

NuMA: An Unusually Long Coiled-Coil Related Protein in the Mammalian Nucleus

Charles H. Yang, Eric J. Lambie, and Michael Snyder

Department of Biology, Yale University, New Haven, Connecticut 06511

Abstract. A bank of 892 autoimmune sera was screened by indirect immunofluorescence on mammalian cells. Six sera were identified that recognize an antigen(s) with a cell cycle-dependent localization pattern. In interphase cells, the antibodies stained the nucleus and in mitotic cells the spindle apparatus was recognized. Immunological criteria indicate that the antigen recognized by at least one of these sera corresponds to a previously identified protein called the nuclear mitotic apparatus protein (NuMA). A cDNA which partially encodes NuMA was cloned from a λ gt11 human placental cDNA expression library, and overlapping cDNA clones that encode the entire gene were isolated. DNA sequence analysis of the clones has identified a long open reading frame capable of encoding a protein of 238 kD. Analysis of the predicted protein sequence suggests that NuMA con-

tains an unusually large central α -helical domain of 1,485 amino acids flanked by nonhelical terminal domains. The central domain is similar to coiled-coil regions in structural proteins such as myosin heavy chains, cytokeratins, and nuclear lamins which are capable of forming filaments. Double immunofluorescence experiments performed with anti-NuMA and antilamin antibodies indicate that NuMA dissociates from condensing chromosomes during early prophase, before the complete disintegration of the nuclear lamina. As mitosis progresses, NuMA reassociates with telophase chromosomes very early during nuclear reformation, before substantial accumulation of lamins on chromosomal surfaces is evident. These results indicate that the NuMA proteins may be a structural component of the nucleus and may be involved in the early steps of nuclear reformation during telophase.

THE eukaryotic nucleus is an organelle that partitions DNA and nuclear processes from the cytoplasm (for review see Gerace and Burke, 1988; Newport and Forbes, 1987). Morphological studies have shown that the nucleus is composed of structural and functional domains that are involved in the various aspects of nuclear function. The nucleolus, which is involved in ribosome biogenesis, and the nuclear membrane which regulates transport between the nucleus and the cytoplasm, are two well studied examples of defined structural domains within the nucleus (Gerace and Burke, 1988; Goessens, 1984). In addition, splicing regions (Wu et al., 1991) and novel subnuclear structures such as nuclear bodies and dots of unknown functions have been described (Ascoli and Maul, 1991; Xie, K., E. F. Lambie, and M. Snyder, manuscript submitted for publication).

These components are often distributed nonrandomly within the nucleus. For example, in *Drosophila* the nuclear envelope interacts with telomeres, centromeres, and specific chromosomal loci (Gruenbaum et al., 1984; Hochstrasser et al., 1988), and in yeast the nucleolus and the spindle pole body (the yeast microtubule organizing center) are localized

at opposite ends of the nucleus (Yang et al., 1989a). Compartmentalization of the nucleus into domains may require a specialized nucleoskeleton. A nucleoskeleton may also be expected to establish the overall morphology of the nucleus and might help regulate the activities of different nuclear processes throughout the cell cycle (Cook, 1988; Verheijen et al., 1988).

To expose structural components of the nucleus (other than chromatin), several protocols for nuclear fractionation have been developed. These procedures, involving sequential nuclease, salt and detergent extractions, have revealed a complex, branched and fibrous network within the nucleus (Berezney and Coffey, 1974, 1977). This putative nuclear matrix has been implicated in the regulation of DNA replication, hnRNA processing, gene expression, and maintenance of higher order chromatin structure (for review see Jackson and Cook, 1988; Verheijen et al., 1988). However, whether these residual filaments represent a bona fide nuclear skeleton or merely preparation artefacts has remained controversial (Cook, 1988).

Molecular analyses of these putative matrices have proved to be difficult due to the biochemically complex nature of this structure. Topoisomerase II (Berrios et al., 1985), actin (Nakayasu and Ueda, 1986), and RNA-associated proteins (Bouvier et al., 1985) are some of the various components

Eric J. Lambie's present address is Laboratory of Molecular Biology, University of Wisconsin, Madison, WI.

that have been identified and implicated in nuclear matrix function and organization. RNA may also be a component of this structure (Fey et al., 1986; Gallinaro et al., 1983; Miller et al., 1978; Xing and Lawrence, 1991).

At present, the best characterized component of the nuclear matrix remains the nuclear lamina, which forms a fibrillar meshwork underlying the nuclear envelope (Aebi et al., 1986) and is primarily composed of lamins A, B, and C (Gerace et al., 1978). Structurally and biochemically, lamins behave similarly to intermediate filament proteins (Aebi et al., 1986; Moir et al., 1990; Parry et al., 1987), and sequence analysis has also shown that lamins are related to this family of proteins (Fisher et al., 1986; McKeon et al., 1986).

Recent studies have indicated that the nuclear matrix which extends into the interior of the nucleus may also be composed of similarly fibrous components. A nuclear protein that is immunologically related to intermediate filament proteins has been identified in the matrix fraction (Beven et al., 1991). In addition, nuclear matrix preparations designed to reduce artefacts have revealed the existence of 10-nm filamentous structures that are morphologically indistinguishable from intermediate filaments (He et al., 1990; Jackson and Cook, 1988).

Whether or not such filamentous structures are composed of intermediate filament-like proteins has not yet been determined. Nonetheless, it raises the possibility that the nuclear interior may be organized by skeletal structures that are composed of fibrous protein subunits in the same manner as the cytoplasm and the nuclear periphery.

Data presented below describe a protein called the nuclear mitotic apparatus protein (NuMA;¹ Lyderon and Pettijohn, 1980) that we suggest may be a structural component of the nucleus. As its name indicates, NuMA has an unusual cell cycle-dependent distribution pattern. In interphase cells, NuMA is present in the nucleus; in mitotic cells, it localizes to the polar regions of the spindle apparatus (Lyderon and Pettijohn, 1980). Biochemical analyses have demonstrated that NuMA is an abundant high molecular mass (240 kD) phosphoprotein possessing an extended, native structure (Price and Pettijohn, 1986). It cannot be extracted from isolated nuclei by treatment with DNAase I and then 2 M NaCl, indicating that NuMA is tightly associated with the nucleus (Lyderon and Pettijohn, 1980). NuMA is present in many species, and autoimmune disease patients sometimes have antibodies directed against this protein (Price et al., 1984). The cellular function of NuMA is unknown.

We have identified human autoimmune sera that contain anti-NuMA antibodies and have used one serum to clone cDNAs that encode the NuMA protein. Analysis of the predicted amino acid sequence of NuMA suggests that this protein contains an unusually long coiled-coil α -helical domain that is similar to the rod domains of fibrous structural proteins. Double immunofluorescence experiments using anti-NuMA and antilamin antibodies demonstrate that NuMA dissociates from condensing chromosomes very early in prophase, before the complete disintegration of the nuclear lamina. At the end of mitosis, NuMA reassociates with telo-

phase chromosomes before substantial accumulation of lamins is apparent on the chromosomes, indicating that NuMA may be important in the early steps of nuclear reassembly.

Materials and Methods

Autoimmune Sera

Screening of a bank of 892 autoimmune sera by indirect immunofluorescence on HT29 cells is described in Yang et al. (1989a). Six sera were identified that stained the spindle apparatus in mitotic cells and the nucleus in interphase cells.

Isolation and Preliminary Analysis of cDNA Clones

A λ gt11 human placental cDNA expression library (Clontech, Palo Alto, CA) was immunoscreened with human serum h240-1 according to procedures described in Snyder et al. (1987). Of 10^6 phage screened, one positive clone, λ 240-1, was identified and purified. Reactive antibodies were affinity purified from λ 240-1 plaques and λ gt11 plaques using the h240-1 serum according to Snyder et al. (1987), and used for indirect immunofluorescence.

E. coli cells were infected with purified λ 240-1 or control λ gt11 phage and plated. Proteins expressed in plaques were adsorbed to nitrocellulose and probed with the six different anti-NuMA-like sera (Snyder et al., 1987). Five sera recognized λ 240-1 proteins; none recognized proteins expressed from λ gt11.

The 2.8-kb EcoRI insert in λ 240-1 was used as a hybridization probe to screen the same human placenta cDNA library, using procedures described in Maniatis et al. (1982). The DNA probe was radiolabeled according to Feinberg and Vogelstein (1983). A screen of 2×10^6 phage yielded one novel positive clone, λ 240-2.

Total HeLa Cell RNA Extraction and Identification of 5' NuMA cDNA by Polymerase Chain Reaction

Amplification of the 5' coding region of NuMA was performed using the RACE (rapid amplification of cDNA ends) protocol (Frohmann et al., 1988) with some modifications. First, HeLa total cell RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction similar to that described in Chomczynski and Sacchi (1987). A 40-mer primer (designated ppl), with the sequence 5'GAGAATTCGGATCCAAGCTTCTATCTGTGCATCCTTCTCG was used to prime HeLa cell total RNA for reverse transcription with AMV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). The 5' 20 nucleotides of this primer contains the EcoRI, BamHI, and HindIII restriction sites, respectively. The 3' 20 nucleotides (underlined) are complementary to nucleotide positions 735-754 of the NuMA open reading frame, and correspond to a region near the 5' end of the λ 240-1 EcoRI insert (see Fig. 7). The reverse transcription product was tailed at its 3' end with terminal deoxynucleotidyl-transferase (Bethesda Research Laboratories, Bethesda, MD) in the presence of dGTP rather than dATP. Polymerase chain reaction (PCR) amplification of the tailed cDNAs was performed with AmpliTaq (Perkin Elmer-Cetus, Norwalk, CT) in a 50- μ l reaction volume using the ppl primer and a 36-mer adaptor-primer 5'-GACTCGAGTCGACATCTAT(C)₁₇ that is complementary to the cDNA synthetic tail. The 5' 19 nucleotides contain the XhoI, SalI, and ClaI restriction sites. The RACE products were extracted with phenol-chloroform, ethanol precipitated, and resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6. The amplified DNA was subsequently digested with the restriction enzymes SalI and EcoRI, and cloned into the corresponding restriction sites of the Bluescript vector (Stratagene, San Diego, CA).

