Kinetochore Structure, Duplication, and Distribution in Mammalian Cells: Analysis by Human Autoantibodies from Scleroderma Patients

SARI BRENNER, DANIEL PEPPER, M. W. BERNS, E. TAN, and B. R. BRINKLEY
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, High Voltage Electron Microscope Laboratory, Madison, Wisconsin 53706, Department of Developmental and Cell Biology, University of California, Irvine, Irvine, California 92717, and Department of Medicine, University of Colorado, Medical Center, Denver, Colorado 80262

ABSTRACT The specificity of the staining of CREST scleroderma patient serum was investigated by immunofluorescence and immunoelectron microscopy. The serum was found to stain the centromere region of mitotic chromosomes in many mammalian cell types by immunofluorescence. It also localized discrete spots in interphase nuclei which we have termed "presumptive kinetochores." The number of presumptive kinetochores per cell corresponds to the chromosome number in the cell lines observed. Use of the immunoperoxidase technique to localize the antisera on PtK_2 cells at the electron microscopic level revealed the specificity of the sera for the trilaminar kinetochore disks on metaphase and anaphase chromosomes. Presumptive kinetochores in the interphase nuclei were also visible in the electron microscope as randomly arranged, darkly stained spheres averaging 0.22 μm in diameter. Preabsorption of the antisera was attempted using microtubule protein, purified tubulin, actin, and microtubule-associated proteins. None of these proteins diminished the immunofluorescence staining of the sera, indicating that the antibody-specific antigen(s) is a previously unrecognized component of the kinetochore region. In some interphase cells observed by both immunofluorescence and immunoelectron microscopy, the presumptive kinetochores appeared as double rather than single spots. Analysis of results obtained using a microspectrophotometer to quantify DNA in individual cells double stained with scleroderma serum and the DNA fluorescent dye, propidium iodide, led to the conclusion that the presumptive kinetochores duplicate in G_2 of the cell cycle.

The centromere or kinetochore region in most animal cells can be seen in the light microscope as a localized constriction appearing on otherwise linear metaphase chromosomes. It is at this region that the sister chromatids, attached along their entire lengths, first separate at the onset of anaphase. The region also functions as the site of spindle fiber (microtubule) attachment. The placement of the centromere on the chromosome may differ from chromosome to chromosome, but there is a constant and recognizable location on homologous chromosomes and a specific pattern within a given species.

In the electron microscope, the mitotic centromere is also recognized by its constricted configuration and by the presence in some species of a distinct, specialized structure called the kinetochore. The ultrastructure of the kinetochore varies in plant and animal species and has been especially well described in mammalian cells (7). Briefly, it appears to have a trilaminar morphology in cross section, with an electron-dense outer plate, lightly staining middle layer, and a darker inner area immediately adjacent to the underlying centromeric heterochromatin. Spindle microtubules are specifically attached to the outer plate. There is also compelling evidence to suggest that kinetochores serve as assembly sites or organizing centers for the chromosomal microtubules (10, 12, 16, 21, 23).

The chemical makeup of the kinetochore is largely unknown,

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 OCTOBER 1981 95-102
© The Rockefeller University Press · 0021-9525/81/10/0095/08 $1.00

although cytochemical studies have shown that DNA (17) and RNA (4, 17, 18) are probably present and that DNA plays a role in maintenance of kinetochore structure (17). Tubulin has also been localized in the mitotic mammalian kinetochore (15) by immunoelectron microscopy and has been found to play an essential role in microtubule assembly (16).

At the end of mitosis, the specialized kinetochore structure as well as the location of the centromere is lost to view by existing techniques for the duration of interphase. Part of our work will make use of a new immunological probe which allows visualization of this area during interphase.

Recently, much attention has been given to the detection of antinuclear antibodies (ANA) in human patient sera for diagnosis of and distinction between certain rheumatic diseases. These diseases include systemic lupus erythematosus, Sjogren's sicca complex, mixed connective tissue disease, and scleroderma (both linear and systemic sclerosis). Many of the sera from patients with these diseases show distinct patterns of nuclear fluorescence when used as indirect immunofluorescent probes on mouse-tissue frozen sections or HEp2 tissue-culture cells. Recent reports (8, 13) have correlated the presence of a centromere specific serum autoantibody with the CREST variant of systemic sclerosis. In the two studies mentioned above, >90% (33 of 35 patients studies) of CREST syndrome sclero-

