Stress response in yeast mRNA export factor: reversible changes in Rat8p localization are caused by ethanol stress but not heat shock

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Accepted 28 April 2004
Journal of Cell Science 117, 4189-4197 Published by The Company of Biologists 2004
doi:10.1242/jcs.01296

Summary
Ethanol stress (10% v/v) causes selective mRNA export in Saccharomyces cerevisiae in a similar manner to heat shock (42°C). Bulk poly(A)+ mRNA accumulates in the nucleus, whereas heat shock protein mRNA is exported under such conditions. Here we investigated the effects of stress on mRNA export factors. In cells treated with ethanol stress, the DEAD box protein Rat8p showed a rapid and reversible change in its localization, accumulating in the nucleus. This change correlated closely with the blocking of bulk poly(A)+ mRNA export caused by ethanol stress. We also found that the nuclear accumulation of Rat8p is caused by a defect in the Xpo1p/Crm1p exportin. Intriguingly, the localization of Rat8p did not change in heat shocked cells, suggesting that the mechanisms blocking bulk poly(A)+ mRNA export differ for heat shock and ethanol stress. These results suggest that changes in the localization of Rat8p contribute to the selective export of mRNA in ethanol stressed cells, and also indicate differences in mRNA export between the heat shock response and ethanol stress response.

Key words: Stress response, mRNA export, Rat8p/Dbp5p, Xpo1p/Crm1p, Ethanol

Introduction
In eukaryotic cells, mRNA is synthesized and processed in the nucleus and exported to the cytoplasm through nuclear pore complexes (NPCs). In Saccharomyces cerevisiae, matured mRNA is exported as messenger ribonucleoprotein complexes (mRNPs) by mRNA export factors including RNA binding proteins (Sub2p, Mud2p, Yra1p, Yra2p, Mex67p, Npl3p, Nab2p and Hrp1p), nucleoporins and NPC-associated proteins (Mtr2p, Gle1p, Gle2p, Rip1p/Nup42p and Rat7p/Nup159p) and DEAD box RNA helicase (Rat8p/Dbp5p). The functions and interactions of these export factors have been well reviewed (Cole, 2000; Zenklusen and Stutz, 2001; Lei and Silver, 2002a). For the efficient nuclear export of mRNA, appropriate pre-mRNA processing steps are necessary (Bentley, 1999; Brodsky and Silver, 2000; Proudfoot, 2000; Lei and Silver, 2002b; Sträßer et al., 2002; Jensen et al., 2003; Reed, 2003). Recently, it has been suggested that the co-transcriptional recruitment of processing and export factors is a mechanism for efficient production of export-competent mRNPs (Lei et al., 2001; Lei and Silver, 2002a; Reed and Hurt, 2002). Several studies also show that polyadenylation during 3'-end processing is coupled to the efficient export of mRNA (Hilleren et al., 2001; Jensen et al., 2001; Hammell et al., 2002; Lei and Silver, 2002b). Both hyperadenylation and defects in polyadenylation lead to the blocking of mRNA export and the apparent accumulation of pre-mRNA at the site of transcription (Brodsky and Silver, 2000; Jensen et al., 2001). Conversely, several mutant strains defective in mRNA export also exhibit hyperadenylation of the 3'-end of mRNA (Hilleren and Parker, 2001).

mRNA export is a target in the regulation of stress. Yeast cells exposed to heat shock (42°C) shut down the synthesis of most proteins with the exception of stress-responsive proteins such as heat shock proteins (HSPs). This effect is partly caused by selective mRNA export. Cells alter not only their transcriptional patterns but also the types of mRNA to be exported from the nucleus in order to adapt rapidly to heat shock stress. Under heat shocked conditions, stress-induced transcripts such as SSA4 encoding HSP70 are efficiently exported, whereas bulk poly(A)+ mRNA accumulates in the nucleus (Tani et al., 1995; Saavedra et al., 1996; Krebber et al., 1999). The mechanisms of this selective mRNA export caused by heat shock seem to be quite complex. Initially, it was proposed that heat shock mRNA (hs mRNA) is exported through a specific pathway defined by the nucleoporin Rip1p/Nup42p (Saavedra et al., 1997; Stutz et al., 1997). The shut-off of mRNA export by stress also involves the dissociation of Npl3p, a heterogeneous nuclear ribonucleoprotein (hnRNP), from mRNA under these conditions (Krebber et al., 1999). However, it has recently been reported that Rip1p also participates in the export of non-hs mRNA at 42°C (Vainberg et al., 2000). It has also been reported that other general mRNA export factors are involved in the export of hs mRNA at elevated temperatures, suggesting that hs mRNA and non-hs mRNA are exported via similar pathways (Vainberg et al., 2000; Hurt et al., 2000). The pathways of mRNA export under conditions of stress remain controversial.

