Asr1p, a Novel Yeast Ring/PHD Finger Protein, Signals Alcohol Stress to the Nucleus*

Christian Betz, Gabriel Schlenstedt, and Susanne M. Bailer‡

From the Universität des Saarlandes, Medizinische Biochemie und Molekularbiologie, Gebäude 44, D-66421 Homburg/Staar, Germany

During fermentation, yeast cells are exposed to increasing amounts of alcohol, which is stressful and affects both growth and viability. On the molecular level, numerous aspects of alcohol stress signaling remain unresolved. We have identified a novel yeast Ring/PHD finger protein that constitutively shuttles between nucleus and cytoplasm but accumulates in the nucleus upon exposure to ethanol, 2-propanol, or 1-butanol. Subcellular localization of this protein is not altered by osmotic, oxidative, or heat stress or during nitrogen or glucose starvation. Because of its exclusive sensitivity to environmental alcohol, the protein was called Asr1p for Alcohol Sensitive Ring/PHD finger 1 protein. Nuclear accumulation of Asr1p is rapid, reversible, and requires a functional Ran/Gsp1p gradient. Asr1p contains two N terminally located leucine-rich nuclear export sequences (NES) required for nuclear export. Consistently, it accumulates in the nucleus of xpo1-1 cells at restrictive temperature and forms a trimeric complex with the exportin Xpo1p and Ran-GTP. Deletion of ASR1 leads to sensitivity in growth on medium containing alcohol or detergent, consistent with a function of Asr1p in alcohol-related signaling. Asr1p is the first reported protein that changes its subcellular localization specifically upon exposure to alcohol and therefore represents a key element in the analysis of alcohol-responsive signaling.

Yeast cells frequently encounter fluctuations in environmental conditions, including changes in osmosis and temperature or availability of nutrients. Specific stress occurs during fermentation, when yeast cells produce increasing amounts of ethanol, the primary fermentation product. Although yeast has developed a high tolerance to ethanol, a concentration above 4–6% is stressful, eventually leading to growth inhibition and to cell death (1). The primary effects of ethanol include changes in membrane fluidity and protein denaturation (1, 2).

In general, environmental changes are communicated to the interior of the cell by a complex network of signaling molecules (3–9) and compensated by transcriptional adaptation (10–13). To restore vital functions upon alcohol stress, a large number of genes is differentially up-regulated, including genes involved in energy metabolism, protein destination, ionic homeostasis, and stress response as well as lipid metabolism (1, 12, 14, 15). However, surprisingly little is known about how alcohol stress is sensed and signaled to its effectors. Global gene expression analysis indicates that ethanol stress feeds into multiple signaling pathways, in particular the general stress response pathway (1, 3, 12). Ethanol may also signal through elements of the high glycerol osmostress responsive pathway, which contrasts with the finding that the Hog1 kinase is not activated by ethanol (12, 16–18). Finally, ethanol stress signaling overlaps with the signaling of cell integrity and morphogenesis (19, 20). Despite these findings, specific alcohol-responsive signaling molecules that target the transcription machinery and other cellular components are likely to exist (12, 20).

Subcellular compartmentalization of molecules is an important way of regulating signaling events (21–24). The exchange of molecules between the cytoplasm and nucleus occurs through the nuclear pore complex, is mediated by soluble transport factors of the importin-β family, and is driven by the Ran/Gsp1p-GTP gradient (25–28). The latter is generated by the nuclear localization of the guanine nucleotide exchange factor RCC1 (Prp20p in Saccharomyces cerevisiae) and the nuclear exclusion of the RanGTPase-activating protein RanGAP1 (Rna1p in S. cerevisiae). As a consequence of signaling, proteins are modified differentially thus altering their affinity for import or export factors. This was reported for the transcription factors Msn2p (29, 30), Pho4p (22), and Yap1p (31, 32) and the mitogen-activated protein kinase Hog1p (16, 33). Alternatively, proteins may preferentially bind to anchor proteins confined to a certain compartment or be released from them upon environmental changes as is the case with Msn2p and Msn4p (34).

We have identified a novel protein of 36 kDa (YPR093C), which constitutively shuttles between the nucleus and cytoplasm, but rapidly and reversibly accumulates in the nucleus upon alcohol stress. Because Asr1p changes its subcellular localization specifically in response to alcohol but not to any other form of stress, and is required for tolerance to media containing alcohol, Asr1p is a novel element of an uncharacterized alcohol-responsive signaling pathway that targets the nucleus for stress compensation.

