Structural elements required for the localization of *ASH1* mRNA and of a green fluorescent protein reporter particle *in vivo*

P. Chartrand, X-H. Meng, R.H. Singer and R.M. Long*

The sorting of the Ash1 protein to the daughter nucleus of *Saccharomyces cerevisiae* in late anaphase of the budding cycle correlates with the localization of *ASH1* mRNA at the bud tip [1,2]. Although the 3′ untranslated region (3′ UTR) of *ASH1* is sufficient to localize a reporter mRNA, it is not necessary, a result which indicates that other sequences are involved [1]. We report the identification of three additional *cis*-acting elements in the coding region. Each element alone, when fused to a *lacZ* reporter gene, was sufficient for the localization of the *lacZ* mRNA reporter to the bud. A fine-structure analysis of the 3′ UTR element showed that its function in mRNA localization did not depend on a specific sequence but on the secondary and tertiary structure of a minimal 118 nucleotide stem–loop. Mutations in the stem–loop that affect the localization of the *lacZ* mRNA reporter also affected the formation of the localization particles, in living cells, composed of a green fluorescent protein (GFP) complexed with *lacZ–ASH1*-3′ UTR mRNA [3]. A specific stem–loop in the 3′ UTR of the *ASH1* mRNA is therefore required for both localization and particle formation, suggesting that complex formation is part of the localization mechanism. An analysis on one of the coding-region elements revealed a comparable stem–loop structure with similar functional requirements.

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Results and discussion

Multiple localization elements in the *ASH1* mRNA sequence

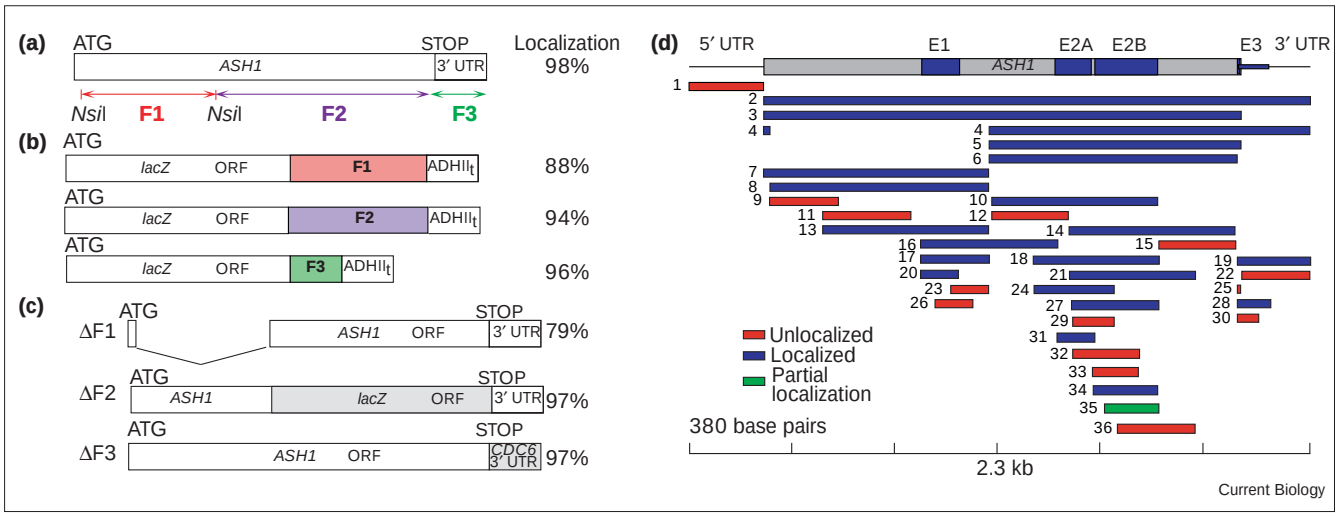
In order to identify all the *cis*-acting determinants for localization in the *ASH1* mRNA sequence, we segregated this mRNA into three fragments: two in the coding sequence (F1 and F2) and one in the 3′ UTR (F3; Figure 1a). Each fragment was fused in-frame to a *lacZ* reporter gene and the cytoplasmic distribution of these reporter mRNAs was analyzed by fluorescent *in situ* hybridization (FISH) [4]. We found that each fusion mRNA was able to localize asymmetrically to the bud just as well as the full-length *ASH1* mRNA (Figures 1b and, below, 3e). Moreover, the deletion of each of these single fragments in the *ASH1* mRNA sequence did not result in a decrease in localization (Figure 1c). These results indicated that at least three distinct *cis*-acting elements were present in the *ASH1* mRNA and although each element was sufficient for localization, none of them was essential.

