The Yeast *RNA1* Gene Product Necessary for RNA Processing Is Located in the Cytosol and Apparently Excluded From the Nucleus

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Abstract. The yeast *RNA1* gene is required for RNA processing and nuclear transport of RNA. The *rna1-1* 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 RNA1 protein. As determined by indirect immunofluorescence localization and organelle fractionation, the RNA1 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 RNA1 antigen does not localize differently under stress conditions. These findings suggest that the RNA1 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 identification and understanding of these gene products. Our 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-prp11* alleles (Rosbash et al., 1981; Fried et al., 1981; previously these alleles were designated *rna2-rna11*), *prp14* (Couto et al., 1987) and several of the *prp17-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 *RNA1* gene resembles the *PRP* genes in that a mutation of this gene, *rna1-1*, affects RNA processing. There is also genetic evidence that relates the *RNA1* gene to the *PRP* genes because mutations of the *SRN1* locus suppress phenotypes of some of the *prp* mutations as well as the *rna1-1* mutation (Pearson et al., 1982; Nolan, S. L. and A. K. Hopper, unpublished observations). However, there are distinctions between the *RNA1* gene and the *PRP* genes. The *rna1-1* 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 *rna1-1* 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 *rna1-1* mutation causes pleiotropic defects in RNA production, it has been

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thought that *RNA1* 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 *RNA1* in RNA processing pathways is to localize the RNA1 protein in yeast. Based upon the proposed models of the RNA1 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 *RNA1* gene. This work provided the tools and information to generate anti-RNA1 sera. Using such antisera, we show that the RNA1 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 RNA1 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 RR1 (F- *pro leu thi lacY Str*^{r} *r*-$_k$ *m*-$_k$ *hsdR hsdM endoI*) was used for all manipulations except for construction of the *OmpF-RNA1-LacZ* fusion. To generate this plasmid, *Escherichia coli* strains MH3000 and TK1046 were used as described by Weinstock et al. (1985). L-broth and YT-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. YEp24 is a *URA3*-containing vector that achieves multiple copies in yeast (Botstein et al., 1979). YEpRNA1 and YEprna1-1 are derivatives of YEp24 that contain genomic fragments encoding the wild-type *RNA1* and mutant *rna1-1* alleles, respectively (Atkinson et al., 1985). YCpRNA1 is a *CEN3*-containing single or low copy plasmid that harbors genomic *RNA1* sequences; YEpcRNA(1.4) was derived from pMac561, a TRP1-containing multicopy vector with a cDNA encoding *RNA1* regulated by the *ADH1* promoter (Atkinson et al., 1985). YEpcRNA (1.4) leads to high expression of *RNA1* 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 (*H2B2*) and *E. coli* β-galactosidase (Moreland et al., 1987). pFB1-7a contains 7 codons of *H2B2* 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 *H2B2* fused in-frame to *LacZ* and encodes a chimeric protein that translocates to yeast nuclei (Moreland et al., 1987).

YEpRNA1(1-187)/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 Sma I and Bam HI. A region extending from a Dra I site upstream of the *RNA1* ORF to a Bam HI site at codon 187 was ligated into the Sma I–Bam HI-digested pFB1-7a. This created an in-frame fusion of *RNA1* 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 *RNA1* gene, and *LacZ*. To generate pORF1-RNA1 a Dpn I–Hinc II restriction fragment from an *RNA1*-containing plasmid was inserted into pORF1 at the Sma I site (Fig. 1, *B* and *C*).

Protein Extraction and Blot Analysis

To purify the OmpF-RNA1-β-galactosidase trihybrid protein, pORF1-RNA1 was transferred to *E. coli* strain TK1046 that has the $OmpR_{cs}$ allele. Trihybrid protein production was induced by temperature shift from 25 to 42°C as described by Weinstock et al. (1983). The OmpF-RNA1-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).

Table I. Yeast Strains

Strain	Genotype	Description/Source
A364a	*MATa gal1 ade1 ade2 ura1 his7 tyr1 lys2*	L. Hartwell; parent ts136: the original strain containing *rna1-1* (Hutchison et al., 1969)
SJ17	*MATα ura3-52 leu2-3, 112 gal4 his4*	J. E. Hopper
AKH1008	*rna1-1 trp1-1* (MAT, leu, his, ura and gal not tested)	Derived from SJ17 and 1C that was derived from ts136
EE1b	*MATa rna1-1 ura3-52 ade1 tryl his7 his4* Gal$^-$	Atkinson et al., 1985; derived from SJ17 and ts136
EE1b-35	*MATa RNA1 rnh1::URA3 ura3-52 his7 his4 ade1 tyr1* Gal$^-$	Traglia et al., 1989; derived from EE1b by gene replacement
EE1b-6	*MATa rna1-1 rnh1::URA3 ura3-52 his7 his4 ade1 tyr1* Gal$^-$	Traglia et al., 1989; derived from EE1b by gene replacement
EE1b-Δ397-407	*MATa rna1-Δ397-407 rnh1::URA3 his7 his4 ura3-52 ade1 tyr1* Gal$^-$	Traglia et al., 1989; *rna1-Δ397-407* allele is a deletion of codons corresponding to the peptide used to generate antipeptide sera; derived from EE1b by gene replacement
EE1b-Δ359-397	*MATa rna1-Δ359-397 rnh1::URA3 his7 his4 ura3-52 ade1 tyr1* Gal$^-$	Traglia et al., 1989; *rna1* allele is a deletion of codons 359–397 that causes temperature-dependent lethality; derived from EE1b by gene replacement
2bx3b	MATa/MATα, *ura3-52/ura3-52, leu2-3,112/ leu2-3,112, rna1-1/rna1-1, tyr1/TYR1,* His$^-$/His$^+$, Gal$^-$, Ade$^-$	Atkinson et al., 1985; derived from SJ17 and ts136