MATERIALS AND METHODS

Yeast Strains and Growth, Microbiological Techniques, and Plasmids—The yeast strains used in this work are listed in Table I. Cells were grown in minimal SDC or YPD medium or as indicated. Minimal SDC medium/plates contained all amino acids and nutrients except those used for selection. To determine the growth properties of asr1::HIS3 cells, logarithmically growing cells were diluted in 10⁻¹ steps, and equivalent amounts of asr1::HIS3 (Δ) or wild type cells were dripped onto YPD plates containing 1% ethanol, 1% butanol, 0.02% SDS, 0.05 µg/ml Blankophor P167% (Bayer Leverkusen), 1 M NaCl, or 1 M sorbitol and incubated at 30 or 37 °C for 3–4 days. Alternatively, cells were dripped onto YP plates containing no carbon source, 2% citrate, 2% glycerol, or 2% pyruvate and incubated at 30 °C for 3–4 days.
Gene manipulation of yeast and general cloning were performed as described earlier (35). The coding region of full-length Asr1p was amplified by PCR using specific 5′ and 3′ primers. To mutate Asr1p NES1 and NES2, a 5′ and a 3′ fragment of the Asr1p coding sequence was amplified by PCR and fused by an inserted BamHI site. In this way, the original amino acid sequence 120–140 of NES1 (QEIPLSER-LGTLVMDTIKI) altered to QEISSMDTIIK. Because NES2 is located in proximity to the metal ligand 2 of Ring/PDH finger 2 of Asr1p, it was intended to keep the spacing of 11 amino acids between metal ligands 2 and 3. Therefore, the NES2 sequence was not deleted but it was intended to keep the spacing of 11 amino acids between metal ligands, and the conservation of distinctly positioned hydrophobic amino acids suggest that the N-terminal export sequence.

RNAi analyses of Gsp1p-GTP (mutant Gsp1p-Q71L) were added as indicated in the figure legends. PBS/KMT was added to a final volume of 200 μl. After incubation for 1 h at 4 °C on a turning wheel, the beads were washed three times with 1 ml of PBS/KMT. Bound proteins were eluted with 60 μl of SDS containing gel loading buffer. Eluates were analyzed by SDS-PAGE and subsequent Coomassie Blue staining or Western blotting using anti-Xpo1p and anti-Gsp1p antibodies (38).

RESULTS

The Hypothetical Open Reading Frame YPR093C Encodes a Novel Ring/PDH Finger Protein That Predominantly Localizes to the Cytoplasm—The open reading frame YPR093C was identified in a yeast 2-hybrid screen using Nup116p as bait. YPR093C is located on chromosome XVI and codes for a protein of 310 amino acids with a calculated mass of 35,988 kDa and a pl of 6.24. Based on the primary amino acid sequence, at least two domains can be distinguished (Fig. 1A). The N-terminal domain of Asr1p (residues 1–188) contains several cysteines and histidines, which could form a zinc binding domain. The consensus sequences C₆H₅C₆ and C₆H₅C₆ (Fig. 1B), the spacing of the metal ligands, and the conservation of distinctly positioned hydrophobic amino acids suggest that the N-terminal half of this protein folds into two Really Interesting New Gene (Ring)- or plant homeodomain (PHD)-type finger domains, one with a high probability within residues 26–69 and a second one with a lower probability in residues 143–189 (Refs. 39, 40, and references therein). Its C-terminal

1 The abbreviations used are: GFP, green fluorescent protein; DAPI, 4′,6′-diamidino-2-phenylindole; GST, glutathione S-transferase; Ring, Really Interesting New Gene; PHD, plant homeodomain; NES, nuclear export sequence.

2 J. Solsbacher and G. Schlenstedt.

6 | Yeast strains
| Strain | Genotype | References |
|--------|----------|------------|
| RS453n | MATA ade2, his3, leu2, trp1, ura3 | 35 |
| asr1::HIS3 | or a, ade2, his3, leu2, trp1, ura3, asr1::HIS3 | This study |
| XPO1 | MATA ura3, trp1, his3, leu2, ade2, can1::kanMX pRS316-XPO1 | 44 |
| xpo1::HIS3 | or a, can1::kanMX pRS311-xpo1 | 44 |
| rna1-1 | MATA ADE2, HIS3, leu2, trp1, ura3, rna1-1 | 49 |

