RNA–protein interactions promote asymmetric sorting of the *ASH1* mRNA ribonucleoprotein complex

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ABSTRACT

In *Saccharomyces cerevisiae*, *ASH1* mRNA is localized to the tip of daughter cells during anaphase of the cell cycle. *ASH1* mRNA localization is dependent on four *cis*-acting localization elements as well as Myo4p, She2p, and She3p. Myo4p, She2p, and She3p are hypothesized to form a heterotrimeric protein complex that directly transports *ASH1* mRNA to daughter cells. She2p is an RNA-binding protein that directly interacts with *ASH1* *cis*-acting localization elements and associates with She3p. Here we report the identification of seven She2p mutants—N36S, R43A, R44A, R52A, R52K, R63A, and R63K—that result in the delocalization of *ASH1* mRNA. These mutants are defective for RNA-binding activity but retain the ability to interact with She3p, indicating that a functional She2p RNA-binding domain is not a prerequisite for association with She3p. Furthermore, the nuclear/cytoplasmic distribution for the N36S and R63K She2p mutants is not altered, indicating that nuclear/cytoplasmic trafficking of She2p is independent of RNA-binding activity. Using the N36S and R63K She2p mutants, we observed that in the absence of She2p RNA-binding activity, neither Myo4p nor She3p is asymmetrically sorted to daughter cells. However, in the absence of She2p, Myo4p and She3p can be asymmetrically segregated to daughter cells by artificially tethering mRNA to She3p, implying that the transport and/or anchoring of the Myo4p/She3p complex is dependent on the presence of associated mRNA.

Keywords: *SHE1/MYO4*; *SHE2*; *SHE3*; *IST2*; *Saccharomyces cerevisiae*; mRNA localization; myosin; RNA-binding protein

INTRODUCTION

Generating cellular polarity requires the asymmetric sorting of specific proteins to distinct regions of a cell. RNA localization is one pathway by which proteins can be asymmetrically sorted, and numerous eukaryotic organisms rely on mRNA localization for the intracellular and intercellular sorting of proteins (Bashirullah et al. 1998; Palacios and St. Johnston 2001; Kloc et al. 2002). The initial event in mRNA localization is the identification of specific *cis*-acting sequences in the mRNA, termed zipcodes, by *trans*-acting mRNA-binding proteins (Kislauskis and Singer 1992; Bashirullah et al. 1998; Palacios and St. Johnston 2001; Kloc et al. 2002). Following specific recognition of the *cis*-acting element, mRNA localization can be achieved through a num-

ber of mechanisms: directed transport, generalized degradation with localized protection, and entrapment/anchoring of RNA at the site of localization (Bashirullah et al. 1998; Palacios and St. Johnston 2001; Kloc et al. 2002).

The sorting of Ash1p to daughter cell nuclei in *Saccharomyces cerevisiae* is a model system for investigating the asymmetric segregation of a cell-fate determinant by mRNA localization (Long et al. 1997; Takizawa et al. 1997). Asymmetric sorting of Ash1p results from transport and anchoring of *ASH1* mRNA to the cortex of daughter cells during anaphase of the cell cycle (Long et al. 1997; Takizawa et al. 1997). In daughter cell nuclei, Ash1p functions as a specific transcriptional repressor of *HO*, restricting mating-type switching to mother cells (Bobola et al. 1996; Jansen et al. 1996; Sil and Herskowitz 1996; Maxon and Herskowitz 2001).

ASH1 mRNA contains four *cis*-acting localization elements: E1, E2A, E2B, and E3 (Chartrand et al. 1999; Gonzalez et al. 1999). Each of these *cis*-acting elements can independently localize a heterologous reporter mRNA to daughter cells (Chartrand et al. 1999; Gonzalez et al. 1999). In

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higher eukaryotic organisms, most *cis*-acting elements reside in the 3'-untranslated region (3'-UTR) of the mRNA (Bashirullah et al. 1998; Palacios and St. Johnston 2001; Kloc et al. 2002). The *ASH1* E3 mRNA localization element is located predominantly in the 3'-UTR, whereas elements E1, E2A, and E2B reside within the *ASH1* open reading frame (ORF; Long et al. 1997; Takizawa et al. 1997; Chartrand et al. 1999; Gonzalez et al. 1999). The sequence as well as the secondary structure for these elements appear crucial for mRNA localization activity (Chartrand et al. 1999; Gonzalez et al. 1999; Chartrand et al. 2002). The position of the *cis*-acting localization elements is not critical for *ASH1* mRNA localization but apparently regulates the translation of Ash1p until its mRNA is properly localized (Chartrand et al. 2002).

In addition to the *cis*-acting localization elements, *ASH1* mRNA localization requires numerous *trans*-acting factors: *KHD1*, *LOC1*, *BUD6/AIP3*, *SHE1-5*, as well as the actin cytoskeleton (Long et al. 1997; Takizawa et al. 1997; Beach and Bloom 2001; Long et al. 2001; Irie et al. 2002). *SHE1/MYO4*, *SHE2*, and *SHE3* appear to have the most direct role in *ASH1* mRNA localization. She1p was previously identified as Myo4p, a type V nonprocessive myosin that directly transports the *ASH1* mRNA ribonucleoprotein particle to the bud tip and colocalizes with *ASH1* mRNA at the distal tip of the bud (Haarer et al. 1994; Jansen et al. 1996; Bertrand et al. 1998; Beach et al. 1999; Münchow et al. 1999; Reck-Peterson et al. 2001). The C-terminal tail of Myo4p interacts with a domain in the N terminus of She3p (Böhl et al. 2000). The Myo4p/She3p complex does not associate with *ASH1* mRNA independent of She2p (Münchow et al. 1999; Takizawa and Vale 2000). Furthermore, asymmetric sorting of Myo4p to daughter cells requires She2p (Münchow et al. 1999).

She2p directly and specifically interacts with each of the *ASH1 cis*-acting localization elements (Böhl et al. 2000; Long et al. 2000). Furthermore, She2p directly interacts with a domain in the C terminus of She3p (Böhl et al. 2000; Long et al. 2000). However, unlike Myo4p and She3p, which colocalize with *ASH1* mRNA at the bud tip, She2p, when expressed at endogenous levels, is uniformly distributed throughout the cell (Böhl et al. 2000). These observations have led to the following model. The RNA-binding protein, She2p, recruits the Myo4p/She3p transport complex to *ASH1* mRNA through interaction with She3p, and once the Myo4p/She3p/She2p/*ASH1* mRNA ribonucleoprotein particle is assembled, it is competent for localization to daughter cells (Böhl et al. 2000; Long et al. 2000).

Apparently, She2p is a novel RNA-binding protein, because homology searches have not identified any known RNA-binding motifs within She2p, and the molecular details for the Myo4p/She3p/She2p/mRNA interactions remained to be characterized. Consequently, to study the assembly and transport of this complex in the absence of bound mRNA and to further characterize She2p RNA-

binding activity, we sought to identify and characterize novel mutants of She2p specifically defective for RNA-binding activity. Using these mutants, we could investigate the various protein-protein interactions required for *ASH1* mRNA localization in the absence of She2p RNA-binding activity. Here we report the identification of seven novel mutations in *SHE2*, resulting in amino acid substitutions N36S, R43A, R44A, R52A, R52K, R63A, and R63K, which are defective for mRNA localization and RNA-binding activity. The nuclear/cytoplasmic distribution for She2p mutants N36S and R63K is not altered, and both mutants retain the ability to interact with She3p, demonstrating that in vivo the She2p/She3p interaction is independent of associated mRNA. Furthermore, though all the protein-protein interactions required for mRNA localization are detected with She2p mutants N36S and R63K, the absence of She2p RNA-binding activity affects the sorting of Myo4p and She3p to daughter cells. However, the sorting of Myo4p and She3p to daughter cells in the absence of She2p can be rescued when RNA is artificially tethered to She3p. Consequently, this work provides new insight into the mechanistic details of mRNA localization in *S. cerevisiae*.

RESULTS

Identification of She2p point mutants defective for *ASH1* mRNA localization

We isolated mutations in She2p defective for *ASH1* mRNA localization by adapting a genetic selection/screen that monitors the asymmetric regulation of the *HO* promoter (Bobola et al. 1996; Jansen et al. 1996). Yeast strain YLM851 is deleted of *SHE2* and contains *ADE2* and *CAN1* regulated by the *HO* promoter. In the absence of *ADE2* expression, this strain appears red on media containing a low concentration of adenine. If *CAN1* is expressed, the growth of this strain is sensitive to the arginine analog canavanine. When this strain was transformed with the vector plasmid YCplac111, the cells appeared red on media containing a low concentration of adenine and were insensitive to canavanine, indicating that both *ADE2* and *CAN1* expression were repressed in mother and daughter cells by the symmetric segregation of Ash1p (Fig. 1A). In contrast, when this strain was transformed with a plasmid containing wild-type *SHE2*, the cells appeared white and were sensitive to canavanine because of the asymmetric regulation of *ADE2* and *CAN1*, respectively (Fig. 1A).

