Heat shock and ethanol stress provoke distinctly different responses in 3’-processing and nuclear export of HSP mRNA in *Saccharomyces cerevisiae*

Shingo IZAWA¹, Takeomi KITA, Kayo IKEDA and Yoshiharu INOUE

Laboratory of Molecular Microbiology, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

Under conditions of heat shock at 42 °C, mRNAs of HSP (heat shock protein) genes are exported out of the nucleus, whereas bulk poly(A)+ (polyadenylated) mRNA shows a nuclear accumulation in *Saccharomyces cerevisiae*. Such a selective mRNA export seems an efficacious strategy of yeast cells to adapt rapidly to stress. Although ethanol stress (10%, v/v) as well as heat shock blocks the export of bulk poly(A)+ mRNA, the differences and/or similarity between heat shock and ethanol stress in the mechanisms of selective mRNA export still remain to be clarified.

We found that ethanol stress induced transcriptional activation of a subset of yeast HSP genes; however, intriguingly, most such transcripts remained in the nucleus in a hyperadenylated state and, as a consequence, were not translated into HSPs. Elimination of ethanol resulted in a rapid shortening of the poly(A) tails of HSP mRNAs, loss of their nuclear retention, and the coincidental synthesis of the respective HSPs. Since HSP mRNAs are selectively exported from the nucleus in heat-shocked cells, yeast cells respond differently to ethanol stress and heat shock in the 3’-processing and transport of HSP mRNAs. Furthermore, these results also suggest that hyperadenylation and nuclear retention of mRNAs might be used as a means to control eukaryotic gene expression under stressed conditions.

Key words: eukaryotic gene expression, HSP42 (Hsp42 gene), HSP104 (Hsp104 gene), hyperadenylation, selective export of mRNAs, SSA4 (Hsp70 gene).

INTRODUCTION

Under heat-shocked (42 °C) conditions, yeast cells change the pattern of protein synthesis by shutting down the synthesis of most proteins with the exception of stress-responsive proteins such as HSPs (heat shock proteins) [1]. This effect is caused in part by stress-responsive transcriptional activation and selective mRNA export, i.e. yeast cells alter not only their transcriptional patterns but also the types of mRNA to be exported from the nucleus upon heat shock [2,3]. It is well recognized that transcriptional activation of HSP genes is quickly induced by heat shock [1,4]. Stress-induced transcripts such as SSA4, encoding one of the Hsp70s, are efficiently exported to the cytoplasm, whereas bulk poly(A)+ (polyadenylated) mRNA accumulates in the nucleus in heat-shocked cells [2,3]. These findings indicate that gene expression in eukaryotic cells under stressed conditions can be affected via changes in the pattern of mRNA export from the nucleus. Additionally, such a selective export of mRNA appears to be an effective cellular response to adapt rapidly to heat shock.

Mechanisms of selective mRNA export under conditions of heat shock seem to be quite complex and are still not well clarified. In *Saccharomyces cerevisiae*, the nucleoporin Riplp/Nup42p was proposed to play an important role in the export of HSP mRNAs under heat-shocked conditions [5,6]. Initially, it was proposed that HSP mRNAs are exported through a specific pathway defined by Riplp [5,6]. However, the pathway for the export of HSP mRNAs upon heat shock is still controversial, since it has been reported that Riplp also participates in the export of non-HSP mRNAs at 42 °C [7]. Furthermore, it is still not clear how the HSP mRNAs and non-HSP mRNAs are distinguished.

There are several reports regarding the mechanisms behind the shutting-off of bulk poly(A)+ mRNA export upon heat shock. Blocking of the export of bulk poly(A)+ mRNA under heat-shocked conditions involves the dissociation of Npl3p (an hnRNP protein) from mRNA [8]. We also reported a relationship between the nuclear accumulation of bulk poly(A)+ mRNA and dissociation of Gle2p (a component of the nuclear pore complex and involved in the export of mRNA) from the nuclear pore complex at 42 °C [9]. Meanwhile, it is known that changes in the processing of pre-mRNA affect competency for nuclear export. Both hyperadenylation and defects in polyadenylation of the 3’-end of mRNA affect the efficiency of mRNA export and cause the nuclear accumulation of pre-mRNA [10,11]. However, it is not clear whether heat shock impairs the processing and transport of bulk poly(A)+ mRNA or cells actively halt the export of bulk poly(A)+ mRNA upon heat shock [9]. The detailed mechanisms underlying the nuclear accumulation of bulk poly(A)+ mRNA in heat-shocked cells are still not well clarified [9].

