

# A Monoclonal Antibody against the Nucleus Reveals the Presence of a Common Protein in the Nuclear Envelope, the Perichromosomal Region, and Cytoplasmic Vesicles

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**Abstract.** A monoclonal antibody that recognizes antigenic determinants on the nucleus of cultured mammalian cells was isolated. Immunofluorescence studies using this antibody showed that the recognized antigen was present not only on the nucleus but also in cytoplasmic vesicles of interphase cells and in the perichromosomal region of mitotic cells. Premature chromosome condensation analysis showed that the

reactive site for this monoclonal antibody could be detected in the perichromosomal region during the G<sub>2</sub> and M phases, but not during the G<sub>1</sub> and S phases. Finally, immunoblot analysis showed that this monoclonal antibody prepared against the nucleus recognized a protein of ~40 kD both in the cytoplasm and in the perichromosomal regions.

THE difficulties encountered in the detection and the identification of nuclear envelope components have limited the studies on the fate of nuclear envelope components during mitotic breakdown and on the origin of the components necessary for the reassembly of the nuclear envelope during the late M and early G<sub>1</sub> phases. However, in the last ten years, several nuclear envelope components were isolated and the use of antibodies prepared against these components has allowed the determination of their locations in the nucleus. The nuclear envelope is composed of three nuclear lamin proteins in addition to nuclear pore complexes. The nuclear lamins are thought to provide a structural framework for the nucleus (4-6). Recently, a study using an anti-nuclear antibody from a patient with systemic lupus erythematosus showed that a 33-kD protein, perichromin, is present both in the interphase nuclear envelope and in the periphery of metaphase chromosomes (12). Monoclonal antibodies provide an excellent tool for detecting specific unknown proteins in the nucleus. We isolated several monoclonal antibodies against the nucleus, and report here that one of these monoclonal antibodies recognizes the nuclear envelope region and vesicles in the cytoplasm of interphase cells and the perichromosomal region in mitotic cells and G<sub>2</sub> cells.

## Materials and Methods

### Production of Monoclonal Antibodies

To prepare the immunogen, 1 ml of packed rat liver nuclei was suspended in 7 ml of PBS containing 0.5% NP-40 and treated with 10 µg/ml DNase

I at room temperature for 15 min. The treated nuclei were centrifuged at 12,000 g for 10 min. The precipitate was suspended in PBS, mixed with purified HVJ (Sendai-virus) (packed volume, 0.2 ml) to a final volume of 10 ml, and glutaraldehyde was added at a final concentration of 0.5%. After 30 min of slow agitation at room temperature, 1 ml of glycine (100 µg/ml) was added to the suspension and the mixture was centrifuged and washed with PBS.

A suspension of 0.1 g of the immunogen (rat liver nuclei-HVJ complex) in 0.1 ml of PBS was injected into the peritoneum of 4-8-wk-old BALB/c mice. The injection was repeated at least 12 times at 1- or 2-wk intervals, and 72 h after the last injection spleen cells were prepared from the immunized mice and fused with mouse myeloma line SP2/0 cells (resistant to 8-azaguanine) using 50% polyethylene glycol (PEG; 4,000 Nakarai) as described by Koeller and Milstein (9, 10). The treated cells were inoculated into 96-well plates and incubated at 37°C in DME containing 15% FCS, 0.1 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine. After colonies had developed in the 96-well plates, hybridomas that produced anti-nuclear antibody were detected by radioimmunoassay as described below and by indirect immunofluorescence assay using the culture medium.

About 1 ml of packed isolated nuclei was suspended in 10 ml of PBS and distributed in 50-µl aliquots into polyvinylchloride microtiter wells. After incubation for 12 h at 4°C, each well was washed four times with borate-buffered saline (BBS).<sup>1</sup> 100 µl of BBS containing 2% horse serum were added, and the plates were incubated for 12 h at 4°C to block nonspecific binding. The polyvinylchloride microtiter plates coated with rat liver nuclei were washed three times with BBS, and after addition of 50 µl of hybridoma culture supernatant, they were incubated for 12 h at 4°C. The wells were then washed three times with BBS and 100 µl of <sup>125</sup>I-labeled goat anti-mouse F(ab)<sup>2</sup> in BBS was added. After 4 h at room temperature, each well was washed five times with BBS, and corresponding radioactivity was counted in a γ counter.

