Nuclear–mitotic apparatus protein: A structural protein interface between the nucleoskeleton and RNA splicing

CHANGQING ZENG*, DACHENG HE*, SUSAN M. BERGET†, AND B. R. BRINKLEY‡

*Department of Cell Biology and †Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

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ABSTRACT Vertebrate splicing factors are localized to discrete domains within the nuclei of somatic cells. The mechanism whereby such nuclear domains, identified as speckles by immunofluorescence microscopy, are generated is unclear. Recent studies suggest that the spatial order within the nucleus is maintained by nuclear matrix factors. Here we show that a protein in the nuclear matrix and mitotic apparatus (nuclear–mitotic apparatus protein, NuMA; Lydersen, B. & Pettijohn, D. (1980) Cell 22, 489–499) colocalizes with splicing factors in interphase nuclei and is associated with small nuclear ribonucleoproteins in a complex immunoprecipitated from HeLa extract with small nuclear ribonucleoprotein antibodies. Moreover, NuMA associates with splicing complexes that are reconstituted in vitro using wild-type pre-mRNA, but not with nonspecific RNA. Cumulatively, these observations suggest a function of NuMA or NuMA-like proteins in interphase cells in providing a bridge between RNA processing and the nucleoskeleton.

The eukaryotic nucleus is a highly organized structure. Electron microscopy has provided excellent morphological evidence for the existence of a complex nucleoskeleton (1,2). An intermediate-filament-like nuclear network termed core filaments has been revealed by a sequential extraction procedure (2,3). These 9- to 13-nm filaments may be the core structure to which other proteins associate to form the nuclear matrix. Many nuclear activities, such as synthesis of DNA and RNA and RNA splicing, have been localized in discrete nuclear domains rather than diffusely distributed throughout the nucleoplasm (4–7). Particularly, the localizations of these specific domains persist after the removal of chromatin, indicating that the nuclear matrix has a key function in maintaining spatial order within the nucleus. Recent microscopic studies have suggested that pre-mRNAs are both processed and transported in discrete nuclear “tracks” that might correspond to nuclear filaments (8–11). In addition, earlier experiments revealed a preferential association of pre-mRNA with the nuclear matrix (12–16). Strong evidence for the relationship of the nucleoskeleton to gene expression, however, has been lacking due to the phenomologenial nature of biochemical matrix preparations. No studies have used reagents directed against a well-characterized element of the nucleoskeleton in an attempt to bridge the gap between nuclear architecture and gene expression.

Nuclear–mitotic apparatus protein (NuMA) (17), also known as centrophilin (18), SPN (19), and SP-H (20), is a 230-kDa nuclear protein that resides at the spindle poles during mitosis. Studies on the primary structure of NuMA have shown that it is a long coiled-coil protein with two globular end domains separated by a discontinuous α-helix (21,22). Analysis of various NuMA cDNA clones has suggested the existence of NuMA isoforms generated by alternate splicing (23), although no correlation has been made between the isoforms and multiple functions for NuMA. In mitotic cells, NuMA appears to play a role in spindle microtubule formation and post-mitotic nuclear assembly as shown by various studies on microtubule inhibition, antibody injection, and expression of truncated NuMA proteins (18–20, 24–27). Beyond its identity as a nuclear matrix protein (17,19), however, little is known of the function of NuMA in the interphase nucleus.

Here we investigate the role of NuMA in interphase nuclei by the ability of NuMA-specific antibodies to stain discrete foci and to immunoprecipitate nuclear assemblies. Specifically, we show that NuMA colocalized with the small nuclear ribonucleoprotein particles (snRNPs) required for processing of pre-mRNAs in interphase. Furthermore, we show that NuMA associates in vitro with both nuclear snRNPs and reconstituted spliceosomes.

MATERIALS AND METHODS

Cell Culture. HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% (vol/vol) fetal bovine serum. Human cervical epidermoid carcinoma cells (CaSkI; American Type Culture Collection) were cultured in RPMI 1640 medium containing 2.5% fetal bovine serum and 7.5% (vol/vol) calf serum.

