The Yeast RNAI Gene Product Necessary for RNA Processing Is Located in the Cytosol and Apparently Excluded From the Nucleus

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Abstract. The yeast RNAI gene is required for RNA processing and nuclear transport of RNA. The rnal-1 mutation of this locus causes defects in pre-tRNA splicing, processing of the primary pre-rRNA transcript, production of mRNA and export of RNA from the nucleus to the cytosol. To understand how this gene product can pleiotropically affect these processes, we sought to determine the intracellular location of the RNAI protein. As determined by indirect immunofluorescence localization and organelle fractionation, the RNAI antigen is found exclusively or primarily in the cytoplasm. Only a tiny fraction of the endogenous protein could be localized to and functional in the nucleus. Furthermore, the RNAI antigen does not localize differently under stress conditions. These findings suggest that the RNAI protein is not directly involved in RNA processing but may modify nuclear proteins or otherwise transmit a signal from the cytosol to the nucleus or play a role in maintaining the integrity of the nucleus.

EuKaryotic genes are generally transcribed as precursor RNAs (pre-RNAs) that are processed to mature RNA species. Processing consists of removal of extra 5', 3', or intervening sequences as well as addition of nucleotides and modification of nucleosides. With the exceptions of processing 20S pre-rRNA to mature 18S rRNA (for review see Warner, 1989), and addition of some modifications of the anticodon loop of tRNAs (for review see Bjork et al., 1987), all processing steps appear to occur before transport of RNAs from the nuclear to the cytosolic compartments.

The gene products that participate in RNA processing are largely undescribed. However, the approaches of purification of enzyme activities (for example Phizicky et al., 1986), use of autoimmune sera (for review see Padgett et al., 1986), and identification of mutations of yeast that block particular processing steps (for review see Woolford, 1989; Culbertson and Winey, 1989) have all contributed to a rapidly growing work concerns the characterization of the wild-type counterpart of yeast genes identified by mutation.

Many Saccharomyces cerevisiae genes encoding products necessary for the processing of mature RNA species have been identified among collections of conditionally lethal yeast mutants (Hartwell et al., 1967; Vijayraghavan et al., 1989). The prp2-prpl1 alleles (Rosbash et al., 1981; Fried et al., 1981; previously these alleles were designated rna2-pral1), prpl4 (Couto et al., 1987) and several of the prpl7-prp27 lesions (Vijayraghavan et al., 1989) affect removal of intervening sequences from pre-mRNAs. Many of the PRP genes encode proteins that either affect the assembly of pre-mRNA onto spliceosomes (Lin et al., 1987) or are components of snRNPs and presumably spliceosomes (Lossky et al., 1987; Chang et al., 1988; Bjorn et al., 1989; Banroques and Abelson, 1989). The protein products of some of these genes have been localized to the nucleus (Chang et al., 1988; Last and Woolford, 1986). The RNAI gene resembles the PRP genes in that a mutation of this gene, rnal-1, affects RNA processing. There is also genetic evidence that relates the RNAI gene to the PRP genes because mutations of the SRNI locus suppress phenotypes of some of the prp mutations as well as the rnal-1 mutation (Pearson et al., 1982; Nolan, S. L. and A. K. Hopper, unpublished observations). However, there are distinctions between the RNAI gene and the PRP genes. The rnal-1 mutation pleiotropically affects all classes of RNA, tRNA at the step of removal of intervening sequences (Knapp et al., 1978; Hopper et al., 1978), ribosomal RNA at the step of processing the primary transcript (Hopper et al., 1978), and all mRNAs at a step(s) that is not well described (Hutchison et al., 1969; Shiokawa and Pogo, 1974; St. John and Davis, 1981). A recent study shows that the poly(A) tails of mRNAs generated at the nonpermissive temperature are increased in length (Piper and Aamand, 1989). The rnal-1 mutation also affects transport of RNA from the nucleus to the cytosol (Hutchison et al., 1969; Shiokawa and Pogo, 1974), although this may be a secondary consequence of RNA processing defects. Conversely, mutations of PRP loci do not appear to affect pre-tRNA splicing (Hopper, A. K., unpublished results) and affect only mRNAs coded for by genes that contain an intron (Rosbash et al., 1981). Because the rnal-1 mutation causes pleiotropic defects in RNA production, it has been
thought that RNAi might code for a component common to several RNA processing reactions, a regulator of genes coding for processing products, or might be involved in the general structure of the nucleus (Hopper et al., 1980; Atkinson et al., 1985).

One approach to understanding the role of RNAi in RNA processing pathways is to localize the RNAi protein in yeast. Based upon the proposed models of the RNAi protein function, we expected this gene product to be found exclusively within the yeast nucleus and perhaps near the inner nuclear membrane. In previous studies we cloned (Atkinson et al., 1985) and sequenced (Traglia et al., 1989) the RNAi gene. This work provided the tools and information to generate anti-RNAi sera. Using such antisera, we show that the RNAi protein is present in the cytoplasm and appears to be excluded from the nuclear compartment. These results are unexpected and have forced us to reevaluate the models of RNAi function in RNA processing.

