Nuf2, a Spindle Pole Body-associated Protein Required for Nuclear Division in Yeast

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Abstract. The *NUF2* gene of the yeast *Saccharomyces cerevisiae* encodes an essential 53-kd protein with a high content of potential coiled-coil structure similar to myosin. Nuf2 is associated with the spindle pole body (SPB) as determined by coimmunofluorescence with known SPB proteins. Nuf2 appears to be localized to the intranuclear region and is a candidate for a protein involved in SPB separation. The nuclear association of Nuf2 can be disrupted, in part, by 1 M salt but not by the detergent Triton X-100. All Nuf2 can be removed from nuclei by 8 M urea extraction. In this regard, Nuf2 is similar to other SPB-associated proteins including Nufl/SPC110, also a coiled-coil protein. Temperature-sensitive alleles of *NUF2* were generated within the coiled-coil region of Nuf2 and such *NUF2* mutant cells rapidly arrest after temperature shift with a single undivided or partially divided nucleus in the bud neck, a shortened mitotic spindle and their DNA fully replicated. In sum, Nuf2 is a protein associated with the SPB that is critical for nuclear division. Anti-Nuf2 antibodies also recognize a mammalian 73-kd protein and display centrosome staining of mammalian tissue culture cells suggesting the presence of a protein with similar function.

THE process of nuclear division in the yeast *Saccharomyces cerevisiae* requires a large number of gene products, many of which have been characterized extensively. The yeast microtubule organizing center, the spindle pole body (SPB)[1], is a large, trilaminar structure embedded in the nuclear envelope (Byers and Goetsch, 1974). Its behavior during the cell-cycle is morphologically well studied (Byers and Goetsch, 1975). At mitosis, the SPB duplicates, with two SPBs initially located side-by-side within the nuclear envelope; this stage is termed a monopolar spindle. The SPBs then separate, establishing a bipolar mitotic spindle across the interior of the nucleus. The duplicated SPB is oriented toward the daughter bud, and the spindle elongates as the nucleus is separated and enters the bud. At its maximum, the bipolar spindle extends from within the daughter bud through the bud neck into the mother cell. The mitotic spindle is responsible for correctly distributing the chromosomal complement to both the mother and daughter cell. Mutants in genes responsible for spindle formation and maintenance often display increases in chromosome loss (i.e., Rose and Fink, 1987; Brown et al., 1993; Goh and Kilmartin, 1993).

Several SPB proteins have been identified biochemically (Rout and Kilmartin, 1990, 1991; Kilmartin et al., 1993). One of these, Nufl/Spc110, is a coiled-coil containing protein (Mirzayan et al., 1992) shown to be a structural component of the SPB. Genetic screens have implicated other genes required for proper SPB duplication. These include *CDC4, CDC31, CDC34,* (Byers and Goetsch, 1975), *KAR1* (Conde and Fink, 1976), *NDC1* (Thomas and Botstein, 1986; Winey et al., 1993), *MPS1,* and *MPS2* (Winey et al., 1991). Cells mutant in these genes are defective in SPB duplication or related processes and arrest with unseparated SPBs. The kinesin-related proteins, Cin8 and Kipl, are important for proper assembly (Roof et al., 1992) and movement of the duplicated SPBs to opposite sides of the nucleus (Saunders and Hoyt, 1992; Hoyt et al., 1992, 1993).

Yeast mutants have been identified which arrest at a stage later than SPB separation, but before spindle elongation. Byers and Goetsch (1974) show that one class of *CDC* mutants displayed a shortened mitotic spindle which did not traverse the maximum length of the nucleus, while other *CDC* mutants arrest with spindles extending the entire length of the nucleus. Mutants of the first class include *CDC13, CDC16, CDC17, CDC20, CDC23,* and *CDC27.* All display an increase in the rate of chromosome loss, while *cdc13* and *cdc17* cells also show an increase in the rate of mitotic recombination (Hartwell and Smith, 1985). All of these

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1. *Abbreviations used in this paper*: SPB, spindle pole body; ts, temperature sensitive.

genes are believed to be involved in chromosome segregation, DNA replication, or DNA repair, features consistent with their stage of arrest.

We report here the identification of a novel gene, *NUF2*, with a mutant phenotype of cell-cycle arrest with an unelongated bipolar spindle. Nuf2 is localized to the nuclear side of the SPB. Nuf2 contains a large region of coiled-coil potential and therefore is a candidate for a structural protein involved in SPB separation and spindle elongation.

Materials and Methods

Yeast Strains and Manipulations

Yeast strains are described in Table I. Yeast growth media were as described in Rose et al. (1990). High efficiency yeast transformation was performed according to Schiestl and Gietz (1989).

To integrate 8 LexA binding sites upstream of the *lacZ* gene, the plasmid pSH18-34-ΔSpe (derived from pSH18-34, a gift of Roger Brent, Harvard Medical School) was cut with Apa I to target the integration to the *URA3* locus of GGY1, yielding PSY190.

A *GAL1* promoter driven *NUF2* integrant was constructed in a diploid by transforming PSY198 with Sac I-digested p42P+*LEU2*+*GAL1-NUF2*$_{1\text{-}359}$ (described in a following section). The integration was verified by Southern blot and the galactose-inducible expression of Nuf2 was detected by immunoblotting.

To integrate the *nuf2*^{ts} genes into the genome, the plasmids YIp*NUF2*^{ts} (pPS553-565) containing the mutant copies of *NUF2* were cleaved with Sna BI (which cuts 3' to *NUF2*), and transformed into PSY450 (a spore of PSY196). Stable Ura$^+$ transformants were obtained and streaked onto YPD plates at 25°C. Several isolated colonies were picked and streaked onto SC containing 1 mg/ml 5-FOA. Colonies were then checked for temperature sensitivity on YPD plates (see Fig. 7, *A-C*). Several of the integrated *nuf2*^{ts} mutants were also checked by transformation with YCp*NUF2*, to verify that the temperature sensitivity could be complemented by the wild-type copy of *NUF2* (Fig. 7 *D*).

PSY498, the *nuf2-61/nuf2-61* homozygous diploid was obtained by mating PSY455 (*nuf2-61*) with PSY481 (a spore of PSY196), sporulating and dissecting, and subsequently obtaining two spores: One *MAT*αAde$^-$ Trp$^+$ (PSY479), and one *MAT*a, Ade$^+$ Trp$^-$ (PSY474). These were mated to generate PSY498, and temperature sensitivity was verified by streaking colonies at 25°C and 36°C.

Two-Hybrid Screening

PSY190 was transformed with both p*lexA*$_{87}$-*NUP1* (pPS363) and three separate pools of the yeast genomic library of Chien et al. (1991). Leu$^-$His$^-$ transformants were selected and replica plated to Leu$^-$His$^-$ sucrose plates containing 5-Bromo-4-Chloro-3-indoyl-α-D-galactoside (X-Gal). After three days at 30°C, blue colonies were identified and restreaked from the master plate. After rechecking on X-gal plates, the cells were grown in Leu$^-$ liquid media to amplify the library plasmid. Plasmid DNA was prepared and used to transform *E. coli* strain MC1061 by electroporation. pGAD plasmids were retransformed into PSY190 along with p*lexA*$_{87}$-*NUP1*. Of six blue colonies identified and confirmed by restreaking from 300,000 transformants, all were blue on retransformation. One of these (pGAD*NUF2*$_{225\text{-}451}$, pPS537) is detailed in this report.

To more rapidly screen the interactions between LexA fusions and Gal4-fusions, the X-gal filter assay was used (Rose et al., 1990).

DNA Manipulations

All DNA manipulations were performed as generally described by Sambrook et al. (1989).

pPS363. The Pml I-Pvu II fragment of pLD1 (Davis and Fink, 1990) encoding amino acids 14-1076 was treated with Klenow fragment and BamHI (12mer) linkers were added. This fragment was ligated into the Bam HI site of pSH2-1 (a gift of Roger Brent), a 2 micron-based yeast plasmid (with the HIS3 selectable marker) encoding the first 87 amino acids of *E. coli* LexA, creating a fusion of LexA and Nup1.

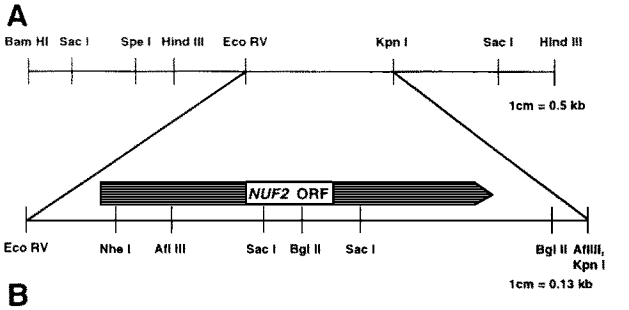
pPS537 (pGADNUF2$_{225\text{-}451}$). The 4.2-kb Sau 3AI fragment inserted in pGAD3R obtained as a putative Nup1-interactor from the screen.

pPS538. The 3.6-kb Hind III fragment of pPS537 was subcloned into pBluescript (KS$^+$, hereafter referred to as pBS, [Stratagene, La Jolla, CA]).