As the quality of the mRNA affects its competency for export (Lei and Silver, 2002b; Jensen et al., 2003; Reed, 2003),
changes in the processing of pre-mRNA may occur and regulate the export competency of mRNA under stressed conditions. It has been reported that splicing is blocked following heat shock (Yost and Lindquist, 1991; Vogel et al., 1995). There is also a possibility that export factors and NPCs affect selective mRNA export under stressed conditions. It is known that the distribution of Npl3p and Hrp1p changes during stress (Kreber et al., 1999; Henry et al., 2003). However, it is still not well understood how the functions of export factors and NPCs are altered in response to stress. At present, it is quite difficult to estimate the contribution of export factors to the selective export of mRNA, as the stress responses of mRNA processing factors and export factors remain to be clarified.

Ethanol stress as well as heat shock causes selective mRNA export. Bulk poly(A)+ mRNA accumulates in the nucleus, whereas mRNA of HSPs is exported from the nucleus in ethanol stressed cells (Saavedra et al., 1996; Kreber et al., 1999). Adverse effects of ethanol on yeast cells are key problems facing brewers and the ethanol stress response of yeast has been investigated with great interest. It has been reported that heat shock and ethanol stress induce an almost identical stress response in yeast (Piper, 1995). Both stresses strongly induce the expression of HSPs and accumulation of trehalase in order to address protein denaturation and membrane disruption (Attfield, 1987; Piper et al., 1994). It is also known that both stresses cause changes in membrane lipid composition and increased fluidity of plasma membranes (Swan and Watson, 1999). However, although pre-exposure to heat shock leads to the acquisition of ethanol tolerance, the converse is not true. Ethanol-treated cells are actually hypersensitive to heat, indicating that there are differences in stress response between heat shock stress and ethanol stress (Piper et al., 1994; Piper, 1995).

The differences between heat shock stress and ethanol stress in the mechanisms of selective mRNA export are completely unknown. In this study, we investigated the changes in mRNA export factors under stressed conditions. We found that the localization of Rat8p/Dhp5p, an essential mRNA export factor, changed in ethanol stressed cells. Rat8p is one of the DEAD box proteins and shuttles between the nucleus and the cytoplasm (Snay-Hodge et al., 1998; Hodge et al., 1999). As it is usually concentrated in the cytoplasmic fibrils of NPCs where it interacts with Rat7p/Nup159p and Gle1p, Rat8p has been suggested to participate in a terminal step of mRNA export through the removal of proteins that accompany mRNA through NPCs (Cole, 2000; Hodge et al., 1999; Strahm et al., 2007). In this study, we found that the intracellular localization of Rat8p changed rapidly and reversibly in response to ethanol stress but not to heat shock. The change correlated with the blocking of bulk poly(A)+ mRNA export caused by ethanol stress. These results suggest that changes in the localization of export factors caused by stress also contribute to the selective export of mRNA, and indicate a difference in mRNA export between the heat shock response and ethanol stress response.

### Materials and Methods

**Yeast strains and medium**

Saccharomyces cerevisiae strains used in this study were W303-1A (MATa ade2-1 ura3-1 leu2-3, 112 trp1-1 his3-1, 15 can1-100), SWY2257 (MATa MTR2-GFP: HIS5 ade2-1::ADE2 ura3-1 leu2-3, 112 trp1-1 his3-11, 15 can1-100) and spox-1 (MATa ade2-1 ura3-1 leu2-3, 112 trp1-1 his3-1, 15 can1-100 spox::LEU2 spox-1::HIS3). SWY2257 and spox-1 were kindly donated by Dr S. R. Wente and Dr K. Weis, respectively (Stade et al., 1997; Strahm et al., 1999). Cells were cultured in 50 ml SD 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.

