The Yeast hnRNP-like Protein Hrp1/Nab4 Accumulates in the Cytoplasm after Hyperosmotic Stress: A Novel Fps1-dependent Response

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The Hrp1/Nab4 shuttling protein belongs to a family of RNA binding proteins that bind to nascent RNA polymerase II transcripts and form hnRNP complexes. Members of this family function in a staggering array of cellular activities, ranging from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA translation and turnover. It has recently been recognized that the yeast stress response can include alterations in hnRNP-mediated mRNA export. We now report that the steady-state localization of Hrp1p rapidly shifts from the nucleus to the cytoplasm in response to osmotic stress. In contrast to a general stress response resulting in a transient relocation, Hrp1p redistribution is specific to hyperosmotic stress and is only reversed after stress removal. Hrp1p relocation requires both the *CRM1/XPO1* exportin and the *FPS1* glycerol transporter genes but is independent of ongoing RNA transcription and protein arginine methylation. However, mutations in the high osmolarity glycerol and protein kinase C osmosensing pathways do not impact the Hrp1p hyperosmotic response. We present a working model for the cytoplasmic accumulation of Hrp1 and discuss the implications of this relocation on Hrp1p function.

INTRODUCTION

In eukaryotic cells, mRNA synthesis takes place in the nucleus, whereas protein synthesis occurs in the cytoplasm. Coordination of these processes depends on the proper exchange of soluble factors between both compartments. One class of nuclear factors required for successful nuclear/cytoplasmic communication is the heterogeneous nuclear ribonucleoproteins (hnRNPs). This family of RNA-binding proteins binds to nascent RNA polymerase II transcripts and forms hnRNP complexes (reviewed in Krecic and Swanson, 1999). Several hnRNPs have been shown to participate in RNA processing events and export of mRNA from the nucleus, whereas others shuttle from the nucleus to the cytoplasm and have roles in translational regulation and mRNA stability.

To cope with the deleterious effects of stress, cells have developed rapid molecular responses to repair the damage and protect against future exposure (reviewed in Mager and De Kruijff, 1995; Estruch, 2000). It has recently been recognized that these responses can include alterations in hnRNP-

mediated mRNA export. In yeast, where this phenomenon has been studied in greatest detail, cells respond to various types of stress by selectively exporting mRNAs encoding heat shock proteins. (Saavedra *et al.*, 1996, 1997; Stutz *et al.*, 1997) with a concurrent cessation in the export of normal mRNAs (Saavedra *et al.*, 1996; Tani *et al.*, 1996). During conditions of stress this regulated transport has recently been attributed to altered functions of the yeast hnRNP Npl3p, also referred to as Nop3p, Mts1p, Nab1p, and Mtr13p (Bossie *et al.*, 1992; Russell and Tollervey, 1992; Ellis and Reid, 1993; Kadowaki *et al.*, 1994; Wilson *et al.*, 1994; Krebber *et al.*, 1999).

Under normal conditions, Npl3 is a major component of an RNP complex required for mRNA export (Lee *et al.*, 1996). However, after the onset of stress Npl3 is no longer RNA associated and is rapidly exported from the nucleus (Krebber *et al.*, 1999). Interestingly, this relocation is transient. As cells adapt to the heat shock Npl3p returns to the nucleus (Krebber *et al.*, 1999; Nanduri and Tartakoff, 2001). In a separate report, the stress-induced export of Npl3p was found to be PKC1 dependent (Nanduri and Tartakoff, 2001).

Given the predominant role of Npl3p in mRNA maturation and export it is likely that additional hnRNPs are required for proper stress response. Among the best-characterized yeast hnRNPs is the abundant mRNA-binding protein Hrp1/Nab4 (Henry *et al.*, 1996). *HRP1* was originally

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Table 1. Plasmids used in this study

Plasmid/primer	Description	Source
Myc-HRP1	Yeast LEU2 centromere vector carrying HRP1-Myc	Henry <i>et al.</i> (1996)
P548	Yeast URA3 centromere vector carrying <i>BCK1</i>	Lee and Levin (1992)
p636	Yeast URA3 centromere vector carrying <i>BCK1-20</i>	Lee and Levin (1992)
YEpfPS1	Yeast URA3 high-copy vector carrying <i>FPS1</i>	Tamas <i>et al.</i> (1999)
YEpfps1-Δ1	Yeast URA3 high-copy vector carrying <i>fps1-Δ1</i>	Tamas <i>et al.</i> (1999)
YEpgDP1	Yeast URA3 high-copy vector carrying <i>GDP1</i>	Larsson <i>et al.</i> (1993)
pHPS13	Yeast LEU2 centromere vector carrying <i>PKC1</i>	Schmitz <i>et al.</i> (2001)

identified in a screen for suppressors of the temperature-sensitive mutant *npl3-1* (Henry *et al.*, 1996). The central region of Hrp1p contains two RNP-type RNA-recognition motifs (RRMs), whereas the carboxy terminus of the protein is arginine and glycine rich. Analogous to Npl3p, the Hrp1 protein is modified in vitro by the yeast arginine methyltransferase (Henry and Silver, 1996). Although Hrp1p localizes to the nucleus at steady state (Henry *et al.*, 1996), it has also been found to efficiently exit the nucleus (Kessler *et al.*, 1997). More recent experiments have established that Hrp1p functions in both the nucleus and the cytoplasm. In the nucleus, Hrp1 is required for proper 3'-end formation (Kessler *et al.*, 1997; Minvielle-Sebastia *et al.*, 1998), whereas in the cytoplasm it is directly involved in modulating the activity of the nonsense-mediated mRNA (NMD) pathway (Gonzalez *et al.*, 2000). Consistent with these dual roles, Hrp1 binds mRNAs containing either the polyadenylation UA-efficiency element or the NMD downstream sequence element (Kessler *et al.*, 1997; Gonzalez *et al.*, 2000).

In this report, we present data that show that the cell responds to hyperosmotic stress by rapidly exporting Hrp1p from the nucleus to the cytoplasm. Significantly, this response is limited to hyperosmotic stress and Hrp1 only returns to the nucleus after the removal of stress conditions. This is in contrast to the transient cytoplasmic relocation of Npl3p, which is observed for Npl3p after a variety of stress conditions. We show that the cytoplasmic accumulation of Hrp1 after hyperosmotic stress requires both the CRM1/XPO1 exportin and the FPS1 glycerol exporter genes. However, neither the high osmolarity glycerol (HOG) nor protein kinase C (PKC)1 osmosensing pathways are required. Together, we propose a simple working model in which after hyperosmotic stress, Hrp1 is exported to the cytoplasm via the Crm1p/Xpo1p pathway and reimport into the nucleus is reduced. We further propose that the glycerol transporter Fps1 plays a role in the process.

