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Kinetochore Components Recognized by Human Autoantibodies Are Present on Mononucleosomes

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We have developed a competitive enzyme-linked immunosorbent assay for solubilized kinetochore components, using human CREST (calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia) scleroderma autoimmune antibodies specific for these kinetochore elements. Using this quantitative assay, we found interphase persistent or "pre-kinetochore" components in low- and moderately high-salt (375 mM salt) extracts of micrococcal nuclease-digested rat liver and chicken erythrocyte nuclei. The release of antigen activity from nuclei under these conditions has been correlated with loss of pre-kinetochore foci as determined by immunofluorescence microscopy. Combined biochemical and competition assay analysis of chicken erythrocyte nuclear extracts indicates that pre-kinetochore components are tightly bound to chromatin of mononucleosome size. The conclusions based on competition assay data are supported by a direct binding assay, which confirms that antigens recognized by CREST sera are present on chromatin. These results raise the possibility that the kinetochore-specific chromosomal antigen(s) we have detected substitutes for "standard" mononucleosome components, such as histone H1. Furthermore, they suggest approaches to the isolation of kinetochore-specific DNA sequences from higher eucaryotes.

Kinetochores are organelles that serve as the attachment points connecting chromosomes to the mitotic apparatus. There is one kinetochore per chromatid. Through the action of spindle mechanics on kinetochores, chromosomes are first aligned on the metaphase plate and then segregated toward the spindle poles in anaphase.

In higher eucaryotes, kinetochores differentiate from diffuse globular structures present in prophase cells to become trilaminar plaques located at the primary constriction of metaphase chromosomes (reviewed in reference 32). A fully differentiated kinetochore is composed of 10.0-nm fibers resembling the bulk of chromatin (6, 34). These fibers may loop back and forth in a highly ordered manner to define the inner and outer layers of the trilaminar plaque (34). Fully differentiated kinetochores either attach to or seed the assembly of a morphologically and biochemically distinct subset of spindle microtubules that is essential for normal chromosome movement (reviewed in references 24, 27, and 30). Chromosomal microtubules are attached perpendicularly and with uniform polarity to the outermost layer of the kinetochore so that their favored assembly ends, as defined by in vitro assay, are all bound to the structure (10, 41).

Recently, Moroi et al. (26) demonstrated that victims of the CREST (calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia) variant of the autoimmune disease scleroderma frequently carry circulating antibodies specific for centromeres. A subsequent study by Brenner et al. (5) localized the binding of anticentromere antibodies in CREST sera to the inner and outer plates of mitotic kinetochores from PtK2 cells. In addition to binding mitotic chromosome kinetochores, antibodies in scleroderma anticentromere sera also bind to small spherical organelles in interphase nuclei. The number of these organelles is consistent with the number of chromosomes within the cell, and the organelles replicate late in the G2 phase of the cell cycle to form doublet foci of indirect immunofluorescent or immunoenzyme staining (5, 11, 25). Since these organelles presumably represent interphase persistent kinetochore components, they have been termed "presumptive kinetochores" or "pre-kinetochores" (5).

It is reasonable to expect that an understanding of the molecular organization of pre-kinetochores will help to answer the many fundamental questions that can be posed concerning higher eucaryotic kinetochore structure and function. These include inquiry into the nature of the underlying chromatin, the mechanism of the reorganization that results in a functional mitotic organelle, and the nature of the interaction of the organelle with microtubules. Accordingly, we have begun an analysis of the structure of pre-kinetochores in higher eucaryotes. Central to this analysis is an immune competition assay that exploits kinetochore-specific CREST scleroderma sera to quantitate soluble antigens. We report here methods for solubilization and assay of interphase persistent kinetochore components, and demonstrate that the solubilized components are tightly bound to chromatin, including mononucleosomes.

MATERIALS AND METHODS

Buffers. Three buffer systems were employed in these studies: buffer A, phosphate-buffered saline (PBS), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-EDTA. The buffer A system was used for the isolation of nuclei, micrococcal nuclease digestion of nuclei, lysis of digested nuclei, application of primary antibodies for immunofluorescence studies, and application of primary antibodies in the solid-phase immunological competition assay of kinetochore antigen. PBS was used for the application of secondary antibodies and washing of solid-phase wells. HEPES-EDTA was used for the low-ionic-strength lysis of nuclei and buffering of sucrose gradients. Buffer A contained 0.22 M sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 1.0 mM EDTA, 0.2 mM EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic

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acid] and 15 mM Tris-hydrochloride (pH 7.4) (15). In addition to the components listed above, various modified forms of this buffer contained an additional 0.2 mM phenylmethylsulfonyl fluoride and 15 mM β-mercaptoethanol (A'), 0.3 M NaCl (A–0.3 M NaCl), 0.475 M NaCl (A–0.475 M NaCl), 0.3 M NaCl, and 0.1% bovine serum albumin (BSA) (A–0.3 M NaCl–BSA), or other components as indicated in the text. PBS was 136 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4, and 1.5 mM H_2PO_4 (pH 7.5). PBS was modified to contain 0.1% BSA and 0.05% Tween 20 (PBS-BSA-Tween), or other components as indicated. HEPES-EDTA was 5 mM HEPES–0.2 mM EDTA (pH 7.4).

Cells. PtK2 (Potoroo kidney epithelial) cells (American Type Culture Collection) and RMCD (normal rat mammary) cells (36) were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum. MSB cells (a line of chicken leukemia cells transformed by Mareks disease virus) were grown in RPMI 1640 medium containing 10% fetal calf serum (33). Media were from GIBCO Laboratories. All cells were grown at 37°C in a humidified atmosphere containing 6% CO_2.

To accumulate mammalian cells in prometaphase, exponentially growing cultures were thymidine blocked for 16 h (1 mM thymidine in medium), released for 8 h, and blocked with 0.06 μg of Nocodazole per ml in medium for 16 h (17, 45). MSB cells were blocked with Nocodazole only.

Purification of nuclei. All operations were performed at 4°C unless otherwise indicated. Nuclei were prepared from adult rat livers, using slight modifications of a previously published procedure (28). Freshly excised, minced livers were homogenized in 8 to 10 volumes of buffer A', using a Potter-Elvehjem apparatus. Nuclei were pelleted at 650 × *g* for 10 min, suspended in A' containing 0.2% Triton X-100, and again pelleted. Triton-washed nuclei were resuspended in A', layered over A' containing 2.1 M sucrose, and centrifuged at 75,000 × *g* for 45 min. Pelleted nuclei were resuspended in A' and centrifuged at 650 × *g* for 10 min in three successive washes to remove sucrose.

Chicken erythrocyte nuclei were prepared by the use of a procedure modified from that of Ruiz-Carrillo et al. (38). Erythrocytes were lysed in buffer A' containing 0.2% Triton X-100, and the nuclei were centrifuged at 650 × *g* for 10 min. A Dounce homogenizer with a loose-fitting pestle was used to resuspend the pelleted nuclei for each of five such washes. The washes in A' containing Triton were followed by two washes with buffer A' to remove detergent.

Frogs (*Xenopus laevis*) were chilled on ice for 30 to 60 min, decapitated, and bled into SSC (0.15 M NaCl, 0.015 M sodium citrate). Erythrocytes were washed with three cycles of resuspension in SSC followed by centrifugation. Erythrocyte nuclei were isolated in buffer A supplemented with 4.5 mM spermidine (5 mM final concentration), 5 mM $MgCl_2$, 15 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. Erythrocytes were suspended once in buffer, centrifuged at 650 × *g* for 10 min, resuspended in buffer, and lysed by the addition of digitonin to 0.1%. Digitonin was added as a 10% stock solution in dimethyl sulfoxide. Nuclei were collected by centrifugation at 650 × *g* for 10 min and were washed by three cycles of resuspension and centrifugation in buffer lacking digitonin. This method of nuclear isolation is adapted from a method for the isolation of metaphase chromosomes (3).

Rat liver and chicken erythrocyte nuclei were stored at −70°C in buffer A' containing 50% (wt/vol) glycerol instead of sucrose. *X. laevis* nuclei were stored at −70°C in isolation buffer containing 50% (wt/vol) glycerol. The A_{260} of hot (100°C, 15 min) 1 M NaCl–1 M perchloric acid extracts was used as a measure of nuclear concentration. One A_{260} unit of chicken erythrocyte nuclei contained 1.6×10^7 nuclei.

Serum sources and partial purification of antibodies. Human autoimmune serum was the kind gift of Eng Tan. Normal human serum was obtained from Cappel Laboratories and from Jackson ImmunoResearch Laboratories. Normal human serum from Cappel Laboratories was used for most of the work described in this paper. Protein A binding immunoglobulins were removed from 0.1-ml aliquots of serum by chromatography on 1.0-ml columns of protein A-Sepharose (13). Serum components not adsorbed to protein A were dialyzed against buffer A containing 0.02% sodium azide. Antikinetochore antibodies present in the human autoimmune serum are concentrated in the protein A nonadsorbed fraction. Immunoglobulin G (IgG) concentrations in the retained dialysates were determined with a noncompetitive sandwich enzyme-linked immunoassay (9).

Indirect immunofluorescence. PtK2 cells grown on glass cover slips were washed once with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at 22°C, washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS, and again washed three times with PBS before the application of primary antibodies. Isolated rat liver, chicken erythrocyte, or *X. laevis* erythrocyte nuclei were centrifuged onto poly-D-lysine-coated cover slips at 2,000 × *g* in buffer A' at 4°C. Nuclei on cover slips were subjected to appropriate treatments as described in the text and figure legends. After treatment, nuclei were fixed with 4% paraformaldehyde in PBS and washed three times with PBS.