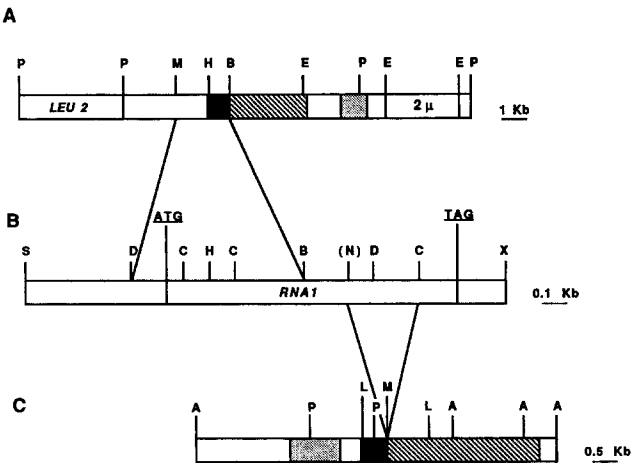


Figure 1. Strategies for generating *RNA1-LacZ* and *OmpF-RNA1-LacZ* chimeric genes. (*A*) Restriction map of pFB1-7a, a yeast shuttle vector that contains the regulatory region and codons for the first seven amino acids for histone H2B (■) fused in-frame to *LacZ* (▧) (Moreland et al., 1987). Lines from Fig. 1, *A* and *B* indicate the Dra I to Bam HI restriction fragment from the *RNA1* locus (*B*) that was used to replace the *H2B2*-containing fragment deleted by digestion of pFB-1-7a by Sma I and Bam HI. The resulting plasmid is YEpRNA1(1-187)/LacZ. (*C*) Restriction map of pORF1 (Weinstock et al., 1983). Lines extending from Fig. 1, *B* to *C* indicate the Dpn I/Hinc II fragment from *RNA1* that was inserted at the Sma I site of pORF1 to generate in-frame fusions with *OmpF* (▨) upstream and LacZ (▧) downstream. The resulting plasmid is pORF1-RNA1 (▨) indicates sequences encoding 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 *RNA1* and only one is indicated); *P*, Pst I; *S*, Sst I; X, *X*ba 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 *RNA1* ORF.

Cell Fractionation

Yeast cells were fractionated by a modification of the method described by Silver et al. (1984). 300 ml of 2b × 3b cells containing YEp24 and pFB 1-67a or YEpRNA1 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 K_2HPO_4-KH_2PO_4, pH 6.5, 0.5 mM $CaCl_2$, 0.5% 2-mercaptoethanol, 0.5 mM PMSF, and the cell walls were removed by digestion at room temperature with Zymolyase-20T (Seikagaka Kogyo Co., Tokyo, Japan) at a final concentration of 0.25 mg/ml. Spheroplasts were collected by centrifugation at 3,500 *g* for 3 min and were resuspended in 2 ml of 0.2% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM PMSF. The spheroplasts were lysed and homogenized in a Dounce homogenizer until nearly all cells appeared broken as determined by light microscopy. A fraction of this lysate was saved and used as a source of total cell protein. Nuclei from the lysed cells were collected by centrifugation at 3,000 *g* for 5 min. The supernatant was saved and used as a source of cytoplasm-enriched proteins. The pellet fraction was resuspended in 30 ml of 50% (wt/vol) Percoll, 40 mM Pipes, pH 6.8, 10 mM $MgCl_2$, 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 K_2HPO_4-KH_2PO_4, pH 6.5 and nuclei were collected by centrifugation at 5,000 *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 Biotec, 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 RNA1 protein were prepared by Pocono Rabbit Farm and Laboratory (Canadenasis, PA). To generate sera against the OmpF-RNA1-β-galactosidase trihybrid 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 RNA1 COOH-terminal peptide, a synthetic peptide of sequence GDLAERLAETEIK conjugated to dipalmityl lysine carrier (Hopp, 1984) was synthesized by Biosearch (San Rafael, CA). 5 mg of this preparation were 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-RNA-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 RNA1 antigen. However, using this serum we were unable to distinguish staining of cells processing an endogenous RNA 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 EE1b-Δ397-407 cells were removed by digestion with Glusulase and Zymolyase 5000 (see immunofluorescence, below). The spheroplasts were resuspended in 1.0 ml 0.73 mM KH_2PO_4, 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 RNA1 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 RNA1 antigen are given in the figure legends.

Results

Three approaches were taken to obtain antisera useful for determining the intracellular location of the RNA1 protein. One approach was to generate *RNA1-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 trihybrid protein could then be purified from *E. coli* and used to generate anti-RNA1 sera. The third approach was to use a synthetic peptide corresponding to a hydrophilic region of the *RNA1* ORF to obtain rabbit sera specific to this peptide.

