. A crude HCl extract of HeLa nuclei (lane 1), peak fraction of CENP-A after first (lane 2) and second (lane 3) reversed-phase HPLC, and CENP-A purified by SDS/PAGE and electro-elution (lanes 4 and 5) are shown. CENP-A monomer (17 kDa) and dimer (34 kDa) are indicated. (*B*) H3/H4 (lanes 1–6), CENP-A/H4 (lanes 7–12), or H4 (lanes 13–18) were subjected to Topo I analyses using the relaxed form of closed circular DNA (pUC-αdimer, 20 ng) with (lanes 1–3, 7–9, and 13–15) or without NAP-1 (lanes 4–6, 10–12, and 16–18). Supercoil ladders were detected by Southern hybridization. H3/H4: 30 ng (lanes 1 and 4), 50 ng (lanes 2 and 5), 75 ng (lanes 3 and 6); CENP-A/H4: 10 ng (lanes 7 and 10), 20 ng (lanes 8 and 11), 30 ng (lanes 9 and 12); H4: 25 ng (lanes 13 and 16), 50 ng (lanes 14 and 17), and 75 ng (lanes 15 and 18). (*C*) Reconstituted core histones (H3/H4/H2A/H2B) (lanes 1–6), CENP-A/core histones (CENP-A/H4/H2A/H2B) (lanes 7–10), or H3(-) core histones (H4/H2A/H2B) (lanes 11–14) were subjected to Topo I analyses with (lanes 1–3, 7, 8, 11, and 12) or without NAP-1 (lanes 4–6, 9, 10, 13, and 14) under the same conditions as in *B*. H3/H4/H2A/H2B: 25 ng (lanes 1 and 4), 35 ng (lanes 2 and 5), 50 ng (lanes 3 and 6); CENP-A/H4/H2A/H2B: 40 ng (lanes 7 and 9), 60 ng (lanes 8 and 10); H4/H2A/H2B: 40 ng (lanes 11 and 13), and 60 ng (lanes 12 and 14).

Topo I Analysis Using the Recombinant CENP-A Purified Under Native or Denaturing Conditions. To analyze extensively the structure of the DNA/CENP-A/core histone complex, recombinant CENP-A was used for further experiments. To obtain the CENP-A/H4 complex under native conditions, histone H4 with the 6× histidine tag at its N terminus (His6H4) and CENP-A were coexpressed in insect cells, and the complex was purified by using an Ni column. Fig. 2*A* shows the mixture of CENP-A/His6 H4 and H2A/H2B (native CENP-A core histones) (lane 2) compared with native core histones (lane 1). Maximum supercoiling activity according to the Topo I assay was obtained with an amount of protein approximately equal to that of DNA (0.1 μg) when native core histones (Fig. 2*B*, lane 4) or native CENP-A/core histones (Fig. 2*B*, lane 8) were used. When CENP-A with the 6× histidine tag at its N terminus (His6CENP-A) was expressed on its own in insect cells and purified under denaturing conditions, renaturation as described in *Methods* was necessary to produce supercoiling with a mixture of His6CENP-A and histone H4. A mixture of the renatured His6CENP-A/H4 and H2A/H2B (His6CENP-A core histones) also exhibited the same amount of supercoiling as shown in Fig. 2*B*, lane 8, but a 2-to 4-fold excess of the proteins was needed (data not shown). The histidine tag at the N terminus of CENP-A was removed by digestion with EK (Fig. 2*C*) during nucleosome formation without any effect on the efficiency of nucleosome formation (data not shown). In each subsequent structural analysis, the maximum extent of nucleosome formation was checked by using the Topo I analysis.