Primary human sera depleted of protein A-binding immunoglobulins were diluted to 0.66 μg of IgG per ml in application buffer. Application buffer was PBS containing 2% BSA, PBS-BSA-Tween, or buffer A–0.3 M NaCl–BSA. Similar results were obtained with fixed samples using any of these application buffers. Cover slips were incubated with 30 μl of primary antibody for 2 h at 22°C. They were washed twice with buffer A–0.3 M NaCl and three times with PBS-BSA-Tween or three times with PBS-BSA-Tween alone. The second antibody was fluorescein-conjugated goat antihuman immunoglobulin diluted 200-fold with PBS-BSA-Tween. Incubation was for 1 h at 22°C. Cover slips were washed five times with PBS-BSA-Tween and twice with water, air dried, and mounted in a glycerol-based medium containing *p*-phenylenediamine (18) or, in later experiments, *n*-propyl gallate (12).

For preparation of chromosome spreads, cells blocked with Nocodazole were washed with 75 mM KCl at 22°C and incubated in 75 mM KCl at 37°C for 15 to 20 min. The cells were then centrifuged onto poly-D-lysine-coated glass cover slips at 300 × *g* for 5 min (22°C). The cover slips were washed with buffer A, incubated for 2 min in buffer A containing 0.2% Triton X-100, washed with buffer A, and incubated for 15 min in buffer A–0.3 M NaCl. Similar results could also be obtained by using PBS in place of buffer A and A–0.3 M NaCl. The cover slips were then incubated with primary antibodies in PBS-BSA-Tween and washed with PBS-BSA-Tween as described above. After two additional washes with PBS, chromosomes were fixed with 2% paraformaldehyde in PBS for 20 min (22°C) and washed twice with PBS and once with PBS-BSA-Tween. The second antibody was affinity-purified and rhodamine-conjugated goat antihuman IgG (Tago) diluted 1,500-fold with PBS-BSA-Tween. Final washes in PBS-BSA-Tween were followed by a 1-min incubation in water containing 1 μg of DAPI (4',6-diamino-2-phenylindole) per ml to stain DNA (44).

Cover slips were then washed with water, dried, and mounted as described above.

Fluorescent images were recorded on Tri-X film (Kodak), using either a Zeiss photomicroscope equipped with III-RS epifluorescence, or a Leitz Ortholux 2 microscope equipped with epifluorescence and Vario-Orthomat camera system. In early experiments, film was developed with Diafine, but later experiments employed Acufine (both products of Acufine Chemicals, Inc.).

Preparation of nuclear extracts containing immune reactive particles. Unless otherwise noted, nuclei were thawed, washed twice with 0 to 4°C buffer A containing 15 mM β-mercaptoethanol, and suspended in digestion buffer at 25 A_{260} units per ml. Digestion buffer was buffer A containing 15 mM β-mercaptoethanol, and 3.2 mM $CaCl_2$. No adjustment was made for changes of pH upon addition of $CaCl_2$. In some experiments, the concentration of spermine and spermidine in the digestion buffer was dropped to one-half the usual concentration to promote extraction of digested chromatin by low-salt buffers (43). After a 5-min preincubation, micrococcal nuclease (Sigma) was added and the nuclei were digested for 20 min at 22 to 23°C. Nuclease activity was measured as described by Heinz et al. (14). Digestions were stopped by the addition of buffered EDTA (pH 7.4) to 10 mM. Nuclei were pelleted at 650 × *g* for 10 min and lysed by the addition of either buffer A–0.3 M NaCl or HEPES-EDTA. Some *Xenopus* chromatin was lost during centrifugations of digested nuclei before lysis. Therefore, in one experiment, chicken erythrocyte and *Xenopus* erythrocyte nuclei were digested at 50 absorbance units/ml and lysed by the addition of salt directly to the digestion mix. Salt was added as an equal volume of buffer A containing an additional 0.6 M NaCl and 10 mM EDTA. This procedure gave results identical to those obtained with our standard A–0.3 M NaCl lysis, except that more 260-nm absorbing material was present in the *Xenopus* extract. Insoluble debris was removed from lysates by centrifugation for 15 min at 1,500 × *g*. Buffer A–0.3 M NaCl extracts were diluted as appropriate and used directly in the immunological competition assay. HEPES-EDTA extracts were brought to buffer A–0.3 M NaCl by the addition of a 3× concentrated stock and used directly. The residual HEPES-EDTA had no effect on the solid-phase assay. Alternatively, chromatin in HEPES-EDTA lysates was precipitated by the addition of one-half volume of 30 mM $MgCl_2$–5 mM HEPES (pH 7.4), chilling on ice for 10 min, and centrifugation for 10 min at 15,600 × *g* (16). Precipitated chromatin was redissolved in either A–0.3 M NaCl or HEPES-EDTA as required. Except where noted, A_{260} was used as a measure of the concentration of soluble chromatin.

Assay of kinetochore-associated antigen. Wells of a Costar 96-well tissue culture plate were coated at 22°C for 30 min with 0.1 mg of poly-D-lysine (Sigma) per ml in water, washed with water, and dried. All remaining operations in the preparation of solid-phase plates were performed at 4°C. Rat liver nuclei in buffer A′ were deposited at 0.25 A_{260} unit per well by centrifugation at 1,000 × *g* for 15 min. The supernatants were removed and replaced with 150 μl of buffer A lacking sucrose but containing NaCl at a final concentration of 415 mM. After 30 min, the plate was again centrifuged at 1,000 × *g* for 15 min. With the supernatants still in place, 150 μl of PBS containing 4% paraformaldehyde and 0.25 M sucrose was added to each well. The plate was centrifuged as described above. The wells were washed three times with PBS, filled with PBS containing 2% BSA and 0.2% sodium azide, and stored at 4°C.

Immediately before use, wells of a solid-phase assay plate were washed three times with buffer A–0.3 NaCl. Unless otherwise specified, wells were incubated for 2 h at 22°C with a total of 50 μl of A–0.3 M NaCl containing 0.05% BSA, soluble nuclear extract, and an appropriate dilution of primary antibody (protein A nonadsorbed fraction). In later experiments, the concentration of BSA was raised to 1% to reduce nonspecific binding of human IgG to the solid phase. After incubation with primary antibody, wells were washed twice with A–0.3 M NaCl and three times with PBS-BSA-Tween. The second antibody was peroxidase-conjugated goat antihuman IgG diluted in PBS-BSA-Tween. Incubation was for 1 h at 22°C. Wells were washed five times with PBS-BSA-Tween and twice with PBS, and developed with 100 μl of 0.01% H_2O_2 and 0.04% *o*-phenylenediamine in citrate phosphate buffer (pH 5.0) (9). Reactions were stopped by the addition of 50 μl of 4 N H_2SO_4, and A_{492} was determined with a Titertek Multiscan (Flow Laboratories).

Preparation of ^{125}I-labeled antihuman IgG. Affinity-purified goat antihuman IgG (Tago) was iodinated to ca. 25 μCi/μg with chloramine T (37). After iodination, the antibodies were affinity purified on columns of human IgG (Sigma) bound to Affigel-10 (Bio-Rad Laboratories). Labeled antihuman IgG was stored at 4°C in PBS containing 5% BSA and 0.05% sodium azide, and was diluted just before use in 5 mM HEPES–25 mM NaCl–0.05% Tween 20–5% BSA (pH 7.4).

Precipitation assay of chromatin-bound antibodies. Aliquots (25 μl) of chicken erythrocyte chromatin (40 A_{260} units/ml in 5 mM HEPES, 25 mM NaCl, 0.05% Tween 20 [pH 7.4]) and primary antibody (in 5 mM HEPES, 25 mM NaCl, 0.05% Tween 20, 5% BSA [pH 7.4]) were mixed and incubated for 1 h at 22°C. Chromatin was precipitated by the addition of 1 ml of PBS-BSA-Tween and was collected by centrifugation. The pellets were twice dissolved in 100 μl of HEPES-EDTA and precipitated with 1 ml of PBS-BSA-Tween. The pellets were then dissolved in 50 μl of HEPES-EDTA, brought to 100 μl with 5 mM HEPES–50 mM NaCl–0.1% Tween 20 (pH 7.4), and mixed with 50 μl (20,000 cpm) of ^{125}I-labeled antihuman IgG. Incubation, precipitation, and washing of the chromatin pellets were as described above, except that an additional 150 μl of 2× PBS was added for the initial precipitation. Samples were counted in a Beckman Gamma 4000 counter. The average values obtained for samples containing goat serum (200-fold final dilution) in place of human serum or human serum fractions were subtracted from the experimental values.

Sucrose gradient centrifugation. HEPES-EDTA lysates of chicken erythrocyte nuclei were concentrated by pressure filtration to ca. 200 absorbance units/ml, using an Amicon apparatus with a PM10 membrane. Aliquots (0.5 ml) were fractionated on linear-log sucrose gradients buffered with HEPES-EDTA and prepared for particles of density 1.3 (4). Centrifugation was for 15 h at 40,000 rpm in an SW41 rotor held at 4°C. Gradients were unloaded by displacement from below with heavy sucrose. Gradient fractions were brought to A–0.3 M NaCl by the addition of one-half volume of a 3× concentrated buffer stock.

