Yeast *NOP2* Encodes an Essential Nucleolar Protein with Homology to a Human Proliferation Marker

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Abstract. We have isolated a gene (*NOP2*) encoding a nucleolar protein during a search for previously unidentified nuclear proteins in the yeast *Saccharomyces cerevisiae*. The protein encoded by *NOP2* (Nop2p) has a predicted molecular mass of 70 kD, migrates at 90 kD by SDS-PAGE, and is essential for cell viability. Nop2p shows significant amino acid sequence homology to a human proliferation-associated nucleolar protein, p120. Approximately half of Nop2p exhibits 67% amino acid sequence identity to p120. Analysis of subcellular fractions indicates that Nop2p is located primarily in the nucleus, and nuclear fractionation studies suggest that Nop2p is associated with the nucleolus. Indirect immunofluorescence localization of Nop2p shows a nucleolar-staining pattern, which is heterogeneous in appearance, and a faint staining of the cytoplasm. The expression of *NOP2* during the transition from stationary phase growth arrest to rapid growth was measured, and compared to the expression of *TCM1*, which encodes the ribosomal protein L3. Nop2p protein levels are markedly upregulated during the onset of growth, compared to the levels of ribosomal protein L3, which remain relatively constant. *NOP2* mRNA levels also increase during the onset of growth, accompanied by a similar increase in the levels of *TCM1* mRNA. The consequences of overexpressing *NOP2* from the *GAL10* promoter on a multicopy plasmid were investigated. Although *NOP2* overexpression produced no discernible growth phenotype and had no effect on ribosome subunit synthesis, overexpression was found to influence the morphology of the nucleolus, as judged by electron microscopy. Overexpression caused the nucleolus to become detached from the nuclear envelope and to become more rounded and/or fragmented in appearance. These findings suggest roles for *NOP2* in nucleolar function during the onset of growth, and in the maintenance of nucleolar structure.

THE nucleolus is the specialized region within the nucleus where the majority of the steps in the complex process of ribosome subunit synthesis are executed (for recent yeast reviews, see Raue and Planta, 1991; Woolford and Warner, 1991). Within the nucleolus RNA polymerase I synthesizes a precursor rRNA, which is processed and modified, but not spliced, in a series of steps to generate mature 5.8 S, 18 S, and 25 S rRNAs. The 5 S rRNA is transcribed from a separate transcription unit by RNA polymerase III. The large subunit is assembled from probably up to 45 different ribosomal proteins and the 5 S, 5.8 S, and 25 S rRNAs, whereas the small subunit contains 32 ribosomal proteins and the 18 S rRNA. The biogenesis of ribosomal subunits in the nucleolus is thought to involve the coordinated formation of a series of subunit precursors consisting of both ribosomal proteins and rRNA intermediates. The nucleolus plays a central role in coordinating, integrating, and regulating the numerous steps in ribosome subunit synthesis.

To facilitate the assembly of ribosomes, the nucleolus consists of a group of proteins and RNAs that are not part of mature cytosolic ribosomes. Nucleolar components characterized to date have been shown to function in: transcription of precursor rRNA; processing and modification of rRNA precursors and intermediates; and assembly of preribosomes. A number of nucleolar protein-encoding genes, including *GAR1*, *NOP1*, *NOP3*, *NOP4 (NOP77)*, *NSR1*, *SOF1*, and *SSB1* (Schimmang et al., 1989; Clark et al., 1990; Henriquez et al., 1990; Lee et al., 1991; Tollervey et al., 1991; Girard et al., 1992; Russell and Tollervey, 1992; Jansen et al., 1993; Berges et al., 1994; Sun and Woolford, 1994), and small nucleolar RNAs (for reviews see Fournier and Maxwell, 1993; Mattaj et al., 1993) have been identified. Certain nucleolar proteins shuttle between the nucleolus and cytoplasm, and may facilitate transport of other molecules to and from the nucleolus. Nucleolar components may also be in-

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volved in the maintenance of nucleolar structure, although this remains an open question at this time.

Certain nucleolar proteins in higher eukaryotic cells are known to undergo a dramatic increase in synthesis during cell proliferation. The nucleolus is typically hyperactive and pleomorphic in malignant cells, and the clinical literature is replete with documentation of the diagnostic value of various nucleolar proteins as markers for cellular proliferation. The nucleolar protein p120 is a proliferation marker that has been the subject of numerous studies (for review see Freeman and Busch, 1991). The expression of p120 is associated with cell proliferation, and p120 has been found in a variety of malignant tumors, as well as in nonmalignant proliferating tissues. The expression of p120 is important for cell proliferation (Valdez et al., 1992), and is regulated during the cell cycle, increasing during G_1 and S phases (Fonagy et al., 1992, 1993). Inhibition of p120 synthesis using an antisense oligonucleotide causes a reduction in cell growth rate, and induces profound changes in nucleolar morphology including an unraveling of nucleolar structure (Perlaky et al., 1993). Clinically, p120 has been shown to be of prognostic significance in the evaluation of human breast carcinoma (Freeman et al., 1991). Here, we report the isolation of *NOP2*, whose gene product, Nop2p, exhibits significant amino acid sequence homology with p120. Our studies in yeast reveal other similarities between Nop2p and p120, supporting the notion of the conservation of an evolutionarily conserved function in these nucleolar proteins.

Materials and Methods

Amino Acid Sequences from Nop2p Cyanogen Bromide Fragments

Nuclei were prepared in batches from a total of ~150 liters of culture of the protease deficient yeast strain BJ2168 as previously described (Aris and Blobel, 1991*b*). The nuclei were digested with DNase I to yield a fraction enriched in the nucleolus and nuclear envelope, which was extracted with 1 M NaCl to release the majority of nucleolar proteins (Aris and Blobel, 1991*b*). The salt extract was diluted with buffer containing 8 M urea, and fractionated on a DEAE ion exchange column, followed by SDS-hydroxyapatite chromatography as described (de Beus, 1992). Preparative SDS-PAGE was done (de Beus, 1992), and amino acid sequence data were obtained from cyanogen bromide fragments isolated by standard methods (de Beus, 1992) using microsequencing (Rockefeller University Biopolymer Facility, New York).

Cloning and Sequencing of NOP2

The coding region for a 15-amino acid stretch (see Fig. 1) from a CnBr fragment was amplified by PCR using genomic DNA from YJPA1, and cloned and sequenced using standard methods (Ausubel et al., 1993). A yeast genomic library in λ-DASH (Clontech, Palo Alto, CA) was screened, and positive λ DNAs were hybridized to a degenerative oligonucleotide mixture derived from an additional stretch of protein sequence of 12 amino acids. Three independently isolated Sal I inserts, from λ9 (~15 kb), λ18 (~18 kb), and λ23 (~14 kb), were subcloned into pBluescript SK+ to yield plasmids pEdB1, pEdB2, and pEdB3, respectively. The *NOP2* loci in pEdB2 and pEdB3 were sequenced on both strands, using standard methods and special nucleotides where necessary as described (Aris and Blobel, 1991*a*). A total of 3,235 bp were sequenced. Protein database and motif searches were conducted using the BLAST and MOTIFS programs within the Genetics Computer Group software package (Devereux et al., 1984) available through the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Plasmid and Yeast Strain Construction

The plasmids and yeast strains used in this study are listed in Table I. The 2.3-kb EcoRI fragment containing *NOP2* was subcloned from pEdB1 into pBluescript to yield pJPA20. The oligonucleotides 5'-TACCCATATGATGTTCCTGATTACGCTCAACGT-3' and 5'-TGAGCGTAATCAGGAACATCATAAGGGTAACGT-3' were annealed and ligated into the unique AatII site of pJPA20, yielding pJPA25 and pJPA26. SalI–BamHI fragments from pJPA20, pJPA25, or pJPA26 were cloned into YEp51 (Broach et al., 1983) to generate pEdB10, pEdB11, or pEdB12, respectively. pEdB11 encodes Nop2p carrying the influenza virus hemagglutinin epitope tag plus an additional arginine (R) residue. The resulting Nop2p amino-terminal sequence is MGSRRYPYDVPDYAQRHKNK. pEdB12 contains the oligonucleotide in the reverse orientation at the AatII site, which introduces three stop codons into the *NOP2* open reading frame. To construct pJPA30, a 5.8-kb XhoI–BglII *NOP2* fragment from pEdb2 was cloned into pRS314 (Sikorski and Hieter, 1989) between the XhoI and BamHI sites.

Yeast cell culture and other manipulations were performed essentially as described (Ausubel et al., 1993). Using convenient restriction sites and standard cloning methods, ~760 bp near the middle of *NOP2* was replaced with the *URA3* gene from YEp25 (see Fig. 3). An EcoRI fragment carrying the *nop2::URA3* disruption was used to transform strain YJPA1 using lithium acetate, and ura^+ colonies were analyzed by Southern blotting.

Blot Analyses of NOP2

Poly A+ selected RNA was prepared, separated in a 1.0% agarose glyoxal gel, transferred to nitrocellulose membrane, and hybridized to probe under conditions of high stringency according to standard methods (Ausubel et al., 1993). Preparation of genomic DNA and Southern blot analysis were done as described (Ausubel et al., 1993). Probes were prepared by random hexamer primer extension (United States Biochem. Corp., Cleveland, OH) from isolated 2.3-kb EcoRI fragment containing *NOP2*, or the EcoRI–NsiI fragment downstream of *NOP2* (see Fig. 1 *C*). A chromosomal blot was hybridized to probe from the 2.3-kb *NOP2* EcoRI fragment according to the supplier (Clontech).