MATERIALS AND METHODS

Plasmids and Strains

Plasmids used in this study are listed in Table 1. The yeast strains used in this study are listed in Table 2. Growth and maintenance of yeast strains, as well as genetic manipulations were performed as described previously (Rose *et al.*, 1990). Liquid sporulation media was prepared as described previously (Kassir and Simchen, 1991). Yeast strains were transformed using the lithium acetate method (Ito *et al.*, 1983) modified according to Gietz *et al.* (1992). Loss of

URA3 plasmids from yeast cells was accomplished by plating on solid media containing 5-fluoroorotic acid (Boeke *et al.*, 1984). Strain MHY705 (*kap104-16*) was generated by curing strain KAP104/*kap104-16* of pRS316-KAP104. Strains MHY834 and MHY836 were constructed by transforming plasmid pHPS13 (*LEU2 PKC1*) into diploid strain 23710, sporulating the resulting transformants and screening for Gent^r spore clones, which were also Leu⁻ (MHY835) or Leu⁺ (MHY837). Strain MHY862 was generated by exchanging plasmid pHPS13 (*LEU2 PKC1*) in strain MHY836 with plasmid p636 (*URA3 BCK1-20*).

Stress Conditions

Cells were routinely grown in defined synthetic complete medium (SC) supplemented with 2% glucose as a carbon source (Rose *et al.*, 1990). When required, strains harboring plasmids were grown in SC media lacking the appropriate amino acids and/or nutrients. When required, cycloheximide was added at a final concentration of 10 μg/ml. To apply a hyperosmotic shock in liquid medium, cells were pregrown in medium supplemented with 2% glucose to 1×10^7 cells/ml. The cells were then sedimented and resuspended in medium containing either 0.7 M NaCl, 0.7 M KCl, or 1.4 M sorbitol. Samples were taken at the time points indicated in the figures. For hypotonic shock, cultures were diluted with 4 volumes of water. For heat stress, cultures were transferred to a shaking water bath at 42°C. For carbon starvation, cells were grown exponentially in SC supplemented with 2% glucose, galactose, or glycerol where appropriate and were then washed and resuspended in SC media lacking a carbon source. For nitrogen starvation, cells were grown exponentially in SC supplemented with 2% glucose, washed, and then resuspended in media lacking yeast nitrogen base. For oxidative stress, cells were resuspended in media containing 0.4 mM H₂O₂. For the growth of *Δpkc1* mutants, all media contained 0.5 M sorbitol to stabilize the cells.

Radiolabeling and Immunoprecipitation

Δhrp1 cells containing the *HRP1*-Myc plasmid (MHY371) were grown in SC glucose medium lacking leucine and methionine. For labeling, 1000 μCi (90 μl) of EXPRE^{35S} protein labeling mix (>1000 Ci/mmol; PerkinElmer Life Sciences) was added to 100 ml of exponential cells (0.5×10^7 cells/ml), and cells were allowed to proceed for 1 h at 30°C. For the *t* = 0 time point, 20 ml of cells was pelleted, washed twice with ice-cold water, and frozen at -20°C. For samples that were chased after labeling, cells were washed twice in SC glucose medium lacking leucine and were resuspended in the original volume of the same medium containing 0.7 M NaCl. Incubation was continued and 20-ml samples were collected as described above at 5, 30, and 60 min. For immunoprecipitation, frozen cell pellets were resuspended in 200 μl of lysis buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (0.5 mM phenylmethylsul-

Table 2. Yeast strains used in this study

Strains	Genotype	Source
W303a BY4741	<i>MATa ura3-1 leu2-3, 112 ade2-1 his3-11, 15 trp1-1</i> <i>MATa his3D1 leu2D0 met15D0 ura3D0</i>	A. Tzagoloff Saccharomyces Deletion Project
Y262 YNR120	<i>MATa rpb1-1 ura3-52 his4-539</i> <i>Mata Δhot1::KanMX Δmsn1::TRP1 Δmsn2::HIS3 Δmsn4-1::TRP1 leu2-1, 112 ura3-1</i> <i>trp1-1 his3-11, 15 ade2-1 can1-100</i>	Nonet <i>et al.</i> (1987) Rep <i>et al.</i> (2000)
MH312	<i>MATa Δssk2::KanMX Δssk22::LEU2 Δsho1::TRP1 Δsln1::HIS3 leu2 ura3 trp1 his3</i>	Van Wuytswinkel <i>et al.</i> (2000)
FPS1 fps1Δ1	<i>MATa ura3-1 leu2-3, 112 ade2-1 his3-11, 15 trp1-1 Δfps1::HIS3 + YEpfps1</i>	Marco Siderius
PSY818	<i>MATa ura3-1 leu2-3, 112 ade2-1 his3-11, 15 trp1-1 Δfps1::HIS3 + YEpfps1-Δ1</i>	Marco Siderius
PSY1105	<i>MATα Δhrp1::HIS3 ura3 ade2 ade8 his3 leu2 lys1Trp + pRS316-Hrp1</i>	Kessler <i>et al.</i> (1997)
KAP104/ Kap104-16	<i>MATα Δxpo1::LEU2 ura3 leu2 trp1 his3 ade2 can1 + PKW457 xpo1-1 HIS3</i> <i>MATa Δkap104::ura3::HIS3 ura3-52 lys2-801 leu2-3, 112 his3Δ200 trp1-1 + pRS316-</i> <i>KAP104 + pRS314-kap104-16</i>	Stade <i>et al.</i> (1997) Aitchison <i>et al.</i> (1996)
23710	<i>MATa/MATα Δpkcl::KanMX/PKC1 leu2D0/leu2D0 ura3D0/ura3D0 met15D0/MET15</i> <i>his3D1/his3D1 lys2D0/LYS2</i>	Saccharomyces Deletion Project
95400	<i>MATa Δhog1::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
1328	<i>MATa Δbck1::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
2487	<i>MATa Δmkk1::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
2112	<i>MATa Δmkk2::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
993	<i>MATa Δslt2::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
1784	<i>MATa Δwsc1::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
1161	<i>MATa Δwsc2::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
6255	<i>MATa Δwsc3::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
5341	<i>MATa Δmid2::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
1531	<i>MATa Δfps1::KanMX his3D1 leu2D0 met15D0 ura3D0</i>	Saccharomyces Deletion Project
3171	<i>MATa his3D1 leu2D0 met15D0 ura3D0 Δhmt1::KanMX</i>	Saccharomyces Deletion Project
5055	<i>MATa his3D1 leu2D0 met15D0 ura3D0 Δlos1::KanMX</i>	Saccharomyces Deletion Project
3694	<i>MATa his3D1 leu2D0 met15D0 ura3D0 Δmsn5::KanMX</i>	Saccharomyces Deletion Project
MHY371 MHY705	<i>MATα Δhrp1::HIS3 ura3 ade2 ade8 his3 leu2 lys1 Trp + Myc-HRP1</i> <i>MATa Δkap104::ura3::HIS3 ura3-52 lys2-801 leu2-3, 112 his3 Δ200 trp1-1 + pRS314-</i> <i>kap104-16</i>	This study This study
MHY834 MHY836	<i>MATa Δpkcl::KanMX leu2D0 ura3D0 met15D0? his3D1 lys2D0?</i> <i>MATa Δpkcl::KanMX leu2D0 ura3D0 met15D0? his3D1 lys2D0? + pMHY247 (PKC1</i> <i>LEU2)</i>	This study This study
MHY862	<i>MATa Δpkcl::KanMX leu2D0 ura3D0 met15D0? his3D1 lys2D0? + p636 (BCK1-20</i> <i>URA3)</i>	This study