a J. Solsbacher and G. Schlenstedt.
Asr1p is a cytoplasmically located Ring/PHD finger protein. A, schematic drawing of Asr1p with Ring/PHD fingers 1 and 2 (hatched). B, comparison of Asr1p Ring/PHD finger domains 1 and 2 with the consensus sequence (cs) of Ring- and PHD-type finger domains. C, localization of GFP-Asr1p expressed in asr1Δ cells from an ARS/CEN plasmid (a and b) or from a 2μ plasmid (c and d). Cells were grown in selective medium at 30 °C and analyzed by indirect immunofluorescence using rabbit anti-GFP antibodies followed by fluorescein isothiocyanate-labeled anti-rabbit antibodies (a) or by fluorescence microscopy (c). Nuclei or cells were visualized by DAPI staining (b) or phase contrast (d). GFP-Asr1p expressed in asr1Δ cells from an ARS/CEN plasmid (top panel, lane 1) or from a 2μ plasmid (lane 2) was analyzed by SDS-PAGE and Western blotting using rabbit anti-GFP antibodies and peroxidase-coupled goat anti-rabbit antibodies.

Asr1p Accumulates in the Nucleus Specifically upon Alcohol Stress—The initial finding that Asr1p interacts with the nucleoporin Nup116p suggested that it contacts the nucleocytoplasmic transport machinery and thus may travel between the cytoplasm and nucleus. Its subcellular localization could therefore be regulated in terms of space and time and altered under special circumstances. In search of conditions that shift the cytoplasmic steady state localization of Asr1p to a predominantly nuclear one, the localization of GFP-Asr1p expressed in logarithmically growing asr1Δ cells was tested following various stress conditions. Exposure of the cells to 7.5% ethanol for 10 min dramatically changed the predominant cytoplasmic localization of GFP-Asr1p to a nuclear one, as is indicated by the DAPI staining (Fig. 2A). Several aliphatic alcohols were tested to find out whether GFP-Asr1p specifically responded to ethanol or whether other alcohols had a similar effect. As shown in Fig. 2A, a 10-min exposure to 5% 2-propanol or 2% 1-butanol led to a nuclear accumulation of GFP-Asr1p comparable with that following exposure to 7.5% ethanol. Obviously, stress generated by various alcohols leads to rapid nuclear accumulation of Asr1p.

Because the stress response to alcohol overlaps other forms of stress (1, 3, 12), the localization of GFP-Asr1p was analyzed...
Asr1p Signals Alcohol Stress to the Nucleus

**Fig. 2.** Asr1p specifically accumulates in the cell nucleus upon alcohol stress. *A*, nuclear accumulation of GFP-Asr1p is induced by various aliphatic alcohols. Localization of GFP-Asr1p expressed in logarithmically growing *asr1Δ* cells before and after exposure to 7.5% ethanol, 5% 2-propanol, and 2% 1-butanol. Nuclei were visualized by DAPI staining. *B*, subcellular localization of GFP-Asr1p expressed in logarithmically growing *asr1Δ* cells following 10 min of heat shock (37°C), of osmotic stress (0.5 M NaCl), of oxidative stress (2 mM H2O2), after nitrogen starvation (starv.), and during the stationary phase.

**Fig. 3.** Nuclear accumulation of Asr1p upon alcohol stress is rapid, reversible, and depends on the Ran/Gsp1p gradient. *A*, nuclear accumulation of GFP-Asr1p upon alcohol stress. Localization of GFP-Asr1p expressed in logarithmically growing *asr1Δ* cells and *asr1Δ* cells exposed to 7.5% ethanol for 5, 10, or 30 min. To analyze the recovery of cytoplasmic localization of GFP-Asr1p, cells were first stressed for 10 min by the addition of 7.5% ethanol. Recovery was analyzed 10 min after removal of the ethanol-containing medium. Nuclei were detected with DAPI. *B*, the *rna1-1* mutant expressing GFP-Asr1p was grown at permissive (25°C) temperature or shifted to restrictive (32°C) temperature for 30 min. GFP-Asr1p was visualized before or after the addition of 7.5% ethanol.

After heat stress at 37°C (Fig. 2B) or 42°C (data not shown), after osmotic stress (0.5 M NaCl, 1 M sorbitol (data not shown)), or after oxidative stress (2 mM H2O2), obviously none of these stressors had any effect on the localization of GFP-Asr1p (Fig. 2B). Nor did GFP-Asr1p change its localization upon nitrogen starvation or during the stationary phase (Fig. 2B). Taken together, Asr1p accumulates in the nucleus upon alcohol stress, a phenomenon that is unique to alcohol and is not observed after any other form of stress.