Anti-Nop2p Antibody Preparation

Antisera were prepared against two 15-amino acid long synthetic peptides selected from the Nop2p sequence based on a number of considerations that influence peptide antigenicity and synthesis efficiency. Peptide A ("acidic", residues 102-116) and B ("basic", residues 269-283) were synthesized with an extra cysteine residue at the carboxyl terminus (Multiple Peptide Systems), and coupled to the carrier protein keyhole limpet hemocyanin with the carbodiimide coupling reagent EDC (Harlow and Lane, 1988). Two New Zealand White rabbits were immunized with 0.5 mg of each conjugate, and antisera were obtained using a standard schedule of boosts (Harlow and Lane, 1988). To affinity purify the peptide-specific IgG, peptides were coupled via the COOH-terminal cysteine to Sulfo-link gel (Pierce, Rockford, IL). IgG was eluted in batches with 100 mM glycine, pH 2.5, rapidly neutralized, and equilibrated with PBS (Harlow and Lane, 1988). Affinity purified polyclonal antibodies, termed APpAb2 and APpAb3, are directed against peptide "B" and peptide "A", respectively.

SDS-PAGE and Immunoblotting

Proteins were separated on 10.5% polyacrylamide gels according to the method of Laemmli as previously described (Aris and Blobel, 1988). Broad range molecular weight standards were from BioRad Labs (Hercules, CA). For immunoblotting, proteins were transferred to nitrocellulose membrane using a semi-dry apparatus (BioRad Labs). Dried blots were rehydrated, probed with primary antibody at a dilution of 1:10,000 unless otherwise indicated, and processed for chemiluminescent detection as described by the supplier (Amersham Corp., Arlington Heights, IL). Peptides A and B were present at a concentration of ~20 μM in competition experiments. The monoclonal antibodies FB2 and 12CA5 were used to detect, respectively, human p120 (Freeman et al., 1988) and the influenza hemagglutinin epitope YPYDVPDYA (Kolodziej and Young, 1991). Noplp was detected with mAb D77 (Aris and Blobel, 1988).

Galactose Induction of Nop2p Synthesis

For overexpression, strain YJPA1 was transformed with pEdB10 or pEdB11, and the plasmids YEp51 or pEdB12 were used as controls (Table I). Trans-

formants were grown for 18 h at 30°C in synthetic raffinose medium containing required supplements (SRaWu) to an OD_{600} = 1. Cells were diluted to OD_{600} = ~0.075 in SRaWu, and grown at 30°C to OD_{600} = ~0.15. One tenth volume of 20% galactose was added and 5-ml aliquots of culture were harvested at 0, 1, 2, 3, and 18 h. Cells were centrifuged and washed with ddH_2O.

For gel electrophoresis, cell pellets were frozen in liquid nitrogen and stored at −70°C. For the 0–3-h time points, cells were resuspended in 100 μl of SDS-PAGE sample buffer, vortexed with 0.1 g of 0.5-mm acid washed glass beads in a glass tube, boiled for 5 min, and microcentrifuged for 5 min, to yield a total cell lysate, which was stored at −20°C. For the 18-h time point, cells were lysed for SDS-PAGE in the presence of TCA as described below.

For electron microscopy, cells were grown in SRaWu medium plus a synthetic mixture of non-essential amino acids (Bio101), collected at the 0 and 3 h time points, and prepared for EM as described (Byers and Goetsch, 1991). Sections were viewed with a Jeol 100CX electron microscope after post-staining with uranyl acetate and lead citrate (Aris and Blobel, 1991*b*). Cells from the same cultures were fixed for cytometry, or were prepared for SDS-PAGE by lysis in the presence of TCA as described below.

Cell and Nuclear Fractionation

The cell fractions studied were prepared as previously described (Aris and Blobel, 1991*b*). Fractionation of isolated yeast nuclei was conducted as previously described (Aris and Blobel, 1991*b*), except that the PSM, PSE, and PEN buffers were adjusted to pH 6.5 at room temperature instead of pH 7.0.

Immunofluorescence Localization

Indirect immunofluorescence localization was done as previously described (Aris and Blobel, 1988, 1989), with the following modifications based on Rout and Kilmartin (1990). Exponentially growing yeast were fixed with 3% freshly prepared paraformaldehyde, digested with a final concentration of 1 mg/ml Zymolyase 20-T in phosphate-citrate buffer, placed on a multiwell slide coated with polylysine, and treated with methanol and acetone. Cells were blocked and incubated with diluted antibody, in PBS plus 0.1% Tween-20 and 5% non-fat dried milk. Monoclonal A66 against Noplp has been previously described (Aris and Blobel, 1988). The mAb C21 recognizes Nsr1p. Confocal images were obtained using a laser scanning confocal microscope (BioRad Labs) using separately optimized channels for fluorescein and rhodamine detection, and displayed on a Focus Graphics image recorder, at the Optical Microscopy Suite of the College of Medicine at the University of Florida.

Determination of NOP2 mRNA and Protein Levels

A standardized procedure was developed for the measurement of protein levels during the onset of growth. On day -3, the strain of interest is revived from a stock kept at −80°C and grown on a YPD plate at 30°C. On day -1, a 3-ml YPD culture is inoculated and grown for about 18 h at 30°C. The saturated culture is diluted 10-fold into YPD at 30°C. At each time point, culture is collected for different analyses. For cytometry, cells are added to an equal volume of 7.4% formaldehyde solution, stored at 4°C, and measured with a hemocytometer. Our comparisons of hemocytometry and optical densities revealed that OD_{600} readings for the two strains we have studied (YJPA1 and YPH501) typically increase a short time (<30 min) before increase in cell number, perhaps because of cell shape changes that precede division.

For protein extraction, cells are combined with 1/5 vol of 50% TCA at −20°C, pelleted, washed with cold 10% TCA, and stored as a pellet at −80°C. Cell pellets from different time points are lysed in parallel in the presence of TCA by vortexing twice for 1 min with 0.5-mm acid-washed glass beads (Reid and Schatz, 1982). TCA precipitates are pelleted in a microcentrifuge, and solubilized in SDS-PAGE sample buffer, boiled for 5 min, sonified in a water bath for ~30 s, microcentrifuged for 2 min, and the supernatant is stored at −20°C. Protein determinations are performed twice, in duplicate, after precipitating proteins with deoxycholate and 10% TCA (Ausubel et al., 1993). Protein samples (40–70 μg) from one time course experiment are subjected to 10.5% polyacrylamide SDS-PAGE on one gel and transferred to nitrocellulose. To detect Nop2p, the Western blot is probed with affinity purified antipeptide antibody APpAb3 as described (Aris and Blobel, 1988). A polyclonal antiserum against Noplp (Henriquez et al., 1990), or mAb 7.1.1 against ribosomal protein L3 (from J. R. Warner), or mAb 1G1 against Pablp (Anderson et al., 1993) were used. Blots are subsequently incubated with ^{125}I-protein A. For Nop2p, the sum of the 70-kD and 90-kD band values was used. Immunoblots are checked for discrepancies in loading and/or transfer by staining proteins with India ink, which may be conveniently done after the collection of data.

For RNA extraction, cells are combined with 1/5 vol of ice at −20°C, pelleted, washed with cold TE buffer, and stored as a pellet at −80°C. Cell pellets from different time points are lysed in parallel as described using a glass bead method (Ausubel et al., 1993). Total RNA is prepared, separated in a 1.2% agarose glyoxal gel, transferred to nitrocellulose membrane, and hybridized to probe using standard methods (Ausubel et al., 1993). Probes are prepared by random hexamer primer extension, or 5′-end labeling, using as a template either a 2.3-kb EcoRI fragment containing *NOP2*, or a HpaI–SalI fragment from *TCM1*, or an antisense oligonucleotide to U3 snRNA (from J. R. Warner).

The radioactivities from the relevant bands on Western or Northern blots are quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and corrected for local backgrounds. Comparison of duplicate experiments indicated that measurements of the relative change in protein and RNA levels varied by less than 10% (data not shown). In our experience, chemiluminescent immunoblot detection methods followed by laser scanning densitometry does not give reproducible or consistent results.

Results

Identification and Characterization of NOP2

The identification of *NOP2* evolved out of an effort to purify a putative yeast nuclear pore complex protein of 110 kD that cross-reacts with monoclonal antibody 414 directed against rat liver nuclear pore complex proteins (Aris and Blobel, 1989). During this effort, we identified a protein containing a stretch of 15 amino acids that was strikingly similar to a stretch of sequence from the human nucleolar protein p120. This amino acid sequence also conformed to the consensus sequence for an ATP-binding site. We decided to clone the corresponding yeast gene because the homology with a human protein offered the prospect of investigating a nucleolar function that has been evolutionarily conserved. To our knowledge, Nop2p is the second yeast nucleolar protein that exhibits homology to a human nucleolar protein. The other yeast nucleolar protein homologous to a human nucleolar protein is Noplp (Aris and Blobel, 1991*a*; Jansen et al., 1991).

The *NOP2* locus was cloned, and an open reading frame of 1,857 bp was identified (Fig. 1 *A*). We have termed this gene *NOP2*, for nucleolar protein gene 2. We introduced the NOP nomenclature with the *NOP1* gene (Henriquez et al., 1990). *NOP2* encodes a protein of 618 residues which contains two stretches of sequence that are identical to the two amino acid sequences obtained from cyanogen bromide fragments of the isolated protein (Fig. 1 *A*). Nop2p has a predicted molecular mass of 69.8 kD, a predicted pI of ~4.9, and contains no long stretches of hydrophobic amino acids. Nop2p contains 33 potential phosphorylation sites: 3 cAMP dependent; 14 protein kinase C; 14 casein kinase II; 2 tyrosine sites; no CDC28 kinase sites. A potential nuclear localization sequence $KKKSK_{54}$, and the consensus for a P-loop ATP-binding site $AAAPGGKT_{360}$ (Saraste et al., 1990) are found in Nop2p.