In Situ Sequential Extraction. CaSkI cells were chosen for core filament preparation (2) because these cells adhere tightly to coverslips after each of the extraction steps. Cells on coverslips were lysed for 2 min in cytoskeleton (CSK) buffer (10 mM Pipes, pH 7.0/100 mM NaCl/300 mM sucrose/3 mM MgCl₂/1 mM EGTA/4 mM vanadyl ribonucleoside complex) containing 0.5% Triton X-100 and multiple protease inhibitors [PLAP: 0.1 mM phenylmethylsulfonyl fluoride/leupeptin (1 µg/ml)/antipain (1 µg/ml)/pepsatin (1 µg/ml)] and then digested with 30 units of RNase-free DNase I (Boehringer Mannheim) in 100 µl of CSK buffer at 32°C for 40 min. Subsequently, cells were extracted with 0.25 M ammonium sulfate on CSK on ice for 5 min. The coverslips were then immersed in 7 ml of CSK in a 100-mm Petri dish and 7 ml of 4 M NaCl stock was added drop by drop with gentle shaking to a final concentration of 2 M. After incubating on ice for 4 min, core filament preparations were fixed in formaldehyde and incubated with antibodies. For further RNA digestion, RNase A (100 µg/ml) was added and the cells were incubated at 33°C for 12 min after fixation.

Immunofluorescence. HeLa or CaSkI cells were grown on glass coverslips, permeabilized for 2 min in PEM (100 mM Pipes, pH 7.0/1 mM EGTA/1 mM MgCl₂) plus 0.5% Triton X-100, and then fixed in 3% (vol/vol) formaldehyde in PEM for 30 min, except for the samples for core filament preparation. Fixed cells were washed with phosphate-buffered

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saline (PBS: 100 mM NaCl/10 mM phosphate, pH 7.4) and stained with primary antibodies for 0.5–3 hr at 37°C. For double-label immunofluorescence, cells were stained in turn, with monoclonal antibody 8.22, rhodamine-conjugated goat anti-mouse IgG, αSm (a snRNPs-specific autoantibody) (28), and fluorescein isothiocyanate-conjugated goat anti-human IgG. OPTIMUS software (Meyer's Instruments, Houston) was used to analyze images and compute pseudocolor.

**Immunoprecipitation in HeLa Extracts.** HeLa cells in one T150 flask were washed twice with Hank's buffer and incubated in 10 ml of methionine-free medium and 1 μCi of [35S]methionine (1 Ci = 37 GBq) and cysteine (ICN) for 6 hr. After washing with PBS, cells were lysed with 0.5% Triton X-100 in PEM with protease inhibitors in 100 ml and then centrifuged and rinsed with PBS. The supernatants were precleared by incubation with 30 μl of agarose, uncoupled and washed 3 times with PBS containing 0.25 M NaCl and twice with PBS. Pellets were subjected to SDS/PAGE and autoradiography. For samples also used for immunoblot after precipitation, nonlabeled 0.5 M salt HeLa extract was prepared. αTMG antibody (30 μl) coupled to agarose, uncoupled protein G-agarose, or 2 μl of CREST antibody (32) was used to perform immunoprecipitation as described above and the complexes were examined by SDS/PAGE and immunoblot analysis using P9 or CREST antibodies.

**Immunoprecipitation with in Vitro-Reconstituted Spliceosomes.** 32P-labeled splicing precursor RNA was transcribed in vitro from the MINX adenovirus mimic genome containing a single intron (16). Precursor RNA was capped during synthesis. HeLa nuclear extract was prepared as described (33) and in vitro splicing was performed as reported (34). All antibodies were affinity-purified from cell culture medium, ascites fluid, or serum. Purified antibodies at concentrations of 3–5 μg were added to each reaction mixture and incubated on ice for 15 min. To precipitate the immunocomplexes, we found that the prewashed streptococcal protein G was much more effective than agarose-conjugated protein G. After incubation with 20 μl of protein G for 15 min on ice, pellets were washed with modified NETS (50 mM Tris-HCl, pH 7.9/150 mM NaCl/0.05% Nonidet P-40/1.5 mM MgCl2/0.5 mM dithiothreitol). After ethanol precipitation, RNA was dissolved in 90% (vol/vol) formamide/10 mM EDTA, boiled, and resolved on a 10% polyacrylamide gel containing 9 M urea.