To identify mutants of She2p defective for *ASH1* mRNA localization, yeast strain YLM851 was transformed with a randomly mutagenized plasmid pool of *SHE2-myc₆*. Canavanine-resistant transformants that displayed the red adenine phenotype were chosen for further characterization. Mutant candidates were screened for full-length expression of She2p-myc₆ by Western blot, and the DNA sequence was determined for candidates that expressed full-length pro-

FIGURE 1. *ASH1* mRNA and *IST2* mRNA are delocalized in cells expressing *She2p* mutants N36S and R63K. (A) Strain YLM851 was transformed with plasmids YCplac111 (vector), pRL199 (*She2p*-wt), pRL322 (*She2p*-R63K), or pRL324 (*She2p*-N36S). Transformants were patched on low adenine media devoid of leucine or streaked for single colonies on media devoid of leucine and arginine in the (+) presence and (–) absence of canavanine. (B) Strain YLM777 was transformed with plasmid C3431 (YEplac195/*ASH1*) and pRL199, pRL322, or pRL324. Transformants were grown in media devoid of leucine and uracil. Cells were processed for in situ hybridization using Cy3-conjugated complementary DNA probes for *ASH1* mRNA. Representative images for in situ hybridization, DAPI staining, and (Nom.) Nomarski optics are shown. (C) Strain YLM777 was transformed with plasmid pRL277 [pHZ18(A)-*IST2*] and pRL199, pRL322, or pRL324. Cells were processed for in situ hybridization using Cy3-conjugated complementary DNA probes for *lacZ*-*IST2* mRNA.

tein. Here we report the characterization of two *She2p* mutants, N36S and R63K. Both mutants displayed the red adenine phenotype and were insensitive to canavanine (Fig. 1A). Additional *She2p* mutants isolated from this selection/screen are listed in Supplemental Table S1, at <http://www.mcw.edu/microbiology/rml/rna2003support.html>.

The growth assays described above are indirect assays for the localization of *ASH1* mRNA and Ash1p sorting. To directly observe the distribution of *ASH1* mRNA in cells expressing the N36S and R63K *She2p* mutants, fluorescent in situ hybridization (FISH) was performed for *ASH1* mRNA (Fig. 1B). Compared with wild-type *She2p*, *ASH1* mRNA was delocalized in cells expressing either the N36S or R63K *She2p* mutants (Fig. 1B; Table 1). From the plate assays and FISH results, we conclude that *ASH1* mRNA localization and Ash1p sorting are defective in cells expressing *She2p* mutants N36S and R63K.

In *S. cerevisiae*, at least one other mRNA, *IST2*, localizes to daughter cells dependent on *She2p* (Takizawa et al. 2000). Consequently, we investigated if *She2p* mutants N36S and R63K also affected *IST2* mRNA localization (Fig. 1C). Identical to the *ASH1* mRNA results, *IST2* mRNA localization was defective in cells expressing either the N36S or R63K mutant, demonstrating that these two *She2p* mutants are defective for a common activity shared by the *ASH1* and *IST2* mRNA localization pathways.

Altering the intracellular concentration of *She2p* could affect the localization of *ASH1* and *IST2* mRNA. Specifically, the N36S and R63K amino acid substitutions could reduce or increase the steady-state level of *She2p*, resulting in the delocalization of mRNA. The steady-state level for wild-type and mutant *She2ps* was determined by Western blot (Fig. 2). When normalized to the tubulin loading control, there was no apparent difference in the steady-state

TABLE 1. *ASH1* mRNA localization in cells expressing She2p mutants

Plasmid/She2p ^a	<i>ASH1</i> mRNA localization ^b
pRL199/She2-wt	48%
pRL324/She2p-N36S	0%
pRL745/She2p-R43K	54%
pRL740/She2p-R43A	18%
pRL746/She2p-R44K	52%
pRL741/She2p-R44A	2%
pRL747/She2p-R49K	46%
pRL742/She2p-R49A	24%
pRL748/She2p-R52K	0%
pRL743/She2p-R52A	2%
pRL322/She2p-R63K	0%
pRL744/She2p-R63A	0%

^aThe indicated plasmids were transformed into strain YLM777 along with plasmid C3431 (YEplac195/*ASH1*).

^bFifty anaphase cells with signal for *ASH1* mRNA were counted. The percent of cells with localized *ASH1* mRNA is indicated.

level of the N36S (Fig. 2, lane 3) and R63K (Fig. 2, lane 4) She2p mutants compared with the wild-type control (Fig. 2, lane 2). Therefore, this result implies that delocalization of mRNA in cells expressing either the N36S or R63K She2p mutants is not a result of differences in the intracellular concentration of She2p. Rather, we hypothesized that the N36S and R63K She2p amino acid substitutions affect She2p RNA-binding activity, the She2p–She3p interaction, or both.

She2p mutants N36S and R63K are able to directly interact with She3p

Proper *ASH1* mRNA localization apparently requires She2p to interact with She3p (Böhl et al. 2000; Long et al. 2000). Therefore, we compared the ability of wild-type and mutant She2p to interact with She3p using an in vitro GST pull-down assay (Fig. 3A). GST (Fig. 3A, lane 1) alone was unable to pull down She3p, whereas wild-type GST-She2p (Fig. 3A, lane 2) as well as GST-She2p mutants N36S (Fig. 3A, lane 3) and R63K (Fig. 3A, lane 4) were able to pre-

FIGURE 2. She2p amino acid substitutions N36S and R63K do not affect the steady-state level of She2p. Yeast strain YLM777 was transformed with plasmids (lane 1) YCplac111 (vector), (lane 2) pRL199 (She2p-wt), (lane 3) pRL324 (She2p-N36S), or (lane 4) pRL322 (She2p-R63K). Transformants were grown in media devoid of leucine, and cell lysates were prepared. Cell lysates were used for Western blotting with anti-myc monoclonal antibody or anti- α -tubulin antibody.

cipitate She3p. We further studied the She2p/She3p interaction in vivo by two-hybrid analysis. She2p mutants N36S and R63K expressed She3p-dependent *lacZ* activity at levels comparable to wild-type She2p (Fig. 3B). These results demonstrate that She2p amino acid substitutions N36S and R63K do not alter the ability of She2p to directly interact with She3p in vitro and in vivo, implying that the overall structure of the N36S and R63K She2p mutants apparently is not altered by these two substitutions.

We further characterized the interaction between the She2p mutants and She3p under physiological conditions. For these experiments, rabbit She2p antiserum was generated (Fig. 3C). Although some cross-reactivity was observed with all yeast lysates tested (Fig. 3C, lanes 1–3), this antiserum recognized a protein corresponding to the predicted molecular mass, 28.3 kD, for She2p in lysates containing endogenous levels of She2p (Fig. 3C, lane 2), and this protein was absent in extracts prepared from cells deleted of She2p (Fig. 3C, lane 1). This result indicated that the antiserum was capable of detecting She2p.

To conclusively demonstrate that the antiserum was detecting She2p, we performed two additional experiments. First, we probed yeast extracts prepared from a *she2Δ* strain expressing a She2p-myc₆ construct (Fig. 3C, lane 3). We did not observe the 28.3-kD form of She2p in this extract. Rather, because of the presence of the myc₆ tag, we observed a slower-migrating form of She2p that was not detected in either of the other yeast lysates tested. In the second experiment, recombinant GST-She2p fusion proteins were purified from *Escherichia coli*, separated by SDS-PAGE, and detected by Western blot with the She2p antiserum. We observed that the antiserum had the ability to detect a protein corresponding to the molecular mass of wild-type GST-She2p (Fig. 3C, lane 5), GST-She2p N36S (Fig. 3C, lane 6), and GST-She2p R63K (Fig. 3C, lane 7), and this protein was absent from the GST negative control (Fig. 3C, lane 4). The sum of these results indicates that the antiserum has the ability to detect She2p in yeast lysates as well as recombinant She2p purified from *E. coli*.

To study the interaction between full-length She3p-myc₆ and mutant She2p, a coimmunoprecipitation experiment was performed. She3p-myc₆ was immunoprecipitated, and coimmunoprecipitating proteins were analyzed for She2p by Western blot with the She2p antiserum (Fig. 3D). Wild-type She2p (Fig. 3D, lane 2) as well as She2p mutants N36S (Fig. 3D, lane 4) and R63K (Fig. 3D, lane 5) coimmunoprecipitated with She3p, indicating that under physiological conditions She2p amino acid substitutions N36S and R63K do not affect the ability of She2p to interact with She3p.

