

# An Exclusively Nuclear RNA-binding Protein Affects Asymmetric Localization of *ASH1* mRNA and Ash1p in Yeast

Roy M. Long,\* Wei Gu,‡ Xiuhua Meng,‡ Graydon Gonsalvez,\* Robert H. Singer,‡ and Pascal Chartrand‡

\*Medical College of Wisconsin, Department of Microbiology and Molecular Genetics, Milwaukee, Wisconsin 53226; and

‡Albert Einstein College of Medicine, Department of Anatomy and Structural Biology, Bronx, New York 10461

**Abstract.** The localization of *ASH1* mRNA to the distal tip of budding yeast cells is essential for the proper regulation of mating type switching in *Saccharomyces cerevisiae*. A localization element that is predominantly in the 3'-untranslated region (UTR) can direct this mRNA to the bud. Using this element in the three-hybrid in vivo RNA-binding assay, we identified a protein, Loc1p, that binds in vitro directly to the wild-type *ASH1* 3'-UTR RNA, but not to a mutant RNA incapable of localizing to the bud nor to several other mRNAs. *LOC1* codes for a novel protein that recognizes double-stranded RNA structures and is required for

efficient localization of *ASH1* mRNA. Accordingly, Ash1p gets symmetrically distributed between daughter and mother cells in a *loc1* strain. Surprisingly, Loc1p was found to be strictly nuclear, unlike other known RNA-binding proteins involved in mRNA localization which shuttle between the nucleus and the cytoplasm. We propose that efficient cytoplasmic *ASH1* mRNA localization requires a previous interaction with specific nuclear factors.

**Key words:** *ASH1* • RNA localization • yeast • nuclear RNA-binding protein • three-hybrid

## Introduction

The ability of a single cell to divide into two daughter cells with different developmental fates is essential for the development of multicellular organisms. For this to occur, the sister cells need to express different genetic programs. Differences in gene expression can occur by the asymmetric segregation of cell fate determinants that affect gene expression. The yeast *Saccharomyces cerevisiae* serves as a model system for studying the asymmetric segregation of cell fate determinants. Haploid yeast cells display two mating types: a or  $\alpha$ . Mother cells are capable of switching mating type whereas daughter cells are not. Mating type switching is regulated by the expression of the HO endonuclease (Nasmyth, 1993), which is expressed in mother cells but not in daughter cells. The expression of HO is repressed in daughter cells by Ash1p, which is asymmetrically distributed to daughter cell nuclei (Bobola et al., 1996; Sil and Herskowitz, 1996; Maxon and Herskowitz, 2001).

Localization of mRNA is one mechanism by which cell fate determinants can be sorted between sister cells. The asymmetric sorting of Ash1p to the daughter cell nucleus results from the localization of *ASH1* mRNA to the distal tip of daughter cells during anaphase of the cell cycle (Long et al., 1997; Takizawa et al., 1997). Localization of

*ASH1* mRNA requires *SHE1-5* and a functional actin cytoskeleton (Long et al., 1997; Takizawa et al., 1997). *SHE1* was previously identified as *MYO4*, a type V myosin in yeast (Haarer et al., 1994; Jansen et al., 1996). Using a live cell assay, Myo4p and *ASH1* mRNA-containing particles were observed to move from mother cells to daughter cells, suggesting that Myo4p apparently has a direct role in transporting *ASH1* mRNA to the bud tip (Bertrand et al., 1998; Beach et al., 1999). She3p was observed to colocalize with these particles implying that She3p might be a structural component of the particle. Recently, She3p was found to interact with Myo4p and to be associated with *ASH1* mRNA via its interaction with She2p (Münchow et al., 1999; Böhl et al., 2000; Long et al., 2000; Takizawa and Vale, 2000). As She2p is a novel RNA-binding protein which binds to the localization elements of the *ASH1* mRNA, it was proposed that She2p recruits the Myo4p/She3p complex to the *ASH1* mRNA (Böhl et al., 2000; Long et al., 2000). *SHE5* was previously identified as *BNI1* (Jansen et al., 1996; Zahner et al., 1996). *she5/bni1* strains are defective in cytokinesis and accumulate *ASH1* mRNA at the bud neck (Jansen et al., 1996; Long et al., 1997). The mislocalization of *ASH1* mRNA in *she5/bni1* strains presumably results from defects in the actin cytoskeleton because these strains display alterations in the organization of the actin cytoskeleton (Kohno et al., 1996; Evangelista et al., 1997). She4p is a novel protein with no significant amino

Address correspondence to Roy M. Long, Medical College of Wisconsin, Department of Microbiology and Molecular Genetics, Milwaukee, WI 53226-0509. Tel.: (414) 456-8423. Fax: (414) 456-6535. E-mail: rlong@mcw.edu

acid homology to other known proteins and is hypothesized to be involved in organizing the actin cytoskeleton (Jansen et al., 1996; Wendland et al., 1996).

*ASH1* mRNA contains four cis-acting localization elements (Chartrand et al., 1999; Gonzalez et al., 1999). Each of these elements is sufficient to localize a heterologous mRNA to daughter cells in budding yeast (Chartrand et al., 1999; Gonzalez et al., 1999). Three of the elements (E1, E2A, and E2B) are located within the coding region of the *ASH1* mRNA, whereas the remaining element (E3) is located primarily within the *ASH1* 3'-UTR (Chartrand et al., 1999; Gonzalez et al., 1999). The E1 and E3 elements were predicted to form RNA secondary structures containing stem-loops (Chartrand et al., 1999; Gonzalez et al., 1999). Disruption of the stem-loop structure destroys the ability of these elements to direct RNA localization (Chartrand et al., 1999; Gonzalez et al., 1999).

During our investigation for the identification and characterization of RNA-binding proteins required for *ASH1* mRNA localization, we identified an uncharacterized yeast protein, Loc1p (for localization of mRNA), that interacted with the E3 localization element. Loc1p is a nuclear protein that associated with full-length *ASH1* mRNA in vivo but also interacted nonspecifically with RNA-containing stem-loop structures in vivo and in vitro. Surprisingly, Loc1p never leaves the nucleus as assayed by three independent approaches. In *loc1* strains, *ASH1* mRNA localization was reduced, and Ash1p was symmetrically distributed between most mother and daughter cells. From these results, we hypothesize that mRNAs destined for localization in the cytoplasm may be first identified in the nucleus and then directed towards the cytoplasmic RNA localization machinery.

## Materials and Methods

### Growth Media, Yeast Strains, and Plasmids

Yeast cells were grown in either synthetic media lacking the nutrients indicated or rich media (Adams et al., 1997). The yeast strains used in this study are listed in Table I. The *LOC1* gene was disrupted in yeast using a *KAN* disruption cassette (Güldener et al., 1996). This cassette was generated by PCR amplification of the *loxP-KAN-loxP* cassette in plasmid pUG6 plasmid using the following primers: *LOC1KAN1*, ATGGCACCAAAGAAA-CCTTCTAAGAGACAAAATCTGAGAAGAGCTGAAGCTTCGTCGC; *LOC1KAN2*, CTATTGAGCAAATGAGACTTTTTTACCT-TCCTTCCTCAGTGCATAGGCCACTAGTGGATCTG.

Yeast cells were transformed with the *loc1::KAN* disruption cassette and plated on YEPD plates containing 200 µg/ml of G418. After replica plating the original transformation plates to fresh YEPD+G418 plates, positive colonies were isolated. Genomic DNA was extracted from the candidate *loc1::KAN* colonies using the DNA-Pure Yeast Genomic Kit (CPG Inc.). The *loc1::KAN* disruption was confirmed by PCR of genomic DNA.

Disruption of *CLA4* in strains K5552 and YLM092 were created in an analogous fashion to that described for the YLM090. The *cla4::KAN* disruption cassette was created using primers *CLA4KAN1* and *CLA4KAN2*: *CLA4KAN1*, CCTTCTAGCCTGAGCCTTGTACAT-GTATTAGCGTTGCCAGGCTGAAGCTTCGTACGC; *CLA4KAN2*, GTAGTATGTATGATATGCTTATAGAAATAGTTGTGTGCTTCG-CATAGGCCACTAGTGGATCT.

Before transformation with the *cla4::KAN* cassette into strain YLM091 the *KAN* marker was removed from the strain. In all of the disruption cassettes described the *KAN* locus is flanked by *loxP* sites. Therefore, the *KAN* locus can be excised by expression of the *cre* recombinase (Güldener et al., 1996). A galactose-inducible *cre* recombinase is contained on plasmid pSH47 along with *URA3*. Strain YLM091 was transformed with pSH47 and expression of the *cre* recombinase induced with galactose. Colonies containing a silent *loc1* disruption were identified and cured of plasmid pSH47, generating strain YLM092. This strain was transformed with the *cla4::KAN* disruption cassette. The *cla4::KAN* disruption was confirmed, generating strain YLM093.

Plasmids used for these studies were constructed using standard techniques. The DNA fragments containing the sequences of the elements E1 (153 bp), E2A (153 bp), and E3 (127 bp from 2,741–2,867) were cloned in the *SmaI* site of plasmid pIII/MS2-2, generating plasmids pXR196, pXR197, and pRL080, respectively. The specific mutants in the element E3 (mutants M9, M9+9A, M13, and M14; Chartrand et al., 1999) were also cloned in the *SmaI* site of pIII/MS2-2 (Table II).

