Identification and Nuclear Localization of Yeast Pre-Messenger RNA Processing Components: *RNA2* and *RNA3* Proteins

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Abstract. Temperature-sensitive mutations in the *RNA2* through *RNAll* genes of yeast prevent the processing of nuclear pre-mRNAs. We have raised antisera that detect the *RNA2* and *RNA3* proteins in immunoblots of extracts of yeast containing high copy number *RNA2* and *RNA3* plasmids. Subcellular fractionation of yeast cells that overproduce the *RNA2* and *RNA3* proteins has revealed that these proteins are enriched in nuclear fractions. Indirect immunofluorescence results have indicated that these proteins are localized in yeast nuclei. These localization results are consistent with the fact that these genes have a role in processing yeast pre-mRNA.

MUCH of the interest in expression of RNA polymerase II transcribed genes is focused on defining the mechanism of processing the primary transcription products of these genes (pre-mRNAs) to cytoplasmic, translatable mRNAs. Accurate splicing of introns from pre-mRNAs is absolutely required for production of functional polypeptides from mosaic genes. The modulation of pre-mRNA processing patterns to generate polypeptide diversity during development of eukaryotic viruses (Nevins, 1982) and metazoan organisms (DeNoto et al., 1981; Schwarzbauer et al., 1983; Periasamy et al., 1985) is widespread, and elucidation of its molecular mechanisms is of great importance. This has become possible since the development of well-defined soluble in vitro systems that are capable of processing intron-containing exogenously added pre-mRNA molecules, using mammalian whole-cell and nuclear extracts (Frendewey and Keller, 1985; Grabowski et al., 1985; Krainer and Maniatis, 1985) or yeast cell extracts (Brody and Abelson, 1985; Lin et al., 1985). Unique species with kinetic properties consistent with pre-mRNA processing intermediates have been identified in in vitro processing reactions (Grabowski et al., 1984; Padgett et al., 1984; Ruskin et al., 1984; Lin et al., 1985). The in vitro pre-mRNA processing mechanism appears to mimic reactions occurring in vivo, since similar intermediates have also been detected in mammalian cells (Wallace and Edmonds, 1983; Zeitlin and Efstratiadis, 1984) and yeast (Domdey et al., 1984; Rodriguez et al., 1984).

Although the budding yeast *Saccharomyces cerevisiae* has a relatively small number of intron-containing genes (Gallwitz and Seidel, 1980; Ng and Abelson, 1980; Rosbash et al., 1981; Fried et al., 1981; Larkin and Woolford, 1983; Miller, 1984), the powerful molecular-genetic techniques available in this organism (Botstein and Davis, 1982), and the rapidly advancing cell biological technology make this an excellent system for studying pre-mRNA processing both in vivo and in vitro. The availability of a yeast in vitro splicing system (Brody and Abelson, 1985; Lin et al., 1985) provides the opportunity to analyze the functions of purified proteins in the pre-mRNA processing reaction.

A unique advantage of studying pre-mRNA processing in yeast is the existence of 10 complementation groups of temperature-sensitive mutants, *rna2* through *rnall* (Hartwell, 1967), that have been shown to be defective in pre-mRNA processing (Rosbash et al., 1981; Larkin and Woolford, 1983; Teem et al., 1983; Last et al., 1984). Two suppressors of *rna* mutations have been reported. The *SRN1* allele suppresses a broad spectrum of mutations in single and double *rna* mutant backgrounds (Pearson et al., 1982). The *SRN2* locus on a medium copy number plasmid suppresses several temperature-sensitive *rna2* alleles, but does not suppress a nonconditionally lethal deletion-insertion allele (Last, R. L., and J. L. Woolford, manuscript submitted for publication). This laboratory is interested in determining the role that the *RNA* and *SRN* genes play in pre-mRNA processing.

We have previously reported the isolation and characterization of the *RNA2*, *RNA3*, *RNA4*, and *RNAll* genes (Last et al., 1984) and presented evidence for functional relationships of the *SRN2* suppressor locus and *RNA2* as well as relationships between *RNA3* and *RNA4* (Last, R. L., and J. L. Woolford, manuscript submitted for publication). J. Abelson and M. Rosbash, using temperature-sensitive mutants, have provided evidence for the roles of many of the *RNA* genes in in vitro pre-mRNA processing (Abelson, J., and M. Rosbash, personal communication). To understand the specific roles of these gene products in pre-mRNA processing it will be necessary to develop assays for them in in vivo and in vitro splicing extracts.

In this paper we report the production of antisera against tribrid proteins that contain portions of the *RNA2* or *RNA3*

open reading frames. These antisera were used to identify polypeptides that are overproduced in yeast strains transformed with high copy number *RNA2* or *RNA3* plasmids. Subcellular fractionation and indirect immunofluorescence results indicate that the *RNA2* and *RNA3* gene products are localized in the nuclei of vegetatively growing yeast cells. This observation is consistent with the expectation that pre-mRNA splicing in yeast takes place in the nucleus, as it does in mammalian cells (Hernandez and Keller, 1983; Krainer et al., 1984).

Materials and Methods

Materials

Reagents were of the highest purity available unless otherwise stated. Unless specifically listed below, reagents were purchased from Fisher Scientific Co., Pittsburgh, PA. Biotinylated donkey anti-rabbit IgG, streptavidin-conjugated Texas red, and FITC were purchased from Amersham Corp., Arlington Heights, IL. Acrylamide, Biogel A-5M, SDS PAGE low molecular mass markers, and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories, Richmond, CA. DNA modification and restriction enzymes, 5-bromo-4-chloro-3-indolyl-a-D-glucopyranoside (X-gal) and pepstatin A were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. 10-well microscope slides were obtained from Carlson Scientific, Peotone, IL. Zymolyase 20,000 and 60,000 were purchased from Miles Laboratories, Inc., Elkhart, IN. Percoll was from Pharmacia Fine Chemicals, Piscataway, NJ. Normal goat serum was obtained from Rockland, Inc., Gilbertsville, PA. Nitrocellulose (0.2-μm pore size) was purchased from Schleicher and Schuell, Inc., Keene, NH. Ampicillin, bovine serum albumin (98-99%), deoxyribonuclease (type I), 4-chloro-1-napthol, 4',6'-diamidino-2-phenylindole (DAPI),[1] β-galactosidase, β-glucuronidase (type H2), glycine, high molecular mass standards for SDS gel electrophoresis, lysozyme, phenylenediamine, phenylmethylsulfonyl fluoride (PMSF), polylysine hydrobromide (>300 kD), Triton X-100, and Tween-20 were purchased from the Sigma Chemical Company, St. Louis, MO. Biotinylated goat anti-rabbit IgG and horseradish peroxidase-conjugated avidin DH-biotin complex reagents were obtained from Vector Labs, Inc., Burlingame, CA. Plasmid pJDB207, which contains a poorly expressed allele of the yeast *LEU2* gene and the 2-μm yeast replicator, was a kind gift of Dr. Jim Hopper (Dept. of Biological Chemistry, Hershey Medical Center, Hershey, PA). This plasmid obtains very high copy number (50–200 copies/cell) in yeast (Beggs, 1981).

