NuMA Is Required for the Proper Completion of Mitosis

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Abstract. NuMA is a 236-kD intranuclear protein that during mitosis is distributed into each daughter cell by association with the pericentrosomal domain of the spindle apparatus. The NuMA polypeptide consists of globular head and tail domains separated by a discontinuous 1500 amino acid coiled-coil spacer. Expression of human NuMA lacking its globular head domain results in cells that fail to undergo cytokinesis and assemble multiple small nuclei (micronuclei) in the subsequent interphase despite the appropriate localization of the truncated NuMA to both the nucleus and spindle poles. This dominant phenotype is morphologically identical to that of the tsBN2 cell line that carries a temperature-sensitive mutation in the chromatin-binding protein RCC1. At the restrictive temperature, these cells end mitosis without completing cytokinesis followed by micronucleation in the subsequent interphase. We demonstrate that the wild-type NuMA is degraded in the latest mitotic stages in these mutant cells and that NuMA is excluded from the micronuclei that assemble post-mitotically. Elevation of NuMA levels in these mutant cells by forcing the expression of wild-type NuMA is sufficient to restore post-mitotic assembly of a single normal-sized nucleus. Expression of human NuMA lacking its globular tail domain results in NuMA that fails both to target to interphase nuclei and to bind to the mitotic spindle. In the presence of this mutant, cells transit through mitosis normally, but assemble micronuclei in each daughter cell. The sum of these findings demonstrate that NuMA function is required during mitosis for the terminal phases of chromosome separation and/or nuclear reassembly.

In higher eucaryotes, the nucleus is completely disassembled at prometaphase and is reassembled in each daughter cell at the end of telophase. This requires that the major nuclear structures (nuclear envelope, lamins, matrix) undergo a mitosis-specific disassembly (or assembly in the case of the chromosomal superstructure), distribution to each daughter cell, and finally reassembly of interphase organization. To date, there are three distinct pathways described for the mitotic segregation of nuclear proteins. The chromosomes (and their associated proteins) condense during mitosis, are captured by microtubules of the mitotic spindle and are deposited to each spindle pole just before cytokinesis (reviewed in Mitchison, 1989). On the other hand, after hyperphosphorylation by cdc2 kinase, the nuclear lamins depolymerize, distribute by passive diffusion, and polymerize around the telophase chromatin mass after dephosphorylation (Fisher, 1987; Gerace et al., 1978; Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985; Glass and Gerace, 1990; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990). The third pathway has been defined for the intranuclear protein NuMA (*Nu*clear protein that associates with the *M*itotic *A*pparatus; Lydersen and Pettijohn, 1980). This abundant nuclear constituent is distributed to each daughter cell through association with the pericentrosomal domain of the spindle microtubules (Lydersen and Pettijohn, 1980; Price and Pettijohn, 1986), followed by nuclear pore-dependent import into the developing daughter nuclei (Compton et al., 1992).

The discovery of this third segregation pathway coupled with the identification of NuMA as a nuclear matrix component have fueled divergent speculation as to NuMA's function within nuclei and at mitosis. A role in mitotic spindle architecture was first proposed based on the segregation of NuMA to the spindle poles and the association of NuMA with asterlike spindle structures induced in cells by treatment with the microtubule assembly-inducing agent taxol (Kallajoki et al., 1992; Maekawa et al., 1991; Tousson et al., 1991). More recently, direct evidence for NuMA contribution to spindle assembly arose from the generation of aberrant spindle structures in cells microinjected with a monoclonal antibody to NuMA (the antigen was called SPN in the initial report [Kallajoki et al., 1991], but subsequently has been shown to be NuMA [M. Osborn, personal communication]). Even more compelling evidence is the collapse of the metaphase spindle in cells microinjected with a rabbit anti-NuMA polyclonal antibody (Yang and Snyder, 1992).

On the other hand, in view of NuMA's abundance (2×10^5 copies/nucleus; Compton et al., 1992), cell cycle dependent localization, and micronucleation in cells after injection of one monoclonal antibody against NuMA (Kallajoki et al., 1991), we (Compton et al., 1992) and others

(Yang et al., 1992; Price and Pettijohn, 1986) have proposed an integral role for NuMA in establishment or maintenance of nuclear structure. This possibility was also attractive by identification of NuMA as a component of the nuclear matrix (Kallajoki et al., 1991), an insoluble proteinaceous scaffold consisting of both the nuclear lamina proteins as well as a collection of other proteins (Lebkowski and Laemmli, 1982), only one of which (topoisomerase II) has been well characterized (Earnshaw et al., 1985). The matrix has been associated with a variety of nuclear processes including transcription (Ciejek et al., 1983; Xing and Lawrence, 1990), splicing (Zeitlin et al., 1987), and replication (Berezney and Coffey, 1977; Pardoll et al., 1980). Moreover, under some conditions the matrix has been observed to contain 8-10 nm filaments (He et al., 1990; Jackson and Cook, 1988) and in light of its long coiled-coil domain it is possible that NuMA is one component of such structures.

To examine directly NuMA's role either in mitosis or in nuclear organization, we have now expressed various segments of the protein in tissue culture cells. Expression of NuMA lacking either its amino-terminal head or carboxyl-terminal tail domains causes defects in mitosis resulting in the assembly of multiple small nuclei (micronuclei). Moreover, in a temperature-sensitive hamster cell line (tsBN2) that spontaneously generates post-mitotic micronuclei after degradation of its endogenous NuMA, forcing the expression of wild-type human NuMA results in restoration of assembly of a single, normal sized nucleus. These data demonstrate that NuMA function is essential for mitotic spindle function, post-mitotic nuclear assembly, or both.

Materials and Methods

Cell Culture

The hamster cell lines BHK-21 and tsBN2 were maintained in DME containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 μg/ml streptomycin. Cells were grown at 37°C (BHK-21) or 33°C (tsBN2) in a humidified incubator in a 5% CO_2 atmosphere. G1/S synchronization of 60–80% was achieved by double hydroxyurea (2 mM) block, and verified by FACS® analysis.

Transfection and Microinjection

Cells were transiently transfected using the calcium-phosphate precipitation protocol described by Graham and van der Eb (1973). Briefly, 5 μg of plasmid DNA was mixed with 15 μg of high molecular weight genomic DNA carrier, and brought to a final concentration of 40 μg/ml in Hepes-buffered saline containing 125 mM calcium chloride. This solution containing the microprecipitate was added to 5 ml of media per 100 mm dish of 50% confluent cells for 6–8 h at 37°C. The cells were then washed with PBS, and grown with fresh media for 12–16 h.

Cells growing on photoetched alpha-numeric glass coverslips (Bellco Glass Co., Vineland, NJ) were microinjected following the procedures of Cleveland et al. (1983) and Capecchi (1980). Interphase cells were microinjected in the nucleus with plasmid DNA at a concentration of 100 μg/ml in 100 mM KCl, 10 mM KPO_4, pH 7.4. Injected cells were found to be expressing protein from the injected plasmid (assayed by immunofluorescence) in as little as 1 h post-injection. The injected cells were followed by phase contrast microscopy until they reached the desired stage of the cell cycle, at which time they were processed for immunofluorescence.

Immunological Techniques

Intracellular localization of NuMA in BHK-21 and tsBN2 cells was determined as described elsewhere (Compton et al., 1991). Cells growing on glass coverslips were fixed by immersion in PBS containing 3.5% paraformaldehyde for 5 min at room temperature. The fixed cells were then extracted with 0.5% Triton X-100 in TBS (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% bovine albumin) for 5 min at room temperature. The cells were then rinsed and maintained through all subsequent steps in TBS at room temperature. Primary antibodies, including the anti-β-galactosidase antibody (Promega Corp., Madison, WI), were added to the appropriate cells, and incubated for 30 min at room temperature in a humidified chamber. Coverslips were washed in TBS and the bound antibodies were detected with fluorescein-conjugated or Biotin-conjugated secondary antibodies in conjunction with Texas-red conjugated streptavidin (Vector Labs, Inc., Burlingame, CA). 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.

