Molecular Cell, Vol. 10, 1319–1330, December, 2002, Copyright ©2002 by Cell Press

Asymmetric Sorting of Ash1p in Yeast Results from Inhibition of Translation by Localization Elements in the mRNA

Pascal Chartrand,[1,2] Xiu Hua Meng,[1] Stefan Huttelmaier,[1] Damiane Donato,[2] and Robert H. Singer[1,3]
[1]Department of Anatomy and Structural Biology
Department of Cell Biology
Albert Einstein College of Medicine
Bronx, New York 10461
[2]Department of Biochemistry
Université de Montréal
Montréal, Quebec H3C 3J7
Canada

Summary

***ASH1* mRNA localizes at the bud tip of late-anaphase yeast, resulting in accumulation of Ash1p in the daughter nucleus. We show that disruption of the secondary structure, but not the protein coding, of all four *ASH1* localization elements resulted in RNA and protein delocalization. Localization of both was incrementally restored by replacement of each of the four elements. However, transposition of the elements to the 3′UTR reinstated the RNA, but not the protein, localization. Interestingly, the mutant *ASH1* mRNA was translated more efficiently, suggesting that asymmetry of Ash1p resulted from translational inhibition by the localization elements. In support of this, Ash1p asymmetry could be rescued by slowing its translation.**

Introduction

Messenger RNA localization is one mechanism by which cell fate determinants can be sorted between sister cells. The localization of a specific mRNA in a cell cytoplasm is generally mediated through the interaction of the localization machinery with one or several *cis*-acting localization element(s), or "zipcodes," present within the mRNA sequence (Kislauskis and Singer, 1992). These localization elements are, in most cases, present in the 3′ untranslated region (UTR) of the mRNA (for review see Bashirullah et al., 1998). But recently a few mRNAs have been reported with zipcodes in their 5′UTR or in their coding sequence (Chartrand et al., 1999; Gonzalez et al., 1999; Thio et al., 2000). A common feature of several localized mRNAs identified in various organisms is the presence of multiple localization elements. While some of these mRNAs contain repeated elements with redundant functions, like the *Vg1* and *Xlsirt* mRNAs in *Xenopus* (Gautreau et al., 1997; Kloc et al., 1993), *bicoid* and *orb* mRNA in *Drosophila* (Macdonald and Kerr, 1997; Lantz and Schedl, 1994), and β-*actin* mRNA in vertebrates (Kislauskis et al., 1994), others contain multiple elements with nonredundant or complementary functions, like in the *nanos*, *oskar*, and *gurken* mRNAs in *Drosophila* (Gavis et al., 1996; Kim-Ha et al., 1993, Thio et al., 2000), *Xcat-2* mRNA in *Xenopus* (Kloc et al., 2000), and *CamKIIα* and *MBP* mRNAs in vertebrates (Mori et al., 2000; Ainger et al., 1997). For most of these localized mRNAs, it is still not known why they require redundant localization elements and which RNA binding proteins interact with their zipcodes.

The yeast *Saccharomyces cerevisiae* protein Ash1 serves as a model system for studying the asymmetric segregation of cell-fate determinants through mRNA localization (see Chartrand et al., 2001). The asymmetric sorting of Ash1p to the daughter cell nucleus correlates with the localization of *ASH1* mRNA to the distal tip of daughter cells during anaphase of the cell cycle (Long et al., 1997; Takizawa et al., 1997) and results in the inhibition of mating-type switching in the daughter cell (Jansen et al., 1996; Sil and Herskowitz, 1996). The core components of the *ASH1* mRNA localization machinery have been recently identified and were shown to form a complex called the "locasome" that includes Myo4p, a type V myosin that was shown to be directly involved in the transport of the *ASH1* mRNA to the bud tip (Bertrand et al., 1998), She2p, an RNA binding protein that binds specifically to the four *ASH1* mRNA zipcodes (Böhl et al., 2000; Long et al., 2000), and She3p, which interacts with the tail of Myo4p and with She2p, therefore acting as a bridge between these two proteins (Böhl et al., 2000; Takizawa and Vale, 2000; Long, et al., 2000).

Of the four *ASH1* mRNA elements, three (E1, E2A, and E2B) are located within the coding region of the *ASH1* mRNA, while the remaining element (E3) includes the termination codon but is located primarily within the *ASH1* 3′UTR. In previous studies, the elements E1 and E3 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). Moreover, the integrity of the stem-loop structure of the element E3 is essential for the binding of She2p (Böhl et al., 2000; Long et al., 2000).

While each localization element is sufficient to localize a reporter mRNA (Chartrand et al., 1999; Gonzalez et al., 1999) to the bud of yeast cells, it is not yet clear why the *ASH1* mRNA requires the presence of four distinct localization elements and why they are present within the coding region of this mRNA. One possibility is that four elements, by potentially involving four myosins, increase the processivity of the myosin-driven translocation. Another is that the elements may control the translation of the Ash1 protein until its mRNA is properly localized; the consequences of a premature translation of Ash1p would be disastrous for mating-type switching. Here we report that mutagenesis of all four localization elements disrupted the localization of both *ASH1* mRNA and Ash1p. Insertion of the elements in the 3′UTR of the mutated *ASH1* mRNA restored the localization of the mRNA but did not fully restore the asymmetric distribution of Ash1p. The protein asymmetry could be rescued by slowing down the ribosome initiation using a stem-loop in the 5′UTR, suggesting that *ASH1* mRNA translation may be temporally regulated by the presence

[3]Correspondence: rhsinger@aecom.yu.edu

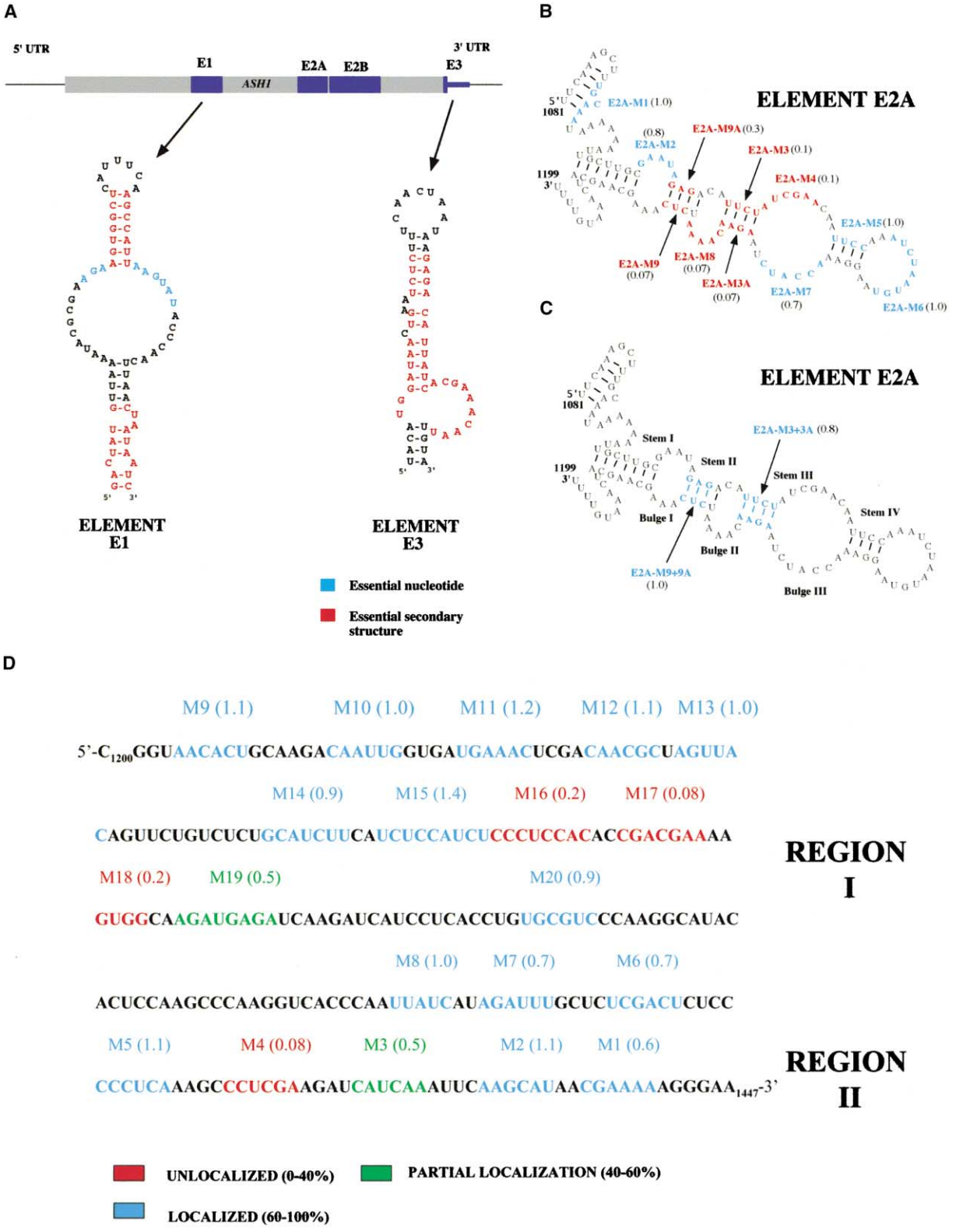

Figure 1. Mutational Analysis of the *ASH1* mRNA Localization Elements E2A and E2B

