Characterization of Nuclear Polyadenylated RNA-binding Proteins in *Saccharomyces cerevisiae*

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Abstract. To study the functions of heterogeneous nuclear ribonucleoproteins (hnRNPs), we have characterized nuclear polyadenylated RNA-binding (Nab) proteins from *Saccharomyces cerevisiae.* Nablp, Nab2p, and Nab3p were isolated by a method which uses UV light to cross-link proteins directly bound to poly(A)$^+$ RNA in vivo. We have previously characterized Nab2p, and demonstrated that it is structurally related to human hnRNPs. Here we report that Nablp is identical to the Npl3p/Nop3p protein recently implicated in both nucleocytoplasmic protein shuttling and pre-rRNA processing, and characterize a new nuclear polyadenylated RNA-binding protein, Nab3p. The intranuclear distributions of the Nab proteins were analyzed by three-dimensional immunofluorescence optical microscopy. All three Nab proteins are predominantly localized within the nucleoplasm in a pattern similar to the distribution of hnRNPs in human cells. The *NAB3* gene is essential for cell viability and encodes an acidic ribonucleoprotein. Loss of Nab3p by growth of a *GAL::nab3* mutant strain in glucose results in a decrease in the amount of mature *ACT1*, *CYH2*, and *TPI1* mRNAs, a concomitant accumulation of unspliced *ACT1* pre-mRNA, and an increase in the ratio of unspliced *CYH2* pre-mRNA to mRNA. These results suggest that the Nab proteins may be required for packaging pre-mRNAs into ribonucleoprotein structures amenable to efficient nuclear RNA processing.

HETEROGENEOUS nuclear RNAs (hnRNAs)[1] are the products of RNA polymerase II transcription, and include polyadenylated and nonpolyadenylated pre-mRNAs and mRNAs as well as several uncharacterized RNAs (Dreyfuss et al., 1993). An array of nuclear factors bind to hnRNAs during transcription, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and small nuclear RNP (snRNP) particles (Amero et al., 1992; Matunis et al., 1993). Numerous studies have established the roles of snRNP particles in a variety of nuclear processes such as pre-mRNA splicing (Green, 1991; Guthrie, 1991; Ruby and Abelson, 1991; Baserga and Steitz, 1993). In contrast, the functions of hnRNPs have remained unclear. HnRNPs are nuclear RNA-binding proteins whose primary and stable RNA-binding site is hnRNA. Current ideas about the functional roles of hnRNPs in pre-mRNA processing have evolved from a variety of in vitro biochemical analyses. These proposed functions include nearly all of the known steps in nuclear mRNA maturation such as pre-mRNA packaging (Chung and Wooley, 1986; Dreyfuss, 1986), constitutive and alternative splicing (Choi et al., 1986; Sierakowska et al., 1986; Mayeda and Krainer, 1992), polyadenylation (Wilusz and Shenk, 1990), and nucleocytoplasmic mRNA shuttling (Piñol-Roma and Dreyfuss, 1992).

Isolation of 30–40 S hnRNP monoparticles by mild treatment of vertebrate cell nucleoplasm with RNases followed by sucrose gradient sedimentation initially indicated that six "core" proteins (A1, A2, B1, B2, C1, and C2) were associated with hnRNPs in a nonsequence-specific manner (Chung and Wooley, 1986; Dreyfuss, 1986). These six proteins are abundant within the nucleus, and are associated with hnRNPs in a manner which makes them relatively resistant to digestion with RNases. These observations led to the suggestion that hnRNPs might package hnRNAs to generate "ribonucleosomes" in a fashion analogous to the packaging of DNA by histones to form nucleosomes (Chung and Wooley, 1986). Subsequent work demonstrated that much larger hnRNP complexes (40 to ≥200 S) can be isolated either by sucrose gradient sedimentation (Sperling et al., 1985; Spann et al., 1989) or by immunopurification (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988) if nucleoplasm is not treated with

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1. *Abbreviations used in this paper*: aa, amino acid; hnRNA and RNP, heterogeneous nuclear RNA and RNP; MBP, maltose-binding protein; Nab, nuclear polyadenylated RNA-binding; nt, nucleotide; rt, room temperature; snRNP, small nuclear RNP.

RNases. Immunopurified hnRNP complexes are composed of more than twenty abundant hnRNA-binding proteins. In contrast to the ribonucleosome model, more recent in vitro analyses demonstrated that many of these hnRNPs bind to RNAs with sequence preference (Swanson and Dreyfuss, 1988*a*, *b*), and different arrays of hnRNPs bind to different pre-mRNAs (Bennett et al., 1992*b*). Moreover, the amphibian hnRNP L protein (Piñol-Roma et al., 1989) and several of the *Drosophila* hrp (hnRNP) proteins (Matunis et al., 1993) associate with nascent RNA polymerase II transcripts in a transcript-specific manner.

HnRNPs may also play an active role in the regulation of particular steps in the pre-mRNA processing pathway in addition to their possible role in pre-mRNA packaging. Early studies indicated that the human hnRNP A, B, and C proteins may be essential factors for constitutive splicing (Choi et al., 1987; Sierakowska et al., 1986). However, hnRNPs do not appear to be components of prespliceosomes or spliceosomes assembled in vitro although they may function at an earlier step in the assembly pathway (Bennett et al., 1992*a*). The observation that the relative concentrations of SF2/ASF and hnRNP A1 appear to influence splice site choice in vitro has led to a proposed role for hnRNPs in alternative or regulated pre-mRNA splicing (Mayeda and Krainer, 1992). Mutations in the conserved AG dinucleotide at the 3′ acceptor splice site have been shown to influence the binding of the A1 protein to the 3′ end of the intron indicating that this hnRNP may bind to subregions of pre-mRNAs in a sequence-specific manner (Swanson and Dreyfuss, 1988*b*). SF2 has been recently shown to be an exonic splicing enhancer binding protein that binds to a purine-rich splicing enhancer in the last exon of bovine growth hormone

Table I. Yeast Strains and Plasmids

Strain/Designation	Genotype/Description	Source
Yeast Strains		
BJ926	*MATa/MATα prb1-1122/prb1-1122 prc1-407/prc1-407 pep4-3/pep4-3 can1/can1 gal2/gal2 his1/HIS1 TRP1/trp1*	YGSC*
YJA501	*MATa/MATα leu2Δ2/leu2Δ2 ura3-52/ura3-52*	Anderson et al., 1993*b*
YSW101	YJA501 *NAB3/nab3Δ2::LEU2*	This study
YSW102	YSW101 *pURA3CEN4NAB3* (pNAB3.14)	
YSW102-1A	*MATα leu2Δ2 ura3-52 nab3Δ2::LEU2 pURA3CEN4NAB3* (pNAB3.14)	
YSW102-1B	*MATa leu2Δ2 ura3-52 NAB3*	"
YSW109	*MATa/MATα leu2Δ2/leu2Δ2 ura3-52/ura3-52 NAB3/nab3Δ2::LEU2 pURA3CEN6GAL1,10NAB3* (pNAB3.18)	"
YSW109-1A	*MATα leu2Δ2 ura3-52 nab3Δ2::LEU2 pURA3CEN6GAL1,10NAB3* (pNAB3.18)	"
YSW109-1B	*MATα leu2Δ2 ura3-52 NAB3*	"
Plasmids‡		
pNAB3.1	1.9-kb EcoRI fragment (starting at nucleotide 408 of the *NAB3* gene) isolated by expression screening of a λ gt11 genomic DNA library and subcloning into EcoRI cut pSP72 (Promega, Madison, WI).	
pNAB3.3	genomic 3.8-kb fragment containing the entire coding region of the *NAB3* gene cloned into EcoRI cut pSP72.	
pNAB3.7	encodes amino acids 117-803 of the NAB3 protein fused in-frame to the maltose-binding protein generated by subcloning the EcoRI fragment from pNAB3.1 into EcoRI cut pMAL-c2 (New England Biolabs, Beverly, MA).	
pNAB3.12	*NAB3* knockout construct generated by subcloning the EcoRI fragment from pNAB3.3, which was blunt-ended, subcloned into PvuII/EcoRV cut pSP72, digested with HindIII, blunt-ended, and the blunt-ended 2.0-kb XhoI/SalI fragment, containing *LEU2* from YEp13, inserted.	
pNAB3.14	YCp50 8.0-kb clone containing the entire *NAB3* gene.	
pNAB3.15	the NsiI fragment from pNAB3.14 was subcloned into PstI cut pSP73 (Promega).	
pNAB3.16	the XhoI/BamHI fragment from pNAB3.15 subcloned into XhoI/BamHI cut pRS314 (Sikorski and Hieter, 1989).	
pNAB3.18	the NciI/BamHI fragment from pNAB3.15 was blunt-ended and subcloned into SmaI cut pRD53 (EcoRI/BamHI fragment containing the *GAL1,10* promoter region subcloned into the SpeI/BamHI site of pRS316 [Sikorski and Hieter, 1989], gift of R. J. Deshaies, University of California, San Francisco, CA).	