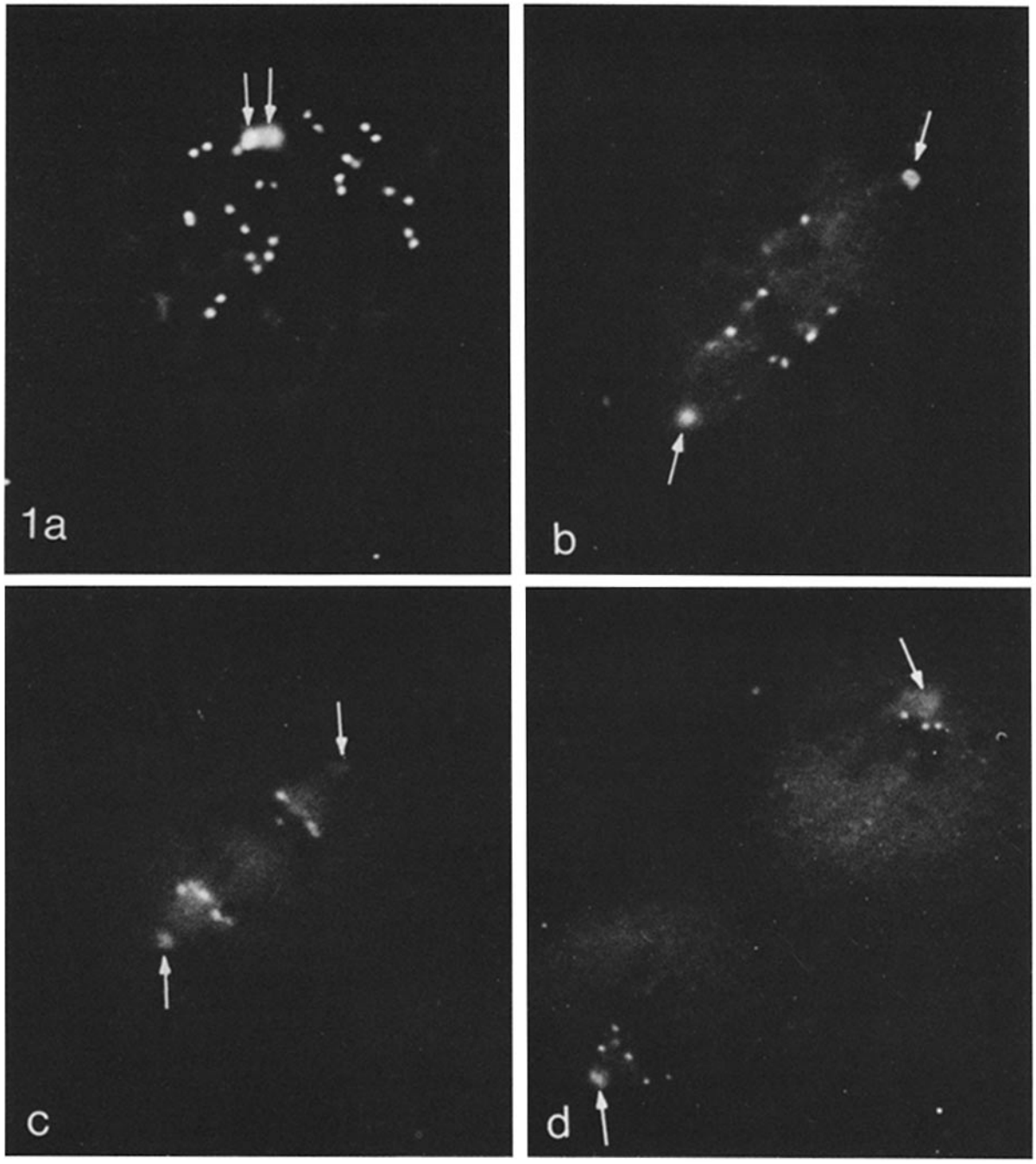

FIGURE 1 Immunofluorescent localization of scleroderma anticentromere serum in PtK_2 cells. All cells have been double-stained with a human anticentrosome serum (arrows point to centrosomes) (5) to provide orientation. (*a*) Prophase PtK_2 cell exhibits a single pair of fluorescent spots on each chromosome. × 2,200. (*b*) At metaphase the chromosomes are lined up at the metaphase plate. × 2,200. (*c*) Anaphase. × 2,200. (*d*) Telophase. × 1,800.

derma patients have this antibody. The staining pattern is quite recognizable in metaphase chromosome spreads, where the centromere region is discretely fluorescent. On tissue sections or tissue-culture cells, the mitotic cells also exhibit centromere fluorescence. A most interesting finding is that the antiserum stains a distinct number of sites in the interphase nuclei of cells. Enzyme digestion experiments presented in one of these studies (13) indicated that the antigen stained by this antibody is a protein bound strongly to DNA at the centromere region.

This study further characterizes CREST scleroderma patient sera by immunofluorescence and immunoelectron microscopy and reveals the specificity of the antiserum from the kinetochore components and absence of localization in other components of the centromere. Also included is a study of the structure and distribution of interphase "presumptive kinetochores" by conventional and high voltage electron microscopy and a study of the effects of microtubule depolymerization by colcemid on kinetochore structure as revealed by this antiserum. An analysis of kinetochore separation during the cell cycle is accomplished using microspectrophotometry to monitor DNA replication.

MATERIALS AND METHODS

Rat kangaroo (PtK_2) and muntjac cells were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) and grown in Ham's F-10 plus 10% fetal calf serum (FCS) plus 2 mM glutamine. Swiss mouse 3T3 cells were also obtained from the ATCC and grown in Dulbecco's minimal essential medium (MEM) plus 10% FCS. Chinese hamster (CHO-Kl) cells were grown in McCoy's 5A (Hsu's modification) supplemented with 2% FCS and 7% horse serum. Mouse L-929 and normal rat kidney (NRK) cells were obtained from the ATCC and were provided by R. Brown (Frederick Cancer Research Center, Frederick, Md.), as were human foreskin fibroblasts at passage 30.

Antisera

Autoantibodies against the centromere were found initially in scleroderma patient sera by Moroi et al. (13). In this study, sera from two patients were used and found to be identical. The antisera were stored frozen or at 4°C with 0.02% sodium azide. For use in immunofluorescence studies, the antiserum was diluted 1:100 to 1:400 with Dulbecco's phosphate-buffered saline (PBS). Fluorescein-labeled anti-human IgGs (Miles-Yeda Laboratories, Elkhart, Ind.) were diluted 1:20 in PBS. Cells on cover slips were prepared for immunofluorescence by rinsing in PBS and fixing in 3% formaldehyde in PBS for 20 min. Cells were then rinsed again and placed in a solution of 0.05% Triton X-100 in PBS for 90 s. After rinsing with PBS, cells were incubated in diluted antisera for 30 min at 37°C, rinsed for 30 min in PBS, and incubated for 30 min at 37°C with the appropriate fluorescently labeled anti-IgGs. After rinsing, cells were mounted in 9:1 glycerol:PBS for observation.

Colcemid Treatment

In some experiments, cell cultures were treated with 0.06 to 0.1 μg/ml colcemid for 1–3 h before fixation.

Preabsorption of Antisera with Tubulin, Actin, and Microtubule-associated Proteins

10 μl of the centromere specific antisera were incubated for 48 h at 4°C with one of each of the following: 70 μl of phosphocellulose purified 6S bovine brain tubulin (tubulin concentration of 1.5 mg/ml) or 25 μl of a 2.5 mg/ml solution of skeletal muscle G-actin, a gift of J. Bryan, or 200 μl of a solution of phosphocellulose purified microtubule-associated proteins (MAPs) (0.9 mg/ml). 6S tubulin and MAPs were purified according to the procedure of Borisy et al. (3). Protein determinations were done by the Schacterle and Pollack modification of the Lowry procedure (11, 20). After 48 h, the sera were diluted to a final volume of 1 ml, giving a final dilution of 1:100, and used as described above for immunofluorescence.

