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

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Abstract. The localization of ASHI 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 ASHI 3′-UTR RNA, but not to a mutant RNA incapable of localizing to the bud nor to several other mRNAs. LOCI codes for a novel protein that recognizes double-stranded RNA structures and is required for efficient localization of ASHI 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 ASHI mRNA localization requires a previous interaction with specific nuclear factors.

Key words: ASHI • 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 regulation 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 α. 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 ASHI mRNA to the distal tip of daughter cells during anaphase of the cell cycle (Long et al., 1997; Takizawa et al., 1997). Localization of ASHI 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 ASHI mRNA–containing particles were observed to move from mother cells to daughter cells, suggesting that Myo4p apparently has a direct role in transporting ASHI 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 ASHI 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 ASHI mRNA, it was proposed that She2p recruits the Myo4p/She3p complex to the ASHI 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 ASHI mRNA at the bud neck (Jansen et al., 1996; Long et al., 1997). The mislocalization of ASHI 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
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 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 loc), 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.

Table I. Yeast Strains Used in This Study

| Strain | Genotype | Source |
|--------|----------|--------|
| L40-coat | MATA, ura3-52, leu2-3,112, his3-200, trpl-1, ade2, 2LYS2::(LexAop)-lacZ, LexA-M52 coat (TRP1) | Sen Gupta et al., 1996 |
| YB1-1 | MATA, ura3-52, leu2-3,112, his3-200, trpl-1, ade2, 2LYS2::(LexAop)-lacZ, LexA-M52 -M52 coat (N55K) | M. Wickens |
| K699 | MATA, ura3, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100 | Jansen et al, 1996 |
| K5552 | MATA, ura3, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, ASH1-myc9 | Long et al., 1997 |
| K4452 | MATA, HO-ADE2 HO-CAN1 leu2-3,112, ura3, his3 | Jansen et al, 1996 |
| YPC001 | MATA, HO-ADE2 HO-CAN1 leu2-3,112, ura3, his3, loc1::KAN | This study |
| YLM090 | MATA, ura5, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, loc1::KAN | This study |
| YLM091 | MATA, ura5, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, ASH1-myc9, loc1::KAN | This study |
| YLM092 | MATA, ura5, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, ASH1-myc9, loc1::KAN | This study |
| YLM093 | MATA, ura5, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, ASH1-myc9, loc1::KAN, cls4::KAN | This study |
| YLM094 | MATA, ura5, leu2-3,112, his3-11, trpl-1, ade2-1, ho can1-100, ASH1-myc9, 413, trls EUE2 | Lee et al., 1996 |
| PSY413 | MATA, ade2, ade3, his3, leu2, ura3, nup49::TRP, plus pUH100-nup49-131 ts LEU2 | Flach et al., 1994 |
| M739 | MATA, leu2-3,112, ura3-52, ade2-101, karl1 | This study |
Table II. Plasmids Used in This Study

| Plasmid | Features | Source |
|---------|----------|--------|
| pUG6    | Plasmid for generating KAN 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| 2μ yeast shuttle plasmid marked with URA3 | R.D. Gietz |
| pESC-URA3| Yeast galactose-inducible expression vector | CLONTECH Laboratories, Inc. |
| C3003   | E. coli plasmid containing myc epitope | K. Nasmyth |
| C3319   | YEPlac181 yeast plasmid (LEU2, 2μ) containing the ASH1 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 Smal E3 ASH1 localization element | This work |
| pRL090  | YCplac33 containing a 1.6-kb PstI/Smal LOCI fragment | This work |
| pRL091  | YCplac33 containing a 1.6-kb PstI/Smal LOCI fragment with a unique BamH1 site at the Loc1p stop codon | This work |
| pRL092  | YEplac195 containing a 1.6-kb PstI/Smal LOCI fragment with a unique BamH1 site at the Loc1p stop codon | This work |
| pRL093  | pRL091 with six copies of the myc epitope subcloned into the unique BamH1 site | This work |
| pRL094  | pRL092 with three copies of the myc epitope subcloned into the unique BamH1 site | This work |
| pRL134  | Loc1-myc coding sequence from pRL093 subcloned into pESC-URA3 | This work |
| pRL168  | pGEM-Z containing element E3 | Long et al., 2000 |
| pRL176  | pGEM-Z containing element E1 | Long et al., 2000 |
| pRL177  | pGEM-Z containing element E2A | Long et al., 2000 |
| pRL179  | pGEM-Z containing element E2B | Long et al., 2000 |
| pRL204  | pIIAM/MS2-2 containing 130 bp from the ADHII 3′UTR | This work |
| pPS811  | 1.5 kb BamH1 fragment from pPS425 containing NPL3 in pCGF-1A | Lee et al., 1996 |
| pXAR192 | Element E3-mutant M9 (118nt) cloned in pIIIA/MS2-2 | This work |
| pXAR193 | Element E3-mutant M9 +9A (118nt) cloned in pIIIA/MS2-2 | This work |
| pXAR194 | Element E3-mutant M13 (118nt) cloned in pIIIA/MS2-2 | This work |
| pXAR195 | Element E3-mutant M14 (118 nt) cloned in pIIIA/MS2-2 | This work |
| pXAR196 | Element E1 (150 nt) cloned in pIIIA/MS2-2 | This work |
| pXAR197 | 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 selection. Transformants were selected on media lacking leucine and tryptophan (Li et al., 1994) and obtained from the American Type Culture Collection and cultured in the S. Elledge (Baylor College of Medicine, Houston, TX) laboratory.

