The Kinetochore Is Part of the Metaphase Chromosome Scaffold

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ABSTRACT   We used antisera from patients with the CREST syndrome of scleroderma (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) to show that an antigenic component of the kinetochore present in metaphase chromosomes is also present in nonhistone chromosome scaffolds isolated following extensive digestion of the DNA and extraction of the bulk of chromosomal protein. All sera from 12 scleroderma CREST patients previously shown by immunofluorescence microscopy to have circulating antikinetochore antibodies recognise a protein of M_r 77,000 (CREST-77) in an immunoblotting assay. 9 of the 12 sera also recognise an antigen of M_r 110,000 (CREST-110). These proteins are present in isolated chromosomes and nonhistone scaffolds derived from them by two different procedures. Sera of five scleroderma CREST patients who are antikinetochore negative (by immunofluorescence) bind to neither protein in immunoblots. These data suggest that CREST-77 (and possibly CREST-110) is a component of the human kinetochore, and that the kinetochore is an integral part of the mitotic chromosome scaffolding.

Chromosomes of most eucaryotes have a laminar plaque structure, the kinetochore, which provides the anchoring point for microtubules of the mitotic spindle. The kinetochore and the condensed chromosomal region where it is found (the centromere) have previously been studied extensively by light and electron microscopy, but protein components unique to this region have never been identified (reviewed in reference 1). The use of nuclease digestion procedures to enrich for mouse centromeres has recently been described (2, 3); however, the degree of enrichment obtained has not yet been sufficient to permit identification of kinetochore proteins, which are expected to be extremely minor chromosomal components.

Recently, a new avenue for study of the kinetochore was opened by the discovery that sera obtained from certain patients with the CREST syndrome of scleroderma (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) contain autoantibodies that bind specifically to a centromere antigen (4) shown by immunoelectron microscopy to be located in the basal region of the kinetochore (5). Early experiments suggested that the antigen recognised by these sera is a protein:DNA complex (it was sensitive to trypsin and DNase I [4]). The sera have been used to show that the kinetochore remains relatively condensed during interphase (5, 6).

More recently, electron microscope examination of nonhistone chromosome scaffolds isolated from nuclease-digested metaphase chromosomes (7-9) suggested that the scaffolds might retain kinetochore components (10). Since scaffolds retain only ~5% of the chromosomal protein (9, 10), they could be substantially enriched for any kinetochore proteins specifically retained in the structure.

In the work reported below, we have used antikinetochore sera from a number of patients with scleroderma CREST to show that some components of the kinetochore are indeed found in the mitotic chromosome scaffold (7-10). We have further identified a protein antigen of M_r 77,000 that is recognised by all kinetochore-positive scleroderma sera we have tested. This protein is likely to be a component of the kinetochore.

MATERIALS AND METHODS

Buffers and Reagents: The buffers referred to below are: RSB, 10 mM Tris:HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl$_2$; D-PBS, 8.06 mM Na$_2$HPO$_4$·7H$_2$O, 1.47 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, 0.68 mM CaCl$_2$, 0.492 mM MgCl$_2$; KB, 10 mM Tris:HCl pH 7.7, 150 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin (Sigma fraction 5); CB, 10 mM triethanolamine:HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl$_2$.

DAPI (Sigma Chemical Co., St. Louis, MO) is 4'6-diamidino-2-phenylindole, a nonintercalating DNA-binding fluorescent dye.

Immunofluorescence of Cells and Chromosomes: HeLa S3 cells were spun down at 1,200 g and resuspended in ⅟₂₀ vol of RSB buffer. After 5 min, 10 μl of cells were placed on a 9 × 9 mm cover slip (precoated with polylysine [11]) that was placed on a filter paper disk in a plastic petri dish. The dish was centrifuged in a Beckman TJ6-R centrifuge for 2.5 min at

2,800 rpm. Cover slips with antigen were washed twice for 5 min in D-PBS, fixed for 10 min in 3% paraformaldehyde in D-PBS, washed twice for 5 min in buffer KB, incubated with 15 μl of the appropriate serum dilution in KB for 30 min at 37°C, washed twice for 10 min with buffer KB, incubated with the appropriate dilution of preadsorbed FITC-coupled goat anti human Ig (Cappel Laboratories, Cochranville, PA) for 30 min at 37°C, washed twice for 10 min at 4°C in KB, blotted dry, and mounted in 2 μl of Mowiwol, pH 8.5 (12).