**Plasmid construction**

Ylp-RAT7-GFP

A 1698 bp fragment encoding the ORF of RAT7 was amplified using primers as follows: 5′-GCTGATACTAAAGAAACTTAGGAAAAGGGC-3′ and 5′-AGCTAATATCAGTGCAAGGCTAATGTC-3′. The amplicon was digested with SfiI/NheI and cloned into the SfiI/NheI sites of pPS1630 to construct Ylp-RAT7-GFP. To integrate the RAT7-GFP gene at the chromosomal RAT7 locus, Ylp-RAT7-GFP was linearized by SalI and introduced into yeast cells.

Ylp-MEX67-GFP

A 971 bp fragment encoding the ORF of MEX67 was amplified using primers as follows: 5′-GCATTAGGCGAATCATGAGCGTTTCGACAAGCAAA-GGGC-3′ and 5′-TCCTTCTCCTTGTCCGACATGCACTGACAAAGGGTGTGTGGATCT-3′. The amplicon was digested with XbaI/NheI and cloned into the XbaI/NheI sites of pPS1630 to construct Ylp-MEX67-GFP. To integrate the MEX67-GFP gene at the chromosomal MEX67 locus, Ylp-MEX67-GFP was linearized with Hpal and introduced into yeast cells.

pAUR-GFP-RAT8

The integrated type GFP-RAT8 plasmid was constructed by cloning the XbaI/KpnI fragment of pCS835 (Snay-Hodge et al., 1998) containing GFP-RAT8 into the XbaI/KpnI sites of pAUR101 (Takara, Kyoto, Japan). pAUR-GFP-RAT8 was digested by BsrWI and introduced into yeast cells to integrate the GFP-RAT8 gene.

pRS426-GFP-RAT8 and pRS426-RAT8

The multicopy type GFP-RAT8 plasmid (pRS426-GFP-RAT8) was constructed by cloning the BamHI/KpnI fragment of pCS835 containing GFP-RAT8 into the BamHI/KpnI sites of pRS426 (Christianson et al., 1992). The multicopy type RAT8 plasmid (pRS426-RAT8) was constructed by cloning the BamHI/KpnI region of the RAT8 gene into the BamHI/KpnI sites of pRS426.

pRS315-GFP-MTR2 was donated by Dr E. Hurt (Santos-Rosa et al., 1998). pTS-RIP1-GFP was provided by Dr Y. Kikuchi (Takahashi et al., 2000). pCS835 (GFP-RAT8) was donated by Dr C. N. Cole (Snay-Hodge et al., 1998). pFS2146 (pHA-YRA1) and pFS2262 (pMyc-YRA2) were provided by Dr F. Stutz (Zenklusen et al., 2001). pKW430 (NLS-NE-GFP) was donated by Dr K. Weis (Stade et al., 1997). pRS-GFP-Yap1 and pRS-GFP-Yap1 cm46A5 was provided by Dr S. Kuge (Kuge et al., 1997). pNPL3-GFP and pCS38 (GFP-Npl3 fusion) were donated by Dr T. Lithgow (Gratzer et al., 2000) and Dr C. Guthrie (Gilbert et al., 2001), respectively.

### In situ hybridization and oligonucleotide probes

The oligo(dT)39 probe was labeled at its 3′-end with digoxigenin using a DIG Oligonucleotide Tailoring Kit (Roche Diagnostics, Mannheim, Germany). The SSA4 probe was labeled as follows: SSA4-1, 5′-GTTAGAGGAAAAACTAGAAGATCCTGCTGACTTACAAGCATTGGTG-3′; SSA4+2, 5′-GAGAAGCTACAAATAGTACTGCAATTGCTAATTACTGATTGTAATATTATATATAT-3′. These probes
were directly labeled with Cy3 fluorescent dye using Label IT Cy3 Labeling Kits (Mirus, Madison, WI, USA). In situ hybridization assays to detect poly(A)+ mRNA or SSA4 mRNA were performed as described previously (Amberg et al., 1992; Long et al., 1995).

