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Autoantibodies from a patient with scleroderma CREST recognized kinetochores of the higher plant *Haemanthus*

(centromere/mitosis/chromosomes/autoimmunity/calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, and telangiectasia)

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ABSTRACT **Human autoantibodies from a patient with scleroderma CREST (calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, telangiectasia) were used to immunostain kinetochores on chromosomes in endosperm of the seed of the monocot *Haemanthus katherinae* Bak. Kinetochores of mitotic chromosomes and prekinetochores of interphase cells were specifically stained using conventional indirect immunofluorescence procedures as well as a nonfading immunogold–silver-enhanced technique and analyzed by fluorescence and video microscopy. In interphase, prekinetochores were either single or double structures depending on the stage of the cell cycle but became quadruple (two distinct stained dots on each chromatid) in mid-to-late prophase. In favorable preparations of prometaphase chromosomes, multiple subunits could be resolved within each sister kinetochore suggesting a compound organization. Western blot analysis demonstrated common epitopes in centromeric peptides of HeLa and *Haemanthus* cell extracts. Although the molecular mass of individual polypeptides differed in the two species, the presence of shared epitopes indicates striking conservation of centromere/kinetochore components throughout evolution.**

The centromere is a specialized domain located at the primary constriction of most eukaryotic chromosomes and is intimately involved in chromosome segregation (for review, see refs. 1 and 2). The spindle fibers (microtubule bundle), which are instrumental for chromosome segregation, associate with a localized segment of the centromere called the kinetochore. The centromere and kinetochore in some plant and animal cells may not be localized at a single focus, instead being diffuse or polycentric in its distribution along the chromosome. Chromosomes lacking functional kinetochores fail to undergo normal movement and are excluded from the telophase nucleus, often forming micronuclei. Structural rearrangements leading to the formation of two centromeres on the same chromosome (dicentrics) are unstable, resulting in an anaphase bridge, breakage, and fusion cycle (3).

Studies of mammalian centromeres/kinetochores have provided the most detailed information on kinetochore structure, composition, and function. The typical mammalian centromere remains unseparated until the onset of anaphase and the kinetochore has a characteristic trilaminar structure with microtubules generally attached to outer-most layer (for review, see refs. 1 and 2).

The discovery that human autoantibodies from patients with the CREST (calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, telangiectasia) variant of scleroderma react with the kinetochore (4–7) and recognize a small family of centromeric proteins (6, 8, 9) has facilitated the biochemical dissection of this segment of the mammalian chromosome. Moreover, CREST autoantibodies are useful immunostaining reagents because they recognize inactive kinetochores in interphase nuclei (prekinetochores) as well as functional kinetochores on mitotic chromosomes (5). Although anti-kinetochore antibodies have been widely used in studies of mammalian kinetochores, they have not been applied to studies of kinetochores in plant cells. Here, we report cross-reactivity of serum from a patient with scleroderma CREST with epitopes on kinetochores in the endosperm of the seed of the monocot *Haemanthus katherinae* Bak and describe several features of plant kinetochore structure, distribution, and duplication. CREST serum could have widespread use in future studies of plant cytogenetics.