Genetic Manipulations

Yeast and *Escherichia coli* were transformed and grown as previously described (Last et al., 1984; Last, R. L., and J. L. Woolford, manuscript submitted for publication).

ompF-RNA-lacZ Tribrid Plasmid Constructions

Standard DNA manipulations were done as previously described (Last et al., 1984). The sources of *RNA2* and *RNA3* genomic clones were purified yeast DNA fragments from pRN2-1 and pRN3-3, respectively (Last et al., 1984). The DNA fragments were sonicated, concentrated by DEAE cellulose chromatography and digested lightly with exonuclease Bal31 (Gray et al., 1982). A population of fragments ranging in size from 300 to 700 bp was purified with preparative acrylamide gel electrophoresis by crushing the gel slice and eluting DNA overnight at room temperature in 0.5 M ammonium acetate, 10 mM Tris, pH 7.5, and 1 mM EDTA (Maxam and Gilbert, 1977). These fragments were ligated into the *SmaI* site of pORF2 (Weinstock et al., 1983). *E. coli* strain MH1000 (F⁻ *araABOIC-leu*)7679 *gall galk* (Δ*lacX-74*) *rpsL thi ompR101* was transformed with the ligation mixtures and cells were plated on Luria broth plates containing 150 μg/ml ampicillin and 0.1 ml per plate of a solution containing 10 μg X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) per milliliter of dimethylformamide. DNA from dark blue colonies was tested for the presence of inserts. Plasmids that contained inserts of 300 bp or larger were transformed into *E. coli* TK1046 (*araD* 139Δ*argF-lac*U169 *rpsL*150 *relA*1 *flbB530l deoC1 ptsF25 malPQ*::Tn5 *ompBcsl*), and extracts were screened as described by Weinstock and co-workers (1983), with inductions for tribrid protein synthesis at 40°C for 1.5–2 h. Plasmids from strains producing inducible proteins larger than the hybrid *ompF-lacZ* protein were used as hybridization probes to blots of fractionated yeast poly(A⁺) RNA to determine whether the insert open reading frame (ORF) DNA was homologous to the respective *RNA2* or *RNA3* transcripts.

Purification of Fusion Polypeptides

All steps were done at 4°C unless otherwise noted. This protocol is for 1 liter of culture. Cells were grown with vigorous agitation at 23°C in 250 ml Luria broth in 1-liter flasks to OD 0.2 (Spectronic 21; Spectronics Corp., Westbury, NY) then shifted to 40°C to induce synthesis of hybrid protein. After a 2-h induction, the cell pellet was incubated with 3 mg lysozyme in 15 ml of ice-cold buffer A (50 mM NaCl, 50 mM Tris-HCl (pH 7.8), 10 mM EDTA (pH 7.5), 1 mM 2-mercaptoethanol, 1 mM PMSF from a freshly prepared 100 mM stock in 100% ethanol). After 30 min the mixture was frozen at −80°C, thawed on ice, and 0.25 ml 1 M $MgCl_2$ was added, followed by 75 μg DNase I. This mixture was left on ice for 30 min, and spun at 12,000 *g* for 10 min. The pellet was resuspended in 25 ml buffer A, homogenized well in a 30-ml Potter homogenizer, and recentrifuged. The buffer A extraction was repeated, followed by two buffer A + 2% Triton X-100 extractions. The final pellet was resuspended in 5 ml of 1% SDS, 10 mM Tris-HCl, pH 8.0, and 50 mM 2-mercaptoethanol in a 15-ml Potter homogenizer and the solution was boiled for 10 min.

The solubilized solution was brought to room temperature and remaining particulate matter was removed by centrifugation in a rotor (model 50Ti, Beckman Instruments, Inc., Fullerton, CA) at 35,000 rpm at 20°C for 1 h. The supernatant was boiled for 2 min, cooled to room temperature, and loaded on a column (70 × 2.5 cm, A-5M; Bio-Rad Laboratories) equilibrated in 0.5% SDS, 10 mM Tris-HCl (pH 8.0), and 50 mM 2-mercaptoethanol at room temperature (Shuman et al., 1980). Fractions containing purified tribrid protein were pooled and lyophilized overnight, resuspended in a minimal volume of H_2O and precipitated with 20 vol of −20°C acetone. The precipitate was resuspended at 0.5–1.0 mg/ml protein in 0.1% SDS, 10 mM Tris (pH 8.0). Protein concentrations were estimated by comparison with commercial β-galactosidase in Coomassie Blue–stained protein gels. This material was used to immunize rabbits.

Isolation of Antisera Against RNA Gene Products

Young female New Zealand albino rabbits were immunized subcutaneously in the back (8–10 sites) and intramuscularly in the thighs (4–6 sites) with a total of 250–750 μg immunogen emulsified with an equal volume of complete Freund's adjuvant for the primary series. A total of 100–500 μg immunogen in incomplete Freund's adjuvant was used for subsequent immunizations. Three to five immunizations were required to obtain serum of high enough antibody titer to yield positive results by immunoblot. Serum was stored, in lyophilized or frozen form, at −20°C.

Affinity Purification of Antitribrid Antibodies

Antibodies that recognized the tribrid proteins were purified as described by Smith and Fisher (1984) with the following modifications. All steps were performed at room temperature. Tribrid protein was diluted to 200 μg/ml in 10 mM Tris-HCl (pH 7.5), 10% methanol. 2 ml of this protein solution was aspirated through a wetted 0.2-μM nitrocellulose disk. The disk was washed two times with 10% methanol and three times with dH_2O. The filter was blocked and incubated at room temperature overnight in a 1:50 dilution of antiserum in PBSa (1% NaCl, 0.025% KCl, 0.15% Na_2HPO4, 0.025% KH_2PO4 (final pH 7.6) containing 10% normal goat serum). The next morning the filter was washed with agitation four times in PBSa with 0.5% Tween-20 for 10 min at a time. Antibodies were eluted by incubating the filter for 30 s in 150 μl 5 mM glycine-HCl (pH 2.3), 500 mM NaCl, 0.5% Tween-20, and 100 μg BSA. The solution was immediately neutralized by adding it to 15 μl of 0.5 M Na_2HPO_4. This treatment was repeated twice, the last time incubating the filter in elution buffer for 90 s before neutralization. The filter was then washed with 150 μl PBSa with 0.5% Tween-20 and this wash was pooled with the neutralized fractions. The purified antibody solution was stored at 4°C in the presence of 0.02% sodium azide.

1. *Abbreviations used in this paper*: DAPI, 4-chloro-1-napthol,4',6'-diamidino-2-phenylindole; ORF, open reading frame; PBSa, 1% NaCl, 0.025% KCl, 0.015% Na_2HPO4, 0.025% KH_2PO4, final pH 7.6, containing 10% normal goat serum; PBSa-BSA, PBSa with 0.1 mg/ml BSA; X-gal, 5-bromo-4-chloro-3-indolyl-a-D-glucopyranoside.

Protein Gel Electrophoresis and Immunoblotting

Electrophoresis of denatured proteins was performed by the method of Laemmli (1970) for 7.5 and 10% gels using 30:0.8 acrylamide/bisacrylamide, or Dreyfuss et al. (1984) for 8 and 10% gels using 33.5:0.3 acrylamide/bisacrylamide. Protein gels were transferred to 0.2-μm pore size nitrocellulose at 90 V for 2 h (in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) in a Trans-Blot apparatus (Hoeffer Scientific Instruments, San Francisco, CA) with internal cooling (Towbin, et al. 1979). These blots were either stained for total protein with India ink (Hancock and Tsang, 1983) or processed for immunological detection of antigen as follows. The blot was blocked at 32°C for 1 h in PBSa containing 25 μg/ml BSA and 10% normal goat serum. Primary antibody incubation was carried out at room temperature for 1.5 h, or at 4°C overnight, using antiserum diluted 1:200–1:300 in PBSa containing 10% normal goat serum. The blot was washed three times at room temperature with agitation in PBSa containing 0.05% Tween-20 for 10 min each time, followed by a 10-min PBSa wash. The blot was incubated at room temperature for 1.5 h with biotinylated goat anti-rabbit IgG diluted 1:2000 in PBSa containing 0.01% BSA. The blot was washed as described above and incubated with avidin-biotinylated horseradish peroxidase complex in PBSa for 1.5 h at room temperature. The avidin-biotin complex was preformed as suggested by the manufacturer and then diluted 1:5 in PBSa containing 0.01% BSA before being used. Antigen-antibody complex was visualized by mixing 20 ml of 3 mg/ml 4-chloro-1-napthol freshly dissolved in cold absolute methanol with 50 μl 30% hydrogen peroxide in 100 ml PBSa. Molecular masses of the *RNA2* and *RNA3* polypeptides were estimated by comparison of their mobilities with commercially available molecular mass markers on 7.5 and 10% (30:0.8% acrylamide/bisacrylamide) SDS polyacrylamide gels (Laemmli, 1970).

