Distribution of Kinetochore (Centromere) Antigen in Mammalian Cell Nuclei

YASUOKI MOROI, ANTHONY L. HARTMAN, PAUL K. NAKANE, and ENG M. TAN

Departments of Medicine and Pathology, University of Colorado School of Medicine, Denver, Colorado 80262. Dr. Moroi's present address is the Department of Internal Medicine and Physical Therapy, University of Tokyo, Tokyo, Japan.

ABSTRACT Antigens associated with mammalian centromeres were localized at the light and electron microscopic levels using the peroxidase-labeled antibody method. The antibody used was of a type naturally occurring in the sera of patients with scleroderma. At the light microscopic level, it reacts specifically with the centromere regions of chromosomes in a variety of mammalian species and strains in discrete foci in interphase nuclei. We find that the number of foci approximates the number of chromosomes present in the various cell types. At the ultrastructural level, the antigenic foci are confirmed to lie in the kinetochore regions of each chromosome. In interphase nuclei, the antigenic foci were usually associated either with the inner surfaces of the nuclear envelope or with the nucleoli. These observations indicate that the centromere regions of the chromosomes in interphase are not randomly distributed within the nucleus but are usually fixed either to the inner surface of the nuclear envelope or to nucleoli.

In the past several years, the morphology of kinetochores (centromeres), especially in relation to microtubule assembly on mitotic chromosomes (12–15, 18), and the significance of kinetochores as the site of initiation and formation of chromosomal microtubules have been well established. Except for their association with tubulin (12) and RNA (14), the precise molecular components of kinetochores are still poorly defined. Several microscopic studies on serial sections of interphase nuclei in plant cells have described centromeres as loose, fibrillar, spherical formations in the nucleus (1, 7, 9). The location of the centromeres during interphase varies from report to report: some reports suggest association with the nuclear envelope (3, 17) and some do not (1, 7, 9). In animal cells, the association of kinetochores with the nuclear membrane has been suggested by autoradiography at the light microscopic level (5). However, the lack of a specific means for the detection of centromeres in interphase cells prevented analysis of these organelles, especially in animal cells.

The discovery of naturally occurring antinuclear antibodies (ANA) of multiple specificities has provided new tools for immunocytochemical investigation of the cell nucleus (16). One of these ANA, anticentromere antibody, was reported recently from this laboratory (10), and the specificity of this antibody was characterized at the light microscopic level by immunofluorescence techniques. This antibody specifically reacted not only with the kinetochore region on mitotic chromosomes but also with discrete foci in interphase cell nuclei of several mammalian species.

To elucidate more precisely the location of kinetochores, especially in interphase cells, we employed an immunoelectronmicroscopic method in this study.

MATERIALS AND METHODS

Sera

Serum from one patient (MB) with scleroderma was selected because of its monospecificity for centromere antigen as judged by the staining pattern on indirect immunofluorescence. Antisera with specificity for other nuclear antigens (histones, nuclear ribonuclear protein (RNP), and nucleoli) and normal human serum (KM) were used as controls (16).

Both normal and autoimmune sera were depleted of complement by heating to 56°C for 30 min and were used at a dilution of 1:1024 for the light and electron microscopic studies. Fab' fragments of IgG were prepared by pepsin digestion (4).

Cultured Cells

A human B lymphoid cell line (Ramos) was maintained in suspension in 200-ml tissue-culture flasks (Corning Glass Works, Corning, N. Y.) in minimal essential medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal calf serum, vitamins, L-glutamine, nonessential amino acids, antibiotics, and sodium pyruvate.

Chinese hamster ovary cells (kindly provided by Dr. F. T. Kao, Eleanor Roosevelt Institute for Cancer Research, University of Colorado) were grown as monolayers in 60-mm plastic tissue-culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in minimal essential medium supplemented as described above.