MATERIALS AND METHODS

Indirect Immunofluorescence. Endosperm cells were centrifuged onto ethanol-cleaned glass microscope slides at 1500 rpm for 3 min with a Cytospin II (Shandon, Pittsburgh). The cells were fixed in 100% (vol/vol) methanol at −20°C for 7 min, rinsed, and rehydrated in two changes of isotonic phosphate-buffered saline (PBS; pH 7.4) for a total of 10 min. The slides were then incubated in CREST serum EK at a dilution of 1:20 for 30 min in a humidified 37°C incubator. After three 5-min rinses in PBS, the slides were incubated in fluorescein isothiocyanate-conjugated goat anti-human IgG (1:20 dilution; Boehringer Mannheim) for 30 min at 37°C. The slides were then rinsed for 5 min in PBS, counterstained for DNA by immersing the slides for 5 min in PBS containing Hoechst 33258 (10 μg/ml; Sigma), and rinsed again for 5 min in PBS. Slides were overlayed with 50 μl of mounting medium [*p*-phenylenediamine at 1 mg/ml in a 1:10 (vol/vol) mixture of PBS/glycerol], coverslipped, blotted, and sealed. Negative controls were processed in the same way using normal human serum RB (1:20 dilution), which does not stain mammalian kinetochores. The slides were viewed with a Nikon Optiphot microscope equipped with epifluorescence and a DAGE MIT series 65 SIT (silicon-intensifier target tube) video camera. Images were video-enhanced by summing 128 frames and performing background subtraction and contrast enhancement using Image 1-AT (image analysis system; Universal Imaging, Media, PA). Images were photographed directly from the video monitor with a 35-mm camera set at 2-sec exposures using Kodak T-Max 100 (ASA 100). Alternatively, photomicrography was performed using a Leitz Orthoplan microscope and Kodak T-Max 400 film push-processed to ASA 1600.

Indirect Immunogold Silver Enhancement. Cells were fixed as described above and incubated with EK CREST serum (1:5 to 1:10 dilution) overnight at room temperature. The cells

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Abbreviations: CREST, calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, telangiectasia; IGSS, immunogold–silver-enhanced staining.

were then incubated with 5-nm immunogold for 6 hr and immunogold–silver-enhanced staining (IGSS) was by the method of Hoefsmit *et al.* (10) as modified for *Haemanthus* by Mole-Bajer and Bajer (11).

Preparations were embedded in Permount (SO-P-15; Fischer), a medium matching the refractive index of chromosomes and nuclei after methanol fixation. Consequently, hardly visible chromosomes did not mask absorption contrast of IGSS. Serial optical sections of video-processed images (background subtraction and contrast enhancement) using Image 1-AT (image analysis system) were recorded with an optical memory laser disc recorder (OMDR; Panasonic model TQ-2025F). The images were displayed on a 9-inch video monitor (Conrac SNA9-C; 1 inch = 2.54 cm) and photographed directly from the video screen with a 35-mm camera at a 1-sec exposure using Kodak Plus-X film.

Micrographs made using the standard microscope were taken with Nikon phase-contrast microscope and a Plan-Apo oil-immersion 100×, N.A. 1.4, objective. Those in VEM (video-enhanced microscopy) were in bright-field optics using polarized light with crossed Glan–Thompson prisms offset 10°. The objective was a Nikon phase-contrast Plan-Apo DM 60× oil-immersion N.A. 1.4.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Endosperm extracts were electrophoresed on 5–12% gradient SDS gels. The separated proteins were then either stained with Coomassie blue or transferred to nitrocellulose (12). The nitrocellulose strips were blocked for 2 hr with 3% (wt/vol) nonfat powdered milk in PBS and then treated with either CREST serum (EK) (1:1000 dilution) or control human serum (RB) (1:1000 dilution) in powdered milk for 2.5 hr at room temperature. The blots were rinsed three times with the milk solution and then incubated for 2 hr at room temperature in 3% powdered milk containing peroxidase-labeled anti-human IgG antibody (1:1000 dilution; Boehringer Mannheim). The nitrocellulose strips were then rinsed three times and developed with 3,3′-diaminobenzidine. HeLa nuclear extracts were prepared as described (13) and electrophoresed in parallel with endosperm extracts.

RESULTS

The chromosomes of the higher plant *Haemanthus katherinae* Bak. ($n = 9$) are large (4 μm thick and up to 30 μm long in metaphase) and relatively easy to observe by conventional light microscopic techniques. However, the centromere/kinetochore region of mitotic chromosomes, depicted by the primary constriction, was relatively structureless when viewed by conventional phase-contrast or bright-field optics. When these same cells were examined by indirect immunofluorescence after exposure to scleroderma CREST antiserum, the kinetochores were clearly detected as discrete granular structures on mitotic chromosomes and as stained punctate bodies in interphase nuclei. By using both conventional indirect immunofluorescence staining procedures and IGSS, several features of kinetochore structure, distribution, and duplication were visualized and some previous observations were confirmed.

