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Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma

William C. Earnshaw[1] **and Naomi Rothfield**[2]

[1] Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA, addressee for reprint requests; [2] Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, Farmington, CT 06032, USA

Abstract. We have examined "preimmune" serum samples from a patient who progressively developed the symptoms of scleroderma CREST over a period of several years. During this period, anti-centromere antibodies (recognized by indirect immunofluorescence) appeared in the serum. Concomitant with the appearance of the anti-centromere antibodies, antibody species recognizing three chromosomal antigens in immunoblots of SDS polyacrylamide gels appeared in the patient's serum. These antigens migrate with electrophoretic mobilities corresponding to $M_r = 17$, 80, and 140 kilodaltons (kd). Affinity-eluted antibody fractions recognizing the antigens have been prepared from sera of three other patients. Indirect immunofluorescence labeling of mitotic cells using these antibody fractions demonstrates that the antigens are centromere components. We designate them CENP (CENtromere Protein) – A (17kd), CENP-B (80kd), and CENP-C (140kd). The three CENP antigens share antigenic determinants. Immunoblotting experiments show that these patients make antibody species recognizing at least three distinct epitopes on CENP-B and two on CENP-C. Sera from different patients contain different mixtures of the antibody species.

Introduction

The centromere is a particularly interesting region in mitotic chromosomes since it possesses the microtubule organizing (MTOC) ability responsible for attachment of the chromosomes to the spindle. Recent genetic advances have permitted isolation and characterization of DNA sequences present at yeast centromeres (Clarke and Carbon 1980, 1983; Bloom and Carbon 1982). Little is known, however, about the proteins present in this region, both because it is difficult to isolate biochemically and because the constituent proteins are very minor chromosomal components.

In recent years it has been found that 57%–98% of patients with the CREST (Calcinosis, Raynauds phenomenon, Esophogeal dysmotility, Sclerodactyly, Telangiectasia) syndrome of scleroderma have circulating anti-centromere antibodies (Moroi et al. 1980; Tan et al. 1980; Fritzler and Kinsella 1980; Tuffanelli et al. 1983; Steen et al. 1984; Tramposch et al. 1984). These sera therefore provide a serendipitous tool for the study of centromere structure and function. Three studies identifying possible candidates for centromere proteins have been reported (Cox et al. 1983; Earnshaw et al. 1984; Guldner et al. 1984). In one case the serum was shown to interfere with the MTOC function of the kinetochore in a lysed cell system (Cox et al. 1983).

We have shown previously that anti-centromere-positive sera obtained from 14 CREST patients contained antibodies to a 77 kd antigen which we designated CREST-77 (Earnshaw et al. 1984). Sera from nine of these patients also recognized a larger antigen, designated CREST-110. Five patients with scleroderma CREST who lacked circulating anti-centromere antibody also lacked anti-CREST-77 and anti-CREST-110. These results suggested that CREST-77 might be the centromere antigen responsible for the specific staining exhibited by our CREST patient sera and that CREST-110 might also be a centromere component (Earnshaw et al. 1984).

In this communication we describe the preparation and characterization of affinity-eluted antibody fractions directed against a number of chromosomal proteins. These experiments have shown the antigens previously designated CREST-110 and CREST-77 to be centromere components and have also identified a new centromere antigen of $M_r = 17$ kd. Characterization of the affinity-eluted antibody fractions indicates that the three proteins share antigenic determinants.

Materials and methods

Reagents. All materials used for chromosome isolation and immunofluorescence microscopy were as described previously (Lewis and Laemmli 1982; Earnshaw and Laemmli 1983; Earnshaw et al. 1984). The sera had titers (maximum dilution at which anticentromere antibody (ACA) could be detected by immunofluorescence) of $\geqq$: JR 1:2,560; SN 1:5,120; and GS 1:10,240. Previously published work indicates that the ACA are IgG (Tramposch et al. 1984).

Preparation of antigens. Chromosomes were isolated by an aqueous method as previously described (Lewis and Laemmli 1982).

Rat HTC cell lysates were obtained as follows. HTC cells growing in MEM plus 10% calf serum were blocked overnight with 0.1 μg/ml Colcemid. Mitotic cells were released by shaking off (from three 125 cm^2 flasks), centrifuged at $900 \times g$ for 3 min, and resuspended to swell in 50 ml RSB buffer (10 mM Tris:HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$). All solutions contained Tras and PMSF

to inhibit proteases. After 5 min at room temperature the solution was centrifuged as above and the supernatant removed by aspiration. To the tube were then added 100 µl solution of 5 mg/ml RNase A (previously boiled for 5 min), and 10 ml lysis buffer (15 mM Tris-HCl pH 7.4, 0.3 mM spermine, 0.75 mM spermidine, 2 mM K-EDTA pH 7.4, 80 mM KCl, 0.1% digitonin). The tube was rapidly vortexed and the suspended cells broken by homogenization in a glass dounce homogenizer. Of this lysate 0.5 ml was sonicated for 15 s on ice; $CaCl_2$ added to 4 mM, micrococcal nuclease added to 80 µg/ml and the solution incubated 20 min at 4° C. The digested solution was then centrifuged for 10 min at 12,000 × g and the pellet resuspended by boiling and sonication in 500 µl SDS sample buffer (Lewis and Laemmli 1982). Of this material 20 µl was loaded per gel lane.

Electrophoresis and procedures for immunoblotting were as previously described (Earnshaw et al. 1984).