THE JOURNAL OF CELL BIOLOGY · VOLUME 90 JULY 1981 254-259

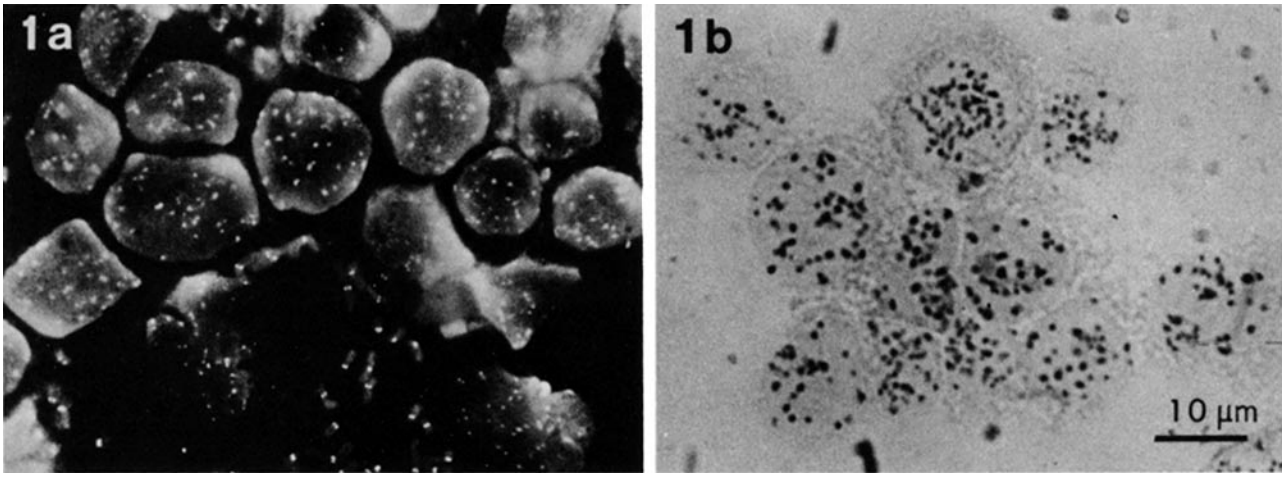

FIGURE 1 Light microscopic localization of kinetochores showing focal nuclear staining of interphase Ramos cells. (*a*) Cells reacted with human anticentromere serum followed by FITC-labeled goat anti-human IgG. (Note the staining present on the chromosomes of a ruptured mitotic cell in the lower portion of the figure.) (*b*) Cells reacted with human anticentromere serum followed by HRPO-labeled rabbit anti-human IgG.

Peroxidase-labeled Antibody Technique

The localization of centromere antigen was accomplished using the horseradish peroxidase (HRP)-labeled antibody technique developed by Nakane and Pierce (11) for light and electron microscopy.

For light microscopy, cell suspension were washed three times in balanced salt solution (BSS), resuspended in BSS, and centrifuged onto glass slides using a cytocentrifuge (Shandon Southern Instruments, Sewickley, Pa). The slides were dried at 37°C for 30 min, fixed for 15 min in periodate-lysine-paraformaldehyde (PLP) fixative (8), incubated with sera for 2 h, washed in BSS, and then treated with the HRP-labeled antiserum for 2 h.

The slides were then immersed in DAB solution (0.02% 3,3'-diaminobenzidine, 0.005% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6). After washing, the slides were dehydrated in graded ethanols and xylene and mounted. In the case of CHO cells, the cells were detached from culture dishes by gentle trypsinization and immediately washed in culture medium once and in BSS twice. In some cases, the cells were fixed with PLP before detachment. All subsequent procedures were the same as those described above.

The HRP-labeled antibody method was compared with indirect immunofluorescence using the same incubation and washing times. Chromosomal spreads for light microscopy were prepared as previously reported (10).