Proteins were analyzed from transiently transfected cells by immunoblot analysis following SDS-PAGE (Laemmli, 1970). Cells were washed three times in ice cold PBS and harvested directly in SDS-PAGE sample buffer. Proteins were separated by size by SDS-PAGE and transferred onto nitrocellulose. This nitrocellulose blot was preincubated for 30 min at room temperature before incubation with primary antibody in TTBS (0.25% Tween 20, 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM NaN_3) containing 5% albumin for 4–12 h. Unbound antibody was removed by washing in TTBS five times for 3 min. Bound antibody was then detected with ^{125}I-labeled goat anti-mouse antibody (Amersham Corp., Arlington Heights, IL).

Construction of NuMA Expression Plasmids

The full-length human NuMA cDNA was assembled from three overlapping cDNA fragments (Fig. 1 *A*) following methods previously described (Maniatis et al., 1982; Ausubel et al., 1989; Sambrook et al., 1989). The 5' 3137 nucleotides were excised as an *EcoRI/HgaI* fragment from cDNA clone 1Fl-2, the central 1567 nucleotides were obtained as a *HgaI/SpII* fragment from cDNA 1Fl, and the 3' 2513 nucleotides were derived from an *SpII/EcoRI* fragment isolated from cDNA clone 1Fl-4. The resulting 7217 bp cDNA (bounded at both ends by *EcoRI* sites) contains the 5' and 3' untranslated sequences along with the entire coding sequence of the human NuMA polypeptide. This *EcoRI* fragment was inserted into a unique *EcoRI* site in a pUC-derived plasmid (pGW1CMV) containing the immediate early gene promoter from the human cytomegalovirus (CMV),[1] followed by the SV40 polyadenylation sequence. The product, CMV/NuMA1-2101, encodes the full-length NuMA under the transcriptional control of the CMV promoter (Fig. 1 *B*). Carboxyl-terminal truncated NuMA (CMV/NuMA1-1545; Fig. 1 *B*) was constructed by joining the 5' 3137 nucleotide *EcoRI/HgaI* fragment from cDNA 1Fl-2 to the central 1752 nucleotide *HgaI/EcoRI* fragment from cDNA 1Fl and inserting both into the *EcoRI* site of pGW1CMV. The amino-terminal deleted NuMA (CMV/NuMAΔ19-208; Fig. 1 *B*) was constructed from CMV/NuMA1-2101 by internally deleting the 567 nucleotides between the *PmlI* and *EcoRV* sites such that the original reading frame is maintained. The β-galactosidase/NuMA fusion gene (CMV/β-gal/NuMA; Fig. 1 *B*) was also constructed in pGW1CMV. A 2785 nucleotide *HindIII/AccI* fragment of the β-galactosidase gene containing an ATG codon modified for efficient eucaryotic translation (from plasmid pZA; kindly provided by R. Kothary, Institute of Animal Physiology, Cambridge, UK) was blunted at the *AccI* site with mung bean nuclease and ligated in frame to the 3'-terminal 1960 nucleotide segment of NuMA carried on an *FspI/EcoRI* fragment from cDNA 1Fl-4. This fragment was inserted into pGW1CMV between the *HindIII* and *EcoRI* sites. All of these plasmids were propagated in *Escherichia coli* strain DH5α.

Results

Amino-terminal Truncated NuMA Inhibits Cytokinesis and Induces Micronucleation

To assay the functional properties of wild-type NuMA or NuMA proteins lacking either the head or tail domains, we constructed three plasmids carrying the entire coding sequence or various portions of human NuMA under the tran-

1. *Abbreviation used in this paper*: CMV, cytomegalovirus.

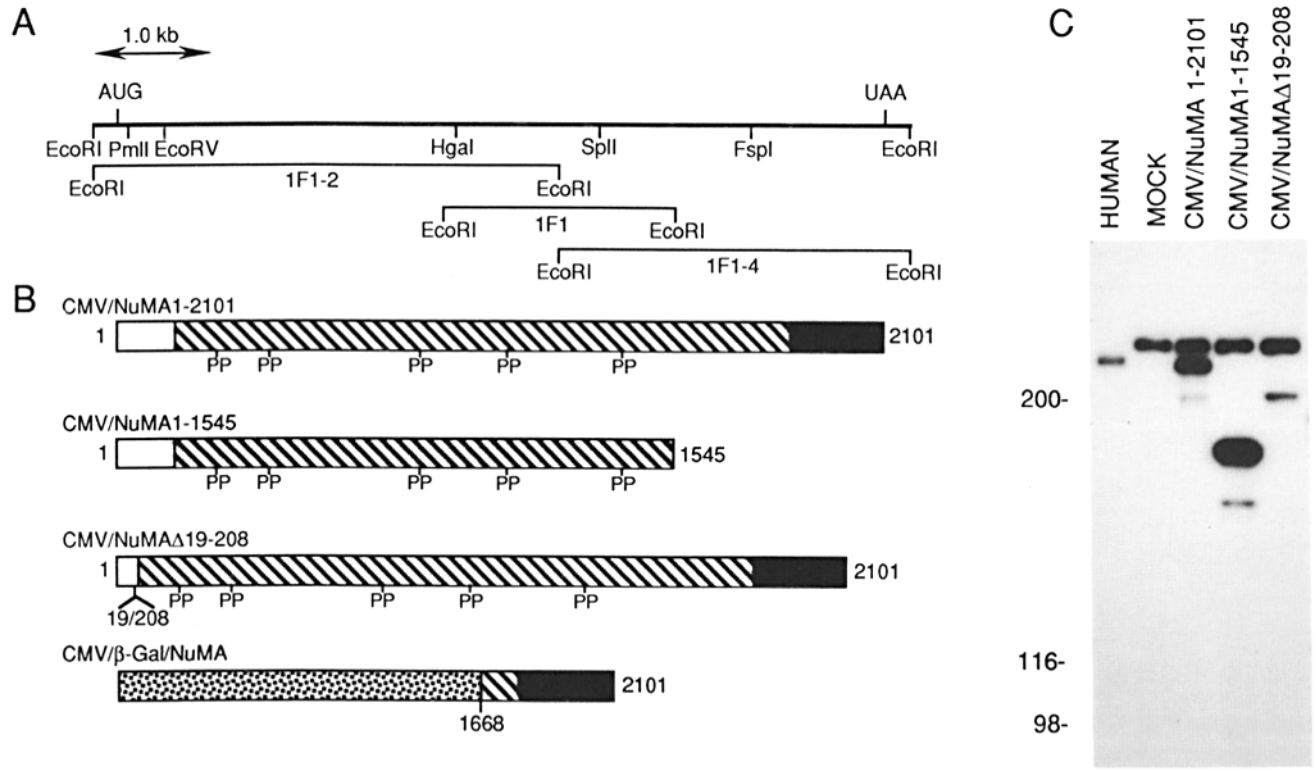


Figure 1. Expression of wild-type and mutant human NuMA in hamster cells. (*A* and *B*) Schematic drawings of hybrid genes encoding wild-type or mutant NuMA proteins. □, NuMA head domain; ▨, NuMA coiled-coil rod domain; ■, NuMA tail domain; ▩, β-galactosidase coding sequences; *PP*, helix disrupting proline residues. (*C*) Immunoblot detection of full-length and truncated human NuMA after transient transfection of hamster BHK-21 cells. An anti-NuMA autoantiserum was used to detect the endogenous hamster and/or the exogenous human NuMA polypeptides in 50 μg of cell extract after electrophoresis on a 5% SDS-polyacrylamide gel. (Lane *1*) Human K562 erythroleukemic cells; (lane *2*) mock transfected BHK-21 cells; (lane *3*) BHK-21 cells expressing full length human NuMA; (lane *4*) tailless human NuMA; or (lane *5*) headless human NuMA. The migration positions of myosin (200 kD), β-galactosidase (116 kD), and phosphorylase b (98 kD) are indicated at left.