Microscopic analysis
The method of immunofluorescence microscopic observation for yeast cells was described previously (Izawa et al., 1999). Anti-nuclear pore complex proteins antibody (mAb414) was purchased from Berkley Antibody, Berkley, CA, USA (Aris and Blobel, 1989). Anti-HA, anti-Myc and anti-GFP antibodies were purchased from Santa Cruz Biotechnology Inc., CA, USA. The secondary antibody (Fluorescent anti-mouse polyvalent immunoglobulins) was purchased from Sigma, St Louis, MO, USA. Fluorescent microscope system was BX60 (Olympus, Tokyo, Japan) with the imaging software IPLab (Scanalytics Inc. Fairfax, VA, USA). Whole cells were visualized by phase-contrast microscopy.

Results
Ethanol affects the localization of nucleoporins
The mechanisms of nucleocytoplasmic transport in yeast under ethanol stressed conditions still remain to be clarified. The transport of macromolecules including mRNA between the nucleus and cytoplasm takes place through nuclear pore complexes (NPCs) that contain nucleoporins. Most of the nucleoporins play critical roles in the nucleocytoplasmic transport of macromolecules (Cole, 2000). Thus, a functional and physiological analysis of nucleoporins should contribute to our understanding of the mechanisms of nucleocytoplasmic transport under ethanol stressed conditions.

We first investigated whether ethanol affects the cellular localization of nucleoporins by immunofluorescence microscopy using the monoclonal antibody mAb414, which can cross-react with yeast nucleoporins (Aris and Blobel, 1989). Under non-stressed conditions, mAb414 stained the rim of the yeast nucleus with a punctate pattern and no staining of internal nuclear components was observed (Fig. 1A). However, under ethanol stressed conditions (10% v/v), internal nuclear components were clearly stained with mAb414 (Fig. 1A). This result indicates that the localization of nucleoporins changes under ethanol stressed conditions, and also suggests that nucleoporins show loss or change of function under such conditions.
It is well known that 10% ethanol as well as heat shock (42°C) inhibits the export of bulk poly(A)+ mRNA (Saavedra et al., 1996). We also observed the accumulation of bulk poly(A)+ mRNA in the nucleus and the selective export of SSA4 mRNA under such conditions (Fig. 1B,C). We inferred from these results that changes in the localization of nucleoporins that function as mRNA export factors may lead to the blocking of the export of bulk poly(A)+ mRNA in ethanol stressed cells. Therefore, we then examined the effects of ethanol stress on the localization of each mRNA export factor, and compared them with the effects of heat shock stress as it is still not clear whether ethanol and heat shock affect mRNA export in the same way.

Localization of mRNA export factors under stressed conditions

Using various GFP fusion proteins and immunofluorescent techniques, we investigated the distribution of mRNA export factors. We observed the localization of Npl3p under stressed conditions and reconfirmed earlier results (Krebber et al., 1999) (data not shown). The localization of Rat7p was also observed and it was not changed by heat shock (42°C) or ethanol stress (10% v/v) (Fig. 2). The distribution of Mtr2p, Rip1p, Yra1p, Yra2p, and Sub2p did not change either (data not shown).

In contrast, the localization of Rat8p clearly changed in response to ethanol stress (Fig. 2). GFP-Rat8p (pCS835) localized within internal nuclear components, indicating that Rat8p is concentrated in the nucleus in ethanol stressed cells. This change happened within 5 minutes of the addition of ethanol (10% v/v) but not in response to heat shock (42°C). We examined the levels of GFP-Rat8p protein with western blotting using anti-GFP antibody and an integrated type plasmid (pAUR-GFP-RAT8), and found no significant change in ethanol stressed cells (data not shown).