### Purification of Monoclonal Antibodies

To purify IgM antibodies, the supernatant of the ascitic fluid containing the specific monoclonal antibody was precipitated with 40% saturated ammo-

1. Abbreviation used in this paper: BBS, borate-buffered saline.

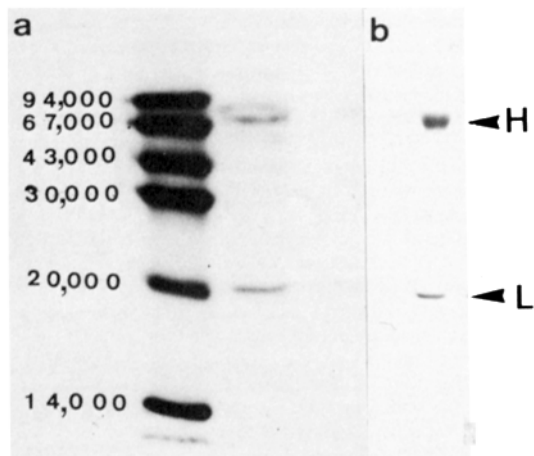
nium sulfate. The precipitate was collected by centrifugation (2,000 rpm for 20 min), dissolved in 3.5 ml of 50 mM borate buffer containing 0.5 M NaCl (pH 8.0), and dialyzed against the same buffer. The antibody solution was centrifuged at 1,200 rpm for 20 min and the supernatant was applied to a Sepharose CL-6B column equilibrated with the buffer described above. After fractionation, the activity of each fraction to stain the cell nucleus was determined by the indirect immunofluorescence method. Fractions with the highest activity were chromatographed once more on Sepharose CL-6B and finally they were purified by affinity chromatography with CNBr-activated Sepharose 4B conjugated with goat anti-mouse IgM. The purified antibody was concentrated and stored at 4°C until use.

### Immunoblot Analysis

NIH 3T3 cells were incubated in 50 mM Tris-HCl (pH 7.4) containing 10 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1% Triton X-100, and 1% NP-40, and then homogenized using a 0.5-ml glass teflon homogenizer. Homogenates were separated into cytoplasmic and nuclear fractions by centrifugation for 5 min at 3,000 rpm. Chromosomes of 3T3 cells were collected according to the method of Sillar and Young (14). The mitochondrial fraction was collected from homogenized 3T3 cells by the following procedure. 3T3 cells suspended in 10 mM Tris containing 1 mM MgCl<sub>2</sub> and 0.25 M sucrose were homogenized, and centrifuged at 1,800 rpm for 5 min. The supernatant was centrifuged at 5,000 g for 10 min and the resulting pellet was taken as the mitochondrial fraction, whereas the remaining supernatant containing other cellular components was centrifuged at 15,000 g for 30 min. The resulting supernatant was designated supernatant of the 15,000 g fraction. Each sample was resuspended in 3% SDS and dialyzed overnight against 50 mM Tris-HCl buffer (pH 6.8) containing 1% SDS, 20% glycerol, and 0.01% phenol red. After addition of 0.1 M DTT and boiling for 5 min, each sample was analyzed by electrophoresis for 1.5 h on a 7.5–20% gradient SDS polyacrylamide gel which was then electrophoretically transferred to nitrocellulose paper as described by Burnette (1). The nitrocellulose paper was incubated in 10 mM Tris-buffered saline (0.9% NaCl) containing 1.5% BSA, 1.5% egg albumin, and 20% calf serum at 37°C for 120 min to block nonspecific binding, and then it was incubated with <sup>125</sup>I-labeled antibody M-108 at 4°C for 12 h. Finally it was washed eight times with 10 mM Tris-buffered saline (pH 7.4) containing 0.05% Tween-20, dried, and autoradiographed.

### Extraction of Antigen M-108 from Chromosomes

Chromosomes of L cells were collected as described above, suspended in 40 µg/ml of DNase 1 solution (10 mM Tris-Cl buffer, pH 7.3, containing 1 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>) and incubated for 30 min at 37°C. After centrifugation at 5,000 g for 5 min, supernatant and precipitate were kept

