INTRODUCTION

The nuclear mitotic apparatus protein (NuMA) was first described by Lyderson and Pettijohn (1980) as a predominantly nuclear protein that is present in the interphase nucleus and is concentrated in the spindle pole of mitotic cells. Recently, several nuclear proteins with an immunofluorescence staining pattern similar to that of NuMA have been defined. These proteins, including centrophilin (Tousson et al., 1991), SPN (Kallajoki et al., 1991), SP-H (Maekawa et al., 1991), 1H1/1F1 (Compton et al., 1991) and W1 (Tang et al., 1993b), have been defined by various monoclonal antibodies or autoimmune sera. According to the amino acid or nucleotide sequences obtained, NuMA (Compton et al., 1992; Yang et al., 1992), SPN (Kallajoki et al., 1993), SP-H (Maekawa and Kuriyama, 1993), 1H1/1F1 (Compton et al., 1992) and W1 (Tang et al., 1993b) represent different names for the same protein.

Recently, cDNA clones that cover the entire coding region of human NuMA have been isolated and sequenced (Compton et al., 1992; Yang et al., 1992; Tang et al., 1993b; Maekawa and Kuriyama, 1993). Secondary structure predictions reveal that NuMA is composed of a long α-helical central core flanked by two globular domains (Compton et al., 1992; Yang et al., 1992; Tang et al., 1993b; Maekawa and Kuriyama, 1993). Interestingly, the central α-helical region of NuMA shares a high degree of sequence similarity with myosin, lamins, intermediate filaments and other coiled-coil proteins. This observation suggests that NuMA belongs to the large coiled-coil protein family.

On the basis of microinjection and cell-cycle-dependent localization experiments, several laboratories have proposed that NuMA may play a role in maintaining or establishing nuclear structure, possibly as a structural component of the nuclear matrix (Lyderson and Pettijohn, 1980; Price and Pettijohn, 1986; Kallajoki et al., 1991; Compton et al., 1992; Yang and Snyder, 1992). Recently, Compton and Cleveland (1993) have shown that expression of human NuMA lacking its globular head or tail domains may induce cells to become micronucleated. Moreover, expression of wild-type human NuMA in tsBN2 cells, in which the endogenous NuMA protein is normally degraded, resulting in micronuclei at a restrictive temperature, was sufficient to suppress the micronucleation phenotype. These results lead them to suggest that NuMA is required for the proper terminal phases of chromosome separation and/or nuclear reassembly during mitosis. In addition, microinjection of anti-NuMA antibodies into early mitotic or metaphase cells prevents the formation or causes the collapse of the mitotic spindle apparatus (Yang and Snyder, 1992), suggesting that NuMA may play an important role during mitosis.

We have recently isolated a series of overlapping cDNA clones that cover the entire coding region of human NuMA (Tang et al., 1993b). Analysis of various cDNA clones revealed that NuMA is composed of at least three isoforms that differ mainly at the carboxy terminus, and are generated by alternative splicing of a common mRNA precursor from a single NuMA gene (J. Cell Sci. (1993) 104, 249-260). Transient expression of human NuMA-l isoform (T33/p230) in Chinese hamster ovary polyoma (CHOP) cells showed that NuMA-l was present in interphase nuclei and was concentrated at the polar regions of the spindle apparatus in mitotic cells. However, expression of two other isoforms (NuMA-m and -s) revealed a distinct subcellular localization. NuMA-m (U4/p195) and NuMA-s (U6/p194) were present in the interphase cytosol and appeared to be mainly located at the centrosomal region. When cells entered into mitosis, however, NuMA-m and -s moved to the mitotic spindle pole. Analysis of a series of linker scanning-mutants and NuMA/β-galactosidase chimeric proteins showed that residues 1972-2007 of NuMA-l constitute a novel nuclear localization signal (NLS) and residues 1538-2115 are necessary and sufficient for spindle association. Further analysis of the NLS by site-specific mutagenesis indicated that Lys1988 is essential for nuclear targeting, whereas Arg1984 is not. These results have allowed us tentatively to assign specific biological activities to distinct structural domains of the NuMA polypeptide.

Key words: nuclear protein, NuMA, centrosome, spindle pole, nuclear localization signal

SUMMARY

We have recently shown that the nuclear mitotic apparatus protein (NuMA) is composed of at least three isoforms that differ mainly at the carboxy terminus, and are generated by alternative splicing of a common mRNA precursor from a single NuMA gene (J. Cell Sci. (1993) 104, 249-260). Transient expression of human NuMA-l isoform (T33/p230) in Chinese hamster ovary polyoma (CHOP) cells showed that NuMA-l was present in interphase nuclei and was concentrated at the polar regions of the spindle apparatus in mitotic cells. However, expression of two other isoforms (NuMA-m and -s) revealed a distinct subcellular localization. NuMA-m (U4/p195) and NuMA-s (U6/p194) were present in the interphase cytosol and appeared to be mainly located at the centrosomal region. When cells entered into mitosis, however, NuMA-m and -s moved to the mitotic spindle pole. Analysis of a series of linker scanning-mutants and NuMA/β-galactosidase chimeric proteins showed that residues 1972-2007 of NuMA-l constitute a novel nuclear localization signal (NLS) and residues 1538-2115 are necessary and sufficient for spindle association. Further analysis of the NLS by site-specific mutagenesis indicated that Lys1988 is essential for nuclear targeting, whereas Arg1984 is not. These results have allowed us tentatively to assign specific biological activities to distinct structural domains of the NuMA polypeptide.

