

A 17-kD Centromere Protein (CENP-A) Copurifies with Nucleosome Core Particles and with Histones

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Abstract. We have detected and begun to characterize a 17-kD centromere-specific protein, CENP-A (Earnshaw, W. C., and N. Rothfield, 1985, *Chromosoma*, 91:313–321). Sera from several humans with CREST scleroderma autoimmune disease (CREST: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) bind this protein in immunoblot assays of HeLa whole cell or nuclear extracts. We have affinity purified the anti-17-kD centromere protein (anti-CENP-A) specific antibodies from immunoblots of HeLa nuclear protein. The antibodies react with epitopes present on CENP-A derived

from humans but apparently do not recognize specific epitopes in either rat or chicken nuclei. Only human nuclear protein is CENP-A positive by immunoblot. Furthermore, human cells show localization of anti-CENP-A antibody to centromeres by immunofluorescence microscopy, whereas rat cells do not. On extraction from the nucleus, CENP-A copurifies with core histones and with nucleosome core particles. We conclude that this centromere-specific protein is a histone-like component of chromatin. The data suggest that CENP-A functions as a centromere-specific core histone.

CHROMOSOME movement during meiosis and mitosis is correlated with the formation of microtubules that connect each chromosome to the spindle pole. Kinetochores, which in mammals are morphologically well defined organelles with a distinct trilaminar structure, are the chromosomal sites of attachment of these spindle microtubules (reviewed by Reider, 1982; Pickett-Heaps et al., 1982). Kinetochores-to-pole microtubules, unlike other spindle microtubules, display the unusual property of cold stability (Brinkley and Cartwright, 1975). This restricted distribution of cold-stable microtubules in the spindle suggests that kinetochores may condition microtubules for cold stability. The protein associated with microtubule cold stability also slides on microtubules, and thus may be involved in the force-generating mechanism which moves chromosomes poleward in anaphase A (Pabion et al., 1984; Margolis et al., 1986).

In vitro, kinetochores associated with crude chromosomal preparations have been observed to both seed microtubule assembly and to capture microtubules seeded by other microtubule organizing centers, but the dominant mechanism by which microtubules become associated with kinetochores in vivo (capture versus seeded growth) has not been conclusively determined (Mitchison and Kirschner, 1985a, b). Determinations of the structural polarity of spindle microtubules have shown that the net assembly ends of kinetochores-to-pole microtubules are kinetochore proximal (Telzer and Haimo, 1981; Euteneuer and McIntosh, 1981), and kinetochore proximal subunit addition has been demonstrated

in vivo by microinjection of biotinylated tubulin into metaphase African green monkey fibroblasts (Mitchison et al., 1986). Subunit addition at the kinetochore implies that a complex machinery must be responsible for proximal assembly and translocation of subunits outward from this site through treadmilling mechanisms. The possibility that kinetochores condition microtubules for properties unique to their function (reflected in cold stability), and the possibility that they encompass a proximal assembly mechanism make kinetochores interesting objects for biochemical study. Further, kinetochores are organelles of interest with respect to chromatin packaging mechanisms, and mechanisms for functional and morphological differentiation during the cell cycle.

Electron microscopy has demonstrated that mitotic kinetochores are composed, at least in part, of chromatin. The kinetochore plates of PtK cell chromosomes stain positive for DNA (Ris and Witt, 1981) and are decondensed by treatment with DNAase I (Pepper and Brinkley, 1980). However, hypotonic treatment reversibly disperses mammalian kinetochores into chromatin fibers which differ in diameter from those of nonkinetochore chromatin, and to which microtubules are directly attached (Brinkley et al., 1980; Ris and Witt, 1981). These latter observations suggest that the composition of kinetochore chromatin differs from that of nonkinetochore chromatin, and that the differences have important functional consequences. The underlying differences at the molecular level between kinetochore and bulk chromatin remain to be elucidated.

It has been demonstrated that antibodies in human autoimmune sera derived from patients with the CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) variant of scleroderma recognize both mitotic chromosome centromeres and interphase persistent centromere structures (Moroi et al., 1980; Fritzier and Kinsella, 1980; Moroi et al., 1981; Brenner et al., 1981). Using whole sera, Brenner et al. (1981) found that specific binding of human anticentromere autoantibodies to the centromeres of PtK cells was exclusively to the inner and outer kinetochore plates. However, it is not clear that all human centromere antigens have cross-reacting homologues in non-human species. In fact, there is disagreement over the number and molecular masses of the antigens recognized by CREST scleroderma sera even in humans, although a point of agreement in several reports is the presence in HeLa cells of an antigen with a mass of ~ 17 kD (Guldner et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; McNeilage et al., 1986). Using antibodies affinity purified from Western blots, Guldner et al. (1984) and Earnshaw and Rothfield (1985) have convincingly shown that this ~ 17 kD protein is centromere specific. Throughout this paper, we shall refer to this protein as CENP-A, as suggested by Earnshaw and Rothfield (1985). Here, we report that CENP-A is a component of chromatin, that it has core histone-like properties of salt and acid solubility, that it fractionates with nucleosome core particles, and that it elutes with H3 and H4 from a cation exchange column under non-denaturing conditions. This evidence suggests that CENP-A functions as a centromere-specific core histone, possibly substituting for H3 or H4.

Materials and Methods

Growth of HeLa Cells and Preparation of Whole-Cell Extracts

HeLa cells were grown in Jocklick's modified MEM or in DME (Gibco, Grand Island, NY). Bovine calf serum (Hyclone Laboratories, Logan, UT) was added to 5%. Spinner culture cells were used for most experiments, but populations enriched for mitotic or interphase cells were obtained by shake-off fractionation of adherent cells blocked for 16 h with 0.1 $\mu\text{g}/\text{ml}$ of nocodazole (Sigma Chemical Co., St. Louis, MO) (Zieve et al., 1980). Cells were washed with PBS and sonicated in SDS gel sample buffer containing 1.5 μM aprotinin, 10 μM leupeptin, and 20 μM phenylmethylsulfonyl fluoride. Extracts were boiled for 2 min and centrifuged before application to polyacrylamide gels.

Isolation of Nuclei and Preparation of Total Nuclear Protein Extracts

Rat liver and chicken erythrocyte nuclei were isolated using buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 1.0 mM EDTA, 0.2 mM EGTA, 15 mM Tris-HCl, pH 7.4) and stored as described (Palmer and Margolis, 1985). HeLa cells were washed in one-fourth concentration buffer A with 30% wt/vol glycerol, protease inhibitors as above, and 15 mM β -mercaptoethanol, and were collected by centrifugation. They were resuspended and swollen in the same buffer supplemented with 0.2% Triton X-100 (4°C, 30 min), and were homogenized (Dounce-A pestle) until the nuclei were largely free of cytoplasm, as determined by phase-contrast microscopy. Nuclei were collected by centrifugation (1,000 g, 10 min), and were stored at -70°C in isolation buffer lacking Triton X-100. Total nuclear protein extracts were prepared as described above for whole-cell extracts.

Preparation of Chromatin and Nucleosome Core Particles

Except where noted, nuclei were washed three times and resuspended at 50

A_{260}/ml in 5 mM Hepes, pH 7.5, containing 1.5 μM aprotinin, 10 μM leupeptin, and 15 mM β -mercaptoethanol. Soluble long chromatin was prepared by three cycles of micrococcal nuclease digestion and extraction. For one digestion and extraction cycle, micrococcal nuclease was added to 40 U/ml and CaCl_2 to 1 mM, and the nuclei digested for 2 min at 37°C . Digestion was stopped by the addition of buffered EDTA to 10 mM. Nuclei were collected by centrifugation, and extracted twice with 5 mM Hepes plus protease inhibitors and β -mercaptoethanol. Digestion and extraction supernatants from the three cycles were pooled and concentrated for use as long chromatin. Other steps in the preparation of nucleosome core particles were as described by Lutter (1978), except that core particles were resolved on linear log sucrose gradients (Brakke and Van Pelt, 1970) buffered with 2.5 mM Hepes, pH 7.5. DNA contained in chromatin fractions was analyzed on polyacrylamide gels containing 8% acrylamide (20:1 acrylamide/bis-acrylamide) and buffered with Tris-borate (Maniatis et al., 1982).