Electrophoresis of chromatin DNA. Chromatin samples in 20 mM NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 8.0) were digested with proteinase K (200 μg/ml, 30 min, 37°C.), dissociated by the addition of sodium dodecyl sulfate to 0.2%, and diluted with an equal volume of 50% (wt/vol) glycerol containing 0.5% bromophenol blue. Samples con-

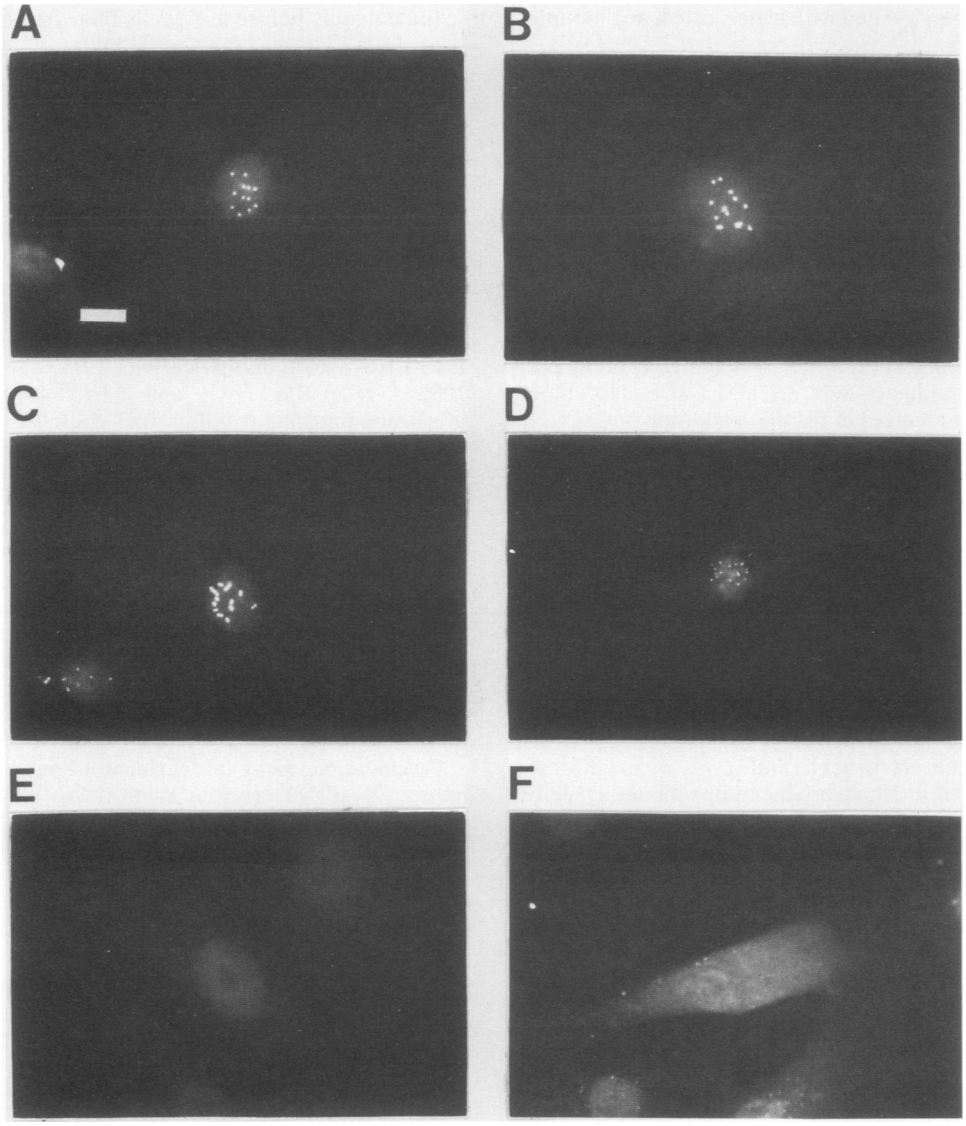

FIG. 1. Staining of PtK2 cells by the indirect immunofluorescence procedure, using CREST antikinetochore serum. Cells were stained with antikinetochore (A to D) or nonimmune (E and F) serum applied in PBS containing 2% BSA. (A, B, and C) Interphase PtK2 cells. These cells illustrate a progression through interphase, culminating in the replication of pre-kinetochores. (D) A metaphase cell; (E) an interphase cell treated with nonimmune antibodies; (F) a metaphase cell treated with nonimmune antibodies. The bar in A represents 10 μm. All figures are the same magnification.

taining 1 to 2 μg of DNA were electrophoresed in 6% acrylamide gels (acrylamide-to-bisacrylamide ratio, 20:1). The electrode buffer was 0.04 M Tris-acetate–0.002 M EDTA (pH 8) (22). *Hae*III restriction fragments of ϕX174 were used as markers (39).

RESULTS

Characterization of antikinetochore antibodies and their specificity. High-titer antikinetochore serum was used at a dilution of 200-fold to stain PtK cells by the indirect immunofluorescence procedure (Fig. 1). Staining of interphase cells was restricted to between 12 and 14 punctate foci, consistent with the karyotype of these cells (American Type Culture Collection). In some cells, these foci appeared as double spots, presumably the result of replication of kinetochore regions in G2. In mitotic cells, the fluorescent foci were collected at metaphase plates. These results are consistent with those obtained by other authors who have used antikinetochore sera (5, 11, 25, 26). They demonstrate the high specificity of antibodies in this serum for kinetochore components.

Applying these antibodies to nuclei from other species, we found punctate staining of nuclei from both rat liver and chicken erythrocytes, but not of nuclei from *X. laevis* erythrocytes (Fig. 2). Foci in rat liver nuclei were intensely stained when secondary reagents that recognize human IgG3 were employed, but staining was greatly reduced or absent if secondary reagents that recognize only IgM, IgA, or the IgG1, IgG2, and IgG4 isotypes of IgG were employed (unpublished data). These results suggested that a majority of these antikinetochore antibodies are IgG3. Therefore, except for the data shown in Fig. 1, all studies reported in this paper employed serum that was depleted of the staphylococcal protein A-binding IgG fractions (IgG1, IgG2, IgG4) by chromatography on protein A-Sepharose. Antikinetochore antibodies were concentrated in the nonadsorbed

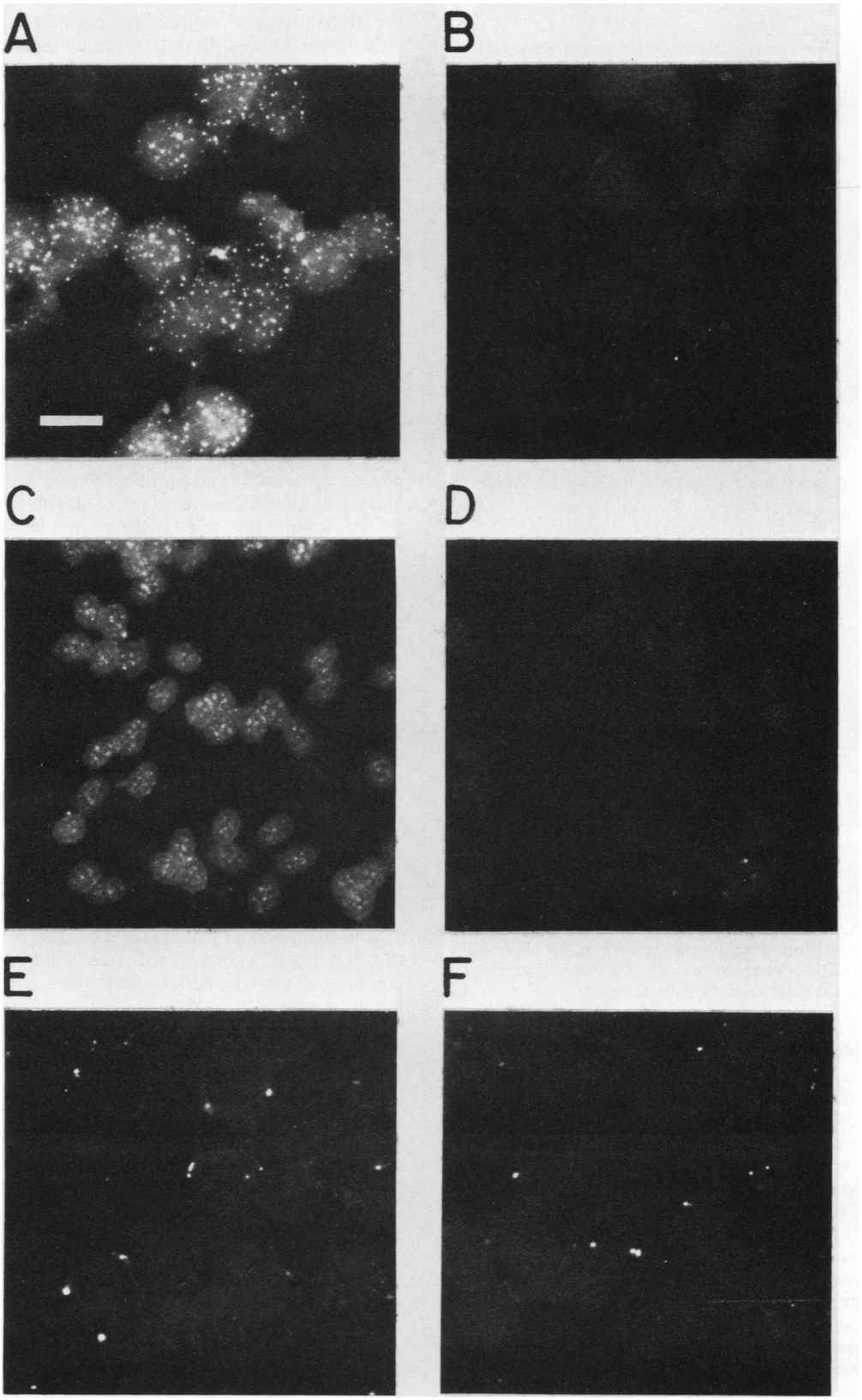

FIG. 2. Indirect immunofluorescence of isolated rat liver, chicken erythrocyte, and *X. laevis* erythrocyte nuclei. (A, C, and E) Nuclei treated with antikinetochore serum; (B, D, and F) nuclei treated with nonimmune serum. Primary antibodies were applied in buffer A–0.3 M NaCl–BSA. (A and B) Rat liver nuclei; (C and D) chicken erythrocyte nuclei; (E and F) *X. laevis* erythrocyte nuclei. The rat liver and chicken erythrocyte nuclei show discrete spots of fluorescence lacking in the *Xenopus* erythrocyte nuclei. The bar in A represents 10 μm. All figures are the same magnification.