Hybrid selection is a screen for RNA dependence. The plasmid expressing expression levels were determined either by X-gal filter assay or liquid assay in vitro.

Further characterizations of Loc1p RNA-binding activity were performed by selecting transformants resulting from low levels of histidine containing 5 mM 3-aminotriazole to reduce background in the selection. Transformants were selected on media lacking leucine and tryptophan (Li et al., 1994) and obtained from the American Type Culture Collection and cultured in the S. Elledge (Baylor College of Medicine, Houston, TX) laboratory (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 1X 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 100X, 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–l, 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 (Scianalytics). 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 pRL34 and pPS811 contain a galactose-inducible cassette of Loc1-myc and Npl3-green fluorescent protein (GFP), 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, the cells were washed again with an equal volume of YEPD and resuspended in SC media for 1 h before being processed for immunofluorescence.

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. At the temperature shift, cultures were fixed and processed for immuno-
fluorescence as described above.

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: 5'-CGGG ATC CCG ATG GCA CCA AAG AAA CCT TCT TAA AGA-3' and 5'-CGC GCC GAA TTC TTT AAT GTC CTT GCT AGT TTG C. The PCR product was digested with BamHI/EcoRI and cloned in the BamHI/EcoRI sites of plasmid pGEX-5X-3 (Amersham Pharmacia Bio-
tech). The GST-LOC1 junction and the LOC1 PCR product were con-
formed by DNA sequencing. The pGEX-Loc1 plasmid was transformed into Escherichia coli BL21, expressed by induction with IPTG, and puri-
fied on a glutathione column following the GST Gene Fusion System Protoc (Amersham Pharmacia Biotech).

Heterokaryon Protein Shuttling Assay

This assay was performed essentially as described previously (Flach et al., 1994). Strain K69 was transformed with one of the following plasmids: pESC-UARA3, pRL134, or pPS811. Transformants were grown overnight at 30°C in SC media containing 2% glucose but lacking uracil. These cul-
tures were diluted into uracil dropout media containing 2% raffinose and grown overnight at 30°C. When the cultures reached a cell density of 3 × 10^7 cells/ml, galactose was added to a final concentration of 2% and cul-
tures were incubated for 1 h at 30°C. Cells were harvested by centrifu-
gation, washed once in YEPD, and resuspended at a density of 2 × 10^7 cells/ml in YEPD. Cultures were incubated for 2 h at 30°C following by mating to strain YMT79 (karl-1). For mating, 3 × 10^5 cells of the K69 transformants were mixed with 3 × 10^5 cells of YMT79 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_g_0.1–0.5 and disrupted with glass beads in breakage buffer (20 mM Tris/His, pH 7.4, 3 mM MgCl_2, 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 centrifuga-
tion, the supernatant was recovered and centrifuged for an additional 30 min at 10,000 g, and the supernatant (containing about 5–10 μg/ml 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 ASHI 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, 32P-labeled RNA was gen-
erated by SP6 RNA polymerase directed in vitro transcription from an

Immunoprecipitation and Reverse Transcription PCR

Yeast cells (strain K69 containing plasmid pRL094) were grown to OD_g_0.1, centrifuged at 4,000 rpm for 10 min at 4°C, and resuspended at 100 OD_g/ml in a breakage buffer (1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluo-
ride, 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-Tag antibody (Roche) were added to 200 μl of extract incubated for 2 h at 4°C. 5 μl of pro-
tein-A-agarose slurry (Immunopure Protein A; Pierce Chemical Co.) was 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 re-
moved from the beads, and the beads were boiled in 200 μl of diethyl py-
rocarbonate (DEPC) water for 5 min, spun, and the supernatant recov-
ered. 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 chloro-
form. 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 first primers were used in the amplification: ASHI PCR 23 (5'-CGCCGGCGGTTGATTTTCTGGTATGTT-3') and ASHI PCR 56 (5'-CGGCGCCGGAATGAAGT-GAAGGTGACAGTGCTATACCACA-3'), which generates a PCR product of 375 bp. For the detection of the other mRNAs, the follow-
ing primers were used: ACTI-PCR-3' 5'-TTGACAAAACACTTACTA-CAACTTACAT-3' and ACTI-PCR-3' 5'-TTAGAAACACTTGTG- GAAACGATT-3', amplifying a 307 bp fragment of ACTI mRNA; ADH2-PCR-3' 5'-CGACCTTCACCAAGAGGAAGAC-3' and ADH2-PCR-3' 5'-TAGGAGATCCTATTGAAATGG-3', amplifying a 399 bp fragment of ADH2 mRNA; PGK1-PCR-3' 5'-AACTCTAGAAGTGGTGTTGCTACT-3' and PGK1-PCR-3' 5'-TATCTTCTTCTCGGATAGAAAGCAAC-3', amplifying a 301 bp fragment of PGK1 mRNA; and SIC1-PCR-5' 5'-CTTCGGAAGAATGAGCCAGAG-3' and SIC1-PCR-3' 5'-TAATCGTCTCCGGAATTGTATTTTC-3', amplifying a fragment of 318 bp of SIC1 mRNA.

