Fractionation and Initial Characterization of the Kinetochore from Mammalian Metaphase Chromosomes

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ABSTRACT We have partially isolated the kinetochore and associated centromeric structures from mammalian metaphase chromosomes. Human autoantibodies from scleroderma CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) patients were used as immunofluorescent probes to monitor fractionation. The procedure includes digestion of total chromosomal DNA with micrococcal nuclease, dehistonization with heparin, and dissociation of the remaining material with detergent and urea. We used a density gradient (metrizamide) to obtain an enriched fraction of stained material (kinetochore). When examined by electron microscopy, the kinetochore fraction is seen to contain numerous small immunoperoxidase-positive masses which are morphologically similar to the centromere/kinetochore region of intact metaphase chromosomes. The particulate fraction that contains kinetochore components represents <5% of total chromosomal proteins and contains <1% of total DNA. Two polypeptides of 18 and 80 kD were identified as kinetochore antigens by immunoblotting with CREST antiserum. In this paper we discuss the distribution of these kinetochore polypeptides with the associated centromeric chromatin.

The genetic information encoded in interphase chromatin is uniformly distributed to the daughter cells in normal mitosis. One specific chromosomal locus, the kinetochore, is believed to be responsible for chromosome attachment to the mitotic spindle and segregation during mitosis and meiosis. Although the cytology and ultrastructure of the kinetochore are well documented (6, 9, 16), relatively little is known of its biochemical composition.

As shown by ultrastructural studies, the typical kinetochore of metaphase chromosomes consists of a trilamellar disk composed of a dense outer layer, a lightly stained middle layer, and a dense inner layer juxtaposed with the centromeric chromatin. The kinetochore-associated microtubules attach to the outermost layer. Although very little is known of the molecular composition of the kinetochore plates, cytochemical studies have suggested the presence of RNA (30), DNA (28), and tubulin (26).

A major advance in the investigation of kinetochores came with the discovery of human autoantibodies from scleroderma CREST[1] (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) patients that selectively stain centromeres of metaphase chromosomes by indirect immunofluorescence (23). Using immunoperoxidase staining at the electron microscopic level, Brenner et al. (5) discovered that CREST antiserum binds specifically to kinetochore plates. In the present study, we have used CREST antiserum as an immunofluorescent probe to monitor the steps of extraction and fractionation of the kinetochore from metaphase chromosomes and to identify some of its polypeptide components by immunoblotting experiments.

MATERIALS AND METHODS

Cultured Cells and Chromosome Isolation: HeLa cells were grown in McCoy's 5a medium supplemented with 8% fetal calf serum. The cells were synchronized with 2.5 mM thymidine (Sigma Chemical Co., St. Louis, MO) added to the medium for 20 h and then treated with colcemid at 0.06 μg/ml for 16 h. Chinese hamster ovary cells (CHO) were grown in Ham's F-10 medium supplemented with 7% fetal calf serum. In this case, cells were partially synchronized by decreasing the serum in the media to 2% for 14 h

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CREST, calcinosis, Raynaud's phenomenon, esophogeal dysmotility, sclerodactyly, telangiectasia; HMG, high mobility group; MNAse, micrococcal nuclease; TEP, 10 mM Tris, pH 7.1, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride.

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and then incubating for 9 h in colcemid at 0.1 μg/ml in media supplemented with 7% fetal calf serum. The mitotic cells in both cases were collected by shake off and centrifugation. A mitotic index of 98% for HeLa and 50–70% for CHO cells was commonly obtained.

For a typical preparation, metaphase chromosomes were isolated from 10^8 mitotic cells by the hexylene glycol method of Wray and Stubblefield (37). After isolation, the crude chromosome suspension was purified by a 30-ml 10–50% (vol/vol) glycerol discontinuous gradient in hexylene glycol buffer (13). The chromosomes were sedimented through the gradient by centrifugation at 1,000 rpm in a Beckman JS-13 swinging bucket rotor (Beckman Instruments Inc., Palo Alto, CA) for 50 min at 4°C. 3-ml fraction were collected from the top of the gradient. Those containing purified chromosomes as determined by phase-contrast microscopy (fractions 4–7, typically) were then pooled. Purified chromosome suspensions containing glycerol were centrifuged in a Beckman JA-20 rotor for 15 min at 5,000 rpm at 4°C. The chromosomes were stored in hexylene glycol buffer at 4°C.

Protocol for Kinetochore Fractionation: Purified chromosomes (either from HeLa or CHO mitotic cells) in hexylene glycol buffer were centrifuged at 3,000 rpm for 10 min at 4°C and resuspended in nuclease digestion buffer (10 mM Tris, pH 7.1, 2 mM $CaCl_2$ containing 1 mM phenylmethylsulfonyl fluoride). The volume of the chromosome suspension was usually 4–5 ml and the A_{260} was 7–10. To this, micrococcal nuclease (MNAse) Worthington Biochemical Corp., Freehold, NJ) was added at a final concentration of 200 U/ml, and the solution was incubated at 37°C for 60 min with occasional gentle swirling. Later, the suspension was centrifuged in a Beckman JA-20 rotor at 5,000 rpm for 10 min at 4°C, and the supernatant was stored at 4°C. The pellet was resuspended in 10 mM Tris, pH 7.1, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (TEP buffer). Heparin (Sigma Chemical Co.) at 2 mg/ml was added, and the solution was incubated at 4°C for 120 min with constant shaking. The suspension was centrifuged at 5,000 rpm for 15 min at 4°C, and the supernatant was kept at 4°C. After this spin the pellet was resuspended in TEP buffer. Nonidet P-40 (BDH Chemicals, Poole, U.K.) plus cholate (Sigma Chemical Co.) both at a final concentration of 1% were added, and the suspension was incubated for 60 min at 4°C with constant shaking. The suspension was then centrifuged as above. The supernatant was stored at 4°C and the pellet was resuspended in TEP buffer and incubated for 10 min at 4°C in the presence of 2 M urea. The suspension was then centrifuged at 10,000 rpm for 15 min at 4°C. The preparation was layered onto a 3 ml 10–50% (wt/vol) metrizamide (Nyegaard Co., Oslo) gradient in TEP buffer and centrifuged at 4°C for 15 min at 8,000 rpm in a Beckman JS-13 swinging bucket rotor. 0.3-ml fractions were collected from the top and checked for kinetochore staining by immunofluorescence and electron microscopy.

Immunofluorescence and Electron Microscopy: The different steps in the protocol for kinetochore isolation were monitored by centrifuging aliquots onto microscope coverslips coated with poly-L-lysine (Sigma Chemical Co.) at 1 mg/ml and staining first with kinetochore antiserum and then with anti-human IgG labeled with fluorescein isothiocyanate. Sometimes the preparations were also stained with the DNA-binding fluorochrome Hoechst 33258 (American Hoechst Corp., Somerville, NJ) or propidium iodide for visualization of DNA. The immunofluorescence procedure was similar to that described by Pepper and Brinkley (27). Whole chromosomes and aliquots at each step of the kinetochore fractionation procedure were processed for electron microscopy by the immunoperoxidase technique as described by Brenner et al. (5).