**RESULTS AND DISCUSSIONS**

**Colocalization of NuMA and snRNPs.** To obtain reagents specific for NuMA, a monoclonal antibody (2D3) specific for primate NuMA (18) was used to screen a cDNA library prepared from the human cell line HT29. A partial cDNA of 1.9 kb was isolated and determined to code for NuMA by sequence analysis (21, 22). A fusion protein containing β-galactosidase was produced and injected into mice for the generation of antibodies. Resulting antibodies were selected for anti-NuMA activity by detection of mitotic polar staining. One monoclonal antibody, 8.22, and one polyclonal antibody, P9, were chosen for further study. It was noted that P9, 8.22, and 2D3 stained spindle poles during mitosis, consistent with the known specificity of all anti-NuMA antibodies. As shown in Fig. 1, all three antibodies reacted with two or more of a set of three proteins of apparent molecular masses 160, 200, and 220 kDa, upon Western blot analysis. The largest protein was recognized by all three antibodies and corresponds to previously characterized NuMA (17). The middle band, detected only with the polyclonal antibody, was a known isoform of NuMA produced by alternative splicing (23) as determined by peptide digestion (35) (data not shown). The 160-kDa protein recognized by all three antibodies had a different protein digestion pattern than the largest bands and, therefore, probably represents a protein other than NuMA containing a common epitope.

Although all three antibodies exhibited identical staining of spindle poles in metaphase, two distinct patterns of staining were observed for interphase nuclei (Fig. 2). Both P9 and 2D3 produced a diffuse nuclear staining pattern like that seen with other NuMA antibodies, whereas 8.22 stained discrete foci or speckles. A similar staining pattern has also been seen in two other studies (17, 23). In their initial report, Lydersen and Pettijohn (17) identified discrete fluorescent spots in the nuclei of HeLa cells stained with anti-NuMA antibody. Another NuMA monoclonal antibody, W1, also produced nuclear speckles in immunofluorescence (23). Since 2D3 and 8.22 resolved the same proteins in immunoblots (Fig. 1), the different fluorescent pattern of the two antibodies is probably due to differential availability of each epitope of NuMA (Fig. 2 a and b). This difference in staining suggests that the epitope recognized by 8.22 antibody resides in a particular nuclear domain distinct from those containing the majority of the NuMA protein. The foci produced by 8.22 were reminiscent of the staining patterns of a number of splicing factors. To further investigate this similarity, cells were stained with both 8.22 and an αSm antibody that reacts with human snRNPs (28) (Fig. 2 c–h). In interphase nuclei, the two staining patterns colocalized with the exception that αSm, but not 8.22, also stained a few distinct spots presumed to be coiled bodies (coilin-containing nuclear foci of unknown function) (36–38) (Fig. 2 c–e). During mitosis, however, the staining pattern of 8.22 changed to a series of discrete, punctate foci, presumably NuMA-like epitope.
FIG. 2. NuMA antibody 8.22 stains nuclear speckles. (a and b) Staining of interphase or mitotic (Insets) HeLa cells with 2D3 (a) and 8.22 (b) generated interphase patterns of diffuse nuclear staining or nuclear foci and speckles, respectively; and mitotic patterns of spindle crescents. P9 gave the same staining pattern as 2D3 (data not shown). (c–f) Nuclear staining of human cervical epidermoid carcinoma cells (CaSki) with 8.22 produced smaller and more numerous nuclear foci and speckles than HeLa cells. (c–h) Computerized pseudocolor of double immunofluorescence with 8.22 (c and f) (green) and human αSm antibody (d and g) (red). The patterns produced by the two antibodies in interphase nuclei are superimposable (e) (yellow). The only exception is the specific staining of one to a few round spots presumed to be coiled bodies (36–38) (arrows, and red in e) by αSm, but not 8.22. (f–h) Mitotic staining by the same procedure with the majority of 8.22 antibody deposited in the polar regions. Most αSm assumed a diffuse localization in the cytoplasm. (i and j) Staining of the nuclear core filament system prepared by DNA digestion and gradient salt extraction (2) with the αNuMA (i) and αSm (j) still colocalized and yielding higher intensity and more foci and speckles, implying more antigens were uncovered by extraction. (k and l) Staining of the core filaments after the preparations in i and j, respectively, were treated with RNase A. Treatment deformed the speckles to blobs but colocalization remained.
pattern of the two antibodies differed considerably. As shown in Fig. 2 f–h, 8.22 antibody transferred to the polar regions, whereas the αSm antibody assumed a diffuse localization in the cytoplasm. Both NuMA and αSm antigens reappeared in the newly formed daughter nuclei at late telophase. The coalescence of snRNP-specific and NuMA-specific antibodies only during interphase indicates that the antibodies are recognizing two distinct epitopes that both occupy a nuclear domain during only one portion of the cell cycle.