Table 1. Results of purification of CENP-A from HeLa cells ($\sim 1 \times 10^{10}$)

Fraction	Total protein	Yield, %	CENP-A, μg	Purity, %
0.6 M NaCl nuclear pellet			33	
Chromatin protein	120 mg	90	30	0.025
1st HPLC	360 μg	45	15	4.2
2nd HPLC	90 μg	30	10	11
SDS/PAGE/electroelution	5 μg	15	5	>95

AFM Observations. The complexes formed with the closed circular DNA and either native core histones or His6CENP-A core histones with EK digestion were isolated with gel filtration and mounted on mica supports. As shown in Fig. 3 *A* and *B*, "beads on a string" structures were clearly detected in both cases, indicating that a CENP-A/core histone complex was actually arrayed on a closed circular DNA.

DNase I Digestion. The complexes were digested with DNase I, and DNA fragments were extracted and labeled with γ^{32}P-ATP and T4 polynucleotide kinase and separated by electrophoresis through an 8% polyacrylamide-urea gel (Fig. 4). DNA ladders with 10- to 11-bp repeats were detected from the digests of both

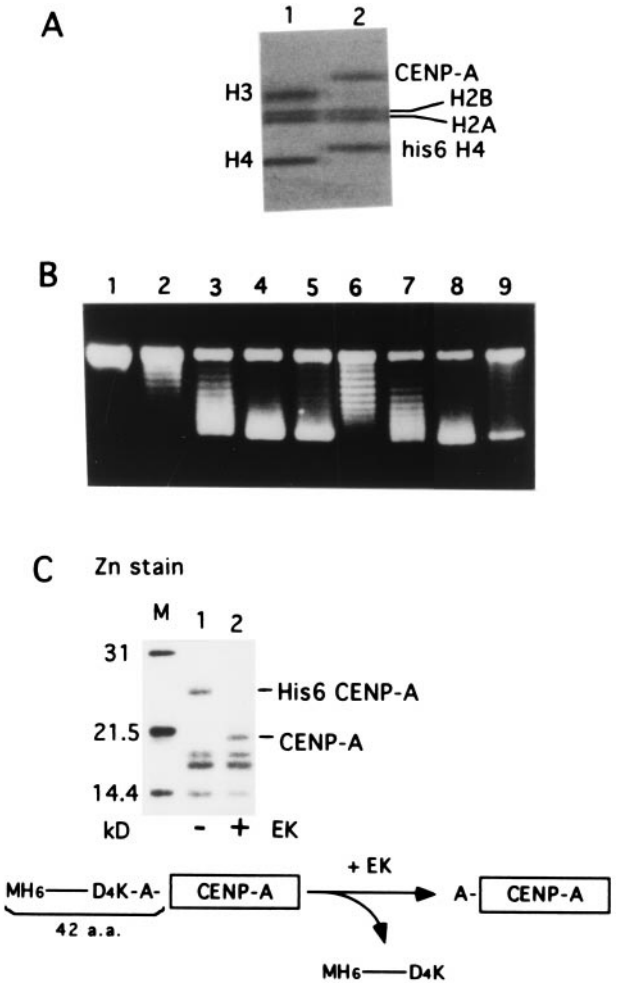

Fig. 2. Topo I analysis using the recombinant CENP-A purified under native condition. (*A*) 4–20% SDS/PAGE of native core histones (0.24 μg, lane 1) and native CENP-A/core histones (0.12 μg, lane 2). The gel was stained with Coomassie brilliant blue after electrophoresis. (*B*) Topo I analysis of the native CENP-A/core histones (lanes 6–9) compared with the native core histones (lanes 2–5). Nucleosome reconstitution was carried out by using the relaxed form of pUC-α11mer DNA (100 ng) and NAP-1 as described in *Methods*. Supercoil ladders were detected with ethidium bromide staining after agarose gel electrophoresis. The amounts of the native core histones were 0 ng (lane 1), 72 ng (lane 2), 98 ng (lane 3), 120 ng (lane 4), and 180 ng (lane 5), and the amounts of His6CENP-A/core histones were 63 ng (lane 6), 100 ng (lane7), 126 ng (lane 8), and 250 ng (lane 9). (*C*) EK digestion of the complex removes the histidine tag at the N terminus of CENP-A. Mononucleosomes were isolated by glycerol density-gradient sedimentation after MNase digestion of the reconstituted complexes formed from His6CENP-A/core histones and DNA with (lane 2) or without (lane 1) EK digestion and separated by 15% SDS/PAGE. The gel was stained with Zn. The primary structure of the N terminus of His6CENP-A is shown at the bottom.

