Primary Structure of NuMA, an Intranuclear Protein That Defines a Novel Pathway for Segregation of Proteins at Mitosis

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Abstract. From a collection of monoclonal antibodies that specifically bind to various parts of the mitotic apparatus in human cells (1991. *J. Cell Biol.* 112: 1083–1097), two (1F1 and 1H1) recognize a >200-kD intranuclear protein that associates with the spindle immediately upon nuclear envelope breakdown and progresses down the spindle microtubules to concentrate ultimately at the pericentrosomal region. At the completion of anaphase this protein dissociates from the spindle microtubules and is imported into the regenerating nuclei through the nuclear pores. Overlapping cDNA clones that span the entire length of the corresponding 7.2-kb mRNA reveal an encoded polypeptide of 236,278 D that is predicted to contain two globular domains separated by a discontinuous α-helix with characteristics for adopting a coiled-coil structure. The corresponding gene is highly conserved but neither the DNA sequence nor the predicted amino acid sequence shows significant homology to any previously reported. Since the cDNA also encodes the epitopes recognized by antibodies specific for two previously described proteins, NuMA and centrophilin, and all three show similar molecular weights and localization during the cell cycle, NuMA, centrophilin, and the 1F1/1H1 antigen represent either the same protein or a family of proteins, for which the original name, NuMA, seems most appropriate. While the function of NuMA remains uncertain, its unusual pattern of segregation at mitosis defines a novel pathway for the segregation of nuclear proteins during cell division.

THE nucleus of higher eukaryotic cells undergoes profound changes during mitosis. In eukaryotic cells that undergo "open" mitosis, the strictly maintained compartmentalization between the nucleus and cytoplasm found during interphase is completely disrupted, only to be restored in each daughter cell at the completion of mitosis. The complex set of logistic problems associated with coordinating the reassembly of a functional nucleus is overcome using several specific mechanisms for the proper distribution of nuclear components during mitosis (e.g., Newport and Forbes, 1987).

The most intensively studied mechanism is the segregation of chromosomes during mitosis (reviewed by Mitchison, 1989; Rieder, 1991). At the onset of mitosis, transcription ceases and the chromatin condenses into chromosomes discernable by light microscopy. Upon nuclear envelope breakdown, the chromosomes attach to the astral microtubules through the kinetochore and begin a complex set of movements, concluding in their precise and coordinated segregation at anaphase (Hayden et al., 1990). This mechanism is intimately associated with microtubule dynamics and results in the direct partitioning of equal amounts of genetic material into each daughter cell (Gorbsky et al., 1987; Koshland et al., 1988). This mode of segregation is not only used for the proper distribution of the genetic material, but a variety of other nuclear proteins are carried to the daughter cells through attachment to the chromosomes. Nuclear proteins involved in chromatin structure, such as the histones and topoisomerases, follow this pathway. In addition, other nuclear proteins that are not intimately associated with chromosome structure (e.g., fibrillarin) also follow this pathway (Reimer et al., 1987).

Another mechanism for the distribution of nuclear components during mitosis is by diffusion throughout each daughter cell after nuclear envelope dissolution at the onset of prometaphase, followed by repartitioning into each daughter nucleus by specific targeting (reviewed by Gerace and Burke, 1988). The nuclear lamins are the most extensively studied proteins that follow this pathway (Gerace and Blobel, 1980). The assembly properties of the nuclear lamina are controlled during mitosis by the reversible phosphorylation of the lamin subunits (Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985). At the onset of mitosis, phosphorylation of the lamin subunits results in the complete dissolution of the nuclear lamina. Dephosphorylation of these proteins at telophase results in the accumulation of the lamins at the chromosomal surface and the subsequent polymerization of the lamina (Burke and Gerace, 1986; Glass and Gerace, 1990). The nuclear envelope also follows such a pathway when it vesicularizes at prometaphase and reassembles with the lamina onto the chromosome surface at the end of anaphase (Zeligs and Wollman, 1979; Pfaller et al., 1991).

Once the lamina is formed and the nuclear membrane is re-established, additional nuclear constituents initially dispersed throughout the cytoplasm are targeted into the regenerating nuclei by active transport through the nuclear pores. This process relies on specific nuclear localization sequences (NLS)[1] within the amino acid sequence of nuclear proteins. One such example is that of the SV-40 large T antigen which carries PKKKRKV as an NLS (Kalderon et al., 1984; Lanford and Butel, 1984). This short sequence can target an otherwise cytoplasmic protein into the nucleus (Goldfarb et al., 1986; Lanford et al., 1986) and recently a pair of cytoplasmic proteins that specifically bind this NLS have been purified and shown to enhance nuclear uptake of proteins containing this sequence (Adam and Gerace, 1991).

In this report, we present a detailed analysis of a nuclear protein that follows a third redistribution pathway at mitosis. After release from nuclei at prometaphase, this protein is segregated to each daughter cell by attachment to each spindle pole. Independent of chromosome movement, the antigen appears to migrate to the minus end of the microtubules of the bipolar spindle apparatus, ultimately concentrating at the pericentrosomal region. Isolation of overlapping cDNA clones that span the entire length of the 7.2-kb RNA transcript that encodes this protein reveals a single open reading frame encoding a novel protein of 236,278 D that is predicted to have a tripartite (globular, helical, globular) structure. Several other large nuclear proteins identified by others (including NuMA [Lydersen and Pettijohn, 1980], centrophilin [Tousson et al., 1991], SP-H [Maekawa et al., 1991], SPN [Kallajoki et al., 1991], and others [Izant et al., 1982; Pepper et al., 1984]) follow a similar cell cycle–dependent localization. Since at least three of these (NuMA, centrophilin, and our 1F1/1H1 antigen) share common epitopes and molecular weights, all probably represent the same protein or family of proteins, for which the original name NuMA seems most appropriate.

Materials and Methods

Cell Lines

Three human cell lines were used for analyzing the antigens recognized by monoclonal antibodies 1F1 and 1H1 (Compton et al., 1991), 2E4 (against NuMA; Lydersen and Pettijohn, 1980), and 2D3 (against centrophilin; Tousson et al., 1991). All three lines accumulate immunoreactive proteins with indistinguishable electrophoretic mobilities and cell cycle distributions. CF-PAC1 (cystic fibrosis pancreatic cancer) cells were selected for most immunolocalization experiments because of their relatively flat morphology during mitosis. Whole cell extracts and chromosome scaffolds were prepared either from suspension cultures of HeLa or K562 erythroleukemic cell (selected for their rapid growth and ease with which large quantities can be obtained).

Immunological Techniques

Intracellular localization of antigens throughout the cell cycle was determined using human CF-PAC1 cells. Cells were grown on coverslips in Iscove's modified Dulbecco's medium containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 μg/ml streptomycin. Coverslips were immersed in microtubule stabilization buffer (MTSB; 4 M glycerol, 100 mM Pipes, pH 6.9, 1 mM EGTA, and 5 mM $MgCl_2$) for 1 min at room temperature, extracted in MTSB + 0.5% Triton X-100 for 2 min, and then returned to MTSB for 2 min. Cells were then fixed in −20°C methanol for 5 min and rehydrated in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% albumin). Rabbit anti-tubulin serum and anti-NuMA monoclonal antibodies (mAb1F1) were added and incubated for 30 min at room temperature in a humidified chamber. Coverslips were washed in TBS and the bound rabbit antibodies were detected with fluorescein-conjugated goat anti-rabbit antibodies (Vector Laboratories, Inc., Burlingame, CA). The bound mouse antibodies were detected with Biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Inc.) followed by Texas Red–conjugated streptavidin (Vector Laboratories, Inc.). DNA was detected with 4′,6-diamidino-2-phenylindole (DAPI; 0.4 μg/ml; Sigma Chemical Co., St. Louis, MO). Coverslips were mounted with Gel/Mount (Biomeda, Foster City, CA) and observed with an Olympus BH-2 microscope equipped for epifluorescence.

Cells were prepared for laser confocal microscopy essentially as described above except for the following modification. Cells were rinsed in MTSB and then fixed directly in 2.0% paraformaldehyde, 0.4% glutaraldehyde, 90 mM Pipes, pH 6.8, 1 mM EGTA, and 5 mM $MgCl_2$ for 5 min at room temperature. Cells were then transferred to TBS + 0.5% Triton X-100 for 5 min and then washed in TBS before antibody application. After antibody incubations and washing, the coverslips were mounted in Testog FITC-guard mounting medium (Testog, Inc., Chicago, IL). Cells were observed using a laser scanning confocal microscope (model MRC600; Bio-Rad Laboratories; Richmond, CA) mounted on a Nikon Optiphot microscope.