RNA1-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

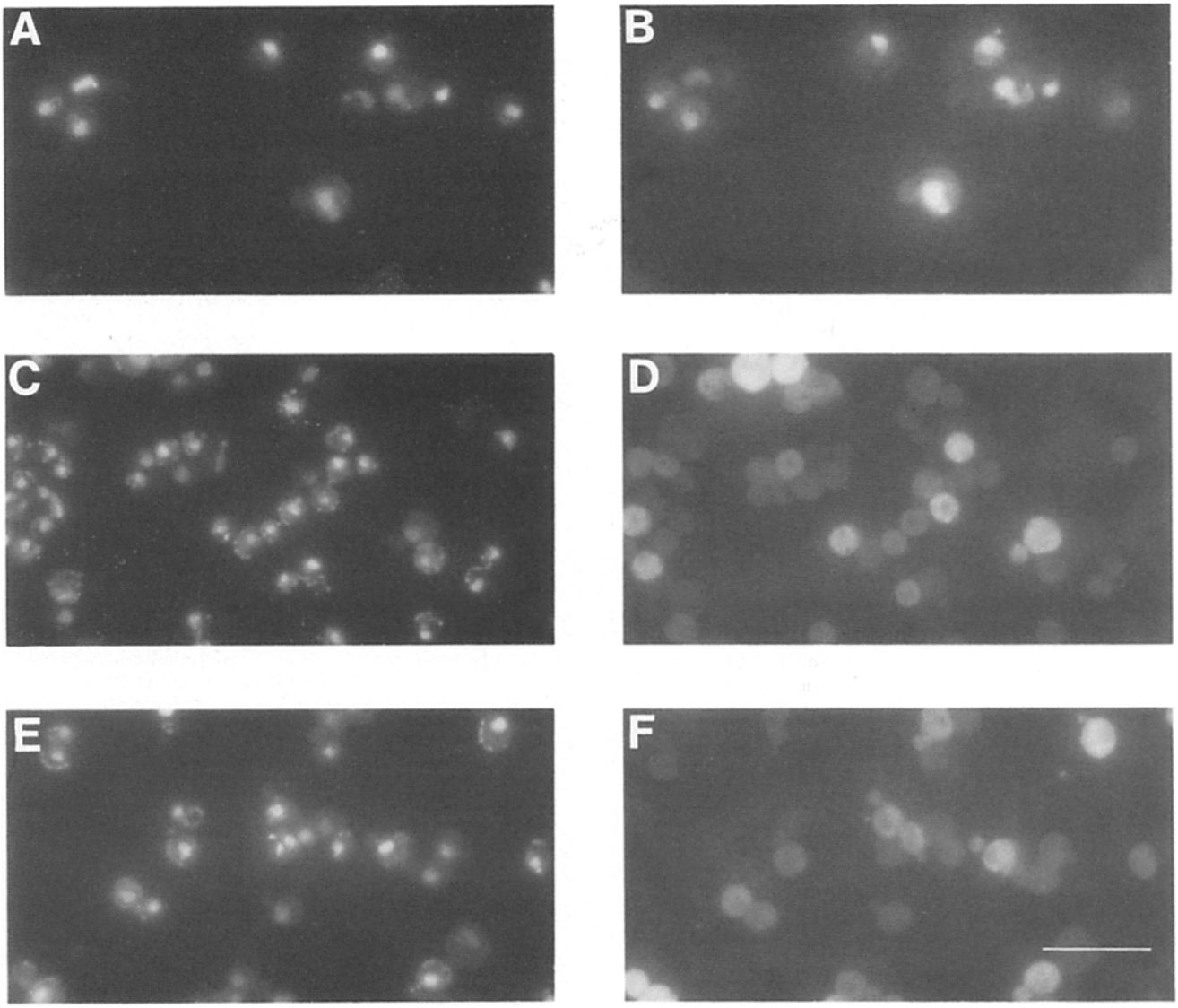


Figure 2. Indirect immunofluorescence using β-galactosidase antibody. Cells were grown under selective conditions and were prepared for indirect immunofluorescence as described in Materials and Methods. (*A* and *B*) Yeast strain SJ17 harboring pFB1-67a that encodes a histone 2B-β-galactosidase protein targeted to nuclei (Moreland et al., 1987). (*C* and *D*) Yeast strain SJ17 harboring pFB1-7a that encodes a histone 2B-β-galactosidase protein unable to translocate to yeast nuclei (Moreland et al., 1987). (*E* and *F*) Yeast strain SJ17 harboring YEpRNA1(1-187)/LacZ that encodes a fusion protein containing the first 187 amino acids of RNA1 fused in-frame to LacZ. *A*, *C*, and *E* cells stained with DAPI to show location of nuclei. *B*, *D*, and *F* cells stained with 1/100 dilution of affinity-purified anti-β-galactosidase IgG as primary antibody and 1/100 dilution affinity-purified FITC-conjugated goat anti-rabbit IgG as secondary antibody. 600×. Bar, 10 μm.

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 *RNA1* to *LacZ* to study the location of the *RNA1* 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-β-galactosidase chimeric protein containing seven amino acids of histone H2B. The *H2B2* sequences of pFB1-7a were substituted by *RNA1* sequences to generate YEpRNA1(1-187)/LacZ. YEpRNA1(1-187)/LacZ contains the *RNA1* 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 YEpRNA1(1-187)/LacZ produces β-galactosidase antigen. The vast majority of the antigen was of the electrophoretic mobility expected for an RNA1-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 goat 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-