Materials and Methods

Strains, Media, and Genetic Methods

The yeast strains used in this study are described in Table I. These strains were grown in either YEPD, complete minus uracil or complete minus uracil and leucine, formulated as described previously (Hurt et al., 1987). Bacterial strain RRI (F- pro leu thi lacI2345 m-1, hsdR17 m-1 end1) was used for all manipulations except for construction of the OmpF-RNAi/-LacZ fusion. To generate this plasmid, Escherichia coli strains MH3000 and TK1046 were used as described by Weinstock et al. (1985). L-broth and TY-amp solid media were formulated as described previously (Maniatis et al., 1982).

Yeast cells and bacteria were transformed according to the method of Ito et al. (1983) and Maniatis et al. (1982) respectively.

DNA Manipulations and Plasmid DNAs

Restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, MD; New England Biolabs, Inc., Boston, MA; Boehringer Mannheim Biochemicals, Indianapolis, IN) were used as prescribed by the manufacturers. Ligations using T4 DNA ligase (Bethesda Research Laboratories) were carried out in the buffer supplied by the manufacturer at 23°C for 4 h. A variety of yeast E. coli shuttle vectors were employed. YEps24 is a URA3-containing vector that achieves multiple copies in yeast (Botstein et al., 1979). YEpRNAi and YEpRNA1l are derivatives of YEp24 that contain genomic fragments encoding the wild-type RNAi and mutant rnal-1 alleles, respectively (Atkinson et al., 1985). YCpRNAi is a CEN3-containing single- or low copy plasmid that harbors genomic RNAi sequences; YEpRNA1(1.4) was derived from pMac561, a TRPl-containing multicopy vector with a cDNA encoding RNAi regulated by the ADH1 promoter (Atkinson et al., 1985). YEpRNA1(1.4) leads to high expression of RNAi in yeast cells. pFB1-7a and pFB1-67a are multicopy plasmids that contain the yeast LEU2 gene and gene fusions of sequences encoding yeast histone H2B and E. coli β-galactosidase (Moreland et al., 1987). pFB1-7a contains 7 codons of H2B fused in-frame to LacZ and encodes a H2B-β-galactosidase chimeric protein that is located in yeast cytoplasm; pFB1-67a contains 67 codons of H2B fused in-frame to LacZ and encodes a chimeric protein that translocates to yeast nuclei (Moreland et al., 1987).

YEpRNA1(187)/LacZ was derived from pFB1-7a as diagrammed in Fig. 1, A and B. The H2B2-containing fragment of pFB1-7a was removed by digestion with Sma1 and Bam HI. A region extending from a Dra I site upstream of the RNAi ORF to a Bam HI site at codon 187 was ligated into the Sma1-Bam HI-digested pFB1-7a. This created an in-frame fusion of RNAi to LacZ.

pORF1-RNA1, a derivative of pORF1 (Weinstock et al., 1983) contains in-frame fusion of E. coli OmpF, an internal portion of the yeast RNAi gene, and LacZ. To generate pORF1-RNA1 a Dpn I-Hinc II restriction fragment from an RNAi-containing plasmid was inserted into pORF1 at the Sma1 site (Fig. 1, B and C).

Protein Extraction and Blot Analysis

To purify the OmpF-RNAi-β-galactosidase trihybrid protein, pORF1-RNA1 was transferred to E. coli strain TK1046 that has the OmpFα allele. Trihybrid protein production was induced by temperature shift from 25 to 42°C as described by Weinstock et al. (1983). The OmpF-RNAi-β-galactosidase hybrid protein was extracted from 500 ml of culture as described by Last and Woolford (1986) except that the purification was terminated after resuspension and boiling of the 2% Triton X-100 washed pellet. As determined by SDS-PAGE analysis the vast majority of protein at this step was the desired fusion protein (data not shown). Yeast cell protein extracts were prepared by modifications of the procedures described by Hopper et al. (1974) or Schultz et al. (1987) as indicated in the figure legends. Protein blot analysis followed the procedure of Towbin (1979).

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<th>Table I. Yeast Strains</th>
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Figure 1. Strategies for generating RNA1-LacZ and OmpF-RNA1-LacZ chimeric genes. (A) Restriction map of pFB1-7a, a yeast shuttle vector that contains the regulatory region and codons for the first seven amino acids for histone H2B (w) fused in-frame to Lacz (w) (Moreland et al., 1987). Lines from Fig. 1, A and B indicate the DraI to Bam HI restriction fragment from the RNAI locus (B) that was used to replace the H2B2-containing fragment deleted by digestion of pFB1-7a by Sma I and Bam HI. The resulting plasmid is YEprRNA1(187)-Lacz. (C) Restriction map of pORF1 (Weinstein et al., 1983). Lines extending from Fig. 1, B to C indicate the Dpn I/Hinc II fragment from RNAI that was inserted at the Sma I site of pORF1 to generate in-frame fusions with OmpF (m) upstream and LacZ (w) downstream. The resulting plasmid is pORF-RNAI (w) indicates sequences encoding an ampicillin resistance. Restriction sites: A, Ava I; B, Bam HI, C, Hinc II; D, Dra I; E, Eco RI; H, Hind III, L, Cla I; M, Sma I; N, Dpn I (There are multiple Dpn I sites in RNAI and only one is indicated); P, Pst I; S, Sst I; X, Xba I. For A and C, only the restriction sites relevant to generating the construction are indicated. ATG and TAG indicate the beginning and end of the RNAI ORF.