Affinity-elution of antibody fractions from nitrocellulose paper. Chromosomes from about 10^9 cells were isolated (Lewis and Laemmli 1982), electrophoresed in 12.5% SDS polyacrylamide gels, and electro-blotted to nitrocellulose paper (Towbin et al. 1979). Affinity elutions were performed using the strategy of Olmsted (1981) as further modified from the protocol of Burke et al. (1982). A 1 cm strip was excised from one side of the blot, incubated with a 1:500 dilution of patient serum for 30 min at room temperature, and processed with ^{125}I-protein A to permit detection of bound antibodies. The remainder of the blot was stored in transfer buffer (20 mM Tris base, 190 mM glycine, 0.1% SDS, 20% methanol). The test strip was developed after 1 h. The rest of the blot was stained with Ponceau S (0.2% in 3% trichloroacetic acid) for 2 min, then destained with distilled water. The stained blot and test strip were aligned and regions binding antibody excised, minced, and placed in 10 ml tubes. (No stained band corresponding to CENP-80 could be identified unambiguously.) The minced blot was preincubated with 5 ml PTX + BSA (10 mM Na-phosphate pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM Na-azide, 0.2% Triton X-100, +4% BSA) for 30 min, and then antibody added to a dilution of 1:500 for overnight incubation. Next morning the antibody solution was removed and saved. The minced strip was then washed as follows: twice with PTX, 5 min, room temperature; twice with PTX, 5 min, 4° C; twice with PBS (PTX minus Triton X-100), 5 min, 4° C; once with 0.4 M KCl in 10 mM Na-phosphate pH 7.5, 2 min 4° C; and then eluted with 1 ml of 3 M K-thiocyanate, 0.5 M NH_4OH, 5 mg/ml BSA (prepared fresh), 5 min 4° C. The elution was repeated with a further 1 ml of this solution for 15 min at 4° C. It was not possible to use standard procedures to elute active antibody from blots, apparently due to the high affinity of anti-CENP-80 for its antigen. Binding was not disrupted by glycine buffer pH 2.8 (Olmsted 1981), 1 or 2 M acetic acid, 1 M NH_4OH, 8 M urea, 4 M $MgCl_2$, or 4 M guanidine-HCl (data not shown). After elution, the antibody was immediately returned to PBS by centrifugation through Sephadex G-25M (in PBS) in 1 ml plastic pipette tips (2 min at 200 × g; 0.5 ml loaded per tip). The flow-through was concentrated 3–4-fold by dialysis against dry sucrose, and dialyzed overnight against PBS at 4° C.

Affinity elutions performed with CREST sera are prone to an artefact which may lead to false identification of certain antigens as centromere components. This artefact arises from: (1) the extremely high titer of the sera, and 2) the high specificity of the fluorescence staining pattern. The false-positive result arise from nonspecific binding of all serum antibodies to sticky regions of blots. If sufficient serum is bound and if the elution is efficient, then a small amount of all antibody species will be released. This gives rise to weakly positive immunofluorescence. The control for this is straightforward – reblotting with the eluted serum is specific only if the primary interaction was specific. By this criterion the anti-CENP and anti-20–25 kd antibody fractions arise from specific interactions while the anti-30 kd fraction was generated by the presence of a sticky protein (H1) at that region of the immunoblot.

Immunofluorescence studies. In all cases the antigen was mitotic HeLa cells centrifuged onto cover slips and processed for immunofluorescence as described (Earnshaw et al. 1984).

Results

Patient sera

We have been using a collection of ACA-positive sera from 45 different patients with rheumatic diseases, including the CREST syndrome of scleroderma, to characterize protein antigens of the centromere. The detailed description of this collection will be presented in a future publication (Earnshaw and Rothfield, in preparation). These sera recognize several antigens of M_r 17, 20.6, 21.6, 23.5, 80, and 140 kd in immunoblots of HeLa chromosomes (figures 1–3). Different sera recognize different subsets of these antigens, though all centromere-positive sera we have tested recognize the 80 kd species (Earnshaw et al. 1984). The 80 and 140 kd species correspond to two polypeptides that were provisionally designated CREST-77 and CREST-110 in a previous publication (Earnshaw et al. 1984). The alteration in M_r of the larger component resulted from use of an additional high molecular weight standard protein which permitted a more accurate extrapolation in the upper region of the gel (where the dependence of mobility on molecular weight is not strictly linear in our gel system).

The experiments reported below have used four sera which recognize different subsets of the above antigens. Of these, JR was chosen because it most appears to resemble the serum used in a previous study of centromere antigens (Cox et al. 1983).

Examination of preimmune sera

One patient followed at our clinic since 1977 was found to develop progressively symptoms of the CREST syndrome of scleroderma including calcinosis and telangiectasias (Tramposch et al. 1984). During this period (9/77–9/80) her serum remained ACA negative. In 8/81 she developed Raynaud's phenomenon. At this point her serum became ACA-positive (titer 1:2,560), and it remained so through 7/83 (date of our most recent serum sample). Immunoblots using eight sera obtained from this patient are shown in Figure 1a–h. In all cases, the antigen tested was isolated HeLa chromosomes. It can be seen that concomitant with the development of the ACA phenotype, the serum acquired new antibody species recognising chromosomal antigens of

