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Misfolded Proteins Are Competent to Mediate a Subset of the Responses to Heat Shock in *Saccharomyces cerevisiae**[S]

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Eleanor W. Trotter‡§, Camilla M.-F. Kao¶‖, Ludmilla Berenfeld, David Botstein‡‡, Gregory A. Petsko**§§, and Joseph V. Gray‡¶¶**

*From the ‡Division of Molecular Genetics, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G11 6NU, United Kingdom, the Departments of ¶Biochemistry and ‡‡Genetics, Stanford University School of Medicine, Stanford, California 94305, and the **Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02454-9110*

Cells may sense heat shock via the accumulation of thermally misfolded proteins. To explore this possibility, we determined the effect of protein misfolding on gene expression in the absence of temperature changes. The imino acid analog azetidine-2-carboxylic acid (AZC) is incorporated into protein competitively with proline and causes reduced thermal stability or misfolding. We found that adding AZC to yeast at sublethal concentrations sufficient to arrest proliferation selectively induced expression of heat shock factor-regulated genes to a maximum of 27-fold and that these inductions were dependent on heat shock factor. AZC treatment also selectively repressed expression of the ribosomal protein genes, another heat shock factor-dependent process, to a maximum of 20-fold. AZC treatment thus strongly and selectively activates heat shock factor. AZC treatment causes this activation by misfolding proteins. Induction of *HSP42* by AZC treatment required protein synthesis; treatment with ethanol, which can also misfold proteins, activated heat shock factor, but treatment with canavanine, an arginine analog less potent than AZC at misfolding proteins, did not. However, misfolded proteins did not strongly induce the stress response element regulon. We conclude that misfolded proteins are competent to specifically trigger activation of heat shock factor in response to heat shock.

Eukaryotic cells respond to heat shock by the induction of a conserved set of proteins, the heat shock proteins, via transcriptional activation of the corresponding genes (1). In the budding yeast *Saccharomyces cerevisiae*, two distinct promoter elements mediate transcriptional activation in response to heat shock (reviewed in Ref. 2). Heat shock elements (HSEs)[1] are found upstream of many heat-induced genes, *e.g. HSP42* and *SSA4*. Heat shock factor binds to HSEs and is required for the induction of HSE-driven genes in response to heat shock. Stress response elements (STREs) are also found upstream of many heat shock-induced genes, *e.g. CTT1* and *DDR1*, and bind the transcription factors Msn2 and Msn4 (2). Loss of both transcription factors compromises heat shock-induced expression of STRE-containing genes. Some heat shock-induced genes contain both HSEs and STREs in their promoters, *e.g. HSP12*, *HSP30*, and *HSP104*. The HSE and STRE regulons constitute the majority (if not all) of the genes that are specifically induced by heat shock (3).

Rapid upshifts in temperature within the permissive growth range of yeast (so-called "temperature upshifts"), *e.g.* 23–36 °C, result in the transient and selective induction of the heat shock genes (both HSE- and STRE-containing) and in the transient and selective repression of the 137 ribosomal protein genes (Ref. 4 and references therein). Heat shocks to nonpermissive temperature (*e.g.* to 42 °C) also cause a global repression of gene expression not seen upon temperature upshift (4). Repression of the ribosomal protein genes by heat shock is dependent on activation of heat shock factor. Because these genes do not contain HSEs, the repression is thought to be indirect (4).

The current model for how cells sense heat shock is as follows (Ref. 6 and references therein). Heat shock is proposed to cause the thermal misfolding of a fraction of cellular protein. Because activation of heat shock factor requires protein synthesis, it is thought that nascent proteins are the most susceptible to thermal denaturation. Misfolded proteins then bind to cytoplasmic Hsp70 chaperones. Prior to heat shock, these chaperones are believed to equilibrate between being bound to heat shock factor (and inactivating it) and being free in the cytoplasm. Because misfolded proteins bind Hsp70 chaperones very tightly, their accumulation upon heat shock is proposed to titrate Hsp70 chaperones, resulting in liberated and active heat shock factor. Consistent with this model, misfolded proteins have been detected in mammalian cells upon heat shock (7). In addition, some HSE-containing genes have been shown to be induced by the accumulation of nascent proteins (8), by failure to degrade misfolded and short-lived proteins (9), and by reduced cytoplasmic Hsp70 function (10). Unfortunately, the extent, specificity, potency, and mechanism of the above inductions are not known. Alternative triggers for activation of heat

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains Table S1.

This paper is dedicated to Prof. Herskowitz.

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‖ Damon Runyon-Walter Winchell Postdoctoral Fellow.

§§ Planted the seeds for the AZC project while a visiting professor in the laboratory of Ira Herskowitz at the University of California at San Francisco.

¶¶ To whom correspondence should be addressed: Div. of Molecular Genetics, Faculty of Biomedical and Life Sciences, University of Glasgow, Anderson College Complex, 54–56 Dumbarton Rd., Glasgow G11 6NU, UK. Tel.: 141-330-5114; Fax: 141-330-4878; E-mail: J.Gray@bio.gla.ac.uk.

[1] The abbreviations used are: HSEs, heat shock elements; STREs, stress response elements; AZC, a racemic mixture of D- and L-azetidine-2-carboxylic acid; WT, wild-type; ORFs, open reading frames; HSF, heat shock factor.

shock factor upon heat shocks have also been proposed, such as heat shock-induced oxidative stress and membrane changes (5). The trigger for induction of the STRE-containing genes upon heat shock is not known (2, 5). If thermally misfolded proteins indeed trigger some or all of the transcriptional responses to heat shock, then induction of misfolded proteins by artificial means in the absence of temperature changes should 1) cause many or all of the gene expression changes caused by heat shock, 2) cause these changes as strongly as does heat shock, 3) selectively cause these changes, and 4) cause these changes by the same mechanism as does heat shock.

Azetidine-2-carboxylic acid (AZC) is a toxic analog of proline and is incorporated into proteins competitively with proline (11). Because the analog has one less carbon atom in its ring than does proline, the conformation of the polypeptide backbone is altered when AZC is incorporated in place of proline. Thus, incorporation of AZC into proteins causes reduced thermal stability or misfolding (12–14). Indeed, AZC-containing proteins bind avidly to Hsp70 chaperones *in vivo* (7). AZC thus affords us an opportunity to study the cell's response to misfolded proteins in the absence of temperature changes.