A 2.1-kb DNA fragment, containing the YFR001w open reading frame with 500 bp of 5' sequence and 1,000 bp of 3' sequence, was amplified by PCR from yeast K699 genomic DNA. This DNA fragment, *LOC1*, was cloned into the *PstI* and *SmaI* sites of YCplac33, generating plasmid pRL090 (Gietz and Sugino, 1988). Plasmid pRL090 was transformed into strain YLM090 and checked for complementation of the *loc1* slow growth phenotype and RNA localization defect. A unique *BamHI* site was introduced before the stop codon of the *Loc1p*, and this *Loc1p* fragment was subcloned in the *PstI* and *SmaI* sites of YCplac33 and YEplac195, resulting in plasmids pRL091 and pRL092. A myc epitope was inserted in-frame to *Loc1p* at the unique *BamHI* site of pRL091 and pRL092, generating plasmids pRL093 and pRL094, respectively. Plasmid C3003 containing three copies of the myc epitope was generously provided by the laboratory of K. Nasmyth (Research Institute of Molecular Pathology, Vienna, Austria). Plasmid pRL093 contains six copies of the myc epitope, whereas pRL094 contains three copies of the myc epitope. Plasmids pRL093 and pRL094 were transformed into strain YLM090 and checked for complementation of the *loc1* slow growth phenotype and RNA localization defect.

A galactose-inducible version of *Loc1p-myc* was constructed by subcloning the *Loc1p-myc* fragment from pRL093 into pESC-URA3 (CLONTECH Laboratories, Inc.). The *Loc1p-myc* coding sequence was amplified by PCR with primers designed to generate an *XhoI* site at the 5' end of the fragment and a *KpnI* site at the 3' end of the fragment. The PCR product was digested with *XhoI/KpnI* and ligated into *SalI/KpnI*-digested pESC-URA3, generating plasmid pRL134.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
L40-coat	<i>Mata, ura3-52, leu2-3,112, his3-200, trp1-1, ade2, LYS2:::(LexAop)-lacZ, LexA-MS2 coat (TRP1)</i>	SenGupta et al., 1996
YBZ-1	<i>Mata, ura3-52, leu2-3,112, his3-200, trp1-1, ade2, LYS2:::(LexAop)-lacZ, LexA-MS2 -MS2 coat (N55K)</i>	M. Wickens
K699	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100</i>	Jansen et al, 1996
K5552	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, ASH1-myc9</i>	Long et al., 1997
K4452	<i>Mata, HO-ADE2 HO-CAN1 leu2-3,112, ura3, his3</i>	Jansen et al., 1996
YPC001	<i>Mata, HO-ADE2 HO-CAN1 leu2-3,112, ura3, his3, loc1::KAN</i>	This study
YLM090	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, loc1::KAN</i>	This study
YLM091	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, ASH1-myc9, loc1::KAN</i>	This study
YLM092	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, ASH1-myc9, loc1</i>	This study
YLM093	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, ASH1-myc9, loc1, cla4::KAN</i>	This study
YLM094	<i>Mata, ura3, leu2-3,112, his3-11, trp1-1, ade2-1, ho can1-100, ASH1-myc9, cla4::KAN</i>	This study
PSY413	<i>Mata, ade2, ade3, his3, leu2, ura3, nup49::TRP, plus pUN100-nup49-313 ts LEU2</i>	Lee et al., 1996
MS739	<i>Mata, leu2-3,112, ura3-52, ade2-101, kar1-1</i>	Flach et al., 1994

Table II. Plasmids Used in This Study

Plasmid	Features	Source
pUG6	Plasmid for generating <i>KAN</i> disruption cassettes by PCR	J.H. Hegemann
pSH47	Yeast plasmid expressing galactose-inducible cre recombinase	J.H. Hegemann
YCplac33	CEN yeast shuttle plasmid marked with URA3	R.D. Gietz
YEplac195	2 $\mu$ yeast shuttle plasmid marked with URA3	R.D. Gietz
pESC-URA3	Yeast galactose-inducible expression vector	CLONTECH Laboratories, Inc.
C3003	<i>E. coli</i> plasmid containing myc epitope	K. Nasmyth
C3319	YEplac181 yeast plasmid ( <i>LEU2</i> , 2 $\mu$ ) containing the <i>ASH1</i> gene	K. Nasmyth
pIIIA/MS2-2	Three-hybrid vector for the expression of MS2-fusion RNAs	M. Wickens
pIII/IRE-MS2	Three-hybrid plasmid expressing MS2-IRE fusion RNA	M. Wickens
pRL080	pIIIA/MS2-2 containing the 127-nt SmaI E3 <i>ASH1</i> localization element	This work
pRL090	YCplac33 containing a 1.6-kb PstI/SmaI <i>LOC1</i> fragment	This work
pRL091	YCplac33 containing a 1.6Kb PstI/SmaI <i>LOC1</i> fragment with a unique BamHI site at the Loc1p stop codon	This work
pRL092	YEplac195 containing a 1.6-kb PstI/SmaI <i>LOC1</i> fragment with a unique BamHI site at the Loc1p stop codon	This work
pRL093	pRL091 with six copies of the myc epitope subcloned into the unique BamHI site	This work
pRL094	pRL092 with three copies of the myc epitope subcloned into the unique BamHI site	This work
pRL134	Loc1-myc coding sequence from pRL093 subcloned into pESC-URA3	This work
pRL168	pGEM-3Z containing element E3	Long et al., 2000
pRL176	pGEM-3Z containing element E1	Long et al., 2000
pRL177	pGEM-3Z containing element E2A	Long et al., 2000
pRL179	pGEM-3Z containing element E2B	Long et al., 2000
pRL204	pIIIA/MS2-2 containing 130 bp from the ADHII 3'UTR	This work
pPS811	1.5 kb BamHI fragment from pPS425 containing <i>NPL3</i> in pCGF-1A	Lee et al., 1996
pXR192	Element E3-mutant M9 (118nt) cloned in pIIIA/MS2-2	This work
pXR193	Element E3-mutant M9+9A (118nt) cloned in pIIIA/MS2-2	This work
pXR194	Element E3-mutant M13 (118nt) cloned in pIIIA/MS2-2	This work
pXR195	Element E3-mutant M14 (118 nt) cloned in pIIIA/MS2-2	This work
pXR196	Element E1 (150 nt) cloned in pIIIA/MS2-2	This work
pXR197	Element E2A (150 nt) cloned in pIIIA/MS2-2	This work

### Three-Hybrid Screen

The components for the three-hybrid selection were provided by the laboratory of M. Wickens (University of Wisconsin, Madison, WI), and the selection was performed essentially as described elsewhere (SenGupta et al., 1996; Zhang et al., 1997). *HIS3* and *lacZ* serve as reporter genes for the three-hybrid assay. In brief, yeast strain L40-coat containing plasmid pRL80 was transformed with an *S. cerevisiae* cDNA activation library prepared in the S. Elledge (Baylor College of Medicine, Houston, TX) laboratory (Li et al., 1994) and obtained from the American Type Culture Collection. Transformants were selected on media lacking leucine and histidine containing 5 mM 3-aminotriazole to reduce background in the assay resulting from low levels of *HIS3* expression. Built into the three-hybrid selection is a screen for RNA dependence. The plasmid expressing the fusion RNA construct is marked with *URA3* and *ADE2*. When the yeast strain L40-coat is transformed with this plasmid and grown under nonselective conditions, this plasmid can be lost from the yeast cells, resulting in red and red/white sectored colonies. Colonies that remain completely white represent candidates that apparently require the fusion RNA for *HIS3* expression on the 3-aminotriazole selection plates. About  $2 \times 10^6$  transformants were screened. After 1 wk at 30°C, ~2,000 colonies were visible. The majority of these colonies were red or red/white sectored, indicating that *HIS3* expression was independent of the fusion RNA. The remaining 66 colonies were initially all white and were studied further. By selection for the loss of the fusion RNA plasmid by plating on media containing 5-fluoroorotic acid, only 4 of 66 colonies expressed *HIS3* and  $\beta$ -galactosidase dependent on the hybrid RNA.  $\beta$ -galactosidase expression levels were determined either by X-gal filter assay or liquid assay using *O*-nitrophenyl- $\beta$ -galactoside (Guarente, 1983).

Further characterizations of Loc1p RNA-binding activity were performed in strain YBZ-1 (provided by the M. Wickens laboratory). YBZ-1 is very similar to L40-coat, except YBZ-1 contains a LexA-MS2-MS2 construct rather than the LexA-MS2. Also, the LexA-MS2-MS2 construct harbors a mutation, N55K, which increases the binding affinity measured in vitro.

