

NuMA Is Required for the Organization of Microtubules into Aster-Like Mitotic Arrays

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Abstract. NuMA (Nuclear protein that associates with the Mitotic Apparatus) is a 235-kD intranuclear protein that accumulates at the pericentrosomal region of the mitotic spindle in vertebrate cells. To determine if NuMA plays an active role in organizing the microtubules at the polar region of the mitotic spindle, we have developed a cell free system for the assembly of mitotic asters derived from synchronized cultured cells. Mitotic asters assembled in this extract are composed of microtubules arranged in a radial array that contain NuMA concentrated at the central core. The organization of microtubules into asters in this cell free system is dependent on NuMA because immunodepletion of NuMA from the extract results in randomly dispersed

microtubules instead of organized mitotic asters, and addition of the purified recombinant NuMA protein to the NuMA-depleted extract fully reconstitutes the organization of the microtubules into mitotic asters. Furthermore, we show that NuMA is phosphorylated upon mitotic aster assembly and that NuMA is only required in the late stages of aster assembly in this cell free system consistent with the temporal accumulation of NuMA at the polar ends of the mitotic spindle *in vivo*. These results, in combination with the phenotype observed *in vivo* after the prevention of NuMA from targeting onto the mitotic spindle by antibody microinjection, suggest that NuMA plays a functional role in the organization of the microtubules of the mitotic spindle.

THE nucleus of a vertebrate cell undergoes profound physical changes during mitosis. These physical changes are necessary for the accurate segregation of the genetic material and include the dissolution of the nuclear envelope, the depolymerization of the nuclear lamina, and the condensation of the chromosomes (Newport and Forbes, 1987; Gerace and Burke, 1988). After the physical rearrangement of these nuclear components, they each follow one of three known segregation pathways for nuclear components during mitosis. First, the chromosomes attach to the microtubules of the mitotic spindle, and the segregation of the sister chromatids into each daughter cell is precisely controlled at anaphase through the dynamics of microtubules and their associated microtubule-dependent motor proteins (Mitchison, 1989a; Rieder, 1991). The second segregation pathway for nuclear components is followed by the nuclear lamins and the nuclear envelope as these two nuclear components are both distributed into each daughter cell through passive diffusion (McKeon, 1991; Nigg, 1992; Wiese and Wilson, 1993). Finally, the NuMA protein (Nuclear protein that associates with the Mitotic Apparatus)¹ defines a third pathway for

the segregation of nuclear components during mitosis, and is equally distributed into each daughter cell by accumulating at the pericentrosomal region of the mitotic spindle (Lydersen and Pettijohn, 1980; for reviews see Compton and Cleveland, 1994; Cleveland, 1995).

The unique cell cycle distribution of NuMA has spawned two broadly defined views for the function of this protein. One view holds that NuMA is performing a structural role for the nucleus during interphase. Support for this functional role comes from the fact that NuMA is a coiled coil-containing protein (Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993) that is localized to a subset of the core filaments of the nuclear matrix (Kallajoki et al., 1991; Zeng et al., 1994), and is localized in the nucleus of terminally differentiated (nonmitotic) cells (Kallajoki et al., 1992; Tang et al., 1993). Further support for this view comes from the induction of multiple small nuclei (micronuclei) following both the microinjection of a NuMA-specific antibody and the expression of truncated forms of the NuMA protein (Kallajoki et al., 1993; Compton and Cleveland, 1993). In each of these experiments, micronuclei formed after mitosis in the absence of any detectable alterations of the mitotic spindle suggesting that the phenotype of postmitotic micronucleation was due to a perturbation of NuMA function during nuclear assembly. Contrary to these results, however, Yang and Snyder (1992) have demonstrated that a morphologically normal nucleus will assemble in the absence

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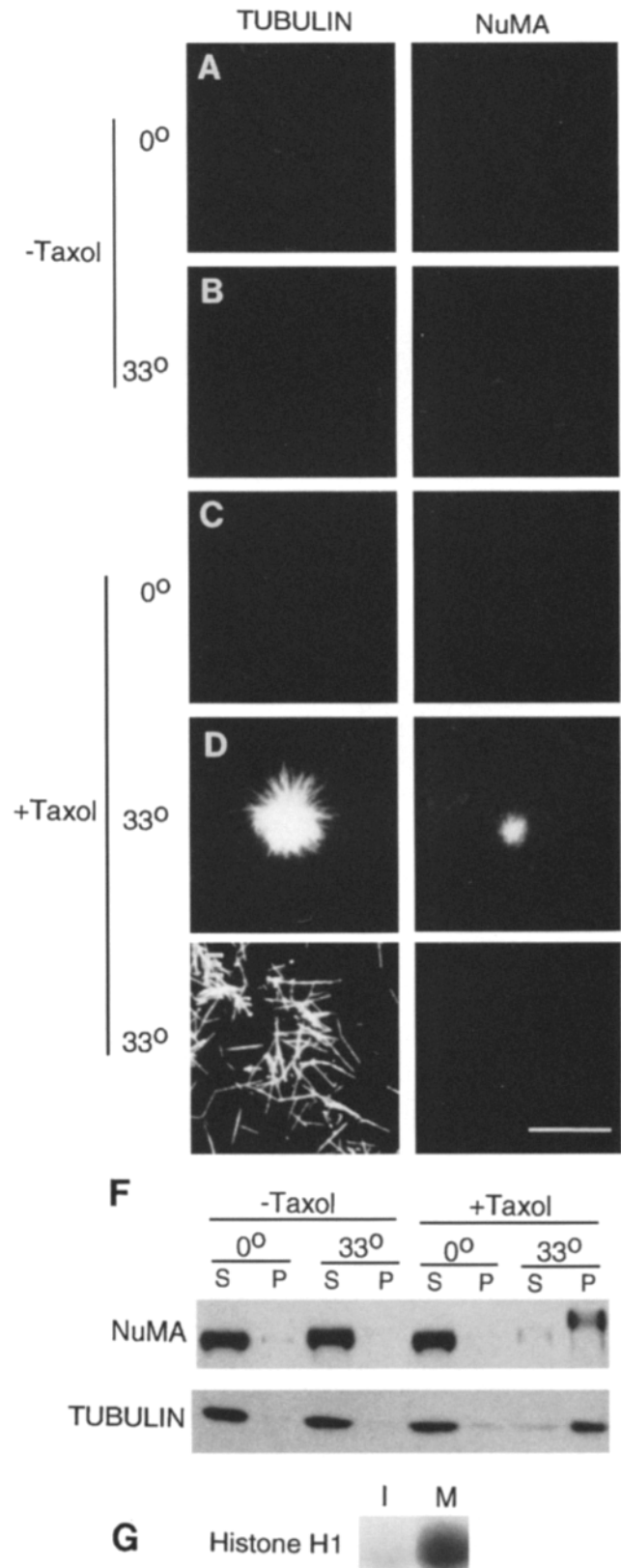
1. *Abbreviation used in this paper:* NuMA, Nuclear protein that associates with the Mitotic Apparatus.

of NuMA after the antibody-induced aggregation of NuMA in the cell cytoplasm. Taken together, these data suggest that NuMA may serve a functional role within the nucleus, but that assembly of a morphologically normal nucleus does not strictly require the NuMA protein.

A second view for the function of the NuMA protein is that it acts as an organizing or stabilizing agent for the minus ends of the microtubules in the mitotic spindle. Support for this proposed function comes from the fact that the localization of NuMA at the polar end of the mitotic spindle requires intact microtubules (Price and Pettijohn, 1986; Kallajoki et al., 1991; Maekawa et al., 1991; Tousson et al., 1991), and that NuMA is associated with the polar ends of mitotic asters that assemble after the removal of the microtubule destabilizing drug nocodazole (Tousson et al., 1991). Further evidence that NuMA may play a functional role in the assembly of the mitotic spindle comes from the failure of cells to assemble a typical bipolar spindle after the microinjection of NuMA-specific antibodies (Kallajoki et al., 1991, 1992; Yang and Snyder, 1992). In fact, Yang and Snyder (1992) demonstrated that microinjection of a NuMA-specific polyclonal antibody into metaphase cells was sufficient to cause the collapse of the preexisting mitotic spindle suggesting that NuMA was necessary for both the assembly and maintenance of the mitotic spindle. Finally, we have recently shown that expression of mutant forms of NuMA that are incapable of targeting to the mitotic spindle induce a dominant negative phenotype that is characterized by the failure to complete mitosis due to the assembly of mitotic spindles that lack clearly defined polar domains (Compton and Luo, 1995). Collectively, these data support the view that NuMA is involved in the organization of the mitotic spindle, however, none of these experiments demonstrate that NuMA has a required role in this process.

In this article we present data using two complementary approaches that support the hypothesis that NuMA functions in the organization of the microtubules of the mitotic spindle. First, we have found that NuMA is accurately targeted to and accumulates at the central core of mitotic asters assembled in a cell free extract prepared from synchronized cultured cells. NuMA is required for the formation of these mitotic asters because depletion of NuMA from the extract using a NuMA-specific antibody results in the assembly of randomly arranged microtubules, and the addition of the purified recombinant NuMA protein to this NuMA-depleted extract is sufficient to reconstitute the assembly of organized mitotic asters. Second, we describe a polyclonal anti-NuMA antibody that prohibits NuMA from binding to the mitotic spindle when microinjected into cultured cells. These microinjected cells are signifi-

Figure 1. Assembly of mitotic asters in a cell free mitotic extract. Mitotic (A–D) and interphase (E) extracts were supplemented with ATP and incubated at either 0° or 33° with or without the addition of taxol as indicated and processed for indirect immunofluorescence (A–E) using a tubulin-specific mouse monoclonal antibody DM1α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). After a 30-min incubation, the extracts were subjected to centrifugation at 10,000 g and separated into soluble (S) and insoluble (P) fractions and subjected to immunoblot analysis (F) using either a tubulin-specific mouse monoclonal anti-



body DM1α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). (G) Histone H1 kinase activity was determined by incubation of histone H1 in either the interphase (I) or mitotic (M) extracts in the presence of γ - ^{32}P ATP followed by size fractionation of the histone H1 by SDS-PAGE and autoradiography. Bar, (A–E) 10 μm .

cantly delayed in their progression through mitosis and have disorganized mitotic spindles. These results suggest that NuMA is necessary for the organization of the mammalian mitotic spindle.

Materials and Methods

Cell Culture

The human HeLa cell line and the monkey CV-1 cell line were both maintained in DME containing 10% FCS, 2 mM glutamine, 100 i.u./ml penicillin, and 0.1 µg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

Antibody Preparation and Purification

The recombinant human NuMA protein was expressed using the baculovirus expression system and purified to ~90% homogeneity (Compton, D. A., P. A. Coulombe, and D. W. Cleveland. 1993. *Mol. Biol. Cell.* 4:80a). This purified protein was used to generate a rabbit polyclonal antibody after the procedures outlined by Harlowe and Lane (1988). Briefly, ~500 µg of protein was suspended in Freund's complete adjuvant and administered to a rabbit by subcutaneous injection. After this initial immunization, the rabbit was boosted with the same quantity of protein in Freund's incomplete adjuvant at three week intervals until there was a strong immune response detectable (three boosts). Serum was collected from the rabbit and stored at -80°C.

The IgG fraction was purified from 1-ml aliquots of this serum by affinity chromatography using protein A-agarose (Boehringer Mannheim, Indianapolis, IN) as described by Harlowe and Lane (1988). The purified IgG fraction was dialyzed into 100 mM KCl, 10 mM KPO₄, pH 7.0, and concentrated using a Centricon-30 (Amicon, Beverly, MA) to ~10 mg/ml.

Microinjection and Immunological Techniques

CV-1 cells growing on photo-etched α -numeric glass coverslips (Bellco Glass Co., Vineland, NJ) were microinjected using the procedures of Compton and Cleveland (1993) and Capecchi (1980). Interphase cells were microinjected in the cytoplasm with either the preimmune IgG or the immune IgG and monitored by phase contrast microscopy as they progressed into mitosis. Cells were followed for up to 4 h after injection, and then processed for immunofluorescence microscopy.