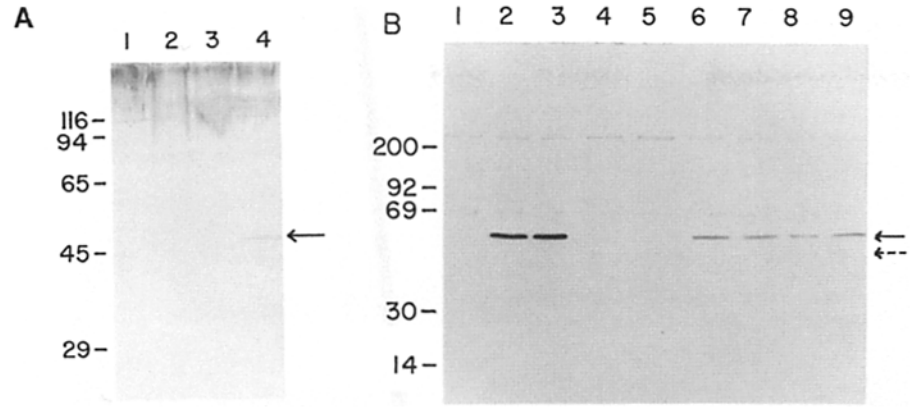


Figure 3. Protein blot analysis of RNA1 protein. (*A*) Analysis using affinity purified anti-RNA1 sera raised against the OmpF-RNA1-β-galactosidase trihybrid fusion protein. Yeast strains were grown under selective conditions to log phase. Lysates were prepared by a variation of the procedure described by Hopper et al. (1974). Cells were broken by homogenizing the following mixture four times for 15 s: 0.2 ml cells at ~1 × 10^9 cells per ml in 0.0625 M Tris(hydroxymethyl)amino methane (pH 6.8), 5% mercaptoethanol 3% SDS, 0.3 mg PMSF per ml, and 0.5 ml glass beads (0.45 mm diameter). Cells were eluted from the 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 1/1,000 dilution. Lanes: *1*, wild-type strain A364a; *2*, strain AKH1008 (*rnal-1 trpl-1*); *3*, strain AKH1008 containing vector pMac561; *4*, strain AKH1008 with YEpcRNA1(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 1/1,000 dilution of sera from rabbit 6142 and then with 1/1,000 dilution of affinity-purified alkaline phosphatase–conjugated goat anti–rabbit IgG. Lanes: *1*, strain EE1b-Δ397-407 that is missing the COOH-terminal epitope; *2*, EE1b harboring YCpRNA1 (single copy plasmid containing *RNA1*); *3*, EE1b harboring YEpRNA1; *4*, EE1b-Δ359-397 (containing a detection causing temperature-sensitive growth) incubated at the nonpermissive temperature (37°C); *5*, same as *4* except cells maintained at permissive temperature; *6* and *7*, EE1b-6 (*rnal-1*) grown at nonpermissive and permissive temperatures respectively; *8* and *9*, EE1b-35 (RNA1) grown at 37° and 23°C, respectively. →, position of RNA1 antigen; --→, position of antigen produced by the deletion allele *rnal-Δ359-397*.

clear location of the H2B1-67-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 YEpRNA1(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 H2B1-7-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 particular 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. Unfortunately, such constructs expressed very low levels of β-galactosidase 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 trihybrid fusion proteins are generated (Weinstock et al., 1983). The *OmpF* sequences served to target the trihybrid 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 $OmpR_{cs}$ allele and trihybrid protein production was induced (Weinstock et al., 1983). The trihybrid 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