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INTRODUCTION

The nuclear mitotic apparatus protein (NuMA) was first described by Lyderson and Pettijohn (1980) as a predominantly nuclear protein that is present in the interphase nucleus and is concentrated in the spindle pole of mitotic cells. Recently, several nuclear proteins with an immunofluorescence staining pattern similar to that of NuMA have been defined. These proteins, including centrophilin (Tousson et al., 1991), SPN (Kallajoki et al., 1991), SP-H (Maekawa et al., 1991), 1H1/1F1 (Compton et al., 1991) and W1 (Tang et al., 1993b), have been defined by various monoclonal antibodies or autoimmune sera. According to the amino acid or nucleotide sequences obtained, NuMA (Compton et al., 1992; Yang et al., 1992), SPN (Kallajoki et al., 1993), SP-H (Maekawa and Kuriyama, 1993), 1H1/1F1 (Compton et al., 1992) and W1 (Tang et al., 1993b) represent different names for the same protein.

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We have recently isolated a series of overlapping cDNA clones that cover the entire coding region of NuMA (Tang et al., 1993b). Analysis of various cDNA clones revealed that
their sequences were identical, except for six sequence blocks (75 bp, 42 bp, 45 bp, 576 bp, 1012 bp and 212 bp), which were either inserted or deleted in individual cDNA clones. Among these, two sequence blocks (75 bp and 42 bp), which encode a 25 and a 14 amino acid peptide, respectively, are located at the middle portion of the NuMA cDNA. In contrast, the other four blocks (45 bp, 576 bp, 1012 bp, 212 bp), some of which carry a translation termination codon, are located at the 3′ end of the NuMA transcript (Tang et al., 1993b).

Interestingly, combinatorial splicing of four sequence blocks located at the 3′ end of the transcript may generate at least three different NuMA isoforms that differ at their carboxy termini. These isoforms are generated from a single RNA precursor by alternative splicing (Tang et al., 1993b). The T33 form of NuMA mRNA codes for 2115 amino acids, corresponding to the longest polypeptide of ~230 kDa (p230). Two other NuMA isoforms, which use different translation termination codons located in different 3′ sequence blocks, would produce polypeptides of lower molecular masses. The U4 form of the mRNA would code for 1776 amino acids, corresponding to p195, and the U6 form would code for 1763 amino acids, corresponding to p194. For convenience in describing these different isoforms, the T33/p320 form is now renamed NuMA-l (large), the U4/p195 form NuMA-m (medium), and the U6/p194 form NuMA-s (small). All the isoforms described here contain both the 75 and 42 bp sequence blocks (Tang et al., 1993b) that encode 25 and a 14 amino acid (aa) peptides, respectively. For the isoform that lacks the 14 amino acid peptide, the name of NuMA-x (-14 aa) may be used, where x represents large (l), medium (m), or small (s). According to this principle, the NuMA-x (-14 aa) represents an isoform that lacks the 14 amino acid peptide, and so on.

To find out whether any particular isoform shows a subcellular localization identical to that reported for the original NuMA, we have now expressed these three different isoforms of NuMA in cultured cells. Expression of the largest isoform (NuMA-l) showed a cell-cycle-dependent distribution indistinguishable from that of the original NuMA in human cells, i.e. present throughout the interphase nuclei but concentrated at the spindle poles during mitosis. To our surprise, expression of the largest isoform (NuMA-l) showed a cell-cycle-dependent distribution indis -

**MATERIALS AND METHODS**

**Cell culture**

The Chinese hamster ovary cell line that expresses polyoma large T antigen (CHOP) was kindly provided by Dr James W. Dennis, Toronto, Canada. CHOP cells were propagated and maintained in αMEM containing 10% fetal calf serum (FCS) and 200 µg/ml of G418 (Gibco) as described previously (Heffernan and Dennis, 1991).

**Construction of deletion and chimeric expression plasmids**

Full-length human NuMA cDNAs were assembled and constructed from overlapping cDNA fragments (Tang et al., 1993b) by standard cloning methods (Aussel et al., 1989; Sambrook et al., 1989). The 3′ end DNA fragments of NuMA-l, -m and -s were derived from T33, U4 and U6 cDNA clones, respectively (Tang et al., 1993b). The EcoRI fragments containing the entire coding sequence for NuMA-l, -m or -s were then inserted into the unique EcoRI site of a eukaryotic expression vector, pSG5 (Strategene), which contains an SV40 early promoter, intron II of the rabbit β-globin gene, and the SV40 polyadenylation signal. These constructs were renamed SV/NuMA-l, -m and -s, respectively (see Fig. 1A). All cDNA constructs contained both the 75 bp and 42 bp sequence blocks (Tang et al., 1993b) in their coding sequences. A series of truncated linker spanning mutants (see Fig. 6A) were generated by partial digestion of SV/NuMA-l cDNA with NarI (L1), XhoI (L12) or StyI (L24, L36, L42), filling the 3′ recessed termini with the Klenow fragment of Escherichia coli DNA polymerase I in the presence of the appropriate dNTPs and ligated with a phosphorylated XbaI linker (Strategene). The linker is a short oligonucleotide (CTAGTCTAGACACTAG) that contains an XbaI restriction site and stop codons in all three reading frames. This construction allowed us to generate a series of truncated mutants.