Cell Fractionation

Yeast cells were fractionated by a modification of the method described by Silver et al. (1984). 300 ml of 2% x 3% cells containing YEpr24 and pFB1-47a or YEprRNA1 and pFB1-67a were grown to early log-phase in media lacking leucine and uracil. The cells were collected at 5,000 g for 10 min and were resuspended in 8 ml of 1 M sorbitol, 20 mM K2HPO4-KH2PO4, pH 6.5, 0.5 mM CaCl2, 0.5 mM 2-mercaptoethanol, 0.5 mM PMSE and the nuclear band was removed from the gradient, diluted 10-fold with buffer (Schultz et al., 1987) and served as the source of nuclear proteins. The pellet fraction was resuspended in 30 ml of 50% (wt/vol) Percoll, 40 mM Pipes, pH 6.8, 10 mM MgCl2, 0.5 mM PMSF, 0.05% Triton X-100 and centrifuged at 21,000 g for 35 min in a rotor (model SS34; Sorvall Div.). The nuclear band was removed from the gradient, diluted 10-fold with 0.01 M K2HPO4-KH2PO4, pH 6.5 and nuclei were collected by centrifugation at 5000 g for 5 min. This pellet was resuspended in 300 μl gel loading buffer (Schultz et al., 1987) and served as the source of nuclear proteins.

Antisera

The following antibodies were obtained commercially: rabbit anti-E. coli β-galactosidase IgG (Cappel Laboratories/Cooper Biomedical, Malvern, PA); mouse anti-E. coli β-galactosidase (Promega Biotech, Madison, WI); alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG, heavy plus light-chain specific (Cappel Laboratories/Organon Teknika and Promega Biotec); FITC-conjugated affinity-purified goat anti-rabbit IgG (Cappel Laboratories/Organon Teknika); and rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN). All commercial antibodies were used as supplied except for rabbit anti-β-galactosidase that was affinity-purified before use (see below).

Antisera specific to the RNAI protein were prepared by Pocono Rabbit Farm and Laboratory (Canadensis, PA). To generate sera against the Ompf-RNA1-β-galactosidase trityrid fusion protein, 50 μg of the semi-purified preparation were injected into two rabbits subcutaneously on day 0 and at 4 wk. The rabbits were injected intravenously at 6 wk and subcutaneously at 14 wk with 10 μg of the protein preparation. The serum obtained from a final bleeding at 16 wk of rabbit 5154 was used for the experiments described.

To generate sera against the RNAI COOH-terminal peptide, a synthetic peptide of sequence GDLAERLAETEIK conjugated to dipalmitoyl lysine carrier (Hopp, 1984) was synthesized by Biosearch (San Rafael, CA). 5 mg of this preparation was injected intradermally into two rabbits at day 0. At 2 wk, 1 mg was injected intradermally. At 4 wk and 8 wk, 1 mg of conjugated peptide was injected subcutaneously. The serum obtained from a bleeding of rabbit 6142 at 10 weeks was used for the studies reported here.

Anti-β-galactosidase and anti-OmpF-RNA1-β-galactosidase sera were affinity purified (Livingston, 1974). CNBr-Sepharose 4B was coupled to the protein ligand as described by Pharmacia Fine Chemicals (Piscataway, NJ). Chromatography proceeded as described by Kilmartin and Adams (1984).

The serum from rabbit 6142 could be used directly in immunofluorescence studies to detect amplified levels of RNAI antigen. However, using this serum we were unable to distinguish staining of cells processing an endogenous RNAI gene from the staining of cells possessing a deletion of the epitope. To improve sensitivity, the serum was purified by preadsorption. The cell walls from 10 ml of log-phase formaldehyde-fixed E. coli were removed by digestion with Glusulase and Zymolyase 5000 (see immunofluorescence, below). The spheroplasts were resuspended in 1.0 ml 0.73 mM KH2PO4, 145 mM NaCl, and 0.1% BSA. Antiserum was mixed with the resuspended fixed spheroplasts at a dilution of 1/100 and incubated with agitation for 4 h at 4°C. The cells and debris were subsequently removed from this pre-adsorbed serum by centrifugation. The specificity and titre of the pre-adsorbed antisera was verified by protein blot analysis (not shown). Preadsorbed sera could be used to detect endogenous levels of RNAI antigen via immunofluorescence.

Indirect Immunofluorescence

Indirect immunofluorescence experiments were carried out by modifications of the procedure described by Kilmartin and Adams (1984) and Moreland et al. (1987) and recently described in detail (Li et al., 1989). Variations upon this procedure for triple labeling experiments or to study heat-dependent location of RNAI antigen are given in the figure legends.

Results

Three approaches were taken to obtain antisera useful for determining the intracellular location of the RNAI protein. One approach was to generate RNAI-LacZ gene fusions that could be expressed in yeast. Antisera directed against β-galactosidase was used to locate the chimeric protein. A second approach was to construct a Ompf-RNA1-LacZ gene fusion that could be expressed in E. coli. This chimeric protein could then be purified from E. coli and used to generate anti-RNAI sera. The third approach was to use a synthetic peptide corresponding to a hydrophilic region of the RNAI ORF to obtain rabbit sera specific to this peptide.

RNAI-β-Galactosidase Fusion Protein Is Apparently Excluded from Yeast Nuclei

Parts of genes encoding proteins that are targeted to organelles have been fused to genes encoding passenger proteins. These fusions have been useful in yeast to determine the location of the first product as well as to map targeting...
information. For example, the E. coli LacZ gene has been used successfully for studies of mitochondrial (Douglas et al., 1984) and nuclear (Hall et al., 1984) targeting in yeast.