Preparation of Yeast Whole Cell Extracts

A 50-ml culture of RL117 (*a/α rna3/rna3 leu2-3,112/leu2-3,112 his4/ + trp1/ + ura3-52/ura3-52*) containing pJDB207 or its *RNA* gene derivatives was grown overnight to late log phase at 23°C in complete medium without leucine (OD at 610 nm, 0.4–0.6 in a Spectronic 21; Spectronics Corp.). All subsequent steps were done on ice. The cells were harvested, washed once in 10 ml H_2O, and resuspended in 0.5 ml glass bead lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.3, 1.5 mM DTT and 1 mM PMSF). The cells were broken for 3 min in a homogenizer (B. Braun Instruments, Burlingame, CA) with nitrogen gas cooling. The extract was brought to a final concentration of 1× SDS sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT, 50% glycerol, 0.002% bromophenol blue), and boiled for 3 min. The boiled extract was cooled to room temperature and spun in an Eppendorf microfuge for 5 min. The decanted supernatant was used for gel electrophoresis.

Yeast Subcellular Fractionation

Percoll gradient nuclear-enriched fractions from 500-ml cultures of strain BJ2407 (RL159) (*a/α pep4-3/pep4-3 prb1-1.1.2/prb1-1.1.2 prc1-407/prc1-407 leu2/leu2 trp1/trp1 ura3-52/ura3-52*) containing pRN2-2 (pJDB207) or pRN3-4 (pJDB207) (Last et al., 1984) grown to mid-log phase (OD at 610 nm of 0.1 in a Spectronic 21) in C-leucine medium were isolated as described by Potashkin et al. (1984).

Triton X-100–extracted nuclear-enriched fractions were obtained by a modification of the method of Fisher et al., 1982 (and Wu, L., and P. Fisher, personal communication). Strain BJ2407 transformed with pRN2-1 or pRN3-4 on pJDB207 was grown in 1 liter of C-leucine medium to late log phase (OD at 610 nm of 0.5–0.7 in a Spectronic 21). All subsequent steps were done at 4°C unless otherwise indicated. The cells were harvested, washed in distilled water, and spheroplasted at 30°C for 30 min in 10 ml of spheroplast buffer (1 M sorbitol, 5 mM $MgCl_2$, 25 mM potassium phosphate, pH 7.8, 25 mM sodium succinate, pH 5.5, 2 mM DTT, 1 mM PMSF, 2 μg pepstatin A/ml, and 1.3 mg Zymolyase 20,000/ml). Spheroplasts were pelleted at 1,000 *g* for 5 min, and the pellet resuspended in 5 ml extraction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, and 2 μg pepstatin A/ml) containing 1% Triton X-100, and extracted with 20 strokes in a Dounce homogenizer. This fraction is called the crude homogenate. The crude homogenate was centrifuged at 12,000 *g* for 10 min to yield the postnuclear supernatant and first nuclear pellet. The pellet was washed twice in 25 ml extraction buffer and centrifuged each time as in the previous step. The supernatant from the first wash is called wash supernatant. The final pellet was resuspended in 5 ml 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ to yield the nuclear pellet fraction.

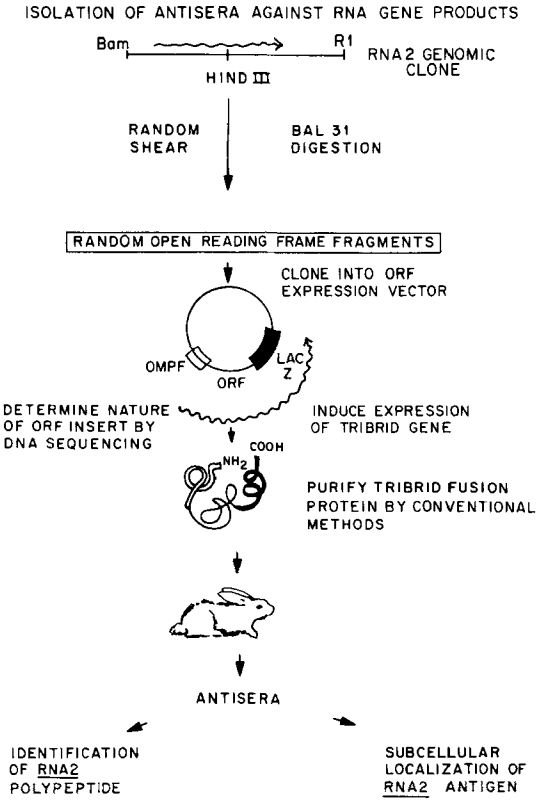


Figure 1. Schematic representation of construction of tribrid proteins containing *RNA2* or *RNA3* open reading frames. Size-fractionated randomly sheared *RNA2* or *RNA3* DNA was ligated into SmaI cleaved pORF2 DNA and the ligation mixture was transformed into *E. coli* strain MH1000 (*ompR101*). Plasmid DNA was prepared from colonies that were dark blue on LB+ampicillin plates containing X-gal. Plasmids containing inserts of >300 bp were transformed into *E. coli* strain TK1046 (*ompBcs1*). DNA from transformants that produced abundant proteins larger than hybrid size were used as hybridization probes to blots of yeast poly(A^+) RNA. Plasmids that hybridized to the *RNA2* or *RNA3* message were identified, and the DNA sequence of one insert of each class was determined. Tribid proteins were purified and used to immunize rabbits.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed on RL117 transformed with pRN2-1(pJDB207) or pRN3-3(pJDB207) by modification of the method of Adams and Pringle (1984). 50 ml of a culture of early log phase cells (OD of 0.05 at 610 nm in a Spectronic 21) grown in C-leucine medium at 23°C was harvested by filtration through a 1.2-μm filter (Millipore Corp., Bedford, MA) and washed three times with 5 ml of 0.1 M potassium phosphate (pH 6.5). The cells were immediately fixed with 3.6% formaldehyde in 0.1 M potassium phosphate (pH 6.5) for 30 min at room temperature. The fixed cells were washed four times with 5 ml 0.1 M potassium phosphate buffer (pH 6.5) and once with 5 ml sorbitol buffer (1.2 M sorbitol, 0.1 M dibasic potassium phosphate, 0.1 M citric acid, final pH of 5.9), collecting the cells by centrifugation at 3,000 *g*. The cells were permeabilized by incubation in 2.5 ml sorbitol buffer containing 10% glusulase and 8 μg Zymolyase 60,000/ml at 30°C for 1.5 h, with occasional agitation. The permeabilized cells were collected by centrifugation at 1,000 *g* and washed twice with 5 ml sorbitol buffer.

These fixed, permeabilized cells were resuspended in 1 ml sorbitol buffer, applied to polylysine-coated 10-well slides, and incubated for 10 min at room temperature. The cells were removed by aspiration and the slides placed into −20°C absolute methanol in a Coplin jar for 6 min, thawed at room temperature for 5 min, and placed into −20°C acetone for 30 s. The slides were blocked at room temperature in PBSa with 25 μg BSA/ml and

10% normal goat serum for 10 min. The blocker was aspirated off and 10–15 μl undiluted affinity-purified primary antibody added. The slides were incubated overnight at 4°C in a dark, moist atmosphere. Unbound antibody was washed three times by 5-min incubations in PBSa with 0.1 mg/ml BSA (PBSa-BSA). A 1:100 dilution of affinity-purified biotinylated donkey anti-rabbit IgG in PBSa-BSA was added to the wells and incubated at room temperature in a dark, moist atmosphere for 1.5 h. The slides were washed three times for 5 min each in PBSa-BSA and incubated with a 1:1000 dilution of streptavidin-conjugated FITC at room temperature in a dark, moist atmosphere for 1.5 h. The slides were washed twice in PBSa-BSA and incubated at room temperature with a solution of 1 μg DAPI/ml dH_2O for 5 min (Williamson and Fennell, 1975), followed by a 5-min wash with PBSa-BSA. The samples were mounted with 1 μg/ml phenylenediamine in 90% glycerol, 10% PBSa adjusted to pH 8.0 with 0.5 M carbonate/bicarbonate, pH 9.0, and observed using a 100× objective on a Zeiss microscope equipped for epifluorescence illumination of FITC, rhodamine (for Texas red), or DAPI.