To assay centromere staining after rapid microtubule depolymerization, cells were treated as described above with the following modifications. Cells growing on coverslips were immersed in TBS + 5 mM $CaCl_2$ for 2 min at room temperature, transferred to TBS + 5 mM $CaCl_2$ + 0.5% Triton X-100 for 2 min, and then fixed in PBS + 3.5% paraformaldehyde for 5 min at room temperature. Antibody staining and detection were done identically as stated above.

Immunoblotting was performed on nitrocellulose strips containing proteins from the human erythroleukemia cell line K562. These cells were grown in RPMI-1640 medium containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 μg/ml streptomycin. Cells were harvested by centrifugation, washed with ice-cold PBS, resuspended in SDS sample buffer, and separated by SDS-PAGE (Laemmli, 1970). Nitrocellulose strips were then preincubated for 30 min before incubation with primary antibody in phosphate-Triton X solution (PTX) (10 mM $NaPO_4$, pH 7.5, 0.2% Triton X-100, 150 mM NaCl, 1 mM EGTA, and 1 mM NaN_3) containing 4% albumin for 4–12 h at room temperature. Unbound antibody was removed by washing in PTX five times for 3 min. Bound antibody was then detected with ^{125}I-labeled goat anti-mouse antibody (Amersham Corp., Arlington Heights, IL).

Microinjections

CF-PAC1 cells were microinjected as described by Cleveland et al. (1983). Prometaphase cells were injected with rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, Inc.) at a concentration of 5 mg/ml in 5 mM Hepes, pH 7.0, 150 mM NaCl. After injection, cells were incubated for 2 h at 37°C, 5% CO_2. Cells were then fixed with 3.5% paraformaldehyde in PBS for 5 min at room temperature, extracted with TBS + 0.5% Triton X-100 for 5 min at room temperature, and rinsed in TBS. Immunofluorescence was then performed exactly as described above, except that a fluorescein-conjugated secondary antibody was used for detection of the bound mouse monoclonal antibody.

Identification of cDNA Clones by Expression Library Screening

A λZAP HeLa cDNA library (Stratagene Inc., La Jolla, CA) was screened using monoclonal antibody 1F1 (Compton et al., 1991). Nitrocellulose filters containing proteins expressed from isopropyl-β-D-thiogalactoside-induced plaques were incubated with antibody under the same conditions described above for immunoblotting. After plaque purification the 1.9-kb cDNA insert was excised into pBluescript SKII in situ by superinfection with helper-phage. This cDNA was then used to rescreen the same library by DNA hybridization. Phage were lysed by treatment with 0.5 N NaOH, 1.5 mM NaCl. After neutralization in 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl, phage DNA was immobilized on nitrocellulose filters by baking at 80°C. Hybridization with the radiolabeled DNA was in 600 mM NaCl, 60 mM Na-citrate, 0.2 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS, 0.04% polyvinylpyrollidone, 0.04% albumin, 0.04% Ficoll, and 100 μg/ml fragmented salmon sperm DNA at 68°C for 18–20 h. The DNA probe was radiolabeled to a sp act 1–2 × 10^8 cpm/μg using random priming as described by Fein-

1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; MTSB, microtubule stabilization buffer; NLS, nuclear localization sequence; R-WGA, rhodamine-conjugated wheat germ agglutinin.

berg and Vogelstein (1983). After hybridization, filters were washed and exposed to Kodak XAR-5 film at −70°C overnight.

DNA Sequencing

A nested series of deletions of both strands of each of the three plasmids (plF1, plF1-2, and plF1-4) was created using ExoIII. These were cloned into M13-mp8 and templates were prepared as described by Sanger et al. (1977) and sequenced using the sequenase enzyme (U.S. Biochemical Corp., Cleveland, OH). The sequence was assembled using the Wisconsin GCG sequence analysis package.

Expression of Recombinant Proteins in Bacteria and Immunization of Mice

The EcoRI fragments of each of the cDNA clones were inserted into the pATH vector series of expression plasmids and transformed into a protease-deficient strain of *Escherichia coli* (CAG 456). Cultures were grown to log phase in M9 medium (Maniatis et al., 1982) and induced with indoleacrylic acid for 4–6 h (see Lopata and Cleveland, 1987, for details). The cells were then pelleted, washed with 10 mM Tris-HCl, pH 7.5, and resuspended in SDS-PAGE sample buffer. The protein obtained by induction of plF1 was partially purified by excising the appropriate gel band from an SDS polyacrylamide gel followed by electroelution. 50 μg of partially purified protein was mixed with Freund's adjuvant and injected intraperitoneally into BALB/c mice at 10-d intervals. Serum was collected by tail bleed and (for the final bleed) cardiac puncture.

Results

Identification of a Novel Pathway for the Distribution of Nuclear Material during Mitosis

We have previously described the isolation of two monoclonal antibodies (mAb1F1 and mAb1H1) both recognizing a >200-kD antigen that is found at or within the nucleus at interphase, but redistributes to the spindle poles during metaphase (Compton et al., 1991). To document more precisely the transit of this protein from the nucleus to the spindle poles at prometaphase and the subsequent reassociation with nuclei at the completion of mitosis, we used indirect immunofluorescence with these monoclonal antibodies. As shown in Fig. 1 *A*, the 1F1/1H1 antigen(s) are exclusively nuclear during interphase, revealing punctate staining throughout the nucleus except for exclusion from the nucleoli.

To characterize further this antigen's localization within the interphase cell nucleus, we used double-label indirect immunofluorescence with an antibody specific for the nuclear lamins (provided by L. Gerace, Scripps Clinic) and laser confocal microscopy to assess the nuclear distribution of the antigen detected by mAb1F1 relative to the nuclear lamina. Fig. 2 *A* shows an optical section of the nucleus of a cell stained with both antisera. These data clearly show that the antigen detected by mAb1F1 is intranuclear and distributed throughout the nucleus, whereas the lamin staining is confined to the periphery of the nucleus.

At prophase, as the interphase microtubule array begins to disassemble and the duplicated centrosomes nucleate two prominent asters, the 1F1 antigen remains nuclear, although its distribution is less uniform than in interphase (Fig. 1 *B*). The progression from prophase to prometaphase is marked by the breakdown of the nuclear envelope and during this period the antigen detected by mAb1F1 redistributes from the nuclear compartment to the astral microtubules (Fig. 1 *C*). It is interesting to note that the antigen is only associated with those astral microtubules that project into the region that was previously occupied by the nucleus, a finding consistent with the rapid association of this antigen with the astral microtubules immediately upon nuclear envelope breakdown. Furthermore, it is also apparent from Fig. 1 *C* that this antigen does not homogeneously decorate the entire length of each microtubule. Since we have observed this staining pattern in prometaphase cells that have been fixed using paraformaldehyde or methanol, with or without prior Triton X-100 extraction, we believe that the observed microtubule association is unlikely to be the result of a fixation artifact.

As the cell progresses through prometaphase, an increasing proportion of the 1F1 antigen is found at the centrosomes (Fig. 1 *D*), and by metaphase the antigen is confined to the pericentrosomal region of both poles of the bipolar spindle (Fig. 1 *E*). In the pericentrosomal region the antigen is not associated with the centrosome itself or the innermost pericentrosomal domains. This was demonstrated by double immunofluorescence localization of the 1F1 antigen relative to the centrosomal antigen detected by the human autoantibody 5051 (provided by S. Doxsey and M. Kirschner, University of California, San Francisco, CA). The antigen defined by the 5051 serum previously has been shown by immunoelectron microscopy to lie proximal to the centriole itself (Calarco-Gillam et al., 1983; Doxsey, S., P. Calarco, P. Siebert, L. Evans, P. Stein, and M. W. Kirschner. 1990. *J. Cell Biol.* 111:179*a*). At the immunofluorescence level, the 5051 antigen localizes only to two focused spots (Fig. 2 *B, center panel*) that are seen as dark "holes" in the pericentrosomal region stained by mAb1F1 (Fig. 2 *B, right panel*). It is thus clear that the 1F1 antigen is associated with the microtubules of the pericentrosomal region of the spindle pole, but is not a component of the centrosome itself.

When the chromosomes segregate at anaphase, the 1F1 antigen remains microtubule associated in the pericentrosomal region (Fig. 1 *F*), although a decrease in fluorescence intensity is seen reproducibly. As the cells progress from telophase into the following interphase and the remnants of the spindle microtubules are transformed into an interphase array, the antigen no longer associates with the microtubules or centrosomes of the daughter cells, but rather re-enters the newly formed nuclei (Fig. 1 *G*). Thus, a striking feature of the cell cycle–dependent localization of this nuclear antigen is its distribution to each daughter cell, apparently mediated by specific association with the spindle microtubules (a possibility initially suggested by Price and Pettijohn [1986] for the NuMA antigen; see below) followed by accumulation at each spindle pole. We propose that this represents a novel pathway for faithful segregation of nuclear components at mitosis.