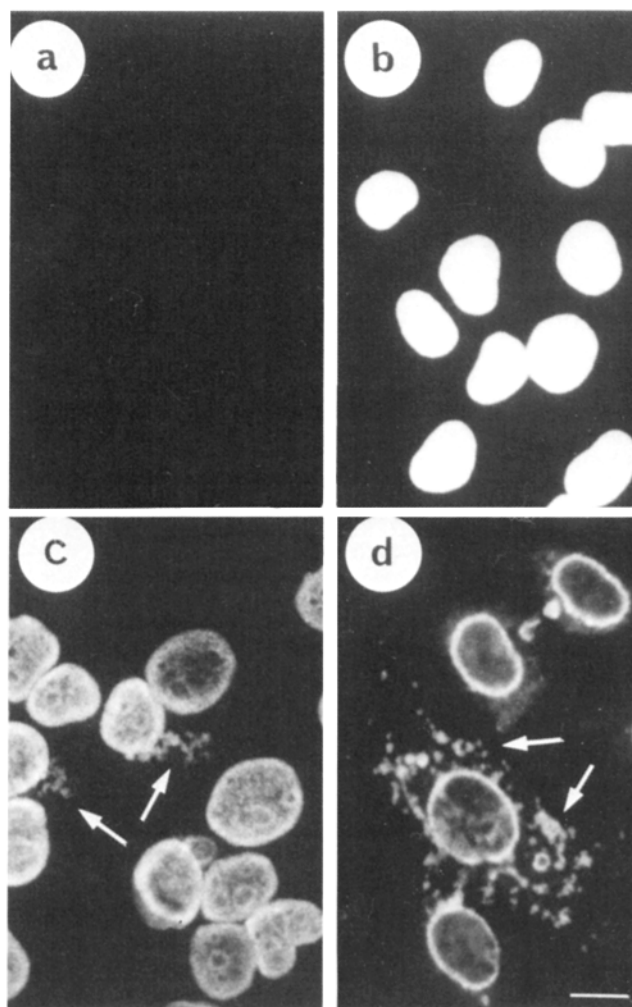


**Figure 1.** (a) Radioactive profile of culture medium from hybridoma No. 108 cells. [<sup>35</sup>S]Methionine was added at a final concentration of 0.2 mCi/ml to the culture medium of hybridoma No. 108 cells. The cells were incubated at 37°C for 5.5 h. The labeled culture medium was collected by centrifugation and 1 µl was analyzed by SDS PAGE. (b) Profile of purified antibody M-108 from ascites. H, Heavy chain; L, light chain.

for the following experiment as samples. One-fifth volume of 0.5 M DTT and 250 mM Tris-HCl buffer, pH 6.8, containing 5% SDS, 50% glycerol, and 0.05% phenol red were added to each sample. After boiling for 5 min, samples were analyzed by electrophoresis for 1.5 h on a 7.5–20% gradient SDS polyacrylamide gel. The protein transfer and immunoblot analysis were carried out as described above.

### Immunofluorescence Microscopy of Interphase Cells

FL (human amnion), L (C3H fibroblast), 3T3 (BALB/c and NIH), PtK<sub>2</sub> (rat-kangaroo) cells, and normal human skin fibroblasts were grown on glass coverslips. Two kinds of fixation procedures were used. In the first case, cells were fixed with 5% TCA at 4°C for 30 min, and then treated with absolute methanol at -20°C for 10 min. In the second case, cells were fixed with 3.7% formaldehyde-PBS (pH 7.0) for 10 min at room temperature and then permeabilized for 10 min in 0.1% Triton X-100. Nonspecific binding was blocked by rinsing the coverslips in PBS containing 20 mM glycine and 3% albumin for ~2 h, they were washed three times with Tris buffer (pH 8.0), and exposed to antibody M-108 for 2–12 h at 4°C. Control samples were exposed to non-immune mouse IgM. The coverslips were washed 10 times with Tris buffer, incubated for 2 h at 37°C with FITC-conjugated goat anti-mouse IgM, washed five times with Tris buffer, stained by Hoechst 33258 (2.0 µg/ml in PBS), and observed by microscopy. Immunofluorescence microscopic profiles obtained by using the first fixation procedure are



**Figure 2.** Immunofluorescence microscopy of interphase cells. *a* and *b* are control experiments. FL cells stained with nonimmune mouse IgM (*a*) and counterstained with Hoechst 33258 (*b*). Interphase FL cells (*c*) and BALB/c 3T3 cells (*d*) were stained with antibody M-108. Arrows in *c* and *d* denote the vesicular staining in the cytoplasm which appeared in some of the interphase cells. Bar, 5 µm.

almost the same as those obtained by using the second fixation procedure. Fluorescence patterns were photographed with Kodak Ektachrome 400 films.

### **Immunofluorescence Microscopy of Mitotic Chromosomes**

FL, 3T3, and L cells were cultured in medium containing 0.1  $\mu\text{g/ml}$  colcemid for 5 h. The mitotic populations were harvested by gently shaking the culture flasks, washed, and incubated in 75 mM KCl at 4°C for 15 min. After centrifugation for 2 min at 1,500 rpm, the cells were fixed with methanol-acetate (3:1) and chromosomal spreads were prepared. They were stained by ethidium bromide (50  $\mu\text{g/ml}$ ) and observed by the indirect immunofluorescence technique as described above.

In addition, double immunofluorescence was carried out. After a 2-h preincubation with rhodamine-conjugated antibody NC-18 (IgG), chromosomes were incubated with antibody M-108 for 2 h at 4°C. Nonspecific binding was blocked as described above and chromosomes were stained with FITC-conjugated goat anti-mouse IgM-specific antibody for 2 h at 4°C. After washing, the coverslips were mounted in Gelvatol and individual metaphase chromosomes were analyzed.