Ethanol stress (10%, v/v) as well as heat shock blocks the export of bulk poly(A)+ mRNA [3,8,9,12]. However, the differences and/or similarity between heat shock and ethanol stress in the mechanisms of mRNA export still remain to be clarified. It has been reported that both heat shock and ethanol stress induce an almost identical response in yeast such as changes in membrane lipid composition, plasma membrane H+-ATPase activity and vacuolar morphology [13,14]. On the other hand, we reported that the intracellular distribution of Rat8p/Dbp5p (an essential DEAD-box protein that plays a role in mRNA export) changes rapidly and reversibly in response to ethanol stress but not to heat shock [12]. Additionally, it has been reported that cells treated with 10% ethanol show a defect in SSA4 mRNA export [15]. These findings suggest that yeast cells partly show different responses regarding mRNA export to heat shock and ethanol stress.

Abbreviations used: DAPI, 4’,6-diamidino-2-phenylindole dihydrochloride; FISH, fluorescent in situ hybridization; GFP, green fluorescent protein; HSP, heat shock protein; ORF, open reading frame; PGK, phosphoglycerate kinase; poly(A)+, polyadenylated; SD, synthetic dextrose.

¹ To whom correspondence should be addressed (email izawa@kais.kyoto-u.ac.jp).
To understand the reality of mRNA export under stressed conditions, we here investigated whether HSP mRNAs are preferentially exported in cells treated with ethanol stress as observed in cells treated with heat shock. Ethanol stress also caused the transcriptional activation of HSP genes, and the level of HSP42, SSA4 and HSP104 mRNAs in the ethanol-treated cells was nearly equal to that in the heat-shocked cells. However, most of the HSP mRNAs synthesized in the ethanol-treated cells accumulated in the nucleus in a hyperadenylated state and, as a consequence, were not translated into HSPs.

EXPERIMENTAL

Yeast strains and medium

The *S. cerevisiae* strains used in this study were W303-1A (MATa his3-11, 15 leu2-3, 112 trpl-1 ade2-1 ura3-1 can1-100) and FY1770 (MATa his3-11, 15 leu2-3, 112 trpl-1 ade2-1 ura3-1 rpl1::kan) [6]. Cells were cultured in 50 ml of SD (synthetic dextrose) minimal medium (2% glucose and 0.67% yeast nitrogen base without amino acids, pH 5.5) with appropriate amino acids and bases at 28°C with reciprocal shaking in 300 ml Erlenmeyer flasks. Exponential-phase cells were prepared by culturing to a D$_{600}$ of 0.3–0.5.

Plasmids

To estimate the protein levels of Hsp42p, Ssa4p and Hsp82p, FLAG-tagged HSP constructs (integrate type vectors) were generated and integrated at the chromosomal locus of each HSP gene. These constructs contain a FLAG sequence, a stop codon and the 3′-flanking region of each HSP gene, and are predicted to have little effect on the 3′-processing, mRNA export and translation. Ylp-SSA4-FLAG, a 1.1 kbp fragment encoding the ORF (open reading frame) of SSA4 was amplified using 5′-GAGGGTAGTCCGACCTACAAGGATGACGATGACAAGTGAATATCGTATCTG-3′ and 3′-TTCGGA TCCGAGTTGAGCCCGT-3′ as primers, and a 0.9 kbp fragment encoding a FLAG sequence, a stop codon and the 3′-flanking region of SSA4 was amplified using 5′-GGTTGCTCTCTAACAAGGATGACGATGACAAGTAGAATATCAAGATGCGATGTAAG-3′ and 5′-TTCGGA TCCGAGTTGGTCTTCTCATAAA-3′. Each amplicon was digested with XbaI/Xhol and Xhol/BamHI respectively, and cloned into the XbaI/BamHI sites of pPS1630 to construct Ylp-SSA4-FLAG. To insert the SSA4-FLAG gene at the chromosomal SSA4 locus, Ylp-SSA4-FLAG was linearized by EcoRI and introduced into yeast cells. Ylp-HSP42-FLAG, a 1.1-kbp fragment encoding the ORF of HSP42 amplified using 5′-GAGGTATCCGATTC-TAGCTTCTCAATAC-3′ and 5′-TTCGGA TCCGAGTTGGTCTTCTCATAAA-3′, and a 0.6 kbp fragment encoding a FLAG sequence, a stop codon and the 3′-flanking region of HSP42 were amplified using 5′-TAAAGATCTACGACTACAAGGATGACGATGACAAGTGAATATCGTATCTG-3′ and 5′-GAGGGTAGTCCGACCTACAAGGATGACGATGACAAGTGAATATCGTATCTG-3′ as primers, and a 0.9 kbp fragment encoding a FLAG sequence, a stop codon and the 3′-flanking region of HSP42 was amplified using 5′-GGTTGCTCTCTAACAAGGATGACGATGACAAGTGAATATCGTATCTG-3′ and 5′-TTCGGA TCCGAGTTGGTCTTCTCATAAA-3′. Each amplicon was digested with XbaI/Xhol and Xhol/BamHI respectively, and cloned into the XbaI/BamHI sites of pPS1630 to construct Ylp-HSP42-FLAG. To integrate the HSP42-FLAG gene at the chromosomal HSP42 locus, Ylp-HSP42-FLAG was linearized by XhoI and introduced into yeast cells. Ylp-HSP82-FLAG, a 1.8 kbp fragment encoding a FLAG sequence, a stop codon and the 3′-flanking region of HSP82 was amplified using 5′-GGTACGACTACAAGGATGACGATGACAAGTAGGAGTAGAATATCGTATCTG-3′ and 5′-TCAAGGCATGATCTTCTACTCGTAGCTACC-3′, and a 0.7 kbp fragment encoding a FLAG sequence, a stop codon and the 3′-flanking region of HSP82 was amplified using 5′-GGTAGCTC-GAGCCGACTACAAGGATGACGATGACAAGTAGGAGTAGAATATCGTATCTG-3′ and 5′-TCAAGGCATGATCTTCTACTCGTAGCTACC-3′. Each amplicon was digested with XbaI/Xhol and Xhol/BamHI respectively, and cloned into the XbaI/BamHI sites of pPS1630 to construct Ylp-HSP82-FLAG. To integrate the HSP82-FLAG gene at the chromosomal HSP82 locus, Ylp-HSP82-FLAG was linearized by EcoRI and introduced into yeast cells. pAdh1-Msn2-GFP (where GFP is green fluorescent protein) was donated by Dr C. Schüller (Medical University Vienna, Max F. Perutz Laboratories, Department of Medical Biochemistry, A-1030 Vienna, Austria) [17]. pAdh1-Msn4-GFP was previously described [18].