* Yeast Genetic Stock Center (Berkeley, CA)
‡ *NAB3* nucleotide numbers refer to Fig. 6.

(Sun et al., 1993). Therefore, it is possible that region and/or sequence-specific binding of these two types of pre-mRNA binding proteins modulates splice-site choice in vivo in conjunction with other *trans*-acting factors, including snRNP particles. Finally, although hnRNPs have been hypothesized to function in polyadenylation (Wilusz and Shenk, 1990) and nucleocytoplasmic shuttling of mRNAs (Piñol and Dreyfuss, 1992), there is no evidence for an essential role of hnRNPs in these functions.

As an alternative to studying hnRNP function using in vitro assays, our goal has been to characterize hnRNP functions in an organism amenable to genetic manipulation. To this end, we have described the isolation of hnRNP-like proteins from *S. cerevisiae*, the nuclear polyadenylated RNA-binding (Nab) proteins (Anderson et al., 1993*b*). All of the Nab proteins are directly associated with nuclear poly(A)$^+$ RNAs in vivo. Nab2p is essential for cell viability, and is required for both correct polyadenylation of pre-mRNAs and mRNA export from the nucleus (Anderson et al., 1993*b*; Anderson, J. T., and M. S. Swanson, unpublished data). We now report the isolation and characterization of Nablp and Nab3p, and study the subnuclear distribution of all three Nab proteins by three-dimensional fluorescence optical microscopy. Our studies show that both Nab2p and Nab3p are essential for growth in yeast, and are required for several different steps in the biogenesis of mRNA within the nucleus.

Materials and Methods

Yeast Strains, Plasmids, and Genetic Manipulations

Table I lists the yeast strains and plasmids used in this study. BJ926 was used for UV cross-linking, preparation of genomic DNA and polyadenylated RNA, and cellular immunofluorescence. *NAB3* disruptions were performed by transforming YJA501 with pNAB3.12 to generate YSW101 (see Table I). Growth experiments in both galactose and glucose with the *GAL::nab3* strain (YSW109-1A) and a sister haploid *NAB3* strain (YSW109-1B) were performed by growing overnight cultures in SG (YSW109-1A) or SG+Ura+Leu (YSW109-1B). Cultures were diluted to a starting OD_{600} (indicated in Fig. 9 *A*) in either SD (YSW109-1A) or SD+Ura+Leu (YSW109-1B). At various times after shift into glucose, aliquots containing 8×10^7 cells were centrifuged, resuspended in cold 10% trichloroacetic acid and disrupted by vigorous vortexing with glass beads, and precipitated proteins fractionated by SDS-PAGE (see below). Growth media for yeast were prepared as described (Rose et al., 1990), and all genetic manipulations were performed using standard techniques (Guthrie and Fink, 1991).

Isolation of UV Cross-linked Polyadenylated RNA-RNP Complexes

BJ926 yeast cells were grown in YPD, and UV cross-linking as well as isolation of polyadenylated RNPs were performed according to a previously described procedure (Anderson et al., 1993*b*).

Polyclonal and Monoclonal Antibody Preparation

Polyclonal antisera reactive against Nablp, Nab2p, and Nab3p were elicited by injecting BALB/c mice with UV light cross-linked polyadenylated RNPs as described previously (Anderson et al., 1993*a*, *b*). The 1E4 monoclonal antibody against Nablp was isolated by fusing spleen cells from one of the mice injected with total cross-linked polyadenylated RNPs with SP2/O myeloma cells as described (Anderson et al., 1993*b*). The resulting hybridomas were screened by both immunoblotting against cross-linked RNPs and cellular immunofluorescence to detect antibodies reactive against nuclear poly(A)$^+$ RNA-binding proteins. The 2F12 monoclonal antibody against Nab3p was prepared by injecting BALB/c mice three times with 1 μg per injection of a Nab3p-maltose-binding protein (MBP) fusion protein containing 80% of Nab3p (prepared using pNAB3.7; Table I).

Nablp Isolation and Protein Sequencing

Repeated attempts to clone the *NAB1* gene by expression screening of both yeast genomic DNA and cDNA libraries using either polyclonal antisera reactive against total crosslinked poly(A)$^+$ RNPs or the 1E4 monoclonal antibody were unsuccessful. Nablp was therefore directly purified by affinity chromatography using the 1E4 anti-Nablp monoclonal antibody, and subjected to protein microsequencing. Briefly, the 1E4 monoclonal antibody was first purified on Protein G-agarose and then covalently coupled using dimethyl pimelimidate (Immunopure Protein G IgG Orientation Kit; Pierce Chemical Co., Rockford, IL). Nablp was purified from 7–10 ml of yeast splicing extract (Cheng et al., 1990), which had been previously shown to contain relatively high levels of Nablp by immunoblotting analysis (Wilson, S. M., J. T. Anderson, and M. S. Swanson, unpublished data). During preparation of the splicing extract, and/or during affinity chromatography, smaller Nablp polypeptides of 50–55 kD, which were still reactive against 1E4, were detectable. Since protein microsequencing initially determined that the amino terminus of full-length Nablp was blocked, the two Nablp putative proteolysis products of 50 and 55 kD were subjected to NH_2-terminal sequencing (model 473A; Applied Biosystems, Foster City, CA).

NAB3 Gene Isolation and DNA Sequencing

The *NAB3* gene was isolated as an expression clone from λgt11 genomic library (Snyder et al., 1987) using a 1:300 dilution of the anti-cross-linked polyadenylated RNP antisera described above. The entire *NAB3* gene was isolated on an 8.0-kb clone from YCp50 genomic library (Yeast Genetic Stock Center, Berkeley, CA) using a 700-bp HindIII/ScaI fragment from pNAB3.1 (Table I). DNA sequences were determined for both strands of chromosomal fragments cloned into pSP72/73 using SP6/T7, or gene-specific, oligonucleotide primers (Anderson et al., 1993*b*). DNA and protein sequence information was analyzed using The University of Wisconsin Genetics Computer Group programs, and database searches were accomplished by using the BLAST network service at the National Center for Biotechnology Information (Altschul et al., 1990).

Genomic DNA and Polyadenylated RNA Blot Analyses

High molecular weight yeast genomic DNAs were isolated and fractionated in a 0.8% agarose gel as previously described (Anderson et al., 1993*b*). Both DNAs and RNAs were transferred by capillary blotting to Hybond-N$^+$ (Amersham Corp., Arlington Heights, IL), and hybridization performed for 12–14 h at 65°C in a hybridization oven (model 1000; Robbins Scientific Corp., Sunnyvale, CA) in 0.25 M Na_2HPO_4 (pH7.5)/7% SDS/1 mM EDTA/1% BSA. Blots were washed three times in 2× SSC(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH7.0])/0.1% SDS at room temperature (RT) for 15 min and twice in 0.5× SSC/0.1% SDS at 65°C for 15 min. The genomic blot hybridization probe was PvuII/EcoRI fragment (nucleotides [nt] 1750 to 2553; see Fig. 6) from *NAB3*. Polyadenylated RNAs were prepared from BJ926, fractionated on a 1.2% glyoxal agarose gel, and transferred to Hybond-N$^+$ (Amersham Corp.). Hybridizations and washes were performed as described above. Hybridization probes for Northern analysis were: (*a*) *ACT1*, either representing the complete intron (nt 663 to 955) or the intron plus second exon (nt 663 to 1396) (Gallwitz and Sures, 1980; Ng and Abelson, 1980); (*b*) *CYH2*, either for the intron (nt 61 to 507) or for the full-length gene (nt 65 to 1065) (Kaufer et al., 1983); and (*c*) *TPI1*, for the full-length gene (nt 971 to 2017) (Alber and Kawasaki, 1982).

Gel Electrophoresis and Immunoblotting

Proteins were resolved by SDS-PAGE using 12.5% (final acrylamide concentration) separation gels (Anderson et al., 1993*b*). After electrophoresis, proteins were transferred to nitrocellulose using a semi-dry electroblotter (Bio-Rad Laboratories, Hercules, CA) for 30 min at 15V. Immunoblotting was performed as described (Anderson et al., 1993*b*) using a 1:500 dilution of 1E4 (anti-Nablp), a 1:250 dilution of 2F12 (anti-Nab3p), a 1:2,000 dilution of 2B1 (anti-Pub2p), and a 1:5,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse secondary antibody. Reactive antigens were visualized by enhanced chemiluminescence (Amersham Corp.) using a film exposure of 1–30 s.