scriptional control of the CMV promoter (Fig. 1, *A* and *B*). Each plasmid was introduced by transient transfection into BHK-21 cells and expression followed by immunoblot analysis with a human autoantiserum that recognizes both the endogenous hamster NuMA and the transfected products (Price et al., 1984). As expected, expression of the wild-type human NuMA (CMV/NuMA1-2101) yielded a protein that migrated indistinguishably from the endogenous NuMA in a human K562 cell extract (Fig. 1 *C*, lanes *1* and *3*), while the wild-type hamster protein was easily distinguished by its slower mobility (Fig. 1 *C*, lane *2*). Transfection with plasmids expressing headless (CMV/NuMAΔ19-208) and tailless (CMV/NuMA1-1545) NuMA produced protein products whose molecular weights were consistent with the expected deletions (Fig. 1 *C*, lanes *4* and *5*). For localization of the human protein in the hamster cells, each recombinant plasmid was microinjected into cells. The resulting human protein was localized before, during, and after the completion of mitosis (Fig. 2) by indirect immunofluorescence using the human-specific anti-NuMA monoclonal antibody 1F1 (Compton et al., 1991). For cells in interphase, the wild-type human NuMA accumulated exclusively in the nucleus of the injected cell, although like the endogenous NuMA, it was excluded from nucleoli (Fig. 2 *A*, *pre-mitotic*). In mitotic cells, the human NuMA concentrated at the pericentrosomal region of the spindle apparatus (Fig. 2 *A*, *mitotic*). Ultimately, in post-mitotic cells, the human NuMA protein was found exclusively in the nuclei of the two daughter cells (Fig. 2 *A*, *post-mitotic*). This cell cycle-dependent distribution of human NuMA exactly parallels that in human cells (Price and Pettijohn, 1986; Compton et al., 1992) as well as the endogenous NuMA of these hamster cells (data not shown).

Localization of the headless human NuMA protein (CMV/NuMAΔ19-208) mimicked the localization of the wild-type human NuMA. Headless NuMA accumulated in the interphase nucleus before and after mitosis (Fig. 2 *B*, *pre-mitotic* and *post-mitotic*) and associated with the pericentrosomal region of the mitotic apparatus (Fig. 2 *B*, *mitotic*). In 12 cells expressing the headless human NuMA protein, however, all 12 displayed a striking and unexpected phenotype: cells failed to complete mitosis normally and assembled a collection of 5–15 small nuclei (micronuclei) of heterogeneous size in the subsequent interphase (Fig. 2 *B*, *post-mitotic*).

The terminal phenotype obtained by expression of the headless human NuMA is similar to the terminal phenotype observed in some cell types that escape mitosis without chromosome segregation (e.g., after inhibition of microtubule as-

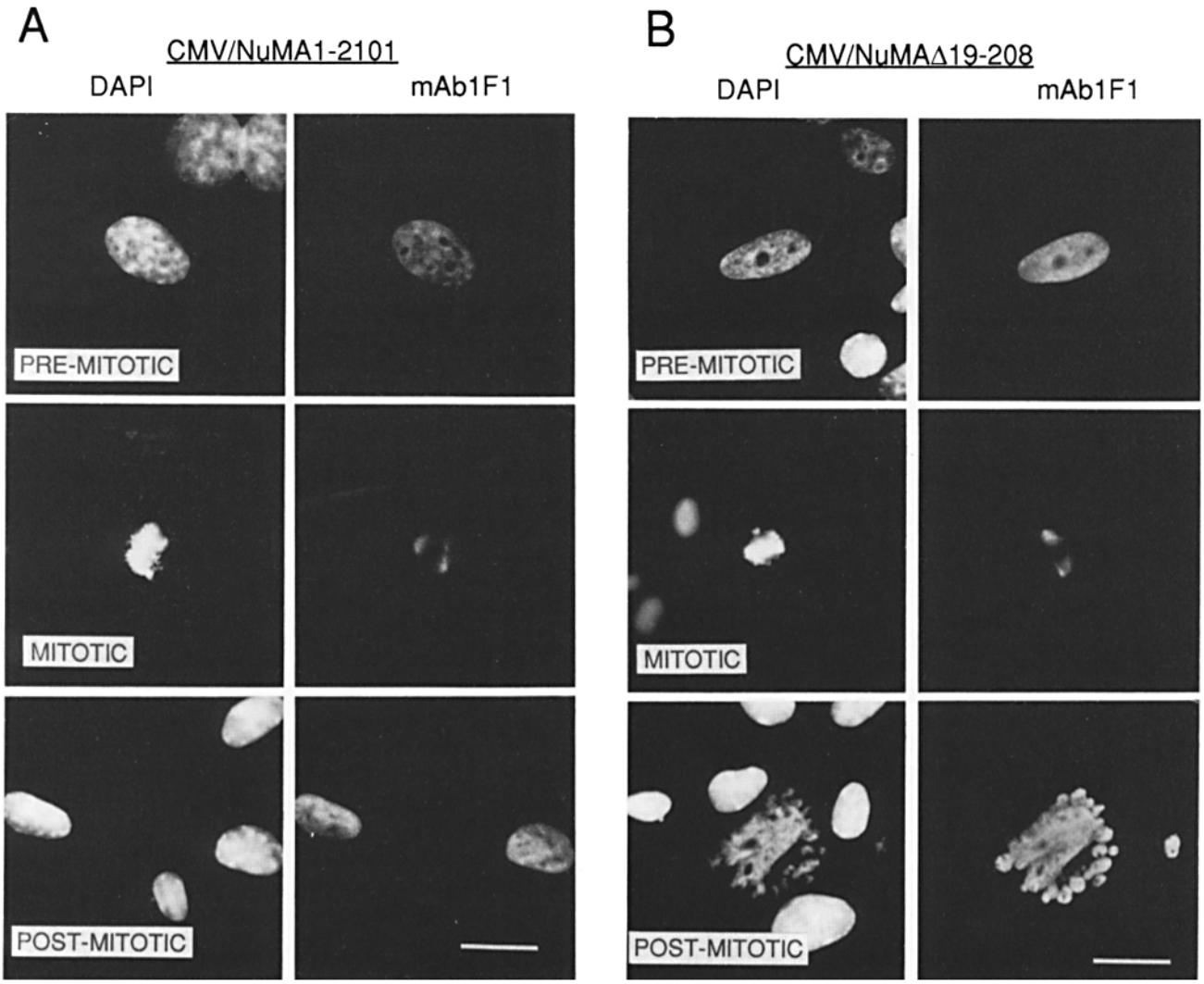


Figure 2. Cellular localization of wild-type and amino-terminally truncated human NuMA expressed in BHK-21 cells. Hamster BHK-21 cells were microinjected with plasmids driving the expression of either (*A*) wild-type (*CMV/NuMA1-2101*) or (*B*) amino-terminal (*CMV/NuMAΔ19-208*) truncated human NuMA protein. Cells were fixed in interphase before mitosis (*pre-mitotic*), during metaphase (*mitotic*), and in interphase after mitosis (*post-mitotic*) and processed for immunofluorescence with a DNA-specific dye (*DAPI*) and a human-specific anti-NuMA monoclonal antibody (*mAb1F1*). Bar, 20 μm.

sembly with microtubule destabilizing drugs). To determine whether the phenotype obtained by expression of headless human NuMA derives from a disruption of microtubules and/or failure of chromosome congression, we examined the spindle organization and chromosome position in mitotic cells expressing the headless NuMA subunit (Fig. 3). Unlike the spindle disruption seen in cells treated with microtubule destabilizing drugs, expression of the headless human NuMA protein does not inhibit the assembly of the mitotic spindle or congression of the chromosomes to the metaphase plate (Fig. 3 *B*).