### In Situ Hybridization and Immunofluorescence

Yeast cells were grown in the appropriate medium to early or mid-log phase, fixed with formaldehyde, and spheroplasted as described previously (Long et al., 1995). For in situ hybridization, yeast spheroplasts were

hybridized with a pool of Cy3-conjugated *ASH1* DNA oligonucleotide probes following the protocol described elsewhere (Long et al., 1995). Immunofluorescence was performed using a protocol described previously (Gorsch et al., 1995) with the following modifications. The anti-myc antibody (Roche Molecular Biochemical) was used in a 1:50 dilution in 1 $\times$  PBS and 0.1% BSA. The secondary antibody was a Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) diluted 1:500 into the same buffer.

Images were captured using the Esprit Image Analysis software (Life Science Resources) with an OlymPix TE cooled 12-bit CCD camera (Life Science Resources) mounted on an Olympus BX-60 fluorescence microscope (Olympus) with a PlanApo 100 $\times$ , 1.35 NA objective (Olympus). Single plane images were captured and processed using the Adobe Photoshop 5.0 software (Adobe Systems). For Fig. 7 B, panels g-1, a three-dimensional data set, composed of 30 images separated by 200 nm in the axial direction, was acquired and deconvolved with an acquired point spread function (PSF) using EPR software (Scanalytics). 7 to 10 planes for both Cy3 and DAPI channels were overlapped to give panels h and k in Fig. 7 B using Adobe Photoshop 5.0 software.

### Shuttling Assay

The nuclear shuttling assay was performed as described previously (Lee et al., 1996). Strain PSY413, provided by P. Silver (Dana Farber Cancer Center, Boston, MA), was transformed with either plasmid pESC-URA3, pRL134, or pPS811. Plasmids pRL134 and pPS811 contain a galactose-inducible cassette of Loc1-myc and Npl3-green fluorescent protein (GFP),<sup>1</sup> respectively. Transformants were grown at 25°C in synthetic complete (SC) media lacking uracil and containing 2% glucose. These cultures were subsequently diluted into SC media lacking uracil and containing 2% raffinose. Raffinose-containing cultures were grown overnight at 25°C. When cultures reached mid-log phase, Loc1p-myc and Npl3-GFP expression was induced by the addition of galactose for 2 h. After the induction with galactose, the cultures were harvested by centrifugation at 3,700 rpm for 10 min at 25°C. The cells were washed with an equal volume of YEPD media and collected by centrifugation. The cell pellets were resuspended in an equal volume of YEPD and incubated at 25°C for 2 h. At this point,

<sup>1</sup>Abbreviations used in this paper: GFP, green fluorescent protein; IRE, iron responsive element; RT, reverse transcription; SC, synthetic complete.

an aliquot of the culture was shifted to 36°C or maintained at 25°C for 5 h. After the temperature shift, cultures were fixed and processed for immunofluorescence as described above. Cycloheximide was used at a final concentration of 10 µg/ml and was added after the wash in YEPD.

### Heterokaryon Protein Shuttling Assay

This assay was performed essentially as described previously (Flach et al., 1994). Strain K699 was transformed with one of the following plasmids: pESC-URA3, pRL134, or pPS811. Transformants were grown overnight at 30°C in SC media containing 2% glucose but lacking uracil. These cultures were diluted into uracil dropout media containing 2% raffinose and grown overnight at 30°C. When the cultures reached a cell density of  $3 \times 10^6$  cells/ml, galactose was added to a final concentration of 2% and cultures were incubated for 90 min at 30°C. Cells were harvested by centrifugation, washed once in YEPD, and resuspended at a density of  $2 \times 10^6$  cells/ml in YEPD. Cultures were incubated for 2 h at 30°C followed by mating to strain YM739 (*kar1-1*). For mating,  $3 \times 10^7$  cells of the K699 transformants were mixed with  $3 \times 10^7$  cells of YM739 in 45 ml of YEPD and incubated at 30°C for 4 h at 100 rpm. After mating, cells were fixed and processed for immunofluorescence as described above.

### Loc1p Purification and Gel Mobility Assays

Yeast strains used for preparing protein extracts are listed in Table I. Yeast cells were grown to OD<sub>600</sub> 1.0–1.5 and disrupted with glass beads in breakage buffer (20 mM Tris/HCl, pH 7.4, 3 mM MgCl<sub>2</sub>, 40 mM KCl, and 1 mM DTT) containing 0.7 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 2,000 g for 10 min, the supernatant was recovered and centrifuged for an additional 30 min at 10,000 g, and the supernatant (containing about 5–10 µg/µl of protein) was stored in aliquots at –80°C.

For in vitro transcription the 126 nucleotide localization element E3, present in the 3'-UTR of the *ASH1* mRNA (Chartrand et al., 1999), was amplified by PCR and inserted into a pSP64.Poly(A) vector (Promega) at the HindIII and Aval sites. For gel shift assays, <sup>32</sup>P-labeled RNA was generated by SP6 RNA polymerase directed in vitro transcription from an Aval linearized construct. The RNA was purified by electrophoresis on a 6% denaturing gel. Unlabeled RNA was purified by DNase digestion, phenol/chloroform extraction, and ethanol precipitation. For RNA affinity purification, polyadenylated transcripts were synthesized in vitro with the SP6 MEGAScript kit (Ambion) from EcoRI linearized constructs. Trace amounts of [<sup>32</sup>P]CTP were added to allow detection and quantitation of transcribed RNA. The transcribed RNA contained the 126 nts of the element E3 RNA and a poly(A) tail of 30 nts.

RNA–protein gel shift assays were performed at room temperature. In brief, 10<sup>5</sup> CPM of <sup>32</sup>P-labeled RNA probe was incubated with 5 µl of yeast protein extract (25–50 µg) for 20 min in 20 µl binding solution containing 20 mM Hepes, pH 7.4, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 2 mM DTT, and 5% glycerol. Unbound RNAs were degraded by a 10-min incubation with 1 U of RNase T1, and nonspecific RNA–protein interactions were minimized by incubation with 5 mg/ml heparin for 10 min. RNA–protein complexes were separated in a 4% native gel and visualized by autoradiography. To establish the specificity of RNA–protein interactions, competition assays were performed by preincubation of the protein extract with unlabeled RNA competitors before the labeled RNA was introduced.

For affinity purification of proteins that interact with the E3 element, 200 µl of poly(U) agarose beads (type 6; Amersham Pharmacia Biotech) were suspended in RNA binding buffer (25 mM Tris/HCl, pH 7.4, 100 mM KCl) and packed into a 2-ml column. About 100 µg of in vitro-synthesized poly(A)-element E3 RNA was added to the column and cycled four times. The efficiency of RNA binding to poly(U) agarose beads was monitored by <sup>32</sup>P-labeled RNA. After binding, the beads were equilibrated with the yeast protein extract buffer, mixed with 5 ml of yeast protein extracts (from strain YLM090 with the plasmid pRL093) containing 50 U/ml RNasin (Promega) and incubated for 1 h at room temperature with gentle shaking. To lower the nonspecific protein binding, yeast tRNA and heparin were added to 50 µg/ml and 5 mg/ml, respectively. The beads were then centrifuged for 2 min at 1,000 g, suspended in binding buffer, and re-packed into the 2-ml column. The column was extensively washed with binding buffer and eluted with 20 mM Hepes buffer, pH 7.4, containing 2 M KCl. The eluted proteins were analyzed by SDS-PAGE and Western blotting. For microsequencing, the eluted protein fraction was concentrated with a Centricon-10 filter and aliquots were resolved in 12% SDS-PAGE. Protein bands were visualized by Coomassie blue staining, excised from the gel and analyzed by mass spectroscopy at Yale University.

For expression and purification of glutathione-S-transferase (GST)-Loc1p, the *LOC1* open reading frame and 3'-UTR was amplified by PCR using the following primers: Loc1-5', CGC GGG ATC CCC ATG GCA CCA AAG AAA CCT TCT AAG AGA; Loc1-3', ATC GCC GAA TTC TTT AGT ATA GTC TCT GCT AGC TTT GTT C.

The PCR product was digested with BamHI/EcoRI and cloned in the BamHI/EcoRI sites of plasmid pGEX-5X-3 (Amersham Pharmacia Biotech). The *GST-LOC1* junction and the *LOC1* PCR product were confirmed by DNA sequencing. The pGEX-Loc1 plasmid was transformed into *Escherichia coli* BL21, expressed by induction with IPTG, and purified on a glutathione column following the GST Gene Fusion System Protocol (Amersham Pharmacia Biotech).

To deplete the bacterial extract of GST-Loc1p, 20 µl of extract containing recombinant GST-Loc1 fusion protein was either incubated with 10 µg of anti-GST (CLONTECH Laboratories, Inc.) for 2 h at 4°C followed by incubation with 5 µl protein G-coupled agarose beads for another 2 h at 4°C, or with 2 µl of glutathione beads (Sigma-Aldrich) for 2 h at 4°C. After extensively washing with 1× PBS, the beads were collected by centrifugation at 5,000 g. The supernatants were recovered and used for RNA mobility shift assay with E3.

For purification of GST-Loc1p, recombinant GST-Loc1p purified with glutathione beads was resolved by SDS-PAGE. After Coomassie blue staining, the protein bands of GST-Loc1p were cut from the gels and placed in an Electro-Eluter (model 422; Bio-Rad Laboratories). The protein was eluted at a constant current of 60 mA and dialyzed against 1× PBS overnight at 4°C. The protein was concentrated with a Centricon-30 filter and used for mobility shift assay with E3.