Elution of Proteins from Nuclei or Chromatin Adsorbed to Hydroxylapatite

All operations were performed at 4°C . HeLa nuclei were washed twice with 5 mM Hepes, 0.2 mM EDTA (pH 7.5) and twice with 5 mM Hepes alone. They were then resuspended in 5 mM Hepes and mixed for 30 min with a slurry of hydroxylapatite (Bio-Gel HTP; Bio-Rad Laboratories, Richmond, CA) at a concentration of $\sim 15 A_{260 \text{ nm}}$ units of nuclei per milliliter of settled hydroxylapatite. Chromatin was similarly bound, except that the application buffer was 5 mM Hepes containing protease inhibitors and β -mercaptoethanol. The hydroxylapatite was then transferred to a column and washed with 10 vol of 25 mM NaPO_4 (pH 7.0). In experiments involving chromatin, and in later experiments with nuclei, this wash was omitted. Nuclear proteins were differentially eluted using slight modifications of the procedure of Philip et al. (1979). Nonhistones and H1 were eluted with 15-column volumes of 0.6 M NaCl in 25 mM NaPO_4 (pH 7.0), after which core histones were eluted using 2.5 M NaCl in 25 mM NaPO_4 (pH 7.0). Elution of protein was monitored by absorbance at 230 nm.

SDS PAGE and Immunoblotting

Unless otherwise indicated, samples were precipitated for subsequent electrophoretic analysis with TCA (15% final concentration, 0°C), or in the case of chromatin, with spermine (5 mM final concentration, 0°C). SDS polyacrylamide gels were Tris-glycine-buffered 5–20% or 10–20% acrylamide gradient gels, with an acrylamide/bisacrylamide ratio of 30:0.8 (Hames, 1981). In later experiments, proteins were resolved using a minigel system (Bio-Rad Laboratories) in SDS 18% polyacrylamide gels prepared according to Thomas and Kornberg (1975). Gels were stained with Coomassie Brilliant Blue R (Hames, 1981). Proteins were transferred to nitrocellulose (BA 83; Schleicher & Schuell, Inc., Keene, NH) according to Towbin et al. (1979), using a Tris-glycine transfer buffer containing 0.1% SDS. Nitrocellulose strips were stained for protein with Amido black 10B (Gershoi and Palade, 1982). For immunodetection of proteins, we used PBS/0.05% Tween 20 as the buffer for blocking, primary and secondary immunoreagent application, and washing steps (Batteiger et al., 1982). In addition, some experiments employed washes of 2 M urea, 10 mM Tris-HCl, pH 7.3 (Earnshaw et al., 1984), and/or PBS containing an additional 2 M NaCl. Blots were autoradiographed at -70°C using an intensifying screen (Cromex Hi Plus; Dupont Co., Wilmington, DE) and Kodak XAR-5 film.

Primary immunoreagents included individual and pooled whole human sera, and affinity-purified human autoantibodies. Except where noted in the figure legends, whole sera were used at a dilution of 1,000-fold, and the concentration of affinity-purified antibodies was not determined. The secondary reagent was ^{125}I -labeled protein A (New England Nuclear, Boston, MA) used at a dilution of 3,500-fold.

Ion Exchange Chromatography of HeLa Nuclear Protein

HeLa nuclei (200 $A_{260 \text{ nm}}$ U) were washed three times with 5 mM Hepes containing protease inhibitors and β -mercaptoethanol, and were extracted with ice-cold 0.25 M HCl. Extracted protein was precipitated with TCA, redissolved in 0.5 ml of 25 mM NaPO_4 (pH 7.0), and applied to a cation exchange column (TSK SP-5-PW; Bio-Rad Laboratories). The column was washed with NaPO_4 (pH 7.0) application buffer at a flow rate of 1 ml/min for 10 min before initiation of a linear gradient of NaCl from 0 to 1.5 M (in NaPO_4 buffer). Fractions of 1 ml and 0.5 ml were collected during the application buffer wash and salt gradient elution, respectively. For all column runs we used an HPLC apparatus (Bio-Rad Laboratories).

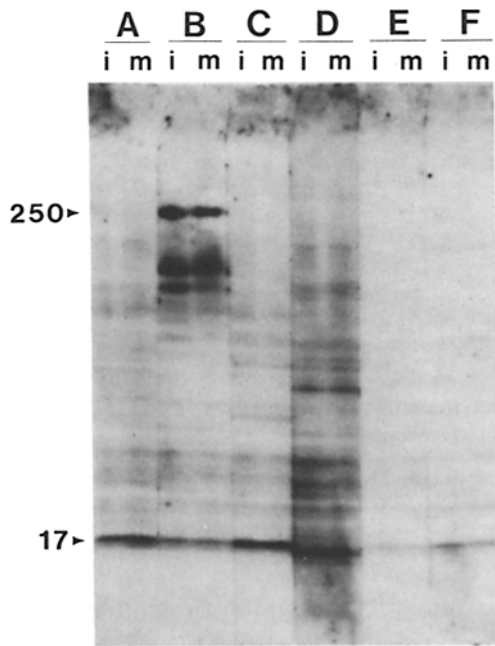


Figure 1. Immunoblotting of mitotic and interphase HeLa cell extracts using anticentromere and control sera. Cycling HeLa cells were blocked overnight with nocodazole and fractionated into the nonadherent mitotic population (lanes *m*) and the residual adherent interphase population (lanes *i*). Whole-cell extracts were fractionated on 5–20% linear acrylamide gradient SDS gels and blotted to nitrocellulose. The nitrocellulose strips were probed using five different anticentromere sera and a pooled normal human serum, each at a dilution of 500-fold. (A) Serum A.J.; (B) serum B.S.; (C) serum G.D.; (D) normal human serum; (E) serum H.W.; (F) serum I.G. All five anticentromere sera recognize a 17-kD antigen not recognized by the normal human serum. In addition, antibodies in the serum from patient B.S. recognize the 250-kD NuMA antigen and its breakdown products (B).

Affinity Purification of Antibodies

Antibodies were eluted from immunoblots as described by Olmsted (1981), except that 5 M NaI, 5 mM sodium thiosulfate was used as the eluting agent. Eluted antibodies were diluted with an equal volume of PBS containing 0.045% BSA, and were buffer exchanged and concentrated using concentrators (Centricon 10; Amicon Corp., Danvers, MA). After concentration, the buffer contained 1% BSA and 0.1% Na₂S₂O₃. The antibodies were stored at –20°C until use.

Indirect Immunofluorescence

HeLa cells and RMCD cells (a rat mammary cell line, see Rosok and Rohrschneider, 1983) were grown on poly-D-lysine-coated glass slides in DME containing 5% fetal calf serum and in RPMI 1640 medium containing 10% fetal calf serum, respectively. Cells were fixed and permeabilized as described elsewhere (Palmer and Margolis, 1985). Primary antibodies were applied in PBS containing 1% BSA. Other steps in indirect immunofluorescence microscopy were as described previously (Palmer and Margolis, 1985), except that the second antibodies were FITC-conjugated anti-human IgGs (Southern Biotechnology Associates, Inc., Birmingham, AL) used at a dilution of 800-fold.