In order to determine the minimal size of the localization elements present in the *ASH1* coding sequence, a systematic analysis was performed on fragments of different sizes derived from the original F1 and F2 fragments (Figure 1d). Each fragment was generated by polymerase chain reaction (PCR), then was fused to the *lacZ* reporter gene and the localization of the fusion mRNA analyzed by FISH [4]. The analysis revealed the presence of three minimal localization elements: element 1 (E1), which comprised the sequence between nucleotides 598 and 750 of the coding sequence (fragment 20); E2A, which comprised the sequence between nucleotides 1044 and 1196 of the coding sequence (fragment 31); and E2B, which comprised the sequence between nucleotides 1175 and 1447 (fragment 34).

Characterization of the localization element in the *ASH1* 3′ UTR

Next, we investigated the element in the 3′ UTR in more detail, to determine its essential features. The MFOLD program for RNA secondary structure [5] predicted that the minimal 118 nucleotide element, termed element E3 (fragment 28; Figure 1d), could fold into a long stem–loop structure formed by two stems separated by an asymmetric bulge (Figure 2b). This stem–loop structure included the last 15 nucleotides of the coding sequence and the stop codon, which formed stem I. In order to define further the sequence(s) and/or structural motif(s) in this stem–loop that could specifically bind *trans*-acting factor(s), we performed PCR mutagenesis on the element (Figure 2). Stem II was particularly affected by mutations. Mutations M9, M9A and M14 completely abolished localization (Figure 2a,c). Interestingly, even if the bulge is required for proper localization (mutant M13), most of its sequence was not essential (see mutant M8 and M12; Figure 2c). Mutations in stem 1, like M1A and M5A, did

Figure 1



Determination of the *cis*-acting determinants involved in the localization of the *ASH1* mRNA. (a,b) Separation of the *ASH1* mRNA into three fragments: F1 (in red, 810 nt), F2 (in purple, 920 nt) and F3 (in green, 267 nt). The second *Nsi*I restriction site serves as the boundary between F1 and F2. The percentage of budding yeast cells that localize asymmetrically to the bud either the wild-type *ASH1* mRNA or each of the three fragments fused to a *lacZ* reporter gene [1] was determined by FISH. (c) Disruption of each of the localization elements in the *ASH1* mRNA sequence. F1 was completely deleted from the *ASH1* sequence, whereas F2 and F3 were replaced respectively by a segment of the *lacZ* coding sequence and by the 3′ UTR of the non-localizing *CDC6* gene [1] (in gray). Each fusion is in-frame with the *ASH1* mRNA coding sequence. (d) Determination of the minimal size of each element (E) that maintains localization to the bud. The fragments tested are numbered from 1 to 36 and colored according to their phenotype (localized, 60–100% of budding cells with localization; partially localized, 40–60% of budding cells with localization; unlocalized, 0–40% of budding cells with localization). The sizes, positions in the *ASH1* mRNA sequence and percentage of localization of each fragment are listed in the Supplementary material published with this paper on the internet.

not have the same impact; only the M1 mutation reduced the level of localization (Figure 2a).

To test structural predictions on the 118 nucleotide element, compensatory mutants were prepared: M1+M1A; M5+M5A; M9+M9A (Figure 2b). If the stem–loop hypothesis were correct, these double mutants should restore the stem–loop structure and localization. Figure 2b shows that in the compensatory double mutant M9+M9A, localization was effectively restored, strongly supporting the putative structure of stem II in this element. Mutations and compensatory mutations in stem I are less convincing, however, because of the difference in the phenotype of M1 versus M1A and M5 versus M5A; such analysis therefore does not strongly support the existence of this stem. Importantly, the double mutants created stems with different sequences than those of the wild-type, indicating that the sequences of these stems were not important for the function of the element. These results support the hypothesis that three-dimensional features at the stem–bulge junction could act as recognition elements for the binding of specific proteins.