Electrophoresis and Immunoblots: Electrophoresis was performed using the buffer system of Laemmli (18). Gels were stained with Coomassie Blue R-250. Proteins from SDS gels were transferred to nitrocellulose paper in 15 mM Tris, pH 8.3, 150 mM glycine, 20% methanol as previously described (35), containing 0.1% SDS. Nitrocellulose strips containing sample proteins were incubated for 3 h at room temperature in Tris-saline (10 mM Tris, pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin (wt/vol) and 5% fetal calf serum. Later, human anti-kinetochore IgG (purified by Affil blue DEAE, Bio-Rad Laboratories, Richmond, CA) was added at dilutions indicated in the figure legends, and incubation was resumed overnight at 4°C. Then the paper was rinsed five times in 50 min with Tris-saline containing 0.05% Tween at room temperature. The nitrocellulose paper was then incubated with either peroxidase-conjugated goat anti-human IgG (Miles Laboratories, Inc., Elkhart, IN) for 2 h at 37°C in Tris-saline containing 0.05% Tween or with ^{125}I-goat anti-human IgG (see figure legends). After being washed as described above, the paper was either incubated for 30 min at a solution of 4 chloro-1-naphthol (30 mg) in Tris saline-methanol-H_2O_2 (50 ml/10 ml/20 μl) and the reaction stopped by rinsing in double-distilled H_2O, or it was used to autoradiographically expose Kodak X-Omat S film.

Protein concentration was quantified from each step of fractionation (supernatants and pellets) by the Lowry method (20). In the case of supernatants, when the concentration of some reagent (such as urea and detergent) was high enough to interfere with the determination, the sample was diluted and the concentration was corrected by the dilution factor. For pellets, the samples were dissolved in 2% SDS and diluted, and protein concentration was determined. The DNA present in each fraction was extracted with phenol plus chloroform, and precipitated with ethanol. DNAs dissolved in Tris-EDTA were quantified by optical density at 260 nm.

RESULTS

Kinetochore Fractionation

Recent electron microscopic examination of whole mount preparations of isolated chromosomes have demonstrated the higher structural stability of kinetochores and centromeric chromatin. Both entities are more resistant than ordinary chromatin to digestion with DNAse (29), hypotonic solutions (21), trypsin, EDTA or NaOH (10), and glycerol or toluene (25). By using human autoantibodies as immunofluorescent probes, it is feasible to determine whether the kinetochore antigen is still associated with the chromosomal material remaining after various digestions. As a result of different pilot experiments with nucleases, proteases, detergents, pH, various ionic strengths, etc., we were able to develop a protocol to produce a chromosomal fraction highly enriched in kinetochore material from HeLa and CHO metaphase chromosomes. The fractionation procedure shown in Fig. 1 begins with metaphase chromosomes isolated in hexylene glycol buffer (37) from cultured cells synchronized in interphase

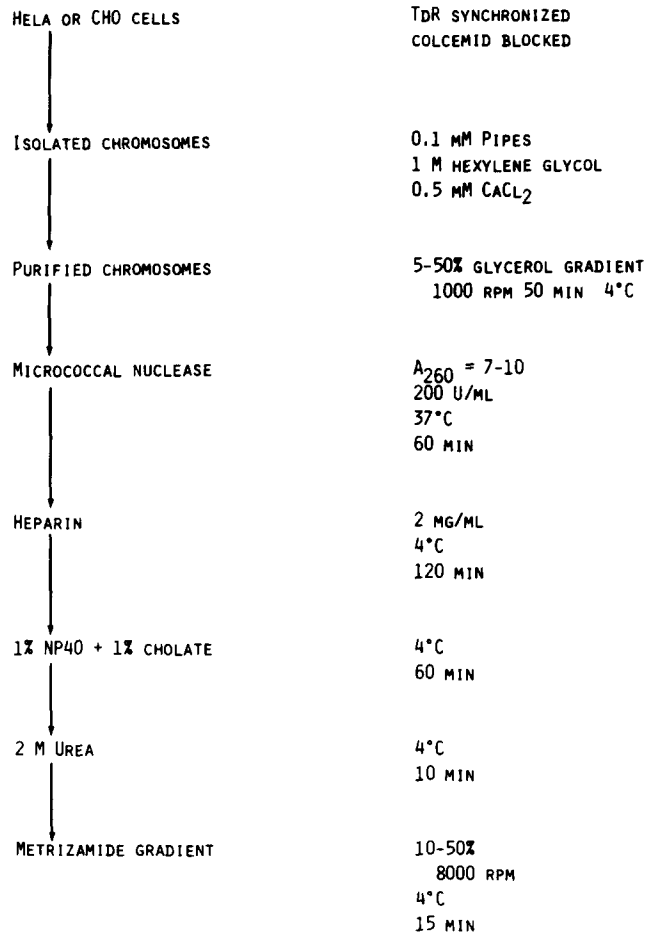

FIGURE 1 Flow diagram showing the fractionation of kinetochores from mammalian metaphase chromosomes. The details of the subchromosomal fractionation are as described in Materials and Methods. NP40, Nonidet P-40. TDR, thymidine.

with high concentrations of thymidine and a subsequent colcemid block in mitosis. Low-speed glycerol gradient centrifugation is used to free chromosomes of soluble cytoplasmic components and more rapidly sedimenting material such as nuclei, aggregated chromosomes, etc. After purification, chromosomes are extensively digested with MNAse to render mononucleosome-size DNA (data not shown). Later, heparin extraction is carried out essentially as described by Bornens (4) to release all histones and reveal chromosome scaffolds or cores. The remaining pellet is further treated with a mixture of Nonidet P-40 and cholate (nonionic and ionic detergents, respectively) to release aggregates of dehistonized chromosomes and remove nonhistone proteins resistant to heparin treatment. Finally, a brief treatment with 2 M urea is used to dissociate the remaining chromosomal structures that contain kinetochore material. This last material that reacts with a specific antiserum for kinetochore plates (5) is further layered onto a metrizamide density gradient to enrich for stained material from other nonstaining chromatin structures and aggregates.