We have recently shown that AZC reversibly inhibits proliferation of budding yeast cells, causing arrest in the G_1 phase of the cell cycle within one to two cell generations (15). AZC treatment and temperature upshift cause G_1 arrest by the same mechanism; arrest is due to lowered G_1 cyclin activity and is dependent on proper activation of heat shock factor in both cases (15). AZC treatment may therefore activate heat shock factor via misfolding a fraction of cellular protein. Phenotypic analyses indicate that AZC treatment does not activate other heat shock-induced responses such as activation of the cell integrity pathway and accumulation of glycogen and trehalose (15). Thus, AZC treatment appears to induce some (but not all) of the phenotypic responses to heat shock. In this study, we examined the effect of AZC treatment on gene expression.

EXPERIMENTAL PROCEDURES

Materials and Chemicals

All chemicals were from Sigma (Dorset, UK). Components of the growth medium were from BD Biosciences and Fisher. DL-AZC was used throughout this work, but L-AZC is the active agent in this racemic mixture.[2] Any given concentration of AZC herein refers to the concentration of the racemic mixture.

Plasmids, Yeast Strains, and Manipulations

Liquid media, both rich and minimal, were prepared as described previously (16). Solid media contained 2% agar. Strains were routinely grown on yeast/peptone/dextrose (YPD) agar plates at 25 °C. Liquid cultures were grown in YPD broth.

The strains used in this study were W303-1A (wild-type (WT): *MAT***a** *ura3-1 lys2 trp1-1 leu2-3,112 his3-11 can1-100 ade2-1*) the laboratory collections; JVG961 (WT S288c: *MATα ra3-52 lys2-801 his3-Δ200 leu2 ade2-101 ho*::*lacZ*) from the laboratory collections; and MH297 (*EXA3-1* strain: *MATα leu2-3,112 lys2 ura3-52 his3-11,15 trp1-Δ1 EXA3-1*) and a corresponding WT strain, DS10 (*MAT***a**), both kind gifts from E. A. Craig (4). The *msn2*Δ *msn4*Δ double mutant (*MAT***a** *STRE-lacZ msn2*Δ::*HIS3 msn4*Δ::*TRP1*) and its congenic WT strain (W303-1A-*STRE-lacZ*) were kind gifts from C. Schüller.

For drug treatments, cells were grown at 30 °C to $A_{600\ nm} = 0.05$, unless stated otherwise. AZC and canavanine were dissolved in water to a stock concentration of 500 mM and added to the growth medium to achieve the final concentrations specified in individual experiments. Ethanol was added to the growth medium to a final concentration of 8% (v/v). Cycloheximide (stock solution of 10 mg/ml in ethanol) was added to a final concentration of 10 μg/ml (and hence, the vehicle (ethanol) to 0.1% (v/v)).

For temperature upshift experiments, cells were grown at 23 °C to $A_{600\ nm} = 0.2$ and added to an equal volume of YPD medium in a conical flask preheated at 36 °C in a water bath. Incubation was continued at 36 °C with agitation for the time course of each experiment. All absorbance measurements were made on a Milton Roy Spectronic 601 spectrophotometer.

Microarray Analysis

RNA Preparation—Yeast strain W303-1A was grown to early log phase ($A_{600\ nm} \sim 0.4$) in 100 ml of YPD medium. AZC was added to a final concentration of 50 mM, and the cells were incubated for up to an additional 5 h. The control sample was treated identically, but did not contain AZC. Cells were harvested by centrifugation for 3 min at 1500 × *g* (7000 rpm in a Beckman tabletop centrifuge). The pellet was resuspended in 1 ml of ice-cold water and microcentrifuged for 10 s at 4 °C. The pellet was then resuspended in 400 μl of TES solution (10 mM Tris-Cl, pH 7.4, 10 mM EDTA, 0.5% SDS), and 400 μl of acid phenol was added with vortexing for 10 s. After a 60-min incubation at 65 °C with occasional brief vortexing, the sample was placed on ice for 5 min and then microcentrifuged at 14,000 rpm for 5 min at 4 °C. The aqueous (top) phase was transferred to a clean 1.5-ml microcentrifuge tube, and the phenol extraction, incubation, and microcentrifugation were repeated. The aqueous phase was then transferred to a clean 1.5-ml microcentrifuge tube, and 400 μl of chloroform was added with vigorous vortexing. The total RNA sample was then microcentrifuged for 5 min at 14,000 rpm at 4 °C. The aqueous phase was transferred to a new tube, and 40 μl of 3 M sodium acetate (pH 5.3) and 1 ml of ice-cold 100% ethanol were added, followed by microcentrifugation at 14,000 rpm for 5 min at 4 °C to precipitate the RNA. The RNA pellet was washed by vortexing briefly in ice-cold 70% ethanol and spun down as before. After resuspension in 50 μl of H_2O, the concentration of RNA was determined spectrophotometrically. mRNA was selected from total RNA using a Poly(A) Olygotex kit (QIAGEN Inc.).

Expression Profiling—For each mRNA sample, 2 μg was used for the cDNA microarray experiments. Changes in the mRNA transcript levels for the 6219 protein-encoding genes of budding yeast were measured by comparing transcript abundance at $t = 1$ and 5 h relative to $t = 0$ (untreated). Fluorescent $t = 0$ RNA was prepared by reverse transcription in the presence of Cy3-labeled dUTP, which fluoresces green (maximum at 532 nm) and was used as the common hybridization reference for the remaining samples. Fluorescent cDNA from the 1- and 5-h samples was synthesized using Cy5-labeled dUTP, which fluoresces red (maximum at 635 nm). The Cy5-labeled cDNA representing mRNA from each time point was mixed with Cy3-labeled $t = 0$ cDNA, and the mixture was hybridized onto a DNA microarray containing ~6200 yeast open reading frames (ORFs). The resulting fluorescence intensities across the array were measured by a laser scanning microscope. For a given array spot, the ratio of Cy3 and Cy5 intensities reflects the transcript levels of the corresponding gene at the time in question relative to $t = 0$, after adjustment by a normalization factor that sets the average of the log transformed ratios from one array to zero.