Reversible change in the localization of Rat8p

Because the localization of GFP-Rat8p changed quickly after the addition of ethanol (within 5 minutes), we examined whether this change is responsive to ethanol concentration. When cells were transferred to fresh medium after treatment with 10% ethanol, the distribution of GFP-Rat8p reverted to normal with a re-localization around the rim of the nucleus within 5 minutes of the change of medium (Fig. 3). This result indicates that the change in the localization of Rat8p is reversible. The export of bulk poly(A)+ mRNA resumed in the fresh medium as the distribution of Rat8p returned to normal (Fig. 3).

We further investigated the concentration-dependent effects of ethanol on mRNA export and the change in the localization of Rat8p. As shown in Fig. 4, the localization of GFP-Rat8p partly changed in response to 6% ethanol and completely changed in response to 9% ethanol. Corresponding to this change, a partial accumulation of bulk poly(A)+ mRNA in the nucleus was observed with 6% ethanol, whereas a complete accumulation was observed with 9% ethanol. The minimum concentration of ethanol (6%) required to cause the accumulation of bulk poly(A)+ mRNA was consistent with the minimum concentration causing a change in the localization of Rat8p. These results indicate that Rat8p reversibly changes its localization according to the ethanol concentration and that this change correlates well with the blocking of bulk poly(A)+ mRNA export caused by ethanol stress. The change in the localization of Rat8p may be one of the reasons for the accumulation of bulk poly(A)+ mRNA in the nucleus caused by ethanol stress.

Function of Xpo1p/Crm1p under ethanol stressed conditions

It has been reported that Rat8p shuttles between the nucleus and cytoplasm (Hodge et al., 1999). Rat8p is exported in a Xpo1p/Crm1p-dependent manner, thus Rat8p is concentrated in the nucleus in xpo1-1 cells at a non-permissive temperature (Hodge et al., 1999), a finding we confirmed here (Fig. 5A). It has also been reported that Rat8p can enter, but not exit, the nucleus in strains with mutations affecting the Ran-GTPase system (Hodge et al., 1999). This indicates that the correct localization of Rat8p is presumably maintained by active export. Thus we investigated whether a defect in Xpo1p caused the
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nuclear accumulation of Rat8p in ethanol stressed cells. In order to examine the function of Xpo1p, we investigated the localization of nuclear localization sequence-nuclear export sequence (NLS-NES)-GFP, an artificial cargo of Xpo1p (Stade et al., 1997). As shown in Fig. 5B, ethanol stress (10% v/v) caused the accumulation of NLS-NES-GFP in the nucleus, indicating that Xpo1p is not functional under conditions of 10% ethanol stress. NLS-NES-GFP partly accumulated in the nucleus in response to 6% ethanol (Fig. 5B) and completely accumulated at 9% ethanol concentration (data not shown). Additionally, the export of NLS-NES-GFP by Xpo1p was resumed upon elimination of the ethanol (data not shown). By contrast, heat shock did not affect the localization of NLS-NES-GFP (Fig. 5B).

We verified that Xpo1p is dysfunctional using another cargo molecule originally exported by Xpo1p in yeast cells. Yap1p, an oxidative stress-responsive transcription factor, is also known to be exported to the cytoplasm by Xpo1p (Kuge et al., 1998; Yan et al., 1998). Since Xpo1p efficiently exports Yap1p under steady state conditions, Yap1p localizes mainly in the cytoplasm under non-stressed conditions. On the other hand, Yap1p is not exported under conditions of oxidative stress (Kuge et al., 1997; Kuge et al., 1998; Yan et al., 1998; Izawa et al., 1999). Yap1p is oxidized and forms a disulfide bond(s) within the NES at its C-terminus under oxidative stress conditions, preventing Yap1p from interacting with Xpo1p and causing it to accumulate in the nucleus (Kuge et al., 1997; Kuge et al., 1998; Kuge et al., 2001). As shown in Fig. 5C, ethanol stress as well as oxidative stress (0.4 mM H2O2) caused the accumulation of GFP-Yap1p in the nucleus, whereas heat shock did not affect the localization of GFP-Yap1p (Fig. 5C). Furthermore, we used the mutant Yap1p cm46A5 to demonstrate that the NES of Yap1p is still functional under 10% ethanol stress. This mutant has threonine

Fig. 3. Reversible change in the localization of GFP-Rat8p. Yeast cells in exponential phase were treated with 10% ethanol for 30 minutes, collected, and transferred to fresh SD medium without ethanol. GFP-Rat8p fluorescence was visualized before ethanol treatment (w/o stress) after ethanol treatment for 30 minutes (10% EtOH) and 5 minutes after the shift to fresh SD medium. Cells at each stage were also fixed for in situ hybridization using Cy3-labeled SSA4 probes to detect poly(A)+ RNA and DAPI staining.