**Nuclear Accumulation of Asr1p upon Alcohol Stress Is Rapid and Reversible and Depends on the Ran/Gsp1p Gradient—**To study the kinetics of the alcohol-induced nuclear accumulation of Asr1p, *asr1Δ* cells transformed with a GFP-Asr1p fusion plasmid were treated with 7.5% ethanol for various periods. Nuclear fluorescence of GFP-Asr1p increased as early as 1 min after the addition of ethanol and reached its maximum between 10 and 30 min (Fig. 3A).

Next we examined whether nuclear accumulation of GFP-Asr1p following alcohol stress is reversible. For this purpose GFP-Asr1p expressed in *asr1Δ* cells was first exposed to 7.5% ethanol for 10 min. Subsequently, the ethanol-containing medium was removed, and the cells were analyzed again 10 min after the removal of the stressor. As is shown in Fig. 3A, the removal of the alcohol-containing medium led to rapid relocation of GFP-Asr1p to the cytoplasm.

To find out whether alcohol-induced nuclear accumulation of Asr1p depends on the Ran/Gsp1p gradient, the subcellular localization of GFP-Asr1p was analyzed in cells impaired in the GTPase-activating protein Rna1p or the GTP/GDP exchange factor Prp20p. Although ethanol treatment of the mutant cells *rna1-1* (Fig. 3B) and *prp20-1* (data not shown) led to rapid nuclear localization of GFP-Asr1p at a permissive temperature, nuclear accumulation of GFP-Asr1p following alcohol stress was impaired at a restrictive temperature. Taken together, these data indicate that the alcohol-induced nuclear accumulation of GFP-Asr1p is a rapid and reversible process that requires a functional Ran/Gsp1p gradient.

**Nuclear Export of Asr1p Is Mediated by Nuclear Export Signals Located within the Ring/PHD Finger Domain—**In search of signal sequences that could mediate active nuclear import or export of Asr1p, two leucine-rich sequences matching the consensus nuclear export sequence (NES) were identified within the N-terminal Ring/PHD finger domain (Fig. 4A (42, 43)). The first sequence located within residues 124–135 and designated NES1 is highly homologous to the NES consensus sequence, whereas homology of the second sequence within residues 145–156 and designated NES2 is less striking. To analyze the potential of these sequences to export Asr1p into the cytoplasm, we generated mutants whereby each of these putative NESs was deleted. Expression of these deletion mutants as GFP fusion proteins in comparison to wild type GFP-Asr1p in an *asr1Δ* strain was analyzed by Western blotting using anti-GFP antibodies (Fig. 4B, lanes 1–3). As analysis by fluorescence microscopy showed, the absence of residues 124–135 led to a strong accumulation of GFP-Asr1p in the nucleus with only minor amounts in the cytoplasm (Fig. 4B, a). Obviously, NES1 has a strong potential to function as a nuclear export signal. Deletion of the second putative export signal in GFP-Asr1p (residues 144–156) resulted in the localization of GFP-Asr1p both in the cytoplasm and nucleus (Fig. 4B, b). These experiments show that Asr1p harbors one or two functional sequences within its N-terminal Ring/PHD finger domain that...
Asr1p Signals Alcohol Stress to the Nucleus

Fig. 4. Nuclear export sequences within Asr1p-N. A, NES1 and NES2 of Asr1p and their homology to other nuclear export sequences. B, fluorescence localization of GFP-Asr1p-NES1A (a), GFP-Asr1p (a, inset), and GFP-Asr1p-NES2Δ (b) in asr1Δ cells. Expression of GFP-Asr1p (lane 1), GFP-Asr1p-NES1Δ (lane 2), and GFP-Asr1p-NES2Δ (lane 3, lower panel) was analyzed as described in the legend to Fig. 1.

Fig. 5. Asr1p shuttles between the nucleus and cytoplasm in an Xpo1p-dependent manner. A, fluorescence localization of GFP-Asr1p (left) and GFP-Yrb1p (right) in XPO1 and xpo1-1 strains at 23°C and after shifting to 37°C for 15 min. B, GST-Asr1p (lanes 1–4), GST-NES(GFP)2 (lanes 5–8), or GST (lanes 9–12) were immobilized on glutathione-Sepharose and incubated for 60 min at 4°C with Xpo1p (lanes 2, 6, and 10), Gsp1p-GTP (lanes 3, 7, and 11), both Xpo1p and Gsp1p-GTP (lanes 4, 8, and 12), or alone (lanes 1, 5, and 9). The load of Xpo1p and Gsp1p-GTP is shown in lanes 13 and 14, respectively. GST and GST fusion proteins are indicated by open circles, and the binding of Xpo1p and Gsp1p-GTP is indicated (from top) by asterisks. Bound material of all reactions was analyzed by SDS-PAGE (top panel) and Western blot using rabbit anti-Xpo1p or anti-Gsp1p antibodies followed by peroxidase-coupled goat anti-rabbit antibodies (lower panels). Molecular mass markers are indicated on the left.

are required for efficient nuclear export. Moreover, these data reveal that Asr1p is constitutively imported into the nucleus under normal growth conditions.