NuMA Is Degraded in a Mutant Cell Line That Spontaneously Forms Post-mitotic Micronuclei

The phenotype generated by expression of amino-terminal truncated NuMA is remarkably similar to the mitotic phenotype in the temperature sensitive hamster cell line tsBN2 (Nishimoto et al., 1978). After the shift to the restrictive temperature (40°C), tsBN2 cells in G1 do not progress further in the cell cycle. However, cells at the G1/S boundary or in S phase at the time of the temperature shift initiate mitosis precociously without completing DNA synthesis. After premature mitotic entry, the entire program of mitotic events (including phosphorylation cascades, nuclear envelope breakdown, chromosome condensation, and mitotic spindle assembly) are activated, but the cells fail to segregate their chromosomes, and ultimately complete the pseudo-mitosis without undergoing cytokinesis (Nishitani et al., 1991). In the subsequent interphase, instead of assembling a single nucleus, a set of 5–15 micronuclei is formed. A missense mutation in the RCC1 gene that encodes a highly conserved chromatin-binding protein has been demonstrated to be responsible for the temperature sensitive phenotype (Kai et al., 1986; Uchida et al., 1990).

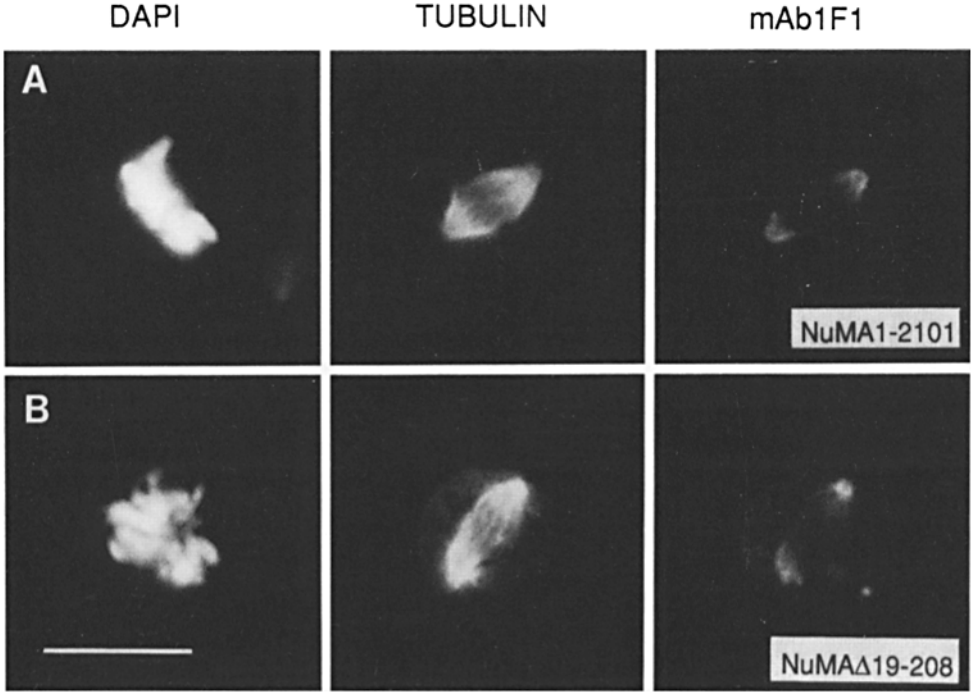
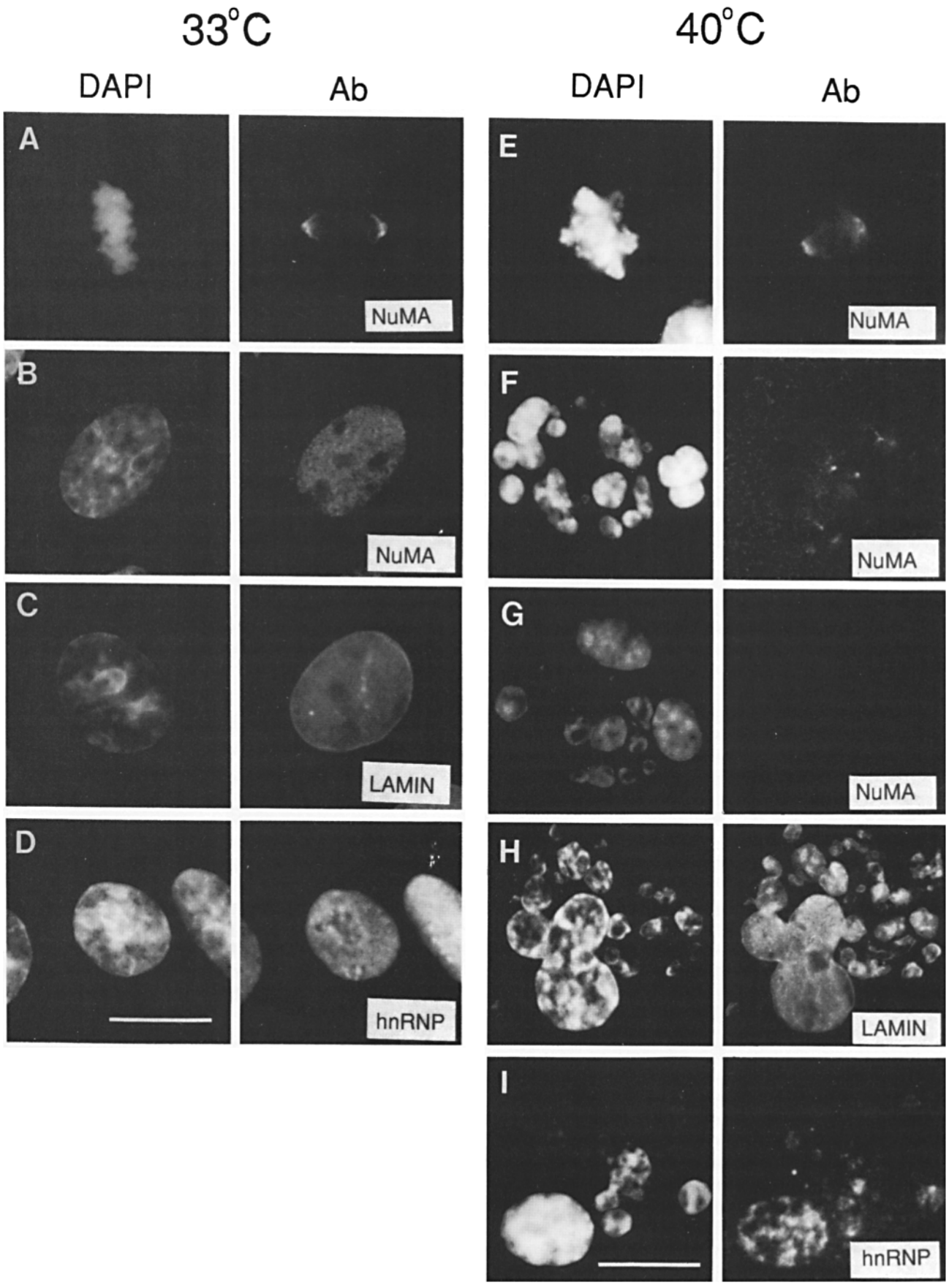


Figure 3. Localization of wild-type and amino-terminal truncated human NuMA relative to the mitotic spindle in metaphase cells. Hamster BHK-21 cells were microinjected with plasmids driving the expression of either (*A*) wild-type (*CMV/NuMA1-2101*) or (*B*) amino-terminal truncated (*CMV/NuMAΔ19-208*) human NuMA. Cells were fixed in metaphase and processed for immunofluorescence with a DNA-specific dye (*DAPI*), rabbit anti-tubulin antibody (*tubulin*), and a human-specific anti-NuMA monoclonal antibody (*mAb1F1*). Bar, 10 μm.