Immunoelectron Microscopy

Cells were processed for electron microscopy by the immunoperoxidase procedure as described by Pepper and Brinkley (15). PtK_2 cells (kangaroo) were grown in 60-mm Lux Permanox dishes (Lux Scientific Corp., Newbury Park, Calif.) or on 11 × 22-mm cover slips. The cells were rinsed in PBS for 5 min., then fixed in 3% formaldehyde in PBS for 20 min. They were then rinsed two times for 4 min in 0.5 mg/ml sodium borohydride and then rinsed two times for 1 min in 50 mM Tris-HCl, pH 7.5. Cells were then mildly lysed for 90 s in 0.05% Triton X-100 in Tris, and washed with buffer. They were subsequently incubated in either anticentromere or control human serum for 30 min at 37°C, washed three times for 10 min in Tris, and incubated in peroxidase-conjugated anti-human IgG (Miles-Yeda Laboratories) for 30 min at 37°C. After a final 30-min Tris wash, cells were fixed in a 2% glutaraldehyde (in Tris), rinsed, and incubated for 5 min in a solution of 0.02% diaminobenzidine (DAB) and 0.02% H_2O_2 in Tris buffer. After a 5-min water wash, the cells were incubated in 1% OsO_4 for 10 min and washed with water. Cells on cover slips were then mounted for light microscopy in glycerol as described above. Cell monolayers grown in Lux dishes for electron microscopy were run through a graded series of ethanols and embedded in a medium soft formulation of Spurr's (22) low viscosity epoxy resin. Individual cells were chosen for thin serial sectioning by processes previously described (6). Some cells were also chosen for thick (0.3–0.5 μm) serial sectioning and were viewed in the high voltage electron microscopy (HVEM) at the University of Wisconsin at Madison. Thick or thin sections were picked up on single-slot (1 × 2 mm) formvar-coated copper grids and examined at 60–80 kV (thin sections) or in the HVEM without lead or uranyl acetate staining.

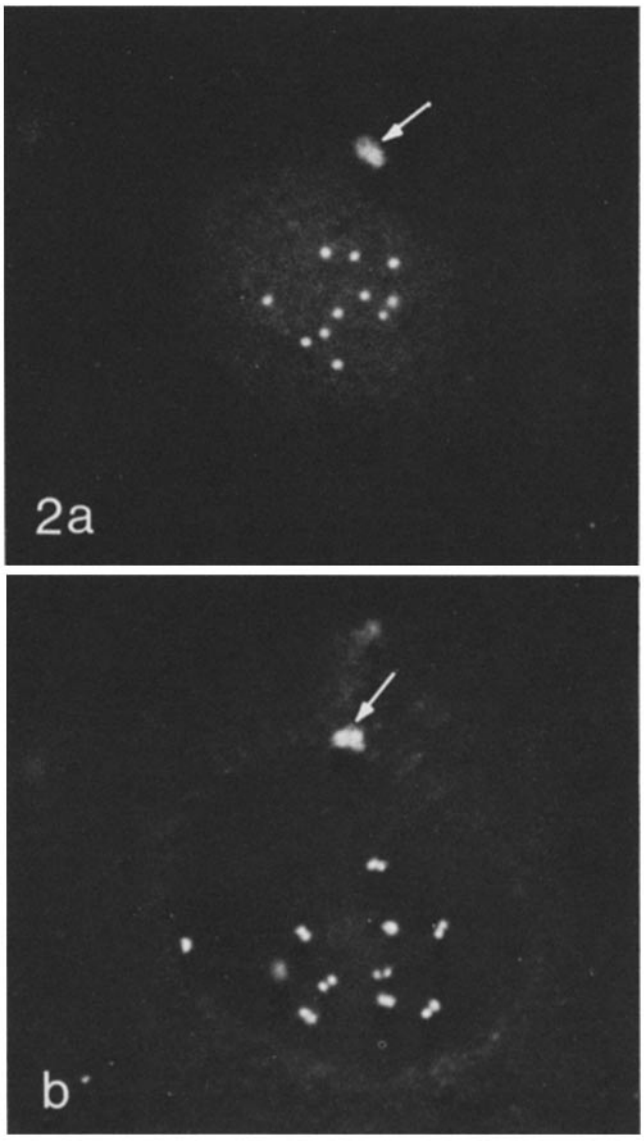

FIGURE 2 Interphase presumptive kinetochores observed by indirect immunofluorescence. The cells have also been double-stained with anticentrosome antisera as in Fig. 1 (arrows). *(a)* Interphase cell with single presumptive kinetochores. Eleven are visible in the focal plane of the photograph. Thirteen can usually be found in PtK_2 (2n = 13). *(b)* Interphase cell with doubled pre-kinetochores. × 2,600.