(A) Distribution of the four *cis*-acting localization elements within the *ASH1* mRNA sequence. Three of the elements are present within the coding sequence of the mRNA: the elements E1 (150 nt), E2A (118 nt), and E2B (250 nt). The fourth element, E3 (118 nt), contains 15 nucleotides of the coding sequence, the stop codon, and 100 nt of the 3′UTR (Chartrand et al., 1999). Effect of mutations in the localization element E2A (B and C) and in the localization element E2B (D) on the localization of a *lacZ* reporter mRNA. Regions mutated are colored according to their phenotype (localized, 60%–100% of budding cells with localization, blue; partially localized, 40%–60% of budding cells with localization,

of the localization elements so that the RNA could be localized before translation was complete. To confirm this hypothesis, we performed in vitro and in vivo kinetics of translation and observed a faster rate of translation of an *ASH1* mRNA with disrupted localization elements compared to a wild-type *ASH1* mRNA.

Results

Mutagenesis of the Elements E2A and E2B

We previously reported the identification of four *cis*-acting localization elements within the sequence of the *ASH1* mRNA (Figure 1A) (Chartrand et al., 1999). Two of these elements, E1 and E3, have been characterized by mutagenesis and found to adopt specific stem-loop structures (Chartrand et al., 1999; Gonzalez et al., 1999). While both elements have a similar function, their essential nucleotides and secondary structures are different. In order to determine the essential sequences and secondary structures of the remaining two elements, E2A and E2B, we performed a similar mutagenesis study on both of them (Figures 1B–1D).

The minimal element E2A is 118 nt long (from nucleotides 1081 to 1199 in the *ASH1* mRNA) and is predicted to fold into a long stem-loop structure by the MFOLD software (Zucker, 1998), with four short stems separated by three asymmetric bulges (Figure 1B). The mutagenesis study revealed that the region overlapping the stems II and III, the bulge II, and part of the bulge III, is essential for the function of the element (mutations E2A-M3, M3A, M4, M8, M9, and M9A; Figure 1B). To test the structural prediction, we created compensatory mutations that changed the original sequence but restored the predicted stems II and III: the mutations E2A-M3+3A and E2A-M9+9A. As shown in Figure 1C, these compensatory mutations restored the localization function of this element to near wild-type levels, suggesting that these two stems may be in the active structure of the element E2A. This result supports but does not prove the predicted RNA secondary structure. Moreover, as in the case of the element E1, specific nucleotides in its bulges may also be essential for E2A function.

The element E2B is the largest of the four, with a size of 247 nt (from nucleotides 1200 to 1447 in the *ASH1* mRNA). Contrary to the other three elements, E2B contains two separate regions: regions I and II (see Figure 1D). These two regions are essential for E2B function since the deletion of either one resulted in a nonfunctional element (Chartrand et al., 1999). The results show that mutations in both regions affect the function of E2B: mutants M16, M17, M18, and M19 in region I, mutants M3 and M4 in region II (Figure 1D). Using the MFOLD program for RNA secondary structure prediction, several conformations were proposed for this element. We tested the structures predicted by using compensatory mutants that restore the proposed stem-loop structures, but none of them restored the localization function of this element (data not shown). Altogether, these results show that the element E2B is more complex than the other zipcodes. Moreover, we cannot rule out that this element may fold into a specific secondary structure, which may be important for its function.

Mutagenesis of the Localization Elements within the *ASH1* mRNA

In order to determine if all four localization elements are required for the localization of the *ASH1* mRNA, we generated mutations inside each of the four elements. The mutations were created in order to disrupt the sequence and secondary structure of each element while maintaining the open reading frame of the Ash1 protein intact (all the mutations are shown in Supplemental Table S3 at http://www.molecule.org/cgi/content/full/10/6/1319/DC1). The resulting *ASH1* mutant gene, called *ASH1-MUT*, was integrated in an *ASH1*Δ yeast strain. The distribution of the *ASH1-MUT* mRNA and Ash1-mut protein were determined by FISH and immunofluorescence, respectively. As shown in Figure 2A, the *ASH1-MUT* mRNA appears evenly distributed between mother and daughter cells (the bright spot in the small daughter cell probably corresponds to the transcription site of the gene). While 98% of the wild-type *ASH1* mRNA was found to be localized into the bud of late-anaphase yeast cells, the *ASH1-MUT* mRNA was always found delocalized in these cells (see Table 1). Moreover, while the wild-type Ash1 protein was specifically sorted to the daughter cell nucleus, the Ash1-mut protein appeared evenly distributed between mother and daughter cell nuclei (Figure 2B). These results showed that the *ASH1* mRNA was completely delocalized when all four localization elements were disrupted. They also indicated that no additional localization element(s) other than those previously identified (see Chartrand et al., 1999; Gonzalez et al., 1999), was present inside the *ASH1* mRNA sequence. Furthermore, these results showed that the localization of the *ASH1* mRNA was the sole determinant of the asymmetric distribution of Ash1p; the protein had no intrinsic sorting capability.

While this experiment demonstrated that mutations in all four *cis*-acting localization elements of the *ASH1* mRNA resulted in its complete delocalization, it was still unclear whether all the elements were necessary. To answer this question, we generated all possible combinations of wild-type localization elements within the *ASH1* mRNA sequence. In all, 16 different *ASH1* constructs were created (see Table 1 for description and numbering). Each construct was integrated at the *LEU2* locus in an *ASH1*Δ yeast strain. The distribution of *ASH1*

green; unlocalized, 0%–40% of budding cells with localization, red) and are referred as E2A-M1 to E2A-M9A for E2A, and M1–M20 for E2B. Numbers in parentheses correspond to the percentage of budding cells with bud-localized *lacZ* mRNA normalized with the wild-type element as 1.0.

(B) Complete mutagenesis of the stem-loop structure of the element E2A.

(C) Double mutants that restore the stem-loop structure of E2A.

(D) Complete mutagenesis of the element E2B.

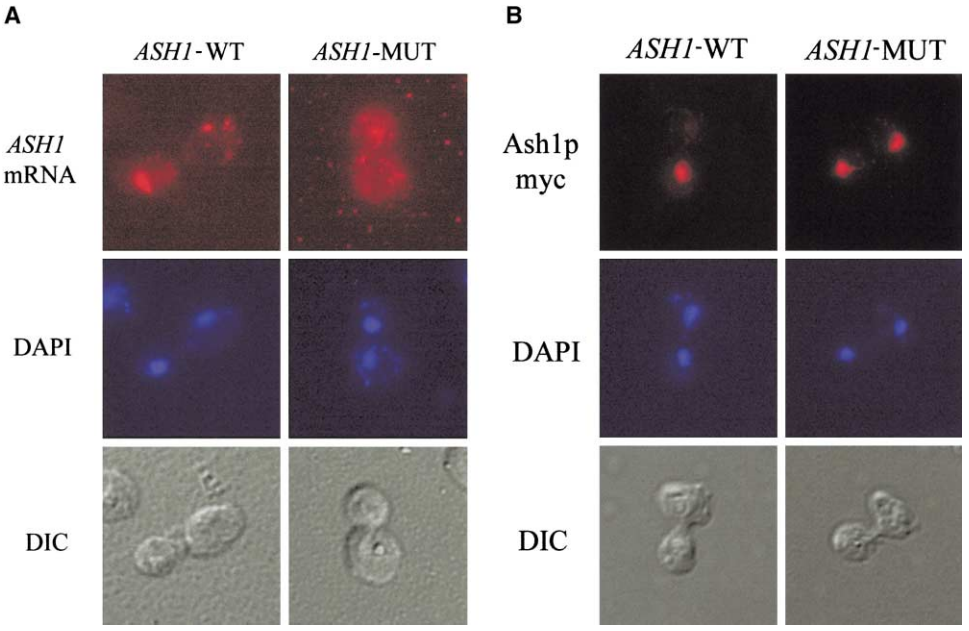

Figure 2. Effect of the Disruption of the Four Localization Elements on *ASH1* mRNA Localization and Ash1p Distribution