The similarities in the mitotic defects found in cells expressing the headless human NuMA and in the tsBN2 cell line prompted us to examine the fate of the endogenous hamster NuMA in tsBN2 cells after temperature-induced premature entry into mitosis. As expected, at the permissive temperature (33°C) these cells grow normally and the endogenous hamster NuMA localizes within the interphase nucleus (Fig. 4 *B*) along with the nuclear lamins (Fig. 4 *C*) and the C protein of the hnRNP complex (Fig. 4 *D*). At mitosis, the hamster NuMA associates with the spindle poles (Fig. 4 *A*). When cultures enriched in G1 cells (after release from nocodazole) were shifted to the restrictive temperature (40°C), the cells arrested, as reported previously, but showed no change in NuMA distribution (data not shown). Cells synchronized at the G1/S boundary (by treatment with hydroxyurea) prematurely entered mitosis following shift to 40°C and, as expected, the endogenous NuMA protein associated with the pericentrosomal region of the spindle apparatus (Fig. 4 *E*). However, at the completion of the precocious mitosis, as the cells re-entered interphase and developed micronuclei, most NuMA was not imported into the developing nuclei, but remained dispersed throughout the cell cytoplasm (Fig. 4 *F*). This failure of NuMA to be imported properly into the daughter nuclei occurred despite successful nuclear targeting and import of other nuclear proteins (such as the lamins [Fig. 4 *H*] and the hnRNP complex C protein [Fig. 4 *I*]) into each micronucleus. Further incubation of the cells at the restrictive temperature resulted in micronucleated cells that by 6–8 h retained no detectable NuMA staining (Fig. 4 *G*). Immunoblot analysis revealed that after temperature shift, hamster NuMA was progressively lost, with appearance of presumptive proteolytic products. The loss of NuMA after mitosis at the restrictive temperature does not appear to be an intrinsic feature of NuMA, because incubation of the parental cell line (BHK-21) at 40°C has no affect on NuMA distribution or integrity (data not shown). This suggests that NuMA might interact with the RCC1 protein or an RCC1-dependent protein, and in the absence of such an interaction NuMA import and/or stability is affected.

Expression of Wild-Type Human NuMA Suppresses Post-mitotic Micronucleation in tsBN2 Cells

Because expression of headless NuMA in normal cells causes a mitotic defect followed by micronucleation and the endogenous hamster NuMA is not imported into developing nuclei and is then degraded as the tsBN2 cells develop micronuclei after mitosis, these findings suggested that at least a portion of the mitotic defect and/or micronucleation phenotype in the tsBN2 cell line could be the result of loss of wild-type NuMA. To test this directly, we microinjected the plasmid encoding the wild-type human NuMA protein (CMV/NuMA1-2101) into semi-synchronous cultures of tsBN2 cells grown at 33°C. This human NuMA accumulated efficiently and localized correctly within the nucleus of the injected cells grown at the permissive temperature (Fig. 5 *A*). Cultures were then synchronized at the G1/S boundary with hydroxyurea, microinjected, shifted to the restrictive temperature, and the fate of each injected cell was followed over the next 4–6 h. In 30 uninjected control cells that were followed as they emerged from premature mitosis, 24 developed post-mitotic micronuclei. In contrast, out of 11 microinjected cells that prematurely entered mitosis (as judged by the rounded morphology and assembly of a metaphase plate observed by phase contrast microscopy), 10 stained positively for the human NuMA and all 10 developed a single nucleus instead of a collection of micronuclei (Fig. 5, *B–D*). The nuclei assembled under these conditions were near normal in size, but were irregularly shaped and the


33°C
40°C
DAPI
Ab
DAPI
Ab
A
NuMA
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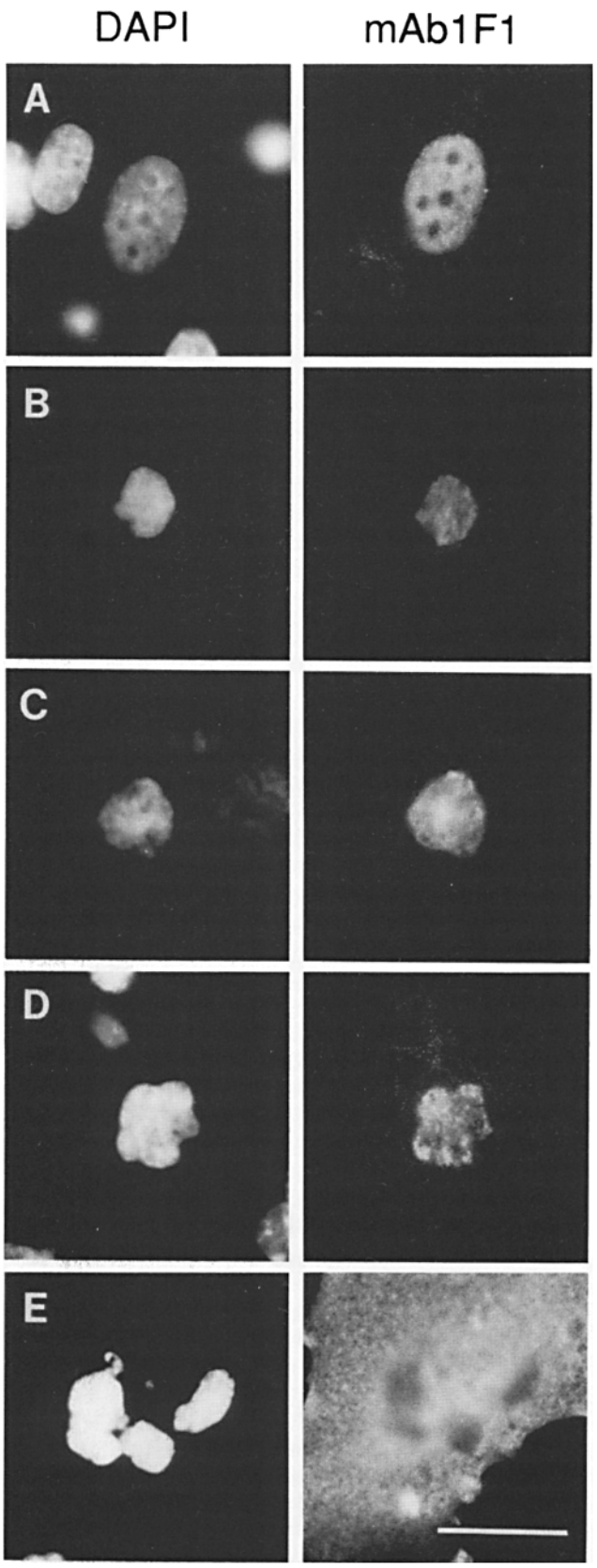

DNA failed to decondense completely. (That the nuclei are not fully wild type is hardly surprising because at least one nuclear protein [RCC1] is inactivated at the restrictive temperature and the cell cycle is arrested following the aberrant mitosis.) Suppression of the micronucleation phenotype is dependent on the accumulation of the wild-type human NuMA because all cells expressing headless (CMV/NuMAΔ19-208; five of which were carefully followed across the mitotic cycle [data not shown]) or tailless (CMV/NuMA1-1545; Fig. 5 *E* [four were carefully followed through mitosis]) human NuMA continued to produce multiple nuclei after the abortive mitosis. Thus, wild-type human NuMA is sufficient to suppress the temperature-dependent micronucleation phenotype in the tsBN2 cells without affecting the RCC1-dependent phenotype of premature entry into mitosis. We cannot distinguish whether suppression of micronucleation derives from an excess of NuMA overcoming inefficient nuclear import or saturation of the degradation pathway.