Similarity of the 1F1/1H1 Antigens to NuMA and Centrophilin

The changes in cell cycle distribution of the 1F1 antigen are remarkably similar to those detected by mono- or polyclonal antibodies reported by four other groups (Lydersen and Pettijohn, 1980; Kallajoki et al., 1991; Maekawa et al., 1991; Tousson et al., 1991). Each of the antibodies in these reports recognizes a >200-kD nuclear antigen that associates with the spindle poles during mitosis. Various names (NuMA, centrophilin, SP-H, and SPN) have been proposed for the corresponding proteins, each of which are presently defined

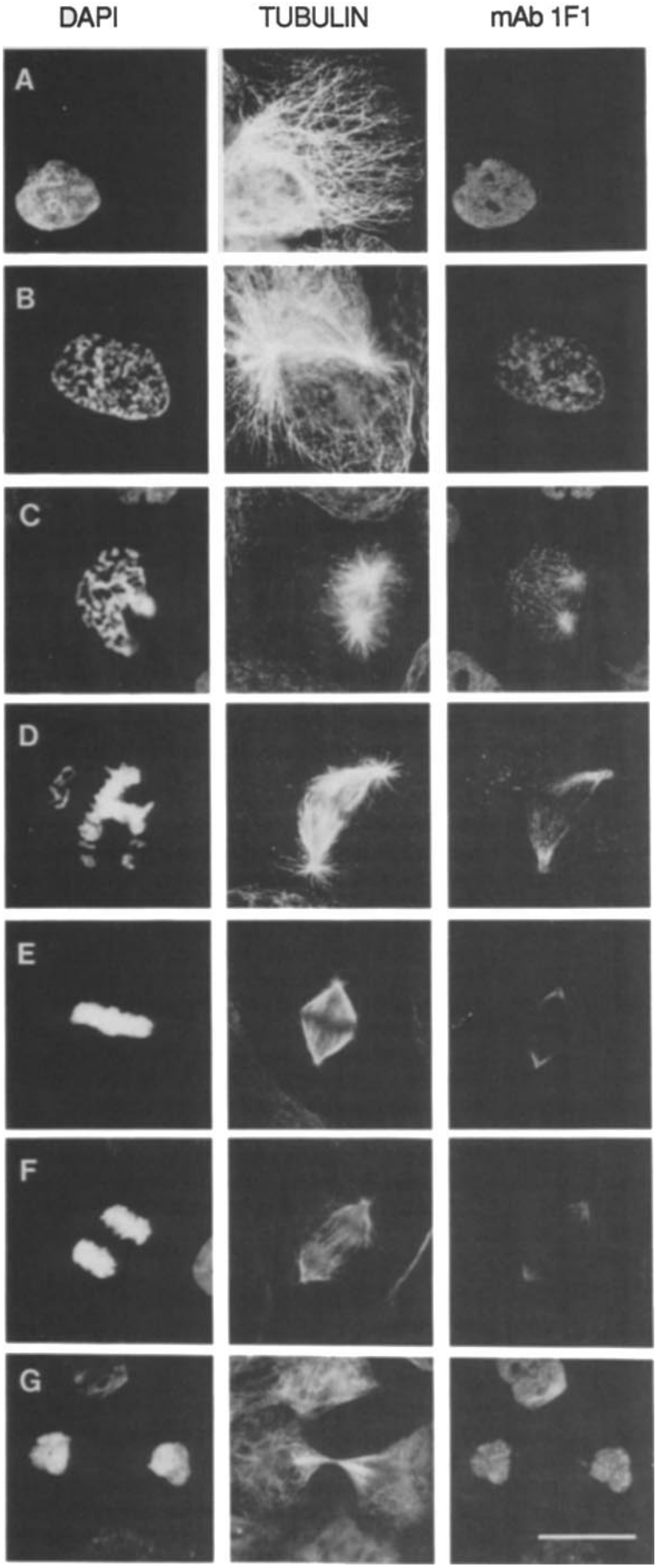

Figure 1. Localization of the 1F1 antigen in human CF-PAC 1 cells traversing the normal cell cycle. Double immunofluorescence staining with rabbit anti-tubulin serum and mAb1F1. (*left panels*) DAPI staining showing nuclear morphology and chromosome position; (*center panels*) tubulin staining; (*right panels*) mAb1F1 staining. Cells shown are in interphase (*A*), prophase (*B*), prometaphase (*C* and *D*), metaphase (*E*), anaphase (*F*), and telophase (*G*). Bar = 20 μm.

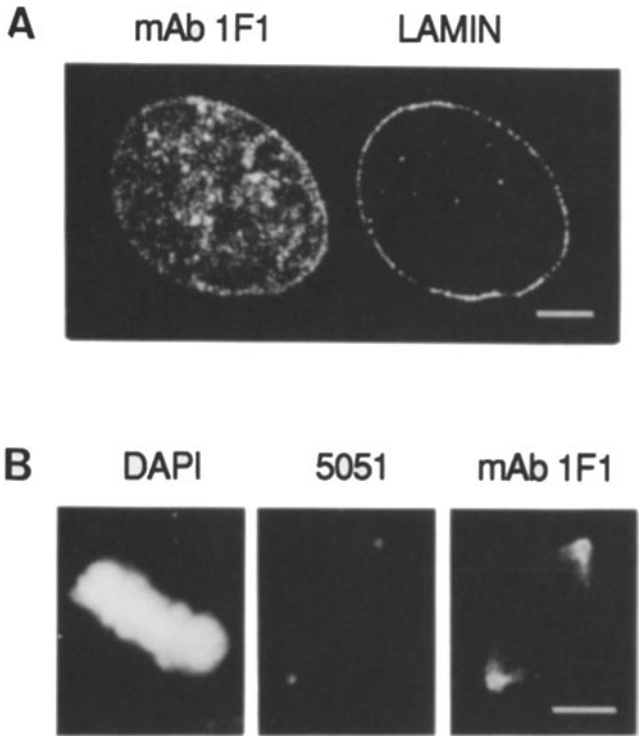

Figure 2. The 1F1 antigen is an intranuclear protein during interphase. Double immunofluorescence staining of CF-PAC1 cells showing the localization of the mAb1F1 antigen relative to the nuclear lamina in an interphase nucleus visualized with laser scanning confocal microscopy (*A*) and the centrosomes of the mitotic spindle poles visualized by conventional epifluorescence (*B*). (*A*) Simultaneous optical sections of an interphase nucleus stained with mAb1F1 (*left*) and an antibody against nuclear lamins (*right*). (*B*) DAPI staining showing chromosomes aligned at the metaphase plate (*left*); autoimmune sera 5051 staining (*center*); and mAb1F1 staining (*right*). Bar = 5 μm.

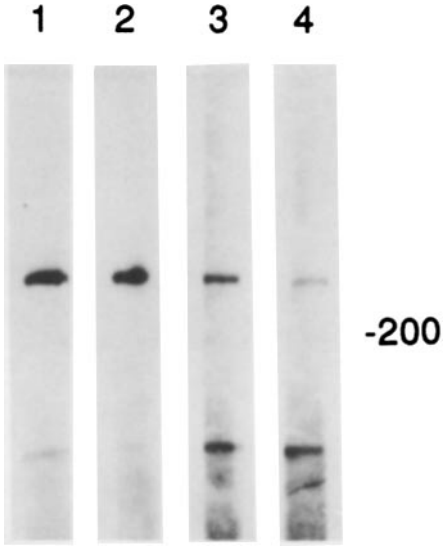

Figure 3. Antibodies to the 1F1 antigen, the 1H1 antigen, NuMA, and centrophilin each recognize a >200-kD polypeptide with the same electrophoretic mobility. Parallel immunoblots, each containing 40 μg of K562 whole cell extract, were prepared after electrophoresis on a 4% polyacrylamide gel. The blots were probed with mAb1F1 (lane *1*), mAb1H1 (lane *2*), mAb2E4 (NuMA) (lane *3*), and mAb2D3 (centrophilin) (lane *4*). The migration position of myosin (200 kD) is shown on the right.

solely by molecular weight and cell cycle–dependent localizations (as determined by antibody binding). For those cases in which the initial antibodies were available to us, we tested the possibility that each antibody might identify the same protein by comparing immunofluorescence patterns and molecular weights of the antigens detected. A comparison of the antigens identified by mAb1F1 and mAb1H1 with those detected with monoclonal antibodies to NuMA (mAb2E4; Lydersen and Pettijohn, 1980) and centrophilin (mAb2D3; Tousson et al., 1991) is shown in Fig. 3. When all four monoclonal antibodies were used to probe immunoblots of total cell proteins from the human erythroleukemia cell line K562, all were found to recognize a cellular protein of an indistinguishable electrophoretic mobility, as well as a smaller polypeptide (~180 kD) that has been previously argued to represent a proteolytic product of the larger protein (Price and Pettijohn, 1986). Similarly, indirect immunofluorescence analysis of cells progressing through the cell cycle revealed staining patterns for each antibody indistinguishable from that seen (Fig. 1) for mAb1F1 (data not shown).