### **Immunofluorescence Microscopy of Premature Chromosome Condensation**

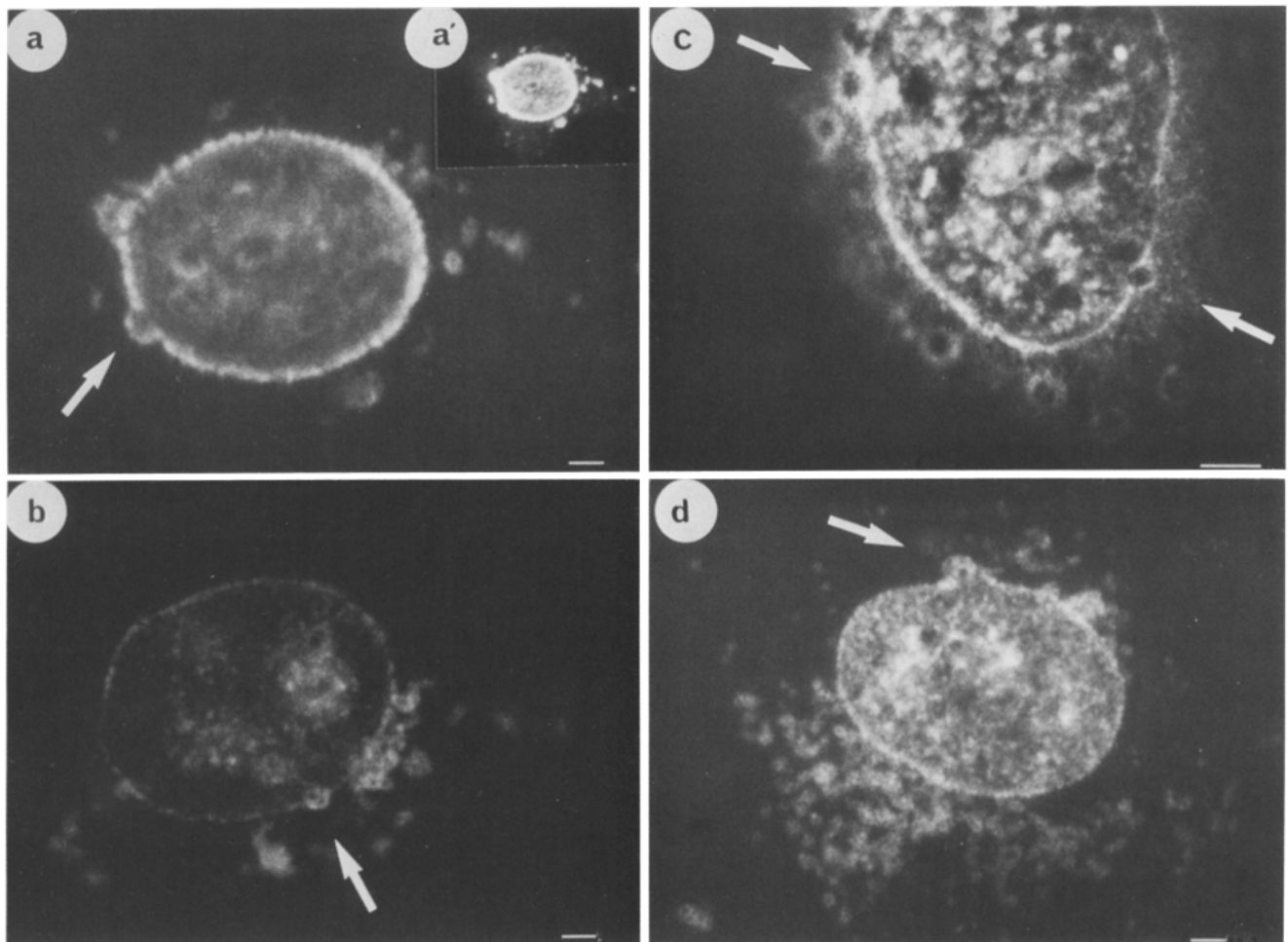
FL cells were cultured in MEM containing 0.1  $\mu\text{g/ml}$  colcemid for 12 h. Mitotic and interphase FL cells were collected separately, washed, and suspended in balanced salt solution. Mitotic FL cells ( $1.1 \times 10^7$ ) and interphase FL cells were mixed, centrifuged, and resuspended in 0.5 ml of

balanced salt solution containing 2 mM  $\text{CaCl}_2$ . 0.5 ml of UV-irradiated HVJ (Sendai-virus) (500 HAU) was added to the cell suspension. The mixture was incubated at 4°C for 7 min, then at 37°C for 30 min, and the reaction was stopped by adding MEM containing 10% calf serum. The cells were collected by centrifugation, resuspended in 75 mM KCl, and incubated at 4°C for 30 min. They were then fixed in methanol-acetate (3:1), mounted on a slideglass, and examined by the indirect immunofluorescence technique using the antibody M-108.