For the competition of Loc1p binding to ASHI mRNA, elements E1, E2, E3, and E3 (Chartrand et al., 1999) were cloned in plasmids pGEM-
3Z at the BamHI and PstI sites (plasmids pRL16, pRL17, pRL177, and pRLL179; Long et al., 2000) and transcribed in vitro using T7 RNA poly-
merase (MegaScript Transcription kit; Ambion). The transcribed RNAs

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of each element were pooled together in an equimolar mix. The 56-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 30 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 50 μM of each dNTP, 2 fmol of template, 5 U Taq DNA polymerase (Boehringer), and 30 pmol of the following primers: YFR001 (5'-GGCGTGCAGGTTGAAGTATAATGATTATATATAGCCAGATTC-3'), and 5'-CCGCGGCAATTCTTTAGTATAGTC-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 pUC18. 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.

**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.

![Figure 1](image-url)  
*A band mobility shift assay identifies proteins that specifically bind the E3 element.*

| Fusion RNA* | Binding activity† | RNA localization activity§ |
|------------|------------------|---------------------------|
| 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†                       |
| ADHII      | 0.02             | 0.10                      |
| IRE        | 1.51             | ND                       |

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

†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.

§Indicates the percentage of budding yeast with lacZ mRNA localized to the bud tip.

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

**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 clones 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+M9A; 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 and various functionalities of the ASH1 3′-UTR localization element, including its specificity for ASH1 mRNA localization and its affinity for Loc1p.
and the strength of its interaction with Loc1p. Loc1p activated β-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 β-galactosidase expression level even greater than any of the ASH1 RNA constructs (Table III). The level of β-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 β-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 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 nonspecific 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.
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, ADHII, PGK1, 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 to compete the binding of Loc1p-myc for the ASH1 mRNA. As shown in Fig. 6 C, the presence of an excess (between 10 and 0.1 μg) 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 ASH1 mRNA by RT-PCR in the pellet after competition with 1 or 0.1 μg 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.
Cells Exhibit Defects in ASH1 mRNA Localization and Ash1p Segregation

The data presented thus far indicate that Loc1p has an affinity for ASH1 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 budding 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 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 plate. 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 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 μm. (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 μm.

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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 by immunofluorescence. In a loc1 strain, the Loc1p-myc construct restored the efficient localization of the ASH1 mRNA and rescued the loc1 slow growth phenotype. From these results we concluded that Loc1p-myc was functional (data not shown).

Surprisingly, we found that Loc1p-myc was present only in the nucleus of yeast cells and apparently absent from the cytoplasm, even when overexpressed and imaged with maximal sensitivity (Fig. 8A). Loc1p-myc was never observed coincident with the mRNA at the bud tip of late anaphase cells. This result suggests that Loc1p is essentially 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 GAL1-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-

**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 μm. (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 μm. (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 μm.
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 ASHI mRNA in the cytoplasm.

**Discussion**

We have used a segment of ASHI 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 ASHI mRNA and was required for its localization. LOC1 is an uncharacterized gene in yeast, and we observed that deletion of LOC1 resulted in a slow-growth phenotype. LOC1 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 ASHI 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 mRNA 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 ASHI 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 ASHI 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 ASHI mRNA specifically, as other mRNAs were only weakly co-precipitated in comparison. Moreover, although in vivo Loc1p binding to the ASHI mRNA was effectively competed by exogenous ASHI 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 ASHI 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 ASHI 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 ASHI mRNA is an indirect effect of the mutation. The association of Loc1p with full-length ASHI 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 ASHI 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 LOC1 with growth rates similar to wild-type cells but still delocalize ASHI mRNA. After screening 9,000 loc1 mutants, we were unable to isolate any revertants of the slow growth defect which still maintained the ASHI mRNA localization defect. Possibly Loc1p may have another role in nuclear RNA metabolism. Our data favor a direct role for Loc1p in ASHI mRNA localization. However, if delocalization of ASHI mRNA in loc1 cells were by an indirect effect, it would likely be through a yet unidentified component of the ASHI 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 Staufen 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 (Ferrandon et al., 1994). Staufen 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 Staufen 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 ASHI gene does not contain any
introns, Loc1p is probably not a splicing factor. Importantly, Loc1p is not required for nuclear export of the ASHI mRNA, as loc1 mutant strains have ASHI mRNA in the cytoplasm. More significantly, ASHI mRNA cannot localize to the bud tip in most cells unless it has been first exposed to Loc1p in the nucleus. This exclusive nuclear localization of Loc1p is in contrast to RNA-binding shuttling proteins such as the Drosophila hnRNPs Sgd, 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 of 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–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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