Production and Affinity-Purification of Anti-NuMA Antibodies

The NuMA protein was overproduced in *E. coli* using two methods. First, based on the assumption that the 2.8-kb insert of λ 240-1 was in frame with *lacZ*, the NuMA DNA fragment was inserted into the EcoRI restriction site of pATH11, a *trpE* expression vector (Koerner et al., 1991). The resulting construct is called pATH::12. Subsequent sequence analysis revealed that the NuMA open reading frame was not in frame with the *lacZ* gene, and consequently the NuMA insert was also out of frame with *trpE* in pATH::12. Nevertheless, a 100-kD NuMA fragment was expressed

1. **Abbreviations used in this paper:** NuMA, nuclear mitotic apparatus protein; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

reasonably well from pATH::12 as a nonfusion protein. Expression of the 100-kD polypeptide is dependent upon the *trpE* gene for transcription as its expression was induced with indoleacrylic acid.

The 100-kD polypeptide was overproduced and an insoluble pellet containing this protein was prepared as described (Snyder, 1989). The pellet was solubilized with SDS sample buffer (see below) and the 100-kD protein was purified by SDS-PAGE. 50 μ g of the purified NuMA polypeptide in complete Freund's adjuvant was used for the primary injection into the intraperitoneal cavity of a mouse, and subsequent boosts with 50 μ g of NuMA protein were carried out after one month and again, two weeks later. Serum was collected two weeks after the final boost.

Two rabbit polyclonal sera specific to polypeptides expressed from two nonoverlapping regions of the NuMA 2.8-kb fragment were also prepared. A 0.65-kb fragment near the 5' region of the λ 240-1 insert, and a 3' 1.6-kb fragment were separately cloned into the expression vector pGEX3 and pGEX1, respectively, to generate glutathione-S-transferase-NuMA fusion proteins (Smith and Johnson, 1988). Both NuMA fragments were derived from the nested deletions used for sequence analysis (see below). The fusion proteins GST::1 (55 kD) and GST::2 (85 kD) of the expected sizes were overproduced and affinity purified as described (Smith and Johnson, 1988). The fusion proteins were subsequently used to immunize two different rabbits in order to generate independent anti-NuMA polyclonal sera (Pocono Rabbit Farm, Canadensis, PA). Primary and boost injections were performed with 250 μ g of fusion protein.

Affinity purification of anti-NuMA antibodies was performed using 100 μ g of *E. coli*-expressed NuMA protein according to Snyder et al. (1989; See also Mirzayan et al., 1992).

Immunofluorescence

African Green monkey CV-1 cells and human HeLa cells were seeded onto glass coverslips and grown for 24–48 h to a density \sim 50% that of confluence. The cells were fixed for 30 min at room temperature in 100 mM Pipes (pH 6.8), 2 mM EGTA, 2 mM MgCl₂, and 4% formaldehyde, then washed twice for 5 min each with PBS (150 mM NaCl, 25 mM sodium phosphate, pH 7.3). The cells were permeabilized for 10 min with PBS containing 0.1% (vol/vol) NP-40, then washed twice for 5 min each with PBS. The cells were next incubated with PBS containing 0.2 M glycine for 15 min, followed by two 5-min washes with PBS. The cells were incubated with primary antibodies for 1.5–2 h, and then washed with PBS, PBS + 0.1% NP-40, then PBS. After incubation in the presence of secondary antibodies for 1 h, cells were washed once with PBS, and twice with PBS + 0.1% NP-40. The cells were then rinsed in distilled water, and mounted in a glycerol solution containing Hoechst 33258 (70% glycerol, 3% *n*-propyl gallate, 0.25 μ g/ml Hoechst 33258).

Primary antibodies were used for immunofluorescence as follows. h240-1 human autoantibodies were used at 1:500 dilutions in PBS, and Dr. Pettijohn's human anti-NuMA antibodies (Price and Pettijohn, 1986) were diluted at 1:50. Affinity-purified mouse or rabbit anti-NuMA antibodies were diluted at 1:500. In double immunofluorescence experiments, cells were stained with human antilamin antibodies, LS-1 ((McKeon et al., 1983); diluted 1:500), or serum 5051 ((Tuffanelli et al., 1983); diluted 1:100). Microtubules were stained with the commercially available mouse monoclonal antitubulin antibody DM-1A (diluted 1:1,000; Sigma Chemical Co., St. Louis, MO).

Secondary reagents used in these experiments included rhodamine-conjugated goat antihuman antibodies, FITC-conjugated goat antimouse antibodies (Cappel Laboratories, Durham, NC), and biotinylated goat anti-rabbit antibodies used in conjunction with FITC-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA).

Electrophoresis and Immunoblot Analysis

HeLa cells were lysed directly in culture dishes using 2 \times sample buffer (125 mM Tris-HCl, pH 6.8, 10% β -mercaptoethanol, 4% [wt/vol] SDS, 20% glycerol, and 0.004% bromophenol blue), and boiled for 5 min. Cellular proteins were separated on 7% SDS polyacrylamide gels according to Laemmli (1970), and blotted onto nitrocellulose (Burnette, 1981). The blots were blocked in TBS containing 20% (wt/vol) FCS, 10% nonfat dry milk, 0.2% sodium azide for 4 h at room temperature, then incubated with human autoantibodies (diluted 1:1,000 in the blocking solution) overnight at 4°C. Immunoreactive polypeptides were detected by incubation with ¹²⁵I-protein A (>30 mCi/mg; Amersham Chemical Co., Arlington Heights, IL; diluted in TBS, 0.1% (wt/vol) BSA).

NuMA-encoded proteins expressed in *E. coli* were analyzed by immunoblot analysis as described above. 0.1 μ g of purified NuMA proteins were

separated in a 10% SDS polyacrylamide gel and blotted onto nitrocellulose. The blots were then incubated with either h240 autoantibodies as described above, with affinity-purified anti-NuMA antibodies (diluted 1:500), or with rodent antibodies (diluted 1:1,000).

Sequence Analysis

The λ 240-1 EcoRI DNA insert was cloned into the replicative form of the M13mp19 vector in both orientations. The cDNA clone PCR1 was introduced into the Bluescript vector (Stratagene, CA). λ 240-2 was cloned into both Bluescript and M13mp19 vectors. To facilitate sequence analyses, a nested set of deletions was generated with T4 DNA polymerase (Cyclone kit; International Biotechnologies, Inc., New Haven, CT) or with exonuclease III using the Erase-a-Base kit (Promega Biotech, Madison, WI). The different clones were sequenced using single-stranded or double-stranded DNA according to Sanger et al. (1977) using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH) and the T7 Sequencing kit (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

Sequence similarity searches of the NBRF-PIR and GENBANK databases were performed with FASTA and tFASTA programs (Pearson, 1990). Prediction of secondary structure was performed using a program based on Garnier algorithms (Pearson and Lipman, 1988).

Confirmation of the Translation Termination Site

A second open reading frame lies at the 3' end of the NuMA coding sequence and extends several hundred base pairs past the predicted termination codon. To ensure that there were no sequencing frameshift errors, the position of the translation termination signal was confirmed in the following manner. A deletion product that contains 1.7 kb of the λ 240-2 cDNA insert spanning the 3' end of the NuMA open reading frame was identified from the deletion clones used for NuMA sequence analysis (described above). The NuMA fragment was situated downstream of the Bluescript T7 RNA polymerase promoter site, and contained an in-frame ATG codon which could serve as a translation initiation site.

This construct was linearized at the XhoI or EcoRI restriction sites, which are located 25 nucleotides upstream, and 0.55 kb downstream, of the putative termination signal, respectively. mRNA was synthesized *in vitro* from the linearized DNAs, and then translated in a rabbit reticulocyte lysate (Stratagene) containing ³⁵S-methionine (ICN Radiochemicals, Irvine, CA). Similar-sized translation products of the expected molecular weight were generated from both constructs, indicating that the in-frame TAA codon located near the XhoI site may serve as an authentic translation termination site (data not shown).

Results

Autoimmune Sera That Recognize a NuMA-like Antigen

In a search for antibodies that recognize interesting components of the mitotic spindle apparatus or nucleus, a bank of 892 human autoimmune sera was screened on human HT29 cells by indirect immunofluorescence (Yang et al., 1989a). Six sera, h240-1 through 6, were identified that recognized an antigen(s) with an unusual cell cycle distribution pattern. In interphase cells, these sera recognized the entire nucleus except the nucleolus. In mitotic cells, they stained the spindle apparatus, preferentially in the polar regions (Fig. 1). This pattern was observed on a wide variety of mammalian cell types, including human HT29 (Fogh, 1975) and HeLa epithelial cells (Gey et al., 1952), human primary fetal skin fibroblast cells (obtained from T. Yang-Feng and M. Liskay, Yale University, New Haven, CT), African Green Monkey kidney CV1 cells (Jensen et al., 1964), and porcine LLC-PK₁ kidney cells (Hull et al., 1976).

