Binding of DEAD-box helicase Dhh1 to the 5′-untranslated region of ASH1 mRNA represses localized translation of ASH1 in yeast cells

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Local translation of specific mRNAs is regulated by dynamic changes in their subcellular localization, and these changes are due to complex mechanisms controlling cytoplasmic mRNA transport. The budding yeast Saccharomyces cerevisiae is well suited to studying these mechanisms because many of its transcripts are transported from the mother cell to the budding daughter cell. Here, we investigated the translational control of ASH1 mRNA after transport and localization. We show that although ASH1 transcripts were translated after they reached the bud tip, some mRNAs were bound by the RNA-binding protein Puf6 and were non-polysomal. We also found that the DEAD-box helicase Dhh1 complexed with the untranslated ASH1 mRNA and Puf6. Loss of Dhh1 affected local translation of ASH1 mRNA and resulted in delocalization of ASH1 transcript in the bud. Forcibly shifting the non-polysomal ASH1 mRNA into polysomes was associated with Dhh1 dissociation. We further demonstrated that Dhh1 is not recruited to ASH1 mRNA co-transcriptionally, suggesting that it could bind to ASH1 mRNA within the cytoplasm. Of note, Dhh1 bound to the 5′-UTR of ASH1 mRNA and inhibited its translation in vitro. These results suggest that after localization to the bud tip, a portion of the localized ASH1 mRNA becomes translationally inactive because of binding of Dhh1 and Puf6 to the 5′- and 3′-UTRs of ASH1 mRNA.

Local translation of particular mRNAs involves dynamic changes in subcellular localization. To this end, cells employ various and complex mechanisms to control specific cytoplasmic localization of mRNAs and their site of translation (1). The budding yeast Saccharomyces cerevisiae has emerged as a good system to study these mechanisms because a substantial number of transcripts have been identified as transporting from the mother cell to the budding daughter cell for such a purpose (2). One of these transcripts, ASH1, is transcribed in the mother cell and then transported to the bud tip, where the protein is translated and translocated into the daughter cell nuclei during late anaphase (3). The asymmetric localization of Ash1 within the daughter cell nuclei represses the transcription of HO endonuclease, which is crucial for the mating-type switch (3–5). The localization of ASH1 mRNA relies on She2, an RNA-binding protein that is co-transcriptionally bound to the cis-acting elements (or “zipcodes”) of the mRNA (6, 7). After transcription, the ASH1 mRNA complex is exported to the cytoplasm, where it interacts with She3, an adaptor protein for the mRNA localization machinery (8). She3 connects the ASH1 mRNA complex to the motor protein She1 (Myo4), which utilizes the actin cytoskeleton to transport the complex to the bud tip (9).

In addition to She2, other RNA-binding proteins, such as Puf6 and Khd1, have been identified as essential for complete localization of ASH1 mRNA complex and subsequent local translation of Ash1. Khd1 was identified in a systematic survey of potential candidate RNA-binding proteins for ASH1 mRNA localization (10). Binding of Khd1 decreased translational initiation of ASH1 mRNA and reduced leakage of Ash1 into the mother cell nucleus (11). Puf6 is a member of the Pumilio/FBF (fem-3 mRNA-binding factor) family of RNA-binding proteins that was purified from She2-associated mRNPs (12). Puf6 directly binds to the 3′-UTR of ASH1 mRNA and represses its translation during the transport process. At the bud tip, phosphorylation of Puf6 releases translational repression of ASH1 mRNA and leads to Ash1 synthesis (13). These studies indicate that translation of ASH1 mRNA is precisely regulated by RNA-binding proteins, and Puf6 and Khd1 could function in the linkage between ASH1 mRNA localization and its translation. Although translational repression of ASH1 mRNA during transport has been well studied, the regulatory mechanism for ASH1 mRNA translation after its proper localization remains to be studied.

In this study, we show that although translation of ASH1 mRNA occurs after localization, a portion of the transcripts remain non-polysomal and therefore could still bind to Puf6. We identified Dhh1, which interacts with untranslated ASH1 mRNA and co-precipitates with Puf6. Deletion of the DHH1 gene not only results in diffusion of ASH1 mRNA in the bud but also...

2 The abbreviations used are: mRNP, messenger ribonucleoprotein; MBP, maltose-binding protein; MCP, MS2-binding coat protein; qPCR, quantitative PCR; CHX, cycloheximide; IP, immunoprecipitation.

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affects translation of the mRNA. Forcibly shifting non-polysomal ASH1 mRNA into polysomes causes dissociation of Dhh1 with the transcript. Dhh1 is not recruited to ASH1 mRNA co-transcriptionally but is possibly associated with the messenger RNA within the cytoplasm. Interestingly, Dhh1 did not directly interact with Puf6 but bound to the 5′-UTR of ASH1 mRNA, and this binding repressed translation of the mRNA in vitro. These results suggest a novel function of Dhh1 in regulating localized ASH1 mRNA translation after its bud-tip localization.