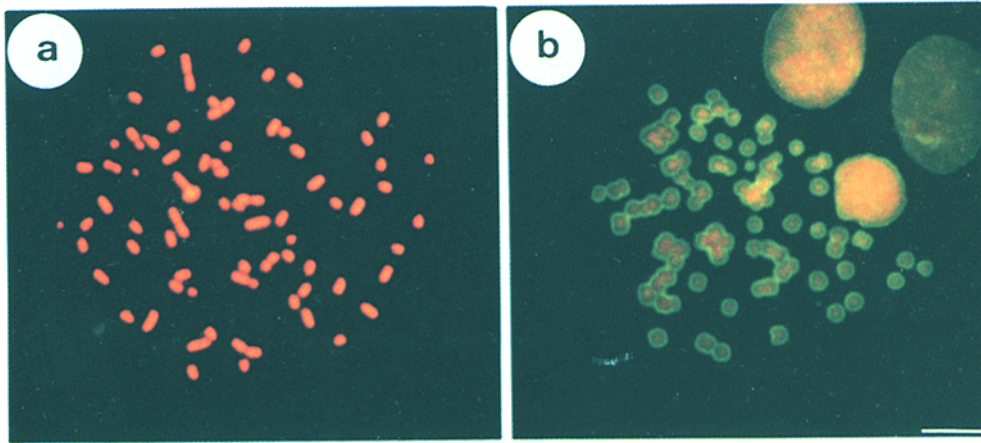
## **Results**

### **Selection of Monoclonal Antibodies**

Several monoclonal antibodies against the nucleus were isolated. These antibodies were used to stain human FL cells by indirect immunofluorescence and it was found that one of them stained the nuclear envelope region and cytoplasmic vesicles. The hybridoma cell line (No. 108) producing this particular antibody was cloned four times and the same immunofluorescence pattern was observed in all cases. Immunodiffusion studies showed that the antibody produced by hybridoma No. 108 was an IgM and it was named antibody M-108. To know whether this hybridoma produced one or more of each heavy and light immunoglobulin chains, hybridoma cells producing the antibody M-108 were labeled with 0.2 mCi/ml of [ $^{35}\text{S}$ ]methionine for 5.5 h and the secret-



**Figure 3.** High magnification of vesicular staining in the cytoplasm. (a) BALB/c 3T3 cell; (a') low magnification of a; (b) BALB/c 3T3 cell; (c) FL cell; (d) NIH 3T3 cell. Some of the vesicular staining (arrow) was connected with the nuclear envelope region while others were not. Bar, 2  $\mu\text{m}$ .



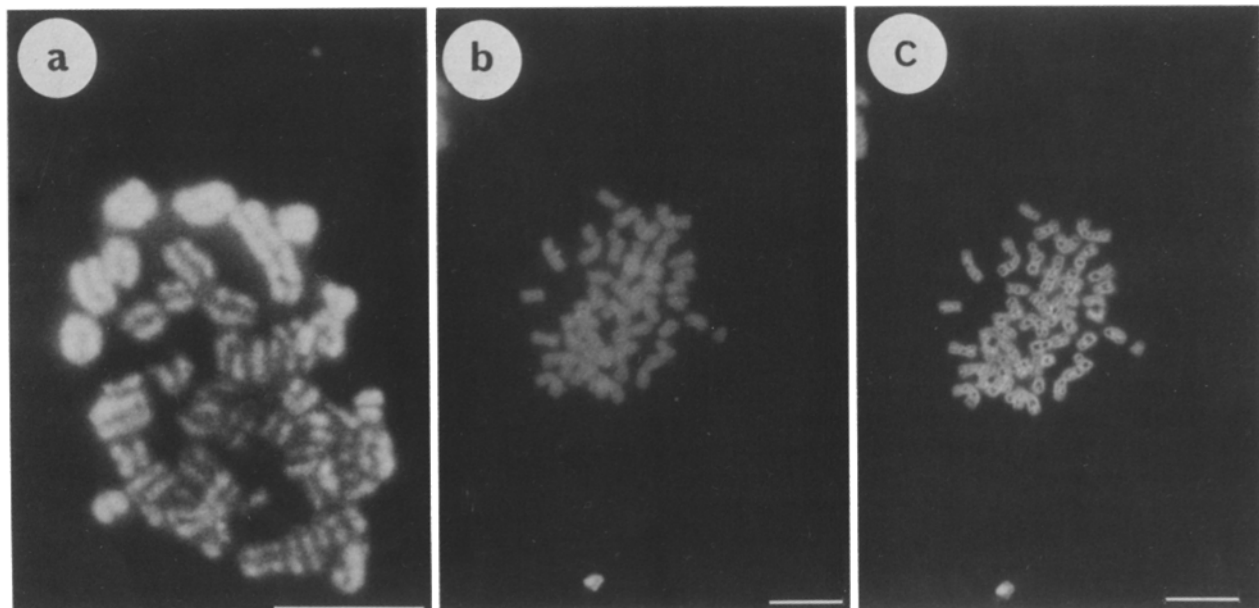
**Figure 4.** Immunofluorescence microscopy of mitotic chromosomes. Metaphase chromosomes double stained by ethidium bromide and indirect immunofluorescence method using nonimmune IgM (a) and antibody M-108 (b). Bar, 5  $\mu$ m.

ed proteins were analyzed. The protein fraction prepared from the culture medium of the labeled hybridoma was analyzed by SDS PAGE in the presence of a reducing agent (Fig. 1 a) and a single radioactive band was observed for each chain. Purified antibody M-108 from mouse ascites also showed a single band for each heavy and light chain (Fig. 1 b).