fonyl fluoride and 3 μg/ml each of pepstatin, leupeptin, aprotinin, and chymostatin). Glass beads (50 μl, 0.5 mm) were added and the cells were lysed by repeated vortexing at 4°C (4× for 30 s each). Lysates were clarified by centrifugation for 10 min at 12,000 × g at 4°C and precleared with a 1-h incubation with Sepharose CL-4B beaded agarose (Sigma-Aldrich) at 4°C. Myc-Hrp1p-antibody complexes were precipitated by addition of 20 μl of a 50% slurry of c-Myc (9E10) antibody agarose conjugate (Santa Cruz Biotechnology) to the precleared lysate followed by incubation for 1 h with rocking at 4°C. Beads were washed three times with lysis buffer, and immunoprecipitated proteins were visualized by separation on a 10% SDS-polyacrylamide gel followed by autoradiography.

Preparation of Rabbit Polyclonal anti-Hrp1 Antiserum

A rabbit polyclonal antiserum against Hrp1 was raised using an affinity-purified glutathione S-transferase (GST)-Hrp1 fusion protein. The plasmid expressing GST-Hrp1 (pGEX-HRP1) has been described previously (Kessler *et al.*, 1997). The protein was expressed in BL21 (DE3) cells (Studier *et al.*, 1990) and purified by glutathione affinity chromatography as specified by the supplier (Amersham Biosciences). Immunization of an elite New Zealand White rabbit with this material was performed by a commercial antibody service (Covance Research Products, Denver, PA).

Immunoblot Analysis

Total cell extracts were prepared (Bossie *et al.*, 1992) from cells collected at each time point. Proteins were resolved by SDS-PAGE, transferred, and immunoblotted as described previously (Bossie *et al.*, 1992). The blots were first exposed to anti-Hrp1 antisera at a concentration of 1:5000, followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibodies at 1:5000. Secondary antibodies were detected with enhanced chemiluminescence Western blotting reagents (Amersham Biosciences).

Indirect Immunofluorescence

Indirect immunofluorescence microscopy was performed as described previously (Sadler *et al.*, 1989). Anti-Hrp1 antisera were used at 1:1000.

RESULTS

Hrp1 Rapidly Relocalizes to the Cytoplasm in Response to Osmotic Stress

When yeast cells are grown continuously in either rich (YEPD) or SC media, Hrp1p/Nab4p is abundant throughout the nucleoplasm and is faintly visible in the cytoplasm at 25–36°C, as judged by indirect immunofluorescence (Figure 1A; Henry *et al.*, 1996; Lee *et al.*, 1996). We know however, that under these conditions Hrp1p is capable of exiting and reentering the nucleus (Lee *et al.*, 1996; Shen *et al.*, 1998). Thus, under steady-state conditions, Hrp1p seems to localize to the nucleus because its rate of reimport into the nucleus after export is quite rapid (Figure 1B). During standard growth conditions the export of Hrp1p is thought to be coupled in some manner to the export of poly(A)⁺ RNA (Kessler *et al.*, 1997; Shen *et al.*, 1998; Valentini *et al.*, 1999).

The ability of *Saccharomyces cerevisiae* to respond to stress is highly regulated (reviewed in Mager and De Kruijff, 1995; Estruch, 2000). Given this tight regulation, it was of interest to analyze the shuttling dynamics and localization of Hrp1 under different types of stress conditions. Accordingly, yeast cells were exposed to heat, salt, peroxide, and carbon or nitrogen starvation (see MATERIALS AND METHODS), and the intracellular localization of Hrp1p was determined by indirect immunofluorescence with anti-Hrp1p antibodies. Of the stress conditions tested, only exposure to high salt (0.7 M) was found to significantly impact the localization of Hrp1 in exponentially growing cells (Figure 2A). After exposure to 0.7 M NaCl, we found that Hrp1p rapidly accumulated in the cytoplasm. Relocalization was complete within 5 min (Figure 2A, top row). It should be noted that a weak cytoplasmic accumulation of Hrp1p was sometimes also observed after glucose depletion (our unpublished data). However, this level was significantly less than that observed after salt exposure. Together, these results led us to postulate that the appearance of Hrp1p in the cytoplasm was specific to osmotic shock rather than to a general stress response.

To confirm that osmotic shock, per se, is responsible for the relocation of Hrp1p, we repeated these experiments with 0.7 M KCl and 1 M sorbitol. Both had effects comparable with those of 0.7 M NaCl (our unpublished data), but strong hypotonic shock did not result in Hrp1p relocalization (Figure 2B). Furthermore, the nuclear export of Hrp1p is an active process that can be inhibited if cells are incubated at 4°C after salt exposure (Figure 2A, bottom row). Hrp1p was

Figure 1. Hrp1p exhibits a steady-state nuclear localization. (A) Localization of Hrp1p at 25–36°C. Wild-type cells were grown to 1×10^7 cells/ml in SC medium at 25°C (top row), 30°C (middle row), or 36°C (bottom row). Cells were then fixed and processed as described for immunofluorescence. Cells were photographed by use of Nomarski optics (right column) and DNA was stained with 4,6-diamidino-2-phenylindole (middle column). Hrp1p was visualized with anti-Hrp1 antisera (left column). (B) Schematic diagram of Hrp1p shuttling and steady-state localization during standard growth conditions. In this diagram, Hrp1p seems to localize to the nucleus because its rate of nuclear import is faster than its rate of export.

found to remain intact throughout the experiment as determined by immunoblot analysis (Figure 2C).