Results

Translation occurs after ASH1 mRNA localization to the bud tip

It has been reported previously that the average time to transcribe a yeast gene is about 25–50 s/kb (14–17), and the transport time for an ASH1 mRNA reporter (from mother to the bud tip) is about 128 s (18). Based on these results, the process from transcription to localization of ASH1 mRNA would be around 4–5 min. Due to difficulties in investigating the local translation of endogenous ASH1 mRNA, we transformed two centromeric plasmids, one expressing HA-ASH1-Myc-MS26 mRNA (a 2.5-kb transcript) under Gal promoter control and another expressing a GFP-MCP (MS2-binding coat protein) fusion protein into an ASH1 gene deletion strain. The fusion protein contains a nuclear localization signal, such that the protein unbound to ASH1 mRNA is sequestered within the nuclei (Fig. 1A). This system allowed us to study the coordinated localization and translation of ASH1 mRNA in the anaphase cells because translational initiation of the protein could be imaged by immunostaining with HA antibodies, and fully translated Ash1 would be translocated into the bud nucleus and be detected by Myc antibodies. Fig. 1B shows representative

![Figure 1. Translation of ASH1 mRNA occurs after bud-tip localization. A, schematic presentation of the centromeric construct used to visualize ASH1 mRNA localization and translation in ash1 cells. The ASH1 gene was placed under the control of a galactose-inducible promoter (Gal1). HA and Myc tags were separately fused onto the N and C termini of Ash1. Six repeats of MS2 stem loops were fused to the 3′-UTR of ASH1 mRNA, which were recognized by GFP-MCP (MS2 RNA coat protein). B, yeast cells were cultured in 2% raffinose medium at 30 °C and induced with galactose (2%). After 0, 5, and 10 min of induction, cells were harvested and subjected to immunostaining assays. Localization of ASH1 mRNA was visualized by GFP-MCP, and translation of Ash1 was detected by Cy3-labeled HA (red) or Cy5-labeled Myc (cyan) antibodies. The arrows indicate the bud-tip-localized ASH1 mRNA. Bar, 3 μm. C, percentage of anaphase cells with localized ASH1 mRNA and translated HA-Ash1 signals after galactose induction from three independent experiments. An average of 50–60 cells were counted each time. D, RT-PCR was used to detect the expression of ASH1 mRNA after 0, 5, and 10 min of galactose induction. GAPDH mRNA was used as a positive control. Right, mean values ± S.D. (error bars) were from three independent experiments. **, p < 0.01. E, full-length Ash1 after 0, 5, and 10 min of galactose induction was detected using anti-Myc antibodies by Western blotting, in which Pgd1 was used as a loading control. Right, mean values ± S.D. (error bars) were from three independent experiments. **, *p < 0.01.]

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A portion of ASH1 mRNAs could be translationally repressed after localization

To examine whether all ASH1 mRNA was translated after localization, we performed sucrose-gradient fractionation of cells expressing an endogenous HA-tagged ASH1-MS26 transcript and a His-tagged Puf6 (Fig. 2A). An A$_{254}$ plot corresponding to the polysomal fractions is shown in Fig. 2B. Total RNAs were isolated from the sucrose fractions and were used to analyze the distribution of ASH1 mRNA by Northern blotting (Fig. 2C). In comparison with GAPDH mRNA that was mostly polysomal, a substantial amount of ASH1 mRNA was detected in non-polysomal fractions (fractions 2 and 3) as well as in the polysomal fractions (fractions 6 – 8). Disrupting the polysomes with EDTA shifted ASH1 mRNA into non-polysomal fractions (supplemental Fig. S1). Because binding of Puf6 repressed ASH1 mRNA translation during transport (12) and release of the protein by phosphorylation resulted in translational activation of the mRNA (13), we analyzed the distribution of Puf6 in
the sucrose fractions. Western blotting demonstrated that Pu6 was present in the non-polysomal fractions (Fig. 2D, fractions 2 and 3 of the top panel). We then used recombinant chimeric MBP-MCP (supplemental Fig. S2A; MBP binds to amylose resin) to precipitate ASH1-MS2\(_6\) mRNA in selected sucrose fractions (Fig. 2E, top). Pu6 co-precipitated with ASH1 mRNA in the non-polysomal fraction 3 but not in the polysomal fraction 7. She2, an essential protein of the ASH1 mRNA localization machinery, was also detected in the precipitates of the non-polysomal fraction. An mRNA-binding and -decapping protein, Dcp1 and an abundant Puf6 (phosphoglycerate kinase 1) were not present in the precipitates of either fraction 3 or fraction 7 (Fig. 2E, bottom). These data suggest that the non-polysomal fractions contained localized and translationally repressed ASH1 mRNA.