She2p mutants N36S and R63K are defective for association with *ASH1* and *IST2* mRNA

Because the She2p/She3p interaction was apparently not altered for the N36S and R63K She2p mutants, we sus-

FIGURE 3. She2p mutants N36S and R36K are able to interact with She3p in vitro and in vivo. (A) In vitro GST pull-down assays were performed with 1 μ g of (lane 1) GST, (lane 2) GST-She2p-wt, (lane 3) GST-She2p-N36S, or (lane 4) GST-She2p-R63K incubated with a C-terminal domain of She3p prepared by coupled in vitro transcription/translation. (B) She2p mutants N36S and R63K are able to interact with She3p by the two-hybrid assay. Strain PJ69-4a was transformed with the following combinations of plasmids: pGBDU-c1 (vector)/pACT2 (vector), pRL418 (pGBDU-c1-She2p-wt)/pACT2, pGBDU-c1/pEP13 (pACT2-She3p), pRL418/pEP13, pRL419 (pGBDU-c1-She2p-N36S)/pEP13, and pRL431 (pGBDU-c1-She2p-R63K)/pEP13. Transformants were grown in liquid culture and processed for β -galactosidase assays. (C) Characterization of the anti-She2p rabbit antiserum. Extracts were prepared from yeast strains YLM777 transformed with (lane 1) YCplac111 (vector), (lane 2) pRL453 (YCplac111-She2p), and (lane 3) pRL199 (YCplac111-She2p-myc₆). Transformants of YLM777 were grown in synthetic media devoid of leucine. Lysates were prepared and used for Western blotting with anti-She2p rabbit antiserum. In addition, 5 ng of purified (lane 4) GST, (lane 5) GST-She2p-wt, (lane 6) GST-She2p-N36S, and (lane 7) GST-She2p-R63K were analyzed by Western blot with anti-She2p rabbit antiserum. The bands corresponding to She2p, She2-myc₆, and GST-She2p are indicated by the arrows. (D) She2p mutants N36S and R63K associate with full-length endogenous She3p in vivo. Yeast strain YLM1320 was transformed with the following combinations of plasmids: (lane 1) YCplac22 (vector)/pRL453 (YCplac111-She2p-wt), (lane 2) pRL460 (YCplac22-She3p-myc₆)/pRL453, (lane 3) pRL460/YCplac111 (vector), (lane 4) pRL460/pRL462 (YCplac111-She2p-N36S), or (lane 5) pRL460/pRL463 (YCplac111-She2p-R63K). Transformants were grown in synthetic media devoid of leucine as well as tryptophan, and lysates were prepared. An aliquot of the total lysate was analyzed by Western blot for She3p-myc₆ and She2p (total). She3p-myc₆ was immunoprecipitated from the lysate using anti-myc antibody, and the immunoprecipitates were analyzed by Western blot for the presence of She3p-myc₆ and She2p (IP). She2p was detected in the immunoprecipitates using anti-She2 rabbit antiserum. Subsequently, the Western blot was stripped and reprobed with anti-myc to detect She3p-myc₆.

pected that these two mutants could be defective for RNA association. To explore this possibility, we first investigated the ability of the She2p mutants to interact with RNA by UV cross-linking (Fig. 4A; Long et al. 2000). As previously observed (Long et al. 2000), a UV cross-link product corresponding to wild-type GST-She2p and the *ASH1* E3 localization element was detected (Fig. 4A, lanes 1,2). Furthermore, to demonstrate that the She2p RNA-binding ac-

tivity is specific for the E3 localization element, we performed the UV cross-linking assay with MS2 RNA. The MS2 RNA, like E3, forms a stem-loop structure (Valegard et al. 1997). However, we observed no UV cross-link product corresponding to an She2p/MS2 RNA complex (Fig. 4A, lane 3). From this result we can conclude that in vitro She2p RNA-binding activity is apparently specific for the E3 stem-loop structure.

FIGURE 4. She2p mutants N36S and R63K are defective for *ASH1* and *IST2* mRNA-binding activity. (A) The UV cross-linking assay was performed by incubating (lane 1) 0.25 μ g of GST-She2p-wt, (lanes 2,3) 0.5 μ g of GST-She2p-wt, (lanes 4,5) 5.0 μ g of GST-She2p-N36S, or (lanes 6,7) 5.0 μ g of GST-She2p-R63K with 1 ng of (lanes 1,2,4,6) 32 P-labeled E3 RNA or (lanes 3,5,7) MS2 RNA. The arrow indicates the position of the GST-She2p/RNA complex. (B) Apparent gel shift of She2p with E3 RNA is not caused by RNA-protein complex formation. (Lane 1) Trace (1 nM) labeled E3 RNA combined with (lane 2) mobility shift buffer (MSB) produces an apparent mobility shift in the absence of protein. Addition of (lanes 3–6) 125 nM She2p with (lanes 4–6) increasing molar equivalents of unlabeled E3 competitor RNA results in an increase, not a decrease, in the amount of the shifted species, and is consistent with the formation of a base-paired RNA duplex, not an RNA-protein complex. (C) She2p RNA-binding activity for mutants N36S and R63K as measured by three-hybrid assay. Strain YLM585 was transformed with the following combinations of plasmids: pIIIA-MS2-2(vector)/pGAD-c1 (vector), pIIIA-MS2-2/pRL445 (pGAD-c1-Shep-wt), pRL80 (pIIIA-MS2-2-E3)/pRL445, pRL80/pRL441 (pGAD-c1-She2p-N36S), and pRL80/pRL442 (pGAD-c1-She2p-R63K). Transformants were grown in liquid culture and processed for β -galactosidase assay. (D) She2p RNA-binding activity for She2p mutants N36S and R63K as measured by IP/RT-PCR. Yeast strain YLM777 was transformed with plasmid C3431 (YEplac195/*ASH1*) and plasmid (lanes 1,2) pRL199 (She2-myc₆-wt), (lane 3) pRL324 (She2-myc₆-N36S), or (lane 4) pRL322 (She2-myc₆-R63K). Transformants were grown in synthetic media devoid of leucine and uracil; lysates were prepared and immunoprecipitations were performed with anti-myc. Immunoprecipitations were subsequently used for RT-PCR reactions with primers specific for *ASH1* mRNA or *IST2* mRNA. RT-PCR reactions corresponding to an aliquot of the lysates prior to immunoprecipitation (total) are shown as well as reactions corresponding to the immunoprecipitation reactions with and without RT (+RT and -RT). Also, an aliquot of the immunoprecipitation was analyzed by Western blot using anti-myc.

Using the UV cross-linking assay, we investigated the *in vitro* RNA-binding activity for She2p mutants N36S and R63K. This experiment was performed identically to that for wild-type She2p with the exception that 10–20-fold more mutant She2p was incubated with the radiolabeled RNAs. We detected a faint UV cross-link product for both

She2p N36S/E3 (Fig. 4A, lane 4) and She2p R63K/E3 (Fig. 4A, lane 6). Furthermore, we investigated if the RNA-binding specificity might be altered for She2p mutants N36S and R63K. Identically to wild-type She2p, we observed no UV cross-link product corresponding to She2p N36S/MS2 (Fig. 4A, lane 5) or She2p R63K/MS2 (Fig. 4A, lane 7). These

results imply that these two amino acid substitutions apparently do not affect the specificity of RNA-binding activity, but greatly impair the She2p/E3 *ASH1* mRNA association, most likely because of a reduced affinity for the RNA substrate.

Given these observations, we sought to determine the relative binding affinities of wild-type She2p and the two She2p mutants for the E3 localization element. Using untagged She2p and in vitro transcribed RNA, we performed an extensive series of RNA-binding studies using a quantitative electrophoretic mobility shift assay (EMSA). However, despite considerable effort, we have been unable to identify a mobility shift that conclusively corresponds to direct and specific binding of She2p to either E1 or E3 RNA. Previously, an electrophoretic mobility shift was observed with GST-She2p and E3 (Böhl et al. 2000), but we now believe this shifted species represents an alternative RNA conformation rather than She2p:E3 binary complex. Notably, this alternative RNA conformation was further stimulated in a self-competition experiment, in which increasing amounts of unlabeled E3 RNA are titrated into a solution containing a trace amount of labeled E3 RNA and She2p (Fig. 4B, lanes 3–6). Normally, unlabeled competitor RNA decreases the intensity of the shifted band through competition for protein binding. However, addition of competitor increases the amount of shifted species (Fig. 4B), indicating that the shifted band is comprised of a dimeric E3 RNA. A similar result was also obtained for the E1 localization element (data not shown). Consequently, because of the inability to detect an She2p:E3 complex by electrophoretic mobility shift assay, we have been unable to determine the relative RNA-binding affinities for purified She2 and the She2p mutants. We conclude that weak She2p interactions with RNA can be trapped by UV cross-linking, but there is no evidence for a high-affinity binary complex between She2p and RNA.