Asr1p Constitutively Shuttles between Nucleus and Cytoplasm in an Xpo1p-dependent Manner—NES sequences as identified in the N-terminal half of Asr1p are recognized by the exportin Xpo1p. To test whether the export of Asr1p is in fact mediated by Xpo1p, GFP-Asr1p was expressed in the export-deficient mutant xpo1-1 or its isogenic wild type strain XPO1 and analyzed for its localization at permissive and restrictive temperatures. For control, GFP-Yrb1p, shown previously to shuttle in an Xpo1p-dependent manner, was expressed in XPO1 and xpo1-1 cells (38, 44). At the permissive temperature or upon expression in the XPO1 wild type strain, GFP-Asr1p predominantly located to the cytoplasm, whereas shifting the xpo1-1 cells to the restrictive temperature for 15 min resulted in a nuclear accumulation of GFP-Asr1p (Fig. 5A, left panel). Similar results were obtained for GFP-Yrb1p (Fig. 5A, right panel). This in vivo observation is consistent with the constitutive shuttling of Asr1p between the nucleus and cytoplasm in an Xpo1p-dependent manner.

Generally, proteins containing a leucine-rich NES form a complex with Xpo1p and Gsp1p-GTP, the GTP-bound form of the yeast Ran (38, 45). To determine whether Asr1p is able to form such an export-competent trimeric complex, GST-Asr1p was expressed and purified from Escherichia coli and tested for interaction in vitro with purified recombinant His6-Xpo1p and His6-Gsp1p (Fig. 5B). Because wild type Gsp1p hydrolyzes GTP, the GTPase-deficient mutant Gsp1pQ71L loaded with GTP was used in this study. Purified GST-Asr1p, GST-NES2-GFP (38) as a positive control, and GST as a negative control were immobilized on glutathione-Sepharose. Xpo1p (lane 13), Gsp1p-GTP (lane 14), or both were added to the resin. Only minor amounts of Xpo1p (lanes 2 and 5) or Gsp1p-GTP (lanes 3 and 7) were bound to GST-Asr1p (lanes 2 and 3) or GST-NES2-GFP (lanes 6 and 7). However, in the presence of both Xpo1p and Gsp1p-GTP, binding is significantly increased and trimeric cargoXpo1p/Gsp1p-GTP complexes were formed with GST-Asr1p (lane 4) or GST-NES2-GFP (lane 8) but not with GST (lane 12). Consistent with nuclear accumulation of GFP-Asr1p in the xpo1-1 strain, Asr1p formed a trimeric complex with the exportin Xpo1p and Gsp1p-GTP, a finding that correlates with the presence of at least one functional nuclear export sequence within Asr1p.

Nuclear Accumulation upon Alcohol Stress Is an Intrinsic Feature of Asr1p—Nucleocytoplasmic exchange of molecules is controlled by modulating the nuclear import or export rate or...
by its retention in a certain compartment. Because the nuclear import of Asr1p is constitutive in xpo1Δ cells, we reasoned that Asr1p accumulates in the nucleus as a consequence of inhibited nuclear export. To further address the molecular mechanism of the alcohol-induced nuclear accumulation of Asr1p, GFP-Yrb1p and an NES-GFP-NLS reporter protein were analyzed following alcohol stress. Both of these proteins shuttle between the nucleus and cytoplasm in an Xpo1p-dependent manner (38) comparable with Asr1p. Following a 10-min exposure of asr1Δ cells expressing GFP-Yrb1p to 7.5% ethanol, the cytoplasmic localization GFP-Yrb1p was unaltered (Fig. 6A). The same was true for the NES-GFP-NLS reporter protein (data not shown). Thus, the ability to accumulate in the nucleus upon alcohol stress is intrinsic to Asr1p and not caused by a general block of the Xpo1p-linked nuclear export system.