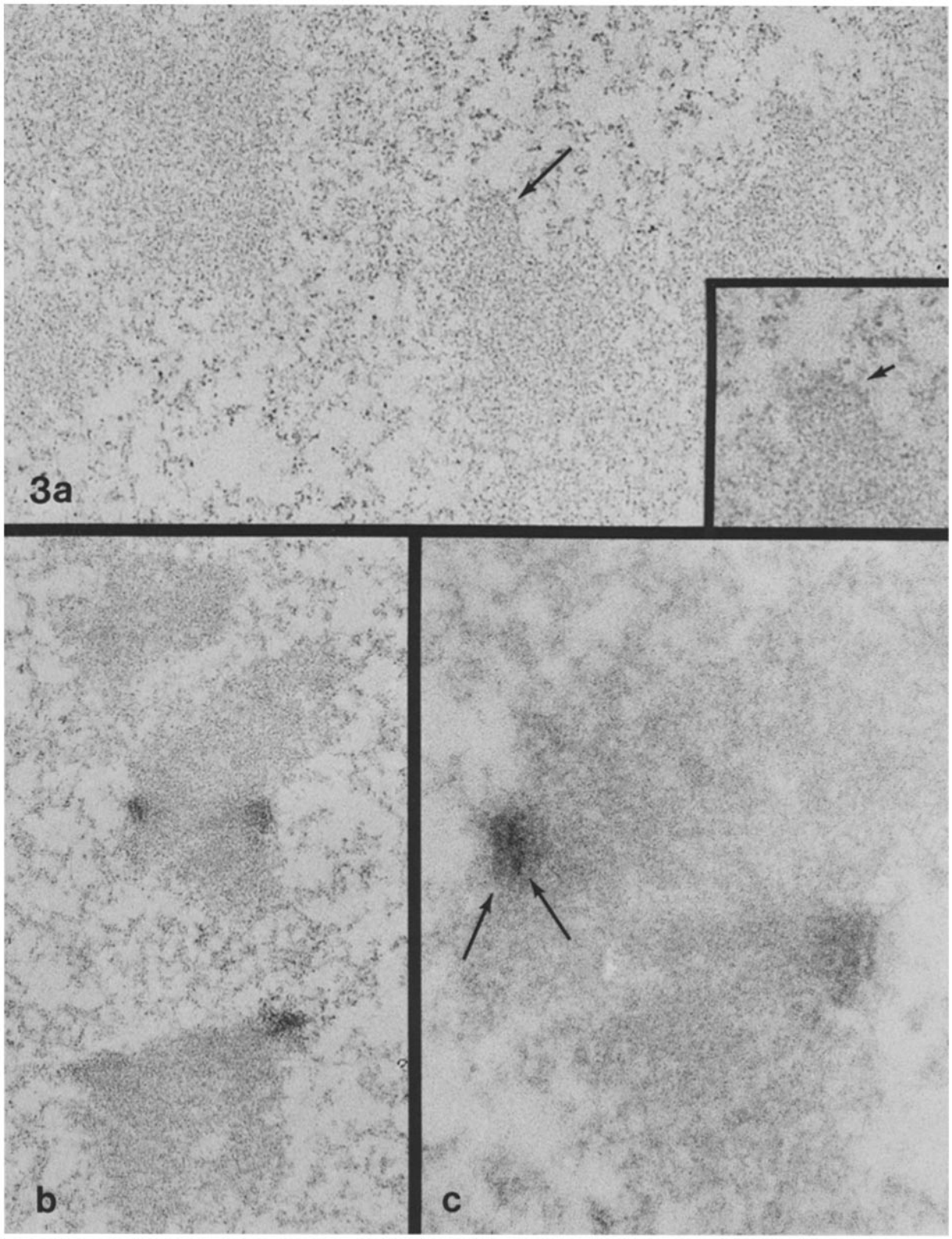

FIGURE 3 Indirect immunoperoxidase localization of scleroderma anticentromere serum by thin-section electron microscopy. (*a*) Control kinetochore in a PtK_2 cell stained by the immunoperoxidase reaction with normal human serum. Note the lack of contrast. Arrow points to kinetochore. × 23,000. *Inset:* kinetochore at higher magnification. × 40,000. (*b*) Metaphase cell stained with scleroderma serum. The kinetochores are specifically and darkly stained in comparison to Fig. 4 *a*. × 15,700. (*c*) Higher magnification of metaphase chromosome showing the plate structure of the kinetochore (arrows). Note the lack of staining of the underlying centromeric chromatin. × 57,200.

Cell Synchronization

Indian muntjac cells were grown in glass Blake bottles. For cell synchrony studies, cells were treated with 0.06 mg/ml colcemid for 2 h and mitotic cells were detached by shake off. The mitotic cells were replated into media without colcemid on 11 × 22-mm glass cover slips. 4 h after attachment, the cells were fixed and stained with scleroderma serum as described above.

Microspectrophotometry

To determine the phase of the cell cycle at which kinetochores duplicate, microspectrophotometric analysis was carried out on individual PtK_2 cells which had been double-stained with propidium iodide (for DNA) (9) and anticentromere sera with fluorescein-conjugated antihuman IgG tag. For microspectrophotometry, cells were fixed for 7 min in 70% ice-cold ethanol, rinsed in PBS, and stained as described above with antiserum and fluorescein-tagged anti-human IgG. Before mounting, cells were incubated for 5 min in 30 μg per ml propidium iodide in PBS and rinsed for 5 min in PBS.

Analysis was done using a Nanospec 10s computerized microspectrophotometer system (Nanometrics, Inc., Sunnyvale, Calif.) fitted to a Zeiss Universal fluorescence microscope. The spectrophotometer incorporates a monochrometer as well as a sensitive photomultiplier capable of detection over the 200–900 nm range. Ten readings per cell were taken and averaged by a Hewlett Packard computer (Packard Instrument Co., Inc., Downers Grove, Ill.) interfaced to the spectrophotometer. The latter was also equipped with a variable slit aperture which was used to delineate the desired area. In this case, the slit was set to a width slightly larger than the diameter of an interphase nucleus. Propidium iodide fluorescence intensity of each individual cell at 620 nm (the experimentally determined maximum emission wavelength) was measured using an excitation filter for fluorescence (excitation peak at 546, barrier filter pass >590), which does not excite fluorescein isothiocyanate. The speckled staining pattern was then quantified by counting the number of discrete fluorescent spots in each cell, using fluorescein excitation filters (excitation 450–490 nm, barrier 520–560 nm). Background of the propidium iodide staining was found to be extremely low as indicated by a near zero reading both with the light to the spectrophotometer blocked and the slit positioned slightly off interphase nuclei.