In Vitro Splicing

Cell extracts competent for in vitro splicing of pre-mRNAs were prepared

from BJ2168 cells as described previously (Lin et al., 1985). Splicing reactions were performed with [^{32}P]GTP-labeled actin pre-mRNAs prepared using T7 polymerase by transcription of a plasmid (constructed by A. Zaug, University of Colorado, Boulder, CO) containing a modified *ACT1* gene (Lin et al., 1985; Schwer and Guthrie, 1991). Splicing products were resolved on 6% sequencing gels, and visualized by autoradiography.

Depletion of Nab3p from whole cell extracts was accomplished by passing extracts in 10 mM Hepes (pH 7.0)/1.5 mM $MgCl_2$/200 mM KCl/0.5 mM DTT through a 2 ml 2F12 affinity column at 4°C followed by dialysis for 3 h against 20 mM Hepes (pH 7.0)/0.2 mM EDTA/0.5 mM DTT/50 mM KCl/20% (vol/vol) glycerol. The 2F12 affinity matrix was prepared by first purifying 2F12 from ascites fluid on a protein G-agarose column followed by covalent cross-linking of the IgG-protein G complex with dimethyl pimelimidate (Immunopure Protein G IgG Orientation Kit; Pierce Chemical Co.). The amount of Nab3p in undepleted and depleted extracts was assayed by immunoblotting. For immunoinhibition studies, 2F12 was purified on a protein G column, eluted with 0.1 M glycine-HCl (pH 2.8), immediately neutralized, and dialyzed against PBS. Cell extracts (4 μl) were treated for 10 min at 4°C with either 1, 5 or 10 μg of purified 2F12 before adding labeled precursor RNA as described previously (Schwer and Guthrie, 1991).

Three-dimensional Cellular Immunofluorescence

Preparation of BJ926 yeast cells for both direct and indirect cellular immunofluorescence was performed as described previously (Anderson et al., 1993*a*,*b*). For indirect cellular immunofluorescence, monoclonal antibodies were diluted in 3% BSA/PBS at 1:500 (1E4, 3F2, and A66), 1:250 (2F12), and 1:5,000 (1G1). Antigen-antibody complexes were detected using a 1:10 dilution of affinity-purified/subclass-specific goat anti-mouse secondary antibodies conjugated with either fluorescein (FITC), rhodamine (RITC), or Texas Red (Southern Biotechnology Associates, Inc., Birmingham, AL) (Anderson et al., 1993*a*).

Three-dimensional fluorescence optical microscopy datasets were collected and analyzed as previously described using instrumentation in the Optical Microscopy Suite of the Center for Structural Biology at the University of Florida (Gainesville, FL) as well as in the laboratories of J. W. Sedat and D. A. Agard of the Howard Hughes Medical Institute at the University of California-San Francisco (HHMI-UCSF, San Francisco, CA) (Anderson et al., 1993*a*). The microscope at the University of Florida essentially duplicates the original microscope at HHMI-UCSF, and was assembled by Applied Precision, Inc. (Seattle, WA).

Results

Nablp and Nab3p Are Associated with Nuclear Polyadenylated RNAs In Vivo

We recently described the isolation and characterization of a novel yeast nuclear pre-mRNA-binding protein, Nab2p (Anderson et al., 1993*b*). Nab2p was identified as a ribonucleoprotein that is localized to the nucleus, and which readily cross-links to polyadenylated RNAs when cells are exposed to UV light. Since our goal was to study the functions of pre-mRNA/mRNA-binding proteins in nuclear RNA processing, and the types of protein-protein and protein-RNA interactions which are important for these functions, additional Nab proteins isolated during this initial study were chosen for further characterization.

As previously described for *NAB2*, the *NAB3* gene was isolated by screening a λgt11 genomic expression library with polyclonal antisera raised against cross-linked poly(A)$^+$ RNA-RNP complexes. Briefly, genomic clones which encoded fusion proteins reactive with antisera against these cross-linked complexes were purified, plated at high density, and the fusion proteins transferred to nitrocellulose filters during induction with isopropyl-β-D-thiogalactopyranoside (IPTG). These filters were then used to affinity purify those antibodies reactive against each fusion protein (Snyder et al., 1987). The affinity-selected antibodies were assayed to determine which clones encoded proteins which not only crosslinked to poly(A)$^+$ RNA in vivo, but were also localized to the nucleus by indirect cellular immunofluorescence. The *NAB3* gene was subcloned into a MBP-fusion protein expression vector, and the Nab3p-MBP fusion protein used to immunize mice for the preparation of the anti-Nab3p monoclonal antibody 2F12.

As an alternative to direct gene isolation by expression screening using polyclonal antisera and production of monoclonal antibodies against Nab-MBP fusion proteins, one of the mice injected with cross-linked RNA-RNP complexes was used to directly prepare a bank of hybridomas (see Materials and Methods). The supernatants from these clones were then screened by indirect cellular immunofluorescence to detect antibodies which specifically reacted against nuclear proteins. The 1E4 monoclonal antibody against Nablp was prepared from one of these clones.

The 2F12 monoclonal antibody against Nab3p recognized a protein which migrated at ~120 kD by SDS-PAGE, and that cross-linked to poly(A)$^+$ RNAs in vivo (Fig. 1, *NAB3*). The 1E4 monoclonal antibody reacted against a 60 kD yeast nuclear protein, Nablp, that also crosslinked efficiently to poly(A)$^+$ RNAs in vivo (Fig. 1, *NAB1*). In contrast to these two Nab proteins, Pub2p, which is a 60 S large ribosomal subunit protein that cross-links to a non-poly(A)$^+$ RNA (Anderson et al., 1993*a*), did not detectably cross-link to poly (A)$^+$ RNAs (Fig. 1, *PUB2*).

The intracellular distributions of all three Nab proteins were investigated by three-dimensional fluorescence optical microscopy. Comparison of the distribution of Nablp (Fig. 2 *a*) with chromosomal DNA, as detected by DAPI staining (Fig. 2 *b*), and the cytoplasmic polyadenylate tail-binding protein (Fig. 2 *c*, *Pablp*) demonstrated that Nablp was primarily detectable within the nucleus. The intranuclear dis-

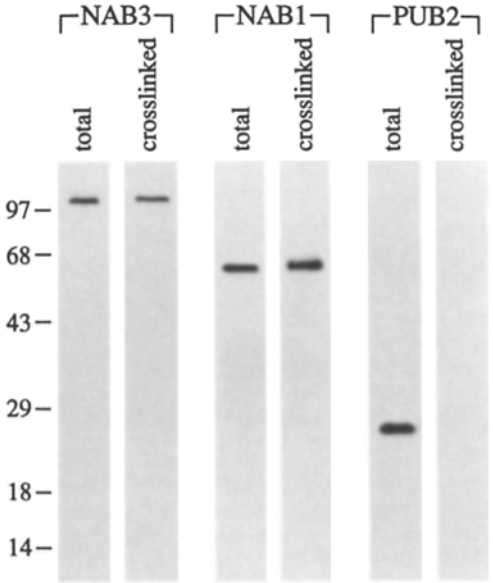

Figure 1. Nablp and Nab3p are associated with polyadenylated RNAs in vivo. Immunoblot analysis of total cell extracts (*total* lanes) or purified polyadenylated RNA-RNP crosslinked complexes (*cross-linked* lanes). Blots were probed with monoclonal antibodies against Nab3p, Nablp, and Pub2p.

