The *NUF1* Gene Encodes an Essential Coiled-Coil Related Protein That Is a Potential Component of the Yeast Nucleoskeleton

Christine Mirzayan, Connie S. Copeland, and Michael Snyder

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

Abstract. In an attempt to identify structural components of the yeast nucleus, subcellular fractions of yeast nuclei were prepared and used as immunogens to generate complex polyclonal antibodies. One such serum was used to screen a yeast genomic λgt11 expression library. A clone encoding a gene called *NUF1* (for *nu*clear *f*ilament-related) was identified and extensively characterized. Antibodies to NUF1 fusion proteins were generated, and affinity-purified antibodies were used for immunoblot analysis and indirect immunofluorescence localization. The NUF1 protein is 110 kD in molecular mass and localizes to the yeast nucleus in small granular patches. Intranuclear staining is present in cells at all stages of the cell cycle. The NUF1 protein of yeast is tightly associated with the nucleus; it was not removed by extraction of nuclei with nonionic detergent or salt, or treatment with RNAse and DNAse. Sequence analysis of the *NUF1* gene predicts a protein 945 amino acids in length that contains three domains: a large 627 residue central domain predicted to form a coiled-coil structure flanked by nonhelical amino-terminal and carboxy-terminal regions. Disruption of the *NUF1* gene indicates that it is necessary for yeast cell growth. These results indicate that NUF1 encodes an essential coiled-coil protein within the yeast nucleus; we speculate that NUF1 is a component of the yeast nucleoskeleton. In addition, immunofluorescence results indicate that mammalian cells contain a NUF1-related nuclear protein. These data in conjunction with those in the accompanying manuscript (Yang et al., 1992) lead to the hypothesis that an internal coiled-coil filamentous system may be a general structural component of the eukaryotic nucleus.

WITHIN the cell nucleus, a variety of essential processes such as DNA replication, RNA transcription, RNA splicing, and tRNA production occur (Newport and Forbes, 1987). Recent evidence indicates that chromosomes and other components within the nucleus are often organized into morphological domains. The nucleolus, nuclear membrane, and splicing centers (or snurposomes) are sites of well-known nuclear functions (Carmo-Fonseca et al., 1991; Spector, 1990). Less well-characterized nuclear domains have also been described in mammalian cells. These include sites of more active gene transcription (defined by the presence of RNA-binding proteins in spread chromosomes [Alberts et al., 1977; Igo-Kemenes et al., 1982]), nuclear "dots" [Ascoli and Maul, 1991; Xie, K., E. Lambie and M. Snyder, manuscript submitted for publication]), and nuclear bodies [Chaley et al., 1983; Vagner-Capodano et al., 1982]). In several species, chromosomes are also nonrandomly organized within the nucleus. For example, in *Drosophila*, polytene chromosomes of salivary glands have a specific configuration (Ellison and Howard, 1981: Hochstrasser et al., 1986; Saumweber, 1987), and in mammalian cells, centromeres can be paired, grouped at one end of the nucleus (Ringertz et al., 1986) or clustered near the nucleolus (Bartholdi, 1991).

Despite its simplicity and small size, the nucleus of the budding yeast *Saccharomyces cerevisiae* has a defined internal organization. The nucleolus occupies a crescent of approximately one third the nuclear volume, abutting the nuclear envelope (Aris and Blobel, 1988; Schimmang et al., 1989; Yang et al., 1989). The nucleolus lies directly opposite the spindle pole body, a microtubule organizing center embedded in the nuclear envelope (Yang et al., 1989). Furthermore, splicing components can be localized in discrete islands within the nucleus (Elliott, D. J., and M. Rosbash, personal communication).

The components that organize the nucleus into distinct morphological regions are not known. Two mechanisms, which are not mutually exclusive, could account for nuclear organization. In one case, chromosomes might assume particular orientations relative to each other and to the nuclear envelope. This might specify the position of other nuclear components such as the nucleolus which resides at the rDNA (Karpen et al., 1988; Warner, 1990). Alternatively, nonchromosomal structural components (RNA and protein) might specify a framework for nuclear organization, or create landmarks within the nucleus. In the latter case, the organization of the DNA, as well as the macromolecular

Connie S. Copeland's present address is Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.

complexes important for replication, transcription, RNA processing, and RNA transport would be specified by interactions with the underlying framework.

One component that may contribute to nuclear organization is the nuclear lamina, which is a network of fibrous proteins underlying the inner nuclear membrane. The proteins comprising this layer, the nuclear lamins (Gerace et al., 1978) are related in sequence to cytoplasmic intermediate filament proteins (Franke, 1987; McKeon, 1991; McKeon et al., 1986). Lamins, like intermediate filaments, are alpha-helical proteins that dimerize into coiled-coil rods, and associate to form higher-order structures (Aebi, et al., 1986; Heitlinger et al., 1991). The resulting lamina is likely to provide structural support for the nucleus (Gerace et al., 1978), and may provide an anchoring site for a few specific chromosomal loci (Paddy et al., 1990). However, the fibrous lamina does not extend any appreciable distance into the nuclear interior, suggesting that other internal components might be important for nuclear organization and/or morphology.

In an attempt to identify other structural components of the nucleus, a number of laboratories have extracted nuclei using various combinations of salts, detergents and nucleases. The remaining insoluble residue has been termed the nuclear matrix or nucleoskeleton (Belgrader et al., 1991; Berezney and Coffey, 1977; He et al., 1990; Lothstein et al., 1985; Potashkin et al., 1984; Staufenbiel and Deppert, 1984; reviewed by Verheijen et al., 1988). Most nuclear matrix preparations contain a filamentous structure that varies greatly in appearance and composition depending on the procedure used to isolate it. Because of this variability, and because of the possibility of aggregation-induced artifacts, the in vivo existence of a nuclear matrix has been called into question (see Cook, 1988). However, despite its controversial status, the nuclear matrix has been suggested as the site of a number of critical nuclear functions, including DNA replication, transcription, and RNA processing (e.g., Carri et al., 1986; Dijkwell et al., 1979; Pardoll et al., 1979, Xing and Lawrence, 1991).

Nucleoskeletons from mammalian cells have also been observed when conditions have been adjusted to reduce artifact formation. Jackson and Cook (1988) extracted agarose embedded cells with detergent at physiological salt concentrations, and then removed DNA by restriction enzyme digestion. This nucleoskeleton preparation retained the capacity to carry out replication and transcription at rates similar to permeabilized cells. The structure they observed by EM was a network of filaments of ~10 nm diameter, with a repeating structure of roughly 23 nm. These dimensions are quite similar to those of cytoplasmic intermediate filaments and nuclear lamins (Heitlinger et al., 1991). Whether a similar structure is a general feature of all eukaryotic nuclei is an important unresolved question.

The nuclei of *S. cerevisiae* carry out the same fundamental functions as those of other eukaryotes, and thus may be expected to have similar structural components, although yeast do not dissolve their nuclear envelope during mitosis. A nuclear scaffold fraction has been described for *S. cerevisiae* when nuclei are treated under certain conditions (Amati and Gasser, 1988; Cardenas et al., 1990). This insoluble structure contains topoisomerase II, which is an abundant, insoluble nuclear protein in interphase *Drosophila* cells (Berrios et al., 1985), and a component of the metaphase chromosome scaffold in vertebrate cells (Berrios et al., 1985; Earnshaw and Heck, 1985; Gasser et al., 1986). In yeast, centromeric DNA and origins of replication can be specifically associated with an insoluble scaffold (Amati and Gasser, 1988). However, for both mammalian and yeast structures, the proteins (or RNAs; see He et al., 1990) comprising the fibrous structures have not been characterized.

In this study, a new essential gene called *NUF1*, (for nuclear filament-related) of *Saccharomyces cerevisiae* has been identified and characterized. The NUF1 gene product localizes to the interior of the nucleus and cannot be easily extracted by detergent, salt, or nucleases. The predicted NUF1 protein sequence is similar to that of coiled-coil proteins such as intermediate filaments, lamins, and myosins. We speculate that the NUF1 protein is part of a novel filamentous nucleoskeleton. Furthermore, anti-NUF1 antibodies recognize an internal nuclear antigen in mammalian cells, suggesting that internal nuclear coiled-coil filamentous systems may be general components of the nucleoskeleton in eukaryotes.

Materials and Methods

Strains and General Techniques

Bacterial media and general DNA cloning methodology is described in Davis et al. (1980) and Sambrook et al. (1989). Yeast media and genetic manipulations are according to Sherman et al. (1986). The yeast strains used in this study are congenic with S288C; their genotype is presented in Table I.

Yeast Subcellular Fractionation

To generate nuclear fractions for immunization, tetraploid yeast nuclei were prepared using procedures adapted from Aris and Blobel (1988) and Hurt et al. (1988). Briefly, spheroplasts were prepared from four liters of tetraploid yeast strain Y558 grown to early log phase in YPD at 30°C. Washed cells were resuspended to 10% wet weight/volume in 1.3 M sorbitol, 0.50 mM KPO_4, pH 7.5, 0.3% β-mercaptoethanol, 0.5 mg/ml Zymolyase 100T (ICN K&K Laboratories Inc., Plainview, NY) and 0.05% (vol/vol) Glusulase (NEN; Dupont, Boston, MA). After a 75-min incubation at 37°C, spheroplasts were transferred to 0°C, washed once in 50 ml 1.3 M sorbitol, 50 mM KPO_4, pH 6.5, and then spun through a cushion of 10 ml 0.6 M sorbitol, 0.6 M sucrose, 2% Ficoll 400 in 20 mM KPO_4, pH 6.5 (5 min at 4,000 rpm, JA20 rotor).

Pellets were combined and resuspended to 10% wt/vol in cold homogenization medium (20% Ficoll 400, 20 mM KPO_4, pH 6.5, 1 mM $MgCl_2$, with protease inhibitors: 1 mM PMSF, 2 μg/ml each of chymostatin, leupeptin, pepstatin, and antipain, 1 mM *p*-aminobenzamidine, 1 mM ϵ-aminocaproic acid, 50 mM *p*-chloromercuriphenyl sulfonic acid). Homogenization was performed at 0° using a Dupont/Sorvall Omni-mixer (usually one or two bursts of 10 s at speed 10). Homogenization was monitored by fluorescence microscopy, by mixing equal volumes of homogenate with either of two multi-stain mixtures (multi-stain A: 1.5 μg/ml DAPI, 200 μg/ml rhodamine-conjugated phalloidin (Molecular Probes Inc., Junction City, OR), 200 μg/ml FITC-wheat germ agglutinin (Polysciences, Inc., Harrington, PA) in 20% Ficoll 400, 20 mM KPO_4, pH 6.5, 1 mM $MgCl_2$; multi-stain B: as for multi-stain A, but substituting rhodamine 6G at 1 μg/ml for rhodamine phalloidin). Homogenization was adjusted until nuclei (visualized by DAPI) were free of digested cell walls (FITC-WGA), actin spots (rhodamine phalloidin) and excess membranes (rhodamine 6G), but before the nuclei appeared torn. After homogenization, DTT was added to a final concentration of 1 mM.