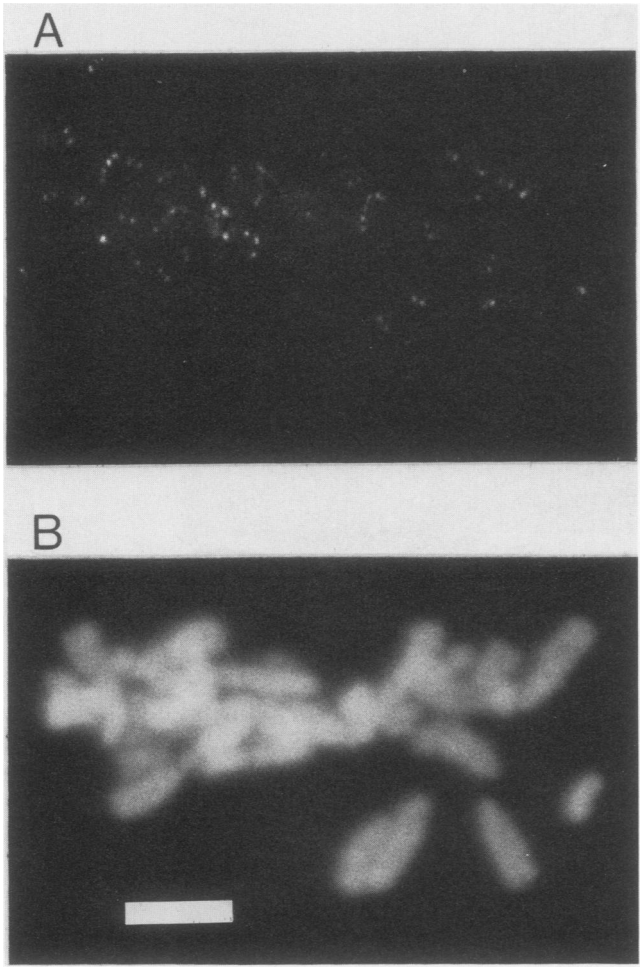

FIG. 3. Binding of partially purified antikinetochore antibodies to rat cell chromosomes. (A) Indirect immunofluorescence of RMCD (normal rat mammary) cell chromosomes, using partially purified (protein A nonadsorbed) antikinetochore antibodies. (B) DAPI (4,6-diamino-2-phenylindole) staining of DNA in the chromosomes shown in A. Doublet foci of immunofluorescence are observed at the primary constrictions of chromosomes. The bar in A represents 2 μm. Both images are the same magnification.

fraction, since most allotypes of human IgG3 do not adsorb to staphylococcal protein A (42). All secondary reagents were anti-IgG.

To confirm that the partially purified antibodies from the CREST serum bound to rat and chicken kinetochores, and to further investigate the specificity of the autoimmune antibodies, we stained chromosome spreads by the indirect immunofluorescence procedure. Doublet foci of fluorescence were readily observed at the primary constriction of both rat (Fig. 3) and chicken (data not shown) cell chromosomes. There was no indication of specific staining in the rest of the chromosome, as would be expected if antibodies directed against DNA, nucleosomes, histones, or scleroderma 70 antigen were present. Staining of kinetochores was not observed when the IgG3 fraction of normal human serum was used (data not shown).

Development of methods for the solubilization and competitive immunoassay of kinetochore components. For evaluation of procedures that might be useful for the solubilization of interphase kinetochore components, rat liver and chicken erythrocyte nuclei were centrifuged onto poly-D-lysine-coated cover slips and subjected to various treatments before fixation and indirect immunofluorescent staining. At mild levels of digestion (see figure legends), micrococcal nuclease treatment had only slight effects on the subsequent indirect immunofluorescent staining of either rat liver or chicken erythrocyte nuclei, although some diminution of staining of rat liver nuclei could be discerned (Fig. 4C and D). However, mild nuclease digestion followed by buffer A–0.3 M NaCl (375 mM salt) extraction completely abolished indirect immunofluorescent staining of interphase kinetochore components (Fig. 4E and F). These results showed clearly that solubilization of kinetochore components was minimal with nuclease digestion alone or with buffer A–0.3 M NaCl extraction alone, but suggested that kinetochore components were being solubilized by the combined nuclease digestion and moderate salt extraction.

We developed and employed a competitive enzyme-linked immunoassay to study solubilized kinetochore components. Undigested rat liver nuclei were extracted with buffer A–0.4 M NaCl (475 mM salt) and were fixed to the wells of poly-D-lysine-coated tissue culture plates to form a solid phase that would bind antikinetochore antibodies (Fig. 5). Human antibodies bound to the solid phase were quantitated by incubating the wells with peroxidase-coupled antihuman IgG, washing, and developing a color reaction, using *o*-phenylenediamine and H_2O_2 as peroxidase substrates. This assay system can be used to quantitate pre-kinetochore-specific antigens in extracts from nuclei through their ability to compete with the solid phase for antibody binding. Figure 6 shows the relationship between color development and the addition of antikinetochore antibodies to the solid-phase wells (in the absence of competing extract). The A_{492} values shown are the observed values for wells incubated with antikinetochore serum minus the observed values for wells incubated with nonimmune serum. IgG concentrations in the experimental and nonimmune serum wells were adjusted to be identical. Nevertheless, values obtained with nonimmune antibodies were usually less than 0.08 absorbance unit. It is clear that color development depends on the addition of human autoimmune antibodies, and that the solid phase can be saturated with the autoimmune antibodies.

In experiments in which nuclear extracts were assayed for their ability to compete with the solid phase for limiting amounts of antikinetochore antibodies, antibodies were diluted to give 75% maximal response in the absence of competing extract. The absolute amount of IgG added per well varied slightly among experiments, depending on the particular preparation of protein A-Sepharose-treated serum employed. Blank values for control (no extract) and experimental wells were obtained by using nonimmune antibodies as described above. Competing activity or "antigen activity" in a given amount of nuclear extract was defined to be the log of the absorbance of a well incubated with antibodies alone minus the log of the absorbance of a well incubated with antibodies plus competing extract (35; Fig. 7).

Buffer A–0.3 M NaCl extracts of micrococcal nuclease-digested rat liver nuclei and chicken erythrocyte nuclei contained competing activities (Fig. 7 and 8). The log of the A_{492} of solid-phase wells decreased linearly with increasing amounts of added competing extract (Fig. 7). This experiment was performed with a chicken erythrocyte nuclear extract. Similar results are obtained with rat liver nuclear extracts (data not shown). This relationship is the basis for the definition of antigen activity. From Fig. 7 it is clear that chicken erythrocyte nuclear extracts will compete for more than 90% of the binding of human autoimmune antibodies to rat liver nuclei.