For generation of NuMA-l/galactosidase expression plasmids, various lengths of NuMA-l DNA fragments were PCR-amplified from the tail region of NuMA-l cDNA by several primer sets. The primers selected for generation of a series of pc NuMA-l/galactosidase constructs (see Figs 7 and 9) were as follows: pc1 (4611-4629/6497-6517), pc6 (4611-4629/5906-5925), pc18 (5514-5533/6268-6287), pc20 (5913-5932/6268-6287), pc21 (5913-5932/6160-6179), pc23 (6087-6106/6160-6179), pc24 (5913-5932/6004-6023), pc25 (6003-6022/6085-6104), pc30 (5940-5960/5964-5983) and pc31 (5940-5960/6004-6023). The numbers in parenthesis indicate the nucleotide positions of NuMA according to the numbering system of Yang et al. (1992). The PCR amplified fragments were first subcloned into the PCR II vector (Invitrogen) for the convenience of direct cloning and restriction site exchange. The PCR-amplified products in PCR II were then released by EcoRI digestion followed by ligation into the unique EcoRI site located downstream of the carboxy terminus of the lacZ gene in pCH110 vector. Because the primers for PCR amplification of NuMA-l cDNA were specifically selected, the final PCR amplified products would be cloned in-frame with the lacZ gene. For generation of the pc7 construct, which encodes a short polypeptide (seven amino acids) of NuMA-l, two oligonucleotides (N3: 5′-GGATCCACGGCAAACGGCTCCGGAATTCCA-3′) and N4: 5′-CGCTTAAGGTCGTCGCGTTTGCCCAGAGGCTTAAGGT-3′), which each carried an EcoRI recognition site at each end and were complementary to each other, were synthesized. Equal amounts of complementary oligonucleotides were then annealed, followed by EcoRI digestion. The EcoRI-released fragment was then subcloned into the unique EcoRI site of the lacZ gene in the pCH110 vector. For construction of site-directed mutagenesis vectors, pc24 was selected as a construction backbone for generation of both pc24N1 and pc24N2 mutant constructs. The primers selected for generation of these vectors were as follows: pc24N1 (5′-CATTCAACCAGGG-CAGCAGC-3′); pc24N2 (GCAGCAGCAGGCGTTACGGTTC). The site-directed mutagenesis clones were generated by using the altered sites in the in vitro mutagenesis system as described by the manufacturer (Promega). The same mutagenesis primer as described for pc24N1 was used to produce two other constructs, pc30 and pc31, which carry a single amino acid substitution at residue 1984 of NuMA-l. The cDNA sequences in all the constructed mutants were confirmed by DNA sequencing.

**Cell transfection and immunoechemical analysis**

CHOP cells growing on glass coverslips were transiently transfected using Lipofectin (Gibco-BRL) reagent as described by the manufacturer. (Lipofectin reagent is a 1:1 (w/w) lysosome formulation of the cationic lipid N-[1-(2,3-dioleoyloxy)-propyl] -N,N,N-trimethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE).) Briefly, 2 µg of plasmid DNA diluted in serum-free medium
was mixed with 7 µl of Lipofectin reagent and incubated at room temperature for 10-15 minutes. The solution containing Lipofectin reagent-DNA complexes was layered over cells at 60% confluency and incubated for 6 hours at 37°C. The cells were then washed with PBS (phosphate buffered saline), and grown in complete medium containing 10% FCS for 18-20 hours. The transfected cells were then fixed in methanol/acetone (1:1, v/v) at −20°C for 5 minutes. Monoclonal antibody W1 (Tang et al., 1993b) was applied to the cells and incubated for 60 minutes at 37°C in a humidified chamber. Coverslips were washed in PBST (PBS containing 0.1% Tween-20) and the bound antibodies were detected with fluorescein-conjugated rabbit anti-mouse antibodies (Cappel Laboratories). DNA was detected with propidium iodide (2.7 µg/ml; Sigma Chemical Co.). For double-labeling experiments, transfected cells were fixed and stained with mAb W1 and anti-tubulin antibody as described (Tang et al., 1993b). Coverslips were mounted and observed with a laser scanning confocal microscope (MRC600 model; Bio-Rad Laboratories) for all immunofluorescence photos described in this paper, except Fig. 8A) or with a Zeiss Axiophot microscope equipped with epifluorescence (for Fig. 8A only).