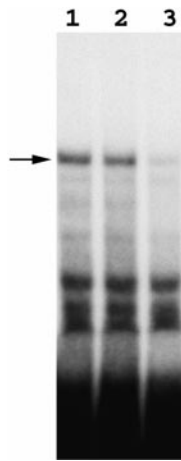
### Immunoprecipitation and Reverse Transcription PCR

Yeast cells (strain K699 containing plasmid pRL094) were grown to OD<sub>600</sub> of 1.0, centrifuged at 4,000 rpm for 10 min at 4°C, and resuspended at 100 OD/ml in a breakage buffer (1× PBS, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin, 0.5 µg/ml chymostatin, and 80 U/ml RNasin). 1 ml of cells (100 OD/ml) were broken with glass beads at 4°C, centrifuged at 14,000 RPM for 1 h at 4°C, and the supernatant used for immunoprecipitation.

For immunoprecipitation, 5 µg of mouse Anti-c-myc antibody (Roche) were added to 200 µl of extract and incubated for 2 h at 4°C. 50 µl of protein-A-agarose slurry (Immunopure Protein A; Pierce Chemical Co.) were added and incubated overnight at 4°C. After centrifugation, the beads were washed three times with 1× PBS, 0.05% Tween 20 for 3 min at 4°C, and centrifuged at 14,000 rpm for 1 min. The supernatant was removed from the beads, and the beads were boiled in 200 µl of diethyl pyrocarbonate (DEPC) water for 5 min, spun, and the supernatant recovered. 1 ml of Trizol (GIBCO BRL) was added to 200 µl of supernatant and mixed well, followed by the addition and mixing of 200 µl of chloroform. This mixture was incubated at 4°C for 5 min, followed by centrifugation at 1,000 rpm for 10 min at room temperature. The mRNA in the aqueous phase was precipitated with half volume of isopropanol. After precipitation, the mRNA was resuspended in 20 µl of DEPC water.

For reverse transcription (RT) and PCR, the RT-PCR kit Ready to Go from Amersham Pharmacia Biotech, one step method, was used. The RT reaction was incubated for 30 min at 42°C. All PCR reactions were performed for 30 cycles. The following primers were used in the amplification: *ASH1* PCR 23 (5'-CGCGCCCGGTAATTGTTTCGTGATAAT-GTCTCTTATT-3') and *ASH1* PCR 56 (5'-CGCGCCCGGGGAAGTA-GAAGGTCCAGTGGCTCATCACCA-3'), which generates a PCR product of 375 bp. For the detection of the other mRNAs, the following primers were used: *ACT1*-PCR-5' (5'-TTGACCAAACACTACTTCAACTCCATC-3') and *ACT1*-PCR-3' (5'-TTAGAAACACTTGTGGT-GAACGATAG-3'), amplifying a 307-bp fragment of *ACT1* mRNA; *ADH2*-PCR-5' (5'-CGACTTCACCAAGAGAAGGACATT-3') and *ADH2*-PCR-3' (5'-TAGGAGATCCGCTTATTAGAAAGTG-3'), amplifying a 399-bp fragment of *ADH2* mRNA; *PGK1*-PCR-5' (5'-AATCTAGAAAGTTGTTTGTGCTACT-3') and *PGK1*-PCR-3' (5'-TTATTTCTTTTCGGATAAGAAAGCAAC-3'), amplifying a 301-bp fragment of *PGK1* mRNA; and *SIC1*-PCR-5' (5'-CTCTCCGAA-AAATGACGCCAGGAG-3') and *SIC1*-PCR-3' (5'-TAATCGTTC-CAGAACTTTTTTTTCA-3'), amplifying a fragment of 318 bp of *SIC1* mRNA.

For the competition of Loc1p binding to *ASH1* mRNA, elements E1, E2A, E2B, and E3 (Chartrand et al., 1999) were cloned in plasmids pGEM-3Z at the BamHI and PstI sites (plasmids pRL168, pRL176, pRL177, and pRL179; Long et al., 2000) and transcribed in vitro using T7 RNA polymerase (MEGAScript Transcription kit; Ambion). The transcribed RNAs



**Figure 1.** A band mobility shift assay identifies proteins that specifically bind the E3 element. Radiolabeled E3 transcripts were incubated with yeast protein extracts, and then with RNase T1 and heparin at room temperature. RNA–protein complexes were resolved in 4% native gel and visualized by autoradiography. Lane 1, E3 transcripts incubated with K699 extract; lanes 2 and 3, E3 transcripts incubated with K699 extracts in the presence of 200× molar excess of pGEM RNA and unlabeled E3 transcripts, respectively.

of each element were pooled together in an equimolar mix. The 56-nt iron responsive element (IRE; Klausner et al., 1993) was cloned in the plasmid pGEM-4Z at HindIII and XmaI sites, and transcribed with T7 RNA polymerase. For the competition, 10, 1, and 0.1 μg of competitor RNA were added to the yeast extract for 30 min at 16°C, then the Loc1p-myc protein was immunoprecipitated as described above. Subsequently, the pellet was submitted to RT and PCR to detect the presence of the *ASH1* mRNA.

### Mutagenesis of *LOC1*

A plasmid pool of *LOC1* mutants was generated by PCR mutagenesis (Zhou et al., 1991). 30 cycles of PCR amplification of a 1.6-kb fragment of the *LOC1* gene were performed in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM Mg Cl<sub>2</sub>, 50 μM of each dNTP, 2 fmol of template, 5 U Taq DNA polymerase (Boehringer), and 30 pmol of the following primers: YFR001 PCR8, 5'-GCGCCTGCAGGGTTGAAAAGTTAGAAAATTAGTATTAATAGC-3'; LOC1-3', 5'-CGCGCCGAATTCTTTAGTATAGTCCTTGCTAGCTTTGTTCC-3'.

This protocol results in a mutation frequency of 60% (i.e., of 100 clones, 60 have a mutation in their sequences; Zhou et al., 1991). The PCR fragments were digested with EcoRI and PstI, and cloned into plasmid YEplac181 (Gietz and Sugino, 1988). After *E. coli* transformation, >9,000 clones were isolated, and 70% of these clones contained the 1.6-kb *LOC1* gene. The library was purified in a large maxiprep (QIAGEN) and transformed in the yeast strain YPC001. Transformants were plated onto selective media-leucine-arginine plates containing 0.03% canavanine and grown at 30°C, in an attempt to isolate *loc1* mutants that affected either *ASH1* mRNA localization or growth, but not both processes.