Results

Construction of RNA2 and RNA3 ORF Clones

To characterize the *RNA* gene products, we have used the previously cloned *RNA2* and *RNA3* genes to obtain antisera that identify these antigens in yeast cells. Tribrid proteins containing regions of the *RNA2* or *RNA3* protein coding regions were created using the ORF vector pORF2, that contains cloning sites between *E. coli ompF* and *lacZ* domains (Weinstock et al., 1983). The scheme employed is diagrammed in Fig. 1. Genomic restriction fragments containing the *RNA2* or *RNA3* genes were isolated from plasmids pRN2-1 or pRN3-3 respectively (Last et al., 1984). These DNAs were sheared and molecules ranging in size from 300–600 bp were purified by preparative gel electrophoresis and blunt end–ligated into the *SmaI* site of pORF2. *E. coli* transformants were identified that expressed β-galactosidase activity associated with the production of a fusion-sized protein. One plasmid, which contained a ~450-bp insert from the *RNA2* gene (*RNA2* ORF1) was characterized. Additionally, plasmids containing inserts of 339 bp (*RNA3* ORF1) and ~280 bp (*RNA3* ORF2) from the *RNA3* gene were identified. The DNA sequences of the two ends of the *RNA2* ORF1 and the entire *RNA3* ORF1 were determined by sequencing both strands from the ends of the inserts (Last, 1986). Each insert was in the same orientation and reading frame as the *E. coli OmpF* and *lacZ* domains. The insert from *RNA2* ORF1 was shown to contain part of the *RNA2* open reading frame, as defined in the laboratory of J. Beggs (personal communication). The *RNA3* ORF1 is colinear with the 1407 nucleotide ORF of *RNA3* from the codon for asparagine 330 to that for leucine 432 of the predicted 469 amino acid *RNA3* polypeptide (Anthony, J., and J. Woolford, unpublished result).

Purification of Fusion Proteins

A rapid protocol for purification of the tribrid protein products from *E. coli* was based upon the relatively large size and

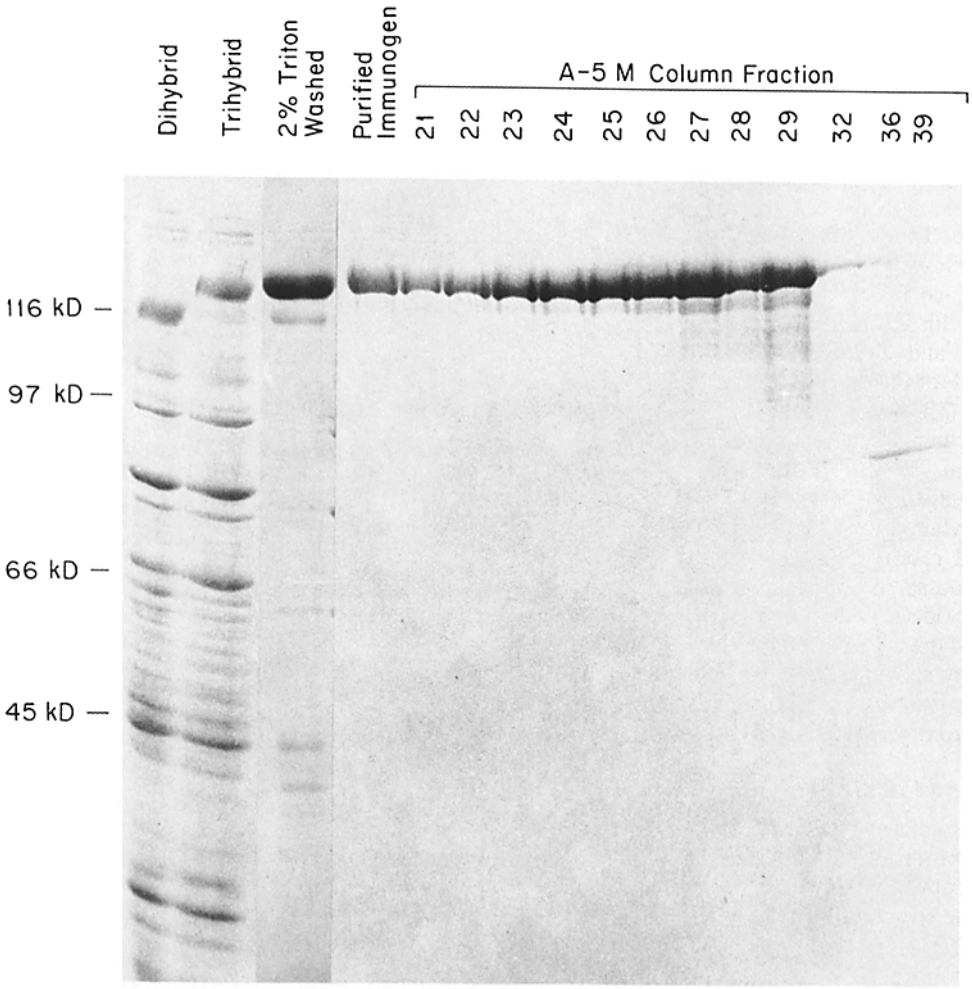


Figure 2. Purification of tribrid fusion proteins. Samples from the purification of the tribrid protein encoded by plasmid *RNA2* ORF1 were run on an SDS-polyacrylamide gel and stained with Coomassie R-250. Whole-cell extracts of *E. coli* strain TK1046 induced for production of hybrid *ompF-lacZ* protein or tribrid *ompF-RNA2* ORF1-*lacZ* protein are shown in the first two lanes. The third lane shows a sample of lysozyme-EDTA treated cells that had been extensively extracted with buffer containing 2% Triton X-100. The fourth lane shows the pooled Biogel A-5M fractions used as immunogen. The remaining lanes show fractions from the A-5M column that shows separation of polypeptides by molecular mass (column fraction numbers are shown above the lanes).

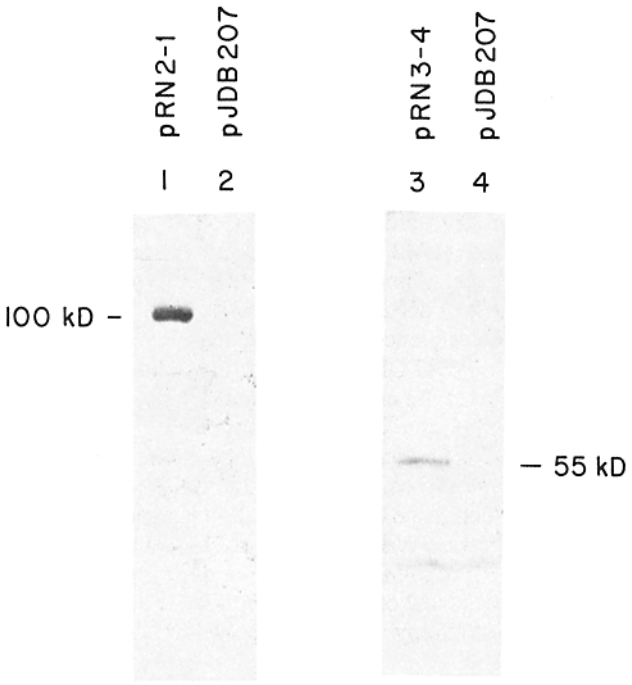


Figure 3. Antisera against tribrid proteins recognize polypeptides in whole-cell extracts from yeast strains containing high copy number plasmids. Whole-cell extracts (10 μl each) from glass-bead broken RL117 yeast cells transformed with the indicated high copy number plasmids were subjected to SDS PAGE (7.5% acrylamide) and immunoblot analysis using peroxidase staining. Lanes *1–2* were treated with anti-*RNA2* ORF1 and lanes *3–4* with anti-*RNA3* ORF1. Lane *1* contains extract from a pRN2-1 (pJDB207) transformant, lane *3* contains an extract from a pRN3-4 (pJDB207) transformant, and lanes *2* and *4* have extracts from a pJDB207 vector transformant.

