A Normally Masked Nuclear Matrix Antigen That Appears at Mitosis on Cytoskeleton Filaments Adjoining Chromosomes, Centrioles, and Midbodies

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Abstract. mAbs were generated against HeLa nuclear matrix proteins and one, H1B2, which selectively stained mitotic cells, was selected for further study. Western blot analysis showed H1B2 antibody detected a protein of 240 kD in the nuclear matrix fractions. The H1B2 antigen was completely masked in immunofluorescently stained interphase cells. However, removing chromatin with DNase I digestion and 0.25 M ammonium sulfate extraction exposed the protein epitope. The resulting fluorescence pattern was bright, highly punctate, and entirely nuclear. Further extraction of the nuclear matrix with 2 M NaCl uncovers an underlying, anastomosing network of 9–13 nm core filaments. Most of the H1B2 antigen was retained in the fibrogranular masses enmeshed in the core filament network and not in the filaments themselves.

The H1B2 antigen showed remarkable behavior at mitosis. As cells approached prophase the antigen became unmasked to immunofluorescent staining without the removal of chromatin. First appearing as a bright spot, the antibody staining spread through the nucleus finally concentrating in the region around the condensed chromosomes. The antibody also brightly stained the spindle poles and, more weakly, in a punctate pattern in the cytoskeleton around the spindle. As the chromosomes separated at anaphase, H1B2 remained with the separating daughter sets of chromosomes. The H1B2 antigen returned to the reforming nucleus at telophase, but left a bright staining region in the midbody. Immunoelectron microscopy of resinless sections showed that, in the mitotic cell, the H1B2 antibody did not stain chromosomes and centrioles themselves, but decorated a fibrogranular network surrounding and connected to the chromosomes and a fibrogranular structure surrounding the centriole.

While the importance of the nonchromatin matrix in nuclear function is increasingly recognized, characterization of the matrix has lagged behind awareness of its functions. Little is known about nuclear matrix proteins and essentially nothing about their assembly into nuclear structure. We have begun addressing these questions systematically using mAbs to individual nuclear matrix proteins. With these, we can determine the biochemical properties of individual proteins, their peptide sequence, and their localization in the matrix. These antibodies detect the rearrangements of nuclear matrix proteins during mitosis, yielding important information about the process of matrix disassembly and assembly.

Knowledge of the nuclear matrix proteins is essential for understanding important aspects of nuclear metabolism. Specific nuclear matrix proteins must conduct the important activities of this structure. These include serving as the site of DNA replication (Berezney and Coffey, 1975; McCready et al., 1980; Pardoll et al., 1980), hnRNA processing (Zeitlin et al., 1987, 1989), and steroid hormone action (Simmen et al., 1984; Barrack, 1983; Barrack and Coffey, 1980; Rennie et al., 1983). Additionally, the matrix is the scaffold determining higher order chromatin architecture (Nickerson et al., 1989). Actively transcribed genes are enriched in the small fraction of DNA remaining after nuclear matrix preparation. This suggests a close association between transcriptionally active chromatin regions and the matrix (Robinson et al., 1982; Ciejek et al., 1983; Hentzen et al., 1984; Small et al., 1985; Thorburn et al., 1988).

Studies of the nuclear matrix have been difficult since it is normally concealed by a much larger mass of tenaciously attached chromatin. Once freed of chromatin, the nuclear matrix can be examined and, if not excessively disrupted by harsh fractionation procedures, its biochemistry can be related to its unique ultrastructure. Removing chromatin from the nucleus without destroying or significantly altering the underlying matrix has been a challenge.

Following the original discovery of the nuclear matrix (Berezney and Coffey, 1974; Berezney and Coffey, 1977), methods for separating it from chromatin have undergone

continuous development (Mirkovitch et al., 1984; Capco et al., 1982; Jackson and Cook, 1985). The gentlest, least disruptive preparation of nuclear matrix so far is that of Capco et al. (1982). This method omits the initial isolation of nuclei; extracting instead a detergent prepared nucleus still associated with the cytoskeleton. After digestion with DNase I, chromatin is eluted with a much lower ionic strength salt (0.25 M ammonium sulfate) than had been used previously. This is sufficient to remove >95% of chromatin while leaving the nuclear matrix intact (Fey et al., 1986).

This procedure prepares a nuclear matrix that appears to retain most of the matrix specific proteins. It consists of a nuclear lamina and of thick, polymorphic fibers connecting the lamina to masses in the nuclear interior. This complex structure can be further fractionated by extraction with 2 M NaCl to uncover a highly branched network of 9- and 13-nm core filaments (He et al., 1990). This filament network retains most of the nuclear RNA and may be the most basic or core element of internal nuclear structure.

While some nuclear matrix proteins are specific to one or a few cell types, many are common to most cells (Fey and Penman, 1988; Stuurman et al., 1990). A very few of the matrix proteins have been characterized. Among these are the lamins A, B, and C of the nuclear lamina (Gerace et al., 1984; Krohne et al., 1987; Fisher et al., 1986) and several internal nuclear matrix proteins including topoisomerase II (Berrios et al., 1985; Nelson and Coffey, 1987) and a calmodulin-stimulated protein kinase (Sahyoun et al., 1984). Also, several well known viral proteins associate with the matrix after infection. These include the adenovirus E1A protein (Chatterjee and Flint, 1986), the Large T antigen of Simian Virus 40 (Schirmbeck and Deppert, 1989), and the trans-activating proteins of both human T-cell leukemia virus type 1 (Slamon et al., 1988) and human immunodeficiency virus type 1 (Müller et al., 1989, 1990).

A more detailed study of nuclear matrix function will require a more complete characterization and high resolution localization of additional nuclear matrix proteins. In this report, we present an approach using monoclonal antibodies to map the matrix, characterizing nuclear matrix proteins both biochemically and topographically. We describe a nuclear matrix–associated antigen that undergoes a striking relocalization during mitosis, associating with elaborate fibrogranular, mitosis-specific networks.

Materials and Methods

Cell Culture

HeLa cells were grown either in suspension culture (CCL 2.2) in Joklik-modified minimal essential medium containing 7% (vol/vol) horse serum or on monolayers (CCL 2) in DME containing 10% (vol/vol) FBS. MCF-7 breast adenocarcinoma cells (HTB 22) and CaSki cervical carcinoma cells (CRL 1550) were cultured in Dulbecco's medium containing 10% (vol/vol) FBS. The W12 cervical dysplasia cell line (Stanley, 1989) was a gift of Dr. Margaret Stanley (University of Cambridge, England). W12 cells were cultured in Dulbecco's medium containing 10% FBS, 0.1 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 10 ng/ml epidermal growth factor and were cocultured with swiss 3T3 feeder cells (CCL 92) that had been treated with 4 μg/ml mitomycin C for 2 h. For studies of mitosis, CaSki cells were synchronized by a single block with 4 mM thymidine for 24 h and used 12 h after the removal of thymidine.