For electron microscopy, suspensions of Ramos cells were washed three times in BSS by centrifugation at 800 rpm for 5 min, suspended in PLP at a concentration of 3×10^6 cells/ml, and 0.1 ml of this suspension was cytocentrifuged onto glass slides coated with 0.1% polylysine in PBS using a cytocentrifuge. After centrifugation, the slides were removed and immediately placed in PLP for 1 h without allowing the cells to air-dry. The fixed slides were washed three times for 10 min in BSS containing 10% sucrose and incubated with diluted sera or Fab' fragments for 12 h in a wet box at room temperature. After incubation with the primary antibody, the slides were washed three times in BSS–10% sucrose for 10 min each, and the slides were then incubated with HRP-labeled rabbit Fab' anti-human immunoglobulin for 3 h. The slides were again washed three times in BSS with 10% sucrose and fixed in 2% glutaraldehyde in BSS for 30 min. After three washes in BSS with 10% sucrose, the slides were immersed in DAB solution without hydrogen peroxide for 30 min and then immersed in the same solution supplemented with 0.005% hydrogen peroxide for 10 min. The slides were washed and incubated with 1% osmium tetroxide in PBS for 1 h, dehydrated in graded ethanols, and embedded in Epon-Araldite. Ultrathin sections were prepared and examined with a JEM 100B electron microscope at 60 kV. Each stained kinetochore was scored as being associated with nuclear envelope, associated with nucleolar material, or free-floating in the nucleoplasm on electron micrographs of all cells that were sectioned through the nucleus.

RESULTS

Light Microscopy

Compared with immunofluorescence staining (Fig. 1 *a*), the HRP-labeled antibody method gave a similar staining pattern for centromere antigen (Fig. 1 *b*). On chromosomal spreads,

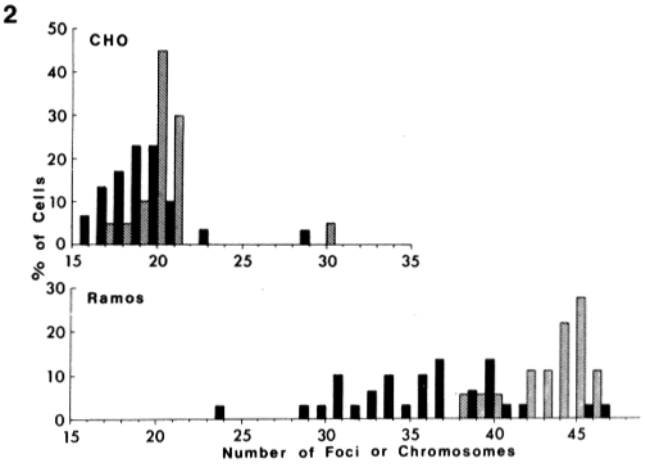

FIGURE 2 Histographs of number of antigenic foci counted per interphase nucleus and number of chromosomes counted per mitotic cell. The number of antigenic foci were determined by counting the foci in each cell which has been stained immunohistochemically with the peroxidase-labeled antibody method. Chromosome counts were made using cytocentrifuge preparations of cells which have been arrested at metaphase by 0.05 μg/ml colcimid. Solid black bars are antigenic foci. Diagonally striped bars, are chromosomes.

the staining was localized to the centromere region of each chromosome and consisted of a pair of spherical structures, presumably the kinetochores. On interphase cells, roundish discrete foci were seen distributed evenly throughout the nucleus. None of the control sera showed this staining pattern, and normal human serum produced no staining at this dilution. The number of foci were counted under the light microscope in 30 interphase cells and compared with the mean chromosome counts for the cell in question (Fig. 2). On Ramos cells, the number of foci ranged from 24 to 47 (mean = 34.4), whereas the mean chromosome number was 43.3. On CHO cells, the number of foci ranged from 16 to 29 (mean = 19.4), whereas the mean chromosome number was 20.5. The size of these foci was uniform in CHO cells; however, in Ramos cells, some foci were larger than others.