Results

The 17-kD Antigen Is Centromere Specific

Whole-cell extracts from mitotic (nocodazole blocked) and interphase HeLa cells were subjected to electrophoresis on

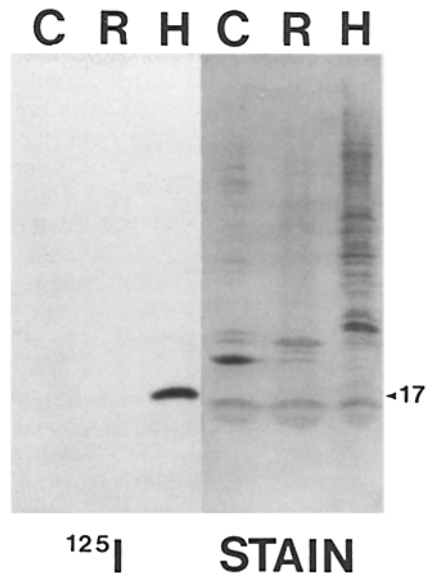


Figure 2. Immunoblotting of rat liver, chicken erythrocyte, and HeLa cell nuclear extracts using affinity-purified anti-17-kD protein antibodies derived from serum G.D. Total nuclear protein extracts representing 4 A_{260 nm} U of nuclei were fractionated on linear 10–20% gradient SDS polyacrylamide gels, transferred to nitrocellulose, and probed using affinity-purified anti-17-kD protein antibodies (¹²⁵I lanes), or a whole normal human serum (not shown). The concentration of the affinity-purified antibodies was not determined. No specific binding of ¹²⁵I-labeled protein A was observed using the normal human serum at a dilution of 500-fold. After autoradiography the nitrocellulose strip was stained for protein using Amido black 10B (STAIN lanes). (Lanes R) Rat liver nuclear protein; (lanes C) chicken erythrocyte nuclear protein; (lanes H) HeLa cell nuclear protein.

linear 5–20% acrylamide gradient SDS gels, blotted to nitrocellulose, and probed with five different anticentromere sera and a pooled normal human serum. All five anticentromere sera recognized a ~17-kD protein, present in similar amounts in both cell populations, which was not recognized by the normal human serum (Fig. 1). No other anticentromere-specific bands were detected, although later experiments have indicated the presence of antigens at both ~80 and ~140 kD in HeLa cells, and ~50 kD in rat, as reported by Earnshaw and Rothfield (1985). Antibodies in the pooled normal human serum (lot No. 291; Jackson Laboratory, Bar Harbor, ME) bound to histone H3, which migrates slightly faster than CENP-A. This binding was not observed with other individual or pooled normal human sera tested (not shown). Whole-cell extracts from cycling HeLa cells have also been probed using these five and seven additional anticentromere sera, three nonanticentromere autoimmune sera, two individual normal human sera, and one additional pooled normal human serum. The results in each case were similar to those reported above (data not shown). CENP-A was recognized by all but one of the anticentromere sera, but by none of the normal human or irrelevant autoimmune sera. These results are consistent with those of other workers (Guldner et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; McNeilage et al., 1986). The single anticentromere serum that did not detectably bind CENP-A may recognize labile epitopes present on proteins

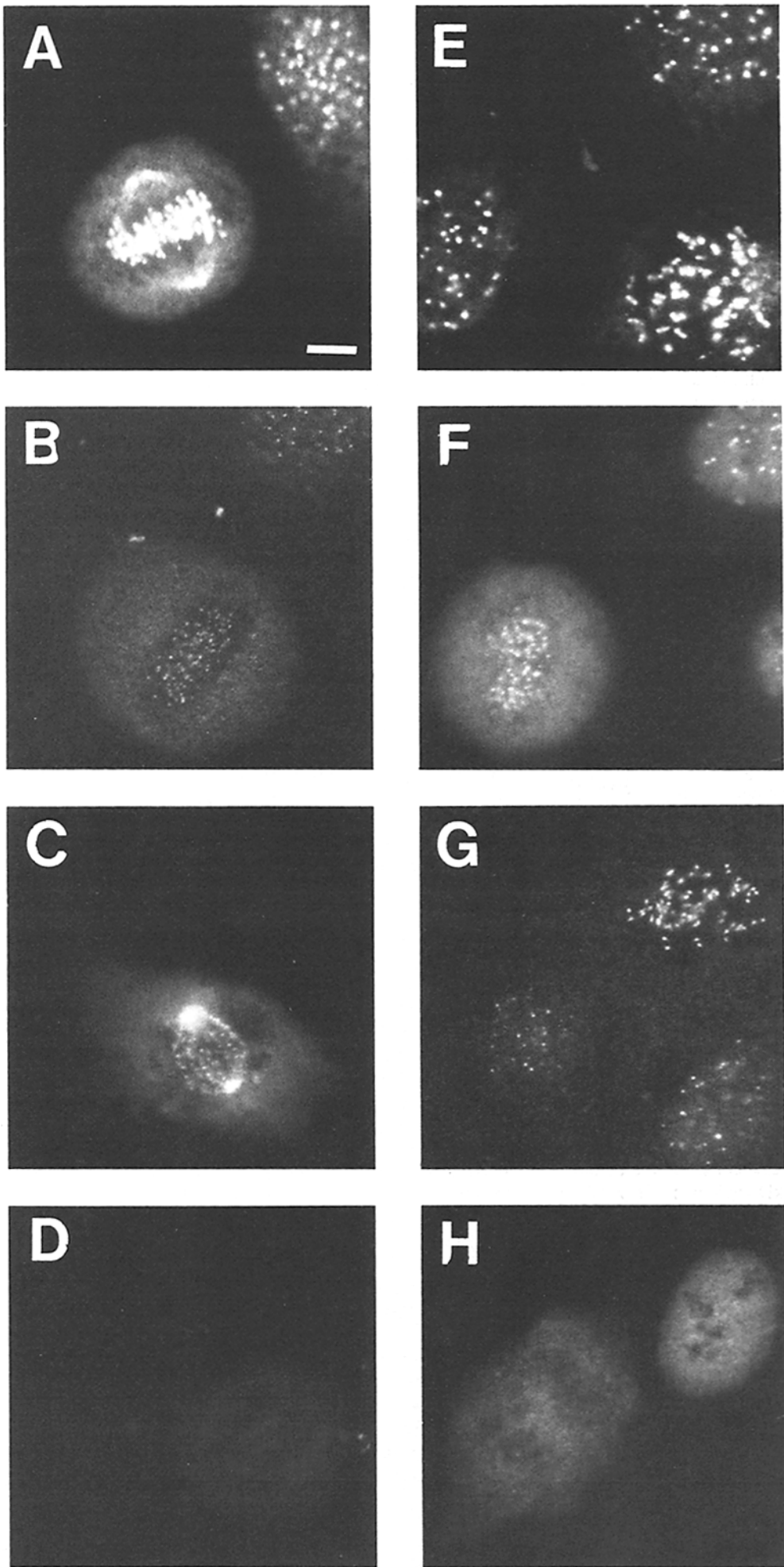


Figure 3. Indirect immunofluorescence staining of human (HeLa) and rat (RMCD) cells using whole sera and affinity-purified anti-17-kD protein antibodies. (A) Serum B.S. on HeLa cells. Both spindle (anti-NuMA) and centromere staining are visible. (B) Affinity-purified anti-17-kD protein antibodies from serum B.S. on HeLa cells. The concentration of the affinity-purified antibodies was not determined. Spindle staining has been lost, but centromere staining is retained. (C) Serum B.S. on RMCD cells. Again, both spindle and centromere staining are visible. (D) Affinity-purified anti-17-kD protein antibodies from B.S. used on RMCD cells. Neither spindle nor centromere staining is detectable. (E) HeLa cell centromere staining using serum G.D. (F) HeLa cell centromere staining using affinity-purified anti-17-kD protein antibodies derived from G.D. The antibodies were used at a concentration of 35 ng/ml. (G) Serum G.D. used on RMCD cells. (H) Affinity-purified anti-17-kD protein antibodies derived from serum G.D. used on RMCD cells. No centromere staining is detectable. Bar, 2 μ m.

or complex molecular assemblies, such as nucleosomes, or centromere-specific proteins present in amounts so small as to be undetectable with our procedures.