In interphase nuclei, stained spots representing prekinetochores could be seen distributed throughout the nucleus (Fig. 1 *A* and *B*). Although some prekinetochores were located near the nuclear envelopes (Fig. 1*C*), through-focus images obtained by ultrathin optical sectioning suggested that many were distributed deeper within the nucleus. In some cells, prekinetochores were grouped as polarized clusters near one side of the nucleus (Fig. 2*E*), showing a typical Rabl orientation (for review, see refs. 14 and 15). This distribution, established by the position of kinetochores near the pole at telophase, was maintained in many interphase cells.

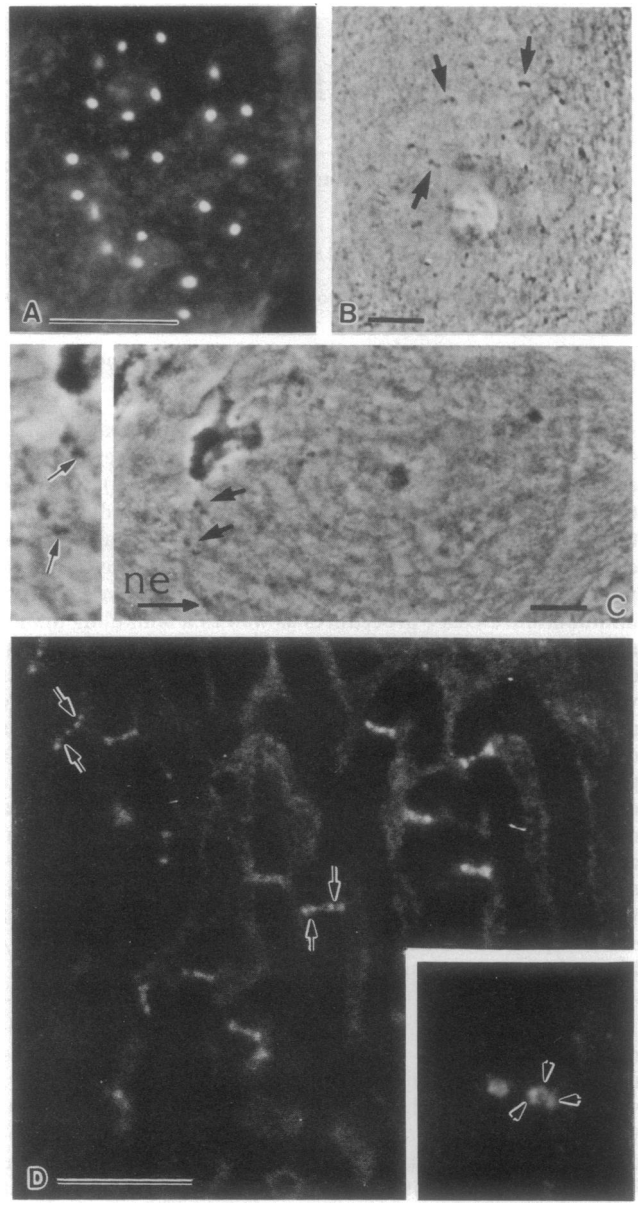

FIG. 1. Kinetochores in *Haemanthus* stained by indirect immunofluorescence using a fluorescein isothiocyanate-conjugated secondary antibody or the IGSS procedure using a secondary antibody conjugated to 5-nm gold. Prekinetochores in interphase nuclei are single (*A*) or double (*B*) (arrows). Further subdivision of kinetochores into quadruple arrangements occurs in prophase (arrows in *C* and *Inset*) and by prometaphase (*D*) (arrows) sister kinetochores on each chromatid contain two fluorescent spots. Favorable images suggest that each sister kinetochore may be composed of even smaller subunits (*D Inset*). Ne, nuclear envelope. (Bars equal 5 μm.)