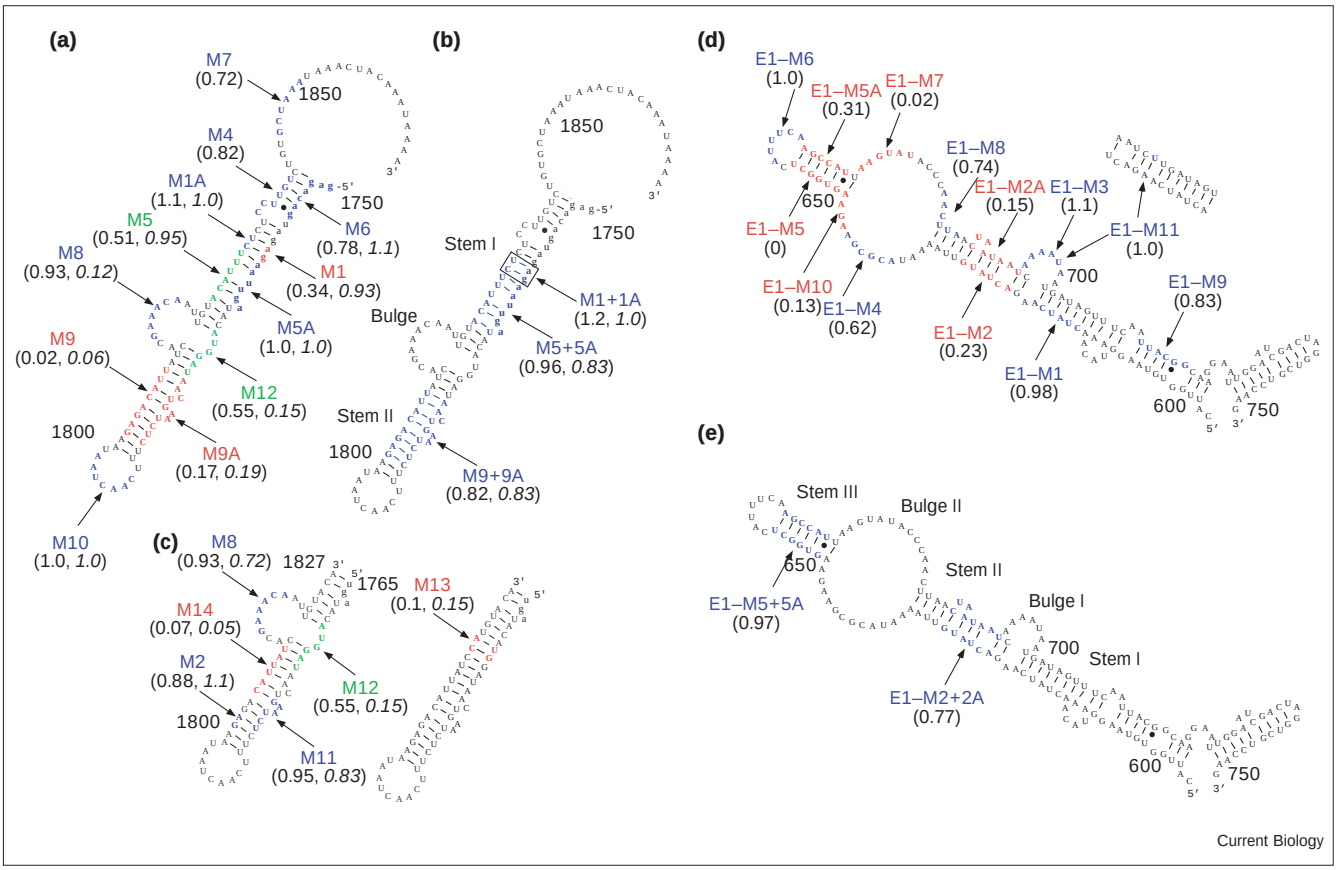
Mechanistic similarities between RNA localization and particle formation

Recently, a technique based on the use of GFP to tag RNA *in vivo*, was developed for the study of mRNA transport and localization in living yeast [3]. Yeast cells are cotransformed with two plasmids which express firstly a GFP fused to the phage MS2 RNA-binding protein, and secondly a *lacZ* reporter mRNA containing six repeated MS2-binding motifs and the *ASH1* 3′ UTR. A bright fluorescent particle, composed of a GFP–*lacZ*–MS2 reporter mRNA complex, localizes to the bud [3].

Particle formation seems to be a peculiarity of the *ASH1* mRNA localization determinants; the presence of non-*ASH1* mRNA sequences did not produce such particles [3]. These results suggested that particle formation and RNA localization could be a manifestation of the same mechanism.