Images were analyzed with ScanAlyze (M. B. Eisen),[3] and fluorescence ratios (along with numerous quality parameters; see ScanAlyze manual) were stored in a custom data base. Single spots or areas of the array with obvious blemishes were flagged and excluded from subsequent analysis.

Northern Probes

Probes were amplified from amplified yeast ORFs (Research Genetics, Huntsville, AL) using universal yeast primers (Research Genetics) by PCR using Reddy-load PCR mixture (Advanced Biotechnologies Ltd., Surrey, UK) according to the manufacturer's instructions. The genes that were probed (and their ORF numbers) are as follows: *ACT1* (YFL039c), *HSP42* (YDR171w), *SSA4* (YER103w), *HSP12* (YFL014w), *HSP30* (YCR021c), *CTT1* (YGR088w), *RPL3* (YOR063w), *RPL30* (YGL030w), and *RPS1a* (YLR441c).

Amplified yeast ORFs were run on 1% agarose gels and purified using the Concert rapid gel extraction system (Invitrogen) according to the manufacturer's instructions. Amplified yeast ORFs were labeled with [^{32}P]dCTP (PerkinElmer Life Sciences) using the Prime-It II random primer labeling kit (Stratagene, La Jolla, CA) and purified using NucTrap purification columns (Stratagene) according to the manufacturer's instructions.

Northern Analysis

Extraction of total RNA was performed as previously described (16). For Northern analysis, 10 μg of total RNA was denatured at 65 °C for 5 min before separation on a 1.3% agarose gel containing 10% formaldehyde. The RNA was transferred overnight to Biodyne B membrane

[2] G. A. Petsko and J. V. Gray, unpublished observation.

[3] Available at www.microarrays.org/software.

(0.45 μm; Pall Corp., Ann Arbor, MI) in 10× SSC. Membranes were baked for 2 h at 80 °C and then prehybridized in 50% formamide, 10× Denhardt's solution, 2% SDS, 5× SSC, and 100 μg/ml denatured salmon sperm DNA for 2 h. The labeled probe was boiled for 5 min before addition to the membrane in the presence of the prehybridization mixture. Membranes were hybridized overnight at 42 °C. After stringent washing (2 × 15 min in 1% SDS and 0.25× SSC at room temperature, 15 min in 0.1% SDS and 0.1% SSC at room temperature, and 30 min in 0.1% SDS and 0.1% SSC at 65 °C), membranes were exposed to x-ray film (Konica Corp., Tokyo, Japan) at −70 °C with intensifying screens for an appropriate length of time.

RESULTS

In this study, we set out to determine whether AZC treatment mimics the effect of heat shock on genome-wide gene expression and, if so, to explore the mechanism by which the analog causes these effects. Laboratory strain backgrounds differ in their sensitivity to AZC, as judged by colony formation on plates (15).[4] The concentrations used herein are those just sufficient to permanently inhibit proliferation of each strain background under study, unless stated otherwise. All experiments were conducted in the presence of normal amounts of proline in the growth medium.

We determined the expression changes of the 6129 protein-encoding genes by microarray analysis (see "Experimental Procedures") using WT W303-1A cells treated with a growth-inhibiting concentration of AZC (50 mM) for 5 h. We also performed an equivalent analysis after 1 h of AZC treatment (50 mM). The result of the latter experiment was qualitatively similar to the 5-h data set, but with less potent expression changes.[4] Our subsequent analysis therefore focused on the 5-h data set.

AZC Treatment Does Not Cause Starvation—AZC is an analog of proline and may thereby interfere with proline uptake or metabolism, resulting in starvation. Nutrient-starved cells or cells treated with rapamycin, an inhibitor of the TOR (target of rapamycin) proteins, stop proliferation in the G_1 phase of the cell cycle and enter quiescence-like, non-proliferating states (17, 20), superficially reminiscent of AZC-arrested cells (15). We found, by microarray analysis, that AZC treatment (5 h) did not induce the gene expression changes characteristic of starving cells (21–24), *e.g. GAP1*, *PUT4*, *SNZ1*, and *SNZ2* were not strongly induced by AZC treatment (0.8-, 1.4-, 1.2-, and 1.1-fold, respectively). We conclude that AZC does not arrest proliferation by causing starvation. Furthermore, expression of the proline utilization genes *PRO3* and *PRO1* was not significantly induced by AZC treatment (1.02- and 1.26-fold, respectively),[4] indicating that AZC treatment does not grossly interfere with proline metabolism.

AZC Treatment Selectively Causes Most of the Genome-wide Gene Expression Changes Caused by Temperature Upshift—Does AZC treatment mimic heat shock? The expression levels of 91.8% of the genes did not vary within a factor of 3 upon AZC treatment, indicating that the analog does not induce the dramatic global changes in gene expression characteristically caused by severe heat shock. Our subsequent analysis focused on a comparison with temperature upshift.

We determined the correlations between the microarray-derived expression profiles of AZC-arrested cells (5 h) (this work) and temperature-upshifted cells (from 23 to 37 °C for 10, 20, and 40 min) (18). This analysis focused on those genes (2470 in total) of known function because data for this subset upon temperature upshift are publicly available (18). We found that the correlation between AZC (5 h) and temperature upshift microarray data sets was highest for the 20-min time point of temperature upshift, peaking at 0.576. This correlation compares well with those between temperature upshift time points: 10 *versus* 20 min, correlation of 0.793; and 10 *versus* 40 min, correlation of 0.582 (18).[2] These data suggest that AZC treatment selectively causes the majority (but not all) of the gene expression changes characteristic of temperature upshift. We conclude that AZC treatment partially mimics temperature upshift.