**Dhh1 binds to bud-tip-localized and translationally inactive ASH1 mRNA**

In yeast, Dhh1 is an abundant DEAD-box helicase, implicated in translational repression and enhancement of mRNA decay (19–21). The role of Dhh1 in storing and maintaining untranslated mRNAs has been well reported (22). To test the possibility that Dhh1 or other P-body proteins could be involved in the regulation of ASH1 mRNA translation, we prepared extracts from WT and puf6 strains expressing an endogenous HA-tagged ASH1-MS2\(_6\) transcript. We then used the recombinant MBP-MCP to pull down the ASH1-MS2\(_6\) mRNA. RT-PCR analysis indicated that ASH1 mRNA was successfully precipitated in both wild-type and puf6 cells (Fig. 3A). The specificity of the experiment was tested by a control strain in which the MS2 stem loops were not fused to ASH1 mRNA (Fig. 3A, NC). Analyzing the proteins co-precipitated with the ASH1 mRNA, we found that in addition to Pu6, Dhh1 also existed in the precipitates of both WT and puf6 cells (Fig. 3B), suggesting that the in vivo binding of the Dhh1 with ASH1 mRNA could be Pu6-independent. Another P-bodies component, a decapping enzyme, Dcp1, was not detected in the precipitates. The interaction of Dhh1 with ASH1 mRNA was confirmed by a reciprocal approach, in which untagged endogenous ASH1 mRNA was detected in the precipitates of Dhh1 antibodies (Fig. 3C). We next precipitated ASH1 mRNA from sucrose fractions 3 and 7 (Fig. 2A) and found that Dhh1 was associated with ASH1 mRNA specifically in the non-polysomal fraction (Fig. 3D). Dcp1 was not detected in the precipitates (not shown). Interestingly, further experiments revealed that although Dhh1 and She2 were both identified to associate with ASH1 mRNA, precipitation of Dhh1-bound ASH1 mRNA in the non-polysomal fraction detected little She2 (Fig. 3E). This indicates that Dhh1 could bind to localized and translationally inactive ASH1 mRNA independent of She2. Using fluorescent in situ hybridization (FISH) assays for ASH1 mRNA in a yeast strain expressing Dhh1-GFP or Dcp1-GFP fusions, we found that ASH1 mRNA was co-localized with Dhh1 in >30% of the budding cells (Fig. 3, F and G).

**The roles of Dhh1 in bud-tip localization, translation, and stability of ASH1 mRNA**

We have shown previously that binding of Pu6 to the PUF consensus sequences in the 3' -UTR of ASH1 mRNA regulated localization and translation of the mRNA (12). Here, we analyzed how the Dhh1-ASH1 mRNA interactions affect the localization and translation of ASH1 mRNA. Whereas >70% of the wild-type budding cells showed localized ASH1 mRNA at the bud tip (red), a diminution of bud-tip localization of ASH1 mRNA (~40%) was observed in dhh1 cells, where about 42% of ASH1 mRNA was diffuse in the bud (Fig. 4A, bottom left). Compared with the phenotype observed in she2 or puf6 cells, where the asymmetric distribution of ASH1 mRNA was completely disrupted (12, 23), deletion of Dhh1 only resulted in delocalizing ASH1 mRNA in the bud.

To examine the potential effect of Dhh1 in ASH1 mRNA translation and stability, we examined the intracellular levels of Ash1 and ASH1 mRNA in the wild-type, dhh1, and dhh1-puf6 strains. The levels of Ash1 were clearly increased in the dhh1 (~1.38-fold) and dhh1-puf6 cells (~1.5-fold), in contrast to the wild-type strain (Fig. 4B). The ASH1 mRNA levels were also relatively higher in the dhh1 (~1.2-fold) and dhh1-puf6 strains (~1.3-fold) when normalized to GAPDH mRNA (Fig. 4C). To analyze whether the increased levels of ASH1 mRNA resulted from DHH1 deletion, we transformed a plasmid expressing ASH1 mRNA under the Gal promoter control into the ash1 and ash1-dhh1 strains. Cells were grown in medium with 2% raffinose to ~0.6 at A\(_{600}\) and were induced with 2% galactose for 10 min. The Gal promoter was repressed by the addition of glucose, and the ASH1 mRNA levels were quantitated using RT-qPCR. The steady-state levels of ASH1 mRNA were not obviously different between the two cell strains. In the dhh1 cells, levels of ASH1 mRNA were decreased by 35% at 5 min and by 62% at 10 min compared with reduction of 46% at 5 min and 70% at 10 min in the control cells (Fig. 4D). To further determine the role of Dhh1 in ASH1 mRNA translation, we transformed a centromeric plasmid expressing Dhh1 protein into dhh1 cells. We found that when Dhh1 was overexpressed, expression of Ash1 was considerably decreased, whereas the levels of ASH1 mRNA were still mildly reduced (Fig. 4, E–G). These results indicate that Dhh1 affects both the levels of ASH1 mRNA and protein. It seems that the impact of Dhh1 on protein levels can at least partially be explained by effects that are distinct from the effects on mRNA levels. One possibility, consistent with previous studies on Dhh1 as a translation repressors (19), is that it can act to repress translation of the ASH1 mRNA.