Cell Extraction

The in situ sequential extraction of cultured cells to reveal the nuclear matrix has been described in previous publications (Capco et al., 1982; Fey et al., 1986). After a wash in PBS, cells were extracted in cytoskeleton buffer: 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl riboside complex, 1.2 mM phenylmethylsulfonyl fluoride, and 0.5% (vol/vol) Triton X-100. In some experiments, the cytoskeletal framework was extracted in RSB-magik (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 4 mM vanadyl riboside complex, 1.2 mM PMSF, 1% (vol/vol) Tween 40, and 0.5% (vol/vol) sodium deoxycholate) for 5 min at 4°C. This step strips away the cytoskeleton leaving nuclei with their attached intermediate filaments. In some experiments this step was eliminated, without affecting the morphology of the nuclear matrix. Chromatin was removed with 20–40 U of RNase-free DNase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN) per 10^6 cells at room temperature or 30°C for 50 min in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl riboside complex, 1.2 mM PMSF, and 0.5% (vol/vol) Triton X-100. Ammonium sulfate was added from a 1 M stock solution to a final concentration of 0.25 M. This removed chromatin leaving a nuclear matrix-intermediate filament scaffold containing nuclear ribonucleoprotein complexes. This is the RNA-containing nuclear matrix. Cells were extracted either in suspension, with gentle centrifugation between steps or on a monolayer by gently adding and removing extraction solutions. Except for the DNase I digestion and 0.25 M ammonium sulfate elution, all steps are done at 4°C.

The complete nuclear matrix could be further fractionated to reveal the core filaments of the nuclear matrix (He et al., 1990) by further extraction with 2 M NaCl. This accomplishes two important ends. The material removed is a good source of nuclear matrix proteins since the 2 M NaCl releases the outer nuclear matrix proteins free of the usually troublesome contamination by intermediate filament proteins. The 2 M NaCl extraction leaves in place the core filaments.

The procedure was modified in the case of mitotic cells to preserve the microtubules. Taxol (5 μg/ml) was added to the culture medium 5 min before extraction. Taxol (5 μg/ml) was also present in the initial washing buffer and during the extraction with Triton X-100.

Protein concentrations were determined by the dye-binding method of Bradford (1976).

mAb Generation

Mouse myeloma cells and hybridoma cells were cultured in RPMI 1640 containing 10% FBS. Balb/C mice were injected at 2-wk intervals with 100–400 μg of HeLa nuclear matrix proteins in the Ribi Adjuvant System (Ribi ImmunoChem Research, Hamilton, MT), which is a mixed adjuvant of monophosphoryl lipid A and trehalose dimycolate. Mouse serum obtained from tail bleeds was assayed by ELISA assay after the third and subsequent injections for antibodies to nuclear matrix antigens. A final booster injection was done without adjuvant 4 d before fusion. Splenocytes from immunized animals were fused with cells from the nonsecreting mouse myeloma line SP2/0-Ag14 using 40% (wt/vol) polyethylene glycol in serum-free medium. Selection for hybrid cells was done in 96-well plates in culture medium containing aminopterin, hypoxanthine, and thymidine. Clones of surviving cells were be screened by ELISA assay using HeLa nuclear matrix proteins absorbed to 96-well micro titre plates, HRP-coupled goat anti-mouse IgG and IgM, and detection by reaction with *o*-phenylenediamine and hydrogen peroxide. Cells from ELISA positive wells were subcloned by limiting dilution and screened by immunofluorescent staining of HeLa CCL 2 cells grown on slides or coverslips. Hybridomas were stored in vials submerged in liquid nitrogen in culture medium containing 10% (vol/vol) DMSO and 30% FBS.

Hybridoma lines were injected into pristane-primed Balb/C and AJ mice for the production of ascites fluid.

Western Blot Analysis of the H1B2 Antigen

HeLa cells were fractionated by the sequential procedure described above. Fractions with either 0.25 M ammonium sulfate or 2 M NaCl were dialyzed against PBS and nuclear fractions were treated with Benzon Nuclease (E. Merck) to remove interfering nucleic acids. Proteins were separated by SDS-PAGE (Laemmli, 1970) before semi-dry transfer to HyBond ECL nitrocellulose (Amersham Corp., Arlington Heights, IL) for 1.5 h at 20 V in 48 mM Tris, 39 mM glycine, 0.13 mM sodium dodecylsulfate, and 20%

(vol/vol) methanol. Nonspecific binding was blocked by incubation of the blot in TBS (25 mM Tris, pH 8.0, 137 mM NaCl, and 2.7 mM KCl) containing 10% (vol/vol) normal goat serum for 1 h and then in TBS containing 10% (wt/vol) nonfat dry milk for 1 h. All further dilutions, incubations, and washes were performed with TBS containing 0.05% (vol/vol) Tween 20 at room temperature. The H1B2 antibody was applied as culture supernatant diluted between 1:200 and 1:10,000, although similar results were obtained using ascites fluid. The second antibody was biotinylated goat anti-mouse IgM μ chain (Vector Laboratories, Inc., Burlingame, CA) used at 1 μg/ml. The blots were then incubated with Vectastain Elite ABC reagent (Vector Laboratories, Inc.) for 30 min. Detection was by enhanced chemiluminescence using a reagent kit and Hyperfilm ELC (Amersham Corp.). The molecular mass standards used were Rainbow Markers (Amersham Corp.).

Light Microscopy

Cells were grown on glass slides. They were extracted as described above before fixation with either 3.7% formaldehyde or 3.7% para-formaldehyde in PBS for 30 min at room temperature. These extracted and fixed cells were washed with PBS and blocked with 10% (vol/vol) goat serum, 10% (vol/vol) FBS, or 2% (wt/vol) BSA in saline for 30 min at room temperature. Staining with monoclonal first antibodies and rhodamine-conjugated goat anti-mouse IgG plus IgM plus IgA (Cappel-Organon Teknika Corp., West Chester, PA) was done at room temperature in saline containing 1% normal goat serum, except when the mAb was present in culture supernatants. After staining, coverslips were mounted with Aquamount mounting medium (Lerner Laboratories, Pittsburgh, PA) before viewing with both epifluorescence and phase contrast optics.

Electron Microscopy

High resolution immunolocalization of antigens was done by resinless section EM of immunostained cells (Nickerson et al., 1990). Cells were grown on Mylar polyester film, a gift from the DuPont Corporation. To briefly summarize the sample preparation procedure, cells extracted to reveal the nuclear matrix were fixed with either glutaraldehyde or paraformaldehyde. After blocking with goat serum, the extracted cell was incubated with the mAb H1B2. The sample was then incubated with the gold bead-conjugated second antibody followed by washing and a second fixation with glutaraldehyde and osmium tetroxide. The sample was embedded in diethylene glycol distearate, thin sections were cut, and the embedding resin was removed before critical point drying and carbon coating. The removable embedding resin diethylene glycol distearate was obtained from Polysciences (Warrington, PA). Gold bead-conjugated goat anti-mouse IgG + IgM second antibodies were obtained from Amersham (Arlington Heights, IL). Resinless sections were examined using a JEOL 1200EX electron microscope with a goniometer attachment. When stereo electron micrographs were taken the total tilt angle was 10°.