Table I. Yeast Strains Used in This Study

Strain		Genotype*	Source
W303	a	*ade2*-1 *trp1*-1 *leu2*-3,112 *his3*-11,15 *can1*-100	A. Tzagoloff
GGY1	α	*ura3*-52 *his3*A *leu2*-3,112 Δ*gal4* Δ*gal80*	Jun Ma
PSY190	α	*ura3*-52 *his3*A *leu2*-3,112 Δ*gal4* Δ*gal80* *URA3*::pSH18-34	This study
CTY10-5d	α	*ura3*-52 *his3*Δ200 *leu2*-3,112 Δ*gal4* Δ*gal80* *trp1*-901 *URA3*::pSH18-34	S. Fields
GJGR156	a	*cdc15-2 ade his ura*	J. Broach
GJH123-9-1	a	*cdc16*-1 *ade his7 leu ura*1	J. Broach
GJ4028 22	a	*cdc17*-1 *ade leu ura*1	J. Broach
GJH160-3-3	a	*cdc27-1 aro arg leu ura*	J. Broach
PSY196	a/α	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *ade2*-1 *ura3*-52 *leu2*-3,112 + +	M. Rose (MS810)
ABYS1	a/α	*pra1 prb1 prc1 cps1 ade*	D. H. Wolf
W303a-42::URA3	a	*NUF2::URA3 ade2*-1 *trp1*-1 *leu2*-3,112 *his3*-11,15 *can1*-100	This study
PSY450	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1	This study
PSY451	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1 nuf2-3	This study
PSY452	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *nuf2*-22	This study
PSY453	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *nuf2*-45	This study
PSY455	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *nuf2*-61	This study
PSY469	a/α	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *ade2*-1 *nuf2*-Δ2::*LEU2* *ura3*-52 *leu2*-3,112 + +	This study
PSY474	a	*ura3*-52 *leu2*-3,112 ade2-1 *nuf2*-61	This study
PSY479	α	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *nuf2*-61	This study
PSY481	α	*ura3*-52 *leu2*-3,112 *ade2*-1	This study
PSY493	a	*ura3*-52 *leu2*-3,112 *trp1*Δ1 *ade2*-1 *nuf2*-Δ2::*LEU2* {YEp*NUF2*$_{myc}$}	This study
PSY494	α	*ura3*-52 *leu2*-3,112 *trp1*Δ1	This study
PSY497	a/α	*ura3*-52 *leu2*-3,112 *trp1*Δ1 ade2-1 *nuf2*-61 *ura3*-52 *leu2*-3,112 + + +	This study
PSY498	a/α	*ura3*-52 *leu2*-3,112 *trp1*Δ1 ade2-1 *nuf2*-61 *ura3*-52 *leu2*-3,112 + + *nuf2*-61	This study

* Resident plasmid is indicated in braces ({}).



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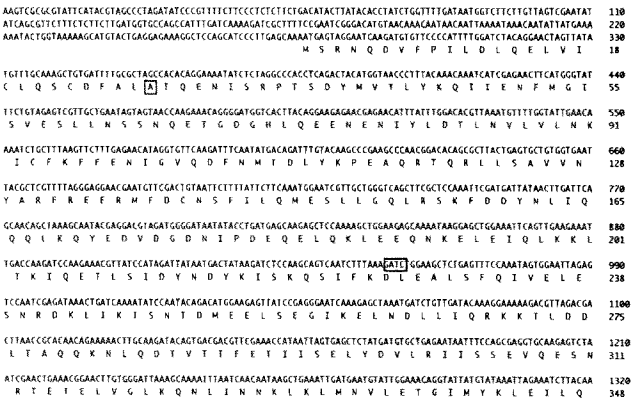

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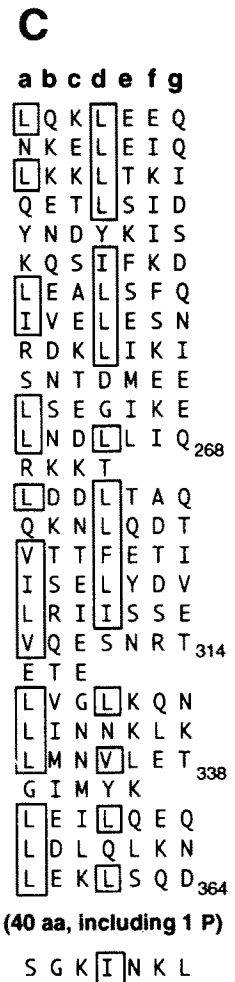

Figure 1. Sequence and restriction map of *NUF2*. (*A*) Restriction enzyme recognition sites of the *NUF2* region of chromosome XV. *NUF2* open reading frame (*ORF*) is indicated. (*B*) DNA and predicted amino acid sequence of *NUF2* and the protein Nuf2. The boxed alanine at residue 26 is the location of the myc epitope insertion. The boxed GATC at nucleotide 952 is the site of the fusion between GAL4 and NUF2 in plasmid pGAD-*NUF2*$_{225\text{-}451}$, which was recovered from the library. (*C*) A potential α-helical alignment of the COOH-terminal region of Nuf2, predicted to form an α helical coiled-coil. Hydrophobic residues in columns *a* and *d* are boxed. See text for details. These sequence data are available from EMBL/GenBank/DDBJ under accession number X72225.

pPS539. The 2.2-kb Sac I fragment of pPS538 was subcloned into the Sac I site of pPS540, a pBS derivative containing the *URA3* gene in the Xba I site (filled in with Klenow) of the polylinker, such that *URA3* is transcribed in the direction from Kpn I toward Sac I.

pPS542. The 3-kb Hind III–Kpn I fragment from pPS541 encoding the complete *NUF2* gene and upstream sequence subcloned into the Hind III and Kpn I sites of a pBS KS$^+$ derivative which had its Sac I site destroyed.

pPS544. The Nhe I–Bgl II fragment of pPS542 replaced by the Xba I/Bam HI fragment containing the *LEU2* gene of pJJ252 (Jones and Prakash, 1990).

pPS546. The coding region of *NUF2*, from amino acids 1 to 359 (of pPS542), amplified by PCR using the primers: 5'GCGGATCCATGAGTAGGAATCAAGATGTC 3' and 5'GCGGATCCATCTTGCGATGTTTTCCAAAT 3'. The PCR product was purified as described by Crowe et al. (1991), digested with Bam HI, and ligated to pBS KS$^+$ cut with Bam HI.

pPS547. The Bam HI fragment of pPS546 inserted into pPS293 such that the *GAL1* promoter will direct transcription from the 5' end of the *NUF2* coding region.

pPS548. The 1.5-kb Afl III fragment (encoding amino acids 85–451 of Nuf2) from pPS542, was inserted into the Bam HI site of pBS KS$^+$.

pPS549. The Bam HI insert of pPS548 inserted into the Bam HI site of pQE9 (Qiagen, Chatsworth, CA), such that amino acids 85–451 are expressed as a fusion protein to six histidine residues at the NH_2 terminus.

pPS551. The Bam HI insert of pPS546 inserted into the Bam HI site of pQE9, such that a fusion protein is produced that has six histidine residues followed by amino acids 1–359 of Nuf2.

pPS552. A derivative of pPS542, in which the 1-kb Eco RV fragment has been deleted and the ends religated.

pPS511. The Bam HI–Kpn I fragment of pPS552 inserted into pRS314 (Sikorski and Hieter, 1989), a centromere-based vector carrying the *TRP1* selectable marker.

pPS553-565. The Bam HI–Kpn I fragment of mutagenized pPS511 inserted into a YIp5 (New England BioLabs, MA) derivative whose Hind III site was converted into a Kpn I site.

42P+LEU2+GAL1+NUF2. A Bst XI–Spe I fragment encoding a region 5' to the Nuf2 coding sequence in pBS (42P) was digested with Eco RV to remove all of the Nuf2 coding region and ligated with Pst I linkers. The *LEU2* gene, on a Pst I fragment from pJJ252 was then inserted. An Eco RI–Hind III fragment of pPS547 was inserted into the Eco RI and Hind III sites. The Sac I sites used to integrate this fragment into the *NUF2* locus are at the 5' end of the polylinker and within the Nuf2 coding sequence.

pPS567-569. *nuf2*-61 was amplified from the genome by PCR with the 5' primer used to generate pPS546 and the 3' primer 5' CCGGATCCATATGGTACCTTCACAT 3'. The PCR products were cleaved with Bam HI and Kpn I and subcloned into pBS KS$^+$.

Recovery of the 5' End of NUF2

As the pGAD insert from the library plasmid contained only the 3' portion of the gene to which Gal4 was fused, the remaining 5' portion was cloned by the integration/excision method of Roeder and Fink (1980). pPS539 was cleaved with Bgl II and used to transform strain W303a. DNA recovered from a stable Ura$^+$ colony was digested with Bam HI, religated, and used to transform *E. coli*. One clone (pPS541) containing 8 kb of DNA 5' of *NUF2*.