Cells were extracted in situ as follows. After the second wash in D-PBS, the cover slips were incubated with 40 μg/ml micrococcal nuclease (Worthington Biochemical Co., Freehold, NJ) in D-PBS for 20 min at 4°C. They were then incubated for 20 min in a solution of 1× lysis mix (with either dextran sulphate:heparin or NaCl) prepared as previously described (7, 9, 10). The extracted cover slips were rinsed twice with D-PBS and then processed as described above, starting with the formaldehyde fixation. To monitor the success of the in situ nuclease digestion procedures, we stained some cover slips prepared in parallel with DAPI (13). Slides were examined in an Olympus BH-2 microscope and photographed on Tri-X pan film, ASA 1000.

Chromosomes and scaffolds were isolated from HeLa S3 cells as described previously (9, 10). Note that the polyanion procedure, wherein chromosomal proteins are extracted in the presence of dextran sulphate and heparin (9), requires low ionic strengths, making it intrinsically different from extraction with 2 M NaCl (see discussion in reference 10).

Chromosomes and scaffolds were centrifuged onto cover slips as follows. A 24-well microtiter plate was cut in half. In each well, 0.6 ml of the appropriate buffer containing 0.1 M sucrose was added on top of a 9-mm square polylysine-coated cover slip. 20–50 μl of the appropriate dilution of chromosomes or scaffolds was layered over the cushion, and the plate was then centrifuged in

the TJ6-R at 2,800 rpm for 20 min. Staining was carried out exactly as described above, except that buffer CB was used for the final two washes instead of buffer KB.

Electrophoretic Techniques: Electrophoresis in 12.5% SDS poly-acrylamide gels was performed as described previously (9, 10). Proteins were electrophoretically transferred onto nitrocellulose paper and processed for final antibody detection with ^{125}I-protein A (14). Note that in these gels there is heavy nonspecific binding to the histones by normal as well as patient sera. This is due to the large amount of protein present in the histone bands. Histones are sticky enough so that even the stringent wash conditions used here (both antibody and protein A were washed with 2 M urea in addition to the more standard Triton X-100–containing buffer) were not sufficient to eliminate all nonspecific binding.

RESULTS AND DISCUSSION

Immunofluorescence Experiments

When HeLa cells arrested in mitosis are centrifuged onto cover slips and incubated with serum GS, from a scleroderma patient, the kinetochore regions are stained specifically (Fig. 1, *a* and *b*). This staining is not observed with normal serum (Fig. 1, *c* and *d*) or with FITC second antibody alone (not shown).