Antibodies were eluted from the 17-kD region of an immunoblot probed with serum G.D., and were in turn used to probe a nitrocellulose blot containing equivalent amounts (as $A_{260\text{ nm}}$ U of nuclei) of HeLa, rat liver, and chicken erythrocyte nuclear extracts. In lanes containing HeLa nuclear extract, the antibodies bound only to the 17-kD region, demonstrating the specificity of these affinity-purified antibodies for CENP-A (Fig. 2). We detected no binding to rat liver or chicken erythrocyte nuclear proteins. Similar results were obtained with rat liver and HeLa cell nuclear proteins using a second affinity-purified anti-17-kD protein antibody. Indeed, we have not observed binding to the 17-kD region of rat or chicken nuclear or whole-cell extract blots, using any of several whole sera tested. Such results suggest that the epitope(s) recognized by these anti-17-kD protein antibodies may be human specific. We have not assayed for the 17-kD protein in other primates.

To demonstrate that the 17-kD protein we have detected is centromere specific, we used affinity-purified antibodies for indirect immunofluorescence. The anti-17-kD antibodies affinity purified from serum G.D. stained the centromeres of mitotic chromosomes (Fig. 3) and interphase-persistent centromere foci in HeLa cells (not shown). The centromeres of mitotic HeLa cell chromosomes stained using whole sera frequently had a dumbbell-like appearance, whereas with affinity-purified anti-17-kD antibodies only punctate foci were observed. In addition, staining was generally less intense than with whole serum. These data may be explained by the presence in whole sera of antibodies that bind to other centromere antigens (Earnshaw et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; McNeilage et al., 1986). These immunofluorescence data confirm previous reports demonstrating the human centromere-specific binding of antibodies affinity purified from Western blots of CENP-A (Guldner et al., 1984; Earnshaw and Rothfield, 1985). Further, they document the properties of affinity-purified anti-CENP-A antibodies from serum G.D., which were used extensively for generating the data presented in this study.

To further demonstrate that anti-17-kD antibodies specifically bind to the centromere, we exploited the fact that one serum (B.S.) contained not only anticentromere antibodies, but also antibodies specific for a 250-kD nuclear and mitotic apparatus-associated protein (designated NuMA) and its degradation products (Fig. 1, see also McCarty et al., 1984; Pettijohn et al., 1984). Immunofluorescent titers of both specificities were $>12,800$ on HeLa cells. We eluted bound antibody from the 17- and 250-kD regions of HeLa whole-cell blots incubated with serum B.S., and used the eluted antibodies for immunofluorescence. Whereas antibodies in whole serum bound to nuclei to give homogeneous nuclear staining, to mitotic spindles (the anti-NuMA specificity), and also to premitotic and mitotic centromeres, antibodies eluted from the 17-kD region bound only to premitotic and mitotic HeLa centromeres, with no detectable NuMA staining (Fig. 3). As in the case of anti-17-kD protein antibodies affinity purified from serum G.D., staining with anti-17-kD protein antibodies from serum B.S. was restricted to punctate foci in both interphase nuclei and mitotic chromosomes. In

contrast to the anticentromere staining observed with anti-17-kD protein antibodies, antibodies eluted from the 250-kD region (NuMA region) of blots probed with serum B.S. showed homogeneous staining of nuclei and staining of mitotic spindles, with no detectable binding to premitotic or mitotic centromeres (data not shown). In all experimental and control cells, cytoplasmic staining was similar and nonspecific.

Consistent with our immunoblotting data, indirect immunofluorescence experiments showed that affinity-purified anti-17-kD protein antibodies from serum B.S. and from serum G.D. failed to stain the interphase precentromeres and mitotic centromeres of rat (RMCD) cells when used at concentrations adequate to stain these structures in HeLa cells (Fig. 3).

Core Histone-like Properties of CENP-A

We have examined conditions required to solubilize the 17-kD protein from nuclei. These studies showed that the antigen is not extracted from nuclei by 0.6 M salt at pH 7.0, a condition which extracts most nonhistone proteins (including the high mobility group proteins) and H1 (Bonner et al., 1968; Goodwin et al., 1978), but that it is extracted by salt concentrations of 2 M NaCl or greater, or by 0.25 M HCl (Fig. 4). The latter conditions extract nucleosome core histones (Bonner et al., 1968; Busch, 1968). Like the core histones, but unlike H1, CENP-A is not extracted from nuclei with 5% TCA, and salt- or acid-extracted antigen is likewise insoluble in 5% TCA (Fig. 4 and data not shown). Similar results have been obtained using 5% perchloric acid (not shown). Antigen is not solubilized from intact nuclei by low ionic strength buffers, but after digestion of the nuclei with micrococcal nuclease or with DNAase I the antigen is extracted with chromatin, and is quantitatively precipitated with chromatin by 10 mM $MgCl_2$ or 5 mM spermine (Fig. 5). These observations indicate that the 17-kD protein is a component of chromatin with core histone-like properties of salt and acid extractability.

Small amounts of CENP-A were extracted from nuclei as chromatin during moderately high salt (≤ 0.6 M NaCl) washes of nuclei in suspension (not shown). Therefore, we turned to hydroxylapatite as a support for nuclei or chromatin during salt fractionation. The salt concentrations required for the elution of proteins from nuclei or chromatin adsorbed to hydroxylapatite have been shown to parallel those required for their extraction from nuclei in suspension (Bloom and Anderson, 1978; Philip et al., 1979). Relative to the histones, apparent recovery of CENP-A after binding of nuclei or chromatin to hydroxylapatite and elution using 2.5 M NaCl was variable, ranging from ~ 50 to 100%, depending on the particular experiment. Apparently incomplete recovery of CENP-A (Fig. 4 B) might reflect denaturation of the epitope(s), since no CENP-A was recovered in washes of nuclei or chromatin using neutral buffers that were at or below 0.6 M salt (Figs. 4 B, 5, and data not shown). Alternatively, incomplete recovery might reflect a failure to completely dissociate CENP-A from residual nuclear material remaining bound to hydroxylapatite.

CENP-A is under-represented in chromatin rendered soluble by brief digestion with micrococcal nuclease, and a substantial fraction of CENP-A is retained in the residual nucleus after even extensive digestion. This micrococcal nucle-