Northern blot analysis

Northern blot analysis was performed using 15 μg of total cellular RNA isolated from yeast cells by the method of Schmitt et al. [19]. The probes were prepared by PCR with the primers listed in Supplementary Table S1 at http://www. BiochemJ.org/bj/414/bj4140111add.htm. Each PCR fragment of these HSP genes was labelled with [α-32P]dCTP using the Random primer DNA labelling kit version 2 (Takara Bio, Otsu, Japan). The oligonucleotides SSA4-P1-P4 and PGK1-P1-P4 (where PGK is phosphoglycerate kinase) (see Supplementary Table S1) were labelled with [γ-32P]ATP using T4 DNA kinase and used as the probes for SSA4 and PGK1.

Western blot analysis

After treatment of cells with various forms of stress in SD medium, a cell-free extract was immediately prepared. Levels of Hsp42p, Hsp82p and Ssa4p were monitored by Western blotting using a monoclonal anti-FLAG M2 antibody purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Levels of Hsp104p were monitored with an anti-Hsp104p antibody purchased from Stressgen Biotechnologies (Victoria, Canada). Levels of Pgk1p were monitored with an anti-Pgk antibody (A-6457) purchased from Molecular Probes (Eugene, OR, U.S.A.). Protein (20 μg) was applied to each lane for SDS/PAGE. Thiolurin experiments were performed by adding thiolurin (Sigma-Aldrich) to a final concentration of 5 μg/ml [20].

FISH (fluorescent in situ hybridization)

In situ hybridization assays were performed as described previously [21]. DNA probes listed in Supplementary Table S1 were directly labelled with the fluorescent dye Cy3 using Label IT Cy3 labelling kits (Mirus, Madison, WI, U.S.A.). A BX60 fluorescence microscope system (Olympus) and the imaging software IPLab (Scanalytics, Fairfax, VA, U.S.A.) were used for the fluorescence microscopic analysis. BrightLine™ CY3-4040A-000 (Semrock, Rochester, NY, U.S.A.) was used as a single band fluorescent filter to detect the Cy3 signal with high sensitivity.

RNaseH assay

RNaseH assays were performed as described by Decker and Parker [22] with modifications. Briefly, total RNA (90 μg) was incubated with digestion oligonucleotides (1800 ng) in 20 μl of hybridization buffer (25 mM Tris/HCl, pH 7.5, 1 mM EDTA and 50 mM NaCl) at 68°C for 10 min, then cooled down gradually to 37°C. Some samples also contained 3600 ng of oligo(dT)$_{25}$. The cooled RNA solution was mixed well with 21 μl of RNaseH cocktail solution [3 units of RNaseH (TOYOBO, RHH-201, Osaka, Japan)], 40 units of RNase inhibitor (Sigma, 075K1887), 40 mM Tris/HCl (pH 7.5), 20 mM MgCl$_2$, 100 mM NaCl, 2 mM
Ethanol stress as well as heat shock elevated levels of HSP mRNAs

Both heat shock (42°C) and ethanol stress (10%, v/v) block the nuclear export of bulk poly(A)⁺ mRNA in yeast [3,8,9,12]. However, there is almost no information about the induction of HSPs under conditions of 10% ethanol stress. Therefore, we first investigated the levels of HSP mRNAs following the treatment with heat shock and 10% ethanol (Figure 1A). In wild-type cells, the level of almost every HSP mRNA examined (except for HSP150) was increased by exposure to heat shock at 42°C. The treatment with 10% ethanol also resulted in increased levels of most HSP mRNAs. On the other hand, the level of PGK1 mRNA was hardly changed by the treatment with heat shock or ethanol stress.