RESULTS

The serum from scleroderma CREST patients was found to specifically bind to the centromere in many diverse cell types: these include CHO-Kl, kangaroo PtK_2, Indian muntjac, mouse 3T3 and L-929, and human fibroblasts. The staining specificity for the centromere region is most obvious in mitotic cells. A series of mitotic PtK_2 cells stained with the scleroderma serum is shown in Fig. 1. At prophase and metaphase, the fluorescence is confined to the centromere region of the chromosome and is visible as paired spots at the primary constriction on every chromosome. At anaphase and telophase, the now single fluorescent regions are localized at the leading portion of the chromatids as they move toward the spindle poles (Fig. 1 *c* and *d*). After telophase, the staining persists in interphase cells (Fig. 2). A majority of cells show discrete spots in the nucleus. A small number of cells show paired spots as illustrated in Fig. 2 *b*.

To determine the relationship between the number of spots in the nucleus and the chromosome number, Indian muntjac ($2n = 7$) cells were synchronized and stained as described in Materials and Methods. The distribution of numbers of nuclear spots in G_1 centered at seven spots per nucleus, corresponding to the diploid chromosome number in muntjac (1).

To determine the ultrastructural specificity of the scleroderma anticentromere serum, the indirect immunoperoxidase method was used to localize the serum at the electron microscopic (EM) level. Figs. 3 and 4, respectively, illustrate thin sections viewed by conventional transmission EM and thick section viewed by HVEM. In both serial thin sections and thick sections, the antiserum was found to stain both the inner and outer plates of the kinetochore, as well as the immediately adjacent chromatin fibers. The staining distinguishes the kinetochores entirely from the underlying unstained centromeric chromatin. More than 120 interphase cells were examined by both conventional and HVEM (see Fig. 5). The antiserum-stained cells displayed small, spherical, electron-dense sites in their nuclei which averaged 0.22 μm in diameter. In view of

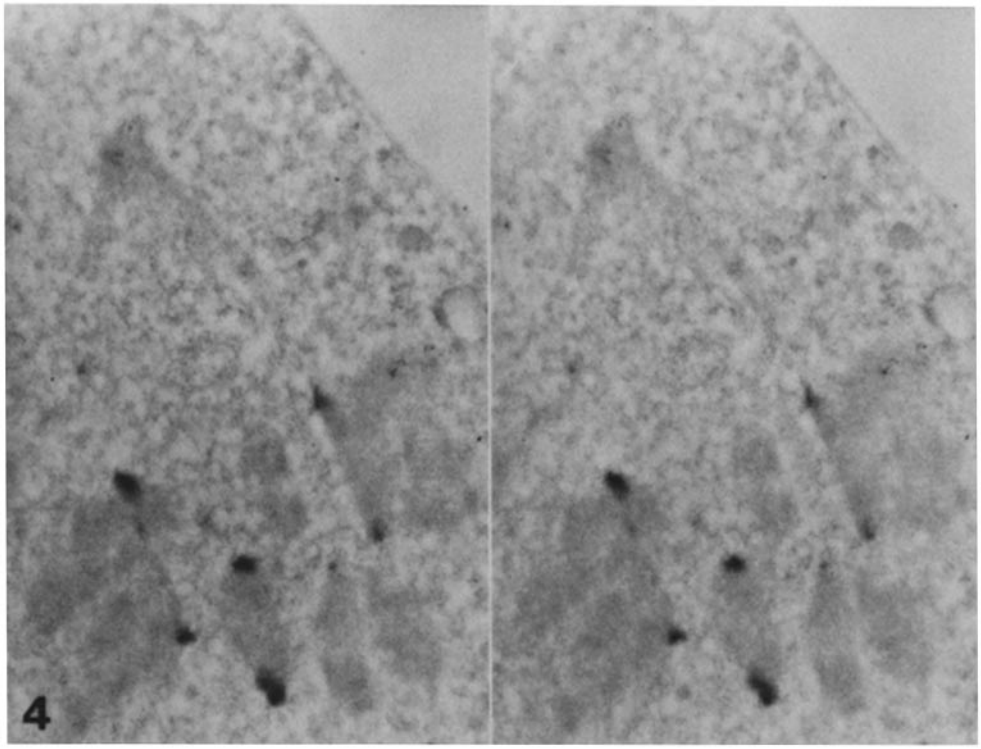

FIGURE 4 High-voltage stereo electron micrographs of a thick-sectioned metaphase cell. The kinetochores are densely and specifically stained. × 7,000.

the amorphous appearance of these sites and their dissimilarity to stained regions on metaphase chromosomes, we termed them "presumptive kinetochores" (pre-kinetochores). There were generally 12–13 pre-kinetochores in each PtK_2 nucleus, corresponding to the 2n chromosome number. The images observed by EM corresponded well to the immunofluorescent structures observed in the nuclei of PtK_2 cells (see Fig. 2). In some interphase cells the pre-kinetochores appeared doubled when viewed by EM (Fig. 5*b*). Subsequently, we shall provide evidence that such cells were probably in very late G_2 phase of the cell cycle.

Special attention was paid to the distribution of these pre-kinetochores in interphase nuclei. In very recent work by Moroi et al. (14) on CHO cells and Ramos human B-lymphoid cell line, pre-kinetochores seemed to be associated with the nuclear membrane. No consistent association could be found between the pre-kinetochores and the nuclear envelope in PtK_2 and, in general, the arrangement of the stained spots appeared random in the interphase nucleus. An exception to this was late telophase/early G_1, when the pre-kinetochores could be seen localized in the area of the nucleus nearest to the previous spindle pole, as might be expected. In addition, one (and sometimes two) pre-kinetochore was often seen closely apposed to the nucleolus (Fig. 5) in many interphase cells. This is possibly a manifestation of the proximity of the centromere and nucleolus organizer to each other on the X chromosome of PtK_2 cells (2), but was also observed by Moroi et al. (14), who used Chinese hamster ovary cells, which do not have centromere and nucleolus organizers in proximity. To further examine the possible role of microtubules in the structural maintenance of the kinetochore in terms of this new kinetochore antigen, colcemid-treated cells were examined by both immunofluorescence and electron microscopic immunoperoxidase procedures. The location and ultrastructure of the interphase pre-kinetochores appeared unaltered by colcemid treatment, as did pre-kinetochores in early prophase cells. Mitotic cells assumed the familiar "c" metaphase configuration (7) and the kinetochore staining was visible as a ring pattern by fluorescence as shown in Fig. 6*a*. The ultrastructural localization of the antiserum is shown in Fig. 6*b*. Note that the staining reveals two obvious layers of the kinetochore, as compared to conventionally stained colcemid-treated kinetochores which show only one distinct layer. The length of the stained kinetochore increased approximately fourfold, a finding also reported in conventional preparations (19).