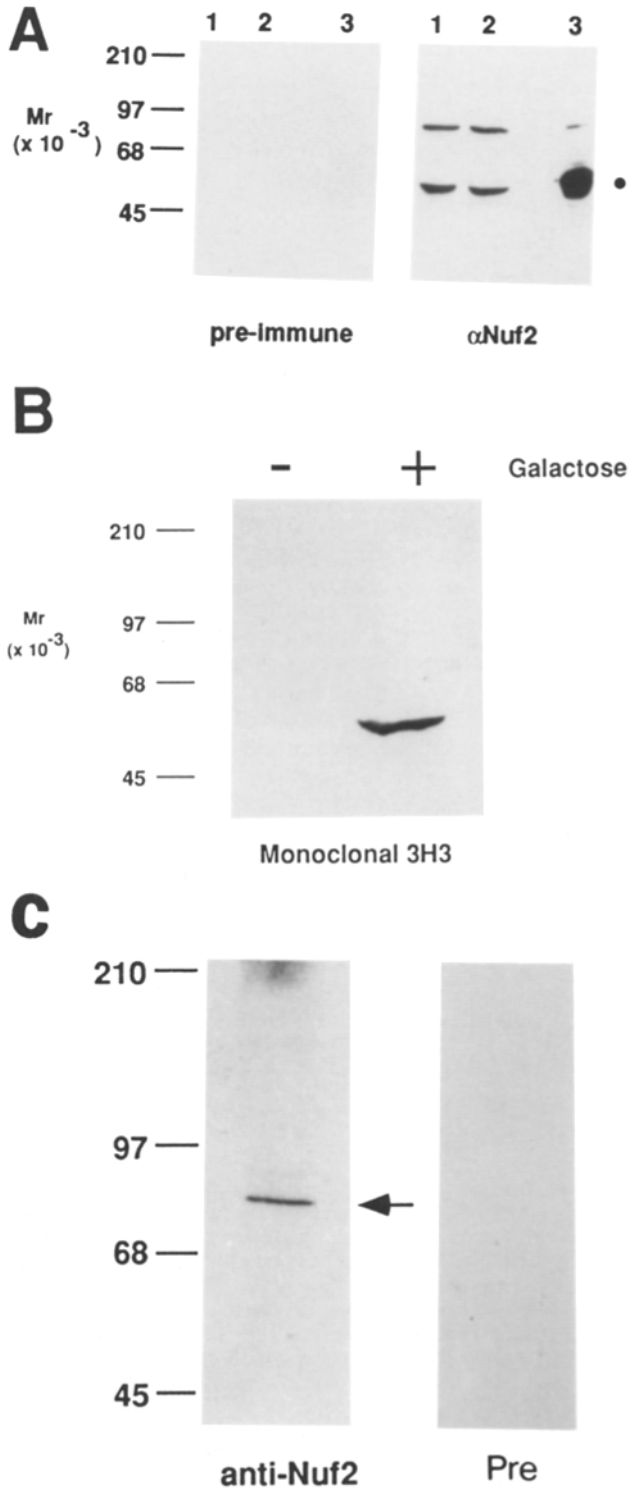

Figure 2. Antibodies to Nuf2 recognize a band of 53,000 M_r in crude yeast extracts. (*A*) (*Left*) Preimmune serum (0.5 μg/ml) and (*Right*) affinity-purified rabbit anti-Nuf2 antibodies (0.5 μg/ml) were used on immunoblots of yeast extracts to detect Nuf2. Lane *1*, W303a yeast extract; lane *2*, W303a containing plasmid YEp*GAL1-NUF2* grown in glucose; lane *3*, W303a containing plasmid YEp*GAL1-NUF2* grown in galactose for 11 h. (*B*) Monoclonal antibody 3H3 was used at 1:100 dilution from tissue culture supernatant. W303a containing plasmid YEp*GAL1-NUF2* grown in glucose (−) and W303a containing plasmid YEp*GAL1-NUF2* grown in galactose for 11 h (+). (*C*) Affinity-purified rabbit anti-Nuf2 antibodies (0.5 μg/ml) were used to an immunoblot of NIH 3T3 nuclear extracts to detect a cross-reacting protein. The right panel is the same extract probed with the preimmune serum.

Sequencing of NUF2

All DNA was sequenced by the chain-termination method of Sanger et al. (1977), using the Sequence 2.0 kit (United States Biochemical, Cleveland, OH). The sequence of the entire region between the Eco RV and Kpn I sites was determined on both strands.

The mutations responsible for the temperature-sensitive growth phenotype of the *nuf2*-61 allele were determined by sequencing of pPS567–569, using unique primers that allowed sequencing of the entire coding region on one strand. The sequence of these primers is available upon request.

Antibody Generation

Plasmids pQE-*NUF2*$_{85-451}$ and pQE-*NUF2*$_{1-359}$ were transformed into *E. coli* strain M15[pREP4] (Qiagen), expression was induced with 1 mM IPTG, and the fusion protein purified by chromatography on Ni^+-NTA resin exactly as described by the manufacturer.

Fractions containing Nuf2 were run on 10% SDS gels and electrotransferred to PVDF. Nuf2 was excised from the filter and eluted with 50 mM Tris pH 9.0, 2% SDS, 1% Triton X-100 for 30 min at room temperature. Roughly 500 μg of purified protein was diluted 1:1 with Freund's complete adjuvant (Gibco Life Technologies, Grand Island, NY) and injected into a New Zealand white rabbit. Anti-Nuf2 antibodies were affinity purified by adsorption to Nuf2 coupled to sepharose.

Mouse monoclonal antibodies were prepared after injection of two BALB/c mice with a purified Nuf2 fragment (amino acids 1–359). Positive subclones were identified by ELISA and tested on immunoblots and for indirect immunofluorescence. Monoclonal antibodies were used at dilutions of 1:100 for immunoblots and 1:1,000 for immunofluorescence.

Anti-Nspl antibodies were prepared from extracts of *E. coli* strain CC9 (containing a malE::Tn10 insertion, a gift of T. Silhavy, Princeton University) carrying pPS274, encoding a MalE-Nspl fusion protein, by amylose chromatography as described by the manufacturer (New England BioLabs). The eluate was treated with factor Xa, run on SDS gels, electroblotted to PVDF, and eluted as described for Nuf2. The protein was injected into a rabbit and antibodies prepared as described for Nuf2. Anti-Nspl IgG preparations were used at 13 μg/ml for immunoblotting. Affinity-purified anti-Nuf1 antibodies were prepared from crude serum exactly as described by Mirzayan et al. (1992) and used at 0.14 μg/ml for immunoblotting. The anti-90-kd and anti-80-kd SPB antibodies were kindly provided by J. Kilmartin (MRC, Cambridge). The anti-centrosome antibody was kindly provided by R. Balczon (U. of S. Alabama).

Indirect Immunofluorescence

Indirect immunofluorescence for anti-tubulin was performed as described (Sadler et al., 1989) with the following modification. Spheroplasting was preceded by treatment of cells with 0.1 M dithiothreitol in 0.01 M Tris pH 9.4 for 15 min at 30°C, followed by washing in P buffer (1.2 M sorbitol, 0.1 M KPi, pH 6.5).

For immunofluorescence with anti-Nuf2, the anti-80-kd, and the anti-90-kd monoclonal antibodies, cells were converted to spheroplasts as above, and processed as described by Kilmartin and Adams (1984). Briefly, cells were fixed in −20°C methanol for 5 min followed by acetone for 30 s at 25°C. Cells were blocked with 5 mg/ml BSA in PBS for 1–4 h and 1° antibodies were incubated at 25°C overnight. After washing, 2° antibodies conjugated to FITC were used at a dilution of 1:50 and Texas red conjugated 2° antibodies were used at 1:1,000; both were incubated for 2 h at 25°C. All secondary antibodies were obtained from Jackson Immunoresearch Labs (West Grove, PA).

Temperature-sensitive mutants were grown overnight in YPD at 25°C to a density of 5×10^7 cells/ml, and then diluted 1:10 in 36°C YPD and grown for 3–5 h. Cells were fixed at 25°C for 20 min in formaldehyde (or converted to spheroplasts and fixed with methanol) and processed as described above.

NIH 3T3 cells were grown on coverslips, fixed in −20°C methanol for 5 min, permeabilized with 0.1% Triton X-100 for 15 min, and washed extensively with PBS. 1° antibodies were incubated overnight at 25°C, 2° antibodies were incubated for 2 h at 25°C.

For standard immunofluorescence cells were viewed at 1000× (yeast cells) and 630× (NIH 3T3 cells) magnification with a Zeiss Axioskope

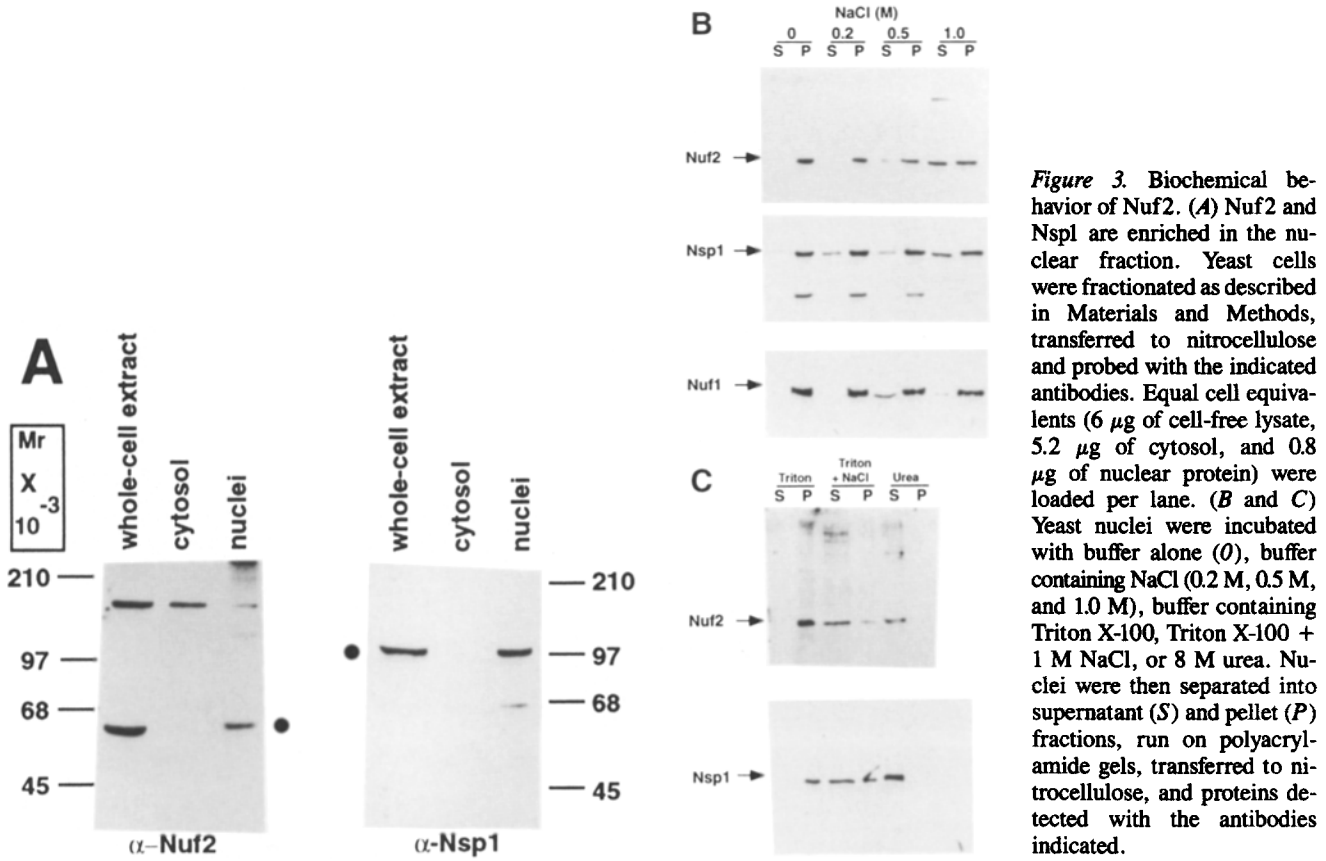

Figure 3. Biochemical behavior of Nuf2. (*A*) Nuf2 and Nspl are enriched in the nuclear fraction. Yeast cells were fractionated as described in Materials and Methods, transferred to nitrocellulose and probed with the indicated antibodies. Equal cell equivalents (6 μg of cell-free lysate, 5.2 μg of cytosol, and 0.8 μg of nuclear protein) were loaded per lane. (*B* and *C*) Yeast nuclei were incubated with buffer alone (*0*), buffer containing NaCl (0.2 M, 0.5 M, and 1.0 M), buffer containing Triton X-100, Triton X-100 + 1 M NaCl, or 8 M urea. Nuclei were then separated into supernatant (*S*) and pellet (*P*) fractions, run on polyacrylamide gels, transferred to nitrocellulose, and proteins detected with the antibodies indicated.

equipped for epifluorescence. Confocal microscopy was performed using a BioRad MRC 600 scanning device with a krypton/argon laser mounted to a Zeiss Axiophot microscope. A Zeiss Plan-neofluor 63× objective lens was used. All images were collected as a 3× scanning zoom and averaged using the Kalmer filter algorithm.