Unbroken cells and cell walls were removed by two centrifugations at 5,000 rpm (JA20 rotor) for 15 min, 0°C. The supernatant was then loaded over six Ficoll step gradients as described by Aris and Blobel (1988), and spun in an SW28 rotor at 11,000 rpm for 75 min with slow acceleration and deceleration. The 30 and 40% layers were harvested separately, and diluted with an equal volumes of 0.6 M sucrose in 20 mM KPO_4, 1 mM $MgCl_2$ with twice the concentration of protease inhibitors listed above. Each mix-

ture was loaded over two step gradients of 5.8 ml 1.3 M sucrose, 9% Ficoll 400, 0.5 mM $MgCl_2$ in 20 mM KPO_4, pH 6.5, and 5.8 ml 1.5 M sucrose 9% Ficoll 400, 0.5 mM $MgCl_2$ in 20 mM KPO_4, pH 6.5. These gradients were centrifuged for 1 h in an SW28 rotor at 15,000 rpm. The pellets were harvested, and contained ~15 mg of nuclear protein. Aliquots of nuclei resuspended in homogenization buffer with protease inhibitors were frozen on dry ice-methanol and stored at −80°C.

For a rapid preparation of crude tetraploid nuclei, the homogenate was cleared of unbroken cells and residual cell walls by two spins of 15 min at 5,000 rpm in a JA20 rotor and then separated into supernatant and pellet fractions by a final spin of 13,000 rpm for 30 min in the same rotor. Nuclei were resuspended in homogenization medium with protease inhibitors. For a similar preparation of haploid nuclei the homogenate was cleared by two centrifugations of 15 min at 7,000 rpm (JA20), and the final spin was 30 min at 12,500 rpm (JA21 rotor).

Immunization of Mice with Subnuclear Fractions

Tetraploid nuclear fractions were prepared using Ficoll gradients followed by Ficoll/sucrose gradients, as described above. Nuclei were diluted with buffered sucrose to a concentration of ~2.5 mg/ml protein in 0.25 M sucrose, 5% Ficoll, 0.25 mM $MgCl_2$. DTT was added to 20 mM and EDTA to 5 mM. Nuclei were mixed by inversion, and pelleted for 30 min at 12.5K rpm, JA18.1 rotor, wide position. The supernatant was removed and the pellet resuspended in 0.25 M sucrose, 20 mM KPO_4, pH 6.5, 0.5 mM $MgCl_2$, and then adjusted to 1% Triton X-100 and 0.2 M NaCl in the same buffered sucrose. Nuclei were vortexed vigorously, incubated on ice for 30 min, and then separated into a supernatant and pellet by spinning 30 min, 12.5K rpm JA18.1 rotor. Fractions were stored frozen at −80°C. The supernatant fraction, called 2S, which contains nuclear pore complex-related proteins, was used to immunize mice. Other mice received the pellet fractions.

For immunization, the 2S fraction was precipitated with cold 10% TCA, washed in ice-cold ethanol/ethyl ether (1:1 vol/vol), resuspended in PBS with 0.1% SDS and heated to 95°C for 5 min. A female Balb/c mouse was primed with 0.4 ml pristane intraperitoneally. 5 wk later the mouse was immunized with the 2S extract from 500 μg of nuclei mixed with an equal volume of Freund's complete adjuvant. Three immunizations followed at 2–3-wk intervals with 250 μg-equivalents of 2S extract also in complete Freund's. A periorbital test bleed was taken 3 d after the second boost. 3 d after the last boost, 5×10^6 SP2/0 myeloma cells were injected IP to initiate ascites tumor formation. Ascites fluid was harvested ~2 wk after myeloma injection.

Extraction of Yeast Nuclei

Nuclei were isolated as described above and resuspended in 10% Ficoll, 20 mM KPO_4, pH 6.5, and 0.5 mM $MgCl_2$ to a final protein concentration of 1 mg/ml; this mixture contained the protease inhibitors PMSF, aprotinin, chymostatin, antipain, pepstatin, and leupeptin (Snyder, 1989). The nuclei were separated into 250-μl aliquots and extracted with 1–4 vol of sucrose extraction (SE) buffer plus salt, nonionic detergent, and/or nucleases (SE = 0.5 M sucrose, 20 mM KPO_4, pH 6.5, 0.5 mM $MgCl_2$, and protease inhibitors). Nine extraction/treatment conditions were used. Shown in Fig. 4 are (*1*) SE + 0.2 M NaCl; (*2*) SE + 0.5 M NaCl; (*3*) SE + 1% Triton X-100; (*4*) SE + 1% Triton X-100 + 0.2 M NaCl; (*5*) SE + 1% Triton X-100 + 1.0 M NaCl; (*6*) 0.25 mg/ml RNAse + 0.25 mg/ml DNAse I. Not shown are (*7*) SE + 1.0 M NaCl; (*8*) SE + 2.0 M NaCl; and (*9*) SE + 100 mM lithium acetate, 10 mM lithium 3′, 5′-diiodosalicylate. As a control, the isolated nuclei were treated with SE buffer alone, and processed in parallel with the extracted samples.

All incubations were performed on ice for 30 min, except for the nuclease treatment which was performed for 1 h. After the incubations, the samples were spun in a cold JA18.1 rotor at 12.5K RPM for 30 min. The supernatants were carefully removed and extracted proteins were precipitated with 10% TCA (final concentration) for 1 h on ice. This mixture was spun at 16,000 *g* for 5 min and the pellet was washed twice with cold ethanol/ether (1:1 vol/vol). The pellets were air dried, resuspended in 40 μl SDS sample buffer, and boiled for 5 min. The insoluble nuclear pellet fractions were resuspended in 40 μl SDS lysis buffer (Sambrook et al., 1989), and boiled for 5 min. 10 μl of each sample was analyzed by immunoblot analysis. 20 μl were analyzed in a parallel Coomassie blue-stained gel. Inspection of the Coomassie blue-stained gel revealed that for the samples extracted with 0.2 M NaCl (+/−detergent) equal amounts of protein were observed in the supernatant and pellet. For samples extracted with higher NaCl concentrations (+/−detergent), greater amounts of protein were present in the supernatant than in the pellet.

Isolation of the NUF1 Gene

A λgt11 yeast genomic DNA expression library was screened with the mouse ascites fluid (Snyder et al., 1987). The ascites had been previously depleted of anti-*E. coli* antibodies and diluted 1:700 in TBS + 20% FCS. After screening 1×10^6 recombinants, two positive clones were identified, called λNUF1.1 and λB. For λNUF1.1, portions of the inserts were subcloned into the bluescript vector (SK^+; Stratagene, La Jolla, CA). One of the subclones of λNUF1.1, contains a 1.9-kb EcoRI fragment and is called pNUF1.1.

To isolate λNUF1.2 and λNUF1.3, a λEMBL3A yeast genomic library (Snyder et al., 1986) was screened using a ^{32}P-labeled 1.9-kb EcoRI fragment of pNUF1.1 (Sambrook et al., 1989).

Preparation of the Glutathione-S-Transferase: NUF1 Fusion Protein and Production of Anti-NUF1 Antibodies

The 0.9-kb EcoRV fragment of λNUF1.1 (see Fig. 1) was cloned into the SmaI site of pGEX1 (Pharmacia Fine Chemicals, Piscataway, NJ) resulting in a fusion of NUF1 coding sequences in the appropriate frame and orientation. Overexpression and production of the fusion protein was performed in the *E. coli* strain RR1 as described in Smith and Johnson (1988). 3 mg of GST:NUF1 fusion protein was purified from two 1L cultures using glutathionine-conjugated agarose beads, and a small sample was analyzed in a polyacrylamide gel containing SDS (Laemmli, 1970) and stained with Coomassie brilliant blue. A single polypeptide of the expected 65-kD molecular mass was observed. Rabbit immunizations were administrated by the Pocono Rabbit Farms and Laboratories (Candesis, PA). A primary injection was followed by three boosts, spaced two weeks apart. Each immunization consisted of 600 μg protein applied subcutaneously in 10 locations.

Affinity purification of antibodies to the GST:NUF1 fusion was performed as described in Snyder (Snyder, 1989). 200 μg of GST:NUF1 fusion protein was adsorbed onto $1cm^2$ of nitrocellulose filter overnight at 4°C. The filter was blocked with PBS + 3% BSA (PBS = 0.15 M NaCl, 50 mM $NaPO_4$, pH 7.2) for 6 h at room temperature and then incubated with the 0.4 ml of rabbit serum + 0.8 ml of PBS overnight. The filters were washed, and affinity-purified antibodies were eluted with 0.2 M glycine pH 2.5. Purified antibodies (~1 ml) were desalted and concentrated by spinning for 15 min, 5,000 *g* at 4°C in Centricon-30 microconcentrators (Amicon) to a final volume of 500 μl. As a negative control, preimmune sera was incubated with GST::NUF1-coated filters treated in an identical fashion. Affinity-purified anti-NUF1 antibodies react strongly with the GST:NUF1 fusion (data not shown). Experiments using the preimmune serum failed to recognize this protein.

Immunoblot Analysis

To test the immunological reactivity of the polyclonal sera, 0.2 μg of the GST:NUF1 fusion protein was heated to 100°C in SDS sample buffer for 10 min, separated in a 7.5% polyacrylamide gel containing SDS, and blotted to nitrocellulose (Burnette, 1981). The blots were blocked in 20% FCS + 10% nonfat milk in TBS (0.15 M NaCl, 50 mM Tris, pH 7.2) for 6 h at room temperature, incubated in affinity-purified antibodies diluted 1:100 in 20% FCS + TBS overnight at 4°, and detected by a 1-h incubation with 5 μCi/ml ^{125}I protein-A (ICN K&K Laboratories) at room temperature. Three 15-min washes were conducted after the primary antibody and ^{125}I protein-A incubations. All washes were performed in 5% nonfat milk, 0.05% NP-40 in TBS at room temperature.

For immunoblot analysis of yeast proteins, spheroplast homogenates, or nuclear and cytoplasmic fractions were prepared from yeast as described above. The quantity of protein from each fraction was determined as described by Bradford (1976) and 40 μg of each fraction was boiled in SDS sample buffer for 10 min and separated in a 7.5% polyacrylamide gel containing SDS (Laemmli, 1970). Gel blots were prepared and treated as described above.