The number, size, and form of immunostained spots varied considerably in interphase nuclei. In some, the prekinetochores appeared as single discrete spots (Fig. 1*A*) and, in the majority, the number of spots was consistent with the chromosome number of 27 (endosperm of *Haemanthus* is a triploid tissue). Some images of prekinetochores appeared to be composed of two smaller subunits that were very tightly bound together. This bipartite appearance was discernible in the microscope but difficult to record on photographic film. In other nuclei, however, the prekinetochores were clearly double (Fig. 1 *B* and *C*) and even quadruple (Fig. 1*C Inset*) in structure. The appearance of double fluorescent spots has been described in cultured mammalian cells and shown to represent kinetochore duplication occurring in the late S or G_2

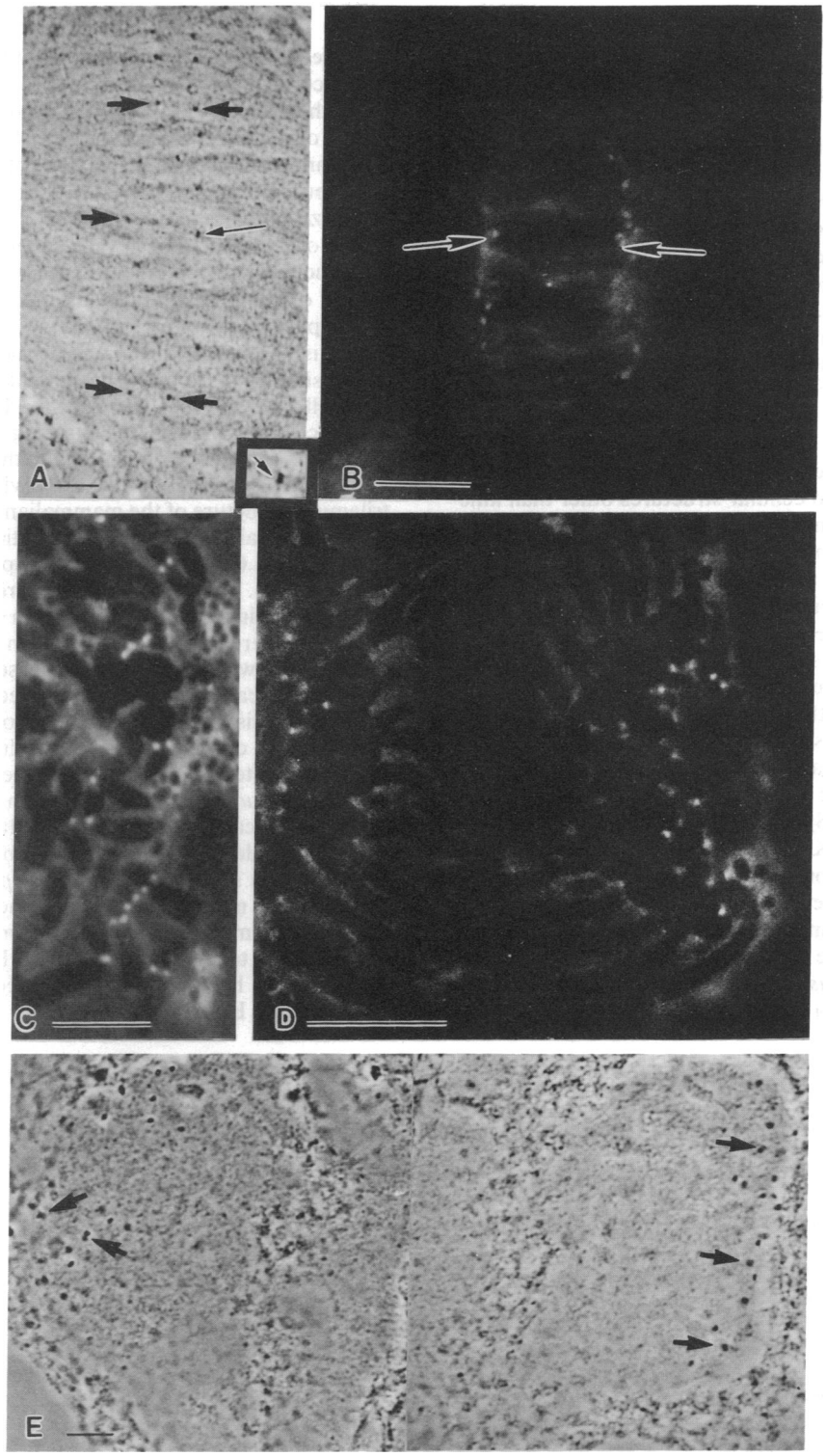

FIG. 2. Kinetochores in metaphase (*A* and *C*), anaphase (*B*), telophase (*D*), and daughter nuclei (*E*). At metaphase (*A*) double sister kinetochores (arrows and *Inset*) are oriented toward opposite poles. The organization of paired kinetochores at metaphase (*B*) (arrows) can also be seen in fluorescence images but kinetochore substructure is not as well resolved as in the IGSS preparations (compare *A* and *E* with *B*, *C*, and *D*). The kinetochores in daughter nuclei are clearly double (*E*) (arrows). (Bars equal 5 μm.)

phase of the cell cycle (5). Our observations suggest that kinetochore duplication also occurs during interphase in plant cells, but other evidence (cited below) indicates that prekinetochores are already bipartite at the time of duplication, resulting in a quadruple organization in late G_2 or mitosis.