The serum from scleroderma CREST patients (the probe used in this assay) binds specifically to the centromere region of the chromosome and is visible by immunofluorescence (Fig. 2*a*) or immunoperoxidase (Fig. 2*b*) as paired structures at the primary constriction of every chromosome. Brenner et al. (5) have demonstrated the specificity of the sera for the

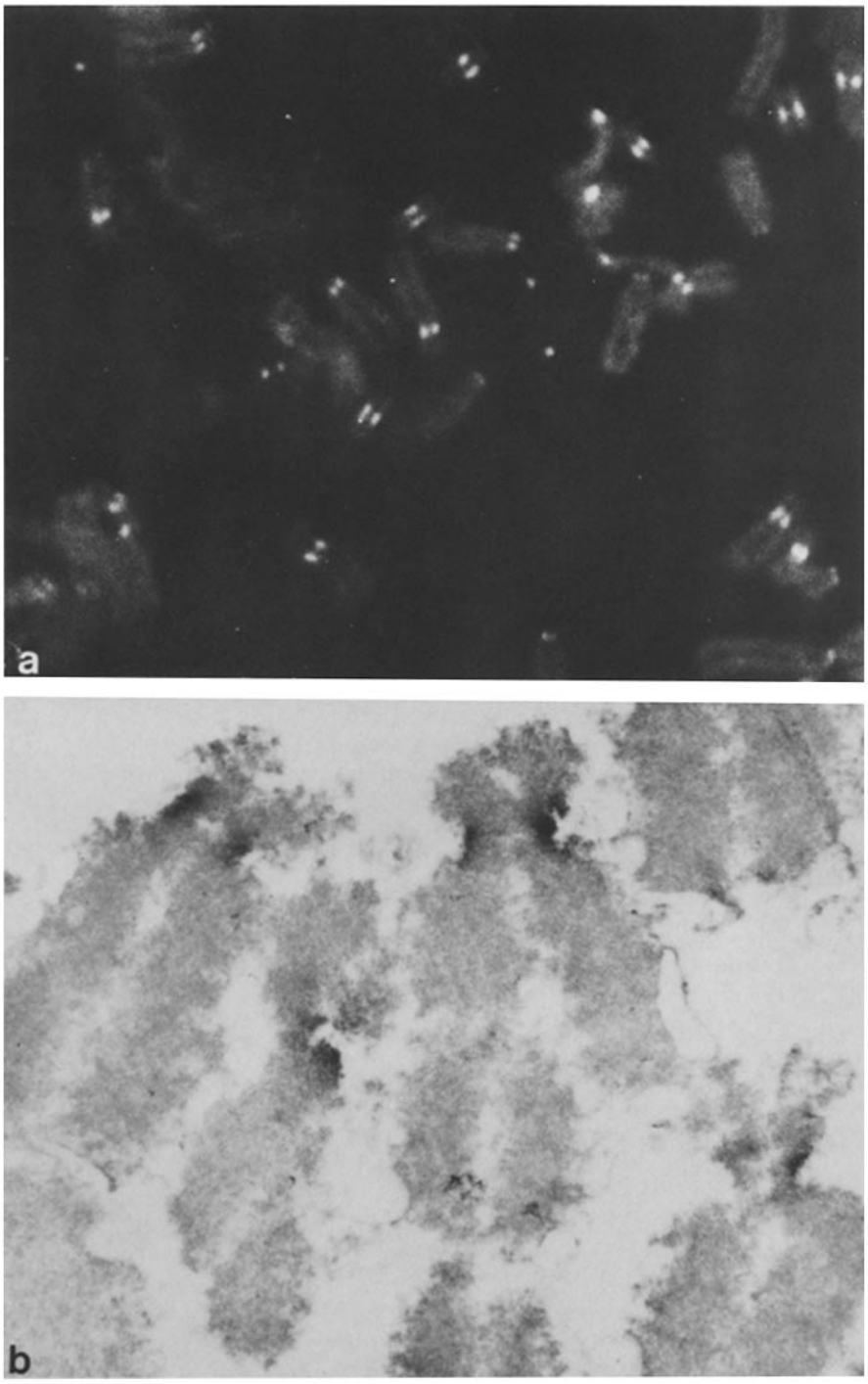

FIGURE 2 (*a*) Indirect immunofluorescence of CHO metaphase chromosomes with human kinetochore antiserum. Dilution of antiserum used was 1:500. × 3,200. (*b*) Electron micrograph of similar preparation stained with immunoperoxidase. × 13,000.

trilaminar kinetochore disks on metaphase chromosomes, and we confirm their findings in this study. When intact chromosomes are digested under different conditions, DNA, histones, and most nonhistone proteins are released, but the remaining material still reacts strongly with the CREST antisera. The results of our fractionation are shown in different steps in Figs. 3 and 4. Each kinetochore is seen as a discrete darkly stained mass on chromatin in early stages of fractionation (Fig. 3, *a–d*). After urea treatment, the kinetochore can still be detected (Fig. 4, *a* and *c*), but the size and shape of stained regions vary considerably, which suggests that there is probably an alteration of kinetochore morphology. We used different urea concentrations and times of digestion and found that some material still reacts with the CREST antisera even after 1 h at 4°C in the presence of 4 M urea. These results demonstrate a pronounced structural stability of some kinetochore components. Several controls shown in Fig. 4, *b* and *d* demonstrate the specificity of the staining reaction. When either nonkinetochore human autoantibodies (e.g., anticentrosome) or nonimmune sera is used as the first antibody, staining is not detected on the final chromosomal material by immunofluorescence or immunoperoxidase. After the urea treatment, a metrizamide gradient is used to enrich for free kinetochore/centromere regions, which appear densely stained by immunoperoxidase. The result of this gradient is shown in Fig. 5. The amount of nonstaining chromosomal material accompanying kinetochores in various fractions of the gradient is small (compare staining in Fig. 3, *c* and *d*, with that in Fig. 5). Examination of the final pellet by immunoelectron microscopy reveals the kinetochores as either electron dense masses or thin plates relatively free of other associated chromosomal material (inset, Fig. 5).

Biochemical Studies

By quantitative analysis, we have determined the amount of protein and DNA present in our most enriched fractions after the metrizamide gradient. Table I shows a quantification of a typical preparation. More than 50% of total chromosomal protein is solubilized after the MNAse and heparin treatment. This is not surprising since it is well known that both treatments together release most of the DNA and histones from the chromatin (4, 15). The final pellet after urea treatment contains only 10% of the total proteins. After sedimentation on the metrizamide gradient, the fractions exhibit free kinetochores, which represent <4% of total chromosomal protein and <1% total DNA (data not shown). A small amount of RNA is also present in the final material. Analysis by one-dimensional SDS gel electrophoresis shows that the kinetochore fraction contains very little, if any, histones (Fig. 7, *f* and *g*). Although this could be an indication that histones are absent from the kinetochore, it is equally likely that histones are present but unnecessary for the staining of kinetochores with CREST antiserum. Similar polypeptide composition of purified kinetochores from both HeLa and CHO metaphase chromosomes was observed. In an earlier article (36), we reported similar polypeptides by two-dimensional gel electrophoresis. The major protein in both was actin (42 kD), which has been previously reported in whole chromosomes (32) and the matrix (8), and more recently chromosomal actin has been reported to be involved in transcriptional events of lampbrush chromosomes (33). Nevertheless, it is difficult to rule out the possibility that actin is a contaminating protein from the cytoplasm. Other major polypeptides of 50–55, 34–40, and 18 kD were present on both HeLa and CHO fractions. We suggest that some of these proteins are constituents of kinetochores that have been enriched through the fractionation, but final proof must await the production of antibodies for their specific localization on kinetochores.