For immunoblot analysis of mammalian proteins, isolated nuclear fractions were prepared according to Davis and Blobel (1986). 100 μg of protein was separated in a 7.5% polyacrylamide gel containing SDS and processed as above. A protein band of ~100 kD in molecular mass was recognized upon long exposures of the blot.

Indirect Immunofluorescence

Yeast. The indirect immunofluorescence procedure was similar to that de-

Table I. Strain List

Strain	Genotype
Y270	*MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1*
	MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1
Y727	*MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 nufl::URA3-1*
	MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 NUF1
Y728	*MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 nufl::URA3-1*
	MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 NUF1
Y558	*MATa ura3-52 LYS2 ade2-101 HIS3 trp1-Δ1 TYR1*
	MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 tyr1
	MATα ura3-52 LYS2 ade2-101 his3-Δ200 trp1-Δ1 TYR1
	MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 TYR1

scribed by Gehrung and Snyder (1990). Three yeast strains were used: (*a*) wild type diploid strain Y270; (*b*) *nufl::URA3-1/NUF1* heterozygous diploid strain Y727; and (*c*) tetraploid strain Y558. Double immunofluorescence was performed using affinity-purified anti-NUF1 antibodies (1:100 dilution) and YOL1/34 (1:100 dilution), a mAb that recognizes tubulin and which served as a positive control (not shown). The secondary antibodies used were Texas red–conjugated goat antirabbit antibodies (1:100 dilution of stock; Amersham Corp., Arlington Heights, IL) and fluorescein-conjugated goat antirat antibodies (1:100 dilution of Cappel Laboratories stock). Visualization of DNA in the same samples was accomplished by the addition of 0.25 μg/ml Hoechst 33258 to the mounting solution (70% glycerol, PBS, 2% *n*-propyl gallate).

Mammalian. CV1 cells were grown on glass cover slips, rinsed once with PBS, and fixed in PBS containing 1% formaldehyde for 10 min. The cells were washed 5 min each in PBS, PBS + 0.1% NP-40, and then PBS. Affinity-purified anti-NUF1 antibody was diluted 1:50 and incubated with the cells for 2 h at room temperature. The cells were washed again, 5 min each, in PBS, PBS + 0.1% NP-40, then PBS, and then incubated with Texas red–conjugated goat antirabbit antibodies at a dilution of 1:100 in PBS. The cells were washed and mounted in solution containing 0.25 μg/ml Hoechst 33258.

DNA Sequence Analysis

The DNA sequence of the *NUF1* gene was determined by the dideoxy chain termination method as described in Sanger et al. (1977) in the presence of [α-^{35}S]dATP (650 Ci/mmol; Amersham Corp.) (Williams et al., 1986). Three general approaches were utilized to sequence both strands of the *NUF1* gene. The 3.0-kb EcoRI piece of clone λNUF1.2 and the 3.7-kb SalI piece of clone λNUF1.3 were subcloned into the bluescript vector (SK$^+$; Stratagene). From both ends of the insert, sets of unidirectional nested deletions were generated by partial digestion with exonuclease III and S1 nuclease (Henikoff, 1987). Several subclones were also made using specific DNA restriction fragments of the *NUF1* gene. Finally, primers were made to eight locations of the gene and used to determine the sequence in specific regions. In areas of ambiguity, multiple subclones and/or primers were prepared and analyzed. Some portions (~20% overall) of the second strand were determined using DNA derived from the λgt11 clone, λNUF1.1; this sequence was identical to that obtained from the λEMBL3A clones. The NBRF databank was searched for similarity to the predicted amino acid sequence of NUF1 using the FASTA program in the ktup-2 mode, and the translated Genbank was searched using tFASTA (Pearson, 1990; Pearson and Lipman, 1988). Secondary structure predictions were made using the Garnier secondary structure program (Pearson, 1990).

Construction and Analysis of a Disruption Mutation in the NUF1 Gene

To construct the *nufl::URA3* allele, the 2.5-kb SacI fragment of the *NUF1* gene was subcloned into DSK$^+$-1. DSK$^+$-1 is a derivative of SK$^+$ whose polycloning site had been truncated by digesting SK$^+$ with BamHI and ClaI. The ends were filled in with the large fragment of *E. coli* DNA polymerase I and dNTPs, and then ligated. The *URA3* gene was isolated on a 1.1-kb HindIII fragment from YEP24 (Carlson and Botstein, 1982) and subcloned into the SK$^+$-1::NUF1 construct at the HindIII site. Subclone *nufl-1::URA3* was recovered with the *URA3* gene transcribed in the opposite orientation as NUF1. *nufl-2::URA3* had the *URA3* gene in the same orientation as NUF1. These constructs were then digested with Sac I, transformed into diploid yeast strain Y270 by electroporation (Becker and Guarente, 1991; Rothstein, 1983). Ura$^+$ transformants could only be recovered for the *nufl-1::URA3* allele and not for *nufl-2::URA3*. Presumably, promoter occlusion prevents efficient transcription of the URA3 gene in the *nufl-2::URA3* construct.

Two *nufl::URA3-1/NUF1* heterozygous diploids were sporulated. 32 tetrads were analyzed from strain Y727. 25 tetrads segregated progeny where 2 spores produced colonies and 2 spores did not (2:2 segregation); 7 tetrads segregated progeny where 1 spore produced a colony and 3 spores did not (1:3 segregation). From strain Y728, 23 tetrads were analyzed, 17 tetrads segregated progeny in which 2 spores each produced colonies and 2 did not; 5 tetrads segregated progeny in which one spore produced a colony and three did not. All haploid progeny that formed colonies were Ura- as determined by replica-plating onto SC plates lacking uracil.

Gel blot analysis of genomic DNA of Y727 using ^{32}P-labeled NUF1 probes confirmed correct substitution at the *NUF1* locus; analysis of two progeny that formed colonies from this strain revealed that they contained only the wild-type *NUF1* allele.

Results

Isolation of the NUF1 Gene

To identify proteins that might be structural components of the nucleus, an immunological approach was undertaken. A variety of yeast subnuclear fractions were prepared and complex polyclonal antibodies were generated in mice. One nuclear fraction, a supernatant obtained after washing yeast nuclei with EDTA, then treating with 0.2 M NaCl and 1% Triton X-100, yielded polyclonal antibodies that recognized five major bands on immunoblots of yeast proteins and a large number of minor species (data not shown). Indirect immunofluorescence indicated that this complex serum recognized a variety of nuclear components including the nuclear envelope, nucleolus and the nuclear interior. This serum was used to screen 1 × 10^6 recombinant phage of a λgt11 yeast genomic library. Two positive clones, λNUF1.1 and λB, were identified and shown to be distinct by comparison of DNA sequences and restriction maps.

Proteins produced from the λgt11 clones were used to affinity purify antibodies from the complex serum. Proteins from λB purified antibodies that recognize a 60-kD cytoplasmic protein as determined by indirect immunofluorescence

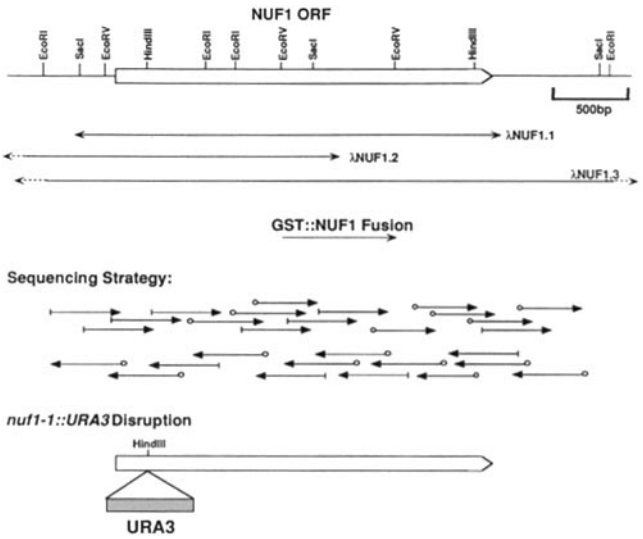

Figure 1. A restriction map of the NUF1 region and sequencing strategy. The positions of yeast coding sequences of the λgt11 clone, λNUF1.1, and the λEMBL3A clones, λNUF1.2 and λNUF1.3, are indicated. λNUF1.2 and λNUF1.3 contain ~15-kb yeast DNA inserts and were mapped only in the NUF1 region; uncharacterized portions extend beyond this region as indicated by the dashed lines. The position of the 900-bp segment used for generating GST::NUF1 fusions is also indicated. For the sequencing strategy, arrows indicate the length and direction of the sequence determined. Arrows beginning with a short vertical line denote sequence derived from the λNUF1.1 clone; arrows starting with a circle denote sequence derived from the λEMBL3A clones. No sequence differences were observed between the λgt11 and λEMBL3A clone. (*Bottom*) The position of the *nuf1-1::URA3* insertion mutation in the *NUF1* gene. A 1.1-kb fragment containing the *URA3* gene (not to scale) was inserted into the HindIII site located at the 5′ end of the NUF1 gene. The open reading frame of *URA3* is in the opposite direction of the *NUF1* ORF.

and immunoblot analysis. Antibodies purified from λNUF1.1 proteins failed to provide definitive results in indirect immunofluorescence. However, λNUF1.1 was studied further because preliminary sequence analysis indicated that the predicted protein encoded by this clone may form a coiled-coil structure (see below). A restriction map of λNUF1.1 is presented in Fig. 1. The encoded gene is called *NUF1* for *Nu*clear *F*ilament-related gene. NUF1 is not encoded as a fusion protein on λNUF1.1 as determined by both immunoblot analysis (not shown) and DNA sequence analysis (see below).

The NUF1 Gene Encodes a 110-kD Polypeptide That Fractionates with the Nucleus

Indirect immunofluorescence or immunoblot analysis failed to provide conclusive results using λNUF1.1 proteins to affinity purify antibodies from the complex serum. We therefore prepared polyclonal antibodies directly to the NUF1 gene product. An internal 0.9-kb coding segment of the *NUF1* gene was cloned into the pGEX1 vector (Fig. 1), and a 65-kD glutathione-S-transferase::NUF1 fusion protein was overproduced in *E. coli*. 55% of the 65-kD fusion protein was encoded by *NUF1* sequence, the remainder by glutathione-S-transferase. Rabbits were immunized with the GST::NUF1 fusion, and NUF1-specific antibodies were affinity purified from the immune serum using the fusion protein.