TABLE I
Selected results from microarray-derived expression changes of known genes after AZC treatment for 5 h (see Table S1 in the Supplemental Material for more data)

ORF	Gene name	Protein function	Induction by AZC
			-fold
Most strongly induced by AZC (and strongly induced by temperature upshift)			
YFL014W	*HSP12*	Heat shock protein	27.96
YLL026W	*HSP104*	Heat shock protein	22.00
YDR171W	*HSP42*	Heat shock protein	19.35
YBL075C	*SSA3*	Heat shock protein	17.52
YER103W	*SSA4*	Heat shock protein	16.63
YDR258C	*HSP78*	Heat shock protein	14.74
YBR169C	*SSE2*	Heat shock protein	14.12
YCR021C	*HSP30*	Heat shock protein	13.67
Most strongly repressed by AZC (and strongly repressed by temperature upshift)			
YLR441C	*RPS1a*	Ribosomal protein	0.05
YJL148W	*RPA34*	RNA polymerase subunit	0.05
YML063W	*RPS1b*	Ribosomal protein	0.05
YPL220W	*RPL1a*	Ribosomal protein	0.05
YJL190C	*RPS22a*	Ribosomal protein	0.05
YGR148C	*RPL24b*	Ribosomal protein	0.05
Strongly induced by temperature upshift, but not by AZC			
YGR088W	*CTT1*	Catalase T	1.70
YIL101C	*XBP1*	Transcriptional repressor	0.49
Strongly induced by AZC, but not by temperature upshift			
YJL077C	*ICS3*	Copper homeostasis	6.11

AZC Treatment Selectively Causes Heat Shock Factor-dependent Gene Expression Changes—Which genes are selectively induced by AZC treatment? Expression of a subset of genes was strongly induced by treatment with AZC (see Table I for a partial list and Table S1 in the Supplemental Material for the complete list). Dramatic induction (3–27-fold) was observed for 217 genes. Of the 50 most strongly induced genes of known function, 46 are also strongly induced after 20 min of temperature upshift (18). Most conspicuous among these highly induced genes are those known, or suspected, to be part of the HSE regulon, *e.g. HSP104*, *HSP82*, *HSP78*, *HSP42*, *HSP30*, *HSP12*, *HSP26*, *SSA3*, *SSA4*, and *SSE2* (3). These data indicate that AZC treatment mimics temperature upshift in selectively inducing expression of the HSE regulon. Thus, AZC treatment may selectively activate heat shock factor.

Activation of heat shock factor directly or indirectly causes repression of the ribosomal protein genes (4). If AZC treatment indeed activates heat shock factor, then we expected treatment with the analog to also repress expression of the ribosomal protein genes. Expression of a subset of genes (293 in total) was repressed by a factor of 3 or more (to a maximum of 20-fold) by AZC treatment (see Table I for a partial list and Table S1 in the Supplemental Material for the complete list). Of the 50 genes of known function that are repressed most strongly by AZC treatment, 47 are also strongly repressed by temperature upshift (18). The majority of these (42 of 47) encode ribosomal proteins, and repression of these genes by severe heat shock is dependent on heat shock factor (4). Our data suggest that AZC treatment, like temperature upshift, selectively activates heat shock factor and thereby causes increased expression of HSE-containing genes and repression of the ribosomal protein genes.

Of the 50 genes of known function whose expression is repressed most strongly by temperature upshift (18), 46 were also

[4] E. W. Trotter and J. V. Gray, unpublished data.

strongly repressed by AZC treatment. Again, the majority of these genes encode ribosomal proteins. Thus, AZC treatment selectively causes the vast majority of the repressions caused by temperature upshift.

AZC Treatment Fails to Strongly Induce Expression of the STRE Regulon—Almost all of the genes whose expression is induced by temperature upshift are components of the HSE or STRE regulon, or both (3). Does AZC treatment also activate the STRE regulon? Expression of a significant number of genes is strongly induced by temperature upshift (18), but not by AZC treatment. Of the 50 genes encoding proteins of known function that are most strongly induced after 20 min of temperature upshift (18), only 25 were strongly induced by AZC treatment.[4] Most notable among the genes whose expression is strongly induced by temperature upshift but not by AZC treatment are targets of the STRE pathway, *e.g. CTT1* and *DDR1* (induced only 1.1- and 2.4-fold, respectively, in response to AZC treatment) and also *HXK1*, *GLK1*, *TPS1*, *TPS3*, *PYK2*, *HXT2*, *HXT5*, *HXT6*, *HXT7*, *PYC1*, *SDH1*, *SDH2*, *SDH4*, *ZWF1*, *ALD4*, *ALD6*, *GIP2*, *GSY1*, and *GPH1* (3). We conclude that the STRE regulon is, at best, weakly activated by AZC treatment. This possibility is supported by our previous finding that AZC treatment fails to cause accumulation of glycogen and trehalose, a phenomenon dependent on activation of the STRE pathway (15). We conclude that the STRE regulon is much less sensitive to AZC treatment than is the HSE regulon. To a first approximation, temperature upshift selectively induces the STRE and HSE regulons (and thereby represses expression of the ribosomal protein genes), whereas AZC treatment selectively induces only the latter.

AZC Treatment Causes Few Gene Expression Changes Not Caused by Temperature Upshift—Although incorporation of AZC into any given protein molecule is likely to cause dysfunction of that protein, the efficiency of incorporation of AZC in place of proline in our experiments is likely to be low (see "Discussion") (15). Hence, most molecules of any particular protein in AZC-treated cells are unlikely to contain AZC and thus are functional. However, proteins that are large, proline-rich, and short-lived are most likely to incorporate at least one residue of AZC in place of a proline. Thus, AZC treatment may preferentially inactivate a particular subset of proteins within the cell and thereby directly cause gene expression changes not caused by temperature upshift. Surprisingly, we found only a handful of genes in this set: *CUP1-1*, *CUP1-2*, and *ICS3* were induced by AZC treatment, but by not temperature upshift; *ACS2*, *RPN8*, *CTS1*, and *BUD2* were repressed by AZC treatment, but not by temperature upshift (Ref. 18; see Table I for a partial list and Table S1 in the Supplemental Material for the complete list of genes whose expression is affected by a factor of 3 or more by AZC treatment). Therefore, the majority of the gene expression changes caused by AZC treatment are not due to selective inactivation of any particular subset of proteins by the analog.

AZC Treatment, Like Temperature Upshift, Does Not Activate the Endoplasmic Reticulum Unfolded Protein Response—If AZC treatment and temperature upshift cause widespread misfolding of cellular proteins, then we would expect these treatments to activate the unfolded protein response pathway of the endoplasmic reticulum. This signaling pathway is activated by the accumulation of misfolded proteins in the endoplasmic reticulum and promotes transcription (and thus expression) of genes containing unfolded protein response elements in their promoters (19). Significantly, we found that genes that were strongly induced by the endoplasmic reticulum unfolded protein response pathway, *e.g. EUG1*, *PDI1*, and *LHS1*, were not strongly induced by AZC treatment (1.15-, 1.7-, and 1.63-fold, respectively).[4] Expression of these genes is also not strongly induced by temperature upshift (18). These findings are consistent with AZC treatment and temperature upshift causing only a low level of protein misfolding in the endoplasmic reticulum (and presumably throughout the cell).