Because new Hrp1p protein is being synthesized during the course of the experiment, the origin of the Hrp1p that relocalizes to the cytoplasm is uncertain. It may either be preexisting cytoplasmic Hrp1p, newly synthesized Hrp1p, or a combination of both. Two experiments were used to answer this question. In the first, a pulse-chase experiment was designed to determine the stability of protein synthesized before high-osmolarity exposure throughout the experiment. The cytoplasmic accumulation of Hrp1 after exposure to osmotic stress conditions is not only due to new protein synthesis. Hrp1p remains stable throughout the incubation period of the experiment; therefore, the cytoplasmic signal is at least partially due to export of preexisting Hrp1p from the nucleus (Figure 2D). In the second experiment, the cytoplasmic accumulation of Hrp1 was deduced to be independent of new protein synthesis because it was unaffected by addition of the protein translational inhibitor cycloheximide (our unpublished data).

Figure 2. Hrp1p rapidly relocates to the cytoplasm in response to hyperosmolarity. (A) Time course of Hrp1p cytoplasmic accumulation after hyperosmotic exposure. Cells were incubated at 30°C, transferred to hyperosmotic conditions (0.7 M NaCl), and then incubated at either 30°C (top two panels) or 4°C (bottom two panels). Hrp1p was assessed 0, 5, 15, 30, and 60 min after hyperosmotic exposure. Hrp1p was also localized in cells grown continuously (>10 generations) in media containing 0.7 M NaCl. Cells from each time point were subjected to immunofluorescence microscopy with anti-Hrp1 antisera. Hrp1p was visualized with fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (top). Cells were photographed by use of Nomarski optics (bottom). (B) Hypotonic shock does not induce Hrp1 cytoplasmic accumulation. For hypotonic shock, cultures grown at 30°C in YEPD were diluted with 4 volumes of water. The Hrp1p protein was assessed at 0, 5, 15, 30, and 60 min after hypotonic shock. Cells from each time point were subjected to immunofluorescence microscopy with anti-Hrp1 antisera. Hrp1p was visualized with FITC labeled anti-rabbit antibody (top). (C) Hrp1p remains intact after hyperosmotic exposure. Yeast

Steady-State Location of Hrp1p Remains Cytoplasmic during High Osmolarity

Interestingly, the steady-state location of Hrp1p remains cytoplasmic with continued exposure to high osmolarity. Once relocated, Hrp1p remained in the cytoplasm at 15, 30, and 60 min after salt exposure (Figure 2A). In fact, Hrp1p remained cytoplasmic in cells grown continuously (>10 generations) in 0.7 M NaCl (Figure 2A). This result is in contrast to the transient relocation observed for several nuclear and nucleolar proteins after hypertonic shock (Krebber *et al.*, 1999; Nanduri and Tartakoff, 2001). As a control, we tested a protein reported to exhibit transitory relocation after hyperosmotic shock, the hnRNP-like protein Npl3. Consistent with previous reports, Npl3p reentered the nucleus within 1 h after hyperosmotic shock (our unpublished data; Krebber *et al.*, 1999; Nanduri and Tartakoff, 2001). In sum, these results indicate that Hrp1p remains in the cytoplasm during hyperosmotic conditions.

Cytoplasmic Hrp1p Relocalizes to the Nucleus after the Removal of Hyperosmotic Conditions

Because hyperosmotic shock triggers Hrp1p relocation to the cytoplasm, we predicted that a return to standard osmotic conditions would result in the nuclear accumulation of Hrp1p. To test this hypothesis, cells grown in 0.7 M NaCl were washed, resuspended in salt-free media, and examined over an extended period. The intracellular localization of Hrp1p was determined by immunofluorescence with anti-Hrp1 antibodies. As expected, Hrp1p relocalized to the nucleus within 5 min of stress removal and remained nuclear at all subsequent time points tested (Figure 3). Similar results were seen in the presence of the protein translational inhibitor cycloheximide, indicating new protein synthesis is not required (our unpublished data).

Nuclear Protein Export and RNA Synthesis

Because the nuclear export of Hrp1p requires ongoing RNA synthesis during standard osmolarity conditions (Shen *et al.*, 1998), we tested whether the same was true during hyperosmotic shock. To accomplish this, we used a temperature-sensitive allele of RNA Pol II termed *rpb1-1* (Nonet *et al.*, 1987). In the *rpb1-1* strain, RNA Pol II transcription is inhibited at the nonpermissive temperature. After a 30-min shift to the restrictive temperature, *rpb1-1* cells were exposed to high osmolarity (0.7 M NaCl), and the localization of Hrp1p was determined by immunofluorescence with anti-Hrp1 antibodies. At both the permissive and restrictive temperatures

extracts were prepared after they were grown to log phase and shifted to hyperosmotic conditions without (–, left gel) or with (+, right gel) cycloheximide addition. The lysates were separated on a 10% SDS-polyacrylamide gel and analyzed on a Western blot with antibodies against Hrp1p. (D) The cytoplasmic accumulation of Hrp1p after hyperosmotic shock is at least partially due to the nuclear export of preexisting Hrp1p. Wild-type cells expressing myc-Hrp1 were grown, radiolabeled, lysed, and immunoprecipitates were prepared. Immune complexes were analyzed by Western blot and autoradiography. Cells were labeled in medium containing glucose (lane 1) followed by a 1-h chase in the same medium containing 0.7 M NaCl (lanes 2–4).

Figure 3. Cytoplasmic Hrp1p is imported into the nucleus after a return to standard osmolarity. Wild-type cells were grown to log phase and stressed with 0.7 M NaCl for 30 min (first row). The NaCl was removed and Hrp1p localization was examined at 15- (second row), 30- (third row), and 60 (fourth row)-min time points. Cells were prepared for immunofluorescence with anti-Hrp1p antisera and then with fluorescein isothiocyanate-labeled anti-rabbit antibody to visualize Hrp1p (top row). Cells were photographed by use of Nomarski optics (bottom row).

for Rpb1 protein function, Hrp1p had relocalized to the cytoplasm within the first examined time point (15 min) (Figure 4, left). These results indicate that RNA Pol II transcription is not required for the export of Hrp1p from the nucleus during hyperosmotic shock.

Figure 4. Hyperosmotic-mediated Hrp1p export is independent of both RNA synthesis and arginine methylation. Wild-type (B, E, and H), *rpb1-1* (A, D, and G), or $\Delta HMT1$ (C, F, and I) cells were grown to log phase in SC medium at 30°C. The *rpb1-1* cells were shifted to 36°C for 30 min (A) and then stressed with 0.7 M NaCl for 15 (D) and 30 (G) min. The wild-type cells were incubated at 30°C in the presence of thiolutin for 15 min (B) and then transferred to medium containing 0.7 M NaCl for 15 (E) and 30 (H) min. The $\Delta HMT1$ cells were incubated at 30°C (C) and then transferred to medium containing 0.7 M NaCl for 15 (F) and 30 (I) min. Cells were prepared for immunofluorescence with anti-Hrp1p antisera and then with fluorescein isothiocyanate-labeled anti-rabbit antibody to visualize Hrp1p.