For immunoblot analysis, CHOP cells were transfected with Transfectam reagent (Promega), since the transfection efficiency for Transfectam in CHOP cells was much higher than that observed for Lipofectin. Transfection efficiency was determined by analysis of 500 CHOP cells that had been transfected with SV/NuMA-l cDNA and detected by mAb W1. In general, 10-20% of the inoculated CHOP cells were positively stained by mAb W1 when the Transfectam reagent was used, whereas only 2-5% positive cells were observed using the Lipofectin reagent. However, CHOP cells transfected with the Transfectam reagent were not suitable for immunofluorescence analysis, because the precipitated DNA on the coverslip increased the background staining when we counterstained the nuclear DNA with propidium iodide.

For immunoblot experiments, 1×10⁶ cells were plated onto 100 mm Petri dishes. After overnight culture, the medium was aspirated and the solution containing Transfectam reagent-DNA complex was overlayed on the cells as described by the manufacturer. After 24 hours of exposure, the cells were trypsinized, washed with PBS and then lysed in 60 µl EBC buffer (Tang et al., 1993b). The cell lysates were prepared and subjected to immunoblot analysis as described (Tang et al., 1993b).

Fig. 1. (A) Schematic representation of various NuMA polypeptides derived from SV/NuMA expression vectors. The numbering system refers to amino acid residues on various NuMA isoforms. The carboxy-terminal 391, 52 and 39 residues are distinct for the NuMA-l, NuMA-m and NuMA-s proteins, respectively. The full-length human NuMA cDNAs that span the entire coding region for NuMA-l, -m and -s were subcloned into a pSG5 expression vector (see Materials and Methods for details). All cDNA constructs contain both the 75 bp and 42 bp sequence blocks, which encode 25 and 14 amino acids (aa), respectively (black boxes). (B) Immunoblot analysis of various SV/NuMA isoforms after transient transfection of CHOP cells. Lane 1, SV/NuMA-s transfected CHOP cells; lane 2, SV/NuMA-m; lane 3, SV/NuMA-l; lane 4, mock-transfected CHOP cells. The low intensity band of ~194 kDa protein shown in lane 1 reflects the fact that the transfection efficiency of SV/NuMA-s is ten times lower than that of SV/NuMA-m or SV/NuMA-l due to an unknown reason. (C) Subcellular localization of human NuMA-l at various cell cycle stages of transfected CHOP cells. CHOP cells were transfected with SV/NuMA-I, fixed, and processed for immunofluorescence with mAb W1 (a, b, c, d) and a DNA-specific dye, propidium iodide (a', b', c', d'). Interphase (a, a'); metaphase (b, b'); anaphase (c, c'); telophase (d, d'). Bar, 25 µm.
were then clarified by centrifugation at 12,000 g for 15 minutes at 4°C. Cell lysates (30 µl) were then mixed with 5× SDS sample buffer and analyzed by 7.5% SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane and incubated with mAb W1 (Tang et al., 1993b). The immunoreactive polypeptides were detected with the Western Exposure Chemiluminescent Detection System as described by the manufacturer (Clontech). Briefly, the immunoreactive polypeptides were detected by the addition of goat anti-mouse IgG-alkaline phosphatase conjugate followed by incubation with chemiluminescent substrate solution for 5 minutes at room temperature. The membranes were then drained and placed in contact with X-ray film to expose the film.

RESULTS

Expression of SV/NuMA-I reveals a subcellular localization pattern that is indistinguishable from that of endogeneous NuMA in human cells

To define the subcellular localization of various NuMA isoforms, we have developed a transient expression system. Three plasmids (SV/NuMA-l, -m and -s) that carry the entire coding sequences of human NuMA-l, -m and -s cDNAs, respectively, were constructed and placed under the control of the SV40 early promoter (Fig. 1A). Each plasmid was introduced via transient transfection into CHOP cells, which were then subjected to immunoblotting and confocal fluorescence microscopic analyses. The basis for detecting the expressed product corresponding to each of the constructs is a monoclonal antibody (mAb W1) originally derived from mice immunized with the nuclear residue from small cells of the bovine esophageal epithelial (BEE) basal layer (Tang et al., 1993a,b, 1994). This monoclonal antibody recognizes a specific epitope of NuMA from human, bovine and murine cells (Tang et al., 1993b), but fails to recognize NuMA proteins of hamster (CHOP) cells on the basis of both immunoblot (Fig. 1B, lane 4) and immunofluorescence analyses (compare Fig. 1C-a with 1C-a’).

Fig. 1B shows that transfected human NuMA-l in CHOP

Fig. 2. Subcellular localization of human NuMA-m at various cell cycle stages of transfected CHOP cells. The SV/NuMA-m transfected cells were double-stained with mAb W1 (left, green) and propidium iodide (center, red). The composite photographs are shown on the right. (a-c) Interphase; (d-f) metaphase; (g-i) telophase. Bar, 25 µm.
**Fig. 3.** Subcellular localization of human NuMA-s at various cell cycle stages of transfected CHOP cells. CHOP cells were transfected with SV/NuMA-s. Cells were fixed and processed for immunofluorescence with mAb W1 (left, green) and propidium iodide (center, red). The composite photographs of mAb W1 and propidium iodide are shown on the right.