(A) Fluorescent in situ hybridization on late-anaphase yeast cells expressing either the wild-type *ASH1* mRNA (*ASH1-WT*, left) or the mutant *ASH1* mRNA (*ASH1-MUT*, right). The bright spot present in the nucleus of both cells corresponds to the transcription site of the *ASH1* gene. (B) Immunofluorescence of Ash1p in post-anaphase yeast cells expressing either the wild-type *ASH1* mRNA (*ASH1-WT*) or the mutant *ASH1* mRNA (*ASH1-MUT*). DAPI, DNA staining; DIC, Nomarski.

mRNA and Ash1p for each was determined by FISH and immunofluorescence, respectively. For the localization of the mRNA, late-anaphase yeast cells were scored according to the quality of the localization: either as a crescent localization at the bud tip or as bud localization (see Figure 3A for description and definition). As shown in Figures 3B and 3C, we observed a trend toward a decrease in the percentage of cells with bud localization and a concomitant tendency in an increased crescent localization of the *ASH1* mRNA proportional to the number of localization elements within the *ASH1* mRNA.

The asymmetric distribution of the Ash1 protein produced by each *ASH1* construct was also determined (Figure 3D). The cells were scored according to whether the distribution of the protein between the mother and daughter nucleus in post-anaphase cells was asymmet-

Table 1. Effect of the Number of Localization Elements on *ASH1* mRNA Localization

Construct Number	Number of Active Elements	Construct[a] Name	Percent of Localization[b]		
			Crescent	Bud	Delocalized
1	0	*ASH1-MUT*	0	0	100
2	1	*ASH1-MUT-E1*	15 ± 7	74 ± 2	11 ± 5
3	1	*ASH1-MUT-E2A*	23 ± 1	65 ± 1	12 ± 2
4	1	*ASH1-MUT-E2B*	14	80	6
5	1	*ASH1-MUT-E3*	20 ± 2	74 ± 2	6 ± 5
6	2	*ASH1-MUT-E1-E2A*	50	48	2
7	2	*ASH1-MUT-E1-E2B*	25 ± 1	69 ± 7	6 ± 6
8	2	*ASH1-MUT-E1-E3*	46 ± 4	51 ± 1	3 ± 3
9	2	*ASH1-MUT-E2A-E2B*	57 ± 3	43 ± 3	0
10	2	*ASH1-MUT-E2A-E3*	43 ± 5	50	7 ± 5
11	2	*ASH1-MUT-E2B-E3*	48 ± 10	50 ± 10	2
12	3	*ASH1-MUT-E1-E2A-E2B*	69 ± 5	31 ± 5	0
13	3	*ASH1-MUT-E1-E2A-E3*	60 ± 16	38 ± 6	2
14	3	*ASH1-MUT-E1-E2B-E3*	68 ± 6	32 ± 6	0
15	3	*ASH1-MUT-E2A-E2B-E3*	64 ± 2	34 ± 2	2
16	4	*ASH1-WT*	71 ± 9	27 ± 9	2

[a] Construct name indicates which localization element is intact, i.e., *ASH1-MUT-E1* contains only the intact element E1; the other three elements are mutated. For *ASH1-WT*, all elements are intact.

[b] Indicates the percentage of anaphase yeast cells with crescent-localized, bud-localized, or delocalized *ASH1* mRNA construct. Bud-localized mRNA is defined as a diffusely localized mRNA in the bud.

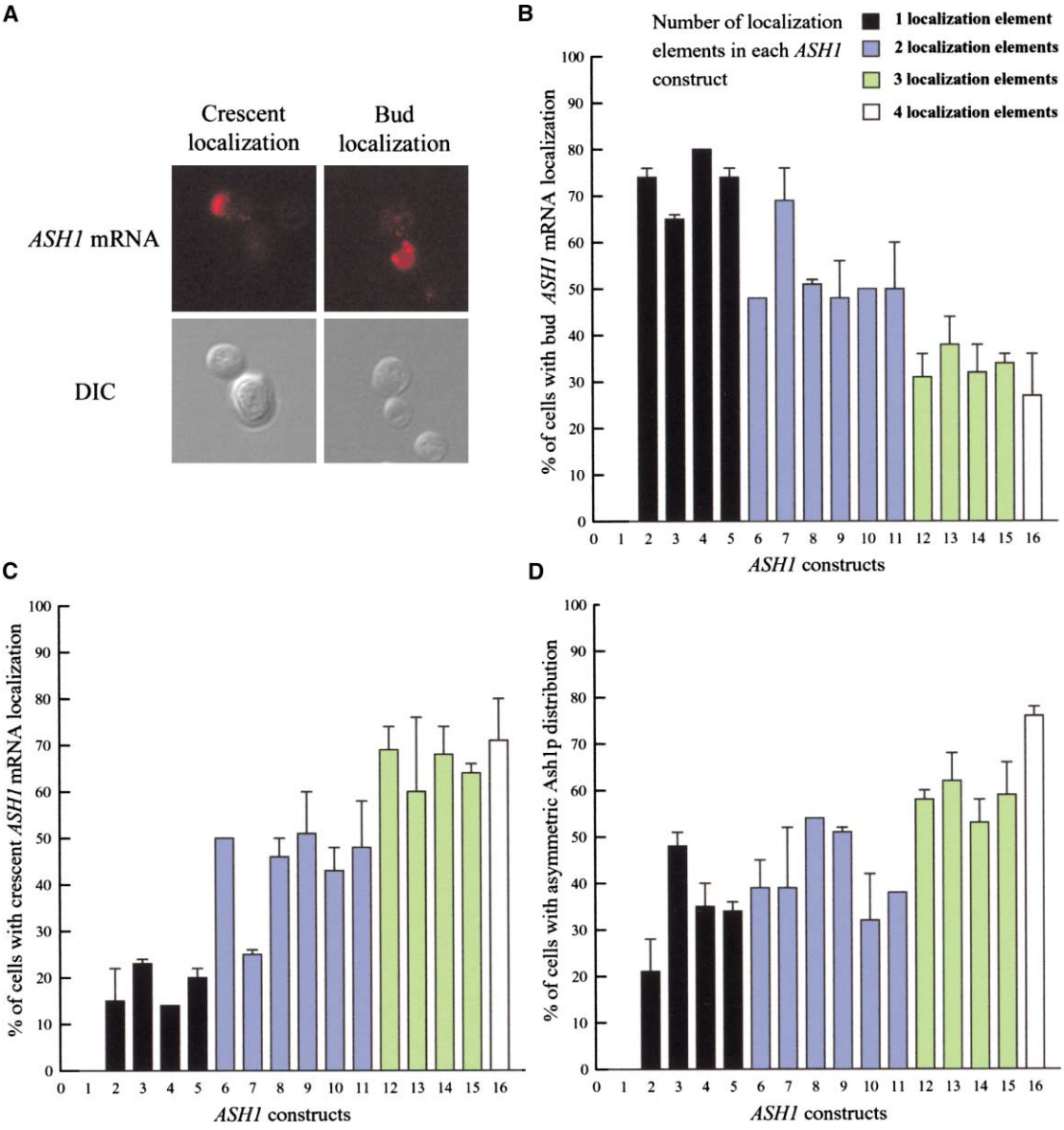

Figure 3. Effect of the Number of Localization Elements on *ASH1* mRNA Localization and Ash1p Distribution

(A) Phenotypes of *ASH1* mRNA localization. Crescent localization corresponded to mRNA localized as a tight crescent at the bud tip. Bud localization corresponded to mRNA diffusely localized in the bud or localized along the sides of the bud, even if part of the RNA localized as a crescent.

(B) Effect of the number of localization elements on the percentage of cells with bud-localized *ASH1* mRNA. The *ASH1* constructs 1–16 are described in Table 1.

(C) Effect of the number of localization elements on the percentage of cells with crescent localized *ASH1* mRNA.