We could not detect clear transitions between stages when double prekinetochores quadrupled. Subunits seemed to stain with different intensities that perhaps reflected some asynchrony in prekinetochore development. By mid-prophase however, each sister kinetochore appeared double and a total of four granules were positioned across the centromere (Fig. 1*C Inset*). At prometaphase most of the chromosomes displayed four discrete granules as shown in Fig. 1*D*. In some preparations, even smaller substructures could be detected (Fig. 1*D Inset*), suggesting that each sister kinetochore was a compound structure made up of multiple subunits. The

quadruple structure was most obvious at prometaphase where four discrete granules (two granules per chromatid) formed a band across the centromere (see arrows in Fig. 1*D*). Each sister kinetochore had one subunit on the outside of the centromere and another positioned on the inner centromere region. By metaphase, the tandem subunits on each chromatid were shifted such that they were arranged parallel to the long axis of the chromosome and perpendicular to the spindle axis (Fig. 2*A Inset*). This orientation coincides with the attachment of a double kinetochore fiber to each sister kinetochore as described (16) and was maintained through anaphase and telophase. It should be noted that the double appearance of sister kinetochores during the metaphase–anaphase transition was best resolved in the IGSS preparations and was less clearly seen in conventional immunofluorescence preparations of cells at metaphase (Fig. 2*C*), anaphase (Fig. 2*B*), and telophase (Fig. 2*D*).

It should also be noted that the silver-enhancement procedure added contrast to cellular structures other than kinetochores. Spurious staining was also noted in control preparations where the primary antibody was omitted from the procedure, suggesting a nonspecific affinity of silver ions for various plant cell proteins. Indeed some nonspecific background staining of undefined cytoplasmic components was also seen in preparations stained by indirect immunofluorescence using only the fluorescein isothiocyanate-conjugated secondary antibody in the staining steps.