Yeast Subcellular Fractionation and Extraction of Nuclei

Cell fractionation was performed as described by Mirzayan et al. (1992) using strain ABYS1. For nuclear extractions, 250 μg of nuclear protein was diluted to 500 μl with 0.5 M sucrose, 20 mM KPi, pH 6.5, 0.5 mM $MgCl_2$, 1 mM PMSF, and 1 μg/ml each chymostatin, antipain, aprotinin, leupeptin, and pepstatin A, or the same buffer containing 0.2 M NaCl, 0.5 M NaCl, 1 M NaCl, 1% Triton X-100, 1 mM NaCl and 1% Triton X-100, 8 M urea, or 0.25 mg/ml RNAse A plus 0.25 mg/ml DNAse I, respectively. The mixtures were incubated on ice for 30 min and centrifuged for 30 min at 4°C.

Generation of Temperature-Sensitive Mutants in NUF2

Temperature-sensitive (ts) mutants of *NUF2* were generated by the methods of Leung et al. (1989) and Caplan et al. (1992). Using the universal (−20) and a unique primer that hybridizes to the region at the Kpn I site of *NUF2* (see Fig. 1 *a*) the complete *NUF2* gene including the promoter could be amplified from plasmid pPS511. Using the mutagenic conditions (0.5 mM $MnCl_2$, 0.04 μM dATP), 10 ng of YCp*NUF2* was amplified by PCR (30 s at 94°C, 30 s at 50°C, 1 min at 72°C) for 30 cycles. The product was split into four parts (10 μl of the 100 μl reaction per tube) and reamplified under standard conditions. As a control, PCR under standard conditions was performed (and reamplified). At this point, the product was split in half. One half of each reaction was used to construct a library by inserting the Bam HI–Kpn I digested PCR product into pRS314 (Sikorski and Hieter, 1989). The libraries were then used to transform yeast strain PSY493 (containing a chromosomal deletion of *NUF2* and plasmid YEp$NUF2_{myc}$).

The second half of the PCR products were mixed with YCp*NUF2* that had been cleaved with Nhe I and Bgl II and used to transform PSY493 by the gap repair method (Caplan et al., 1992). All yeast transformants were replica plated to two SC (−Trp) containing 1 mg/ml 5-FOA, and incubated at 25 or 36°C. $NUF2^{ts}$ mutants were tested for temperature sensitivity on 5-FOA-containing plates after retransformation of the recovered plasmid DNA into PSY493.

To determine the rapidity of the cell-cycle arrest phenotype, *wild-type* and *nuf2-61* mutant cells were grown at 25°C to a density of 1.5×10^7 cells/ml and treated with α-factor for 5 h at 25°C. They were then centrifuged, washed with fresh YPD, recentrifuged and resuspended in YPD, and incubated at 25°C or 36°C for 4 h, at which time they were fixed with formaldehyde and stained with DAPI to visualize their DNA.

Preparation of yeast cells for flow cytometry was exactly as described by Meluh (1993), which is a modification of the method of Hutter and Eipel (1978).

Chromosome loss was assayed in a manner similar to Spencer et al. (1990) by comparing the mating ability of PSY498 and PSY196 Mat a/α diploids with mating type tester strains. Cells were grown from a single colony at either 23°C or 30°C in YPD to 10^7 cells/ml, patched onto YPD plates, and uniform lawns allowed to develop at either 23 or 30°C. Cells were then replica-plated onto lawns of mating type tester strains and scored for the presence of *MATa* and *MATα* cells.

Mating efficiency was measured by comparing the ability of *nuf2-61* strains (both of a and α mating types) to mate with mating type tester strains as well as with each other. As a positive control, a *kar1-1* mutant strain was used. Cells were mated at 25°C for 12 h and 24 h before being replica plated to selective media. Benomyl sensitivity and resistance was determined by streaking mutant and wild-type strains onto several YPD plates containing 5, 10, 20, 30, 40, and 50 μg/ml benomyl.

Results

Identification of NUF2

We originally sought to identify proteins that interact with the nucleoporin Nup1 (Davis and Fink, 1990) through the use of the two-hybrid system (Fields and Song, 1989; Chien

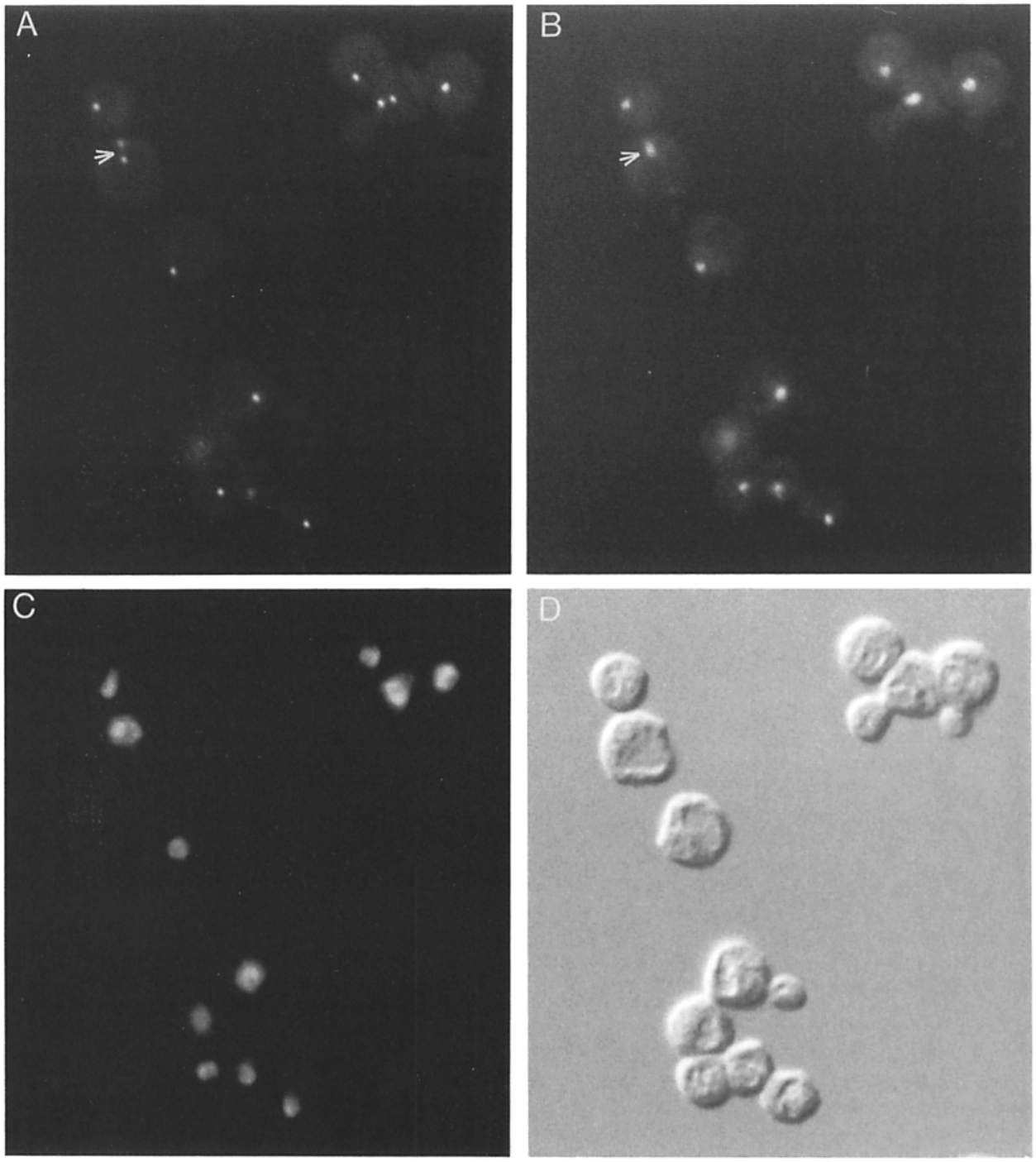

Figure 4. Nuf2 is located at the spindle pole body as determined by immunofluorescence. Yeast cells were prepared for immunofluorescence and probed with antibodies to localize Nuf2 and other SPB-associated proteins. (*A*) *A*—cells stained with anti-90 kd monoclonal antibodies; *B*—the same cells probed with anti-Nuf2 rabbit polyclonal antibodies; *C*—the same cells stained with DAPI; and *D*—the same cells viewed by Nomarski. (*B*) Confocal laser scanning microscopy of the same cells as in *A*. Nuf2 is labeled green, while the 90-kd SPB is red. (*C*) *A*—cells stained with DAPI; *B*—cells stained with anti-Nuf2 monoclonal antibody 3H3; and *C*—the same cells viewed by Nomarski.

et al., 1991). Fusions between the bacterial DNA binding protein LexA and amino acids 14–1076 of Nup1 (p*lexA*$_{87}$-*NUP1*) were constructed and their expression verified with anti-lexA antibodies (data not shown). Six potential interactors were isolated (as described in Materials and Methods) and one of these, Nuf2, is described in detail here.