Indirect immunofluorescence microscopy was performed essentially as described by Compton et al. (1991). Cells were immersed in microtubule stabilization buffer (MTSB; 4 M glycerol, 100 mM Pipes, pH 6.9, 1 mM EGTA, and 5 mM MgCl₂) 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 10 min and rehydrated in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% albumin and all antibody incubations and washes were performed in TBS + 1% albumin. NuMA was detected with either a human-specific mouse monoclonal antibody (mAb1F1; Compton et al., 1991), a human autoimmune serum (courtesy of Dr. D. Pettijohn, University of Colorado), or the rabbit polyclonal antibody described here. Tubulin was detected using the monoclonal antibody DM1 α (Blöse et al., 1984). Each primary antibody was incubated on the coverslip for 30 min followed by 5-min washes in TBS + 1% albumin, and the bound antibodies were detected using either fluorescein- or Texas red-conjugated species-specific secondary antibodies at dilutions of 1:500 (Vector Labs, Burlingame, CA). The DNA was detected using DAPI (4',6-diamidino-2-phenylindole) at 0.4 µg/ml (Sigma Chemical Co., St. Louis, MO). After a final wash the coverslips were mounted in FITC-guard-mounting medium (Testog, Inc., Chicago, IL) and observed on a Nikon Optiphot microscope equipped for epifluorescence.

Proteins were analyzed by immunoblot analysis after SDS-PAGE (Laemmli, 1970). Cells were washed three times in ice-cold PBS and lysed directly in SDS-PAGE sample buffer, and proteins from mitotic extracts were solubilized directly with SDS-PAGE sample buffer. Proteins were separated by size using SDS-PAGE and transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membranes were blocked in TBS containing 5% nonfat milk for 30 min at room temperature, and the primary antibody incubated for 6 h at room temperature in TBS containing 1% nonfat milk. Nonbound primary antibody was removed by washing

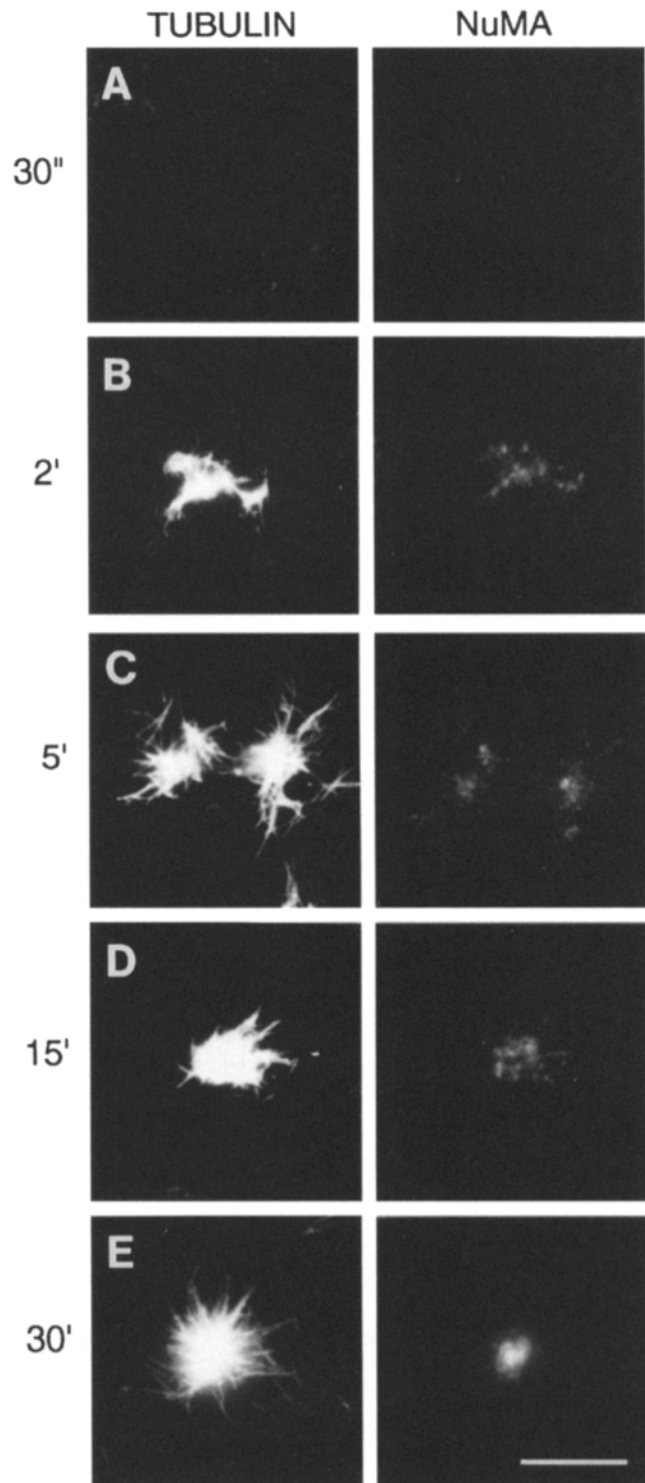


Figure 2. Kinetics of mitotic aster assembly in the cell free mitotic extract. Mitotic asters were assembled under optimal conditions with taxol and ATP at 33° for the indicated times and processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). Bar, 10 µm.

five times for 3 min each in TBS and the bound antibody was detected using either horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad Co., Hercules, CA). The nonbound secondary reagent was removed by washing five times for

3 min each in TBS and the signal detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Preparation of Mitotic Extracts for Mitotic Aster Formation

HeLa cells were synchronized in the cell cycle by double block with 2 mM thymidine. After a release from thymidine block, the cells were allowed to grow for 6 h and then nocodazole was added to a final concentration of 10 μ g/ml. The mitotic cells that accumulated over the next 4 h were collected by mitotic shake off and incubated for 30 min at 37°C with 20 μ g/ml cytochalasin B. The mitotic index of the population of cells isolated in this fashion was >95% and we typically obtained between 10^7 and 10^8 cells from five 150-cm² tissue culture flasks. The cells were then collected by centrifugation at 1,500 rpm and washed twice with cold PBS containing 20 μ g/ml cytochalasin B. Cells were washed one last time in cold KHM buffer (78 mM KCl, 50 mM Hepes, pH 7.0, 4 mM MgCl₂, 2 mM EGTA, 1 mM DTT; Burke and Gerace, 1986) containing 20 μ g/ml cytochalasin B, and finally Dounce homogenized (tight pestle) at a concentration of $\sim 3 \times 10^7$ cells/ml in KHM buffer containing 20 μ g/ml cytochalasin B, 20 μ g/ml phenylmethylsulfonyl fluoride, and 1 μ g/ml each of chymostatin, leupeptin, antipain, and pepstatin. The crude cell extract was then subjected to sedimentation at 100,000 g for 15 min at 4°C (Beckman airfuge, 25 psi). The supernatant was recovered and supplemented with 2.5 mM ATP or AMP-PNP (prepared as Mg⁺⁺ salts in KHM buffer) and 10 mM taxol. The total protein concentration of the extract prepared in this fashion was routinely ~ 5 mg/ml, and the mitotic asters were stimulated to assemble by incubation at 33°C for 30 min. After incubation, 5 μ l of the extract was diluted into 25 μ l of KHM buffer and spotted onto a poly-L-lysine-coated glass coverslip, fixed by immersion in -20°C methanol, and then processed for indirect immunofluorescence microscopy as described above. The remainder of the extract containing the assembled mitotic asters was subjected to sedimentation at 10,000 g for 15 min at 4°C. The supernatant and pellet fractions were both recovered and solubilized in SDS-PAGE sample buffer for immunoblot analysis.

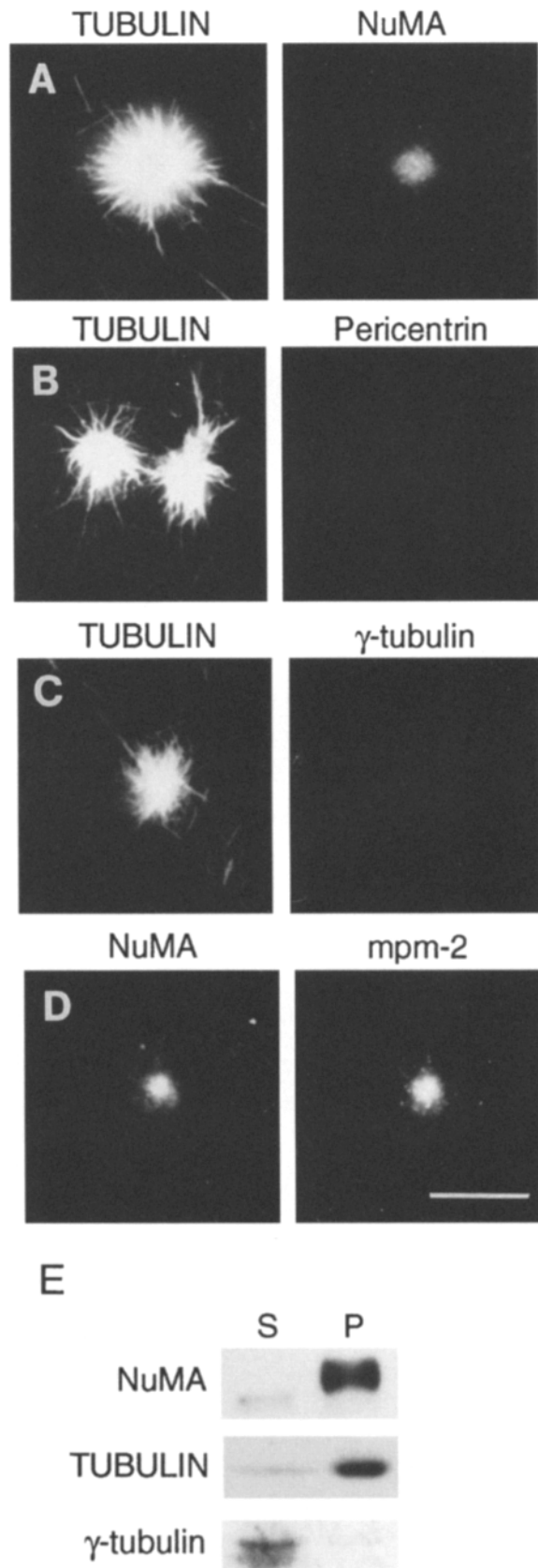
To deplete NuMA from the extract before mitotic aster assembly, 10 μ g of either the preimmune IgG or the anti-NuMA immune IgG were adsorbed onto ~ 25 μ l of protein A-conjugated agarose. The antibody-coupled protein A-agarose was washed in KHM buffer, and then packed by centrifugation to remove the excess fluid. The agarose was then resuspended with the mitotic extract and incubated with agitation for 2 h at 4°C. After this incubation the agarose was removed from the extract by sedimentation at 15,000 g for 10 s. The extract was then collected and supplemented with ATP and taxol and treated as described above.

Protein Phosphorylation and Kinase Assays

Histone H1 kinase activity was measured using 2.5 μ g of the cell extract in a reaction containing 0.2 mg/ml histone H1, 20 mM Tris, pH 7.2, 2 mM DTT, 1 mM EGTA, 10 μ M MgCl₂, 10 μ M ATP, and 2 μ Ci γ -[³²P]ATP (3,000 Ci/mmol; Amersham Corp.). Reactions were incubated at 30° for 15 min and stopped by the addition of SDS-PAGE sample buffer. The histone was separated from the nonincorporated nucleotide on a 12.5% SDS-PAGE, the gel dried and exposed to X-ray film. Quantitation of the histone H1 kinase activity present in the extract was performed by densitometric scanning of multiple different film exposures.