Repeating the analysis done for the sequence and/or motif in the 118 nucleotide element in the *ASH1* 3′ UTR that causes RNA localization, we next determined which sequences affected the formation or the localization of the particle. The MS2-binding sites were introduced into the mutants of the 118 nucleotide element previously assessed for localization of the *lacZ* reporter mRNA. Each mutant was coexpressed in yeast cells with the GFP–MS2 fusion protein, and particle formation and localization to the bud were scored. Figure 2a–c shows the effect of specific mutations in the 118 nucleotide element of the

Figure 2



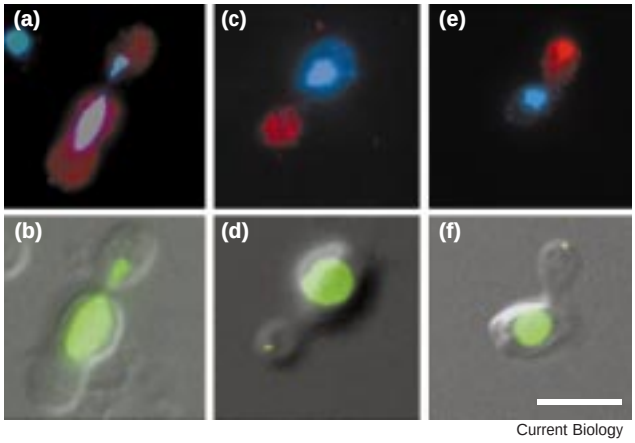
Effect of mutants in the 118 nt localization determinant in element 3 (a–c) and in the 150 nt localization determinant in element 1 (d,e) on the localization of a reporter mRNA. Regions mutated are colored and referred as M1–M14 for the E3 and E1–M1 to E1–M11 for E1. All the sequences of the mutations are listed in the Supplementary material. The color code is as in Figure 1d and corresponds to the data from *lacZ* mRNA localization by FISH. Numbers in parentheses correspond to the percentage of budding cells with bud-localized *lacZ* mRNA (numbers in plain) or bud-localized GFP particle (numbers in italics). Numbers have been normalized, using the wild-type as 1.0. In (a–c), nucleotides in lowercase are part of the coding sequence, whereas nucleotides in uppercase are part of the 3′ UTR. (a,d) Complete mutagenesis of (a) the stem–loop structure of E3 and (d) E1. (b,e) Double mutants that restore the stem–loop structure of (b) E3 and (e) E1. The mutant E1–M11 results in the deletion of the 7 nt bulge, which is replaced by a single base-pair. (c) Mutagenesis of stem II of E3. The mutant M13 results in the deletion of the 10 nt bulge structure, which is replaced by two base-pairs.

ASH1 3′ UTR on particle formation. As with RNA localization, mutations in stem I did not affect particle formation (Figure 2a). Further, the same mutations in stem II (M9, M9A and M14) and the bulge (M13) that abolished RNA localization also disrupted particle formation (Figures 2a,2c,3a,3b). The mutation M12, which partially affects localization, had a stronger inhibitory effect on particle formation, however (Figure 2c), suggesting that particle formation is more sensitive to mutations in the stem II–bulge region. The construct containing both mutations, M9+9A, restored RNA localization and particle formation (Figures 2b,3c,3d). These results suggest that the *trans*-acting factors that recognize this stem–loop are involved in both the localization of the mRNA and the formation of a major RNA–protein complex, observed *in vivo* as the GFP particle.

Structural requirements for the function of the localization element E1

A fragment of 150 nucleotides, derived from the coding sequence of the *ASH1* gene, was found to be sufficient to maintain the localization function of E1 (fragment 20; Figure 1d). Using the MFOLD program for RNA secondary structure prediction [5], this fragment can be folded in a stem–loop structure containing three stems (stems I to III; Figure 2e) that are separated by two asymmetric bulges (bulges I and II; Figure 2e). We followed a similar mutagenesis approach to that used in the study of E3, in order to investigate the structural features required for the function of E1. Mutations in both stems II and III affect the localization (Figure 2d). To test the structural prediction on E1, we generated compensatory mutants in stems II and III (mutants E1–M2+M2A and E1–M5+M5A, respectively;

Figure 3



An image illustrating that the disruption of the stem–loop structure in E3 by the M9 mutant also abolishes *lacZ* mRNA localization (a) and particle formation (b) in the same cell. The restoration of the stem–loop structure by the double-mutant M9+9A restores *lacZ* mRNA localization (c) and particle formation (d). Wild-type element E3 as control (e,f). FISH on the *lacZ* mRNA, in red; GFP–*lacZ* mRNA particle, in green; DAPI, in blue. Size bar: 5 μm.