The complete *NUF2* open reading frame (Fig. 1 *A*) was sequenced and found to encode a protein of 451 amino acids (Fig. 1 *B*), with a predicted M_r of 53,000 and pI of 4.4.

NUF2 Is Similar to Coiled-coil Containing Proteins

Comparison of the *NUF2* open reading frame to protein sequence databases reveals significant similarities to proteins

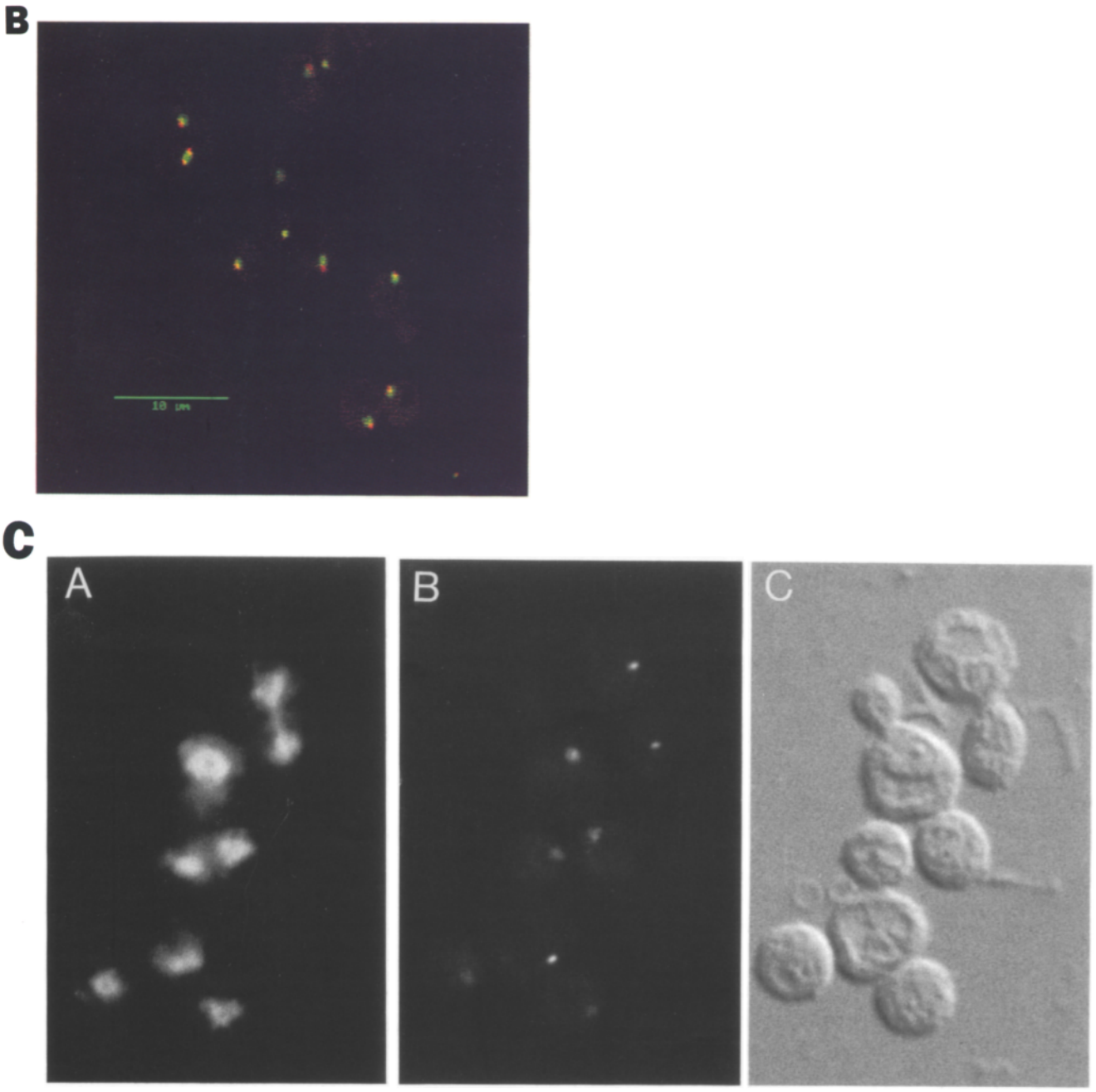

Figure 4.

containing α-helical coiled-coil structures. This similarity is restricted to amino acids 160–451; no significant similarities are observed within the first 160 amino acids. The presence of a coiled-coil structure is further supported by the algorithm described in Lupas et al. (1991), which predicts four separate regions of Nuf2 with high ($P \geq 0.95$) likelihood of α-helicity (residues 181–221, 260–297, 340–371, and 401–433).

A proposed structural alignment of the heptad repeats in the coiled-coil region is shown in Fig. 1 *C*. Hydrophobic amino acids in positions *a* and *d*, when present in an α-helix, would face another hydrophobic surface. Columns *a* and *d* contain predominantly (69%) hydrophobic or uncharged amino acids, while positions *b*, *c*, and *f* contain a large number of polar residues (77%). These percentages are in agreement with those proposed for coiled-coil-containing proteins (Cohen and Parry, 1986, 1990). Due to its similarities in predicted structure and subcellular localization to another yeast nuclear filamentous protein, Nufl (Mirzayan et al., 1992), we propose the name Nuf2 (Nuclear filament-containing protein).

NUF2 Is Essential for Cell Viability

To determine if *NUF2* is an essential gene, a disruption was constructed that replaced codons 26–451 with the *LEU2* gene. The DNA fragment was integrated into a diploid strain and the proper gene replacement at the genomic *NUF2* locus was confirmed by Southern blotting. Sporulation and dissection of 20 tetrads yielded only two viable spores per tetrad.

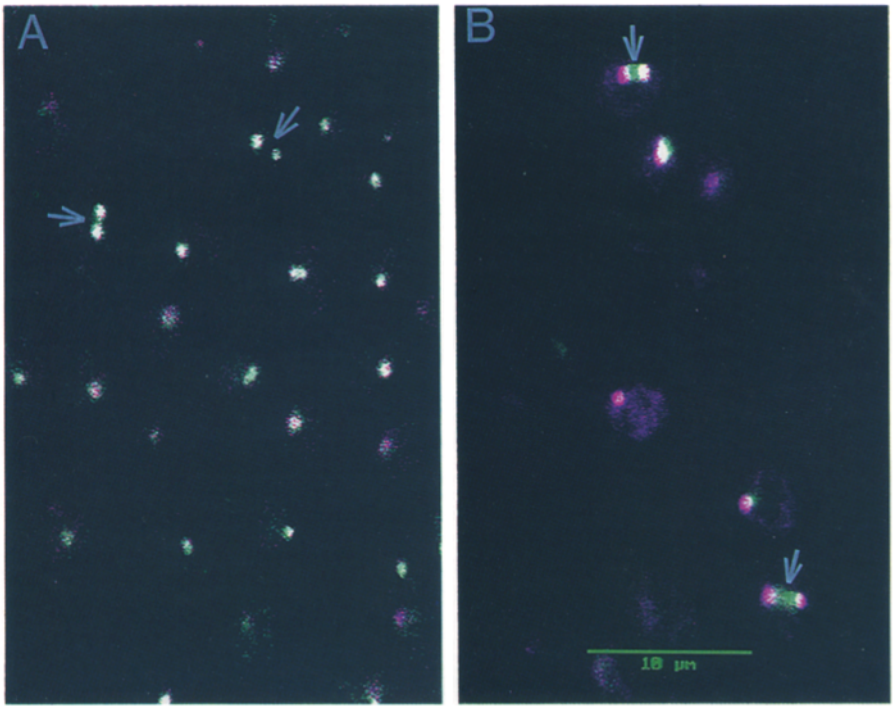

Figure 5. Colocalization of Nuf2 with the 80-kd SPB antigen. Cells were prepared for immunofluorescence as described in Materials and Methods, and costained with anti-Nuf2 polyclonal antibodies followed by FITC-conjugated anti-rabbit antibodies (*green*), and with anti-80 kd mouse monoclonal antibodies followed by Texas red-conjugated anti-mouse antibodies (*purple*), and viewed by confocal laser scanning microscopy. A white signal indicates where the two overlap. (*A*) W303a wild-type cells; (*B*) *nuf2-61* cells after 3.5 h at 36°C. Arrows in both panels indicate recently divided SPBs.

Each viable spore was Leu$^-$, indicating that it contained the undisrupted copy of *NUF2*. Thus, *NUF2* is essential for yeast cell growth.

Localization of Nuf2 in Yeast and Mammalian Cells

Antibodies were generated to Nuf2 protein fragments expressed in *E. coli*. Fusions to Nuf2 amino acids 85–451 and 1–359 were made to include six histidine residues at the NH_2 terminus, allowing purification of the fusion proteins over a Ni$^+$-NTA resin (see Materials and Methods). Both affinity-purified rabbit polyclonal antibodies and mouse monoclonal antibodies were prepared. Immunoblots of yeast extracts from wild-type cells (Fig. 2 *A*, *right panel*, lane *1*) and cells containing overproduced Nuf2 (Fig. 2 *A*, *right panel*, lanes *2* and *3*) were probed with the antibodies. Both the affinity-purified rabbit anti-Nuf2 antibodies (Fig. 2 *A*) and the monoclonal antibody 3H3 (Fig. 2 *B*) recognize the 53-kd Nuf2 protein on immunoblots whereas the rabbit preimmune serum did not (Fig. 2 *A*, *left panel*). Although 3H3 is unable to detect wild-type levels of Nuf2 (Fig. 2 *B*, [−]), the 53-kd band is visualized when *NUF2* is placed under control of the strong *GAL1* promoter and induced in galactose (Fig. 2 *B*, [+]). The rabbit antibody also recognizes a band migrating at ~85–90 kd under these conditions. However, only the 53-kd band increases upon galactose induction of *NUF2* (Fig. 2 *A*, *right panel*, lane *3*).