Alignment of Nop2p with human p120 points to the evolutionary conservation of a large domain that consists of approximately half of Nop2p (Fig. 1 *B*). The stretch of 290 residues from 260–549, which is 47% of the total of 618 residues in Nop2p, is 67% identical to human p120 (Fonagy et al., 1989). Over the entire alignment, from 8–581, the to-

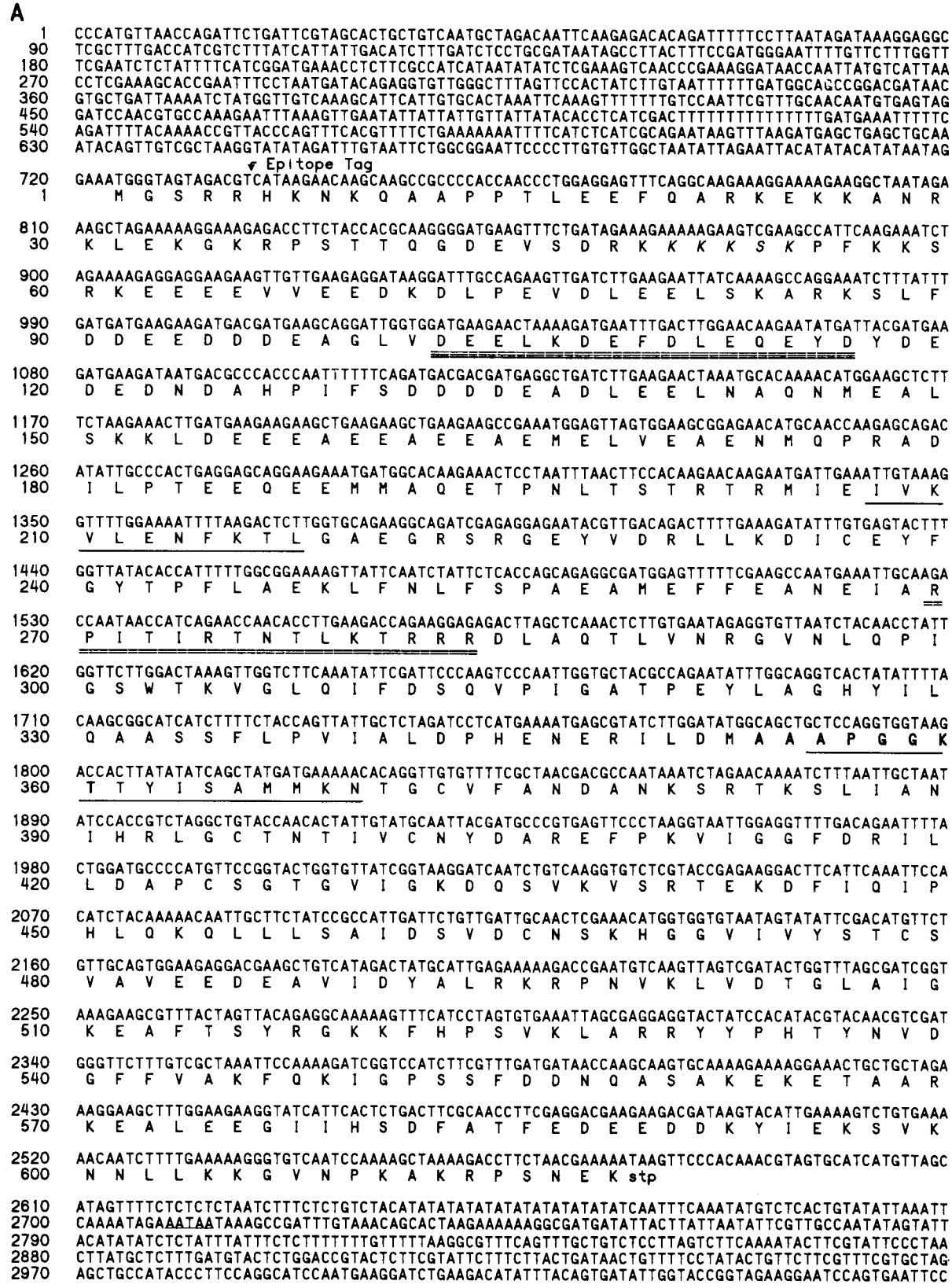

Figure 1.

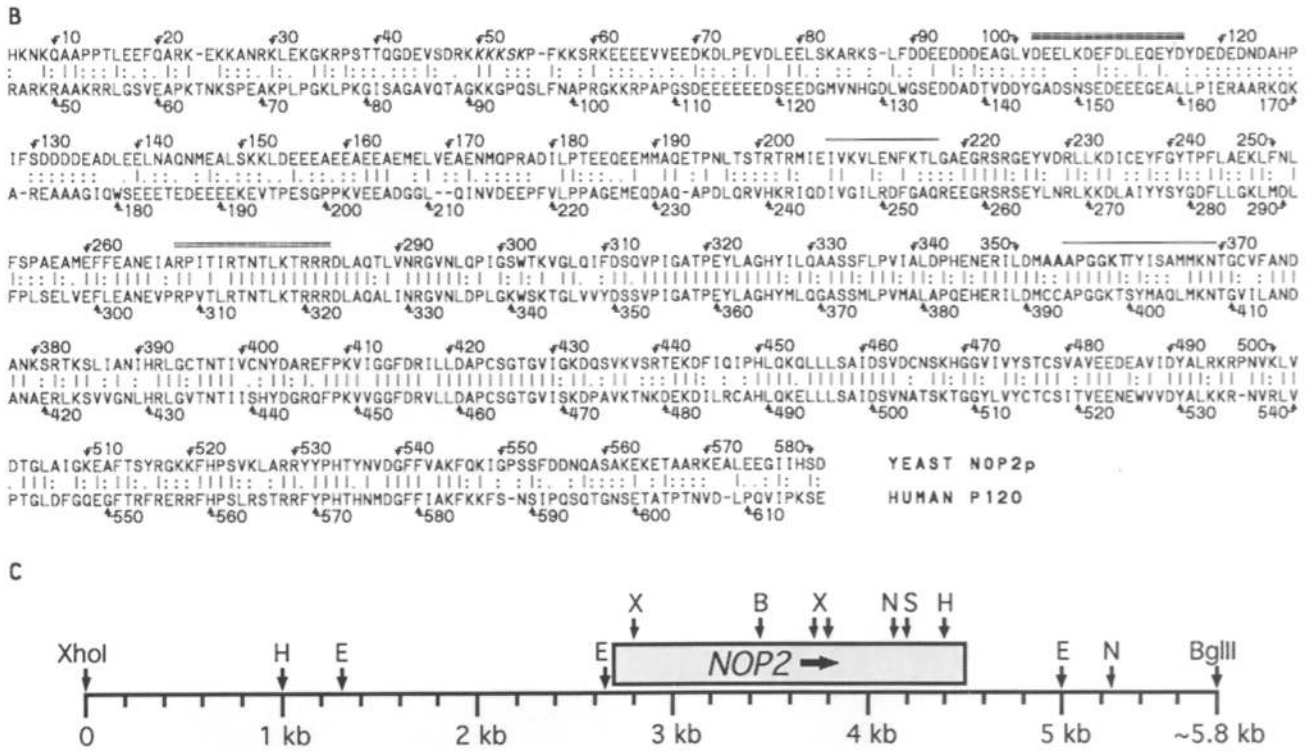

Figure 1. (*A*) Sequence of *NOP2* and predicted sequence of Nop2p. The open reading frame consists of 1,857 bp of the 3,059 bp shown, and encodes 618 amino acids. The point of insertion of the epitope tag is indicated. Peptide A is triply underlined (APpAb3 antigen). Peptide B is doubly underlined (APpAb2 antigen). Protein sequences confirmed by microsequencing are singly underlined. A potential nuclear localization signal is in italics, and a potential ATP-binding site is identified by bold type. (*B*) Protein sequence alignment of Nop2p with human p120. The alignment extends from amino acids 8–581 for a total identity of 47%. The stretch between positions 260 and 549 is 67% identical. Stretches of sequence are overlined as described above. (*C*) Restriction map of the *NOP2* locus: *B*, BstBI; *E*, EcoRI; *H*, HindIII; *N*, NsiI; *S*, SpeI, *X*, XbaI. The *NOP2* sequence has been assigned the accession number X82656.

tal identity is 47%. The only other protein to emerge from database searches for proteins similar to Nop2p is mouse p120 (data not shown), which is very similar to human p120 (Valdez et al., 1992). All other proteins that exhibit sequence similarity to Nop2p have stretches of acidic or basic residues that result in modest similarity with the amino-terminal region of Nop2p (data not shown). The nucleolar proteins nucleolin/C23 (Lapeyre et al., 1987) and NO38/B23 (Schmidt-Zachmann et al., 1987), and the yeast nucleolar protein Nsrlp (Lee et al., 1991), were among those detected in database searches for this reason.

Northern blot, Southern blot, and chromosomal blot analyses were done to characterize *NOP2*. A restriction map was created for these analyses (Fig. 1 *C*). Northern blot analysis indicates that *NOP2* mRNA is 2.0 kb (Fig. 2 *A*), which is consistent with the length of the *NOP2* open reading frame plus the typical length (~75 nt) of a polyA tail in yeast. Southern blot analysis of yeast genomic DNA shows hybridization of probe spanning all of *NOP2* to DNAs of the predicted size (2.3 kb EcoRI band, 3.4 kb HindIII band, 1.1 kb NsiI band, 0.9 kb XbaI band) based on the occurrence of one copy of *NOP2* per haploid genome (Fig. 2 *B*). Hybridization of a chromosomal blot with radiolabeled *NOP2* probe indicates that *NOP2* is located on chromosome XIV (Fig. 2 *C*).