(D) Effect of the number of localization elements on the percentage of cells with asymmetric Ash1p distribution.

ric or symmetric. As shown in Figure 3D, there was a correlation between the number of localization elements present in the *ASH1* mRNA and the percentage of post-anaphase cells with an asymmetric distribution of the Ash1 protein. In general, the more localization elements within the *ASH1* mRNA sequence, the more asymmetric the localization of the mRNA and the protein. These results are consistent with a model that the presence of incorrectly localized *ASH1* mRNA could lead to the synthesis of Ash1 protein that can enter the mother cell nucleus before the end of cytokinesis. In contrast, Ash1p originating from tightly localized *ASH1* mRNA at the bud tip would enter the bud nucleus preferentially.

Effect of the Position and Identity of the Localization Elements on *ASH1* mRNA and Ash1p Localization

Most localized mRNAs identified in various organisms have their localization elements present only in the 3′UTR (Bashirullah et al., 1998). However the *ASH1* mRNA zipcodes are dispersed throughout the coding

sequence (Chartrand et al., 1999; Gonzalez et al., 1999). This suggested that the position of these elements inside the *ASH1* mRNA sequence had some functional significance. In order to address this, we inserted each single element into the 3′UTR of the *ASH1-MUT* gene to obtain a multimer E1-E2A-E2B-E3 (E1-2A-2B-3). With all four localization elements now in the 3′UTR, we could analyze how it affected the localization of the *ASH1-MUT* mRNA and the distribution of Ash1p. We also multimerized each single element in order to have four copies of each in the 3′UTR of the *ASH1-MUT* mRNA. In this way, we could confirm whether the elements were redundant and if there was any effect on the efficiency of localization of both *ASH1* mRNA and Ash1p.

As shown in Figure 4A, we observed that each single element, when multimerized and inserted in the 3′UTR of the *ASH1-MUT* mRNA, could reach near wild-type levels of crescent localization. A similar level of RNA localization was also observed with the four elements together (E1-E2A-E2B-E3). However, their effect on the asymmetric distribution of Ash1p was clearly different (Figure 4B). Although the E2A(4×) multimer demonstrated near wild-type levels of Ash1p localization, the others were significantly below wild-type levels (64%–75% of wild-type). Most interestingly, the multimer E1-E2A-E2B-E3 was significantly below (60% of wild-type) levels of Ash1p asymmetric distribution, although it localized the *ASH1-MUT* mRNA as well as the *ASH1* wild-type. These results suggested that the four elements were functionally and quantitatively redundant for *ASH1* mRNA localization; four copies of any single element were necessary to reach near wild-type *ASH1* mRNA localization. Nor was their position within the *ASH1* mRNA essential for their localization ability; they worked as well in the 3′UTR as they did in their wild-type positions. Strikingly, the position of the localization elements had a major effect on the asymmetric distribution of Ash1 protein. When the elements were moved to the 3′UTR, they no longer efficiently sorted the Ash1 protein asymmetrically as the wild-type *ASH1* mRNA, even though the RNA localization was normal. This result suggested that their presence within the *ASH1* mRNA coding sequence may affect Ash1p distribution.

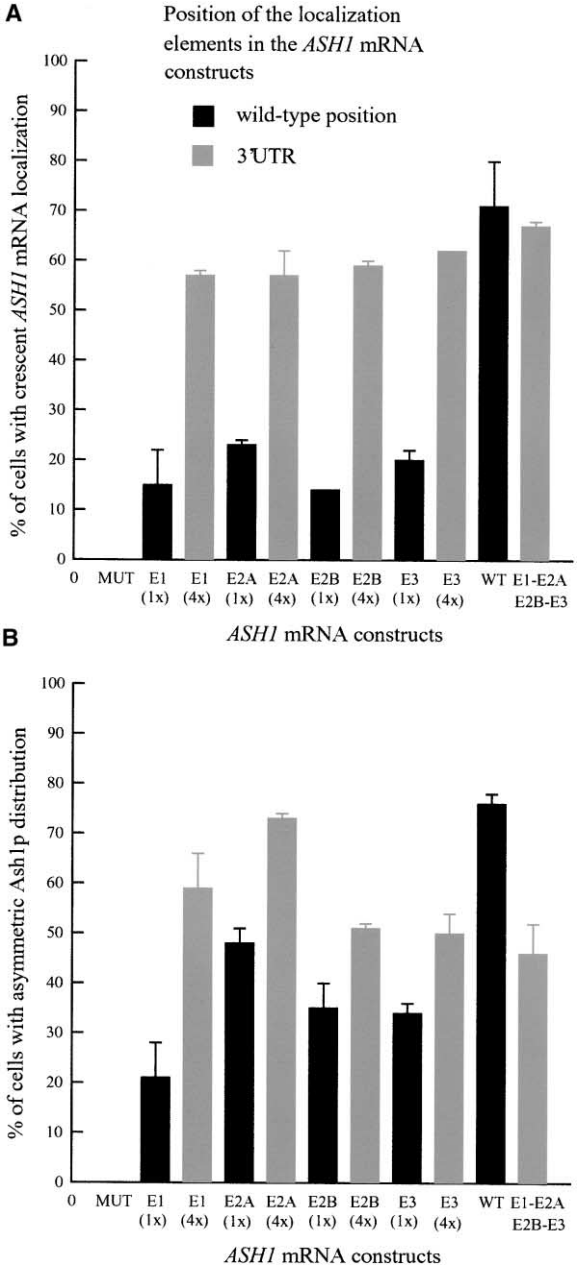

Figure 4. Effect of the Position and Number of Localization Elements on *ASH1* mRNA Localization and Ash1p Asymmetric Distribution

ASH1 constructs containing one copy (1×) or four copies (4×) of a single element, either at its wild-type position within the *ASH1* sequence (black bars) or in the 3′UTR of the mRNA (gray bars). MUT, all elements are mutated. WT, wild-type *ASH1* mRNA. E1-E2A-E2B-E3, all four elements are present in the 3′UTR only.

A Decrease in mRNA Translation Results in an Increase in Ash1p Localization

We have shown that a tightly localized *ASH1* mRNA resulted in an Ash1 protein asymmetrically distributed to the bud nucleus. This would only occur if the mRNA was translated after it was localized. A reduction in asymmetric Ash1p localization likely resulted from translation of some poorly localized mRNAs close to the mother cell nucleus. Therefore, the discrepancy in the localization of the RNA and the protein when the localization elements were transposed to the 3′UTR could have resulted from translation of the *ASH1* mRNA before it localized. Because the expected time for Ash1p synthesis (10 aa/s) is close to the time of localization (Bertrand et al., 1998), the faster the translation, the more likely the resulting protein would enter the mother nucleus. To test this hypothesis we reduced the rate of translation of the Ash1 protein, thereby increasing the probability that *ASH1* mRNA would reach the bud tip before it was translated. This should therefore result in a suppression of the protein distributed to the mother nucleus with a concomitant increase in asymmetric distribution of the protein to the bud nucleus.

We inserted a short stem-loop structure in the 5′UTR of the *ASH1* mRNA in order to reduce its level of transla-

tion. Several studies in yeast have shown that a strong stem-loop structure in the 5′UTR of an mRNA can significantly reduce or inhibit the translation of this mRNA, while shorter stem-loops have a lower impact on translation (Vega Laso et al., 1993; Sagliocco et al., 1993). The stem-loop structure was designed to be short enough to avoid a complete inhibition of Ash1p synthesis, but long enough so that it can affect the rate of *ASH1* mRNA translation. Using a stem-loop structure of 6 bp (see Figure 5A), we could still detect Ash1p in yeast cells by immunofluorescence (data not shown). The presence of the stem-loop did not affect the localization of the *ASH1* mRNA constructs (data not shown). This stem-loop was inserted in weakly localized *ASH1* constructs, each containing only one of the four localization elements, and its effect on Ash1p asymmetric distribution was determined by immunofluorescence. The insertion of this stem-loop in the 5′UTR of these *ASH1* constructs resulted in a partial decrease in Ash1p expression (Figure 5B). As shown in Figure 5C, we observed a significant increase in the asymmetric distribution of Ash1p for each *ASH1* construct containing the stem-loop structure. We measured a 50% increase in Ash1p asymmetric distribution for *ASH1* mRNA constructs containing either the element E2A, E2B, or E3, and more than a 100% increase in the construct with only the element E1. As we expected, when the stem-loop was inserted in the 5′UTR of the *ASH1-MUT* mRNA with the four localization elements in the 3′UTR (E1-2A-2B-3), Ash1p asymmetric distribution was restored to wild-type levels, while the same mRNA without the stem-loop resulted in a 40% decrease in Ash1p localization (Figure 5C). These results suggest a temporal relationship between the localization and the translation of the *ASH1* mRNA, and this relationship is important for the optimization of Ash1p asymmetric distribution. This work emphasizes the importance of the position of the localization elements within the *ASH1* mRNA coding sequence on the efficiency of Ash1p sorting.