AZC Treatment Strongly and Robustly Causes Heat Shock Factor-dependent Gene Expression Changes—We wished to confirm the salient features of the gene expression changes detected from microarray profiling by Northern blot analysis. We also wished to compare the magnitudes of these expression changes with those caused by temperature upshift. For this analysis, we used a WT haploid strain of the S288C background whose proliferation on YPD medium is inhibited by 10 mM AZC (15). We determined the expression level of the following genes as a function of time after addition of AZC (10 mM) or upon temperature upshift (from 23 to 36 °C): *ACT1* as a loading control and a gene sensitive to the global repression characteristic of severe heat shock; the HSE-containing genes *HSP42* and *SSA4*; the HSE- and STRE-containing genes *HSP12* and *HSP30*; the STRE-containing gene *CTT1*; the ribosomal protein genes *RPS1a* and *RPL30*, containing Rap1-binding sites in their promoters; and the ribosomal protein gene *RPL3*, containing an Abf1-binding site in its promoter. The mechanism(s) regulating expression of the ribosomal protein genes are poorly understood. However, the promoters of these genes fall into two classes, those containing potential binding sites for the Rap1 transcription factor and those containing potential binding sites for the Abf1 transcription factor. We included representatives of both subclasses for completeness. Equal amounts of total RNA were loaded on each lane, and blots were hybridized with the same probe at the same time and exposed to the same film for equal amounts of time for each probing.

The results of the Northern analysis are shown in Fig. 1 (*A* and *B*). In agreement with the results from the microarray analysis described above, we found that 1) expression of the HSE-containing genes *HSP42*, *SSA4*, *HSP12*, and *HSP30* was strongly induced by AZC treatment, comparable with, if not more profoundly than, the peak transient induction of each gene upon temperature upshift; 2) expression of the STRE-driven gene *CTT1* was, at best, weakly induced by AZC treatment, but was more strongly induced by temperature upshift; and 3) expression of the ribosomal protein genes *RPL3*, *RPL30*, and *RPS1a* (the probe also detected the homologous gene *RPS1b*) was strongly repressed by AZC treatment, comparable with, if not more profoundly than, the peak transient repression caused by temperature upshift. Our Northern analysis thus confirmed the observations made by microarray analysis on the gene expression changes caused by AZC treatment. Our data also demonstrate that the gene expression changes caused by treatment with a growth-inhibiting concentration of AZC were at least as strong as the peak inductions caused by temperature upshift, which transiently inhibited proliferation. Treatment with AZC thus causes heat shock factor-dependent gene expression changes as strongly as does temperature upshift.

As expected for continuous incorporation of AZC into newly synthesized proteins, expression changes caused by treatment with the analog were persistent and not transient (as is the case for temperature upshift) and developed slowly. Induction of *HSP42*, *SSA4*, and *HSP30* in response to temperature upshift peaked at the 15-min time point. In contrast, induction of *HSP42* and *SSA4* by AZC treatment had only begun at the 30-min time point. Curiously, *HSP30* was significantly induced after only 60 min of analog treatment. Such differences in the kinetics of induction of HSE-containing genes may reflect different sensitivities of the promoters to the activity of heat

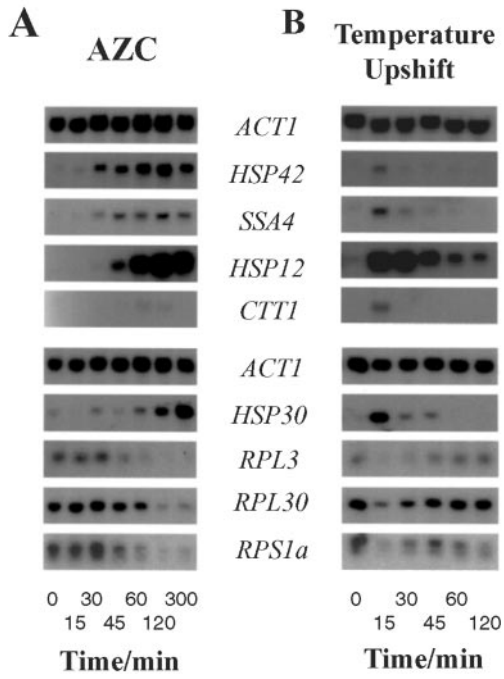

FIG. 1. **AZC treatment mimics temperature upshift.** *A*, AZC treatment strongly induces expression of HSF-dependent genes and strongly represses expression of the ribosomal protein genes. JVG961 (WT S288c) cells were grown to logarithmic phase on YPD medium and treated with AZC (10 mM) at 30 °C. Total RNA was prepared from samples collected as a function of time after AZC addition. Northern blots (10 μg of RNA/sample) were probed for expression of the indicated genes. *B*, temperature upshift induces HSF- and STRE-dependent genes and represses the ribosomal protein genes. For temperature upshift, JVG961 cells were grown to logarithmic phase on YPD medium at 23 °C and subjected to temperature upshift (from 23 to 36 °C). Samples were prepared and analyzed as described for *A*. Blots were probed with the same probes and exposed to the same film and for the same times for each probe.

shock factor revealed only when activation of the transcription factor is slow.

The microarray and Northern analyses were performed on different strain backgrounds, yet the results are in excellent agreement. Although different concentrations of AZC were required to permanently arrest proliferation of the two different strain backgrounds used (50 mM for W303 *versus* 10 mM for S288c), the cellular responses appeared identical in both strain backgrounds. We infer that the different sensitivities of the strain backgrounds to AZC does not reflect any fundamental differences in how the cells respond to the analog once it is incorporated into protein. Rather, the different sensitivities to the analog are likely to result from differences in the rate of uptake or efflux of the analog, in the efficiency of incorporation of the analog into protein, or in the size of the intracellular pool of proline.