The phosphorylation of NuMA in the mitotic extract was determined by adding γ -[³²P]ATP (6,000 Ci/mmol; Amersham Corp.) into 100 μ l of the extract at a final concentration of 0.8 μ M in the presence of either 1.6 mM ATP or AMP-PNP. The reaction was incubated at 33° for 30 min and separated into 10,000 g soluble and insoluble (i.e., asters) fractions. The

Figure 3. Mitotic asters assembled in a cell free mitotic extract do not contain known centrosomal components. Mitotic asters were assembled under optimal conditions with taxol and ATP for 30 min at 33° and processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*), and either a rabbit NuMA-specific antibody (*NuMA*), a rabbit pericentrin-specific antibody (*Pericentrin*), a rabbit γ -tubulin-specific antibody (*γ -tubulin*), or the mouse mpm-2 monoclonal antibody (*mpm-2*). After a 30-min incubation, the extracts were subjected to centrifugation at 10,000 g and separated into soluble (S) and insoluble (P) fractions and subjected to immuno-



blot analysis (E) using either a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*), a rabbit NuMA-specific antibody (*NuMA*), or a rabbit γ -tubulin-specific antibody (*γ -tubulin*). Bar, (A-D) 10 μ m.

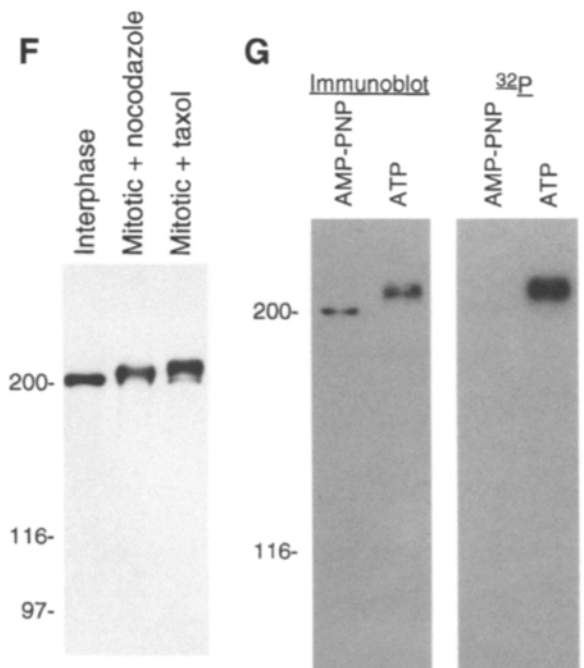
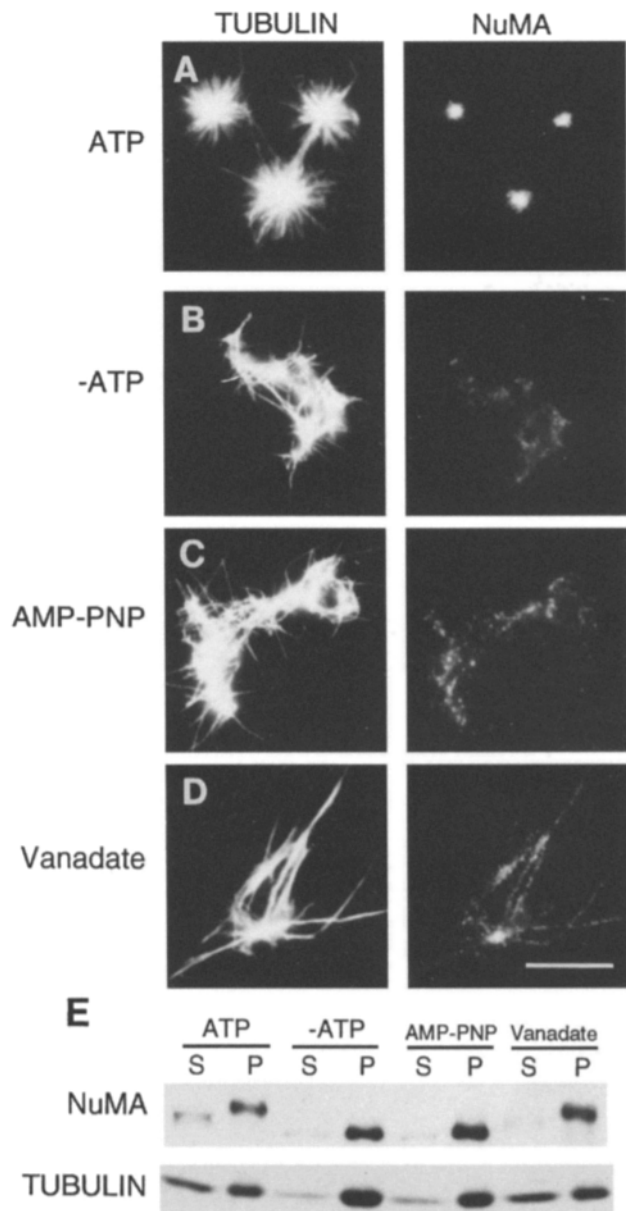


Figure 4. Mitotic aster assembly in the cell free extract requires ATP. Mitotic asters were assembled under optimal conditions with taxol for 30 min at 33° either with (A) or without (B) the addition of ATP or in the presence of AMP-PNP (C) or 50 μM vanadate (D) and processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). (E) After the incubation, the extracts were subjected to centrifugation at 10,000 g and separated into soluble (S) and insoluble (P) fractions and subjected to immunoblot analysis using either a tubulin-specific mouse monoclonal antibody DM1α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). (F) Total cell extracts from cells in interphase (*Interphase*), mitosis in the presence of nocodazole (*mitotic + nocodazole*), or mitosis in the presence of taxol (*mitotic + taxol*) were harvested and subjected to immunoblot analysis using a rabbit NuMA-specific antibody. (G) Asters were assembled in the mitotic extract with γ -[³²P]ATP in the presence of ATP or AMP-PNP as indicated, the microtubule structures collected by sedimentation at 10,000 g, the proteins solubilized, and NuMA isolated by immunoprecipitation. The immunoprecipitates were size fractionated on a 5% SDS-PAGE

and the proteins identified by immunoblot (left panel) followed by autoradiographic exposure for incorporated ³²P (right panel). The migration position of myosin (200), β-galactosidase (116), and phosphorylase B (97) are indicated on the left of panels F and G and are indicated in kD. Bar, (A–D) 10 μm.

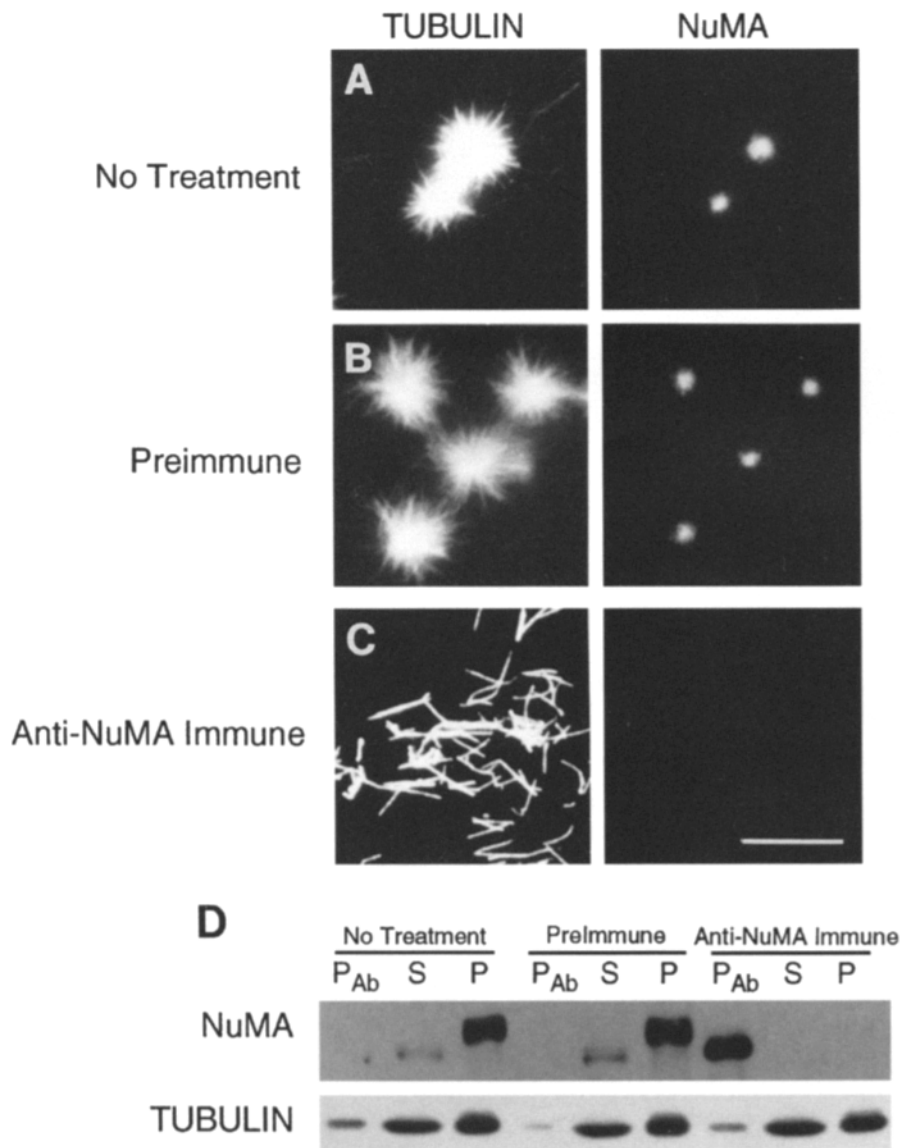
insoluble fractions were resuspended in 150 μl of extraction buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 mM Sodium fluoride, and 10 mM sodium pyrophosphate) and heated to 100° two times for 3 min each with vigorous agitation between each incubation. After this extraction, the samples were cooled to room temperature and diluted eightfold with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 10 mM sodium pyrophosphate, 3.4% Triton X-100. 10 μg of rabbit anti-NuMA IgG was added to each and incubated at 4° for 12 h with gentle agitation. After this incubation, 50 μl of protein A-conjugated agarose (50% slurry in TBS) was added and the extracts were incubated for 2 h at 4° with gentle agitation. The protein A-conjugated agarose containing the IgG was then collected by centrifugation at 10,000 g for 15 s, washed three times with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 30 mM sodium pyrophosphate, 1.5% Triton X-100, washed twice in 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 30 mM sodium pyrophosphate, 1.5% Triton X-100, and finally solubilized in 50 μl SDS-PAGE sample buffer. The immunoprecipitated material was then subjected to size fractionation on 5% SDS-PAGE and processed for immunoblotting using enhanced chemiluminescence (exposure times varied from 5 to 30 s at room temperature without an

intensifying screen). The chemiluminescence signal was then allowed to extinguish overnight and the blot subsequently exposed to film at -70° with an intensifying screen for 2–10 h.

Results

NuMA Is a Component of Mitotic Asters Assembled in a Cell Free Mitotic Extract

To test directly if NuMA participates in the organization of microtubules into mitotic arrays, we analyzed whether NuMA was a component of mitotic asters induced with taxol in a cell free mitotic extract prepared from synchronized HeLa cells. HeLa cells were synchronized in mitosis with nocodazole after a release from thymidine block and collected by shake-off. The cells were chilled on ice,



washed twice in PBS to remove the nocodazole, homogenized, and separated into 100,000 g soluble and insoluble fractions. The 100,000 g soluble fraction was retained and had histone H1 kinase activity 10 times higher than the activity of an extract prepared under the same conditions from nonsynchronized cells (Fig. 1 G). A majority of the tubulin and NuMA are recovered in the 100,000 g soluble fraction, while the chromosomes and other particulate matter from the cell are discarded in the 100,000 g pellet.