We used gene fusions of RNAI to LacZ to study the location of the RNAI gene product. These fusions used plasmid pFB1-7a (Moreland et al., 1987). pFB1-7a is an E. coli/yeast multicopy shuttle vector in which the histone H2B regulatory region directs synthesis of a histone-H2B-galactosidase chimeric protein containing seven amino acids of histone H2B. The H2B2 sequences of pFB1-7a were substituted by RNAI sequences to generate YEpRNAI(1-187)/LacZ. YEpRNAI(1-187)/LacZ contains the RNAI regulatory region and codons for the first 187 amino acids fused in-frame with LacZ (see Materials and Methods and Fig. 1).

Yeast strain SJ17 (relevant genotype: leu2-3,112) harboring YEpRNAI(1-187)/LacZ produces β-galactosidase antigen. The vast majority of the antigen was of the electrophoretic mobility expected for an RNAI-β-galactosidase chimeric protein as determined by protein blot analysis (not shown).

We used indirect immunofluorescence using affinity-purified rabbit anti-β-galactosidase and FITC-conjugated affinity-purified anti-rabbit antibody to localize the fusion protein within yeast. Yeast containing a related plasmid, pFB1-67a, containing the first 67 amino acids of histone H2B (Moreland et al., 1987) show subcellular staining to a compartment that also stains with DAPI, a stain specific to DNA (Fig. 2, A and B). As the nuclear targeting sequences for yeast histone H2B are between amino acids 28–33, the nu-
beads by three washes with 0.2 ml of the same buffer, immediately boiled for 3 min and frozen at -20°C. Proteins were resolved on a 5% stacking, 10% running SDS polyacrylamide gel. The blot was blocked, reacted with a 1/50 affinity-purified antifusion protein antibody, washed and then reacted with alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG at 1/1000 dilution. Lanes: 1, wild-type strain A364a; 2, strain AKH1008 (rnal-trpl-1); 3, strain AKH1008 containing vector pMac561; 4, strain AKH1008 with YEpcRNAI(1.4). (B) Analysis using anti-RNA1 COOH-terminal peptide. Cells were grown to midlog phase under selective conditions. If heat treatment was applied, the culture was split and one-half of the culture was maintained at 23°C and the other half was transferred to 37°C. After 1 h, the cells were quickly collected, washed one time with 23 or 37°C water (as appropriate), suspended in breaking buffer (Schultz et al., 1987) and quickly frozen. Preparation of cell lysates was as described by Schultz et al., (1987) except that supernatant and pellet fractions were not separated. Equal micrograms of proteins (except for lanes 4 and 5, which have twice the quantity) were loaded onto two 5% stacking/10% running SDS polyacrylamide gels; one gel was stained with Coomassie blue and the proteins on the other were electrophoretically transferred to membranes. The blots were first reacted with 1/1000 dilution of sera from rabbit 6142 and then with 1/1000 dilution of affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG. Lanes: 1, strain EElb-Δ359-397 that is missing the COOH-terminal epitope; 2, EElb harboring YEpcRNAI (single copy plasmid containing RNA1-); 3, EElb harboring YEpcRNAI; 4, EElb-Δ359-397 (containing a deletion causing temperature-sensitive growth) incubated at the nonpermissive temperature (37°C); 5, same as 4 except cells maintained at permissive temperature; 6 and 7, EElb-6 (rnal-1) grown at nonpermissive and permissive temperatures respectively; 8 and 9, EElb-35 (RNA1) grown at 37°C and 23°C, respectively. →, position of RNA1 antigen; ↔, position of antigen produced by the deletion allele rnal-Δ359-397.

clear location of the H2BI-67-β-galactosidase fusion protein is consistent with previous studies (Moreland et al., 1987). As previously reported, yeast containing the parental plasmid pFB1-7a that does not contain information for nuclear targeting show immunofluorescence staining in the cytosol (Moreland et al., 1987), but very little staining in the nucleus (Fig. 2 D), a staining pattern we refer to as "black holes." Because whole cells are used in these immunofluorescence studies one views the nucleus through the cytosol. Thus, even if there is no antigen in the nucleus, some staining is expected from the cytosolic contribution above and below the nucleus. A similar staining pattern is seen for cells harboring YEpcRNAI(1-187)/LacZ. Although the cytosol stained intensely (Fig. 2 F), the nuclear region (Fig. 2 E) consistently possessed very little cross reactivity to anti-β-galactosidase. That is, it appeared that the RNA1-β-galactosidase fusion protein like the H2BI-7-β-galactosidase fusion protein was present in the cytosol, but not in the nucleus.

It is possible that the RNA1-β-galactosidase chimeric protein is not localized to the same compartment as authentic RNA1 protein. The fusion may not have included sequences, should they exist, of RNA1 that would specify nuclear targeting. Nuclear targeting sequences are not restricted to partic-
ular regions of proteins and indeed some have been localized to the carboxy terminus (for review see Dingwall and Laskey, 1986). Alternatively, since the RNA1-LacZ sequences were contained on a multicopy yeast plasmid, overexpression of this sequence could lead to mislocalization in the cell. To address both of these problems we constructed chromosomally located fusions containing the entire RNA1 gene. Unfortu-

nately, such constructs expressed very low levels of β-galac-
tosidase and were insufficient for immunofluorescence studies.