Carboxyl-terminal Truncated NuMA Induces Micronucleation in the Absence of Additional Mitotic Defects

Unlike the wild-type and headless NuMA, tailless human NuMA protein (CMV/NuMA1-1545) did not accumulate in interphase nuclei before or after mitosis (Fig. 6, *pre-mitotic* and *post-mitotic*). This was true even in cells expressing the highest level in which (like the cell shown) the cytoplasmic NuMA assembled into unusual sheetlike aggregates. At mitosis, this tailless human NuMA did not interact specifically with the mitotic spindle apparatus, but remained diffusely distributed throughout the cytoplasm (Fig. 6, *mitotic*).

A dominant phenotype was consistently observed during the terminal phases of mitosis or the earliest stages of interphase in cells expressing the tailless NuMA: in 18 out of 18 cells, despite the apparently normal chromosome segregation through telophase and a seemingly normal cytokinesis, post-mitotic nuclear reformation was disrupted leaving each daughter cell with a set of 5–10 heterogeneously sized micronuclei (Fig. 6, *post-mitotic*). Unlike micronucleation induced by expression of the headless human NuMA or temperature shift-induced mitosis in the tsBN2 cell line (both of which also block cytokinesis), micronucleation in the presence of the tailless human NuMA occurred without affecting any other observable aspect of mitosis. Analysis of spindle

Figure 5. Suppression of micronucleation in tsBN2 cells by expression of wild-type human NuMA. Semi-synchronous tsBN2 cells growing at 33°C were microinjected with plasmids driving the expression of either wild-type human NuMA or tailless human NuMA. (*A*) Cells expressing wild-type human NuMA and maintained at 33°C; (*B–D*) cells expressing wild-type human NuMA or (*E*) tailless human NuMA following completion of mitosis induced by incubation at 40°C. Cultures were fixed, and processed for immunofluorescence with a DNA-specific dye (*DAPI*) and a human-specific anti-NuMA monoclonal antibody (*mAb1F1*). Bar, 20 μm.

Figure 4. Post-mitotic loss of endogenous NuMA in tsBN2 cells following mitosis at the restrictive temperature. (*Right portion of each panel*) (*A, B, E–G*) The endogenous hamster NuMA, (*C, H*) the nuclear lamins, and (*D, I*) the hnRNP complex C protein were localized by indirect immunofluorescence in tsBN2 cells grown (*A–D*) at 33°C or for (*E, F, H, I*) 4 h or (*G*) 6 h at 40°C. (*Left portion of each panel*) DNA staining (using DAPI) in the same cells as in the right panels. Bar, 20 μm.

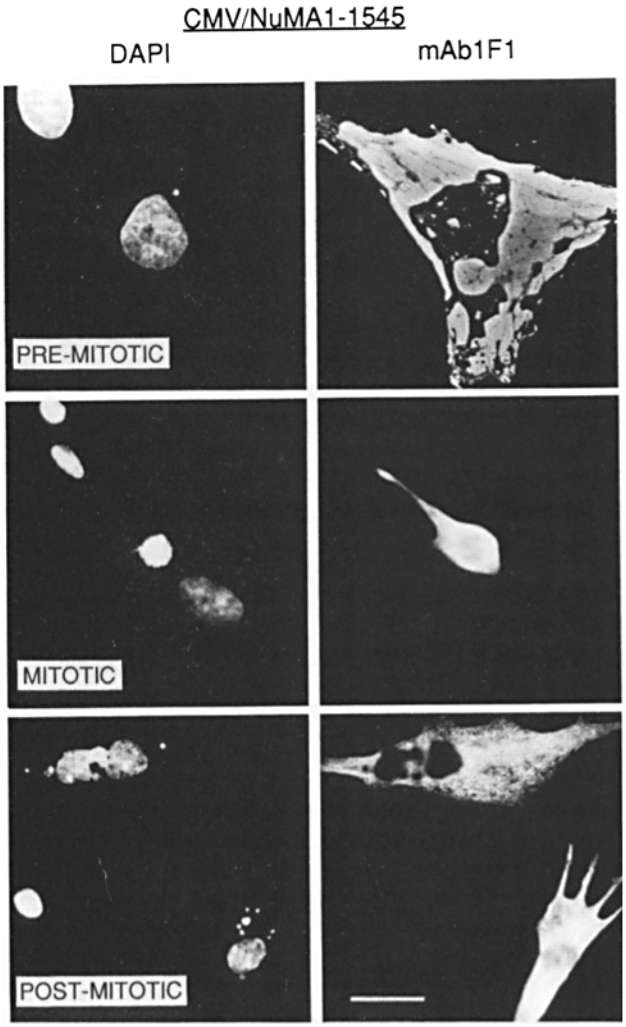

Figure 6. Cellular localization of carboxyl-terminal truncated human NuMA expressed in BHK-21 cells. Hamster BHK-21 cells were microinjected with a plasmid (*CMV/NuMA1-1545*) driving the expression of the carboxyl-terminal truncated human NuMA protein. Cells were fixed in interphase before mitosis (*pre-mitotic*), at metaphase (*mitotic*), and in interphase after mitosis (*post-mitotic*) and processed for immunofluorescence with a DNA-specific dye (*DAPI*) and a human-specific anti-NuMA monoclonal antibody (*mAb1F1*). Bar, 20 μm.

organization and chromosome position of mitotic cells expressing tailless human NuMA confirmed the absence of a detectable affect on metaphase spindle assembly, chromosome congression, anaphase chromosome movement, or chromosome positioning at telophase (Fig. 7), suggesting that defective assembly (or maintenance) of a single nucleus must derive from loss of NuMA function either at the latest stage of mitosis or earliest stage of the subsequent interphase.

The NuMA Tail Contains the Domains Necessary for Nuclear Localization and Spindle Association

That headless NuMA targets correctly to nuclei and mitotic spindle poles, whereas tailless NuMA does neither, suggested that the tail is sufficient for both of these targeting functions. To test this directly, we constructed a plasmid that encodes 100 kD of β-galactosidase linked to the 50-kD carboxyl-terminal tail of NuMA (Fig. 1 *B*). Plasmids containing this fusion or wild-type β-galactosidase (under the transcriptional control of the CMV promoter) were introduced into BHK-21 cells by nuclear microinjection and the resulting proteins localized by indirect immunofluorescence using an anti-β-galactosidase monoclonal antibody (Fig. 8). While the bulk of the β-galactosidase localized diffusely in the interphase cell cytoplasm (Fig. 8 *A*) in all 22 cells analyzed, the β-gal/NuMA fusion accumulated exclusively in the nucleus in each of the 14 cells examined (Fig. 8 *C*). In mitotic cells, neither β-galactosidase alone (eight cells were examined) nor the β-gal/NuMA fusion protein (10 cells were examined) associated with the mitotic spindle apparatus (Fig. 8, *B* and *D*) suggesting that, despite the absence of a conventional nuclear localization sequence (Kalderon et al., 1984; Lanford and Butel, 1984), the carboxy-terminal globular domain of NuMA is necessary and sufficient for nuclear targeting, and necessary but not sufficient for associating with the mitotic spindle apparatus.

Discussion

We show here that expression of mutant NuMA deleted of either the amino-terminal head or carboxyl-terminal tail domains results in dominant defects in mitosis resulting in micronucleated cells. Moreover, we demonstrate that the endogenous NuMA protein is degraded in a temperature sensitive cell line that spontaneously generates micronuclei after mitosis induced at the restrictive temperature. Expression of wild-type human NuMA in these mutant cells is sufficient to complement this micronucleation phenotype, leading us to conclude that NuMA is essential during mitosis for the reassembly of daughter cell nuclei. Whether NuMA is required for nuclear assembly per se, or whether it acts indirectly through the mitotic spindle to stabilize nuclear reassembly against fragmentation is not yet established (see below), although it is clear that general nuclear assembly processes (e.g., chromatin decondensation, lamin deposition, and import of the C protein of the hnRNP complex) are not disrupted in cells expressing truncated NuMAs that efficiently lead to micronucleation.