We also examined the effect of a complete RNA synthesis block on RNA export. To accomplish this, we used the antifungal agent thiolutin, an inhibitor of all three RNA polymerases in yeast (Tipper, 1973). When cells are exposed to 0.7 M NaCl in the presence of thiolutin, Hrp1p rapidly relocalizes to the cytoplasm at a rate equivalent to that of cells lacking thiolutin (Figure 4, middle). This result indicates that after hyperosmotic shock the export of Hrp1p is no longer coupled to RNA synthesis.

Effect of Methylation on the Nuclear Export of Hrp1p after Exposure to Hyperosmotic Conditions

Because arginine methylation facilitates the nuclear export of Hrp1p during standard osmolarity conditions (Shen *et al.*, 1998), we tested whether the same was true during hyperosmotic shock. As shown in Figure 4 (right), when $\Delta HMT1$ cells are exposed to high osmolarity (0.7 M NaCl), Hrp1p rapidly relocalizes to the cytoplasm at a rate equivalent to that of wild-type cells (Figure 3, middle). Furthermore, the nuclear accumulation of Hrp1p after a return to standard osmolarity was also found to be independent of arginine methylation. When a $\Delta HMT1$ strain initially grown at high osmolarity was washed and resuspended in salt-free media, cytoplasmic Hrp1p relocalizes to the nucleus within 5 min (our unpublished data). Together, these data demonstrate that cellular Hmt1 arginine methyltransferase activity does not effect Hrp1p localization after hyperosmotic exposure.

Hrp1p Relocalization by Hyperosmotic Stress Does Not Occur through the HOG Pathway

To properly adapt to hyperosmotic conditions, the cell has to sense osmotic changes and transmit the signal to the nucleus. Many, if not all, eukaryotic cells use mitogen-activated protein (MAP) kinase pathways for this purpose. The primary osmosensing MAP kinase pathway in *S. cerevisiae* is the HOG pathway outlined in Figure 5A (reviewed in O'Rourke *et al.*, 2002). Because this pathway is stimulated by hypertonic shock, we predicted that it would be required for the cytoplasmic accumulation of Hrp1p. To test this hypothesis, we examined the effects of 0.7 M NaCl on the localization of Hrp1p in strains deleted for key genes of the HOG1 pathway. Unexpectedly, Hrp1p still relocalized to the cytoplasm in a strain lacking either 1) the *HOG1* gene itself, 2) the *SHO1* and *SLN1* transmembrane osmosensors, or 3) four putative terminal transcription factors (*MSN1*, *MSN2*, *MSN3*, and *HOT1*) (Figure 6A). Strains harboring deletions of other HOG pathway genes (*STE11* and *PBS2*), yielded similar results (our unpublished data). In sum, these data indicate that the cytoplasmic accumulation of Hrp1p after hyperosmotic stress is independent of the HOG MAP kinase cascade.

To complete our analysis of the HOG pathway we also tested whether these factors were required to reimport Hrp1p into the nucleus after a return to standard osmolarity. As noted in Figure 6A (bottom), when HOG-pathway mutants initially grown at high osmolarity are washed and resuspended in salt-free media, cytoplasmic Hrp1p relocalizes to the nucleus within 5 min. These data demonstrate that the nuclear reimport of Hrp1p after a shift from high to standard osmolarity is also independent of the HOG pathway.

A. HOG Pathway **B. PKC1 Pathway**

Figure 5. Schematic representation of the main components of the *S. cerevisiae* Hog1p and Pkc1p pathways. (A) The HOG pathway. Two osmosensors, Sho1p and Sin1p, stimulate the HOG MAP kinase cascade by different mechanisms. Activation of this MAP kinase cascade leads to the phosphorylation of the Hog1 MAP kinase, which is then imported into the nucleus. Four putative nuclear targets of Hog1p (Msn1p, Msn2p, Msn3p, and Hot1p) are also shown. (B) The Pkc1p pathway. Dotted arrows indicate activation of the Pkc1 pathway in which the detailed mechanisms of signal transduction are not yet known.

Hyperosmotic Localization of Hrp1 Is Pkc1p Independent

The PKC pathway (Figure 5B) is a second osmosensing pathway in yeast that has been shown to affect expression of genes after an osmotic shift (Davenport *et al.*, 1995). Interestingly, Nanduri and Tartakoff, 2001 have recently reported that Pkc1p is required for the transient relocation of several nuclear proteins after hyperosmotic shock. Because Hrp1p was not examined in this study, we monitored its localization in a $\Delta pkc1$ strain after an osmotic shift. Hrp1p rapidly relocalized to the cytoplasm in both a $\Delta pkc1$ strain (Figure 6B, left) and a $\Delta pkc1$ strain harboring wild-type *PKC1* on a plasmid (Figure 6B, right). In these experiments, cells were initially grown in 0.5 M sorbitol and then shifted to 1.4 M sorbitol. The starting concentration of sorbitol (0.5 M) provided the osmotic support required for $\Delta pkc1$ cell viability yet did not induce relocalization (Figure 6B, top). The final sorbitol concentration (1.4 M) chosen closely mimics the osmolarity provided by 0.7 M NaCl used in previous experiments. This data suggests that Hrp1p relocalization does not require Pkc1p function.

Pathway Components Upstream and Downstream of Pkc1p Are Not Required for the Hyperosmotic Relocalization of Hrp1

Although Pkc1p seemed not to be required for the cytoplasmic accumulation of Hrp1p, it was possible that downstream kinases (refer to Figure 5B) could play a role independent of Pkc1p. However, isogenic strains lacking either Bck1p, Mkk1p, Mkk2p, or Slt2p showed Hrp1p relocalization after hypertonic shock (our unpublished data). Furthermore, in these mutants cytoplasmic Hrp1p reentered the nucleus at a rate equivalent to wild-type cells after salt

removal (our unpublished data). Thus, the kinase cascade downstream of Pkc1p is also not necessary for the hyperosmotic localization of Hrp1p.

The absence of required mediators downstream of Pkc1p allowed us to reexamine the role of Pkc1p in the absence of any complications attributable to the presence of osmotic support. To accomplish this, we used a constitutively active allele of *BCK1* termed *BCK1-20* (Lee and Levin, 1992). In a *BCK1-20* $\Delta pkc1$ double mutant strain, partial suppression of the $\Delta pkc1$ allele permits growth in the absence of osmotic stabilizers. Both wild-type and *BCK1-20* $\Delta pkc1$ cells were exposed to high osmolarity (0.7 M NaCl), and the localization of Hrp1p was determined by indirect immunofluorescence with anti-Hrp1 antibodies. In both strains, Hrp1p had relocalized to the cytoplasm by the first time point examined (30 min) (Figure 6C, left). These results confirm our previous results that Hrp1p relocalization does not require Pkc1p function.