**Authentic RNA1 Protein Cannot be Detected in Nuclei by Indirect Immunofluorescence**

Due to problems in localizing the RNA1 protein by employing fusion proteins, we sought to obtain sera that would recognize authentic RNA1 protein. First, a RNA1 fusion that could be expressed in E. coli was generated. The pORF1 vectors are designed so that foreign sequences are inserted between bacterial OmpF and LacZ sequences such that trityrid fusion proteins are generated (Weinstock et al., 1983). The OmpF sequences served to target the trityrid protein to the bacterial outer membrane to simplify purification. pORF1-RNA1 contains a Dpn I-Hinc II restriction fragment encoding 102 codons of the RNA1 gene (see Materials and Methods and Fig. 1). This fragment was chosen because it would create in-frame fusions with both OmpF and LacZ sequences and because the peptide was predicted to be hydrophilic (Kyte and Doolittle, 1982). The resulting plasmid was transferred to E. coli strain TK1046 that has the OmpR, allele and trityrid protein production was induced (Weinstock et al., 1983). The trityrid protein was purified to near homogeneity and used to inject rabbits.

The serum from one rabbit, 5154, showed cross-reactivity with RNA1 protein when overexpressed. Proteins extracted from strain A364a (relevant genotype: RNA1), AKH1008 (relevant genotype rnal-1 trpl-1) or AKH1008 containing vector (pMac561) sequences showed no cross-reactivity with the antibody (Fig. 3, lanes 1, 2, and 3). However, proteins
from strain AKH1008 containing YEpcRNA1(1.4) that carries a cDNA copy of RNA1 inserted into pMac561 (Atkinson et al., 1985) showed a cross-reacting species at the molecular weight calculated for the RNA1 ORF (Fig. 3 A, lane 4). Therefore as analyzed by protein blot analyses of crude extracts, the antisera can recognize the RNA1 protein only when it is amplified in yeast cells.

Serum 5154 was affinity-purified and used as the primary antibody for indirect immunofluorescence studies. No RNA1-specific staining could be detected when cells containing only the chromosomal rna/-1 allele were used (Fig. 4 B). However, the same antibody did stain cells harboring the multicopy YEpcRNA1 plasmid. The staining pattern showed black holes (Fig. 4 D) in the nuclear region (Fig. 4 C) and was indistinguishable from the pattern obtained using anti-/3-galactosidase to detect RNA/-/3-galactosidase chimeric protein. Therefore, the results of studies using antibody to the authentic protein also indicated that the vast majority of RNA1 protein was located in the cytosol.

Although the antibody raised against the trihybrid protein allowed detection of the authentic RNA1 protein, it was not of sufficient titer to detect endogenous levels of protein from a chromosomal copy of RNA1. Thus another strategy to obtain high-titer sera was tried. Previously we reported that COOH-terminal codons 397-407 of the RNA1 ORF were non-essential (Traglia et al., 1989). A synthetic peptide corresponding to these codons was employed to raise anti-RNA1 sera for two reasons. First, this peptide corresponds to a hydrophilic COOH-terminal portion of the RNA1 ORF (Kyte and Doolittle, 1982). Second, if a high-titer sera was obtained, then yeast cells possessing an rna/- allele deleted for these residues (i.e., RNA1Δ397-407) would generate protein lacking the epitope of this peptide and, therefore, would serve as a negative control for localization studies.

The peptide GDLAERLAETEIK was used to obtain specific sera (see Materials and Methods). We used a congeneric set of strains to verify the specificity of sera from rabbit 6142 and to localize the RNA1 antigen by indirect immunofluorescence and organelle fractionation. Yeast strains EEIb-Δ397-407, EEIb-35, EEIb-6, and EEIb-Δ359-397 are variants of EEIb (relevant genotype: rna/- 1 ura3-52; Table I) that were derived by gene replacements. EEIb-Δ397-407 harbors the rna/- allele that is deleted for the COOH-terminal codons corresponding to the peptide used to generate the an-
tisera, EEIb-35 harbors the wild-type RNAI allele, EEIb-6 contains the rnal-1 allele, and EEIb-Δ359-397 possesses a deletion of codons 359-397 (Traglia et al., 1989). EEIb strains containing YCpRNA1 and YEpRN possess single (or low) copy and multicopy vectors (5-20 copies), respectively harboring genomic RNAI sequences. Strains that harbor YEpcRNA1(1.4), a highly expressed cDNA copy of RNAI, have also been employed and results analogous to those for YEpcRNA1 were obtained (not shown). Proteins extracted from cells harboring the rnal-359-407 allele did not contain an antigen recognized by this sera (Fig. 3 B, lane 1). However, proteins extracted from EEIb-35 wild-type cells (Fig. 3 B, lanes 8 and 9) or EEIb-6 cells harboring the rnal-1 allele (Fig. 3 B, lanes 6 and 7) contained a cross-reacting species of the mobility expected for the RNAI protein. This signal increased in intensity when strain EEIb contained either low-copy YCpRNA1 (Fig. 3 B, lane 2) or multicopy YEpcRNA1 (Fig. 3 B, lane 3) plasmids. Furthermore, proteins extracted from EEIb-Δ359-397 cells possessing an internal deletion of 38 amino acids, produces a signal at an appropriate lower molecular weight (Fig. 3 B, lanes 4 and 5).

We conclude that serum from rabbit 6142 is specific for the RNAI protein and of high titer.