Microtubule assembly was stimulated in the extract by the addition of 10 μ M taxol and incubation at 33°C. Under these conditions the microtubules polymerized and spontaneously organized into radial arrays \sim 10 μ m in diameter (i.e., asters) that contained NuMA concentrated at the central core (Fig. 1 D). If the extract was incubated in the absence of taxol at either 0°C or 33°C or with taxol at 0°C there was no detectable microtubule polymerization and the NuMA protein was not found in concentrated aggregates that could be detected by immunofluorescence microscopy (Fig. 1, A–C). The organization of the microtubules into astral arrays appears to be an intrinsic property

of an extract prepared from mitotic cells since only randomly arranged microtubule arrays were found in extracts prepared under identical conditions from interphase cells (Fig. 1 E). The lack of staining for NuMA in the interphase extract is due to the fact that NuMA is in the interphase nucleus and is discarded as a component of the 100,000 g insoluble material during the preparation of the extracts. Based on previous experiments demonstrating that the microtubules in taxol-induced mitotic asters are oriented with their minus ends inward and their plus ends extending outward (Maekawa et al., 1991; Verde et al., 1991), our results support the conclusion that NuMA is localizing to the minus ends of the microtubules of the mitotic asters formed in these extracts.

To determine the efficiency of aster assembly under these conditions, we subjected the extract to sedimentation at 10,000 g after the assembly reaction. The extract was then separated into 10,000 g soluble and insoluble fractions, the proteins solubilized in SDS-PAGE sample buffer and subjected to immunoblot analysis for both tubulin and NuMA. Consistent with the immunofluores-

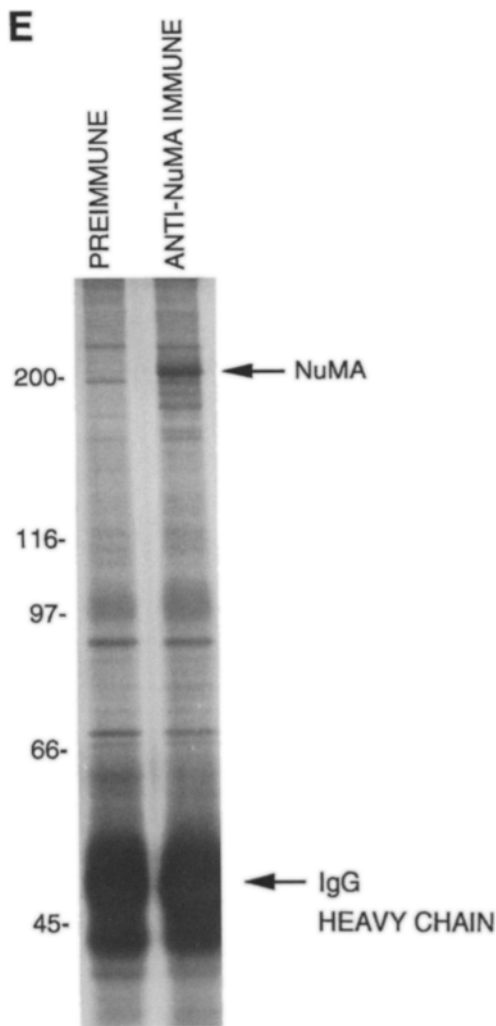


Figure 5. NuMA is required for the formation of mitotic asters in the cell free mitotic extract. The cell free mitotic extract was treated with protein A-conjugated agarose alone (*No Treatment*), protein A-conjugated agarose containing IgG from the preimmune rabbit serum (*Preimmune*), or protein A-conjugated agarose containing IgG from the NuMA-specific rabbit serum (*Anti-NuMA Immune*). After incubation at 4° for 2 h, the protein A-conjugated agarose was removed and the mitotic asters assembled under optimal conditions with taxol and ATP for 30 min at 33° and processed for indirect immunofluorescence (*A–C*) using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). After these treatments, the protein A-conjugated agarose was recovered (*P_{Ab}*) and the remainder of the extracts were separated into soluble (*S*) and insoluble (*P*) fractions by centrifugation at 10,000 *g* and subjected to immunoblot analysis (*D*) using either a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). The proteins bound to the protein A-conjugated agarose containing either the IgG from the preimmune rabbit serum (*Preimmune*) or the anti-NuMA immune serum (*Anti-NuMA Immune*) were analyzed by silver staining of SDS-PAGE (*E*). The migration position of myosin (200), β -galactosidase (116), phosphorylase B (97), bovine serum albumin (66), and ovalbumin (45) are indicated on the left of panel *E* and are indicated in kD. Bar, (*A–C*) 10 μ m.

cence data, the conversion of both tubulin and NuMA from a soluble form to an insoluble form is dependent on the presence of taxol and incubation at 33°C (Fig. 1 *F*). Elimination of taxol and/or incubation at 0°C prevented microtubule assembly, and neither NuMA nor tubulin were found in the insoluble fraction. Based on the conversion of both NuMA and tubulin from a soluble form into an insoluble form, we have calculated that the average efficiency of mitotic aster assembly in these extracts is ~80%, however, there is some variability between preparations that yield assembly efficiencies that range between 50 and 95%. These data indicate that the solubility of NuMA in these extracts is dependent on the assembly state of the microtubules, and strongly suggest that the fraction of NuMA observed in the 10,000 *g* pellet derives from the concentration of NuMA at the core of the mitotic asters that are observed by immunofluorescence microscopy.

Next, we analyzed how these structures assemble as a function of time. For this experiment we induced microtubule assembly under optimal conditions, and then fixed and processed the extract for immunofluorescence to localize both NuMA and tubulin at various time intervals during the assembly reaction. The first signs of aster assembly are observed as the accretion of both NuMA and tubulin into aggregates detectable by immunofluorescence microscopy as early as 2 min after the initiation of incubation at 33°C (Fig. 2 *B*). Indeed, there is a nearly perfect correlation between the appearance of NuMA staining and tubulin staining although no clearly identifiable microtubules are detectable at this early time point. Over time, these aggregates seem to “mature” by accumulating further NuMA staining, extending the microtubules, and organizing the microtubules into aster-like arrays (Fig. 2, *C–D*). After 30 min of incubation the assembly of the mitotic asters is complete and is characterized by the organization of the microtubules into radial arrays concomitant with the concentration of NuMA at the central core (Fig. 2 *E*).

The accretion of both NuMA and tubulin during the early stages of aster assembly (i.e., 2 min; Fig. 2 *B*) suggests that these proteins may be associating with one or more components of the centrosome present in the extract. To test this possibility we performed immunofluorescence analysis on the mitotic asters using antibodies against the centrosomal components pericentrin (Doxsey et al., 1994; antibody courtesy of S. Doxsey) and γ -tubulin (Stearns and Kirschner, 1994; Joshi et al., 1992; antibody courtesy of H. Joshi) at antibody concentrations that yield pronounced centrosomal staining in cultured cells. In both cases, there was no detectable staining of the taxol-induced mitotic asters assembled in this extract (Fig. 3, *B* and *C*). Furthermore, immunoblot analysis of the 10,000 *g* soluble and insoluble fractions prepared after the assembly of the mitotic asters demonstrates that, contrary to NuMA and α -tubulin, the γ -tubulin present in our extract is not enriched in the insoluble fraction containing the assembled mitotic asters (Fig. 3 *E*). Conversely, the core of these mitotic asters did contain an antigen(s) recognized by the monoclonal antibody mpm-2 (courtesy of P. Rao) indicating that an additional protein(s) that contain mitosis-specific phosphoepitopes is present on these structures (Fig. 3 *D*). Thus, while we cannot discount the possibilities that minor, undetectable quantities of either pericentrin or

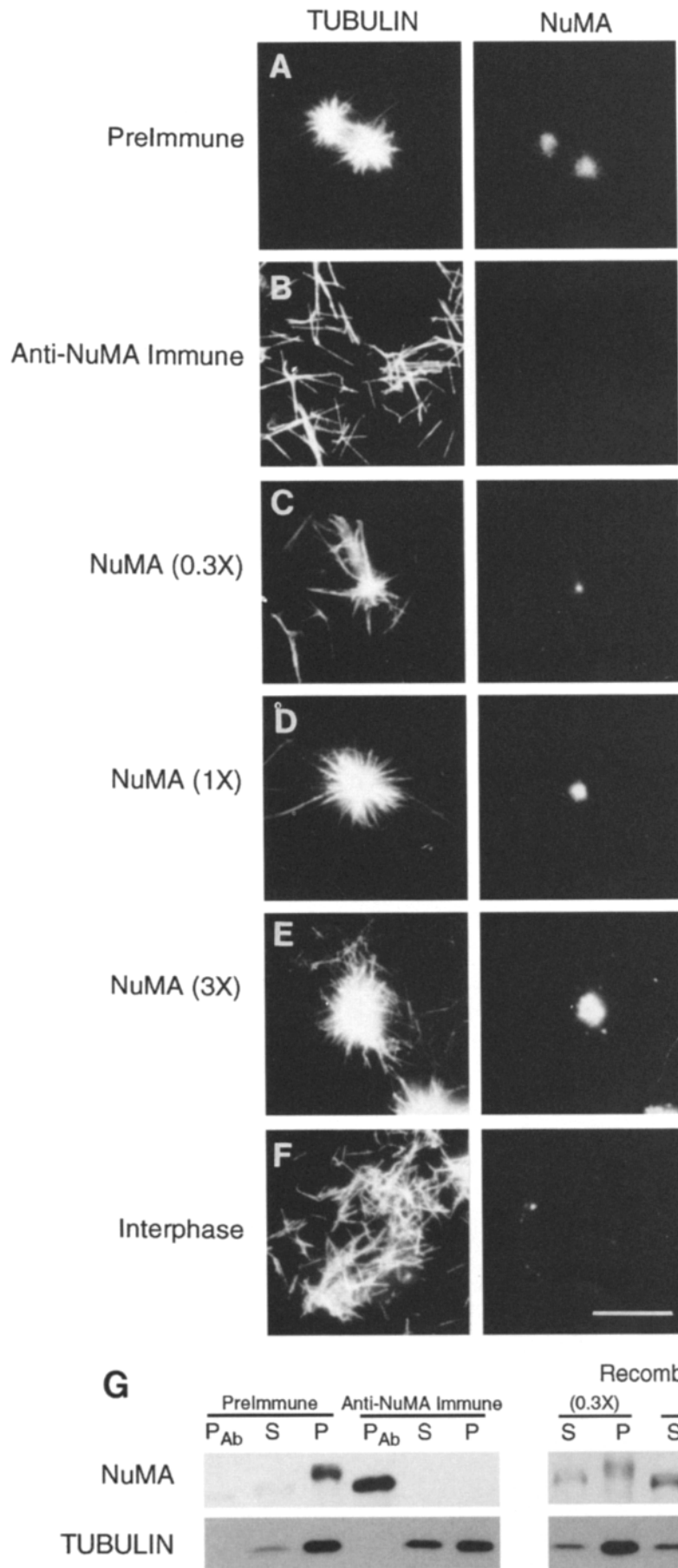


Figure 6. Reconstitution of mitotic aster formation in the NuMA-depleted mitotic extract using the purified recombinant NuMA protein. A cell free extract was prepared from either interphase cells (*F*) or mitotic cells (*A–E*). The cell free mitotic extract was treated with protein A-conjugated agarose containing IgG from the preimmune rabbit serum (*A*) or protein A-conjugated agarose containing IgG from the NuMA-specific rabbit serum (*B–E*). After incubation at 4° for 2 h the protein A-conjugated agarose was removed and the mitotic asters assembled under optimal conditions with taxol and ATP for 30 min at 33° in the absence (*A* and *B*) or the presence of various concentrations of the purified recombinant NuMA protein (*C–E*). After the incubation, the extracts were processed for indirect immunofluorescence (*A–F*) using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). The protein A-conjugated agarose was recovered (*P_{Ab}*) and the remaining extracts were separated into soluble (*S*) and insoluble (*P*) fractions by centrifugation at 10,000 g and subjected to immunoblot analysis (*G*) using either a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). Bar, (*A–F*) 10 μ m.

γ -tubulin are localized at the core of these structures or that additional (as yet unidentified) centrosomal proteins are participating in the assembly of these mitotic asters, these results demonstrate that these asters lack conventional centrosomes consistent with the centrosome-independent assembly of taxol-induced mitotic asters observed *in vivo* (Maekawa et al., 1991; Maekawa and Kuriyama, 1991; Kallajoki et al., 1992).