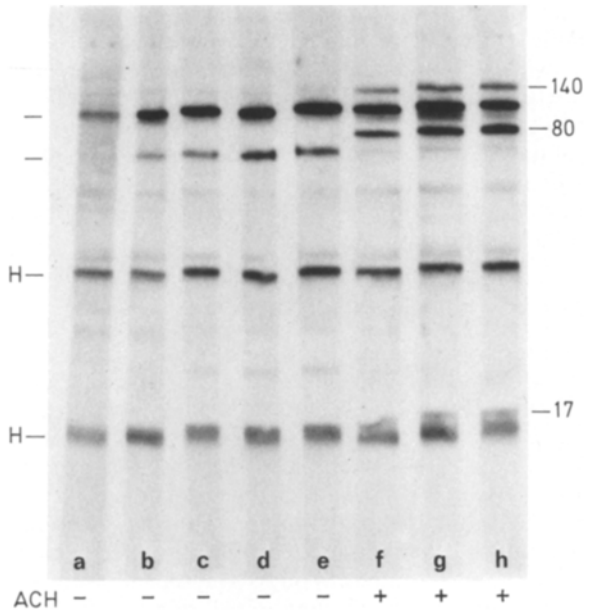

Fig. 1. Immunoblotting analysis of isolated HeLa chromosomes using sera from a patient undergoing progressive development of the CREST syndrome. In this and Figures 2 and 3 identical samples (isolated chromosomes) were electrophoresed in all lanes and in all lanes bound antibody was detected with ^{125}I-labelled protein A. Lanes **a–e** are from ACA negative sera used at a dilution of 1:250. Lane **f–h** use ACA positive sera at dilutions (**f** 1:500, **g**, **h** 1:250). The dates of the serum samples are as follows: **a** 9/77; **b** 6/78; **c** 1/80; **d** 7/80; **e** 9/80; **f** 8/81 (ACA positive); **g** 3/82; **h** 7/83). The molecular weights of chromosomal antigens recognised by the sera (in kilodaltons) are indicated

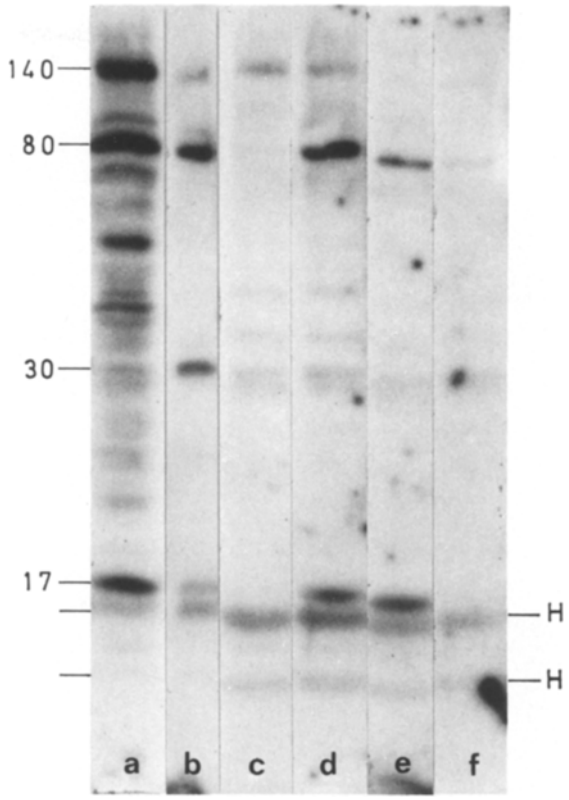

Fig. 2. Immunoblotting analysis of chromosomal antigens recognized by serum GS and antibody fractions affinity-eluted from it. Lane **a** In this case the antigen was whole cells boiled in sample buffer. The antibody was whole serum GS. Lanes **b–f** proteins of isolated chromosomes probed by immunoblotting. The sera used were: lane **b** whole serum GS (1:1,000); lane **c** anti-140 kd antibody fraction (i.e. the antibody fraction affinity-eluted from the 140 kd region of a preparative immunoblot. All affinity-eluted fractions were used at a dilution of 1:20); lane **d** anti-80 kd antibody fraction; lane **e** anti-17 kd antibody fraction; lane **f** anti-30 kd fraction. There is some artefactual binding of antibodies and protein A to histones H3 and H4, indicated by 'H' in the figure

M_r 17, 80, and 140 kd. Other antibody species recognizing antigens of about 110, 30, and 15 kd remained constant over the time period of the examination. However examination of the total proteins present on Ponceau S – stained blots of chromosomes suggests that the latter two species may arise from nonspecific binding of antibody to histones H1 and H3, respectively. Lastly, at the time of development of positive ACA an antibody species recognizing an antigen of about 70 kd was either lost or greatly diminished. This 70 kd antigen may be Sc1–70, a chromatin antigen previously described using sera of scleroderma patients lacking ACA (Douvas et al. 1979). We have no explanation for the sudden disappearance of this antibody species concomitant with the appearance of ACA.

Characterization of affinity-eluted antibody fractions

Affinity eluted antibody fractions were prepared from regions excised from preparative immunoblots of HeLa chromosomes as described in Materials and methods. For each serum a number of molecular weight ranges of antigen were used. These were: serum GS – 17 kd, 25 kd (control), 30 kd, 50 kd (control), 80 kd, and 140 kd; serum JR – 20–25 kd, 80 kd, 140 kd; serum SN – 15 kd (histone H3), 17 kd, 30 kd, 80 kd, 140 kd (control). Antigens listed above as controls correspond to regions of blots where no bound antibody was detected with ^{125}I-protein A.

Figure 2a shows that the antigens recognized by serum GS in isolated chromosomes are also detected in whole cells that have been centrifuged from suspension culture and resuspended directly in boiling SDS-PAGE sample buffer. Antigen species of 17 kd, 80 kd and 140 kd are detected as well as several other antigens. It therefore appears that the antigen spectrum is not greatly altered by proteolysis during chromosome isolation, although certain (presumably cytoplasmic) antigens are eliminated.

Figure 2b–f presents the results of immunoblotting experiments using serum GS and four antibody fractions affinity-eluted from it. In each case the source of the antigens was isolated HeLa chromosomes, of which a Coomassie blue stained gel is shown in Figure 3a. Whole serum GS recognises four antigens in isolated HeLa chromosomes (Fig. 2b). These are of M_r 17 kd, 30 kd, 80 kd, and 140 kd. The 30 kd species comigrates with histone H1. In panels b–f (Fig. 2) binding is also seen in the region of histones H3 and H4, presumably due to the large amount of protein present in this region. Much of this nonspecific binding, shown in Figure 2c–f, may be due to denatured immunoglobulins present as a result of the harsh chemical treatment used for the elution procedure.