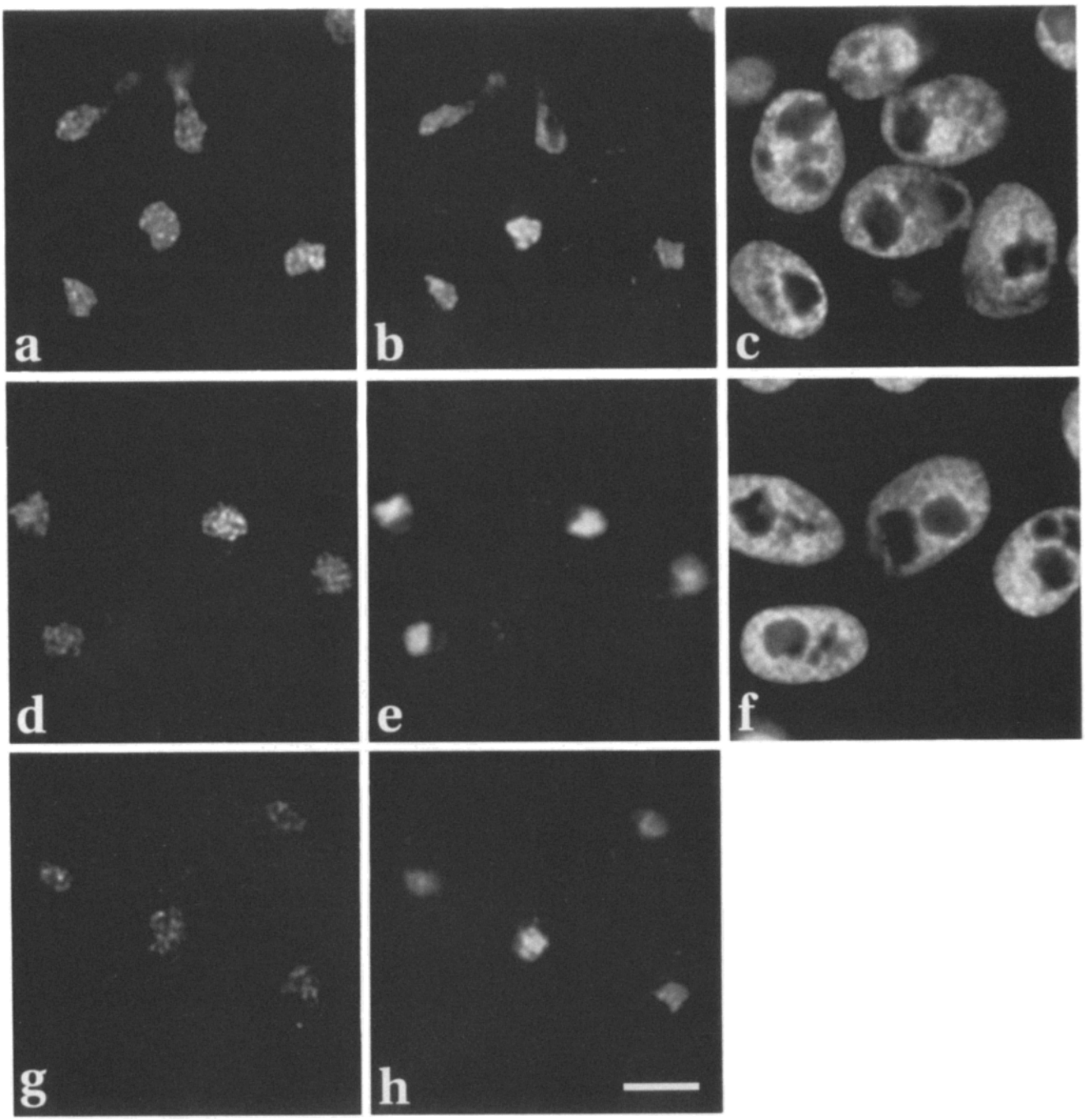

Figure 2. Nablp, Nab2p, and Nab3p are all localized to the nucleus. The subcellular distributions of all three Nab proteins were determined by three-dimensional fluorescence optical microscopy using monoclonal antibodies against Nablp (*a*), Nab2p (*d*), and Nab3p (*g*). Also shown for each set of cells is the distribution of DNA, visualized with DAPI (*b*, *e*, and *h*), and the cytoplasmic polyadenylate tail-binding protein, Pablp (*c* and *f*) for the Nablp and Nab2p data sets. The distribution of Pablp is not shown for the Nab3p data set (*g* and *h*) since the intense cytoplasmic fluorescence for the very abundant Pablp protein contributes to the much weaker cytoplasmic signal for Nab3p. Antigens were localized using affinity-purified and subclass-specific secondary antibodies conjugated with either rhodamine or Texas red (Nab proteins) or fluorescein (Pablp). Bar, 4 μm.

tribution of Nab2p (Fig. 2 *d*) was also compared with chromosomal DNA (Fig. 2 *e*) and Pablp (Fig. 2 *f*), and was very similar to Nablp indicating that these proteins have a similar distribution within the yeast nucleus. Finally, Nab3p also appeared to be predominantly nuclear (Fig. 2, *g* and *h*). Weak cytoplasmic staining was also detectable for both Nab2p and Nab3p. Immunoblot analysis indicated that Nab3p was approximately tenfold less abundant than Nablp (Wilson, S. M., J. T. Anderson, and M. S. Swanson, unpublished data), which prohibited double-staining with anti-Pablp antibodies due to the extremely strong cytoplasmic Pablp immunofluorescence signal.

A variety of studies using both light and electron microscopy have demonstrated that the nucleolus occupies a discrete crescent-shaped subregion within the yeast nucleus (Sillevis Smitt et al., 1973; Aris and Blobel, 1988). Since it was conceivable that the Nab proteins might also function in nucleolar pre-rRNA metabolism, their intranuclear distribution was compared to an abundant nucleolar protein, Noplp (Aris and Blobel, 1988). Noplp, which is also referred to as yeast fibrillarin, is one of the proteins associated with U3 snRNP involved in nucleolar pre-rRNA processing, and has been shown to be localized throughout the nucleolus (Aris and Blobel, 1988). Comparison of the nuclear distributions of Nablp (Fig. 3 *a*), Nab2p (Fig. 3 *d*), Nab3p (Fig. 3 *g*) with the intranucleolar localization of Noplp (Figs. 3, *b*, *e*, and *h*, compare the distribution of Noplp with DAPI staining) suggested that the Nab and Nop proteins were localized differently within the nucleus. In cells doubly stained for Nablp/Noplp (Fig. 3 *c*), Nab2p/Noplp (Fig. 3 *f*), and Nab3p/Noplp (Fig. 3 *i*), the nuclear area occupied by the Nab proteins (*red* staining) appeared to be predominantly separate from the Noplp nucleolar region (*green* staining) although apparent colocalization was detectable (*yellow* staining). To extend this observation, three-dimensional reconstructions of the subnuclear localization of Nab2p and Noplp were produced using both volume- and surface-rendering methods (Paddy et al., 1990). These analyses indicated that the distribution of Noplp was highly reticulated and interwoven with Nab2p staining along the surfaces where the two proteins were juxtaposed (Paddy, M. R., unpublished data). None of the Nab proteins specifically colocalized with Noplp, indicating that it is unlikely they are appreciably distributed within the nucleolus. In summary, the RNA cross-linking and intracellular distribution studies demonstrate that Nablp, Nab2p, and Nab3p are authentic nuclear polyadenylated RNA-binding proteins.

Nablp Is Identical to Npl3p/Nop3p

Repeated attempts, using several different protocols, to isolate the gene encoding Nablp by immunoscreening two different λgt11 genomic expression libraries, as well as a λZAP cDNA expression library (Anderson et al., 1993*b*), were unsuccessful. We therefore decided to first purify the Nablp protein by affinity chromatography using the 1E4 monoclonal antibody, determine the amino acid sequence of several different peptides by protein microsequencing, and isolate the *NABI* gene by hybridization with degenerate oligonucleotides if the protein had not been previously characterized. Preliminary studies indicated that the amino terminus of the full-length 60-kD protein was blocked. Fortunately, the isolation of Nablp from yeast splicing extracts (Cheng et al., 1990) resulted in partial proteolysis products of 50 and 55 kD which were recognized by 1E4. NH_2-terminal sequencing of both of these peptides (Fig. 4, *bold underlines*) demonstrated that Nablp was identical to the previously characterized Npl3p/Nop3p protein (Bossie et al., 1992; Russell and Tollervey, 1992). Npl3p/Nop3p has been suggested to be both a factor involved in nuclear import of proteins (Bossie et al., 1992), and a nucleolar protein that plays a role in pre-rRNA processing (Russell and Tollervey, 1992). Our results demonstrated that Nablp was bound to nuclear poly(A)$^+$ RNA in vivo and was primarily localized within nonnucleolar regions of the nucleus suggesting that it may function in pre-mRNA processing.