To determine the size and subcellular location of the NUF1 protein in yeast, immunoblotting experiments were performed on subcellular fractions. Homogenates of yeast spheroplasts were separated into nuclear and cytoplasmic fractions, and equal amounts of protein from each sample were separated in an SDS-polyacrylamide gel. Immunoblots were prepared and probed with affinity-purified anti-NUF1 antibodies. As shown in Fig. 2, a protein of 110 kD is recognized in the spheroplast homogenate; this protein is highly enriched in the nuclear fraction. Two less intense bands of lower molecular mass are also detected in the nuclear fraction. These smaller proteins probably represent degradation products since they are not observed under conditions that reduce proteolysis (not shown). No bands are observed in experiments using preimmune serum. Thus, the 110-kD nuclear polypeptide corresponds to the NUF1 gene product.

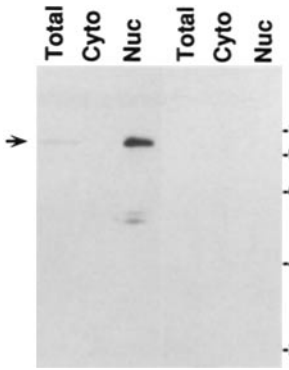

Figure 2. Immunoblot of yeast spheroplast proteins and proteins from nuclear and cytoplasmic fractions probed with anti-NUF1 antibodies. Equal amounts of proteins prepared from whole yeast cells (*Total*), yeast nuclear (*Nuc*) and cytoplasmic (*Cyto*) fractions were separated in a polyacrylamide gel containing SDS. Immunoblots were prepared and probed with affinity-purified anti-NUF1 antiserum and preimmune sera. (*Left*) Affinity-purified antibodies. (*Right*) Preimmune serum. The arrowhead indicates the position of the 110-kD NUF1 polypeptide.

The NUF1 Gene Product Localizes to the Nucleus by Indirect Immunofluorescence

To independently test the nuclear location of the NUF1 protein, indirect immunofluorescence experiments were carried out on diploid yeast cells using affinity-purified anti-NUF1 antibodies. Anti-NUF1 staining is visible throughout the nucleus, and is not uniform, but appears somewhat granular (Fig. 3). The NUF1 protein is not present in the nucleolar region, which is recognizable as the weak DAPI-staining portion of the nucleus. Furthermore, nuclear staining is observed in all cells regardless of the stage of the cell cycle; unbudded, small budded, and large budded cells (including mitotic cells with dividing nuclei) all stain with similar intensity. Wild type tetraploids, diploids and heterozygous *nuf1/NUF1* diploid mutants each stain well. No staining above background was observed when preimmune sera was used (Fig. 3 *e*). Thus, subcellular fractionation and immunofluorescence experiments both indicate that the NUF1 protein is a nuclear protein.

The NUF1 Protein Is Tightly Associated with Yeast Nuclei

To understand the nature of the association of the NUF1 protein with the nucleus, biochemical extraction experiments were performed. Yeast nuclei were prepared and extracted with a buffer containing either 0.2, 0.5, 1.0, or 2.0 M NaCl, or with 1% Triton X-100 in combination with increasing salt concentrations (see Materials and Methods). The insoluble

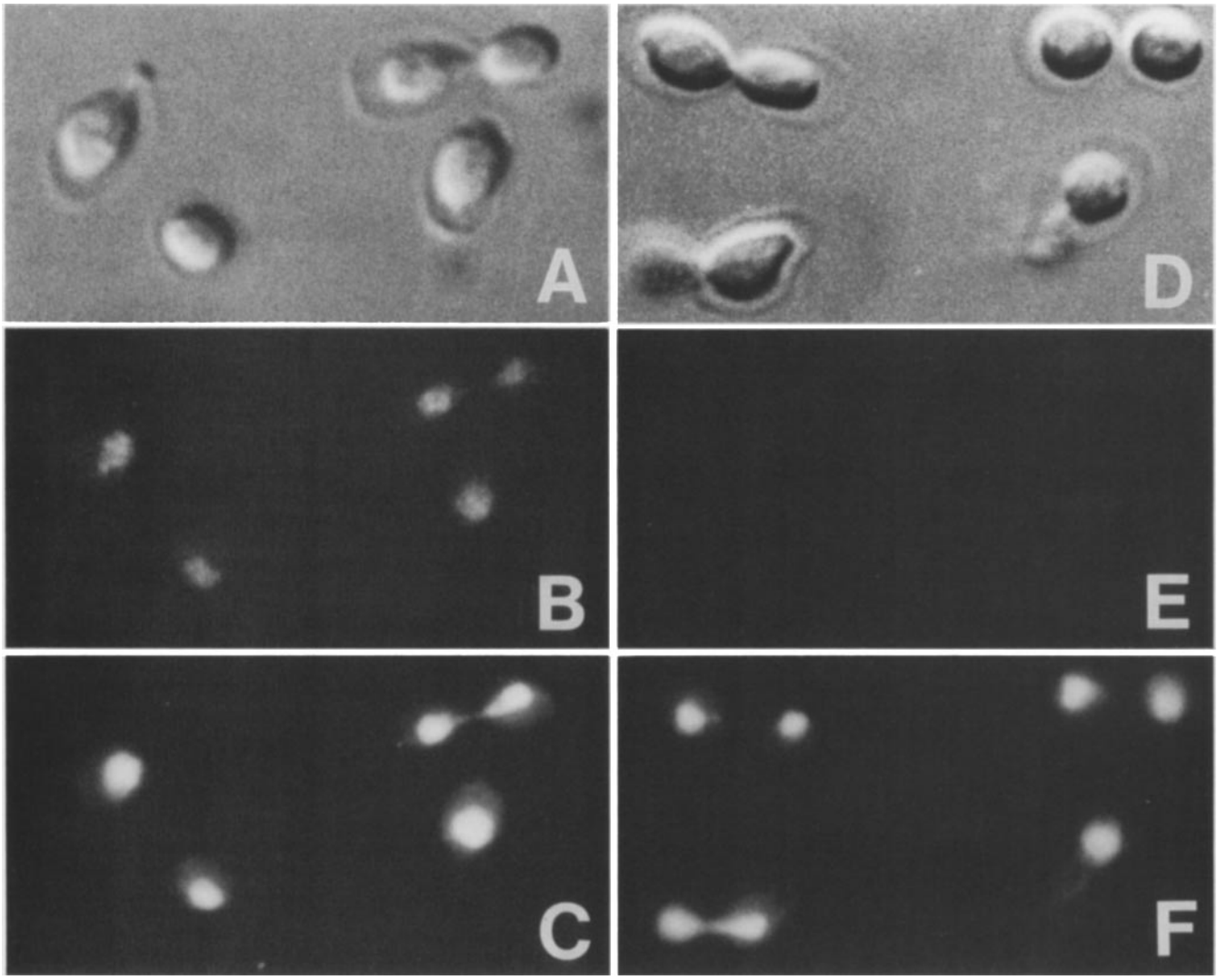

Figure 3. Indirect immunofluorescence of diploid yeast cells using affinity-purified anti-NUF1 antibodies (*A–C*) and control preimmune antibodies (*D–F*). (*A* and *D*) Differential interference contrast microscopy of the stained cells. (*B* and *E*) Staining with anti-NUF1 antibodies (*B*) or preimmune antibodies (*E*). (*C* and *F*) Hoechst 33258 DNA staining of the same samples. Note that each sample contains unbudded, small budded, and mitotic cells.

nuclear material was pelleted by centrifugation; the extracted proteins remained in the supernatant. Control nuclei received only buffer treatment. As shown in Fig. 4, the NUF1 protein and presumed degradation products are not extracted with either 0.2 or 0.5 M NaCl, and extraction with up to 1.0 or 2.0 M NaCl also failed to remove the NUF1 protein from the nucleus (data not shown, identical to that observed for samples A–D). Furthermore, the majority of the NUF1 protein remained in the pellet after extraction with 0.2 or 1.0 M NaCl in the presence of 1.0% Triton X-100, although small amounts of the NUF1 protein are released under these membrane solubilization conditions. Extraction of yeast nuclei with 10 mM lithium diiodosalicylate which removes histones (Mirkovitch et al., 1984), also does not remove the NUF1 protein from the nucleus (data not shown; identical to that of samples A–D, Fig. 4). These experiments indicate that the NUF1 protein is tightly associated with the yeast nucleus and does not depend upon membrane integrity or chromatin for this association.

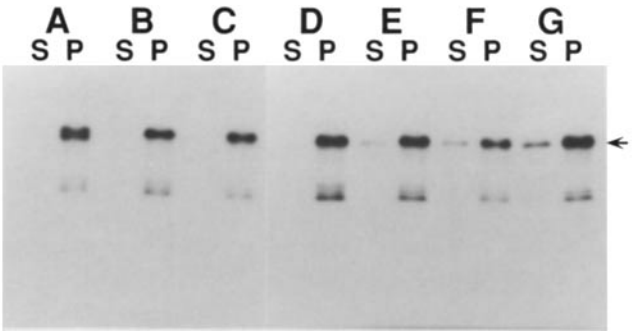

Figure 4. Immunoblots of nuclear fractions after extraction with salt, Triton X-100, and/or nuclease treatment. Yeast nuclei were extracted with low salt buffer (*A*), or buffer plus 0.2 M NaCl (*B*), 0.5 M NaCl (*C*), RNAse A plus DNAase I (*D*), 1% Triton (*E*), 1% Triton plus 0.2 M NaCl (*F*), or 1% Triton plus 1.0 M NaCl (*G*). The extracted proteins recovered from the supernatant (*S*) and residual pellet proteins (*P*) were separated in a 7.5% polyacrylamide gel, blotted, and probed with anti-NUF1 antibodies.

To test whether NUF1 is associated with RNA or DNA, the yeast nuclei were treated with RNAse and DNAase (Fig. 4, sample D). No detectable NUF1 protein was released from the nuclei, indicating that intact DNA and RNA are not required for maintaining NUF1 nuclear association.

The Predicted NUF1 Protein Sequence Contains a Large Domain with Similarity to Coiled-Coil Proteins

In an effort to learn more about the function of the NUF1 gene product, the DNA sequence of the *NUF1* gene was determined. The entire NUF1 open reading frame proved to be encoded by λNUF1.1, although this clone contained very little flanking sequence (Fig. 1). To isolate a clone encoding the entire *NUF1* gene with additional flanking sequences, a 1.9-kb segment of the *NUF1* gene was used as a probe to screen a λEMBL3A library containing ~15 kb segments of yeast genomic DNA. Two positive clones, λNUF1.2 and λNUF1.3 were identified. λNUF1.2 contains the 3′ end of the *NUF1* gene, and λNUF1.3 contains the entire *NUF1* reading frame plus adjacent sequences (Fig. 1).