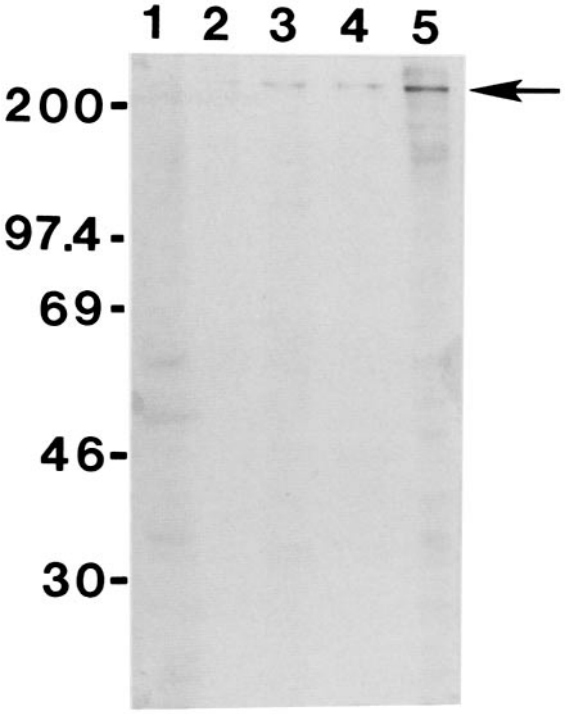

Figure 1. Western blot of H1B2. HeLa cell fractions were electrophoresed on 7% polyacrylamide gels before semi-dry transfer to nitrocellulose, antibody staining, and detection by enhanced chemiluminescence as described in Materials and Methods. Each lane has the protein from 3.6×10^5 cells. Cells were sequentially fractionated with 0.5% Triton X-100 to remove soluble proteins (lane *1*) and with RSB-magic to remove most of the cytoskeleton (lane *2*). Chromatin (lane *3*) was removed by digestion with DNase I and extraction with 0.25 M ammonium sulfate. This leaves the complete nuclear matrix, still connected to the intermediate filaments of the cytoskeleton. The outer nuclear matrix proteins (lane *4*) were removed by 2 M NaCl extraction. The core filament network and nuclear lamina remained in the final pellet (lane *5*). The H1B2 antibody stained a protein of 240 kD which was primarily in the final fraction (lane *5*). The numbers to the left are the sizes of molecular mass markers in kilodaltons.

Results

The H1B2 mAb was prepared using mice immunized with purified nuclear matrix proteins from HeLa (CCL2.2) cells. Hybridomas were screened first by ELISA using isolated HeLa nuclear matrix proteins, and then by immunofluorescent staining of HeLa (CCL2) cells grown on microscope slides. Hybridoma clones producing ELISA-positive antibodies with interesting fluorescence patterns were selected for further cloning and study.

H1B2, an IgM, was chosen for further study because it reacted strongly in ELISA assays and, under appropriate conditions, selectively stained the nuclear matrix of HeLa cells. The antigen, present in many different human epithelial cell lines, showed a striking redistribution during mitosis (to be discussed below). While many nuclear matrix proteins are specific to cell type and stage of differentiation, others are invariant from cell to cell (Fey and Penman, 1988; Stuurman et al., 1989; Stuurman et al., 1990; Dworetzky et al., 1990). The H1B2 antigen has been found in HeLa, MCF-7, CaSki, SiHa, and W12 cells and may be a universal component of the basic nuclear matrix.

Identification of the H1B2 Antigen

Western blots of HeLa cell fractions showed that the H1B2 antigen was a nuclear matrix protein of ~240 kD (Fig. 1). A small fraction of this protein was removed with the chromatin by DNase I digestion and 0.25 M ammonium sulfate extraction (Fig. 1, lane *3*). More was removed by 2 M NaCl extraction of the complete nuclear matrix (Fig. 1, lane *4*), but most of the high molecular weight H1B2 antigen was retained with the HeLa core filament network (Fig. 1, lane *5*).

The location of the H1B2 antigen was entirely nuclear as shown by immunofluorescent light microscopy and immunogold EM (Figs. 2–4). Staining cells at different stages of our sequential fractionation confirmed the results of Western blots. The antigen was primarily in the nuclear matrix fraction and not in the chromatin. Furthermore, the antigen appeared largely associated with the core filament subfrac-

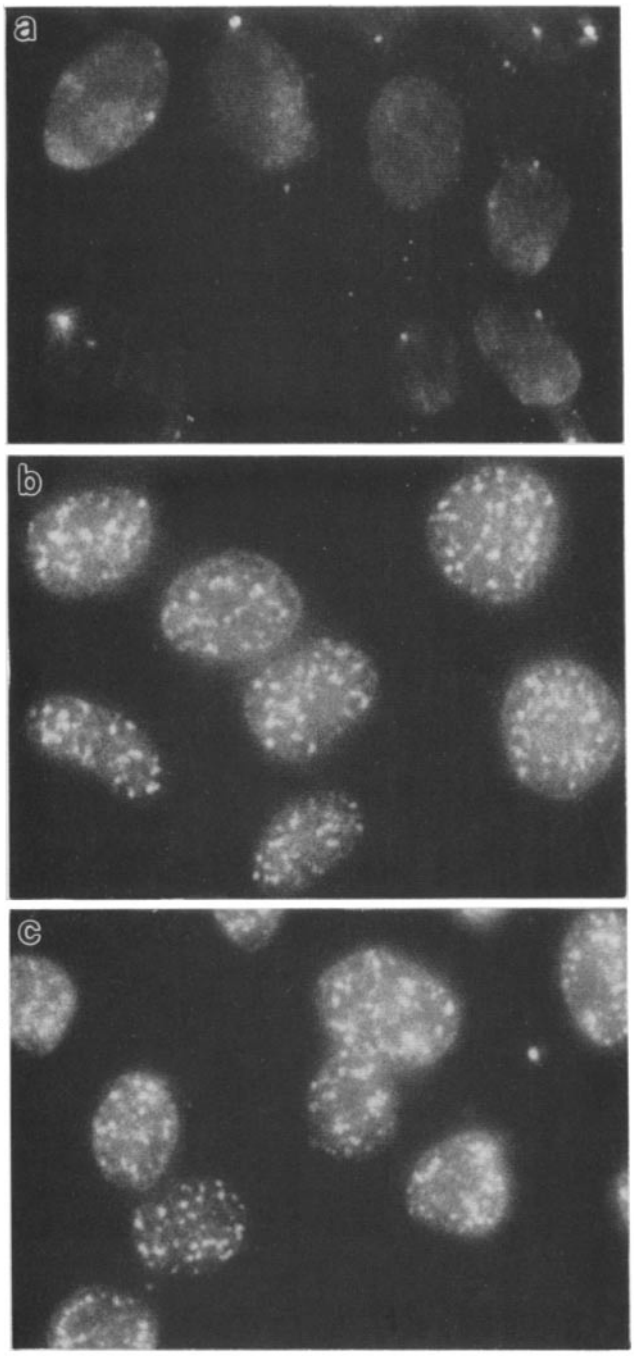

Figure 2. Immunofluorescence localization of H1B2 in CaSki cell interphase nuclei. All the immunofluorescence photomicrographs were taken and printed at the same exposure to make quantitative comparison possible. (*a*) When cells were extracted with 0.5% Triton X-100 in cytoskeletal buffer the nuclei were only slightly stained. (*b*) To uncover the nuclear matrix, chromatin was removed from 0.5% Triton X-100 extracted cells by digestion with DNase I and extraction with 0.25 M ammonium sulfate. After this treatment, H1B2 stained the nucleus with a punctate pattern. (*c*) Cells extracted to reveal the complete nuclear matrix as in part b were further extracted with 2 M NaCl to uncover the network of core filaments (He et al., 1990). The pattern and intensity of H1B2 immunofluorescence was unchanged in most cells. A few cells had less intense, finer, and more dispersed spots of fluorescence.

tion of the nuclear matrix since at least half of the antigen resisted 2 M NaCl extraction in MCF-7 cells and nearly all in CaSki and HeLa cells.

Immunofluorescence Localization of the H1B2 Antigen in Interphase Nuclei

The H1B2 antibody did not stain the nuclei of unextracted cells since the antigen is masked during interphase. Extraction of cells with 0.5% Triton X-100 removed soluble proteins but did not unmask the nuclear antigen (Fig. 2 *a*). Only a low intensity, uniform nuclear fluorescence was detected. Occasional nuclei had a more intense fluorescence in a single spot or a small region of the nucleus. As shown below these are probably cells nearing mitosis.