Notably, levels of HSP42, SSA4 and HSP104 mRNAs in cells exposed to 10% ethanol were comparable with those in cells treated at 42°C. These three genes contain multiple STREs (stress-responsive elements; consensus sequence is 5′-CCCCT-3′ or 5′-AGGGG-3′) in their promoter regions. It is well established that Msn2p and Msn4p, stress-responsive transcription factors, accumulate in the nucleus and induce the activation of STRE-dependent promoters under various stressful conditions [23,24]. Indeed, Msn2p-GFP and Msn4p-GFP accumulated in the nucleus under conditions of 10% ethanol stress and heat shock (Figure 1B), indicating that Msn2p and Msn4p were activated. We verified that GFP alone did not accumulate in the nucleus under stressed conditions (Figure 1B). These results show that 10% ethanol and heat shock at 42°C induce essentially identical responses in the transcriptional activation of these HSP genes.

Since Rip1p is proposed to play a role in the export of HSP mRNA under heat-shocked conditions [5,6], we also investigated the levels of HSP mRNAs in the rip1Δ cells (FSY17). Although the level of SSA4 mRNA in the rip1Δ cells was increased by the treatment with heat shock and 10% ethanol stress (Figure 1A), the rip1Δ cells showed lower levels of most HSP mRNAs than wild-type cells under stressed conditions, as partly reported by Stutz et al. [6].

Ethanol did not elevate protein levels of HSPs

Next, we investigated the changes in protein levels of HSPs upon 10% ethanol stress by conducting Western blot analysis (Figure 2A). We monitored the levels of Hsp42p, Ssa4p, Hsp82p, and Hsp104p, since increased levels of their mRNAs except...
HSP82 were almost the same in both heat-shocked cells and 10 % ethanol-treated cells (Figure 1A). As expected, the protein levels of HSPs were markedly increased by heat shock in the wild-type cells (Figure 2A). In contrast, a very slight increase in the levels of these HSPs was observed in the ethanol-treated wild-type cells (Figure 2A). These results suggest that the expression of HSP genes is repressed or inhibited by post-transcriptional events in 10 % ethanol-treated cells. Interestingly, yeast cells showed greater resistance to 10 % ethanol stress than heat shock at 42 °C without increased protein levels of HSPs (Figures 2A and 2B), indicating that increased expression of these HSP genes is dispensable for the resistance to 10 % ethanol stress.

In the rip1Δ cells, the protein level of Ssa4p was little increased even by heat shock (Figure 2A). This is accounted for by the nuclear retention of SSA4 mRNA at 42 °C [5,6,25–27]. Additionally, the rip1Δ cells expressed less Hsp42p, Hsp82p, and Hsp104p upon heat shock at 42 °C, as Saavedra et al. [5] reported. In response to 10 % ethanol, the rip1Δ cells showed almost no increase in the protein levels of HSPs other (Figure 2A).

Ethanol reduced the efficiency of the nuclear export of HSP mRNAs

Although the levels of HSP mRNAs were increased, the protein levels of HSPs were little increased by 10 % ethanol (Figures 1A and 2A). Since these findings suggest that 10 % ethanol affects the post-transcriptional process, we next investigated the transport of HSP mRNAs in ethanol-treated cells by in situ hybridization. The HSP42, SSA4 and HSP104 mRNAs were all increased and exported to the cytoplasm in the wild-type cells at 42 °C (Figure 3), as previously reported regarding SSA4 by Saavedra et al. [3]. In the rip1Δ cells at 42 °C, SSA4 mRNA was detected in bright intranuclear foci, as previously reported by others [5,26,28]. Since our results reproduced those reported by other groups, we judged the technical quality of the in situ hybridization to be trustworthy.