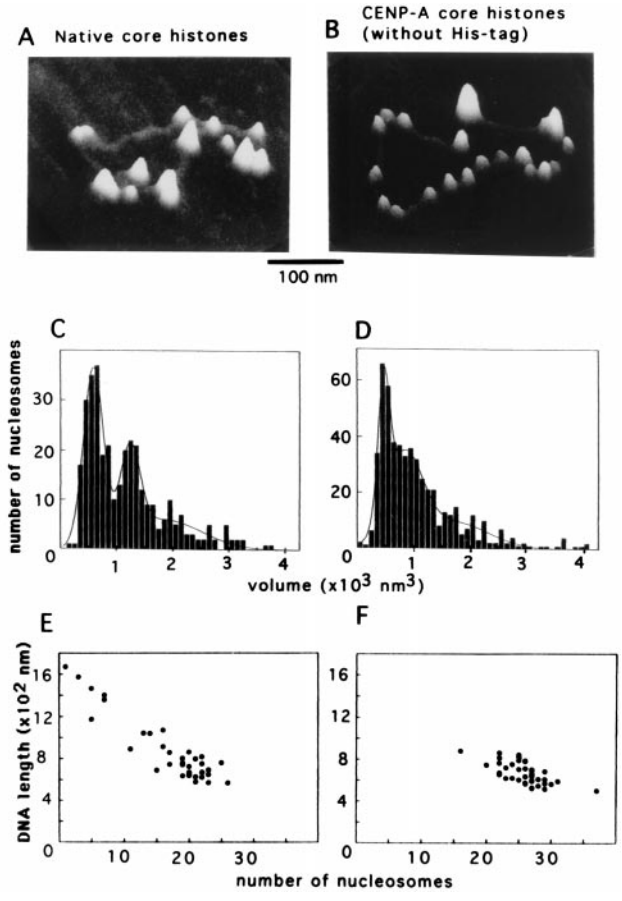

Fig. 3. Analysis of the reconstituted nucleosomes by AFM. Nucleosomes were reconstituted with pUC-α11mer DNA and native core histones (*A*, *C*, and *E*) or His6CENP-A/core histones with EK digestion (*B*, *D*, and *F*) and observed by AFM. (*A* and *B*) Nucleosomes formed on circular DNA. (Bar indicates 100 nm.) (*C* and *D*) Histogram of the number of nucleosomes in each volume class. (*E* and *F*) Plot of the contour length of each circular DNA molecule against the number of nucleosomes on it.

the CENP-A/core histone/DNA complex (Fig. 4, lanes 4–9) and the native core histone/DNA complex (Fig. 4, lanes 1–3). These results indicated that the DNA is wrapped around the CENP-A/core histone complex in the same way as it is around the native core histone complex.