Induction of HSP42 and SSA4 by AZC Treatment Is Dependent on Heat Shock Factor—Does AZC treatment, like temperature upshift, activate heat shock factor? Heat shock factor is an essential protein, abrogating the possibility of treating mutants deleted for *HSF1* with AZC. Temperature-sensitive alleles of *HSF1* are not useful for probing responses in the absence of temperature change. The *EXA3-1* allele of *HSF1* encodes a mutant form of heat shock factor with a single amino acid residue change in the DNA-binding domain of the protein (4). *EXA3-1* mutants display delayed transcriptional activation of heat shock factor-regulated genes (by 20 min) and delayed repression of the ribosomal protein genes upon heat shock to nonpermissive temperatures (4). The slow time course of AZC incorporation would preclude accurate detection of such subtle kinetic delays. However, *EXA3-1* mutants are also altered in the extent of induction of HSE-driven transcripts: some transcripts are induced more strongly in the mutant than in the WT strain, whereas others are induced more weakly (25). If AZC treatment activates heat shock factor, then we expect the *EXA3-1* allele to alter the extent, and possibly the persistence, of activation of HSE-driven transcripts in response to treatment with the analog.

Curiously, the strain background containing the *EXA3-1* mutation appeared to be resistant to AZC even up to a concentration of 100 mM, as judged by colony formation on YPD plates.[4] As noted above, different strain backgrounds have different sensitivities to AZC treatment. However, treatment of the equivalent WT strain with AZC led to a transient inhibition of proliferation in liquid culture reminiscent of the transient arrest caused by temperature upshift (15). We therefore monitored expression of *HSP42* and *SSA4* in the *EXA3-1* mutant and in its WT strain as a function of time upon AZC treatment (40 mM) and upon temperature upshift. As shown in Fig. 2 (*A* and *B*), we found that these HSE-containing genes were indeed transiently induced in this strain background in response to AZC treatment. Furthermore, we found that expression of *HSP42* was induced more strongly in the *EXA3-1* mutant compared with the WT strain in response to both AZC treatment and temperature upshift. Expression of *HSP42* was also more persistent in the mutant in response to both treatments. Although there may be subtle effects of the *EXA3-1* mutation on the extent of *SSA4* induction in response to both AZC treatment and temperature upshift, expression of this gene was clearly more persistent in the mutant in response to both treatments. The *EXA3-1* mutation in *HSF1* thus affects the extent or persistence (or both) of induction of *HSP42* and *SSA4* in response to AZC treatment and temperature upshift. These data indicate that AZC treatment and temperature upshift cause induction of *HSP42* and *SSA4* (and by inference, all HSE-containing genes) by the same mechanism, namely activation of heat shock factor.

The induction of *HSP42* and *SSA4* by AZC treatment was clearly transient for the WT strain used in Fig. 2, in contrast to the equivalent data for the WT S288c strain shown in Fig. 1. The persistence of heat shock factor activation in response to AZC treatment in these strain backgrounds clearly parallels the persistence of proliferation arrest caused by the analog, which gratifyingly is also dependent on heat shock factor (15). Although the reason for the different responses of these two strain backgrounds to the analog is not known, it is likely to be due to some combination of differences in the rate of uptake or efflux of the compound, to different efficiencies of incorporation of the analog into protein, or to different capacities to degrade analog-containing peptides. From our data in Figs. 1 and 2, we cannot compare the relative expression levels of *HSP42* and *SSA4* between these two strain backgrounds because different batches of labeled probes were used for each experiment.

Induction of HSP12 by AZC Treatment Is Partly Dependent on Msn2 and Msn4—As determined by the microarray analysis described above, STRE-containing genes were, at best, poorly induced by AZC treatment (Table I and Table S1 in the Supplemental Material).[4] This observation suggests that Msn2 and Msn4, the redundant transcription factors required for induction of the STRE regulon, are relatively insensitive to protein misfolding in the cell. It is known that *HSP12* is a member of both the HSE and STRE regulons. Given that *HSP12* expression was very strongly induced by AZC treatment (Fig. 1 (*A* and *B*) and Table I), we set out to determine whether Msn2 and Msn4 contribute to induction of *HSP12* upon AZC treatment.

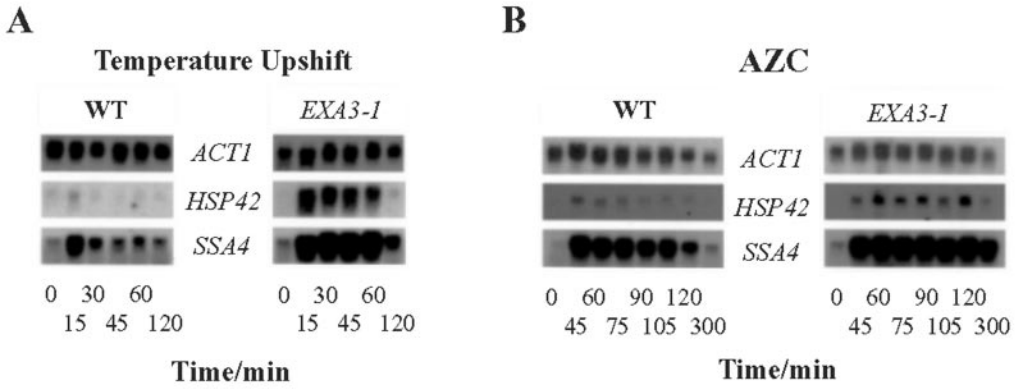

FIG. 2. **Induction of *HSP42*, *SSA4*, and *HSP30* by AZC treatment is dependent on heat shock factor.** *A*, the *EXA3-1* mutation affects the extent and timing of induction of HSF-dependent genes upon temperature upshift. The WT (DS10) and *EXA3-1* (MH297) strains were grown to logarithmic phase on YPD medium and subjected to temperature upshift (from 23 to 36 °C). Total RNA was prepared as a function of time. Samples (10 μg of RNA each) were Northern-blotted, and the blots were probed for expression of the indicated genes. *B*, the *EXA3-1* mutation affects the extent and timing of induction of HSF-dependent genes upon AZC treatment. The WT (DS10) and *EXA3-1* (MH297) strains were grown to logarithmic phase on YPD medium and treated with AZC (40 mM) at 23 °C. Samples were prepared and analyzed as described for *A*.

We found that the induction of *HSP12* by AZC treatment and temperature upshift was significantly reduced in a strain deleted for both *MSN2* and *MSN4* relative to its congenic WT strain (Fig. 3, *A* and *B*). We conclude that Msn2 and Msn4 (and by inference, the STRE regulon) may indeed be activated by AZC treatment, but only partially.