To determine whether the kinetochore antigen might be a component of the nonhistone chromosome scaffold as previ-
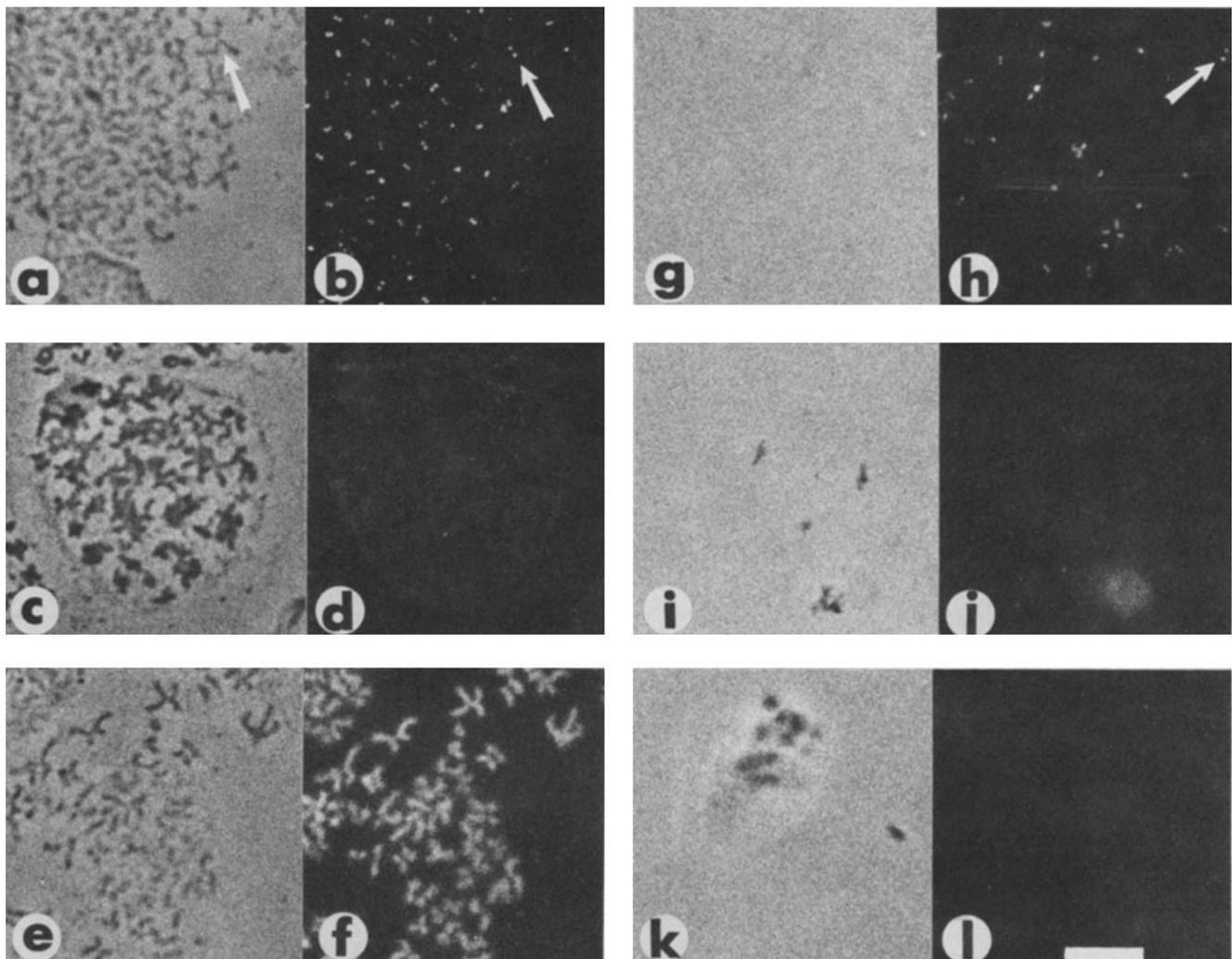
FIGURE 1 The kinetochore antigen is retained in scaffolds formed from mitotic cells on cover slips. (*a–f*) cells centrifuged onto cover slips and stained as follows: (*a* and *b*) patient serum GS, (*c* and *d*) normal serum, WE, and (*e* and *f*) DAPI. (*g–l*) Cells centrifuged onto cover slips, nuclease digested and extracted as described in Materials and Methods, then stained as follows: (*g* and *h*) patient serum GS, (*i* and *j*) normal serum WE, and (*k* and *l*) DAPI. Bar, 10 μm. × 1,130.

ously suggested (10), we subjected cells centrifuged onto cover slips to the treatments used to isolate scaffolds in solution (7–10). These treatments remove all discrete structures observable under phase contrast (Fig. 1g) as well as the bulk of the cellular DNA detected by fluorescent staining with DAPI (in Fig. 1, compare e and f with k and l). Nevertheless, the kinetochore-specific staining by serum GS remains (Fig. 1h). Such staining is not observed when normal serum is used (Fig. 1, i and j). Our results contrast with those of an earlier report, where DNase 1 treatment was found to abolish specific staining (4). The persistence of twin spots arising from sister kinetochores suggests strongly that discrete structures and not debris give rise to the staining.

When extractions are performed using cells attached to cover slips, it is not possible to characterize the extracted structures biochemically. We therefore repeated the above experiments using chromosomes isolated by an aqueous procedure (9). These chromosomes were treated to isolate nonhistone scaffolds as described previously (10), and then centrifuged onto cover slips. SDS PAGE indicated that the histones and the bulk of the nonhistone proteins were solubilized by the procedure (for example, see Fig. 3c).