## Results

### A Novel RNA-binding Protein, *Loc1p*, Identified by Three-Hybrid Analysis

To verify that RNA-binding proteins required for *ASH1* mRNA localization existed, we used the localization element E3 in a gel mobility assay with yeast extracts (Fig. 1). The gel shift revealed an RNA-specific band which could not be competed with heterologous RNA. We next used the element as an affinity ligand to isolate binding proteins; however, the proteins isolated by this approach were not specific, being mainly ribosomal proteins (data not shown). Consequently, we initiated an in vivo approach for the identification of RNA-binding proteins required for *ASH1* mRNA localization. We used the three-hybrid system, which is a molecular genetic approach for the identification and characterization of RNA-binding proteins in vivo (SenGupta et al., 1996). In this assay, formation of a specific RNA–protein complex in yeast results in transcriptional activation of the reporter genes *HIS3* and *lacZ*.

```

1  MAPKKPSKRQNLRRREVAPEVFQDSQARNQLANVPHLTEKSAQRKPSKTKV
51  KKEQSLARLYGAKKDKKGYSEKDLNIPTLNRAIVPGVKIRRGKKGKKFI
101  ADNDTLTLNRLITIGDKYDDIAESKLEKARRLEEIRELKRKEIERKEAL
151  KQDKLEEKKDEIKKSSVARTIRRNKRNDMLKSEAKASESKTEGRKVKKV
201  SFAQ

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**Figure 2.** Amino acid sequence of *Loc1p* as predicted from the Yeast Genome Sequencing project. One remarkable feature of *Loc1p* is the abundance of arginine and lysine in this protein. *Loc1p* contains 20.1% lysine and 9.8% arginine. The pI value of 10.79 for *Loc1p* is a reflection of the abundance of positive charge in this protein.

A plasmid expressing a fusion RNA containing two copies of the MS2 coat protein stem-loop motif and one copy of the *ASH1* 3'-UTR localization element (E3) was transformed into the three-hybrid host yeast strain, L40-coat. The yeast strain expressing the fusion RNA was transformed with a yeast cDNA library fused to the Gal4p activation domain, and the cells were plated onto selection media. From the 2,000 colonies that grew on the selection plates, only four colonies were found to express the reporter genes dependent on the fusion RNA. The identity of the cDNA inserts was determined by DNA sequencing. From the *S. cerevisiae* genome database, two cDNAs corresponded to the yeast ribosomal protein L9B/L9A whereas the remaining two cDNAs corresponded to the uncharacterized open reading frame YFR001w (Fig. 2). We have given YFR001w the name *LOC1*. *LOC1* is predicted to encode a 26-kD protein rich in arginine and lysine. It does not demonstrate any homology to known proteins.

We investigated the specificity of the *Loc1p*–RNA interaction by three-hybrid analysis using the *ASH1* cis-acting localization elements E1, E2A, and E3 as bait as well as point mutants in the element E3 (M9, M13, M14, and M9+9A; Chartrand et al., 1999; Table III). Assuming that the various fusion RNAs were expressed equivalently, the level of β-galactosidase expression was indicative of the strength of the *Loc1p*–RNA interaction for the respective elements. From this analysis, we observed a correlation between mRNA localization activity of elements E1 and E3

**Table III.** *Loc1p* Binding Activity with the Localization Elements E1, E2A, and E3

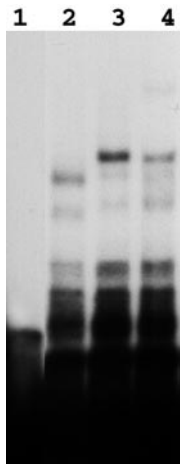
Fusion RNA*	Binding activity <sup>‡</sup>	RNA localization activity <sup>§</sup>
E3/wt	1.00	1.00
E2A/wt	0.13	1.10
E1/wt	0.97	1.00
E3/M9+M9A	0.65	0.82
E3/M9	0.27	0.02
E3/M14	0.30	0.07
E3/M13	0.22	0.10
Vector	0.28	ND <sup>  </sup>
<i>ADH1</i>	0.02	0.10
IRE	1.51	ND

\**ASH1* cis-acting localization elements analyzed for interaction with *Loc1p* and RNA localization activity.

<sup>‡</sup>*Loc1p* binding activity for the various elements measured by β-galactosidase expression in the three-hybrid assay. β-galactosidase levels are normalized to *Loc1p* with the E3/wt element.

<sup>§</sup>Indicates the percentage of budding yeast with *lacZ* mRNA localized to the bud tip. Values are normalized with the E3/wt element.

<sup>||</sup>Not determined.



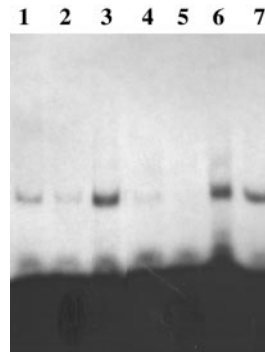
**Figure 3.** The formation of the specific RNA–protein complex is dependent on Loc1p. Band mobility shift assays were performed as mentioned in the legend to Fig. 1. Lane 1, E3 RNA probe only, no protein extract. Lanes 2 and 3, E3 transcripts were incubated with yeast extracts of YLM090 (*loc1*) and K699 (wild-type) strains. Lane 4, E3 transcripts incubated with extracts of YLM090 (*loc1*) complemented with a *LOCI-myc* plasmid.

and the strength of its interaction with Loc1p. Loc1p activated  $\beta$ -galactosidase expression equally from elements E1 and E3, whereas the expression was reduced in cells containing any of the mutations (M9, M13, and M14) predicted to disrupt the secondary structure of the element E3 and its localization function. The double mutant M9+9A, which is predicted to restore the secondary structure and the localization function of the element E3, also partially restored the interaction with Loc1p. These results suggest that the elements E1 and E3 are functionally redundant with Loc1p, and this interaction apparently depends on the integrity of the secondary structure of these elements. In contrast, element E2A appears to interact weakly with Loc1p, implying that Loc1p may function differently with element E2A. Alternatively, the expression level of the E2A three-hybrid fusion RNA may be lower than either E1 or E3.

To test whether Loc1p could interact with any RNA containing strong secondary stem-loop structures, we used a construct containing the IRE (SenGupta et al., 1996). The interaction between Loc1p and the IRE RNA resulted in a  $\beta$ -galactosidase expression level even greater than any of the *ASH1* RNA constructs (Table III). The level of  $\beta$ -galactosidase expression observed with the vector control most likely represents Loc1p–RNA binding activity for the two MS2 stem loop structures found in this plasmid. Binding of the MS2 coat protein to one stem-loop and binding of Loc1p to the second stem-loop could create a functional transcriptional activator, resulting in some  $\beta$ -galactosidase expression. These data suggest that Loc1p has affinity for double-stranded RNA.

### *Loc1p Directly Binds Double-stranded RNA and Associates with ASH1 mRNA In Vivo*

We more closely investigated Loc1p RNA-binding activity by mobility shift assay using E3 RNA probe and extracts prepared from wild-type and *loc1* strains (Fig. 3). An RNA–protein complex of the same electrophoretic mobility was not detected from the *loc1* extract (Fig. 3, lane 2) compared with the wild-type extract (Fig. 3, lane 3). However, a new complex of lower molecular weight appeared from the *loc1* extract, possibly coming from a nonspecific RNA-binding protein. After incubating element E3 with an extract prepared from a *loc1* strain expressing Loc1p-myc from a multicopy plasmid (Fig. 3, lane 4), we observed that the specific

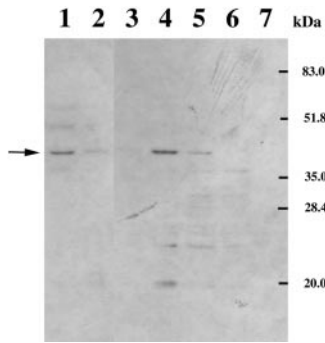


**Figure 4.** Recombinant Loc1p-GST fusion binds to E3. Lanes 1 and 2, E3 transcripts incubated with bacterial extracts containing recombinant GST-Loc1p but immunodepleted by GST antibody or glutathione beads, respectively. Lane 3, E3 transcripts incubated with the extract before immunodepletion. Lanes 4, 5, and 6, E3 transcripts incubated with bacterial extract containing recombinant GST-Loc1p in the presence of 100 $\times$  excess of unlabeled E3, IRE, and pGEM 3Z RNAs, respectively. Lane 7, E3 transcripts incubated with SDS-PAGE purified recombinant Loc1p-GST fusion. The arrow indicates the E3/Loc1p-GST complex.

RNA–protein complex was restored. This result indicates that Loc1p is required for the formation of the higher molecular weight RNA–protein complex. However, these results do not distinguish whether Loc1p is a component of the complex or if Loc1p directly binds element E3.