Figure 2d). As predicted from the secondary structure, both mutants E1–M2+M2A and E1–M5+M5A restored the localization. Whereas bulge I does not seem to be important for localization (mutant E1–M11), specific mutations in the sequence of bulge II result in a reduced localization (E1–M7, E1–M10; Figure 2d). These results differ from those obtained on E3, for no specific sequences were found to be required for its function.

The identification of stem–loop structures involved in mRNA localization is not unique to *ASH1* mRNA. Other mRNAs in *Drosophila*, such as the *bicoid* mRNA [6,7], the *K10* mRNA [8], and β actin mRNA in fibroblasts [9] contain stem–loop structures in their 3′ UTRs that mediate their localization. As in *ASH1* mRNA, their integrity is essential for function. Interestingly, the *ASH1* 3′ UTR element and the *bicoid* 3′ UTR element share other common properties: both form ribonucleoprotein particles *in vivo* [3,6] and both require an intact stem–loop, and not a specific sequence, for their function [6].

Furthermore, the work presented here demonstrates that *ASH1* mRNA localization in yeast differs from most mRNA localization in other eukaryotic systems, such as chicken fibroblasts, *Drosophila* or *Xenopus*, which contain *cis*-acting sequences that form stem–loops responsible for localization only in their 3′ UTR [10–12]. The presence of elements with redundant localization function does not mean that they necessarily have redundant structures and bind the same *trans*-acting factors. Our analysis of E1 and E3 revealed some similarities and differences in their structural motifs that are important for localization. These results suggest that the two elements could interact with different RNA-binding proteins, which could require the She1–5 proteins for their function. Moreover, an analysis of E2A and E2B shows no sequence similarity with E1 or E3 (data not shown), but they also contain stem–loop structures, which could possibly act as localization determinants, binding yet additional proteins. Interestingly, all *cis*-acting elements have some or all key components in the coding region, which suggests that there may be some relationship between RNA translation and localization, and perhaps even RNA stability. Future work in this fertile system will probably clarify this intricate regulation.

Supplementary material

Additional methodological details and details about the different mutants are published with this paper on the internet.

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Supplementary material

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Table S1

Efficiency of localization of fragments derived from the *ASH1* gene sequence.

Fragment	Length (nucleotides)	First and last nucleotides*	Localization†
1	250	–250 to +1	–
2	2016	+1 to 2016	+
3	1767	+1 to 1767	+
4	958	+1 to 36, 846 to 1767	+
5	922	846 to 1767	83%
6	896	846 to 1741	98%
7	846	+1 to 846	75%
8	810	37 to 846	82%
9	248	37 to 284	2%
10	602	846 to 1447	98%
11	302	237 to 538	6%
12	251	846 to 1096	–
13	610	237 to 846	73%
14	595	1147 to 1741	71%
15	499	598 to 1096	74%
16	295	1447 to 1741	–
17	249	598 to 846	89%
18	455	993 to 1447	90%
19	268	1750 to 2017	96%
20	153	598 to 750	90%
21	452	1147 to 1598	80%
22	253	1765 to 2017	–
23	153	694 to 846	4%
24	307	993 to 1299	94%
25	15	1750 to 1765	–
26	149	647 to 795	12%
27	301	1147 to 1447	77%
28	118	1750 to 1867	85%
29	153	1147 to 1299	2%
30	80	1750 to 1829	–
31	153	1044 to 1196	82%
32	232	1147 to 1378	4%
33	204	1175 to 1378	2%
34	273	1175 to 1447	70%
35	226	1222 to 1447	42%
36	299	1300 to 1598	7%

*These numbers correspond to the first and the last nucleotides of the *ASH1* gene present in each fragment. The nucleotides are numbered starting from the adenosine of the start codon as +1. †Indicates the percentage of budding yeast cells with bud-localized *lacZ* mRNA. The + and – signs indicate that the majority of cells have, respectively, localized or delocalized *lacZ* mRNA.