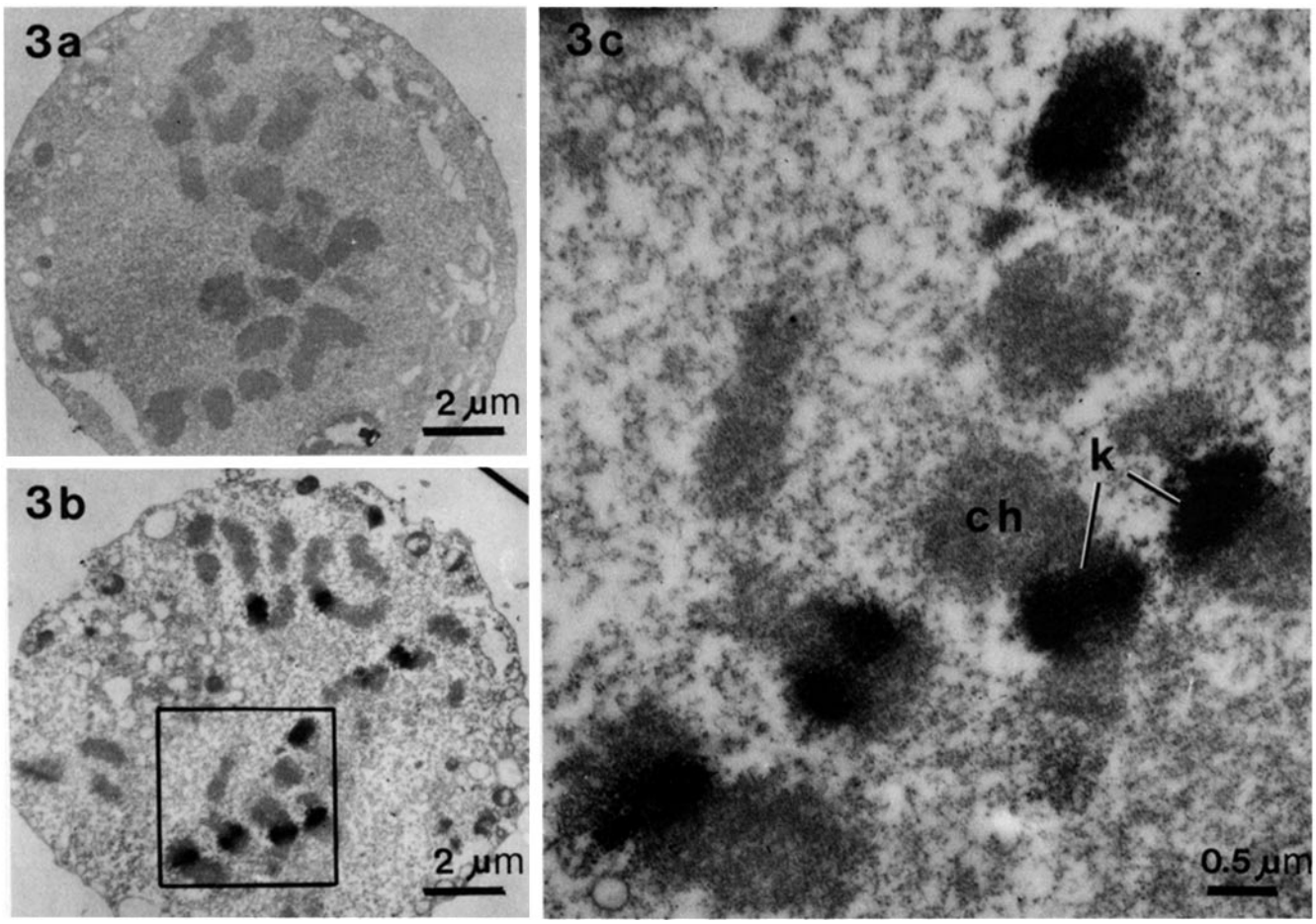

FIGURE 3 Electron micrographs of mitotic Ramos cells. (*a*) Control cell reacted with normal human serum. (*b*) Stained cell reacted with human anticentromere serum. (*c*) Higher magnification of the boxed area in Fig. *b*. ch, chromosome, and k, kinetochore.