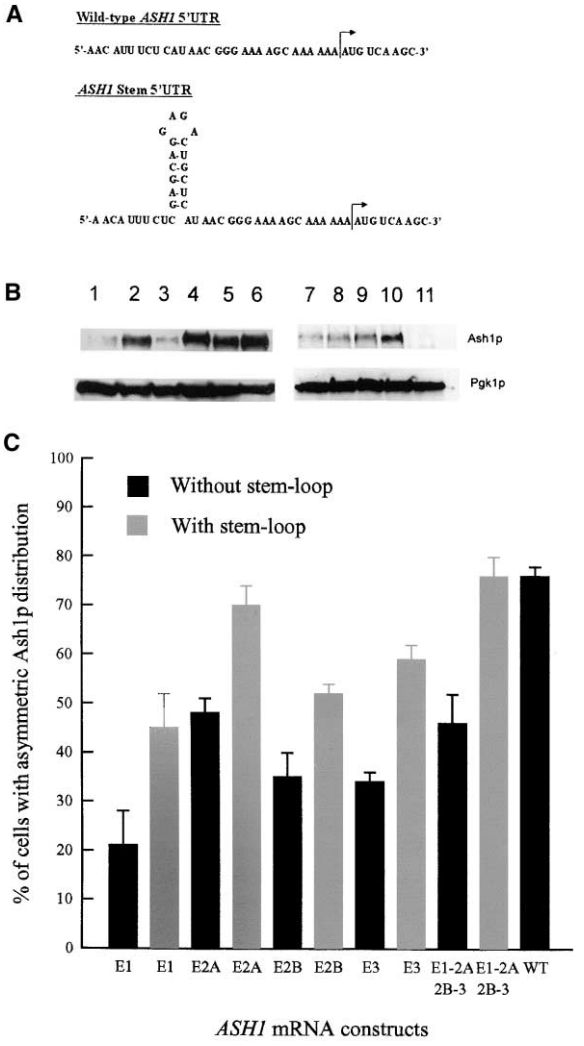

Figure 5. Effect of a Stem-Loop in the 5′UTR on the Translation and Asymmetric Distribution of Various Constructs of *ASH1-MUT* mRNA

(A) Description of the stem-loop structure inserted in the 5′UTR of the *ASH1-MUT* constructs.

(B) The *ASH1-MUT* constructs studied contained only one wild-type localization element; either E1 (*ASH1-MUT-E1*), E2A (*ASH1-MUT-E2A*), E2B (*ASH1-MUT-E2B*), or E3 (*ASH1-MUT-E3*) at their normal position or all four localization elements in the mRNA 3′UTR (*ASH1-MUT-E1-2A-2B-3*). Western blot showing the effect on Ash1p expression of the stem-loop (SL) in the 5′UTR of the *ASH1* constructs: 1, *SL-ASH1-MUT-E1*; 2, *ASH1-MUT-E1*; 3, *SL-ASH1-MUT-E2A*; 4, *ASH1-MUT-E2A*; 5, *SL-ASH1-MUT-E2B*; 6, *ASH1-MUT-E2B*; 7, *SL-ASH1-MUT-E3*; 8, *ASH1-MUT-E3*; 9, *SL-ASH1-MUT-E1-2A-2B-3*; 10, *ASH1-MUT-E1-2A-2B-3*; and 11, Control K4452-*ash1*.

(C) Effect of the stem-loop on Ash1p asymmetric distribution from the *ASH1* constructs in (5B). WT, wild-type *ASH1* mRNA; E1-2A-2B-3, all four elements are present in the 3′UTR only.

The Disruption of the Localization Elements Results in an Increased Rate of Translation of *ASH1* mRNA

The previous results suggested that the presence of the localization elements within the coding sequence of the *ASH1* mRNA might interfere with the elongation of the ribosomes during the translation. In order to test this hypothesis we performed in vitro and in vivo kinetics of translation of both *ASH1* wild-type (*ASH1-WT*) and the *ASH1* construct with the four localization elements disrupted (*ASH1-MUT*), and compared the rate of synthesis of the Ash1 protein coded by these mRNAs. A priori, the disruption of the four localization elements in the *ASH1-MUT* construct should not affect its rate of translation (compared to the *ASH1-WT*) since the mutations resulted in the replacement of only 26 codons (of 588 total), including 5 rare codons that were replaced, while 4 new rare codons were inserted. Moreover, both mRNAs code for an identical Ash1 protein (588 amino acids).

We cloned the two genes, with myc tags at the C terminus of the proteins, under the control of the galactose-inducible *GAL1* promoter and integrated the constructs in the yeast genome. The yeast were grown in a noninducible, nonrepressive medium (lactic acid/glycerol) and induced by the addition of 3% galactose. After induction, samples were taken at different time points, cell extracts were prepared from each sample, and the appearance of the Ash1p-myc protein was detected by Western blot. As shown in Figures 6A and 6B, the Ash1p-myc protein synthesized from the *ASH1-MUT* mRNA appeared about three times faster after induction than

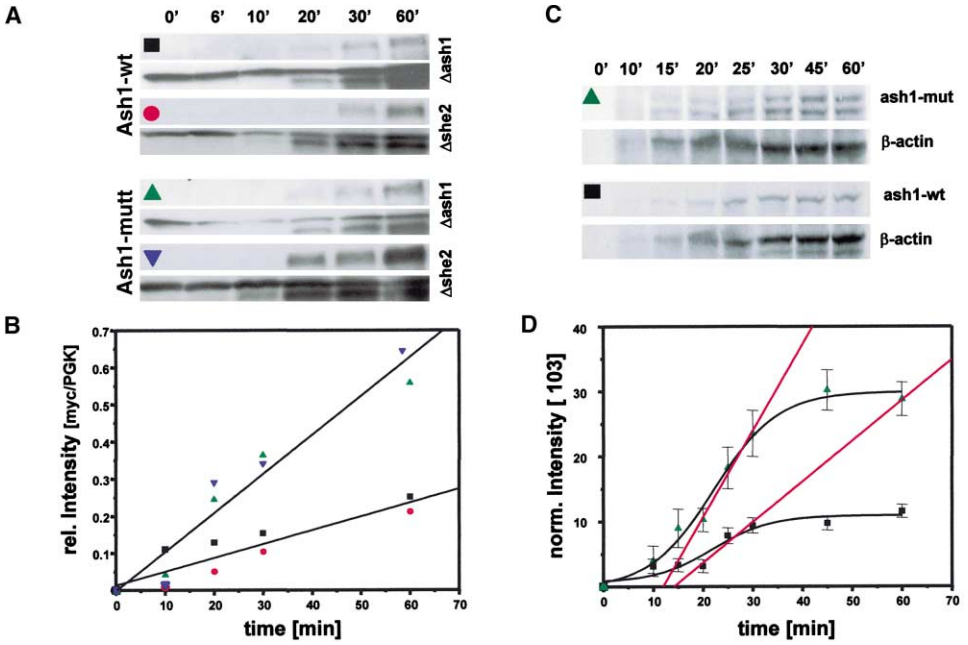

Figure 6. Effect of the Disruption of the Four *cis*-Acting Localization Elements (E1, E2A, E2B, and E3) on the Translation of *ASH1* mRNA In Vivo and In Vitro

(A) Western blots showing the expression of myc-tagged Ash1p-WT versus Ash1p-MUT at various time points after galactose induction in an *ash1* or *she2* yeast strain. Ash1p-myc signal intensities (upper panels) were quantified and normalized with Pgk1p signal (lower panels).

(B) Relative intensities of Ash1p-myc versus Pgk1p were plotted at various time points: ■, *ASH1-WT* (*ash1*); ●, *ASH1-WT* (*she2*); ▲, *ASH1-MUT* (*ash1*); and ▼, *ASH1-MUT* (*she2*). A linear fit function for Ash1p-WT or Ash1p-MUT in both yeast strains is indicated (Δslope [Ash1p-MUT to Ash1p-WT] ~2.8).