To determine the intracellular location of Nuf2, cell fractionation and immunofluorescence experiments were performed. As a control for localization of the proteins of the nuclear pore complex, rabbit polyclonal antibodies were raised to the yeast nucleoporin Nsp1 (Hurt, 1988; Nehrbass et al., 1990; see Materials and Methods). As expected, the antiserum recognizes a band of ~100 kd in yeast extracts (Fig. 3 *A*, *right panel*) and decorates the yeast nuclear envelope in a punctate manner in immunofluorescence experiments (Schlenstedt et al., 1993). To determine the location of the Nuf2 protein, yeast cells were fractionated into whole-cell, cytosol, and nuclear fractions and assayed by immunoblot. Nuf2 (Fig. 3 *A*, *left panel*) and Nsp1 (Fig. 3 *A*, *right panel*) both cofractionate with nuclei and neither protein is detected in the crude cytosolic fraction. (The higher molecular weight protein, which cross-reacts with anti-Nuf2 antibodies, fractionates with the cytosol.)

To localize Nuf2 within the nucleus, indirect immunofluorescence was performed. Conventional formaldehyde fixation of yeast cells, even for times as brief as five minutes resulted in an undetectable Nuf2 fluorescence signal. However, using the methanol fixation conditions of Kilmartin and Adams (1984), bright staining was observed in a dot eccentric to the nucleus with both the affinity-purified rabbit polyclonal anti-Nuf2 antibodies (Fig. 4 *A*, *upper right, B*) and the monoclonal 3H3 anti-Nuf2 antibody (Fig. 4 *C*, *middle panel B*), which only reacts with Nuf2 (Fig. 2 *B*).

The bright perinuclear dot resembled the staining of SPB proteins (Rout and Kilmartin, 1990) so we performed a double-labeling experiment with both the rabbit anti-Nuf2 antibodies and monoclonal antibodies directed against a SPB protein of 90 kd. The results indicate that Nuf2 (Fig. 4 *A*, *upper right panel B*) and the 90-kd antigen (Fig. 4 *A*, *upper left panel A*) are closely associated. However, in cells where the SPBs have duplicated but are still closely spaced, the anti-90-kd mAb gives two distinct dots while the anti-Nuf2 antibodies show a continuous region of fluorescence (as indicated by the arrowheads in the Fig. 4 *A*; *A* and *B*), suggesting that Nuf2 may only, in part, colocalize with or be immediately adjacent to the 90-kd antigen.

Confocal laser scanning microscopy further defines the spatial relationship between the 90-kd protein and Nuf2. As shown in Fig. 4 *B*, the red staining of the 90-kd antibody is frequently observed to flank the Nuf2 staining (green). This is also seen in cells where the SPBs have recently duplicated.

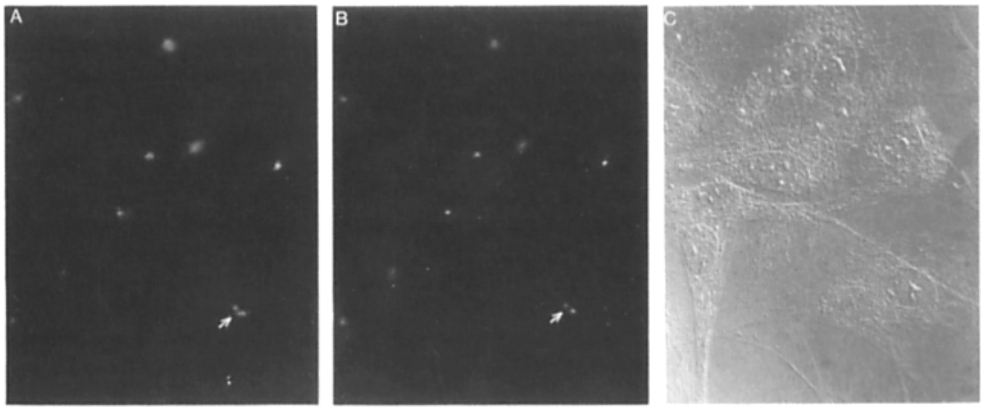

Figure 6. Anti-Nuf2 antibodies recognize a related antigen in mouse cells. NIH 3T3 cells were processed for immunofluorescence as described in Materials and Methods and probed with human anti-centrosomal antibodies followed by Texas-red conjugated anti-human 2° antibodies (*A*) and with affinity-purified anti-Nuf2 antibodies followed by FITC-conjugated anti-rabbit 2° antibodies (*B*). *C* is the same cells viewed by Nomarski. The arrows indicate two positively staining centrosomes adjacent to each other in the same cell.

Once they have separated, the 90-kd and Nuf2 staining are very close together and sometimes appear partially coincident (yellow indicates the area of overlap of staining by the two antibodies in Fig. 4 *B*). In all cases where the nucleus was visible, Nuf2 was on the nuclear side of the 90-kd protein. The anti-90 kd monoclonal antibody recognizes a protein localized by immuno-electron microscopy to both the outer and inner electron-dense laminar structures of the SPB just beyond the nuclear membrane (Rout and Kilmartin, 1990).

Because of the lack of precise coincident localization, another antibody, recognizing a SPB antigen of 80-kd, was tested for colocalization with Nuf2. The 80-kd protein is located proximal to the laminar structure at the nuclear side of the SPB (Rout and Kilmartin, 1990). Nuf2 and the 80-kd SPB protein appear to colocalize. When cells simultaneously probed with both anti-Nuf2 and anti-80 kd antibodies were viewed by standard epifluorescence (data not shown) or confocal laser scanning microscopy, the staining was completely coincident (Fig. 5 *A*; Nuf2 staining is green, 80 kd staining is purple and the overlap is white). The arrows in Fig. 5 *A* indicate recently separated SPBs where the staining with the two antibodies is still coincident. This is distinct from what is observed in *nuf2*^{ts} mutants (Fig. 5 *B*) where Nuf2 staining (green) is no longer completely coincident with the 80-kd staining (purple). This will be discussed further (see below). As the 80-kd protein has been localized towards the end of microtubules at the SPB, this seems likely to also be the subnuclear location for Nuf2. These results are in agreement with those of Rout and Kilmartin (1990) with regard to the relative location of the 80-kd and 90-kd SPB-associated proteins.

Using the same fixation conditions, mouse NIH 3T3 cells were stained with rabbit anti-Nuf2 antibodies. As shown in Fig. 6 *B*, the antibodies stain a dot near the nucleus. Occasionally, cells were observed to possess two dots, usually both flanking the nucleus (*arrows* in Fig. 6, *A* and *B*). This staining pattern is similar to that reported for centrosomal proteins. To confirm this, the same cells were probed with a human autoimmune antibody known to react with centrosomes (Osborn et al., 1982; Balczon and West, 1991). The staining with the anti-centrosome antisera (Fig. 6 *A*) was exactly coincident with the anti-Nuf2 staining (Fig. 6 *B*). Mouse NIH 3T3 cells probed with the preimmune sera or with only the secondary antibodies showed no staining. In mitotic cells, anti-Nuf2 staining remained at the centrosomes (data not shown). Immunoblotting of NIH 3T3 cell nuclear extracts revealed one major immuno-reactive band of ~73 kd and occasionally a faint higher molecular weight band of ~90 kd (Fig. 2 *C*, *left lane*). The major 73-kd band was detected in nuclear extracts. The preimmune serum from the same rabbit showed no reactivity with mammalian nuclear extracts (Fig. 2 *C*, *right lane*).

Nuf2 Is Tightly Associated with the Yeast Nucleus

To determine the nature of the association of Nuf2 with the nuclear compartment, nuclei were extracted with salt and detergent and the supernatant and pellet fractions assayed by immunoblotting. Nuf2 is tightly associated with nuclei, as it is only partially extractable by 1 M NaCl (Fig. 3 *B*, *top panel*). Its extraction profile is largely similar to that of Nspl (Fig. 3 *B*, *middle panel*; Hurt, 1988), but it is more readily removable from nuclei than the SPB protein Nufl/Spc110 (Fig. 3 *B*, *lower panel*; Mirzayan et al., 1992). Nufl/Spc110, Nuf2, and Nspl are all completely extracted by 8 M urea (Fig. 3 *C* and data not shown for Nufl). Neither Nuf2 nor Nspl are removed from nuclei by treatment with nucleases (data not shown), as shown for Nufl (Mirzayan et al., 1992).

Phenotypic Characterization of Temperature-Sensitive Mutants of NUF2

To learn more about the function of Nuf2, *ts* mutants in *NUF2* were generated as described in Materials and Methods. Of the 90 candidate *ts* mutants identified initially, 70 remained *ts* after rescue of the plasmid in *E. coli* and retransformation into PSY493. As an independent test, *ts* cells were transformed with a plasmid encoding wild-type *NUF2* or vector alone. Cells reverted to temperature-resistance only with wild-type *NUF2*, verifying that the *NUF2* insert carries the determinant of temperature-sensitivity.

To confirm that the *NUF2* coding region had been mutagenized, the plasmids containing several of the *nuf2*^{ts} alleles were partially sequenced. All contained multiple *NUF2* mutations, confirming the mutagenesis (Osborne, 1993).