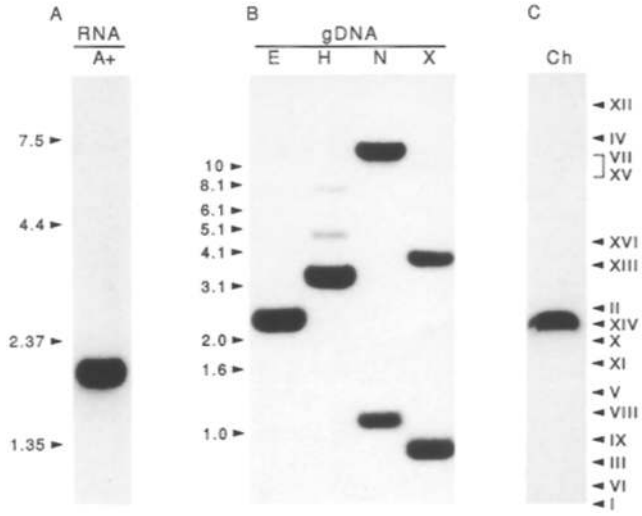

Figure 2. Blot analysis of *NOP2*. (*A*) Northern blot analysis of polyA+ (A+) selected RNAs. (*B*) Southern blot analysis of genomic DNA (*gDNA*) digested with the restriction endonucleases EcoRI (*E*), HindIII (*H*), NsiI (*N*), or XbaI (*X*). (*C*) Chromosomal blot analysis, with the positions of yeast chromosomes indicated. Each blot was hybridized to probe from the *NOP2* EcoRI fragment. RNA and DNA standards are in kb.

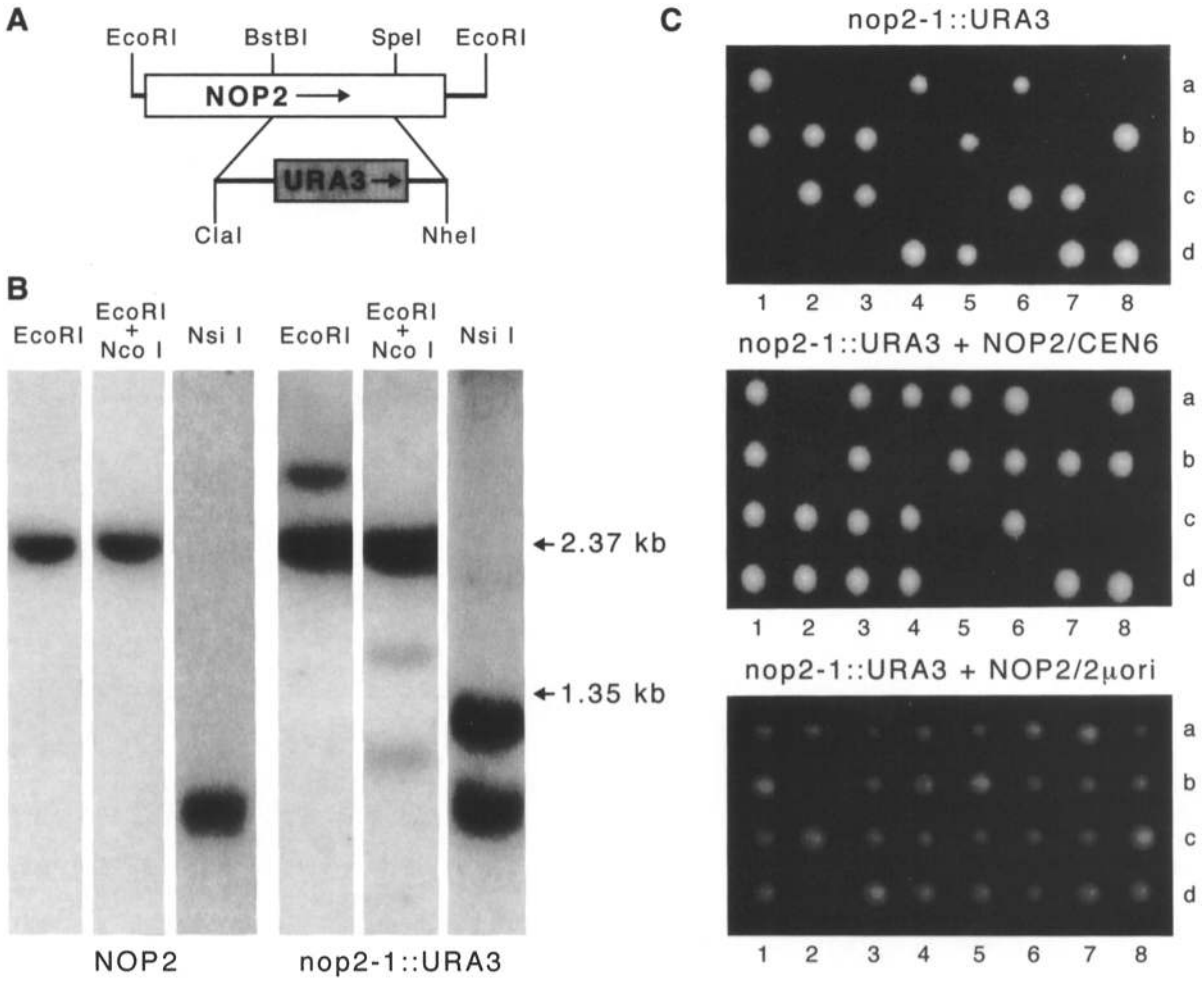

Figure 3. Disruption and complementation of *NOP2*. (*A*) Strategy for interrupting *NOP2* with *URA3*. (*B*) Genomic DNAs from parental YJPA1 (NOP2) or disrupted YJPA10 (nop2-1::URA3) strains were prepared and digested with EcoRI, or EcoRI and NcoI, and analyzed by Southern blotting with probe prepared from the EcoRI fragment carrying *NOP2*. DNAs digested with NsiI were hybridized to probe prepared from an EcoRI–NsiI fragment downstream of *NOP2*. (*C*) Tetrad analysis. Analysis of 8 tetrads (1–8), from which were dissected four spores each (*A–D*). The strains YJPA10 (nop2-1::URA3), YJPA10 carrying pJPA30 (NOP2/CEN6), and YJPA10 with pEdB10 (GAL-NOP2/2 μ ori) were examined.

Table I. Yeast Plasmids and Strains Used in This Study

Plasmid	Relevant functional DNA	Comments
pEdB10	*GAL10::NOP2*, *LEU2*, 2 μ ori	YEp51 derivative with *NOP2* open reading frame under *GAL* promoter control.
pEdB11	*GAL10::NOP2*-epitope tag, *LEU2*, 2 μ ori	Similar to pEdB10. Nop2p with influenza virus hemagglutinin epitope tag near amino terminus.
pEdB12	*GAL10::NOP2*-reverse tag, *LEU2*, 2 μ ori	Similar to pEdB11. Epitope coding sequence in reverse orientation, which introduces three stops.
pJPA30	*NOP2*, *CEN6*, *TRP1*	5.8-kb fragment containing *NOP2*, including 2.7 kb of 5′ flanking sequence, cloned into pRS314.
Strain	**Genotypic description**	**Source**
YJPA1	*MATa/MATα ade5/ade5 can1/+ gal2/+ +/his7-2 leu2-3/leu2-3 leu2-112/leu2-112 trp1-289$_a$/ trp1-289$_a$ ura3-52/ura3-52 [Kil-o]*	Mating of CG378 and CG379, from the Yeast Genetic Stock Center.
YJPA10	YJPA1, *nop2-1::URA3/+*	Transformation of YJPA1 with EcoRI fragment containing *nop2-1::URA3*.

NOP2 Is an Essential Gene

To disrupt *NOP2*, ~760 bp near the middle of the gene was replaced with *URA3*, generating YJPA10 (Table I and Fig. 3 *A*). Southern blotting detects a 3.0-kb EcoRI band, containing an NcoI site, which verifies the presence of the correct disruption construct in the genome (Fig. 3 *B*). The detection of a 1.3-kb NsiI band using probe downstream of *NOP2* verifies recombination at the correct locus (Fig. 3 *B*). Asci dissected from YJPA10 produced a 2:2 distribution of viable and inviable spores (Fig. 3 *C*). Similar results were obtained with dissections of tetrads from two other disruption isolates (data not shown). All of the viable colonies required uracil for growth, and inviable spores germinated and produced 2–5 cells after up to 5 d of growth on YPD plates (data not shown). YJPA10 was transformed with pJPA30, which carries *NOP2* and its endogenous promoter on a *CEN* plasmid (Table I). pJPA30 complements the *nop2-1* disruption, but does not appear to segregate with high fidelity during meiosis, yielding some 3:1 and 2:2 dissection results (Fig. 3 *C*). pEdB10 was constructed by placing *NOP2* under control of the *GAL10* promoter in a yeast episomal plasmid (Table I). Good complementation of the disruption was observed with pEdB10, and most dissections generated a 4:0 distribution of viable to inviable spores (Fig. 3 *C*).

NOP2 Encodes a Protein with an Apparent Molecular Mass of 90 kD

To identify Nop2p, affinity purified polyclonal antibodies, termed APpAb2 and APpAb3, were prepared against a basic peptide (peptide B) and an acidic peptide (peptide A), respectively (see Materials and Methods, and Fig. 1 for peptide sequences).

The principal protein recognized by APpAb2 and APpAb3 on immunoblots of yeast nuclear proteins is a protein of ~90 kD (Fig. 4 *A*). Binding of each antibody to the 90-kD protein is abolished by incubation in the presence of purified peptide. Preimmune antisera did not detect a 90-kD protein (data not shown). APpAb2 and APpAb3 recognize a 90-kD band on immunoblots of proteins from a whole cell lysate of strain YJPA1 (Fig. 4 *B*, lane *C*). A 90-kD protein is also detected in a lysate from a different strain used for immunofluorescence localization (Fig. 4 *B*, lane *F*). A protein of 90 kD is also detected in extracts from cells in which *NOP2* is overexpressed from the GAL10 promoter on a multicopy plasmid (Fig. 4 *B*, lane *2p*↑). *NOP2* overexpression is discussed below in conjunction with Figs. 5 and 8, but is mentioned here to demonstrate that the overexpressed protein comigrates with Nop2p expressed from its endogenous promoter.

Since peptide B is similar to a stretch of the human p120 sequence (13/15 identities, plus I:V and I:L), an immunoblot of proteins from HeLa cell nuclei was probed with the APpAb2 antibody directed against this peptide. APpAb2 detects a band that comigrates in a 1D SDS gel with a band detected by the mAb FB2 directed against p120 (Fig. 4 *C*). The apparent size of this protein is ~105 kD in our gel system. The predicted size of p120 is 85 kD, although it has been observed to migrate at 120 kD on SDS gels (Freeman et al., 1988).