Preabsorbed 6142 serum was used for indirect immunofluorescence studies to localize the RNAI antigen within yeast cells. Cells harboring the rnal-359-407 allele show very little FITC staining (Fig. 5 B). The faint staining that can be detected appears to be localized to the cytosol and has a granular appearance. We have been unable to completely eliminate the FITC signal in these negative control cells. Wild-type cells (Fig. 5 D) or cells harboring the chromosomal rnal-1 mutation (not shown) show a staining pattern similar to that obtained in Figs. 2 and 4. That is, there is staining of the cytosol but the nuclear region (Fig. 5 C) is devoid of FITC signal. Although the RNAI antigen is spread throughout the cytosol, in some cells (arrows, Figs. 5, D and F) it appears to be concentrated around the nucleus. The signal increases as the number of the RNAI copies increases (compare Fig. 5, D, F, and H). With increased RNAI copy number, the signal appears more disperse and the black holes become more prominent. We have evidenced cell to cell variation in staining of cells with both RNAI and β-galactosidase specific antibodies (also see Figs. 2 and 6). This is due to variation in plasmid copy number, plasmid loss, and differential permeability of the cells to antibody.

To be certain that the region devoid of signal was the nucleus, we employed triple labeling procedures. Diploid strain 2b × 3b (relevant genotype; rnal-1/ranl-1, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112) was transformed with two plasmids: either YEp24 vector containing the UR33 gene and pFL1-67a that contains the histone H2B2-67 LacZ fusion and the LEU2 gene or YEpcRNA1 containing the RNAI and UR3 genes in combination with pFL1-67a. Cells were first stained with preabsorbed rabbit anti-RNAI peptide antibody and affinity-purified mouse anti-β-galactosidase and then with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG. The cells were also stained with DAPI. The region devoid of FITC (RNAI) stain appears larger than the region stained by DAPI (compare Figs. 6, A and D to C and F) as if the DNA does not occupy the entire nucleus. However, rhodamine (histone-β-galactosidase) stain occupies nearly the entire region not stained by FITC (compare in Figs. 6, B and E to C and F). Thus FITC and rhodamine stain mutually exclusive regions of the yeast cell. We conclude that the region apparently depleted of RNAI antigen is the nucleus.

RNA Antigens Do Not Fractionate with Nuclei

Cell fractionation was used to obtain an independent assessment of the subcellular location of the RNAI protein. Two yeast strains were used. The first strain is the variant of the diploid 2b × 3b that contains pFB1-67a, harboring LEU2 and 67 codons of H2B2 fused in-frame to LacZ, and vector YEp24 harboring URA3. The second strain is the 2b × 3b variant containing pFB1-67a and the RNAI multicopy plasmid, YEpcRNA1, instead of YEp24. Proteins from cell lysates and semipurified nuclei (see Materials and Methods) from the yeasts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with rabbit antibodies specific to β-galactosidase, the COOH-terminal peptide of the RNAI protein or the RNAI region incorporated into pORFI-RNAI. Alkaline phosphatase–conjugated goat anti-rabbit IgG was used as the secondary antibody. As can be seen in Fig. 7 A, lanes 6-8 and 10-12 and Fig. 7 B, lanes 6-8, semi-purified nuclear fractions, cytoplasm-enriched fractions, and total cell extracts all contain β-galactosidase antigen. Fig. 7 A also shows the fractionation of endogenous levels of RNAI protein as detected by the anti-peptide serum from rabbit 6142. The cytoplasm-enriched fraction (lanes 3 and 11) and total cell extracts (lanes 4 and 12) contain antigen, but there is no antigen detected in the purified nuclear fraction (lanes 1 and 10). Fig. 7 B shows the location of the RNAI antigen recognized by the antibody raised to the protein encoded by pORFI-RNAI. Because this antibody does not detect endogenous levels of RNAI antigen, the cells used for these fractionation studies contained a multicopy plasmid harboring the RNAI gene. Consistent with the results in Fig. 7 A, the antibody to the OMPF-RNAI-β-galactosidase fusion protein can detect RNAI antigen in cytoplasm-enriched fractions, and total cell extracts, but not in the nuclear fraction. Therefore, as assessed by two different RNAI-specific antisera, the location of endogenous or overproduced levels of the RNAI protein in subcellular fractions is cytoplasmic; no nuclear form of the RNAI antigen was detected in these studies. Thus, the results of the cell fractionation studies confirm the results obtained by indirect immunofluorescence.

The RNA Antigen Does Not Change Subcellular Distribution in Response to Heat Shock

There is precedence for proteins relocating within cells under certain stress conditions (Baeuerle and Baltimore, 1988; for review see Lindquist, 1986). We applied stress conditions to the yeast cells to determine if RNAI protein would translocate to a different subcellular location. Fig. 8 shows the results of a treatment of cells for 50 min at 37°C. As expected, cells possessing the rnal-359-407 COOH-terminal deletion allele show very little cross-reactivity with the preabsorbed antipeptide serum (Fig. 8, A and B). The location of wild-type RNAI protein is similar for cells grown at 23°C and heat treated cells (Fig. 8, E and F). Even though by pro-
Indirect immunofluorescence employing anti-β-galactosidase and anti-RNA1 COOH-terminal peptide affinity-purified antibodies. Yeast strain 2b × 3b harboring both pFB1-67a (H2B/β-gal fusion) and YEpRNA1 were grown in complete minus uracil and leucine medium to middle log phase, fixed, and prepared for immunofluorescence (see Materials and Methods). The cells were stained with 1/200 dilution affinity-purified, mouse anti-β-galactosidase and 1/100 dilution of preadsorbed 6142 serum as primary antibodies. 1/200 dilution of rhodamine-conjugated goat anti-mouse IgG and 1/200 dilution FITC-conjugated goat anti-rabbit IgG were used as secondary antibodies. After staining with the first and second antibodies, the cells were stained with DAPI. A, DAPI stain; B, rhodamine stain; C, FITC stain; D, E, and F, same as for A, B, and C except that a different field was photographed. 600×. Bar, 10 μm.