The six sera were used for immunoblot analysis of proteins isolated from HeLa whole cell extracts. Two sera, h240-1 and h240-2, recognized a high molecular mass polypeptide of \sim 240 kD (Fig. 2). No prominent bands were recognized

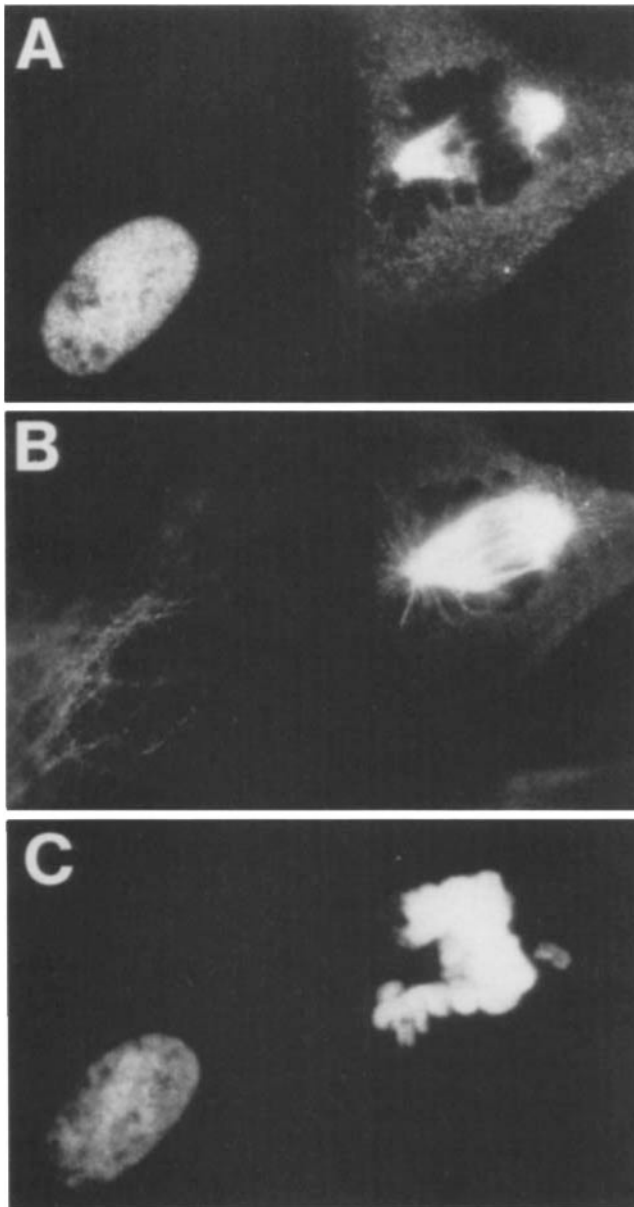


Figure 1. Double immunofluorescence experiments of the p240 antigen and tubulin in mammalian cells. African Green Monkey kidney CV-1 cells were simultaneously stained with (A) human autoimmune serum h240-1, (B) DM-1A, an antitubulin mAb, and (C) Hoechst 33258 to visualize the DNA. In interphase cells, p240 localizes only at the nucleus; in mitotic cells, p240 concentrates at the spindle apparatus, but preferentially in the polar region.

using two other sera. The remaining two sera recognized polypeptides of ~70 kD (data not shown).

The apparent size of the p240 antigen and its cell cycle distribution pattern is similar to the NuMA protein, first described by Lyderson and Pettijohn (1980). Immunological data presented below indicates that they are the same protein.

Identification of cDNAs That Encode the p240 Antigen

To learn more about p240, cDNA clones encoding this pro-

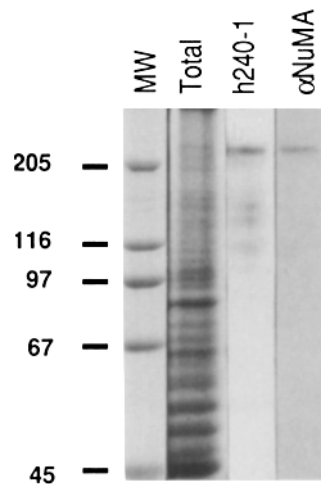


Figure 2. Immunoblot of total HeLa cell protein probed with h240-1 and anti-NuMA antibodies. (MW) Molecular weight markers. (Total) Total HeLa cell protein were separated on 7.5% SDS polyacrylamide gel and stained with Coomassie blue. The separated proteins were blotted onto nitrocellulose and probed with (h240-1) serum h240-1 or (αNuMA) a human autoimmune serum containing anti-NuMA antibodies (Price et al., 1984). Both h240-1 and anti-NuMA antibodies recognize a similar 240-kD polypeptide.

tein were isolated. Serum h240-1 was used to screen a λgt11 human placenta cDNA expression library (Snyder et al., 1987). Of 10⁶ phage screened, one positive clone, λ240-1, containing a 2.8-kb cDNA insert was identified. The six NuMA-like autoimmune sera were used to probe proteins expressed from λ240-1 using a plaque assay (see Materials and Methods); five of the six sera recognized the λ240-1 protein (data not shown). None of the sera reacted with proteins expressed from λgt11.

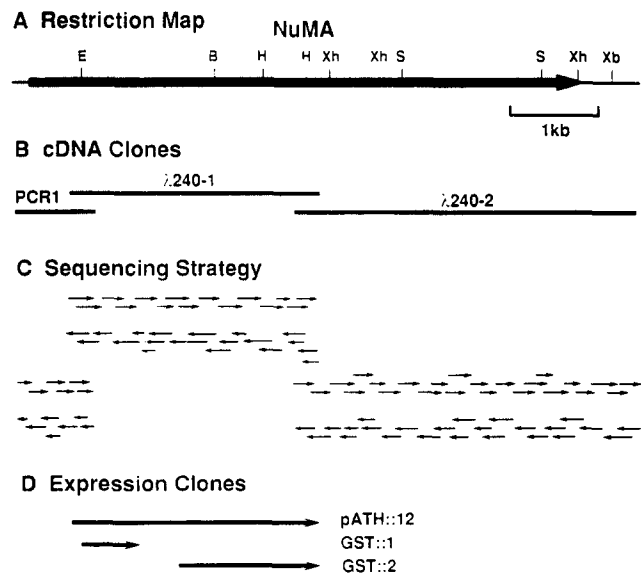


Figure 3. Restriction map and sequencing strategy of the overlapping NuMA cDNAs. (A) The restriction map of the NuMA coding region. E, EcoRV; B, BstEII; H, HindIII; Xh, XhoI; S, SphI; Xb, XbaI. (B) The relative positions of the overlapping NuMA cDNAs. The cDNAs were identified as described (see text). (C) Sequencing strategy. The arrows indicate the direction and extent of individual sequencing samples. Overlapping regions between the cDNAs were found to be identical. (D) Portions of NuMA were over-expressed in *E. coli* and used to generate anti-p240 polyclonal sera (see Fig. 4 and text). pATH::12 was expressed from the entire 2.8-kb λ240-1 insert using the expression vector pATH11. GST::1 and GST::2 were overproduced from nonoverlapping 0.65 and 1.6-kb DNA fragments derived from the λ240-1 insert, respectively, and were expressed as glutathione-S-transferase::p240 fusion proteins.

To further test whether the cDNA insert in λ 240-1 encoded a portion of the p240 antigen, proteins produced from λ 240-1 were overproduced in *E. coli* and used to affinity purify reactive antibodies from serum h240-1 (Snyder et al., 1987). In control experiments, proteins produced from the λ gt11 vector were tested. Antibodies affinity purified from λ 240-1 exhibited the same staining pattern as serum h240-1; antibodies purified from the λ gt11 control failed to exhibit any staining above background (data not shown). Thus, the protein encoded by λ 240-1 is antigenically related to the NuMA-like antigen.

It is possible that the cloned protein shares common epitopes with, but does not correspond to, the NuMA-like protein. To test this possibility, three different polyclonal sera were prepared to the protein encoded by the λ 240-1 insert. The entire 2.8-kb insert was subcloned into the *E. coli* expression vector pATH11 (Koerner et al., 1991). A 100-kD protein of the λ 240-1 insert was overproduced (although not as a fusion protein, see Materials and Methods) and used to generate a mouse polyclonal serum called m240-A.

Similarly, two nonoverlapping regions of the 2.8-kb fragment (see Fig. 3) were separately subcloned into the pGEX expression vector (Smith and Johnson, 1988). The resulting GST::p240.1 and GST::p240.2 fusion proteins were used to generate anti-p240 rabbit polyclonal sera called r240-B and r240-C, respectively. Immunoblot analysis indicates that each of the different sera specifically recognize the expected λ 240-derived proteins (Fig. 4, A and B). Preimmune sera do not recognize these proteins.

Double immunofluorescence experiments of CV1 cells with each of the three immune sera yielded the cell cycle immunostaining pattern that was indistinguishable from that of serum h240-1 (see Fig. 5 for the staining pattern of mouse serum m240-A as compared to bona fide NuMA antibodies; rabbit serum r240-C is shown below in Figs. 10 and 11); the nucleus was stained in interphase cells and the spindle apparatus was recognized in mitotic cells. Preimmune sera failed to exhibit any such staining. The NuMA-like staining pattern is unlikely to represent a fortuitous reaction that is due to a common epitope shared by λ 240-1 and the NuMA-like antigen, because the rabbit r240-B and r240-C polyclonal sera, which were prepared against proteins derived from different parts of the cloned DNA, stain the same antigen by immunofluorescence. (The NuMA regions used to prepare the r240-B and r240-C sera do not exhibit any primary sequence similarity above that expected for a coiled-coil protein.) Thus, we conclude that the protein encoded by λ 240-1 corresponds to the antigen observed by indirect immunofluorescence. Moreover, since all three sera stained both the nucleus and the spindle apparatus, it is likely that the staining at these two sites corresponds to a single protein.

p240 Is Probably the NuMA Protein

Immunological tests were performed to determine whether λ 240-1 encoded the bona fide NuMA protein. First, the reactivity of anti-NuMA antibodies obtained from Dr. Pettijohn (University of Colorado, Boulder, CO) (Price et al., 1984) and the h240-1 or (m240-A) antibodies were compared by both indirect immunofluorescence experiments and immunoblot analysis. Double immunofluorescence experiments of CV1 cells using the mouse m240-A serum and anti-NuMA antibodies showed that both antibodies yielded identical im-