The H1B2 epitope was revealed by the removal of chromatin. The cells were digested with DNase I and extracted with 0.25 M ammonium sulfate. This procedure removed essentially all of the chromatin leaving only the nuclear matrix in the nuclear interior (Fey et al., 1986; He et al., 1990). Fig. 2 *b* shows the punctate staining pattern of the H1B2 antigen in the nuclear matrix prepared in this way.

This complete nuclear matrix can be separated into two distinct parts. The thick nuclear matrix fibers are comprised of a complex set of proteins, some of which are cell type specific. These are assembled around a highly branched network of 9- and 13-nm core filaments which may serve as cores in the thick filaments. In a previous report (He et al., 1990), we showed that extracting the complete nuclear matrix with 2 M NaCl removes most of the outer proteins of the interior nuclear matrix, revealing the inner network of core filaments. This filament fraction, with a relatively simple protein composition, contains nearly all of the nuclear RNA. There are at least two components, the filament network itself and dense masses of fibrogranular material enmeshed in the filaments. These dense masses contain the hnRNP proteins and may be the location of hnRNA.

Much of the H1B2 antigen was associated with the nuclear matrix core filament network. The percentage remaining after 2 M NaCl extraction to uncover the core filaments varied with cell type. In MCF-7 cells about half of the total fluorescence remained in the core filaments while in CaSki cells the core filaments retained nearly all the fluorescence (Fig. 2c). In HeLa cells, as shown by Western blotting (Fig. 1), most of the antigen is retained with the core filaments. Immunoelectron microscopy (Fig. 4) of CaSki cells extracted to reveal the nuclear matrix core filaments showed that the H1B2 antigen was associated principally with the granular material enmeshed in the core filament network and not with the core filaments themselves.

Immunoelectron Microscopy H1B2 Antigen at Interphase

The conventional technique for high resolution localization of structural proteins by EM is to stain epon or Lowicryl embedded thin sections with antibodies. However, while traditional embedded-section EM is an adequate technique for viewing organelles and membrane systems which are best seen in cross-section, it is not suitable for studying the cytoskeleton or nuclear matrix. In embedded sections only the stained surface of the section is seen; the filaments of the structural networks appear only in cross-section unless they

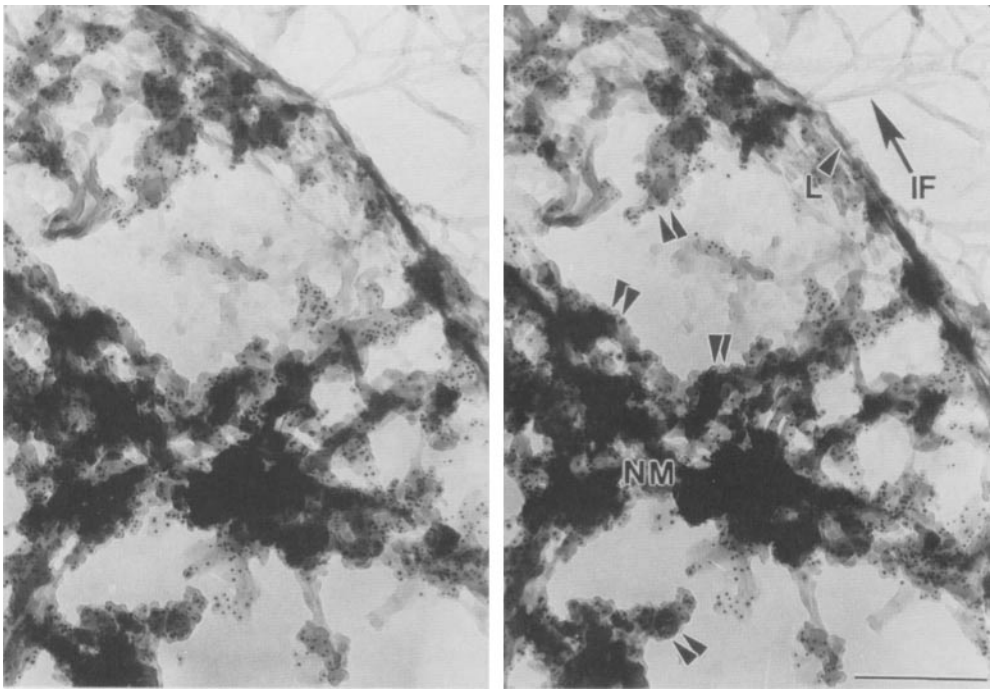

Figure 3. Stereo electron micrograph of H1B2 immunolocalization in the nuclear matrix of W12 cervical dysplasia cells. W12 cells were extracted with 0.5% Triton X-100 in cytoskeletal buffer and their chromatin was removed by DNase I digestion and 0.25 M ammonium sulfate extraction. After fixation, the matrix preparation was treated with H1B2 antibody and then with a colloidal gold-coupled second antibody. This resinless section shows part of the nucleus bounded by the nuclear lamina (*L*). The thick fibers of the nuclear matrix were strongly decorated with 5-nm gold beads showing the location of the H1B2 antigen. The cytoskeleton, consisting principally of intermediate filaments (*IF*s), was not stained. Bar, 0.2 μm.

are both close to and parallel to the surface of the section, something which occurs rarely.

Sample preparation techniques developed in this laboratory are better suited for electron microscopy of the filamentous skeletons of detergent extracted cells (Capco et al., 1982). In this procedure, adapted from that of Wolosewick (1978), extracted cells are embedded in the removable resin diethylene glycol distearate, sectioned and the embedding