The dominant, post-mitotic effect of mutant NuMA is most likely achieved either through mutant subunit competition for binding to other components with which NuMA normally interacts or through oligomerization of the truncated subunits with the endogenous wild-type NuMA, thereby poisoning the wild-type function. This latter view is particularly attractive in view of the presence in NuMA of a long α-helical coiled-coil domain, a motif frequently used for oligomerization. Precedent for dominant disruption of α-helical coiled-coil oligomerization comes from the intermediate filament family of proteins (e.g., keratins [Albers and Fuchs, 1987], neurofilaments [Wong and Cleveland, 1990], and lamins [Loewinger and McKeon, 1988], et cetera) where expression of truncated proteins collapses the entire endogenous filamentous array.

Role of NuMA during Mitosis

How might NuMA normally act to ensure reassembly of a single nucleus? One possibility is that NuMA is acting as an

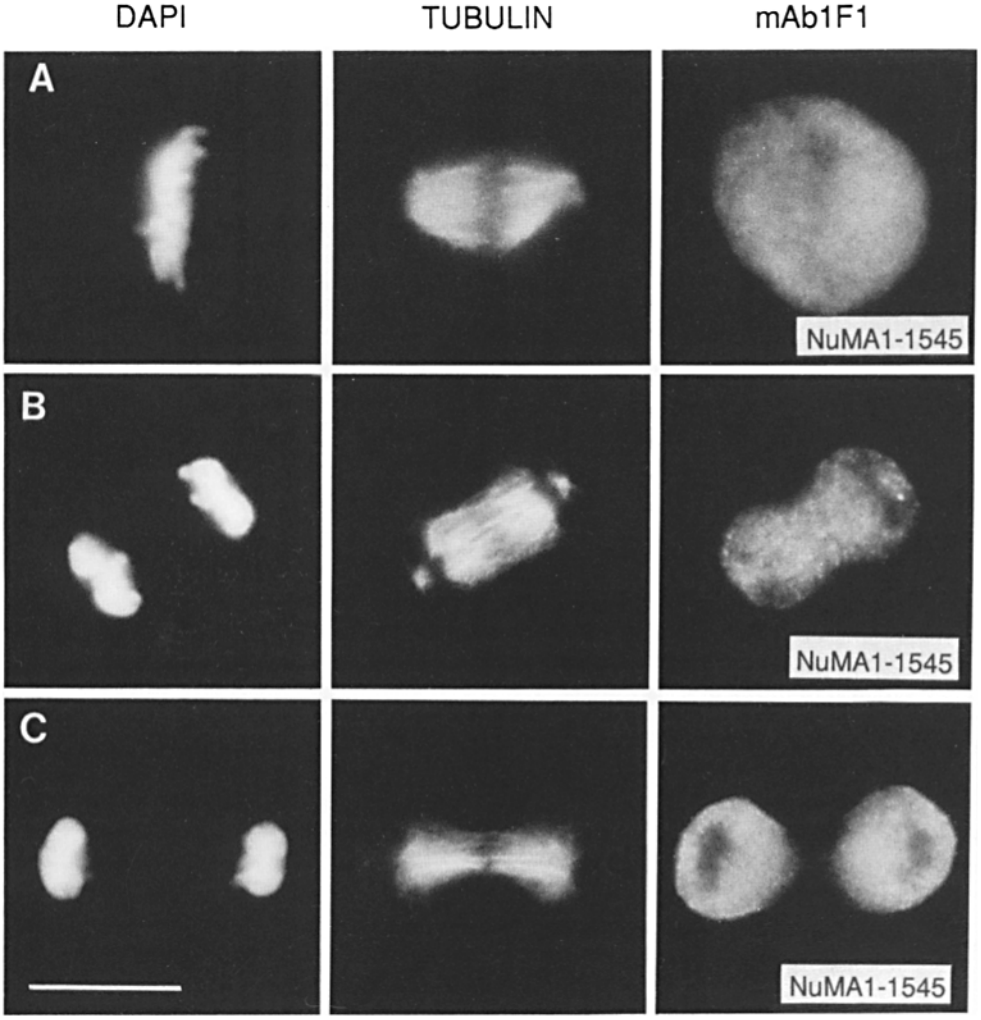

Figure 7. Localization of carboxyl-terminal truncated human NuMA relative to the mitotic spindle apparatus in mitotic BHK-21 cells. Hamster BHK-21 cells were microinjected with a plasmid driving the expression of carboxyl-terminal truncated (*CMV/NuMA1-1545*) human NuMA. Cells were fixed in (*A*) metaphase, (*B*) anaphase, or (*C*) telophase, and processed for immunofluorescence with a DNA-specific dye (*DAPI*), rabbit anti-tubulin antibody (*tubulin*), and a human-specific anti-NuMA monoclonal antibody (*mAb1F1*). Bar, 10 μm.

internal structural component of the nucleus. Perturbation of the wild-type function would lead to nuclei that were unstable and that subsequently fragmented. Evidence for this proposal comes from the fact that NuMA is predicted (Compton et al., 1992; Yang et al., 1992) to assemble into α-helical coiled-coil filaments similar to those observed in the nuclear matrix (He et al., 1990; Jackson and Cook, 1988) and which have been hypothesized to participate in nuclear structure. In addition, the demonstration that expression of tailless NuMA results in the dominant phenotype of post-mitotic

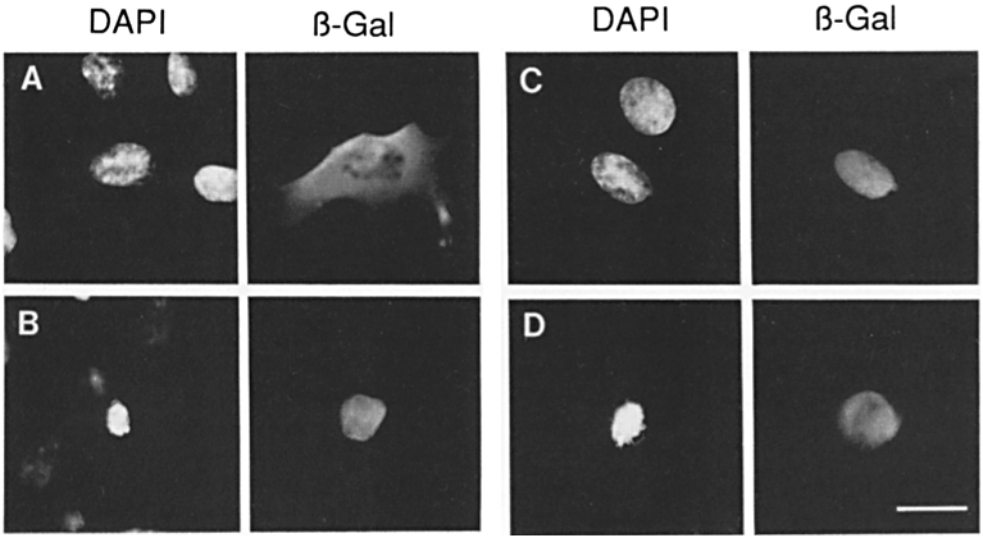

Figure 8. Cellular localization of β-galactosidase and a β-gal/NuMA fusion protein in BHK-21 cells. Hamster BHK-21 cells were microinjected with plasmids driving the expression of either (*A*, *B*) wild-type β-galactosidase or (*C*, *D*) a β-gal/NuMA fusion protein. Cells were fixed (*A*, *C*) in interphase before mitosis or (*B*, *D*) metaphase and processed for immunofluorescence with a DNA-specific dye (*DAPI*) and an anti-β-galactosidase monoclonal antibody (*β-Gal*).

micronucleation without affecting any other observable aspect of mitosis supports this role of NuMA in nuclear reassembly. While the formal possibility that subtle, unobserved defects in spindle architecture before telophase could yield partially functional spindles at the end of mitosis, the simplest view is that tailless NuMA acts (by competing for other binding components) to disrupt a specific function of NuMA in nuclear reassembly at terminal telophase. Arguing against a required role for NuMA in nuclear structure, however, is the recent demonstration that daughter cells can assemble morphologically normal nuclei despite the sequestration of the bulk of endogenous NuMA at the centrosomes after microinjection of anti-NuMA antibody into anaphase cells (Yang and Snyder, 1992).