As previously mentioned, it was noticed during examination of large numbers of interphase PtK_2 or muntjac cells that a very small percentage of cells (<0.5%) exhibited paired fluorescent spots (see Fig. 2*b*). To correlate the doubling of pre-kinetochores with the cell cycle in PtK_2, cells were double-stained with propidium iodide and antikinetochore serum. Individual cells were analyzed with the microspectrophotometer as described in Materials and Methods. Late telophase/early interphase cells (containing the G_1 or 2n,2c amount of DNA) or prophase, metaphase, and early anaphase cells (which contain the G_2 or 2n,4c amount of DNA) were measured as calibration standards on each slide. Fig. 7 shows the distribution of fluorescence intensities at 620 nm (the emission maximum for propidium iodide) of late telophase/early interphase cells (G_1) and of prophase, metaphase or early anaphase (G_2) cells. Interphase cells were then analyzed by counting the pre-kinetochores in each individual cell using the fluorescein excitation filters. The number of pre-kinetochores was plotted against the propidium iodide fluorescence by using propidium

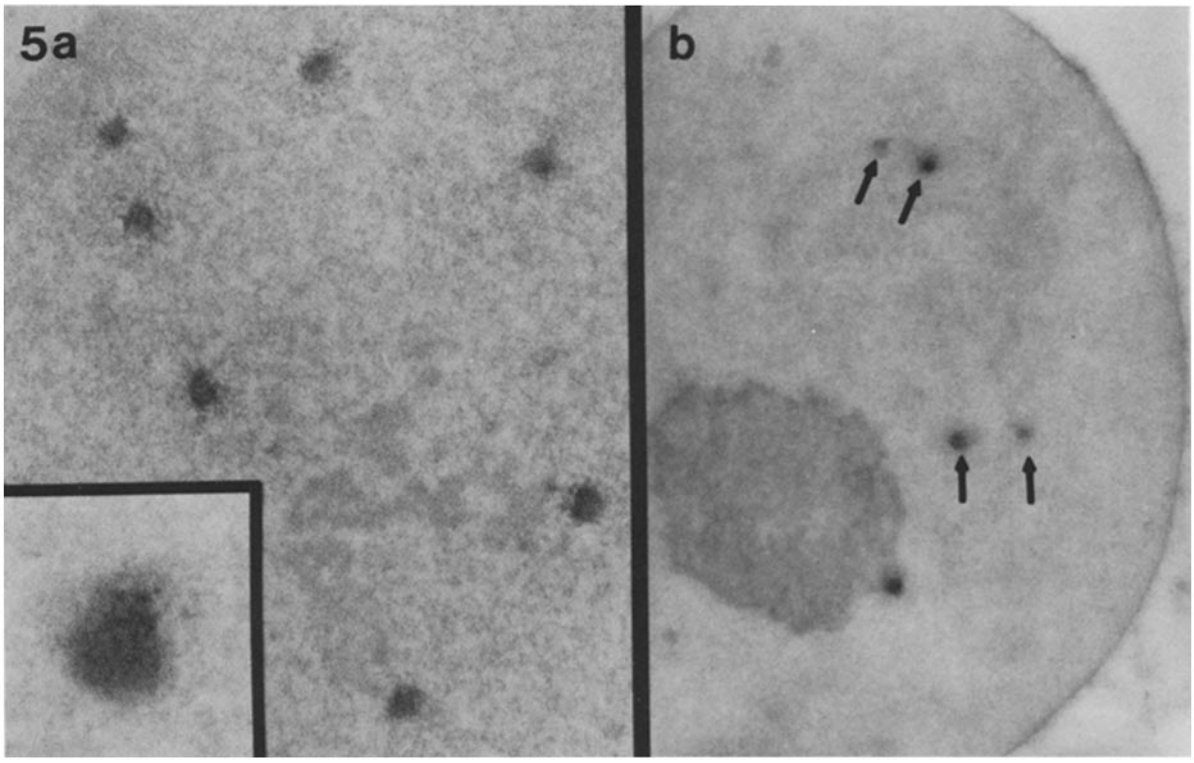

FIGURE 5 Immunoperoxidase localization of interphase presumptive kinetochores. (*a*) Low and high magnification (*inset*) thin-section micrographs of interphase pre-kinetochores. Note the pre-kinetochores associated with the nucleolus in both *a* and *b*. (*a*) × 20,300. *Inset:* × 60,000. (*b*) High voltage electron micrographs of interphase pre-kinetochores showing doubled pre-kinetochores (arrows). All pre-kinetochores in this cell were paired and a total of 12 pairs were counted in serial sections. × 8,000.