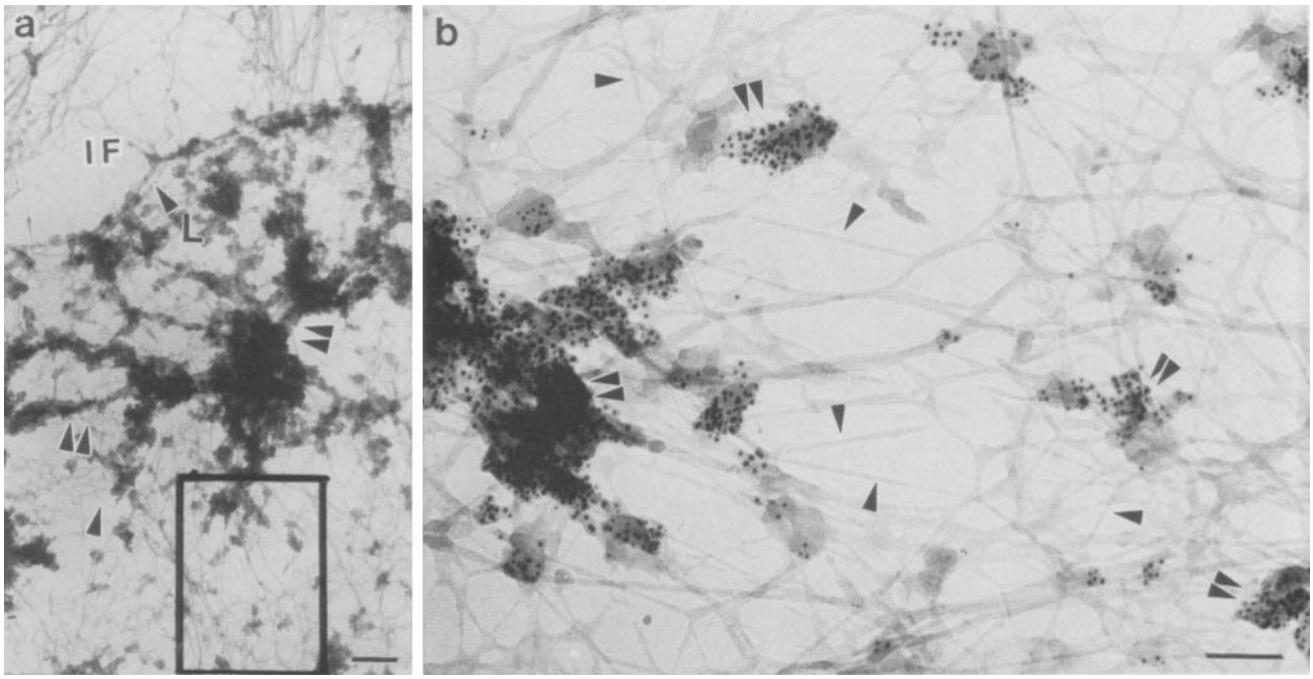

Figure 4. H1B2 distribution in the network of nuclear matrix core filaments as seen in immunostained resinless sections. The low magnification electron micrograph (*a*) of a CaSki cell nuclear matrix made by the DNase I-0.25 M ammonium sulfate method which was further extracted with 2 M NaCl to reveal the network of 10 nm core filaments. A higher magnification view of the same cell is shown in *b*. The double arrowheads point to residual masses that were retained with the network and stained heavily with H1B2 antibody and second antibody conjugated 5-nm gold beads. Single arrowheads show the 10-nm core filaments which were, with few exceptions, devoid of stain. The core filament network was connected to the nuclear lamina (*L*) which also anchored the intermediate filaments of the cytoskeleton (*IF*). The marked rectangle in panel a shows the position of the higher magnification view shown in *b*. Bars: (*a*) 0.2 μm; (*b*) 0.1 μm.

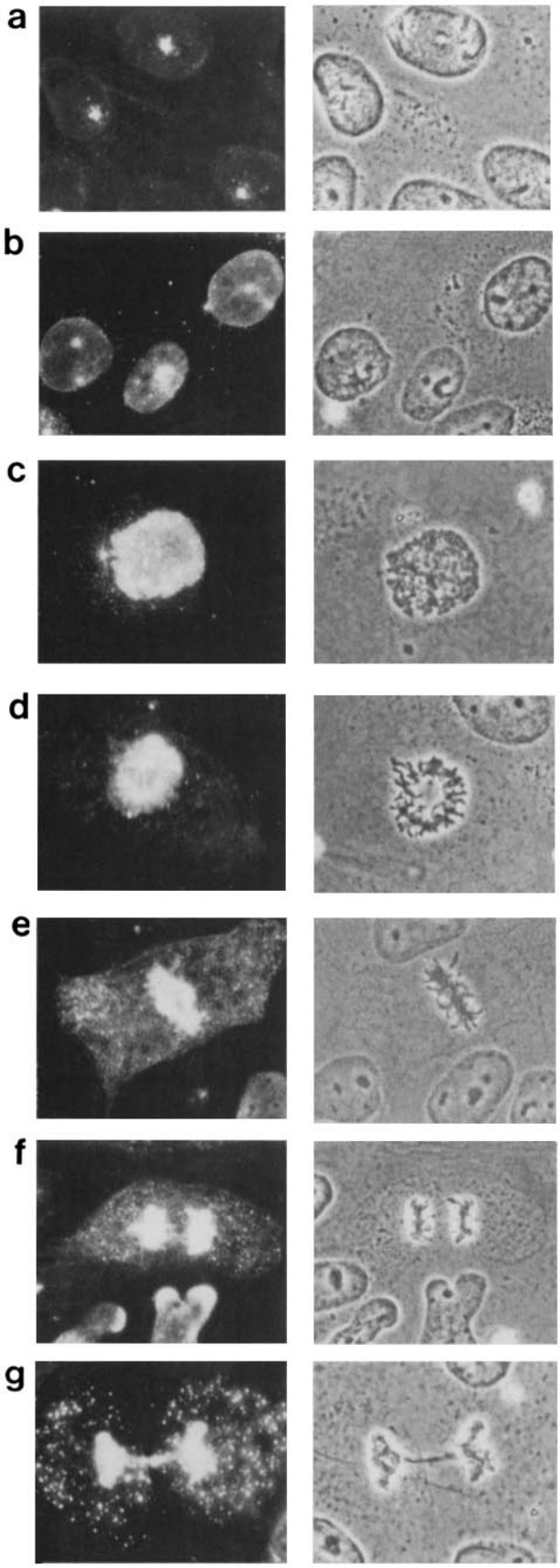

Figure 5. The dynamic redistributions of the H1B2 antigen through mitosis in CaSki cells. CaSki cells were synchronized by single thymidine block and were extracted with 0.5% Triton X-100 in cytoskeletal buffer before fixation and immunofluorescent staining. The pairs of micrographs illustrate different mitotic stages seen in immunofluorescence and in phase contrast. Cells in interphase which were extracted in this way had very weak nuclear staining (Fig. 2 *a*). Only removal of chromatin by the DNase I-0.25 ammonium sulfate procedure uncovered the antigen (Fig. 2 *b*). As seen in this figure, the H1B2 antigen was uncovered in mitotic cells without this extraction. (*a*) Cells approaching prophase had only a single small area of the nucleus stained. (*b*) At a more advanced premitotic stage cells had more nuclear staining. There was a range of different staining patterns from cells showing only a single nuclear spot to cells with staining of larger nuclear areas to cells with staining over the whole nucleus. (*c*) In prophase, as chromosomes began to condense, the labeling was very intense throughout the whole nucleus. Note the strong staining of the aster to the left of the nucleus. (*d*) At prometaphase there was intense H1B2 staining in the area around the condensed chromosomes. The staining was not exactly coincident with the chromosomes. While the chromosomes appeared to form a halo, there was H1B2 staining in the center where chromosomes were absent. (*e*) In metaphase, as the sets of chromosomes lined up at the metaphase plate, intense H1B2 staining could be seen around the chromosomes. The area around the centriole at the spindle poles was stained and there was a punctate staining in the cytoskeletal region. (*f*) In anaphase cells the perichromosomal region retained a high degree of stain. The punctate cytoskeletal staining remained. (*g*) In cells at cytokinesis there was H1B2 staining in the reforming daughter nuclei. The punctate cytoskeletal staining was stronger than at earlier mitotic stages; there were fewer but larger and more intensely stained spots. The midbody between the daughter cells stained intensely.

material removed. For brevity, the method has been designated the "DGD" procedure. With no opaque embedding resin, the entire thickness of the unstained section can be imaged. We have recently reported a technique for immunostaining these resinless sections, thereby localizing antigens in the nuclear matrix and cytoskeleton in three dimensions and throughout the whole section (Nickerson et al., 1990).

The immunogold-stained resinless section of Fig. 3, presented as a stereo pair and best seen with a stereo viewer, shows the complete nuclear matrix prepared by detergent extraction, DNase I digestion, and 0.25 M ammonium sulfate extraction. The H1B2 antigen was located on the thick fibers of the nuclear matrix. This section was cut through the nucleus of a W12 cervical dysplasia cell and stained with H1B2 and a second antibody conjugated with colloidal gold. The nuclear matrix visible in this section consisted of the nuclear lamina, connected to the 10-nm intermediate filaments of the cytoskeleton and the thick nuclear matrix fibers in the nuclear interior. H1B2 staining was confined to the thick fibers of the nuclear matrix; neither the lamina surrounding the nucleus nor the remaining cytoskeleton, consisting mostly of intermediate filaments, was stained. This was consistent with the immunofluorescent staining pattern of H1B2 (Fig. 2 *b*).