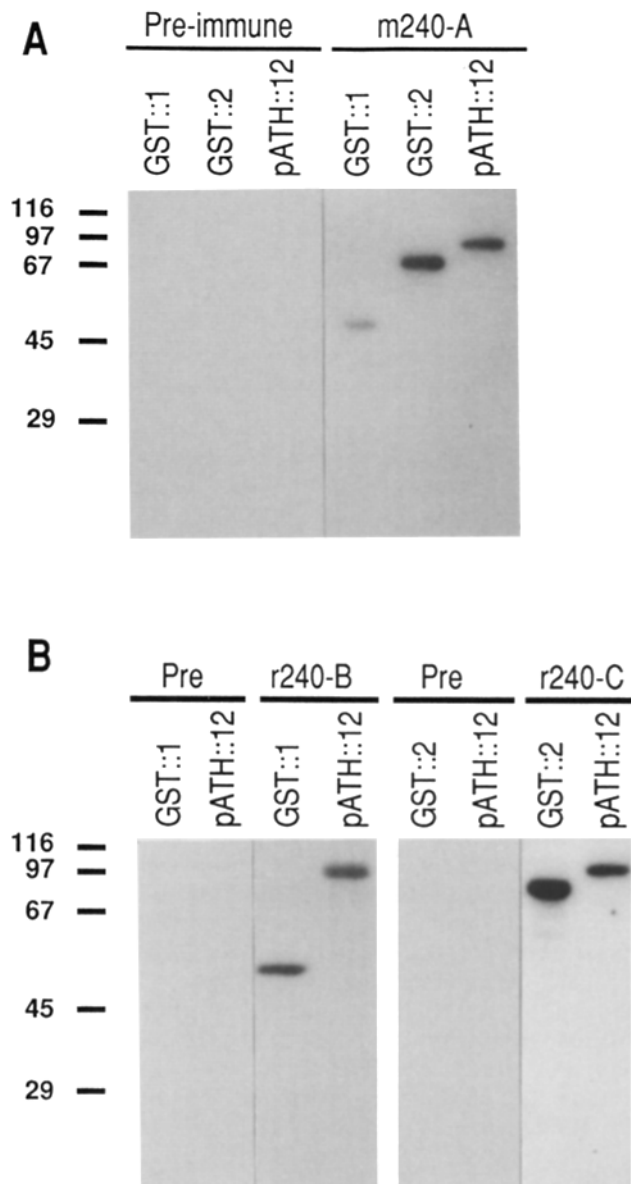


Figure 4. Immunoblot analyses of mouse and rabbit anti-NuMA polyclonal sera. A mouse anti-NuMA polyclonal serum m240-A was generated against a 100-kD nonfusion polypeptide (designated pATH::12) expressed from pATH::12 (see Fig. 3 and text). The rabbit anti-NuMA polyclonal sera, r240-B, and r240-C were generated against 53- and 85-kD glutathione-s-transferase::p240 fusion proteins GST::1 and GST::2, respectively. The GST fusions were derived from nonoverlapping regions of the λ 240-1 DNA insert. (The GST::1, GST::2, and pATH::12 proteins were expressed and purified as described in Materials and Methods.) (A) Affinity-purified antibodies from serum m240-A recognize the 100-kD pATH::12 polypeptide. The antibodies also recognize the GST::1 and GST::2 fusion proteins, as expected. Preimmune sera do not recognize these polypeptides. (B) Affinity-purified antibodies from the rabbit sera r240-B and r240-C recognize the fusion proteins GST::1 and GST::2, respectively. Both sera also recognize the pATH::12 polypeptide. Preimmune sera do not recognize these polypeptides.

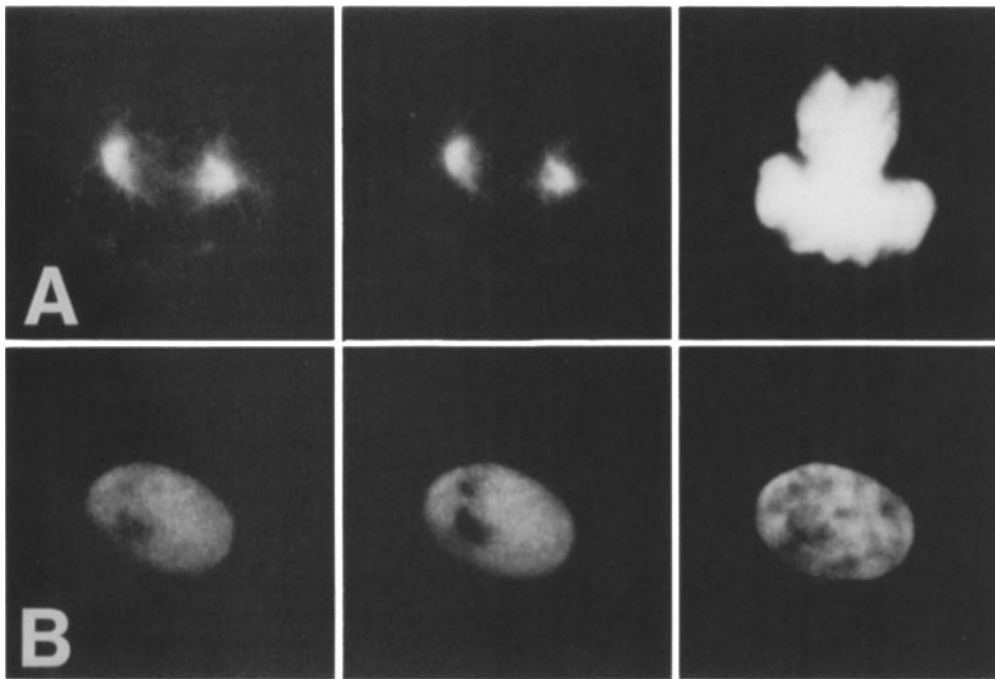


Figure 5. Costaining of mouse anti-p240 and anti-NuMA antibodies. Double immunofluorescence experiments were performed using the mouse serum m240-A and anti-NuMA antibodies (Price et al., 1984). (A and B) left, m240-A staining; center, anti-NuMA staining; right, Hoechst 33258-stained DNA. (A) A metaphase cell. Both m240-A and anti-NuMA antibodies similarly recognize the polar regions of the spindle apparatus. (B) An interphase cell. Both sera similarly recognize the nucleolus, except the nucleolus. All possible combinations using human sera (h240-1 and anti-NuMA) and rodent sera (rabbit r240-B, r240-C, or mouse m240-A serum) yielded identical results (data not shown).

munofluorescence patterns in interphase and mitotic cells (Fig. 5). Identical staining patterns were also observed when HeLa cells were used (data not shown). Second, immunoblot analysis against HeLa whole cell extracts using both anti-NuMA antibodies and h240-1 antibodies revealed that both recognize a similar-sized 240-kD polypeptide (Fig. 2). Finally, both anti-NuMA antibodies and h240-1 antibodies recognize the three proteins expressed from various portions of the p240 gene (Fig. 6). Since two proteins derived from different parts of the λ 240-1 clone are recognized by these sera, crossreaction is unlikely to be due to a single common epitope. The combination of these results indicate that the anti-NuMA and h240-1 antibodies recognize the same p240 protein, which is partially encoded on the 2.8-kb λ 240-1 insert.

Sequence Analysis of the NuMA Gene

To further understand the role of NuMA, we determined the sequence of its coding region. To perform this analysis, additional cDNA clones were isolated that collectively encode the entire protein coding region. This was accomplished by two means. 3' NuMA coding sequences were cloned by screening the λ gt11 human cDNA library with a probe prepared with the 2.8 insert of λ 240-1. From a screen of 2×10^6 phage, one positive clone, λ 240-2 was identified; this clone contained a 4.0-kb insert (Fig. 3). Hybridization screens of the human cDNA library were unsuccessful in isolating cDNAs encoding 5' coding sequences. We therefore employed a second cloning strategy that utilized a RACE PCR strategy, described in Materials and Methods. Briefly, a NuMA gene-specific primer, ppl (see Fig. 7), complementary to a region near the 5' end of the 2.8-kb NuMA sequence was used to prime HeLa cell total RNA for reverse transcription. The resulting cDNA was "tailed" at its 3' end with dGTP using terminal deoxynucleotidyl-transferase. This modified cDNA was then amplified by PCR using a (dC)₁₇

and the ppl primers. Examination of the PCR reaction products by gel electrophoresis revealed a single 0.9-kb cDNA fragment (data not shown), which was subsequently cloned (PCR1; Fig. 3).

The DNA sequence of the three overlapping cDNAs that span the entire NuMA coding sequence was determined (Fig. 7), and a 7,154 nucleotide continuous DNA sequence was obtained. The putative initiation ATG is located at

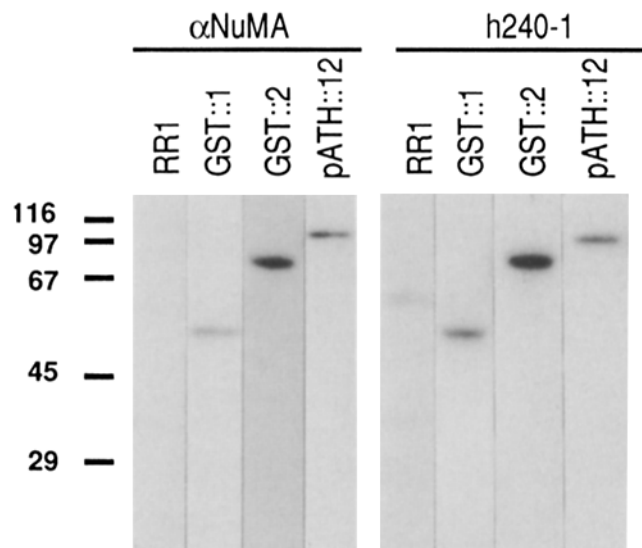


Figure 6. Anti-NuMA and h240-1 antibodies recognize proteins expressed from the λ 240-1 clone. The GST::1, GST::2, and pATH::12 proteins were expressed in the *E. coli* strain RRI and prepared for immunoblot analyses as described above. RRI proteins were also processed for immunoblot analysis as a control. Both anti-NuMA and h240-1 sera specifically recognize all three proteins derived from the λ 240-1 DNA insert.

nucleotide position 157, and is followed by an open reading frame 6,345 nucleotides in length. The stop codon at position 6,502 is likely to be the correct translation termination signal as deduced using the criteria described in Materials and Methods. The large size of the NuMA message predicted from sequence analysis of the cDNA clones is consistent with the large ~7.5-kb message observed by RNA blot analysis of HeLa cell RNA using the 2.8-kb probe of λ 250-1 (data not shown).