TABLE I
Location of Antigenic Foci in the Nucleus

	Number of cells examined	Number of antigenic foci			
		Total	Adjacent nuclear envelope	Adjacent nucleolus	Free in nucleoplasm
			%	%	%
Ramos	50	194	100 (51)	79 (41)	15 (8)
CHO	25	67	28 (42)	16 (24)	23* (34)

* In two of the nuclei counted (which contained a total of nine stained foci free in the nucleoplasm), nuclear pores were prominent in the nuclear envelope, which suggests that the plane of section was near the surface of the nuclei. If these nine foci were counted as adjacent to the nuclear membrane, the results would be 55, 24, and 21% of the foci adjacent to the nuclear envelope, adjacent to the nucleolus, and free in nucleoplasm, respectively.

Electron Microscopy

On sections of mitotic human B cells (Fig. 3), staining was observed in the kinetochore region of the chromosomes. In interphase cells, more areas of staining adjoined the nuclear membrane or associated with nucleoli. Over 90% of the stained areas in Ramos cells and approximately two-thirds of the stained areas in CHO cells were found to be associated with either the nuclear envelope or nucleoli. Some of the stained areas scored as being free in the cytoplasm may have been associated with either nuclear membrane or nucleoli, particularly in those nucleoli tangentially sectioned near the surface (Table I). In Fig. 4 there are three areas of staining adjacent to the nuclear membrane and two associated with a nucleolus.

At higher magnification (Fig. 5), these areas of staining appeared as rounded structures lying against the inner surface of the nuclear membrane. The nuclear membrane interspace was well preserved at the points of kinetochore attachment. Many areas of staining associated with the nucleolus appeared to be on or near the surface of the nucleolus.

DISCUSSION

This investigation reinforces the usefulness of naturally occurring antibodies which are directed against particular cellular components. One such antiserum which appeared to react with the centromere region of metaphase chromosome was indeed found at the ultrastructural level to react with the kinetochore region of the metaphase chromosome. In the interphase nuclei, the antibody reacted with well-defined foci which were usually located near or on the surfaces of nucleoli and the inner surface of the nuclear membrane.

When the number of antigenic foci were counted in whole interphase cells at the light microscopic level, the mean number of foci observed was less than the mean chromosome number. This discrepancy was small in cell lines having a low chromosome number (19.4 vs. 20.5 for CHO) and increased markedly as the chromosome number increased (34.4 vs. 43.3 for Ramos). The reason for the discrepancy could be due to the inaccuracy of counting resulting from either two foci adjacent to one another being counted as one, or else the overlapping of two or