Nab3p Is An Acidic Nuclear Ribonucleoprotein with an RNA-binding Domain Related to the hnRNP C Proteins

The full-length *NAB3* gene (Fig. 5 *A*) was isolated from a YCp50 genomic DNA library by hybridization using pNAB3.1 (Table I). *NAB3* appeared to be a single-copy gene

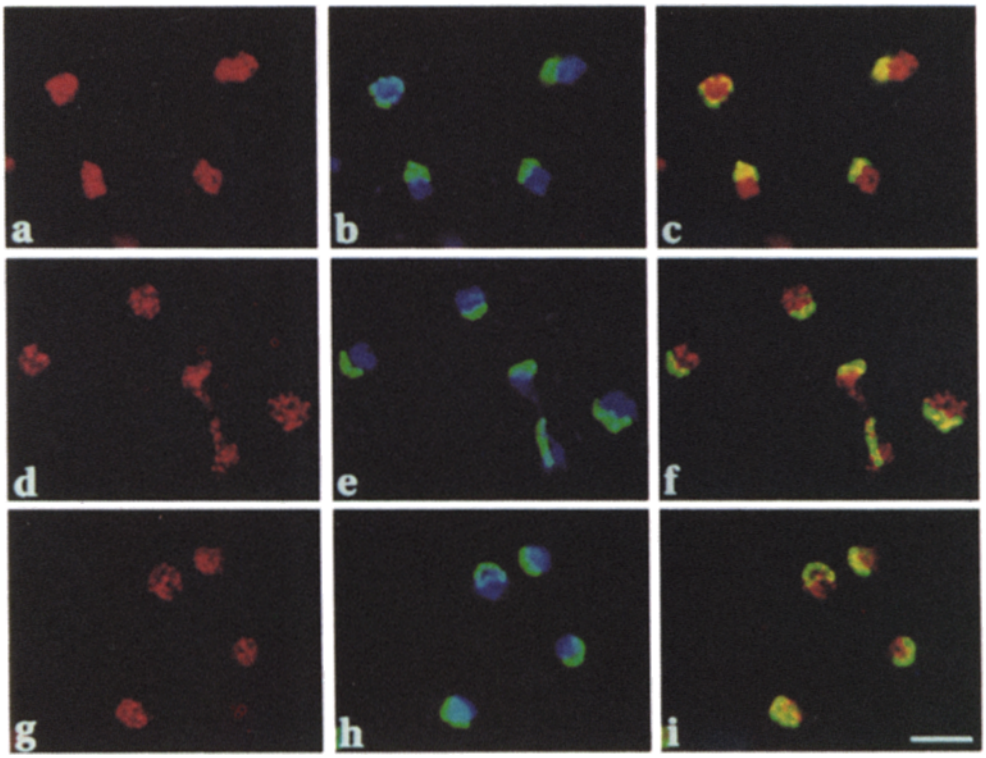

Figure 3. The subnuclear distribution of the Nab proteins is significantly different from fibrillarin, a major nucleolar protein. Three-dimensional fluorescence optical microscopy was performed as described in the legend to Fig. 2. The distribution of the Nablp (*a*), Nab2p (*d*), and Nab3p (*g*) proteins are shown in red. Also shown are two component immunofluorescence images comparing the distribution of DNA by DAPI staining (*blue*) with Noplp (*green*) (*b*, *e*, and *h*), and the distribution of the Nablp (*c*), Nab2p (*f*), and Nab3p (*i*) proteins in red with Noplp in green. Bar, 4 μm.

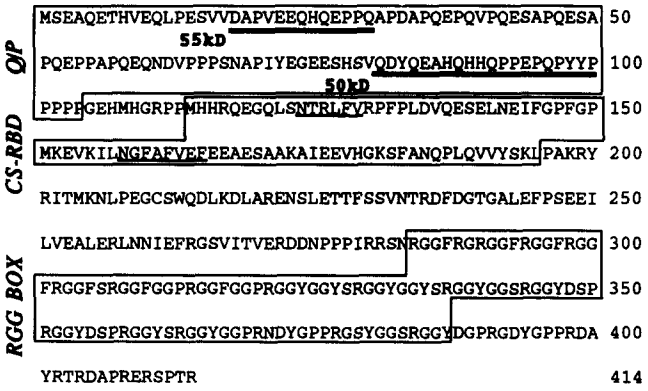

Figure 4. Nablp is identical to Npl3p/Nop3p. Nablp was isolated by affinity chromatography using the 1E4 monoclonal antibody and peptides, probably resulting from proteolysis during isolation, were subjected to protein microsequencing. The amino termini of these two polypeptides (labeled 55 and 50 kD) were sequenced, and determined to be identical to the published sequences of Nop3p (Russell and Tollervey, 1992) and Npl3p (Bossie et al., 1992). Indicated on the previously published Npl3p/Nop3p sequence are the two RNA-binding motifs, the ribonucleoprotein consensus sequence RNA-binding domain (*CS-RBD*) and the RGG box, and a region at the amino terminus rich in glutamine and proline (*Q/P*).

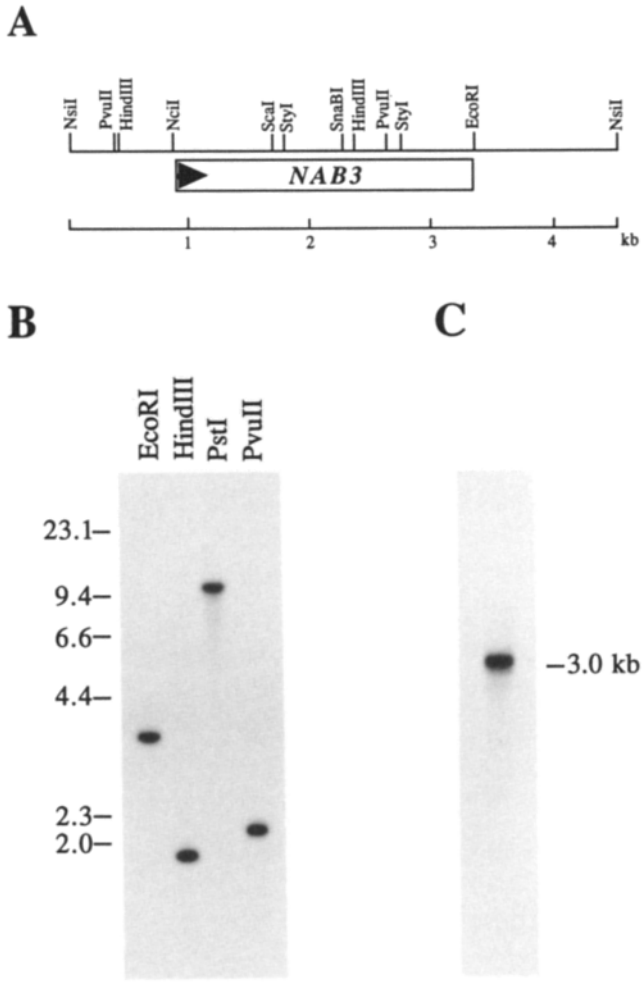

Figure 5. Characterization of the *NAB3* gene. (*A*) Restriction map of the 4.3-kb genomic clone (pNAB3.15) containing the *NAB3* gene. The arrow within the open box marked *NAB3* indicates the direction of transcription. (*B*) Genomic DNA blot analysis using the PvuII/EcoRI 800-bp restriction fragment as a hybridization probe. The presence of a single band in genomic DNA digested with four different restriction enzymes indicates that the *NAB3* gene is single copy. (*C*) Polyadenylated RNAs were isolated, and fractionated by glyoxal gel electrophoresis, blotted onto a nylon membrane and probed as described in *B*.

by genomic blot analysis (Fig. 5 *B*), and produced a 3.0-kb poly (A)$^+$ RNA (Fig. 5 *C*). The deduced amino acid sequence determined from *NAB3* indicated an extremely acidic protein (pI = 4.22) of 802 amino acids with a calculated molecular weight of 90,468 (Fig. 6). Although Nab3p migrated at an estimated molecular weight of ~120,000 by SDS-PAGE (Fig. 1), both the Nablp/Nop3p/Npl3p (Russell and Tollervey, 1992; Bossie et al., 1992) and Nab2p (Anderson et al., 1993*b*) proteins, and a majority of hnRNPs from metazoan cells (Dreyfuss et al., 1993), migrate aberrantly by SDS-PAGE.

Nab3p possesses three recognizable protein structural motifs (Fig. 6). The amino terminus is extremely acidic, and contains a run of 37 aspartic and glutamic acid residues interrupted by only a single asparagine (Fig. 6, amino acids [aa] 101-137). This aspartic/glutamic acid-rich region is reminiscent of an acidic region of nucleolin (Lapeyre et al., 1987). Near the middle of Nab3p is a single ribonucleoprotein consensus sequence RBD (Fig. 6, aa 322-401), a motif that defines a large family of RNA-binding proteins (Bandziulis et al., 1989; Keene and Query, 1991). Finally, the carboxy terminal region is rich in glutamine and proline residues (Fig. 6, aa 568-785) similar to a putative RNA-binding protein involved in spermatogenesis in *Drosophila* (Karsch-Mizrachi and Haynes, 1993).