Under conditions of 10 % ethanol, HSP mRNAs showed nuclear retention in both the wild-type and rip1Δ cells (Figure 3). In contrast with our previous report [12], SSA4 mRNA was also retained in the nucleus and detected as bright foci in the ethanol-treated cells. In this study, we improved the probes for SSA4 and used a new fluorescent filter to specifically detect the Cy3 signal with improved sensitivity (see the Experimental section). These improvements and careful investigation led us to revise the previous observation and conclude that a large proportion of SSA4 mRNA as well as HSP42 and HSP104 mRNAs shows nuclear retention in cells treated with 10 % ethanol. Regarding the nuclear retention of SSA4 mRNA under conditions of 10 % ethanol stress, we reconfirmed earlier results by Rollenhagen et al. [15]. We here found that HSP42 and HSP104 mRNAs also show nuclear retention in the 10 % ethanol-treated cells. These results clearly indicate that ethanol stress and heat shock differ in their effects on the export of HSP mRNAs, and that ethanol stress reduces the efficiency of the nuclear export of HSP mRNAs.

Of course, we cannot exclude the possibility that HSP mRNAs are partially exported to the cytoplasm in the ethanol-treated cells based only on the results of an in situ hybridization analysis. Indeed, the protein levels of HSPs were slightly increased in the wild-type cells after 60 min of 10 % ethanol stress treatment.
(Figure 2A), indicating that the blocking of the export of HSP mRNAs is leaky. However, the rip1Δ cells, in which SSA4 and HSP104 mRNAs are retained in the nucleus at 42 °C [5,6,26], also showed a slight increase in the protein levels of Ssa4p and Hsp104p with treatment at 42 °C for 60 min (Figure 2A). Therefore, the preferable interpretation of these results is that 10% ethanol reduces the efficiency of the nuclear export of HSP mRNAs and causes the nuclear retention of the majority of HSP mRNAs.

**Hyperadenylation of HSP mRNAs was caused by ethanol stress**

It has been reported that controlling the length of poly(A) tails through 3′-processing is linked to the nuclear export of mRNA. Several mutants with defects in the mRNA export machinery possess hyperadenylated transcripts and blocking the export of mRNA causes hyperadenylation of poly(A) tails [11,25,26,28]. It has also been demonstrated that 3′-processing affects the competency for mRNA export [29–31]. These studies suggest that the poly(A) tail is an important determinant of mRNA metabolism and that a proper tail added by the 3′-processing machinery is necessary for the efficient export of mRNA out of the nucleus [32,33]. Since 10% ethanol caused the export of HSP mRNAs to be blocked, we investigated the effects of ethanol on the length of poly(A) tails of HSP mRNAs. We observed that wild-type cells possessed HSP mRNAs with shorter poly(A) tails than did the rip1Δ mutant at 42 °C (Figure 4) and reconfirmed earlier results [25,26]. However, wild-type cells had longer poly(A) tails of HSP mRNAs in the presence of 10% ethanol than under heat-shocked conditions (Figure 4), indicating that hyperadenylation of HSP mRNAs was caused by 10% ethanol stress. These results were well correlated with the localization of HSP mRNAs, suggesting the hyperadenylation of HSP mRNAs to play a role in the nuclear retention of HSP mRNAs in cells treated with 10% ethanol. It is worth noting that the poly(A) tails of HSP mRNAs in the wild-type cells treated with 10% ethanol were slightly shorter than those in the rip1Δ cells under conditions of heat shock or 10% ethanol stress (Figure 4). The length of the poly(A) tail of PGK1 mRNA was not affected by the treatment with ethanol stress or heat shock (Figure 4).

We next investigated whether the ethanol concentration affects the length of poly(A) tails and nuclear export of HSP mRNAs. Levels of HSP mRNAs increased depending on the ethanol concentration and were maximal at approx. 9% ethanol (Figure 5A). In contrast, protein levels of HSPs reached a maximum at around 6% ethanol and were inversely correlated with the concentration at between 6% and 10% ethanol (Figure 5B).

The length of the poly(A) tail of each HSP mRNA clearly increased with the ethanol concentration (Figure 5C), indicating that ethanol has a concentration-dependent effect on the 3′-processing of HSP mRNAs. Indeed, the majority of HSP mRNAs in cells treated with 9–10% ethanol were distinctly longer than those in cells treated with 5% ethanol (Figure 5C), indicating that cells treated with 9–10% ethanol possess mostly hyperadenylated HSP mRNAs. Non-hyperadenylated HSP42 and SSA4 mRNAs clearly decreased with the ethanol concentration (Figure 5C). On the other hand, poly(A) tails of HSP mRNAs of various lengths were observed in the presence of 6–8% ethanol (Figure 5C). Cells treated with 6–8% ethanol still contained poly(A) tails of the same size as cells treated with 5% ethanol, indicating that cells treated with 6–8% ethanol possess both hyperadenylated and non-hyperadenylated HSP mRNAs.

Additionally, the export of HSP mRNAs was also affected by the concentration of ethanol. The nuclear accumulation of HSP mRNAs was not observed in cells treated with 5% ethanol, and

![Figure 4](image)

**Figure 4** Ethanol stress but not heat shock caused hyperadenylation of HSP mRNAs

Cells were treated with heat shock (42 °C) or ethanol stress (10%, v/v) for 30 min, and subsequently total RNA was prepared. The length of poly(A) tails of HSP mRNAs and PGK1 mRNA was monitored by RNaseH assay. The position of the deadenylated mRNAs was indicated by treatment with oligo dT.