From these data, combined with the finding (see below) that an epitope recognized by each of these antibodies is present on a 236-kD polypeptide encoded by a common mRNA, we conclude that all three recognize the same cellular protein or closely related members of a family of antigenically related polypeptides. Since the first of the proposed names accurately describes the cell cycle–dependent localization, we have adopted that nomenclature and shall refer to this protein (or protein family) as NuMA (*Nu*clear protein that associates with the *M*itotic *A*pparatus).

Post-mitotic Nuclear Import Occurs via Transit through the Nuclear Pores

The unusual pathway that NuMA utilizes for segregation at mitosis provoked examination of how this protein re-enters nuclei at the end of telophase. Two possibilities were consistent with the localization data. If NuMA were released from the polar microtubules and then actively imported into the regenerating nucleus through the nuclear pores, then injection of wheat germ agglutinin will inhibit this nuclear reaccumulation, as has previously been demonstrated for import of other nuclear components (Finlay et al., 1987; Newmeyer and Forbes, 1988; Benavente et al., 1989). Alternatively, if NuMA was an important component in reformation of intact nuclei, it might associate with these reforming nuclei before nuclear envelope assembly and independent of transit through nuclear pores. To distinguish between these, metaphase cells were microinjected with rhodamine-conjugated wheat germ agglutinin (R-WGA) to impair nuclear pore permeability and block the later steps of nuclear assembly (Finlay et al., 1987; Yoneda et al., 1987; Newmeyer and Forbes, 1988; Benavente et al., 1989).

Four cells that were mock injected during mitosis and another four injected with R-WGA were carefully monitored after injection. An example of one such experiment is shown in Fig. 4. 2 h after mock injection of a metaphase cell, the injected cell was examined for normal karyokinesis, cytoki-

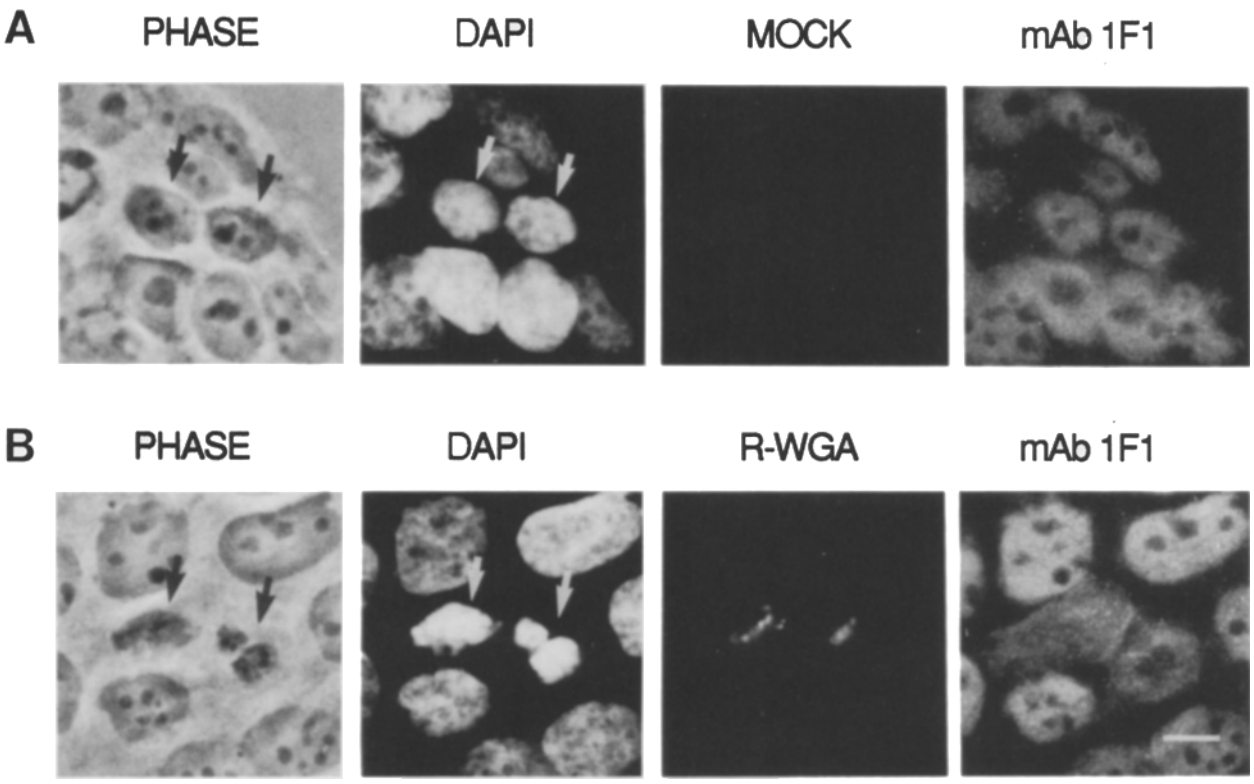

Figure 4. Re-entry of NuMA into nuclei at the end of mitosis is blocked by microinjection of wheat germ agglutinin. 2 h after injection of R-WGA into mitotic cells, the localization of NuMA was determined with indirect immunofluorescence using mAb1F1. Daughter cells (*arrows*) after mock injection (*A*) or injection with R-WGA (*B*) viewed by post-fixation phase contrast microscopy (*left*), DAPI staining (*left center*), rhodamine channel (*right center*), and mAb1F1 staining (*right*). Bar = 10 μm.

nesis, and NuMA localization (Fig. 4 *A*). DAPI staining revealed that nuclear compartmentalization was completely re-established in the two daughter cells, with the chromatin decondensing to yield the typical interphase appearance and NuMA localized exclusively within each daughter nucleus (indistinguishable from that seen in uninjected cells; Fig. 1 *A*). In contrast, 2 h after a mitotic cell was injected with R-WGA normal cytokinesis was completed on schedule, but nuclear assembly after karyokinesis was partially blocked (Fig. 4 *B*). Chromatin was compartmentalized within the newly forming nuclei (Fig. 4 *B, DAPI panel*), but did not fully decondense. Staining with the mAb1F1 to localize NuMA revealed that R-WGA injection resulted in the exclusion of a majority of NuMA from the two daughter nuclei (Fig. 4 *B, 1F1 panel*). In the absence of uptake into nuclei, NuMA was distributed throughout the cytoplasm of the two daughter cells, but did not associate with microtubules, the injected R-WGA, or any other readily identifiable cellular structure. Blockage of NuMA import was also seen in three other independent injections of R-WGA, while three other mock injected cells showed normal reimport of NuMA. These data indicate that the import of most, perhaps all, NuMA into the regenerating daughter nuclei requires proper nuclear pore function.

Is NuMA a Kinetochore Component?

Despite our demonstration that NuMA is a nuclear protein that is localized to the pre-centrosomal domain as mitosis progresses, our monoclonal antibodies (1F1 and 1H1) were initially isolated by their strong binding to the centromere/kinetochore region of isolated chromosomes (Fig. 5 *A*; see also Compton et al., 1991). In such chromosome preparations, both antibodies decorate the centromere/kinetochore domain in a "double-dot" pattern that lies more peripheral (Fig. 5 *A, middle*) to the DNA axis than does CENP-B (Fig. 5 *A, right*). Since CENP-B has been shown to bind to the centromeric heterochromatin (Cooke et al., 1990), this suggested that NuMA (i.e., the 1F1/1H1 antigen) might be a component of the kinetochore or its associated corona. If so, why is no such localization of NuMA seen in cells normally progressing through the mitotic cycle? One obvious possibility might be the occlusion of antibody binding by spindle microtubules. Alternatively, centromeric localization on isolated chromosomes (prepared from cells treated for extended periods with microtubule drugs that block spindle assembly) might arise as the consequence of the redistribution of NuMA during a prolonged, microtubuleless prometaphase arrest.