Fig. 4. Concentration-dependent effects of ethanol on the localization of Rat8p and export of bulk poly(A)+ mRNA. Yeast cells in exponential phase were treated with ethanol (6-9% v/v) for 15 minutes and the localization of GFP-Rat8p was monitored. Cells at each ethanol concentration were also fixed for in situ hybridization using Cy3-labeled SSA4 probes to detect poly(A)+ RNA and DAPI staining.
substitutions at Cys598 and Cys629 and an alanine substitution at Cys620, and can be exported by Xpo1p even under conditions of oxidative stress because it does not form a disulfide bond within the NES (Kuge et al., 1997; Kuge et al., 1998). Actually, GFP-Yap1p cm46A5 did not accumulate in

![Image](https://example.com/image.png)

the nucleus under oxidative stress (Fig. 5D). However, ethanol stress did cause this mutant to accumulate in the nucleus (Fig. 5D), indicating that the accumulation of Yap1p in ethanol stressed cells was likely to be caused by a dysfunction of Xpo1p rather than by a defect in the NES. Taken together, these results indicate that the mislocalization of Rat8p in ethanol stressed cells partly arises from a defect in the exportin Xpo1p/Crm1p.

**Overexpression of Rat8p attenuated nuclear accumulation of bulk poly(A)+ mRNA in ethanol stressed cells**

Overexpression of Rat8p completely prevents the nuclear accumulation of poly(A)+ mRNA in xpo1-1 cells (Hodge et al., 1999). Since xpo1-1 cells overexpressing Rat8p show a drastic increase in the level of cytoplasmic Rat8p even at the non-permissive temperature of 37°C, it has been suggested that the primary function of Xpo1p for mRNA export may be to maintain Rat8p outside the nucleus and that Rat8p on the cytoplasmic face of NPCs is essential for mRNA export (Tseng et al., 1998; Hodge et al., 1999; Schmitt et al., 1999). Thus, we investigated the effect of overexpression of Rat8p on mRNA export in wild-type cells under ethanol stressed conditions. As shown in Fig. 6A, wild-type cells overexpressing Rat8p also showed a drastic increase in the level of cytoplasmic Rat8p. Little GFP-Rat8p was observed in the nucleus under non-stressed conditions. On the other hand, a certain amount of overexpressed Rat8p accumulated in the nucleus in ethanol stressed cells, but most of it still localized in the cytoplasm, indicating that these cells contain abundant cytoplasmic Rat8p (Fig. 6A). Enrichment of cytosolic Rat8p means that a certain amount of Rat8p exists on the cytosolic face of NPCs and can interact with nuclear pore complex proteins (Nups) such as Rat7p and Gle1p even under ethanol stressed conditions.

We further found that overexpression of Rat8p affected mRNA export under conditions of ethanol stress. The nuclear accumulation of bulk poly(A)+ mRNA in ethanol stressed cells was attenuated by overexpression of Rat8p (Fig. 6B). Most, but not all cells overexpressing Rat8p continued the export of bulk poly(A)+ mRNA under

![Fig. 5. Function of Xpo1p/Crm1p under ethanol stressed conditions.](https://example.com/image.png)