insolubility of these polypeptides. The purification of the *RNA2* ORF1 tribrid protein is demonstrated by SDS PAGE in Fig. 2. Cells induced to produce fusion proteins were treated with lysozyme, extracted with 2% Triton X-100, and the pellet solubilized with SDS and 2-mercaptoethanol. Tribrid protein represented ~95% of the Coomassie-stainable solubilized material (Fig. 2; 2% Triton washed). The hybrid protein was purified further by chromatography through Biogel A-5M (Shuman et al., 1980). The final product was nearly homogeneous as judged by Coomassie Blue and silver staining of SDS-polyacrylamide gels (Fig. 2; purified immunogen and unpublished results). Fusion protein preparations showed varying amounts of species that comigrated with wild-type β-galactosidase protein and might represent products of proteolysis of the tribrid proteins (Fig. 2; purified immunogen and data not shown). These purified immunogens were used to raise antisera that recognized the *RNA2* and *RNA3* gene products from yeast cell extracts.

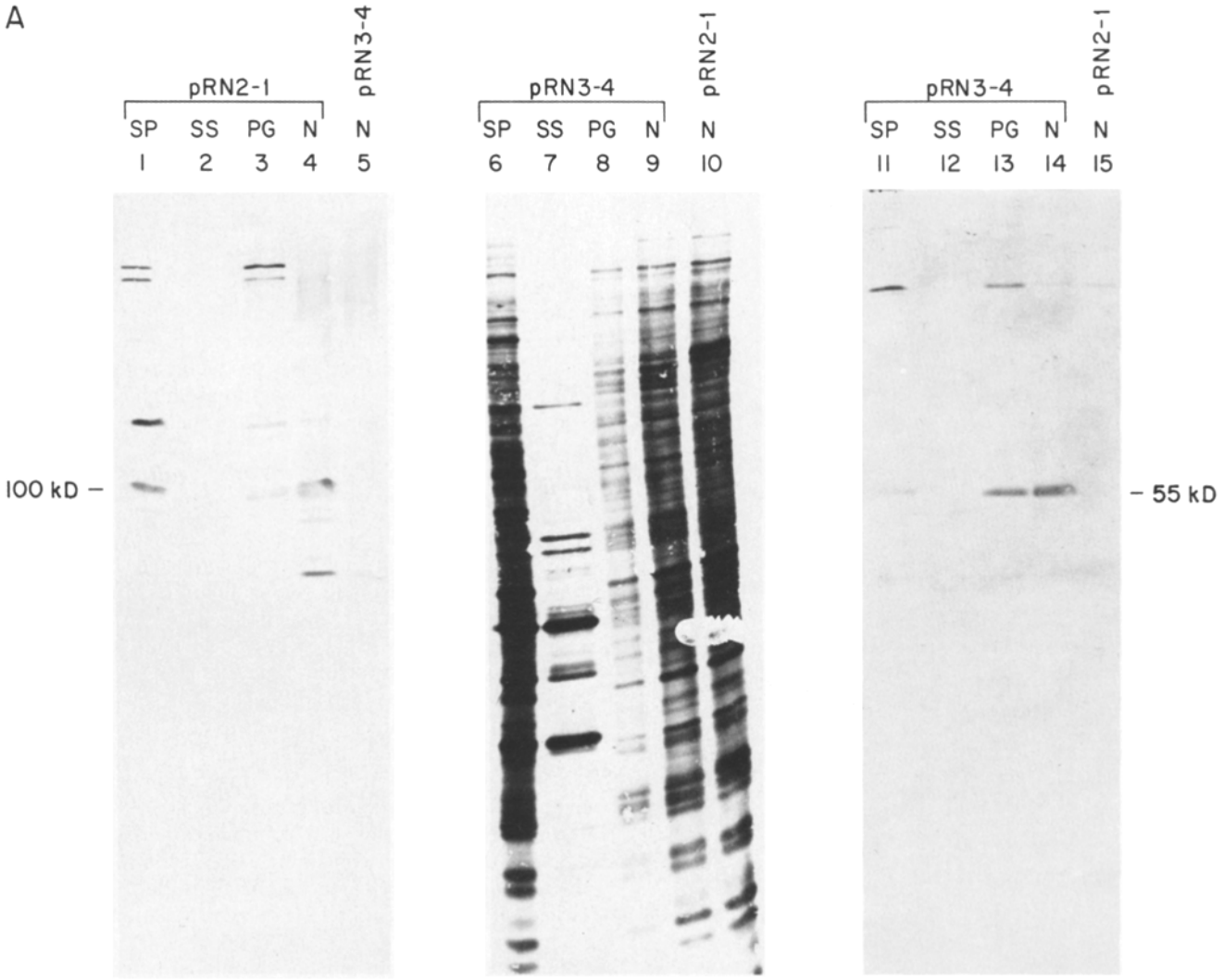
Identification of RNA2 and RNA3 Proteins in Yeast Whole-Cell Extracts

RNA2 and *RNA3* mRNAs are overproduced 100–200-fold in strains transformed with the high copy number recombinant plasmids containing the appropriate structural genes pRN2-1 (pJDB207) and pRN3-3 or pRN3-4 (pJDB207) (Last et al., 1984, Last, R. L., and J. L. Woolford, submitted and unpublished results). The data in Fig. 3 demonstrate that the *RNA2* and *RNA3* proteins are also overproduced in the high copy number transformants. Whole-cell extracts from high copy number recombinant plasmid and vector-transformed strains were subjected to SDS PAGE and immunoblot analysis (Towbin et al., 1979). A polypeptide of 95–100 kD apparent molecular mass is overproduced in RL117 transformed with pRN2-1 (pJDB207) (cf. Fig. 3, lanes *1* and *2*) and a protein of 55–58 kD apparent molecular mass is overproduced in RL117 transformed with pRN3-4 (pJDB207) (cf. lanes *3* and *4*). These polypeptides were not detectable with preimmune sera (Last, 1986). The responses of these sera are not strong enough to unambiguously detect cross-reactive material in extracts made from cells producing wild-type levels of protein. This made it necessary to use high copy number transformants in all subsequent experiments.

The RNA2 and RNA3 Proteins are Localized in the Yeast Nucleus

The subcellular localization of the *RNA2* and *RNA3* polypeptides in cells overproducing them was probed by biochemical fractionation of yeast cells. Subcellular fractions enriched for nuclei (i.e., fractions containing DNA as assayed by DAPI staining) were isolated by two methods that rely on different separation principles (Potashkin et al., 1984; Fisher et al., 1982, and Wu, L., and P. Fisher, personal communication). The nuclear enrichment protocol reported by Potashkin and co-workers (1984) subjects spheroplasts lysed in a hypotonic buffer of pH 3 to centrifugation in Percoll. In the modification of the protocol of Fisher et al., 1982 (and Wu, L., and P. Fisher, personal communication) spheroplasts were lysed in a buffer containing 1.0% Triton X-100 and extracted thoroughly by Dounce homogenization. A pellet enriched for nuclei was obtained by centrifugation of this extract.