To discriminate between these possibilities, spindle microtubules in cells normally cycling through metaphase were rapidly disassembled by cell lysis in the presence of 5 mM calcium. The distributions of NuMA and tubulin, and the positions of centromeres (identified by centromere-specific CREST autoantiserum M; Sullivan and Glass, 1991) were tracked by indirect immunofluorescence. Our rationale was that if microtubules blocked antibody binding to kinetochore-associated NuMA, then rapid in vitro disassembly of these microtubules should obviate such antibody occlusion and reveal centromeric NuMA. As expected, cell lysis under these

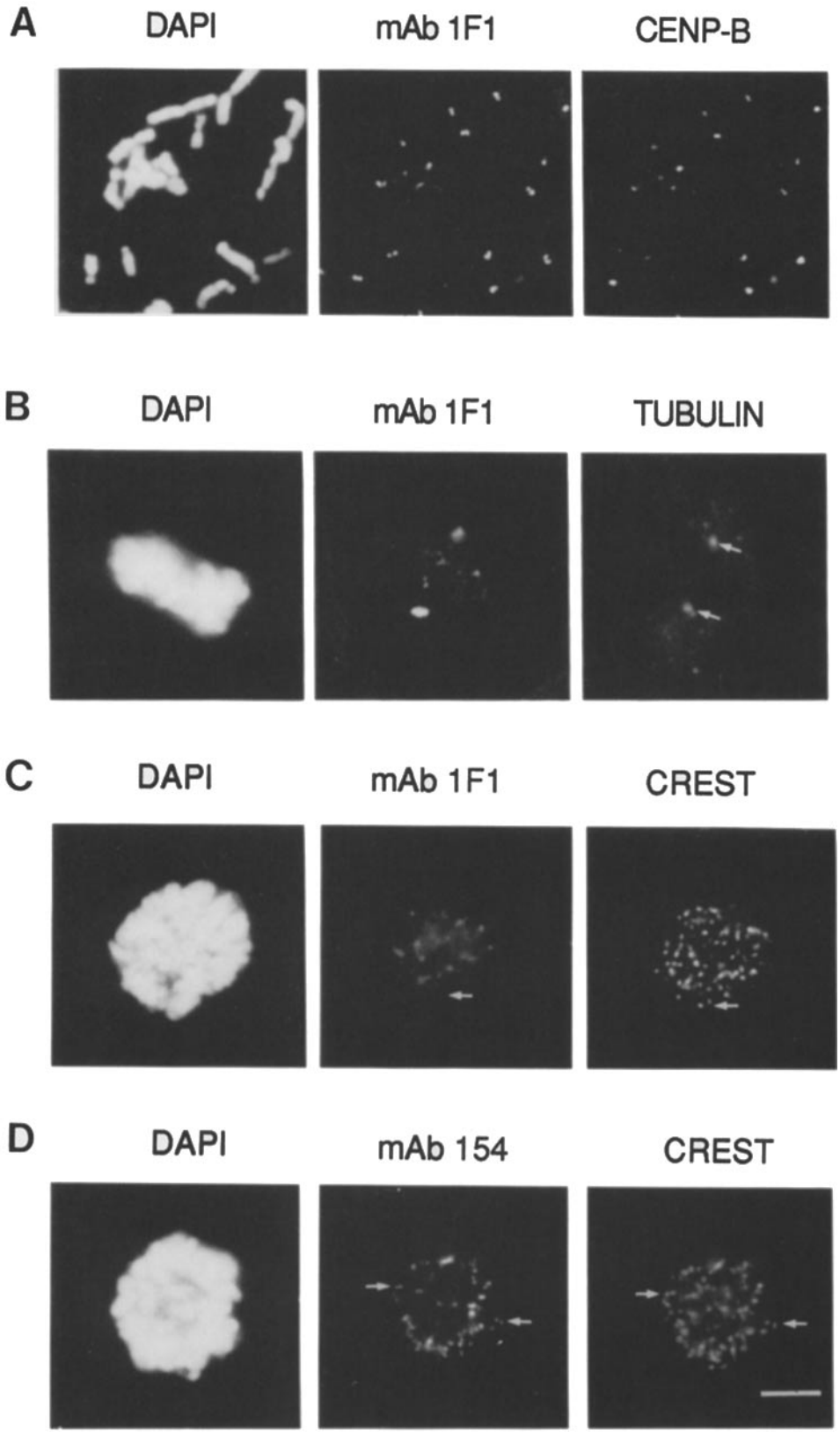

Figure 5. Localization of NuMA after rapid, in vitro spindle disassembly induced by calcium. Double immunofluorescence of mitotic cells treated with colcemid (*A*), or 5 mM $CaCl_2$ (*B–D*) to disrupt the microtubules. (*A*) Chromosomes of a mitotic cell arrested with 1 μg/ml colcemid showing DAPI staining (*left*), mAb1F1 staining (*center*), and anti-CENP-B staining (*right*). (*B*) Metaphase cells lysed with 0.5% Triton X-100 in the presence of 5 mM $CaCl_2$: (*left*) DAPI staining, (*center*) mAb1F1 staining, and (*right*) tubulin staining of residual centrosomes (*arrows*). (*C*) Metaphase cell lysed in the presence of 5 mM $CaCl_2$: (*left* DAPI staining, (*center*) mAb1F1 staining, and (*right*) CREST serum staining of the centromere domains (*arrows*). (*D*) Metaphase cell lysed in the presence of 5 mM $CaCl_2$: (*left*) DAPI staining, (*center*) staining of an authentic kinetochore/centromere antigen detected by mAb154, and (*right*) CREST serum staining of the centromere domains (*arrows*). Bar = 5 μm.

conditions completely disrupted metaphase spindles, with residual tubulin staining primarily at the centrosomes (Fig. 5 *B*). The pericentrosomal staining pattern typically observed for NuMA in metaphase cells (Fig. 1 *E*) was also clearly displaced after this rapid calcium-induced microtubule disassembly (Fig. 5, *B* and *C*), with remaining NuMA now found on both the chromosomes and the residual centrosomes. However, the chromosomal staining was not at the

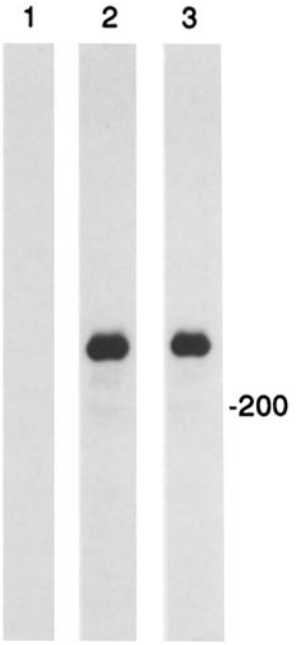

Figure 6. Antibodies produced against the protein encoded by a putative NuMA cDNA clone specifically react with a >200-kD polypeptide in human cell extracts. Immunoblot analyses of 40 μg of K562 cellular proteins after fractionation on 4% SDS-PAGE. (lane *1*) preimmune serum (1:1,000); (lane *2*) serum from a mouse immunized with the bacterially expressed protein encoded by cDNA 1F1 (1:1,000); (lane *3*) mAb1F1. The migration position of myosin (200 kD) is shown at the right.

centromeres, as comparison of the staining pattern seen with the CREST serum revealed that the two were not superimposable (Fig. 5 *C, arrows*). Other centromere antigens, such as CENP-E (Yen et al., 1991), remain centromere associated (Fig. 5 *D*), demonstrating that this authentic centromere/kinetochore antigen remains associated with this domain when the microtubules are rapidly disrupted by calcium.

In view of the failure to detect any centromere-associated NuMA even in the absence of potential microtubule-dependent occlusion of antibody binding, we conclude that the centromere association of NuMA in drug-arrested mitotic cells represents the inappropriate redistribution of this normally pericentrosomal component.

Primary Structure of NuMA

To determine the primary structure of NuMA, we used mAb1F1 to screen an expression library of HeLa cell cDNAs constructed in λZAP. From 250,000 plaques screened, one clone was plaque purified based on its reactivity with the antibody. This clone contained 1.9 kb of cDNA and encoded an immunoreactive protein of 75 kD that was recognized by both monoclonal antibodies 1F1 and 1H1 (data not shown). To establish that this clone encodes the authentic antigen to which the 1F1/1H1 antibodies were directed, we immunized mice with the gel-purified, bacterially produced fusion protein. The mice developed a high titer response against the bacterial protein (not shown) and the sera contained antibodies that in K562 whole cell extracts specifically recognized a protein of >200 kD (Fig. 6, lane *2*) that was not detected by the preimmune sera (Fig. 6, lane *1*). This immunoreactive protein has the same apparent molecular weight as the protein detected by the mAb1F1 (Fig. 6, lane *3*). This murine serum also produced an immunofluorescence pattern in cycling cells indistinguishable from the pattern seen with the monoclonal antibodies (data not shown). These results tentatively confirmed that this cDNA clone encodes an authentic segment of the NuMA protein.