To verify that *NOP2* encodes the 90-kD protein, *NOP2* was placed under control of the GAL10 promoter. In addition, an epitope tagged construct under GAL10 promoter control was made by introducing the influenza virus hemagglutinin epitope tag into the open reading frame at amino acid position 5. For the galactose induction experiment, YJPA1 was transformed with pEdB10 or pEdB11, and for control purposes, with pEdB12 or YEp51 (Table I). After 1,

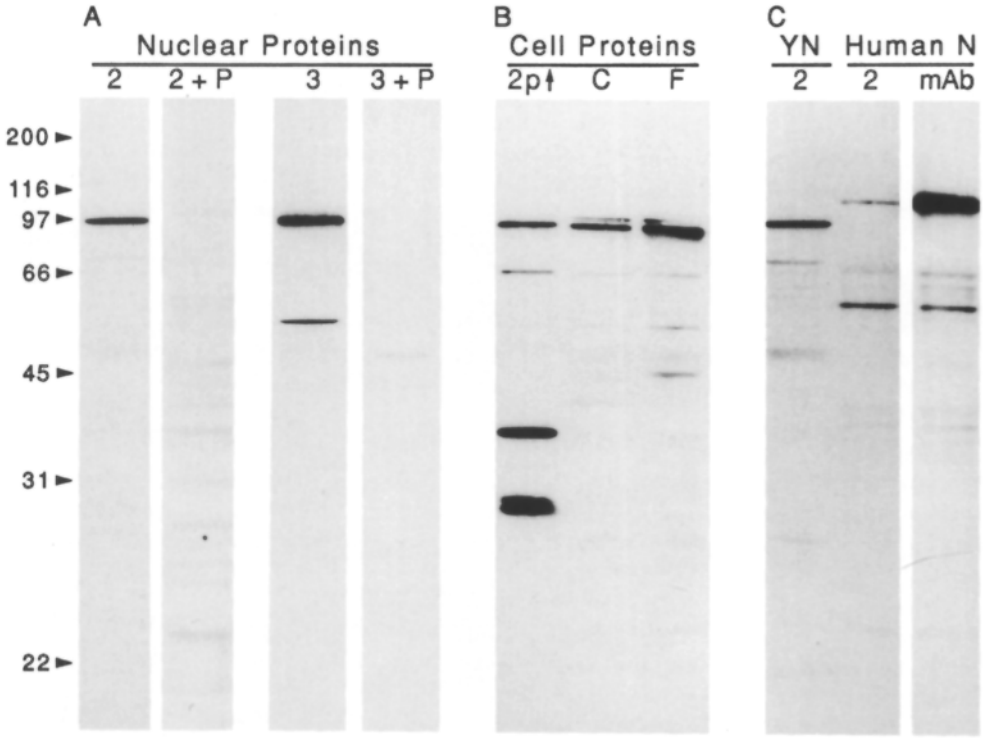

Figure 4. Immunoblot identification of Nop2p. (*A*) Proteins from isolated nuclei were analyzed by immunoblotting with APpAb2 (*2*), or APpAb2 plus 20 μm peptide B (*2 + P*), or APpAb3 (*3*), or APpAb3 plus 20 μm peptide A (*3 + P*). Positions of molecular mass markers are indicated (kD). (*B*) Total cell protein lysates from YJPA1 overexpressing Nop2p (*2p*↑), or YJPA1 (*C*), or the yeast strain used for immunofluorescence localization in Fig. 7 (*F*) were analyzed with APpAb3. (*C*) Nuclear proteins from yeast (*YN*) and human (*HN*) were incubated with APpAb2 or monoclonal antibody FB2 (*mAb*) directed against human p120.

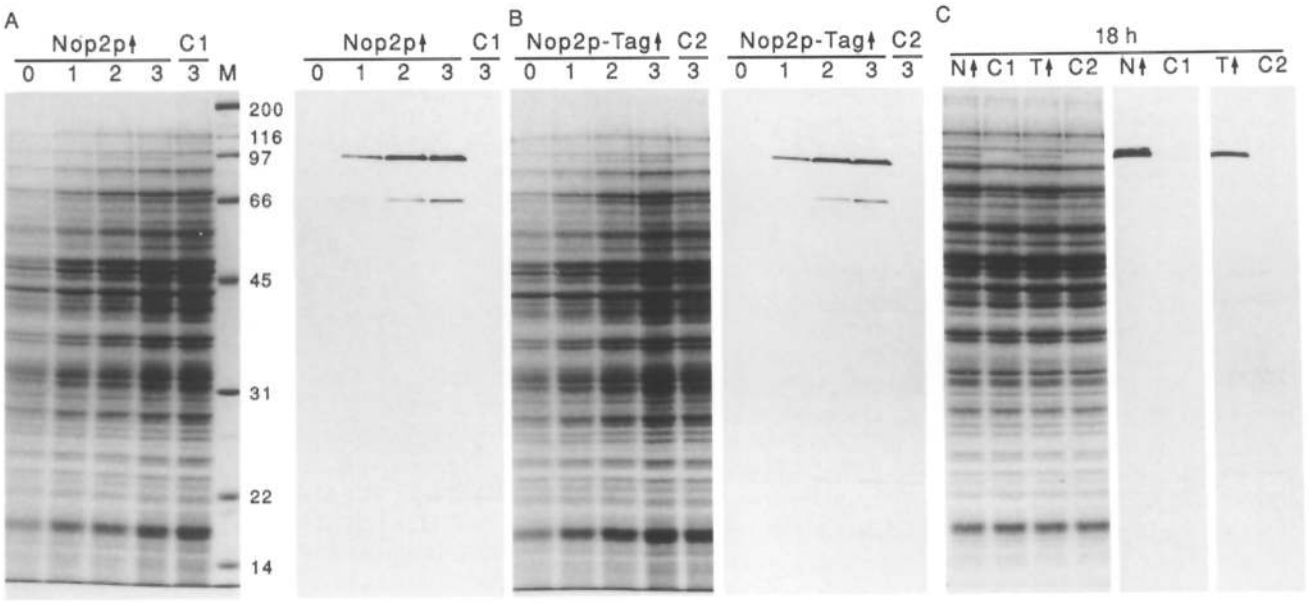

Figure 5. Galactose promoter induced expression of *NOP2*. (*A*) Total lysates from yeast YJPA1 containing pEdB10 (*GAL::NOP2*, 2 μ ori) were prepared at 0, 1, 2, or 3 h after galactose addition. Proteins from equal volumes of yeast culture were separated by SDS-PAGE and analyzed by Coomassie blue staining or by immunoblotting with APpAb3. Molecular mass markers are shown (*M*, in kD). At 3 h, a lysate was prepared from yeast harboring YEp51 (2 μ ori) as a control (*C1*). (*B*) A similar analysis was performed with yeast carrying pEdB11 (NOP2-epitope tag, 2 μ ori). A monoclonal antibody to the hemagglutinin epitope tag was used for immunoblotting. pEdB12 (NOP2-reverse tag, 2 μ ori) was used as a control (*C2*). (*C*) Lysates were prepared from the same four strains cultured for 18 h (*N*, Nop2p; *T*, Nop2p-epitope tag). Immunoblotting was done with APpAb3.

2, or 3 h of growth on galactose-containing medium, the 90-kD protein is readily detected by immunoblotting with APpAb3 (Fig. 5 *A*). The major band detected with the anti-epitope tag mAb also migrates as a 90-kD protein (Fig. 5 *B*). Similar results were obtained with cultures grown 18 h on galactose-containing medium (Fig. 5 *C*). A minor band of 70 kD was also detected.

The 90-kD apparent molecular mass of Nop2p is 20 kD greater than the predicted size of 70 kD. This discrepancy may be due to anomalous migration during SDS-PAGE, and/or posttranslational modification(s). Bands smaller than 90 kD (~55–75 kD) that were weakly detected by the antisera on immunoblots in Figs. 4 and 5 may represent proteolytic fragments of Nop2p. We observed that SDS-PAGE sample preparation influences the mobility of immunoprecipitates obtained with affinity purified antisera (data not shown). The detection on immunoblots of proteins below 55 kD has not been consistent or reproducible, and may result from variable cross-reactivity of the antisera used.

Cell and Nuclear Fractionation Studies

We examined the intracellular distribution of Nop2p by analyzing isolated yeast cell fractions (Aris and Blobel, 1988; Aris and Blobel, 1991*b*). The fractions employed are purified nuclei (N), low (L) and high (H) density membrane fractions, and a soluble (S) fraction. These fractions are prepared from a spheroplast lysate (C) cleared of unlysed cells and cell wall debris by a medium speed centrifugation step (Aris and Blobel, 1991*b*). Virtually all of the proteins of the yeast cell are represented in the cell lysate, with the exception of cell wall components. The majority of the 90-kD protein appears in the nuclear fraction, with a small fraction of Nop2p associated with membrane-containing fractions L and H (Fig. 6 *A*). For comparison, Noplp and histones are convenient markers that are visible in isolated yeast nuclei.

Nuclear fractionation studies were done to characterize interactions between Nop2p and other nuclear constituents. This approach is valuable because nucleolar proteins such as Noplp exhibit a typical extraction behavior (Aris and Blobel, 1988; Aris and Blobel, 1991*b*). Specifically, most of Noplp remains sedimentable after digestion of nuclei with DNase I and treatment with EDTA, which solubilizes most of the histones, and most of Noplp can be liberated from the sedimentable fraction by exposure to high salt (Fig. 6 *B*, Aris and Blobel, 1991*b*). An analysis of the distribution of Nop2p shows that Nop2p occurs principally in the pellet fraction after DNase I digestion and treatment with EDTA (Fig. 6 *B*). Essentially all of the Nop2p present in this pellet fraction is released into the supernatant fraction after extraction with high salt. Thus, the fractionation behavior of Nop2p is consistent with nucleolar localization.