(a-c) Interphase; (d-f) metaphase; (g-i) telophase. Bar, 25 µm.
Fig. 4. Phenotype of transfected CHOP cells that expressed various amounts of NuMA-s. CHOP cells were transfected with SV/NuMA-s and double-labeled with mAb W1 (left, green) and anti-tubulin antibody (center, red). The composite photographs of mAb W1 and anti-tubulin antibody are shown on the right. (a-c) Low-level expression of NuMA-s; (d-f) medium-level expression; (g-i) high-level expression. Bar, 10 μm.
cells (lane 3) produced a protein with $M_r \approx 230,000$ (p230), which migrated according to the predicted size of the endogenous human NuMA ($\approx 230,000$). The band observed in Fig. 1B (lane 3) with a molecular mass lower than p230 was probably due to degradation; this band was not detected in extracts derived from mock-transfected CHOP cells (lane 4).

Similarly, the predicted polypeptides corresponding to p194 and p195 were also observed when CHOP cells were transfected with SV/NuMA-s (lane 1) or SV/NuMA-m (lane 2), respectively.

The subcellular localization of human NuMA isoforms expressed in CHOP cells was further analyzed by fluorescence confocal microscopy using mAb W1. For cells at interphase, NuMA-I, like NuMA in human U251 cells (Tang et al., 1993b), accumulated in the nucleus of transfected CHOP cells but was excluded from the nucleoli (Fig. 1C-a). Note that several untransfected CHOP cells seen by staining with a DNA-specific dye (propidium iodide) (Fig. 1C-a’) did not react with mAb W1 (Fig. 1C-a). When cells entered metaphase, NuMA-I concentrated in the polar regions of the spindle apparatus, forming a characteristic crescent shape (Fig. 1C-b). From metaphase to anaphase (Fig. 1C-c), the crescent staining of human NuMA-I diminished gradually but still remained at the spindle poles. Finally, in telophase cells, the human NuMA-I protein associated with the re-forming nucleus and was subsequently redistributed throughout the nucleus again (Fig. 1C-d).

Taken together, the cell-cycle-dependent distribution of human NuMA-I in transfected CHOP cells is indistinguishable from that in human cells.

**Fig. 5. Low-level expression of human NuMA-m in transiently transfected CHOP cells.** CHOP cells were transfected with SV/NuMA-m and double-labeled with mAb W1 (A) and anti-tubulin antibody (B). The composite photograph is shown in (C). Bar, 25 µm.

**NuMA-m and NuMA-s are present in the interphase cytosol, mainly clustered at the centrosomal region and move into the polar regions of the mitotic apparatus**

When we transfected SV/NuMA-m and SV/NuMA-s into CHOP cells, a quite distinct subcellular localization was observed in interphase cells. In contrast to NuMA-I, which was concentrated exclusively in the interphase nuclei, NuMA-m and NuMA-s were unevenly distributed in the cytosol. In the positive-staining interphase cells, NuMA-m and NuMA-s were present in the cytoplasm, but were prominently labeled as a small round dot (Fig. 2a, NuMA-m; Fig. 3a, NuMA-s). These dots were most frequently seen in the perinuclear region, and sometimes they were observed above or below the nucleus (data not shown).

When the cells entered metaphase, both NuMA-m (Fig. 2d) and NuMA-s (Fig. 3d) concentrated in the polar regions of the spindle apparatus, a staining pattern similar to that observed for NuMA-I (Fig. 1C-b). However, unlike the prominent crescent staining observed for NuMA-I, staining in the shape of a round dot was commonly found in NuMA-m and NuMA-s transfected cells. These results are illustrated in Fig. 2d-f (NuMA-m) and Fig. 3d-f (NuMA-s), which represent double fluorescence analyses using mAb W1 (Figs 2d, 3d) and the DNA-specific dye, propidium iodide (Figs 2e, 3e). Composite, double-colored micrographs are shown in Figs 2f and 3f. As the cells progress through metaphase to telophase, the separated daughter chromosomes arrive at the poles and each group of chromosomes starts to form two daughter interphase nuclei. The prominent labeling of the small round dot remained in the perinuclear position; the most likely interpretation of these observations is therefore that the labeled dot-like structures correspond to the centrosomal region.
The phenomenon of NuMA-s association with the centrosomal region is best illustrated by a comparison of a series of photographs that represent a simultaneous analysis of the staining patterns of transfected CHOP cells that expressed various amounts of NuMA-s, after staining with mAbW1 (Fig. 4, left) and anti-tubulin antibody (Fig. 4, center). Fig. 4a shows that in transfected cells that express low levels of NuMA-s many dot-like structures appear to be concentrated at the interphase centrosome (microtubule organization center) (Fig. 4a-c). However, in medium-level expression cells, the dot-like staining gradually aggregated and finally clustered at the region around the centrosome (Fig. 4d-f). Interestingly, in high-level expression cells, NuMA-s appears to be assembled into a large sheetlike aggregate within the cells (Fig. 4g). The large blurred staining pattern detected by anti-tubulin antibody (4e and 4h) that was superimposed upon the staining of mAb W1 (4d and 4g) could be a cross-reactive signal caused by overproduction of NuMA-s within the cells, since this signal was not detected in low-level expression cells (Fig. 4b). In general, about 5-10% of transfected cells expressing NuMA-s showed a fluorescence staining pattern (a small dot-like structure) similar to that in Fig. 3a. Of the remaining cells expressing NuMA-s, ~10% showed a low-level expression pattern (as in Fig. 4a-c), ~50% showed a medium-level pattern (as in Fig. 4d-f), and ~30% showed a high-level pattern (as in Fig. 4g-h). A similar staining pattern was also observed when we transfected SV/NuMA-m into CHOP cells that expressed NuMA-m (Fig. 5).