Fig. 4 shows protein blots of subcellular extracts of yeast strains overproducing *RNA2* or *RNA3* antigens probed with anti-*RNA2* or -*RNA3* serum, or stained with India ink. Several major India ink staining species that were present in the whole spheroplast extracts were greatly reduced in representation in the Percoll gradient nuclear-enriched fraction (Fig. 4 *a*). These nuclear-enriched fractions also contained approximately two- to threefold less total protein than did spheroplast fractions. The higher molecular mass species detected by the anti-*RNA2* sera in the spheroplast lysate and pregradient fractions of strains overproducing *RNA2* antigens were also detected by anti-*RNA3* sera in the identical fractions of strains overproducing *RNA3*. These extra bands were not detected when affinity-purified antisera were used, suggesting they are not specific to *RNA2* or *RNA3*. We estimate that the *RNA2* and *RNA3* antigens are present in two- to threefold higher levels in these nuclear-enriched extracts. Nuclear-enriched fractions prepared by extraction of spheroplasts in 1.0% Triton X-100 do not show dramatic differences in the spectrum of proteins as compared with spheroplast lysate (Fig. 4 *b*). The total amount of stainable protein is approximately fivefold lower in the nuclear fractions than in the spheroplast lysate and the detectable antigen levels are one- to twofold higher. Thus, both techniques produced nuclear-enriched fractions that are enriched 5–10-fold for the *RNA2* and *RNA3* antigens compared with those seen in total spheroplast lysates. The India ink staining results are quite similar



to those seen in Coomassie-stained gels of fractions from both extraction protocols (Last, 1986).

Indirect immunofluorescence microscopy (Adams and Pringle, 1984) was used to obtain further information about the subcellular localization of the *RNA* gene products. Anti-tribrid protein antibodies were affinity-purified using nitrocellulose-bound fusion proteins (Smith and Fisher, 1984). When these antibody preparations were used as primary antibodies for indirect immunofluorescence microscopy, the *RNA2* or *RNA3* antigens were detected in strains that overproduced the appropriate protein. Figs. 5 and 6 show that the FITC staining of *RNA2* and *RNA3* antigens is localized to the nuclei of the yeast cells, which are stained by DAPI. No nuclear immunofluorescence staining was observed in cells when primary or secondary antibody incubations were omitted from the protocol (data not shown), or in cells that did not harbor the appropriate high copy number plasmids, i.e., cells overproducing *RNA2* or *RNA3* that were incubated with anti-*RNA3* or anti-*RNA2* serum, respectively (data not shown).

Discussion

A detailed understanding of the mechanism of pre-mRNA processing requires identification of macromolecules that participate in the attendant reactions. The yeast *RNA2* through *RNA11* gene products represent good candidates for proteins involved in pre-mRNA processing, since temperature-sensitive *rna* mutants are unable to process nuclear-encoded pre-mRNAs at the nonpermissive temperature. To continue our analysis of the roles of these gene products in pre-mRNA processing, we have raised antisera that react with the *RNA2* and *RNA3* gene products. These sera have been used to identify the *RNA2* and *RNA3* proteins in yeast extracts and to demonstrate that they are localized to the yeast nucleus. Nuclear localization of *RNA2* and *RNA3* antigens was demonstrated by two different subcellular fractionation techniques, and by indirect immunofluorescence microscopy.

The conclusion that the immunologically identified polypeptides correspond to the *RNA2* and *RNA3* gene products is based upon a number of criteria. The antisera were raised against *ompF-RNA-lacZ* tribrid proteins whose only known yeast epitopes are from the *RNA2* or *RNA3* protein coding regions (Last, 1986). Laboratory strains of *S. cerevisiae* do not have detectable β-galactosidase activity or cross-reactive material (Silver et al., 1984). The *RNA2* and *RNA3* polypeptides were overproduced in strains transformed, respectively, with the high copy number plasmids pRN2-1 or pRN3-4 (pJDB207). These plasmids contained only one detectable

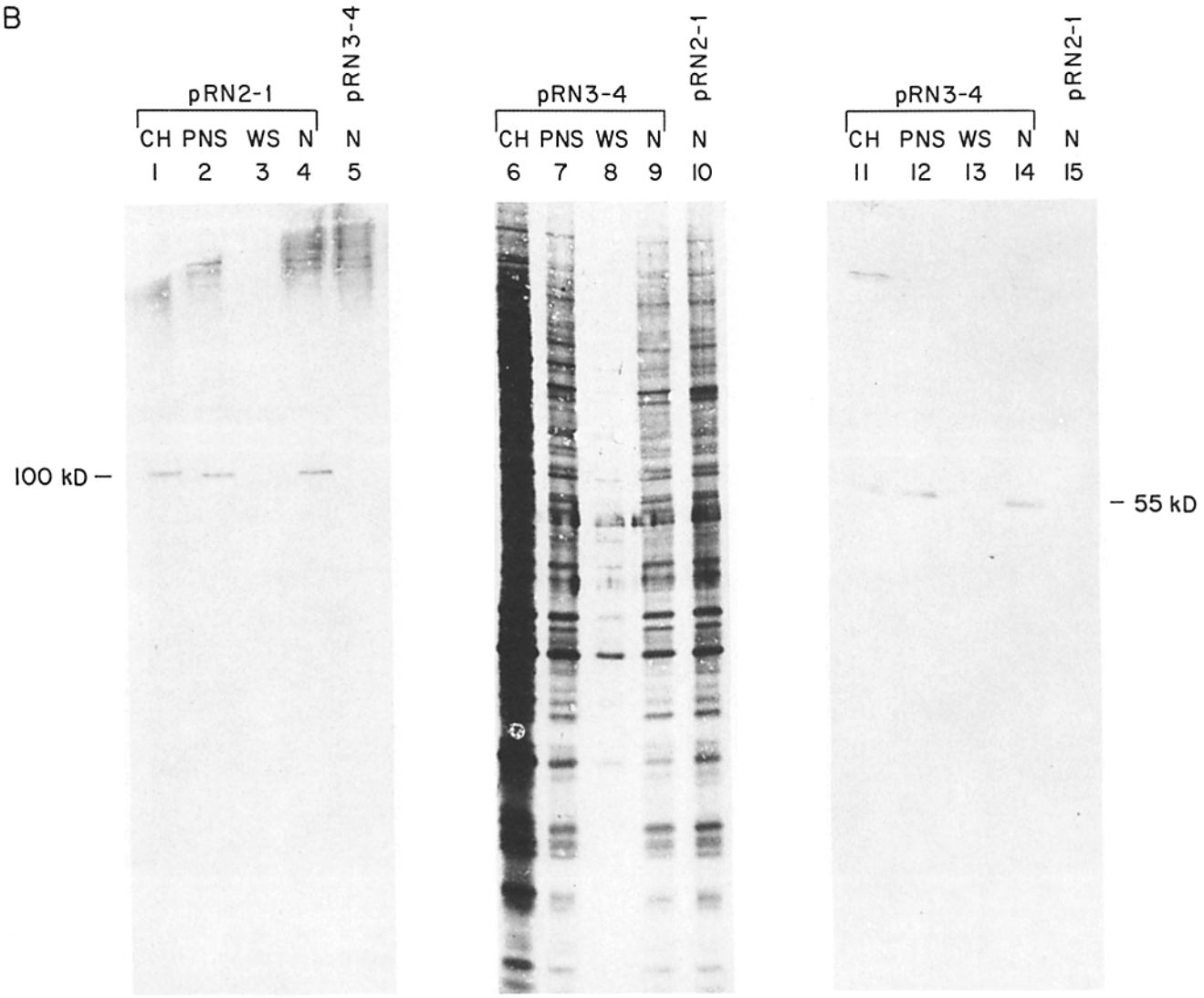


Figure 4. The *RNA2* and *RNA3* proteins are enriched in nuclear-enriched extracts. Subcellular fractions were subjected to electrophoresis on 8% (lanes *1–5*) or 10% (lanes *6–15*) SDS-polyacrylamide gels and blotted to nitrocellulose. The blots were then immunologically treated for detection of *RNA2* antigen (lanes *1–5*), or *RNA3* antigen (lanes *11–15*) using peroxidase staining. Lanes *6–10* were treated with India ink to visualize total protein. (*A*) Fractions from the acid-hypotonic lysis and Percoll gradient method. Lanes *1*, *6*, and *11*, 20 μl (the equivalent of 0.8 ml culture) of whole spheroplast lysate (SP); lanes *2*, *7* and *12*, 40 μl (the equivalent of 1.6 ml culture) of spheroplast supernatant (SS); lanes *3*, *8* and *13*, 20 μl of prePercoll gradient fraction (PG), and lanes *4*, *5*, *9*, *10*, *14*, *15*, 20 μl (the equivalent of 6 ml culture) of nuclear-enriched fraction (N). It is impossible to estimate the relative volume of culture contributed by the PG since boiling the Percoll-containing sample in sample buffer created a gelatinous material. This led to loss of a large amount of sample volume. (*B*) Fractions isolated by the differential solubilization method of Wu and Fisher. Lanes *1*, *6* and *11*, 5 μl (the equivalent of 8 ml culture) crude homogenate (CH); lanes *2*, *7* and *12*, 5 μl (the equivalent of 8 ml culture) postnuclear supernatant (PNS); lanes *3*, *8* and *13*, 5 μl (the equivalent of 2 ml culture) first wash supernatant (WS1), and lanes *4*, *5*, *9*, *10*, *14* and *15*, 5 μl (the equivalent of 8 ml culture) nuclear-enriched pellet (N).