Comparison of the primary structures of the three Nab proteins indicated that the only motif which they had in common was a region rich in glutamine and proline residues, which varies considerably in sequence between these proteins (Fig. 7 *A*). Distinct features of the Q/P-rich region within the three Nab proteins are four repeats of APQE in Nablp (Bossie et al., 1992; Russell and Tollervey, 1992), a Nab2p tetrapeptide repeat of QQQP that is variable in length from two to nine repeats between yeast strains (Anderson et al., 1993*b*), and long consecutive runs of both glutamine and proline ($P_{1\text{-}5}Q_{3\text{-}16}$) in Nab3p. The Nab proteins also possess different combinations of RNA-binding motifs with Nablp containing one RBD and several RGG boxes, Nab2p an RGG box and seven repeats of a C_3H motif, and Nab3p a single RBD. This RBD within Nab3p is most closely related to the single RBD of the hnRNP C1/C2 proteins from *Xenopus* (38% identical residues) and human (36% identity) (Fig. 7 *B*). The frog and human hnRNP C proteins are also acidic ribonucleoproteins (Swanson et al., 1987; Preugschat and Wold, 1988).

NAB3 Encodes a Protein Essential for Cell Growth

Previous work has demonstrated that expression of the *NAB1/NOP3* and *NAB2* genes is required for cell growth (Russell and Tollervey, 1992; Anderson et al., 1993*b*). To determine if *NAB3* was also required for cell viability, a

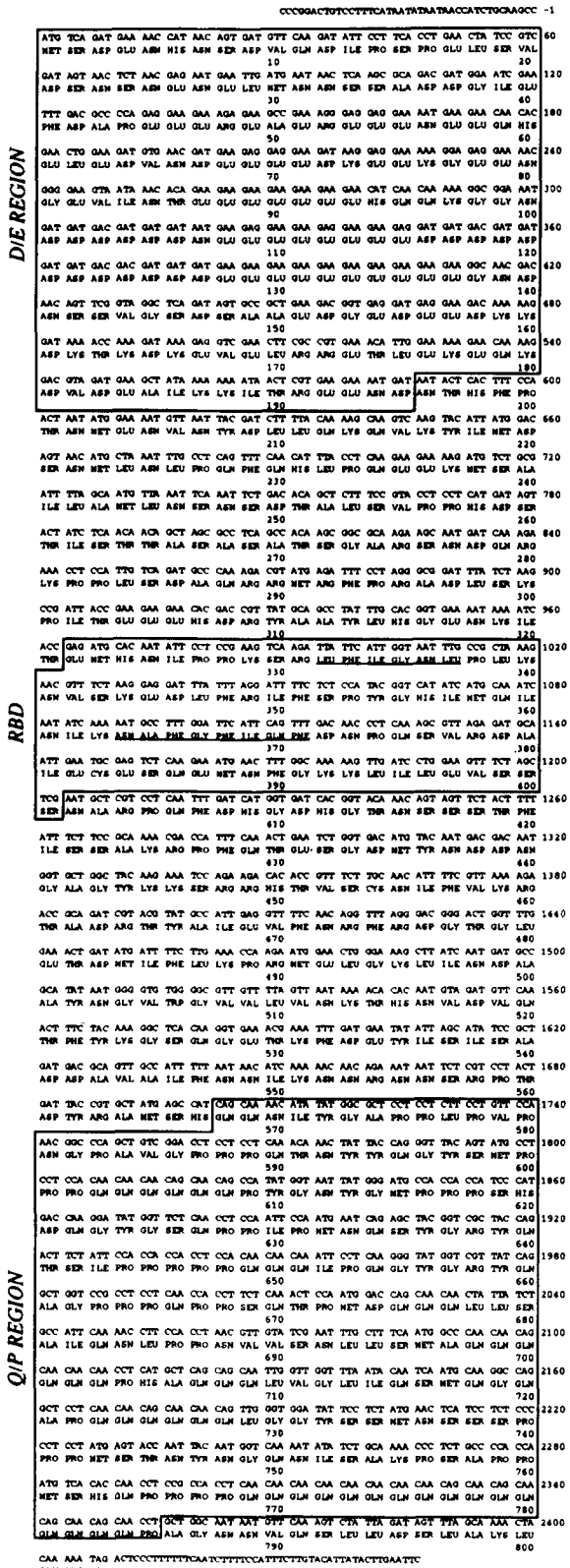

Figure 6. DNA and deduced amino acid sequence of *NAB3*. The genomic DNA sequence from NciI to EcoRI (Fig. 5 *A*) is shown. Open boxes highlight three protein structural motifs within Nab3p including the acidic aspartic/glutamic acid (*D/E*) region, the ribonucleoprotein consensus sequence RNA-binding domain (*RBD*), and a glutamine/proline-rich (*Q/P*) region near the carboxyl terminus. The underlined sequences within the RBD correspond to RNP1 and RNP2 (Bandziulis et al., 1989). These sequence data are available from EMBL/GenBank/DDBJ under accession number U05314.

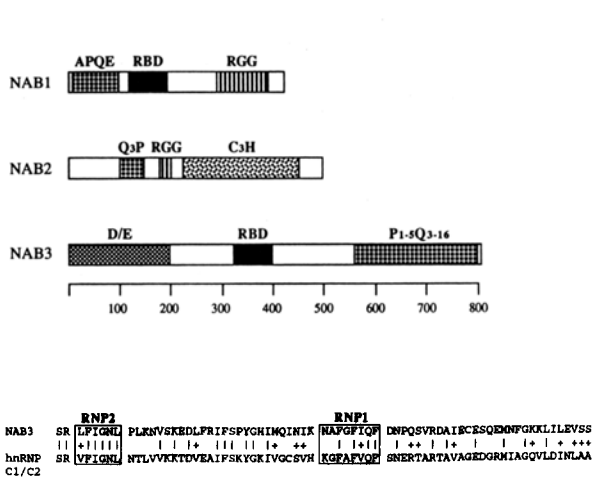

Figure 7. Protein structural motifs in the Nab proteins. (*A*) A comparison of the RNA-binding motifs and auxiliary domains of Nablp, Nab2p, and Nab3p. In Nablp, the auxiliary domain is characterized by a glutamine/proline (Q/P)-rich region consisting of four repeats of alanine-proline-glutamine-glutamic acid (*APQE*), and the RNA-binding motifs are a single ribonucleoprotein consenses sequence RNA-binding domain (*RBD*) and an RGG box region. In Nab2p, the Q/P-rich region consists of a variable length repeat of three glutamines and a proline (Q_3P) while two RNA-binding domains are present, an RGG box region and a motif related to the zinc finger nucleic acid binding motif (C_3H). Two auxiliary domains exist in Nab3p, a Q/P-rich domain composed of variable repeats of adjacent prolines and glutamines ($P_{1\text{-}5}Q_{3\text{-}16}$) and an aspartic/glutamic-rich (*D/E*) region, and a single RNA-binding motif (*RBD*). (*B*) The RBD from Nab3p is compared to that of the hnRNP C proteins. Also shown is the consensus RBD (Bandziulis et al., 1989).

NAB3 null allele was created by replacing ~60% of the *NAB3* protein encoding region with the selectable marker *LEU2* (Fig. 8 *A*). This construct was used to transform YJA501 to generate a leucine prototroph, YSW101 (Table I). Genomic blot analysis demonstrated that one of the *NAB3* alleles had been transplaced with the *nab3::LEU2* allele (Fig. 8 *B*). Sporulation of YSW101, followed by tetrad analysis, resulted in 50 out of 54 tetrads with two viable and 2 nonviable spores (Fig. 9 *C*). All viable spores were leucine auxotrophs. To determine if the growth defect in the *nab3::LEU2* cells could be complemented, YSW101 was transformed with pNAB3.14, a YCp50 clone carrying the entire *NAB3* gene and *URA3* as a selectable marker, to create YSW102. Sporulation of YSW102, and analysis of 16 tetrads gave four with four viable spores, four with three viable and one nonviable, and eight with two viable and two nonviable. Of the 15 spores which were Ura^+/Leu^+, none were able to grow in the presence of 5-fluoro-orotic acid that is selectively toxic to cells expressing *URA3*. We conclude from these studies that expression of *NAB3* is essential for cell growth.