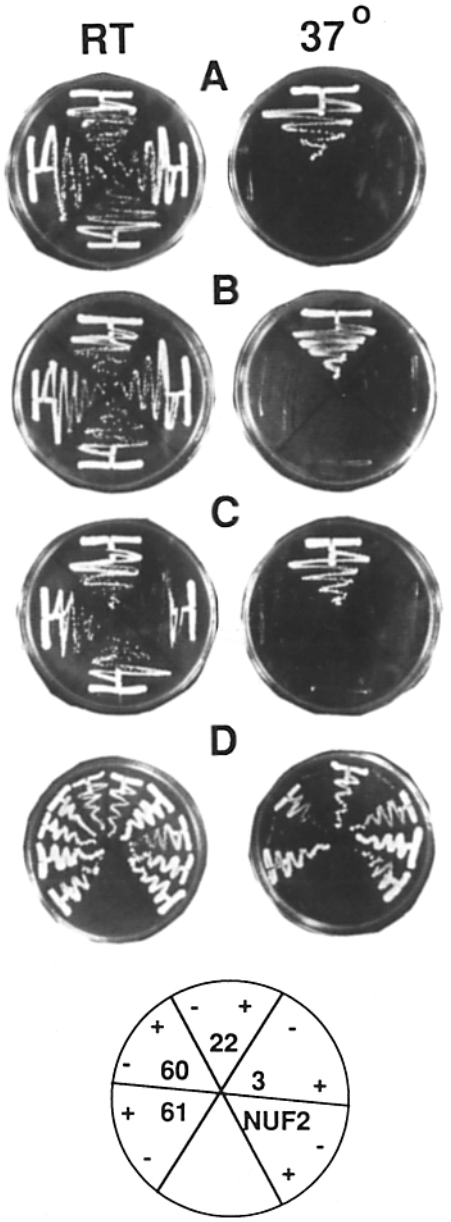

Figure 7. NUF2 temperature-sensitive mutants. Strains PSY450 (*NUF2*), PSY451 (*nuf2-3*), PSY452 (*nuf2-22*), PSY453 (*nuf2-45*), PSY454 (*nuf2-60*), PSY455 (*nuf2-61*), PSY457 (*nuf2-12*), PSY458 (*nuf2-13*), PSY459 (*nuf2-23*), and PSY460 (*nuf2-76*) were streaked on YPD plates at 37°C and 25°C (*RT*) and incubated for 3 d. The top (*12 o'clock position*) of each pair of plates is PSY450 (*NUF2*). Counter clockwise from the left (*A*) *nuf2-3*, *nuf2-12*, and *nuf2-13*); (*B*) *nuf2-22*, *nuf2-23*, and *nuf2-45*; (*C*) *nuf2-60*, *nuf2-61*, and *nuf2-76*; (*D*) *nuf2* mutants can be rescued by YCp*NUF2*. The drawing at the bottom of the figure indicates the *NUF2* allele number (NUF2 is wild-type). [+] indicates the presence of YCp*NUF2*, (−) indicates no plasmid. Cells were streaked out on YPD plates at the temperatures indicated.

To analyze the behavior of the *NUF2* mutants, the extreme thermophobes were introduced into the chromosomal copy of *NUF2*. Nine chromosomal *nuf2*^{ts} alleles were obtained (Fig. 7, *A–C*). To confirm that the temperature-sensitivity is due to a *NUF2* mutation, the ts strains PSY451, 452, 453, and 456, were transformed with pPS511. All mutants were complemented by the plasmid, as judged by their ability to grow at 36°C (Fig. 7 *D*), demonstrating that the ts phenotype was due to *NUF2* and also showing the recessivity of the mutations.

The *nuf2*^{ts} mutants were grown at 25°C, and then shifted to 36°C for varying amounts of time. In all mutants tested, after three hours at 36°C, cells displayed a cell-cycle arrest with large buds (Fig. 8 *B*) and partially divided nuclei (Fig. 8 *A*). Since all mutants examined were similar, further analysis was carried out with *nuf2-61*, which gave a uniform arrest phenotype, with 70–90% of all cells arrested with large buds after 4 h at 36°C. To identify the specific amino acid changes responsible for conferring the *ts* phenotype, the *NUF2* coding region from the *nuf2-61* mutant was amplified by PCR and subcloned. The *NUF2* ORF was completely sequenced from three independent PCR-generated subclones and eight mutations were found (Table II). Seven of eight mutations are A to G or C to T transitions, while there is one A to T transversion. All eight mutations are located within the COOH-terminal proposed coiled-coil region.

The growth of *nuf2-61* and wild-type cells was monitored at 36°C by cell counting. *Nuf2-61* cells stopped growing after 3 h, while wild-type cells continued to divide (data not shown). The cell-division-cycle defect observed in *nuf2-61* cells is observed within the first division after temperature shift, as α-factor arrested cells released from α-factor then arrest at 36°C with large buds after 3 h. Wild-type cells appear normal, as cells with no buds or small buds are frequently observed (data not shown).

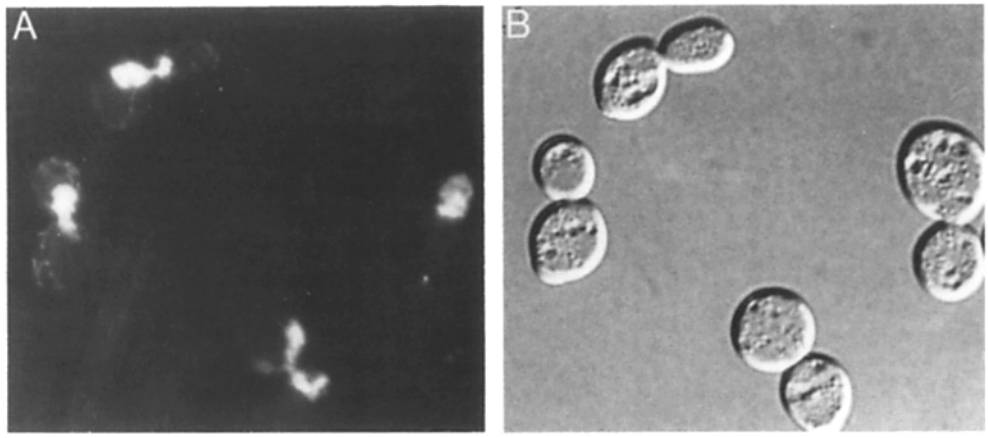

Figure 8. nuf2-61 mutant cells arrest uniformly at 36°C. PSY498 (*nuf2-61/nuf2-61*) were shifted from 25°C to 36°C for 3 h, fixed with formaldehyde for 20 min, and incubated with DAPI for 5 min. *A*–DAPI staining and *B*–cells viewed by Nomarski optics.

Table II. Mutations Found within nuf2-61

Position*	Mutation‡	Amino acid change§
1215/313	A → G	none
1288/338	A → C	T → P
1294/340	A → T	I → K
1424/383	A → G	Y → C
1491/405	T → C	none
1505/410	T → C	L → S
1588/441	A → T	K → I
1612/446	A → T	M → L

* Nucleotide #/amino acid #.
‡ Nucleotide change (sense strand).
§ Single-letter code abbreviations for amino acids changed by mutation.

After a 3-h temperatures shift to 36°C, both *NUF2* and *nuf2-61* cells were processed for immunofluorescence. DAPI-staining of DNA reveals that the nucleus is located at or within the bud neck (Fig. 9 *E*), and staining with anti-tubulin antibodies shows that the arrested cells possess a short spindle contained within the nucleus, which has migrated to the bud neck (Fig. 9 *D*). Staining with antibodies directed against the nuclear proteins Npl3 (Bossie et al., 1992) and Nspl showed the nuclei to have an abnormally elongated shape and to be located within the bud neck (data not shown). From this data, it would appear that *nuf2*^{ts} mutants have a nuclear division defect.

In a separate experiment, *nuf2-61* cells were processed for immunofluorescence with anti-Nuf2 and anti-80 kd antibodies. Localization of Nuf2 along the spindle occurred in cells shifted to 36°C for 3.5 h (Fig. 5 *B*). This is best demonstrated when Nuf2 localization is compared to the distribution of the 80-kd protein (Nuf2 is green, the 80-kd protein is purple and the overlap of the two is white in Fig. 5). Nuf2 is partially colocalized with the 80-kd protein at the non-permissive temperature. Most of the Nuf2 protein is located at the spindle between the poles (see *arrows* in Fig. 5 *B*).

The DNA of *nuf2-61* cells arrested at the non-permissive temperature has replicated, as determined by flow-activated cell sorting (Fig. 10, *lower panel*) and as compared to similarly treated wild-type cells (Fig. 10, *upper panel*). There are also cells with greater than 2N DNA content in the *nuf2-61* cell population. Chromosome loss and mitotic recombination appear to be no greater in the *nuf2-61/nuf2-61* strain than the PSY196 parent, as determined by mating ability when cells were grown at the permissive temperature of 23°C. However, *nuf2-61/nuf2-61* homozygous diploids cultivated at the semi-permissive temperature of 30°C demonstrated a 10–30-fold increase over similarly grown otherwise isogenic *NUF2/NUF2* diploids in their ability to mate as *MATa* or *MATα* cells. This indicates an increased loss of chromosome III in *nuf2-61* cells at the semi-permissive temperature and is consistent with the presence of cells with greater than 2N DNA content after a shift to the non-permissive temperature.

Karyogamy assays indicate that both a and α mating types of *nuf2-61* mutants mate normally with each other and with wild-type strains. Benomyl sensitivity and resistance was indistinguishable from wild-type cells. Alpha-mating hormone does not alter the steady state level of Nuf2 (data not shown).