We next tested the sensitivity of the assembly of the mitotic asters to ATP and nonhydrolyzable ATP analogs. In the absence of any exogenously added ATP both the efficiency of microtubule polymerization (Fig. 4 E) and the histone H1 kinase levels were similar to those found in an extract containing ATP (data not shown). NuMA associated with the microtubules, however, it did not concentrate at any one position that resembled the central core of a mitotic aster and the microtubules were arranged in poorly organized aggregates rather than astral arrays (Fig. 4 B). A similar pattern of microtubule organization and NuMA distribution was found if the nonhydrolyzable analog of ATP, AMP-PNP, was added to the extract (Fig. 4 C). These results indicate that the organization of microtubules into astral arrays in our extracts, like centrosome assembly and mitotic aster formation in *Xenopus* egg extracts (Felix et al., 1994; Verde et al., 1991), is an ATP-dependent process.

Based on the results of Verde et al. (1991) who demonstrated that cytoplasmic dynein is required for the assembly of taxol-induced mitotic asters in extracts prepared from *Xenopus* eggs, we next tested whether vanadate would inhibit mitotic aster assembly in this system. Fig. 4 D shows

that in the presence of 50 μ M vanadate the mitotic asters fail to organize correctly, NuMA fails to concentrate efficiently at a central core, and the microtubules tend to be highly bundled. The effect of vanadate was dose dependent and would not alter mitotic aster formation unless the concentration was greater than 10 μ M. While we cannot discount any nonspecific effects of vanadate on protein phosphatases and other microtubule-dependent motor proteins, these results are consistent with the findings of Verde et al. (1991) and suggest that cytoplasmic dynein is playing a functional role in the organization of microtubules into mitotic asters in both egg and somatic extracts.

Finally, we have noticed a consistent retardation in the mobility of the NuMA protein in SDS-PAGE when it becomes associated with the mitotic asters (Figs. 1 and 4). This alteration in NuMA's mobility in SDS-PAGE appears to be a microtubule-dependent phosphorylation because it is only observed after the stimulation of microtubule polymerization, it is only detected in the fraction of the NuMA protein that is associated with the microtubules (i.e., 10,000 g pellets), and it is inhibited by the presence of AMP-PNP or the absence of ATP (Fig. 4 E). To verify that this retardation in NuMA's mobility is not an artifact arising from the assembly of the mitotic asters under these *in vitro* conditions, we examined the mobility of NuMA by immunoblot analysis using total cell protein derived from cells with varying degrees of mitotic spindle assembly (Fig. 4 F). Cells were harvested in interphase, mitosis in the presence of nocodazole, mitosis after the removal of nocodazole (data not shown), and mitosis after the removal of nocodazole with the addition of taxol and analyzed for

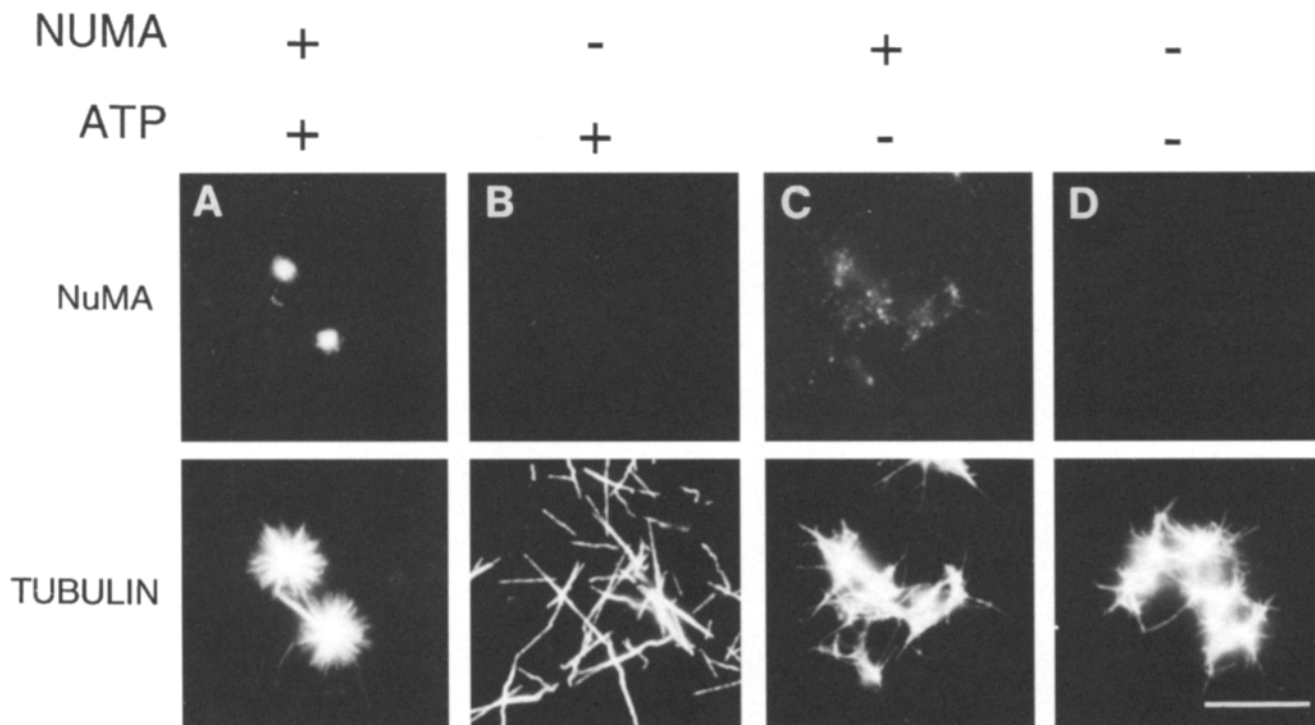


Figure 7. Mitotic aster assembly requires both ATP and NuMA. Mitotic asters were assembled under optimal conditions with taxol and incubation for 30 min at 33° in the presence (A and B) or absence (C and D) of ATP and with (A and C) or without (B and D) the NuMA protein and processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). Bar, 10 μ m.

the relative mobility of NuMA by immunoblot. The results demonstrate that NuMA is subjected to two discrete molecular weight shifts *in vivo*, one of which is dependent on the assembly state of the microtubules (Fig. 4 *F*; compare mitotic+nocodazole with mitotic+taxol) similar to the molecular weight shift that is observed in our *in vitro* extract after the induction of mitotic aster assembly (Figs. 1 *F* and 4 *E*). To test directly if NuMA is subjected to phosphorylation upon aster assembly, we added γ -[32 P]ATP into the extract in the presence of either ATP or AMP-PNP, induced microtubule assembly, and subsequently isolated the NuMA protein that was associated with the microtubule structures in each case by immunoprecipitation. Fig. 4 *G* shows that approximately equal quantities of NuMA are immunoprecipitated from the microtubule structures formed in the presence of either ATP or AMP-PNP (Fig. 4 *G*, immunoblot), but that only the NuMA protein isolated from the extract containing ATP shows a retarded mobility in SDS-PAGE and is phosphorylated (Fig. 4 *G*, 32 P). These results are consistent with the modification of NuMA by at least two discrete phosphorylation events during mitosis. The first phosphorylation event oc-

urs at the transition from interphase to mitosis (Compton and Luo, 1995; Price and Pettijohn, 1986), and the second phosphorylation event occurs upon NuMA's association with microtubules. The identity of the protein kinases responsible for these phosphorylation events is unknown at this time and currently under investigation.

NuMA Is Required for Mitotic Aster Formation

To determine if the formation of mitotic asters in these extracts requires the NuMA protein, we examined whether the mitotic asters assembled normally after the specific depletion of NuMA from the extract. In control samples we added either protein A-conjugated agarose alone or protein A-conjugated agarose coupled with IgG isolated from preimmune rabbit serum. Treatment of the extracts with these two reagents did not alter either the morphology of the mitotic asters that form after microtubule polymerization or the distribution of the NuMA protein at the core of these structures (Fig. 5, *A* and *B*). Also, these treatments did not affect the histone H1 kinase levels in the extract (data not shown) or the assembly efficiency of the extract

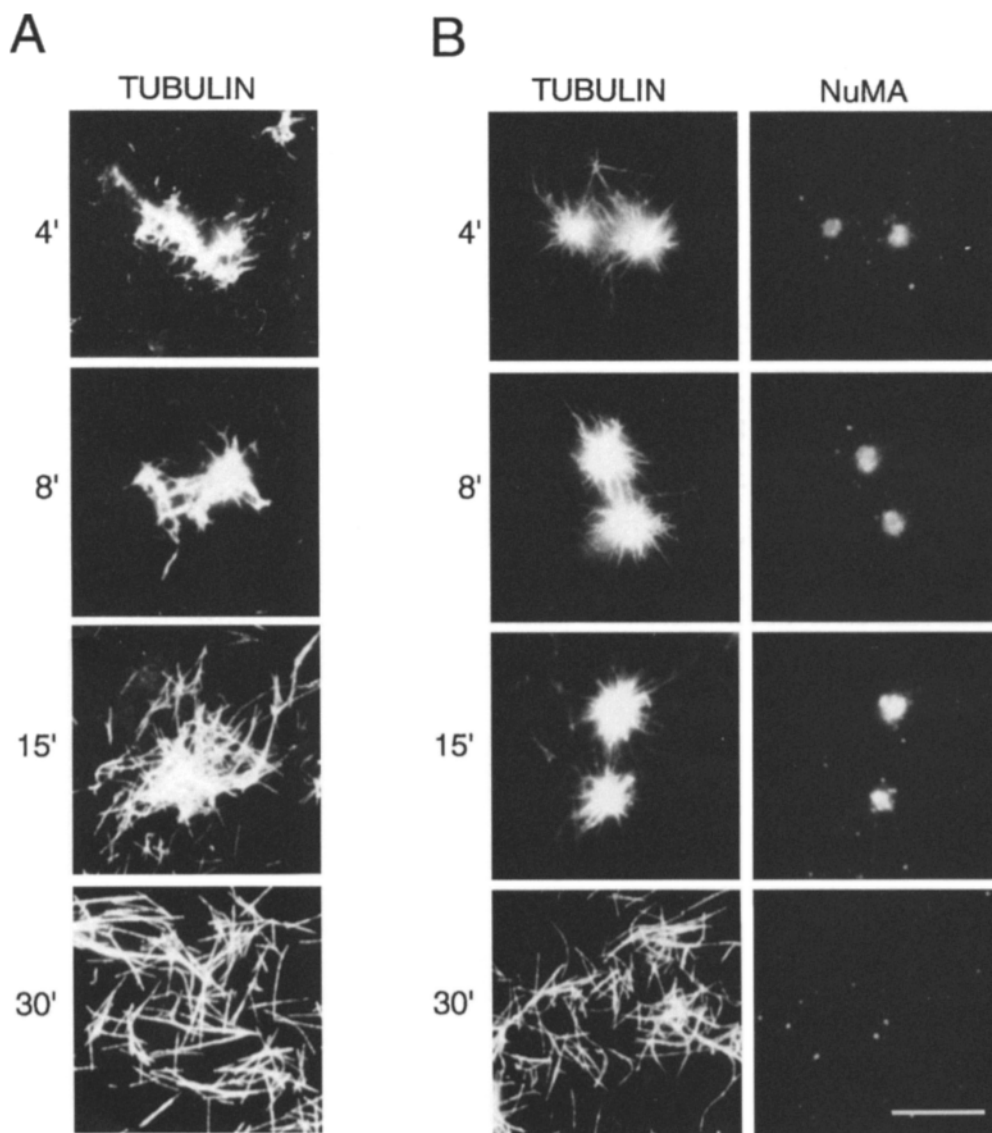


Figure 8. NuMA is required late in the assembly of mitotic asters. (*A*) Mitotic asters were assembled in the NuMA-depleted mitotic extract under optimal conditions with taxol and ATP at 33° for the indicated times and processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*). (*B*) Mitotic asters were assembled in the NuMA-depleted mitotic extract under optimal conditions with taxol and ATP at 33° and the purified recombinant NuMA protein was added at the indicated times after the initiation of microtubule polymerization. 30 min after the addition of the purified recombinant NuMA protein to the assembly reactions, the samples were processed for indirect immunofluorescence using a tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*) and a rabbit NuMA-specific antibody (*NuMA*). Bar, 10 μ m.

as judged by the sedimentation of both NuMA and tubulin at 10,000 g after the assembly reaction (Fig. 5 D). Treatment of the extract with protein A–conjugated agarose coupled with the IgG purified from the anti-NuMA serum, however, inhibited the assembly of the mitotic asters and yielded randomly arranged microtubules (Fig. 5 C). Neither the histone H1 kinase levels (data not shown) nor the polymerization efficiency of the microtubules in the absence of NuMA were appreciably altered, but the microtubules failed to organize into recognizable mitotic asters (Fig. 5, C and D). As the immunoblot demonstrates (Fig. 5 D), the depletion of NuMA from the extract using this procedure is nearly 100%. We failed to detect any NuMA in the 10,000 g soluble or insoluble fractions after the depletion step even after gross overexposure of the film. Thus, these results demonstrate that NuMA (or a NuMA containing complex) is required for the organization of microtubules into aster-like arrays in these extracts.