(A) GFP-Rat8p accumulates in the yeast cell nucleus at the non-permissive temperature of 37°C in xpo1-1 cells. (B) Yeast cells expressing NLS-NES-GFP in exponential phase at 28°C were treated with ethanol (6 or 10% v/v) or heat shock (42°C) for 15 minutes, and then the cellular localization of NLS-NES-GFP-fusion protein was monitored and compared to non-stressed cells (w/o stress). (C,D) Cells expressing GFP-Yap1p (C) or GFP-Yap1p-cm46A5 (D) in exponential phase were treated with stress (10% ethanol, heat shock at 42°C or 0.4 mM H₂O₂) for 15 minutes and the cellular localization of GFP-fusion proteins was monitored.
conditions of 10% ethanol stress for at least 30 minutes, whereas cells without overexpression of Rat8p showed the nuclear accumulation of bulk poly(A)+ mRNA. These results indicate that Rat8p on the cytosolic face of NPCs enables cells to export bulk poly(A)+ mRNA under conditions of ethanol stress, and that ethanol stressed cells usually do not maintain Rat8p on the cytoplasmic face of NPCs. Most of the free Rat8p in the cytosol seems to have no effect on the export of mRNA, since overexpression of Rat8p did not affect the export of mRNA and growth of non-stressed cells (data not shown). Bulk poly(A)+ mRNA gradually accumulated in the nucleus even in cells overexpressing Rat8p after 30 minutes of ethanol stress (data not shown). This seems quite reasonable as other factors besides Rat8p must affect the export of bulk poly(A)+ mRNA in ethanol stressed cells.

**Discussion**

Under steady state conditions, Rat8p mainly localizes on the cytoplasmic face of NPCs and associates directly with the Rat7p complex (Tseng et al., 1998; Hodge et al., 1999; Schmitt et al., 1999). We demonstrate here that ethanol stress leads to changes in the localization of Rat8p and that the nuclear accumulation of Rat8p arises partly from a defect in the exportin Xpo1p/Crm1p. Rat8p rapidly accumulated in the nucleus in response to ethanol stress (Figs 2-4), but the accumulated Rat8p soon returned to the original position (within 5 minutes) upon elimination of the ethanol (Fig. 3). The nuclear accumulation of Rat8p correlated closely with the blocking of the export of bulk poly(A)+ mRNA (Figs 3, 4). Furthermore, it has been reported that Rat8p also accumulates in the nucleus in a variety of mutant strains defective in mRNA export (Hodge et al., 1999). These findings indicate that the blocking of bulk poly(A)+ mRNA export caused by ethanol stress partly arises from the nuclear accumulation of Rat8p. As with Npl3p and Hpr1p (Krebber et al., 1999; Henry et al., 2003), Rat8p is an example of a mRNA export factor whose distribution changes in response to stress. Since the distribution of Rat8p changes in response to ethanol, it may be possible to regard Rat8p as an ethanol-responsive mRNA export factor. Rat8p is likely to regulate the efficiency of mRNA export via a change in its localization. If changes in the localization of Rat8p are directly correlated with the functional activity of Rat8p on the cytoplasmic face of NPCs, cells may regulate the mRNA export through rapid changes in Rat8p activity in response to ethanol stress.

As Rat8p shuttles between the nucleus and cytoplasm in an Xpo1p-dependent manner, Rat8p accumulates rapidly in the nucleus when the function of Xpo1p is blocked (Hodge et al., 1999). It is known that xpo1-1 cells accumulate bulk poly(A)+ mRNA in the nucleus at the non-permissive temperature of 37°C (Stade et al., 1997). We show here the possibility that ethanol stress causes a defect in Xpo1p (Fig. 5), and that overexpression of Rat8p enables cells to export bulk poly(A)+ mRNA under conditions of ethanol stress (Fig. 6). These results clearly indicate that ethanol stressed cells usually cannot maintain Rat8p on the cytoplasmic face of NPCs. It has been suggested (Hodge et al., 1999) that Xpo1p does not play a direct role in mRNA export and Neville and Rosbash also show (Neville and Rosbash, 1999) that the NES-Xpo1p pathway is not a major route of mRNA export in S. cerevisiae. Our findings suggest that the mislocalization of Rat8p is presumably one of the reasons why dysfunctional Xpo1p in xpo1-1 cells and ethanol stressed cells leads to a blocking of the export of bulk poly(A)+ mRNA. This idea is supported by the data of overexpression of Rat8p presented here (Fig. 6) and elsewhere (Hodge et al., 1999).