(C) Autoradiography showing ^{35}S-methionine labeled Ash1p after translation from capped *ASH1-MUT* mRNA (▲) or *ASH1-WT* (■) mRNA in rabbit reticulocyte lysates at various time points. Ash1p signal intensities were normalized to an internal human β-actin control (capped full-length human β-actin mRNA).

(D) Normalized signal intensities of Ash1p translated from *ASH1-MUT* mRNA (■) or *ASH1-WT* mRNA (▲) were plotted over time. Black curves represent a sigmoidal fit of all data shown. Red curves show a linear fit function over data from 20 to 30 min (Δslope [Ash1p-MUT to Ash1p-WT] ~3.5).

the protein synthesized from the *ASH1-WT* mRNA. These results suggested that the presence of the zip-codes inside the coding sequence of the *ASH1* mRNA were affecting ribosome elongation.

Another possibility is that the binding of the localization machinery (Myo4p, She2p, and She3p) to these localization elements may interfere with ribosome elongation. To test this hypothesis, we repeated the in vivo translation kinetics in a strain with a deletion of the RNA binding protein She2p, which interacts with each of the four localization elements (Böhl et al., 2000; Long et al., 2000). As shown in Figures 6A and 6B, this deletion did not affect the difference in Ash1p-myc synthesis between *ASH1-MUT* and *ASH1-WT* mRNAs, suggesting that the binding of the localization machinery to the *ASH1* mRNA had no detectable effect on its translation. This result does not rule out the possibility that factors not part of the localization mechanism could also be involved in the translational control of this mRNA (Irie et al., 2002).

In order to confirm and extend these results, we used a cell-free translation assay to measure the difference between *ASH1-WT* and *ASH1-MUT* mRNA translation. As shown in Figures 6C and 6D, an in vitro translation assay, using in vitro-transcribed and -capped mRNA in a rabbit reticulocyte lysate, shows that *ASH1-MUT* mRNA was translated more efficiently than the *ASH1-WT* mRNA. We observed an increase of 3.5 times the rate of synthesis of Ash1p from the *ASH1-MUT* mRNA compared to the *ASH1-WT* mRNA, approximately the same as occurred in vivo. In contrast to the Ash1p synthesized from wild-type mRNA, two bands were observed for Ash1p from the mutant mRNA. The upper band represents the full-length product suggesting that the lower band is due to degradation or a translational stop at a rare codon.

We also repeated the in vitro translation assay and added purified GST-She2p to the extracts. We observed no effect on the translation of either *ASH1-WT* or *ASH1-MUT* mRNA (data not shown); GST-She2p was verified to bind to the *ASH1-WT,* whereas no binding was observed for the *ASH1-MUT* message (data not shown). Altogether, these results strongly support the data obtained from the in vivo translation assay and implicate the position of the zipcodes in translational control of *ASH1* mRNA.

Discussion

A common feature of several localized mRNAs identified in various organisms is the presence of multiple localization elements (Bashirullah et al., 1998). We directly addressed

the importance of multiple localization elements on the localization of an mRNA and its impact on the sorting of the resulting protein. Several insights into the localization process came from this analysis. First, the presence of multiple localization elements increased the quality of localization of an mRNA (crescent versus bud localization of the *ASH1* mRNA). Second, this quality of localization is essential for the proper sorting of the protein, but it is not sufficient. Other factors, like the timing of the translation of the mRNA, also affect the asymmetric distribution of the protein.

The disruption of the four localization elements resulted in the complete delocalization of *ASH1* mRNA. Moreover, Ash1p produced from this mutant mRNA was symmetrically distributed between mother and daughter cell nuclei. These results clearly demonstrate that the asymmetric distribution of Ash1p is totally RNA controlled and does not require any protein-encoded information. This is in contrast to localization of a cell fate determinant, the *Drosophila* Numb/Pon, which can sort apparently only at the protein level (Lu et al., 1999). These results do not rule out the possibility that the amino acid sequence of Ash1p could be involved in some specific aspect of *ASH1* mRNA localization, such as mRNA anchoring (Gonzalez et al., 1999).

The presence of the four elements was necessary to have an optimal level of *ASH1* mRNA localization at the bud tip of budding yeast cells. We found that the quality of localization (as a crescent at the bud tip) increased with the number of zipcodes present in the mRNA, irrespective of their position. More important, this improvement in the quality of *ASH1* mRNA localization correlated with an increase in the asymmetric distribution of Ash1p. This suggested that the presence of weakly localized *ASH1* mRNA close to the bud neck could lead to Ash1p entering the mother nucleus before cytokinesis, thereby resulting in a more symmetric distribution of Ash1p. *ASH1* mRNA tightly localized at the bud tip, far from the bud neck and close to the bud nucleus, prevented Ash1p from entering the mother nucleus. This is a different phenomenon from the diffusion of another protein, Ist2p, localized to the membrane compartment of the bud via its mRNA (Takizawa et al., 2000). In this case, the protein presumably is inserted into the membranes and is restricted from diffusing back to the mother by the septin barrier at the bud neck. No such barrier appears to exist between the mother and the bud with respect to Ash1p. It is still unclear how the increase in the number of zipcodes led to an increase in crescent *ASH1* mRNA localization. One possibility could be that more localization elements in the mRNA recruited more myosin Myo4p, and thereby increased the rate or productivity of the transport of mRNA. This would be supported by recent work on yeast myosins 2 and 4, indicating that their duty ratios require approximately four motors in concert to promote continuous cargo movement (Reck-Peterson et al., 2001). Another possibility is that the anchoring of the *ASH1* mRNA at the bud tip depended on the number of zipcodes.

The level of crescent *ASH1* mRNA localization was independent of the identity of the zipcodes present, suggesting that the four zipcodes are functionally redundant. These results are also consistent with the fact that each element interacted with the same RNA binding protein, She2p (Böhl et al., 2000; Long et al., 2000), suggesting that each localization element recruited the same She2p/She3p/Myo4p complex to the *ASH1* mRNA. However, the zipcodes were not redundant with respect to their efficiency of Ash1p asymmetry. For instance, the element E2A alone in the *ASH1* mRNA led to twice as much Ash1p asymmetric distribution than the element E1 alone. E2B and E3 were 50% more efficient than E1 in their level of Ash1p localization. Similar results were obtained when each element was multimerized and inserted in the mutant *ASH1* mRNA 3′UTR. While the multimerized E2A zipcode reached near wild-type levels of asymmetric localization of Ash1p, the other elements were less effective, even if they were as efficient in crescent localization. Interestingly, the insertion of the four elements in the 3′UTR of the mutant *ASH1* mRNA resulted in a 40% decrease in Ash1p asymmetric distribution compared to wild-type *ASH1*, even if both mRNAs have a similar level of crescent localization. This result suggested that the position of the elements within the sequence of the *ASH1* mRNA were more important when all four elements were present. These differences among zipcodes implied that they functioned in the regulation of Ash1p expression, possibly by playing a role in the translation of the *ASH1* mRNA, particularly since three of the four localization elements were found within the coding sequence of the *ASH1* mRNA and could therefore have a direct impact on ribosome elongation. Because localization in the cell population was measured, slight differences in the kinetics of transport of the mRNAs among the various mutants may not have been evident. A more thorough analysis of mutant mRNAs will require kinetic measurements in living yeast cells, as in Bertrand et al. (1998).

Yeast cell fate determination appears to be mediated by the timing of Ash1p translation. Its role in asymmetric distribution was supported by the experiment where a stem-loop inserted in the 5′UTR of an mRNA decreased the level of initiation of the ribosomes (Vega Laso et al., 1993; Sagliocco et al., 1993). While Ash1p asymmetric distribution decreased when the four localization elements were in the *ASH1* mRNA 3′UTR, wild-type levels of Ash1p localization were recovered by reducing the translation of this mRNA. This result points to a relationship between the timing of the localization of the *ASH1* mRNA and its translation, and this interaction appears to be important for optimizing the asymmetric distribution of Ash1p. Supporting this view, the rate of synthesis of Ash1p, both in vitro and in vivo, was faster from the *ASH1-MUT* mRNA than the *ASH1-WT* mRNA, suggesting that the presence of the secondary structures formed by the localization elements within the coding sequence of the *ASH1* mRNA interfered with ribosome elongation. We propose that the secondary structures, the stem-loops, renatured after each ribosome passes, thereby exerting a cumulative effect on the synthesis rate.