To determine whether Loc1p directly binds the E3 element *in vitro*, we expressed and purified recombinant GST-Loc1p fusion protein. We observed that an extract containing GST-Loc1p recombinant protein could form a complex with the E3 element in a mobility shift assay (Fig. 4, lane 3). The complex was much reduced when GST-Loc1p was specifically removed from the extract by immunodepletion (Fig. 4, lanes 1 and 2). When incubated with excess unlabeled E3 (Fig. 4, lane 4) or IRE (Fig. 4, lane 5), the complex was effectively competed. However, this complex was not competed with pGEM RNA (Fig. 4, lane 6). Finally, GST-Loc1p eluted from a denaturing gel (Fig. 4, lane 7) could still bind E3 in a two component system. These data indicate that recombinant GST-Loc1p is able to directly bind element E3 and recapitulate the *in vivo* binding observed in the three-hybrid system.

To confirm further that Loc1p is a component of the RNA–protein complex *in vivo*, we passed a protein extract containing Loc1p-myc over an E3 RNA chromatography column and eluted the bound proteins from the RNA column with a high salt solution. From amino acid sequencing of the purified proteins, Loc1p was not detected as one of the abundant proteins. Therefore, the affinity-purified protein fractions were analyzed by Western blot to detect Loc1p-myc using anti-myc antibody (Fig. 5). Loc1p-myc was detected in the high salt elution fraction (Fig. 5, lane 4) but not in the wash fraction (Fig. 5, lane 3). However, if we used a column that contained the element E3 RNA with a mutation that disrupted its secondary structure and its localization (the M9 mutant; Chartrand et al., 1999), we found a significantly reduced level of Loc1p-myc in the high salt fraction (Fig. 5, lane 5). This result is consistent with the *in vitro* binding assay (using mutant M13, data not shown) and the three hybrid results. When using an affinity column with the non-specific pGEM 3Z RNA, no Loc1p-myc was found in the high salt fraction (Fig. 5, lane 6). All these results indicate that Loc1p can interact with localization element E3, and this interaction correlates closely with the structural integrity required of element E3 for *ASH1* mRNA localization.

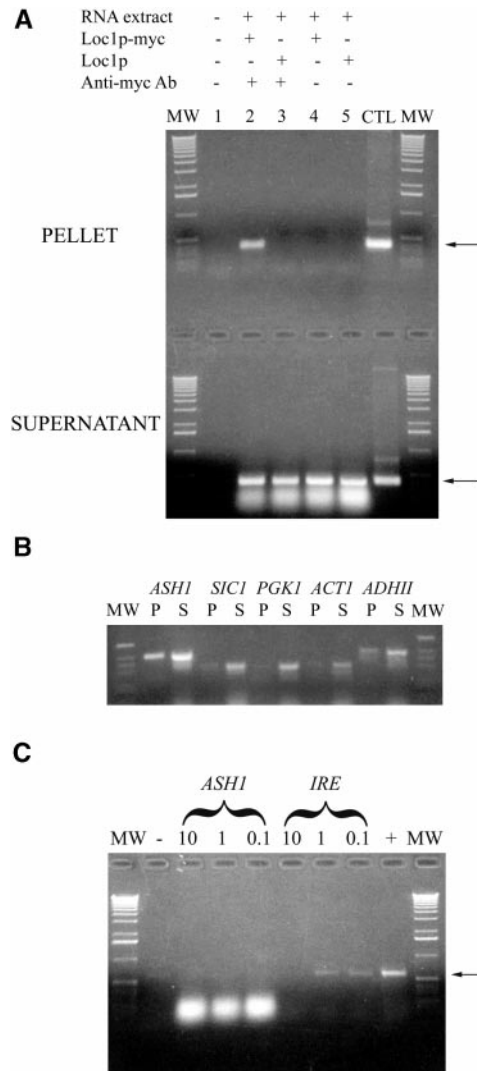


**Figure 5.** Affinity purification of Loc1p-myc from an E3 RNA column. RNA affinity-purified fractions were analyzed by Western blot with anti-myc antibody. Lane 1, starting extracts; lane 2, flowthrough fraction; lane 3, wash fraction; lane 4, elution fraction with high salt; lanes 5 and 6, elution fractions from RNA affinity columns using M9

and pGEM 3Z RNA as ligands, respectively; and lane 7, protein extract from strain K699 (no myc-tagged protein). Arrow, Loc1p-myc protein.

The above observations suggested that Loc1p associates with full-length *ASH1* mRNA in vivo. We investigated this potential association using a combination of immunoprecipitation and RT-PCR (Fig. 6 A). A *loc1* deletion strain was transformed with a plasmid expressing either Loc1p-myc or Loc1p. Cell lysates were prepared from these two strains and used for immunoprecipitation in the presence or absence of anti-myc monoclonal antibody 9E10. By RT-PCR analysis of the immunoprecipitations, we only detected endogenous *ASH1* mRNA in the precipitation product from Loc1p-myc containing extracts. However, the control supernatants contained *ASH1* mRNA. We did not detect *ASH1* mRNA by this assay using lysates from wild-type cells or lysates containing Loc1p-myc without the addition of anti-myc antibody. These results suggest that in yeast cells, Loc1p associates with full-length *ASH1* mRNA in vivo. We also investigated if Loc1p associates in vivo with several other endogenous yeast mRNAs. From the Loc1p-myc immunoprecipitation pellet, we performed RT-PCR reactions with primers specific for the *ACT1*, *ADH1*, *PGKI*, and *SIC1* mRNAs. By comparing the ratio of RNA between the pellet and supernatant in Fig. 6 B, it indicates that immunoprecipitation of Loc1p-myc still precipitates these mRNAs but not as efficiently as *ASH1* mRNA.

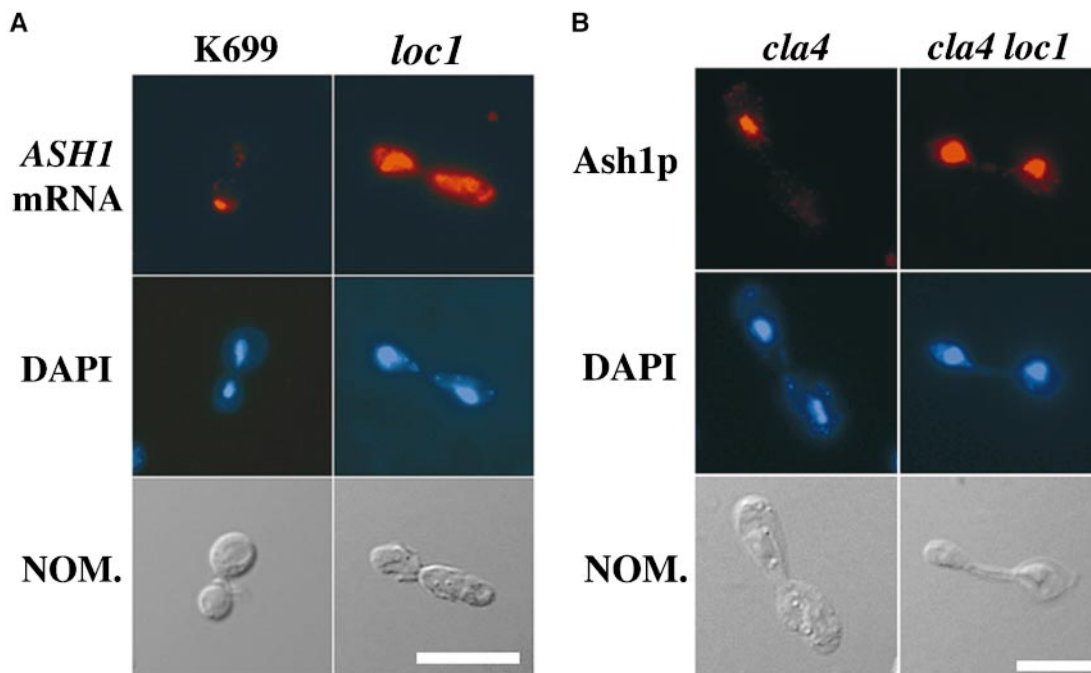
The fact that Loc1p seems to interact, albeit weakly, with other endogenous mRNAs in vivo and to double-stranded RNA in vitro raises the question of the specificity of Loc1p binding to the *ASH1* mRNA and whether Loc1p binds nonspecifically to any RNA containing stem-loop structures. To address this concern, we repeated the immunoprecipitation/RT-PCR experiments and included exogenous competitor RNAs in the yeast extract in order to compete the binding of Loc1p-myc for the *ASH1* mRNA. We added, before the immunoprecipitation of Loc1p-myc, either a mix of the four *ASH1* localization elements (E1, E2A, E2B, and E3) or IRE RNA, which also folds into a stem-loop structure (Klausner et al., 1993). As shown in Fig. 6 C, the presence of an excess (between 10 and 0.1  $\mu$ g) of the four *ASH1* localization element RNAs in the yeast extract effectively competed the binding of Loc1p-myc to the *ASH1* mRNA, which cannot be detected in the pellet after the immunoprecipitation. However, the addition of the IRE RNA at similar concentrations to the yeast extract did not result in an efficient competition of Loc1p-myc binding to the *ASH1* mRNA, as we could still detect the



**Figure 6.** *ASH1* mRNA coimmunoprecipitates specifically with Loc1p-myc. (A) RT-PCR amplification of *ASH1* mRNA extracted from pellet (top) or supernatant (bottom) after immunoprecipitation. Lane 1, RT-PCR reaction without RNA extract. Lanes 2 and 4, immunoprecipitation from strain expressing Loc1p-myc with (lane 2) or without (lane 4) anti-myc antibody. Lanes 3 and 5, immunoprecipitation from strain expressing Loc1p with (lane 3) or without (lane 5) anti-myc antibody. CTL, control PCR amplification of the *ASH1* gene. MW, molecular weight marker. Arrows indicate the *ASH1* PCR fragment of 375 bp. (B) RT-PCR amplifications of various mRNAs after immunoprecipitation of Loc1p-myc. P, RNA extracted from the pellet; S, RNA extracted from the supernatant; MW, molecular weight marker. (C) Competition of the immunoprecipitation of *ASH1* mRNA by the localization elements. Yeast extract was incubated with either 10, 1, and 0.1  $\mu$ g of an equimolar mix of the four *ASH1* localization elements (*ASH1*) or the IRE RNA (*IRE*) before immunoprecipitation of the Loc1p-myc protein and RT-PCR of the *ASH1* mRNA. MW, molecular weight marker; -, negative control (no yeast extract); +, positive control (IP of *ASH1* mRNA and RT-PCR without competition).

*ASH1* mRNA by RT-PCR in the pellet after competition with 1 or 0.1  $\mu$ g of IRE RNA. These results suggest that in vivo, Loc1p interacts more strongly with the *ASH1* mRNA localization elements than to other double-stranded RNA.





**Figure 7.** The *ASH1* mRNA is delocalized in a *loc1* yeast strain. (A) Fluorescent in situ hybridization for *ASH1* mRNA in wild-type (K699) and *loc1* (YLM090) yeast cells. *ASH1* mRNA is expressed from a multicopy plasmid (YEPlac181). DAPI, DNA staining; NOM., Nomarski. The *ASH1* mRNA is tightly localized at the bud tip of a wild-type cell, whereas it is delocalized in the cytoplasm of a late anaphase *loc1* yeast cell. Bar, 10  $\mu$ m. (B) Immunofluorescence detection of the Ash1p-myc in postanaphase YML094 (*cla4*) and YML093 (*cla4 loc1*) yeast cells. DAPI, DNA staining; NOM., Nomarski. Note the elongated bud neck caused by the deletion of the *CLA4* gene. In the majority of cells, Ash1p-myc is symmetrically distributed in the *loc1* strain. Bar, 10  $\mu$ m.

### *loc1* Cells Exhibit Defects in *ASH1* mRNA Localization and Ash1p Segregation

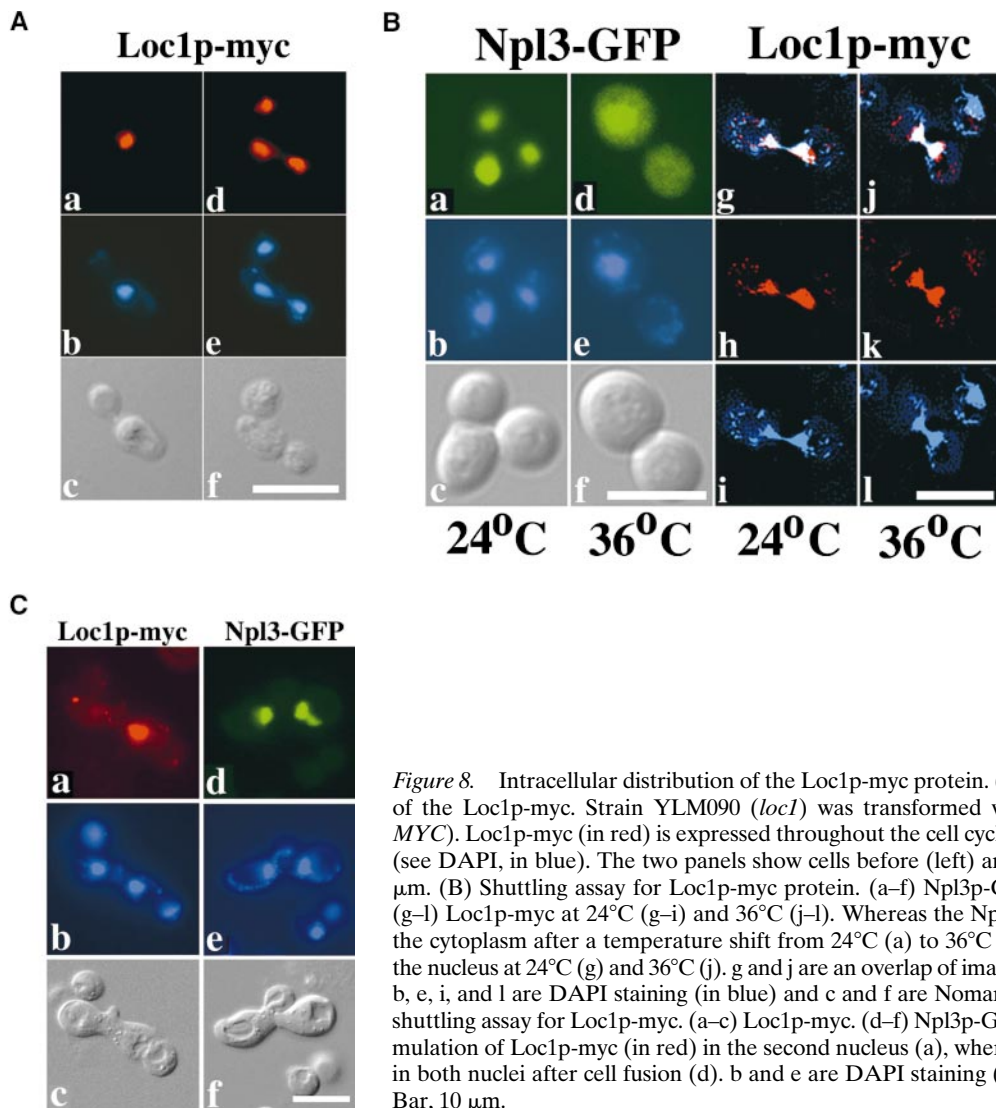