The 1.9-kb cDNA clone hybridized to a single RNA transcript of ~7,200 nucleotides in poly(A+) RNA purified from logarithmically growing K562 cells (data not shown). The size of this RNA transcript easily exceeds that required to encode a protein of ~200 kD. To obtain a full-length cDNA clone, we rescreened the HeLa λZAP expression library using the 1.9-kb cDNA as a hybridization probe. This probe yielded five positive clones, one of which (1F1-2) contained cDNA that extended an additional ~3 kb in the 5′ direction, and another (1F1-4) that extended 3′ and terminated at a consensus polyadenylation signal sequence (Fig. 9 *A*). Both of these cDNA clones also identified a 7.2-kb mRNA on RNA blots (data not shown). When subcloned into the correct translational frame of an expression plasmid, each cDNA was found to encode a protein that was immunoreactive with mAb1F1 (Fig. 7, lanes *1* and *2*). The 5′-most clone (1F1-2) yielded an immunoreactive protein of ~160 kD, along with a series of presumptive degradation products. Clone 1F1-4 expressed two immunoreactive proteins, the largest of which was ~90 kD. Immunoblot analysis of these same bacterially derived samples and mAb1H1 revealed a similar pattern of reactive proteins encoded by the 1F1-2 cDNA (Fig. 7, lane *4*), although no reactivity to the protein expressed from 1F1-4 was observed (Fig. 7, lane *3*). That the proteins expressed from these cDNA clones contain distinct (albeit closely spaced) epitopes recognized by the independent monoclonal antibodies 1F1 and 1H1 is further evidence that the cDNAs encode the NuMA protein.

As further evidence that NuMA had been successfully cloned, and to demonstrate that the nuclear antigen previously identified as centrophilin also represents that same polypeptide or an antigenically related family member, we examined whether the epitopes for the original NuMA (2E4; Van Ness and Pettijohn, 1983) and centrophilin (2D3; Tousson et al., 1991) antibodies were encoded by these cDNAs (Fig. 7, lanes *5–8*). Both antibodies were found to bind avidly to the 1F1-2 polypeptide (Fig. 7, lanes *6* and *8*). We conclude that each of these antibodies, along with our monoclonal antibodies 1F1 and 1H1, bind to NuMA.

The nucleic acid and deduced amino acid sequence of NuMA are shown in Fig. 8. DNA sequencing of the three overlapping cDNAs revealed a nucleotide sequence of 7,218 bases that contains a single open reading frame of 2,101 amino acids. The first ATG codon is found at nucleotide 258 and is preceded by several in-frame translation terminators. The open reading frame is followed by a 657-base 3′ untranslated region, terminating 17 bases 3′ to a consensus sequence for polyadenylation (AATAAA).

The long open reading frame encodes a polypeptide with the predicted molecular mass of 236,278 D and a pI of 5.6. Using the method of Chou and Fasman (1974), the deduced amino acid sequence is predicted to have three structural domains, with two proline-rich globular segments separated by a discontinuous ~1,500 amino acid α-helical region (drawn schematically in Fig. 9). The protein is thus likely to be highly asymmetric, as predicted by Price and Pettijohn (1986). The central helical domain contains several segments of hydrophobic heptad repeat, a motif characteristic of proteins that fold into a coiled-coil configuration. These helical segments are punctuated by five pairs of closely spaced helix-disrupting proline residues. Blotting of genomic human, mouse, chicken, and *Drosophila* DNA revealed a pattern of fragments consistent with a single, highly con-

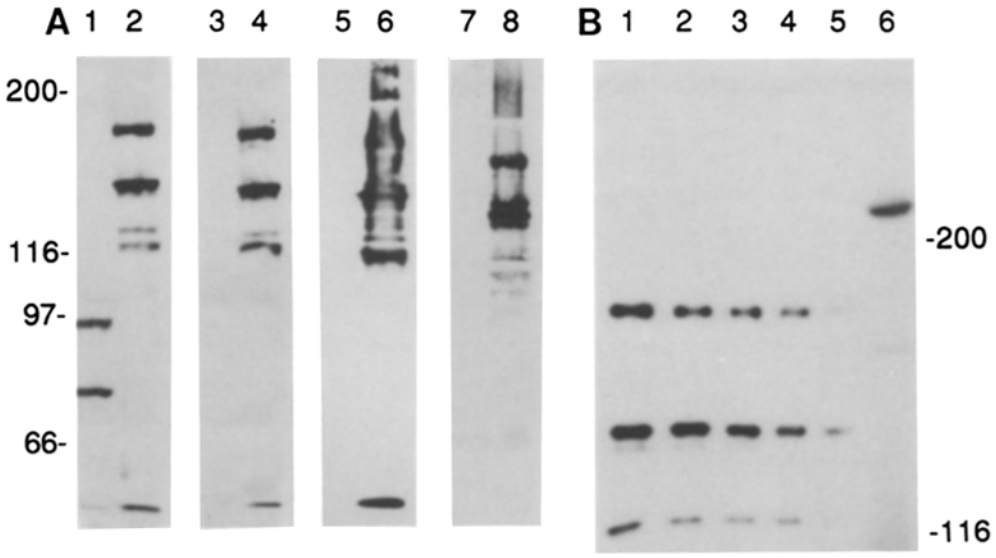

Figure 7. Bacterially expressed proteins encoded by 1F1 cDNA clones contain the epitopes recognized by mAb1F1, mAb1H1, NuMA antibody 2E4, and centrophilin antibody 2D3. (*A*) Extracts from bacteria expressing cDNA 1F1-4 (lanes *1*, *3*, *5*, and *7*) and 1F1-2 (lanes *2*, *4*, *6*, and *8*) were size fractionated by 7.5% SDS-PAGE and analyzed with monoclonal antibodies 1F1 (lanes *1* and *2*), 1H1 (lanes *3* and *4*), 2D3 (centrophilin; lanes *5* and *6*), and 2E4 (NuMA; lanes *7* and *8*). The migration positions of myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), and bovine serum albumin (68 kD) are indicated for each gel. (*B*) Quantitation of the cellular abundance of NuMA. Serial dilutions of an extract from bacteria expressing cDNA 1F1-2 (lanes *1–5*) along with a whole cell extract from 2.5×10^5 K562 cells (lane *6*) were size fractionated by 5% SDS-PAGE and then immunoblotted with mAb1H1. (Lanes *1–5*) Bacterial extracts containing 300 ng (lane *1*), 250 ng (lane *2*), 200 ng (lane *3*), 150 ng (lane *4*) and 100 ng (lane *5*) of the 160-kD 1F1-2 product. (lane *6*) K562 cell extract.

served gene encoding NuMA in each of these genomes (data not shown).

Using the FASTA algorithm (Wilbur and Lipman, 1983) to search the Genbank and NBRF databases for DNA or protein sequences similar to the NuMA sequence reported here, no significant sequence similarities were detected, except for the presence of part of a human Alu repetitive sequence in the 5′-untranslated region between nucleotides 130 and 230 (underlined in Fig. 8). No sequence motifs such as a basic nuclear localization sequence (e.g., PKKKRKV; Kalderon et al., 1984; Lanford and Butel, 1984) or known microtubule binding sites are found in NuMA. These results demonstrate that NuMA is a novel protein, a finding consistent with its unique cell cycle distribution and pathway of segregation during cell division.

Abundance of NuMA: 2 × 10⁵ Copies per Cell

With bacterially produced NuMA protein available, the normal abundance of NuMA was examined as a means of gaining some insight into its in vivo function(s). With known amounts of bacterially made NuMA as standards, we determined NuMA's abundance by immunoblotting known amounts of whole cell proteins from logarithmically growing K562 erythroleukemic cells (Fig. 7 *B*, lanes *1–5*). (The amount of the bacterially produced protein was determined by comparing the intensity of Coomassie blue staining of the bacterially derived protein with known amounts of albumin.) Densitometric scanning of the resulting autoradiogram revealed $\sim 2 \times 10^5$ copies of the NuMA protein per cell (i.e., per nucleus). This value is in close agreement with estimates for the quantities of topoisomerase II (1.3×10^5 copies/cell; Gasser et al., 1986) and a matrix/scaffold attachment region binding protein (1×10^5 copies/cell; von Kries et al., 1991).

Discussion

A New Pathway to Segregate Nuclear Components during Mitosis

As summarized schematically in Fig. 10, the chromosomes, the lamins, and NuMA each follow a distinct route from the parental nucleus to the daughter nuclei. The chromosomes follow the classic microtubule-mediated segregation pathway. The lamins, on the other hand, are distributed to each daughter cell through passive diffusion and most lamins are reimported (Loewinger and McKeon, 1988) through nuclear pores (although a small proportion may be directly targeted to the telophase chromosome mass [Glass and Gerace, 1990]).