Immunofluorescence Localization of Nop2p

Indirect immunofluorescence localization was done using modifications of procedures that we have used in the past. Double immunolabeling was done to compare the distribution of Nop2p to that of well known nucleolar proteins such as Noplp (Aris and Blobel, 1988, 1991*b*); and Nsrlp (Lee et al., 1991). The affinity purified antipeptide antibody APpAb3 produced bright staining of the yeast cell nucleolus (Fig. 7, *a* and *i*). The APpAb2 produced a less intense, but apparently identical nucleolar-staining pattern (Fig. 7 *e*). Both of

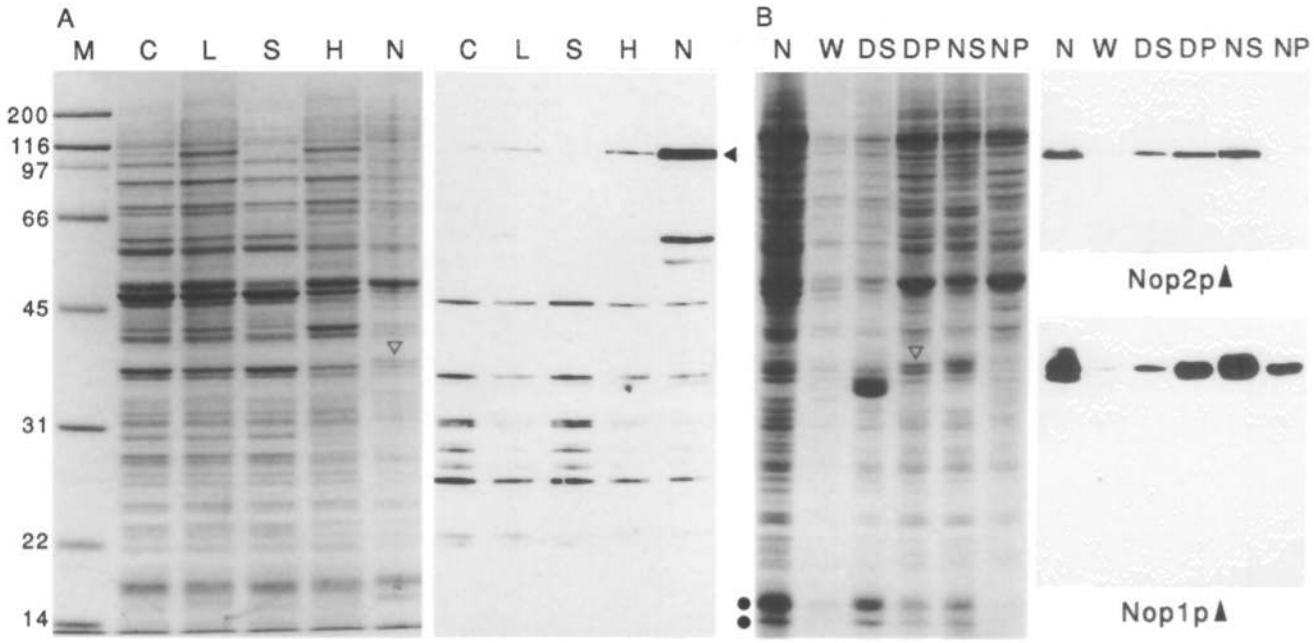

Figure 6. Cell and nuclear fractionation of Nop2p. Proteins from different fractions were separated by SDS-PAGE and analyzed by Coomassie blue staining or by immunoblotting. (*A*) The following subcellular fractions were examined: a cell lysate (*C*), low (*L*), and high (*H*) density membrane fractions, a soluble (*S*) fraction, and purified nuclei (*N*). Molecular mass markers are indicated (*M*, kD). The Nop2p band detected by APpAB3 is designated with a closed arrowhead. Noplp is marked with an open arrowhead. A long exposure of the blot shows minor amounts of Nop2p in nonnuclear fractions. (*B*) Nuclei (*N*) were diluted in buffer with a low Mg^{++} concentration and centrifuged to generate a wash supernatant (*W*). Washed nuclei were digested with DNase I, incubated with EDTA, and centrifuged to generate supernatant (*DS*) and pellet (*DP*) fractions. Twice the amount of the *DP* fraction shown was resuspended in buffer, adjusted to 1 M NaCl, and subjected to centrifugation to yield supernatant (*NS*) and pellet (*NP*) fractions. Nop2p or Noplp were detected with APpAb3, or mAb D77, respectively. Closed circles mark the positions of histones.

the staining patterns obtained with APpAb2 and APpAb3 were largely coincident with the staining obtained with mAb A66 against Noplp (Fig. 7, *b* and *f*). The distribution of Nop2p also appears coincident with the nucleolar protein Nsrlp (Fig. 7, *i* and *j*), which was detected by mAb C21 (Buber, T., and J. P. Aris, unpublished data). A diagnostic feature of nucleolar staining is presence of signal intensity adjacent to and partially overlapping the chromatin staining, here achieved by counter staining with the fluorescent dye DAPI (Fig. 7, *c* and *g*), which also stains mitochondrial DNA at the perimeter of the cell. This pattern is accounted for by the largely mutually exclusive distribution of nucleolar and chromatin domains within the nucleus. To better relate the Nop2p- and Noplp-staining patterns, double-labeled specimens were viewed with a confocal laser scanning imaging system. Within an optical section of ~0.25 μm, the distribution of Nop2p appears to be almost exactly like that of Noplp (Fig. 7, *k* and *l*).

The main differences between Nop2p staining and the staining of Noplp and Nsrlp is that speckles of Nop2p immunofluorescence appear in the cytoplasm and that the Nop2p nucleolar pattern is more heterogeneous. Parallel incubations with the two secondary antibodies alone did not produce visible cytoplasmic staining (data not shown). The faint cytoplasmic staining is consistent with the detection of a small amount of Nop2p in membrane-containing subcellular fractions by immunoblotting (Fig. 6 *A*). The heterogeneous-staining pattern suggests localization of Nop2p to a nucleolar substructure. These differences aside, the majority of Nop2p is clearly localized to the nucleolus in yeast.

Overexpression of Nop2p Results in Altered Nucleolar Ultrastructure

In an attempt to identify a phenotype associated with *NOP2*, we generated an overexpression mutant strain. Strain YJPA1 harboring the plasmid pEdB10 (Table I) was grown under inducing conditions essentially identical to that for Fig. 5, and cells were viewed by EM. To estimate the overexpression of Nop2p, SDS-PAGE sample size was adjusted to give immunoblotting signals of the same intensity from overproducing and control cell lysates. We estimate that the level of Nop2p after 3 h of induction is at least 20-fold increased compared to the YEp51 control (data not shown).

Interestingly, most cells from a culture overproducing Nop2p contained a nucleolus with altered morphology. The most striking aberrant morphology was one in which the nucleolus had separated from the nuclear envelope and had become rounded in cross-section (Fig. 8, *a* and *b*). Rounded nucleoli often contained an area of reduced staining, giving an appearance more like that typical of the nucleolus of higher eukaryotes. Nucleoli that were not rounded usually appeared more fragmented, and less closely associated with the nuclear envelope (Fig. 8 *c*). Nucleoli in cells harboring