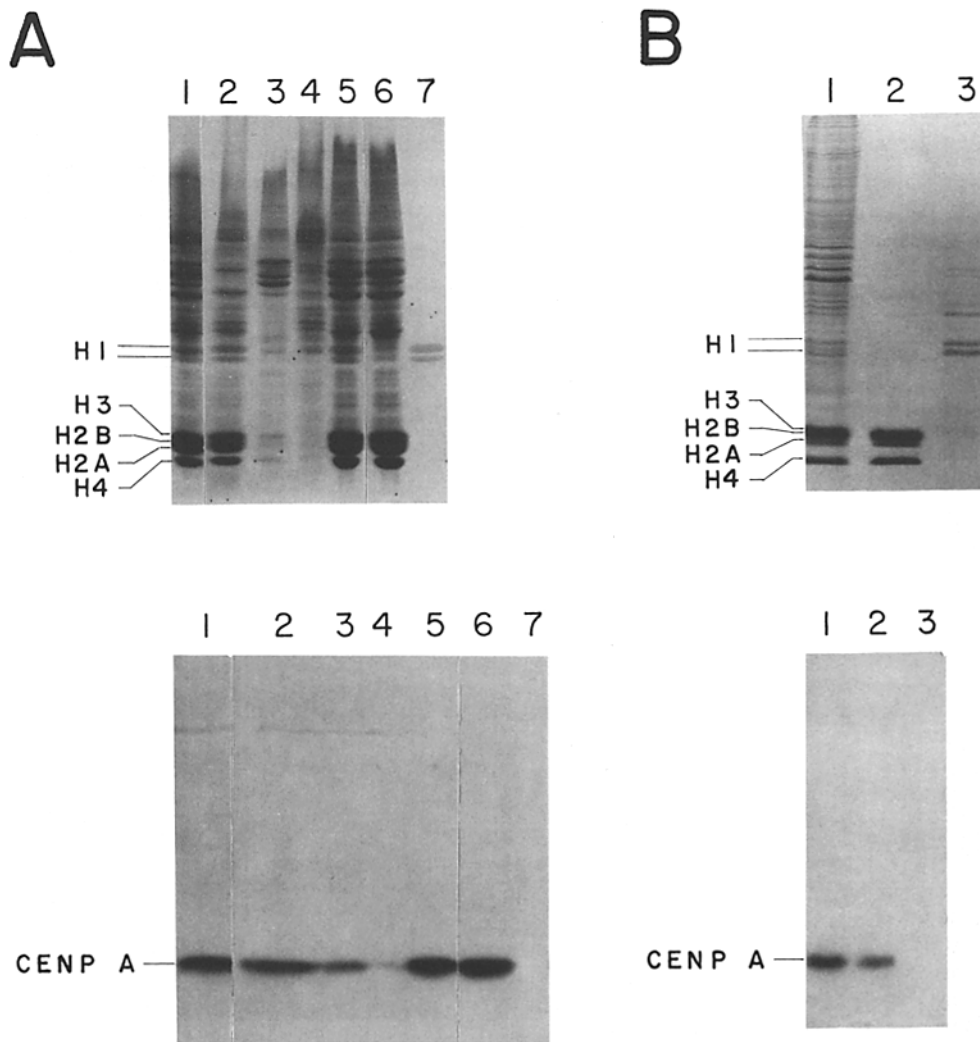


Figure 4. Salt and acid extractability and solubility properties of CENP-A. (A) Extraction of HeLa cell nuclei. (Top) Coomassie Blue-stained 10–20% acrylamide gradient SDS gel loaded with total nuclear protein and protein fractions. Each lane represents protein obtained from 1.5 $A_{260\text{ nm}}$ U of nuclei. (Bottom) Autoradiogram showing the distribution of CENP-A in the protein fractions. Immunoblotting analysis was performed on a duplicate of the stained gel, except that each lane was loaded with protein representing 5 $A_{260\text{ nm}}$ U of nuclei. The immunoblot was probed with serum G.D. (Lane 1) Total nuclear protein; (lane 2) nuclear protein extracted with 2M NaCl; (lane 3) residual protein after 2M NaCl extraction; (lane 4) residual protein after extraction of nuclei with 0.25 M HCl; (lane 5) nuclear protein extracted with 0.25 M HCl; (lane 6) HCl-extracted nuclear protein precipitated with the addition of TCA to 5%; (lane 7) HCl-extracted nuclear protein remaining soluble in 5% TCA. These data show that CENP-A is extracted from nuclei by high salt (2 M NaCl) or 0.25 M HCl, and is insoluble in 5% TCA. (B) Extraction of HeLa nuclei adsorbed to hydroxylapatite. (Top) Coomassie Blue-

stained 18% acrylamide SDS minigel loaded with total nuclear protein and nuclear protein fractions. Each lane represents protein obtained from 0.2 $A_{260\text{ nm}}$ U of nuclei. (Bottom) Autoradiogram showing CENP-A in the protein fractions. Immunoblotting analysis was performed on a duplicate of the stained gel, except that each lane was loaded with protein representing 0.4 $A_{260\text{ nm}}$ U of nuclei. The immunoblot was probed with affinity-purified anti-CENP-A from serum G.D. (Lane 1) Total nuclear protein. (Lane 2) Protein released by 2.5 M NaCl, 25 mM NaPO_4 (pH 7.0) from nuclei that had been previously adsorbed to hydroxylapatite and washed first with 25 mM NaPO_4 (pH 7.0), and then with 0.6 M NaCl, 25 mM NaPO_4 (pH 7.0). Before adsorption to hydroxylapatite, the nuclei were washed with 5 mM Hepes, 0.2 mM EDTA, followed by 5 mM Hepes alone (both buffers at pH 7.5). Low ionic strength washes extract many nuclear proteins, but the extracted proteins do not contain CENP-A (Fig. 5 A and data not shown). (Lane 3) Protein released from low ionic strength washed and adsorbed nuclei using 0.6 M NaCl, 25 mM NaPO_4 (pH 7.0). The H1 bands are prominent. These data show that CENP-A is not extracted from nuclei by 0.6 M NaCl, but that it is extracted by very high salt (2.5 M). Similar results have been obtained with chromatin adsorbed to hydroxylapatite.

ase-resistant fraction is observed whether the nuclei are digested repeatedly with low concentrations of nuclease (Fig. 5 A), or only once but with high concentrations of nuclease or long digestion times (data not shown). However, with residual CENP-A we also observed residual core histones (Fig. 5 A, lane 9). Note that as performed here, DNAase I digestion and low ionic strength extraction solubilizes both core histones and CENP-A essentially quantitatively (Fig. 5 B). Therefore, we believe the residual fraction of CENP-A does not represent a nonchromatin subset, but rather a fraction present in chromatin which is refractory to micrococcal nuclease digestion under the conditions we employ.

To further examine the role of the 17-kD protein in chromatin structure, we asked if it is a component of nucleosome core particles. Salt-washed (450 mM NaCl) long chromatin was further digested with micrococcal nuclease to optimize the production of core particles (Lutter, 1978), and the digest was fractionated on linear log sucrose gradients buffered with 5 mM Hepes as described in Materials and Methods. Fractions reflecting major features of the distribution of 260 nm absorbing material in the gradient were pooled into five separate samples as shown in Fig. 6 A. Peak *a* presumably contained nucleotides released by the micrococcal nuclease digestion of the salt-washed long chromatin, since it contained no detectable oligomeric DNA and little protein (see