In addition to *in situ* staining, human autoantibodies from scleroderma CREST patients were also useful probes for identifying centromeric proteins by Western blot analysis after separation by SDS/gel electrophoresis. In HeLa cells, serum from patient EK recognized nuclear proteins of 52 kDa, 80 kDa, and two or three minor bands of 140–200 kDa (Fig. 3, lane 3). A protein of 110 kDa was also observed in normal human serum and was thus discounted as a kinetochore protein. When the same procedure was used to analyze proteins of *Haemanthus* endosperm cells, we detected proteins of 65 and 135 kDa (Fig. 3, lane 6).

DISCUSSION

The use of human autoantibodies directed against centromeric components has provided considerable knowledge about the structure, distribution, and duplication of kinetochores of animal chromosomes and has helped to identify a small family of proteins associated with the centromere (6, 8, 9). To our knowledge, however, the present study is the first to utilize autoantibody probes to characterize the kinetochores of higher plants. In view of the universal similarity in the structure and outcome of mitosis in eukaryotic cells, the finding of centromeric proteins with common epitopes between plant and animal cells is not, in itself, unexpected. There is, however, surprising variation in the molecular composition and structural design of kinetochores in eukaryotic cells (for review, see ref. 2). The centromeric DNA sequences identified in the budding yeast *Saccharomyces cerevisiae* display little homology to those of the fission yeast *Schizosaccharomyces pombe* (for review, see ref. 17) and the trilaminar structure of the mammalian kinetochore is distinct from the "ball and cup" organization of kinetochores in *Haemanthus* (18) and other plant species (for review, see refs. 1 and 2). Other structural features in *Haemanthus* also differ, including the early separation of sister chromatids at the centromere and the organization of sister kinetochores into at least two distinct granules as seen in light microscopy (19) but not easily discernible by electron microscopy. It is not clear if this reflects the "half-chromatid" organization of *Haemanthus* chromosomes seen in living cells (20).

The bipartite and even quadripartite nature of kinetochores in *Haemanthus* has been described in other plant species. By using aceto-carmine- or Feulgen-stained squash preparations, Lima-de-Faria (21, 22) described the kinetochores of *Tradescantia* species and *Allium cepia* as being compound structures in metaphase chromosomes consisting of four to eight chromomeres (two to four chromomeres per chromatid) arranged as a tandem reverse repeat. Evidence for compound kinetochores has also been presented for mammalian chromosomes by Brinkley *et al.* (23).