**Translational initiation of ASH1 mRNA is accompanied with the dissociation of Dhh1 from the transcript**

We hypothesized that binding of Dhh1 to the non-polysomal ASH1 mRNA could lead to regulation of its localized translation. To address this, we treated yeast cells with low levels of cycloheximide (CHX), which inhibited translational elongation but not initiation (24), to force the shift of ribosome equilibrium toward the polysomes (25). Using the HA-ASH1-Myc-MS2\(_6\) reporter, we showed that in CHX-untreated growing cells, matured Ash1 (red) was synthesized and translocated into the bud nucleus, and ASH1 mRNA was tightly localized at the bud tip (top panels of Fig. 5, A and C, respectively). However, in CHX-treated cells, strong HA signal was mostly seen in the bud cytoplasm, whereas very little matured Ash1 was seen in the nucleus (Fig. 5A, bottom panels). Repression of Ash1 elongation was confirmed by Western blotting using Myc antibodies,
Figure 3. Dhh1 binds to localized and translationally inactive ASH1 mRNA. A, ASH1 mRNA was precipitated from extracts of WT and pu6 cells by MBP-MCP. The precipitates were analyzed by RT-PCR. Arrow, amplified ASH1 cDNA. NC, negative control in which the MS2 motif was not fused into ASH1 mRNA. B, the potential interaction of ASH1 mRNA with Puf6, Dhh1, or Dcp1 was analyzed by immunoblotting with the precipitates from A. C, co-IP experiments were used to analyze the association of endogenous ASH1 mRNA with Dhh1 using Protein A beads conjugated with Dhh1 antibodies in wild-type cells. The precipitates were subjected to Western blotting and RT-PCR analyses. NC, negative control using Protein A beads unconjugated with the antibodies. All Western blotting experiments were carried out at least two times. D, ASH1 mRNA was precipitated with MBP-MCP from total cell extracts (input) and the sucrose fractions 3 and 7 (Fig. 2B). Dhh1 was associated with ASH1 mRNA in the input and non-polysomal fraction. E, co-IP experiments were performed to analyze the binding status of ASH1 mRNA with Dhh1 and She2 in cell extracts (Input) and non-polysomal fraction (#3) using Protein A beads conjugated with Dhh1 antibodies. The precipitates were subjected to Western blotting and RT-PCR analyses. F, FISH was performed to detect the localization of ASH1 mRNA in yeast strains expressing Dhh1-GFP or Dcp1-GFP. The arrowhead in the representative images indicates colocalization of ASH1 mRNA with Dhh1-GFP. Bar, 3 μm. G, the percentage of ASH1 mRNA colocalizing with Dhh1 or Dcp1 was shown from three independent experiments. On average, 40–50 cells were counted in each experiment. Error bars, S.D.
Localization and translational regulation of ASH1 mRNA

A

ASH1 mRNA
Dapi
DIC
WT
dhh1

WT

B

Ash1
Pgk1

WT
puf6-dhh1
dhh1

65
40

WT
puf6-dhh1
dhh1

65
40

C

WT
puf6-dhh1
dhh1

ASH1 mRNA
GAPDH mRNA

D

Relative levels of ASH1 mRNA

WT
puf6-dhh1
dhh1

0 5 10 15 20

E

kD

WT
dhh1
dhh1(Dhh1 exp.)

Dhh1
Pgk1
Ash1

F

Relative Ash1 expression

WT
dhh1
(Dhh1 Exp.)

G

Relative ASH1 mRNA levels

dhh1
WT
dhh1
(Dhh1 Exp.)
Localisation and translational regulation of ASH1 mRNA

Figure 5. Activation of translational initiation results in disassociation of Dhh1 with ASH1 mRNA. A, fluorescent experiments were performed in the cells without or with CHX treatment. The top panels show a representative normal cell, in which Ash1 was fully translated and translocated into the cell nucleus, whereas in a CHX-treated cell, detected Ash1 was mostly cytoplasmic, due to the inhibition of protein elongation (bottom panels). Bar, 3 μm. B, Western blots showing the expression of Ash1 and GAPDH in normal and CHX-treated cells. C, FISH analyses showed ASH1 mRNA that was localized at the bud tip in a normal growing cell (top panels) but was diffuse in the bud in a CHX-treated cell (bottom panels). Bar, 3 μm. D, extracts of yeast cells were prepared after treatment with cycloheximide for 20 min and were fractionated in 10–50% linear sucrose gradients. An A_{260} plot corresponding to the polysomal fractions is shown. Total RNAs were isolated from the input and sucrose fractions. An equal amount of RNA from each fraction was subjected to RT-PCR to detect the distribution of ASH1 mRNA and GAPDH mRNA in the sucrose gradient (arrows). E, immunoprecipitation assays to analyze the association of Dhh1 with ASH1 mRNA in the cells treated with CHX. ASH1 mRNA was co-purified with Dhh1 in normal growing cells but little in CHX-treated cells (right). NC, a negative control in which no antibody was used. F, ASH1 mRNA was pulled down by MBP-MCP (top). Western blotting indicated that Dhh1 and Pu6 were co-precipitated in normal growing cells but not in CHX-treated cells (bottom panels). NC, a negative control using empty beads. The results are representative of three independent experiments.

Involvement of Dhh1 in translational regulation of other bud-localized mRNAs

Reports have shown that a number of bud-localized transcripts, including MID2 (YLR332w) and SRL1 (YOR247w) mRNAs, were associated with Pu6 post-transcriptionally (1).
Interestingly, these two mRNAs were also shown to complex with Dhh1 in vivo (27). To determine whether blocking of translational elongation of mRNAs would also result in the disassociation of Pu6 and Dhh1 with the two transcripts, we treated a TAP-tagged Pu6 strain (12). After treatment with CHX, we immunoprecipitated Pu6 and analyzed the precipitates by Western blotting and RT-PCR. The results indicated that the two transcripts (MID2 and SLR1) as well as Dhh1 co-precipitated with Pu6 in normal growing cells. However, in CHX-treated cells, the binding capability of Pu6 to the transcripts and Dhh1 was dramatically reduced (supplemental Fig. S4A). This suggests that in addition to ASH1 mRNA, Dhh1 could also be involved in the translation of other bud-localized mRNAs.