A 3.7-kb segment containing the *NUF1* gene was sequenced using the dideoxy sequencing method (Fig. 5). A single large open reading frame (1–2,835 bp) was found, capable of encoding a 112-kD protein. The *NUF1* coding region lacks a TACTAAC sequence found near the 3′ ends of most yeast introns (Teem et al., 1984), suggesting that no intervening sequences interrupt the open reading frame. Furthermore, the size of the protein predicted from the DNA sequence is 112 kD, consistent with the results obtained by immunoblot analysis. The predicted NUF1 protein contains many charged amino acids:lysine (12.9% of total residues) arginine (7.3%), glutamic acid (13%), and aspartic acid (7.5%). However, the number of acidic and basic residues is approximately equal, and they are generally well distributed throughout the protein, except at the carboxy terminus which is enriched in arginines.

The predicted NUF1 protein sequence was compared to amino acid sequences in the NBRF-PIR database using the FASTA programs and to translated sequences in the Genbank database using the tFASTA programs (Pearson and Lipman, 1988). The NUF1 protein was found to have significant sequence similarity to proteins capable of forming coiled-coil structures such as myosins, nuclear lamins, and cytoplasmic intermediate filaments. Sequence similarity is strongest in the central NUF1 region extending from amino acid residues 164 to 791. When this region, which comprises 627 amino acids (66.4%) of the predicted NUF1 protein, is compared with known sequences, up to 21% sequence identity and 68% sequence similarity is found with the coiled-coil class of proteins (see Fig. 6 for comparison with the myosin heavy chain of *Dictyostelium discoideum*). These comparisons suggest that the NUF1 protein is a coiled-coil protein.

Further indication that NUF1 is a coiled-coil protein is derived from secondary structure analysis of the predicted protein sequence. Coiled-coil regions are generally alpha helical with heptad periodicity; hydrophobic residues are usually found in the first and fourth positions of the heptad (positions a and d when amino acid residues in each heptad are labeled *a–g*), and hydrophilic residues often occupy the remaining positions. This configuration generates a hydrophobic face on one side of the α-helix; interaction of two subunits occurs at their hydrophobic faces to form a coiled-coil dimer (Cohen and Parry, 1990).

Secondary structure analysis (Pearson and Lipman, 1988) of the NUF1 protein sequence predicts an alpha helical domain in the central region (Fig. 7). This region contains regular heptad repeats with predominantly hydrophobic residues in the a and d positions (Fig. 6). Within these positions, most (57%) of the hydrophobic residues are leucines. As is characteristic of coiled-coil proteins, the other positions of the heptad contain a high number of charged amino acid residues with those in positions e and g containing the highest percentage (61% charged amino acids in e and g as compared to 44% charged residues in c, d, f). Thus, both homology searches and secondary structure predictions indicate that the NUF1 protein could be a filamentous protein in the cell.

Closer inspection of the predicted secondary structure indicates that the 627 amino acid central region contains seven short insertions 1–11 amino acids long that interrupt the heptad periodicity (Figs. 6 and 7). However, we expect that the entire region forms a single coiled-coil, because the small insertions that separate the eight coiled-coil segments do not contain prolines and are expected to only cause minor shifts in the phasing of the heptads (see Discussion).

The amino- and carboxy-terminal domains that flank the central coiled-coil domain are 164 and 153 amino acid residues long, respectively. All of the proline residues fall into these domains. The amino-terminal domain contains two potential phosphorylation sequences for the cdc2/CDC28 protein kinase (Shenoy et al., 1989), which has been implicated in nuclear lamin disassembly (Heald and McKeon, 1990). One of these sequences lies at residues 36–39 (SPTK) and the other at 91–93 (SPR). A less perfect recognition sequence for this enzyme is found at serine 116. No cdc2/CDC28 phosphorylation consensus sequences are found in the carboxy terminus. As noted above, the carboxy-terminal end has an abundance of arginine residues. An unusually high number of tyrosine residues are also found in this region (13% of the total amino acids in this region are tyrosine). Additional features of the predicted NUF1 protein sequence are presented in the discussion.

In summary, the NUF1 protein can be divided into three general domains: an amino-terminal domain with potential phosphorylation sites, an arginine-tyrosine–rich carboxy-terminal domain, and a central alpha helical region predicted to form a coiled-coil rod.

The NUF1 Gene Is Essential for Cell Growth in Yeast

To determine whether the NUF1 gene product is essential for yeast cell growth, the *NUF1* gene was disrupted in vivo. The *URA3* gene of yeast was inserted near the 5′ coding region of the *NUF1* gene such that only 74 amino acids of the NUF1 protein can be translated upstream of the insertion (see Fig. 1). The *nuf1-1::URA3* allele was introduced into a diploid yeast strain by one-step gene transplacement (Rothstein, 1983). Correct substitution at the *NUF1* locus was confirmed by gel blot analysis of yeast genomic DNA. This transformant and a second independent transformant were sporulated and 55 total tetrads were analyzed. 42 tetrads each yielded two haploid progeny that formed colonies and two that did not; the remaining 13 tetrads each yielded one progeny that

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AATTCGGGAGCTCCTTGTAGACATTATTTCATATGTTATACTTAAACTAGATAGAGACGTTTTATTTGTACTAAGCTGAACATTACCCTAATTACGCGTTATAAGTTTTAATCCTTCATTTTG
AAGGAAAACAAATAAACAAAAAGTTGATGTGGAACAAATACAATGTGCGCGGATATCTTAAGCAACTTCGTTAGTTACAGTCCACACAAAGCGAAAAAGGGCTGATAAACACTC

1    ATG GAC GAA GCG TCA CAT CTC CCA AAT GGG AGC TTG AAG AAC ATG GAA TTT ACG CCT GTA GGA TTT ATC AAA TCC AAG CGA AAC ACC ACG
1    M   D   E   A   S   H   L   P   N   G   S   L   K   N   M   E   F   T   P   V   G   F   I   K   S   K   R   N   T   T

91   CAA ACA CAA GTT GTA TCG CCT ACT AAG GTT CCA AAT GCC AAT AAT GGT GAT GAG AAC GAA GGC CCT GTT AAG AAA AGG CAG AGA AGA AGC
31   Q   T   Q   V   V   S   P   T   K   V   P   N   A   N   N   G   D   E   N   E   G   P   V   K   K   R   Q   R   R   S

181  ATT GAT GAT ACA ATT GAC TCC ACA AGG CTA TTT AGT GAA GCT TCA CAA TTC GAT GAC AGC TTT CCA GAA ATT AAG GCT AAC ATT CCG CCT
61   I   D   D   T   I   D   S   T   R   L   F   S   E   A   S   Q   F   D   D   S   F   P   E   I   K   A   N   I   P   P

271  AGT CCA AGG TCA GGC AAT GTT GAC AAA AGT CGC AAG AGA AAT TTG ATT GAT GAT TTG AAG AAA GAT GTG CCA ATG TCT CAG CCC TTG AAA
91   S   P   R   S   G   N   V   D   K   S   R   K   R   N   L   I   D   D   L   K   K   D   V   P   M   S   Q   P   L   K

361  GAA CAA GAA GTA AGA GAA CAC CAA ATG AAG AAA GAG CGA TTT GAC CGT GCT TTA GAG AGT AAA TTA CTA GGA AAA AGA CAC ATA ACA TAC
121  E   Q   E   V   R   E   H   Q   M   K   K   E   R   F   D   R   A   L   E   S   K   L   L   G   K   R   H   I   T   Y

451  GCA AAT TCT GAT ATT TCT AAT AAG GAA CTT TAC ATT AAT GAA ATC AAG AGT TTG AAG CAT GAA ATC AAA GAA TTA AGA AAG GAA AAA AAC
151  A   N   S   D   I   S   N   K   E   L   Y   I   N   E   I   K   S   L   K   H   E   I   K   E   L   R   K   E   K   N

541  GAT ACT CTC AAT AAT TAT GAT ACC CTT GAA GAA GAA ACA GAT GAC TTG AAG AAC AGA TTA CAA GCG CTG GAA AAA GAG CTG GAC GCC AAA
181  D   T   L   N   N   Y   D   T   L   E   E   E   T   D   D   L   K   N   R   L   Q   A   L   E   K   D   V   D   A   K

631  AAT AAA ATT GTG AAT TCA AGA AAA GTA GAT GAT CAT TCT GGA TGC ATA GAA GAA CGT GAA CAA ATG GAA AGA AAG TTG GCT GAA TTA GAA
211  N   K   I   V   N   S   R   K   V   D   D   H   S   G   C   I   E   E   R   E   Q   M   E   R   K   L   A   E   L   E

721  AGA AAA CTG AAA ACT GTG AAA GAC CAA GTG CTA GAA TTA GAG AAT AAT AGT GAC GTA CAA AGT TTA AAA TTG AGA TCT AAG GAG GAT GAA
241  R   K   L   K   T   V   K   D   Q   V   L   E   L   E   N   N   S   D   V   Q   S   L   K   L   R   S   K   E   D   E

811  TTG AAG AAT TTA ATG AAT GAG TTG AAT GAA TTG AAG AGC AAT GCA GAA GAA AAG GAT ACA CAG TTG GAA TTC AAG AAA AAT GAA CTG AGG
271  L   K   N   L   M   N   E   L   N   E   L   K   S   N   A   E   E   K   D   T   Q   L   E   F   K   K   N   E   L   R

901  AAA CGA ACA AAT GAA TTA AAT GAG TTG AAA ATC AAG TCT GAT GAG ATG GAT TTA CAA CTA AAA CAA AAA CAA AAT GAA TCA AAA AGA TTA
301  K   R   T   N   E   L   N   E   L   K   I   K   S   D   E   M   D   L   Q   L   K   Q   K   Q   N   E   S   K   R   L

991  AAA GAT GAA TTA AAT GAG CTT GAA ACC AAA TTC AGC GAA AAT GGT TCT CAG TCT TCT GCA AAA GAA AAT GAA TTG AAA ATG CTG AAA AAT
331  K   D   E   L   N   E   L   E   T   K   F   S   E   N   G   S   Q   S   S   A   K   E   N   E   L   K   M   L   K   N

1081 AAA ATA GCC GAG CTA GAG GAA GAG ATT AGC ACG AAA AAT TCA CAG TTA ATC GCA AAA GAA GGT AAG TTA GCA TCA TTA ATG GCT CAG CTA
361  K   I   A   E   L   E   E   E   I   S   T   K   N   S   Q   L   I   A   K   E   G   K   L   A   S   L   M   A   Q   L