transcription unit large enough to encode proteins of the observed size (Last et al., 1984).

The anti-*RNA2* sera identified a polypeptide of 95–100 kD as the *RNA2* polypeptide. This molecular mass is consistent with the 2,500–3,000 nt size of the mRNA reported in the literature (Last et al., 1984; Lee et al., 1984; Soltyk et al., 1984). Lee et al. (manuscript submitted for publication) have identified a protein of 97–105 kD using either antisera directed against a peptide from the predicted *RNA2* amino terminus, or antisera raised against fusion protein containing 151 amino acids from the middle third of the predicted *RNA2* ORF. Our *RNA2* ORF1 fusion protein contains a region of the *RNA2* protein distinct from those used by Lee et al. (J. Beggs, personal communication). Thus, antibodies raised against three separate parts of the *RNA2* polypeptide react with a protein species of similar molecular mass. The 55–58 kD molecular mass of the *RNA3* antigen identified in this report is consistent with that predicted from the *RNA3* transcript size of 1,500 nt (Last et al., 1984), and the size of the ORF within the *RNA3* gene (56 kD), identified by DNA sequence analysis (Anthony, J., and J. Woolford, unpublished results). Thus, we are confident that the immunologically identified proteins are indeed the *RNA2* and *RNA3* products.

Subcellular fractionation of yeast spheroplasts was undertaken by two methods. The first technique employed centrifugation of lysed spheroplasts through Percoll (Potashkin

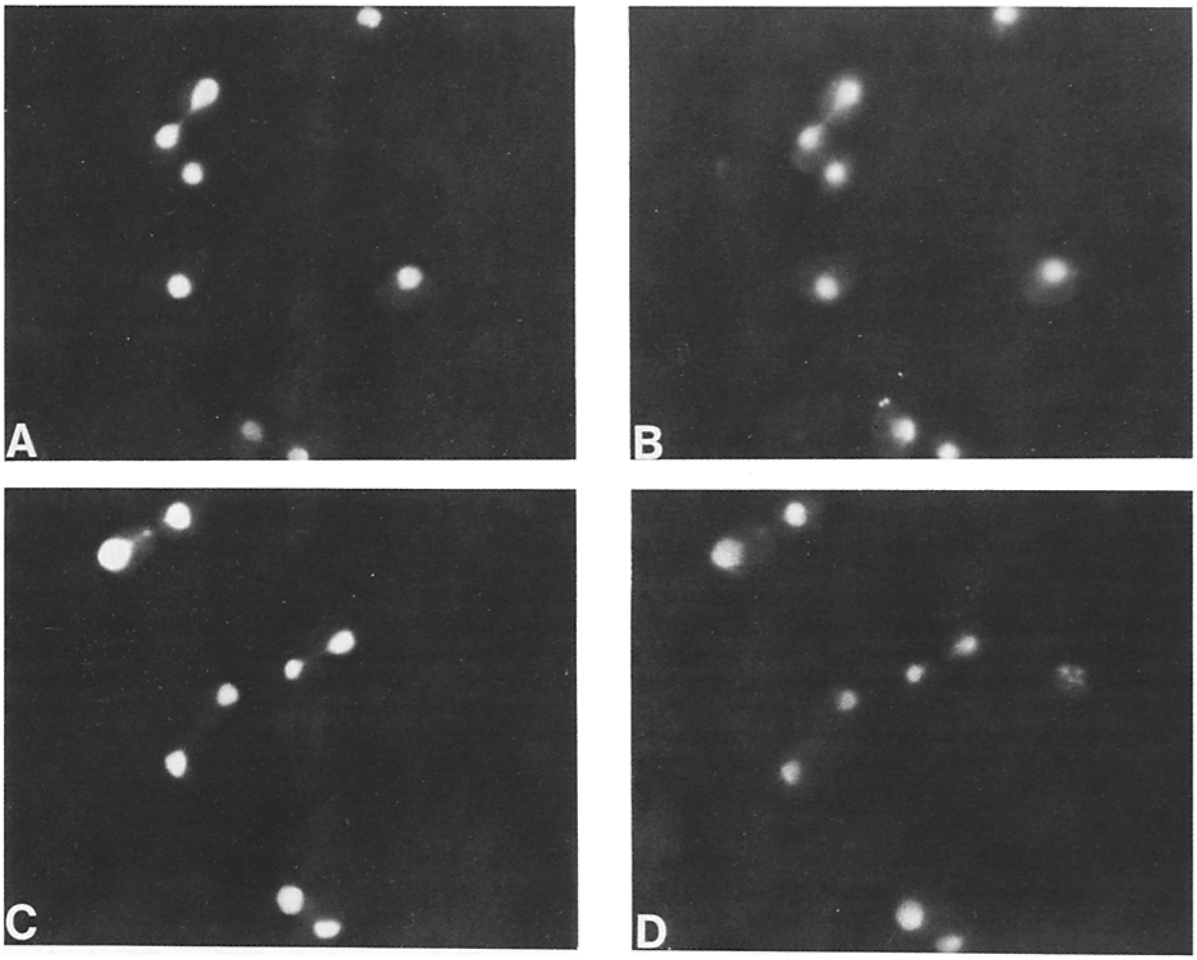


Figure 5. Immunofluorescence microscopy of yeast cells showing nuclear-associated *RNA2* antigen. Yeast cells that were overproducing the *RNA2* antigen were prepared for indirect immunofluorescence microscopy and treated with affinity-purified rabbit anti-*RNA2* ORF1, followed by biotinylated anti-rabbit IgG, and then incubated with FITC-conjugated streptavidin to identify the *RNA2* protein (*A* and *C*). Cells in *B* and *D* were also treated for DAPI fluorescence to identify the cell nuclei. The strain employed was RL117 containing pRN2-1 (pJDB207).

et al., 1984). The method of Wu and Fisher (personal communication) employed differential extraction with 1% Triton. Both methods yielded a 5–10-fold enrichment of *RNA2* and *RNA3* antigens in nuclear-enriched fractions. Unlike the Percoll gradient method, the postnuclear supernatant obtained by centrifugation of spheroplasts lysed at high cell density in 1% Triton X-100 contained significant quantities of *RNA2* and *RNA3* antigens. It is possible that some fraction of the nuclei lysed during this extraction, causing solubilization of the nuclear contents. Alternatively, the centrifugation and washing conditions used for nuclear pellet preparation might not have efficiently separated the particulate from soluble material. Comparison of the nuclear-enriched fractions obtained by the two methods indicate that the Triton extraction method was not as efficient at removing soluble proteins as the Percoll gradient procedure. Thus, there is probably a significant level of cross-contamination of cytoplasmic and nuclear components in the Triton extraction method fractions.