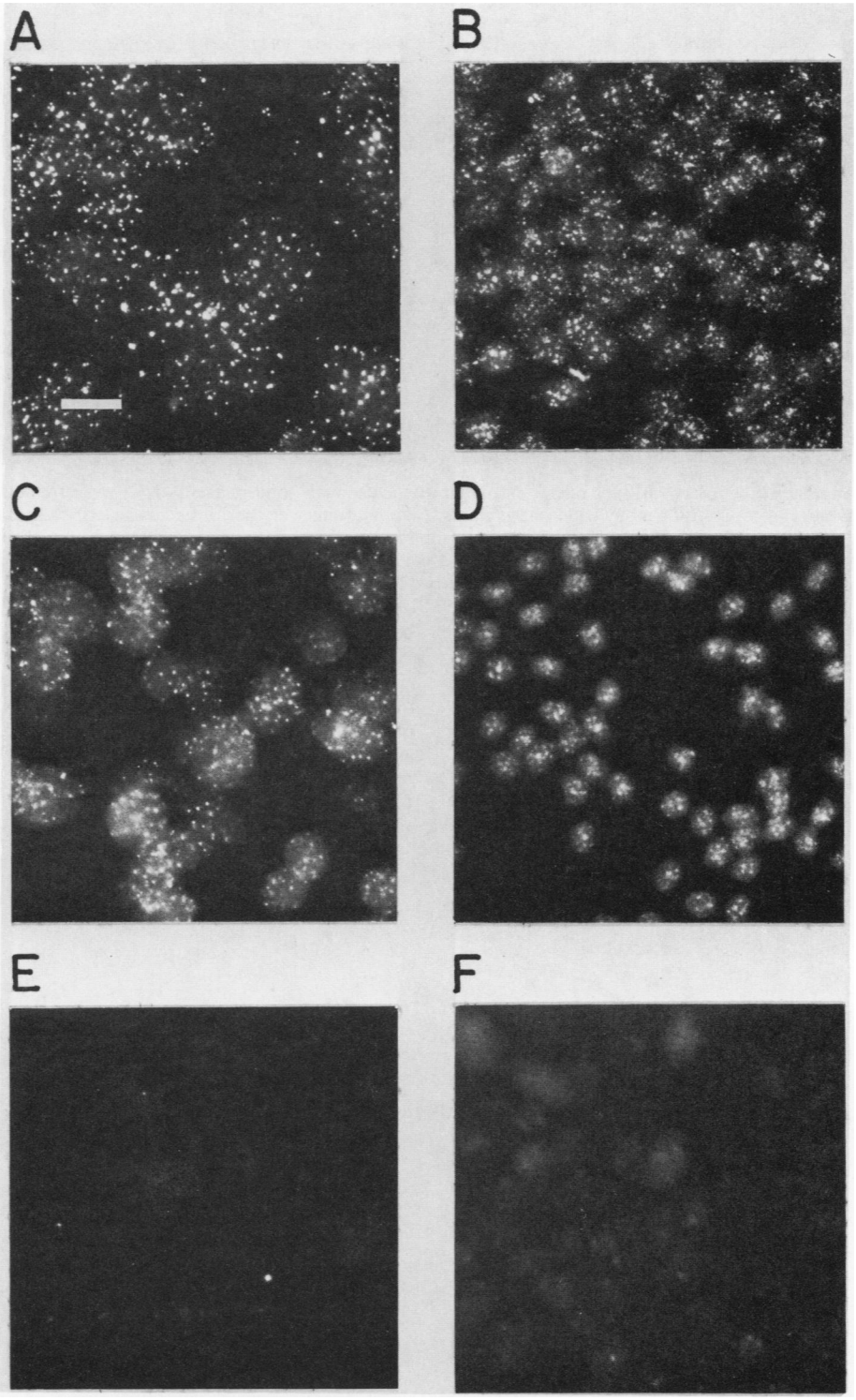

FIG. 4. Micrococcal nuclease digestion followed by salt extraction will abolish antikinetochore antibody binding to nuclei. Nuclei on cover slips were treated as described below. They were then fixed and stained with antikinetochore antibodies. Primary antibodies were applied in buffer A–0.3 M NaCl–BSA. (A, C, and E) Rat liver nuclei; (B, D, and F) chicken erythrocyte nuclei. A and B show nuclei extracted with buffer A–0.3 M NaCl. Interphase kinetochore components are clearly visible. C and D show micrococcal nuclease-digested nuclei subsequently "extracted" with buffer A. Nuclei were digested for 20 min at 22°C in buffer A containing 15 mM β-mercaptoethanol–3.2 mM $CaCl_2$, plus (C) 12.5 U of micrococcal nuclease per ml or (D) 50 U of micrococcal nuclease per ml. Staining of interphase kinetochore components is still apparent. E and F show nuclei digested with micrococcal nuclease as described for C and D, but extracted with A–0.3 M NaCl as described for A and B. In contrast to A through D, staining of kinetochore components is not observed. The bar in A represents 10 μm. All figures are the same magnification.

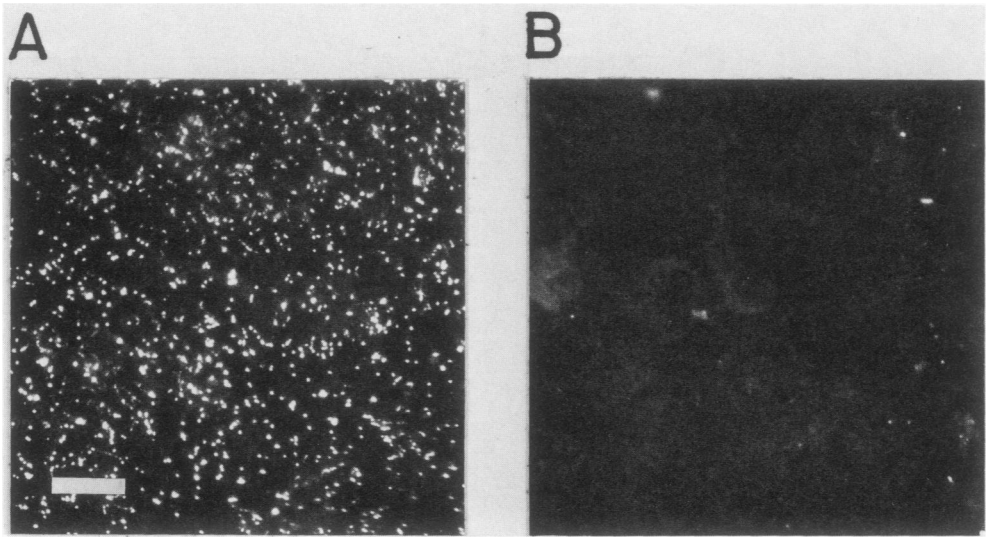

FIG. 5. Indirect immunofluorescence of rat liver nuclei extracted as for use as a solid phase. Nuclei were treated as described for the preparation of the immunological competition assay solid-phase plates. All operations were performed as described for that procedure, except that nuclei were centrifuged onto poly-D-lysine-coated glass cover slips rather than onto poly-D-lysine-coated wells of a microtiter plate. Primary antibodies were applied in buffer A–0.3 M NaCl–BSA. (A) Nuclei treated with antikinetochore antibodies; (B) nuclei treated with nonimmune antibodies. The bar in A represents 10 μm. Both figures are the same magnification.

Consistent with the results of immunofluorescence, the presence of antigen activity in rat liver and chicken erythrocyte nuclear extracts depended both on micrococcal nuclease digestion (Fig. 8 and 9) and on extraction of the digested nuclei with moderately high-salt buffers (Fig. 9). Under our standard digestion conditions, increases in the concentration of micrococcal nuclease during digestion up to ca. 80 U/ml gave increases in the antigen activity present in both rat liver and chicken erythrocyte nuclear extracts. However, with additional increases in micrococcal nuclease concentration, antigen activity in the extracts declined (Fig. 8). Maximum release of antigen activity coincided with maximum release of chromatin DNA, and decreases in antigen activity occurred coincident with decreases in chro-

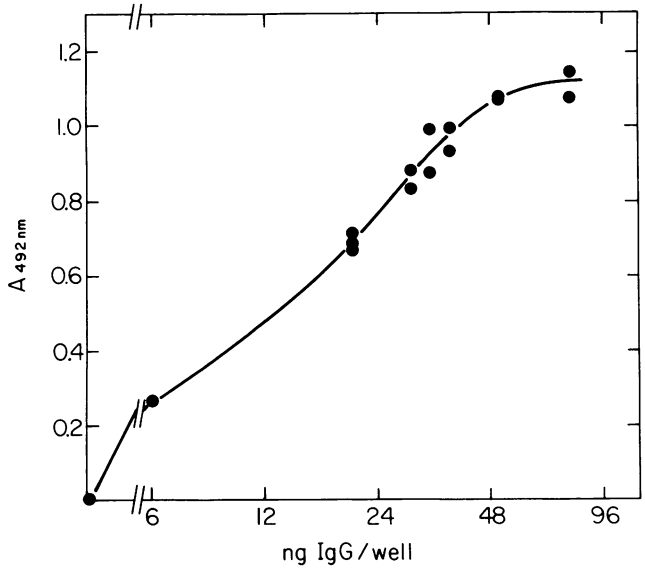

FIG. 6. Titration of the immunological competition assay solid phase with antikinetochore antibodies in the absence of competing antigen. A_{492} is plotted against the amount of human autoimmune IgG added per well. Buffer A–0.3M NaCl was added in place of a competing extract. To obtain each assay point, values obtained with nonimmune antibodies (uniformly less than 0.08 absorbance unit) were subtracted from the corresponding values obtained with antikinetochore antibodies.

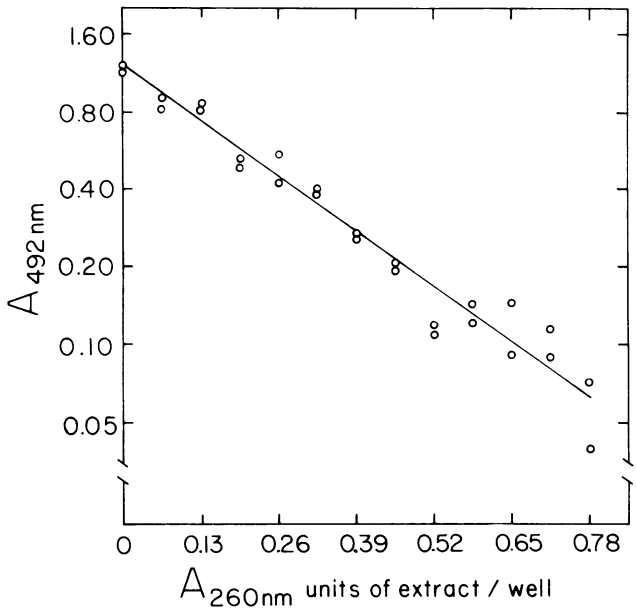

FIG. 7. Dependency of the 492-nm absorbance in immunological competition assay wells on the quantity of added competing nuclear extract. Chicken erythrocyte nuclei were digested with 200 U of micrococcal nuclease per ml and extracted with A–0.3 M NaCl. The extract was diluted with A–0.3 M NaCl, and samples were assayed as described in the text. A_{492} is plotted on a log scale versus the A_{260} (perchloric acid procedure as described for nuclei; see the text). The log of the absorbance depends linearly, with negative slope, on the amount of added nuclear extract.

matin DNA (Fig. 8 and 10). Generally, however, decreases in antigen activity exceeded decreases in chromatin DNA (Fig. 8 and 10).