Further extraction of the complete nuclear matrix with 2 M NaCl reveals an underlying fibrogranular network of 9- and 13-nm nuclear core filaments (He et al., 1990; Fig. 4). Immunogold staining of resinless sections with the H1B2 antibody decorated the masses enmeshed in the filament network and not the core filaments themselves. Thus, the H1B2 antigen is not a protein of the core filaments but is a component of those residual masses.

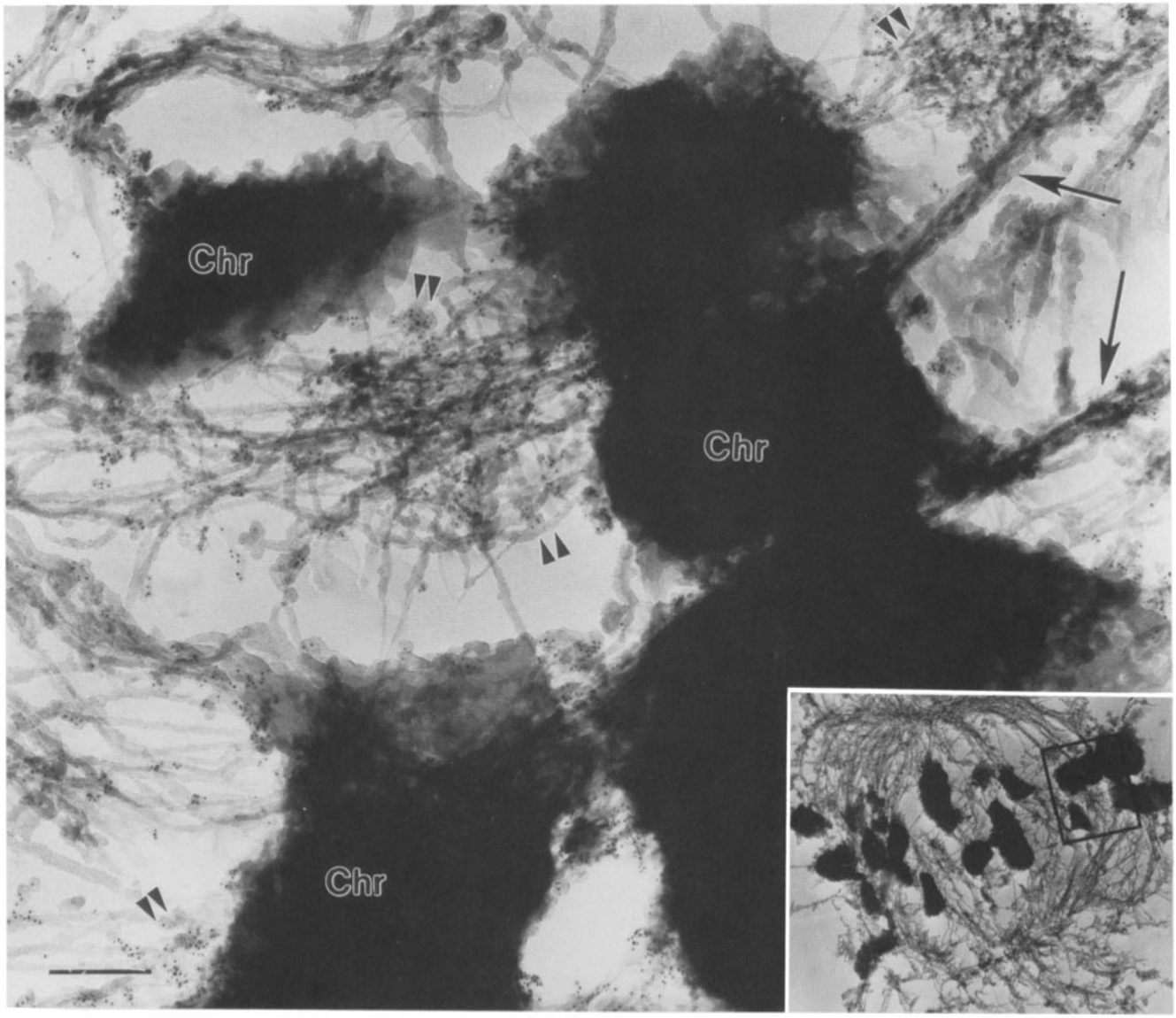

Figure 6. High resolution localization of H1B2 antigen in the perichromosomal region of an anaphase cell. CaSki cells were extracted with 0.5% Triton X-100 in cytoskeletal buffer before fixation, immunogold staining with H1B2 and a 5-nm gold bead–conjugated second antibody, and resinless section EM. The chromosomes (*Chr*) were enmeshed in a fibrogranular network (*double arrowheads*) which was intensely stained with the H1B2 antibody. Spindle microtubules, connected to the chromosomes and marked by arrows, were unstained except where they connected to the fibrogranular network. Note the fine detail of the microtubule- chromosome connections at the kinetochore afforded by the resinless section technique. The insert shows the same cell at lower magnification with a rectangle marking the location of the higher magnification view. Bar, 0.2 μm.

Unmasking of the H1B2 Antigen as Cells Approach Prophase

A previous study (Chaly et al., 1984) demonstrated that nuclear matrix proteins redistributed in a variety of patterns during mitosis. Some were associated with the chromosomes at mitosis, including one on the periphery of the chromosomes, while others dispersed throughout the cytoplasm.

The behavior of H1B2 antigen, studied in synchronized CaSki cells, was unlike that of any previously described nuclear matrix protein. First, the normally masked antigen became increasingly accessible as the cell approached mitosis. During interphase, the H1B2 antigen could be stained only after DNase I digestion and 0.25 M ammonium sulfate extraction had unmasked the antigenic site. As cells approached prophase, the antigen became increasingly exposed and could be immunostained without salt extraction (Fig. 5). The antigen was first seen in one small region of the nucleus (Fig. 5 *a*). The position of the H1B2 staining did not correspond to the nucleoli, as seen in the corresponding phase-contrast image, and may represent a previously uncharacterized premitotic nuclear structure.

In a few cells a second H1B2 spot appeared (Fig. 5 *b*) and in some cells the fluorescence had spread to larger regions of the nucleus. Finally, regions of H1B2 immunofluorescence filled the whole nucleus. Later in mitosis, when the chromosomes condensed in prophase, as seen by phase-contrast mi-

croscopy, the H1B2 staining surrounded but was not limited to the chromosomes (Fig. 5 *c*). This was more clearly seen in the prometaphase CaSki cell of Fig. 5 *d* which has a ring of condensed chromosomes and an empty central region. The H1B2 immunofluorescence was present in the central region as well as in the doughnut of surrounding chromosomes, so even at this low resolution the antigen surrounded but was not coincident with the chromosomes. The asters, precursors of the centrioles, were stained with H1B2. This is clearly seen in the prophase cell of Fig. 5 *c*. Pericentriolar staining persisted throughout the rest of mitosis.

Redistribution of the H1B2 Antigen during Mitosis: Identification of Perichromosomal Filaments

At metaphase, as the chromosomes assembled at the metaphase plate, the H1B2 immunofluorescence appeared to surround the chromosomes (Fig. 5 *e*). Some antigen was also present in many small, bright specks throughout the cell. This punctate material must be bound to the cytoskeleton since the extraction with 0.5% Triton X-100 had not released it.

As the chromosomes separated in anaphase, H1B2 fluorescence separated into two distinct perichromosomal regions with an unstained space between the separating daughter chromosomes (Fig. 5 *f*). The images suggest that the antigen containing material moved with the two daughter sets of chromosomes. At cytokinesis (Fig. 5 *g*), as the two daughter nuclei were reforming, H1B2 was distributed throughout the new nuclei, but small patches of fluorescence remained associated with the cytoskeleton. These may have derived from the more numerous and less intensely staining cytoplasmic spots of the metaphase cell (Fig. 5 *e*). The smaller spots of the metaphase cell may have coalesced into the larger, more intensely stained spots of the anaphase cell. A striking example of the relocation of H1B2 at mitosis was the bright fluorescence that remained in the midbody, the last connection between the daughter cells.