To test whether the NuMA protein that is precipitated from the extracts using this antibody is contained in a multi-protein complex, we analyzed the immunoprecipitate by SDS-PAGE followed by silver staining. On both 7.5% (Fig. 5 E) and 12.5% SDS-PAGE (data not shown), we were unable to detect any proteins that appeared to be in a 1:1 stoichiometric complex with NuMA, although we cannot rule out the presence of a ~50-kD protein due to the antibody heavy chain. The minor protein species observed at the 180–200-kD size range are either proteolytic products of the full-length NuMA protein or the putative isoforms of the NuMA protein (Tang et al., 1993) because they are all reactive with a NuMA antibody by immunoblot analysis (data not shown). Furthermore, there was no detectable protein kinase activity associated with these immunoprecipitates as judged by autophosphorylation (data not shown). These results corroborate the findings of Kalajoki et al. (1993), and demonstrate that the NuMA protein purified from extracts prepared from nocodazole-treated mitotic cells is not present in a 1:1 stoichiometric complex with any additional detectable proteins.

Finally, to test directly if NuMA is the only functional component depleted from the extract using the polyclonal antibody, we tested if the purified recombinant NuMA protein would reconstitute mitotic aster formation in the NuMA-depleted extracts. As described above, extracts treated with protein A–conjugated agarose coupled with IgG from preimmune serum had no effect on mitotic aster formation whereas protein A–conjugated agarose coupled with IgG from the immune serum prohibited the assembly of mitotic asters (Fig. 6, A and B). When the NuMA-depleted extract was supplemented with the purified recombinant human NuMA protein mitotic aster formation was restored. The restoration of mitotic aster formation by the recombinant NuMA protein was dose dependent (Fig. 6, C–E), and cell cycle-dependent because mitotic aster formation was not induced if the purified recombinant protein was added to an extract prepared from interphase cells (Fig. 6 F). The purified recombinant NuMA protein was required in the NuMA-depleted extract at a concentration of ~3.3 µg/ml to fully restore mitotic aster formation (Fig. 6 D). This concentration of the recombinant NuMA protein (equivalent to 2.8×10^5 copies of NuMA per cell equivalent in our extract) is very similar to the

concentration of the endogenous NuMA protein (estimated to be 2.5×10^5 copies per cell based on quantitative immunoblot analysis; Compton et al., 1992). Addition of the recombinant NuMA protein into the NuMA-depleted extract at quantities below the normal endogenous levels led to the formation of mitotic aster-like structures that contain a small core of NuMA with only a few associated microtubules (Fig. 6 C). Addition of the recombinant NuMA protein into the NuMA-depleted extracts at quantities in excess of the normal endogenous levels led to mitotic asters that are fairly typical, although the central core staining pattern of NuMA is noticeably larger and more intense (Fig. 6 E). Finally, we have verified the dose-dependent reconstitution of mitotic aster formation in these NuMA-depleted extracts using the purified recombinant NuMA protein by immunoblot analysis of the 10,000 g soluble and insoluble fractions after mitotic aster assembly (Fig. 6 G). The results of this immunoblot demonstrate that the quantity of the recombinant protein associated with the mitotic asters (i.e., 10,000 g pellets) varies with the amount added, and further shows that the recombinant protein is, like the endogenous protein (Figs. 1 and 4), subjected to a specific modification that retards its mobility in an SDS-PAGE. These results demonstrate that NuMA is the only functional component of the extract that is depleted by our NuMA-specific antibody, that the purified recombinant NuMA protein is capable of functionally replacing the endogenous NuMA protein, and that NuMA is functionally required for the organization of the microtubules into ordered mitotic asters.

So far, our results appear to define two distinct phases in the assembly of mitotic asters in this cell free system: an ATP-dependent phase (Fig. 4) and a NuMA-dependent phase (Figs. 5 and 6). To test if there is a temporal order between these two phases of mitotic aster formation, we performed two experiments. In the first experiment we examined the products of the mitotic aster assembly reaction performed in the absence of both NuMA and ATP (Fig. 7). In the absence of NuMA the microtubules are randomly dispersed (Figs. 5 C and 7 B) and in the absence of ATP the microtubules form disorganized aggregates (Figs. 4 B and 7 C). In the absence of both NuMA and ATP the microtubules form disorganized aggregates that are indistinguishable from the microtubule aggregates formed in the absence of ATP alone (Fig. 7 D). Similar to previous findings, the extract remained mitotic under all of these experimental conditions as judged by the histone H1 kinase levels (data not shown). In the second experiment we tested if the purified recombinant NuMA protein was capable of reconstituting mitotic aster formation in the NuMA-depleted extract at various times after the stimulation of microtubule polymerization. The assembly of mitotic asters in the NuMA-depleted mitotic extract proceeds through a series of steps that resemble the assembly of the mitotic asters in the presence of NuMA until the microtubule aggregates disperse into random microtubule arrangements (compare Fig. 8 A with Fig. 2). The purified recombinant NuMA protein was capable of reconstituting mitotic aster formation in the NuMA-depleted extract if it was added as late as 15 min after the initiation of microtubule polymerization (Fig. 8 B). However, despite the fact that the extract remained mitotically active throughout

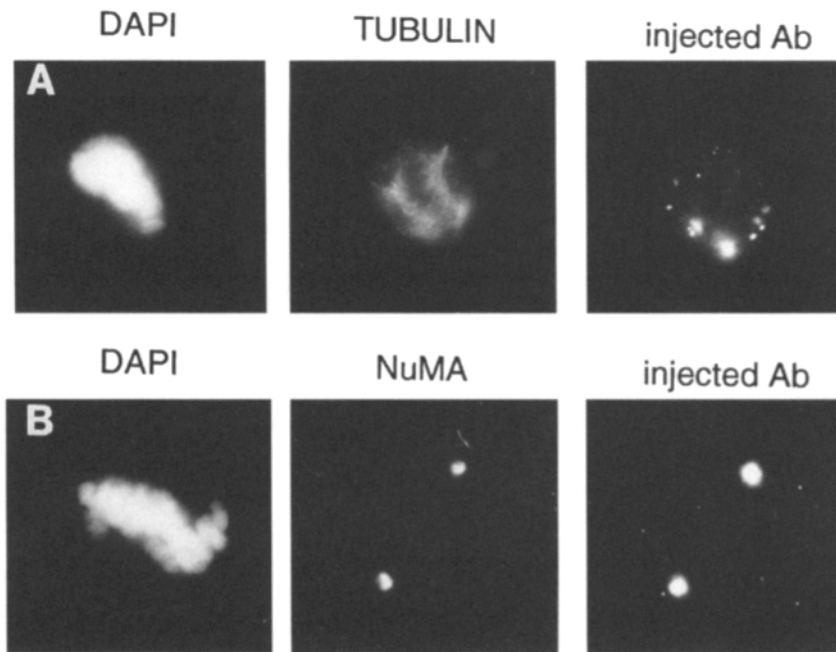


Figure 9.

this experiment as judged by the histone H1 kinase levels (data not shown), the purified recombinant NuMA protein did not restore mitotic aster formation if it was added 30 min after the stimulation of microtubule polymerization, a time at which the microtubules had become completely dispersed (Fig. 8 B). Taken together, these data show that NuMA is required late in the process of mitotic aster assembly and further suggest that an ATP-dependent step in the process of mitotic aster formation may precede the NuMA-dependent phase of mitotic aster formation.

The Mitotic Spindle Is Disorganized in the Absence of NuMA

To test if NuMA plays a required role in the assembly of the mitotic spindle *in vivo*, we have microinjected a NuMA-specific antibody into cultured cells and determined the effect of that antibody on mitotic spindle structure and the distribution of NuMA. The polyclonal rabbit serum used in these experiments was raised against the purified recombinant NuMA protein and detects a single protein species of ~210 kD in total cell extracts prepared from human as well as cells from other primate species. In addition, this antibody decorates interphase nuclei and the polar region of the mitotic spindle in the canonical NuMA-like pattern by immunofluorescence microscopy (data not shown). To test if this antibody would affect the function of the NuMA protein *in vivo*, we microinjected the purified IgG fraction from this serum into monkey CV-1 cells and followed the progression of these cells through mitosis. At concentrations of ~12 mg/ml this antibody significantly delayed the cells in mitosis. In 80 cells that entered mitosis after microinjection, 64 cells (80%) failed to complete mitosis within the subsequent 3 h after their entrance into mitosis. In contrast, in 41 cells injected with the IgG fraction purified from the preimmune serum (~14 mg/ml)

only four cells failed to complete mitosis within an hour. None of these four cells had detectable alterations in the organization of the mitotic spindle. Thus, at high concentration the IgG from the NuMA-specific polyclonal serum delays the completion of mitosis.

To localize the injected antibody and examine the morphology of the mitotic spindle in these cells, we processed the cells for indirect immunofluorescence using Texas red-conjugated goat anti-rabbit and a mouse monoclonal anti-tubulin antibody (DM1 α). In the mitotically blocked cells, the injected antibody consistently localized to between 1 and 15 brightly staining Triton X-100 insoluble aggregates within the cell cytoplasm (Fig. 9 A). If the injected cells were fixed before extraction with Triton X-100, the brightly staining aggregates were still detectable, but there was diffuse cytoplasmic staining due to the excess injected antibody. A majority of the mitotically blocked cells lacked a typical bipolar mitotic spindle as compared to a control injected mitotic cell (Fig. 9, compare A and G), and in most cells the brightly staining aggregates of the injected antibody did not show a consistent association with the microtubules (Fig. 9 A).