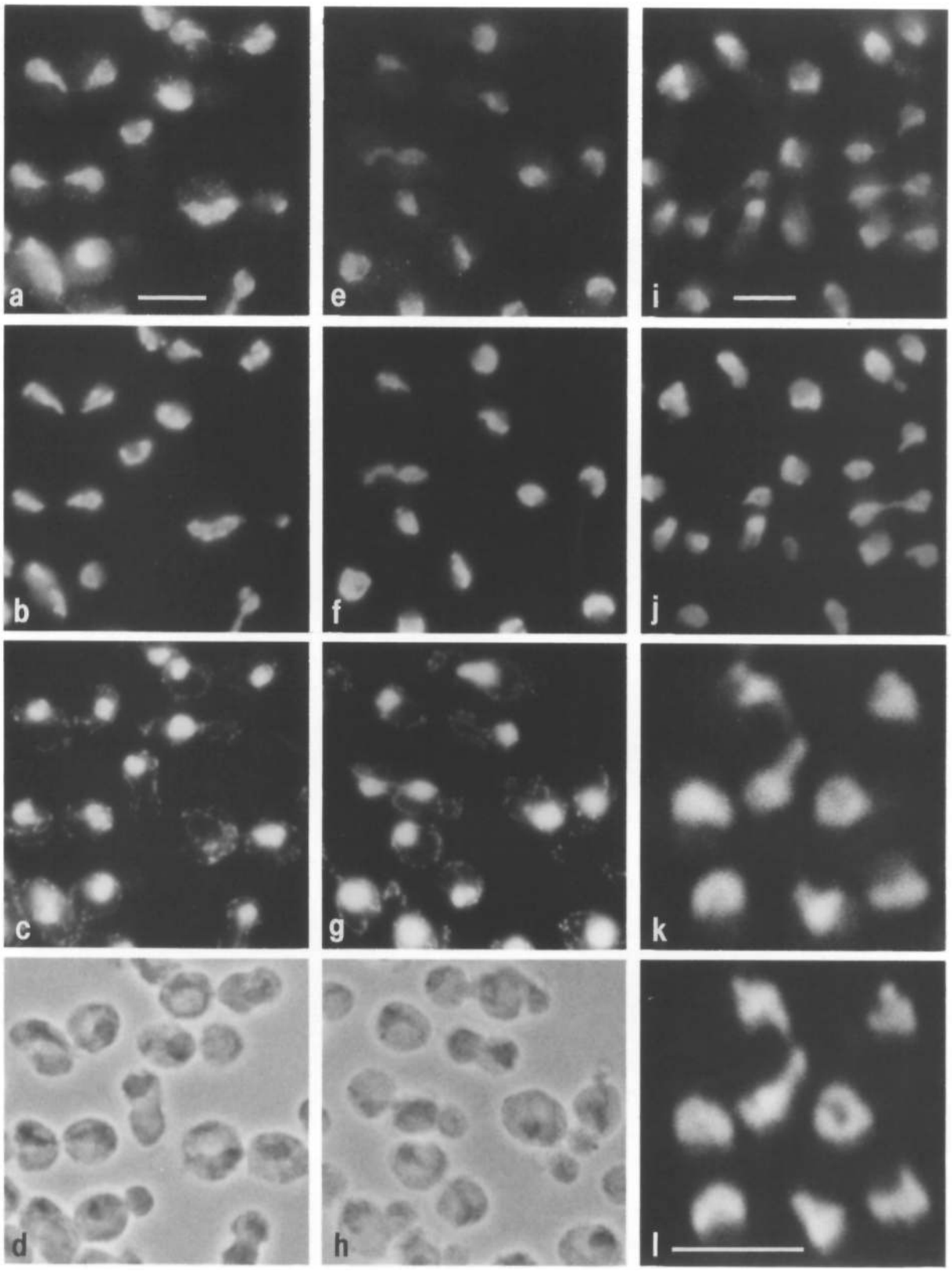

Figure 7. Immunofluorescence localization of Nop2p. Micrographs are organized in a top-to-bottom fashion. The same fields of yeast cells are shown in *a–d*, *e–h*, *i–j*, or *k–l*. Staining of Nop2p is shown in *a*, *e*, *i*, and *k*. Staining of Noplp is shown in *b*, *f*, and *l*. *j* shows staining of Nsrlp. DAPI staining (*c* and *g*) and phase contrast (*d* and *h*) images are also presented. Affinity purified polyclonal antipeptide antibodies APpAb2 (*e*) or APpAb3 (*a*, *i*, and *k*), and fluorescein-conjugated secondary antibody were used. Staining of Noplp (*b*, *f*, and *i*) or Nsrlp (*j*) was visualized with rhodamine-conjugated secondary antibody. Confocal images (*k* and *l*) are shown at a higher magnification. Bars, 1 μm.

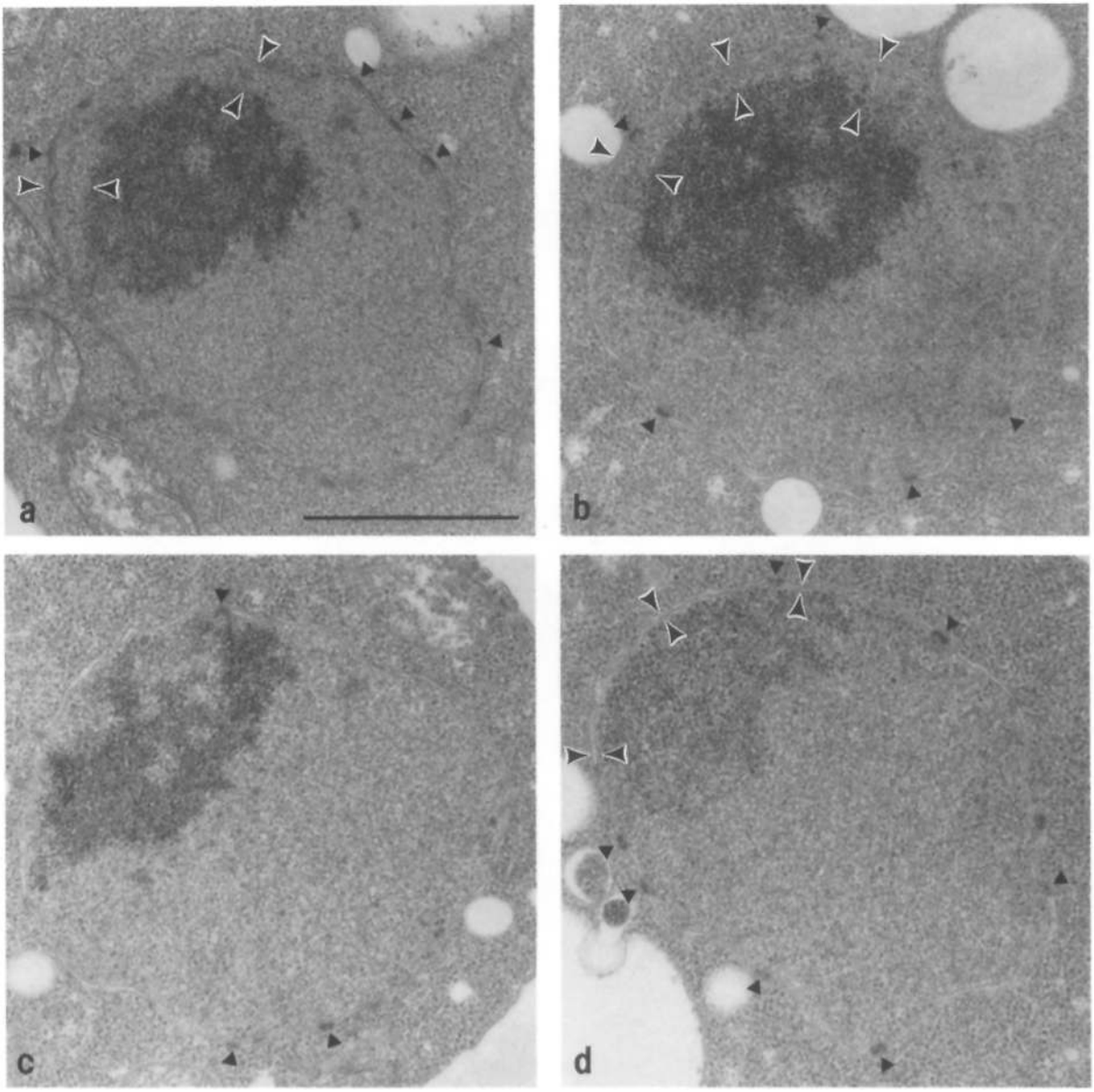

Figure 8. Overexpression of Nop2p induces ultrastructural changes in the nucleolus. Yeast YJPA1 harboring pEdB10 (*GAL::NOP2*, 2 μ ori) or YEp51 were grown in galactose-containing medium for 3 h as described for Fig. 5. Under these conditions, Nop2p expression increases at least 20-fold (see Results). Yeast were prepared for electron microscopy using standard methods. Small arrowheads designate nuclear pore complexes. Large arrowheads mark the proximity of the nucleolus to the nuclear envelope. (*A–C*) Micrographs of cells from cultures overexpressing Nop2p contain nucleoli that are typically rounded, separated from the nuclear envelope, or fragmented in appearance. (*D*) Micrograph of a cell containing YEp51, which does not overexpress Nop2p. Bar, 1 μm.

a control plasmid, YEp51, did not exhibit any unusual features, and looked normal in appearance (Fig. 8 *d*). Specifically, in control cells, nucleoli were found in close proximity to the nuclear envelope, and compact in appearance.

Although cells overproducing Nop2p contained altered nucleoli, we did not detect significant differences in growth rate between control and overproducing cultures. Growth rates of control and overproducing cultures were compared in galactose-containing medium as follows: over the 3-h induction time course; over 2 d of continuous culture; at 16°C, 25°C, and 37°C; during recovery from, and entrance into stationary phase (data not shown). A small difference was observed on a reproducible basis: the overproducing strain attained saturation at an OD_{600} value 5% less than that of control. Cell morphology was also examined without noticeable differences. Ribosome subunit synthesis was analyzed using polysome gradient profiles (Baim et al., 1985). No significant differences were observed in the 40 S, 60 S, 80 S, or polysome peaks, or ratios between peaks, during