Induction of HSP42 by AZC Treatment Is Dependent on Protein Synthesis—If AZC exerts its effects *in vivo* by misfolding proteins into which it is incorporated, then the expression changes caused by AZC treatment should require ongoing protein synthesis. We therefore determined the effect of cycloheximide addition on the ability of AZC treatment to induce expression of *HSP42*. Treatment of WT S288c cells with cycloheximide alone or vehicle alone did not alter expression of *HSP42*. In contrast, we found that the presence of cycloheximide prevented induction of *HSP42* by AZC treatment (Fig. 4*A*). We infer that ongoing protein synthesis is required for AZC treatment to induce the HSE regulon, consistent with the analog functioning via misfolding nascent proteins into which it is incorporated.

Ethanol Treatment Mimics AZC Treatment—If AZC acts via misfolding cellular proteins, then we expect other treatments that misfold proteins in the cell to induce the same spectrum of gene expression changes as that caused by AZC treatment. Ethanol can disrupt protein folding by a mechanism distinct from that of AZC (5). The results of the Northern analysis of ethanol-treated cells are shown in Fig. 4*A*. These blots and those for canavanine-treated cells (see below and Fig. 4*B*) were prepared identically to and probed with the same batch of labeled probe at the same time and exposed to the same film for the same length of time as the blots shown in Fig. 1 (*A* and *B*). Thus, the data shown in Figs. 1 and 4 are directly comparable. The concentration of ethanol used in this experiment (8% (v/v)) was just sufficient to stop proliferation of the strain used (WT S288C).[4] We found that ethanol treatment mimicked AZC treatment: 1) in strongly inducing the expression of genes regulated by heat shock factor, *e.g. HSP42*, *SSA4*, *HSP12*, and *HSP30*; 2) in failing to strongly induce *CTT1* (an STRE-driven gene), in agreement with the very weak activation of STRE-driven genes by ethanol reported previously (26); 3) in failing to repress *ACT1* (*i.e.* no global repression); and 4) in strongly repressing the expression of the ribosomal protein genes tested. We conclude that ethanol and AZC treatments, both of which can misfold proteins, but by very distinct mechanisms, cause similar gene expression changes attributable to activation of heat shock factor.

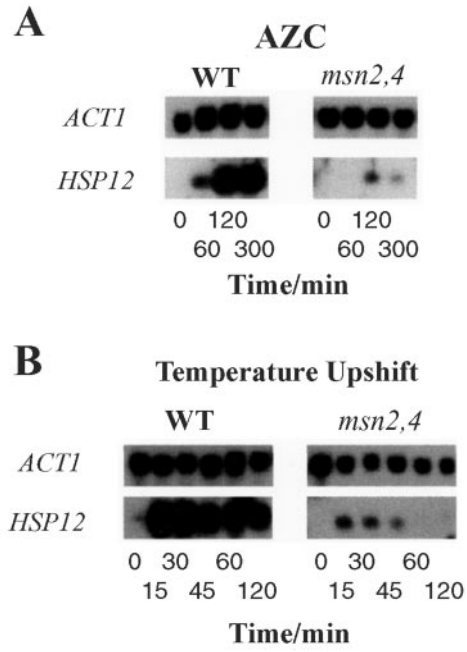

FIG. 3. **Induction of *HSP12* expression by AZC treatment or temperature upshift is partially dependent on Msn2 and Msn4.** *A*, induction of *HSP12* expression by AZC treatment partially requires Msn2 and Msn4. The *msn2*Δ *msn4*Δ double mutant (*msn2,4*) and its congenic WT strain (W303-1A-*STRE-lacZ*) were grown to logarithmic phase on YPD medium at 23 °C and treated with AZC (50 mM). Total RNA was prepared as a function of time. Samples (10 μg of RNA each) were Northern-blotted, and the blots were probed for expression of the indicated genes. *B*, induction of *HSP12* expression by temperature upshift partially requires Msn2 and Msn4. The *msn2*Δ *msn4*Δ double mutant and its congenic WT strain (W303-1A-*STRE-lacZ*) were grown to logarithmic phase on YPD medium at 23 °C and subjected to temperature upshift (from 23 to 36 °C). Total RNA was prepared as a function of time. Samples (10 μg of RNA each) were Northern-blotted, and the blots were probed for expression of the indicated genes.

Canavanine Treatment Does Not Mimic AZC Treatment—Canavanine is an arginine analog that, like AZC, is incorporated into protein competitively with the corresponding natural amino acid (27). Canavanine differs from arginine in the structure of its side chain and, as such, is not expected to significantly alter the conformation of the polypeptide backbone into which it is incorporated (relative to the same protein containing arginine at the equivalent position(s)). Thus, treatment