We also tested upstream components of the Pkc1p pathway (Figure 5B). Several putative cell membrane sensors have been linked to the Pkc1p pathway. These include Slg1p/Wsc1 (Gray *et al.*, 1997; Jacoby *et al.*, 1997; Verna *et al.*, 1997), Wsc2p-Wsc4p (Verna *et al.*, 1997), and Mid2p (Ono *et al.*, 1994). During shifts from standard to high osmolarity and back to standard osmolarity, the localization of Hrp1p in these cells was equivalent to that of wild-type cells (our unpublished data). Deletion of additional proteins linked to the Pkc1p pathway (Bni1p, Fks1p, Rho2p, Rom2p, and Skn7p) also did not affect Hrp1p relocalization (our unpublished data).

Hyperosmotic Localization of Hrp1 Requires the Glycerol Export Protein Fps1

A universal strategy for cellular osmoregulation is the accumulation of a compatible solute to control the osmolarity of

Figure 6. The Hog1p and Pkc1 kinase cascades are not required for the hyperosmotic localization of Hrp1p. (A) Localization of Hrp1p in strains deleted for components of the PKC1 pathway. Hrp1p was localized 0 (first row), 15 (second row), and 60 min (third row) after exposure to hyperosmolarity, as well as 10 min (fourth row) after the return to standard osmolarity. (B) Localization of Hrp1p in wild-type and $\Delta pkc1$ cells. Wild-type (MHY745) and $\Delta pkc1$ (MHY746) cells were collected at 0 (first row), 30 (second row), 60 (third row), and 120 min (fourth row) after the sorbitol level was increased from 0.5 to 1.4 M. (C) Localization of Hrp1p in wild-type and $\Delta pkc1$ BCK1-20 cells. Hrp1p was localized 0 (first row), 15 (second row), 60 (third row), and 120 min (fourth row) after exposure to 0.7 M NaCl. All cells were prepared for immunofluorescence with anti-Hrp1p antisera and then with fluorescein isothiocyanate-labeled anti-rabbit antibody to visualize Hrp1p.

the cytosol (Yancey *et al.*, 1982). In yeast, the compatible solute primarily used is glycerol (Brown, 1978). The accumulation of glycerol is controlled by both the rapid closure of the glycerol channel Fps1p and elevated de novo synthesis of glycerol (Figure 7A). In wild-type cells, the rate of glycerol export is decreased by hyperosmotic shock and is increased by hypoosmotic shock (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). Interestingly, although the glycerol transport rate is rapidly regulated by external osmolarity, it seems to be independent of the HOG and PKC signaling pathways (Tamas *et al.*, 1999). Given the unique role of Fps1p in osmoregulation, we inquired whether this protein might also be required for the hyperosmotic relocation of Hrp1p. Indeed, as shown in Figure 7B (first column), a strain deleted for *FPS1* was unable to relocate Hrp1p from the nucleus to the cytoplasm after hypertonic shock. The hyperosmotic-dependent localization of Hrp1p was restored in this strain when transformed with a plasmid encoding wild-type *FPS1* (Figure 7B, second column).

Given that $\Delta fps1$ and wild-type strains are reported to accumulate equivalent amounts of glycerol after osmotic shock (Luyten *et al.*, 1995; Tamas *et al.*, 1999), we reasoned that Hrp1p relocation was not linked to intracellular glycerol levels. To test this hypothesis, we determined whether increasing the intracellular glycerol levels of cells grown at standard osmolarity could trigger Hrp1p relocation. Glycerol levels were increased by overexpression of Gdp1p. High-copy expression of Gdp1p in a wild-type

strain leads to a twofold increase in glycerol content compared with a wild-type strain carrying a control plasmid. (Philips and Herskowitz, 1997; Tao *et al.*, 1999). As shown in Figure 7B (fourth column), Hrp1p is not relocated to the cytoplasm in cells grown at standard osmolarity, although they contain twofold more intracellular glycerol than wild-type cells.

As a second test, we examined a strain expressing an N-terminal truncation of Fps1p ($\Delta 13$ –230) in which glycerol export is constitutive. This truncation results in an inability to accumulate significant glycerol levels within 3 h after a shift to high osmolarity (Tamas *et al.*, 1999). Thus, although intracellular levels are significantly decreased in this strain, Hrp1p relocation was unaffected (Figure 7B, third column). In sum, these results indicate that though Hrp1 relocation requires Fps1p function, it is independent of intracellular glycerol levels. Furthermore, the data suggests that the amino terminal portion of Fps1p (aa 13–230) is not required for osmolarity-dependent Hrp1 relocation.

Hrp1 Export after Hypertonic Stress Is Crm1/Xpo1 Dependent

We next investigated the nuclear export pathway of Hrp1p after hyperosmotic shock. Within the amino-terminal 60 aa of Hrp1, aa 42–52 (LAALQALSSSL) are predicted to form an amphipathic helix with leucine residues predominantly on one face. Such a structure is characteristic of a leucine-rich

Figure 7. Hyperosmotic localization of Hrp1p requires the glycerol export protein, Fps1, but is independent of intracellular glycerol levels. (A) Glycerol efflux is controlled by the activity of the Fps1p channel protein. Glycerol is produced as the product of a side branch of glycolysis. DHAP is converted into glycerol-3-P by the actions of Gpd1p and Gpd2p. The glycerol-3-P is subsequently dephosphorylated by Gpp1p and Gpp2p. Under conditions of increased osmolarity, the Fps1p glycerol facilitator is closed enabling the cells to accumulate intracellular glycerol. When glycerol is produced during normal growth conditions, however, glycerol is leaked through the Fps1 channel to the outside medium. (B) Localization of Hrp1p in Δ fps1 cells (first column), Δ fps1 + FPS1 plasmid (second column), fps1 Δ 1 (third column), and wild-type + 2 μ M GPD1 (fourth column) after hyperosmotic shock for 0, 15, or 120 min. Cells were prepared for immunofluorescence with anti-Hrp1p antisera and then with fluorescein isothiocyanate-labeled anti-rabbit antibody to visualize Hrp1p.

nuclear export signal (Rittinger *et al.*, 1999), which acts as the ligand for the export receptor Crm1p/Xpo1p (Fornerod *et al.*, 1997; Stade *et al.*, 1997). This prompted us to focus on the Crm1p export receptor. To test for the involvement of this receptor, we monitored Hrp1 localization in the *crm1* (*xpo1-1*) mutant (Stade *et al.*, 1997). After a 30-min shift to the