To further explore the UV cross-linking results, the association between the She2p mutants and the E3 element was monitored in vivo by three-hybrid analysis. In the three-hybrid assay, the She2p RNA interaction was specific for the *ASH1* E3 *cis*-acting element (Fig. 4C; data not shown) and was independent of Myo4p and She3p (data not shown). Furthermore, the level of *lacZ* expression was at least 10-fold lower for the N36S and R63K She2p mutants when compared with wild-type She2p (Fig. 4C). Therefore, these results are consistent with the in vitro UV cross-linking data demonstrating that She2p mutants N36S and R63K are defective for interaction with E3 *ASH1* mRNA.

The experiments described above monitor She2p association with a single *cis*-acting localization element, E3, which is one of four *cis*-acting localization elements found in *ASH1* mRNA. It therefore remained a formal possibility that She2p mutants N36S and R63K could remain associated with full-length *ASH1* mRNA in vivo. Consequently, we investigated if She2p mutants N36S and R63K could

associate with full-length *ASH1* mRNA using an IP/RT-PCR assay (Fig. 4D). Compared with wild-type She2p (Fig. 4D, lane 2), we observed a reduction in the amount of the *ASH1*-specific IP/RT-PCR product coprecipitating with She2p mutants N36S (Fig. 4D, lane 3) and R63K (Fig. 4D, lane 4). Together, these results indicate that She2p mutants N36S and R63K are severely impaired for association with *ASH1* mRNA compared with wild-type She2p. Furthermore, because these mutants retain the ability to interact with She3p in vivo, we can conclude that She2p association with RNA is not required for interaction with She3p.

Given that both *ASH1* and *IST2* mRNA localization were defective in cells expressing either She2p N36S or R63K mutants, we investigated if these mutants were also defective for association with *IST2* mRNA (Fig. 4D). Using the IP/RT-PCR assay, *IST2* mRNA coprecipitated with wild-type She2p (Fig. 4D, lane 2) but not with She2p mutants N36S (Fig. 4D, lane 2) or R63K (Fig. 4D, lane 4). Therefore, we conclude that She2p mutants N36S and R63K are unable to associate with either *ASH1* mRNA or *IST2* mRNA.

She2p RNA-binding activity is also dependent on arginine residues 43, 44, and 52

The observation that R63 is important for She2p RNA-binding activity provided a potential clue for defining an RNA-binding motif within She2p. Several RNA-binding motifs, the arginine-rich motif and the arg-gly-gly (RGG) box, contain arginine residues that are critical for RNA-binding activity. Five of the seven arginines in She2p reside in a relatively arginine-rich region encompassing amino acids 43–63 (Fig. 5A). To determine whether these arginines are important for She2p RNA-binding activity, we individually converted R43, R44, R49, and R52 to alanine or lysine, and arginine residue 63 to alanine. The RNA-binding activity for each mutant was determined by three-hybrid analysis (Fig. 5B). When compared with wild-type She2p, we observed that *lacZ* expression was reduced at least 10-fold for She2p mutants R52A, R52K, and R63A and twofold to threefold for She2p mutants R43A and R44A. We observed very little, if any, difference in the level of *lacZ* expression for She2p mutants R43K, R44K, R49A, and R49K compared with the wild-type control. Importantly, each of these mutants retains the ability to interact with She3p (data not shown). These results demonstrate that She2p residues R52 and R63 are critical for She2p RNA-binding activity; R43 and R44 are less important for She2p RNA-binding activity; and R49 apparently has very little, if any, role in She2p RNA-binding activity. Analogous to arginine 63, a positive charge at position 52 is not sufficient for She2p RNA-binding activity because the R52K She2p mutant is defective for RNA-binding activity. These observations imply that arginine residues 52 and 63 participate in RNA binding in a manner that cannot be mimicked by lysine residues.

FIGURE 5. R43, R44, and R52 are required for She2p association with *ASH1* mRNA. (A) Amino acid sequence for She2p with amino acids N36, R43, R44, R49, R52, and R63 indicated by the black arrowheads. The black rectangle indicates the region of She2p corresponding to the predicted leucine zipper motif. (B) She2p RNA-binding activity for mutants R43K, R43A, R44K, R44A, R49K, R49A, R52K, R52A, and R63A as measured by three-hybrid assay. Strain YLM585 was transformed with the following combinations of plasmids: pRL80 (pIIIA-MS2-2-E3)/pRL445 (pGAD-c1-She2p-wt), pRL80/pRL552 (pGAD-c1-She2p-R43K), pRL80/pRL669 (pGAD-c1-She2p-R43A), pRL80/pRL553 (pGAD-c1-She2p-R44K), pRL80/pRL670 (pGAD-c1-She2p-R44A), pRL80/pRL554 (pGAD-c1-She2p-R49K), pRL80/pRL671 (pGAD-c1-She2p-R49A), pRL80/pRL555 (pGAD-c1-She2p-R52K), pRL80/pRL672 (pGAD-c1-She2p-R52A), and pRL80/pRL673 (pGAD-c1-She2p-R63A). Transformants were grown in liquid culture and processed for β -galactosidase assays.

We next wanted to investigate whether She2p mutants that reduced the expression of *lacZ* in the three-hybrid assay also affected *ASH1* mRNA localization (Table 1). We observed that *ASH1* mRNA localization was reduced to varying degrees in cells expressing She2p mutants R43A, R44A, R52K, R52A, and R63A. Collectively, these results indicate a trend between reduced She2p RNA-binding activity and the ability to localize *ASH1* mRNA. However, it is not a strict correlation because She2p mutants R43A and R44A have very similar, if not identical, RNA-binding activity (Fig. 5B), whereas the R44A mutant is apparently more impaired for *ASH1* RNA localization activity than the R43A mutant (Table 1). Furthermore, although we observed no reduction in *lacZ* expression in the two- and three-hybrid assays for She2p mutant R49A (Fig. 5B; data not shown), we observed a modest reduction in the ability of this She2p mutant to localize *ASH1* mRNA (Table 1). Therefore, although the two- and three-hybrid assays can reveal deficiencies in She2p protein–protein interactions and She2p RNA-binding activity, these assays may not completely recapitulate the in vivo physiology for She2p in *ASH1* mRNA localization.

Amino acid substitutions N36S and R63K do not affect the nuclear/cytoplasmic distribution of She2p

In summary, we have investigated all of the known characteristics associated with She2p and have determined that amino acid substitutions N36S and R63K specifically affect only She2p RNA-binding activity. Using these two mutants

of She2p, we were poised to investigate mechanistic details of *ASH1* mRNA localization that were previously addressed only by deletion of *SHE2* or large truncations in She2p.

Under normal physiological conditions, Myo4p and She3p colocalize with *ASH1* mRNA at the cortex of the bud and are excluded from the nucleus (Jansen et al. 1996; Bertrand et al. 1998; Münchow et al. 1999). In contrast, under identical conditions, She2p is not excluded from the nucleus and is uniformly distributed between mother and daughter cells (Jansen et al. 1996; Bertrand et al. 1998; Böhl et al. 2000). Furthermore, it has recently been shown that an N-terminal truncation of She2p, Δ N70, which affects both RNA-binding activity and the ability to interact with She3p, accumulates in the nucleus (Kruse et al. 2002). Using our She2p point mutants, we were able to specifically address if nuclear accumulation of She2p is a result of the loss of RNA-binding activity, the loss of interaction with She3p, or both. Immunofluorescence analysis revealed that the intracellular distribution of She2p mutants N36S and R63K was no different from that of wild-type She2p (Fig. 6A), indicating that the absence of RNA-binding activity has no effect on the intracellular distribution of She2p.