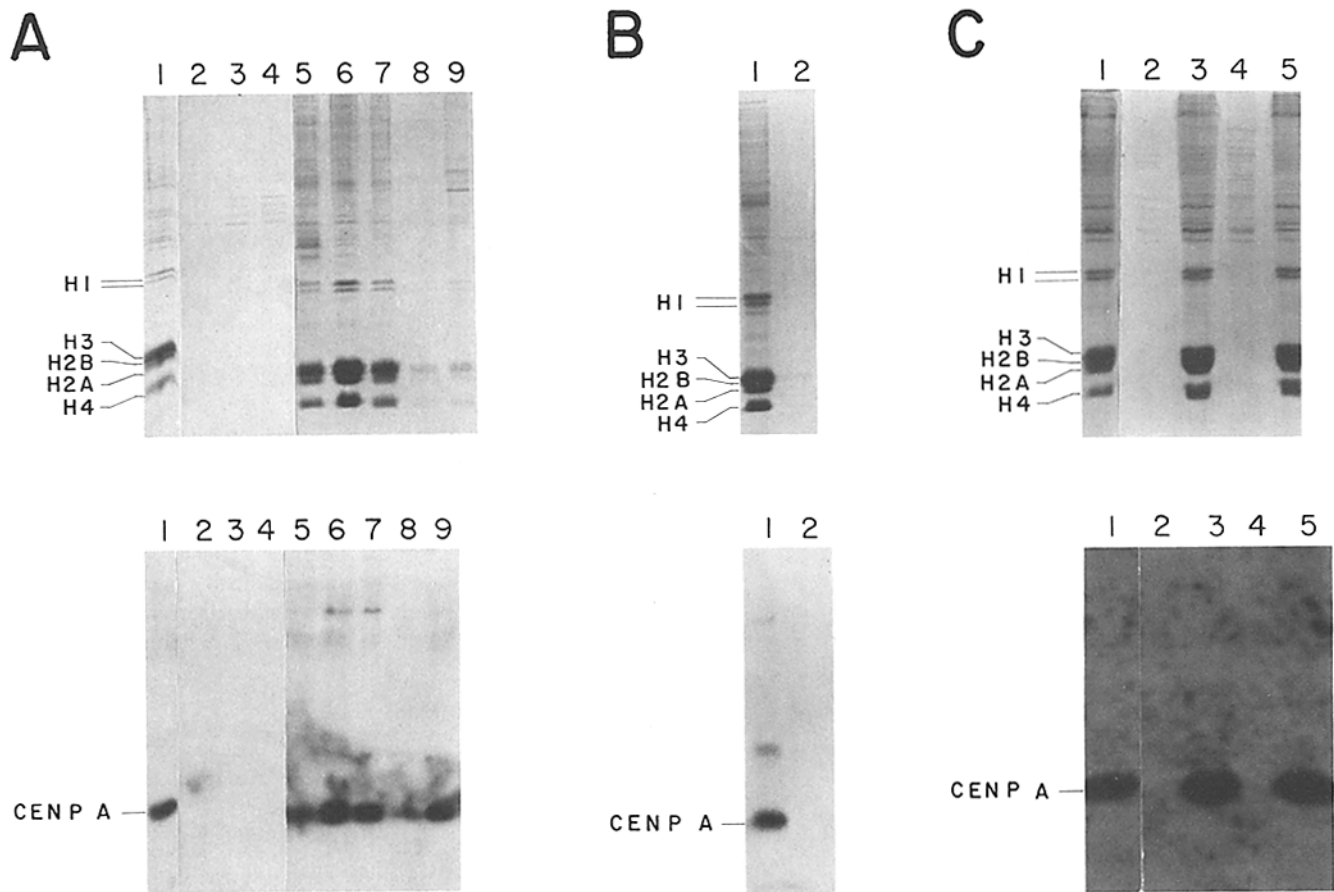


Figure 5. CENP-A is a component of chromatin from HeLa nuclei. (*Top*) Coomassie Blue-stained 18% acrylamide SDS minigels. Corresponding immunoblots (*bottom*) reveal the distribution of CENP-A. Gels for staining and immunoblotting were loaded with protein representing 0.15 and 0.5 $A_{260\text{ nm}}$ U of nuclei/lane, respectively. (*A*) The effect of digestion of nuclei with micrococcal nuclease on the extraction of CENP-A with low ionic strength conditions. Nuclei were washed three times with 5 mM Hepes, pH 7.5, containing β -mercaptoethanol and protease inhibitors (low ionic strength buffer) as described in Materials and Methods. They were then repeatedly digested with micrococcal nuclease (40 U/ml, 2 min) and extracted with low ionic strength buffer as described, except that a fourth digestion (30-min duration) and subsequent extraction were performed. The gel lanes represent protein obtained in each of the sequential treatments of the nuclei. The immunoblot was probed with serum G.D. (*Lane 1*) Total nuclear protein; (*lane 2*) protein extracted with the first low ionic strength wash before nuclease digestion; (*lane 3*) second low ionic strength wash; (*lane 4*) third low ionic strength wash; (*lane 5*) protein obtained from the first nuclease digestion and low ionic strength extraction; (*lane 6*) second nuclease digestion and extraction; (*lane 7*) third digestion and extraction; (*lane 8*) fourth digestion and extraction; (*lane 9*) residual nuclear protein. Extraction of CENP-A by low ionic strength buffer is dependent on nuclease digestion, but some CENP-A and core histone remains in a micrococcal nuclease-resistant fraction. (*B*) Extraction of chromatin and CENP-A from nuclei after digestion with DNase I. Nuclei were digested and extracted as described in Materials and Methods for micrococcal nuclease, except that 2 mM $MgCl_2$ was substituted for $CaCl_2$, and digestion was for 20 min at 22°C. The immunoblot was probed with serum G.D. (*Lane 1*) Nuclear protein extracted by low ionic strength buffer after digestion of HeLa nuclei, 50 U/ml of DNase I; (*lane 2*) residual nuclear protein. CENP-A and core histones are quantitatively extracted with low ionic strength buffer after digestion with DNase I under these conditions. (*C*) CENP-A and chromatin precipitate with $MgCl_2$ or spermine. Soluble long chromatin prepared by micrococcal nuclease digestion and low ionic strength extraction was made 10 mM in $MgCl_2$ or 5 mM in spermine, allowed to stand on ice for 15 min, and centrifuged. Proteins in the supernatant fractions were precipitated with 15% TCA. (*Lane 1*) Total chromatin proteins (15% TCA precipitated); (*lane 2*) 10 mM $MgCl_2$ -soluble proteins; (*lane 3*) $MgCl_2$ -insoluble proteins; (*lane 4*) 5 mM spermine-soluble proteins; (*lane 5*) spermine-insoluble proteins. CENP-A precipitates with the chromatin histones. The immunoblot was probed with affinity-purified anti-CENP-A from serum G.D.

6 B). Electrophoretic analysis of the DNA contained in samples *c* and *d* showed that sample *c* contained DNA of the size expected for nucleosome core particles (146 bp) and traces of subnucleosomal (~ 120 bp) and compact dimer DNA (~ 280 bp) (Lutter, 1978), whereas sample *d* contained predominantly 280-bp compact dimer DNA, with some contaminating 146-bp material (Fig. 6 A, insert). The protein and antigen contents of samples were analyzed by SDS PAGE and immunoblotting. Fig. 6 B shows the distribution of pro-

tein in the samples, as determined by Coomassie staining of an SDS polyacrylamide gel, and Fig. 6 C the distribution of 17-kD antigen as determined by immunoblotting. It is clear that the distribution of the 17-kD protein parallels the distribution of histone in the gradient, which was maximal in sample *c*, and that the amount of antigen relative to the histones is similar for the gradient fractions *c* and *d*, whole nuclei, and salt-washed long chromatin. The amount of CENP-A in the samples does not reflect the distribution of nonhistones