Phase-contrast and fluorescence microscopy of isolated chromosomes stained with serum GS is shown in Fig. 2, a and b. Kinetochore specific staining is observed, but not when normal serum is used (Fig. 2, c and d). Scaffolds isolated by either polyanion or high salt procedures retain the kinetochore-specific staining (Fig. 2, e–h). Again, no specific staining was seen with normal serum and no DNA could be detected in the scaffolds by DAPI staining (data not shown).

Protein Analysis

We used the protein blotting procedure (14) to identify antigens recognized by serum GS in both chromosomes and scaffolds. This serum recognized two proteins of M_r 110,000 and M_r 77,000 (Fig. 3). Because these two antigens are recognised by a number of independent sera from patients with scleroderma CREST (see below), we refer to them as CREST-110 and CREST-77. Both were present in chromosomes and scaffolds isolated by either polyanions or 2 M NaCl (Fig. 3).

CREST-110 and CREST-77 appear to be distinct from a number of antigens and chromosomal components identified with either scleroderma patient sera or by biochemical fractionation of chromosomes. (a) CREST-77 appears to be different from Scl-70, a protein present in rat liver nuclei that is immunoprecipitated by sera from certain scleroderma patients (15). First, immunofluorescence performed with antisera exhibiting anti-Scl-70 activity shows diffuse nuclear rather than punctate anticentromere staining. Second, Scl-70 cofractionates with histone H1 through several extraction steps (15) while CREST-77 is tightly associated with the chromosome scaffold, a structure that totally lacks H1. (b) Our sera do not appear to bind to any of the antigens recognised by the sera reported by Cox et al. (16). We cannot explain this discrepancy, although it could be simply due to diversity in the scleroderma patient population. (c) CREST-110 and CREST-77 are distinct from the two previously identified high molecular weight components of the metaphase chromosome scaffold (9), Scl (M_r 170,000) and Sc2 (M_r 135,000 [see Fig. 3c]).