The carboxy terminus of NuMA-l contains the signal necessary for nuclear localization and spindle association

The finding that NuMA-l is targeted correctly to interphase nuclei, whereas NuMA-m and -s are not, suggests that the carboxy terminus of NuMA-l contains the signal necessary for nuclear targeting. The NuMA-l tail containing the nuclear targeting domain has also been observed by Compton and Cleveland (1993). In an attempt to map the peptide region involved in nuclear localization, a series of linkers that contain stop codons in all three reading frames was introduced into the 3′ end of SV/NuMA-l cDNA (Fig. 6A). The expression of the

![Diagram of NuMA-l protein](image)

**Fig. 6.** Expression of human NuMA-l carboxy-terminal deletion mutants in CHOP cells. CHOP cells were transfected with various linker scanning mutant cDNAs (A). The cells were fixed and processed for immunofluorescence with mAb W1. (B) Transfected cells at the interphase stage; (C) transfected cells at the metaphase stage. Bar, 25 μm.
truncated linker scanning mutants in CHOP cells was analyzed with mAb W1 by confocal fluorescence microscopy.

Fig. 6 shows the subcellular localization of a series of carboxy-terminal deletion mutants of NuMA-I. Linker 12 (L12) and linker 1 (L1) mutants both targeted correctly to interphase nuclei (Fig. 6B) and mitotic spindle poles (Fig. 6C). However, when the carboxy-terminal end was removed up to or after linker 24 position, the nuclear staining disappeared. Instead, a prominent cytosolic localization was noticed (Fig. 6B, L24). Some bright spots distributed in the cytosol were also observed in L42-transfected cells (Fig. 6B). Interestingly, the spindle association gradually diminished in L36 mutants (Fig. 6C) and was completely lost in L42 mutants (Fig. 6C) and in other mutants after linker 36 (data not shown). These results suggest that the 279 amino acid peptide located between L1 and L24 is necessary for nuclear targeting, whereas the peptide domain (residues 1516-1667) located between L42 and L36 appears to be necessary for spindle association.

**Subcellular localization of the carboxy terminus of NuMA-1/β-galactosidase chimeric proteins**

To define more precisely the carboxy terminus of NuMA-1 involved in nuclear localization and spindle association, a series of NuMA-1/β-galactosidase expression vectors were constructed (Fig. 7). β-Galactosidase (β-gal), a protein encoded by the lacZ gene of E. coli was chosen as a cytosol localization marker. The expression of chimeric proteins that encode various carboxy-terminal proteins of NuMA-1 fused to β-gal in CHOP cells was analyzed with a monoclonal antibody specific against β-gal. Consistent with earlier reports, wild-type β-gal was detected predominantly in the cytoplasm with no nuclear expression and no spindle association (data not shown). However, pc11 chimeric protein (NuMA1538-2115/β-gal), consisting of the entire carboxy-terminal region of NuMA-1 (Fig. 7), was expressed primarily in the nucleus but not in the nucleolus (Fig. 8A-a), a pattern similar to that observed in NuMA of human cells (Tang et al., 1993b). Analysis of pc6 chimeric protein (NuMA1538-1975/β-gal) displayed a mostly cytoplasmic expression pattern (Fig. 8A-b). Interestingly, pc11 chimeric protein was targeted to the spindle pole in mitotic cells (Fig. 8B-i). However, spindle-pole association was not observed in cells transfected with pc6 (Fig. 8B-j), pc20 or pc25 chimeric proteins (data not shown). Taken together, these results suggest that residues between 1975 and 2115 of NuMA-1 contain a functional signal for nuclear localization, whereas the entire tail region (1538-2115) of NuMA-1 is necessary and sufficient for spindle association (Fig. 7).

To narrow down further the region involved in nuclear localization, we then transfected several expression vectors (Fig. 7, pc7, pc18, 20, 21, 23, 24, and 25) that each encoded a smaller peptide of the NuMA-1 tail fused to β-gal in CHOP cells. Chimeric proteins consisting of residues 1839-2095 (Fig. 7, pc18; Fig. 8A-c), 1972-2095 (Fig. 7, pc20; Fig. 8A-d), or 1972-2059 (Fig. 7, pc21; Fig. 8A-e) of the NuMA-1 tail revealed a predominantly nuclear pattern of localization. In contrast, chimeric proteins containing either residues 2030-2059 (Fig. 7, pc23; Fig. 8A-f) or 2002-2034 (Fig. 7, pc25; Fig. 8A-g) fused to β-gal displayed a mostly cytoplasmic expression pattern. These results suggest that residues 1972-2007 of NuMA-1 constitute a functional NLS. Indeed, when we transfected pc24, which contains only 36 amino acid residues (1972-2007) of the NuMA-1 tail fused to β-gal, into CHOP cells, we did observe prominent nuclear staining (Fig. 8A-h).