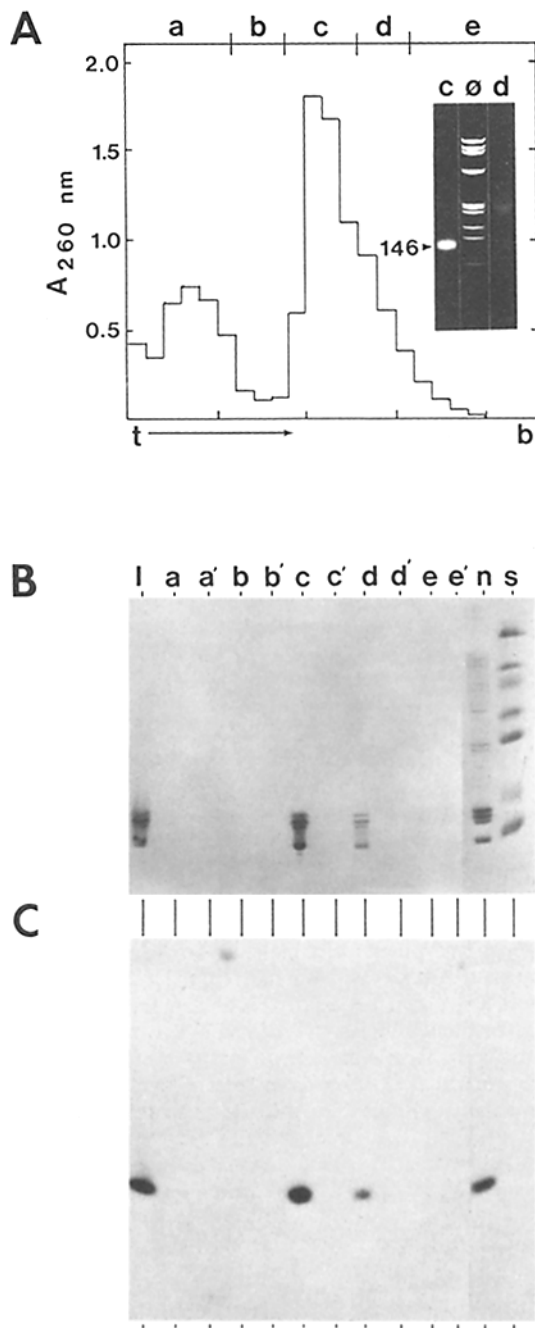


Figure 6. Analysis of chromatin and nucleosome core particles. (A) Fractionation of a micrococcal nuclease digest of salt-washed (450 mM NaCl) long chromatin in a linear log sucrose gradient. The distribution of 260 nm absorbing material in the gradient was used to guide the pooling of fractions to form samples *a-e*, as shown above the gradient profile. (Insert) Size analysis by polyacrylamide gel electrophoresis of the DNA in sucrose gradient samples *c* and *d*. The standard was a Hae III digest of Φ X174 DNA. (B) Protein composition of HeLa nuclei, salt-washed chromatin, and sucrose gradient samples as determined by SDS PAGE (18% polyacrylamide, minigel apparatus). (Lane *l*) Salt-washed long chromatin. (Lanes *a-e*) protein from sucrose gradient samples *a-e*, which precipitated with the addition of spermine to 5 mM (0°C). Pellets were brought to equal volumes in SDS gel application buffer, and equal volumes were loaded. All four core histones, as well as some histone proteolysis products, are present in samples *c* and *d*, but no higher molecular weight nonhistones are detected. From the size analysis of DNA contained in these samples, we can conclude that sample

(compare *c* with *n*). Together, these data indicate that the 17-kD protein is a centromere-specific chromatin component, and suggest that it functions as a nucleosome core histone.

Further evidence for the histone-like character of CENP-A was obtained by fractionation of an acid extract of HeLa cell nuclear protein on a cation exchange column, using a gradient of NaCl from 0 to 1.5 M in 25 mM NaPO₄ (pH 7.0) to elute bound proteins. Fractions were analyzed for their histone and CENP-A composition using an immunodot blotting assay (not shown), SDS PAGE, and immunoblotting. The results are shown in Fig. 7. CENP-A was not detected in the acidic proteins of the flow-through, but eluted from the column at a NaCl concentration of ~1.45 M. This concentration is substantially greater than that required to elute H2A and H2B (~1.20 M), and is similar to but slightly greater than that required to elute even H3 and H4 (H3 and H4 concentration was maximal in sample *j*; CENP-A was maximal in sample *k*). These data indicate that CENP-A itself has highly basic domains, or else is a component of protein complexes with basic domains. Similar results have been obtained using native core histones obtained by elution from nuclei adsorbed to hydroxylapatite (data not shown).

Using these nondenaturing elution conditions, H2A (and its ubiquitinated adduct) and H2B elute from the ion exchange column as a single peak, as do H3 and H4. Furthermore, the distribution of H3 and H4 across their peak is uniform and apparently equimolar (Fig. 7, fractions *h-l*). These data suggest the eluting species are H2A-H2B dimers and H3-H4 tetramers (Isenberg, 1979). The fact that CENP-A elutes with H3 and H4 suggests that it may be part of H3-H4 tetramer-related complexes. We are currently engaged in applying histone complex reconstitution methods to determine if CENP-A might substitute for H3 or H4 in a tetrameric complex.

Discussion

Our data show clearly that CENP-A is a histone-like chromatin component, and further, that it is a component of chromatin fragments with the solubility and sedimentation properties characteristic of nucleosome core particles. One possible explanation of these results is that the antigen is a component of completely novel structures that fractionate with classical core particles. A second possibility is that CENP-A is found in core particles (defined as containing 146 bp of DNA and an octamer of the standard histones H2A, H2B, H3, and H4) in addition to the standard protein octamer set. A third possibility is that CENP-A is a posttranslationally modified standard histone; and a fourth is that it is a pri-

c consists almost entirely of nucleosome core particles, and *d* largely of compact dimers. (Lanes *a'-e'*) Protein in samples *a-e*, which did not precipitate with spermine, but which did precipitate with the subsequent addition of TCA to 20% (0°C). (Lane *n*) HeLa whole nuclear protein. (Lane *s*) Molecular mass standards. Sample *c* contained 0.06 260 nm absorbance units of chromatin, and samples *l* and *n* were adjusted to contain an amount of histone equivalent to that of sample *c*. Protein was stained with Coomassie Brilliant Blue R. (C) Immunoblotting analysis of samples described in B, above. All loadings were proportional to those shown in B, but the samples analyzed by immunoblotting were eight times as large. The immunoblot was probed with affinity-purified anti-17-kD protein from serum G.D.