So far at least, the mechanism by which ethanol stress causes the defect in Xpo1p is completely unknown. One possibility is that Xpo1p cannot properly interact with NPCs in ethanol...
stressed cells. It was recently reported (Shulga and Goldfarb, 2003) that aliphatic alcohols induce the reversible dissociation of several structural nucleoporins (Nup82p, Nup85p, Nup120p, and Nup188p) and FG-nucleoporins (Nup53p, Nup59p, and Nup116p). This report indicated that aliphatic alcohols including ethanol cause reversible changes in the conformation of NPCs. Taking this finding together with the results presented here, we speculate that conformational changes of NPCs caused by ethanol block the proper association between Xpo1p-cargo complexes and NPCs (Lindsay et al., 2001). Attempts to clarify the interaction between Xpo1p-cargo complexes and nucleoporins in ethanol stressed cells are currently underway.

Recently, Rat8p has been shown to associate physically with components of transcription factor IHI (TFIHH) (Estruch and Cole, 2003). These authors propose a model in which Rat8p is involved in rearranging RNA-protein interaction during the early steps of transcription in the nucleus (Estruch and Cole, 2003). This model suggests that the nuclear accumulation of Rat8p may have pleiotropic effects on mRNA export. If Rat8p were to play a role in the early steps of transcription, an excess of Rat8p in the nucleus caused by ethanol stress would have aberrant effects on the processing of pre-mRNA and the quality of mRNA. The mislocalization of Rat8p may lead to unusual aberrant effects on the processing of pre-mRNA and the quality of mRNA. This idea may be supported by a report that a defect in Rat8p causes hyperadenylation of the 3′-end of mRNA (Hilleren and Parker, 2001).

Our results showed several differences in mRNA export between heat shock and ethanol stress. Notably, Rat8p and Xpo1p showed different stress responses to the two forms of stress. We demonstrated that the nuclear accumulation of Rat8p is caused by ethanol stress but not by heat shock (Fig. 2). Our findings indicate that blocking mRNA export by heat shock stress is independent of Rat8p, and suggests that the manner in which the blocking occurs differs between heat shock stress and ethanol stress. Additionally, yeast cells showed the different adaptive response in the export of bulk poly(A)+ mRNA to heat shock and ethanol stress. All wild-type strains we tested induced adaptation to 42°C in the export of bulk poly(A)+ mRNA by pretreatment with mild heat stress (37°C for 1 hour), as described (Tani et al., 1995), i.e., cells pretreated at 37°C for 1 hour can export bulk poly(A)+ mRNA even at 42°C. On the other hand, yeast cells did not induce adaptation of mRNA export to ethanol stress. Cells pretreated with mild ethanol stress (1-5%) or mild heat stress (37°C) for 1 hour still showed the nuclear accumulation of bulk poly(A)+ mRNA under ethanol stressed conditions (6-10%) (data not shown). These results indicate that ethanol stress is probably more potent than heat shock in inducing nuclear accumulation of bulk poly(A)+ mRNA. Different effects of heat shock and ethanol stress on the export of heat shock mRNA have been reported (Vainberg et al., 2000). Therefore, it seems quite reasonable that cells show different responses in the export of mRNA to heat shock and ethanol stress. The biological significance of our findings is not clear, however, cells may contain various regulatory systems for mRNA export specific to the kind of stress. In the response to heat shock, other mRNA export factors may exhibit a change in function and/or localization to regulate mRNA export. Our results may facilitate an understanding of the contribution of export factors to the regulation of the selective export of mRNA under stressed conditions.

We are grateful to Drs C. N. Cole, C. Guthrie, E. Hurt, Y. Kikuchi, S. Kuge, T. Lithgow, P. A. Silver, F. Stutz, K. Weis, and S. R. Wente for providing plasmids and strains. We also thank Drs C. N. Cole and R. Parker for advice regarding the study. This research was supported by a grant from the Noda Institute for Scientific Research, a grant from Bio-oriented Technology Research Advancement Institution (BRAIN), and a research grant from the Japanese ministry of education, culture, sports, science and technology (No. 14035229).

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