**Dhh1 does not directly interact with Pu6 but binds to the 5′-UTR of ASH1 mRNA**

Because Pu6 binds to the 3′-UTR of the ASH1 mRNA (12) and Dhh1 co-precipitated with Pu6 and ASH1 mRNA (Fig. 3), we tested whether the two proteins could interact with each other. The results show that although Dhh1 antibodies could pull down Pu6 in WT strain, it co-precipitated much less Pu6 in ashl cells (Fig. 6A). The small amount of coprecipitated Pu6 could indicate the interaction of Dhh1 and Pu6 with other mRNAs, such as MID2 and SLR1. Furthermore, when yeast extracts and the non-polysomal fraction 3 (Fig. 2) were treated with RNase A and RNase One, Dhh1 was not able to co-precipitate with Pu6 (Fig. 6B, bottom panels), indicating that association of the two proteins was mediated by ASH1 or perhaps the other mRNAs. We then prepared yeast strains expressing ASH1 WT mRNA (5′-UTR-ASH1ORF-3′-UTR) and ASH1 mutant (ADH2-5′-UTR-ASH1ORF-3′-UTR), in which the 5′-UTR of ASH1 mRNA was replaced by the 5′-UTR of ADH2 mRNA (Fig. 6C, top panels), and performed co-IP assays using Dhh1 antibodies. ASH1 mRNA was co-precipitated with Dhh1 in the extracts of WT cells but not in the mutant cells when the 5′-UTR of the ASH1 mRNA was absent (Fig. 6C, bottom panels). Precipitation of Pu6 was also greatly decreased in ASH1 mutant mRNA mutant cells (Fig. 6C, middle panels). Because Pu6 binds to the 3′-UTR of ASH1 mRNA (12), the result suggested that Dhh1 could bind to the 5′-UTR of ASH1 mRNA. To further address this, we fused MS2 repeats into the 3′-ends of 5′UTR-ASH1ORF-3′UTR (WT) and ADH2-5′-UTR-ASH1ORF-3′UTR (5′mut) constructs and used recombinant chimeric MBP-MCP to precipitate ASH1-MS2 repeats mRNA in the cell extracts. Pu6 was co-precipitated with both WT and mutant ASH1 mRNA, whereas Dhh1 was not present in the precipitates of the mutant ASH1 mRNA (supplemental Fig. S4B). These results confirmed that Dhh1 bound to the transcript through the 5′-UTR and that the association of Dhh1 with Pu6 required both 5′-UTR and 3′-UTR of ASH1 mRNA.

**Dhh1 is not recruited on ASH1 mRNA co-transcriptionally**

Pu6 is co-transcriptionally recruited onto nascent ASH1 mRNA (1). To test whether Dhh1 could also be recruited onto ASH1 mRNA during transcription, ChIP assays were per-
formed using strains expressing endogenous C-terminal TAP-tagged Dhh1, She2, or Puf6 (supplemental Fig. S5A). In these assays, chromatin that was associated with She2, Puf6, or Dhh1 was immunopurified by using IgG-Sepharose, and the enriched specific regions of the ASH1 gene were measured by qPCR. Two specific amplicons from the ASH1 gene were analyzed; one was 5′-UTR, and the other was 3′-UTR, which contained the She2- and Puf6-binding motifs and was used as an internal control (Fig. 6D). As shown in the figure, control amplicons of the 3′-UTR were specifically enriched after Pu6 and She2 CHIPs, whereas no enrichment of the 3′-UTR was identified in Dhh1 CHIP. There was also no enrichment for the 5′-UTR amplicon in all ChIP assays. In contrast, ASH1 mRNA was successfully co-precipitated with Dhh1-TAP from the cell extracts (supplemental Fig. S5B). These results suggest that Dhh1 was not co-transcriptionally recruited on the 5′-UTR of the ASH1 gene.

Loss of Dhh1 function affects the HO promoter activity

To examine the physiological relevance of the DHH1 gene to the cell’s ability to control mating-type switch, we tested the influence of Dhh1 on the regulation of the HO promoter. DHH1 was deleted in a strain in which the endogenous CAN1 gene was under the control of the HO promoter (12, 28). The cells were sensitive to canavanine when the gene was under the control of the HO promoter (12, 28). The translation in vitro

Binding of Dhh1 to the 5′-UTR of ASH1 mRNA represses its translation in vitro

We used in vitro RNA mobility-shift experiments to further identify the binding ability of Dhh1 to the 32P-labeled 5′-UTR of ASH1 mRNA. An RNA-protein complex was formed when the RNA probe was incubated with the extracts of WT, pu6, and dep1 cells. However, the complex formation was significantly attenuated upon incubation with the extracts of dhh1 cells (Fig. 7A; complex is indicated by the arrow), indicating that the formation of the complex relied on Dhh1. We next expressed His-tagged Dhh1 in Escherichia coli and used the purified Dhh1 (supplemental Fig. S2B) to perform binding assays. Incubation of 32P-labeled 5′-UTR of ASH1 mRNA with recombinant Dhh1 formed a distinct RNA-protein complex, which was effectively competed by excess amounts of the unlabeled probe (Fig. 7B). The complex formation was specific because a supershift band was observed when Dhh1 antibody was added into the reaction (arrow). Dhh1 did not interact with the 3′-UTR of ASH1 mRNA because the 3′-UTR of ASH1 mRNA did not show any competition for the complex formation (Fig. 7C). Thus, the in vitro experiments supported the in vivo binding of Dhh1 to the 5′-UTR of untranslated ASH1 mRNA.