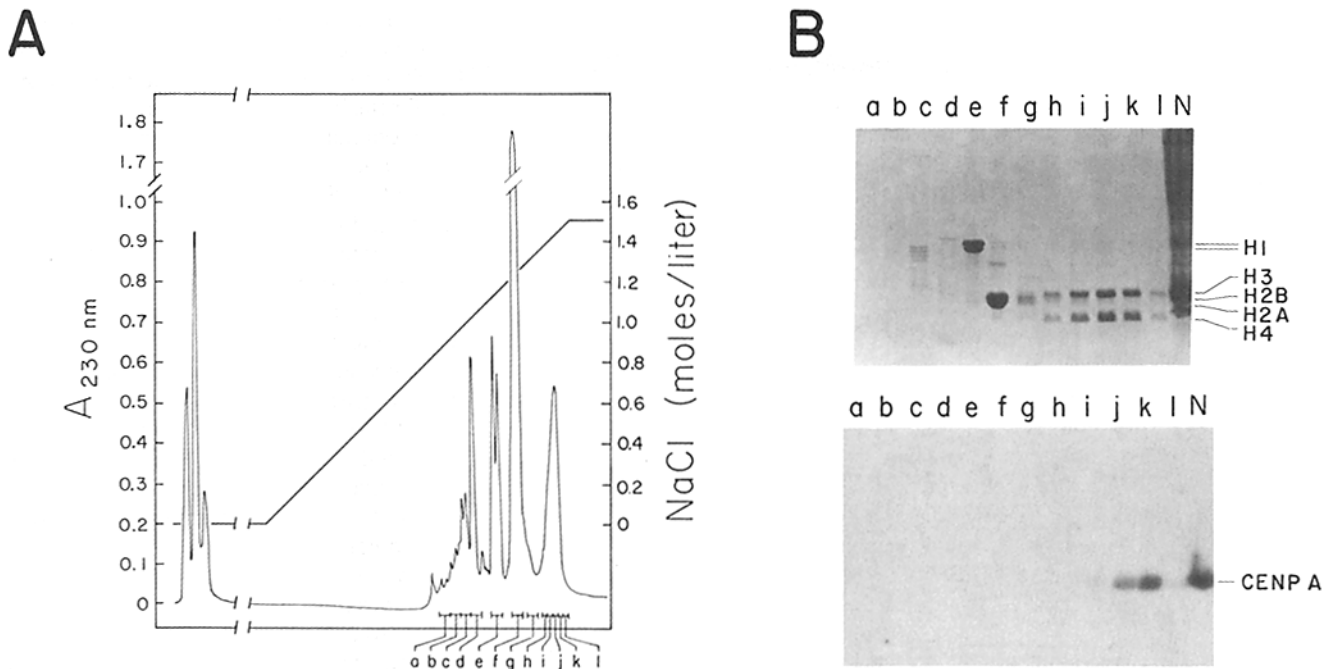


Figure 7. Ion exchange chromatography of HeLa histones. Low ionic strength buffer-washed HeLa nuclei (200 $A_{260\text{ nm}}$ U) were extracted with 0.25 M HCl. The extracted proteins were precipitated with TCA, redissolved in 0.5 ml of 25 mM NaPO_4 (pH 7.0), and applied to a cation exchange column (TSK SP-5-PW) coupled to an HPLC system (Bio-Rad Laboratories). Nonadsorbed proteins were eluted with application buffer at a flow rate of 1 ml/min, and 1-ml fractions were collected. 10 min after injection, a linear gradient of NaCl from 0 M to 1.5 M (in 25 mM NaPO_4 , pH 7.0) was initiated. Half-milliliter fractions were collected and analyzed using an immunodot blotting assay (not shown), SDS PAGE, and immunoblotting. (A) Elution of HeLa nuclear proteins. The line indicating NaCl concentration refers to concentration at the pump, 30 mM greater than the buffered NaCl containing the eluted histone. The indicated pooled (a-g) or individual (h-l) fractions were subjected to SDS PAGE and immunoblotting. (B) Histone and CENP-A distribution in protein fractions. (Top) Coomassie Blue-stained 18% polyacrylamide minigel of samples a-l as indicated in A, and whole nuclear protein (N). (Bottom) Corresponding immunoblot indicating the distribution of CENP-A. The samples applied for immunoblotting were three and a half times those applied for staining. The immunoblot was probed with an affinity-purified anti-CENP-A. Sample f contains predominantly H2A and H2B, and samples h-l contain H3 and H4 in uniform and apparently equimolar proportions. These results suggest that H2A and H2B are eluting as dimers, and H3 and H4 as tetramers (Isenberg, 1979). CENP-A is found predominantly in samples j and k, and is maximal in sample k. Thus, CENP-A elutes at a concentration of NaCl that is close to, but slightly greater than that required to elute the bulk of H3 and H4 (maximal in sample j). These data indicate that either CENP-A itself has highly basic domains, or is a component of protein complexes with basic domains. The fact that CENP-A elutes with H3 and H4 further suggests that it may be part of H3-H4-like complexes.

mary sequence variant which substitutes for a standard histone. While the first two possibilities seem unlikely, precedence for either of the latter two possibilities is abundant. Histones are known to be modified posttranslationally in a variety of ways (reviewed by West and Bonner, 1980a), and the covalent addition of ubiquitin to H2A, H2B, and their primary sequence variants results in the production of proteins with remarkably different physical properties, but which nevertheless are components of histone octamers and nucleosomes (West and Bonner, 1980a, b). The existence of quantitatively minor histones, which differ extensively in primary sequence from standard histones yet substitute for them in nucleosomes, is also well established. For instance, such sequence variants of H2A are found in animals as diverse as *Tetrahymena*, *Drosophila*, and vertebrates (reviewed by Donahue et al., 1986). In view of the unusual packing properties of kinetochore chromatin (Brinkley et al., 1980; Ris and Witt, 1981), nucleosomes containing CENP-A may be important for the induction of a unique higher order structure in chromatin.

Our data show that CENP-A has core histone-like properties of salt and acid solubility. Another property character-

istic of histones is that they have isoelectric points >9.0 , although ubiquitinated H2A and H2B are exceptions (Goldknopf and Busch, 1978; Isenberg, 1979; West and Bonner, 1980b). We have not yet determined the isoelectric point of CENP-A. However, we have found that CENP-A elutes from a cation exchange column at essentially the same NaCl concentration as H3 and H4, suggesting that it too is highly basic.

More fundamental to the definition of a histone than its isoelectric point is its ability to interact with other histones to form well defined complexes, H2A-H2B dimers, H3-H4 tetramers, and hexamers and octamers containing all four histones (Isenberg, 1979). The fact that CENP-A elutes with H3 and H4 from an ion exchange column under nondenaturing conditions suggests that CENP-A may substitute for H3 or H4 in complexes. Experiments are currently under way to test this hypothesis.

Anticentromere antibodies present in whole sera localize to centromere-specific components in nuclei from rat and chicken tissues or cultured cells (Fig. 3, and Palmer and Margolis, 1985). Surprisingly, affinity-purified anti-17-kD protein antibodies did not bind to Western blotted pro-

teins from rat liver or chicken erythrocyte nuclei, nor, as determined by immunofluorescence microscopy, did they localize to centromeres in rat fibroblasts. If the anti-CENP-A antibodies have a much greater avidity of binding to the human protein than to nonhuman homologues, then it is possible that we used the affinity-purified antibodies at concentrations insufficient to detect the nonhuman homologues. However, the fact that we detect no signal from immunoblots of rat or chicken protein probed with affinity-purified anti-CENP-A antibodies, even on long overexposure of the human-specific signal, suggests that there is little or no binding to any rat or chicken protein. It seems likely, therefore, that the staining we observe in rat and chicken nuclei using whole sera reflects the binding of antibodies to centromere-specific epitopes other than those detected by the affinity-purified anti-CENP-A antibodies. These epitopes might be labile to the conditions we use to prepare samples for SDS PAGE, or might be present in amounts so low as to be undetectable with our immunoblotting procedures. They could reside in proteins other than CENP-A, or even in complex molecular assemblies, such as nucleosomes (Tahourdin and Bustin, 1980).

Our failure to detect nonhuman homologues does not imply that no structurally and functionally similar nonhuman homologues exist, it means only that they do not bind the anti-CENP-A antibodies purified using our immunoblotting procedures. This could occur if the recognition epitopes were limited in number and free to evolve rapidly. The existence of a centromere-specific 17-kD protein in Chinese hamster ovary (CHO) cells has been reported (Valdivia and Brinkley, 1985). It is not clear, however, that the reported CHO cell 17-kD protein is the same protein we have studied, since we have never detected anti-CENP-A cross-reactivity with nonhuman species.

An ~80-kD centromere antigen recognized by human autoimmune sera has been reported in HeLa cells (Earnshaw et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985; McNeilage et al., 1986), CHO cells (Valdivia and Brinkley, 1985), and *Leishmania tropica* (McNeilage et al., 1986). Some anticentromere sera we have used (other than G.D. and A.J., which were used for the bulk of the work presented here) have given relatively strong antibody binding to an ~80-kD protein from HeLa cells. Using these same sera to probe immunoblots of rat liver nuclear protein, we do not detect an 80-kD protein, but we do detect an ~50 kD rat protein (Palmer, D. K., K. O'Day, and R. L. Margolis, unpublished data), as reported by Earnshaw and Rothfield (1985). We have not yet attempted to confirm that the 50-kD rat protein is centromere specific, as reported. Earnshaw and Rothfield (1985) showed that affinity-purified anti-CENP-A antibodies from one serum bound to the 80-kD human protein; and that anti-80-kD protein and anti-50-kD protein antibodies from the same serum bound to CENP-A. Although we have not detected similar cross-reactivity, differences in the primary sera used might account for the differences in our results.

The 17-kD centromere autoantigen, while histone-like, is associated with only a minor fraction of genomic DNA. The mechanism by which this restriction is accomplished is unknown, but may involve direct recognition of particular DNA sequences by the 17-kD antigen. Another possibility is sequence recognition mediated by other centromere-specific

autoantigens (Earnshaw et al., 1984; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985) or as yet undiscovered centromere components. In the yeast *Saccharomyces cerevisiae*, phasing of centromere proximal nucleosomes over long stretches involves signals present in the centromere proximal DNA, but the phasing signals are not simple repeating DNA sequences (Bloom and Carbon, 1982). In other studies, Rhodes and Klug (1986) have shown the presence of structural repeats that are important to the binding of the RNA polymerase III transcription factor IIIA, and which are not obvious by casual inspection, in the DNA of the binding site. Therefore, if CENP-A binds directly and specifically to DNA, it is nevertheless possible that the specific recognition sequences will prove to be complex.

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