MNase Digestion and Characterization of Mononucleosomes Isolated by Glycerol-Gradient Sedimentation. The reconstituted complexes were digested with MNase, and the extracted DNA fragments were subjected to agarose gel electrophoresis (Fig. 5*A*). DNA ladders typical for nucleosome formation were detected in the His6CENP-A core histone/DNA complex (Fig. 5*A*, lanes 5–8) as well as in the native core histone/DNA complex (Fig. 5*A*, lanes 1–4). Although a kinetic pause in MNase digestion of DNA was observed with the native core histone/DNA complex at 145 nt (Fig. 5*A*, lanes 2–4), which is the same size observed with nucleosomes *in vivo*, no such pause was obvious with the His6CENP-A/core histone/DNA complex, in which the size of the DNA gradually decreased from 150 bp to 120 bp or less (Fig. 5*A*, lanes 5–8). EK digestion of the complex during nucleosome formation produced no change in the MNase digestion patterns (data not shown). The MNase digests of the complexes were subjected to glycerol-gradient sedimentation (Fig. 5*B Bottom*). MNase digests of HeLa nuclei (Fig. 5*B Top*) and the native core histone/DNA complex (Fig. 5*B Middle*) also were centrifuged in parallel as controls. The monomer complexes formed with DNA and reconstituted His6CENP-A/core histones sedimented at the position centered at fraction 14–15 in Fig. 5*B* (*Bottom*) (relative molecular mass, 200 kDa) and sedimented a little slower than other monomer complexes (Fig. 5*B Top* and *Middle*). The change of sedimentation rate of the monomer complex was undetectable after EK digestion (data not shown). Fig. 5*C* showed that the mononucleosomes contained approximately equimolar amounts of His6CENP-A, H4, H2A, and H2B. From these results we conclude that the mononucleosome of the DNA/CENP-A/core histone complex consists of an octameric CENP-A/H4/H2A/H2B complex with 120–150 bp of DNA wrapped around it.

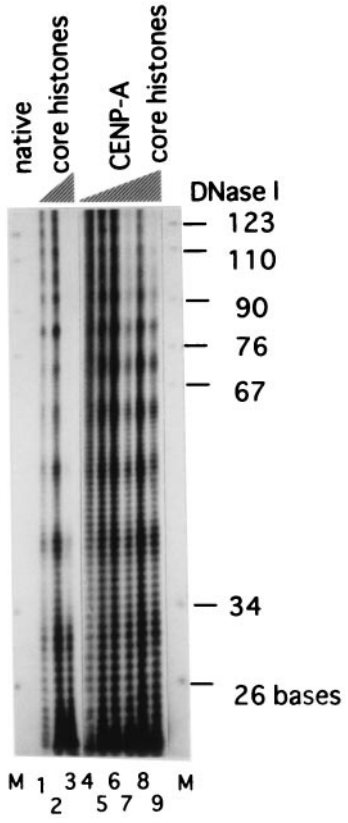

Fig. 4. DNase I digestion of the nucleosomes. The nucleosome complexes were digested with 0.17 unit of DNase I for 4, 10, and 20 min (lanes 1–3) for native core histones and 2, 4, 6, 8, 10, and 15 min (lanes 4–9) for His6CENP-A/core histones at 37°C. Lane M shows an *Msp*I digest of pBR322 as size markers.