To evaluate whether binding of Dhh1 to the 5′-UTR of ASH1 mRNA could affect translation, we performed in vitro cell-free translation assays in a rabbit reticulocyte lysate system using two R-luciferase reporters; one contained the 5′-UTR of ASH1 mRNA (160 bp), and the other contained the 5′-UTR of ADH2 mRNA (150 bp). A progressive reduction of translation was observed when the 5′-UTR of ASH1 reporter was incubated with increasing amounts of recombinant Dhh1. The efficiency of translation was reduced by > 50% when the molar ratio of the reporter RNA/Dhh1 reached 1:16, whereas no obvious reduction was observed when Dhh1 was incubated with the 5′-UTR of ADH2 reporter (Fig. 7D). In addition, the translational efficiency was not changed when the 5′-UTR of ASH1 reporter was incubated with increased amounts of recombinant Dcp1 (Fig. 7E). Recombinant Dhh1 repressed in vitro translation of the ASH1 5′-UTR reporter was also observed in a yeast extract prepared from dhh1 cells (supplemental Fig. S6). The translational inhibition did not result from selective RNA degradation, because the amount of the reporter RNA in the reactions did not change with increasing Dhh1 levels (Fig. 7F). Thus, binding of Dhh1 to the 5′-UTR of ASH1 mRNA conferred repression of translation.

Discussion

We have previously reported that binding of Pu6 to the 3′-UTR of ASH1 mRNA repressed its translation during transport, and this repression could be released by CK2 phosphorylation in the N-terminal region of Pu6 when the mRNA was localized to the bud tip (12, 13). Our new observations showed that although translation occurred after localization, a fraction of ASH1 mRNA was still associated with Pu6 and was translationally inactive. The 5′-UTR of the untranslated ASH1 mRNA was simultaneously bound to Dhh1, which served to maintain the non-polysomal status of the mRNA and cause the mRNA to be tightly localized at the bud cortex.

Post-transcriptional control of mRNA localization and translation was achieved through the concerted action of RNA-binding factors and the translation machinery (10, 29–31). The fact that Dhh1 coordinates with Puf6 to regulate the translation of the ASH1 mRNA was confirmed by the in vivo overexpression analysis and the in vitro translation assays. Binding of Dhh1 to the 5′-UTR of ASH1 mRNA was most likely due to the involvement of the protein in the regulation of translational initiation. This could be partly supported by the finding that Ash1 expression was decreased when Dhh1 was overexpressed and that increasing translational initiation by CHX treatment resulted in the dissociation of the protein from ASH1 mRNA. Because phosphorylation of Pu6 at the bud tip by CK2 decreased its binding ability to ASH1 mRNA and eliminated the translational repression (13), it is possible that CK2 could also act on Dhh1 to release its binding.

ASH1 mRNA contains four localization elements for She2 binding, which make the localization of the mRNA more precise and efficient (7). Localized translation of ASH1 mRNA
could also be finely controlled. This explains why Dhh1 is involved in the regulation of localized translation of ASH1 mRNA. However, compared with puf6 cells, in which ASH1 mRNA was widely delocalized in both the mother and daughter cells (12), the mRNA in the dhh1 cells was mostly diffusely localized in the buds. As a result, we observed a milder effect of Dhh1 on the regulation of the HO promoter when compared with the mutation in puf6. In addition, unlike Puf6 that was recruited into ASH1 mRNA during transcription (1), Dhh1 was shown to associate with the transcript within the cytoplasm, suggesting that Dhh1 might be more involved in the regulation of ASH1 mRNA translation after localization. We hypothesized that after bud-tip localization, some ASH1 mRNA could be programmed for immediate translation, whereas other ASH1 mRNA could be stored. In this case, binding of Dhh1 to translationally inactive ASH1 mRNA could primarily result in temporal repression, storage, and then decay if no more protein synthesis is required.

In S. cerevisiae, Dhh1 is a DEAD-box RNA helicase and is also a major P-body component involved in translational repression and mRNA decapping (20). Recent studies indicated that Dhh1 represses mRNA translation in a way that does not rely on the translation initiation factors and that Dhh1 connects translation to mRNA decay by monitoring codon optimality (32, 33). Because Dhh1 binds to the 5'-UTR of ASH1 mRNA, we feel that Dhh1 could be more implicated in the control of translational initiation of the mRNA. Deletion of the DHH1 gene not only resulted in a defect in mating through the down-regulation of Ste12 expression (34) but also affected the mating-type switch due to the dysregulation of HO expression. In addition, the roles of the Dhh1 family in translational repression and enhancement of mRNA degradation have also been well studied in higher eukaryotes (19, 21). Dhh1 homologs in Drosophila (Me31B), Caenorhabditis elegans (CGH-1), and Xenopus (Xp54) have been implicated in translational repression and storage of maternal mRNAs (35–37).
A recent global analysis of yeast mRNPs failed to identify consensus sequences for Dhh1 binding (27); however, an in vitro analysis has indicated that recombinant Dhh1 showed a strong preference for binding to adenine-rich nucleotides (38). We found that Dhh1 interacted with the 5'–UTR of ASH1 mRNA that is not adenine-rich. Although the exact sequence element for Dhh1 binding has yet to be identified, our results indicated that interaction of Dhh1 with the 5'–UTR of ASH1 mRNA is required for regulation of Ash1 translation. Binding of the Dhh1 to ASH1 mRNA did not result from using the system of MS2-tagged RNA and MCP-GFP (39), because the binding was also confirmed when the mRNA was not MS2-tagged and when the MCP-GFP was not used. Our future work will be to identify the signal that determines the fate of untranslanted ASH1 mRNA: to be translated or to be stored for degradation.