Immunofluorescence light microscopy showed H1B2 staining around, but not exactly coincident with, the chromosomes (Fig. 5). When seen at the higher resolution made possible by EM (Fig. 6), the localization of H1B2 in the perichromosomal region of mitotic cells was both on and around the chromosomes. The chromosomes were surrounded by a fibrogranular network containing the H1B2 antigen. The filaments of the fibrogranular material were ~9–14 nm in diameter and were clearly distinct from the microtubules of the spindle apparatus. These CaSki cells were treated with 5 μg/ml taxol before and during extraction to preserve the spindle microtubules. The H1B2 antigen was not associated with those microtubules but was, instead, located in the fibrogranular network surrounding the chromosomes and occasionally at the surface of the chromosomes. The absence of the H1B2 antigen in the microtubule spindle was verified by immunostaining HeLa cells with rabbit anti-tubulin and monoclonal mouse H1B2 antibodies. The tubulin and H1B2 staining patterns were not coincident.

The fibrogranular network containing the H1B2 antigen (Fig. 6) consisted, in part, of 9–14 nm filaments which interconnected adjacent chromosomes and were occasionally decorated with the H1B2 antibody. A similar network of filaments and associated granular material surrounded the centrioles (Fig. 7). This network was also stained by the H1B2 antibody. This fibrogranular material may comprise the pericentriolar "cloud" seen indistinctly in the conventional epon-embedded section.

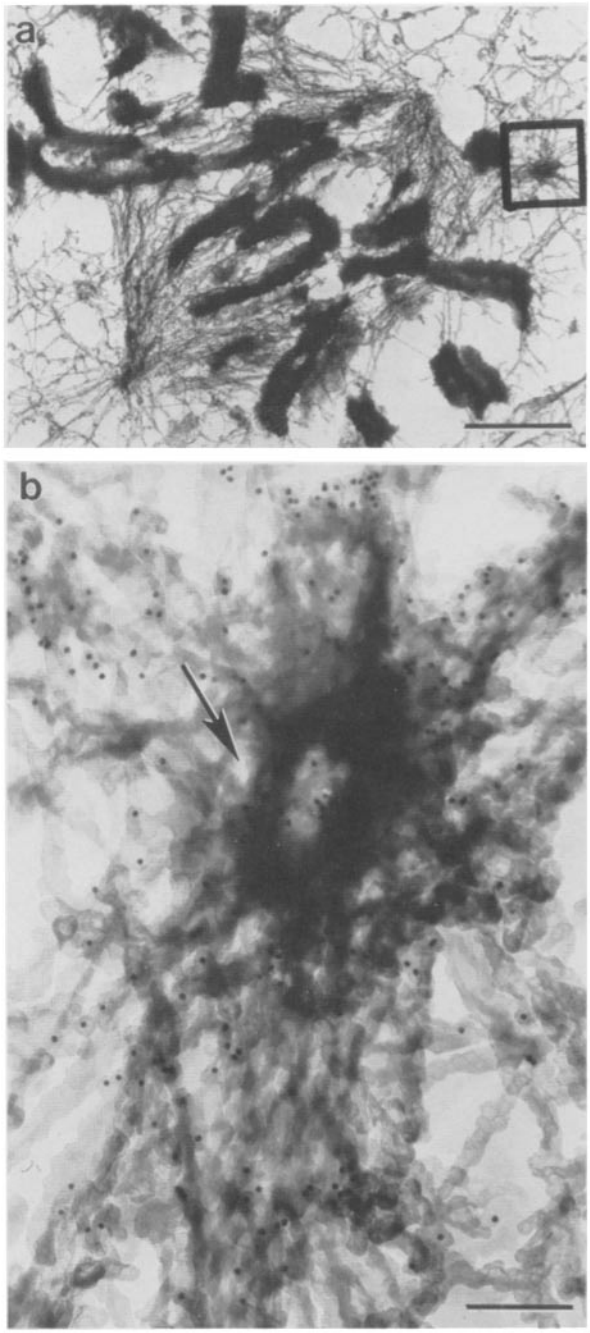

Figure 7. High resolution localization of the H1B2 antigen at the centriole. CaSki cells were extracted with 0.5% Triton X-100 in cytoskeletal buffer, fixed, and immunogold stained with H1B2 and a 10-nm gold bead–conjugated second antibody. Samples were then processed for resinless section EM. *a* is a low magnification view of an anaphase cell. The marked rectangle shows the position of the centriole seen at higher magnification in *b*. *b* shows the centriole, marked by an arrow, and the surrounding fibrogranular material which were strongly stained with the H1B2 antibody. Bars: (*a*) 2 μm; (*b*) 0.2 μm.