The data presented thus far indicate that Loc1p has an affinity for *ASH1* mRNA localization elements. We next addressed whether *ASH1* mRNA localization requires Loc1p. A *loc1::KAN* strain was constructed, and this strain was viable but grew more slowly than the wild-type parental strain at all temperatures tested. The slow growth phenotype may explain why *LOC1* was not identified in the original *she* mutant selection (Jansen et al., 1996). We analyzed the intracellular distribution of *ASH1* mRNA in a *loc1* strain by fluorescent in situ hybridization (FISH; Fig. 7 A) and observed a diminution of mRNA forming a tight crescent at the bud tip (89% in the wild-type to 13% in *loc1*) for endogenous *ASH1* mRNA. The delocalization of *ASH1* mRNA in a *loc1* strain was also observed in cells overexpressing *ASH1* mRNA (62% in the wild-type to 13% in *loc1*). These results indicate that Loc1p is important for efficient localization of *ASH1* mRNA.

The delocalization of the *ASH1* mRNA in a *loc1* strain should also affect the asymmetric distribution of Ash1p to daughter cell nuclei. Consequently, we investigated the sorting of Ash1p to daughter cell nuclei by immunofluorescence in a *loc1 cla4* strain (Fig. 7 B). The *cla4* strain has an elongated bud neck, due to a cytokinetic defect (Cvrckova et al., 1995), which allows an easier identification of postanaphase budding cells. The deletion of *CLA4* does not affect the asymmetric distribution of the Ash1p (Bobola et al., 1996). We observed that Ash1p is symmetrically distributed between mother and daughter nuclei in 78% of *cla4 loc1* budding cells compared with 26% of *cla4* bud-

ding cells. These results demonstrate that Loc1p functions to segregate Ash1p efficiently to daughter cell nuclei through the localization of the *ASH1* mRNA.

We investigated whether the *loc1* slow growth phenotype could be separated from the *ASH1* RNA localization defect. To screen for mutants of *LOC1* which delocalize the *ASH1* mRNA but still maintain a normal growth rate at 30°C, we modified a genetic screen developed by Jansen et al. (1996). This screen relies on the transcriptional repression activity of Ash1p for the *HO* promoter. In this assay, a yeast strain containing the *CAN1* gene under the control of the *HO* promoter (*HO-CAN1*) and capable of asymmetric *ASH1* mRNA localization will grow very slowly on a plate containing the toxic drug canavanine. However, a mutant yeast strain (*HO-CAN1*) that delocalizes *ASH1* mRNA will grow normally on media containing 0.03% canavanine (Jansen et al., 1996). Therefore, a yeast strain (*HO-CAN1*) with a *loc1* mutation that results in a delocalized *ASH1* mRNA should display a normal growth rate on media containing 0.03% canavanine plate. In contrast, *loc1* mutants which disrupt *ASH1* mRNA localization and have a slow growth phenotype should grow very slowly on media containing 0.03% canavanine. We generated by PCR mutagenesis a plasmid library of *loc1* mutants using the error-prone Taq DNA polymerase (Zhou et al., 1991), transformed this library into yeast strain YPC001 (*HO-CAN1 loc1*), and plated the transformants on 0.03% canavanine plates in order to isolate clones which exhibit mRNA localization defects but still maintain normal growth at 30°C. However, we observed that no yeast colonies were able to grow on these plates, suggest-





**Figure 8.** Intracellular distribution of the Loc1p-myc protein. (A) Immunofluorescence detection of the Loc1p-myc. Strain YLM090 (*loc1*) was transformed with the plasmid pRL094 (*LOC1-MYC*). Loc1p-myc (in red) is expressed throughout the cell cycle and appears to be strictly nuclear (see DAPI, in blue). The two panels show cells before (left) and during (right) anaphase. Bar, 10  $\mu$ m. (B) Shuttling assay for Loc1p-myc protein. (a–f) Npl3p-GFP at 24°C (a–c) and 36°C (d–f). (g–l) Loc1p-myc at 24°C (g–i) and 36°C (j–l). Whereas the Npl3p-GFP (in green) accumulates in the cytoplasm after a temperature shift from 24°C (a) to 36°C (d), Loc1p-myc (in red) remains in the nucleus at 24°C (g) and 36°C (j). g and j are an overlap of images h and i, and k and l, respectively. b, e, i, and l are DAPI staining (in blue) and c and f are Nomarski. Bar, 10  $\mu$ m. (C) Heterokaryon shuttling assay for Loc1p-myc. (a–c) Loc1p-myc. (d–f) Npl3p-GFP. Binuclear yeasts show no accumulation of Loc1p-myc (in red) in the second nucleus (a), whereas Npl3p-GFP (in green) appears in both nuclei after cell fusion (d). b and e are DAPI staining (in blue) and c and f are Nomarski. Bar, 10  $\mu$ m.

ing that the *loc1* slow growth phenotype and RNA localization defect are linked.

### *Loc1p Is a Strictly Nuclear Protein*

As Loc1p is required for efficient *ASH1* mRNA localization, we reasoned that Loc1p could be involved in the transport and/or anchoring of this mRNA to the bud tip. If Loc1p is associated with the *ASH1* mRNA, it should also be present at the bud tip of late anaphase cells. To determine the intracellular location of Loc1p in yeast cells, we tagged this protein with six myc epitopes and detected Loc1p-myc<sub>6</sub> by immunofluorescence. In a *loc1* strain, the Loc1p-myc<sub>6</sub> construct restored the efficient localization of the *ASH1* mRNA and rescued the *loc1* slow growth phenotype. From these results we concluded that Loc1p-myc<sub>6</sub> was functional (data not shown).

Surprisingly, we found that Loc1p-myc<sub>6</sub> was present only in the nucleus of yeast cells and apparently absent from the cytoplasm, even when overexpressed and imaged with maximal sensitivity (Fig. 8 A). Loc1p-myc was never observed coincident with the mRNA at the bud tip of late anaphase cells. This result suggests that Loc1p is essen-

tially nuclear; however, this experiment did not rule out the possibility that Loc1p could shuttle between the nucleus and the cytoplasm.

To address this possibility, we used a nuclear shuttling assay that uncoupled nuclear protein export from nuclear import (Lee et al., 1996). This assay used a yeast strain containing a temperature sensitive mutation (*nup49-313*) in the nucleoporin *NUP49*. At the nonpermissive temperature of 36°C, protein import into the nucleus is defective (Schlenstedt et al., 1993; Doye et al., 1994). However, nuclear export appears normal in this strain at the nonpermissive temperature. When incubated at 36°C, shuttling proteins can be exported from the nucleus but not reimported and will accumulate in the cytoplasm, whereas exclusively nuclear proteins will be retained in the nucleus. Loc1p-myc was cloned downstream of a *GALI*-inducible promoter and transformed into the *nup49-313* strain. After a transient induction with galactose, followed by repression with glucose at 25°C, the cells were shifted to 36°C for 5 h in presence or absence of cycloheximide. Immunofluorescence was subsequently performed on these cells. At the permissive temperature, Loc1p-myc was present exclusively in the nucleus; a shift to the nonper-

missive temperature did not result in significant cytoplasmic accumulation of Loc1p-myc in any of the cells (Fig. 8 B, panels g–l). Under similar conditions, the shuttling protein Npl3p-GFP (Lee et al., 1996) was exported from the nucleus and accumulated in the cytoplasm (Fig. 8 B, panels a–f). A formal possibility is that cytoplasmic Loc1p may be unstable for extended periods of time. Consequently, Loc1p shuttling may not be observed in this case. Therefore, we studied shuttling using a second approach which relies on the formation of heterokaryons in yeast (Flach et al., 1994). In this assay, shuttling proteins are observed to move from one nucleus to a second nucleus in the heterokaryon. Again, Loc1p-myc was observed never to shuttle between the nuclei through the cytoplasm (Fig. 8 C). The results from these two approaches strongly suggest that Loc1p functions entirely within the nucleus and this ultimately affects the localization of the *ASH1* mRNA in the cytoplasm.