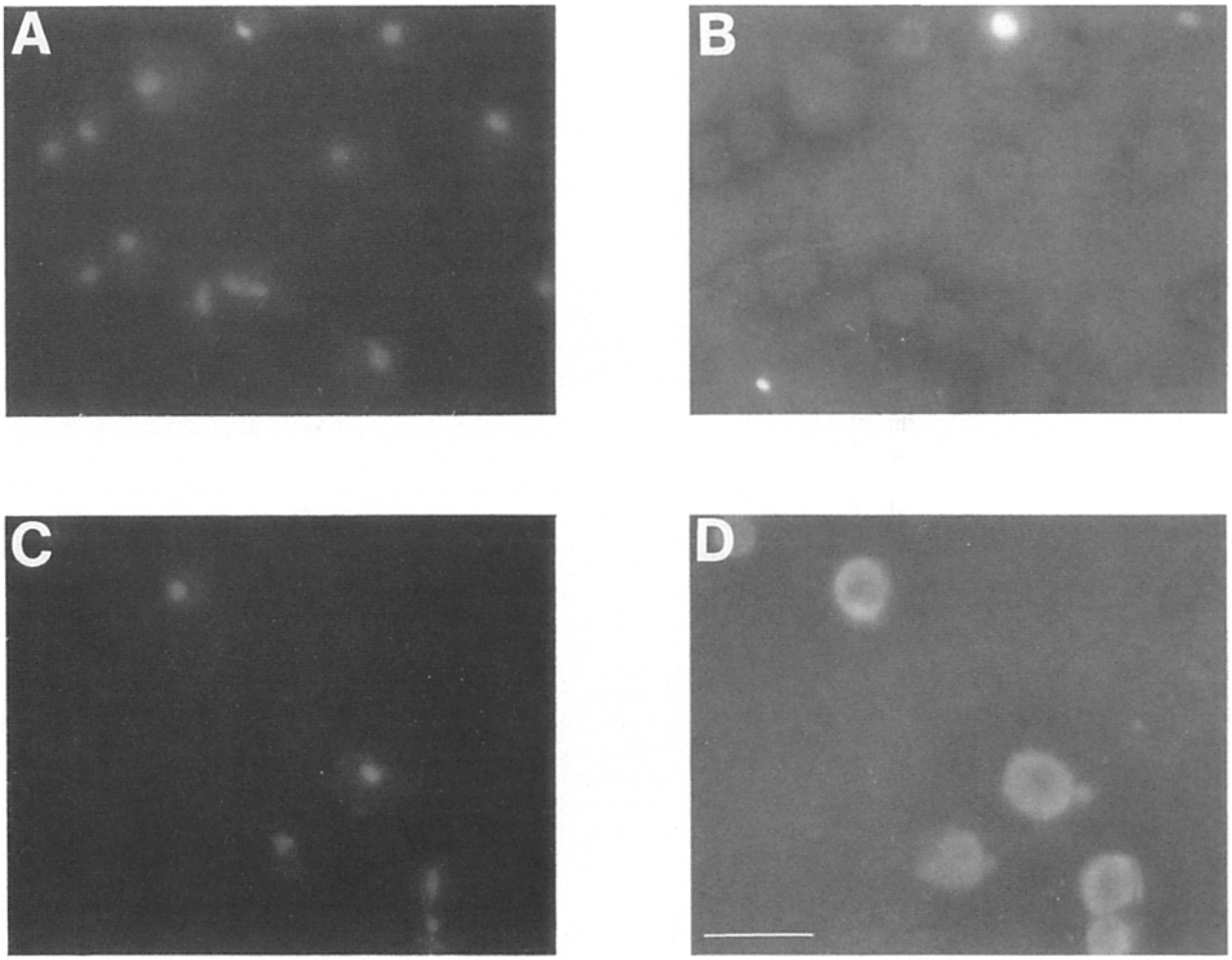


Figure 4. Indirect immunofluorescence using affinity-purified antibody generated against OmpF-RNA1-β-galactosidase fusion protein. (*A*) EE1b cells stained with DAPI; (*B*) same cells as in *A*, but treated with 1/100 affinity-purified anti-RNA1 fusion protein primary antibody and 1/100 FITC-conjugated affinity-purified goat anti-rabbit IgG secondary antibody; (*C*) EE1b containing YEpRNA1 treated with DAPI stain; (*D*) same cells as in *C* reacted with 1/100 anti-RNA1 fusion protein antibody and 1/100 FITC-conjugated affinity-purified goat anti-rabbit IgG. 1,000×. Bar, 5 μm.

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 *rna1-1* allele were used (Fig. 4 *B*). However, the same antibody did stain cells harboring the multicopy YEpRNA1 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-β-galactosidase to detect *RNA1*-β-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 unessential (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 *rna1* allele deleted for these residues (i.e., *rna1-Δ397-407*) 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 congenic 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 EE1b-Δ397-407, EE1b-35, EE1b-6, and EE1b-Δ359-397 are variants of EE1b (relevant genotype: *rna1-1 ura3-52*; Table I) that were derived by gene replacements. EE1b-Δ397-407 harbors the *rna1* allele that is deleted for the COOH-terminal codons corresponding to the peptide used to generate the an-

tisera, EE1b-35 harbors the wild-type *RNA1* allele, EE1b-6 contains the *rna1-1* allele, and EE1b-Δ359-397 possesses a deletion of codons 359–397 (Traglia et al., 1989). EE1b strains containing YCpRNA1 and YEpRNN possess single (or low) copy and multicopy vectors (~5–20 copies), respectively harboring genomic *RNA1* sequences. Strains that harbor YEpcRNA1(1.4), a highly expressed cDNA copy of *RNA1*, have also been employed and results analogous to those for YEpRNA1 were obtained (not shown). Proteins extracted from cells harboring the *rna1-Δ397-407* allele did not contain an antigen recognized by this sera (Fig. 3 *B*, lane *1*). However, proteins extracted from EE1b-35 wild-type cells (Fig. 3 *B*, lanes *8* and *9*) or EE1b-6 cells harboring the *rna1-1* allele (Fig. 3 *B*, lanes *6* and *7*) contained a cross-reacting species of the mobility expected for the RNA1 protein. This signal increased in intensity when strain EE1b contained either low-copy YCpRNA1 (Fig. 3 *B*, lane *2*) or multicopy YEpRNA1 (Fig. 3 *B*, lane *3*) plasmids. Furthermore, proteins extracted from EE1b-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 RNA1 protein and of high titer.

Preadsorbed 6142 serum was used for indirect immunofluorescence studies to localize the RNA1 antigen within yeast cells. Cells harboring the *rna1-Δ397-407* 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 *rna1-1* 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 RNA1 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 *RNA1* copies increases (compare Fig. 5, *D*, *F*, and *H*). With increased *RNA1* 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 RNA1 and β-galactosidase specific antibodies (also see Figs. 2 and 6). Presumably this is due to variation in plasmid copy number, plasmid loss, and differential permeability of the cells to antibody.