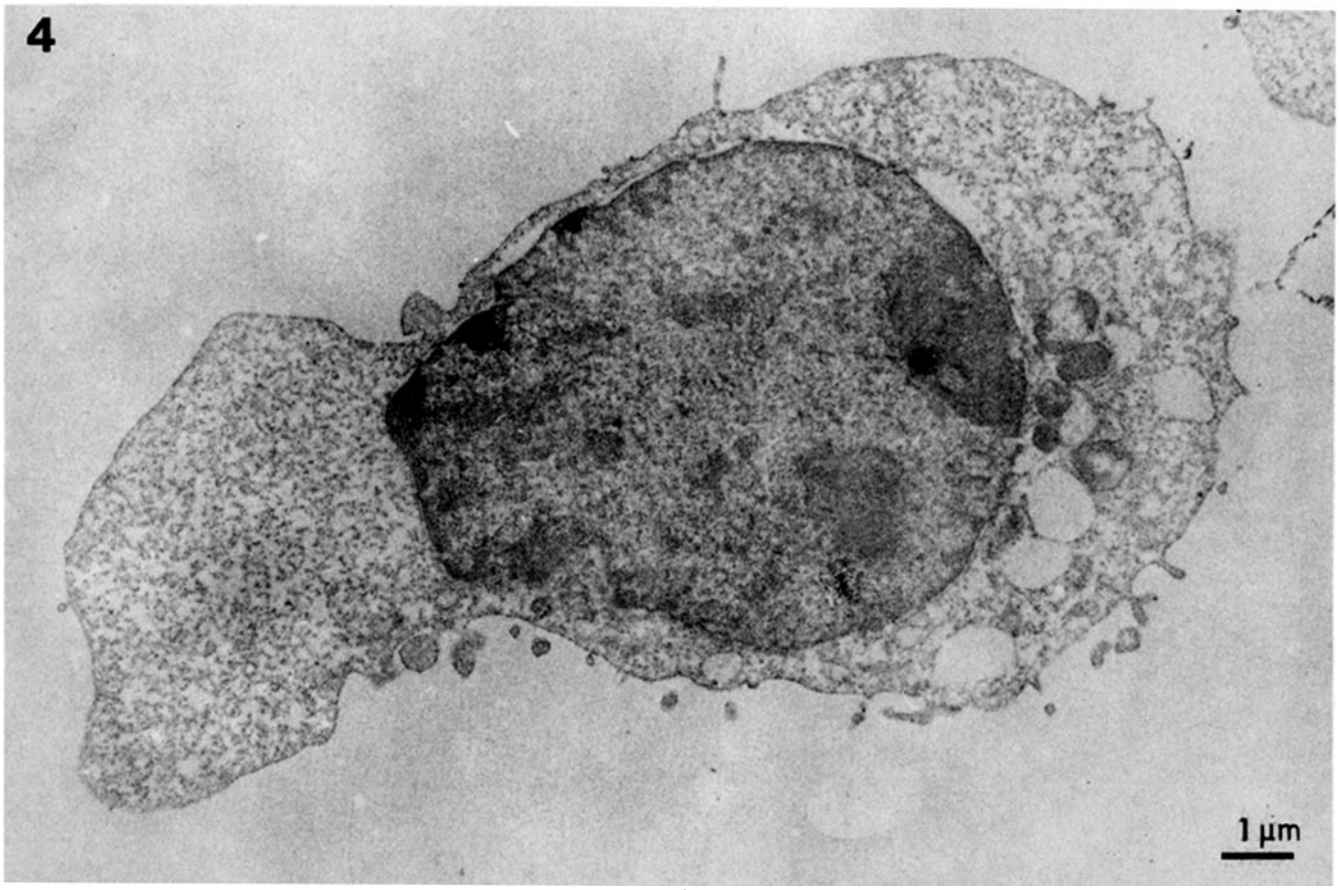

FIGURE 4 Electron micrograph of interphase CHO cell. Note the localization of kinetochore antigen along the nuclear membrane and near the surface of nucleoli.