## Discussion

We have used a segment of *ASH1* mRNA, shown to be sufficient for localization of this mRNA to the bud, in a three-hybrid assay to characterize Loc1p, a protein that associated with full-length *ASH1* mRNA and was required for its localization. *LOCI* is an uncharacterized gene in yeast, and we observed that deletion of *LOCI* resulted in a slow-growth phenotype. *LOCI* encodes a highly basic 26-kD protein that did not have homology to any known proteins. In contrast to the *she* mutants where cells were not observed with localized *ASH1* mRNA, Loc1p seems to act as an efficiency factor which significantly increases the percentage of tightly localized mRNA at the bud tip. This Loc1p-dependent RNA localization was important for the proper asymmetric distribution of Ash1p, as in a *loc1* strain we observed a significant reduction in the asymmetric sorting of Ash1p to the nucleus of the daughter cells.

We have shown by different approaches that Loc1p interacts with *ASH1* mRNA sequences: three-hybrid, affinity purification, immunoprecipitation/RT-PCR, and mobility shift assays. Mutations that disrupt the secondary structure of the element E3 and which result in the loss of its localization function also affect the binding of Loc1p. These results suggest a potential link between Loc1p binding to the element E3 and the localization function of this element. Moreover, Loc1p was also found to interact with element E1, which is predicted to contain a stem-loop structure (Chartrand et al., 1999). These multiple interactions of Loc1p with several localization elements in the *ASH1* mRNA sequence could explain the negative effect of the *loc1* deletion on the localization of this mRNA. Loc1p can also bind the IRE stem-loop structure, but not unstructured RNA. In mobility shift assays, the specific RNA–protein complex could be competed by IRE RNA, suggesting that Loc1p is a double-stranded RNA-binding protein. However, binding of Loc1p to any stem-loop structure in an mRNA is insufficient for localization, as only specific stem-loops are recognized by the cytoplasmic components of the localization pathway, such as She2p (Chartrand et al., 1999; Böhl et al., 2000; Long et al., 2000). Additionally, in vivo Loc1p appears to recognize *ASH1* mRNA specifically, as other mRNAs were only weakly co-

precipitated in comparison. Moreover, although in vivo Loc1p binding to the *ASH1* mRNA was effectively competed by exogenous *ASH1* localization element RNAs, another double-stranded competitor, the IRE RNA, must be present in greater excess to effectively compete the binding. These results suggest that in vivo Loc1p has a higher affinity for *ASH1* mRNA than with other mRNAs. However, the three-hybrid results suggest that Loc1p has affinity for any double-stranded RNAs, but due to overexpression of the three-hybrid components as well as potential differences in steady state levels of the fusion RNAs, the three-hybrid results must be regarded as a qualitative. Consequently, the immunoprecipitation/RT-PCR experiments using either *ASH1* elements or IRE as competitors most likely reflect the true differences in affinity of Loc1p for these various RNA substrates.

From the slow growth defect and altered cell morphology observed for *loc1* cells, it could be argued that delocalization of *ASH1* mRNA is an indirect effect of the mutation. The association of Loc1p with full-length *ASH1* mRNA, both in vivo and in vitro, argues against this. Furthermore, the abundance of She1p, She2p, and She3p is not altered in *loc1* cells compared with wild-type cells (data not shown), and therefore the delocalization of *ASH1* mRNA in *loc1* cells is not an indirect effect through alterations in the steady state levels of She1-3p. Also, no gross abnormalities in the organization of the actin cytoskeleton in *loc1* cells (data not shown) were observed, suggesting that She4p and She5p are also functional in *loc1* cells. We also screened for mutants of *LOCI* with growth rates similar to wild-type cells but still delocalize *ASH1* mRNA. After screening 9,000 *loc1* mutants, we were unable to isolate any revertants of the slow growth defect which still maintained the *ASH1* mRNA localization defect. Possibly Loc1p may have another role in nuclear RNA metabolism. Our data favor a direct role for Loc1p in *ASH1* mRNA localization. However, if delocalization of *ASH1* mRNA in *loc1* cells were by an indirect effect, it would likely be through a yet unidentified component of the *ASH1* mRNA localization pathway, making *loc1* cells a valuable tool for identifying additional novel components of the RNA localization pathway.

Loc1p is not the first double-stranded RNA binding protein found to be involved in mRNA localization. In *Drosophila* oocytes, the protein Staufén is important for the localization of the *bicoid* and *oskar* mRNA to the anterior and posterior pole, respectively (St. Johnston et al., 1991). This protein recognizes specific double-stranded structures in the 3'-UTR of the *bicoid* mRNA in vivo (Ferandon et al., 1994). Staufén does not display any specificity in binding double-stranded RNA in vitro (St. Johnston et al., 1992). It is possible that accessory proteins can provide some specificity for the binding of Staufén and Loc1p.

We have found that Loc1p is a nuclear protein that does not shuttle between the nucleus and cytoplasm. We showed that this protein is exclusively nuclear by three approaches: a ts mutant (*nup49*; Lee et al., 1996) which allows nuclear export but not import, did not accumulate Loc1p in the cytoplasm, a heterokaryon assay (Flach et al., 1994) where only a single nucleus retained Loc1p, and high sensitivity imaging where Loc1p was never detected in the cytoplasm. As the *ASH1* gene does not contain any

introns, Loc1p is probably not a splicing factor. Importantly, Loc1p is not required for nuclear export of the *ASH1* mRNA, as *loc1* mutant strains have *ASH1* mRNA in the cytoplasm. More significantly, *ASH1* mRNA cannot localize to the bud tip in most cells unless it has been first exposed to Loc1p in the nucleus. This exclusive nuclear location of Loc1p is in contrast to RNA-binding shuttling proteins such as the *Drosophila* hnRNP *Sqd*, required for the localization of *gurken* mRNA in oocytes and *fushi tarazu* mRNA in embryos (Lall et al., 1999; Norvell et al., 1999), or other hnRNPs implicated in RNA localization (Ross et al. 1997; Hoek et al., 1998; Cote et al., 1999).

However the nuclear location, even if temporary, for all these proteins suggests that nuclear events may be essential. Possibly they recognize the mRNA to be localized in the nucleus before it gets exported to the cytoplasm (Lall et al., 1999; Cote et al., 1999). In the cytoplasm, this protein-mRNA complex can now be targeted to the localization pathway. The finding that an exclusively nuclear protein is important for mRNA localization indicates that exposure to factors exclusively nuclear can play an important role in subsequent events in the cytoplasm which do not require the continued nuclear protein-RNA interaction. It is possible that this role involves packaging the RNA correctly in the nucleus with appropriate proteins so that the RNA may be efficiently recognized in the cytoplasm or transported there by the correct pathway.

These results demonstrate the increasingly complicated characteristics of the mRNA localization mechanism in yeast, which begins most likely when the RNA to be localized is transcribed and involves a growing cast of RNA and protein determinants on its journey to the bud tip.

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