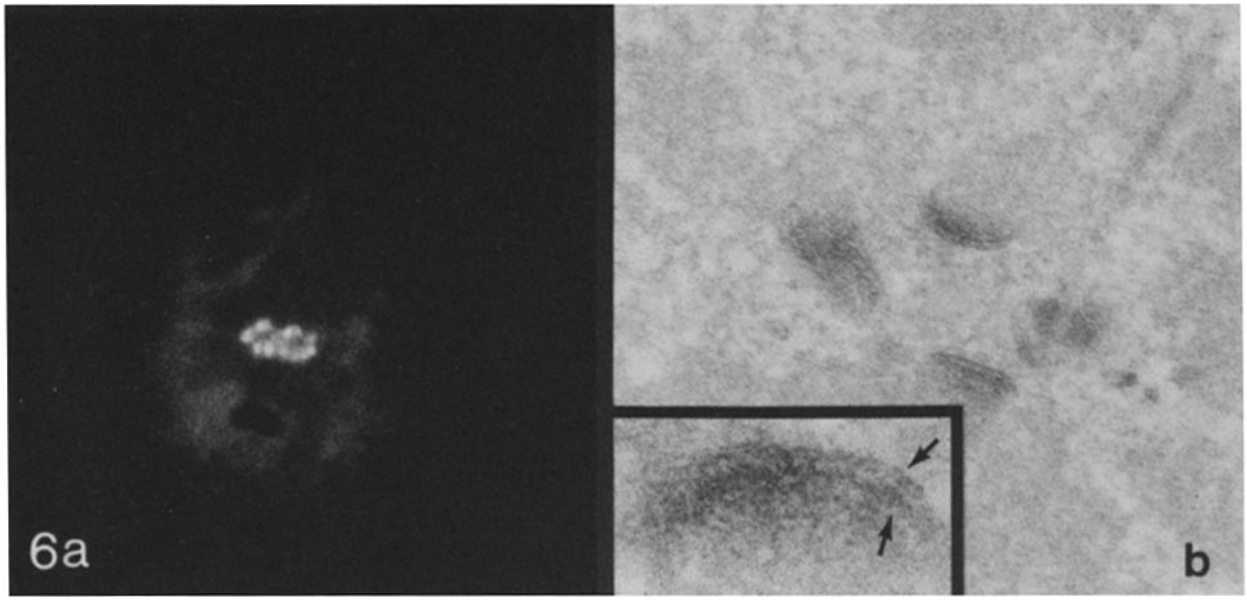

FIGURE 6 Localization of antikinetochore serum in colcemid-treated PtK_2 cells by immunofluorescence and immunoelectron microscopy. (*a*) Immunofluorescence distribution of kinetochores in colcemid-treated "C" metaphase cell. × 2,000. (*b*) "C" metaphase kinetochores stained with the antikinetochore serum by the immunoperoxidase procedure. × 21,000. *Inset:* high magnification of "C" metaphase kinetochore. Note the double-layered appearance of the kinetochore (arrows). × 62,400.

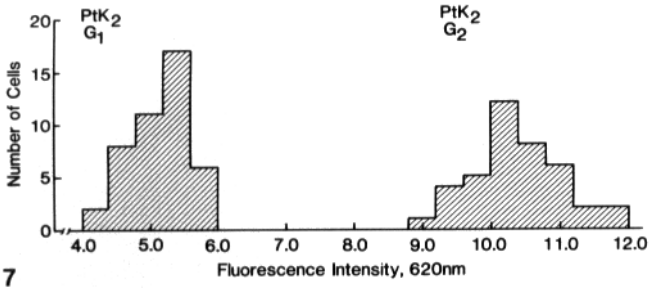

FIGURE 7 Standard distribution of propidium iodide fluorescence in G_1 (late telophase daughter nuclei) and G_2 (metaphase cells).

iodide filters. Fig. 8 shows the results of this analysis. Interphase cells containing 11–15 single pre-kinetochores appeared to have DNA contents corresponding to all phases of the cell cycle—G_1, S, and G_2. The few cells observed with double pre-kinetochores always had the G_2 amount of DNA. It should be noted that Fig. 8 does not represent a random sample of interphase cells. Cells with double pre-kinetochores were selected for measurement of DNA fluorescence and are thus shown in a disproportionate number relative to their general frequency in the population.

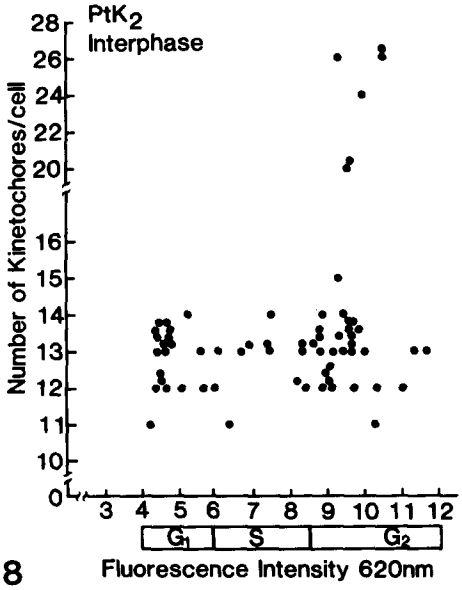

FIGURE 8 Graph of the number of visible pre-kinetochores in metaphase cells vs. the fluorescence intensity of propidium iodide. G_1, S, and G_2 boundaries are determined as in Fig. 7. Note: many cells with single pre-kinetochores have the G_2 amount of DNA.

DISCUSSION

The serum from CREST scleroderma patients has been found to stain the centromere region by indirect immunofluorescence in cells of several mammalian species. When the staining was localized at the electron microscopic level, it was found to be highly specific for the inner and outer kinetochore plates and the immediately adjacent chromatin. The specific staining of the peroxidase reaction is especially visible in thick sections examined by HVEM and illustrate the utility and feasibility of HVEM immunocytochemistry. These findings indicate that the kinetochore antigen is a highly conserved species, and provide the first evidence of a unique kinetochore constituent. The specificity of the electron microscopic localization also affords a clear distinction between the centromere and the kinetochore in that this antigen is distributed only to the kinetochore plates and not to the underlying centromeric chromatin.

In addition to the ability to localize kinetochores on mitotic chromosomes, it is now possible to define and localize what we call interphase pre-kinetochores. The conclusion that the fluorescent and electron-dense spots seen randomly arranged in interphase nuclei are truly equivalent with mitotic kinetochores is supported by several pieces of evidence. First, the distribution of the numbers of pre-kinetochores present in interphase nuclei corresponds to the chromosome number in the particular species. This has been shown here in synchronized muntjac cells

and also found to be true in PtK_2 cells. Secondly, when early prophase and prometaphase cells are stained by the antiserum and observed in the electron microscope (data not shown), the morphology of the stained areas corresponds to the kinetochore mutation sequence as described by Roos (19).

The failure to diminish the fluorescence staining of the kinetochore after preabsorption of the antisera with microtubule proteins, 6S tubulin, MAPs, or actin indicates that the antigen is distinct from these proteins. This finding, along with the previously published enzyme data (13), indicates that the antigen for which the scleroderma antibody is specific is a previously unrecognized unique component of the kinetochore.