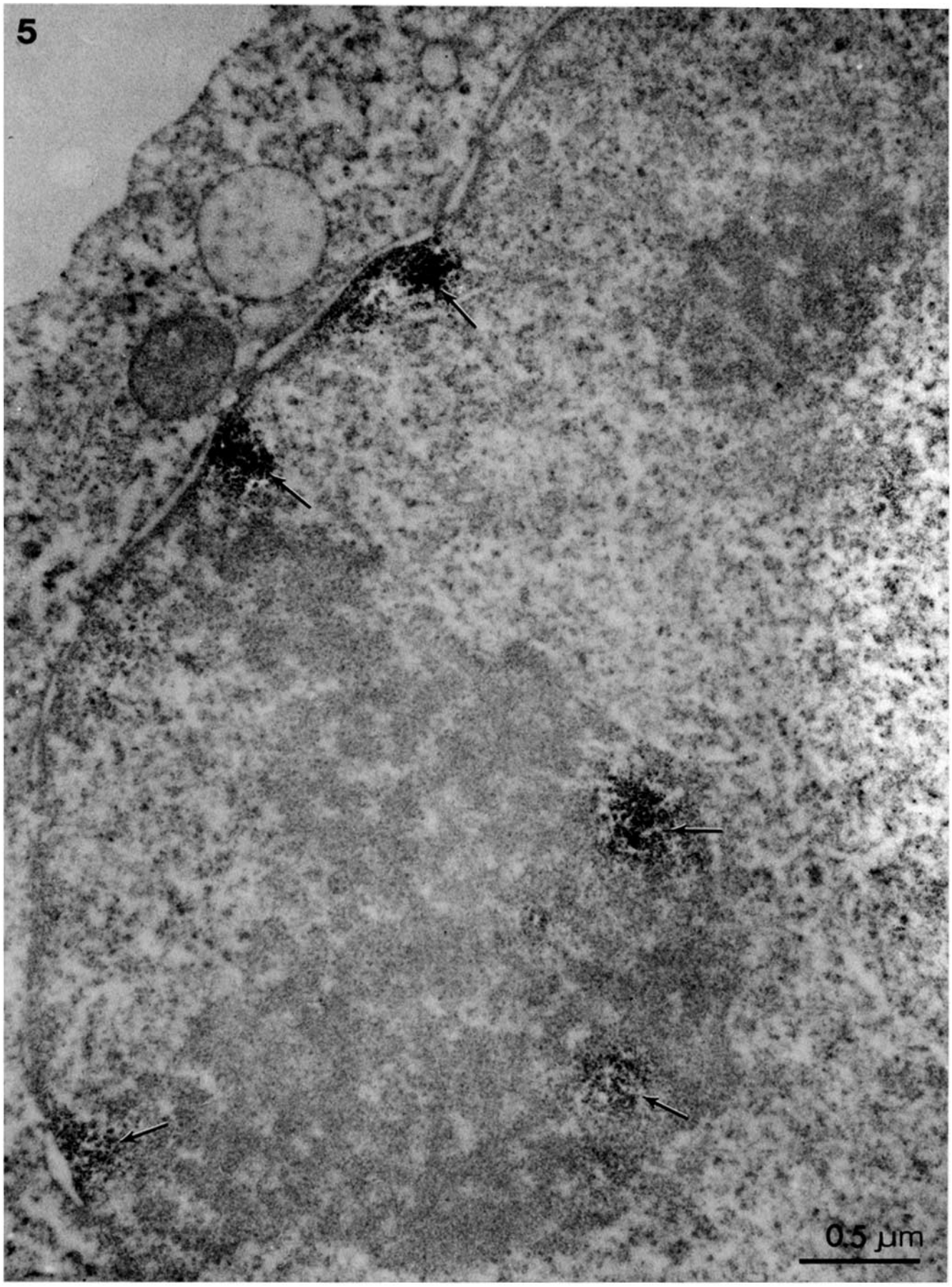

FIGURE 5 Electron micrograph of an interphase CHO cell. Arrows indicate the kinetochore regions of interphase nucleus.