#### **Immunofluorescent Localization of Antigen M-108 in Interphase Cells**

Human FL cells and BALB/c mouse 3T3 cells were stained by the indirect immunofluorescence staining method using either the culture medium of hybridomas or the purified antibody M-108 from mouse ascites. The immunofluorescence pattern consisted of either fine granules covering homogeneously the nucleus or the nuclear envelope region. Some of the FL cells and especially the 3T3 cells, in which the nucleus was stained, also showed many circular or vesicular stainings in the cytoplasm (Fig. 2, c and d). Then the circular

or vesicular stainings in the cytoplasm were observed at higher magnification. Fig. 3 shows that the stained cytoplasmic vesicles were clearly observed in BALB/c 3T3 cells (Fig. 3, a, a', and b), in FL cells (Fig. 3 c), and in NIH 3T3 cells (Fig. 3 d). Some of these vesicles were connected with the nuclear envelope region (arrow), while others were not. These cytoplasmic vesicles existed in 46 of 391 cells tested. The frequency was  $\sim$ 12%. This antibody M-108 gave similar staining patterns when tested with other cultured cells, such as C3H mouse L cells, rat kangaroo PtK<sub>2</sub> cells, and normal human skin fibroblasts. In control assays, using nonimmune mouse IgM, neither the nucleus nor the cytoplasmic vesicles previously observed were stained in any of the cells (Fig. 2, a and b). To rule out the relationship between mitochondria and the cytoplasmic vesicles detected with antibody M-108, FL cells were double stained by indirect immunofluorescence and rhodamin 6G. The result showed that these cytoplasmic vesicles were not associated with mitochondria (data not shown).



**Figure 5.** (a) Monoclonal antibody NC-18 recognized the entire region of mitotic chromosomes. Chromosome spreads were incubated with NC-18 and stained with FITC-conjugated anti-mouse IgG. Bar, 5  $\mu$ m. (b and c) Double immunofluorescence of mitotic chromosomes using NC-18 and M-108. Chromosomes were stained with rhodamine-conjugated NC-18 (b). M-108 antibody was visualized by incubating the chromosomes with FITC-conjugated goat anti-mouse IgM-specific antibody (c). Bar, 5  $\mu$ m.

### Immunofluorescent Distribution of Antigen M-108 on Chromosomes

The distribution of the antigen detected by antibody M-108 was examined on the chromosomes of mitotic cells. As shown in Fig. 4 *b*, the periphery of metaphase chromosomes was recognized specifically by antibody M-108. Nonimmune mouse IgM did not stain the perichromosomal region (Fig. 4 *a*). To rule out fixation and/or staining procedure artifacts, we carried out double immunofluorescence using antibody M-108 and NC-18. Antibody NC-18 is one of the anti-nucleus monoclonal antibodies prepared in our laboratory that recognized entire chromosomes (15) (Fig. 5 *a*). When chromosomes were incubated in rhodamine-conjugated antibody NC-18 before antibody M-108, antibody NC-18 stained entire chromosomes of FL cells (Fig. 5 *b*), while antibody M-108 recognized periphery of the chromosomes (Fig. 5 *c*). This pattern was similar to that described by McKeon et al. using a polyclonal antibody from a patient with systemic lupus erythematosus (12). In addition, antibody M-108 also stained the periphery of mitotic chromosomes of L cells and 3T3 cells (data not shown).

To examine whether prematurely condensed interphase chromosomes were stained with antibody M-108, premature chromosome condensation was performed on FL cells. Morphological observation showed that chromosomes in the G<sub>1</sub> and S phases had no perichromosomal fluorescence (Fig. 6, *a* and *b*). Staining of perichromosomal regions began to appear during the G<sub>2</sub> phase (Fig. 6 *c*) and became stronger during the M phase.

To determine the conditions necessary for releasing or solubilizing the antigen M-108 from chromosomes, isolated chromosomes of cultured cells were treated with staphylococcal nuclease, DNase I, RNase A, or NP-40. The treated chromosomes were examined by indirect immunofluorescence staining. It showed that after treatment with staphylococcal nuclease (40 µg/ml) or DNase I (40 µg/ml), the antigen M-108 on the perichromosomal region had disappeared; but treatment with RNase A (40 µg/ml) or NP-40 (5%) did not have that effect (data not shown).

### Immunoblot Analysis on Different Cellular Fractions

As described above, the antibody M-108 reacted with the perichromosomal region, the nuclear envelope, and cytoplasmic vesicles of cultured mammalian cells. To examine whether a common protein was recognized by antibody M-108, different subcellular fractions were analyzed by the immunoblotting technique. As shown in Fig. 7, the labeled antibody M-108 reacted exclusively with one polypeptide of ~40 kD in the cytoplasmic fraction and in the chromosome fraction. When chromosomes were treated with DNase I and divided into supernatant and precipitate by centrifugation, they were also examined by immunoblot analysis. Antibody M-108 reacted dominantly with a 40-kD protein in the supernatant, but not in the precipitate (compare lanes *e* and *f*). This is consistent with the results of indirect immunofluorescence of DNase I-treated chromosomes. The same treatment did not solubilize this antigen from the nucleus (data not shown).