NuMA Is Predicted to be a Large Coiled-Coil Protein

Analysis of the NuMA DNA sequence predicts that the NuMA protein is very large, 2,115 amino acids in length. The predicted molecular weight of 238,085 D is consistent with that observed from SDS polyacrylamide gel analysis (Fig. 2).

The NuMA protein sequence was compared to other protein sequences contained in the Genbank and NBRF databases using the FASTA and tFASTA programs (Pearson, 1990). A central 1,485 amino acid region (residues 216–1,700) was found to have significant sequence similarity to proteins such as myosins, tropomyosins, intermediate filaments and nuclear lamins. NuMA shared the highest sequence similarity with myosin heavy chains. When compared to human cardiac myosin, the NuMA sequence exhibited 22% identity over a 623 amino acid region, and when compared to chicken embryonic skeletal myosin, 19.6% identity was found over a 1,112 amino acid region (data not shown). All of the proteins that exhibit strong sequence similarity to NuMA contain similar secondary structures. These proteins have been shown (or predicted) to form long coiled-coils. In a coiled-coil, long α -helices of two molecules dimerize through hydrophobic interactions. The basis of this interaction is generated in the primary amino acid sequence of the α -helix which contains heptad (seven amino acids, a through g) repeats. The first (a) and the fourth (d) residue in each of these heptad units are predominantly hydrophobic, and they generate a spine along one side of the α -helix which is capable of interacting with the hydrophobic spine of a second molecule to form a coiled-coil dimer (Cohen, 1990; Cohen and Parry, 1986). The sequence similarity of NuMA with coiled-coil proteins suggests that NuMA may be a member of this family.

Analysis of the predicted NuMA protein sequence is consistent with the interpretation that NuMA is a coiled-coil protein. Secondary structure predictions (Garnier et al., 1978; Pearson and Lipman, 1988) indicate that the NuMA protein has three domains: a very long α -helical central domain of 1,485 amino acids flanked by nonhelical ends (Fig. 8). Examination of the amino acid sequence of the central α helical domain reveals that it contains heptad periodicity (Fig. 7). Approximately 69 and 60% of the amino acids in positions a and d, respectively, are hydrophobic residues, respectively; in contrast ~20% of the residues in each of the other positions are hydrophobic. Positions a and d also contain very few charged residues; most of the charged residues are found in positions b, c, e, f, and g such that ~40% of the residues in these positions are lysine, arginine, aspartic acid or glutamic acid. Thus, this region is predicted to form a long helix with a hydrophilic face on one side, and a hydrophobic face on the other. Interactions along the hydrophobic faces of two NuMA proteins may form a coiled-coil rod.

The pattern of the heptad repeats in the central α -helical region is interrupted by six short segments that contain prolines. Since prolines usually disrupt an α -helix, the central region of the NuMA protein may be divided into seven α -helical segments. Also interrupting the heptad periodicity is the presence of one, three, or four amino acid insertions. These small insertions lack prolines and may cause subtle phase shifts in the α -helix, but probably do not disrupt the helix. Small insertions have been observed in most coiled-coil proteins, for example intermediate filaments, paramyosin, and myosins (Cohen 1990; discussed in more detail in Mirzayan et al., 1992). In summary, both secondary structure predictions and sequence similarity analysis indicate that the NuMA protein contains a very long central rod domain. The rod region may be organized into seven coiled-coil segments that are separated by short nonhelical linkers.

The NuMA protein sequence contains a number of other interesting sequence motifs. A potential nuclear localization signal, RRLKK, is located at the beginning of the α -helical region (position 227–231) and is similar to the nuclear localization signal consensus sequence K-R/K-X-R/K (X = any amino acid; Chelsky et al., 1989). Many protein kinase recognition sequence motifs are also present, principally in the carboxy-terminal domain. For example, two potential phosphorylation sites, RRAS and RRQS for cAMP-dependent protein kinase (RRXS; Feramisco et al., 1980) are found at positions 1,966–1,969 and 2,044–2,047, two recognition sites, REASLR and RPMPTR, for PKC (RXXS/TXR; Kemp and Pearson, 1990) are located at positions 592–596 and 2,012–2,016, four cdc2 kinase recognition sequence motifs (S/TPZX; where Z equals zero or one residue; Shenoy et al., 1989), TPRD, TPCK, SPRI, and TPRA are found at positions 2,015–2,018, 2,055–2,058, 2,087–2,090, and 2,106–2,109, respectively, and 19 sites for Ca^{2+} /calmodulin kinases (RXXS/T; Kemp and Pearson, 1990) are dispersed through the rod and carboxy-terminal domains. Thus, any one of a large number of sites in the NuMA protein are potentially modified by phosphorylation.

Although the NuMA protein is predicted to be slightly acidic overall (pI 5.5), the carboxy-terminal domain contains two particularly basic regions. One is located at position 1,830–1,908 (net charge = +7), and 2,029–2,116 (net charge = +19), which are separated by a linker (position 1,909–2,030). The possible significance of this feature is discussed below.

Cell Cycle Distribution of NuMA

Previous immunofluorescence studies using anti-NuMA antibodies demonstrated that NuMA associates early with the reforming nucleus at telophase (Price and Pettijohn, 1986). It has been suggested that NuMA may be important in the proper reassembly of the postmitotic nucleus. Lamins (particularly lamins A and C), which disassemble during prophase and are released into the cytoplasm, are among the first components known to reassemble in the reforming nucleus after mitosis (Burke and Gerace, 1986; see Discussion). We therefore examined the distribution of NuMA relative to nuclear lamins throughout mitosis, using r240-C (the rabbit anti-NuMA antibody) and LSI (a human antibody that recognizes lamins A and C; McKeon et al., 1983) in double immunofluorescence experiments of CV1 cells (Fig. 9). In parallel experiments, double immunofluorescence was per-

4501 ACTGCCCGGGAGCTGGAGGTGACTGCCAAGTATGAGGGTGCAGGTCAAGGTCCTGGAGGAGGACGCGTTCAGGAAAGAGAGGCAAACTCACTGCCAGGTGGAGCAGCTAGAGGATTTTCAGAGAGAGCAAACTAAGCAG
1501 **T A R R L L V N T A K Y E G A K V K V L L R R Q R P F O E R O K L T A Q V E Q L L E V F O R R E O T K O**

4651 GTGGAAGAACTGAGTAAAGAACTGGCTGACTCTGACCAAGCCAGCAAGGTGCAGCAGCAGAAGCTGAAGGCTCTCCAGGCTCAGGGAGGGGAGGAGCCAGGAGCCCGCCCTCCAGCCCGCACTGAATGAAGTCAAGCCAGCTG
1551 **V E E L L R K K L A D A D A D A S K V O Q O K L K A V Q A O G G R S O O E A Q R L Q A Q E N R L Q A Q I**

4801 AGCCAGAAGGAGCAGCCAGCTGAGCATAAAGCTGCAGATGCAGAAAGCCAAAACACATATGATGCCAAGAAGCAGCAGCAACAGAGCTGCAGGAGCAGCTGCCGAGCCCTGGAGCAGCTGCAGAAAGAAAACAAAGAGCTGCCAGCT
1601 **R O K R O A A R R Y E L O M E K A K T R Y D A K K O O L L O L R L O L R L E L L R A**

4951 GAAGCTGAACGGCTGGCCATGACCTACAGCAGGCTGGGTGAAGACCAAGGAGGCTGAACAGACCTGCCGCCACTTACTGCCAGTGGCCAGCTGGAGGCACAGTTGCCGATGCAGACAGCAGCTTCGAGACCTGGCAATTC
1651 **R A K R L R L R A Q V R R L R A Q V R A F A D Q L R D L G K F**

5101 CAGGTGGCAACTGATGCTTTAAAGACCGCTGAGCCCGAGCTAAGCCCCAGCTGGACTTGATTTGACAGCTGGATCTGAGCTGGAGGAGGGGACCCCACTCAGTATCACCAGCAAGCTGCCTCGTACCCAGCCAGCAGCCAGC
1701 Q V A T D A L K S R E P Q A K P Q L D L S I D S L D L S C E E G T P L S I T S K L P R T Q P D G T S

5251 GTCCTGGAAACCGCTCACCTATCTCCAGCGCTGCCCGCCAGTGAATCCCTGGAGAGTCTTACTTCACTCCATCCCTGCTGGAGTCAGGCCCCCTGGAGAGCAGCTGGACTCCCTGGGAGACGCTTCAGGACTCG
1751 V P G E P A S P I S Q R L P P K V E S L E S L Y P T P I P A R S Q A P L E S S L D S L G D V F Q D S

5401 GGTGTAAGACCGCTCCGCTCGTGGCCGACCAAGCAGATCATCAATCACCATGACCAAGAAGCTAGATGGAAGAGCCAGCAGGCGCAACTCATCGTTCTACAGCAGCGGCTGCTCTGCTGCCAGCTAGCTGGAGCC
1801 G R K T R S A R R R T T Q I I N I T M T K K L D V E E P D S A N S S F Y S T R S A P A S Q A S L R A