Figure 5. Indirect immunofluorescence using preadsorbed anti-RNA1 C-terminal peptide from rabbit 6142. (A) EEIb-Δ397-407 cells (deleted for the COOH-terminal peptide) with DAPI stain; (B) EEIb-Δ397-407 cells reacted with 1/100 6142 antibody; (C and D) EEIb-35 (RNAI) cells stained as in A and B; E and F, EEIb cells harboring YCpRNA1 (~1 copy/cell) stained as A and B; (G and H) EEIb cells harboring YEpRNA1 (5–20 copies/cell) stained as for A and B. The photographs for B, D, and F were all exposed and developed for the same time. H was exposed less time to accommodate the more intense staining. 600×. Bar, 10 μm. Arrows in D and F point to cells that show apparent preferential staining around the nucleus.
tein blot analysis we can detect no difference in rna1-1 protein in cells grown at 23°C or treated for 50 min at 37°C (Fig. 3 B, lanes 6 and 7), by immunofluorescence we detect very little rna1-1 protein after heat shock (Fig. 8, C and D). However, in no case have we been able to detect a nuclear location for wild-type or mutant RNA1 proteins.

**Discussion**

The RNA1 protein was expected to be localized in the yeast nucleus for two reasons: (a) the rna1-1 mutation is known to affect only nuclear processes, pre-tRNA and pre-rRNA processing, mRNA production, and RNA export to the cytosol; and (b) wild-type counterparts of other genes that generate mutant phenotypes similar to rna1-1 encode nuclear-localized proteins. However, by using indirect immunofluorescence and organelle fractionation studies, we have been unable to detect any RNA1 antigen within yeast nuclei. Rather, the antigen is located in the cytosol with only a hint of preferential concentration on the periphery of nuclei.

There are several possible interpretations of our results. First, one could propose that the majority of RNA1 protein is localized to nuclei only under special conditions. Such is the case for other gene products such as heat-shock proteins that translocate to nuclei under certain temperature regimes (for review see Lindquist, 1986) and NF-κB, a DNA binding protein, that translocates to the nucleus upon treatment of cells with phorbol esters (Baehner and Baltimore, 1988). Since rna1-1 and rna1-Δ359-397 cause heat-sensitive growth (Hutchison et al., 1969; Traglia et al., 1989), we used a heat-shock regime to determine whether wild-type or mutant RNA1 protein might become localized to the nucleus at elevated temperatures. The location of protein encoded by the wild-type RNA1 allele is not temperature dependent. The protein encoded by the mutant rna1-1 allele, was barely detectable by immunofluorescence after incubation of the mutant cells for 1 h at the nonpermissive temperature. It is not known why protein encoded by rna1-1 is not detected at high temperatures. However, it is possible that the mutant rna1-1 protein is denatured at high temperatures so that it cannot be recognized by antibody unless it is unfolded by the conditions used for PAGE analysis. Alternatively the protein may be unstable and degraded before it is fixed by formaldehyde. There is evidence of cellular redistribution of gene products at specific points in the cell cycle (for review see Hunt, 1989). It is unlikely that the RNA1 protein is located in nuclei only at specific points in the cell cycle because we have used nonsynchronous populations of cells; regardless of bud size the RNA1 antigen is located in the cytosol. It could be argued that the wild-type RNA1 protein might translocate to the nucleus upon some heretofore untested condition. Although this remains a formal possibility, it seems unlikely because the RNA1 protein is an essential product that is needed for normal mitotic growth (Atkinson et al., 1985).

Second, one could propose that the majority of RNA1 protein is localized to nuclei only under special conditions. Such is the case for other gene products such as heat-shock proteins that translocate to nuclei under certain temperature regimes (for review see Lindquist, 1986) and NF-κB, a DNA binding protein, that translocates to the nucleus upon treatment of cells with phorbol esters (Baehner and Baltimore, 1988). Since rna1-1 and rna1-Δ359-397 cause heat-sensitive growth (Hutchison et al., 1969; Traglia et al., 1989), we used a heat-shock regime to determine whether wild-type or mutant RNA1 protein might become localized to the nucleus at elevated temperatures. The location of protein encoded by the wild-type RNA1 allele is not temperature dependent. The protein encoded by the mutant rna1-1 allele, was barely detectable by immunofluorescence after incubation of the mutant cells for 1 h at the nonpermissive temperature. It is not known why protein encoded by rna1-1 is not detected at high temperatures. However, it is possible that the mutant rna1-1 protein is denatured at high temperatures so that it cannot be recognized by antibody unless it is unfolded by the conditions used for PAGE analysis. Alternatively the protein may be unstable and degraded before it is fixed by formaldehyde. There is evidence of cellular redistribution of gene products at specific points in the cell cycle (for review see Hunt, 1989). It is unlikely that the RNA1 protein is located in nuclei only at specific points in the cell cycle because we have used nonsynchronous populations of cells; regardless of bud size the RNA1 antigen is located in the cytosol. It could be argued that the wild-type RNA1 protein might translocate to the nucleus upon some heretofore untested condition. Although this remains a formal possibility, it seems unlikely because the RNA1 protein is an essential product that is needed for normal mitotic growth (Atkinson et al., 1985).