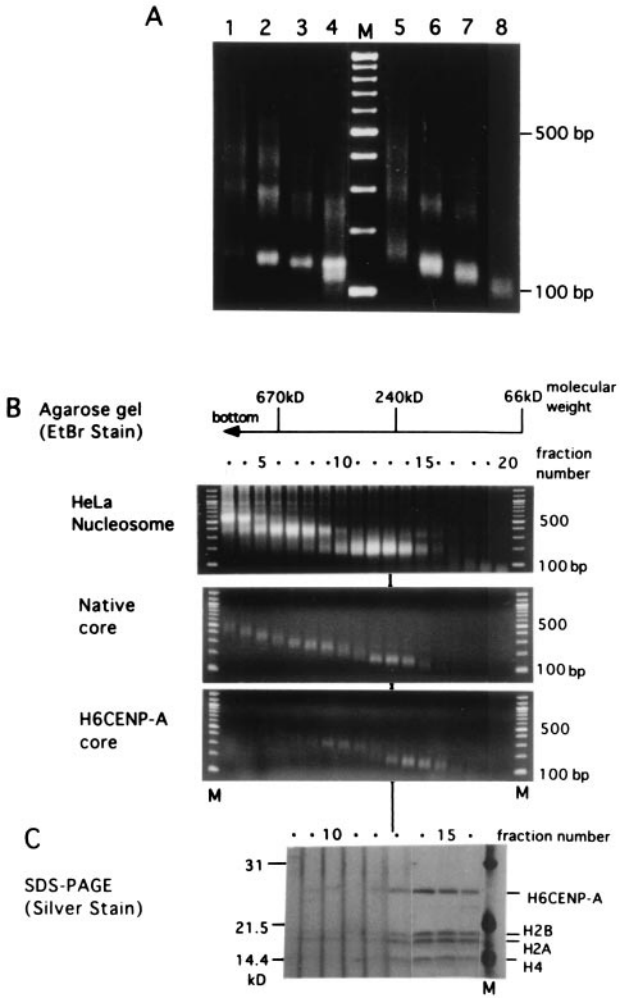

Fig. 5. MNase digestion of the reconstituted nucleosomes and glycerol gradient sedimentation of the MNase digests. (*A*) Nucleosomes were formed from native core histones (lanes 1–4) or His6CENP-A/core histones (lanes 5–8). Aliquots of 8 μl were digested with 0.01 unit (lanes 1 and 5), 0.03 unit (lanes 2 and 6), 0.1 unit (lanes 3 and 7), or 0.3 unit (lanes 4 and 8) of MNase for 10 min at 37°C. The DNA of each sample was electrophoresed through a 1.7% agarose gel and detected by ethidium bromide fluorescence. Lane M shows a 100-bp ladder. (*B*) Glycerol density gradient sedimentation of the MNase digests of the nucleosomes from HeLa nuclei (*Top*), reconstituted with native core histones (*Middle*), or His6CENP-A core histones (*Bottom*). Each 150-μl aliquot was fractionated from the bottom, and 20 μl of each fraction was electrophoresed through 1.7% agarose gel after proteinase K digestion. Each lane of *B* and *C* was numbered according to the fraction number of the glycerol gradient. The position of each molecular mass marker (albumin, 66 kDa; catalase, 240 kDa; thyroglobulin, 670 kDa) was marked at the top. M, 100-bp ladder. (*C*) The remaining 130 μl of glycerol gradient fractions 8–16 (*B Bottom*) were precipitated with acetone and separated by 15% SDS/PAGE. The protein bands were detected with silver staining.

Discussion

Using CENP-A purified from HeLa cells, we have shown that a mixture of CENP-A, H4, H2A, and H2B (CENP-A/core histones) could introduce supercoils into relaxed closed circular DNAs in the presence of Topo I and NAP-1, which suggested nucleosome formation. Using the recombinant CENP-A, we have investigated the structure of the reconstituted nucleosomes and compared them with those formed with standard core histones and have shown that CENP-A could substitute for histone H3 in nucleosome formation with other histones (H4, H2A, and H2B) and DNA. The basic structure of the reconstituted nucleosomes was the same as that formed by wrapping DNA around an octamer of the four standard core histones (H3, H4, H2A, and H2B). Considering our data, together with previous reports that CENP-A was recovered in mononucleosome fractions (9) and locates only to active centromeres (10), active centromeres may be indeed defined by formation of nucleosomes containing CENP-A, which we propose to call centromere-specific nucleosomes. This work is a step toward a biochemical understanding of the function of centromere-specific nucleosomes.

Purification of CENP-A. Because of its high hydrophobicity, CENP-A was difficult to solubilize and liable to be lost during purification. We have succeeded in purifying CENP-A by recovering it from the gel after SDS/PAGE. The partial amino acid sequence of the fragment produced by V8 protease digestion of the purified protein was determined, and this confirmed that it was CENP-A (data not shown). From the results summarized in Table 1, the number of CENP-A molecules per cell was estimated to be 120,000. As the number of CENP-B molecules per cell was reported to be 20,000 (37), the ratio of CENP-A to CENP-B was 6. As each CENP-A nucleosome contains a CENP-A dimer, the ratio of CENP-A nucleosomes to CENP-B molecules would be 3. According to the centromere-specific nucleosome model proposed by us (25), the ratio of CENP-A nucleosomes to CENP-B should be 2, close to the observed ratio.