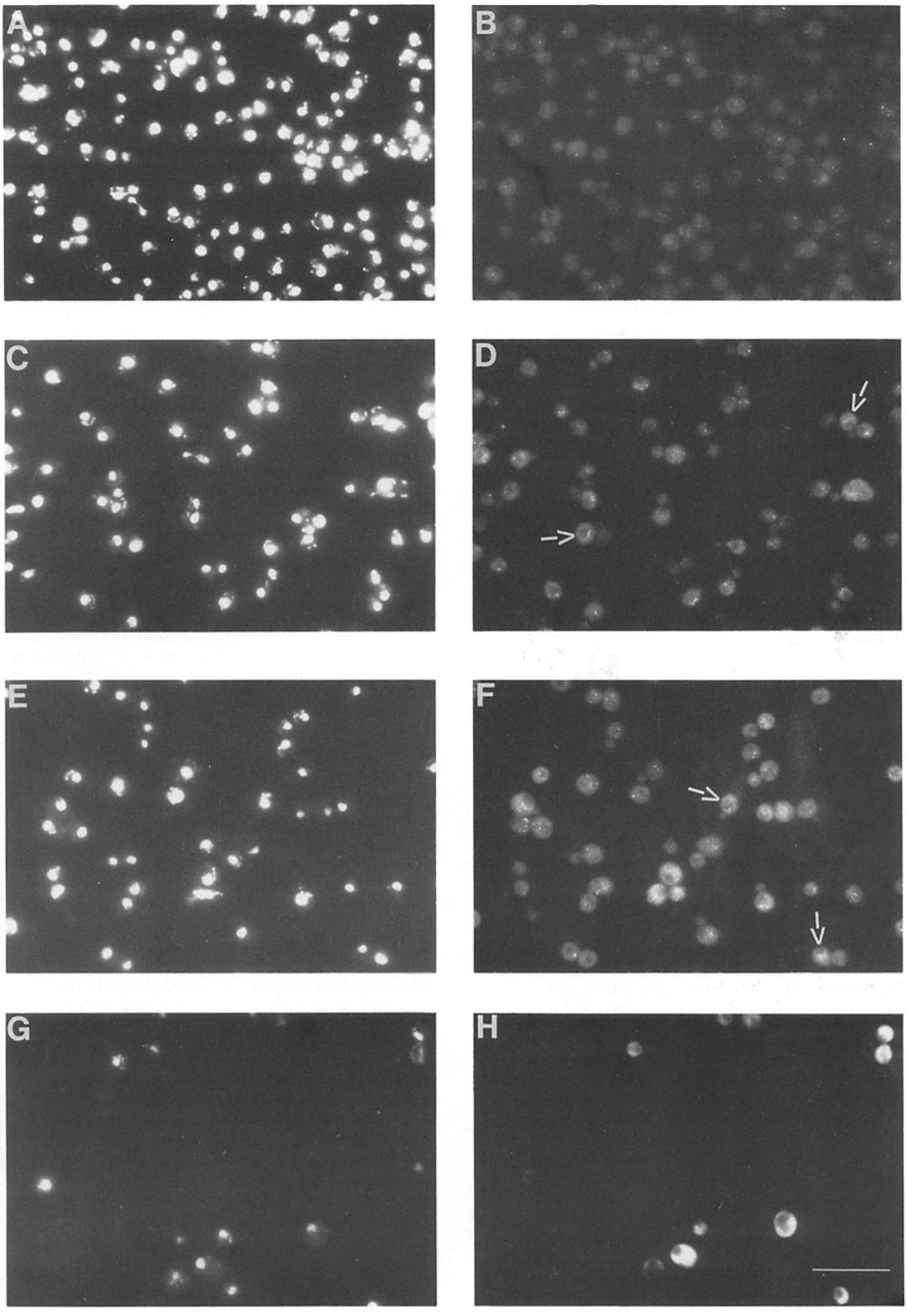
To be certain that the region devoid of signal was the nucleus, we employed triple labeling procedures. Diploid strain 2b × 3b (relevant genotype; *rna1-1/rna1-1, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112*) was transformed with two plasmids: either YEp24 vector containing the *URA3* gene and pFB1-67a that contains the histone *H2B1-67-LacZ* fusion and the *LEU2* gene or YEpRNA1 containing the *RNA1* and *URA3* genes in combination with pFB1-67a. Cells were first stained with preadsorbed rabbit anti-RNA1 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 (RNA1) stain appears larger than the region stained by DAPI (compare Fig. 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 Fig. 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 RNA1 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 RNA1 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 RNA1 multicopy plasmid, YEpRNA1, 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 RNA1 protein or the RNA1 region incorporated into pORF1-RNA1. 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 RNA1 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 RNA1 antigen recognized by the antibody raised to the protein encoded by pORF1-RNA1. Because this antibody does not detect endogenous levels of RNA1 antigen, the cells used for these fractionation studies contained a multicopy plasmid harboring the *RNA1* gene. Consistent with the results in Fig. 7 *A*, the antibody to the OMPF-RNA1-β-galactosidase fusion protein can detect RNA1 antigen in cytoplasm-enriched fractions, and total cell extracts, but not in the nuclear fraction. Therefore, as assessed by two different RNA1-specific antisera, the location of endogenous or overproduced levels of the RNA1 protein in subcellular fractions is cytoplasmic; no nuclear form of the RNA1 antigen was detected in these studies. Thus, the results of the cell fractionation studies confirm the results obtained by indirect immunofluorescence.