It remained a formal possibility that in vivo She2p mutants N36S and R63K retain a sufficiently low level of RNA-binding activity that results in a wild-type distribution of She2p. Mex67p is required for nuclear export of poly(A)⁺ RNA (Hurt et al. 2000), and it has recently been shown that when *ASH1* mRNA is overexpressed, She2p accumulates in the nuclei of *mex67* cells shifted to a nonpermissive temperature (Kruse et al. 2003). We reasoned that if She2p

FIGURE 6. (A) The intracellular distribution of She2p mutants N36S and R63K is not altered. Representative images for the intracellular distribution of She2p-myc₆. Yeast strain YLM777 was transformed with pRL453 (control), pRL199 (She2p-myc₆-wt), pRL324 (She2p-myc₆-N36S), or pRL322 (She2p-myc₆-R63K). Transformants were grown in synthetic media minus leucine; subsequently, the cells were fixed and processed for immunofluorescence using anti-myc. (B) She2p does not accumulate in the nucleus in the absence of mRNA export. Yeast strain YLM1769 was transformed with the following combinations of plasmids: pRL718 (pESC-URA-ASH1)/YCplac22 (vector), pRL718/pRL676 (YCplac22-She2p-myc₆-wt), pRL718/pRL677 (YCplac22-She2p-myc₆-N36S), and pRL718/pRL678 (YCplac22-She2p-myc₆-R63K). Transformants were grown at the permissive and nonpermissive temperatures as described in Materials and Methods, Temperature Shift Experiment. For immunofluorescence, anti-myc antibody was used and ASH1 mRNA was detected by FISH.

mutants N36S and R63K contain low levels of RNA-binding activity, then these two mutants should also accumulate in the nuclei of *mex67* cells shifted to the nonpermissive temperature. The intracellular distribution of both wild-type and mutant She2p was determined by immunofluorescence, and the subcellular location of ASH1 mRNA was simultaneously detected by FISH in *mex67* cells grown at permissive and nonpermissive temperatures (Fig. 6B). As expected, we observed ASH1 mRNA (Fig. 6B) and poly(A)⁺ RNA (data not shown) in the cytoplasm of *mex67* cells grown at the permissive temperature and in the nuclei of

mex67 cells shifted to the nonpermissive temperature. Furthermore, in *mex67* cells expressing wild-type She2p grown at the permissive temperature, we observed localized ASH1 mRNA and a fraction of wild-type She2p colocalized with ASH1 mRNA at the bud tip (Fig. 6B), consistent with previous observations (Böhl et al. 2000). In *mex67* cells expressing She2p RNA-binding mutants, we did not observe localized ASH1 mRNA, and no She2p was observed at the bud tip (Fig. 6B). At the nonpermissive temperature, we observed ASH1 mRNA accumulating in nuclei. In contrast to ASH1 mRNA, we did not observe nuclear accumulation of wild-type She2p in *mex67* cells grown at the nonpermissive temperatures (Fig. 6B). Our observation is not consistent with those previously reported (Kruse et al. 2002). Furthermore, the intracellular trafficking of She2p between the nucleus and cytoplasm in *mex67* cells is not altered in the absence of She2p RNA-binding activity (Fig. 6B). These results support the conclusions that nuclear export of She2p is independent of mRNA transport to the cytoplasm and that subcellular trafficking of She2p between the nucleus and cytoplasm is not dependent on RNA-binding activity.

She2p RNA-binding activity is required for proper asymmetric sorting of Myo4p and She3p

Myo4p and She3p are asymmetrically sorted to daughter cells (Jansen et al. 1996; Bertrand et al. 1998; Münchow et al. 1999). Sorting of Myo4p to daughter cells during anaphase is dependent on She2p (Münchow et al. 1999). However, because this observation was made with

a strain deleted of *SHE2*, it remained to be determined if proper Myo4p sorting to daughter cells is dependent on She2p RNA-binding activity, the She2p–She3p interaction, or both. We investigated by immunofluorescence if She2p RNA-binding activity is required for proper asymmetric sorting of Myo4p and She3p to daughter cells. The intracellular distribution of Myo4p-myc₁₃ was determined in yeast cells expressing either wild-type She2p or She2p mutants N36S and R63K (Fig. 7A). As previously reported, Myo4p-myc₁₃ was symmetrically distributed in *she2* deletion cells compared with the wild-type control. We further

FIGURE 7. (Legend on next page)

pensable for tight polarization of Myo4p to daughter cells, because tethering RNA to She3p (Long et al. 2000) rescues asymmetric sorting of Myo4p and She3p to daughter cells (data not shown).

DISCUSSION

Previous studies demonstrated that She2p directly interacts with She3p and is a novel RNA-binding protein that directly and specifically interacts with each of the *ASH1* mRNA *cis*-acting localization elements (Böhl et al. 2000; Long et al. 2000). However, She2p does not exhibit any obvious characteristics of known RNA-binding motifs, and the details of the She2p/She3p *in vivo* interaction remained largely uncharacterized. To begin a molecular dissection of She2p function, we sought to identify novel alleles of *SHE2* that delocalize *ASH1* mRNA by specifically altering She2p RNA-binding activity or the She2p/She3p interaction, but not both. Initially, we identified two individual amino acid substitutions in She2p, N36S and R63K, that result in the delocalization of *ASH1* mRNA. These two mutants retain the ability to interact with She3p as determined by three independent assays: GST pull-down reactions, two-hybrid analysis, and coimmunoprecipitation experiments (Fig. 3). However, these two mutants are compromised for association with *ASH1* mRNA as determined by three different assays: UV cross-linking, three-hybrid analysis, and IP/RT-PCR (Fig. 4). From our observations, we conclude that *in vivo* the She2p/She3p interaction is independent of a functional She2p mRNA-binding domain.

Interestingly, our *in vitro* work using purified components indicates that She2p may bind *ASH1* mRNA too weakly to be measured by EMSA (Fig. 4B), but it interacts long enough to be photochemically trapped by UV cross-linking (Fig. 4A). The UV cross-linking results are not dependent on the GST portion of GST-She2p, because we observe a specific cross-link product with untagged She2p (data not shown). These results are consistent with a model in which the She2p/*ASH1* mRNA interaction is stabilized by other cellular factors as part of a cooperatively assembled

ribonucleoprotein complex. In support of this model, the U snRNA nuclear export complex involves cooperative interactions between the cap-binding complex (CBC), phosphorylated adaptor for RNA export (PHAX), and capped RNA for the nuclear export of U snRNA (Ohno et al. 2000). Alternatively, She2p association with RNA may be regulated by posttranslational modification. The RNA-binding activity of AKIP1, abscisic-acid-activated-protein-kinase-interacting protein, for dehydrin mRNA is positively regulated by phosphorylation (Li et al. 2002). Although She2p is phosphorylated *in vivo* (data not shown), the functional significance of She2p phosphorylation with respect to RNA-binding activity and RNA localization remains to be determined.

How might N36 and R63 function in She2p RNA-binding activity? Both of these amino acids could be located within a domain that directly contacts RNA. Alternatively, these amino acid substitutions could affect the She2p:RNA interaction through disruption of protein–protein contacts required for RNA binding. The region of She2p containing amino acids 16–46 is predicted to form a leucine zipper motif (Johnson and McKnight 1989). Several proteins require oligomerization through leucine zipper domains for RNA-binding activity (McAfee et al. 1996a,b; Wu et al. 1998; Tan et al. 2001). We propose that She2p RNA-binding activity requires oligomerization dependent on the leucine zipper motif and substitution of serine for asparagine at position 36 alters the structure of the leucine zipper motif, preventing dimerization of She2p. In support of this, She2p has been observed to interact with itself by two-hybrid analysis (Ito et al. 2001). However, definitive biochemical proof for dimerization of She2p is lacking.

R63 might be directly involved with She2p RNA-binding activity, because several RNA-binding motifs, including the RNA recognition motif (RRM), the arginine-rich motif (ARM), the RGG box, and the double-stranded RNA-binding motif (dsRBD), all contain conserved arginine residues (Burd and Dreyfuss 1994). Inspection of the She2p amino acid sequence revealed that five out of seven arginine residues are clustered between amino acids 43 and 63, indicat-