To localize the endogenous NuMA protein relative to both the injected rabbit antibody and the mitotic spindles in these mitotically blocked cells, we performed indirect immunofluorescence microscopy using a NuMA-specific human autoimmune sera (courtesy of Dr. D. Pettijohn). When we stained these cells for both the injected antibody and the endogenous NuMA protein, we found that the endogenous NuMA protein consistently colocalized with the insoluble aggregates that are identified by staining for the injected rabbit antibody (Fig. 9 B). When we compared the localization of the endogenous NuMA protein with the mitotic spindles in these cells, we found that in some of these cells (26.5%) the endogenous NuMA protein appeared to colocalize with the pericentrosomal region of the mitotic spindle although it was aggregated and did not

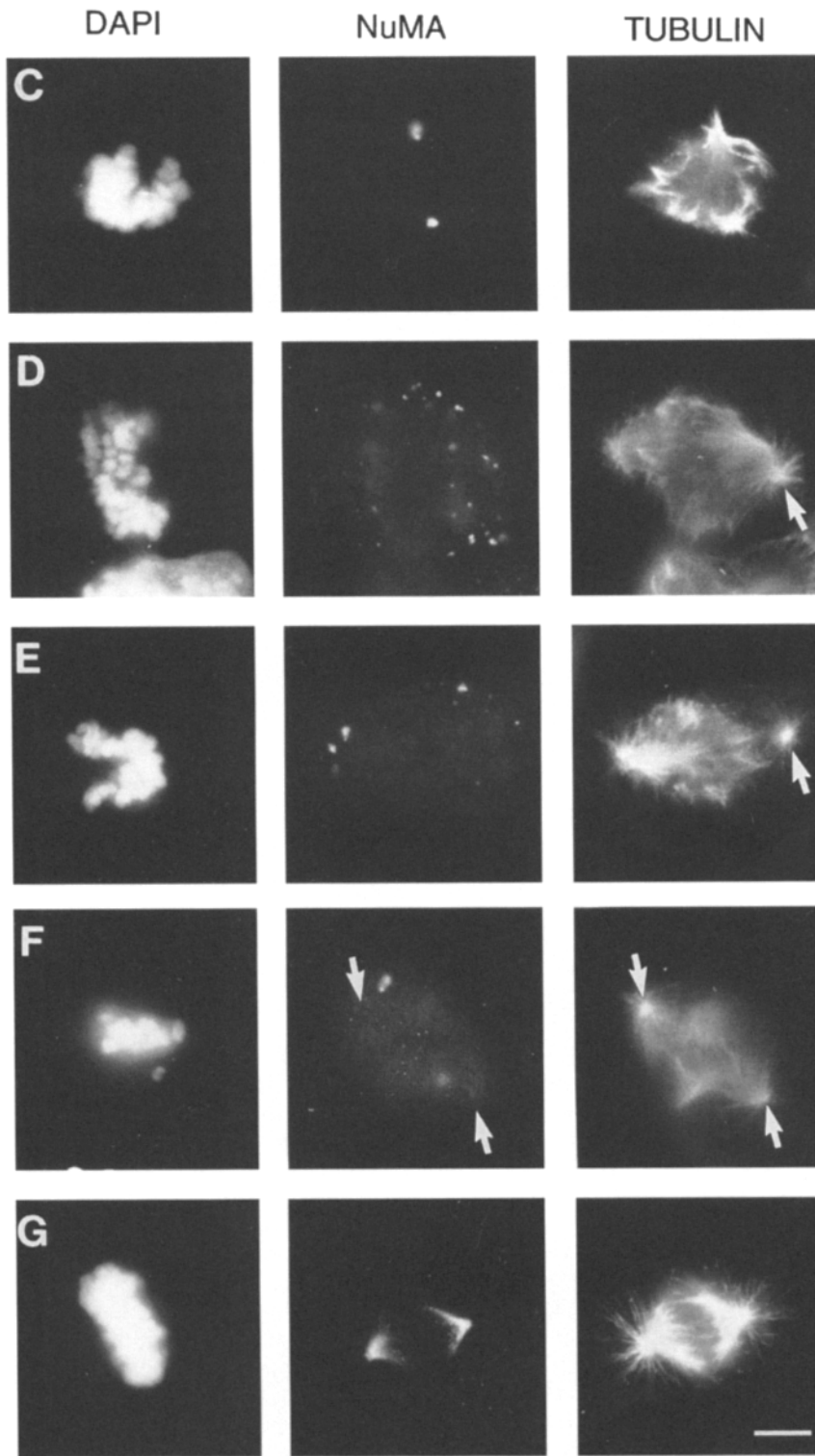


Figure 9. Organization of the mitotic spindle requires the pericentrosomal localization of NuMA. Monkey CV-1 cells were monitored as they progressed through mitosis after microinjection of a NuMA-specific rabbit antibody (A–F) or a preimmune control antibody (G). The mitotic cells were fixed and processed for indirect immunofluorescence using a DNA-specific dye (*DAPI*), the tubulin-specific mouse monoclonal antibody DM1 α (*TUBULIN*), Texas red-conjugated goat anti-rabbit (*injected Ab*), and a human NuMA-specific autoimmune serum (*NuMA*). Bar, 10 μ m.

show the typical crescent shaped staining pattern (compare Fig. 9, B and C with G) similar to the results of antibody injections performed by Yang and Snyder (1992). In contrast, however, in a majority (73.5%) of these mitotically blocked cells the antibody-induced aggregates of NuMA were not in the proximity of the centrosomes of the cell (Fig. 9, D–F). The centrosomes are clearly visible in these cells as the microtubule organizing centers and appear to be nucleating microtubules normally (Fig. 9, D–F,

arrows), but the mitotic spindles are disorganized and do not have a typical bipolar morphology compared to a control-injected mitotic cell (Fig. 9 G). Thus, unlike the results of previous anti-NuMA microinjection experiments that resulted in the aggregation of NuMA on the centrosomes (Yang and Snyder, 1992; Kallajoki et al., 1991), the results of the antibody microinjection experiments shown here indicate that the mitotic spindle is not properly organized if NuMA (and any associated protein[s]) is prohibited from

interacting with the microtubules at the polar region of the mitotic spindle.

Discussion

The most striking feature of the NuMA protein is that it is strictly localized in the cell nucleus during interphase and interacts with microtubules of the mitotic spindle after nuclear envelope breakdown at prometaphase. During the course of events in mitosis the centrosomes nucleate and organize the microtubules into astral arrays during prophase and prometaphase before the release of NuMA from the nuclear compartment. When the nuclear envelope breaks down NuMA associates with the microtubules and becomes concentrated at the polar ends of the mitotic spindle during prometaphase concomitant with the convergence of the microtubules toward the chromosomes into the typical fusiform array. The results of our microinjection experiments show that if NuMA is prevented from localizing onto the microtubules of the developing mitotic spindle *in vivo*, then the centrosomes continue to nucleate microtubules leading to typical astral arrays that fail to progress to form fusiform spindles (Fig. 9, D–F). Furthermore, by using taxol to stimulate microtubule assembly in our *in vitro* system we obviate the need for centrosomes as microtubule-organizing centers and show that NuMA is required for the organization of aster-like mitotic arrays. Collectively, these data support a model in which, upon nuclear envelope breakdown, NuMA concentrates at the polar ends of the microtubules emanating from the centrosomes where it is involved in a second level of microtubule organization that is necessary for the formation of a typical bipolar fusiform microtubule array. This proposed functional activity of NuMA is summarized in Fig. 10 which shows how NuMA, either alone (Fig. 10 A) or in combination with other cellular proteins (Fig. 10 B), may be stabilizing the parallel arrays of microtubules of the mitotic spindle by forming a matrix or lattice through which the microtubules extend from the centrosomes to the chromosomes.

Interestingly, recent experiments using grasshopper spermatocytes have pointed to nonchromosomal components of the nucleus, the so-called “nuclear sap,” that are involved in the stability of the mitotic spindle (Zhang and Nicklas, 1995a). These authors found that the mitotic spindle was stable if the chromosomes were removed from the spindle by micromanipulation after nuclear envelope breakdown, but similar to the findings of Sluder et al. (1986) working with echinoderm embryos, the spindle failed to form if the nucleus was removed from the cell before nuclear envelope breakdown. Based on our findings, NuMA may be an active component of the nuclear sap that is necessary for the stability of the spindle in this system after the removal of the chromosomes. However, in this context NuMA (and/or other components of the nuclear sap) may not be sufficient to organize the mitotic spindle in the absence of chromosomes because Zhang and Nicklas (1995b) have found that the spindle failed to form if they removed the chromosomes but not the nuclear sap from the prophase nucleus before nuclear envelope breakdown.

It is important to emphasize that the functional ability of NuMA to organize microtubules appears to act indepen-

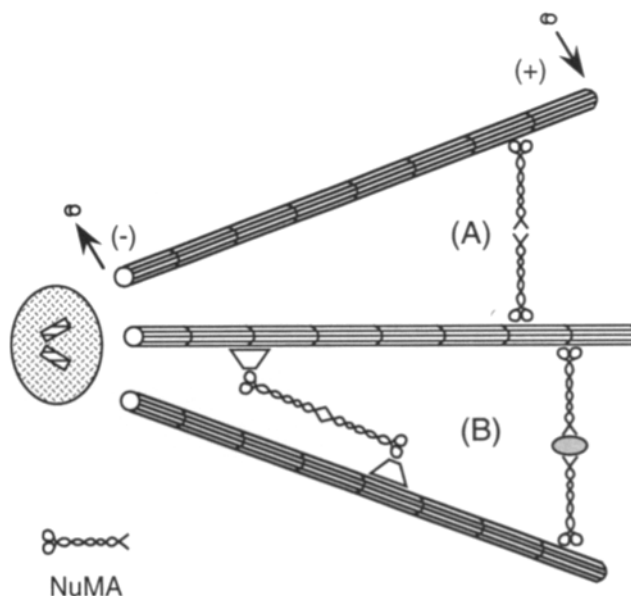


Figure 10. Model for NuMA's function in the assembly of the polar region of the mitotic spindle. NuMA accumulates adjacent to the centrosomes of the mitotic spindle and participates in the organization of the microtubules into the highly ordered array as a component of the mitotic spindle matrix. NuMA may interact with the microtubules directly (*option A*) or be one component of a multi-protein complex that associates with the microtubules (*option B*; only two of many different possibilities are depicted). Assembly of the mitotic spindle matrix is likely to structurally stabilize the mitotic spindle permitting the release of the minus ends of the microtubules from the centrosome for tubulin subunit exchange during poleward microtubule flux.

dently of the centrosomes which have the primary responsibility for microtubule nucleation (and orientation). Several lines of evidence are consistent with this conclusion. First, NuMA is a nuclear protein during interphase and does not localize to the polar ends of the mitotic spindle until it is released from the nuclear compartment after nuclear envelope breakdown at prometaphase. Second, NuMA is not an integral component of the centrosome because NuMA does not colocalize with the centrosome in mitotic cells as determined by double-label immunofluorescence microscopy (Maekawa and Kuriyama, 1991; Maekawa et al., 1991; Kallajoki et al., 1992; Compton et al., 1992; Yang et al., 1992), and NuMA requires intact microtubules to concentrate at the polar end of the mitotic spindle (Price and Pettijohn, 1986; Tousson et al., 1991). Third, in cells where NuMA is prevented from localizing onto the mitotic spindle (Fig. 9, D–F), the centrosomes continue to nucleate microtubules normally into astral arrays, although those arrays do not organize correctly into a bipolar mitotic spindle. Finally, the organization of microtubules into mitotic asters in the presence of taxol is independent of the centrosomes and requires NuMA both *in vitro* and *in vivo* (Kallajoki et al., 1992). Therefore, because NuMA is required for the organization of microtubules into mitotic arrays, and is not a component of the centrosome per se, it satisfies the criteria as a functional component of the hypothesized “mitotic spindle matrix.”

The existence of such a spindle matrix has been pro-

posed by several investigators based on theoretical arguments (McIntosh, 1980; Rebhun and Pallazzo, 1988; Pickett-Heaps et al., 1982), and recent experimental evidence has emerged in support of such a spindle matrix or lattice. Mitchison and coworkers (Mitchison, 1989b; Sawin and Mitchison, 1991, 1994) have demonstrated through the process of poleward microtubule flux that both the plus and minus ends of the microtubules in a mammalian metaphase spindle remain free to exchange tubulin subunits. An inherent prediction of this poleward microtubule flux, however, is that the cell must have a mechanism to maintain the overall spindle organization (i.e., spindle matrix) while permitting tubulin subunit exchange at both the plus and minus ends of the microtubules. Additional support for spindle matrix or lattice comes from spindle severing experiments (Nicklas et al., 1989). These authors demonstrated the existence of a minus-end organizing function within the body of the mitotic spindle by showing that the mitotic spindle remained well organized despite the physical removal of the centrosome from the spindle leaving the minus ends of the microtubules free.