5551 ACCTCTTACTCAGTCTTCTAGCTCGCTGGTTCCTCCGATATGGCAACTCAGCCCTGCTCAGCTTGCTGGCTACCGCCCACTCAGCTGCTGCTGCTGCCAGGCCGGGTCCAGTGGGGCCCTCCAGGAAGGAAC
1851 T S S T Q S L A R L G S P D Y G N S A L L S L P G Y R P T T R S S A R R S Q A G V S S G A P P G R N

5701 AGTCTTACATGGGACTTGCAGSAGTGCAGCTGAGCAGCTGGATGACTGAACCGCATTCAGAGCTGCAGCAGCGCAATGGAGTGTGCCCGCAGATCTGAAGACCTGCTATCCCTGGAGTCCAGGCTTCCTGAGCTGGGCACC
1901 S F Y M G T C Q D E P E Q L D D W N R I A E L Q Q R N R V C P P H L K T C Y P L E S R P S L S L G T

5851 ATCACAGATGAGGAGATGAAAACGGAGACCCCAAGAGACCCCTGGCCGAGCCAGCATGCAGCAATCCAGATAGCCAGGGCACTGGCATCACCACCCGAGCAGCGTTAAAGGGTCTCCCTAGAGCCCCACAGGCCCTGGAACT
1951 I T D E E M K T G D [Q E T L R R A S M Q P I Q I A E C T G I T T R Q Q R K R V S L E P H Q G P G T

6001 CCTGAGTCTAAGAAGGCCACCAGCTGTTCCACGCCCATGACTCCCGAGACCGACATGAAGGGCCAAACAGAGCACTACTGAGGCCAGAAGAAGCAGCTCCAGCTTCTACTAAACAGGCTGACCGCCAGCTCGATGGCCCTC
2001 P E S K K A T S C F P R P M T P R D R H E G R K Q S T T E A Q K K A A P A S T K Q A D R R Q S M A F

6151 AGCATCTCAACACACCCAAAGAGCTAGGAAACAGCCTTCTGGCGGGGAGCCTCAAAAGCCCTGTCGAAGGCTTCCCCAACACTCGCATGGAACCCCGCTTCTCCGCGCATTCGCCACACACAGCAGCCCGCCACTGCT
2051 S I L N T P K K L G N S L L R R E A S K K A L S K A S P N T R S G T R R S P R I A T T T A S A A T A

6301 GCCGCCATTTGCTGCCACCCCTCGAGCCAAAGGCAAGGCCAAAGCACTAA
2101 A A I G A T P R A K G K A K H Z

6349 AGGGCAGTACCAGTGGTGGCCCACTGTGTCCCGATGCTGCGGTCACTGGTCTCCCGCTACTGTCCCTCTCAGTGCCTTCTCAGCTCCAGGCCAAGAGTGGCAAAACCCCTAGAGACAGTGCCTGCCCGCACCCCTGGC
6499 CTGGTCCCTGGTCTTCACTGGCCCTTCTCGGAGCTGGCCAGGGCCCTGGAGCATGGACAGTGTGGGCGCTCTCCCTACTTGCCTCTTTTCTTAAAGCAAGTCACTTCTGCATCACAAACAGATTTGAGGCTGGTTTTGATG
6649 GCTGGTCTTGGGCTGGCCACTTCTCTTAGCCTTGGATCTAGAAGGGACATAAGAGGAGTAGGCCCTGGTCTCCTGCTGCTCGTGGTGGCCAGCAGGGGCCCTCACTTGAAGTCCAGACTGGGCTGACCTGGTGG
6799 GAGCACTGCCAGAGGATGCTCTTCCAGGACGGATGGCCCTGTGTCTCAGGAGTGGGGTGGGGGACAGCCTTCAGCAGCAGCTCACACCCCTACTTCCCAGACTTGCATGGGTGGGATTTGGAGTGATGGAAAGTTTTAAG
6949 CGCCGGGATGGATCTTTCTAAATGTTACTTGTAAATCCCGAATTC

Figure 7.

formed with r240-C and 5051, an anticentrosome antibody (Tuffanelli et al., 1983), in order to examine the distribution of NuMA relative to the centrosome (Fig. 10). The stages of the cell cycle were assessed by the state of chromosome condensation. At least 30 cells were viewed at each of the stages described below.

In interphase cells, NuMA is present throughout the nucleus, except in the nucleolus (Fig. 9 A). No immunostaining of the centrosome is observed, even after the centrosomes have duplicated and separated in preparation for mitosis (Fig. 10 A).

The mitotic redistribution of the NuMA protein com-

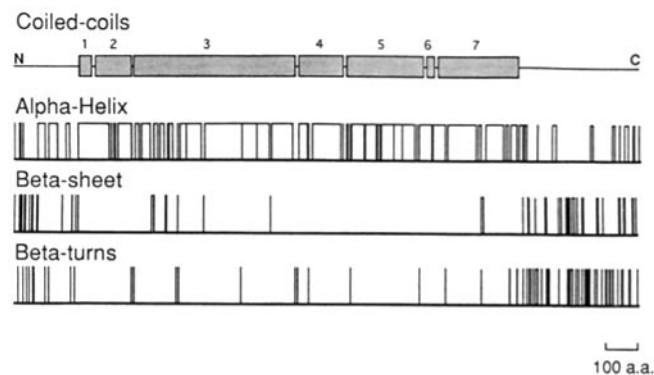
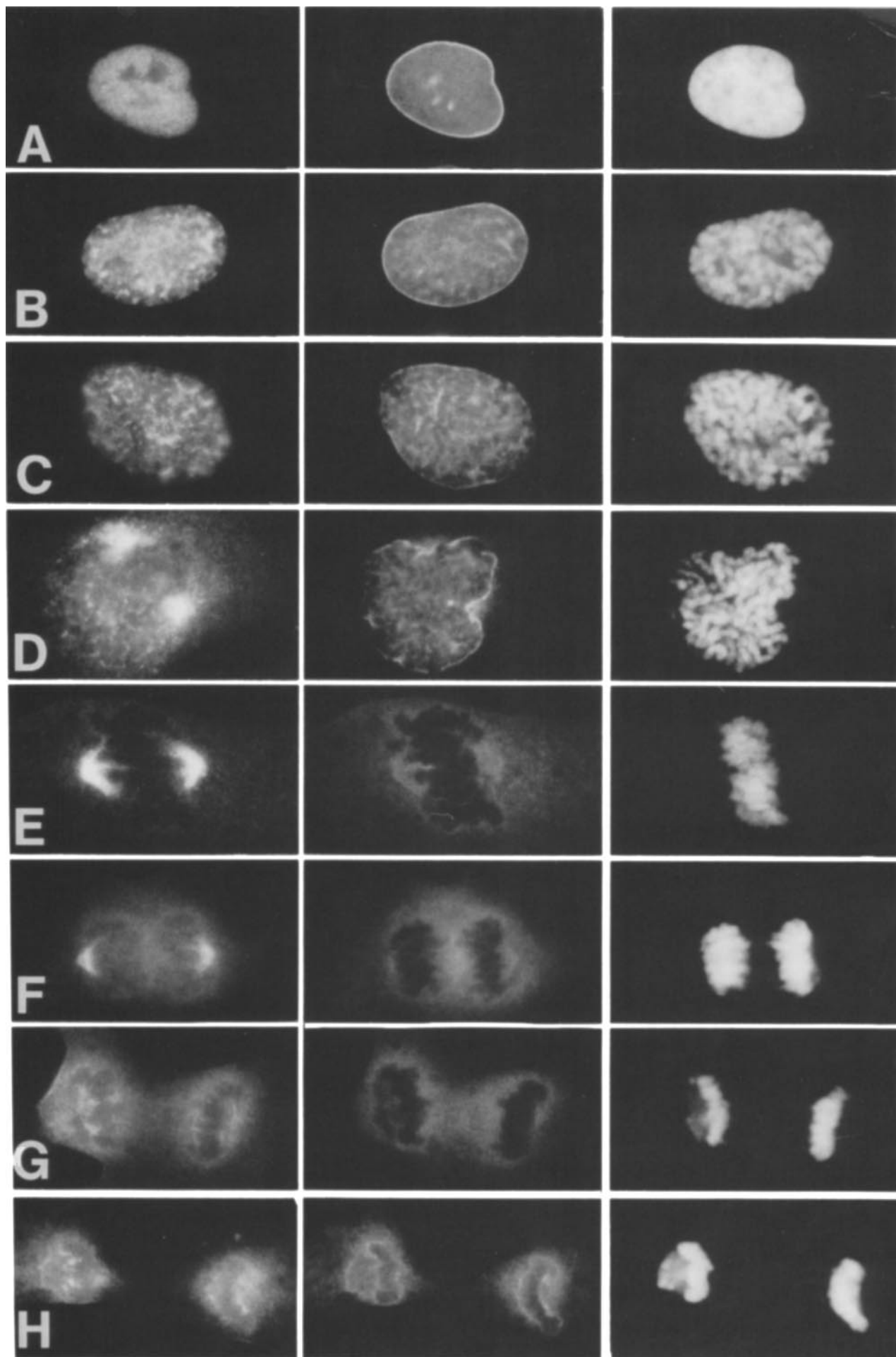


Figure 8. The predicted secondary structure of the NuMA protein. The propensity of the predicted NuMA protein sequence to form α -helix, beta-sheet, or beta-turns was calculated using programs derived by Garnier et al. (1978). The regions of the NuMA protein predicted to be involved in forming coiled-coil dimers are depicted in the top panel.

mences very early in prophase, and appears to be coincident with chromosome condensation. However, NuMA is not a component of the mitotic chromosome; rather it is excluded into the interchromosomal space (Fig. 9 B). Interestingly, the nuclear lamina appears to be largely intact at this early stage of mitosis, although a small reduction in fluorescence signal is evident. By late prophase, NuMA is still in the nuclear region; the intensity of the lamina signal at the nuclear rim is considerably diminished and partly overlaps the NuMA immunostaining pattern (Fig. 9 C).