Table S2

All the mutations generated in the 118 nucleotide fragment of the *ASH1* 3′ UTR.

Mutant	Mutation*
M1	$A_{1760}G_{1761}\rightarrow TC$
M1A	$C_{1831}T_{1832}\rightarrow AG$
M1+M1A	$A_{1760}G_{1761}\rightarrow TC$; $C_{1831}T_{1832}\rightarrow AG$
M2	$G_{1851}A_{1852}\rightarrow CT$
M3	$A_{1760}GAATT_{1765}\rightarrow GAATTC$
M4	$C_{1835}CTTGT_{1840}\rightarrow GAATTC$
M5	$A_{1825}CATTT_{1830}\rightarrow GAATTC$
M5A	$A_{1762}ATTGAT_{1768}\rightarrow GATTTAC$
M5+M5A	$A_{1825}CATTT_{1830}\rightarrow GAATTC$; $A_{1762}ATTGAT_{1768}\rightarrow GATTTAC$
M6	$G_{1750}AGACAG_{1756}\rightarrow GAATTC$
M7	$G_{1845}CTAAA_{1850}\rightarrow GAATTC$
M8	$G_{1815}AAACA_{1820}\rightarrow ATCGAT$
M9	$G_{1801}AGACATT_{1808}\rightarrow TGAATTCG$
M9A	$A_{1777}ACTGAATCTC_{1787}\rightarrow CGCAACATTCA$
M9+M9A	$G_{1801}AGACATT_{1808}\rightarrow TGAATTCG$; $A_{1777}ACTGAATCTC_{1787}\rightarrow CGCAACATTCA$
M10	$C_{1791}AACTAA_{1797}\rightarrow AGAATTC$
M11	$G_{1781}AATCT_{1786}\rightarrow ATCGAT$
M12	$A_{1771}TGGAT_{1776}\rightarrow GAATTC$
M13	$A_{1812}CGAAACAAT_{1821}\rightarrow CA$
M14	$C_{1805}ATTAT_{1810}\rightarrow GAATTC$

*The nucleotides are numbered starting from the adenosine of the start codon as +1.

Table S3

All the mutations generated in the 150 nucleotide fragment of E1.

Mutant	Mutation*
E1–M1	$C_{618}TATCA_{623}\rightarrow ATCGAT$
E1–M2	$A_{626}CTATG_{631}\rightarrow GAATTC$
E1–M2A	$C_{686}TAATAAT_{693}\rightarrow GAATTC$
E1–M2+M2A	$A_{626}CTATG_{631}\rightarrow GAATTC$; $C_{686}TAATAAT_{693}\rightarrow GAATTC$
E1–M3	$A_{695}AAATA_{700}\rightarrow ATCGAT$
E1–M4	$A_{639}CGCGA_{644}\rightarrow GAATTC$
E1–M5	$G_{648}TGGCT_{654}\rightarrow GAATTC$
E1–M5A	$A_{662}GCCAT_{667}\rightarrow GAATTC$
E1–M5+M5A	$G_{648}TGGCT_{654}\rightarrow GAATTC$; $A_{662}GCCAT_{667}\rightarrow GAATTC$
E1–M6	$A_{656}TTTCA_{661}\rightarrow GAATTC$
E1–M7	$A_{669}AGTAT_{674}\rightarrow GAATTC$
E1–M8	$A_{679}ACTTA_{684}\rightarrow TTGAAT$
E1–M9	$T_{714}TACGG_{719}\rightarrow GAATTC$
E1–M10	$A_{645}GAA_{648}\rightarrow GCGC$
E1–M11	$A_{695}AAATAA_{701}\rightarrow T$

*The nucleotides are numbered starting from the adenosine of the start codon as +1.

Supplementary materials and methods

Yeast genotype

All experiments were done with W303, Mata, *ura3–1, leu2–3, his3–11, trp1–1, ade2–1, can1–100*.

Plasmid constructions

The three fragments from the *ASH1* gene were generated by PCR with primers containing *Sma*I sites. The fragment F1 is 810 nucleotides long and comprises the region between nucleotides 36 and 846 (nucleotides are numbered starting from the adenosine of the start codon as +1). F2 is 920 nucleotides long and comprises the region between nucleotides 847 and 1766. F3 is 268 nucleotides long and comprises the region between nucleotides 1750 and 2017. Each PCR product was cloned in-frame with the *lacZ* gene at the *Sma*I site of the plasmid pXR2 [S1]. Fragments 1–36 were also generated and cloned the same way. Table S1 in this section gives a list of all the fragments and the first and last *ASH1* gene nucleotides present in each fragment. All constructions were confirmed by sequencing.