In a separate immunoelectron microscopy study, we found NuMA to be a component of core filaments, the skeleton of the nuclear matrix consisting of heterogeneous proteins and RNAs (2). To further document the colocalization of NuMA and snRNPs, core filaments were prepared by DNA digestion and gradient salt extraction (Fig. 2 i and j). A portion of this material was treated further with RNase A (Fig. 2 k and l). The two antigens remained colocalized after both treatments, despite the changes each treatment caused to nuclear structure. After DNase I and salt treatment, both antigens retained the same pattern as the control except for more intensive staining. After RNase treatment, which disrupted the core filament system, the speckled pattern was replaced by large patches. These patches were stained equally with NuMA and snRNP antibodies. These results are consistent with the presence of NuMA and snRNPs in the same subnuclear regions and suggest that the association of NuMA and snRNPs is relatively stable. In addition, NuMA, but not the 160-kDa protein, could be detected in a core filament preparation on immunoblots with NuMA antibodies (data not shown). The 160-kDa protein, therefore, may not exist in core filaments.

NuMA Associates with snRNPs. To address further whether NuMA antigens are stably associated with snRNPs, we asked whether we could detect NuMA in preparations of snRNPs that had been immunoprecipitated from nuclear extracts with either the αSm antibody or with an antibody (αTMG) specific for the trimethylguanosine cap associated with snRNAs (31) (Fig. 3). The αSm, αTMG, 2D3, and 8.22 antibodies all immunoprecipitated a doublet of ~200 kDa from nuclear extracts of HeLa cells that had been labeled with [35S]methionine (Fig. 3 a and b). Immunoblot analysis of the αTMG immunoprecipitate with the polyclonal NuMA antibody P9 indicated that this large doublet was indeed related to NuMA (Fig. 3 c). As controls, a centromere/kinetochore-specific CREST antibody (32) could neither precipitate NuMA (Fig. 3 c, lane 2) nor detect CREST antigens in an αTMG precipitate (Fig. 3 c, lane 5). Therefore, the association of NuMA with snRNPs is specific and sufficiently strong to survive immunoprecipitation. It should be noted that the 160-kDa protein that was observed in Fig. 1 contain NuMA-like epitopes was not present in any immunocomplex as examined by either [35S]methionine labeling or immunoblot analysis. Thus, the 160-kDa appeared not to be associated with snRNP under these experimental conditions.

NuMA Associates with In Vitro-Reconstituted Splicing Complexes. Immunoprecipitation of NuMA by snRNP antibodies suggested that NuMA is associated with snRNPs in interphase nuclei. To address whether NuMA might be a part of the actual splicing apparatus, we turned to in vitro extracts competent for splicing exogenously provided pre-mRNAs (33). This type of extract is prepared by extracting nuclei in 0.4 M salt and contains both NuMA and multiple snRNPs.

To address the association of NuMA with reconstituted splicing complexes (spliceosomes), we asked whether NuMA antibodies could immunoprecipitate radiolabeled pre-mRNA added to a splicing reaction. A wild-type one-intron pre-mRNA derived from adenovirus was used as the pre-mRNA (16); a similar-length plasmid RNA was used as a negative control RNA. Both RNAs were added to complete splicing

![Fig. 3](image-url) NuMA antibodies are associated with snRNPs.  
(a) Immunoprecipitation of NuMA with anti-snRNP antibodies. An [35S]methionine-labeled 0.5 M NaCl HeLa extract was immunoprecipitated with αSm monoclonal antibody Y12 (lane 1) or agarose-conjugated αTMG monoclonal antibody K121 (31) (Oncogene Science, lane 2). (b) Immunoprecipitation of NuMA with anti-NuMA antibodies. An extract prepared with 0.5 M salt and 0.08% SDS was precipitated with protein G-agarose-conjugated 2D3 (lane 1) or 8.22 (lane 2). Arrows mark an identical doublet of high molecular mass. The lower bands in b are due to the nonspecific binding of protein G-agarose (data not shown). (c) Immunodetection of NuMA in the anti-αTMG immunoprecipitate. Various immunoprecipitates of nuclear extract were analyzed on an immunoblot with NuMA and control antibodies. Lanes: 1, protein G-agarose precipitate probed with the P9 NuMA antibody; 2, a CREST autoantibody (32) immunoprecipitate probed with P9 NuMA antibody; 3, HeLa extract probed with the P9 NuMA antibody; 4, αTMG immunoprecipitate probed with the P9 NuMA antibody; 5, the αTMG immunoprecipitate probed with the CREST autoantibody. The bands of ~50 and 25 kDa in lane 4 are the IgG subunits of the monoclonal antibody used in immunoprecipitation visualized by an anti-mouse secondary antibody.