Affinity-eluted antibody fractions were prepared as described in Materials and methods and tested by reblotting against isolated chromosomes (Fig. 2c–f). For the anti-17 kd, anti-80 kd, and anti-140 kd species the affinity-eluted

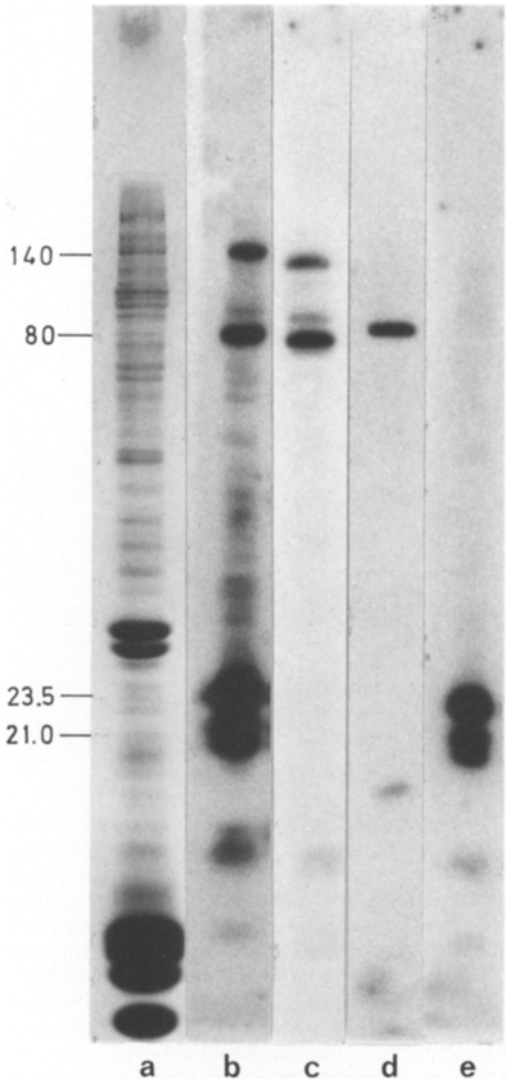

Fig. 3. Immunoblotting analysis of chromosomal antigens recognised by serum JR and antibody fractions affinity-eluted from it. In all cases the antigen was isolated HeLa chromosomes. Lane **a** coomassie-blue-stained gel. The antibody fractions used to probe the blots were as follows: Lane **b** whole serum JR (1:750); Lane **c** anti-140 kd fraction (1:22); Lane **d** anti-80 kd fraction (1:22); Lane **e** anti-20–25 kd fraction (1:25)

fractions were found to rebind specifically to the regions of the gel from which they were derived. The patterns of cross-reactivity observed between these antibody species are discussed below. The anti-30 kd antibody behaved differently. This did not rebind to the 30 kd region of the second immunoblot, but rather presented an extremely weak pattern reminiscent of whole serum. This suggests that the signal seen at 30 kd on track b was due to nonspecific adsorption of serum by the substantial amount of histone H1 present in chromosomes. This is consistent with the observation that no antigen is detected at 30 kd when the proteins of whole cells are probed by immunoblotting (Fig. 2a). In the whole cell, H1 forms a much smaller percentage of the total protein.

Figure 3b–e shows the results of immunoblotting experiments performed with serum JR and three antibody fractions affinity-eluted from it. In this case the anti-80 kd and anti-20–25 kd antibody fractions appear to rebind only to the specific molecular weight regions from which they were derived, while the anti-140 kd fraction shows substantial cross-reaction with the 80 kd antigen. When an immunoblot similar to that shown in Figure 3d is exposed for a longer period of time (Fig. 6c) then some binding of the anti-80 kd antibody to the 140 kd antigen is observed. The cross-reacting species appears to comprise only a small fraction of the anti-80 kd antibody.

Identification of centromere antigens

We have used indirect immunofluorescence to examine the location of the major antigens recognized by the scleroderma sera. In Figure 4 the data are presented as pairs of views of the same microscope field for each antibody fraction. In each case the top panel shows staining of the DNA with DAPI (Williamson and Fennel 1975) while the bottom panel shows the location of the antigen recognized by each antibody fraction (detected with rhodamine-conjugated anti-human second antibody). The second antibody showed some nonspecific binding to cell debris, which we have been unable to remove by preabsorbtion. Therefore we have shown images where the chromosomes spilled out of the cell during centrifugation onto the cover slip. This explains why the chromosome structure sometimes appears distorted (top panels of Fig. 4a, b), but it has no effect on localization of the centromere antigens.

In Figure 4 the antibody fractions used in immunofluorescence were derived from serum GS, and were the same fractions used in the immunoblotting experiment shown in Figure 2. The various antibody fractions used were: (a) whole serum; (b) anti-140 kd; (c) anti-80 kd; (d) anti-55 kd; (e) anti-30 kd; and (f) anti-17 kd. Panels (a–c) and (f) are ACA positive, while (d, e) are negative.

Figure 5 shows the results of examining the antibody fractions used for the immunoblotting experiment of Figure 3 by indirect immunofluorescence. These fractions were derived from the serum of patient JR. The various antibody fractions tested were: (b) whole serum; (d) anti-80 kd; and (f) anti 20–25 kd. Only panels (b, d) are ACA positive. Panels (a, c, e) show phase contrast views of the chromosomes shown in (b, d, f).

We conclude that the 17, 80, and 140 kd antigens are located at the centromere region of HeLa chromosomes. We will henceforth refer to these three proteins as CENP-A (CENtromere Protein 17 kd), CENP-B, and CENP-C. Antibody fractions eluted from chromosomal proteins in the 20–25, 30, and 55 kd molecular weight regions failed to bind to the centromere as detected by the indirect immunofluorescence procedure.