1171 ACT CAA TTG GAG AGT AAA CTT AAT CAA AGA GAC TCC CAG TTG GGC TCA AGG GAA GAA GAA TTG AAA AAA ACA AAC GAT AAG CTA CAA AAA
391  T   Q   L   E   S   K   L   N   Q   R   D   S   Q   L   G   S   R   E   E   E   L   K   K   T   N   D   K   L   Q   K

1261 GAT ATC AGG ATA GCA AGA GAG GAA ACA GTT TCA AAG GAT GAA CGG ATA ATT GAT CTT CAA AAA AAG GTT AAA CAG CTA GAA AAT GAC TTA
421  D   I   R   I   A   R   E   E   T   V   S   K   D   E   R   I   I   D   L   Q   K   K   V   K   Q   L   E   N   D   L

1351 TTT GTG ATA AAA AAA ACG CAC AGT GAG TCT AAA ACT ATT ACT GAT AAT GAA CTA GAA TCT AAA GAT AAA CTT ATT AAA ATT TTA GAA AAC
451  F   V   I   K   K   T   H   S   E   S   K   T   I   T   D   N   E   L   E   S   K   D   K   L   I   K   I   L   E   N

1441 GAT TTA AAG GTT GCA CAA GAG AAG TAC TCT AAA ATG GAA AAA GAG CTC AAA GAA AGG GAA TTT AAC TAT AAA ATT TCC GAA TCA AAG TTG
481  D   L   K   V   A   Q   E   K   Y   S   K   M   E   K   E   L   K   E   R   E   F   N   Y   K   I   S   E   S   K   L

1531 GAA GAT GAA AAG ACC ACG CTA AAT GAA AAA ATT TCT AAC TTA GCC GCA GAA AAC TCA CAG CTA AAA AAT AAA ATA GAG GAC AAT TCG ACT
511  E   D   E   K   T   T   L   N   E   K   I   S   N   L   A   A   E   N   S   Q   L   K   N   K   I   E   D   N   S   T

1621 GCC ACT CAC CAT ATG AAA GAA AAC TAT GAG AAG CAG TTA GAA TCG CTA AGG AAA GAT ATT GAA GAG TAC AAA GAA AGC GCA AAA GAT TCT
541  A   T   H   H   M   K   E   N   Y   E   K   Q   L   E   S   L   R   K   D   I   E   E   Y   K   E   S   A   K   D   S

1711 GAA GAC AAA ATT GAG GAA CTA AAA ATT AGG ATT GCT GAA AAT TCT GCT AAA GTA TCG GAG AAA AGA TCA AAG GAT ATA AAA CAA AAA GAT
571  E   D   K   I   E   E   L   K   I   R   I   A   E   N   S   A   K   V   S   E   K   R   S   K   D   I   K   Q   K   D

1801 GAA CAG ATC AGC GAC CTC ACT CAG AAT CTA AAA CTA CAA GAA GAT GAG ATA AGC TCA TTA AAA TCC ATA ATT GAC AGG TAC AAA AAA GAT
601  E   Q   I   S   D   L   T   Q   N   L   K   L   Q   E   D   E   I   S   S   L   K   S   I   I   D   R   Y   K   K   D

1891 TTC AAT CAA TTG AAA TCT GAA CAG AGT AAT ATC CAA CAT GAC CTA AAT TTA CAA ATA CTA AAT CTG GAA AAT AAG TTA ATA GAG AGC GAG
631  F   N   Q   L   K   S   E   Q   S   N   I   Q   H   D   L   N   L   Q   I   L   N   L   E   N   K   L   I   E   S   E

1981 GAT GAA TTA AAG TCA CTA AGA GAT TCT CAA AAA ATT GAA ATA GAA AAC TGG AAG AGA AAG TAT AAC AAT CTT TCA CTG GAA AAT GAC AGA
661  D   E   L   K   S   L   R   D   S   Q   K   I   E   I   E   N   W   K   R   K   Y   N   N   L   S   L   E   N   D   R

2071 TTG TTG ACA GAA AAA GAA TCC GCA TCA GAC AAA GAG CGC GAG ATA TCC ATC TTG AAC AGA AAA CTT GAT GAA ATG GAT AAA GAA AAA TGG
691  L   L   T   E   K   E   S   A   S   D   K   E   R   E   I   S   I   L   N   R   K   L   D   E   M   D   K   E   K   W
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Figure 5. Nucleotide and predicted amino acid sequences of the *NUF1* locus. The predicted NUF1 translation product is depicted below the nucleotide sequence. Numbers adjacent to the DNA sequence refer to the nucleotide position. Numbers adjacent to the predicted amino acid sequence refer to the amino acid position. These sequence data are available from EMBL/Genbank/DDBJ under accession number Z11582.

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2161 AAC TTA CAG GAA TCT AAA GAA AAA TAT AAA CGC GAA CTA CAG AAG GTG ATT ACT GCT AAT GAT CGT TTG AGA AGA GAG AAA GAG GAG TTG
721 N  L  Q  E  S  K  E  K  Y  K  R  E  L  Q  K  V  I  T  A  N  D  R  L  R  R  E  K  E  E  L

2251 AAT GAA AAC AGC AAT AAT ATT CGT ATC ATG GAA GAC AAG ATG ACT AGG ATA AAA AAA AAT TAT TTG AGT GAA ATC ACT TCT TTA CAA GAG
751 N  E  N  S  N  N  I  R  I  M  E  D  K  M  T  R  I  K  K  N  Y  L  S  E  I  T  S  L  Q  E

2341 GAA AAT AGG AGA CTT GAA GAA CGC CTC ATA TTA AAT GAG AGG CGT AAA GAC AAT GAT TCA ACT ATG CAA TTA AAT GAC ATC ATA AGC TAT
781 E  N  R  R  L  E  E  R  L  I  L  N  E  R  R  K  D  N  D  S  T  M  Q  L  N  D  I  I  S  Y

2431 TAT AAA TTG AAG TAT CAC TCA GAA GTA AGA CAC AAC AAC GAT CTT AAG GTG ATC AAT GAT TAT TTA AAC AAG GTT CTT GCT TTG GGG ACC
811 Y  K  L  K  Y  H  S  E  V  R  H  N  N  D  L  K  V  I  N  D  Y  L  N  K  V  L  A  L  G  T

2521 CGT CGT TTA AGA TTG GAC ACA AGG AAA GGT GAG CAC AGT CTA AAC ATT TCA CTT CCA GAC GAT GAT GAA CTA GAT CGT GAT TAC TAC AAT
841 R  R  L  R  L  D  T  R  K  G  E  H  S  L  N  I  S  L  P  D  D  D  E  L  D  R  D  Y  Y  N

2611 AGC CAT GTT TAT ACA AGG TAT CAT GAC TAT GAA TAC CCG CTT AGA TTC AAT TTA AAC AGA AGA GGT CCC TAT TTC GAA CGC CGC TTA AGC
871 S  H  V  Y  T  R  Y  H  D  Y  E  Y  P  L  R  F  N  L  N  R  R  G  P  Y  F  E  R  R  L  S

2701 TTC AAA ACA GTT GCC CTT TTA GTG CTT GCT TGT GTG AGG ATG AAA AGG ATT GCT TTT TAC AGG AGA TCA GAC GAT AAC AGA TTG CGA ATA
901 F  K  T  V  A  L  L  V  L  A  C  V  R  M  K  R  I  A  F  Y  R  R  S  D  D  N  R  L  R  I

2891 CTA AGA GAT AGA ATT GAG AGT AGC AGC GGG CGT ATA TCT TGG TAG
931 L  R  D  R  I  E  S  S  S  G  R  I  S  W

TATCGCTTACTCTATCATCATATTTCTCGTATGTACATCGACTCCTACGCAGACTGATCTTCAAGCGAGCCACCGTGAATGTGGAAGCATAAACAGATGAGAAACCTTTTTGTTTCTGAGATCG
TGACCTTAGATTGTAGTGTGCTAATCTCCCTAGTATTGACTACTTTGCGTTTATTCATTTCTTTCTCCAATAGAAATACAGCATAAAACATACGTAGGGTATATCTATGTATGTGGTATATTTG
TATTATAAGTTACTTAGATTTACTGGCGCCCTTTTTTAAGCTATTGTAATTATTTACGGCCTCTGCGATTCTTTCCTTGAAAGTATCTGTTATTATCGCCAGTTGGTTTAACATTTCATAGTGA
TTTTCCAACGTATCGCATTTTTCAAGGAGTTCTTTGAACTTCGCAATATTTTTATCTATGGTGTTTTCAATAATACCATTCACC
```

Figure 5.

formed a colony and three that did not. All progeny that formed colonies were Ura⁻. Microscopic inspection of the progeny that did not form colonies revealed two to eight large cells, indicating that the spores had germinated and the vegetative cells had divided one to three times before death. Approximately 80% of the terminal cells were budded. Thus, *NUF1* is necessary for vegetative growth.