Nucleosome Assembly *in Vitro*. We have succeeded in reconstituting nucleosomes efficiently *in vitro* in the presence of NAP-1.

CENP-A nucleosomes could be formed by using salt dialysis, although the efficiency was low. In the NAP-1 system, the efficiency of nucleosome formation is highest when the ratio (by weight) of DNA to native core histones is approximately 1; an excess of histones apparently inhibits nucleosome formation (25). We obtained the same relative activity with the native CENP-A/core histone complex (Fig. 2*B*), which suggested that the increase of linking number per CENP-A nucleosome was the same as that for a standard nucleosome. From this value 25–30 nucleosomes were estimated to be formed on a DNA molecule (pUC119-α11mer). Twenty to 30 nucleosomes were observed in AFM figures (Fig. 3 *E* and *F*). MNase digestion of the reconstituted nucleosomes (Fig. 5*A*) and glycerol-gradient sedimentation of the mononucleosomes (Fig. 5*B*) suggested that there were some structural differences between CENP-A nucleosomes and standard nucleosomes. DNA termini might be more loosely bound in CENP-A nucleosomes than in standard nucleosomes, but it remains to be shown whether or not these differences are found in CENP-A nucleosomes *in vivo*.

AFM. The molecular volume of the nucleosomes seemed to be variable (Fig. 3 *A* and *B*). When the number of nucleosomes in each size class was plotted (Fig. 3 *C* and *D*), three discrete peaks were observed at 580 nm^3, 1240 nm^3, and 1840 nm^3 in native core nucleosomes (Fig. 3*C*); because the ratio of these volumes was 1:2.1:3.2, we considered them to be mono-, di- and trinucleosomes, respectively. The molecular volume of a mononucleosome obtained by x-ray crystallography was 532 nm^3 (38). There was a negative correlation between contour length of a circular DNA and the number of nucleosomes on the DNA (Fig. 3 *E* and *F*), which supports the view that the DNA is wrapped around the protein complex. CENP-A nucleosomes were located nonspecifically on the DNA circle, indicating a lack of sequence dependence for this assembly reaction (Fig. 3*B*).

Centromeric Heterochromatin Structures and Localization of CENP-A Nucleosomes to α-Satellite DNA. α-Satellite sequences are the main components of human centromeres (17), and introduction of α-satellite DNA into cells resulted in the formation of a minichromosome containing the introduced α-satellite sequence as its centromere (39). When α-satellite DNAs with and without CENP-B boxes were ligated with human telomere sequences and introduced into human cells, only the α-satellite DNAs with CENP-B boxes were efficiently maintained as artificial minichromosomes, suggesting that the α-satellite sequence with CENP-B boxes is proficient in establishing an active centromere (40). We previously have shown that the CENP-B dimer binds two CENP-B boxes on the α-satellite sequence to fold the DNA (23, 24, 37) and to promote nucleosome positioning at the centromere region, and thus CENP-B/CENP-B-box complexes seemed to promote formation of the highly organized chromatin structures (25, 41). It has been shown by chromatin immunoprecipitation that CENP-A preferentially localizes to α-satellite DNA (42) or more specifically to α-satellite DNA with CENP-B boxes on all human chromosomes except the Y chromosome (S.A. and K.Y., unpublished work). The CENP-A nucleosome reconstitution system reported here did not show any DNA sequence specificity for nucleosome assembly, suggesting that CENP-A core histones themselves do not recognize the α-satellite sequence (Fig. 3*B* and unpublished data). Therefore, this reconstitution system will provide a good *in vitro* assay system to identify the factors that confer the targeting specificity to CENP-A core nucleosomes as well as molecular mechanisms of kinetochore assembly in general.

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