The centromere proteins share antigenic determinants

The data in Figures 2 and 3 indicate that antibodies eluted from the 17, 80, and 140 kd regions of preparative immunoblots show patterns of cross-reactivity which vary when two patient sera are compared. Presumably the sera recognize a range of epitopes and the two patient sera contain antibodies against overlapping but not identical subsets of these. Thus anti-CENP-C from JR crossreacts with CENP-B while anti-CENP-C from GS does not (Figs. 2c and 3c).

The patient-to-patient variability between the anti-centromere antibodies is seen clearly especially when binding

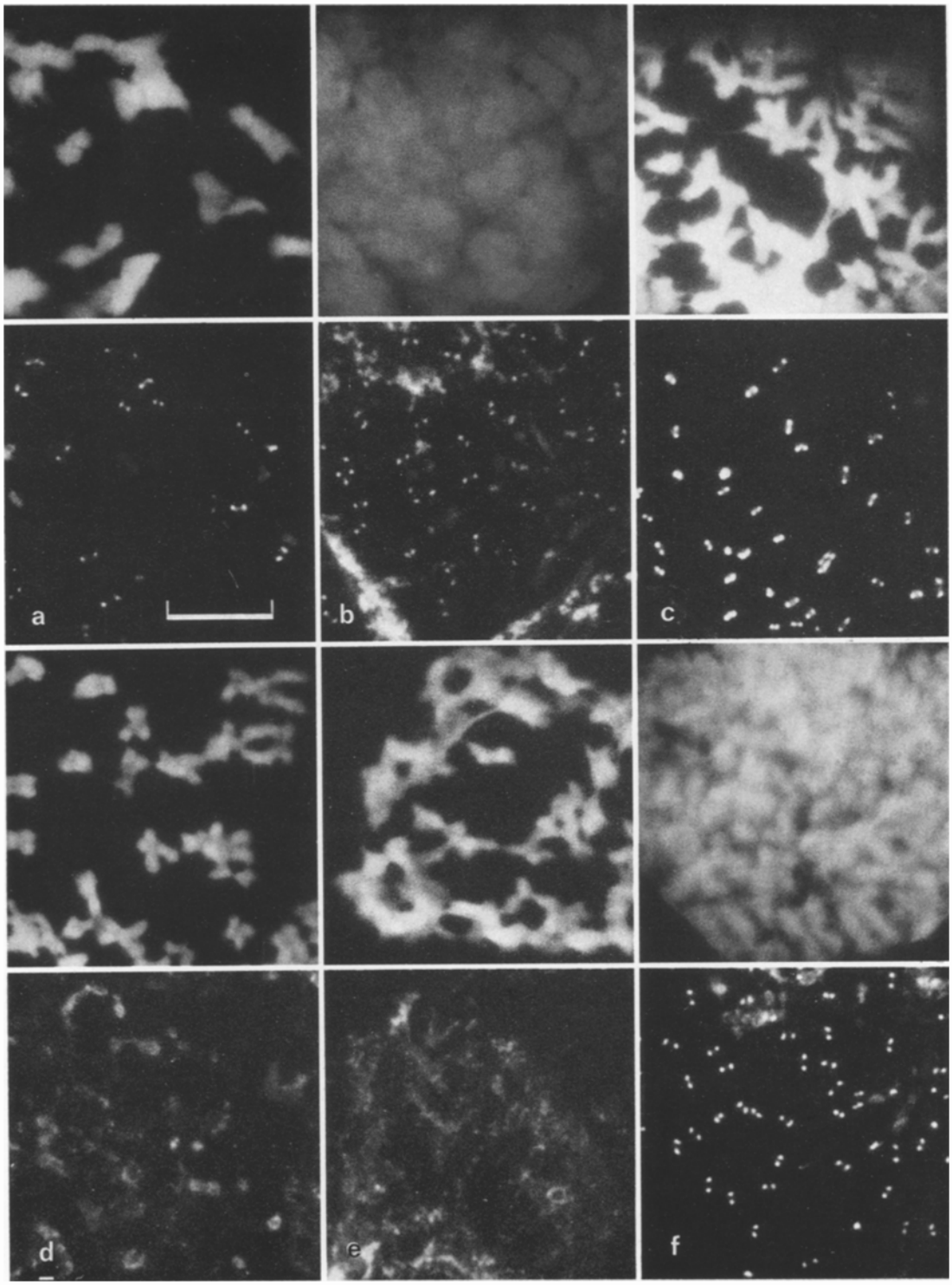

Fig. 4. Indirect immunofluorescence analysis of HeLa mitotic cells with whole serum GS and five antibody fractions derived from it. In each case the upper panel presents staining of the chromosomal DNA with DAPI (Williamson and Fennel 1975) and the lower represents detection of the human antibody with rhodamine-coupled anti-human second antibody. The panels present staining with: panel **a** whole serum GS 1:2,000; panel **b** anti-140 kd fraction; panel **c** anti-80 kd fraction; panel **d** anti-55 kd fraction; panel **e** anti-30 kd fraction; panel **f** anti-17 kd fraction. All affinity-eluted antibody fractions were used undiluted. We consistantly observed some binding of second antibody to cytoskeletal elements, even after adsorbtion of the serum with mitotic cells. This nonspecific binding accounts for the staining observed in panels **d** and **e**. Bar represents 10 μm