FIGURE 7. The asymmetric distribution of Myo4p and She3p in anaphase cells is dependent on She2p mRNA-binding activity, but formation of the Myo4p/She3p complex is independent of She2p RNA-binding activity. (A) Representative images for the intracellular distribution of Myo4p-myc₁₃ in cells devoid of She2p, expressing wild-type She2p, She2p mutant N36S, and She2p mutant R63K. Strain YLM1309 was transformed with plasmid YCplac111 (vector), pRL453 (YCplac111-She2p-wt), pRL462 (YCplac111-She2p-N36S), or pRL463 (YCplac111-She2p-R63K). Transformants were grown in synthetic media minus leucine; subsequently, the cells were fixed and processed for immunofluorescence using anti-myc. Then 100 anaphase cells with signal corresponding to Myo4p were counted; *below* each panel, the percentage of anaphase cells with asymmetrically sorted Myo4p is indicated. (B) Representative images for the intracellular distribution of She3p-myc₁₃ in cells devoid of She2p, expressing wild-type She2p, She2p mutant N36S, or She2p mutant R63K. Strain YLM1368 was transformed and processed for immunofluorescence identically to strain YLM1309 described above. Then 100 anaphase cells with signal corresponding to She3p were counted; *below* each panel, the percentage of anaphase cells with asymmetrically sorted She3p is indicated. (C) The Myo4p/She3p complex can form in the absence of She2p RNA-binding activity. Strain YLM921 was transformed with plasmids (lane 1) pRL460 (YCplac22-She3p-myc₆)/pRL453 (YCplac111-She2p-wt). Yeast strain YLM1320 was transformed with the following combinations of plasmids: (lane 2) vector (YCplac22)/pRL453, (lane 3) pRL460/pRL453, (lane 4) pRL460/YCplac111 (vector), (lane 5) pRL460/pRL462 (YCplac111-She2p-N36S), or (lane 6) pRL460/pRL463 (YCplac111-She2p-R63K). Transformants were grown in synthetic media minus leucine and tryptophan. Cells were harvested, lysates were prepared, and an aliquot of the total lysate was saved for Western blotting. She3p-myc₆ was immunoprecipitated from the remaining portion of each lysate with anti-myc. The yeast lysates (total) and immunoprecipitates were analyzed by Western blot for the presence of She3p-myc₆ and Myo4p-3HA.

ing that this region of She2p may represent an ARM motif. Mutational analysis of She2p revealed that arginine residues 52 and 63 are critical for She2p association with RNA (Figs. 4, 5B). Our finding that She2p mutants R52K and R63K are defective for association with *ASH1* mRNA indicates that a positive charge at either of these positions is not sufficient for She2p RNA-binding activity. Because arginine has more atoms capable of forming hydrogen bonds with RNA compared with lysine (Calnan et al. 1991; Burd and Dreyfuss 1994), we propose that R52 and R63 make specific interactions that cannot be mimicked by lysine residues. In contrast, a positive charge at positions 43 and 44 is apparently sufficient for She2p association with RNA because amino acid substitutions R43K and R44K have no apparent effect on She2p RNA-binding activity, whereas alanine substitutions are not tolerated at these positions (Fig. 5B). Possibly, a positive charge at R43 and R44 increases nonspecific affinity for RNA and could facilitate the search for high-affinity binding sites requiring amino acids R52 and R63.

As expected, from the two- and three-hybrid results for She2p mutants R43A, R44A, R52K, R52A, and R63A (Fig. 5B; data not shown), we observed that *ASH1* mRNA was delocalized in cells expressing these mutants (Table 1). Unexpectedly, we observed a modest reduction for *ASH1* mRNA localization in cells expressing She2p mutant R49A (Table 1), even though this mutant is capable of interacting with She3p and associating with RNA (Fig. 5B; data not shown). It is possible that the sensitivity of the two- and three-hybrid assays is not sufficient to detect subtle differences in She2p interactions that could result in delocalization of *ASH1* mRNA. However, if these two assays accurately reflect the ability of She2p to interact with She3p and associate with RNA localization elements, the inability of She2p mutant R49A to localize *ASH1* mRNA implies that She2p may have an additional unidentified function in the *ASH1* mRNA localization pathway.

Recently, an N-terminal deletion mutant of She2p, Δ N70, that lacks RNA-binding activity was observed to accumulate in the nucleus, and wild-type She2p was observed to accumulate in nuclei only when poly(A)⁺ mRNA nuclear export is inhibited (Kruse et al. 2002). These two observations led Kruse and colleagues to propose that She2p RNA-binding activity is required for She2p nuclear export. Consequently, if nuclear export of She2p is dependent on mRNA-binding activity, we would predict amino acid substitutions N36S and R63K should result in nuclear retention of these two She2p mutants. However, in wild-type cells, the nuclear/cytoplasmic distribution of She2p mutants N36S and R63K is indistinguishable from wild-type She2p (Fig. 6A). Furthermore, in an *mex67* strain, we did not observe nuclear accumulation of wild-type She2p or either She2p mutant even after confirming accumulation of *ASH1* mRNA and poly(A)⁺ RNA in the nucleus of *mex67* cells grown at the nonpermissive temperature (Fig. 6B; data not shown). Therefore, we favor the idea that nuclear export of

She2p is independent of RNA-binding activity. How might these differing observations be explained?

With respect to the *mex67* experiments, subtle differences in the expression level of She2p could explain the discrepancy between the observed results. The *SHE2* construct used in this report contains the wild-type *SHE2* promoter and 3'-UTR with insertion of the myc₆ tag immediately before the She2p termination codon. Kruse and coworkers placed a myc₉ tag into the *SHE2* genomic locus by homologous recombination, which results in the replacement of the wild-type 3'-UTR. It is known that the 3'-UTR can influence the level of expression of the corresponding protein. Consequently, we propose that the different 3'-UTRs affect the level of She2p expression. Whereas the construct used by Kruse and coworkers was integrated at the *SHE2* locus, ensuring only a single copy of She2p-myc per cell, our She2p-myc₆ was harbored on a centromere (CEN)-containing plasmid. Although this difference between the two experiments might alter the level of expression of She2p, we observe very little, if any, difference in expression level between endogenous She2p and She2p expressed from a CEN plasmid (data not shown).

Besides a difference in expression levels between the N36S and R63K She2p mutants and Δ N70, other intrinsic properties of these proteins could affect the steady-state distribution of these molecules. For example, if the nuclear import rate of the N36S and R63K are slower than Δ N70, the N36S and R63K RNA-binding mutants could appear uniformly distributed throughout the cell. However, we favor the hypothesis that a unique property of Δ N70 leads to nuclear accumulation of this protein. Because Δ N70 is defective for *ASH1* mRNA binding activity and for association with She3p, we hypothesize that Δ N70 more effectively interacts with nuclear proteins, resulting in nuclear accumulation (Kruse et al. 2002). In support of this proposal, two-hybrid experiments indicate that She2p can interact with several nuclear proteins, including Hmo1p, Tye7p, and a putative protein encoded by the yeast ORF YJL048c (Uetz et al. 2000; Ito et al. 2001; R.M. Long, unpubl.). We predict that Δ N70 retains the ability to interact with these nuclear proteins.

By identifying mutations in She2p that specifically affect RNA-binding activity, we were able to address a mechanistic detail required for *ASH1* mRNA localization. Given that She2p is required for the asymmetric sorting of Myo4p to daughter cells, proper sorting of Myo4p and She3p to daughter cells could be solely dependent on the formation of the heterotrimeric Myo4p/She3p/She2p complex and independent of any associated mRNA (Münchow et al. 1999). The in vivo data presented here demonstrate that the protein-protein interactions required for the formation of the Myo4p/She3p/She2p complex are detectable in cells expressing the She2p RNA-binding mutants N36S or R63K (Fig. 7). However, even though the protein-protein interactions are maintained, Myo4p and She3p are not properly

sorted to daughter cells during anaphase of the cell cycle (Fig. 7). Consequently, polarization of Myo4p and She3p to daughter cells is dependent on the presence of associated mRNA. While our manuscript was in preparation, an article reaching a similar conclusion was published using different experimental approaches (Kruse et al. 2002).

These observations are extremely informative toward understanding the mechanism of mRNA localization. Even though the protein–protein interactions required for *ASH1* mRNA localization are maintained in the absence of She2p RNA-binding activity, the lack of associated mRNA apparently restricts the transport and/or anchoring of the protein complex. In the absence of associated mRNA, we hypothesize that the Myo4p/She3p complex may not properly anchor at the cortex of daughter cells, because in small budded cells we can detect Myo4p and She3p sorting to daughter cells (Münchow et al. 1999; data not shown). We propose that the Myo4p/She3p complex contains or interacts with a molecular sensor that is sensitive to the presence of mRNA. This sensor could be a novel protein at the bud cortex that anchors the ribonucleoprotein complex when it arrives at its destination. The sensing mechanism apparently is not directly attributed to She2p, because we have previously demonstrated that RNA can be localized to daughter cells when artificially tethered to She3p (Long et al. 2000). Furthermore, both Myo4p and She3p are asymmetrically sorted to daughter cells in the absence of She2p when mRNA is artificially tethered to She3p (Kruse et al. 2002; data not shown). The suggestion that the transport of the She3p/Myo4p complex is sensitive to the presence/absence of mRNA cargo is supported by reports that transport by ad-

ditional molecular motors is regulated by the cargo (for review, see Karcher et al. 2002). With respect to RNA transport and localization, subtle changes in RNP cargo affect the efficiency of motor transport in *Drosophila* embryos (Bullock et al. 2003). Regulating the transport of molecular motors by sensing the appropriate cargo further illustrates that eukaryotic cells have developed sophisticated mechanisms for regulating complex cellular processes.