```
NUF1   IKSLKHEIKELRKEKNDTLNNYDTLEEETDDLKNRLQALEKELDAKNKIVNSRKVDDHSGCIEEREQMERKL  236
MYOSIN EQLAKSNVEKQKKKVELDLEDKSAQLAEETAAKQALDKLKKKLEQELSEVQTQLSEANNKNVNS----DSTN 1261

AELERKLKTVKDQVLELENNSDVQSLKLRSKEDELKNLMNELNELKSNAEEKDTQLEFKKNELRKRTNELNELKIKSDEM  316
KHLETSFNNLKLELEAEQKAKQALEKKRLGLESELKHVNEQLEEEKKQKESNEK----RKVDLEK---EVSELKDQIEE- 1334

DLQLKQKQNESKRLKD-ELNELETKFSENGSQSSAKENELKMLKNKIAELEEEISTKNSQLIAKEGKLASLMAQLTQLES  395
EVASKKAVTEAKNKKESELDEIKRQYADVVSSRDKSVEQLKTLQAKNEELRNTAEEAEGQLDRAERSKKKAEFDLEEAVK 1414

KLNQRDSQLGSREEELKKTNDKLQKDIRIAREETVSKDERIIDLQKKVKQLENDLFVIKKTHSESKTITDNELESKDKLI  475
NLEEETAKKVKAEKAMKKAETDYRSTKSELDDAKNVSSEQYVQI-KRLNEELSELRSVLEEADERCNSAIKAKKTAESAL 1494

KILENDLKVAQEKYSKMEKELKEREFNYKISESKLEDEKTTLN-EKISNLAAENSQLKNKIEDNSTATHHMKENYEKQLE  554
ESLKDEIDAANNAKAKAERKSKELEVRVAELEESLEDKSGTVNVEFIRKKDAEIDDLRARL-DRETESRIKSDEDKKNTR 1574

SLRKDIEEYKESAKDSEDKIEELKIRIAENSAKVSEKRSKDIKQKDEQISDLTQNLKLQEDEISSLKSIIDRYKKDFNQL  634
KQFADLEAKVEEAQREVVTIDRLKKKLESDIIDLSTQLDTETKSR---IKIEKSKKKLEQTLAERRAAEEGSSKAADEEI 1651

KSEQSNIQHDLNLQILNLENKLIESEDELKSLRDSQKIEIENWKRKYNNLSLENDRLLTEKESASDKEREISILNRKLDE  714
RKQVWQEVDELRAQLDSERAALNASEKKIKSLVA----EVDEVKEQLEDEILAKDKLVKAKRAL---EVELEEVRDQLEE 1724

MDKEKWNLQESKEKVKRELQKVITANDRLRREKEELNENSNNIRIMEDKMTRIKKNYLSEITSLQEENRRLEERLIL  791
EEDSRSELEDSKRRLTTEVEDIKKKYDAEVEQNTKLDEAKKK---LTDDVDTLKKQLEDEKKKLNESERAKKRLESE 1792
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Figure 6. Alignment of the predicted coiled-coil sequence of the NUF1 protein (*top*) with the myosin heavy chain of *Dictyostelium discoideum* (*bottom*). This alignment, as determined by a search of the NBRF-PIR database using the FASTA program, is representative of an average identity score (19.5%) and similarity score (67.5%) for myosins. Two dots represent amino acid identity; one dot represents amino acid similarity. Similarity was calculated to be 34.1% using more stringent criteria (identities plus $E = D$, $K = R$, $Q = N$, $I = L = A = V = M$, $F = Y$). The numbers on the right represent amino acids positions of the NUF1 protein (*top*) and of the myosin heavy chain of *D. discoideum* (*bottom*). The first and fourth residues of the NUF1 heptad repeats are indicated by *a* and *d*. The seven underlined regions represent short interruptions in the predicted coiled-coil of the NUF1 protein.

NUF1 Antibodies Recognize a Related Antigen in the Nucleus of CV1 Cells

As shown in the accompanying manuscript (Yang et al., 1992) coiled-coil–related proteins are also present in the nuclear interior of mammalian cells. To determine whether NUF1-related proteins are present in mammalian cells, African Green Monkey Kidney (CV1) cells were stained with affinity-purified anti-NUF1 antibodies using indirect immunofluorescence. The results, shown in Fig. 8, indicate that the nucleus stains intensely. The staining is somewhat granular and is not present in the nucleolus, consistent with NUF1 protein localization in yeast. Very weak staining is detected in the cytoplasm. In mitotic cells, the NUF1 stain is dis-

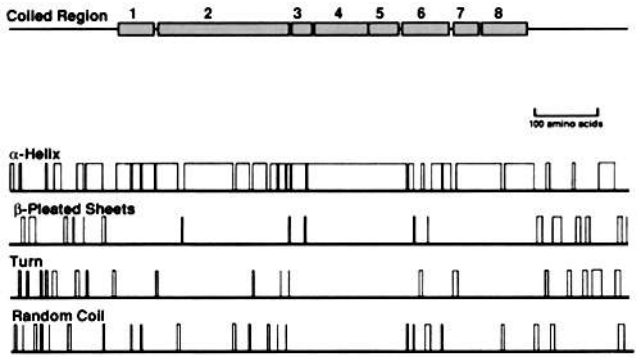

Figure 7. Secondary structure predictions of the NUF1 protein and predicted coiled-coil regions. The predicted central coiled-coil domain is depicted as eight regions that are separated by minor phase shifts. Secondary structure of the NUF1 protein was predicted by the Garnier program (Garnier et al., 1978). α-helix is predicted to comprise most of the coiled-coil region.

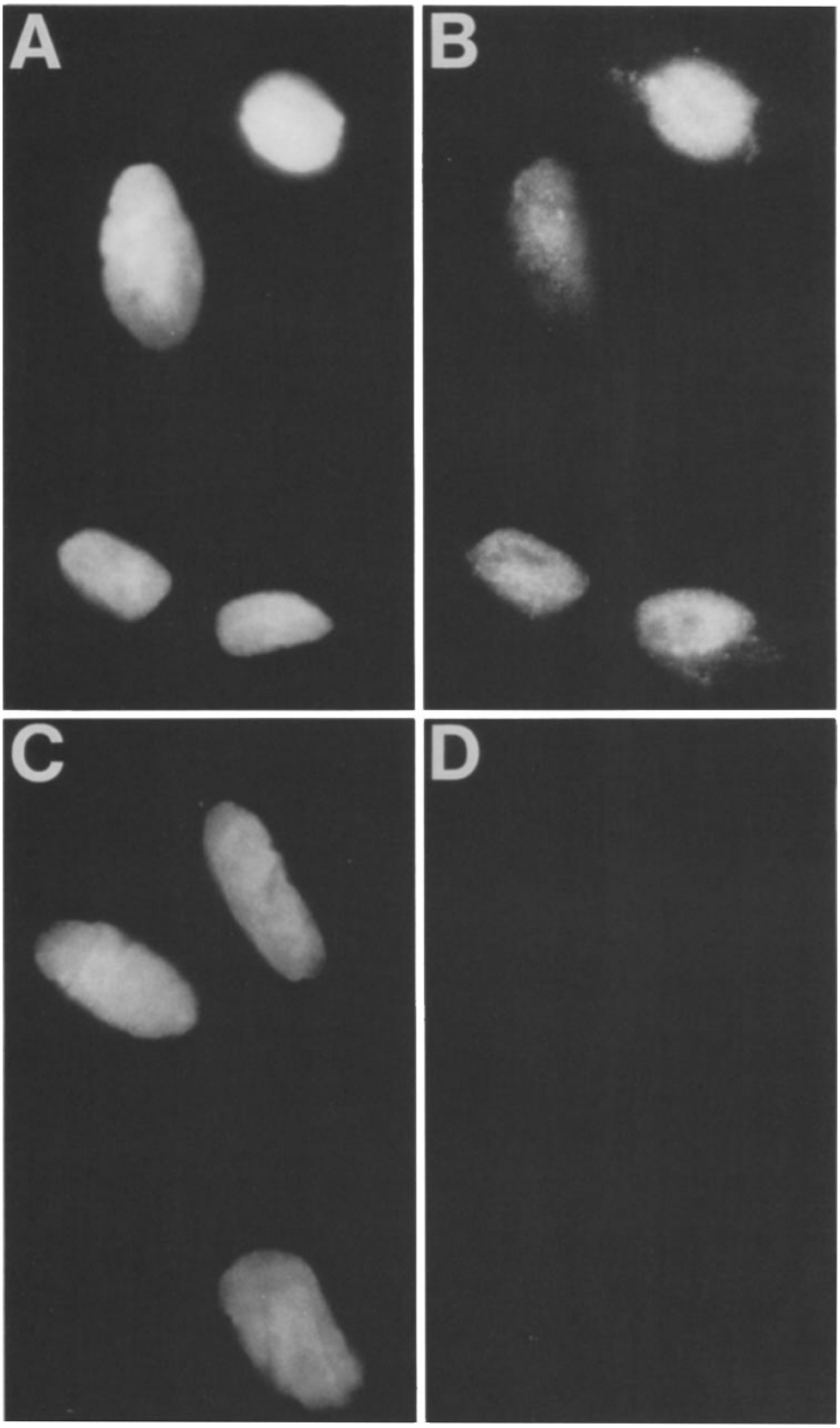

Figure 8. Indirect immunofluorescence of African green monkey (*CV1*) cells using affinity-purified anti-NUF1 antibodies. (*Panels*) Cells were stained with affinity-purified NUF1 antibodies (*B*) or preimmune sera (*D*). Hoechst 33258 staining of DNA in the same cells are shown in *A* and *C*.

persed in the cytoplasm (data not shown). Thus, the mammalian NUF1 analogue has a different localization than NuMA, a coiled-coil related protein that is present in the nucleus of interphase cells and at the mitotic apparatus during mitosis (Yang et al., 1992).

Discussion

In the cytoplasm, a filamentous cytoskeleton provides a structural framework for cellular functions and overall cellular organization. Although a lamina of intermediate filament–related proteins underlies the nuclear envelope, whether a network of filaments extends into and organizes the interior of the nucleus is a matter of great interest and controversy. Intranuclear fibrous elements can be observed after various extraction procedures (Berezney and Coffey, 1977; Jackson and Cook, 1988; Lothstein et al., 1985), but the molecular components of such a potential nucleoskeleton have not yet been defined biochemically. The present study and the companion paper describe two polypeptides that are candidates for components of an internal nuclear filament network.

In this report we describe the identification of a novel gene in yeast called *NUF1*. Subcellular fractionation experiments and immunofluorescence results indicate that the NUF1 protein is a 110-kD nuclear protein that is tightly associated with the yeast nucleus. Based on the predicted secondary structure, the NUF1 protein is expected to have a long coiled-coil region in its central domain, 627 amino acid residues in length. All proteins predicted to form long coiled-coil structures, when analyzed, have been shown to form filaments in vivo or in vitro (Cohen and Parry, 1990; Steinert and Penman, 1985*b*). Hence, we expect that the NUF1 protein will form a filament as well. Gene disruption experiments indicate that the *NUF1* gene is essential for yeast cell growth. These results and their interpretation are discussed in further detail below.

The NUF1 Protein Is Tightly Associated with the Yeast Nucleus

Indirect immunofluorescence experiments using affinity-purified anti-NUF1 antibodies indicate that the NUF1 protein is present in the yeast nucleus in a granular pattern that excludes the nucleolus. Staining is clearly not peripheral, and is present at all stages of the cell cycle. In mammalian cells, similar granular staining is observed for sites of DNA synthesis, which copurify with insoluble nuclear matrix preparation (Nakayasu and Berezney, 1989).

The complex antiserum used to isolate the *NUF1* gene was of interest because it recognized many nuclear components, including the nuclear interior. The mouse was immunized with a soluble fraction from yeast nuclei proteins extracted with 1% Triton X-100 plus 0.2 M NaCl. Later biochemical extraction experiments using affinity-purified anti-NUF1 fusion protein antibodies confirmed the presence of small amounts of NUF1 protein in this fraction. Thus, the NUF1 protein released from nuclei probably provided one of the initial immunogens.