As expected from immunofluorescence data, no competing activity was found in extracts of *X. laevis* erythrocyte nuclei. We obtained these results even though the *Xenopus* nuclear extracts contained quantities of chromatin comparable to those found in chicken extracts that contained activity (Fig. 10). The antigen activity of chicken erythrocyte nuclear extracts was not inhibited in mixtures containing *Xenopus* extract (data not shown). Therefore, it is unlikely that antigen activity in *Xenopus* extracts was obscured by an inhibitor of antibody-antigen interactions. This result shows that the presence of antigen activity in nuclear extracts correlates with the presence of immune reactive foci in the original nuclei, and that antigen activity in extracts likely does not reflect any competitive inhibition of binding of antibodies to DNA, histones, nucleosomes, or other nuclear components that are not kinetochore specific.

In other control experiments, solid-phase wells preincubated with chicken erythrocyte nuclear extract bound as much antibody as control wells, and doubling the time of coincubation of antibodies with chicken erythrocyte nuclear extract did not reduce the capacity of the antibodies to bind the solid phase (data not shown). These results demonstrated that enzymatic degradation of the solid phase by the extracts was insignificant, and suggested that degradation of the primary antibodies was also an unlikely explanation of our results.

Kinetochore-specific epitopes are present on nucleosome multimers and monomers. Similarities in the kinetics of release from nuclei of antigen activity and chromatin DNA suggested that kinetochore-specific epitopes might be present on chromatin. An analysis of the antigen activity relative to

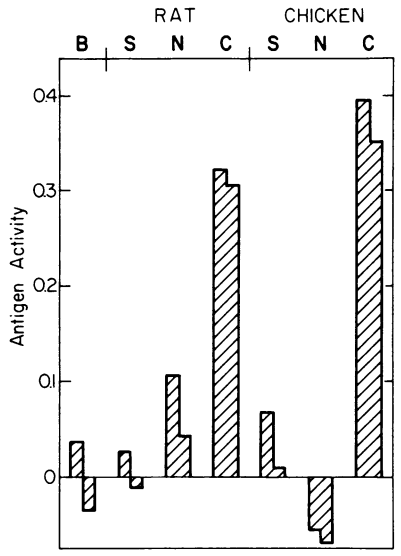

FIG. 9. Release of antigen activity into nuclear extracts is dependent on both micrococcal nuclease digestion and salt extraction. Undigested and nuclease-digested rat liver and chicken erythrocyte nuclei were extracted with buffer A alone (digested nuclei) or with buffer A–0.3 M NaCl (undigested and digested nuclei). Digestion employed 500 U of micrococcal nuclease per ml and standard conditions (see the text). Volumes of extract representing equal A_{260} units of starting nuclei were assayed for antigen activity, also under standard conditions. Each category illustrated in the histogram has two values, representing duplicate determinations of a single extract. Abbreviations: B, blank: values obtained for control wells; S, salt: antigen activity in A–0.3 M NaCl extracts of undigested (no Ca^{2+} or nuclease) nuclei; N, nuclease digested: antigen activity in buffer A extracts of digested nuclei; C, combined treatment: antigen activity in A–0.3 M NaCl extracts of digested nuclei.

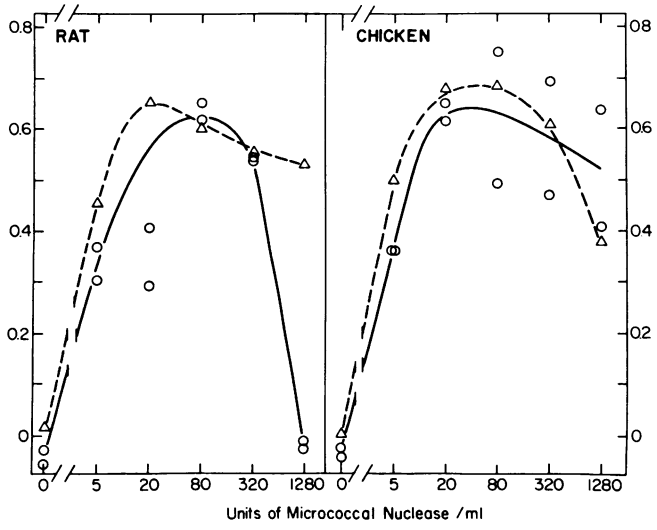

FIG. 8. Comparison of the release of antigen activity and chromatin DNA from rat liver nuclei and chicken erythrocyte nuclei. Rat liver and chicken erythrocyte nuclear preparations containing similar quantities of DNA were digested with micrococcal nuclease, and the nuclei were lysed with buffer A–0.3 M NaCl. Nuclear extracts were analyzed for antigen activity (○) and chromatin DNA (A_{260} [△]). The rat liver nuclei and chicken erythrocyte nuclei both released antigen activity into solution. Increasing digestion resulted in the release of increasing amounts of both antigen activity and chromatin DNA. However, with extensive nuclease digestion, the antigen activity in solution dropped—greatly in the case of the rat.

DNA of chicken erythrocyte nuclear extracts showed that antigen-specific activity was decreased in extracts made by using more than 80 to 100 U of nuclease per ml. However, the decrease in specific activity observed with increasing nuclease concentration was gradual, and the antigen-specific activity of an extract made with 1,280 U of nuclease per ml was still 65 to 70% of the maximum observed value. A majority of the DNA fragments in this extract contained 146 to 160 base pairs, and more than 95% of the fragments contained less than 210 base pairs (data not shown). These data suggested that kinetochore-specific epitopes are chromatin associated and might be present on mononucleosomes.

To determine whether antigen activity has the solubility properties of chromatin, we asked whether antigen activity is soluble in low-ionic-strength buffers and whether it is precipitated by 10 mM $MgCl_2$, as is chromatin (16). Micrococcal nuclease-digested chicken erythrocyte nuclei were extracted with buffer A–0.3 M NaCl or with 5 mM HEPES–0.2 mM EDTA (pH 7.4) (low-ionic-strength buffer). Low-ionic-strength extracts were brought to buffer A–0.3 M NaCl by the addition of a 3× concentrated stock. Alternatively, low-ionic-strength extracts were made 10 mM in $MgCl_2$ by the addition of 5 mM HEPES–30 mM $MgCl_2$ (pH 7.4). Precipitated material was then collected by centrifugation and redissolved in A–0.3 M NaCl. Antigen activity was present in A–0.3 M NaCl extracts, in low-ionic-strength extracts brought to A–0.3 M NaCl, and in 10 mM $MgCl_2$-insoluble material precipitated from low-ionic-strength extracts (Tables 1 and 2). Magnesium chloride quantitatively

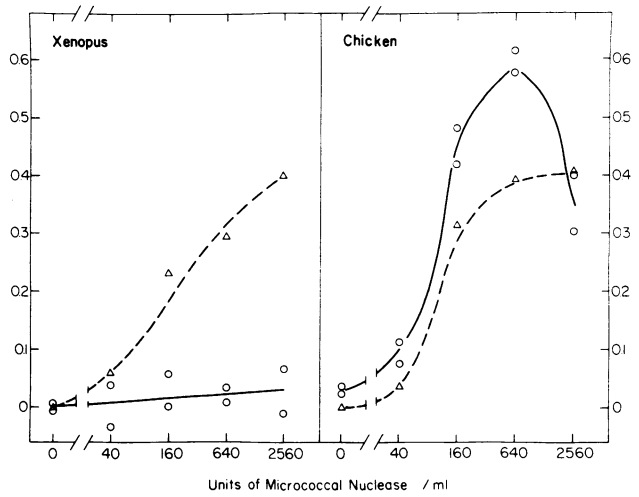

FIG. 10. Assay of antigen activity and chromatin DNA released from *Xenopus* erythrocyte and chicken erythrocyte nuclei by micrococcal nuclease digestion and salt extraction. Nuclei were suspended in buffer A supplemented to contain 5 mM spermidine, 5 mM $MgCl_2$, 3.2 mM $CaCl_2$, and 15 mM β-mercaptoethanol. This modified buffer A helped to stabilize the *Xenopus* nuclei but necessitated the use of higher concentrations of micrococcal nuclease to effect release of competing activity from chicken erythrocyte nuclei than that used in our standard digestion buffer. Micrococcal nuclease was added to the concentrations indicated, and the nuclei were digested for 20 min at 22°C. Digestion was stopped, and the nuclei were lysed by the addition of an equal volume of buffer A containing an additional 0.6 M NaCl and 10 mM EDTA. With this modified procedure, chromatin otherwise lost from *Xenopus* nuclei to the digestion buffer was quantitatively retained for analysis. After centrifugation to remove nuclear debris, extracts were assayed for antigen activity (○) and 260-nm absorbing material (△). Antigen activity was released into solution from the chicken erythrocyte nuclei but not from the *Xenopus* nuclei.

precipitated both chromatin DNA and antigen activity, which could be recovered by dissolving the precipitate in A–0.3 M NaCl (Table 2). Similar results were obtained when 5 mM spermidine was used in place of 10 mM $MgCl_2$ (data not shown). These data show that antigen activity in chicken erythrocyte nuclei has the solubility properties of chromatin.