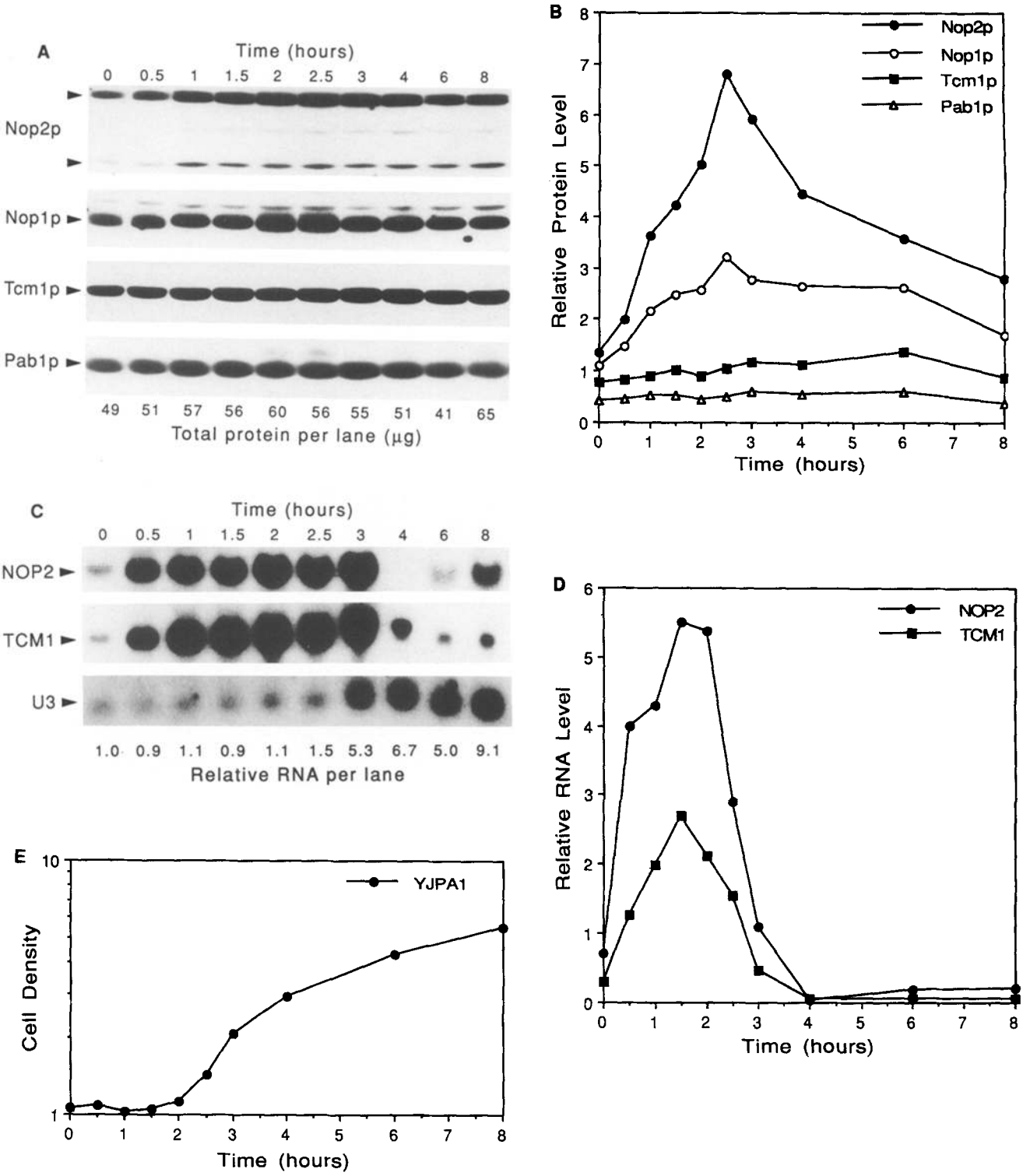

Figure 9. NOP2 expression during the onset of growth. (*A*) Immunoblotting was performed to measure Nop2p levels in total yeast cell lysates. Portions of autoradiograms show the amounts of Nop2p, Noplp, Tcmlp (*L3*), or Pablp at different times after addition of fresh YPD medium to a starter culture of YJPA1. The total amount of protein loaded per lane is indicated. (*B*) The levels of Nop2p, Noplp, Tcmlp (*L3*), or Pablp during growth onset were quantitated using ^{125}I-protein A and analysis with a PhosphorImager. Ratios of the amount of radioactivity per μg of protein loaded per gel lane are plotted in arbitrary units. (*C*) Northern blotting reveals the relative amounts of *NOP2* mRNA in total cell lysates. Portions of autoradiograms show the amounts of *NOP2*, *TCM1*, or U3 RNAs. The amount of U3 snRNA was normalized to the zero hour time point to give a measure of relative RNA per lane. (*D*) mRNA levels were quantitated using a PhosphorImager, and the ratio of the radioactivity value per U3 RNA value serves as an arbitrary unit. (*E*) Cell densities (per ml × 10^7) at each time point were determined using a hemocytometer.

Nop2p overexpression (data not shown). Immunoblotting was used to confirm Nop2p overproduction in all cultures used for comparisons.

Protein and mRNA Levels during the Onset of Growth

Due to the homology with p120, we were interested in investigating *NOP2* expression during the onset of cell growth. For this, *NOP2* protein and mRNA levels were measured during the transition from stationary phase growth arrest to logarithmic growth. A routine procedure for these measurements was developed to standardize our growth experiments (see Materials and Methods).

The relative amount of Nop2p within cells undergoes a large increase as yeast prepare to divide. Nop2p levels begin to increase by 30 min after initiation of growth, although cell division commences after ~2 h (Fig. 9, *A*, *B*, and *E*). The peak in Nop2p abundance occurs at 2.5 h, and represents a fivefold increase in Nop2p level. The growth rate peaks at 2.5 h, and by 3 h the growth rate slows, and is paralleled by a decrease in the level of Nop2p. To investigate the possibility that this pattern of expression is typical of nucleolar proteins in general, we examined the levels of the nucleolar protein Noplp. The abundance of Noplp shows a less pronounced increase with cell growth rate, undergoing a threefold increase over 2.5 h. Furthermore, the levels of Noplp remain little changed between 1.5 and 6 h. The levels of the ribosomal protein L3 (Tcmlp) and the cytoplasmic polyadenylated RNA-binding protein Pablp (Adam et al., 1986) were also determined for control purposes. The levels of L3 (Tcmlp) and Pablp show only minimal fluctuation with change in growth rate, increasing ~50% over 6 h.

Both L3 (Tcmlp) and Pablp are abundant cellular proteins, and may not exhibit dramatic changes in level because of their relative abundance. Because of this, the measurement of *TCM1* expression, and its comparison to the expression of *NOP2*, requires an analysis of transcriptional activity. An analysis of *TCM1* mRNA levels is particularly relevant because the *TCM1* gene product may be considered a "substrate" for nucleolar function. For these experiments, we have taken as an internal standard the amount of the U3 snRNA, which is thought to be present at relatively constant levels during the yeast growth cycle. Northern blot analyses indicate that *NOP2* mRNA levels increase sharply during the first 30 min of growth, and peak at 1.5–2.0 h (Fig. 9, *C* and *D*). *TCM1* mRNA levels show an increase that parallels *NOP2* mRNA. *NOP2* and *TCM1* mRNA levels drop precipitously by 2 h of growth, a time preceding cell division and log phase growth. Interestingly, this decrease anticipates the reduction in growth rate that begins at the 3-h time point, and the growth arrest that occurs much later.

Discussion

Yeast *NOP2* encodes a protein with homology to the nucleolar protein p120 found in human and mouse. Approximately half of Nop2p exhibits high (~67%) amino acid sequence identity with p120. The significant degree of evolutionary conservation of primary structure between Nop2p and p120 suggests the conservation of an important function in eukaryotic cells. Cell fractionation, nuclear fractionation, and immunofluorescence localization results indicate that the majority of Nop2p resides in the nucleolus. To our knowledge, Nop2p is the second example of a nucleolar protein that appears to be conserved from yeast to human. The nucleolar protein Noplp is also evolutionarily conserved from yeast to human (Aris and Blobel, 1991*a*; Jansen et al., 1991).

The amino terminal portion of Nop2p is less similar to human p120 than the middle and carboxy-terminal regions. Likewise, the corresponding amino-terminal portion of mouse p120 exhibits the lowest sequence similarity to human p120 (Valdez et al., 1992). The amino-terminal regions of Nop2p, human p120, and mouse p120 are similar insofar as they contain clusters of acidic and basic amino acids. Stretches of Nop2p rich in charged residues exhibit modest similarity with a number of other nucleolar proteins, including yeast Nsrlp (Lee et al., 1991), vertebrate NO38/B23 (Schmidt-Zachmann et al., 1987), and nucleolin/C23 (Lapeyre et al., 1987). At present, the function(s) of these charge clustered regions remain(s) unclear. Charge clusters in nucleolin are thought to play a role in regulating chromosomal condensation via an interaction with histone H1 (Kharrat et al., 1991), and have been shown to be responsible for binding silver ion in nuclear specific silver stains (Roussel et al., 1992), but not for nucleic acid binding (Ghisolfi et al., 1992). Negative charge clusters in certain nucleolar proteins might interact with the positively charged side chains found in unipartite nuclear localization sequences (Meier and Blobel, 1990; Lee et al., 1991; Xue et al., 1993).

Yeast cells overexpressing Nop2p contain nucleoli with altered morphology. Many nucleoli appear rounded or fragmented, and more separated from the nuclear envelope. This observation could be accounted for by either direct or indirect effects of Nop2p on nucleolar ultrastructure. Effects on nucleolar morphology, including an unraveling of nucleolar structure, are also observed in human tumor cells after inhibition of p120 synthesis (Perlaky et al., 1993). The noticeable separation between the nucleolus and the nuclear envelope in cells overexpressing Nop2p raises questions about the organization of the nucleolus. A connection between the nucleolus and nuclear envelope is present in many eukaryotic cell types, and in yeast, this association persists during "closed" mitosis. This raises the question: what role does this association play in ribosome synthesis, nucleolar function, or nucleolar biogenesis?

Nop2p synthesis increases with the onset of growth in yeast. This increase is not unexpected considering that the output of ribosome subunits from the nucleolus must increase if cell growth is to commence and continue. In addition to the upregulation of *NOP2*, other genes required for ribosome synthesis are likewise upregulated during the onset of growth. The levels of the mRNA for ribosomal protein L3 increase with the same kinetics as *NOP2* mRNA. Unfortunately, the expression of other nucleolar proteins during the growth cycle in yeast has not been examined, which obviates comparison with Nop2p. In the case of p120, expression increases dramatically during initiation of growth. p120 expression is upregulated in proliferating cells and in transformed cells, and is subject to regulation during the cell cycle, with p120 levels increasing in mid-G_1, and peaking in S phase (Freeman et al., 1988; Freeman and Busch, 1991; Fonagy et al., 1992, 1993; Valdez et al., 1992). Interestingly, Nop2p is also downregulated a significant length of time before reduction in growth rate and eventual growth ar-

rest. This suggests that *NOP2* is regulated by a mechanism sensitive to the number of divisions a cell will execute before growth arrest. This aspect of *NOP2* regulation is consistent with recent studies of ribosome biogenesis in yeast, which indicates that ribosome synthesis is regulated on the basis of the cell's estimate of the potential for growth (Ju and Warner, 1994). In general, changes in *NOP2* expression appear to be tied to the changes in growth rate that accompany progression through the yeast growth cycle.

Although the relationship between *NOP2* expression and the yeast growth cycle is only correlative at this time, it is consistent with the regulation of nucleolar function by a mechanism sensitive to the growth state of the cell. Considering the number of gene products that participate in the assembly of ribosome, and the metabolic cost that ensues, it seems likely that nucleolar function is regulated on the basis of the position of the cell in the growth cycle. Aberrations in nucleolar regulation or function may have consequences for cell growth, and certain nucleolar proteins have been implicated in transformation and tumorigenesis. For example, the zinc finger-containing nucleolar protein, LYAR, has been identified from a mouse T cell leukemia line as a novel nucleolar oncoprotein (Su et al., 1993). This raises the question: what role does the nucleolus play in the coordination ribosome synthesis with cell growth? Further investigation of Nop2p function in yeast may provide answers to this and other questions pertaining to nucleolar structure and function.

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