Figure 8. Hrp1p export after hypertonic stress is CRM1/XPO1 dependent. Localization of Hrp1p in *xpo1^{ts}* cells at 36°C (first column) and 25°C (second column), Δ los1 (third column), Δ msn5 (fourth column) after hyperosmotic shock for 0, 15, and 120 min. Cells were prepared for immunofluorescence as described previously.

restrictive temperature, cells were exposed to high osmolarity (0.7 M NaCl) and the localization of Hrp1p was determined by indirect immunofluorescence with anti-Hrp1 antibodies. Under these conditions, Hrp1p remained in the nucleus in the majority of the cells at all time points tested (Figure 8). This result is not attributable to a lack of protein synthesis because, when the assay was performed in the presence or absence of the protein synthesis inhibitor cycloheximide, Hrp1p accumulated in the cytoplasm at equal levels (our unpublished data). In contrast, Hrp1p rapidly relocalized to the cytoplasm in strains lacking either the Los1p or Msn5p exportins. (Figure 8; Arts *et al.*, 1998; Hellmuth *et al.*, 1998; Kutay *et al.*, 1998; Blondel *et al.*, 1999; DeVit and Johnston, 1999). In sum, these results suggest that after exposure to high osmolarity Hrp1p, export from the nucleus requires functional Crm1p/Xpo1p.

Cytoplasmic Accumulation of Hrp1 Is Consistent with Reduced Nuclear Reimport

One explanation for the cytoplasmic accumulation of Hrp1p after hyperosmotic stress is that its rate of nuclear import is simply decreased relative to its rate of export. Consistent with this idea, Hrp1p rapidly relocalizes to the cytoplasm in a strain carrying a temperature-sensitive allele of its import

Figure 9. The Kap104 importin protein is a likely candidate to mediate hyperosmotic Hrp1p cytoplasmic accumulation. (A) Hrp1p accumulates in the cytoplasm of a *kap104-16* mutant strain after a shift to the restrictive temperature. Cells were incubated at 25°C (top two panels) and then shifted to 36°C (top two panels) for 60 min. Cells from both time points were subjected to immunofluorescence microscopy with anti-Hrp1 antisera. Hrp1p was visualized with fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (left). Cells were photographed by use of Nomarski optics (right). (B) Time course of Nab2p cytoplasmic accumulation after hyperosmotic exposure. Cells were incubated at 30°C and then transferred to hyperosmotic conditions (0.7 M NaCl). Nab2p was localized at 0, 30 min, and 10 generations after hyperosmotic exposure. Hrp1p was also localized 5 min after the removal of hyperosmotic conditions. Cells from each time point were subjected to immunofluorescence microscopy with anti-Nab2p antisera. Nab2p was visualized with fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody (left) and cells were photographed by use of Nomarski optics (right). (C) Cytoplasmic Hrp1p does not accumulate in the nuclei of *crm1* (*xpo1-1*) cells at the restrictive temperature. Cells incubated at the permissive temperature of 25°C were transferred to hyperosmotic conditions (0.7 M NaCl) and Hrp1p was localized at 0 and 30 min after hyperosmotic exposure (top two panels). The cells were then shifted to 36°C and Hrp1p was localized at 15, 60, and 120 min (bottom three panels). Cells from each time point were subjected to immunofluorescence microscopy with anti-Hrp1p antisera. Hrp1p was visualized with FITC-labeled anti-rabbit antibody (right) and cells were photographed by use of Nomarski optics (left).

receptor (*KAP104*) when it is shifted to the restrictive temperature (Aitchison *et al.*, 1996; Figure 9A). We reasoned that if the cytoplasmic accumulation of Hrp1p is due to reduced Kap104p-mediated import, then other nuclear proteins that share this receptor would also shift to the cytoplasm after hyperosmotic shock. The Kap104 receptor is known to mediate the nuclear import of only one other protein; the Nab2 RNA-binding protein (Aitchison *et al.*, 1996). Thus, we compared the cellular localization of both Nab2p and Hrp1p during a shift from standard osmolarity, to high osmolarity (0.7 M NaCl), and back to normal osmolarity. At the time points examined, the localization of Nab2p was equivalent to that previously observed for Hrp1p (Figure 9B). Furthermore, Nab2 reentered the nucleus within 5 min after the salt removal (Figure 9B). Three additional nuclear/nucleolar proteins tested (Npl3p, Nop1p, and Nsr1p) whose import is Kap104p-independent did not share this relocalization pattern (our unpublished data). Thus, these results are consistent with the idea that the cytoplasmic accumulation of the

Hrp1 and Nab2 proteins could both be controlled by reduced Kap104p-mediated import.

To examine this further, we treated *crm1* (*xpo1-1*) cells with 0.7 M NaCl for 15 min to induce an increase in cytoplasmic Hrp1p, raised the temperature, and then monitored Hrp1p localization at 15, 60, and 120 min (Figure 9C). As previously reported in this work, Hrp1p nuclear export is blocked in *crm1* (*xpo1-1*) cells grown at the restrictive temperature. Thus, if the cytoplasmic accumulation of Hrp1p after hyperosmotic stress results from an increase in its nuclear export, we expected Hrp1p to become trapped in the nucleus of *crm1* (*xpo1-1*) cells. However, if Hrp1p cytoplasmic accumulation is due to a reduction or inhibition of Hrp1p nuclear import the protein would remain cytoplasmic. As shown in Figure 9C, the Hrp1 protein remains cytoplasmic after a shift of the *crm1* (*xpo1-1*) cells to the restrictive temperature. This result indicates that Hrp1p nuclear import is either completely or partially blocked during conditions of high osmolarity.

DISCUSSION

The cellular response to stress provides a rich opportunity to understand the regulation of mRNA metabolism because it is transcriptionally defined, rapid, and well conserved. Recent reports have shown that the behavior of both mRNA and major mRNA shuttling proteins are modulated by the stress response (Saavedra *et al.*, 1996, 1997; Stutz *et al.*, 1997; Krebber *et al.*, 1999; Nanduri and Tartakoff, 2001). However, all the proteins required and their precise role(s) in mRNA processing and transport after exposure to stress remain an open question. In the yeast *S. cerevisiae*, HRP1/NAB4 encodes an essential and abundant mRNA binding protein that shuttles between the nucleus and the cytoplasm (Henry *et al.*, 1996; Kessler *et al.*, 1997). Interestingly, Hrp1p functions in both cellular compartments. In the nucleus Hrp1p is required for proper 3'-end formation (Kessler *et al.*, 1997; Minvielle-Sebastia *et al.*, 1998), whereas in the cytoplasm it is directly involved in modulating the activity of the NMD pathway (Gonzalez *et al.*, 2000). Given the varied roles of Hrp1p in RNA metabolism, cellular stress is likely to modulate some or all of its activities.