The RNA1 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 RNA1 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 *rna1-Δ397-407* COOH-terminal deletion allele show very little cross-reactivity with the preadsorbed antipeptide serum (Fig. 8, *A* and *B*). The location of wild-type RNA1 protein is similar for cells grown at 23°C and heat treated cells (Fig. 8, *E* and *F*). Even though by pro-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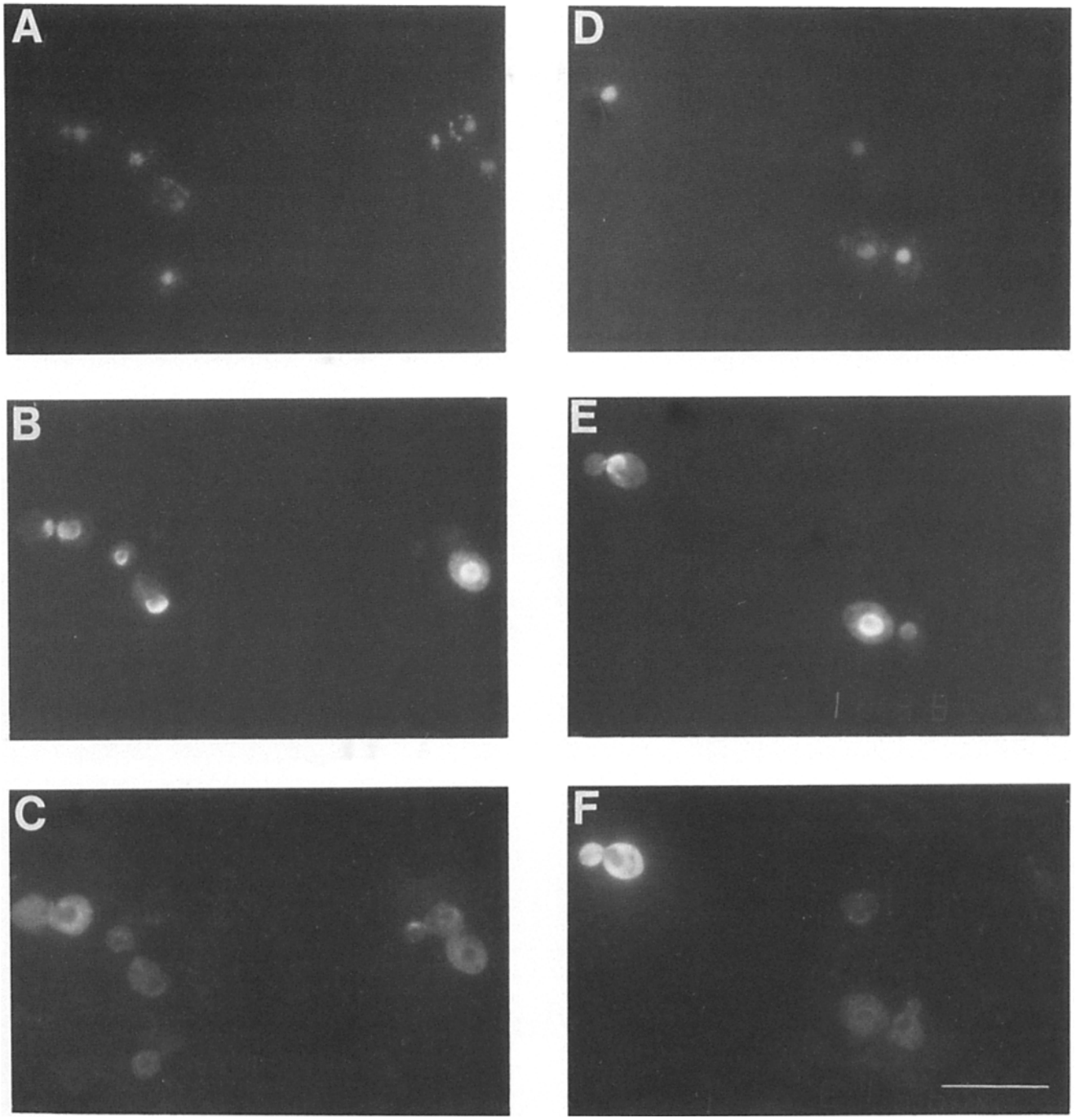

Figure 6. Indirect immunofluorescence employing anti-β-galactosidase and anti-RNA1 COOH-terminal peptide affinity-purified antibodies. Yeast strain 2b × 3b harboring both pFB1-67a (H2B/β-gal fusion) and YEpRNA1 were grown in complete minus uracil and leucine medium to middle log phase, fixed, and prepared 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*) EE1b-Δ397-407 cells (deleted for the COOH-terminal peptide) with DAPI stain; (*B*) EE1b-Δ397-407 cells reacted with 1/100 6142 antibody; (*C* and *D*) EE1b-35 (RNA1) cells stained as in *A* and *B*; *E* and *F*, EE1b cells harboring YCpRNA1 (~1 copy/cell) stained as *A* and *B*; (*G* and *H*) EE1b 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.

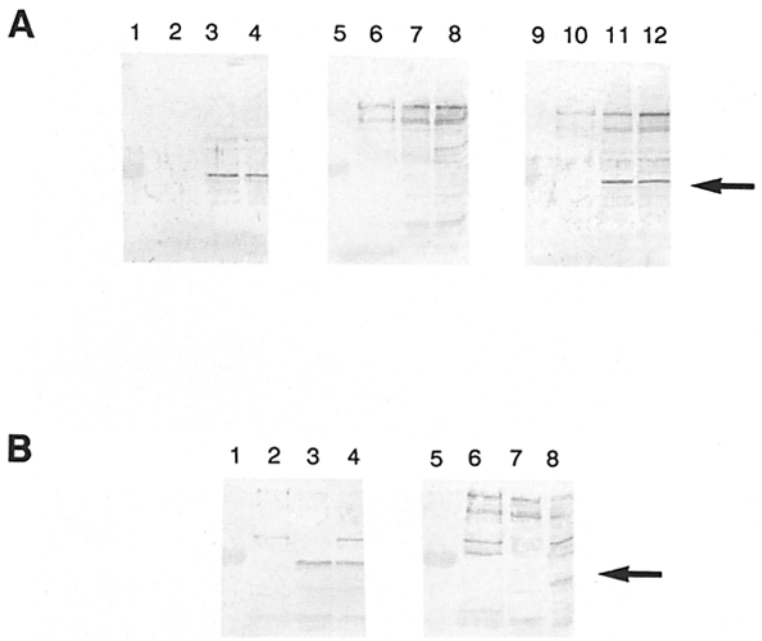

Figure 7. Location of RNA1 antigen in subcellular fractions. Cell fractions were prepared from 2b × 3b containing YEp24 (vector alone) and pFB1-67a (*A*) or 2b × 3b containing YEpRNA1 and pFB1-67a (*B*). Each panel contains four lanes: the first lane contains prestained fumarase, a protein of similar 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 electroelution 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).

tein blot analysis we can detect no difference in rnal-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 rnal-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 *rnal-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 *rnal-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 in yeast nuclei, but is in a cryptic form not recognized by any of our antisera. This possibility is unlikely because by using three different antisera to the *RNA1*-β-galactosidase fusion, the RNA1 peptide included in the pORF1-RNA1 fusion, and the COOH-terminal region, we have sampled a large portion of RNA1 product. Furthermore, using two different methods for detection of protein, immunofluorescence and PAGE analyses, we obtain the same results. It seems unlikely that none of these antisera and methods would have detected a cryptic form of RNA1 protein, should it exist.