Loss of Nab3p Affects Pre-mRNA Processing

Pre-mRNA splicing requires that various snRNPs bind to

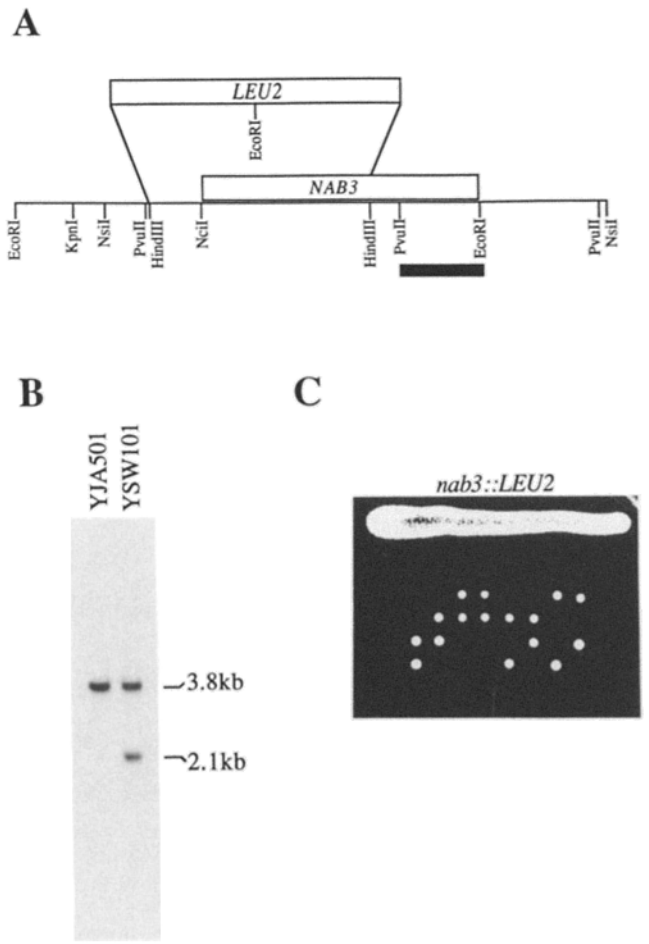

Figure 8. NAB3 is essential for cell viability. (*A*) The one-step transplacement of the *NAB3* gene with *LEU2* is illustrated. (*B*) Genomic DNA was isolated from either YJA501 or the leucine prototroph, YSW101, digested with EcoRI, and hybridized with the PvuII/EcoRI restriction fragment highlighted by the black box in *A*. (*C*) An example of the segregation pattern of dissected tetrads from YSW101. The YPD plate shown was incubated for 3 d, however the segregation pattern remained the same after 10 d of growth at 30°C. Replica plating demonstrated that all growing haploids were leucine auxotrophs.

pre-mRNAs in a defined order (Green, 1991; Guthrie, 1991; Ruby and Abelson, 1991; Rymond and Rosbash, 1992; Baserga and Steitz, 1993). In yeast, this snRNP addition pathway was elegantly characterized in vivo by depletion of individual snRNPs using *GAL*-regulated expression of various snRNA genes (for review see Rymond and Rosbash, 1992). We therefore investigated the function of Nab3p by subcloning the *NAB3* gene into pRD53, a *CEN6URA3-GAL1,10* vector which allows for regulated expression. Growth of YSW109 in galactose induces an ~fivefold increase in Nab3p with no apparent effect on growth rate (Wilson, S. M., J. T. Anderson, and M. S. Swanson, unpublished data). In contrast, repression of *GAL::nab3* expression by growth of haploid strain YSW109-1A in glucose resulted in a gradual loss of Nab3p with a subsequent loss of growth rate (Fig. 9, *A* and *B*). Nab3p was barely detectable after 24 h of growth in glucose, and cell division of YSW109-1A had ceased. The effects of Nab3p loss on the steady state levels of both pre-mRNAs and mRNAs were examined by Northern blot analysis for both unspliced (triose phosphate isomerase, *TPI1*) and spliced (actin, *ACT1* and ribosomal protein L29, *CYH2*) pre-mRNAs (Fig. 9 *C*). Loss of mRNAs produced from both unspliced and spliced pre-mRNAs paralleled the disappearance of Nab3p. In contrast, unspliced *ACT1* pre-mRNA accumulated as Nab3p was depleted (Fig. 9 *C*, *ACT1* intron). However, the *ACT1* pre-mRNA accumulation visualized at 24 h in *GAL::nab3* cells was less than that observed in a temperature-sensitive strain known to be defective in pre-mRNA splicing, *prp2-1* (Fig. 9 *C*, autoradiography for *GAL::nab3* was approximately four times longer than for *prp2-1*). Prp2p is an RNA-dependent ATPase required for the first transesterification reaction (Kim and Lin, 1993). Although unspliced *CYH2* pre-mRNA did not accumulate, the ratio of unspliced pre-mRNA to mature mRNA significantly increased between 0 and 24 h following shift to glucose (approximately 15-fold by PhosphorImager quantitation).

The accumulation of *ACT1* pre-mRNAs during incubation of *GAL::nab3* cells in glucose suggested that Nab3p may be an essential factor for pre-mRNA splicing. In collaboration with Christian Siebel and Christine Guthrie (University of California, San Francisco, CA), the ability of Nab3p-depleted extracts to splice a labeled actin precursor RNA in vitro was tested. Cell extracts were either depleted of Nab3p by affinity chromatography on a 2F12 (anti-Nab3p) monoclonal antibody column or extracts were directly treated with varying concentrations of purified 2F12 (see Materials and Methods). Either immunodepletion or immunoinhibition led to nearly complete degradation of actin precursor RNAs. This result suggests that Nab3p might be required for pre-mRNA stability in vitro although the effect on stability might be indirect (Siebel, C. W., C. Guthrie, and M. S. Swanson, unpublished data). Nevertheless, the extensive precursor RNA degradation precluded a determination of whether Nab3p was involved in pre-mRNA splicing in vitro.

Previous work has demonstrated that cells carrying the *prp2-1* allele accumulate both *ACT1* and *CYH2* pre-mRNAs at the nonpermissive temperature (Vijayraghavan et al., 1989; Forrester et al, 1992). In contrast, cells possessing mutant alleles of *PRP20* accumulate unspliced *ACT1* pre-mRNA and larger extended transcripts, but show a dramatic decrease of unspliced *CYH2* pre-mRNA (Vijayraghavan et al., 1989; Forrester et al., 1992). Although the Prp20p is structurally similar to the human RCC1 protein involved in chromosome condensation, its function in vivo is unclear since mutations in *PRP20* cause a variety of defects in mRNA biogenesis including transcriptional initiation, 3′ end formation and nucleocytoplasmic mRNA export (Aebi et al., 1990; Forrester et al., 1992; Amberg et al., 1993). However, since depletion of Nab3p also resulted in accumulation of *ACT1*, but a loss of *CYH2*, pre-mRNAs, we determined if loss of Nab3p resulted in other defects in the mRNA processing pathway. As opposed to *prp20* mutants, loss of Nab3p had no detectable effect on the distribution of polyadenylated RNA between the nucleus and cytoplasm or on poly(A) tail length in vivo, and did not result in the generation of significant levels of extended *CUP1* or *CYH2* transcripts (Wilson, S. M., J. T. Anderson, and M. S. Swanson, unpublished data). In summary, these results suggested that Nab3p function is required for efficient splicing in vivo, but does not appear to play an essential role in either 3′ end formation or nucleocytoplasmic mRNA shuttling.