MATERIALS AND METHODS

Yeast strains, media, and plasmids

The yeast strains used in this study are listed in Table 2. The plasmids used in this study are listed in Table 3; they were constructed using standard molecular techniques (Sambrook et al. 1989). Details for plasmid constructions are available upon request. Yeast cells were transformed using lithium acetate (Ito et al. 1983) and were grown either in rich media or in defined synthetic media lacking the nutrients indicated (Rose et al. 1990). As described previously, gene deletions were created with PCR products generated from plasmid pUG6 (Güldener et al. 1996). Silent deletions were subsequently created using plasmid pSH47 (Güldener et al. 1996). For tagging endogenous yeast genes with the myc₁₃ or 3HA epitopes, DNA cassettes containing the respective epitope were generated by PCR with gene-specific primers (Longtine et al. 1998).

Genetic selection/screen for She2p point mutations

To isolate She2p point mutants defective for *ASH1* mRNA localization, plasmid pRL199 was initially propagated in *E. coli* strain *mutD5*, which contains a defect in the 3' → 5' exonuclease activity

TABLE 2. Yeast strains used in this study

Strain	Genotype	Source
YBZ-1	<i>MATa, ade2, his3-200, leu2-3, 112, trp1-1, ura3-52, LYS2:: (LexAop)-lacZ, LexA-MS2-MS2 coat (N55K)</i>	Zhang et al. 1997
PJ69-4a	<i>MATa his3-200, leu2-3, 112, trp1-90, ura3-52, gal4, gal80, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ</i>	James et al. 1996
K699	<i>MATa, ade2-1, his3-11, leu2-3,112, ura3, trp1-1, ho, can1-100</i>	Jansen et al. 1996
K4452	<i>MATα, ade2-1, leu2-3, trp1-1, ura3, HO-ADE2, HO-CAN1</i>	Jansen et al. 1996
RS453	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-52, can1-100, GAL+</i>	Segref et al. 1997
YLM585 ^a	<i>MATa, ade2, his3-200, leu2-3,112, trp1-1, ura3-52, LYS2:: (LexAop)-lacZ, LexA-MS2-MS2 coat (N55K), she2::kanMX</i>	Long et al. 2000
YLM777 ^b	<i>MATa, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, she2Δ silent</i>	This study
YLM851 ^c	<i>MATα, ade2-1, leu2-3, trp1-1, ura3, HO-ADE2, HO-CAN, she2::kanMX</i>	This study
YLM921 ^b	<i>MATa, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, she2Δ silent, she3::kanMX</i>	This study
YLM946 ^b	<i>MATa, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, trp1Δ silent, SHE3-myc13:TRP1</i>	This study
YLM1309 ^b	<i>MATa, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, she2Δ silent, SHE1-myc13:kanMX6</i>	This study
YLM1320 ^b	<i>MATa, ade2-1, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, she2Δ silent, she3Δ silent, SHE1-3HA: kanMX6</i>	This study
YLM1368 ^b	<i>MATa, ade2-, his3-11, leu2-3,112, trp1-1, ura3, ho, can1-100, trp1Δ silent, SHE3-myc13:TRP1, she2::kanMX</i>	This study
YLM1769 ^d	<i>MATa, ade2, his3, leu2, trp1, ura3, mex67::HIS3, she2::kanMX, pUN100-mex67-5</i>	This study

^aYBZ-1 genetic background.

^bK699 genetic background.

^cK4452 genetic background.

^dRS453 genetic background.

TABLE 3. Plasmids used in this study

Plasmid	Description	Source
pACT2	Yeast vector for expressing Gal4p activation domain fusion proteins	Li et al. 1994
pET15b	<i>E. coli</i> vector for generating His ₆ -tagged fusion proteins	Novagen
pESC-URA	Yeast multicopy plasmid marked with <i>URA3</i> for galactose-regulated expression	Stratagene
pGAD-c1	Yeast vector for expression of Gal4p activation domain fusion proteins	James et al. 1996
pGBDU-c1	Yeast vector for expression of Gal4p DNA-binding domain fusion proteins	James et al. 1996
pGEX-2T	<i>E. coli</i> vector for generating GST-tagged fusion proteins	Pharmacia
pHZ18(A)	Yeast plasmid containing galactose-inducible lacZ	Long et al. 1995
pHMTc-She2p	<i>E. coli</i> expression vector for His ₆ -MBP-TEV-She2p	This work
YCplac22	Yeast single-copy shuttle plasmid marked with <i>TRP1</i>	Gietz and Sugino 1998
YCplac111	Yeast single-copy shuttle plasmid marked with <i>LEU2</i>	Gietz and Sugino 1998
YEplac112	Yeast multicopy shuttle plasmid marked with <i>TRP1</i>	Gietz and Sugino 1998
C3431	YEplac195 containing <i>ASH1</i>	Long et al. 1997
pGEM-4Z	Vector for <i>in vitro</i> transcription	Promega
pUG6	Plasmid for generating KAN disruption cassettes by PCR	Güldener et al. 1996
pSH47	Plasmid containing a galactose-inducible cre recombinase construct	Güldener et al. 1996
pIIIA/MS2-2	Three-hybrid vector for the expression of MS2-fusion RNAs	Zhang et al. 1997
pEP13	pACT2 containing entire She3p open reading frame	Long et al. 2000
pRL80	pIIIA/MS2-2 containing the 127-nt <i>Smal</i> E3 <i>ASH1</i> localization element	Long et al. 2000
pRL104	pET15b containing a C-terminal domain of She3p	This work
pRL168	pGEM-3Z containing element E3 (118 nt)	Long et al. 2000
pRL172	pGEX-2T containing the open reading frame corresponding to She2p	Long et al. 2000
pRL199	YCplac111 containing She2p-myc ₆	This work
pRL266	pET15b containing the open reading frame corresponding to She2p	This work
pRL270	Identical to pRL172 except carrying the R63K amino acid substitution in She2p	This work
pRL273	Identical to pRL172 except carrying the N36S amino acid substitution in She2p	This work
pRL277	pHZ18(A)/IST2 2098-3141	This work
pRL322	Identical to pRL199 except carrying an R63K amino acid substitution in She2p	This work
pRL324	Identical to pRL199 except carrying an N36S amino acid substitution in She2p	This work
pRL418	pGBDU-c1 containing wild-type She2p	This work
pRL419	pGBDU-c1 containing N36S She2p	This work
pRL431	pGBDU-c1 containing R63K She2p	This work
pRL441	pGAD-c1 containing N36S She2p	This work
pRL442	pGAD-c1 containing R63K She2p	This work
pRL445	pGAD-c1 containing wild-type She2p	This work
pRL453	YCplac111 containing wild-type She2p	This work
pRL460	YCplac22 containing She3p-myc ₆	This work
pRL462	YCplac111 containing N36S She2p	This work
pRL463	YCplac111 containing R63K She2p	This work
pRL552	pGAD-c1 containing R43K She2p	This work
pRL553	pGAD-c1 containing R44K She2p	This work
pRL554	pGAD-c1 containing R49K She2p	This work
pRL555	pGAD-c1 containing R52K She2p	This work
pRL669	pGAD-c1 containing R43A She2p	This work
pRL670	pGAD-c1 containing R44A She2p	This work
pRL671	pGAD-c1 containing R49A She2p	This work
pRL672	pGAD-c1 containing R52A She2p	This work
pRL673	pGAD-c1 containing R63A She2p	This work
pRL676	YCplac22 containing wild-type She2p-myc ₆	This work
pRL677	YCplac22 containing N36S She2p-myc ₆	This work
pRL678	YCplac22 containing R63K She2p-myc ₆	This work
pRL679	pGEM-4Z containing MS2 cloned from pIIIA/MS2-2	This work
pRL718	pESC-URA containing <i>ASH1</i>	This work
pRL740	YCplac111 containing R43A She2p-myc ₆	This work
pRL741	YCplac111 containing R44A She2p-myc ₆	This work
pRL742	YCplac111 containing R49A She2p-myc ₆	This work
pRL743	YCplac111 containing R52A She2p-myc ₆	This work
pRL744	YCplac111 containing R63A She2p-myc ₆	This work
pRL745	YCplac111 containing R43K She2p-myc ₆	This work
pRL746	YCplac111 containing R44K She2p-myc ₆	This work
pRL747	YCplac111 containing R49K She2p-myc ₆	This work
pRL748	YCplac111 containing R52K She2p-myc ₆	This work

of the polymerase III holoenzyme (Echols et al. 1983; Schaaper 1988). The randomly mutagenized pool of plasmid was transformed into yeast strain YLM851. All yeast transformants were initially selected using defined synthetic media lacking leucine. Yeast transformants were recovered from minus leucine plates, and 500 colony-forming units were replated onto synthetic media containing 0.03% canavanine but lacking arginine and leucine. Canavanine-resistant colonies were isolated and screened for the red adenine phenotype. Cell lysates were prepared from colonies displaying the correct phenotypes, and cells expressing full-length mutant *She2ps* were identified by Western blot using anti-myc monoclonal antibody 9E10 (Roche), goat anti-mouse conjugated with horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories, Inc.), and SuperSignal West Pico Chemiluminescent Substrate (Pierce). The plasmids corresponding to the full-length mutant *She2p* clones were recovered from yeast, and the DNA was sequenced to determine the *She2p* mutation.