Nuclear accumulation could be the consequence of masking the N terminally located nuclear export sequences of Asr1p. To test whether Asr1p has the potential to homodimerize, an asr1Δ strain carrying two separate plasmids encoding ProtA-Asr1p and myc-Asr1p was generated. ProtA-Asr1p was affinity-purified using IgG-Sepharose and analyzed for associated myc-Asr1p. ProtA-Asr1p was subjected to affinity-purification on IgG-Sepharose. Homogenate (H) (lanes 1 and 4) and eluate (E) (lanes 2 and 3) were analyzed by SDS-PAGE followed by Western blotting using anti-ProtA antibodies (left panel) and anti-myc antibodies (right panel).

ASR1 Is Required for Tolerance to Alcohol—To gain insight into the function of Asr1p, the asr1Δ strain was compared with the wild type strain under various growth conditions. Replacement of the YPR089C open reading frame with the auxotrophic marker HIS3 leads to a strain fully viable on YPD at all temperatures (Fig. 7A, data not shown). Thus, ASR1 is not essential for growth under normal conditions.

As a protein responding to environmental alcohol, Asr1p could be part of a regulatory network that coordinates the metabolism of various sugars and nonfermentable compounds (13). To determine whether Asr1p is required for the utilization of various carbon sources, the growth of asr1Δ cells was tested on medium containing either no carbon source at all, 2% citrate, 2% glycerol, 2% pyruvate (Fig. 7B), 2% raffinose, 2% galactose, or 2% sucrose (data not shown). Yet, no difference in the use of fermentable and nonfermentable carbon sources by the asr1Δ strain could be detected.

To investigate whether asr1Δ cells are sensitive in growth on media containing alcohols, the wild type and the asr1Δ strain were spotted on YPD media containing 1% ethanol or 1% butanol. Interestingly, although both wild type and asr1Δ cells grew normally on a medium containing 1% ethanol, growth of the disruption strain was severely compromised on 1% butanol (Fig. 7C). Because sensitivity to alcohol could be caused by a compromised cell wall or plasma membrane (19), we analyzed the asr1Δ cells for growth on medium containing the detergent SDS or the chemical Blancophor P167%, an analog of Calcofluor white, which interferes with cell wall assembly (46). Although the isogenic wild type grew normally on media containing either compound, the asr1Δ cells were highly sensitive to the presence of 0.02% SDS but insensitive to Blancophor P167% (Fig. 7B). Consistently, asr1Δ cells grow normally on hyperosmotic medium containing 1 M NaCl or 1 M sorbitol (Fig. 7B). We thus conclude that asr1Δ cells maintain cell wall integrity but are sensitive to chemicals that interfere with plasma membrane integrity. Taken together, our data are consistent with a function of Asr1p in the signaling of alcohol-induced plasma membrane perturbations.

**DISCUSSION**

Alcohol stress is signaled by several pathways leading to a rather pleotropic phenotype which is naturally difficult to analyze (1, 12). We have identified and characterized a novel protein encoded by the open reading frame YPR089C, which we here call Asr1p for Alcohol Sensitive Ring/PHD finger 1 protein. Asr1p constitutively shuttles between the nucleus and cytoplasm under normal growth conditions but accumulates in the nucleus upon alcohol stress, a process that is active, rapid, and reversible. To our knowledge, Asr1p is the first yeast protein described that changes its subcellular localization exclusively upon alcohol stress. Several stress-protective proteins accumulate in the nucleus upon various forms of stress including environmental ethanol e.g. Msn2p (47). However, unlike Msn2p, Asr1p does not respond to heat stress, osmotic stress, or glucose starvation (29, 47), nor does Asr1p respond to oxidative stress or nitrogen starvation. Thus, Asr1p represents a key element in identifying novel signaling elements that specifically respond to alcohol stress.

Consistent with our finding that Asr1p accumulates in the...
nucleus upon alcohol stress but not in response to other environmental changes, cells lacking ASR1 easily tolerate high temperature and osmotic stress, and expression of heat shock genes is unaltered in asr1Δ cells compared with wild type cells. Moreover, ASR1 is not essential for the use of various carbon sources, for butanol-induced filamentation, or for the response to calcium stress (13, 20). Instead, asr1Δ cells are sensitive in growth on medium containing butanol or SDS. Cell insensitivity to growth in ethanol is probably because of the rather low concentration of ethanol in the medium. Unexpectedly, asr1Δ cells are not sensitive to Calcofluor white, a chitin antagonist that indicates perturbations of the cell wall (46). Because alcohols and detergents partition into the bilayer, thus changing its fluidity, we propose that Asr1p is functionally linked to a signaling system that senses changes in membrane fluidity. In this context it is worth mentioning that Asr1p changes its subcellular localization in response to progressively lower concentrations of ethanol, propanol, and butanol, as their aliphatic chain length, their hydrophobicity, and thus their ability to partition into the membrane increase (1). Interestingly, the lipid composition of the plasma membrane is altered upon alcohol stress, and adaptation is crucial for tolerance to alcohol (1, 48). We therefore propose a primary function for Asr1p in either signaling or compensating membrane stress but not cell wall perturbation.