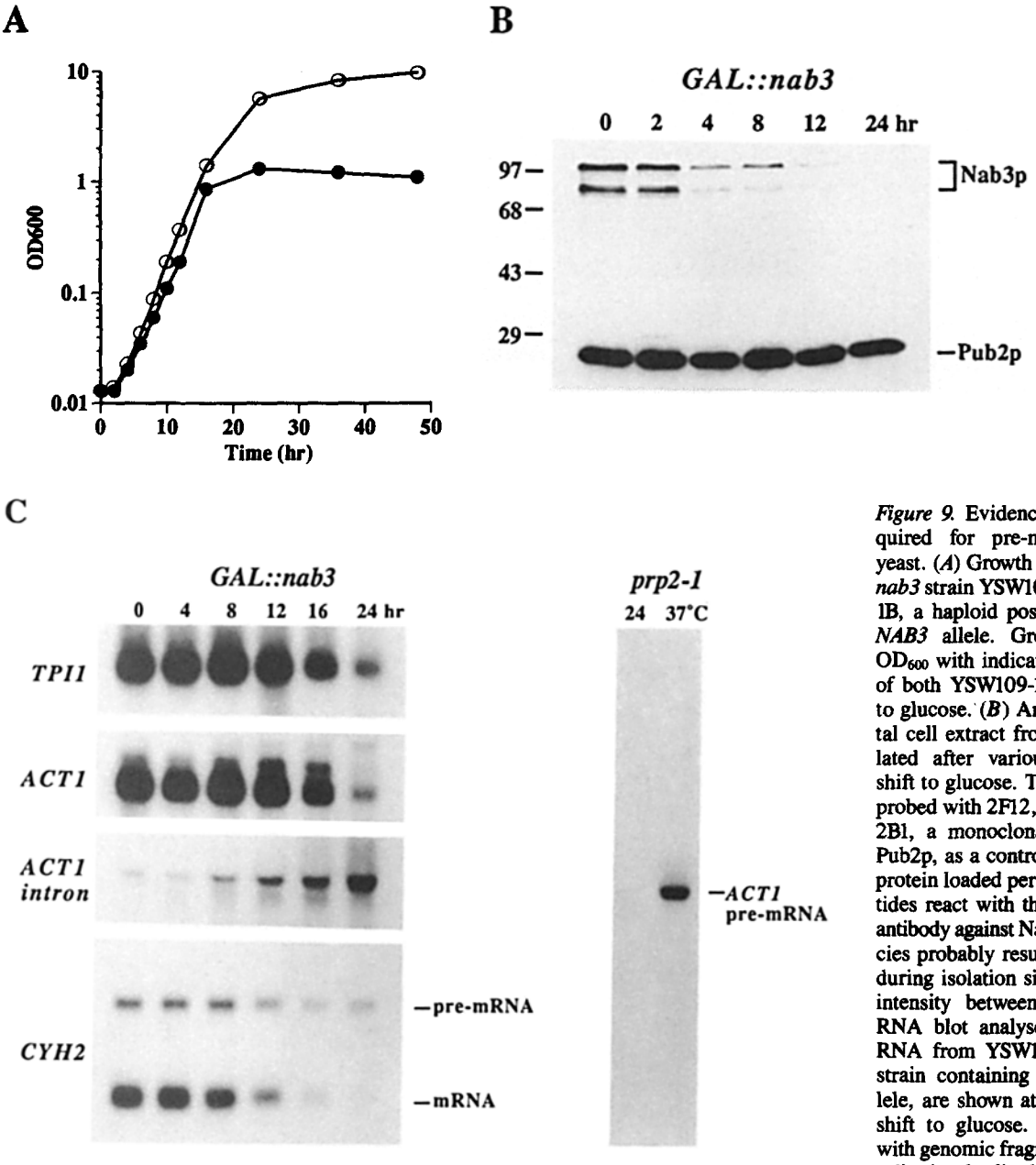

Figure 9. Evidence that Nab3p is required for pre-mRNA splicing in yeast. (*A*) Growth curves of the *GAL::nab3* strain YSW109-1A and YSW109-1B, a haploid possessing a wild-type *NAB3* allele. Growth is shown by OD_{600} with indicated times after shift of both YSW109-1A and YSW109-1B to glucose. (*B*) An immunoblot of total cell extract from YSW109-1A isolated after various times following shift to glucose. The immunoblot was probed with 2F12, against Nab3p, and 2B1, a monoclonal antibody against Pub2p, as a control for the amount of protein loaded per lane. Two polypeptides react with the 2F12 monoclonal antibody against Nab3p. The lower species probably results from proteolysis during isolation since it is variable in intensity between experiments. (*C*) RNA blot analyses of total cellular RNA from YSW109-1A, the haploid strain containing the *GAL::nab3* allele, are shown at various times after shift to glucose. Blots were probed with genomic fragments from both unspliced and spliced genes. These probes included triose phosphate isomerase (*TPI1*), exon and intron regions of actin (*ACT1*), the actin intron alone (*ACT1* intron), and exon and intron regions of the ribosomal gene L29 (*CYH2*). Also shown is an RNA blot probed with the *ACT1* intron probe in which total cellular RNAs were isolated from the *prp2-1* thermal-sensitive strain grown at either 24°C or after shift to 37°C for 45 min. The film exposure time for the *prp2-1* blot was four times less than for *GAL::nab3*. ●, *GAL::nab3*; ○, *NAB3*.

Discussion

We have isolated and characterized a family of nuclear pre-mRNA/mRNA binding proteins that appear to be required for several steps in the pre-mRNA processing pathway. The three Nab proteins described here are indistinguishable from metazoan hnRNPs by several criteria: (*a*) they efficiently cross-link to polyadenylated RNAs in vivo; (*b*) they are localized within the nucleus with a predominantly non-nucleolar distribution; (*c*) their RNA-binding motifs and auxiliary domains are structurally related to hnRNPs from a variety of vertebrate and invertebrate cells; and (*d*) individual Nab proteins are required for particular steps in the pre-mRNA processing pathway in vivo including splicing, polyadenylation, and nucleocytoplasmic export of mRNA.

An important question which remains unanswered is why we have not been able to isolate yeast proteins structurally related to the abundant metazoan A/B proteins that have been characterized in a variety of vertebrate and invertebrate cells (Dreyfuss et al., 1993). One possibility is that our isolation procedure, which depends on UV light–induced cross-

linking of RNA-binding proteins to polyadenylated RNAs, may have failed to detect this group of yeast RNA-binding proteins. However, we have not been successful in isolating a distinct 30–40 S monoparticle from yeast nucleoplasm by sucrose gradient sedimentation under a variety of experimental conditions, supporting the idea that this type of hnRNP A/B-rich nuclear particle may not exist in *Saccharomyces cerevisiae* (Oberdorf, A. M., and M. S. Swanson, unpublished data). Alternatively, the A/B group of proteins may function in a nuclear activity which is not common between higher eukaryotic cells and *S. cerevisiae*. One of the most prominent differences in the pre-mRNA processing pathway between metazoan and *S. cerevisiae* cells is that only 2–5% of the characterized nuclear-encoded genes of budding yeast possess introns, and there are no examples of alternatively spliced pre-mRNAs (Rymond and Rosbash, 1992). Since the relative concentrations of SF2/ASF and the hnRNP A1 protein have been shown to regulate splice site choice in vitro (Mayeda and Krainer, 1992; Sun et al., 1993), the abundant A/B proteins may be a specialized subgroup of hnRNPs in metazoan cells that function as alternative splicing factors.

What are the cellular functions of the Nab proteins? Nablp possesses several types of RNA-binding motifs, and is bound to nuclear polyadenylated RNAs in vivo. Nablp has been previously characterized as Npl3p (Bossie et al., 1992) and Nop3p (Russell and Tollervey, 1992). Whether *NPL3/NOP3* is an essential gene for cell growth appears to be highly dependent on yeast strain background. *NPL3* was identified during a screen for mutants able to grow on glycerol as a result of a defect in the import pathway of proteins into the nucleus (Bossie et al., 1992). These mutants grew because they no longer exclusively imported chimeric proteins, composed of the SV-40 large T antigen nuclear localization sequence fused to the mitochondrial $F_1\beta$-ATPase, into the nucleus. Our data do not directly address the possibility that Nablp is involved in the import of proteins into the nucleus. Since a metazoan hnRNP has been shown to shuttle between the nucleus and cytoplasm (Piñol-Roma and Dreyfuss, 1992), it is possible that Nablp performs multiple functions in both RNA processing within the nucleus and protein import into the nucleus. *NOP3* was isolated during a low stringency hybridization screen using a region of *NOP1* which encodes a conserved RNA-binding motif, the GAR domain or RGG box (Russell and Tollervey, 1992). Growth of a *GAL::nop3* strain on glucose results in the loss of Nop3p and subsequent impairment of both nucleolar pre-rRNA processing and the production of cytoplasmic ribosomes. Our analysis of the subnuclear distribution of Nablp by three-dimensional optical microscopy indicates that the vast majority of this protein is not localized within the nucleolus. However, this observation does not rule out the possibility that Nablp is an integral component of the pre-rRNA processing machinery. To explain the various effects of *NPL3/NOP3* mutations, we postulate that the primary and essential function of Nablp is in the processing of RNA polymerase II transcripts. If Nablp is required for pre-mRNA processing then loss of Nablp function might ultimately lead to loss of mRNAs that encode proteins required for a wide array of cellular functions including pre-rRNA processing and import of proteins into the nucleus.

We have recently discovered that Nab2p is required for both the regulation of poly(A) tail length and nucleocytoplasmic export of mRNAs in vivo (Anderson, J. T., and M. S. Swanson, unpublished data). In contrast, Nab3p appears to be required for efficient splicing of both *ACT1* and *CYH2* pre-mRNAs in vivo, but plays no detectable role in polyadenylation or nucleocytoplasmic transport of mRNAs. Several observations are consistent with the idea that Nab3p plays a role in pre-mRNA splicing. Nab3p is a nuclear polyadenylated RNA-binding protein in vivo whose depletion correlates with an accumulation of unspliced pre-mRNA. Second, the relative amount of Nab3p in cells is significantly lower than either Nablp or Nab2p. This lower abundance is consistent with a factor which may be involved in pre-mRNA splicing because a limited number of nuclear genes produce transcripts that are spliced. However, we favor the hypothesis that Nab3p functions at some other level in the pre-mRNA processing pathway and indirectly influences pre-mRNA splicing efficiency. For instance, if Nab2p and Nab3p bind to different regions within nascent transcripts to form distinct chromatin-associated RNP complexes removal of one of these proteins might have profound, but differential, effects on subsequent processing events. The surprising finding that loss of Nab3p influences pre-mRNA levels within the nucleus while loss of Nab2p results in alterations in both poly(A) tail length and nucleocytoplasmic export of mRNAs is consistent with this idea.

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