However, the majority of NUF1 protein is pelleted under a number of extraction conditions, leading to the conclusion that the NUF1 protein is tightly associated with the nucleus. It is almost completely insoluble under membrane disrupting conditions and under high salt conditions that disrupt ionic interactions between proteins. Therefore, it is unlikely that the NUF1 protein is freely soluble in the nucleoplasm in vivo. The NUF1 protein cannot be removed by treatments with nuclease or lithium diiodosalicylate (which removes histones; Mirkovitch et al., 1984), indicating that intact chromatin and RNA structure is not critical for maintaining NUF1 nuclear association. These results strongly support the hypothesis that the NUF1 protein is part of a structural framework in the yeast nucleus. Whether the NUF1 protein associates with nuclei by virtue of self-association or by association with other insoluble nuclear structures is an important issue to be addressed in the future.

NUF1 Is Predicted to Be a Large Coiled-Coil Protein

Secondary structure predictions based on the coding sequence indicate that the NUF1 protein is comprised of a long alpha helical rod domain flanked by nonhelical regions. The central helical region contains canonical heptad repeats with predominantly hydrophobic residues occupying the first and fourth positions (a and d). In coiled-coil proteins the a and d residues of an α-helix form a hydrophobic face which allows dimerization via hydrophobic interactions (Cohen and Parry, 1990; Dibb et al., 1989; Geisler and Weber, 1982; Kagawa et al., 1989; McKeon et al., 1986; Steven et al., 1988). Coiled-coil dimers are thought to be additionally stabilized by ionic interactions between charged amino acids which are prevalent at positions e and g (Cohen et al., 1987; Dibb et al., 1989). Approximately 60% of the e and g residues of the NUF1 protein are charged. Thus, the heptad periodicity and distribution of charged residues indicate that the NUF1 protein may form a coiled-coil dimer.

Consistent with the secondary structure analysis, searches of sequence databases with the predicted NUF1 protein sequences revealed the NUF1 protein is homologous to proteins of the coiled-coil family, including myosins, lamins and intermediate filaments. The highest similarity was with myosin heavy chain sequences which contain a coiled-coil rod of ~900 amino acids in length. However, unlike myosin, the NUF1 protein lacks a nucleotide binding site and consequently is probably not a motor protein.

The NUF1 protein is similar to intermediate filaments proteins in that it is predicted to have a large central coiled-coil domain flanked by nonhelical terminal domains (Geisler and Weber, 1982; Hanukoglu and Fuchs, 1983; Marchuk et al., 1984; Steinert and Liem, 1990; Steinert et al., 1985*a*). The terminal domains of intermediate filaments are thought to be important for regulating the assembly of intermediate filaments and other specific functions (Geisler et al., 1983). Phosphorylation in these domains is responsible for regulating the process of filament assembly and disassembly (Geisler and Weber, 1988; Heald and McKeon, 1990; Peter et al., 1989; Ward and Kirschner, 1990). It is possible that the NUF1 protein is also regulated by phosphorylation events. The predicted NUF1 protein contains many (12.8%) serine and threonine residues throughout its sequence. Two of the serine residues in the amino terminus of the NUF1 protein have contexts very similar to nuclear lamins and in vitro substrates of the cdc2/CDC28 protein kinase (Heald and McKeon, 1990; Peter et al., 1989; Shenoy et al., 1989; Ward and Kirschner, 1990).

In the nuclear lamins, a putative nuclear localization signal is found in the nonhelical domain (Loewinger and

McKeon, 1988; Vorburger et al., 1989). The NUF1 protein contains three sequences enriched in basic residues that are candidates for nuclear localization signals (Hall et al., 1984; Kalderon et al., 1984*a*; Kalderon et al., 1984*b*). Two (KEKYKR and RLRREK) lie at the end of the putative coiled-coil (positions 726–731 and 742–747, respectively). The other (KKRQRR) lies in the amino-terminal domain at position 54–59. Lamin B also contains an acidic stretch at its carboxy terminus which has been proposed to bind chromatin (Peter et al., 1989; Vorburger et al., 1989). NUF1 contains a similarly sized acidic stretch at its carboxy terminus (residues 860–865) which might also serve to interact with chromatin.

Detailed Structure of the Predicted Rod Domain

In spite of the similarities between NUF1 and members of the intermediate filament family, there are three major differences. First, the length of the coiled-coil rod domain of the NUF1 protein is twice that of intermediate filaments: 627 amino acids residues in the NUF1 protein as compared to 310 amino acids for intermediate filament proteins (or 352 for nuclear lamins). Second, the NUF1 protein does not contain the conserved amino acids residues found in the first and last coils of intermediate filaments (McKeon et al., 1986; Weber et al., 1988, 1989). Third, the NUF1 protein differs with respect to the number and position of breaks in the central coiled-coil rod domain. Intermediate filaments contain three highly conserved breaks of defined sizes (L1:8-14, L12:16-17, L2:8 amino acids long) (Steinert et al., 1985*a*); these breaks often contain prolines which are expected to interrupt the α-helix. Consequently, the intermediate filament rod contains four discrete segments 35, 101, 19, and 121 amino acids long. In contrast, NUF1 contains seven short interruptions (1–11 amino acids long) which result in eight coiled-coil segments 56, 203, 28, 84, 42, 70, 35, 70 amino acids long. All of the interruptions result in a phase shift of either one, three, or four amino acid residues; since an α-helix is 3.6 residues per turn (Cohen and Parry, 1990), the three or four residues insertions should only shift the position of the hydrophobic spine by $\sim$0.5 residues. Since all of these interruptions lack prolines and probably cause only a slight phase shift, we expect the α-helix of the central domain to be maintained as one single coiled-coil.

There is precedent for short interruptions in coiled-coil proteins. In fact, short insertions are found in all coiled-coil molecules except for tropomyosin (Phillips et al., 1986). Regular stutters of one amino acid are found in paramyosin and sarcomeric myosin (Cohen et al., 1987; Kagawa et al., 1989). Four amino acid interruptions are found in the intermediate filaments and lamins. These interruptions have been proposed to modulate the pitch of the coils as they twist about one another (Dibb et al., 1989).

Additional modulation of the α helix can be derived from insertion of polar residues in positions a and d. For example, polar residues are found in these positions in myosin and have been proposed to provide flexibility in bending of the coil (Dibb et al., 1989). In intermediate filaments, specific a and d positions that contain polar residues are well conserved, indicating that irregularities in the heptad periodicity have functional significance (Lewis et al., 1984). The heptad repeats in the predicted NUF1 protein sequence sometimes contain charged residues in positions a and d. In 86 heptads, there is one charged amino acid at position a and 28 charged residues in position d. As postulated for myosin, such residues might provide flexibility to the long NUF1 rod.

Correlations between the length of rod domains, measured by direct observation, and the length of a predicted alpha helical segment have been made for a number of coiled-coil proteins, including the paramyosin of *Schistosoma mansoni*, nuclear lamins (Aebi et al., 1986; Heitlinger et al., 1991), and intermediate filaments (Cohen et al., 1987). If the NUF1 protein forms an alpha helix with similar pitch to the *S. mansoni* paramyosin, which packs an 823 coiled-coil amino acid sequence into a 122-nm molecule, the NUF1 rod would be $\sim$93 nm in length. If figures for intermediate filaments are used, a similar length is predicted. These structural predictions can be tested using in vitro assembly experiments with the NUF1 protein.

The NUF1 Protein as a Possible Component of a "Nucleofilament" System

Using EM, Cook and Jackson visualized a network of filaments in the nuclei of extracted HeLa cells (Jackson and Cook, 1988). The filaments were $\sim$10 nm in diameter with a repeating unit 23 nm in length. Cook and Jackson noted the similarities to the repeat structure of intermediate filaments (Milam and Erickson, 1982; Steinert and Penman, 1985*b*). Coiled-coil dimers of intermediate filaments have been postulated to overlap by 50%, accounting for a $\sim$21–25-nm periodicity.

The extraction experiments and sequence homologies noted above suggest that the NUF1 protein is a candidate to comprise insoluble intranuclear system inside the yeast nucleus. However, the longer length of the NUF1 protein would necessitate a different spacing or overlap arrangement to the periodicity seen in intermediate filaments, perhaps achieved by a three-quarters overlap. An alternative role for NUF1 could be in crosslinking filaments or anchoring other molecules. A crosslinking role has been proposed for plectin, a coiled-coil protein that interacts with cytoplasmic intermediate filaments (Wiche et al., 1991).

NUF1 and Other Coiled-Coil Nuclear Proteins

Anti-NUF1 antibodies recognize a related protein in the nucleus of mammalian CV1 cells. Preliminary immunoblot analysis on isolated CV1 nuclei indicate that this protein is similar in size to the yeast NUF1 gene product. The NUF1 polypeptide is also similar in size to a recently reported 92-kD plant nuclear antigen recognized by mAbs to intermediate filaments (Beven et al., 1991). Since mAbs can recognize proteins that share a common epitope but are otherwise unrelated, the potential homologies to intermediate filaments must be interpreted cautiously. Nevertheless, the plant antigen, mammalian NUF1 antigen, and the yeast NUF1 protein may all be related.

To our knowledge, other than NUF1 and NuMA (see Yang et al., 1992), no alpha-helical proteins have been described that are candidates for coiled-coil nuclear filaments. In the yeast *Schizosaccharomyces pombe*, the nuc2 gene product is thought to be an insoluble intranuclear protein, but is not predicted to form long coiled-coils. Instead, it has a unique 34 amino acid repeat structure that can form amphipathic helices and may serve as a DNA binding domain (Hirano et al., 1988, 1990).

In the accompanying manuscript (Yang et al., 1992), we describe a large coiled-coil related protein called NuMA that is present in the nucleus of interphase cells and in the spindle apparatus of mitosis. Because the size of the mammalian NUF1-related protein and its subcellular distribution during mitosis differs from that of NuMA, we conclude that the NUF1-related protein is different from NuMA. It is likely that more than one type of coiled-coil protein is located in the interior of mammalian nuclei; we speculate that these proteins are components of an entire network of filamentous nuclear structures.

We thank C. Yang for assistance throughout this work, and C. Costigan, R. Heald, B. D. Page, R. Padmanabha, and S. Sobel for critical comments on the manuscript.

This research was supported by National Institutes of Health grant GM36494 to M. Snyder, and used equipment purchased with the help of Pew Scholars Funds. C. S. Copeland was supported by a Damon Runyon-Walter Winchell Cancer Research Fund fellowship, DRG-957.

Received for publication 10 October 1991 and in revised form 30 December 1991.

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