more foci, rather than the dissolution of some kinetochores in interphase. Nevertheless, regardless of the reason for the discrepancy, the observation suggests that many, if not all, kinetochore regions remain intact throughout the cell cycle. The association of kinetochore regions with nucleoli during interphase is in agreement with the reports (2, 6) that the nucleolar organizer regions are situated adjacent to the centromere (kinetochore) on human chromosomes. It may be speculated that the centromeres of chromosomes with nucleolar organizer regions remain associated with the nucleolus during interphase. The association of the kinetochore region with the inner surface of the nuclear membrane suggests that during interphase the chromatin is bound at these sites and that the kinetochore may play an important role throughout the cell cycle. It is of interest to determine whether or not a particular chromosome becomes attached (associated) to a particular area of the nuclear membrane. The behavior of the kinetochore region during the cell cycle has not yet been fully explored, and questions concerning the point in the cell cycle when the duplication of centromere regions takes place remain to be answered. The chemical composition of the antigens presented in this study has not been fully established although preliminary studies have suggested that it is protein in nature (10). The number of antigens recognized by this antiserum has not been established. However, a remote possibility exists that different antigens are recognized at different stages of the cell cycle. If this is the case, the different antigens must be present in tandom as antigen foci in nuclei because no interruption or change in the staining pattern is observed during the cell cycle. The discovery of antibodies against various cellular components in autoimmune diseases is encouraging and should enable one to study further the immunochemical composition and organization of the nucleus and cytoplasm.

This work was supported in part by grants AI-09109, CA-15823, and AM-20705 from the National Institutes of Health and a gift from the R. J. Reynolds Industries, Inc.

Received for publication 22 January 1981, and in revised form 1 April 1981.

REFERENCES

1. Church, K., and P. B. Moens. 1976. Centromere behavior during interphase and meiotic prophase in Allium fistulosum from 3-D, E. M. reconstruction. *Chromosoma (Berl.).* 56: 249–263.
2. Evans, H. J., R. A. Buckland, and M. L. Pardue. 1974. Location of the genes coding for 18S and 28S ribosomal RNA in the human genome. *Chromosoma (Berl.).* 48:405–426.
3. Fussell, C. P. 1975. The position of interphase chromosomes and late replicating DNA in centromere and telomere regions of Allium cepa L. *Chromosoma (Berl.).* 50:201–210.
4. Grey, H. M., and H. G . Kunkel. 1964. H chain subgroups of myeloma proteins and normal 7S γ-globulin. *J. Exp. Med.* 120:253–266.
5. Harrisson, C. M. H. 1971. The arrangement of chromatin in the interphase nucleus with reference to cell differentiation and repression in higher organisms. *Tissue Cell.* 3:523–550.
6. Henderson, A. S., D. Warburton, and K. C. Atwood. 1972. Location of ribosomal DNA in the human chromosome complement. *Proc. Nat. Acad. Sci. U. S. A.* 69:3394–3398.
7. Lafontaine, J. G., and B. T. Luck. 1980. An ultrastructural study of plant cell (*Allium porrum*) centromeres. *J. Ultrastruct. Res.* 70:298–307.
8. McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.
9. Moens, P. B., and K. Church. 1977. Centromere sizes, positions, and movements in the interphase nucleus. *Chromosoma (Berl.).* 61:41–48.
10. 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.
11. Nakane, P. K., and G. B. Pierce. 1966. Enzyme-labeled antibodies: preparation and application for localization of antigens. *J. Histochem. Cytochem.* 14:929–931.
12. 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.
13. Pepper, D. A., and B. R. Brinkley. 1979. Microtubule initiation of kinetochores and centromeres in lysed mitotic cells. Inhibition of site-specific nucleation by tubulin antibody. *J. Cell Biol.* 82:585–591.
14. Rieder, C. L. 1979. Localization of ribonucleoprotein in the trilaminar kinetochore of PtK_2. *J. Ultrastruct. Res.* 66:109–119.
15. Synder, J. A., and J. R. McIntosh. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* 67:744–760.
16. Tan, E. M. 1979. Autoimmunity to nuclear antigens in the cell nucleus. *In Chromatin*, Part D. H. Busch, editor. Academic Press, Inc. New York, 7:457–477.
17. Tancey, R. C. 1975. A new source of evidence for the polarized nucleus in maize. *Can. J. Genet. Cytol.* 17:245–252.
18. Telzer, B. R., M. J. Moses, and J. L. Rosenbaum. 1975. Assembly of microtubules onto kinetochores of isolated mitotic chromosomes of HeLa cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:4023–4027.