**Site-specific mutational analysis of the nuclear localization signal**

The 36 amino acid peptide (residues 1972-2007) of the NuMA-I protein contains four basic residues clustered within positions 1984 to 1989 (Fig. 9A), a feature similar to other known NLS. One mutation with the (K)Lys1988 of NuMA-I substituted by glutamic acid (E) in pc24N2 clone (Fig. 9A) did abolish nuclear localization (Fig. 9B-b). However, another mutation (pc24N1), which has (R)Arg1984 replaced by glycine (G), did not prevent accumulation of the chimeric protein in the nucleus (Fig. 9B-a). These results suggest that Lys1988 is essential for nuclear targeting, whereas Arg1984 is not. To our surprise, the pc7 chimeric protein (NuMA1985-1991/β-gal) consisting of only seven amino acid residues (QQKRKVS) that cover all three basic residues (RKR), located at amino acid positions 1987-1989 (Fig. 9A), was mostly expressed in the cytoplasm (Fig. 9B-c). However, when we transfected with two other constructs (pc30 and pc31), which encode residues 1981-1994 or 1981-2007 of NuMA-I, respectively, fused to β-gal, these two chimeric proteins were homogeneous and distributed equally.
between nucleus and cytoplasm (pc30, Fig. 9B-d; pc31, data not shown). Taken together, these results suggest that residues 1972-2007 (Fig. 9A, pc24), located at the carboxy-terminal domain of NuMA-1, are necessary and sufficient for nuclear...
targeting, whereas residues 1985-1991 (pc7) alone are not sufficient.

**DISCUSSION**

**NUMA is composed of multiple isoforms heterogeneous in size and subcellular localization**

NUMA was originally reported as an intranuclear protein that is concentrated at the spindle pole during mitosis (Lyderson and Pettijohn, 1980). More recently, however, we have shown that NUMA consists of multiple isoforms of various sizes and amounts in a specific cell type (Tang et al., 1993b). The major difference is located at the carboxy terminus. NUMA-1, -m, and -s contain 391, 52, and 39 amino acids, respectively (Fig. 1A), attached to a common polypeptide backbone at the position of amino acid residue 1724 (numbering system of Yang et al., 1992). This finding raises questions concerning the subcellular localization and function of each specific isoform. In this report, we have developed a transient expression system in which only transfected human NUMAs, but not endogenous hamster NUMA, can be detected by the monoclonal antibody (mAb W1) that we use. Our results show that the localization of the largest isoform, NUMA-1 (~230 kDa), mimics that of the endogenous human NUMA (Lyderson and Pettijohn, 1980; Tousson et al., 1991; Kallajoki et al., 1991; Compton et al., 1991; Yang et al., 1992; Tang et al., 1993b). In contrast, two other isoforms (NUMA-m and NUMA-s) with lower molecular masses reveal a distinct subcellular distribution. They appear to be located in the interphase cytosol, mainly clustered at the centrosomal region, but move to the mitotic spindle pole during mitosis.

If NUMA-m and NUMA-s are present in the interphase centrosomal region, why was their presence not observed in previous fluorescence studies? One possible explanation is that the shorter forms of NUMA (p195 and p194) may exist as antigenically inactive precursors or homologues, which were barely detected by earlier reported antibodies. The other possibility is that NUMA-m and NUMA-s isoforms are expressed at a level too low to be detected by the immunofluorescence studies. Indeed, overexpression of these two isoforms in CHOP cells has permitted easy analysis of their subcellular localization.

**The carboxy terminus of NUMA-1 contains a peptide domain for spindle association**

Analysis of a series of NUMA-1 deletion mutants (Fig. 6) revealed that the tail region of NUMA-1 (residues 1516-1667) must possess a signal necessary for spindle association. However, when we transfected the pc6 construct that encodes residues 1538-1975 of NUMA-1 (including the 42 bp encoded peptide) fused to β-gal, or transfected pc20 (NUMA1972-
2095/β-gal) into CHOP cells (Fig. 7), no spindle association was observed. From these results, it is reasonable to speculate that the spindle-association signal is located between residues 1516 and 1538. Unfortunately, this appears not to be the case, since Compton and Cleveland (1993) have shown that a tailless human NuMA protein (CMV/NuMA1-1545), consisting of residues from 1 to 1559 based on our and Yang’s numbering system (Yang et al., 1992), is not targeted to the spindle pole. (The Compton construct, CMV/NuMA1-1545, lacks the 42 bp encoded peptide, therefore residue 1545 is equivalent to 1559, based on our and Yang’s numbering system.) Their observation excludes the possibility that residues 1516-1538 of NuMA-l contain the signal for spindle association. To our surprise, if we transfected the pc11 construct (Fig. 7) encoding the entire tail region of NuMA-l (residues 1538-2115) fused to β-gal, it did reveal a spindle association in mitotic cells (Fig. 8B-i), although the intensity of the signal was weaker than that observed in wild-type SV/NuMA-l (Fig. 1C-b). Altogether, these results suggest that the pc11 chimeric protein contains a peptide domain that is necessary and sufficient for spindle association, whereas other sequences shorter than this region are insufficient for spindle targeting.