We describe here a new route for the segregation of nuclear material during mitosis. The intranuclear protein NuMA is distributed throughout the nucleus in interphase, but is distributed to each daughter cell by association with the astral microtubules immediately upon nuclear envelope breakdown at the prophase/prometaphase transition. While it is not clear whether this protein is associated with the chromatin within the nucleus during interphase, it is obvious (Fig. 1) that its localization is reorganized during prophase and at prometaphase it associates with the astral microtubules independent of chromosomes. NuMA then associates with the microtubules and progressively accumulates at the spindle poles before chromosome segregation at anaphase. Presumably, it remains sequestered at the spindle poles throughout the saltatory chromosome movements and microtubule instability and subunit flux experienced during prometaphase and metaphase (Mitchison, 1989). Even at anaphase, NuMA remains bound to the spindle poles, although the immunofluorescence intensity diminishes. It is plausible that this diminution in immunofluorescence inten-

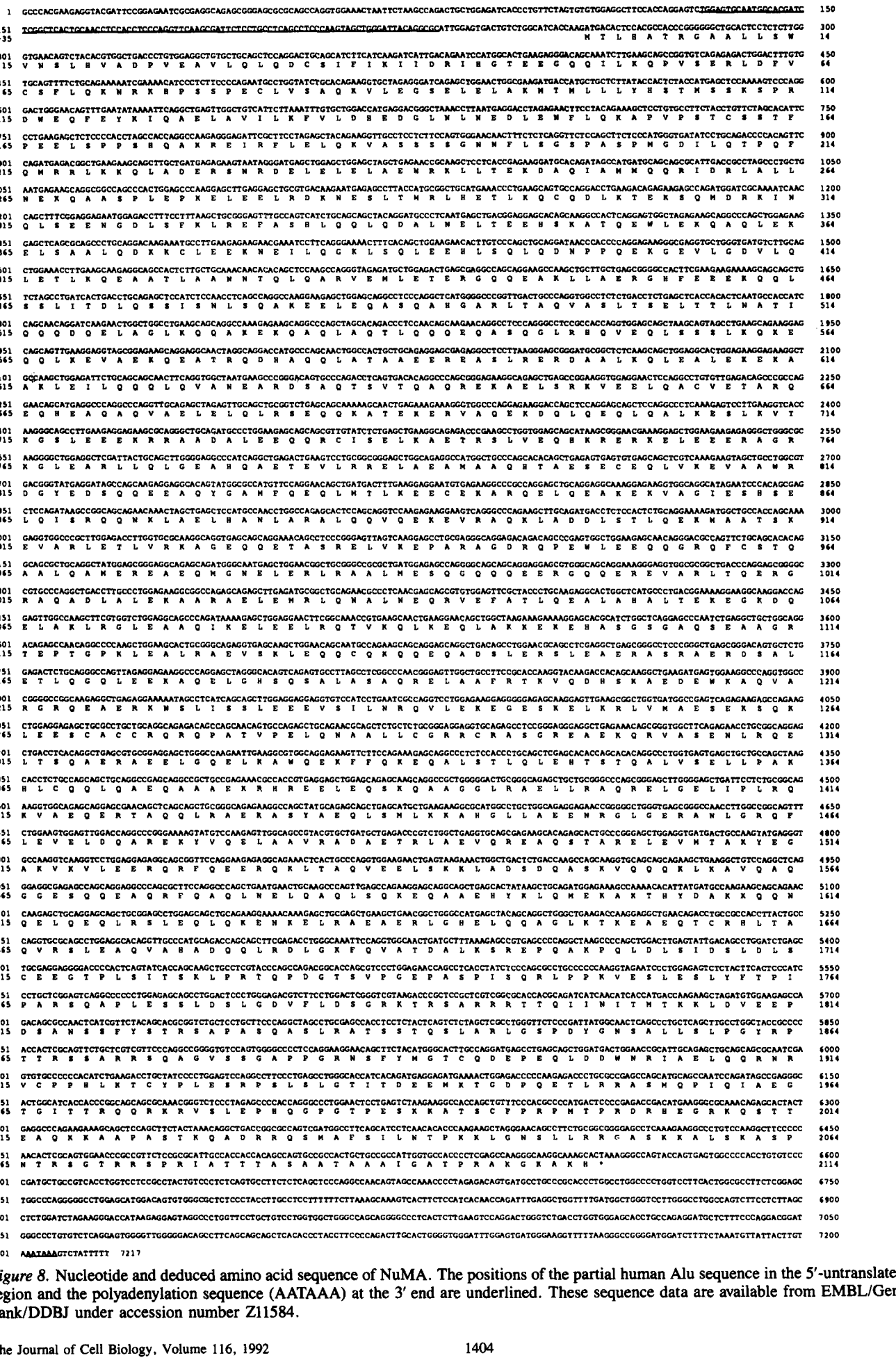

Figure 8. Nucleotide and deduced amino acid sequence of NuMA. The positions of the partial human Alu sequence in the 5′-untranslated region and the polyadenylation sequence (AATAAA) at the 3′ end are underlined. These sequence data are available from EMBL/Genbank/DDBJ under accession number Z11584.

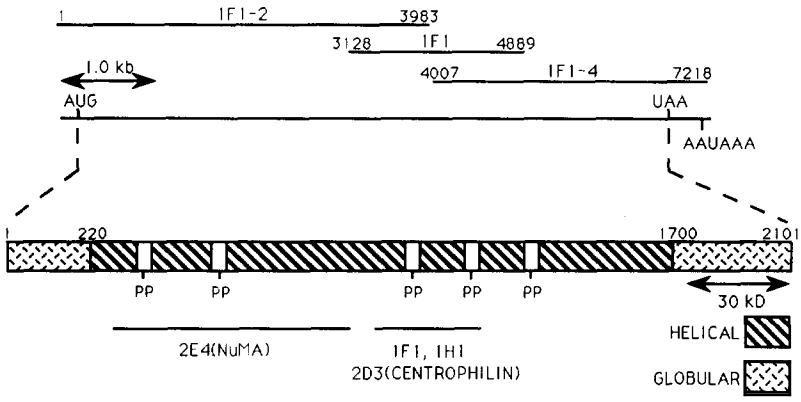

Figure 9. Schematic summary of the NuMA cDNA clones, the predicted secondary structure of NuMA, and the approximate localization within the polypeptide of the epitopes recognized by mAbs 1F1, 1H1, 2D3, and 2E4. (*Top*) Schematic drawing of each of the three overlapping cDNA clones that span the entire 7,218-base length of the NuMA mRNA transcript. The positions of the translation start (AUG) and stop (UAA) codons, and polyadenylation signal sequence (AAUAAA) are indicated. (*Bottom*) Schematic of NuMA's predicted secondary structure indicating the positions of the two globular domains (*stippled regions*) and the α-helical coiled-coil domains (*hatched regions*). Interruptions in the helical domain by pairs of proline (PP) residues are indicated by open spaces. Bars at the bottom represent the intervals of NuMA to which the epitopes for the indicated monoclonal antibodies have been localized. (Since mAb 1F1 recognizes the polypeptides encoded by p1F1-2 and p1F1-4 [Fig. 7 *A*], but the two clones do not overlap, the epitope recognized must be repeated. The most likely candidate sequence is a five amino acid duplication (AQREL) located at amino acid positions 1,188 and 1,402.)

sity results from the release of NuMA from the rapidly depolymerizing astral and kinetochore fibers. This would be consistent with some NuMA staining around the telophase chromatin mass that has been observed by other investigators (Price and Pettijohn, 1986; Tousson et al., 1991). Finally, after the early stages of nuclear reformation, NuMA is released from microtubules and most, possibly all, is actively reimported into the regenerating daughter nuclei by transit through the nuclear pores.

Although a nuclear pore–associated import process has been shown for other proteins to rely on the presence of a NLS, the primary sequence of NuMA does not reveal a putative NLS similar to that defined for SV-40 large T antigen or the variation of this NLS found in nucleoplasmin (Robbins et al., 1991). Future transfection experiments with various segments of the protein expressed from the cloned cDNA should allow examination of the primary sequence requirements for targeting NuMA into the regenerating nuclei.