Comparison of the bands obtained by immunoblot analysis and by SDS PAGE showed that the radioactive 40-kD protein corresponded to a minor protein band, although many major

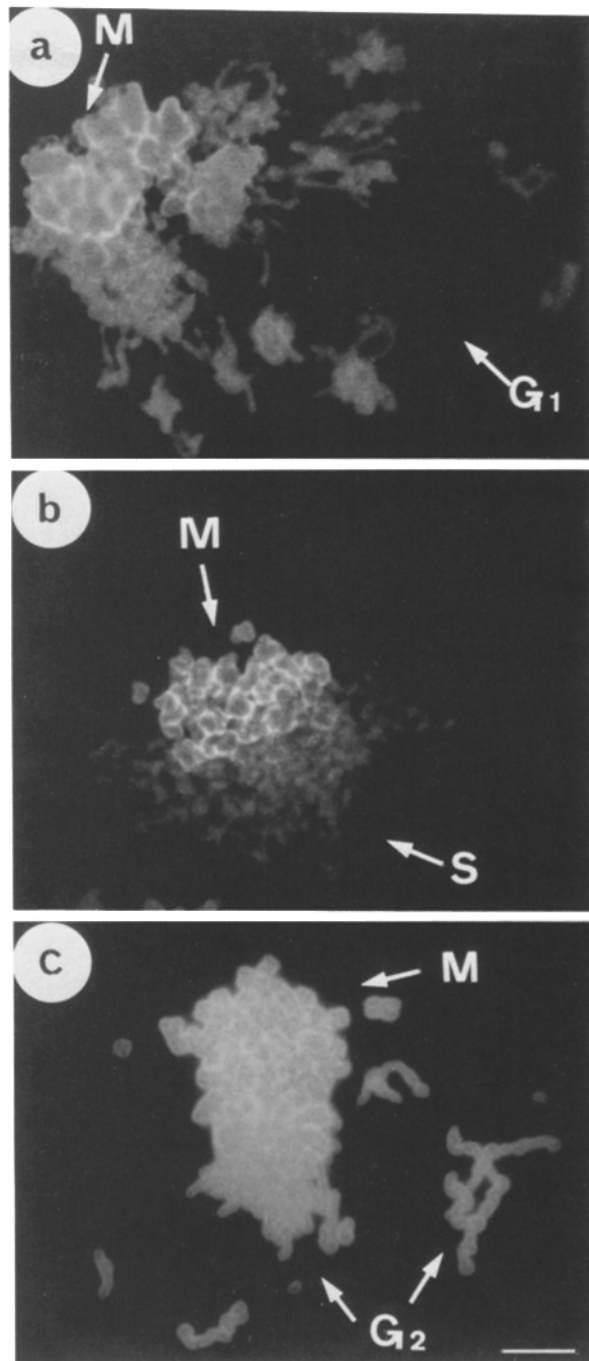
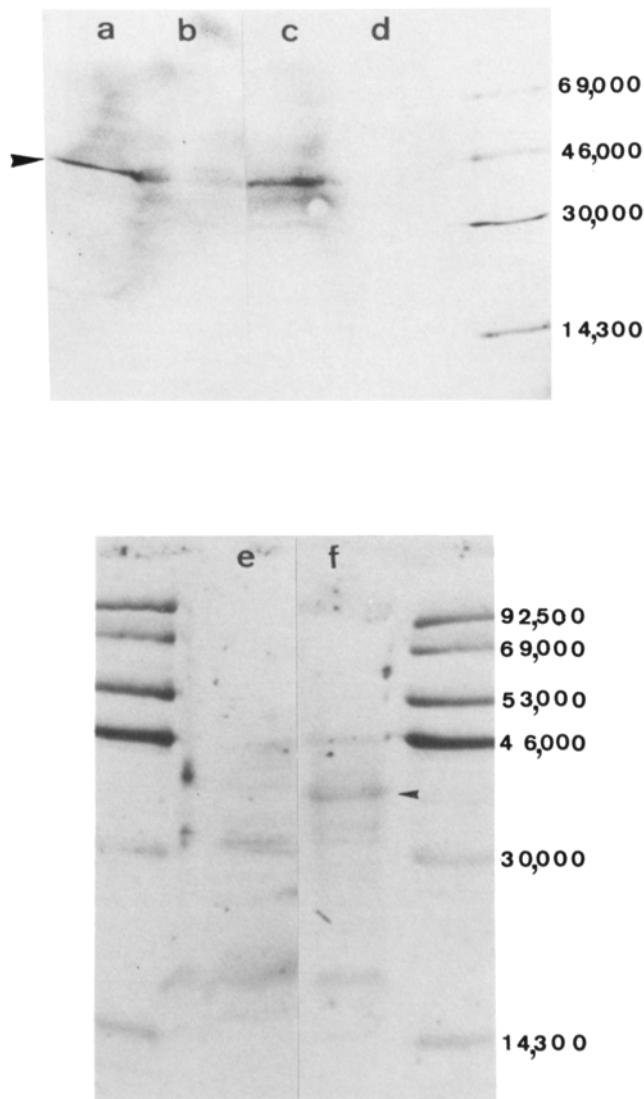