The construct ΔF1 was made by cutting the plasmid PCY 235 with the enzyme *Nsi*I to remove the 810 nucleotide F1 fragment and religating it. PCY 235 was created by cloning the *ASH1* gene under the control of the *GAL1* promoter (from plasmid C3348 [S2], a gift of K. Nasmyth), into the *Sal*I–*Spe*I sites of the YCPlac111 plasmid. To prepare the construct ΔF2, a 812 nucleotide fragment was amplified by PCR from the *lacZ* coding sequence. This PCR fragment, which contains nucleotides 385–1197 of the *lacZ* open reading frame, was digested with *Kpn*I and *Nco*I and subcloned between the *Kpn*I and *Nco*I sites of the *ASH1* gene in the PCY 235 plasmid, to give ΔF2. To prepare the construct ΔF3, the plasmid YEPlac181–*ASH1*–Myc–*CDC6* (a gift from K. Nasmyth), which contains the *CDC6* 3′ UTR at the 3′ end of the *ASH1* coding sequence, was cut with *Nco*I and *Nsi*I to give a 2.4 kb fragment. This fragment, which contains the 3′ end of the coding sequence of *ASH1*, nine Myc tags and the *CDC6* 3′ UTR, was cloned between the *Nco*I and *Pst*I sites of the PCY 212 plasmid, to give PCY 212–Myc–*CDC6* (PCY 212 is a derivative of PCY 235). To remove the Myc tags from PCY 212–Myc–CDC6, this plasmid was cut with *Bam*HI, which removes the 1.7 kb fragment of the *ASH1* coding sequence and the Myc tags. The1.7 kb *ASH1* fragment was then religated to the *Bam*HI cut plasmid to give ΔF3.

All the mutants were generated by PCR using the splicing through overlap extension strategy [S3]. Basically, four primers were used per mutant, which gave rise to two PCR fragments that had an overlapping sequence containing the mutated sequence. These PCRs were performed using the *lacZ*–118 nucleotides *ASH1* 3′ UTR–*ADHII* gene on plasmid pXR63 (a derivative of pXR55 [S1]) as a template. These fragments served as templates for a second PCR in order to amplify the final fragment which contains the mutated 118 nucleotide element of the 3′ UTR and the *ADHII* terminator. This fragment was finally cloned in the *Sac*I site of the *lacZ* gene in the pXR2 plasmid [S1]. All the mutations inserted in the 118 nucleotide fragment of the 3′ UTR are listed in the Table 2 of this section. Mutations inserted in the 150 nucleotide fragment of the E1 are listed in the Table S3 of this section. Mutations were confirmed by restriction digest and sequencing. No stop codons were inserted by the mutagenesis process.

To prepare the plasmids containing the *lacZ* reporter gene with six MS2-binding sites, the plasmid p*GAL–lacZ*–MS2–*ASH1*/*URA* [S1] was digested with *Bgl*II, which produce a 600 bp fragment containing the six MS2 binding sites. This fragment was then subcloned in the *Bgl*II site, between the *lacZ* ORF and the *ASH1* fragments, in all the pXR2 based plasmids constructed above.

In situ *hybridization*

Yeast cells were processed for *in situ* hybridization according to the protocol of Long *et al.* [S4].

Imaging

Images were captured using the Esprit Image Analysis software on an Olympix PII workstation (Life Science Resources) with an Olympix TE cooled 12 bit CCD camera (Life Science Resources) mounted on an Olympus BX-60 fluorescence microscope (Olympus) with a PlanApo 60x, 1.4 NA objective (Olympus). Single plane images were captured and processed using the Adobe Photoshop 3.0 software (Adobe Systems).

Measurement of localization

To obtain quantitative data on the localization of each *lacZ* fusion mRNA constructed, yeast cells with visible bud (cells between G2 and M phase of the cell cycle) 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 is considered as localized when it is predominantly in the bud (either full bud or tight cortical localization). An mRNA is considered as delocalized when it is equally distributed between bud and mother cell. For each mutant, a single experiment was performed, in which 50–100 budding yeast cells were scored, with a variation of 10–15% in the measurements between two independent experiments.

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