The kinetochore plaques comprise a tiny percentage of total chromosome mass; thus, kinetochore constituents would be expected to be extremely minor chromosome components. Consistent with this, no prominent stained bands are seen in Coomassie-Blue-stained gels at mobilities corresponding to CREST-110 or CREST-77. Further, when 5 mg of chromosomes were electrophoresed in an SDS polyacrylamide gel, transferred to nitrocellulose paper (14), and the region con-
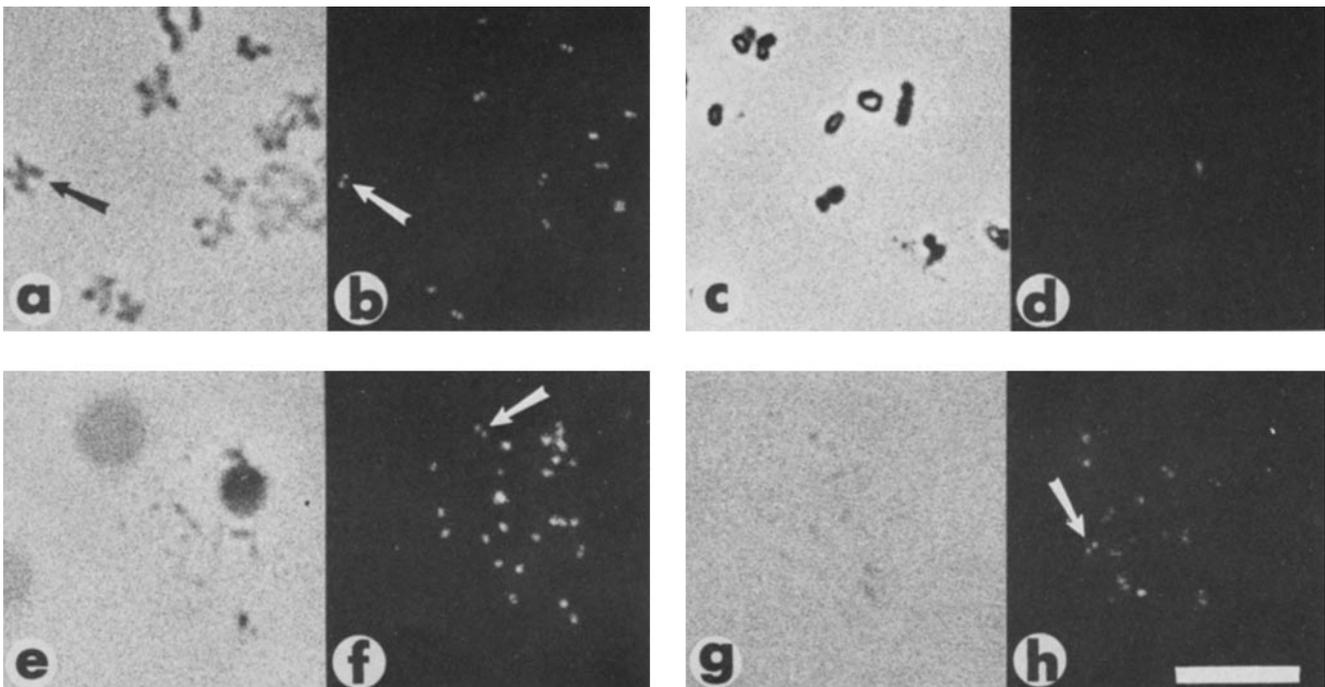
FIGURE 2 The kinetochore antigen is retained in the chromosome scaffold. (a–d) HeLa chromosomes stained with (a and b) patient serum GS and (c and d) normal serum WE. (e and f) Scaffolds made by the polyanion procedure (5, 6) centrifuged onto a cover slip, and stained with patient serum GS. (g and h) Scaffolds made by the 2 M NaCl procedure and stained with patient serum GS. Control experiments with normal serum were performed for the material of e–h but are not shown since nothing was seen by either phase-contrast or fluorescence microscopy. Bar, 10 μm. × 1,640.
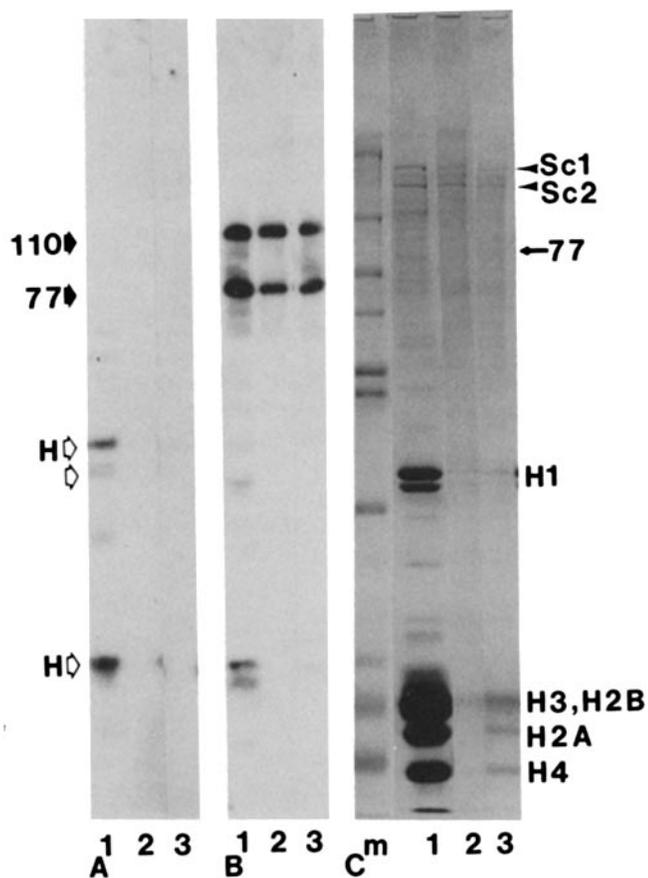
taining CREST-77 was excised, this region neither bound sufficient antibody for use in subsequent staining experiments (17), nor could it deplete the serum of kinetochore-staining activity. (Note, however, that the titer of serum GS is 1:10,240.) Alternatively, our inability to affinity purify anti-CREST-77 in this way may be due to its high binding affinity, since this binding is little affected by standard procedures used for antibody elution (data not shown).