Indirect immunofluorescence results obtained with affinity-purified tribrid antibodies support the contention that most of the anti-*RNA2* ORF and anti-*RNA3* ORF cross-reactive materials are present in yeast nuclei. The heterogeneous staining of cells treated with anti-*RNA3* ORF1 might be due to incomplete permeabilization of the cell walls or the nuclear membranes of these cells, or differences in levels of antigens in individual cells due to variations in copy number of pRN3-3 (pJDB207) plasmid. Since cytoplasmic and nuclear tubulin staining were normal in cells prepared identically to those shown in Fig. 5, we feel it is probable that permeabilization of the cell walls and nuclei was fairly uniform (unpublished result).

The antisera described in this study were capable of yielding detectable reactions in immunoblot and immunofluorescence experiments only when strains that overproduced the *RNA2* or *RNA3* gene products were used. We attribute this to a combination of low titer antibodies and a low abundance of the *RNA2* and *RNA3* proteins in wild-type yeast cells; the *RNA* transcripts represent only 0.01–0.001% of polyadenylated RNA (Last et al., 1984). Thus, it has not been possible to determine the subcellular localization of these proteins in wild-type yeast cells.

Although it seems quite unlikely, our results for the localization of the *RNA* gene products could possibly be biased if these overproduced proteins existed in nonphysiological forms. Overproduction of proteins in *E. coli* is known to

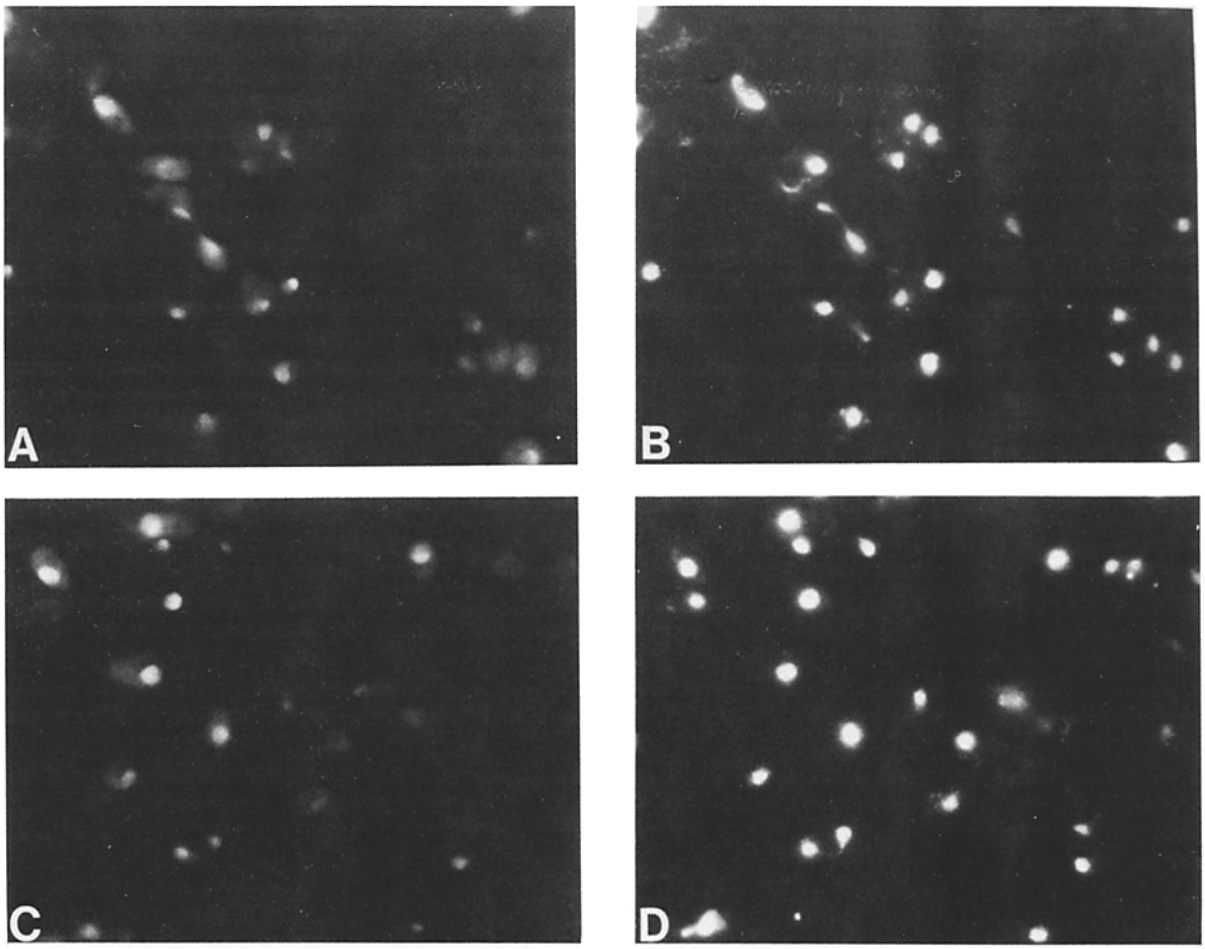


Figure 6. Immunofluorescence microscopy of yeast cells showing nuclear-associated *RNA3* antigen. Yeast cells that were overproducing the *RNA3* antigen were prepared for indirect immunofluorescence microscopy and treated with affinity-purified rabbit anti-*RNA3* ORF1, followed by biotinylated anti-rabbit IgG, and then incubated with FITC-conjugated streptavidin to identify the *RNA3* protein (*A* and *C*). Cells in *B* and *D* were also treated for DAPI fluorescence to identify the cell nuclei. The strain employed was RL117 containing pRN3-3 (pJDB207).

sometimes cause them to be found in an insoluble form. Chaotropic agents are required to render these normally soluble proteins soluble and active (Krippl et al., 1984). While it is possible that overproduction of the *RNA2* protein might cause it to become insoluble, it seems unlikely that much of the overproduced *RNA2* protein is being affected in this manner. R.-J. Lin has shown that extracts from cells containing the high copy number *RNA2* plasmid pRN2-1 (pJDB207) may be diluted 50–200-fold more than untransformed extracts and still complement the in vitro temperature-sensitivity of *rna2-1* extracts (Lin, R.-J., and J. Abelson, personal communication). No harsh treatment was required to render the excess *RNA2* activity accessible to the in vitro pre-mRNA processing components. Since pJDB207 recombinant plasmids commonly reach copy numbers of 50–200 per cell, it is unlikely that most of the overproduced protein is in the highly insoluble form characteristic of some overproduced proteins in *E. coli*.

Although we are not aware of any example of a nonnuclear yeast protein that shows inappropriate nuclear location when overproduced, this possibility cannot be ruled out. Analysis of the subcellular localization of *RNA2* and *RNA3* proteins in wild-type cells must await the isolation of higher quality antibody preparations. Higher titer antisera will also be necessary to improve the sensitivity of the assay for *RNA2* and *RNA3* proteins in splicing extracts. Members of this laboratory are currently attempting to raise higher titer antisera against the *RNA2* and *RNA3* proteins using immunogens that contain most of the *RNA* gene ORFs.

By analogy to mammalian systems, it is likely that most or all of the reactions in yeast pre-mRNA processing are associated with the nucleus (Hernandez and Keller, 1984; Krainer et al., 1984). The subcellular localization results obtained in this study are consistent with the *RNA2* and *RNA3* gene products being involved in pre-mRNA processing, but do not directly address the issue. The localization of the two proteins in the same compartment is also consistent with genetic evidence that many of the *RNA* genes have related functions in the yeast cell (Pearson et al., 1982; Last, R. L., and J. L. Woolford, manuscript submitted for publication). Now that yeast extracts capable of processing pre-mRNA in vitro and antisera against the *RNA2* and *RNA3* proteins are available it should be possible to determine whether these proteins are required for pre-mRNA processing. It will also be of interest to localize these polypeptides in the yeast cell more precisely, using biochemical and ultrastructural tech-

niques. We thus hope to gain a better understanding of the mechanism and site of pre-mRNA processing in eukaryotic cells.

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