To summarize this hypothesis, we propose the following model. A ribosome begins translating the RNA immediately upon exit of the transcript from the nucleus. When the ribosome reaches the first element, E1, it is slowed or stopped by the secondary structure of the element (and maybe the complex She2p/She3p/Myo4p bound to it). Presumably, it will process through each of the elements, eventually unfolding all the RNA secondary structures and removing all the complexes, so

there will be a competition between translation and localization. When the localization elements are in the 3′UTR, translation wins the race, and Ash1p is symmetrically distributed. When all four zipcodes are present within the coding sequence, the localization wins, and Ash1p is asymmetrically distributed. Because it takes only about a minute for the mRNA to localize (Bertrand, et al., 1998) and it takes the ribosome around 10 s to translate 100 amino acids (Bonven and Gullov, 1979), the 588 aa Ash1p would result in a tie between the synthesis of the first protein molecule and the localization of the mRNA, assuming there were no elements in the mRNA to slow down the ribosome. We propose this is why the protein is not optimally distributed to the bud nucleus when all the localization elements are located in the 3′UTR. When the elements are in the coding region, the ribosome is impeded and the mRNA localizes before the proteins are completed.

This hypothesis is compatible with the recent finding that Khd1p, a novel RNA binding protein with three KH domains, has been found to interact with the *ASH1* mRNA and is required for efficient localization of this mRNA (Irie et al., 2002). Overexpression of Khd1p decreases the level of Ash1p, suggesting that Khd1p could be involved in the translational control of the *ASH1* mRNA during its transport to the bud tip. It is possible that the binding of both Khd1p and the She1p/She2p/She3p complex within the coding sequence of the *ASH1* mRNA may have a cumulative effect, resulting in a decrease in the rate of *ASH1* mRNA translation. Altogether, these results underline the importance of translational control in *ASH1* mRNA localization and its effect on Ash1p asymmetric distribution.

We propose that other localization systems may also rely on the timing between localization and translation, perhaps evolving a variety of methods to slow down translation. Several examples are known in which translational control is coupled to mRNA localization. For instance, the translation of the *oskar* mRNA is repressed by the binding of the Bruno protein in the mRNA 3′UTR during its transport to the posterior pole of *Drosophila* oocytes (Kim-Ha et al., 1993; Gunkel et al., 1998). Only localized *oskar* mRNA will produce Oskar protein. Other localized mRNA, like *nanos* and *gurken*, are also controlled at the translation level (Smibert et al., 1996; Saunders and Cohen, 1999). We have shown that delocalized *ASH1* mRNA is translated; therefore, there is no translation repression of delocalized *ASH1* mRNA. The short distance traveled in yeast may make this type of a mechanism possible, but the longer distances traveled in higher eukaryotes may be compensated somewhat by the higher velocity of the microtubule motors (Brendza et al., 2000; Schnorrer et al., 2000).

Experimental Procedures

Growth Media and Yeast Strains

Yeast cells were grown in either synthetic growth media lacking the nutrients indicated or rich media (Rose et al., 1990). Yeast strains used in this study and all the sequences of the mutations on the localization elements E2A and E2B are listed in Supplemental Tables S1–S5 at http://www.molecule.org/cgi/content/full/10/6/1319/DC1. K4452-*ash*1 was used for the FISH experiments (it has a lower background than other strains) and K6278-*cla4* was used for immunofluorescence (a *CLA4*Δ strain improves the identification of post-anaphase yeast cells; see Long et al., 2001). Transformation was performed according to the protocol of Gietz and Schiestl (1995). Yeast gene disruption cassette was created by PCR amplification of the *loxP*-KAN-*loxP* construct in plasmid pUG6 and primers specific for the gene of interest (Güldener et al., 1996). Specific disruption was confirmed by PCR analysis of genomic DNA. Plasmids constructions are described in the supplemental material at http://www.molecule.org/cgi/content/full/10/6/1319/DC1.

Western Blots

Extracts were obtained from 2 ODs of yeast cells grown overnight and processed with glass beads mixed with 50 μl of 50 mM Tris (pH 8.0), 384 mM glycine, 0.2% SDS, and heated to 95°C for 5 min. After vortexing, the extract was centrifuged, and the supernatant was recovered and kept at −20°C. For the Western blot, 20 μl of yeast extracts was loaded on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated first with a mouse anti-myc antibody (Clontech), then with a goat anti-mouse HRP-labeled antibody (Jackson Laboratories) and revealed with the ECL kit (AmershamPharmacia). The Pgk1 protein was detected with a mouse anti-Pgk1 antibody (Molecular Probes). Western blots were quantified using the Image Quant software 5.2 (Molecular Dynamics). Shown regions of each lane were quantified and c-*myc* signal intensities were normalized to Pgk1 signals at each time points (indicated as relative signal intensities, c-*myc* to Pgk1).

In Vitro Translation

Full-length *ASH1-WT* or *ASH1-MUT* were cloned in pcDNA3.1 from the original plasmids pXR193 and pXR192. Capped mRNA was transcribed from ClaI linearized plasmids using a T7 Message Machine kit (Ambion). In vitro translation was performed using the Flexi Rabbit Reticulocyte Lysate System (Promega) and ^{35}S-methionine as a tracer to monitor protein production. Full-length human β-actin was used as an internal control. Samples were loaded to 10% SDS-Page gels and transferred to nitrocellulose membrane. Signal intensities were determined by a Phosphoimager (Molecular Dynamics) and quantified using the Image Quant 5.2 software. Ash1p signal intensities of the shown regions of each lane were quantified and normalized to β-actin signal intensities at each time point (indicated as normalized total signal intensities). For the Ash1p-MUT, both bands were quantified. However, even if only the upper band was quantified for the in vitro translation of Ash1p-MUT (Figure 6C), higher translation rates (approximately two times) were observed compared to Ash1p-WT.

In Situ Hybridization and Immunofluorescence

Yeast cells were processed for in situ hybridization and immunofluorescence according to the protocols described in Chartrand et al. (2000). For in situ hybridization, yeast spheroplasts were hybridized with a pool of Cy3-conjugated *ASH1* DNA oligonucleotide probes. For immunofluorescence, a 1:50 dilution of a mouse anti-myc antibody (Boehringer-Mannheim) was used as primary antibody. For the secondary antibody, a 1:1000 dilution of a donkey anti-mouse Cy3-conjugated antibody (Jackson Laboratories) was used.

Measurement of mRNA Localization and Ash1p Distribution

To obtain quantitative data on the localization of each of the *lacZ* mRNA fused to either the element E2A or E2B mutants, 100 yeast cells with visible bud (i.e., cells between G2 and M phase) were scored for localized or delocalized *lacZ* mRNA (the *ASH1* mRNA and the *lacZ* fusion mRNA can localize as early as the bud starts to appear in S phase; data not shown). An mRNA was considered as localized when it was predominantly in the bud (either full bud or crescent localization). An mRNA was considered as delocalized when it was equally distributed between bud and mother cell. We observed a variation of 10%–15% in the measurements between two independent experiments. All mutants showing more than 40% decrease in localization were counted twice.

To obtain quantitative data on the localization of each *ASH1* mRNA constructs, only late-anaphase yeast cells were scored. For each construct, two independent experiments were performed, where 50 budding yeast cells were scored. An mRNA was consid-

ered as crescent localized when it localized only as a tight crescent at the bud tip, occupying around 1/3 of the bud volume. An mRNA was defined as bud localized when it was diffusely localized in the bud or localized along the sides of the bud, even if part of the RNA localized as a crescent. An mRNA was considered as delocalized when it was equally distributed between bud and mother cell.

To determine the asymmetric distribution of the various Ash1p constructs, only post-anaphase cells were counted. For each construct, two independent experiments were performed, where 50 budding yeast cells were scored. The Ash1p was considered as distributed asymmetrically when it was predominantly present in the nucleus of the daughter cell. It was considered as symmetrically distributed when it was present equally in both mother and daughter cell nucleus.

Acknowledgments

This work was supported by a Canadian Institutes of Health Research Postdoctoral Fellowship to P.C. and by the National Institutes of Health grant GM57071 to R.H.S.

Received: March 15, 2002
Revised: October 4, 2002

References

Ainger, K., Avossa, D., Diana, A.S., Barry, C., Barbarese, E., and Carson, J.H. (1997). Transport and localization elements in myelin basic protein mRNA. J. Cell Biol. *138*, 1077–1087.