To further explore the possibility that either CREST-110 or CREST-77 (or both) is the kinetochore antigen, we probed protein blots with sera from eleven other scleroderma patients (Fig. 4). In all cases, heavy binding to the histones was

FIGURE 3 Binding of scleroderma sera to electrophoretically separated components of isolated chromosomes and scaffolds. In all panels the lanes are as follows: 1, chromosomes; 2, scaffolds made with 2 M NaCl; 3, scaffolds prepared with dextran sulphate:heparin lysis mix. (A) Normal serum WE 1:500.(B) Patient serum GS 1:500.(C) Coomassie-Blue-stained gel with twice as much sample loaded per lane. The additional lane has marker proteins myosin (M_r 200,000), phosphorylase b (M_r 95,000), bovine serum albumin (M_r 68,000), catalase (M_r 60,000), actin (M_r 43,000), aldolase (M_r 40,000), carbonic anhydrase (M_r 29,000), beta lactoglobulin (M_r 18,400), myoglobin (M_r 16,900), cytochrome c (M_r 11,200). Nonspecific binding of antibody is indicated by open arrows (see legend to Fig. 4). The positions of histones are indicated by H in the immunoblots and are more fully labeled inpanel c. Sc1 and Sc2, labeled in c, are the major protein components of HeLa metaphase chromosome scaffolds (9). Note that, due to the different solvent conditions required for immunoblotting and Coomassie Blue staining, the positions of proteins in c are slightly different from those in a and b.
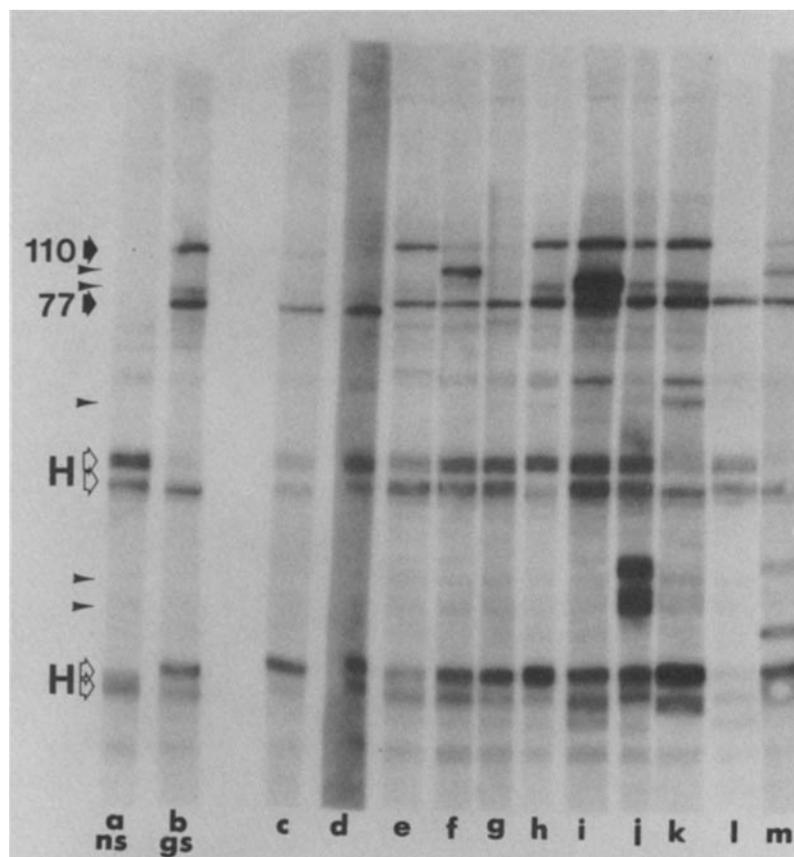