Temperature shift experiment

With few modifications, the *mex67* temperature shift experiment was performed as described previously (Kruse et al. 2002). Yeast cells were grown to exponential phase overnight at 25°C in synthetic media lacking uracil and tryptophan containing 2% raffinose. The cells were harvested by centrifugation and resuspended in YEP media containing 2% galactose. The cells were grown at 25°C for an additional 3 h in YEP galactose media to induce expression of *ASH1* mRNA. Subsequently, half of the culture was shifted to the nonpermissive temperature, 37°C, by addition of an equal volume of 49°C YEP galactose media, and incubation was continued at 37°C for an additional 30 min. An equal volume of 25°C YEP galactose media was added to the remaining half of the 25°C YEP galactose culture, and incubation was continued at 25°C for an additional 30 min. Following the 30-min incubation, cells were harvested, fixed, and processed for in situ hybridization and immunofluorescence.

Two- and three-hybrid analysis

For two-hybrid analysis, yeast strain PJ69–4a (James et al. 1996) was used, and for three-hybrid (SenGupta et al. 1996) analysis, yeast strain YLM585 was used. Transformants were grown in synthetic media devoid of leucine and uracil. Quantitative β -galactosidase expression levels were determined by liquid assay using *O*-nitrophenyl- β -D-galactopyranoside (ONPG; Guarente 1983). Cells were grown overnight in 50 mL of synthetic liquid media to mid-log phase and harvested by centrifugation. The cell pellets were resuspended in 5 mL of buffer Z (60 mM Na₂HPO₄, 40 mM NaHPO₄, 10 mM KCl, 1 mM MgSO₄, and 50 mM β -mercaptoethanol, adjusted to pH 7.0), and the OD₆₀₀ was determined in triplicate. An aliquot of the cell suspension was analyzed in triplicate for β -galactosidase activity in 1 mL of 60 mM Na₂HPO₄, 40 mM NaHPO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, 0.0025% SDS, and 5% chloroform by vortexing the cell suspension for 10–15 sec, followed by equilibration at 30°C for 15 min and incubation with 0.67 mg/mL ONPG at 30°C for 30 min. Reactions were terminated with 300 mM Na₂CO₃ and centrifuged for 5 min at 1100g. The OD₄₂₀ and OD₅₅₀ of the supernatant were determined. β -Galactosidase activity was calculated using the for-

mula $U = 1000 \times [(OD_{420}) - (1.75 \times OD_{550})]/(\text{time in min.}) \times (\text{volume of aliquot of cell suspension used in assay in milliliters}) (OD_{600})$. The reported β -galactosidase expression levels represent the average of three independent experiments.

Immunoprecipitation/RT-PCR analysis

With few modifications, immunoprecipitation of *She2p*-myc and detection of associated mRNAs was performed essentially as described previously (Irie et al. 2002). Briefly, exponentially growing yeast cultures corresponding to 3×10^8 cells were harvested by centrifugation, and the cells were disrupted with glass beads in 300 μ L of breaking buffer: 25 mM HEPES-KOH (pH 7.5), 150 mM KCl, 2 mM MgCl₂, 200 U/mL RNasin (Promega), 0.1% NP-40, 1 mM DTT, 0.2 mg/mL heparin, 0.1 μ g/mL chymostatin, 2 μ g/mL aprotinin, 1 μ g/mL pepstatin, 0.5 μ g/mL leupeptin, and 0.01 μ g/mL benzamidine. The cell lysates were cleared by centrifugation at 4000g at 4°C for 10 min, and immunoprecipitation was performed at 4°C for 1 h using anti-myc monoclonal antibody 9E10. Anti-myc immune complexes were purified by incubation with Protein-A-agarose (Pierce) at 4°C for 1 h. Protein-A-agarose complexes were recovered by centrifugation and washed three times with 500 μ L of wash buffer: 25 mM HEPES-KOH (pH 7.5), 150 mM KCl, and 2 mM MgCl₂. Protein-RNA complexes were eluted from protein-A-agarose by incubation at 65°C for 10 min in 100 μ L of elution buffer: 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and 1% SDS. A small aliquot of the eluted material was solubilized with Laemmli buffer and analyzed by Western blot. RNA was extracted from the remainder of the immunoprecipitate using phenol/chloroform/isoamyl alcohol, and the RNA was precipitated with ethanol. The RNA pellet was resuspended in 20 μ L of nuclease-free water and analyzed with the Access/RT-PCR kit (Promega) using primers specific for either *ASH1* mRNA or *IST2* mRNA. RT-PCR reactions were performed as described by the manufacturer for 25 cycles. Under these conditions, the RT-PCR experiments were in the linear range of the assay.

Coimmunoprecipitation assays

Exponentially growing cultures corresponding to 3×10^8 yeast cells were harvested, and soluble lysates were prepared as described in Supplemental materials. Protein complexes were purified by the addition of 7.5 μ g of anti-myc 9E10 monoclonal antibody. The antibody was incubated with the lysate at 4°C for 1 h. The protein-antibody complexes were subsequently purified using Protein-A-agarose (Pierce). The matrix was washed four times with 500 μ L of wash buffer: 50 mM HEPES-KOH (pH 7.3), 50 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, and 5% glycerol. The bound proteins were eluted by boiling in Laemmli buffer. Equivalent amounts of cell extract (total fraction) and precipitated sample (IP fraction) were separated by SDS-PAGE and analyzed by Western blot. Proteins containing the 3HA epitope were detected using anti-HA monoclonal antibody 16B12 (Babco).

UV cross-linking and electrophoretic mobility shift assays

UV cross-linking reactions were performed with 0.25 μ g, 0.5 μ g, and 5.0 μ g of recombinant protein and 1 ng of ³²P-labeled E3 RNA

(Long et al. 2000). Recombinant protein and RNA were incubated at 30°C for 10 min in 20 mM HEPES (pH 7.9), 33 mM KCl, 3.2 mM MgCl₂, 6% glycerol, and 4 mM dithiothreitol. Subsequently, reaction mixtures were UV cross-linked at 4°C for 20 min using a 254-nm UV lamp (Spectroline, EF-180C) at a distance of 9 cm, followed by treatment with 1.0 mg/mL RNase A at 37°C for 15 min. Reactions were analyzed by SDS-PAGE and autoradiography.

For electrophoretic mobility shift assays, E3 RNA was folded by heating to 90°C for 2 min and immediately transferring the samples to ice. Successful folding of E3 RNA was monitored by comparing the mobility of prefolded and folded samples by gel shift (data not shown). Trace (1 nM) ³²P-labeled E3 RNA and unlabeled competitor E3 RNA were combined in a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 125 µg/mL acetylated BSA (Invitrogen), and 0.01% igepal CA-630 prior to adding purified She2p to 125 nM. Reactions were equilibrated at 30°C for 2.5 h and loaded onto a running 8% (29:1) native gel. Samples were electrophoresed for 2 h at 250 V, dried, and subjected to PhosphorImager analysis.

Pull-down assays between GST-She2p and in vitro transcribed and translated She3p

Radiolabeled She3p was produced by coupled in vitro transcription-translation using plasmid pRL104 with the TnT Coupled Reticulocyte Lysate Kit (Promega). The reaction was assembled using ³⁵S-methionine according to the manufacturer's directions. An aliquot of the transcription-translation reaction was incubated with 1 µg of GST or 1 µg of GST-She2p (wild type or mutant) bound to glutathione Sepharose. The binding reaction was performed at room temperature for 90 min in 500 µL of binding buffer: 50 mM HEPES-KOH (pH 7.3), 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, and 5% glycerol. The matrix was recovered by centrifugation and washed four times with 500 µL of binding buffer. The bound proteins were eluted by boiling in Laemmli buffer and separated by SDS-PAGE, and She3p was visualized by autoradiography.

Supplemental Materials

Experimental protocols for soluble lysate preparation, production of anti-She2p rabbit serum, She2p recombinant protein expression and purification, in vitro transcription, in situ hybridization, and immunofluorescence as well as in vitro mutagenesis are available at <http://www.mcw.edu/microbiology/rml/rna2003support.html>.

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