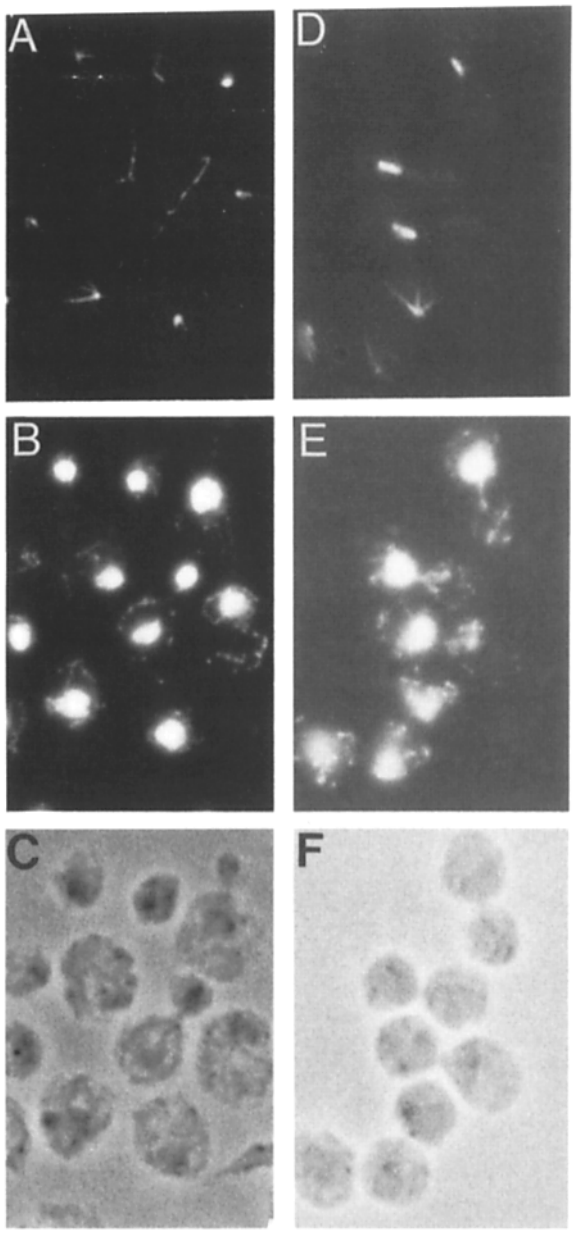

Figure 9. Nuf2-61 mutants arrest with short bipolar spindles. Cells from strains PSY450 (*NUF2* [*A–C*]) and PSY455 (*nuf2-61* [*D–F*]) were grown overnight at 25°C, and then shifted to 36°C for 3 h, and fixed then processed for immunofluorescence. For *A* and *D* rat anti-tubulin antibody was used at 1:200 dilution, and TRITC-anti-rat secondary antibody was used at 1:1,000. *B* and *E*–DAPI staining and *C* and *F*–cells viewed by Nomarski optics.

Discussion

We have characterized *NUF2*, an essential gene of the yeast *Saccharomyces cerevisiae*, which encodes a novel protein associated with the spindle pole body. The COOH terminus of the 53-kd Nuf2 protein is predicted to form an α-helical coiled-coil, and mutations conferring temperature-sensitivity are located in this region. Temperature-sensitive mutants in *NUF2* display uniform cell division-cycle arrest with a partially divided nucleus and a short bipolar spindle. In wild-type cells, Nuf2 colocalizes with the previously

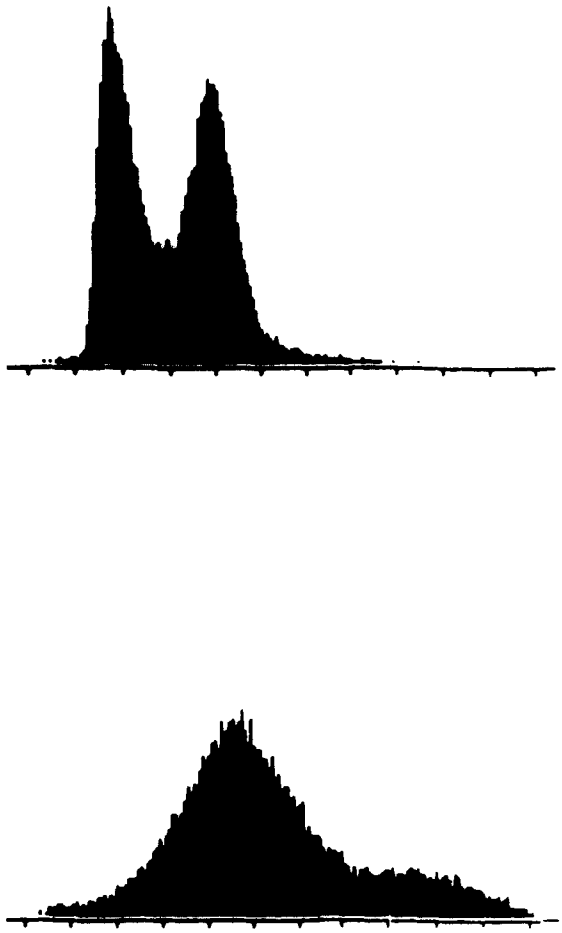

Figure 10. Arrested *nuf2-61* mutants have replicated their DNA. PSY196 (*NUF2/NUF2*) and PSY498 (*nuf2-61/nuf2-61*) cells were grown overnight in YPD, and then shifted to 36°C for 3 h, fixed in 70% ethanol, and stored at −20°C overnight. Cells were stained with propidium iodide immediately before sorting. 25,000 cells of each strain were counted. (*Top*) PSY196 (*NUF2/NUF2*) (*Bottom*) PSY498 (*nuf2-61/nuf2-61*).

identified 80-kd SPB antigen on the nucleoplasmic face of the SPB. Nuf2 contains a region conserved in evolution, as anti-Nuf2 antibodies recognize a related antigen in mammalian cells.

Nuf2 was identified through a genetic interaction with the nuclear pore complex protein Nup1 (Davis and Fink, 1990). Although it remains a formal possibility that Nuf2 and Nup1 interact, the placement of Nuf2 at the SPB under the conditions used here and not the nuclear pore complex appears to make this unlikely. One possibility is that there is some transient interaction of nucleoporins such as Nup1 with the SPB during nuclear pore assembly. In this regard, yeast mutants defective in RNA export have shown clustering of pore complexes at the SPB (Amberg, D., C. Copeland, M. Snyder and C. Cole, personal communication). Alternatively, the isolation of *NUF2* may reflect a particular bias in the two-hybrid screen. Along this line, other putative Nup1 interaction proteins identified in this screen also possess regions of coiled-coil potential (Schlendstedt, G., and P. Silver, unpublished results). Attempts to show a direct association between Nup1 and Nuf2 by other methods have, thus far, been unsuccessful.

The location of Nuf2 at the SPB was detected via coimmunofluorescence staining with other known SPB components. Rout and Kilmartin (1990) have purified yeast SPBs and raised antibodies against several of its major protein components. Antibodies against the 110-, 90-, and 80-kd proteins were used to confirm their SPB location by immunofluorescence. Immunoelectron microscopy further localized each antigen to distinct locations within the SPB. We now present the results of double-labeling experiments with anti-Nuf2 and either the anti-90 kd or anti-80 kd antibodies. There is costaining with the 80-kd and Nuf2 proteins, whereas the Nuf2 and 90-kd proteins were often located side-by-side as determined by confocal microscopy. The colocalization of Nuf2 and the 80-kd SPB protein then localizes Nuf2 to the nucleoplasmic side of the SPB, at the end of the microtubules. This location suggests that Nuf2 may be involved in SPB function, e.g., in microtubule dynamics. This suggestion is supported by the phenotype of mutants with temperature-sensitive mutations in *NUF2*.

There has been ambiguity with regard to the subcellular location of several potential SPB proteins. This appears to be due to problems with the level of expression and the fixation conditions used. Recently, Nufl, initially identified as a nuclear matrix component (Mirzayan et al., 1992), has been shown to be the 110-kd protein of the SPB (Kilmartin et al., 1993). Nufl/SPC110, like Nuf2, contains a large region of coiled-coil structure and deletion of the coil region alters the structure of the SPB. Similarly, during our initial analysis of Nuf2 (which employed formaldehyde fixation), we could only see overproduced protein in the nucleus. It was only when we modified the fixation conditions to those of Rout and Kilmartin (1990) that we could visualize wild-type levels of Nuf2 at the SPB. This raises the possibility that some proteins such as Nufl and Nuf2 may be redistributing during the various fixation processes. Thus, it is also possible that Nuf2 is actually a nuclear microtubule-associated protein. Since most of the microtubules appear to disassemble under the fixation conditions used, the observed Nuf2 staining might be due to colocalization with residual nuclear microtubules. One protein similar to Nuf2 in the GenBank/EMBL database is Mlp1 (Kolling et al., 1993), which was localized to "dots" adjacent to the nucleus in cells overexpressing the protein. These dots resemble SPB staining; however, no costaining with SPB antibodies was done to confirm this possibility.

Anti-Nuf2 antibodies were shown to cross-react with a 73-kd mammalian nuclear protein and to stain centrosomes in mammalian tissue culture cells. Based on these results, it is tempting to speculate that there is a functional Nuf2 homologue present in mammals. If there is indeed conservation between the yeast SPB and the mammalian centrosome, one might be able to identify additional centrosomal proteins based on similarity to yeast proteins.

Temperature sensitive mutants in *NUF2* behave in a manner similar to a subset of previously described *CDC* mutants, which arrest uniformly with a large partially divided nucleus. Mutants in this class include *cdc16, cdc17, cdc20, cdc23,* and *cdc27.* However, the phenotype of *nuf2*^{ts} mutant cells is different from other mutants in SPB components.

Mutants in *mps1*, *mps2* (Winey et al., 1991), *kar1* (Rose and Fink, 1987), *cdc31*, *cik1* (Page and Snyder, 1992), *ndc1* (Thomas and Botstein, 1986; Winey et al., 1993), and *cdc4* arrest with a monopolar spindle or no spindle, whereas *nuf2-61* cells have a partially elongated bipolar spindle. This may indicate that Nuf2 is involved in a process distinct from that of these other SPB proteins.

Little is known about the molecular requirements for separation of duplicated SPBs. Because of its predicted coiled-coil structure, Nuf2 may form filaments that serve as a framework or scaffold for other proteins that perform the work of separation of duplicated SPBs. The location of Nuf2 at the nuclear extreme of the SPB makes it a candidate for such a protein. Force generating proteins such as Kar3, Cin8, and Kip1 may also be required in this process (Roof et al., 1992; Saunders and Hoyt, 1992). Perhaps as yet unidentified proteins exclusively required for SPB separation may bind to Nuf2. Nuf2 is, in some ways, similar to the previously described mammalian NuMA protein (Lyderson and Pettijohn, 1980), which is located at centrosomes in mitotic cells (Compton et al., 1992). Both are potential coiled-coil proteins and NuMa is important for establishment of the mitotic spindle apparatus (Yang and Snyder, 1992). Similar to Nuf2, NuMa has also been suggested to have a structural role during mitosis. Future genetic analysis of Nuf2 and other SPB associated proteins will further elucidate the mechanism of SPB duplication.

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