By using sera from different patients with scleroderma CREST, we have characterized the antigens of the kinetochore. As shown in Fig. 6, the Western transfer technique is a useful approach to differentiate between kinetochore specific antisera and other nonkinetochore human autoantibodies. Immunoblots of isolated HeLa chromosomes suggest that the antigens recognized by kinetochore antisera (Fig. 6, lanes *M*, *E*, and *J*) are two polypeptides of 18 and 80 kD. Some nonspecific binding to histones is suggested by the fact that another human serum (reactive with centrosomes by immunofluorescence and not with kinetochores) reacts with histones but not with the 18-kD protein, as shown in Fig. 6*C*, lane *P*). A control with a second antibody (Fig. 6*A*, lane *C*) and with nonimmune serum (Fig. 6*A*, lane *N*) depicts the specificity of this method.

Are the two antigens present in our final kinetochore-enriched material, or are they associated differently with the centromere? To answer to this question, we assayed each step of our fractionation procedure by the Western transfer technique using kinetochore antiserum. In the left panel of Fig. 7, the 80-kD antigen is associated with the final kinetochore material and is partially resistant to 1 and 2 M urea digestion. The CREST antiserum recognizes the 80-kD polypeptide in both supernatant and pellet after urea treatment. However, in most of our preparations the 18-kD polypeptide is present in the supernatant after treatment with heparin (very faint on this gel), which suggests that this antigen is less resistant to the treatment with MNase and heparin. In some preparations, we found a small amount of the 18-kD polypeptide in the pellet after 2 M urea (data not shown). Fig. 7 suggests that the staining found in the final kinetochore material is mostly due to the 80-kD polypeptide and not to the 18-kD polypeptide. Other antigens present perhaps in smaller amounts than the 18- and 80-kD could also be targets for kinetochore antisera. Since these antigens might be less reactive with CREST antiserum after denaturation with urea, SDS, etc., they would therefore go undetected on the Western method. Finally, we have found by immunofluorescence and electron microscopy that our method for fractionating centromeres/kinetochores works well for CHO and HeLa chromosomes, which indicates that the relative resistance of kinetochores to harsh treatments is probably a general characteristic of any

TABLE I. *Quantification of a Typical Chromosomal Preparation*

Fraction	Total protein	Percent of total chromosomal proteins
	mg	
Whole chromosomes	92.00	100.0
MNAse supernatant	5.25	5.7
Heparin supernatant	45.75	49.7
Detergent supernatant	22.20	24.1
Urea supernatant	9.00	9.7
Urea pellet	10.00	10.8
Metrizamide fractions	3.20	3.4

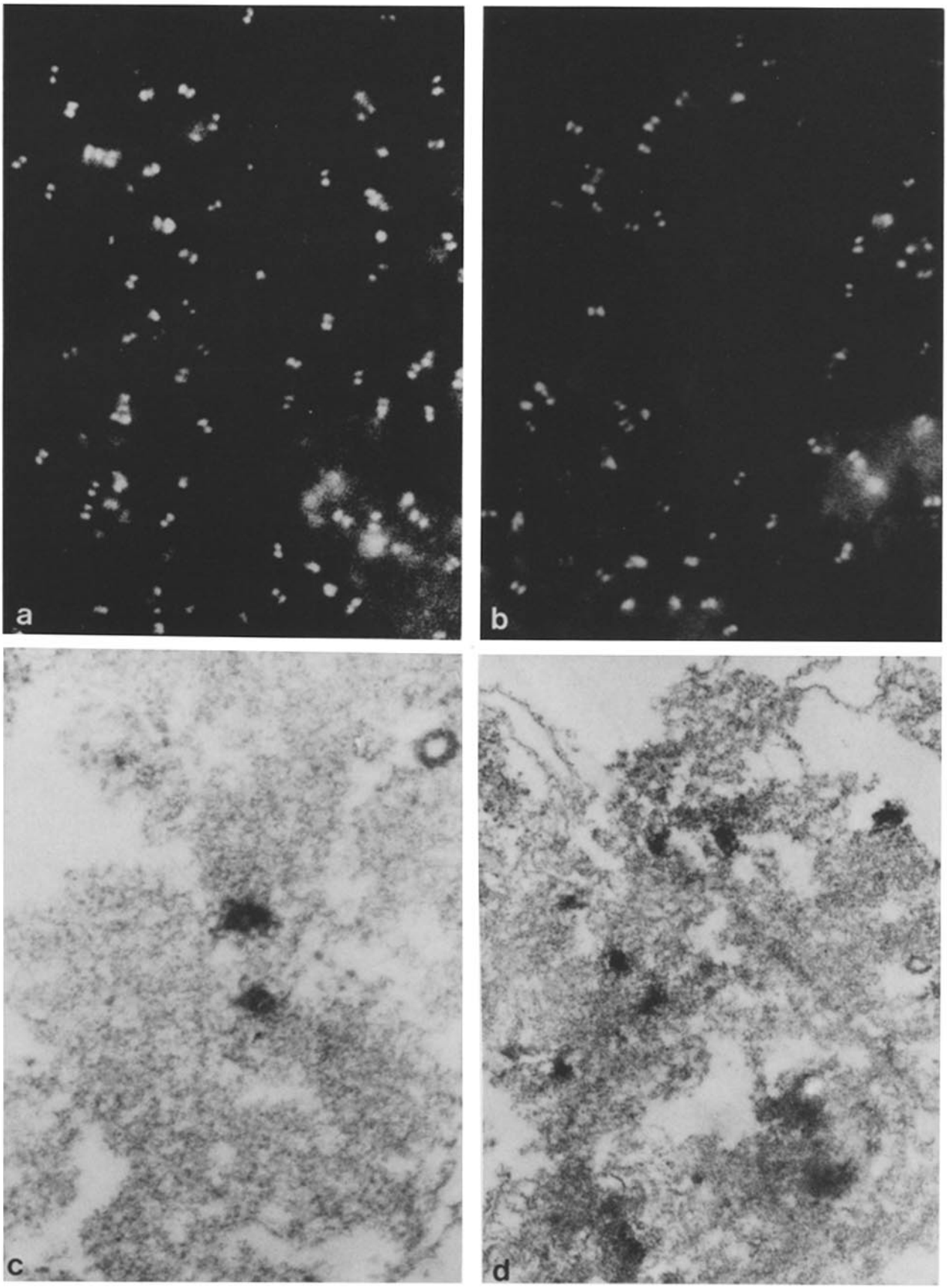

FIGURE 3 Indirect immunofluorescence (*a* and *b*) and immunoperoxidase electron microscopy (*c* and *d*) of CHO metaphase chromosomes after digestion with MNAse and heparin (*a* and *c*) and MNAse heparin and detergents (*b* and *d*). In both cases human kinetochore antiserum at 1:500 dilution was used as the first antibody. (*a*) × 3,000. (*b*) × 3,100. (*c*) × 34,600. (*d*) × 24,200.