In conclusion, we identified a potential regulatory mechanism for localized ASH1 mRNA translation in which not all of the mRNA was translated after localization. Untranslated mRNA was repressed and temporarily stored through the interaction with Dhh1 and Puf6. Thus, translation of localized ASH1 mRNA could be precisely regulated by Dhh1, which bound to the 5'–UTR, and Puf6, which bound to the 3'–UTR of the mRNA. Interference of the coordination due to the lack of Dhh1 could affect ASH1 mRNA localization and translation.

Experimental procedures

Growth medium, yeast strains, and plasmids

Strains used in this study are listed in Table 1. Yeast cells were grown in either synthetic medium lacking the nutrients indicated or rich medium. Transformation was performed according to the protocol of Gietz et al. (40). Unless otherwise stated, standard genetic techniques were used. Tagging, insertion, and deletion were performed by PCR-based homologous recombination using cassettes described previously (12, 41). The deletion was verified by colony-PCR analysis to confirm that the insertion and replacement were in the expected locus.

Plasmid construction

Plasmids used in this study were constructed using standard techniques. The plasmids YCP111-Gal-HA-ASH1-Myc-MS2 and YCP111-HA-ASH1-MS2 were derived from plasmid YCP111-Gal-MS2-LacZ-E3. These plasmids contain full-length 5'–UTR as well as the coding region of ASH1 mRNA and six MS2 sites after the 3'–UTR of ASH1 mRNA for MCP binding. Plasmid YCP33-GFP-MCP was constructed previously (18). Plasmids YCP111-GFP-Dhh1 and YCP111-GFP-MCP were derived from plasmid YCP33-GFP-MCP. Plasmids YIP211-HAASH1-MS2, YIP211-5'UTR-ASH1-3'UTR, and YIP211-ADH2-5'UTR-ASH1-3'UTR were derived from the plasmid pXR193 (42). Plasmid pSP64-ASH-5'UTR was derived from plasmid pSP64-ASH1-3'UTR (12).

In situ hybridization and immunofluorescence

Yeast cells were grown in the appropriate culture medium to early or mid-log phase and were processed for FISH as described previously (12). To perform FISH, yeast spheroplasts were hybridized with a pool of Cy3-conjugated ASH1 DNA oligonucleotide probes or Cy3-conjugated MS2 probes (3, 43). Immunofluorescence was performed using a protocol described previously (42). For detecting HA-tagged or Myc-tagged Ash1 proteins, the mouse Cy3-conjugated anti-HA or Cy5-conjugated anti-Myc antibodies (Roche Applied Science) were used in a 1:200 dilution in 1% PBS and 0.1% BSA.

Preparation of yeast extracts

Yeast cells were cultured to A495 1.0 and were rapidly harvested by centrifugation at 3,200 × g for 5 min at 4 °C. Where indicated, cycloheximide (50 μg/ml) was added to the cultures for 20 min. All subsequent steps were carried out in a cold room. After washing with 10 ml of cold buffer (50 mM Tris–HCl, 100 mM NaCl, 30 mM MgCl2, or 50 μg/ml), the cells were pelleted again and resuspended in 800 μl of the buffer containing 20 units/ml RNasin and 2 × protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml aprotinin, 100 μg/ml pepstatin, 100 μg/ml leupeptin) in 2–ml tubes containing 800 μl of glass beads. Cells were lysed in a FastPrep (MP Biomedicals) 4 times for 60 s each time. Cell debris and beads were removed from the extract by centrifugation for 15 min at 8,000 × g. To prepare the extracts for in vitro translation.
assays, the obtained supernatant was subjected to centrifugation (4 °C, 15,000 × g, 30 min) again.

Sucrose-gradient fractionation

500 μl of yeast extracts were loaded onto 10-ml, 10–50% linear sucrose gradients and fractionated at 35,000 rpm for 2 h in an SW41 rotor (Beckman). Fractions (1.25 ml each) were collected from top to bottom, and the A<sub>254</sub> profile was monitored.

Northern and Western blotting

For Northern blotting, total RNA was extracted, and equal amounts of total RNA were separated by agarose gel electrophoresis. RNA was transferred to a Hybond membrane and incubated with dCTP-32P-labeled cDNA probes for the ASH1 gene and the GAPDH gene. Hybridization and autoradiography were performed as described previously (44). For immunoblotting, equal amounts of cell extracts were resolved into a 4–12% gradient SDS-PAGE (Invitrogen), followed by electroblotting onto a nitrocellulose membrane. Western blotting was performed using one of the following primary antibodies: anti-His, anti-HA, anti-Myc, anti-Dhh1, anti-Dcp1, anti-She2, anti-GAPDH, or anti-Pgk1. After incubation with HRP-conjugated secondary antibodies, proteins were detected with an enhanced chemiluminescence detection kit (Millipore). The intensity of the protein bands was quantified using ImageJ software (NIH). Relative protein levels were determined after normalization to an internal protein control.

Expression and purification of recombinant MBP-MCP and Dhh1

Recombinant MBP-MCP protein was expressed and purified as described previously (45). A His<sub>6</sub>-tagged cDNA encoding Dhh1 or Dcp1 protein was PCR-amplified from yeast genomic DNA and cloned into a donor plasmid pET23a plasmid (Novagen) into the Ndel and BamHI sites. The plasmid, after DNA sequence analysis, was transformed into E. coli (DE21) cells and expressed. The method for purifying recombinant His<sub>6</sub>-tagged Dhh1 or Dcp1 protein was as mentioned previously (45).