Figure 6. Immunofluorescence detection of antigen M-108 on various preparations of premature chromosome condensation observed after fusion of mitotic FL cells with interphase FL cells. Induction of premature chromosome condensation of a G<sub>1</sub>-type nucleus (*a*), an S-type nucleus (*b*), and a G<sub>2</sub>-type nucleus (*c*). Arrows denote the phase in which the condensation of chromosomes was induced. M, Metaphase chromosomes; G<sub>1</sub>, induced condensation of chromosomes in G<sub>1</sub>; S, S phase; G<sub>2</sub>, G<sub>2</sub> phase. Bar, 5 µm.

proteins were contained in each fraction. Antibody M-108 did not react with any protein of the mitochondrial fraction. These experiments showed that the antigen M-108 is present in the perichromosomal region and in cytoplasmic vesicles but not in mitochondria.



**Figure 7.** Identification of antigen M-108 in different fractions of cultured mammalian cells by immunoblot analysis using monoclonal antibody M-108. Each cell fraction was prepared as described in Materials and Methods. Electrophoresis and immunoblotting were carried out as described in Materials and Methods. (Lane *a*) Chromosome fraction of NIH 3T3 cells. (Lane *b*) Supernatant of the 15,000 *g* fraction of 3T3 cells. (Lane *c*) Cytoplasmic fraction of 3T3 cells. (Lane *d*) Mitochondrial fraction of 3T3 cells. (Lane *e*) Precipitate of DNase I treated L cell chromosomes. Lane *f*) Supernatant of DNase I-treated L cell chromosomes. In lanes *a-c* and *f*, the antibody reacted selectively with a protein of 40,000 D (arrow). In lanes *d* and *e*, no antigen was detected by antibody M-108.

## Discussion

This report describes the isolation of a monoclonal antibody, M-108, against the nucleus. It recognizes a protein of ~40 kD both in cytoplasmic vesicles and in the perichromosomal regions of cultured mammalian cells. This 40-kD protein is detected by antibody M-108 in the perichromosomal regions during the G<sub>2</sub> and M phases. The molecular mass of the recognized antigen M-108 (~40 kD) is higher than that of perichromin (33 kD) (12) or of antigen P1 (27–30 kD) (2), which have been detected in the perichromosomal region.

Therefore, the perichromosomal region is probably composed of more than two kinds of proteins. Since the molecular mass of antigen M-108 is similar to that of actin, it was verified that antibody M-108 did not react with actin (data not shown). Antigen M-108 differs from lamin proteins, because during interphase lamin proteins are located exclusively in the perinuclear region and during metaphase they become diffusely distributed throughout the cytoplasm (4–6, 8).

The immunofluorescence experiments (Fig. 2 and 3) show that in interphase nuclei, the antigen detected by antibody M-108 localizes mainly in the nuclear envelope. But in some cells, the antigen appears to be dotted in intranuclear space. This suggests that in interphase nuclei, the antigen may associate with both nuclear envelope and chromatin.

From the results of premature chromosome condensation analysis (Fig. 6), antigen M-108 is visible in the perichromosomal region during G<sub>2</sub> and M phases.

Based on our results, we may propose at least two possibilities about the localization of antigen M-108 during the cell cycle. One possibility is that the antigen localizes on nuclear envelope during G<sub>1</sub> and S phases, then moves from the nuclear envelope to the perichromosomal region during G<sub>2</sub> phase, and during M phase this perichromosomal structure becomes clearer and at the same time the nuclear envelope begins to break down. Another is that the antigen exists in both nuclear envelope and the perichromosomal region throughout the cell cycle and can not be visualized in the perichromosomal region until the chromatin becomes well condensed.

Much evidence has already accumulated for the association of nuclear envelope components with mitotic chromosomes during mitosis (2, 3, 7, 12). Moreover, it was recently shown that the formation of the nuclear envelope in amphibian eggs requires the interaction of cytoplasmic components and chromatin (11, 13). Our results suggest a similar relationship between these components in cultured mammalian cells. However, they do not provide information on the function of antigen M-108 in chromosome organization and nuclear envelope formation. Studies on the translocations of antigen M-108 between cytoplasmic vesicles, the nuclear envelope and the perichromosomal region may help to understand the function of antigen M-108. From our numerous staining experiments, we believe that the cells containing stained cytoplasmic vesicles are probably G<sub>2</sub> phase, since their chromosomes are often observed to be slightly condensed. But, it is also unknown whether the cytoplasmic vesicles are synthesized and transported to the nuclear envelope or budded off from the nuclear envelope. Therefore, further studies on the morphological structures, the components, and the functions of the cytoplasmic vesicles are required.

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