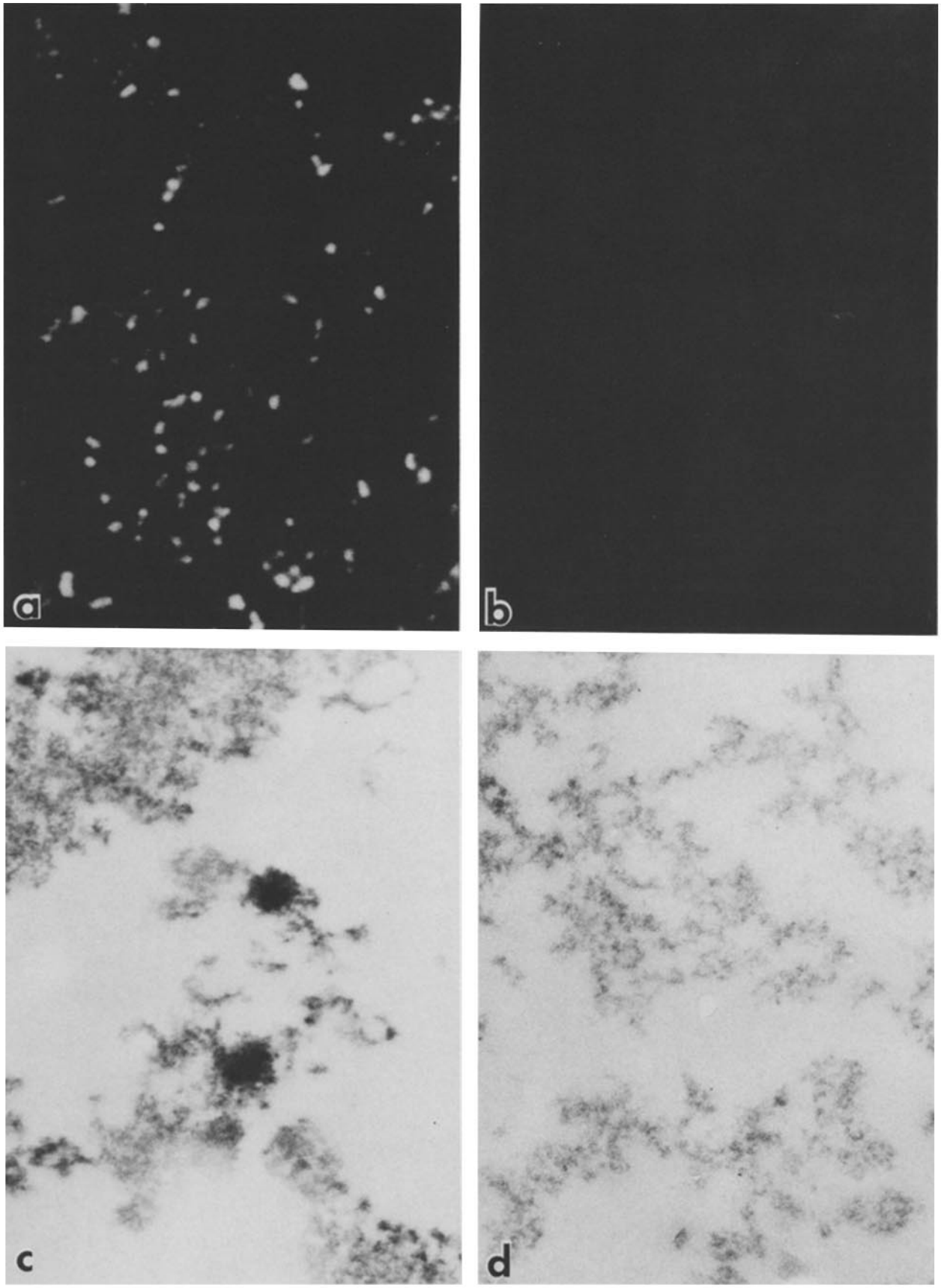

FIGURE 4 Indirect immunofluorescence (*a* and *b*) and immunoperoxidase electron microscopy (*c* and *d*) of CHO metaphase chromosomes after digestion with MNAse, heparin, detergents, and urea as described in Materials and Methods. Human kinetochore antiserum was used in *a* and *c* but omitted from the controls in *b* and *d*. (*a*) × 3,800. (*c* and *d*) × 55,000.

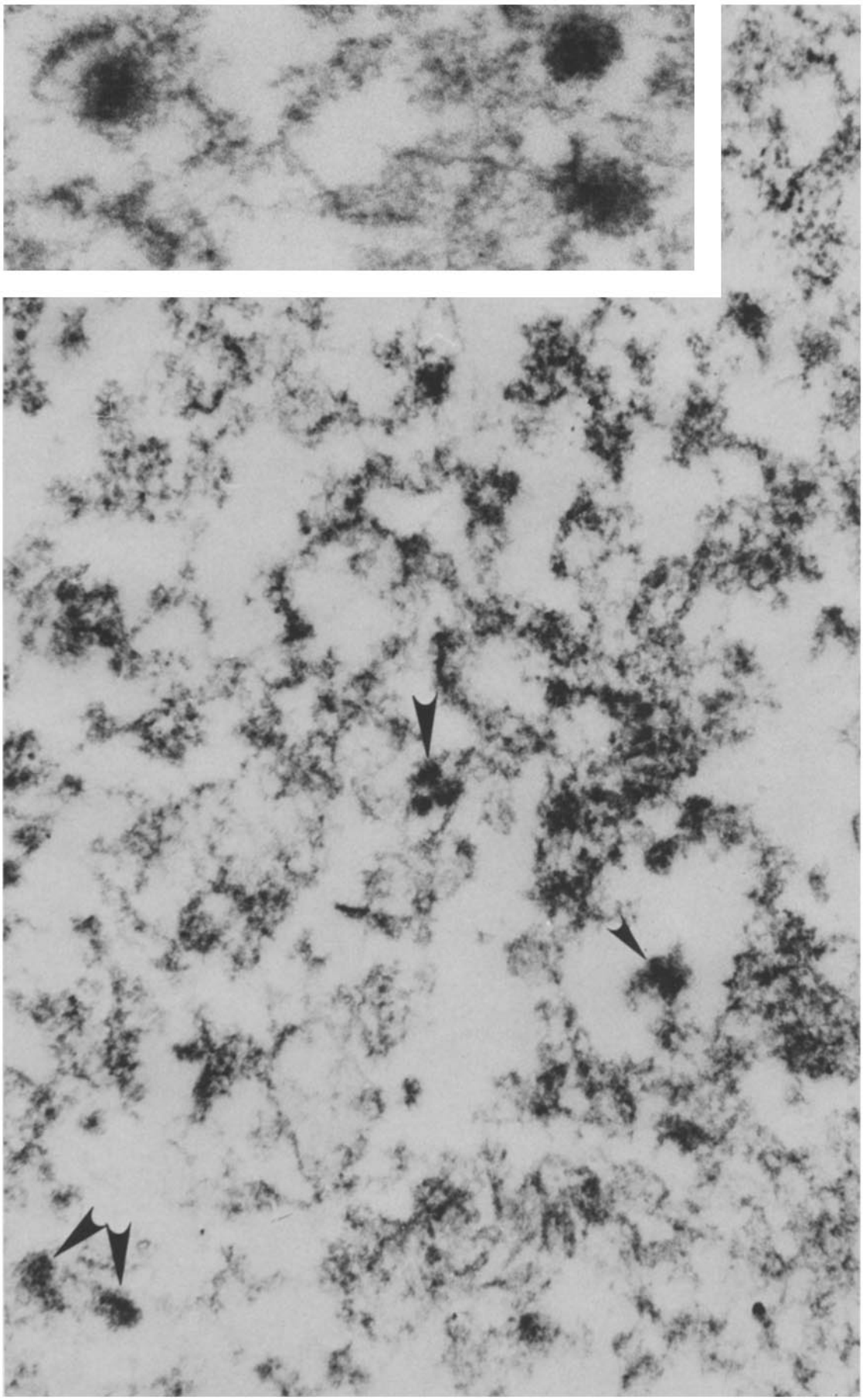

FIGURE 5 Immunoperoxidase electron microscopy of purified kinetochores from CHO metaphase chromosomes after sedimentation on a metrizamide gradient as described in Materials and Methods. HeLa kinetochore staining is identical (not shown). Arrowheads indicate stained kinetochores. × 24,400. The inset shows isolated kinetochores at higher magnification (80,000).