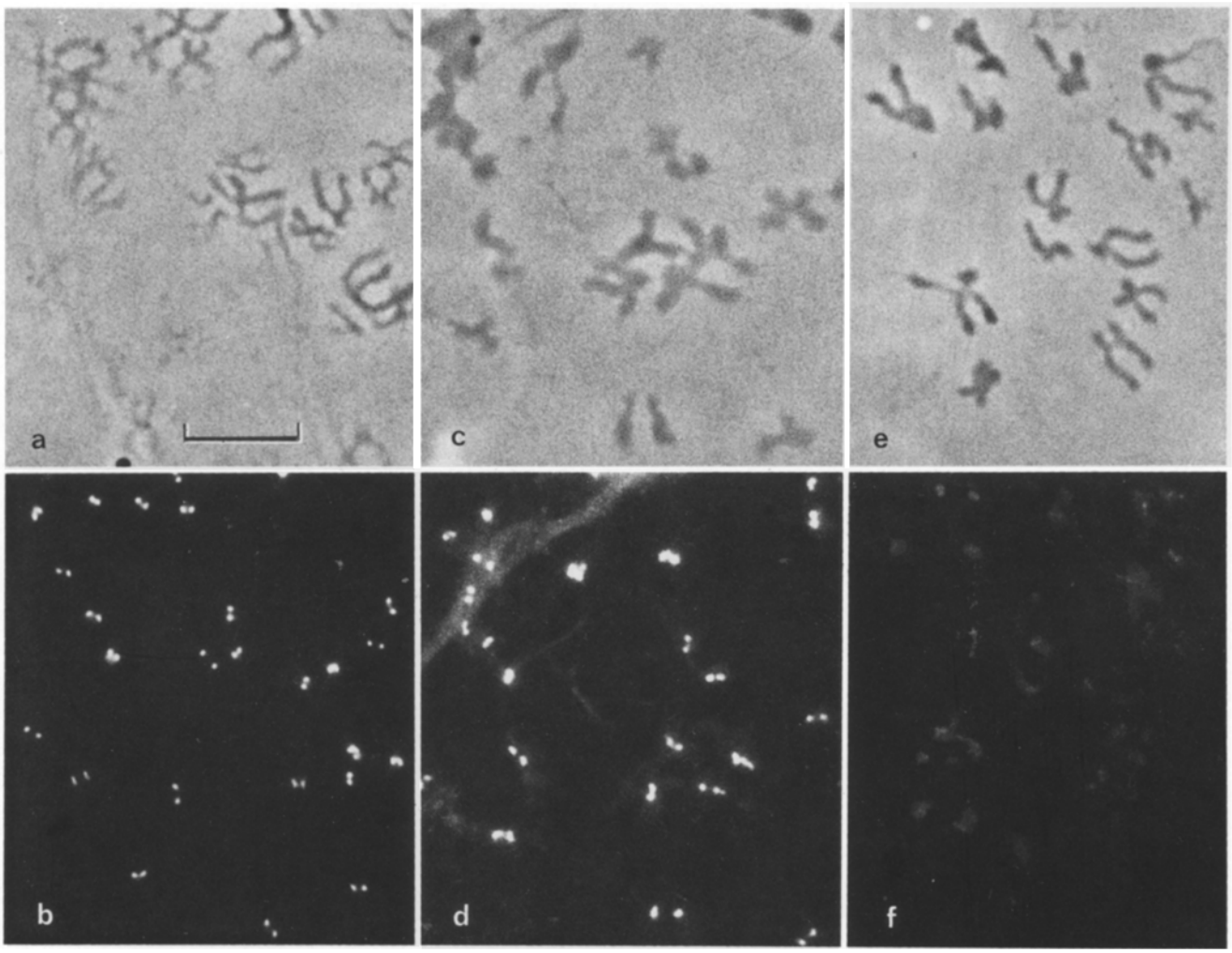

Fig. 5. Indirect immunofluorescence analysis of mitotic HeLa cells with whole serum JR and antibody fractions affinity-eluted from it. Panels **a**, **c** and **e** are phase contrast micrographs. Panels **b**, **d** and **f** present detection of the human antibody with fluorescein-conjugated second antibody. **a**, **b** whole serum JR 1:3,000. **c**, **d** anti-80 kd fraction, undiluted. **e**, **f** anti-20.6–23.5 kd fraction, undiluted

of affinity-eluted antibody fractions to human and rat antigens is compared. As shown in Figure 6, antibodies affinity-eluted from CENP-B recognize a single protein of M_r about 50 kd in rat cells. That the 50 kd rat species is a centromere protein is shown by the experiment illustrated in Figure 7, in which an antibody fraction affinity-eluted from it was used to probe HeLa mitotic cells by indirect immunofluorescence. Note also that occasionally when a Hela chromosome is stretched during centrifugation onto the cover slip (arrow, Fig. 7a) the centromere may be split into two distinct "half centromeres" (arrowheads, Fig. 7b).

As stated above, while all four affinity-eluted antibody fractions presented in Figure 6 recognise CENP-B (and show various degrees of cross-reactivity with CENP-A and CENP-C) the only antigen recognized in the rat cell lysates has a M_r of ca 50 kd. We cannot exclude the possibility that this antigen might arise from proteolytic cleavage of a larger molecule. Antibody fractions eluted from the rat antigen rebind to human CENP-A and CENP-B (Fig. 6b). Anti-CENP-B from patients GS and SN recognises the rat antigen, but that from patient JR apparently does not (Fig. 6c). In fact, whole serum JR fails to bind strongly to any rat protein of greater than 23.5 kd (data not shown).

Discussion

A family of centromere proteins

These experiments identify a family of antigens located at the centromere region of human chromosomes. We have designated these antigens CENP-A CENP-B, and CENP-C. CENP-B and CENP-C correspond to two antigens provisionally designated CREST-77 and CREST-110 in a previous publication (Earnshaw et al. 1984). That the three comprise a related group is supported by the observation that when a patient changed from being ACA-negative to ACA-positive she developed antibodies to all three simultaneously.

That the CENP antigens are proteins has been determined by digestion of chromosomes with protease. Low levels of digestion cause the antigens to migrate with altered mobilities in SDS polyacrylamide gels, while higher levels cause disappearance of the antigens from immunoblots (data not shown).