A clear breach in the integrity of the nuclear lamina is quite evident at prometaphase (Fig. 9 D). In these cells, the lamina appears as a discontinuous structure, and some dispersion of the chromosomes into the surrounding cytoplasm is also apparent. NuMA begins to accumulate at the centrosomes at this stage. As shown in Figs. 9 D and 10 B, NuMA is apparent at both the centrosomes and the interchromosomal spaces during this transitional period. By metaphase and through anaphase, NuMA assumes a crescent-shaped distribution about the polar regions of the spindle apparatus (Figs. 9, E and F; 10 C; also compare with Fig. 1).

A dramatic redistribution of NuMA follows at late anaphase/early telophase and proceeds rapidly (Figs. 9, G and H, 10, D and E). At late anaphase some staining about the spindle pole region is visible (Fig. 10 D), however, by early telophase, little, if any, NuMA immunostaining can be detected at the spindle poles (Fig. 10 E). In addition, at the early stages of nuclear reformation, NuMA clearly begins accumulating at the periphery of the chromosomes, while the nuclear lamins have not yet concentrated in this region (Fig. 9 G). Lamins remain excluded from the surfaces of the chromosomes until the later stages of telophase or cytokinesis



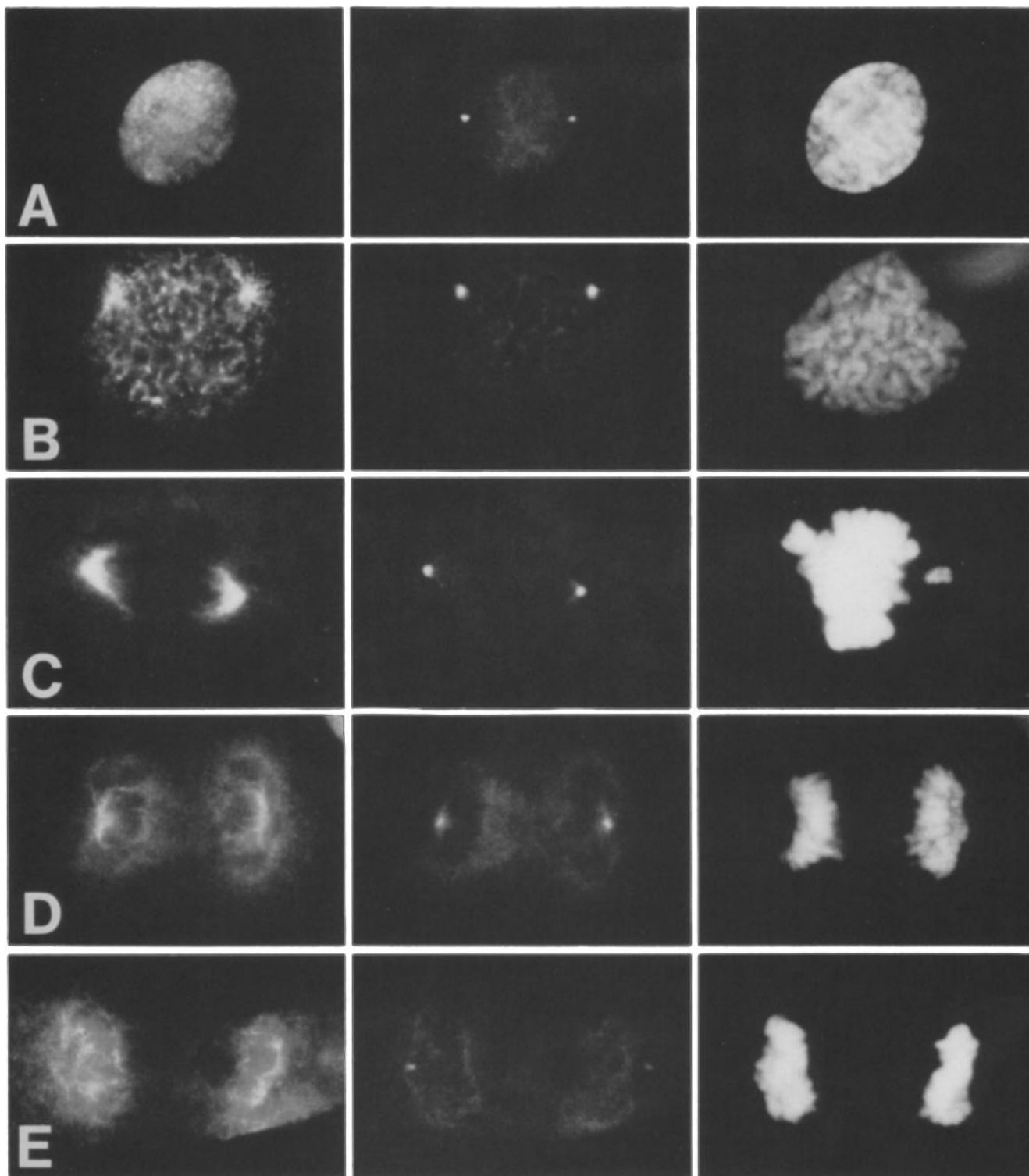


Figure 10. Double immunofluorescence with rabbit anti-NuMA antibodies (r240-C) and human antacentrosome antibodies (5051). (*A-E*) *left*, anti-NuMA staining; *center*, antacentrosome staining; *right*, Hoechst 33258-stained DNA. A representative cell of each stage of cell cycle is depicted (*A*) Prophase. (*B*) Prometaphase. (*C*) Metaphase. (*D*) Late anaphase. (*E*) Telophase.

Figure 9. Double immunofluorescence with rabbit anti-NuMA antibodies (r240-C) and human antilamin antibodies (LS-1). (*A-H*) *left*, anti-NuMA staining; *center*, antilamin staining; *right*, Hoechst 33258-stained DNA. A representative cell at each stage of the cell cycle is presented. (*A*) Interphase. (*B*) Early prophase. (*C*) Late prophase. (*D*) Prometaphase. (*E*) Metaphase. (*F*) Anaphase. (*G*) Late anaphase. (*H*) Telophase.

(Fig. 9 H). At these later times, a bright evenly distributed lamin fluorescence signal emanates from the entire periphery of the chromosomes. Although NuMA is also localized to the periphery of the chromosomal mass of late telophase cells, NuMA fluorescence signal also appears as intense patches and streaks that project into the interchromosomal regions. Thus, at this level of resolution, bulk redistributions of the NuMA protein appear to precede large changes in the lamins A and C localization in prophase, and the NuMA protein concentrates to the reforming nucleus before the nuclear lamins.

Discussion

We have identified several human sera that recognize an antigen(s) that undergoes an unusual cell cycle-dependent distribution pattern. Immunological criteria indicate that this antigen is the NuMA protein, first described by Lyderson and Pettijohn as a large insoluble nuclear protein that localizes to the polar regions of the spindle apparatus during mitosis (Lyderson and Pettijohn, 1980). Overlapping cDNAs that encode the NuMA protein were isolated and analyzed. The cDNAs contain a long open reading frame capable of encoding a protein of 2115 amino acids with a molecular mass of 238,085 D. Analysis of the predicted protein sequence suggests that NuMA is structurally similar to a class of fibrous proteins such as myosins and intermediate filaments that are capable of forming coiled-coil dimers *in vitro* and can aggregate into supramolecular fibrous structures in the cell. Based on these results, NuMA is probably a structural component of the nucleus; perhaps as a component of the eukaryotic nucleoskeleton. Its early association with the reforming nucleus suggests that it may play a role in nuclear assembly. These interpretations are discussed more fully below.

NuMA Is a Large Coiled-Coil-related Protein

NuMA is predicted to possess a three domain structure, consisting of a very long α -helical central domain of 1485 amino acids that is flanked by nonhelical end domains. The central domain is marked by the presence of repeating heptad units in which hydrophobic residues predominate at the first and fourth positions of each unit. In other coiled-coil proteins, the apolar residues align on one side of the α -helix and form hydrophobic interactions with the α -helix of another molecule to form a coiled-coil dimer (Cohen, 1990; Cohen and Parry, 1986). NuMA might also form a coiled-coil dimer via similar interactions in the α -helical domain. Sedimentation analysis indicates that native NuMA has an asymmetric structure, a feature expected of a coiled-coil protein (Price and Pettijohn, 1986).

Consistent with the secondary structure predictions, searches of sequence databases indicate that the predicted NuMA sequence is similar to that of other coiled-coil type proteins, including myosins, nuclear lamins, and intermediate filaments. The NuMA rod domain shares ~20% identity with the rod domains of the proteins of this class. Highest similarity scores were obtained with the myosins, because these proteins contain the longest coiled-coil regions. The 1,485 amino acid central coiled-coil region of NuMA is longer than myosin rods, which are ~900–1,000 amino acid residues in length, and the recently described plectin, which has a coiled-coil 1,400 residues long (Wiche et al., 1991).

In fact, NuMA contains the longest coiled-coil region described to date. We note that NuMA, unlike myosins, is not predicted to have an ATP binding domain and therefore is probably not a motor protein.

Although heptad periodicity is evident throughout the NuMA rod domain, short 5–19 amino acid stretches containing prolines interrupt this domain. Since prolines often disrupt α -helices, these linkers probably disrupt the NuMA central region into seven coiled-coil segments. Because coiled-coil dimers are probably most stable when the overlap between interacting α -helices forms the most hydrophobic interactions (Aebi et al., 1988), two NuMA molecules may interact in parallel and in register to form a dimer, such that the helical and nonhelical portions of the rod domains are correspondingly matched. Other coiled-coil type proteins which possess such aperiodically spaced linkers, except the cytokeratins, have been shown to dimerize in this manner (Hatzfeld et al., 1987). The nonhelical linkers may be important for interactions with other proteins, and/or for higher order structures of NuMA dimers (see below).