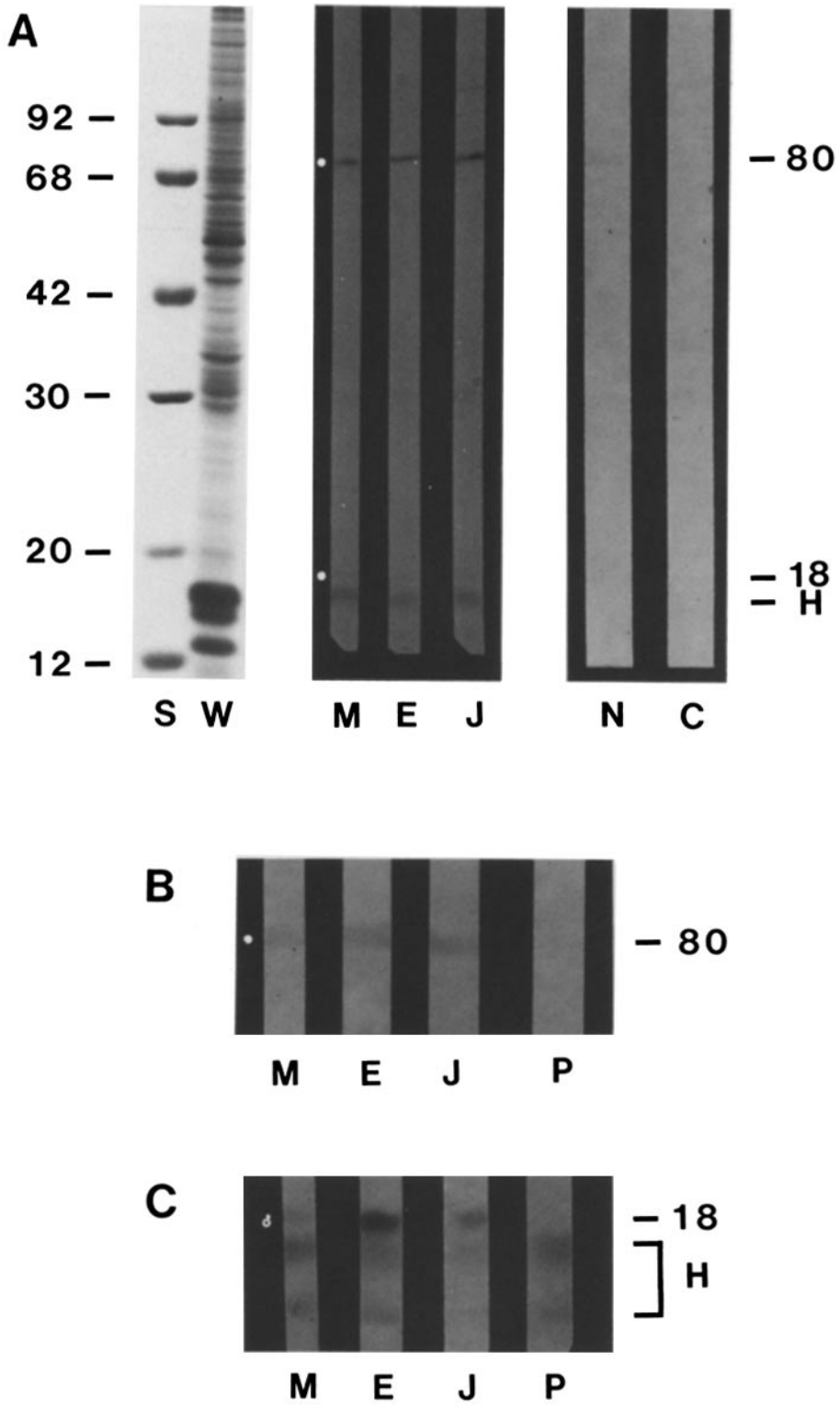

FIGURE 6 Immunochemical characterization of kinetochore antigens using human CREST antisera. A total of three kinetochore antisera were used (lanes *M*, *E*, and *J*) (*A*) HeLa chromosomal proteins were electrophoresed and stained with Coomassie Blue in lane *W*. Control tests for the immunoblots were performed with nonimmune human serum (lane *N*) and the removal of first antibody (lane *C*). Each of the three kinetochore antisera reacts with an 80- and an 18-kD antigen. Some nonspecific binding was observed with core histones (band *H*) Lane *S*, positions of standard protein electrophoresed in the same gel. (*B*) A nonkinetochore scleroderma antiserum (anticentrosome, diluted 1:500) was used as a control (lane *P*) for the 80-kD polypeptide. All three kinetochore antisera (lanes *M*, *E*, and *J*) were used at a 1:2,000 dilution. (*C*) The kinetochore antisera react with an 18-kD polypeptide which runs in this SDS PAGE gel ahead of core histones. The centrosome antiserum does not react with the 18-kD protein but does with the histone region. The second antibody used was peroxidase-conjugated anti-human IgG (Miles Laboratories Inc.).

species of chromosome. We have been attempting to learn if the 80-kD polypeptide is responsible for the staining in our final fraction in both HeLa and HCO cell lines. Fig. 8 indicates that this is the case. The final material contains on 80-kD antigen reactive with the CREST antiserum (see Figs. 7 and 8). However, the 18-kD polypeptide in both cell lines is revealed as a chromatin-associated protein released by treatment with MNAse and heparin.