![Fig. 4](image-url) NuMA antibodies immunoprecipitate in vitro-reconstituted spliceosomes. In vitro splicing reactions were set up using a radiolabeled 238-nt nonspecific plasmid RNA (lanes N) or a 220-nt single-intron pre-mRNA from adenovirus (lanes A). After a 10-min incubation at 30°C, each reaction mixture was split into five parts and immunoprecipitated with IgG fractions of 2D3 (lanes 1 and 2), 8.22 (lanes 3 and 4), no antibody (lanes 5 and 6), anti-snRNP antibody Y12 (lanes 7 and 8), or a normal human serum as a control (lanes 9 and 10). Immunoprecipitates were separated on a denaturing polyacrylamide gel followed by autoradiography. Bands corresponding to the two RNA substrates employed are indicated to the left.
reaction mixtures and incubated for 10 min at 30°C. This time interval is sufficient to permit assembly of the first ATP-dependent splicing complex but is not sufficient to permit the appearance of splicing intermediates and products. Reaction products were immunoprecipitated with IgG fractions of NuMA antibodies and with the αsM monoclonal antibody Y12. As shown in Fig. 4, both 2D3 and 8.22 antibodies immunoprecipitated the pre-mRNA, but not the control RNA, as effectively as the anti-snRNP antibody, suggesting that NuMA protein is associated with in vitro-reconstituted spliceosomes.

The results shown here demonstrate a correlation between the nucleoskeleton and the pre-mRNA splicing apparatus. Antibody 8.22, which is generated against a portion of NuMA, may represent a specific group of NuMA antibodies, which stain discrete domains in interphase nuclei. Therefore, some epitopes of NuMA appear to be masked in the nucleus and not recognized by all NuMA antibodies. The following points support our conclusion that the speckle pattern of 8.22 is contributed by a specific form of NuMA: (i) If, for example, the nuclear speckles represent the 160-kDa protein and not NuMA, 2D3 antibody should stain both a speckle pattern and a diffuse staining pattern. Neither 2D3 and 8.22 resolve the same 160-kDa band in immunoblots. (ii) In core filament preparations, the 160-kDa protein disappeared from the immunoblots, whereas the fluorescent speckle pattern of 8.22 remained the same as the control cell. (iii) Other NuMA antibodies have been reported to produce a speckle staining pattern (17, 23). Most likely then, 8.22 antibody, a polyclonal antibody of NuMA, and W1 antibody, recognize a special structural or functional state of NuMA within discrete nuclear domains. Such heterogenous properties of NuMA may be due to posttranslational or posttranscriptional modifications (21–23) or conformational changes of NuMA within the nucleus. The regions stained by 8.22 and snRNP antibodies, known as speckles, appear to be actively involved in pre-mRNA processing. Elegant microscopic visualization of cellular RNAs undergoing transcription, processing, and transport indicate that pre-mRNA passes through these snRNP-rich speckles as they mature (8–11). Our results suggest that the processing centers are attached to the nuclear substructure through a nuclear core filament protein. Such attachment could poise a pre-mRNA for transport via continued association with nuclear filaments.

Anti-NuMA antibodies also detected a biochemical association between NuMA antigens and both nuclear snRNPs and in vitro-reconstituted spliceosomal complexes. Given the filamentous nature of NuMA (21, 22), it seems unlikely that NuMA plays a functional role in the splicing process. More likely, NuMA is a nuclear structural component involved in association of the splicing apparatus with elements of the nucleoskeleton. Electron microscopic visualization of purified in vitro-reconstituted splicing complexes has revealed the presence of a fibrous extension from a large globular complex that could represent NuMA or a NuMA-like protein (39). In this light, alternatively spliced NuMA gene products may represent a subset of NuMAs associated specifically with the splicing apparatus.

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