**Figure 7.** Location of RNA1 antigen in subcellular fractions. Cell fractions were prepared from 2b × 3b containing YEp24 (vector alone) and pFB1-67a (B) or 2b × 3b containing YEpRNA1 and pFB1-67a (B). Each panel contains four lanes: the first lane contains prestained fumarase, a protein of similar mobility to the RNA1 protein; the second lane contains proteins from semipurified nuclei (see Materials and Methods); the third lane contains proteins from a cytoplasm-enriched fraction (see Materials and Methods); and the fourth lane contains total cell extracts. Note that the nuclear fraction contains about one-tenth the amount of proteins as do the other two fractions. Proteins were resolved on a 5% stacking/8% running SDS polyacrylamide gel (Laemmli, 1970). The proteins were transferred by electrophoresis to nitrocellulose. The blots were preincubated for 4 h at room temperature in NET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.25% gelatin, 0.05% NP-40, 2% BSA). The blots were incubated with 5 ml of the following antibodies: (A) lanes 1-4, 1/5,000 dilution in NET buffer of 6142 antisera, specific to the RNA1 COOH-terminal peptide; lanes 5-8, 1/5,000 dilution affinity-purified rabbit anti-β-galactosidase; lanes 9-12, 6142 serum plus anti-β-galactosidase; (B) lanes 1-4, 1/250 dilution affinity-purified anti-RNA1 sera raised against the OmpF-RNA1-β-galactosidase trihybrid fusion protein, lanes 5-8, 1/5,000 dilution affinity purified anti-β-galactosidase. The blots were washed four times with PBS plus 0.1% NP-40 and 0.1% BSA. The blot was then incubated with 1/5,000 dilution in NET buffer of affinity-purified alkaline phosphatase- conjugated goat anti-rabbit IgG washed four times and then developed to detect the location of alkaline phosphatase (Towbin et al., 1979).
Figure 8. Location of the RNA1 antigen after heat-shock treatment. 20-ml cultures of EEib-Δ397-407, EEib transformed with YEpRNA1 and EEib transformed with YEpRNA1 cells in complete minus uracil medium were grown to early log phase. The cultures were split into two equal volumes. One half was maintained at 23°C and the other half was shifted to 37°C. 50 min later, the cells were quickly collected by centrifugation, resuspended in 23 or 37°C buffer, as appropriate and fixed with formaldehyde. Heat-shocked cells were fixed for 90 min at 30°C (a nonpermissive temperature for RNA1; higher temperatures resulted in poor antigen staining for all cells including β-galactosidase controls). Cells grown at 23°C were fixed for 2 h at room temperature. Immunofluorescence proceeded as described in Materials and Methods. (A) EEib-Δ397-407, 23°C; (B) EEib-Δ397-407, 37°C for 50 min; (C) EEib plus YEpRNA1, 23°C; (D) EEib with YEpRNA1, 37°C, 50 min; (E) EEib with YEpRNA1, 23°C; (F) EEib plus YEpRNA1, 37°C, 50 min.
Third, it is possible that a small percentage of the RNA1 gene product is localized to the nucleus and performs an essential function there. Even though we have been unable to locate any RNA1 protein in the nucleus we cannot conclude that a small fraction of the RNA1 protein, beyond our means of detection, does not reside in this compartment. There is precedence for proteins that are dually located in the nucleus and the cytosol with the majority of the protein residing in the cytosol. Ribosomal proteins are one such example. These proteins are structural components of cytosolic ribosomes, but also translocate to nuclei where they participate in ribosome assembly (for review see Warner, 1989). The RNA1 protein could also have such a dual function. For example, it could serve as a "chaperon" to target RNA out of the nucleus or to target components essential for pre-RNA processing into the nucleus.

Among the possible explanations for our data we favor models based on the premise that the major function of the RNA1 protein is where the majority of the antigen is located; that is, it is most likely that RNA1 protein functions in the cytosol. How might a cytosolic protein affect production and nuclear export of RNA? The RNA1 protein might modify and thereby regulate nucleus-targeted proteins or might otherwise be involved in transmitting a signal from the cytosol to the nucleus. If the RNA1 protein is involved in modification of nuclear proteins, the modification would need to be short lived, as the pre-RNA processing defect of rna/-1 cells is detected shortly after shift to nonpermissive temperature (Hutchison et al., 1969; Shiokawa and Pogo, 1974; Hopper et al., 1978). The RNA1 protein could serve as a cytoplasmic anchor that holds "nuclear" proteins in the cytosol until needed (for review see Hunt, 1989). In this case, also, one would need to propose that the quantities of RNA processing activities be tightly controlled in order to accommodate the rapid defect of rna/-1 cells. The RNA1 protein could also be involved in the integrity of nuclear structure. Alteration of the nuclear membrane would be expected to have pleiotropic consequences upon RNA metabolism and hence such a model could accommodate the pleiotropic defects in RNA metabolism inherent in rna/-1 cells.

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