Colcemid treatment of cells did not alter the fluorescence or ultrastructural localization of kinetochore organizers in interphase or in early prophase. Mitotic cells in the metaphase configuration showed a ring of fluorescent kinetochores. This arrangement is consistent with electron microscopy studies which have shown that the kinetochores in colcemid-treated mitotic cells are radially distributed about the centrosome (7). The immunoelectron microscopic localization of the kinetochore antigen in "C" metaphase cells is of special note because it reveals the persistence of the trilaminar kinetochore morphology. This morphology is not revealed by conventional electron microscope staining.

The ability to follow the pre-kinetochores through the cell cycle has made it possible to time the duplication of the kinetochore region, at least in terms of this antigen. We conclude from the microspectrophotometric studies that the pre-kinetochore becomes visibly duplicated during G_2 phase of the cell cycle. It is impossible to determine the exact time of duplication in G_2. We can, however, speculate, on the basis of the extremely low frequency of cells with doubled pre-kinetochores, that the pre-kinetochores become visibly separated very late in G_2. Doubled pre-kinetochores were never seen in cells with DNA content less than the G_2 amount, whereas cells with single pre-kinetochores displayed all values of DNA content in the G_1, S, and G_2 range.

In summary, scleroderma CREST anticentromere serum has been found to be a highly specific probe for the kinetochore of many types of mammalian cells. This type of autoimmunological probe and others like it (5) promise to provide much new information about the composition and function of cell components. In this study, the anti-kinetochore serum has been employed to localize mitotic kinetochores and distinguish them from other components of the centromere. In addition, we have identified structures termed the presumptive kinetochores in interphase cells. Through the use of this probe, we have been able to examine the visible duplication of the pre-kinetochores, and have concluded that duplication occurs during late G_2 phase of the cell cycle.

We would like to thank Donna Turner and Linda Wible for technical assistance, Dr. Joe Bryan for helpful comments, and Debbie Connatser for typing the manuscript.

This research was supported by Public Health Service grant CA 23022 awarded to B. R. Brinkley by the National Cancer Institute, Department of Health and Human Services. Work was also supported by grants GM 23445 and Biotechnology Resource Center grant RR-01192 to M. W. Berns, and Biotechnology Resource Grant RR-00570-11 to the High Voltage Electron Microscope Laboratory at Madison, Wisconsin.

Received for publication 23 March 1981, and in revised form 22 May 1981.

REFERENCES

1. Ahman, P., and D. D. Katz, editors. 1976. Cell Biology. Federation of American Societies for Experimental Biology, Maryland.
2. Branch, A. D., and M. W. Berns. 1976. Nucleoli and ploidy in *Potorous* cells (PtK_2) *in vitro*. *Chromosoma (Berl.)* 56:33–40.
3. Borisy, G. G., J. M. Marcum, J. B. Olmsted, D. B. Murphy, and K. A. Johnson. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly *in vitro*. *Ann. N. Y. Acad. Sci.* 253:107–132.
4. Braselton, J. P. 1980. Ribonucleoprotein staining of *Luzula* kinetochores. *Can. J. Genet. Cytol.* 22:7–10.
5. Brenner, S., D. A. Pepper, D. Turner, A. E. Boyd, and B. R. Brinkley. 1980. Autoantibodies in human serum selectively bind to the centriole region in cultured cells. *J. Cell Biol.* 87: 240*a*.
6. Brinkley, B. R., D. Murphy, and L. C. Richardson. 1967. Procedure for embedding in situ selected cells in vitro. *J. Cell Biol.* 35:279–283.
7. Brinkley, B. R., and E. Stubblefield. 1970. Ultrastructure and interaction of the kinetochore and centriole in mitosis and meiosis. *In* Advances in Cell Biology. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton Century-Crofts, New York. 119–198.
8. Fritzler, M. J., and T. D. Kinsella. 1980. The CREST Syndrome: A distinct serologic entity with anticentromere antibodies. *Am. J. Med.* 69:520–526.
9. Freid, J., A. G. Perez, and B. D. Clarkson. 1976. Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. *J. Cell Biol.* 71:173–181.
10. Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* 73:601–615.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
12. McGill, M., and B. R. Brinkley. 1975. Human chromosomes and centrioles as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin. *J. Cell Biol.* 67: 189–199.
13. Moroi, Y., C. Peebles, M. J. Fritzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. U. S. A.* 77:1627–1631.
14. Moroi, Y., A. Hartman, P. Nakane, and E. Tan. 1981. Distribution of kinetchore (centromere) antigen in mammalian cell nuclei. *J. Cell Biol.* 90:254–259.
15. Pepper, D. A., and B. R. Brinkley. 1977. Localization of tubulin in the mitotic apparatus of mammalian cells by immunofluorescence and immunoelectron microscopy. *Chromosoma (Berl.).* 60:223–235.
16. Pepper, D. A., and B. R. Brinkley. 1979. Microtubule initiation at kinetochores and centrosomes in lysed mitotic cells. Inhibition of site-specific nucleation by tubulin antibody. *J. Cell Biol.* 85:585–591.
17. Pepper, D. A., and B. R. Brinkley. 1980. Tubulin nucleation and assembly in mitotic cells: evidence for nucleic acids in kinetochores and centrosomes. *Cell Motility* 1:1–15.
18. Reider, C. L. 1979. Ribonucleoprotein staining of centrioles and kinetochores in newt lung cell spindles. *J. Cell Biol.* 80:1–9.
19. Roos, U. P., 1977. The fibrillar organization of the kinetochore and the kinetochore region of mammalian chromosomes. *Cytobiologie* 16:82–90.
20. Schacterle, G. R., and R. L. Pollack, 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* 51:654–655.
21. Snyder, J., and J. R. McIntosh, 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* 67:744–760.
22. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31–43.
23. Telzer, B. R., and J. L. Rosenbaum. 1979. Cell cycle-dependent in vitro assembly of microtubules onto the pericentriolar material of HeLa cells. *J. Cell Biol.* 81:484–497.