FIGURE 4 Detection of protein antigens of isolated chromosomes recognised by 12 different scleroderma patient sera. The chromosomes were run in a single wide track in a 12.5% SDS polyacrylamide gel and blotted onto a single nitrocellulose sheet (14). The sheet was cut into strips and stained with the following sera: a, normal serum WE; b, serum GS; c, G2016; d, E186, e, B4706; f, F2248; g, G1942; h, Pav; i, G857; j, G2442; k, G536; l, H345; m, H352. All sera were used at a dilution of 1:500. Bound antibody was detected with ^{125}I-protein A. In all immunoblotting experiments the histones were found to exhibit nonspecific binding to antibody and protein A. This is likely to be due both to the large amount of histone present and to the intrinsic stickiness of the histones. Nonspecific binding is indicated by open arrows, with the histones labelled H. Various specific antigens recognised by individual sera are indicated by arrowheads.
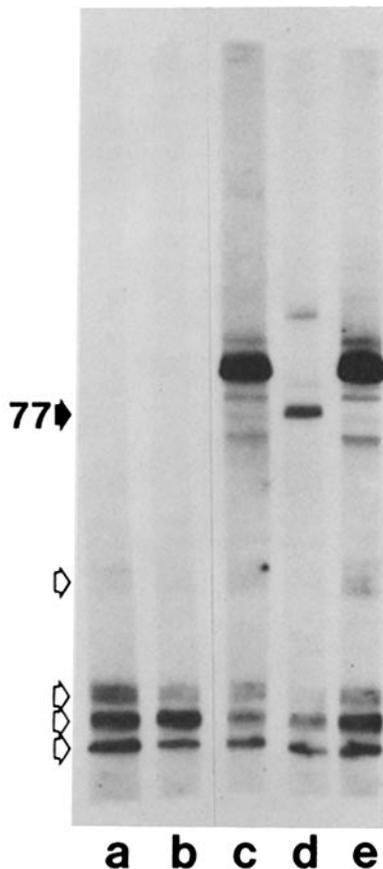
FIGURE 5 Centromere-negative sera from patients with scleroderma CREST do not recognise CREST-110 or CREST-77 in immunoblots. Chromosomes were run in a single wide track in a 7.5% polyacrylamide gel and the proteins electrophoretically transferred to nitrocellulose paper that was then cut into strips and probed with the following sera: a, normal serum WE; b, E2042 (identical patterns were obtained with sera F1145 and H1040); c, W2555; d, centromere-positive serum GS; e, H231. All sera were used at a dilution of 1:500. Nonspecific binding is indicated by open arrows.

observed since the gels had to be heavily loaded to obtain sufficient signal in the specifically labeled bands (see Materials and Methods). While differing sera showed specific binding to a number of different protein species, all sera showed strong binding to CREST-77 and nine showed some binding to CREST-110. All 12 sera show specific kinetochore staining. In addition, we probed protein blots with sera from five patients with sclerodemra CREST who lacked the antikinetochore antibody as detected by immunofluorescence microscopy, and with sera from nonscleroderma patients having speckled, diffuse and peripheral antinuclear antibody. None of these eight control sera showed binding to CREST-77 or CREST-110. Fig. 5 shows representative immunoblots from these experiments.

The most likely interpretation of these results is that CREST-77 is a component of the kinetochore. CREST-110 may also be a kinetochore component, though it does not show the absolute correlation with kinetochore staining that we have observed for CREST-77.

SUMMARY

We have shown that the kinetochore is an integral part of the chromosome scaffold, a network of nonhistone proteins pos-

tulated to play a role in establishment and maintenance of the condensed metaphase morphology (7–9, 18). Since attachment of the spindle to the kinetochore has been shown in micromanipulation experiments to be mechanically robust, surviving extensive stretching of the chromatids (19), it is not surprising that the mitotic apparatus is anchored to the scaffold.

We have found that two proteins of M_r 110,000 and M_r 77,000 (CREST-110 and CREST-77) are recognized in blots of SDS polyacrylamide gels by serum GS. Two lines of evidence suggest that CREST-77 may be the kinetochore antigen recognized in immunofluorescence experiments. First, it is a component of isolated chromosomes and scaffolds, as is the kinetochore antigen. Second, when a series of 12 different patient sera were compared in blotting experiments, a number of other antigens were recognized, but all sera recognized CREST-77. All twelve sera were selected on the basis of their positive reaction in the antikinetochore fluorescence assay. CREST-110 may also be a kinetochore component.