DISCUSSION

In this study, we have developed a procedure for preparing kinetochores/centromeres from mammalian metaphase chromosomes. Sequential extraction of isolated chromosomes yields this region with minor contamination from other chromosomal structures, probably scaffold-related components (12). Scaffold contamination will be hard to avoid due perhaps to the integral character of kinetochores with scaffold, as Earnshaw et al. (12) have proposed. Our fractionation procedure relies on the relative structural resistance of kinetochores to strong dissociating conditions (nuclease, heparin, detergents, urea). Even in the absence of surrounding chromatin (<1% total chromosomal DNA and the absence of histones

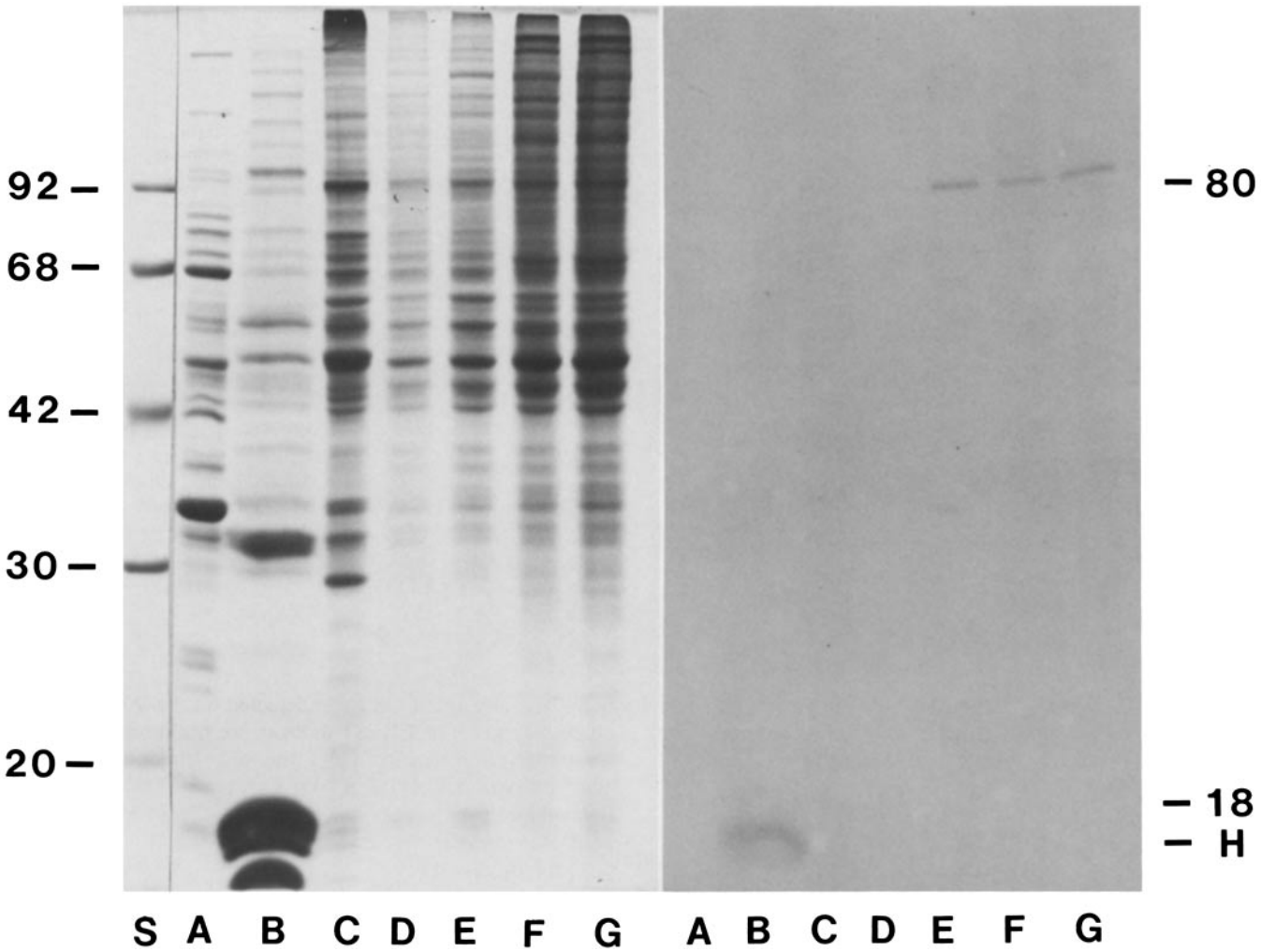

FIGURE 7 SDS PAGE analysis of HeLa metaphase chromosomal fractions and immunoblots using kinetochore antiserum. Lanes: *A*, material released in the supernatant by MNAse treatment; *B*, the supernatant after heparin treatment (note the presence of core histones and histone H_1); *C*, the supernatant polypeptides released by detergent treatment; *D*, the polypeptides released by 1 M urea; *E*, the polypeptides released by 2 M urea; *F*, the remaining material insoluble after 1 M urea treatment; and *G*, the remaining material insoluble after 2 M urea. (This material was layered on a metrizamide gradient to enrich for the kinetochore material shown in Fig. 5.) Metrizamide fractions demonstrate identical electrophoretic and immunoblot patterns as urea-denatured ones (data not shown). Kinetochore antiserum (dilution 1:1,000) reacts slightly with two polypeptides of 18 and 80 kD, respectively (*right*). Some nonspecific binding with histones was also present due to the high amount loaded of these proteins. Positions of standard protein electrophoresed on the same gel are indicated in lanes *S*. The second antibody used was peroxidase-conjugated anti-human IgG (Miles Laboratories Inc.) diluted 1:1,000. *H*, core histones.

on SDS gels) the kinetochore region strongly reacts with the CREST antisera. Examination by immunofluorescence and immunoelectron microscopy suggests that the kinetochores are preserved and identifiable throughout the isolation until the urea step. This is indicated by the presence of paired dots at both sides of the centromere region after treatment with MNAse heparin, and detergent. However, after urea, this "paired dots" organization is lost. Adolph et al. (1) previously reported a similar disorganization of the scaffold region after urea treatment.

The fact that the final kinetochore-enriched material strongly reacts with the CREST antisera suggests that a considerable amount of the antigen is still present. Since other proteins (including histones) are not present in the final material (compare whole chromosomal proteins with final pellet in Fig. 7), and since the Western transfer results indicate that there is no release of the 80-kD polypeptide through the purification, we can state that some kinetochore components are truly enriched in the final material. Supporting this, we have been unable to detect the 80-kD polypeptide in intact chromosomes, which suggests that it represents a small percentage of total chromosomal protein, but the polypeptide is detected by the immunoblotting technique for our fractionated material. The ability of kinetochores to assemble or associate with microtubules would be an ideal assay to determine if they are functional in various stages of purification. Unfortunately, the ability of kinetochores to organize microtubules in vitro has been difficult to demonstrate (2, 27, 34). We, too, have been unable to induce microtubule association with isolated chromosomes in the presence of pure brain tubulin. Therefore, the functional capacity of our kinetochore preparation remains untested.