One obvious possibility is that CENP-A and CENP-B arise from cleavage of CENP-C. In the absence of purified proteins or, indeed even of recognizable bands on SDS

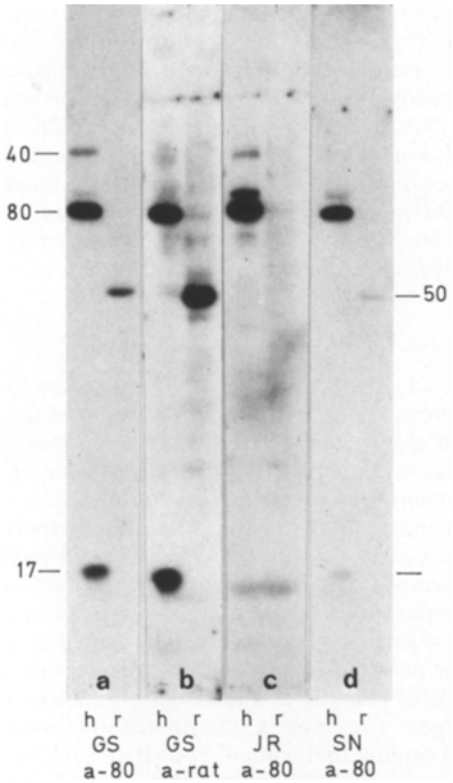

Fig. 6. Immunoblotting analysis of isolated HeLa mitotic chromosomes (*lanes h*) and rat HTC mitotic cell lysates (*lanes r*). Each panel was incubated with the affinity-eluted antibody fraction indicated and processed as for Fig. 1. Panel **a** GS antibody fraction eluted from CENP-B; Panel **b** GS antibody fraction eluted from the ca 50 kd antigen of rat liver nuclei; Panel **c** JR antibody fraction eluted from CENP-B; Panel **d** SN antibody fraction eluted from CENP-B. Whole serum SN shows negligible binding to ag-140 kd (Earnshaw et al. 1984, Fig. 4g)

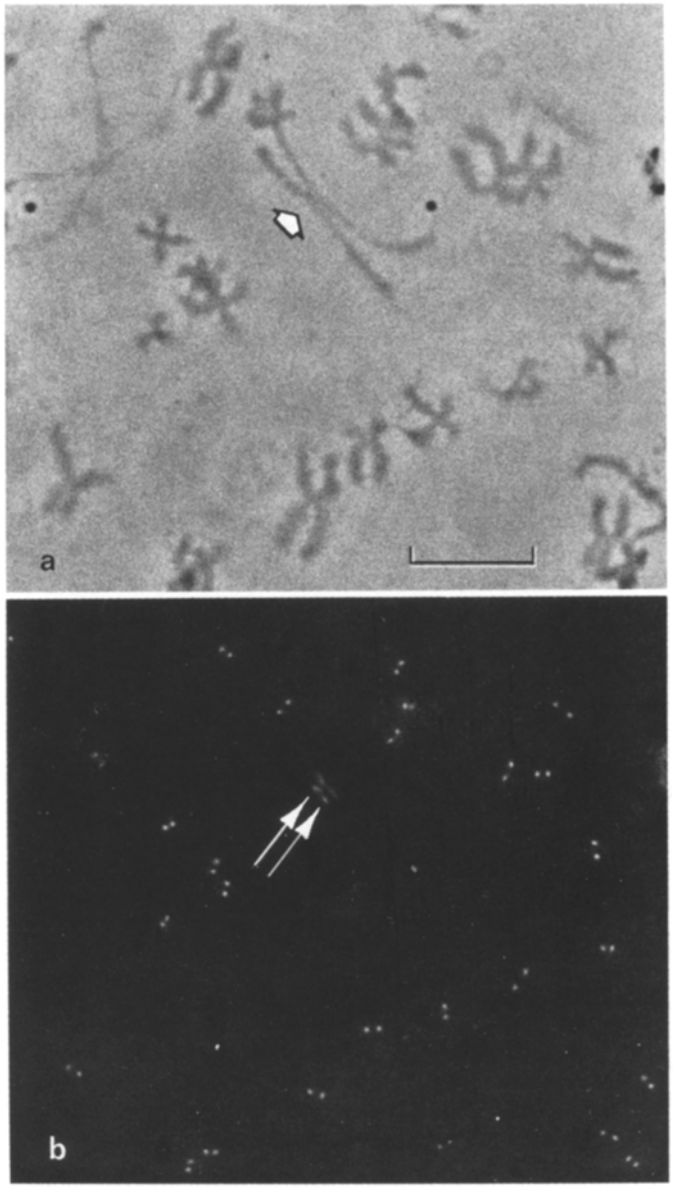

Fig. 7. Immunofluorescence analysis of mitotic HeLa cells using an antibody fraction from patient GS eluted from rat liver ca 50 kd antigen (antibody used undiluted). *Arrowheads* in **b** indicate a centromere which has been split into two "half-centromeres" as a result of stretching of the chromosome indicated by an *arrow* in the phase contrast image of panel **a**

polyacrylamide gels, this question cannot be answered unambiguously. We have shown, however, that all three antigens are present in intact cells (Fig. 2a), so that any proteolysis must occur in vivo and not arise during chromosome isolation. The existence of epitope classes present on CENP-B but absent on CENP-C (see below) also argues against the three antigens being related through simple proteolytic processing, since in general precursor (larger) antigens would be expected to contain all epitopes present on product (smaller antigens).

Examination of the data of Figure 6 will show that at least three distinct epitopes are recognized by these sera on CENP-B, with two being observed on CENP-C. These arguments are summarized in Table 1.

Epitope (a) is present on CENP-A, CENP-B, and CENP-rat, but apparently not on CENP-C. Note that binding to CENP-A and CENP-rat is comparable in each case (strongest in Fig. 6b, weakest in Fig. 6d, not detected in Fig. 6c).