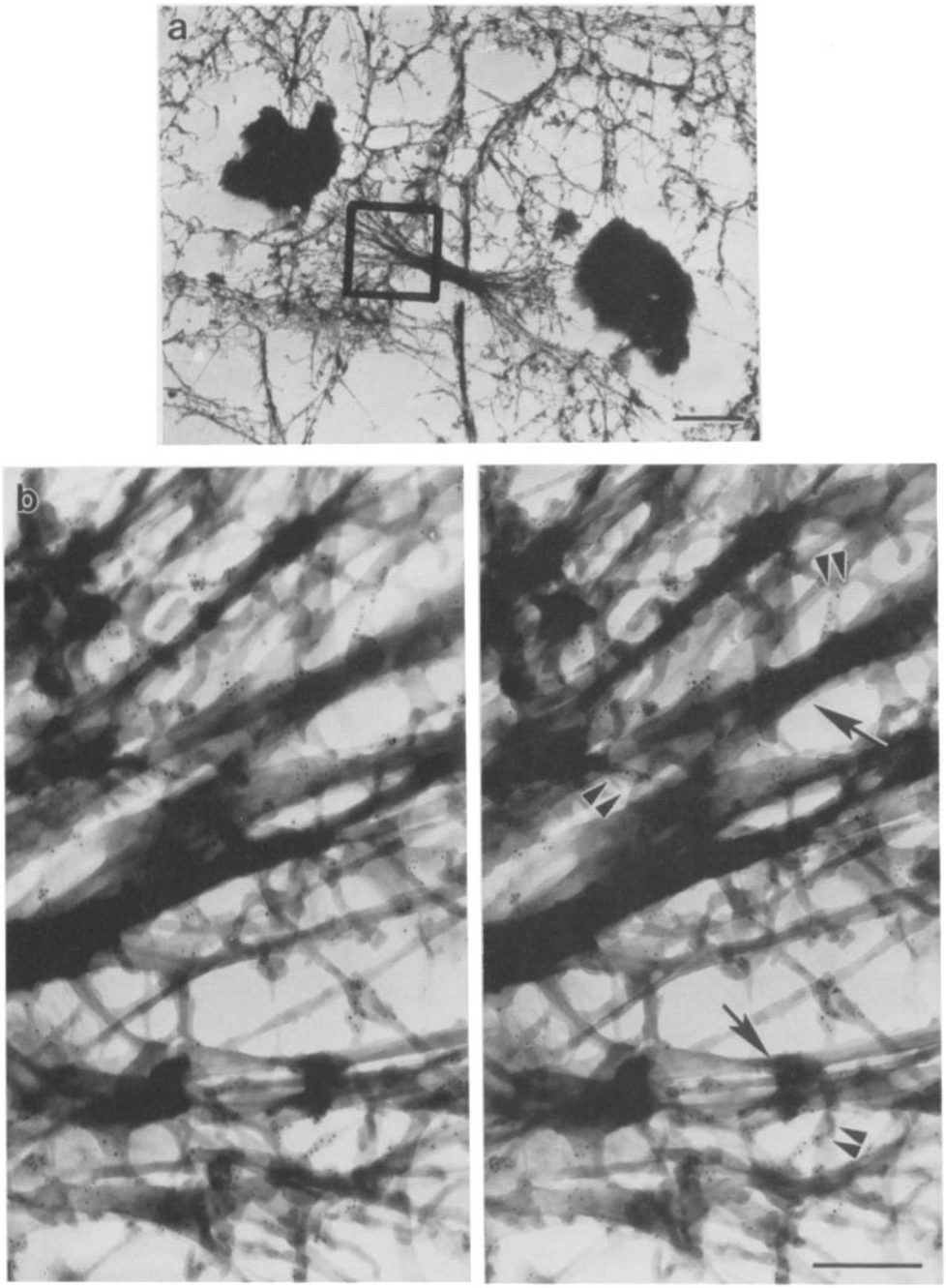

Figure 8. Stereo resinless section view of H1B2 staining at the periphery of the midbody. These CaSki cells were extracted with 0.5% Triton X-100 in cytoskeletal buffer, fixed, and immunogold stained with H1B2 and a 5-nm gold bead-conjugated second antibody. *a* shows at a lower magnification two daughter cells at cytokinesis. The rectangle marks the area shown at higher magnification in panel b. The higher magnification micrograph (*b*) was taken of an area at the periphery of the midbody. Closer to the center of the midbody the structure was too dense to see details of gold bead localization. H1B2 stained the thin filaments (*double arrowheads*) which interconnected the thicker fibers and microtubules (*arrows*). The resinless section technique enables the whole thickness of the section to be seen and not just the surface; this is best seen in stereo. Bars: (*a*) 2 μm; (*b*) 0.2 μm.

Immunofluorescence light microscopy showed H1B2 staining in the midbody between daughter cells at cytokinesis. The midbody, connecting cells, contains remnants of the pole to pole spindle fibers and, as shown by the stereoscopic micrograph of Fig. 8, also contained thinner filaments of ~12–17 nm containing H1B2. The stereoscopic view shows clearly that these filaments are distinct from the microtubule bundles passing through the midbody and represent a previously undetected mitotic structure.

Altered Lability of H1B2 at Mitosis

The appearance of H1B2 immunofluorescence in unextracted nuclei was an unambiguous sign of approach to mitosis. This emergence of the H1B2 epitope appeared to reflect a profound reorganization of cell architecture and not simply a conformational change of H1B2 itself. Another observation supports this view; much of the H1B2 antigen became extractable with 0.25 M ammonium sulfate, even without a DNase I digestion, as cells approached mitosis. Later in mitosis, extraction of CaSki cells with 0.25 M ammonium sulfate removed the fluorescence from the chromosome region, even without a prior DNase I digestion. Not all of H1B2 was removed by this salt treatment; some remained specifically at the midbody and in the pericentriolar filaments near the spindle poles.

Discussion

Nuclear matrix architecture is complex; its characterization

will require the coordinated use of microscopic, biochemical, and immunological techniques to reveal the organization of the cell nucleus. These same techniques, applied to mitotic cells, may detect previously unseen structural networks that facilitate the disassembly and reassembly of nonchromatin nuclear structures at mitosis.

Our approach to nuclear matrix characterization is to generate mAbs against nuclear matrix proteins and to use those antibodies to characterize the antigen. The antibodies also serve to localize the antigen in the cell by immunofluorescence microscopy and, at much higher resolution, by immunogold EM of resinless sections. Conventional EM can show little of the nuclear interior and there were previously no completely satisfactory methods for fractionating the nucleus. The more recently developed techniques of embedment-free EM and nuclear fractionation (Capco et al., 1982, 1984; Fey et al., 1986, He et al., 1990) have given us a clearer understanding of nuclear matrix structure and composition. More recently we have used antibodies and colloidal gold-conjugated second antibodies to label nuclear proteins in resinless sections of extracted cells (Nickerson et al., 1990). This report describes our first application of this combination of techniques for the study of nuclear architecture.

The 240-kD H1B2 protein, normally masked in the detergent-extracted interphase nucleus, was uncovered and made accessible to the antibody either by digestion with DNase I and extraction with 0.25 M ammonium sulfate or by entrance into mitosis. Once the nuclear matrix had been exposed by DNase I digestion and 0.25 M ammonium sulfate extraction, it stained with the H1B2 antibody in a speckled pattern. The mechanism by which the DNase I- 0.25 M ammonium sulfate treatment unmasked H1B2 is unknown. It seems likely that the extraction removed structural components that normally cover the H1B2 antigen.

The rearrangements of the H1B2 antigen with the cell cycle may show the redistribution of the nuclear matrix during mitosis. The H1B2 antigen gradually became unmasked and could be stained without the removal of chromatin as the cell approached prophase. The structural rearrangements preparing the cell for mitosis were also uncovering the antigen. Fluorescence appeared first in a small, single region of the nucleus and later spread throughout the entire nucleus. In some cells a second spot was seen, but whether this is a normal or requisite stage is not known.

When the nuclear envelope disassembled into vesicles and the nuclear matrix rearranged at prometaphase, the H1B2 protein was found in a fibrogranular network surrounding the chromosomes and in a pericentriolar structure surrounding the mitotic spindle pole. This latter fibrogranular structure can be seen in conventional embedded sections only as a pericentriolar "cloud". Previous reports from this laboratory (Capco and Penman, 1983; Wagner et al., 1986) have described nonmicrotubular filaments associated with the spindle. The H1B2 antigen was associated with extensive perichromosomal and pericentriolar networks containing thin filaments and granules, providing the first clue as to their composition.

As the cell began cytokinesis H1B2 fluorescence was, as expected, seen in the reforming daughter cell nuclei. More surprising was the H1B2 staining of the midbody. The midbody consists of at least 35 proteins (Mullins and McIntosh, 1982). Some midbody proteins are associated with other mitotic structures before telophase. A mAb raised against a mitotic scaffold fraction (Cooke et al., 1987) stained peptides of 135 and 155 kD which relocalized from the chromosome scaffold to the midbody during telophase. Two proteins of CHO cells, with molecular masses of 95 and 105 kD, are located throughout the spindle, although at higher concentrations in equatorial regions, and these become midbody proteins at telophase (Sellitto and Kuriya, 1988).

The protein detected by the H1B2 mAb is a component of the nuclear matrix. The presence of the H1B2 antigen in the matrix was shown by Western blots (Fig. 1), immunofluorescence light microscopy (Fig. 2), and immunogold EM (Fig. 3). Furthermore, the H1B2 antigen was retained with the network of 9 and 13 nm core filaments that underlies the complete nuclear matrix (He et al., 1990). Treatment of the complete nuclear matrix with 2 M NaCl leaves most of the H1B2 protein with the core filaments in CaSki and HeLa cells. Western blotting of HeLa fractions with the H1B2 mAb reproducibly detected a band at 240 kD (Fig. 1). This high molecular protein is present in all nuclear fractions, but principally in the core filament network (Fig. 1, lane *5*).

The characterization and localization of structural nuclear proteins will provide important information about nuclear matrix architecture and function. These studies will also yield useful insights into the ultrastructural dynamics of the cell in mitosis.

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