TABLE 1. Antigen activity and chromatin DNA in moderately high and low salt extracts of digested chicken erythrocyte nuclei

Extraction buffer	Amt of micrococcal nuclease (U/ml)[a]	A_{260} units of extract per 10^7 nuclei	Antigen activity per 10^7 nuclei[b]
Moderate salt: bufferA–0.3 M NaCl, 3.3 mM HEPES (pH 7.4)	100	0.198	0.123
	500	0.146	0.074
Low salt: 5mM HEPES, 0.2 mM EDTA (pH 7.4)[c]	100	0.336	0.170
	500	0.247	0.103

[a] Nuclei were digested in a modified buffer A that contained one-half the usual concentrations of spermine and spermidine, and 3.2 mM $CaCl_2$.

[b] Each value of antigen activity per 10^7 nuclei is the average of two determinations made on a single extract. Activity is expressed as the log of the absorbance of a well incubated with antibodies alone minus the log of the absorbance of a well incubated with antibodies plus competing extract (see the text).

Confirmation of a close association of antigen activity with chromatin was obtained by sucrose gradient centrifugation. Nuclei were digested with 400 U of micrococcal nuclease per ml and were extracted with 5 mM HEPES–0.2 mM EDTA (pH 7.5). The low-ionic-strength extract was concentrated by pressure filtration and fractionated on low-ionic-strength linear-log sucrose gradients. Gradient fractions were raised in salt concentration to A–0.3 M NaCl and were assayed for both antigen activity and chromatin DNA. Antigen activity comigrated with both multimeric nucleosomes and mononucleosomes (Fig. 11A). Interestingly, the antigen activity per unit of DNA was less in mononucleosomes than in nucleosome multimers. Furthermore, antigen activity per unit of DNA was reduced in the trailing edge of the mononucleosome peak (Fig. 11A and B). This may reflect the fact that the trailing edge contains a higher percentage of monomers with core particle DNA (146 base pairs) than does the bulk of the mononucleosome peak (Fig. 11C and D). Gradient fractions containing multimers or mononucleosomes were pooled, concentrated, and again fractionated on sucrose gradients. Antigen activity in the original mononucleosome peak again comigrated with mononucleosomes (Fig. 11B). Likewise, antigen activity in the multimer peak comigrated with multimers (data not shown). These data demonstrate that antigen activity is stably associated with nucleosome multimers and monomers in low-ionic-strength buffers. Antigen activity extracted with A–0.3 M NaCl also migrated with chromatin in sucrose gradients formed in A–0.3 M NaCl (data not shown). This salt-resistant association suggests that artifactual aggregation of a kinetochore antigen with chromatin is unlikely, and that the association of antigen with chromatin is probably real and specific.

Direct binding assays show that antibodies in CREST sera bind to chromatin. The preceding experiments have used an indirect competition assay to detect levels of the pre-kinetochore antigen in solubilized chromatin. To confirm by direct means that antigens recognized by CREST scleroderma antikinetochore sera are present on chromatin, we also

TABLE 2. Effect of $MgCl_2$ precipitation on antigen activity in low-salt nuclear extracts

Sample assayed[a]	Amt of micrococcal nuclease (U/ml)[b]	% of total A_{260} units[c]	% of total antigen activity[c,d]
Low-salt extract: 5 mM HEPES, 0.2 mM EDTA (pH 7.4)	100	100	100
	500	100	100
10 mM $MgCl_2$-insoluble fraction	100	85	108
	500	108	126
10 mM $MgCl_2$-soluble fraction	100	1	−3
	500	1	−10

[a] Samples were brought to A–0.3 M NaCl before assay (see the text).

[b] Nuclei were digested in a modified buffer A that contained one-half the usual concentration of spermine and spermidine, and 3.2 mM $CaCl_2$.

[c] The values of percentage of total A_{260} units and percentage of total antigen activity are relative to the appropriate control low-salt extract (i.e., extracts made with 100 or 500 U of nuclease per ml and not treated with $MgCl_2$).

[d] Each value of percentage of total antigen activity is the average of two determinations on a single extract or subfraction. Control experiments demonstrated that $MgCl_2$ in the supernatant fractions did not influence the measured antigen activity.

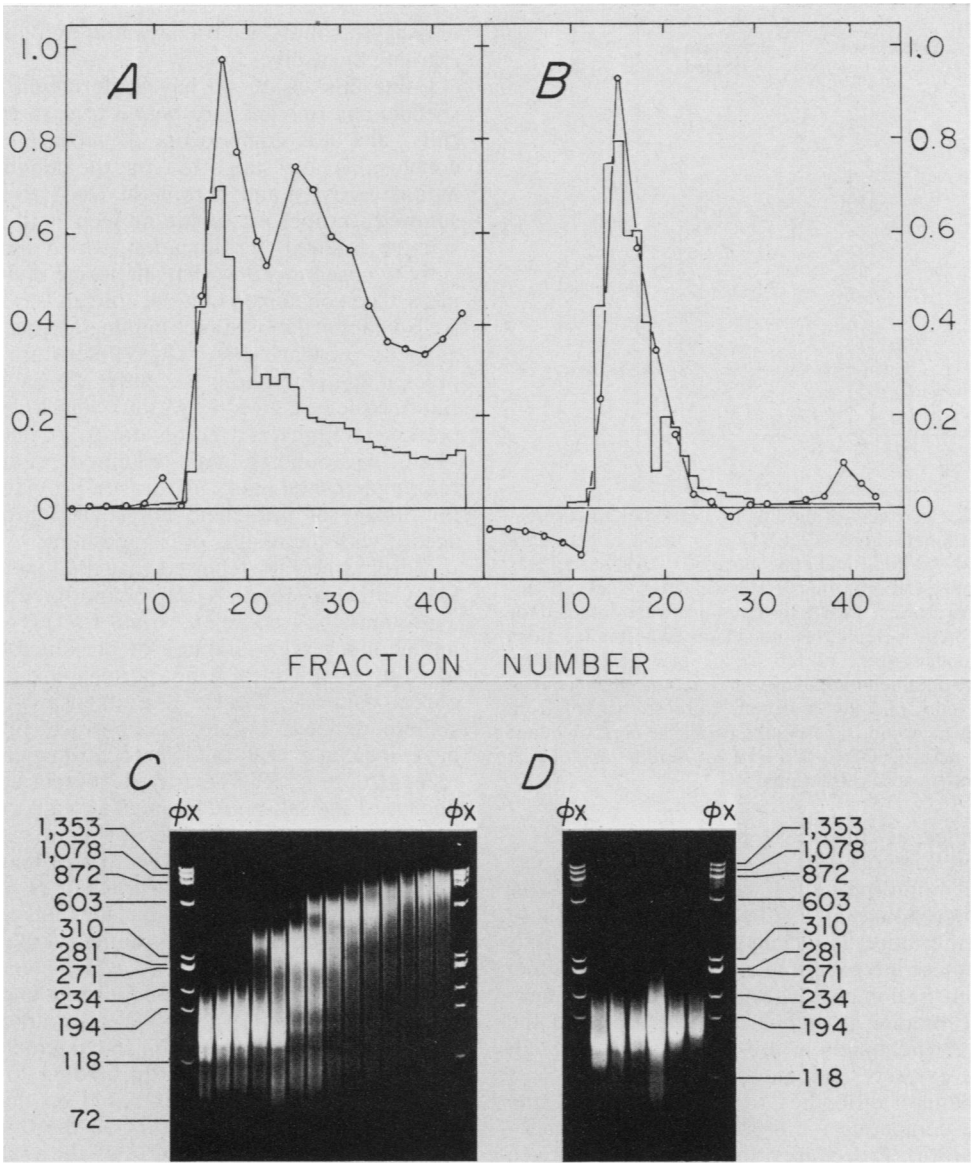

FIG. 11. Antigen activity and chromatin DNA in sucrose gradients of chicken erythrocyte nuclear extracts. (A) Linear-log sucrose gradient of a chicken erythrocyte nuclear extract. Nuclei were digested with 400 U of micrococcal nuclease per ml and were extracted with HEPES-EDTA. The extract was fractionated on linear-log sucrose gradients buffered with HEPES-EDTA. Gradient fractions were raised in salt concentration to A–0.3 M NaCl and assayed for antigen activity (○) and chromatin DNA (A_{260} [⊓⊓]). (B) Linear-log sucrose gradient of mononucleosomes. Mononucleosomes from three sucrose gradients run in parallel with the gradient analyzed in A were pooled, concentrated, and again centrifuged through a linear-log gradient. Fractions were assayed for antigen activity and chromatin DNA as described above. (C) Polyacrylamide gel electrophoresis of DNA from sucrose gradient fractions analyzed in A. DNA samples (1.5 μg) were electrophoresed in 6% polyacrylamide gels. The marker DNAs were *Hae*III restriction fragments of φX174. (D) Polyacrylamide gel electrophoresis of DNA samples from the gradient analyzed in B. Electrophoresis was as described for C.

demonstrated their presence with a precipitation assay. Antikinetochore antibodies bind well to fixed nuclei at salt concentrations as low as 25 mM, a concentration at which chromatin remains soluble (data not shown). Therefore, it was possible to incubate primary and secondary antibodies with soluble chromatin, and to use PBS (or Mg^{2+}; data not shown) to specifically precipitate chromatin and chromatin-bound antibodies (see above). Figure 12 shows the substantially greater binding to chromatin of antibodies from both whole and protein A-Sepharose-fractionated CREST antikinetochore serum than that observed with similarly treated normal human sera. Similar results have also been obtained with a different antikinetochore serum (data not shown).