Epitope (b) is present on CENP-B and CENP-C, but apparently not on CENP-A. This is observed by the cross-reaction of anti-CENP-B with CENP-C (Fig. 2c, Fig. 6a, c) and by the cross-reaction of anti-CENP-C with CENP-B (Fig. 3c).

Epitope (c) is present only on CENP-B. This is inferred from the strength of binding of anti-CENP-B to CENP-B as seen most clearly in Figures 3d and 6d.

Epitope (d) is present only on CENP-C (Fig. 2c).

The epitopes shared by the CENP species are also unlikely to reflect simple post-translational modification of a group of otherwise unrelated proteins, since at least three distinct epitopes are recognised on CENP-B alone. This argues against the determinant being a phosphate group (Davis et al. 1983) or other amino acid modification. It seems therefore most likely that the CENP species are either related by some more complex form of processing, or that they are encoded by a family of related genes.

Independent confirmation that interphase kinetochores contain a predominant polypeptide of $M_r = 80$ kd has been

Table 1. Distinct epitopes present on CENP antigens

Epitope	Present on			
	CENP-A	CENP-B	CENP-C	CENP-rat
a	+	+	−	+
b	−	+	+	−
c	−	+	−	−
d	−	−	+	−

obtained by McKeon and Kirschner, who observed this species along with several others in preparations of highly purified interphase kinetochores (F. McKeon and M. Kirschner, University of California at San Francisco, personal communication). They analyzed the proteins present in the isolated kinetochore fraction by SDS-polyacrylamide gels and autoradiography following labeling of the proteins in vivo with ^{35}S-methionine.

Other chromosomal antigens

Two other reports have suggested that the human centromere proteins recognized by CREST sera are of lower molecular weight (M_r = 14, 20, 23, and 34 kd – Cox et al. 1983; 19.5 kd – Guldner et al. 1984). We feel it likely that CENP-A corresponds to the 14 and 19.5 kd antigens identified in the previous studies. The 19.5 kd antigen has been shown to be a centromere component by affinity-elution experiments performed using a strategy similar to that described above (Guldner et al. 1984).

The major centromere antigens described by Cox et al. (1983) were in the 20–25 kd weight range. Our subcellular fractionation studies indicate that the 20–25 kd antigen group recognized by serum JR is composed of chromosomal proteins. In addition, immunoblotting experiments indicate that these antigens are found in metaphase chromosome scaffolds (data not shown). Both of these results repeat findings of Cox et al. (1983). However, antibody eluted from the 20–25 kd region of immunoblots appears not to recognize any native antigens in intact chromosomes. Therefore the exact localization of this antigen group cannot be determined by this method. A similar negative result was found by Guldner et al. (1984), using affinity-eluted antibodies recognising two antigens of 23 and 25.5 kd.

Neither our experiments nor those of Guldner et al. (1984), prove that the 20–25 kd antigen group are not centromere components. However the data do permit us to conclude that these antigens cannot generally be the antigens eliciting the ACA in CREST patients, since these antibodies were observed in only 3 of 45 ACA positive sera examined by us (Earnshaw and Rothfield, in preparation) and 5 of 18 sera examined by Guldner et al. (1984).

The data of Figure 2f indicate that highly charged proteins such as histone H1 may bind antibody tightly enough to survive a 2 M urea wash. This, combined with the substantial amounts of histone H1 present in nuclei and chromosomes, necessitates cautious interpretation of immunoblotting in this region of the gel. This emphasizes the importance of using affinity-eluted antibody fractions and the necessity for retesting these by a second round of immunoblotting (see discussion of this point in Materials and methods).

We cannot explain why the immunoblotting experiments of Cox et al. (1983) and Guldner et al. (1984) failed, to identify any antigens in the 80 kd weight range, since the antigen was recognized by 45/45 ACA-positive sera tested by us (Earnshaw and Rothfield, in preparation). Since we have detected CENP-B using both ^{125}I-protein A and peroxidase-conjugated anti-human IgG, it seems most likely that differences in either antigen preparation or electrophoresis and transfer to nitrocellulose are responsible for the discrepancy between the various laboratories.

Implications of the results

It is currently the subject of much debate whether kinetochores initiate microtubule growth or capture tubules that have been initiated elsewhere (Rieder 1982). Regardless of the mode of kinetochore function, it is clear that the kinetochore contains proteins that are responsible for attachment of mitotic chromosomes to the spindle. In future experiments it should be possible to use the affinity-eluted antibody fractions described above to ask which kinetochore components are responsible for mediating this attachment.

It will also be of interest to use the affinity-eluted antibodies to probe the distribution of the centromere antigens within the kinetochore plate by immunoelectron microscopy. That the antigens may not be distributed uniformly is suggested by the finding that while CENP-B and CENP-C are found in metaphase chromosome scaffolds CENP-A apparently is not (Earnshaw et al. 1984).

The anti-CENP antibodies are correlated with a rather specific set of symptoms in scleroderma patients. If the antinuclear antibody response arose as a non-specific secondary effect following generalized cell death, the distribution of antibodies produced might be expected to reflect the relative abundance of nuclear antigens. Such a broad spectrum of antibodies is more like that observed in systemic lupus erythematosus (Reichlin and Mattioli 1974; Notman et al. 1975; McDuffie and Bunch 1977). In scleroderma CREST, the immune response is more restricted. In fact at least three apparently distinct sites on CENP-B are recognized by autoantibodies, even though this protein is an extremely minor component of the cell. This suggests that there is a specific selection of the CENP proteins as antigens in scleroderma CREST, and therefore that the CENP antigens may be involved significantly in the pathogenesis of the CREST syndrome. In future experiments it will be important to attempt to trace the source of CENP antigen in scleroderma patients. In these studies affinity-eluted antibodies such as those described above should prove valuable reagents.

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