In this study, we demonstrate that the steady-state location of Hrp1p rapidly shifts from the nucleus to the cytoplasm after hyperosmotic stress. We were able to elucidate the requirements of this transfer by monitoring the distribution of Hrp1p in mutant strains defective for components in the nucleocytoplasmic shuttling and osmotic stress pathways. Unexpectedly, cells disrupted for genes in either the HOG1 or PKC1 osmotic signal transduction pathways had no discernable effect on the redistribution of Hrp1 during hyperosmotic conditions. However, under these conditions the cytoplasmic accumulation of Hrp1p required both the CRM1/XPO1 exportin and the FPS1 glycerol transporter genes. We show that Hrp1p remains cytoplasmic with continued exposure to high osmolarity but rapidly returns to the nucleus after a reduction in osmolarity. We also provide indirect evidence that links the importin Kap104 protein to the process. This importer mediates the nuclear import of Hrp1p and Nab2p, both of which exhibit similar localization responses to hyperosmolarity.

We propose a working model in which the membrane-bound Fps1 protein senses osmotic changes in the media, which in turn directly or indirectly results in either a decrease or inhibition of Hrp1p nuclear import. The mechanism of this inhibition is unclear but likely involves the Kap104 importer protein. Possible scenarios that may disrupt import include a decreased interaction between Kap104p and either Hrp1p, importin alpha, or the nuclear pore. In contrast, although Hrp1p import seems impaired during hyperosmotic conditions, Hrp1p can still exit the nucleus by using an Xpo1/Crm1-dependent export pathway. Furthermore, Xpo1p/Crm1p is likely also required for Hrp1p export during standard growth because Xpo1p/Crm1p and Hrp1p interact during these conditions (Hammell *et al.*, 2002). Ongoing transcription and protein arginine methylation are also important for the nuclear export of Hrp1p during standard growth conditions (Shen *et al.*, 1998). However, in this study we observed that the nuclear export of Hrp1p after hyperosmotic shock was unaffected by either the inhibition of RNA synthesis or the loss of Hmt1 arginine methylation. A plausible interpretation of these results is that after hyperosmotic stress Hrp1p uses an alternate

Hmt1-independent export pathway that does not require active RNA transcription. This preliminary model is easily testable and should serve to guide future experiments.

Recently, the stress induced export of a related yeast hnRNP protein, Npl3, has been proposed to dissociate from mRNA before its nuclear export (Krebber *et al.*, 1999). Although we demonstrate that Hrp1p exits the nucleus after hypertonic stress independent of ongoing transcription, this work does not directly address whether Hrp1p departs the nucleus associated with mRNA. Although intriguing, it may be unwise to draw too many comparisons between these two proteins. In spite of their structural similarities, Hrp1 is functionally distinct because it responds only to hyperosmolarity and translocates to the cytoplasm permanently.

To date, the steady-state cytoplasmic localization of Hrp1p during hyperosmotic conditions is unique among nuclear stress induced proteins. Although Nanduri and Tartakoff (2001) recently observed that hypertonic shock relocated several nuclear and nucleolar proteins to the cytoplasm, the translocation of these proteins was only transient. For instance, on continued incubation in hypertonic medium they observed that the nucleolar propyl isomerase Fpr3 protein reentered the nucleus within 1 h (Nanduri and Tartakoff, 2001). Furthermore, in agreement with Krebber *et al.* (1999), we have observed that the stress-induced relocalization of Npl3p to the cytoplasm is also transient. The Npl3 protein returns to the nucleus within 1 h after the initial exposure to the stress (Krebber *et al.*, 1999; our unpublished data).

Why does Hrp1p exit the nucleus and remain in the cytoplasm during exposure to hyperosmotic conditions? One interesting interpretation is that the removal of Hrp1p from the nucleus serves to alter mRNA polyadenylation during these stress conditions. It has previously been proposed that Hrp1p regulates the use of alternative 3' end cleavage sites in a concentration-dependent manner (Minvielle-Sebastia *et al.*, 1998). It is therefore conceivable that Hrp1p nuclear depletion could lead to the production of specific transcripts with altered 3' ends. Alternative polyadenylation has been observed for a variety of yeast genes, and in a number of cases these genes give rise to transcripts that terminate within the open reading frame. Thus, alternative polyadenylation can affect both transcript stability and translation. In the best-characterized example, *CBP1* produces two mRNAs that are regulated reciprocally by a carbon source (Mayer and Dieckmann, 1989; Sparks *et al.*, 1997; Sparks and Dieckmann, 1998). Although *CBP1* transcripts are not alternatively polyadenylated during hyperosmotic conditions (our unpublished data), it is possible that genes necessary for the hyperosmotic response could be affected at the level of mRNA 3' end formation. Alternatively, it is possible that additional Hrp1 protein is required in the cytoplasm for optimal cell growth during hypertonic conditions. During standard growth conditions, Hrp1p is also present in the cytoplasm at low levels where it functions in the NMD pathway, monitoring premature translation and degrading aberrant mRNAs (Gonzalez *et al.*, 2000). If the level of aberrant mRNA increases during hyperosmotic stress, it may be beneficial to increase the cytoplasmic levels of Hrp1p.

Given that Hrp1p is required for nuclear mRNA polyadenylation, it is surprising that the cell survives Hrp1p relo-

calization to the cytoplasm during hypertonic conditions. A simple explanation is that a small percentage of Hrp1p remains in the nucleus during conditions of hypertonic stress. Consistent with this idea, Hrp1p does not seem to be excluded from the nucleus during these conditions as judged by indirect immunofluorescence. Presumably, this level of Hrp1p would be sufficient to carry out its nuclear function. A second possibility is that Hrp1p is not required for mRNA polyadenylation under these stress conditions. Previous studies concerning the role of Hrp1p in mRNA polyadenylation have been limited to standard growth conditions (Kessler *et al.*, 1997; Minvielle-Sebastia *et al.*, 1998).

Earlier studies on the response to high osmolarity identified two integral membrane proteins, Sho1p and Sln1p, as likely osmosensors for the HOG MAP kinase cascade (Maeda *et al.*, 1994; Posas *et al.*, 1996; Posas and Saito, 1997; Raitt *et al.*, 2000). These two proteins differ structurally in both sequence as well as in their number of membrane-spanning regions and likewise have great functional variations. More recently, a third potential osmosensor, Msb2p, has been reported (O'Rourke *et al.*, 2002). This protein functions in the absence of Hog1p and is partially redundant with Sho1p. We now report that Fps1p is required for cells to relocalize Hrp1p after exposure to hyperosmotic conditions. Due to its integral membrane location, it has the potential to be a fourth osmosensor in yeast. The precise role of Fps1p in this process will be examined in further studies.

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