DISCUSSION

We have described procedures that permit the solubilization and quantitative assay of kinetochore-associated components from avian and mammalian nuclei. The assay procedure that we have developed, a competitive enzyme-linked immunoassay, employs a solid phase of salt-extracted rat liver nuclei reacting with partially purified antikinetochore antibodies. The specificity of this assay for kinetochore-associated epitopes is demonstrated by several observations. (i) Immunofluorescence microscopy shows that the human autoimmune antibodies used in these studies bind to the salt-extracted rat liver nuclei at discrete foci. These foci

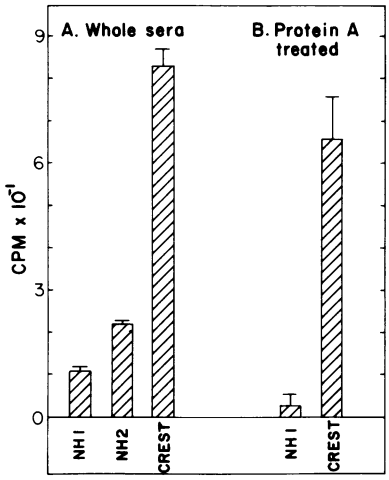

FIG. 12. Precipitation assay of binding of antibodies to chromatin. (A) Whole antikinetochore (CREST) or normal human sera (NH1, NH2) diluted 400-fold (final concentration). NH1 serum was obtained from Cappel Laboratories; NH2 was from Jackson ImmunoResearch Laboratories. Two determinations were obtained for each serum. (B) Protein A-Sepharose nonadsorbed serum fractions used at a final concentration of 6.6 μg of IgG per ml. Four determinations were obtained for each serum fraction. Mean control values of 60 (±4) cpm and 90 (±4) cpm were subtracted from experimental values in A and B, respectively. These control values were obtained by using 200-fold-diluted goat serum in place of human serum or human serum fractions.

have been identified as interphase persistent kinetochore components or pre-kinetochores on the basis of their characteristic number in interphase PtK cells, and their characteristic doublet morphology in a G2 subpopulation of these cells (5, 25). Furthermore, the same partially purified antibodies that stain these interphase foci also stain doublet foci at the primary constriction of PtK, rat, and chicken chromosomes. Since the binding of human autoantibodies to the solid phase is overwhelmingly at pre-kinetochores (Fig. 5), and since nuclear extracts compete for greater than 90% of the binding of human antibodies to the solid phase, the extracts must be competing for the binding of antikinetochore antibodies. (ii) Procedures that solubilize antigen activity from nuclei also extinguish the immunofluorescent staining of pre-kinetochores. These procedures include combined micrococcal nuclease digestion and moderately high salt extraction (this publication), or high salt extraction alone (unpublished data). Conversely, at the concentrations of nuclease and salt used in the studies presented here, neither treatment alone solubilized substantial antigen activity or abolished immunofluorescent staining of pre-kinetochores. (iii) Only those animal species that show immunofluorescent staining of interphase kinetochore components yield nuclear extracts that compete with the solid phase for binding of the human autoimmune antibodies. Therefore, it is unlikely that antibodies to nuclear components that are not kinetochore specific contribute to the binding curve. Such antibodies (e.g., antibodies directed against DNA, nucleosomes, histones, ribonucleoprotein and scleroderma 70 antigen) occur frequently in some types of human autoimmune disease, but are rare in CREST scleroderma (40). The observation that mammalian and avian chromosomes stain specifically only at their kinetochores reinforces this idea, and in addition rules out any possibility that species-specific nonkinetochore antichromatin antibodies account for our results. (iv) Control experiments rule out potential artifacts of assay attributable to enzymatic activities in the nuclear extracts themselves.

Using this assay, we have determined that kinetochore-specific determinants are bound to chromatin in interphase cells, and are components of mononucleosomes. These conclusions are supported by the kinetics of release of antigen activity and chromatin DNA from nuclei, by the solubility properties of the antigen, and by the results of sucrose gradient centrifugation, which showed that antigen activity cosediments with nucleosome multimers and monomers. The conclusion that the antigen is bound to chromatin is also supported by direct binding assays, which show that antigens recognized by CREST sera are present in PBS-precipitable chromatin. Recently, we have found that high salt treatment (e.g., 550 mM salt) causes chicken erythrocyte pre-kinetochores and chromatin to codisperse and form a "halo" surrounding lamin-delimited residual nuclear material (unpublished data). This result provides visual evidence for linkage of pre-kinetochores and chromatin, and is in accord with the results presented here.

Moroi et al. (26) reported that the kinetochore antigen is likely either a protein tightly bound to centromeric DNA or a protein-DNA complex, since CREST serum-dependent immunofluorescent staining of pre-kinetochores was abolished by treatment of fixed, permeabilized cells with micrococcal nuclease, DNase I, or trypsin in the presence of sodium dodecyl sulfate. In contrast, recent publications have indicated that the kinetochore-specific determinants recognized by CREST sera are protein components of the nuclease- and salt-resistant nuclear matrix and chromosomal scaffold (7, 8). Our data are in accord with the findings of Moroi et al. However, we wish to note that we have worked only with nuclei, and that the procedures we have applied do not permit the isolation of nuclear matrix (1). Specifically, our nuclear isolation and lysis buffers contain EDTA, EGTA, and β-mercaptoethanol, agents which cause the dissociation of Ca^{2+}, Cu^{2+}, and disulfide-dependent nuclear and chromosomal structures (19–21). We also note that our assay procedures detect only solubilized kinetochore antigens, and that although we detect little binding to residual nuclear material, this assessment is based on fluorescence procedures and is not quantitative. On the other hand, we can conclude that more than 90% of the epitopes in rat liver nuclei that are recognized by the CREST antibodies we have used are competed for by epitopes present in both rat liver and chicken erythrocyte chromatin. Furthermore, the quantity of chromatin required to compete for 50% binding is roughly comparable to the quantity of rat liver nuclei in the solid-phase wells (Fig. 7; other data not shown). This suggests that chromatin prepared according to our procedures carries a majority of the pre-kinetochore-specific epitopes from rat liver and chicken erythrocyte nuclei that are recognized by the human autoimmune antibodies.

Our findings are consistent with available data on the structure of mammalian kinetochores. The kinetochore plates of PtK cell chromosomes stain positive for DNA (34) and are decondensed by treatment with DNase I (29). Hypotonic treatment will reversibly disperse mammalian kinetochores into chromatin fibers, to which microtubules are directly attached (6, 34). Furthermore, kinetochore chromatin fibers react to changes in salt concentration differently from the way the bulk of chromatin does, suggesting that the composition of kinetochore chromatin differs from that of the typical chromatin fiber (34). Thus, sufficient evidence exists for the conclusion that mitotic chromosome kinetochores are composed, at least in part, of chromatin. Our work

demonstrates that this is also true of interphase persistent pre-kinetochores. Furthermore, our work demonstrates that an immunologically detectable difference between pre-kinetochore-specific and bulk chromatin exists at the fundamental unit of chromatin organization, the mononucleosome.

For both chromosomes III and XI of the yeast *Saccharomyces cerevisiae*, it has been established that 3.0 to 3.5 kilobases of DNA flanking the short, genetically defined essential centromere sequences are organized into chromatin with centromere-specific properties (2). This centromeric chromatin is characterized by highly phased micrococcal nuclease cutting sites at nucleosome-sized, 160-base pair intervals. The characteristic pattern of nuclease sensitivity is abolished by prior extraction of the chromatin with 1 M NaCl, indicating that it is a function of chromatin proteins. In hybrid plasmids reintroduced into yeast, only yeast centromeric DNA is organized into chromatin with the characteristic pattern of nuclease sensitivity. This suggests that specific signals in the yeast centromeric DNA are also involved in establishing the centromere-specific chromatin structure. An important question that may now be addressed is whether mammalian and avian kinetochore chromatins defined by the presence of the CREST antigen contain similar signals.

We note that the antigen-specific activity (relative to DNA) of mononucleosomes is less than the antigen-specific activity of nucleosome multimers. Our mononucleosome peak in sucrose gradients was heterogeneous, but contained mostly particles with DNA fragments of 146 to 160 base pairs. If kinetochore-specific determinants are bound only when DNA is in excess of the 146 base pairs of the nucleosome core particle, then the low specific activity of the mononucleosome peak is understandable, as is the drop in specific activity observed as the micrococcal nuclease concentration is increased during digestion. This suggests that the antikinetochore antibodies recognize a molecule with binding sites for DNA in excess of the 146 base pairs of the core particle, raising the possibility that there is also a preference for specific signals in the DNA. The question of loss of immune reactivity on clipping to core particle size is currently under investigation.

The nature of the kinetochore-specific determinants recognized by the serum we have used is of considerable interest, as is their relationship to determinants recognized by other CREST sera. Two recent publications have indicated that proteins are recognized by CREST sera on immunoblotting, but they have implicated different sets of polypeptides (7, 8). The reasons for this discrepancy are unknown. Our attempts to identify the antigens present in rat liver and chicken erythrocyte nuclei by immunoblotting have been unsuccessful, despite the fact that several variations in protocol have been tried. It may be that exposure of the determinants to denaturing conditions causes loss of immune reactivity, making their accurate identification difficult. Similar effects have been observed with other antigens, including some nuclear autoantigens (23, 31). Recently, we have obtained antigen activity in DNA-free, ca. 850 mM salt extracts of otherwise untreated erythrocyte nuclei. The gel filtration and ion-exchange properties of this activity are similar but not identical to those of H1 and H5, suggesting that the antigen is histone-like in structure. Importantly, the same activity peaks are detected by all three antikinetochore sera tested, but are not recognized by normal sera or irrelevant autoimmune sera (unpublished data). Therefore, we believe we are detecting determinants recognized by most, if not all, antikinetochore sera. Efforts to further characterize these determinants are currently in progress.

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