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-$_k$B, a DNA binding protein, that translocates to the nucleus upon treatment of cells with phorbol esters (Baeuerle and Baltimore, 1988). Since *rnal-1* and *rnal-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 *rnal-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 *rnal-1* is not detected at high temperatures. However, it is possible that the mutant rnal-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).

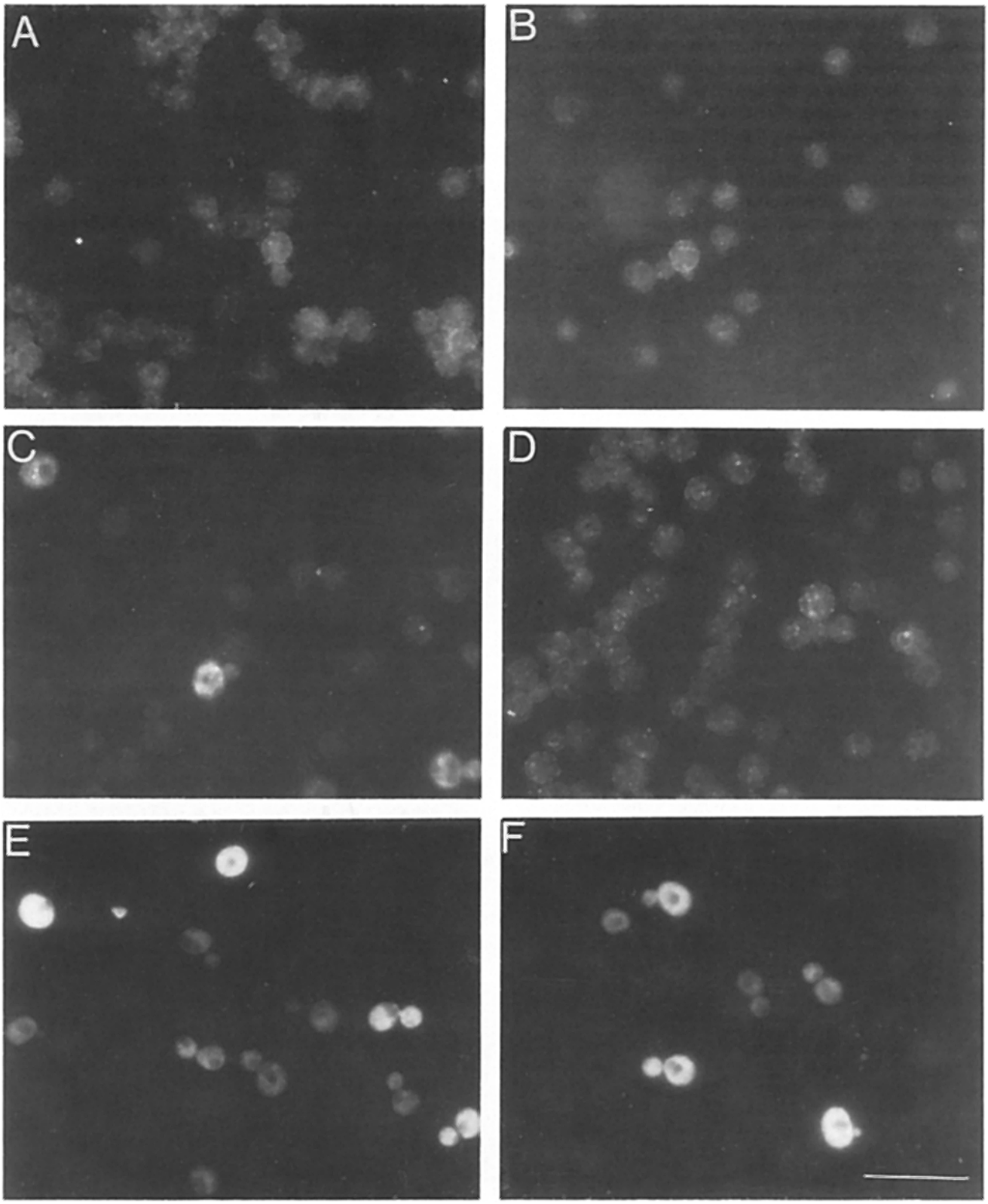

Figure 8. Location of the RNA1 antigen after heat-shock treatment. 20-ml cultures of EE1b-Δ397-407, EE1b transformed with YEprnal-1 and EE1b transformed with YEpRNA1 cells in complete minus uracil medium were grown to early log phase. The cultures were split into two equal volumes. One half was maintained 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 *rnal-1*; 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*) EE1b-Δ397-407, 23°C; (*B*) EE1b-Δ397-407, 37°C for 50 min; (*C*) EE1b plus YEprnal-1, 23°C; (*D*) EE1b with YEprnal-1, 37°C, 50 min; (*E*) EE1b with YEpRNA1, 23°C; (*F*) EE1b 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 *rna1-1* 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 *rna1-1* 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 *rna1-1* cells.

This manuscript is dedicated to the memory of Herschel Roman, a mentor, role model, and friend.

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