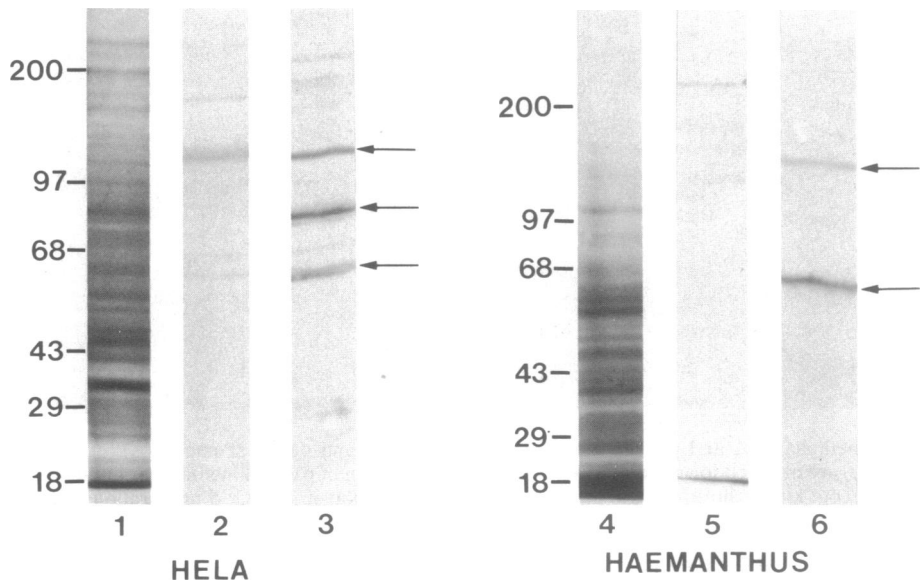

FIG. 3. Western blots of HeLa nuclear extracts and *Haemanthus* total cell extracts. Lane 1: Coomassie-stained gel of HeLa cell nuclear proteins. Lane 2: negative control blot using normal human serum RB (1:1000 dilution). Note reaction with protein(s) of 110 kDa. Lane 3: HeLa cell nuclear extract blotted with CREST serum EK (1:1000 dilution). The arrows to the right of lane 3 represent antigens that are consistently recognized with this serum (≈52, 80, and 110 kDa). Minor bands ≈140 kDa can also be seen. Lane 4: Coomassie-stained gel of *Haemanthus* whole cell extract. Lane 5: immunoblot of *Haemanthus* whole cell extract with normal control human serum RB (1:1000 dilution). Lane 6: immunoblot of *Haemanthus* whole cell extract using EK serum (1:1000 dilution). The arrows to the right of lane 6 represent the bands that are most consistently recognized by this serum. The approximate molecular mass of the proteins are 65 and 135 kDa. Molecular masses in kDa are indicated.

The bipartite nature of kinetochores in *Haemanthus* has functional significance and is reflected in the substructure of the bundle of microtubules (kinetochore fiber) that engage the kinetochore throughout the course of mitosis (17). Microtubules bind to kinetochores at their positive or fast-assembling ends (24) and, during prometaphase movement, the kinetochore fiber forms a double structure becoming less pronounced in later stages of mitosis (17). Thus, each kinetochore subunit appears to bind a separate bundle of microtubules extending from the spindle poles. Apparently, the polar-directed forces acting through the kinetochore fibers shift the subunit orientation from a side-by-side arrangement on each prometaphase chromatid, to one where the subunits are arranged one on top of the other facing the same pole at metaphase. The shift in subunit orientation along with the observation that a double bundle of spindle fibers insert into the kinetochore, strongly suggests that both kinetochore subunits are indeed functional.

Whereas conventional staining techniques limit observations of kinetochores to mitotic or meiotic stages, immunostaining provides a unique opportunity to detect kinetochores at all stages in the cell cycle. Collectively, our observations indicate that the kinetochores of *Haemanthus* chromosomes undergo structural and numerical changes during the cell cycle, much the same as kinetochores of mammalian chromosomes (5). At telophase each kinetochore is a double unit but, later in the interphase cycle, this doubled appearance becomes less distinct and kinetochores appear as single spots. At an even later stage, presumably late S or G_2 phase, the prekinetochores are duplicated and each fluorescent spot again becomes double. Further subdivision is seen as cells progress into prophase or prometaphase where four subunits (two per chromatid) are arranged in a line across the centromere. In some orientations, each sister kinetochore appeared to be composed of three to four smaller subunits as suggested in the tandem inverse repeat model of plant kinetochores proposed by Lima-de-Faria (21, 22) and the compound kinetochore model of mammalian chromosomes proposed by Brinkley *et al.* (23).

Sera from 57–90% of all patients with the CREST variant of scleroderma recognize a small family of proteins found in the centromere of mammalian chromosomes (6). Although some variation exists between patients, centromere proteins of 17–18 kDa (CENP-A), 80 kDa (CENP-B), and 140 kDa (CENP-C) have been consistently reported by Western blot analysis (6, 9). Another protein of ≈50 kDa (CENP-D) has been identified by sera from some patients (7). The EK serum used in the present study recognized epitopes on proteins of 52 and 80 kDa and several minor bands of ≈140 kDa from HeLa nuclear extracts, when tested in parallel with *Haemanthus* extracts. Although the molecular mass of proteins in *Haemanthus* nuclei (65 and 135 kDa) differ significantly from HeLa, the presence of common epitopes between these two widely divergent species indicates a striking conservation of protein components throughout evolution. Although the molecular organization and architecture of kinetochores may vary considerably in different species, evidence for common epitopes on nuclear proteins is in keeping with the conservation of function in this essential component.