The nuclear residence of Asr1p following alcohol stress suggests a function for Asr1p in this compartment. A great number of proteins responding to environmental stress are transcription factors, and future analysis is required to reveal its nuclear targets.

How Asr1p enters the nucleus, whether by direct binding to an importin-β-like transport factor or by a piggy-back mechanism, remains to be determined. Relocation of Asr1p to the cytoplasm is mediated by the exportin Xpo1p, which recognizes one or two N terminally located NESs. Although NES1 fits perfectly the NES consensus sequence, NES2 is more divergent. Although our aim was to conserve the spacing between the metal ligands of the second Ring/PHD finger, the mutagenesis of NES2 may interfere with the folding of this structure and consequently with the mobility of this protein.

In principle, nuclear accumulation upon alcohol stress could be the result of enhanced nuclear import or inhibited nuclear export. Our data show that the nuclear import of Asr1p is constitutive and that the nuclear export machinery involving Xpo1p is fully functional during alcohol stress. Although we cannot exclude at this point that a posttranslational modification of Asr1p as a consequence of alcohol stress signaling could further enhance nuclear import, we favor nuclear retention as a mechanism for its nuclear accumulation. Molecularly, this could be caused by obscuring the export signals through posttranslational modification, by conformational change, or through protein-protein interaction. Interestingly, both nuclear export sequences nest within the Ring/PHD fingers of Asr1p, domains that mediate homo- or heterodimerization (Refs. 39–41 and references therein), and we show that Asr1p is indeed able to homodimerize in vitro. Furthermore, Asr1p could be retained in the nucleus by forming hetero-oligomers with a nuclear target. We thus propose that regulated homo- or heterodimerization is important for subcellular localization and the function of Asr1p.

In conclusion, we have identified and characterized a novel protein that responds to alcohol stress and is required for tolerance to alcohol. Therefore Asr1p represents a highly promising candidate for unraveling at least part of the complex signaling that allows the cell to cope with alcohol stress.

Acknowledgments—We thank C. Balduf for initial help in this project. We thank O. Gadal, Institut Pasteur, Paris cedex, France, P. Milkeriet, University of Regensburg, Germany, M. Montenarh and H. Sahl, University of Salzburg, Austria, for their generous support and suggestions.