However, we have biochemically validated our procedure for fractionating centromeres/kinetochores. The fractionation

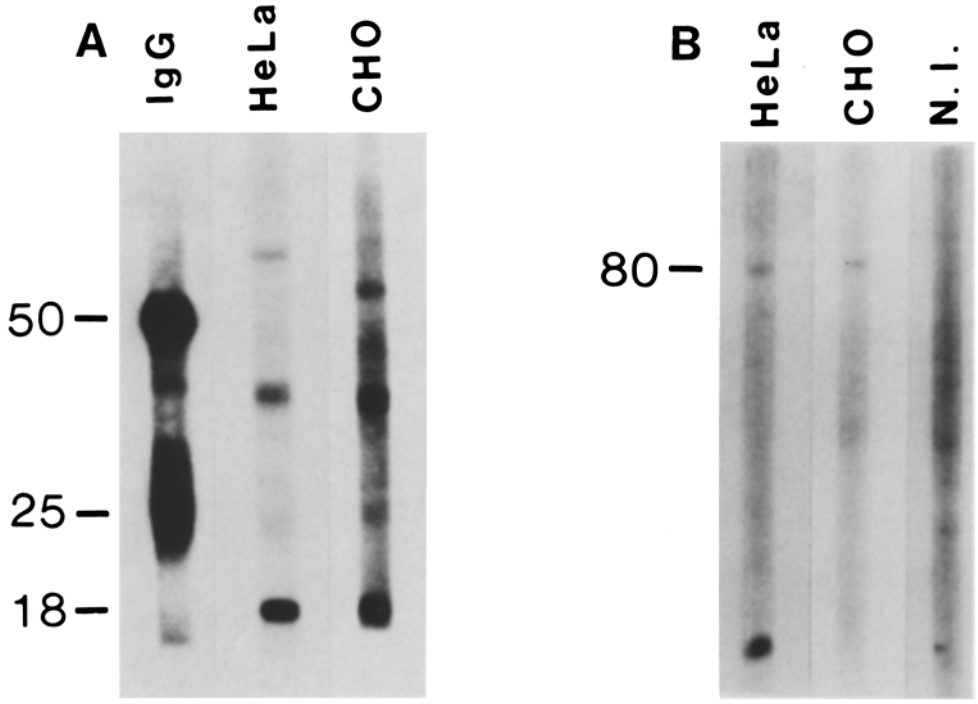

FIGURE 8 Autoradiographic immunoblot identification of HeLa and CHO kinetochore antigens by CREST antiserum (diluted 1:500) and ^{125}I-anti-human IgG. *A*, immunoblots of HeLa and CHO supernatants after MNAse and heparin extraction. Lane *IgG*, a molecular weight standard using iodinated IgG. *B*, immunoblots for HeLa and CHO of the final material after sedimentation on a metrizamide gradient. Lane *N.I.*, a control trial using nonimmune human serum (Miles Laboratories Inc.) as the first antibody.

yields kinetochore material in acceptable quantities (5% yield from 10^8 cells). As compared with whole chromosomes, our final fraction consists of only 8% total chromosomal proteins, <1% of total DNA, and only traces of RNA.

More important, we have been able to identify two polypeptides as antigens recognized by two different human autoantibodies specific for kinetochores. One of these antigens, an 18-kD polypeptide, seems more closely associated with centromeric chromatin because it is released together with histones and DNA after heparin and nuclease digestion. Therefore, the 18-kD polypeptide may be associated with nucleosomes of centromeric chromatin.

The other antigen, an 80-kD polypeptide, is more tightly bound to the material highly resistant to all treatments, even when the chromosomal scaffold is dissolved by 2 M urea. These data suggest that even if the centromere is the part of the chromosome most resistant to nuclease digestion and dehistonization, some of its components are more labile than others. The 80-kD protein is probably the antigen responsible for the staining observed in the last step of our fractionation (Fig. 8).

Several researchers have recently used human autoantibodies produced by CREST patients to identify kinetochore polypeptides. Cox et al. (11) suggested that a kinetochore antiserum reacts with polypeptides of 14, 20, 23, and 34 kD in immunoblots and concluded that these polypeptides may be responsible for binding microtubules. Earnshaw et al. (12) found that antisera from nine CREST patients react with polypeptides of 77 and 114 kD. Guldner et al. (14) demonstrated that 18 different CREST antisera react with a 19.5-kD nonhistone chromosomal polypeptide from HeLa cells. Like our 18-kD polypeptide, this polypeptide runs on SDS PAGE gels close to core histones and probably represents the same protein. There are several possible explanations for the obvious discrepancy between these reports. Each research group used different antisera with different titers. Antisera from unique patients could stain the kinetochore yet recognize different antigens. Furthermore, with different titers of antibody, some bands could go undetected on the Western blot. Of course, since the CREST antisera are not monospecific, it is also possible that some of the polypeptides shown by Western blots are not from kinetochores. We believe that CREST antiserum recognizes the kinetochore in its native configuration and that after the effect of SDS, most of its antigenicity is lost. However, the results from various laboratories suggest that some kinetochore determinants are reactive with CREST antiserum as shown by the Western transfer technique (11, 12, 14, 36). If so, the experimental variability in maintaining kinetochore antigenicity can further explain the discrepancies among various groups and indicate the importance of the conditions under which immunoblots are performed (e.g., in the presence of SDS).

Several reports have suggested that the chromatin structure in centromeric region differs from noncentromeric chromatin. Bloom et al. (3) examined centromeric chromatin of chromosomes III and XI in yeast by using cloned centromeric DNAs as labeled probes. They found that the centromeric nucleosomal subunits are resolved into more distinct ladders than are those from the bulk of the chromatin. Centrometric nucleosomes have a discrete protected region of 220–250 base pairs of DNA instead of 160 base pairs of the flanking nucleosomal chromatin. They suggest that the centromere region was protected from the micrococcal nuclease digestion by specific proteins. In another report Musich et al. (24) reported that the chromatin in a fraction of highly repetitive DNA (satellite DNA) of the African green monkey had an altered configuration of protein–DNA complexes. They suggested that perhaps tightly bound proteins determine the organization of highly condensed satellite heterochromatin and render this chromatin less susceptible to nuclease treatment. In a report on the structure of mammalian kinetochores, Ris and Witt (31) suggested that the outer kinetochore layer represents a different arrangement of chromatin continuous with the body of the chromosome and probably does not contain typical nucleosomes.

The 18-kD polypeptide runs in SDS gels ahead of core

histones, similar to high mobility group (HMG) proteins 14 and 17. These two proteins, although associated with active genes, have also been localized in inactive, highly repetitive heterochromatin from centromeric DNA (19) and satellite DNA (22). In addition, HMG 17 is localized in the linker region where MNAse preferentially cuts. In line with this, the 18-kD polypeptide is released after nuclease and heparin treatment (see Figs. 7 and 8). The HMG proteins are absent from mature sperm (17), surprisingly the only mammalian cells that we have been unable to stain with antikinetochore antiserum. Whether the 18-kD polypeptide is related to or represents an HMG-like protein associated with a specific DNA sequence on centromeric chromatin awaits careful investigation. It is interesting that Bustin et al. (7), by using antisera from autoimmune diseases, have found anti-HMG 17 in several forms of scleroderma. They concluded that HMG-17 is found only on a subset of nucleosomes and that one of the immunogens in systemic lupus erythematosus may be a subset of those nucleosomes that contain specialized DNA sequences. This may also be the case for the origin of CREST kinetochore antiserum.

We do not yet know the molecular mechanism by which centromeric chromatin is, if it is, genetically inactivated. If the kinetochore also contains dormant DNA, it could be important not only for cell biologists who study microtubule binding sites, but also for molecular biologists who study nonexpressed regions of the genome.

Our kinetochore-enriched fraction may provide a unique opportunity for obtaining immunological probes and further identifying the molecular components of the kinetochore plates.

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