It is interesting to note that both NuMA-m and -s also target to the mitotic spindle. By comparison of the sequences of the spindle association domain (residues 1538-2115) defined in NuMA-l with the entire coding region of NuMA-m (residues 1-1776) and -s (residues 1-1763), a short segment located at residues 1538-1725 is identical in all three NuMA isoforms. However, the pc6 chimeric protein (NuMA1538-1975/β-gal) that includes this region is insufficient to drive the chimeric protein into the spindle (Fig. 7, pc6). These results suggest that a particular amino acid sequence may not be directly involved in targeting of NuMA-m and -s to the mitotic spindle pole. Instead, the overall protein tertiary structure or post-translational modification of some specific residues in NuMA polypeptides may play a role. In addition, the presence of a negative regulatory sequence located within residues 1538-1795, which inhibits the spindle association, cannot be excluded.

Furthermore, we have shown that the truncated L36 (1-1667) and L24 (1-1766) polypeptides of NuMA-l isoform are present at the spindle pole, but not at the interphase centrosomal region. In contrast, both NuMA-m and NuMA-s isoforms are localized to the centrosomal region in interphase and mitotic cells. This observed differential subcellular localization might be partially due to the presence of the unique polypeptide located at the carboxy termini of NuMA-m and NuMA-s.

Fig. 10. Schematic representation of the structural (upper) and functional (lower) domains of human NuMA-l. The wavy-line box represents the α-helical coiled-coil domain. The cross-hatched box (25aa and 14aa) represents an alternative-spliced sequence block. L, leucine zipper motif.
Nuclear mitotic apparatus protein

1401

phase centrosomal region (cdc2 and NuMA-m) and the mitotic spindle pole region (cdc2, NuMA-l and NuMA-m) suggest that some NuMA isoforms could potentially be phosphorylated and regulated by p34<sup>cdc2</sup> kinase. In addition, γ-tubulin has been reported to be a centrosomal protein (Zheng et al., 1991; Stearns et al., 1991) required for cell-cycle-dependent microtubule nucleation (Joshi et al., 1992). The coexistence of the γ-tubulin with shorter NuMA isoforms (NuMA-m and -s) in the interphase centrosomal region and the mitotic spindle pole suggests a possible connection between these proteins. Further analysis of the protein-protein interactions among various centrosome-associated proteins may shed some light on their functions.

**NuMA-l tail contains a novel nuclear localization signal**

Our results show that residues 1972-2007 of NuMA-l (pc24) contain a signal that is sufficient for nuclear targeting. When we analyzed this 36 amino acid peptide sequence using the GCN computer program MOTIFS described by Dr Amos Bairoch (University of Geneva), no specific peptide motifs were identified. However, a short basic peptide sequence (RQQRKK, residues 1984-1989) containing a high proportion of positively charged amino acids (lysine and arginine) was noted within this region, which has been described to be one of the important characters for NLS (reviewed by Garcia-Bustos et al., 1991; Whiteside and Goodbourn, 1993). Substitution of Lys1988 in this region (Fig. 9, pc24N2) with an acidic glutamic acid residue did abolish nuclear localization, whereas replacement of Arg1984 (Fig. 9, pc24N1) with a non-basic, non-hydrophobic glycine residue did not. These results suggest that three basic amino acids (RKR) located at residues 1987-1989 may be important for nuclear targeting. To our surprise, the pc7 (NuMA1985-1991/β-gal) chimeric protein, which comprises only seven residues (QQRKRVS) including RKR, was found to have a mostly cytosolic staining pattern. These results suggest that Lys1988 is essential. However, the basic residues (RKR) alone are not sufficient for targeting the chimeric protein into the nucleus.

Recently, it has been reported that the rate of nuclear translocation of SV40 large T antigen depends on the phosphorylation of the serine residue located at the casein kinase II site near the NLS (Rihs et al., 1991). In yeast SW15 protein, phosphorylation of the serine residue located at the potential cdc2 kinase recognition sites within its NLS may also regulate the intracellular localization of SW15 (Moll et al., 1991). However, neither the potential cdc2 nor casein kinase II recognition sites are present within the 36 amino acid peptide in NuMA-l. Moreover, no sequence similar to the bipartite nuclear localization domain reported in nucleoplasmin (Robbins et al., 1991) could be identified within this region. Taken together, the above findings suggest that the 36 amino acid peptide located at the middle portion of the carboxy terminus (1972-2007) of NuMA-l constitutes a novel functional NLS. This nuclear localization signal is distinguished from most previously described NLSs by its large size and its absence of any known phosphorylation sites for cdc2 and casein kinase II. In addition, its short basic peptide alone is insufficient for nuclear targeting. Further analysis of nuclear targeting by this novel NLS will be needed to elucidate the general mechanism of nuclear protein translocation.

In summary, we have shown that NuMA is composed of multiple isoforms heterogeneous in size and subcellular localization. In this report, we have also defined several functional domains of NuMA-l, and presented a structural and functional model for human NuMA-l (Fig. 10), which may provide some important insights into our understanding of the function of various NuMA isoforms.

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