An alternative explanation for how mutant NuMAs induce micronucleation is that normal NuMA function is required to tether together the telophase bundle of chromosomes. An attractive possibility is that as the chromosomes are translocated to the poles, they interact with pole associated NuMA, either through direct interactions of NuMA with chromosomes or by NuMA-dependent stabilization of the parallel arrays of kinetochore microtubules emanating from the centrosomes. Perturbation of NuMA would lead to an unstable or disorganized array of spindle microtubules, resulting in a loosely packed telophase chromatin mass that would fail to assemble into a single nucleus. This possibility is supported by correlative data showing that NuMA's localization to the spindle poles requires intact microtubules (Price and Pettijohn, 1986), NuMA associates with microtubules in vitro (Maekawa et al., 1991; Kallajoki et al., 1992), NuMA is deposited at the spindle poles after centrosome duplication and aster microtubule nucleation (Compton et al., 1992), and NuMA associates with the minus ends of parallel arrays of microtubules induced in mitotic cells with taxol (Maekawa et al., 1991; Kallajoki et al., 1992).

That NuMA can stabilize the spindle before anaphase has been established by Kallajoki et al. (1991) and Yang and Snyder (1992), both of whom observed aberrant mitotic spindles in cells that had been microinjected with anti-NuMA antibodies. Our results add that overexpression of truncated NuMA subunits can also result in aberrant telophase followed by micronucleation. The affects of these mutant NuMA proteins on the mitotic spindle are particularly obvious in cells expressing the headless NuMA, whose accumulation apparently compromises the metaphase mitotic spindle sufficiently that chromosome segregation is inhibited. A role in spindle stabilization could also explain how wild-type human NuMA can suppress the micronucleation phenotype in the tsBN2 cell line. Excess wild-type NuMA could stabilize the metaphase microtubule array so that the chromosomes are more tightly packed at the metaphase plate (compare Fig. 4, *A* with *E*) restoring reformation of a single nucleus, despite the absence of anaphase. (Unfortunately, we can not directly confirm this explanation due to the rounded nature of these cells during mitosis and their unusually short, stubby mitotic spindles.)

In any event, the data presented here demonstrate that the NuMA protein is required for the normal completion of mitosis. Although it is not settled if NuMA functions directly in the nuclear assembly process or indirectly through stabilization of the mitotic spindle (or both), further analyses using the purified protein in in vitro microtubule and nuclear assembly assays should clarify this point.

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References

Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791–806.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. *In* Current Protocols in Molecular Biology. John Wiley & Sons, Inc. New York.

Berezney, R., and D. S. Coffey. 1977. Nuclear matrix: isolation and characterization of a framework structure from rat liver nuclei. *J. Cell Biol.* 73:616–637.

Capecchi, M. R. 1980. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell.* 22:479–488.

Ciejek, E. M., M.-J. Tsai, and B. W. O'Malley. 1983. Actively transcribed genes are associated with the nuclear matrix. *Nature (Lond.).* 306:607–609.

Cleveland, D. W., M. F. Pittenger, and J. R. Feramisco. 1983. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. *Nature (Lond.).* 305:738–740.

Compton, D. A., I. Szilak, and D. W. Cleveland. 1992. Primary structure of NuMA, an intranuclear protein that defines a novel pathway for segregation of proteins at mitosis. *J. Cell Biol.* 116:1395–1408.

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.

Earnshaw, W. C., B. Halligan, C. Cooke, M. M. S. Heck, and L. Liu. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.

Fisher, P. A. 1987. Disassembly and reassembly of nuclei in cell-free systems. *Cell.* 48:175–176.

Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell.* 19:277–287.

Gerace, L., H. Blum, and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. *J. Cell Biol.* 79:546–566.

Glass, J. R., and L. Gerace. 1990. Lamins A and C bind and assemble at the surface of mitotic chromosomes. *J. Cell Biol.* 111:1047–1057.

Graham, F. L., and A. J. ver der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456–467.

He, D., J. A. Nickerson, and S. Penman. 1990. Core filaments of the nuclear matrix. *J. Cell Biol.* 110:569–580.

Heald, R., and F. McKeon. 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly at mitosis. *Cell.* 61:579–589.

Jackson, D. A., and P. R. Cook. 1988. Visualization of a filamentous nucleoskeleton with a 23 nm axial repeat. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3667–3677.

Kai, R., M. Ohtsubo, T. Sekiguchi, and T. Nishimoto. 1986. Molecular cloning of a human gene that regulates chromosome condensation and is essential for cell proliferation. *Mol. Cell. Biol.* 6:2027–2032.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear localization. *Cell.* 39:499–509.

Kallajoki, M., K. Weber, and M. Osborn. 1991. A 210 kD nuclear matrix protein is a functional 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.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly at the head of the bacteriophage T4. *Nature (Lond.).* 227:680–682.

Lanford, R. E., and J. S. Butel. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell.* 37:801–813.

Lebkowski, J. S., and U. K. Laemmli. 1982. Non-histone proteins and long-range organization of HeLa interphase DNA. *J. Mol. Biol.* 156:325–344.

Loewinger, L., and F. McKeon. 1988. Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2301–2309.

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., R. Leslie, and R. Kuriyama. 1991. Identification of a minus end-specific microtubule-associated protein located at the mitotic poles in cultured mammalian cells. *Eur. J. Cell Biol.* 54:255–267.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 545 pp.

Miake-Lye, R., and M. W. Kirschner. 1985. Induction of early mitotic events in a cell free system. *Cell.* 41:165–175.

Mitchison, T. J. 1989. Mitosis: basic concepts. *Curr. Opin. Cell Biol.* 1:67–74.

Nishimoto, T., E. Ellen, and C. Basilico. 1978. Premature chromosome condensation in a tsDNA mutant of BHK cells. *Cell.* 15:475–483.

Nishitani, H., M. Ohtsubo, K. Yamashita, H. Iida, J. Pines, H. Yasudo, Y. Shibata, T. Hunter, and T. Nishimoto. 1991. Loss of RCCI, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1555–1564.

Ottaviano, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamina during interphase and mitosis. *J. Biol. Chem.* 260:624–632.

Pardoll, D. M., B. Vogelstein, and D. S. Coffey. 1980. A fixed site of DNA replication in eucaryotic cells. *Cell.* 19:527–536.

Peter, M., J. Nakagawa, M. Doree, J. C. Labbe, and E. A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinases. *Cell.* 61:591–602.

Price, C. M., G. A. McCarty, and D. E. Pettijohn. 1984. NuMA protein is a human autoantigen. *Arthritis Rheum.* 27:774–779.

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.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Tousson, A., C. Zeng, B. R. Brinkley, and M. M. Valdivia. 1991. Centrophilin, a novel mitotic spindle protein involved in microtubule nucleation. *J. Cell Biol.* 112:427–440.

Uchida, S., T. Sekiguchi, H. Nishitani, K. Miyauchi, M. Ohtsubo, and T. Nishimoto. 1990. Premature chromosome condensation is induced by a point mutation in the hamster RCC1 gene. *Mol. Cell Biol.* 10:577–584.

Xing, Y., and J. B. Lawrence. 1990. Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by in situ hybridization coupled with biochemical fractionation. *J. Cell Biol.* 112:1055–1063.

Ward, G. E., and M. W. Kirschner. 1990. Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell.* 61:561–577.

Wong, P., and D. W. Cleveland. 1990. Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NF-M. *J. Cell Biol.* 111:1987–2003.

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.

Zeitlin, S., A. Parent, S. Silverstein, and A. Efstratiadis. 1987. Pre-mRNA splicing and the nuclear matrix. *Mol. Cell Biol.* 7:111–120.