Autoimmune reactions are believed to be antigen driven by immunogens localized in dynamic subcellular particles whose epitopes are either active or functional regions of the immunogen (25, 26). These and other studies have presumed that the immunogens were of human origin. The present study along with a report (27) that scleroderma autoantibodies recognize components of the nucleolus in meristematic cells of onion and soybean roots indicate that autoantigens are more widely distributed in nature than previously thought. Nucleoproteins from plant tissues should now be added to the list of possible immunogens associated with this autoimmune disease.

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1. Rieder, C. L. (1982) *Int. Rev. Cytol.* **79,** 1–58.
2. Brinkley, B. R., Valdivia, M. M., Tousson, A. & Balczon, R. D. (1989) in *Mitosis: Molecules and Mechanisms.* eds. Hyams, J. & Brinkley, B. R. (Academic, London), pp. 77–118.
3. McClintock, B. (1938) *Genetics* **23,** 315–376.
4. Moroi, Y., Peeples, C., Fritzler, M. J., Steigerwald, J. & Tan, E. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1627–1631.
5. Brenner, S., Pepper, D., Berns, M. W., Tan, E. & Brinkley, B. R. (1981) *J. Cell Biol.* **91,** 95–102.
6. Earnshaw, W. C. & Rothfield, N. F. (1985) *Chromosoma (Berlin)* **92,** 290–296.
7. Kingwell, B. & Rattner, J. B. (1987) *Chromosoma (Berlin)* **95,** 403–407.
8. Earnshaw, W. C., Sullivan, K. F., Machlin, P. S., Cooke, C. A., Kaiser, D. A., Pollard, T. D., Rothfield, N. R. & Cleveland, D. W. (1987) *J. Cell Biol.* **104,** 817–829.
9. Valdivia, M. M. & Brinkley, B. R. (1985) *J. Cell Biol.* **101,** 1124–1134.
10. Hoefsmit, E. C. M., Korn, C., Blijleven, N. & Ploem, J. S. (1986) *J. Microsc. (Oxford)* **143,** 161–169.
11. Mole-Bajer, J. & Bajer, A. S. (1988) *Protoplasma Suppl.* **1,** 99–112.
12. Towbin, H., Staehlin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
13. Mitchison, T. J. & Kirschner, M. W. (1985) *J. Cell Biol.* **101,** 755–765.
14. Avivi, L. & Feldman, M. (1980) *Hum. Genet.* **55,** 281–295.
15. Fussell, C. P. (1987) in *Meiosis,* ed. Moens, P.B. (Academic, New York), pp. 275–299.
16. Bloom, K. & Yeh, E. (1989) *Curr. Opin. Cell Biol.* **1,** 526–532.
17. Bajer, A. S. (1987) *Eur. J. Biol.* **43,** 23–34.
18. Bajer, A. S. & Mole-Bajer, J. (1969) *Chromosoma (Berlin)* **27,** 488–484.
19. Ostergren, G. (1951) *Hereditas* **35,** 85–156.
20. Bajer, A. S. & Ostergren, G. (1961) *Hereditas* **47,** 563–598.
21. Lima-de-Faria, A. (1956) *Hereditas* **42,** 85–160.
22. Lima-de-Faria, A. (1958) *Int. Rev. Cytol.* **7,** 123–157.
23. Brinkley, B. R., Valdivia, M. M., Tousson, A. & Brenner, S. L. (1984) *Chromosoma (Berlin)* **91,** 1–11.
24. Mitchison, T. J. (1988) *Annu. Rev. Cell Biol.* **4,** 527–549.
25. Tan, E. M. (1989) *Adv. Immunol.* **44,** 93–151.
26. Tan, E. M. (1989) *J. Clin. Invest.* **84,** 1–6.
27. Vaughn, M. A. (1987) *Histochemistry* **86,** 533–535.