The essential question regarding the mechanism that NuMA employs to organize the microtubules is whether it is interacting with the microtubules directly (Fig. 10, option A) or whether it interacts with the microtubules indirectly in association with other cellular proteins to form a complex that organizes the mitotic spindle (Fig. 10, option B). Based on the fact that we have reconstituted mitotic aster formation in the NuMA-depleted extract by the addition of the purified recombinant NuMA protein to levels as high as 7× the normal endogenous level of the NuMA protein (data not shown), we favor option A. However, if a functional complex containing NuMA is formed during the assembly of the mitotic spindle (option B), then it must be actively forming in our extracts, because both we and others (Kallajoki et al., 1992) have shown that NuMA is not part of a multi-protein complex in extracts prepared from nocodazole-treated mitotic cells. In any event, the most likely candidates for proteins that might cooperate with NuMA in organizing the mitotic spindle are the microtubule-dependent motor proteins kinesin (and kinesin-like proteins) and cytoplasmic dynein, and several investigators have proposed an interaction between NuMA and various microtubule-dependent motors (Sawin et al., 1992; Compton et al., 1992; Yang and Snyder, 1992). The kinesin-like protein Eg5 has been shown to be necessary for the organization of mitotic spindles assembled in vitro using extracts prepared from frog eggs (Sawin et al., 1992), and the phenotype of the spindles that formed in the absence of Eg5 led those investigators to speculate on the presence of a spindle matrix. In addition, cytoplasmic dynein has been shown to be necessary for the formation of a normal bipolar spindle in vivo (Vaisberg et al., 1993) and mitotic asters in vitro (Verde et al., 1991), and may be required for the assembly of mitotic asters assembled in our somatic cell extracts (see Fig. 4 D). Interestingly, the model for mitotic aster formation proposed by Verde et al. (1991) for the role of cytoplasmic dynein in the assembly of mitotic asters explicitly mentioned the need for an insoluble proteinaceous material to anchor cytoplasmic dynein during force production and microtubule sliding. Thus, as a component of the spindle matrix, NuMA may

be providing a solid support to counter-balance the force production on microtubules for any one of these motor proteins.

Finally, despite our efforts to reduce the problem of mitotic spindle assembly by inducing mitotic asters that lack both centrosomes and chromosomes, we have found that the assembly of mitotic asters in our cell free system is a multi-step reaction that may be subdivided into at least three functional steps. The first step is represented by the nucleation of microtubules in small bundles or aggregates. The microtubules in these aggregates continue to polymerize and begin to reorganize in the second step which is ATP dependent. The ATP in this phase of mitotic aster assembly is probably acting both energetically to power microtubule-dependent motors and chemically in protein kinase reactions that are required during the assembly process. The final phase of the assembly of these mitotic asters is dependent on the functional activity of NuMA. The microtubules are actively reoriented into astral arrays with their minus ends inward and their plus ends outward that will dissociate and disperse in the absence of NuMA. Thus, consistent with the temporal accumulation of NuMA at the polar ends of the mitotic spindle in vivo, NuMA function is required relatively late in the process of mitotic aster formation in vitro and appears to "hold" the microtubules together in an astral array. Given that the organization and dynamics of the minus ends of the microtubules in the mitotic spindle has recently been compared in complexity to the organization and dynamics of the plus ends of the microtubules at the kinetochores (Sawin and Mitchison, 1994), we feel that the cell free system developed here will allow us to identify and characterize other proteins of the mitotic spindle matrix that participate with NuMA in organizing the microtubules at the polar end of the mitotic spindle.

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References

- Blose, S. H., D. I. Melzer, and J. R. Feramisco. 1984. 10-nm filaments are induced to collapse in living cells microinjected with a monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* 98:847–858.
- Burke, B., and L. Gerace. 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell.* 44:639–652.
- Capecchi, M. R. 1980. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell.* 22:479–488.
- Cleveland, D. W. 1995. NuMA involvement in nuclear structure, spindle assembly, and nuclear reformation. *Trends Cell Biol.* 5:60–64.
- Compton, D. A., and D. W. Cleveland. 1993. NuMA is required for the proper completion of mitosis. *J. Cell Biol.* 120:947–957.
- Compton, D. A., and D. W. Cleveland. 1994. NuMA, a nuclear protein involved in mitosis and nuclear reformation. *Curr. Opin. Cell Biol.* 6:343–346.
- Compton, D. A., and C. Luo. 1995. Mutations in the predicted p34^{cdc2} phosphorylation sites in NuMA impair the assembly of the mitotic spindle and block mitosis. *J. Cell Sci.* 108:621–633.
- Compton, D. A., T. J. Yen, and D. W. Cleveland. 1991. Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* 112:1083–1097.
- Compton, D. A., I. Szilak, and D. W. Cleveland. 1992. Primary structure of

- NuMA, an intranuclear protein that defines a novel pathway for the segregation of proteins at mitosis. *J. Cell Biol.* 116:1395–1408.
- Doxsey, S. J., P. Stein, L. Evans, P. D. Calarco, and M. Kirschner. 1994. Pericentrin, a highly conserved protein involved in microtubule organization. *Cell.* 76:639–650.
- Felix, M.-A., C. Antony, M. Wright, and B. Maro. 1994. Centrosome assembly in vitro: role for γ -tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* 124:19–31.
- Gerace, L., and B. Burke. 1988. Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* 4:335–374.
- Harlowe, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. pp. 726.
- Joshi, H. C., M. J. Palacios, L. McNamara, and D. W. Cleveland. 1992. Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature (Lond.)* 356:80–83.
- Kallajoki, M., K. Weber, and M. Osborn. 1991. A 210 kD nuclear matrix protein is a function part of the mitotic spindle; a microinjection study using SPN monoclonal antibodies. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3351–3362.
- Kallajoki, M., K. Weber, and M. Osborn. 1992. Ability to organize microtubules in taxol-treated mitotic PtK2 cells goes with the SPN antigen and not with the centrosome. *J. Cell Sci.* 102:91–102.
- Kallajoki, M., J. Harborth, K. Weber, and M. Osborn. 1993. Microinjection of a monoclonal antibody against SPN antigen, now identified by peptide sequences as the NuMA protein, induces micronuclei in PtK2 cells. *J. Cell Sci.* 104:139–150.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly at the head of the bacteriophage T4. *Nature (Lond.)* 227:680–682.
- Lydersen, B. K., and D. E. Pettijohn. 1980. Human specific nuclear protein that associates with the polar region of the mitotic apparatus: distribution in a human/hamster hybrid cell. *Cell.* 22:489–499.
- Maekawa, T., and R. Kuriyama. 1991. Differential pathways of recruitment for centrosomal antigens to the mitotic poles during bipolar spindle formation. *J. Cell Sci.* 100:533–540.
- Maekawa, T., and R. Kuriyama. 1993. Primary structure and microtubule-interacting domain of the SP-H antigen: a mitotic map located at the spindle pole and characterized as a homologous protein to NuMA. *J. Cell Sci.* 105:589–600.
- Maekawa, T., R. Leslie, and R. Kuriyama. 1991. Identification of a minus-end specific microtubule-associated protein located at the mitotic spindle poles in cultured mammalian cells. *Eur. J. Cell Biol.* 54:255–267.
- McIntosh, J. R. 1980. Microtubule polarity and interaction in mitotic spindle function. In *International Cell Biology*. H. G. Schweiger, editor. Springer, Berlin. pp. 359–368.
- McKeon, F. 1991. Nuclear lamin proteins: domains required for nuclear targeting, assembly, and cell-cycle-regulated dynamics. *Curr. Opin. Cell Biol.* 3:82–86.
- Mitchison, T. J. 1989a. Mitosis: basic concepts. *Curr. Opin. Cell Biol.* 1:67–74.
- Mitchison, T. J. 1989b. Poleward microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. *J. Cell Biol.* 109:637–652.
- Newport, J. W., and D. J. Forbes. 1987. The nucleus: structure, function and dynamics. *Annu. Rev. Biochem.* 56:535–565.
- Nicklas, R. B., G. M. Lee, C. L. Rieder, and G. Rupp. 1989. Mechanically cut mitotic spindles: clean cuts and stable microtubules. *J. Cell Sci.* 94:415–423.
- Nigg, E. A. 1992. Assembly-disassembly of the nuclear lamina. *Curr. Opin. Cell Biol.* 4:105–109.
- Pickett-Heaps, J. D., D. H. Tippit, and K. R. Porter. 1982. Rethinking mitosis. *Cell.* 29:729–744.
- Price, C. M., and D. E. Pettijohn. 1986. Redistribution of the nuclear mitotic apparatus protein (NuMA) during mitosis and nuclear assembly. *Exp. Cell Res.* 166:295–311.
- Rebhun, L. I., and R. E. Palazzo. 1988. In vitro reactivation of anaphase B in isolated spindles of the sea urchin egg. *Cell Motil. Cytoskeleton.* 10:197–209.
- Rieder, C. L. 1991. Mitosis: towards an understanding of chromosome behavior. *Curr. Opin. Cell Biol.* 3:59–66.
- Sawin, K. E., and T. J. Mitchison. 1991. Poleward microtubule flux in mitotic spindles assembled in vitro. *J. Cell Biol.* 112:941–954.
- Sawin, K. E., and T. J. Mitchison. 1994. Microtubule flux in mitosis is independent of chromosomes, centrosomes, and antiparallel microtubules. *Mol. Biol. Cell.* 5:217–226.
- Sawin, K. E., K. LeGuilic, M. Philippe, and T. J. Mitchison. 1992. Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature (Lond.)* 359:540–543.
- Sluder, G., F. J. Miller, and C. L. Rieder. 1986. The reproduction of centrosomes: nuclear versus cytoplasmic controls. *J. Cell Biol.* 103:1873–1881.
- Stearns, T., and M. Kirschner. 1994. In vitro reconstitution of centrosome assembly and function: the central role of γ -tubulin. *Cell.* 76:623–637.
- Tang, T. K., C. C. Tang, Y.-L. Chen, and C.-W. Wu. 1993. Nuclear proteins of the bovine epithelium. II. The NuMA gene gives rise to multiple mRNAs and gene products reactive with monoclonal antibody W1. *J. Cell Sci.* 104:249–260.
- Toussou, A., C. Zeng, B. R. Brinkley, and M. M. Valdivia. 1991. Centrophilin, a novel mitotic protein in the mammalian cell nucleus. *J. Cell Biol.* 112:427–440.
- Vaisberg, E. A., M. P. Koonce, and J. R. McIntosh. 1993. Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *J. Cell Biol.* 123:849–858.
- Verde, F., J.-M. Berrez, C. Antony, and E. Karsenti. 1991. Taxol-induced microtubule asters in mitotic extracts of *Xenopus* eggs: requirement for phosphorylated factors and cytoplasmic dynein. *J. Cell Biol.* 112:1177–1187.
- Wiese, C., and K. L. Wilson. 1993. Nuclear membrane dynamics. *Curr. Opin. Cell Biol.* 5:387–394.
- Yang, C. H., and M. Snyder. 1992. The nuclear-mitotic apparatus protein (NuMA) is important in the establishment and maintenance of the bipolar mitotic spindle apparatus. *Mol. Biol. Cell.* 3:1259–1267.
- Yang, C. H., E. J. Lambie, and M. Snyder. 1992. NuMA: an unusually long coiled-coil related protein in the mammalian cell nucleus. *J. Cell Biol.* 116:1303–1317.
- Zeng, C., D. He, and B. R. Brinkley. 1994. Localization of NuMA protein isoforms in the nuclear matrix of mammalian cells. *Cell Motil. Cytoskeleton.* 29:167–176.
- Zhang, D., and R. B. Nicklas. 1995a. The impact of chromosomes and centrosomes on spindle assembly as observed in living cells. *J. Cell Biol.* 129:1287–1300.
- Zhang, D., and R. B. Nicklas. 1995b. Chromosomes initiate spindle assembly upon experimental dissolution of the nuclear envelope in Grasshopper spermatocytes. *J. Cell Biol.* In press.