CREST-77 and CREST-110 are also of interest because they provide immunological markers for metaphase chromosome scaffolds. While the existence of a chromosome scaffold in vivo remains the subject of some controversy, recent experiments obtaining scaffolds of simple protein composition (9) and defined morphology (10) suggest that scaffolds isolated in vitro do not arise from nonspecific protein aggregation (20, 21). Our discovery that a discrete chromosome substructure, the kinetochore, is retained in the scaffold further supports the idea that the scaffold is held together by specific nonhistone protein associations.

REFERENCES

1. Rieder, C. L. 1982. The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.
2. Rattner, J. B., G. Krystal, and B. A. Hamkalo. 1978. Selective digestion of mouse metaphase chromosomes. *Chromosoma (Berl.).* 66:259–268.
3. Lica, L., and B. Hamkalo. 1983. Preparation of centromeric heterochromatin by restriction endonuclease digestion of mouse L929 cells. *Chromosoma (Berl.).* 88:42–49.
4. Moroi, Y., C. Peebles, M. S. Fritzler, J. Steigerwald, and E. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA.* 77:1627–1631.
5. Brenner, S., D. Pepper, M. W. Berns, E. Tan, and B. R. Brinkley. 1981. Kinetochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J. Cell Biol.* 91:95–102.
6. Moroi, Y., A. L. Hartman, P. K. Nakane, and E. M. Tan. 1981. Distribution of kinetochore (centromere) antigen in mammalian cell nuclei. *J. Cell Biol.* 90:254–259.
7. Adolph, K. W., S. M. Cheng, and U. K. Laemmli. 1977. Role of nonhistone proteins in metaphase chromosome structure. *Cell.* 12:805–816.
8. Adolph, K. W., S. M. Cheng, J. R. Paulson, and U. K. Laemmli. 1977. Isolation of a protein scaffold from mitotic HeLa cell chromosomes. *Proc. Natl. Acad. Sci. USA.* 74:4937–4941.
9. Lewis, C. D., and U. K. Laemmli. 1982. Higher-order metaphase chromosome structure: evidence for metalloprotein interactions. *Cell.* 29:171–181.
10. Earnshaw, W. C., and U. K. Laemmli. 1983. Architecture of metaphase chromosomes and chromosome scaffolds. *J. Cell Biol.* 96:84–93.
11. Mazia, D., G. Schatten, and W. Sale. 1975. Adhesion of cells to surfaces coated with polylysine: applications to electron microscopy. *J. Cell Biol.* 66:198–200.
12. Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. *Methods Cell Biol.* 24:97–132.
13. Williamson, D. H. and D. J. Fennell. 1975. The use of fluorescent DNA-binding agent

for detecting and separating yeast mitochondrial DNA. *Methods Cell Biol.* 12:335–351.

14. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 7:4350–4354.

15. Douvas, A., M. Achten, and E. M. Tan. 1979. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. *J. Biol. Chem.* 254:10514–10522.

16. Cox, J. V., E. A. Schenk, and J. B. Omsted. 1980. Human antibodies to centromeric proteins. *J. Cell Biol.* 87(2, Pt. 2):240a. (Abstr.)

17. Newman, P. J., R. A. Kahn, and A. Hines. 1981. Detection and characterization of monoclonal antibodies to platelet membrane proteins. *J. Cell Biol.* 90:249–253.

18. Laemmli, U. K., S. M. Cheng, K. W. Adolph, J. R. Paulson, J. A. Brown, and W. R. Baumbach. 1978. Metaphase chromosome structure: the role of nonhistone proteins. *Cold Spring Harbor Symp. Quant. Biol.* 42:351–360.

19. Begg, D. A., and G. W. Ellis. 1979. Micromanipulation studies of chromosome movement. I. Chromosome-spindle attachment and the mechanical properties of chromosomal spindle fibers. *J. Cell Biol.* 82:528–541.

20. Okada, T. A., and D. E. Comings. 1980. A search for protein cores in chromosomes: is the scaffold an artefact? *Am. J. Hum. Genet.* 32:814–832.

21. Hadlaczky, G., A. T. Sumner, and A. Ross. 1981. Protein depleted chromosomes. II. Experiments concerning the reality of chromosome scaffolds. *Chromosoma (Berl.).* 81:557–567.