A partial retention of HSP mRNAs in the nucleus was observed in cells treated with 7% ethanol (Figure 5D). It is probable that a certain amount of non-hyperadenylated HSP mRNA was exported from the nucleus and translated in cells treated with 7% ethanol. On the other hand, cells treated with 10% ethanol showed a clear nuclear accumulation of HSP mRNAs (Figure 3). It is likely that the little non-hyperadenylated HSP mRNA in cells treated with a higher ethanol concentration was one of the reasons for the clear nuclear retention and serious translational failure of HSP mRNAs.

**Poly(A) tails of HSP mRNAs were quickly shortened on elimination of ethanol stress**

We also examined whether the hyperadenylation of HSP mRNAs caused by ethanol is a reversible modification or not. Effects of the shift from SD medium with 10% ethanol to fresh SD medium without ethanol on the length of poly(A) tails of HSP mRNAs were investigated (Figure 6A). When cells were transferred to fresh medium after treatment with 10% ethanol, poly(A) tails of HSP mRNAs were shortened quickly (within 5 min) (Figure 6A). Protein levels of HSPs were also apparently increased by the shift to fresh SD medium (Figure 6B). Additionally, in the in situ hybridization analysis, there was no nuclear retention of HSP mRNAs following the shift to fresh SD medium for 10 min (Figure 6C). Protein synthesis of HSPs after the removal of ethanol was rapidly carried out even under conditions where further transcription was blocked by thiolutin (Figure 6D), indicating that HSP mRNAs synthesized and retained in the nucleus during the treatment with 10% ethanol were exported and underwent translation after the elimination of ethanol stress. These results suggest that hyperadenylated HSP mRNAs might be easily converted to normal forms that contain poly(A) tails of the proper length and are competent in nuclear export after the elimination of ethanol.
Figure 5  Dose-dependent effects of ethanol on the length of poly(A) tails of HSP mRNAs

Cells were treated with ethanol (EtOH) stress (5–10 %, v/v) for 30 min, and subsequently total RNA was prepared. (A and B), Levels of HSP mRNAs and HSPs were monitored by Northern blotting (A) and Western blotting (B) respectively. (C) The length of poly(A) tails of HSP mRNAs was analysed by RNaseH assay. The position of the deadenylated mRNAs was indicated by treatment with oligo dT (with dT). (D) The intracellular localization of HSP mRNAs in cells treated with 5 or 7 % ethanol stress for 30 min was monitored by FISH and nuclear DNA was stained with DAPI. The white bar represents 1 μm.

DISCUSSION

It has been thought that cellular responses induced by heat shock and ethanol stress are similar and overlap considerably [13,14]. Indeed, both heat shock and ethanol stress induce the transcriptional activation of HSP genes (Figure 1A) [34] and block the export of bulk poly(A)+ mRNA [3,8,9]. However, we previously reported that the mislocalization of Rat8p is caused by ethanol but not by heat shock [12]. Furthermore, we demonstrate in the present study that ethanol stress causes different responses from heat shock in the nuclear export and 3′-processing of HSP mRNAs (HSP42, SSA4 and HSP104). HSP mRNAs induced by 10 % ethanol were hyperadenylated and retained in the nucleus, whereas HSP mRNAs induced by heat shock were preferentially exported to the cytoplasm without hyperadenylation (Figures 3 and 4). Rollenhagen et al. [15] have also reported the nuclear accumulation of SSA4 mRNA upon ethanol stress. These findings clearly indicate that yeast cells respond to the two types of stress differently, and suggest diversity in the export and 3′-processing of HSP mRNA under different stressed conditions.

Ethanol had a concentration-dependent effect on the length of the poly(A) tail and the nuclear export of each HSP mRNA (Figure 5C). Thus a partial retention of HSP mRNAs in the nucleus was caused by 7 % ethanol (Figure 5D), and most of the HSP mRNAs were retained in the nucleus in a hyperadenylated state by 10 % ethanol (Figures 3 and 4). As a consequence of the nuclear retention of HSP mRNAs, there was an inverse correlation between protein levels of HSPs and the ethanol concentration (Figure 5B). It is presumable that changes in protein levels of HSPs largely reflect the nuclear export of HSP mRNAs in cells treated with 5–10 % ethanol. Although hyperadenylation itself is not sufficient for the nuclear retention of mRNA and not a general consequence of defects in mRNA export [11,30,35], the poly(A) tail is an important determinant of mRNA metabolism and a proper poly(A) tail added by the 3′-processing machinery...
Yeast stress responses in HSP mRNA export