The inhibition of reimport of NuMA after microinjection of wheat germ agglutinin (Fig. 4) demonstrates not only that the nuclear envelope assembles before NuMA association, but also documents the failure of NuMA to interact with interphase microtubules. Prohibiting its entrance into daughter nuclei resulted in a distribution throughout the cytoplasm but it did not appear to associate with the interphase microtubule networks. This suggests that the affinity of NuMA for

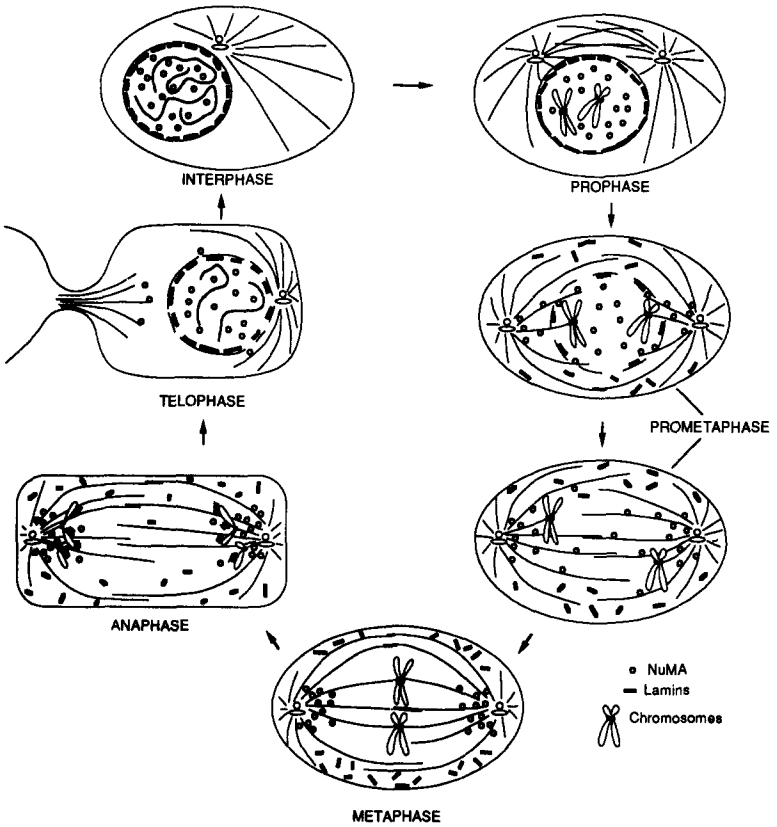

Figure 10. Summary of the cell cycle distribution of chromosomes, lamins, and NuMA relative to the interphase and spindle microtubule arrays.

microtubules is affected by some reversible mitosis-specific modification. Since the primary sequence of NuMA does not contain any previously described microtubule binding sequences, it seems likely either to bind directly to the spindle microtubules through a novel microtubule binding motif or to associate with other cellular microtubule binding proteins in a reversible, mitosis-specific manner.

After attachment of NuMA to spindle microtubules during prometaphase it progressively accumulates at the spindle poles. The most obvious mechanism for this centrosomal accumulation is through transit along the spindle microtubules (although proof of this awaits experimental verification). If this is correct, the directionality of this movement is consistent with that of the minus end–directed microtubule motor cytoplasmic dynein, a small proportion of which has been argued to be associated with the mammalian mitotic apparatus (Pfarr et al., 1991; Steuer et al., 1991). In fact, the staining pattern observed by Pfarr et al. (1991) and Steuer et al. (1991) with antibodies against dynein reveals a strikingly similar distribution at the spindle poles of prometaphase cells compared with the pattern observed here with antibodies against NuMA. If the segregation of NuMA were mediated by dynein (or a related motor), then it could represent a simplified version of mitotic segregation as compared with anaphase chromosome movement, which has also been proposed to be powered by cytoplasmic dynein (Rieder and Alexander, 1990; Pfarr et al., 1991; Steuer et al., 1991). A major difference between such motor-dependent movement of NuMA and chromosomes is that NuMA is not initially congressed to a midplate. The cDNA clones we have reported here should be useful in determining whether NuMA interacts directly with the astral microtubules, or as the cargo of a microtubule motor such as dynein.

NuMA, Centrophilin, SP-H, and SPN Are Probably Different Names for the Same Protein

For the antibodies used to define NuMA, centrophilin, and our 1F1 and 1H1 antigens, we have shown here that all recognize polypeptides of indistinguishable mobility and bind to a single cloned 236-kD polypeptide. Not surprisingly in light of their binding to a common protein, all report essentially indistinguishable patterns of antigen localization throughout the cell cycle. Since there are no other known properties that distinguish among the antigens recognized, it is probable that all of the antibodies identify the same protein, for which we have adopted the name NuMA. We cannot exclude the possibility that NuMA consists of a family of related proteins, although no current evidence supports this possibility. Two additional antigens (named SPN [Kallajoki et al., 1991] and SP-H [Maekawa et al., 1991]) have been shown by monoclonal antibodies and autoimmune sera to have cell cycle distributions and molecular weights strongly reminiscent of NuMA. Despite the arguments by those authors that NuMA antibodies yield subtly differing immunofluorescence images and molecular weights, our view is that these discrepancies are easily explained by minor differences in fixation methods, epitope availability for the various antibodies, or the interpretation of the images obtained. It seems likely that, like centrophilin and our mAb1F1 and mAb1H1 antigens, the SPN and SP-H antigens are in fact NuMA.

Function of NuMA

The primary sequence of NuMA indicates that it contains three discrete structural domains (Fig. 9). Only the central helical domain, based on its segments of heptad repeat, is clearly similar to any previously published protein. The predicted structure of NuMA does, however, suggest that the protein interacts with multiple other cellular components similar to the way the kinesin heavy chain acts to bind microtubules at one domain and cargo to be transported at the opposite domain (Yang et al., 1989). In addition, the presence of segments of hydrophobic heptad repeat within the central domain suggests that this protein forms oligomers in a coiled-coil configuration.

One function that has been proposed for NuMA (called centrophilin in that report but shown here to be the same protein as NuMA) is as a nucleation protein for microtubules during mitosis (Tousson et al., 1991). This conclusion was drawn on purely correlative data, and no direct test of nucleating activity was presented. Our data (Fig. 1 *B*) argue clearly against NuMA acting as such a nucleating protein for microtubules at prophase, prometaphase, or interphase: the centrosomes in cells at prophase nucleate the two sets of astral microtubules *before* the release of NuMA from the nuclear compartment. Moreover, when the bulk of NuMA is prevented from entering the daughter nuclei by microinjection of wheat germ agglutinin, NuMA does not appear to associate with the cytoplasmic microtubules (Fig. 4). Our data, therefore, are not readily consistent with this protein acting directly as a microtubule nucleator. However, microinjection of one antibody to a NuMA-like protein (called SPN in that report, but probably identical to NuMA; see above) has revealed that many cells arrest at prometaphase or metaphase (condensed chromosomes and aberrant spindles but without formation of a mitotic midplate), while others become micronucleated (Kallajoki et al., 1991). This could be the result of a direct requirement for NuMA in spindle function or a less specific effect arising from antibody complexes interfering indirectly with one or more mitotic events. (Five other SPN antibodies had no effect on cell cycle progression [Kallajoki et al., 1991]; neither did microinjection of mAb1F1 or mAb1H1 [data not shown].) If NuMA has any function at all on microtubule dynamics in the mitotic spindle, perhaps the most likely role is as a linking protein between the parallel arrays of microtubules as suggested by Maekawa et al. (1991) (assuming that the antigen SP-H is identical to NuMA as we have proposed; see above). It is unclear, however, why a protein with such a function should be sequestered inside the nucleus during interphase.

Lydersen and Pettijohn (1980) and Kallajoki et al. (1991) proposed that NuMA is part of the nuclear matrix, the salt and nuclease resistant residue of interphase nuclei. However, contrasting work has found NuMA solubilized by low salt-containing buffers (Price and Pettijohn, 1986). Whatever the resolution of NuMA's matrix association, our laser confocal immunofluorescence microscopy (Fig. 2 *A*) clearly localizes NuMA within the interior of the nucleus in a position consistent with possible matrix interaction. In any event, even if NuMA were found to be matrix associated, this does not offer particularly helpful insight as to its underlying function.

A final proposed function for NuMA is in post-mitotic nu-

clear assembly (Price and Pettijohn, 1986). This suggestion was based on the localization of this protein at the spindle poles. We have now shown that disruption of nuclear pore function (with R-WGA) prohibits normal post-mitotic nuclear regeneration and blocks the bulk of NuMA from entering the regenerating daughter nuclei (Fig. 4). One possible function for NuMA could be in the organization and architecture of the chromatin within the nucleus. In this possibility, NuMA could be acting as a structural protein organizing the chromatin within the highly insoluble nuclear matrix. This would be consistent with its intranuclear localization and its apparent exclusion from the chromosomes as they condense in prophase (Fig. 1 *B*; Price and Pettijohn, 1986). In addition, the abundance of NuMA (2×10^5 copies per cell) closely parallels the abundance of two other nuclear proteins that have known functions in chromatin structure. Finally, in this context, an attractive hypothesis is that NuMA utilizes the unique mitotic segregation mechanism described here as a means of preventing continued association with chromosomes during mitosis. A potential test of this idea is now feasible by assaying chromosome behavior in a cell expressing a segment of NuMA that does not have the domain(s) necessary for microtubule association.

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