The general features of NuMA, a three domain-structure and short linkers that disrupt the α -helical coiled-coil, are similar to those of intermediate filaments. However, NuMA does not appear to be closely related to the intermediate filament family, because it lacks the conserved organization of α -helical segments and linkers in its rod domain, as well as particular sequence motifs that are characteristic of the intermediate filaments (McKeon et al., 1986; Steinert and Roop, 1988).

The function of the NuMA nonhelical terminal domains is not known, but they may be important in the assembly of higher order structures. A head-to-tail interaction between the terminal domains of intermediate filament dimers has been suggested to be important in the formation of lamin A and B filaments (Heitlinger et al., 1991; Moir et al., 1991). Interestingly, since NuMA has an inherent polarized charge distribution, it is possible that the basic carboxy nonhelical domain may associate with the more acidic amino domain to form a similar higher order structure. The carboxy-terminal basic domain may have other functions as well, such as binding to DNA or microtubules as is described below.

Phosphorylation of NuMA

Phosphorylation has been shown previously to be important in regulating the assembly of other fibrous coiled-coil proteins, including myosins and intermediate filaments (Evans, 1988; Inagaki et al., 1988; Kuczmarzski and Spudich, 1980; Pasternak et al., 1989). In particular, the mitotic reorganization of the nuclear lamina and vimentin intermediate filaments is believed to be specifically regulated by phosphorylation of the cdc2 protein (Chou et al., 1990; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990), a kinase that controls the entry into mitosis (for review see Draetta, 1990). In the case of the lamins, independent biochemical and mutation analyses have indicated that phosphorylation of conserved serines that flank the ends of the α -helical domain by the cdc2 kinase initiates the process of lamina disassembly (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990).

The NuMA protein has been previously shown to be phosphorylated *in vivo* (Price and Pettijohn, 1986). A survey of

the NuMA protein sequence reveals numerous potential kinase target sequences including at least four potential cdc2 recognition site sequences in the carboxy terminal domain. 11 other possible sites not matching this consensus sequence, but containing motifs that still might be recognized by cdc2 kinase (see Draetta, 1990; Moreno and Nurse, 1990) are present in the amino- and carboxy-terminal NuMA sequence. These cdc2 phosphorylation sites may be important in the regulation of the disassembly/assembly of NuMA protein from higher order structures during mitosis in a manner analogous to nuclear lamins. Regulation by the cdc2 kinase is consistent with the dramatic rearrangement of NuMA that occurs during prophase.

NuMA Redistributes during Mitosis and Assembles Very Early with the Reforming Nucleus at Telophase

Double immunofluorescence experiments with anti-NuMA and antilamin antibodies were performed to study the dynamics of NuMA localization through the cell cycle. Because NuMA may be a novel nuclear structural protein, we were particularly interested in examining the redistribution of this antigen at prophase and telophase to gain possible insights into the process of nuclear breakdown and reformation. Since lamins undergo dramatic rearrangements at these periods, it is also of interest to know the temporal order of events in which nuclear envelope rearrangements and NuMA redistribution occur. The results of these experiments indicate that as cells enter mitosis, the redistribution of the NuMA antigen is an early prophase event, which is concomitant with chromosome condensation. At this level of analysis, dramatic rearrangements in the distribution of NuMA appears to precede significant disassembly of the nuclear lamins. The accumulation of the NuMA protein at the spindle poles only becomes apparent after complete dissolution of lamins A and C, and NuMA remains concentrated about the spindle pole region through metaphase and anaphase.

Surprisingly, the redistribution of NuMA onto the segregating chromosomes at telophase appears to precede substantial binding of lamins onto the surfaces of the chromosomes. The NuMA fluorescence signal at the periphery of the chromosomal mass becomes readily apparent while lamins A and C staining is still considerably more diffuse in the cell.

The result is intriguing. At present, the complex process of postmitotic nuclear reformation is not well understood, but appears to involve several distinct phases (Benavente, 1991). In the first step, condensed chromosomes become enclosed by an intact nuclear envelope that partitions the nucleus and the cytoplasm. In the second step, nuclear components are incorporated into the reforming nucleus by transport through the nuclear pore complexes. Finally, chromosome decondensation and nucleolus formation occur resulting in an interphase-like nucleus.

It is presently unclear whether lamins participate in the earliest step of nuclear assembly or slightly later ones. In vitro experiments with CHO extracts indicate that lamins A and C bind to mitotic chromosomes (Burke, 1990; Glass and Gerace, 1990). Subsequent binding by membrane-bound lamin B is thought to promote assembly of the nuclear membrane (Burke, 1990; Burke and Gerace, 1986; Gerace and Blobel, 1980). However, in vivo experiments in PtK₂ cells indicate that inhibition of nuclear transport blocks the ac-

cumulation of lamins in the nucleus (Benavente et al., 1989), suggesting that these proteins may assemble after the envelope has already formed. In addition, Newport et al. (1987, 1990) have shown that in *Xenopus*, a nuclear vesicle receptor may bind chromosomes and initiate independent nuclear reassembly prior to assembly of the nuclear lamina. Thus, it remains unclear as to whether lamins stimulate the early steps of nuclear assembly or assemble in later events.

Our result that NuMA associates with condensed chromosomes before lamins suggests that NuMA may play an early role in nuclear reformation. To date, only one other protein, the putative 54-kD nuclear lamin receptor, has been shown to reassociate with chromosomes before lamins during nuclear reformation (Bailer et al., 1991). However, because NuMA is a component of the nuclear interior, it may participate in internal nuclear organization rather than as a surface receptor for participating in nuclear envelope assembly. NuMA may even bind DNA via the basic regions in the carboxy terminus (Churchill and Travers, 1991) and thereby initiate nuclear assembly events.

Involvement of NuMA with the Mitotic Spindle Apparatus

One mechanism by which NuMA may associate with the chromosomes very early in nuclear reassembly is via its interaction with the mitotic spindle apparatus. NuMA concentrates at the poles; when the chromosomes are drawn to this region, NuMA may be able to immediately bind to the chromosomes and begin nuclear reassembly.

Interaction of NuMA with the polar region of the spindle apparatus may also reflect a functional role of this protein during mitosis. NuMA might enhance the nucleation or stabilization of microtubules, or play some other, as yet unknown, role.

Although NuMA associates with the mitotic spindle apparatus, it does not appear to contain any known microtubule-binding motifs. At present, the microtubule-binding regions of kinesin (Yang et al., 1989b), MAP2 (Himmler et al., 1989), tau (Lewis et al., 1988), MAPIB (Noble et al., 1989), and 205-kD MAP (Irminger-Finger et al., 1990) have been identified. The predicted NuMA protein sequence does not contain similarity to any of these proteins. However, NuMA contains a very basic carboxy domain. This region might be important in forming electrostatic interactions with the acidic regions of tubulin (Maccioni et al., 1988). Alternatively, NuMA may bind to other components that comprise or interact with microtubules and thereby associate with the spindle apparatus.

NuMA as a Possible Component of the Mammalian Nucleoskeleton

As discussed above, analysis of the predicted amino acid sequence suggests that NuMA is a structural coiled-coil type protein. Like myosins, intermediate filaments, and other coiled-coil proteins, NuMA may form fibrous higher-order structures. Thus, the predicted structure of NuMA as well as its tight association with the nucleus (Lyderson and Pettijohn, 1980) suggest that NuMA is a structural component of the mammalian nucleoskeleton. A putative nuclear matrix composed of filamentous structures has been described by various groups (Berezney and Coffey, 1974, 1977). Most re-

cently, Jackson and Cook (1988), and He et al. (1990) have described the visualization of 10-nm-diameter nuclear filaments which exhibit a 23-nm axial repeat. These dimensions are very similar to those of cytoplasmic intermediate filaments and the nuclear lamina. The 23-nm repeat is thought to be the result of hierarchical organization in intermediate filament structure in which two dimers containing 45-nm rod domains interact laterally in a half-staggered array (Aebi et al., 1988).

Based on the characteristic dimensions of α -helices (Creighton, 1984; also see Mirzayan et al., 1992), we predict that the NuMA rod domain is \sim 200 nm in length. If NuMA is capable of assembling into higher-order structures in a manner similar to that of intermediate filaments, a \sim 10% stagger between NuMA dimers would be required in order to achieve a 23-nm axial repeat. Whether NuMA is capable of such an organization is not known. Alternatively, NuMA may not be a component of the 23-nm axial repeat fibers. Rather, NuMA might function as a cross-linking molecule between various filaments in a similar capacity as cytoplasmic intermediate filament-associated proteins such as plectin (Wiche et al., 1991).

Other coiled-coil type proteins may be present in the nucleus. In the accompanying manuscript (Mirzayan et al., 1992) an essential yeast protein, the NUF1 protein, is described and is also predicted to encode a large coiled-coil protein. Anti-NUF1 antibodies recognize a related protein in mammalian cells. However, the distribution of the mammalian NUF1 protein is different from that of NuMA, since the mammalian NUF1 antigen is not present at the spindle apparatus. Thus, there are probably more than one coiled-coil proteins in the mammalian nucleus, raising the possibility that many proteins of this type may function in organizing the eukaryotic nucleoskeleton.

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Note Added in Proof. Recently, B. R. Brinkley and co-workers have identified centrophilin, a protein with a similar subcellular distribution as that of NuMA (Tousson et al., 1991). Comparison of 450 nucleotides of a cDNA clone encoding centrophilin (B. R. Brinkley, personal communication) with that of the NuMA DNA sequence reveals that they are the same. Thus, NuMA and centrophilin appear to be the same protein.

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