In vitro pulldown experiments, RT-PCR, and Western blotting

Yeast extracts from exponentially growing cell cultures were prepared as described above. For precipitation of Dhh1-associated proteins, a total of 100 μl of extracts were incubated with 2 μg of anti-Dhh1 IgG (Santa Cruz Biotechnology, Inc.) or control IgG and 20 μl of Protein A beads (Sigma) for 4–6 h at 4 °C with gentle shaking. Where indicated, RNase A (10 μg/100 μl) and RNase One (100 units; Promega) were added. After washing extensively with DEPC-treated PBS, the beads were suspended in 40 μl of water, and the proteins were eluted by boiling for 5 min. Alternatively, His-tagged recombinant Dhh1 was attached to nickel-nitrioltriocetic acid beads for pulldown experiments. The methods for precipitation of the ASH1 mRNA complex using MBP-MCP recombinant protein were performed as mentioned previously (45). For reverse transcription, TRIzol-extracted RNA was used as template. All PCRs were performed for 25–30 cycles using the primers specific for ASH1 mRNA and an internal control. The intensity of the DNA bands was quantified using Image software (National Institutes of Health). Relative levels of ASH1 mRNA were normalized to the levels of the control. Primers used for RT-PCR and PCR experiments are shown in Table 2.

ChIP

Puf6-TAP and She2-TAP strains were established previously, and the construction of the C-terminal insertion cassette for tap-tagging of the DHH1 gene was described previously (12). Expression of Dhh1-Tap, Puf6-Tap, and She2-TAP was from their corresponding endogenous loci. For each ChIP, three independent 50-ml early log phase cultures were used. To increase the number of ASH1 transcripts, cells were synchronized for 2 h using Nocodazole at the final concentration of 15 μg/ml. IgG-Sepharose 6 Fast Flow (GE Healthcare) was used for the experiments, and the procedure for CHIP assays was as mentioned previously (1). Quantification of the immunoprecipitated DNA was performed by RT-qPCR (S1000TM thermal cycler Bio-Rad) using the SYBR Green PCR core reagent system. Each 20-μl PCR contained 10 μl of qPCR mix, 2 μl of DNA, and a 300 nM concentration of each of the primers. PCR was performed in the following conditions: activation of the reaction for 30 s at 95 °C, followed by 35 cycles of 10 s at 95 °C and then 30 s at 57 °C for the annealing and extension steps. Cycle thresholds (Ct) for each triplicate of samples were averaged, and ChIP enrichment was calculated by dividing the amount of ChIP DNA over control DNA using the formula, 2<sup>ΔΔCt</sup>.

HO promoter activity assay

The effect of DHH1 on HO expression was determined as described by Jansen et al. (28). Briefly, 10-fold serial dilutions of exponentially growing wild-type (K4535), puf6, or dhh1 cells were spotted on YPD or SD medium contain 0.03% canavanine and incubated for 2 and 5 days at 30 °C, respectively.

Gel mobility-shift assay

A 150-bp cDNA fragment encoding 5′-UTR of ASH1 mRNA was PCR-amplified and subcloned into pSP64 plasmid (Pro-

### Table 2

| Gene   | Forward primers | Reverse primers |
|--------|-----------------|-----------------|
| ASH1 5′-UTR | GCTCTCCCTCTCAAAAAAGAG | GCTCTGCTCTCCAGAGCTG |
| ASH1 3′-UTR | TACATCGCAATGAATCTCTTC | TGGAGGATCCGCGAGCTAG |
| ASH1 mRNA | GACTATGCTCTCTGCAGTC | TGGATTCTCATGGACATT |
| GAPDH | GAGTCACGGATTTCGCCTC | GTTACTTCCTCTTTCAGG |
| DHH1 | TCCATTAAGGAAAGAAGACG | GCTCTGCTCTTTACGAC |
| DCP1 | GTGACCTCTGCAAGATTTC | GTGAGGACGCCCTCTGATAC |
| ADH2 5′-UTR | TCTGCTCTCTGCTATTTAG | CTTACAACTTTACCTATAG |

Localization and translational regulation of ASH1 mRNA
Localization and translational regulation of ASH1 mRNA

In vitro translation assays

In vitro translation assays were performed as described previously (12). Briefly, capped mRNA reporters with the 5′ UTR of ASH1 mRNA or the 5′ UTR of ADH2 mRNA were transcribed from the plasmids, pC3.1-5′-UTR and pS64-ASH1 3′-UTR constructs. Transcribed RNA probes were purified after resolving in a 6% denaturing gel. RNA-protein gel-shift assays were performed at room temperature as described previously (12). The RNA-protein complexes formed were separated by electrophoresis in a 4% native gel and visualized by autoradiography. To establish the specificity of RNA-protein interactions, competition assays were performed by preincubating the cell extracts or the recombinant Dh1 with unlabeled specific or nonspecific RNA competitors.

Analysis of ASH1 mRNA and Ash1 localization

The method of quantitative measurement on the localization of ASH1 mRNA has been described previously (42). By microscopy, yeast cells in late anaphase were scored for localized or delocalized ASH1 mRNA in each experiment. ASH1 mRNA was considered as bud-tip-localized when it was predominantly in the bud tip (crescent localization). ASH1 mRNA was considered as diffuse in the bud when it was not tightly localized at the bud tip. For each experiment, ~100 budding cells were scored.

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