Figure 6 The poly(A) tails of HSP mRNAs were rapidly shortened on elimination of ethanol

After treatment with ethanol (EtOH) stress (10%, v/v) for 30 min, cells were collected and suspended in fresh SD medium without ethanol and incubated for 0–15 min. Cells treated with 10% ethanol for 45 min (w/o shift) were used as the control sample. (A) The length of poly(A) tails of HSP mRNAs was analysed by RNaseH assay. The position of the deadenylated mRNAs was indicated by treatment with oligo dT. (B) Protein levels of HSPs were monitored by Western blotting. (C) The intracellular localization of HSP mRNAs was monitored by FISH, and nuclear DNA was stained with DAPI. Cells treated with ethanol stress (10%, v/v) for 30 min were incubated for 10 min in fresh SD medium without ethanol. The white bar represents 1 μm. (D) After treatment with ethanol stress (10%, v/v) for 30 min, cells were further incubated for another 25 min with thiolutin (5 μg/ml) and 10% ethanol, and then suspended in fresh SD medium without ethanol but containing thiolutin (5 μg/ml) and incubated for 0–15 min. The control sample (w/o thiolutin) was treated with the same procedure but without thiolutin.

is necessary for the efficient export of mRNA out of the nucleus [11,25,26,28,29,31]. Taking all this into account, it is probable that changes in the 3′-processing caused by ethanol stress have negative effects on the smooth export of HSP mRNAs and is one of the reasons for their nuclear retention in the ethanol-treated cells.

Several recent studies suggested that various forms of stress inhibit deadenylation and initiate a general stabilization of multiple mRNAs in eukaryotic cells [36–40]. It seems no wonder that ethanol stress also inhibits deadenylation and stabilizes multiple mRNAs. Indeed, the amount of HSP mRNA, once having increased upon ethanol stress, did not decrease during the period of the experiment (Figure 1A). The HSP mRNAs might be stabilized through the inhibition of deadenylation upon ethanol stress. Intriguingly, the length of the poly(A) tails of HSP mRNAs changed reversibly according to the ethanol concentration (Figures 5C and 6A). Thus mRNA export and protein synthesis of HSPs were quickly restored when ethanol stress was eliminated (Figures 6B and 6C). These results suggest the activities of deadenylase and 3′-processing factors to be modified by ethanol in a concentration-dependent and reversible manner.

Furthermore, the results presented in the present paper suggest that hyperadenylation and nuclear retention of mRNAs serve as a means to control gene expression in ethanol-treated cells; i.e. stabilized HSP mRNAs in a hyperadenylated state are pooled in the nucleus upon ethanol stress but, when ethanol is eliminated, they are exported via shortening of the poly(A) tail and then translated. Since mRNA is never multiplied downstream from transcription, the intervening steps between transcription and translation can function to block or delay the gene expression during the response to stress. Additionally, eukaryotic cells have more complicated steps between transcription and translation (e.g. polyadenylation and the nuclear export of mRNA) than prokaryotic cells. Therefore it is possible that the hyperadenylation and nuclear retention of mRNAs are regulatory
mechanisms of gene expression in eukaryotic cells to adapt to environmental conditions. Although the precise mechanism by which the hyperadenylation and nuclear retention of HSP mRNAs are induced in ethanol-treated cells is unknown, control of the length of the poly(A) tail via the 3′-processing machinery seems more important in the cellular stress response than we supposed.

Since the expression of HSP genes was negatively regulated downstream of transcription in the ethanol-treated cells, the transcriptional activation of HSP genes caused by ethanol is deceptively futile. Such futility may imply that stress-responsive transcriptional activation is not always timely, and also suggests to us that the expression of a certain number of genes is repressed in spite of transcriptional activation under particular conditions. On the other hand, such untimely transcriptional activation may imply that eukaryotic cells can afford to pool transcripts for the future after getting through a crisis of stress. Interestingly, yeast cells showed sufficient resistance to 10% ethanol without increased protein levels of HSPs (at least Hsp42p, Ssa4p, Hsp82p and Hsp104p) (Figures 2A and 2B), indicating that increased expression of these HSP genes is not vital to survival in the presence of 10% ethanol. These HSPs may play important roles in the recovery from the damage caused by ethanol stress, since their protein levels rapidly increased when ethanol was eliminated (Figure 6B). The results may support the view that nuclear retention of HSP mRNAs, is a reasonable response for cells treated with 10% ethanol. They also imply that other mRNAs, excluding these HSP mRNAs, are preferentially exported to the cytoplasm upon ethanol stress. If this is the case, the supposed mRNAs would have non-hyperadenylated poly(A) tails under conditions of ethanol stress. The analysis of mRNAs preferentially exported in ethanol-stressed cells may provide a clue as to the mechanisms of selective mRNA export and high tolerance to ethanol [41], and further investigation to identify such mRNAs is now underway.