REFERENCES

1. Fiper, P. (1995) FEBS Microbiol. Lett. 134, 121–127
2. Rose, A. H. (1993) J. Appl. Bacteriol. 74, suppl. S110–S118
3. Estruch, F. (2000) FEBS Microbiol. Rev. 24, 469–486
4. Ellis, E. A. (2001) J. Cell Sci. 114, 3967–3978
5. van Drogen, P., and Peter, M. (2002) Curr. Biol. 12, 55–55
6. De Nadel, E., Alepuz, P., and Posas, F. (2002) EMBO Rep. 3, 735–740
7. O’Shea, S., Herskowitz, I., and O’Shea, E. (2002) Trends Genet. 18, 465–472
8. Hohmann, S. (2002) Microbiol. Mol. Biol. Rev. 66, 300–372
9. Carroll, A., and O’Shea, E. (2002) Trends Biochem. Sci. 27, 87–93
10. Gasch, A. P., Spellman, P. T., Kad, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Mol. Cell. 11, 4241–4257
11. Causton, H. C., Ren, B., Koh, S. S., Barrison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) Mol. Cell. 12, 323–337
12. Alexandre, H., Ansany-Galeote, V., Dequin, S., and Blondin, B. (2001) FEBS Lett. 498, 98–103
13. Schuller, H.-J. (2003) Curr. Genet. 43, 139–160
14. Aranda, A., Querol, A., and Olmo, M. d. (2002) Arch. Microbiol. 177, 304–312
15. James, T., Campbell, S., Donnelly, D., and Bond, U. (2002) J. Appl. Bacteriol. 94, 432–448
16. Ferrugia, P., Posas, F., Koepp, D., Saito, H., and Silver, P. A. (1998) EMBO J. 17, 5606–5614
17. Tamas, M., Rep, M., Thevelein, J., and Hohmann, S. (2000) FEBS Lett. 427, 159–165
18. Alepuz, P., Jovanovic, A., Reiser, V., and Ammerer, G. (2001) Mol. Cell. 7, 767–777
19. Takahashi, T., Shimoi, H., and Ito, K. (2001) Mol. Genet. Genomics 265, 1112–1119
20. Lenzen, M. C., Cutler, N. S., and Heitman, J. (2000) Mol. Cell. 11, 183–199
21. Garner, W., Schuller, C., and Ruis, H. (1999) Biol. Chem. 380, 147–150
22. Ruis, A., and O’Shea, E. (2000) Curr. Opin. Cell Biol. 12, 355–360
23. Cyert, M. S. (2001) J. Biol. Chem. 276, 20805–20808
24. Schuller, C., and Ruis, H. (2002) Results Prob. Cell Differ. 35, 169–189
25. Gierach, L., and Kutanu, U. (1999) Ann. Rev. Cell Dev. Biol. 15, 607–670
26. Danelin, M., Silver, P., and Corbett, A. (2002) Methods Enzymol. 351, 587–697
27. Weiss, K. (2002) Curr. Opin. Cell Biol. 14, 328–335
28. Fried, H., and Kutay, U. (2003) Cell Mol. Life Sci. 60, 1659–1688
29. Garner, W., Durchschlag, E., Wolf, J., Brown, E. L., Ammerer, G., Ruis, H., and Schuller, C. (2002) EMBO J. 21, 135–144
30. Mayordomo, I., Estruch, F., and Sanz, P. (2002) J. Biol. Chem. 277, 35650–35656
31. Kuge, S., Jones, N., and Nomoto, A. (1997) EMBO J. 16, 1710–1720
32. Kuge, S., Toda, T., Iizuka, N., and Nomoto, A. (1998) Genes Cells 3, 521–532
33. Reiser, V., Ruis, H., and Ammerer, G. (1999) Mol. Biol. Cell 10, 1147–1161
34. Beck, T., and Hall, M. N. (1999) Nature 402, 699–702
35. Balder, S. M., Ballest, C., and Hurt, E. (2001) Mol. Cell. 12, 7944–7955
36. Komeili, A., Wedaman, K. P., O’Shea, E. K., and Powers, T. (2000) J. Cell Biol. 151, 863–876
37. Solasbacher, J., Maurer, P., Bischoff, F. R., and Schlenstedt, G. (1998) Mol. Cell. 18, 6805–6815
38. Maurer, P., Redd, M., Solasbacher, J., Bischoff, F. R., Greiner, M., Podtelejnikov, A. V., Mann, M., Stade, K., Weis, K., and Schlenstedt, G. (2001) Mol. Cell 12, 539–549
39. Capilli, A. D., Schultz, D. C., Rauscher, F. J., III, and Borden, K. L. (2001) EMBO J. 20, 165–177
40. Aravind, L., Iyer, L., and Koonin, E. (2001) Cell Cycle 2, 123–126
41. Gozani, O., Karuman, P., Jones, D., Ivanov, D., Cha, J., Lugovskoy, A., Baird, C., Zhu, H., Field, S., Lessnick, S., Villasenor, J., Mehrotra, B., Chen, J., Rao, V., Brugge, J., Ferguson, C., Payrastre, B., Myonk, D., Cantley, L., Wanger, G., Dvecha, N., Prestwich, G., and Yuan, J. (2003) Cell 114, 99–111
3 C. Betz and S. M. Balder, unpublished results.
Asr1p Signals Alcohol Stress to the Nucleus

42. Segref, A., Sharma, K., Deyo, V., Hellwig, A., Huber, J., Lührmann, R., and Hurt, E. (1997) *EMBO J.* 16, 3256–3271
43. Yamaga, M., Fujii, M., Kamata, H., Hirata, H., and Yagisawa, H. (1999) *J. Biol. Chem.* 274, 28537–28541
44. Künzler, M., Gerstberger, T., Stutz, F., Bischoff, F. R., and Hurt, E. (2000) *Mol. Cell. Biol.* 20, 4295–4308
45. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) *Cell* 90, 1041–1050
46. Klis, F., Mol, P., Hellingwerf, K., and Brul, S. (2002) *FEMS Microbiol. Rev.* 26, 239–256
47. Gorner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schuller, C. (1998) *Genes Dev.* 12, 586–597
48. Alexandre, H., Rousseaux, I., and Charpentier, C. (1994) *FEMS Microbiol. Lett.* 124, 17–22
49. Amberg, D. C., Fleischmann, M., Stagljar, I., Cole, C. N., and Aebi, M. (1993) *EMBO J.* 12, 233–241