Bashirullah, A., Cooperstock, R.L., and Lipshitz, H.D. (1998). RNA localization in development. Annu. Rev. Biochem. *67*, 335–394.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H., and Long, R.M. (1998). Localization of *ASH1* mRNA particles in living yeast. Mol. Cell *2*, 437–445.

Böhl, F., Kruse, C., Frank, A., Ferring, D., and Jansen, R.-P. (2000). She2p, a novel RNA-binding protein tethers *ASH1* mRNA to the Myo4p-myosin motor via She3p. EMBO J. *19*, 5514–5524.

Bonven, B., and Gullov, K. (1979). Peptide chain elongation rate and ribosomal activity in *Saccharomyces cerevisiae* as a function of the growth rate. Mol. Gen. Genet. *170*, 225–230.

Brendza, R.P., Serbus, L.R., Duffy, J.B., and Saxton, W.M. (2000). A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. Science *289*, 2120–2122.

Chartrand, P., Meng, X.-H., Singer, R.H., and Long, R.M. (1999). Structural elements required for the localization of *ASH1* mRNA and of a green fluorescent protein reporter particle *in vivo*. Curr. Biol. *9*, 333–336.

Chartrand, P., Singer, R.H., and Long, R.M. (2000). Sensitive and high-resolution detection of RNA in situ. Methods Enzymol. *318*, 493–506.

Chartrand, P., Bertrand, E., Singer, R.H., and Long, R.M. (2001). RNP localization and transport in yeast. Annu. Rev. Cell Dev. Biol. *17*, 297–310.

Gautreau, D., Cote, C.A., and Mowry, K.L. (1997). Two copies of a subelement from the Vg1 RNA localization sequence are sufficient to direct vegetal localization in Xenopus oocytes. Development *124*, 5013–5020.

Gavis, E.R., Curtis, D., and Lehmann, R. (1996). Identification of cis-acting sequences that control nanos RNA localization. Dev. Biol. *176*, 36–50.

Gietz, R.D., and Schiestl, R.H. (1995). Transforming yeast with DNA. Methods Mol. Cell. Biol. *5*, 255–269.

Gonzalez, I., Buonomo, S.B.C., Nasmyth, K., and von Ahsen, U. (1999). *ASH1* mRNA localization in yeast involves multiple secondary structural elements and Ash1 protein translation. Curr. Biol. *9*, 337–340.

Güldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J.H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. *24*, 2519–2524.

Gunkel, N., Yano, T., Markussen, F.H., Olsen, L.C., and Ephrussi, A. (1998) Localization-dependent translation requires a functional interaction between the 5′ and 3′ ends of oskar mRNA. Genes Dev. *12*, 1652–1664.

Irie, K., Tadauchi, T., Takizawa, P.A., Vale, R.D., Matsumoto, K., and Herskowitz, I. (2002). The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of *ASH1* mRNA in yeast. EMBO J. *21*, 1158–1167.

Jansen, R.-P., Dowzer, C., Michaelis, C., Galova, M., and Nasmyth, K. (1996). Mother cell-specific *HO* expression in budding yeast depends on the unconventional myosin Myo4p and other cytoplasmic proteins. Cell *84*, 687–697.

Kim-Ha, J., Webster, P.J., Smith, J.L., and Macdonald, P.M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. Development *119*, 169–178.

Kislauskis, E.H., and Singer, R.H. (1992). Determinants of mRNA localization. Curr. Opin. Cell Biol. *4*, 975–978.

Kislauskis, E.H., Zhu, X., and Singer, R.H. (1994). Sequences responsible for intracellular localization of beta-actin messenger RNA also affect cell phenotype. J. Cell Biol. *127*, 441–451.

Kloc, M., Spohr, G., and Etkin, L.D. (1993). Translocation of repetitive RNA sequences with the germ plasm in Xenopus oocytes. Science *262*, 1712–1714.

Kloc, M., Bilinski, S., Pui-Yee Chan, A., and Etkin, L.D. (2000). The targeting of Xcat2 mRNA to the germinal granules depends on a cis-acting germinal granule localization element within the 3′UTR. Dev. Biol. *217*, 221–229.

Lantz, V., and Schedl, P. (1994). Multiple cis-acting targeting sequences are required for orb mRNA localization during Drosophila oogenesis. Mol. Cell. Biol. *14*, 2235–2242.

Long, R.M., Singer, R.H., Meng, X., Gonzalez, I., Nasmyth, K., and Jansen, R.-P. (1997). Mating type switching in yeast controlled by asymmetric localization of *ASH1* mRNA. Science *277*, 383–387.

Long, R.M., Gu, W., Lorimer, E., Singer, R.H., and Chartrand, P. (2000). She2p is a novel RNA-binding protein that recruits the Myo4p-She3p complex to *ASH1* mRNA. EMBO J. *19*, 6592–6601.

Long, R.M., Gu, W., Meng, X., Gonsalvez, G., Singer, R.H., and Chartrand, P. (2001). An exclusively nuclear RNA-binding protein affects asymmetric localization of *ASH1* mRNA and Ash1p in yeast. J. Cell. Biol. *153*, 307–318.

Lu, B., Ackerman, L., Jan, L.Y., and Jan, Y.N. (1999). Modes of protein movement that lead to the asymmetric localization of partner of numb during *Drosophila* neuroblast division. Mol. Cell *4*, 883–891.

Macdonald, P.M., and Kerr, K. (1997) Redundant RNA recognition events in bicoid mRNA localization. RNA. *3*, 1413–1420.

Mori, Y., Imaizumi, K., Katayama, T., Yoneda, T., and Tohyama, M. (2000). Two cis-acting elements in the 3′untranslated region of alpha-CamKII regulate its dendritic targeting. Nat. Neurosci. *3*, 1062–1064.

Reck-Peterson, S.L., Tyska, M.J., Novick, P.J., and Mooseker, M.S. (2001). The yeast class V myosins, Myo2p and Myo4p, are nonprocessive actin-based motors. J. Cell Biol. *153*, 1121–1126.

Rose, M.D., Winston, F., and Hieter, P. (1990). Methods in Yeast Genetics. A Laboratory Course Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sagliocco, F.A., Vega Laso, M.R., Zhu, D., Tuite, M.F., McCarthy, J.E.G., and Brown, A.J.P. (1993). The influence of 5′-secondary structures upon ribosome binding to mRNA during translation in yeast. J. Biol. Chem. *268*, 26522–26530.

Saunders, C., and Cohen, R.S. (1999). The role of oocyte transcription, the 5′UTR, and translation repression and derepression in Drosophila *gurken* mRNA and protein localization. Mol. Cell *3*, 43–54.

Schnorrer, F., Bohmann, K., and Nusslein-Volhard, C. (2000). The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of Drosophila oocytes. Nat. Cell Biol. *2*, 185–190.

Sil, A., and Herskowitz, I. (1996). Identification of asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the yeast HO gene. Cell *84*, 711–722.

Smibert, C.A., Wilson, J.E., Kerr, K., and Macdonald, P.M. (1996).

Smaug protein repress translation of unlocalized *nanos* mRNA in the Drosophila embryo. Genes Dev. *10*, 2600–2609.

Takizawa, P.A., and Vale, R.D. (2000). The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. Proc. Natl. Acad. Sci. USA *97*, 5273–5278.

Takizawa, P.A., Sil, A., Swedlow, J.R., Herskowitz, I., and Vale, R.D. (1997). Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. Nature *389*, 90–93.

Takizawa, P.A., DeRisi, J.L., Wilhelm, J.E., and Vale, R.D. (2000). Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. Science *290*, 341–344.

Thio, G.L., Ray, R.P., Barcelo, G., and Schupbach, T. (2000). Localization of gurken RNA in Drosophila oogenesis requires elements in the 5′ and 3′ regions of the transcript. Dev. Biol. *221*, 435–446.

Vega Laso, M.R., Zhu, D., Sagliocco, F., Brown, A.J.P., Tuite, M.F., and McCarthy, J.E.G. (1993). Inhibition of translational initiation in the yeast *Saccharomyces cerevisiae* as a function of the stability and position of hairpin structures in the mRNA leader. J. Biol. Chem. *268*, 6453–6462.

Zucker, M. (1998). On finding all suboptimal foldings of an RNA molecule. Science *244*, 48–52.