We are sincerely grateful to Dr R. Parker, Dr C. Schüller, Dr P. Silver and Dr F. Stutz for providing materials, constructive discussion and advice. This study was supported by research grants from the Mitsubishi Chemical Corporation Fund and the Japanese Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

20 Dower, K. and Rosbash, M. (2002) T7 RNA polymerase-directed transcripts are processed in yeast and link 3′ end formation to mRNA nuclear export. RNA 8, 686–697
Yeast stress responses in HSP mRNA export


37 Bénard, L. (2004) Inhibition of 5′ to 3′ mRNA degradation under stress conditions in Saccharomyces cerevisiae: from GCN4 to MET16. RNA 10, 458–468


Received 16 November 2007/21 April 2008; accepted 28 April 2008
Published as BJ Immediate Publication 28 April 2008, doi:10.1042/BJ20071567
SUPPLEMENTARY ONLINE DATA

Heat shock and ethanol stress provoke distinctly different responses in 3′-processing and nuclear export of HSP mRNA in Saccharomyces cerevisiae

Shingo IZAWA1, Takeomi KITA, Kayo IKEDA and Yoshiharu INOUE

Laboratory of Molecular Microbiology, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

Table S1 List of primers and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer/oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers used for DNA probes of Northern blot analysis</td>
<td></td>
</tr>
<tr>
<td>HSP10-N1</td>
<td>5′-CTGATAGAAAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP10-N2</td>
<td>5′-GGTAAATGGGAGATCTTGTCAGTG-3′</td>
</tr>
<tr>
<td>HSP12-N1</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP12-N2</td>
<td>5′-AAACCTGTCGGTCG-3′</td>
</tr>
<tr>
<td>HSP26-N1</td>
<td>5′-CTGATAGAAAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP26-N2</td>
<td>5′-GGTAAATGGGAGATCTTGTCAGTG-3′</td>
</tr>
<tr>
<td>HSP30-N1</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP30-N2</td>
<td>5′-AAACCTGTCGGTCG-3′</td>
</tr>
<tr>
<td>HSP42-N1</td>
<td>5′-CTGATAGAAAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP42-N2</td>
<td>5′-GGTAAATGGGAGATCTTGTCAGTG-3′</td>
</tr>
<tr>
<td>HSP78-N1</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP78-N2</td>
<td>5′-AAACCTGTCGGTCG-3′</td>
</tr>
<tr>
<td>DNA probes for in situ hybridization</td>
<td></td>
</tr>
<tr>
<td>SSA4-1</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>SSA4-2</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>SSA4-3</td>
<td>5′-AAACCTGTCGGTCG-3′</td>
</tr>
<tr>
<td>HSP42-1</td>
<td>5′-CTCTTTGCTGATCAGTATTTCTCTCTCTCTGCTGCCTTCTG-3′</td>
</tr>
<tr>
<td>HSP42-2</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP42-3</td>
<td>5′-AAACCTGTCGGTCG-3′</td>
</tr>
<tr>
<td>HSP104-N1</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>HSP104-N2</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>Oligonucleotides for RNaseH digestion</td>
<td></td>
</tr>
<tr>
<td>SSA4-D</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>HSP42-D</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>HSP104-D</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>PGK1-D</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>Oligo dT</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>Oligonucleotides for detection of HSP mRNA bands in RNaseH assay</td>
<td></td>
</tr>
<tr>
<td>SSA4-P1</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>SSA4-P2</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>SSA4-P3</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP42-P1</td>
<td>5′-CTCTTTGCTGATCAGTATTTCTCTCTCTGCTGCCTTCTG-3′</td>
</tr>
<tr>
<td>HSP42-P2</td>
<td>5′-GAGACAGGCAATGTGTCG-3′</td>
</tr>
<tr>
<td>HSP42-P3</td>
<td>5′-CTCTTTGCTGATCAGTATTTCTCTCTCTGCTGCCTTCTG-3′</td>
</tr>
<tr>
<td>HSP104-P1</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>HSP104-P2</td>
<td>5′-GTTAAGAGGAAGAAGTGATGTCGATCTTCATGCACTATTTG-3′</td>
</tr>
<tr>
<td>PGK1-P1</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>PGK1-P2</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>PGK1-P3</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
<tr>
<td>PGK1-P4</td>
<td>5′-GGCAACACCTGACCGCTGCAAATAATTACT-3′</td>
</tr>
</tbody>
</table>

Received 16 November 2007/21 April 2008; accepted 28 April 2008
Published as BJ Immediate Publication 28 April 2008, doi:10.1042/BJ20071567

1 To whom correspondence should be addressed (email izawa@kais.kyoto-u.ac.jp).