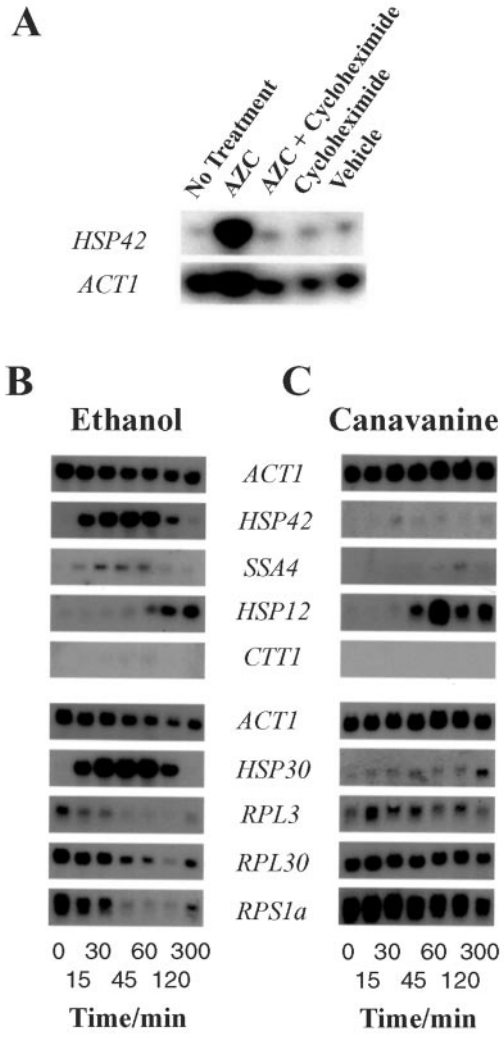

FIG. 4. **Induced expression of HSF-dependent genes correlates with protein misfolding.** *A*, induction of *HSP42* expression by AZC treatment requires protein synthesis. JVG961 cells were grown to logarithmic phase on YPD medium at 30 °C and treated with AZC (10 mM), cycloheximide (10 μM), both, or vehicle (0.1% (v/v) ethanol) alone for 2 h at 30 °C. Total RNA was prepared. The Northern blot (10 μg of RNA/lane) was probed for expression of the indicated genes. *B*, ethanol treatment causes similar gene expression changes as does AZC treatment. JVG961 cells were grown to logarithmic phase on YPD medium at 30 °C and treated with 8% (v/v) ethanol at 30 °C, and total RNA was prepared as a function of time. Northern blots (10 μg of RNA/lane) were probed for expression of the indicated genes. *C*, expression changes caused by canavanine treatment do not closely mimic those caused by AZC treatment. JVG961 (WT S288c) cells were grown to logarithmic phase on YPD medium at 30 °C and treated with an inhibitory concentration of canavanine (10 mM) at 30 °C, and total RNA was prepared from the samples as a function of time. Samples were prepared and analyzed as described for *B*. Blots were probed with the same radioactive probes at the same time and autoradiographed on the same film for the same length of time as each other and as the blots in Fig. 1.

with canavanine is not expected to cause protein misfolding, at least not to the same extent as does AZC treatment.

We examined the effect of canavanine at a sublethal concentration (10 mM), just sufficient to inhibit cell proliferation, on gene expression by Northern analysis. Our results are shown in Fig. 4*B*. In contrast to ethanol and AZC treatments, canavanine treatment did not significantly induce expression of the HSE-containing genes *HSP42* and *SSA3*. Canavanine treatment also failed to significantly repress expression of the ribosomal protein genes. We infer that canavanine treatment does not strongly activate heat shock factor. Canavanine treatment also failed to induce expression of *CTT1* and is thus unlikely to strongly activate the STRE regulon. It should be noted that, in our experiments, both AZC and canavanine were present in the growth medium at concentrations sufficient to inhibit proliferation,[4] yet only AZC treatment induced the HSE regulon. Thus, activation of heat shock factor by AZC treatment is not a consequence of growth inhibition *per se*. Rather, the ability of the analogs (AZC and canavanine) to activate heat shock factor correlates with their relative capacity to misfold proteins into which they are incorporated.

Curiously, canavanine treatment robustly induced expression of *HSP12* and *HSP30*, the latter only slowly and weakly. Although we do not know the mechanistic basis for these inductions, it is tempting to speculate that canavanine treatment weakly activates both heat shock factor and Msn2/4, sufficiently to induce expression of genes regulated by both pathways (*HSP12* and *HSP30*), but too weakly to drive expression of genes dependent on one or the other system.

DISCUSSION

AZC Causes Protein Misfolding in Vivo—Multiple lines of evidence indicate that AZC exerts its effects on yeast cells via incorporation into cellular protein. First, L-AZC, which can be incorporated into cellular protein, is the active agent in racemic mixtures, and not D-AZC, which cannot be incorporated.[2] Second, AZC inhibits growth of many (but not all) organisms. AZC-sensitive organisms contain aminoacyl-tRNA synthetases that can charge $tRNA^{Pro}$ with the analog, whereas AZC-resistant organisms do not (reviewed in Ref. 27). Third, the ability of AZC to arrest cell proliferation is not determined by the absolute concentration of the analog, but rather by the ratio of AZC to proline in the medium.[4] Our microarray data indicate that AZC does not interfere with proline metabolism *per se*, consistent with the analog competing with proline for incorporation into cellular protein. Fourth, induction of the heat shock factor-regulated gene *HSP42* by AZC treatment is abolished in the presence of cycloheximide. Thus, activation of heat shock factor by AZC treatment requires ongoing protein synthesis, consistent with the analog acting via incorporation into cellular protein. Finally, *ubc4 ubc5* mutants, which are defective in the ubiquitin-dependent degradation of short-lived and analog-containing polypeptides, are hypersensitive to AZC treatment (15). The simplest explanation for this observation is that, at a given concentration of AZC, the amount of analog-containing protein in the cell is higher when these proteins are stable than when these proteins are unstable. Thus, a concentration of AZC that is insufficient to arrest cell proliferation in a WT cell would be sufficient to arrest a cell lacking Ubc4 and Ubc5. Taken together, these arguments strongly indicate that AZC acts via incorporation into cellular protein.

Given that incorporation of AZC into protein is known to cause reduced thermal stability or misfolding (12–14), the effects of the analog on gene expression are most likely due to its misfolding proteins. Two lines of evidence support this notion. First, induction of the HSE regulon was also strongly and selectively caused by treatment with ethanol, another agent capable of misfolding proteins, but by a mechanism different from that of AZC. Second, canavanine, an arginine analog that is incorporated into protein competitively with arginine, did not induce the HSE regulon, whereas AZC did so efficiently. Canavanine incorporation is not expected to disrupt protein folding as efficiently as does incorporation of AZC (27). Thus, the relative capacity of the analogs to induce the HSE regulon correlates with their relative capacity to misfold proteins into which they are incorporated.

AZC Treatment Selectively Activates Heat Shock Factor—AZC treatment selectively causes the gene expression changes attributable to activation of heat shock factor. First, the expression level of only a small fraction of the protein-encoding genes

was altered by a factor of 3 or more after 5 h of treatment with an inhibitory concentration of AZC (8.2% affected in total: 3.5% induced and 4.7% repressed). Hence, AZC treatment did not cause any global changes in gene expression, but selectively affected expression of a discrete subset of genes. Second, HSE-containing transcripts predominated among those induced by AZC treatment. Third, the ribosomal protein genes (and co-regulated genes encoding components of the translation apparatus) composed the vast majority of the genes that were strongly repressed by AZC treatment. Repression of the ribosomal protein genes by heat shock is known to be dependent on activation of heat shock factor (4).

We have confirmed that AZC treatment activates the HSE regulon (induction of HSE-containing genes and consequent repression of the ribosomal protein genes) by Northern analysis. Furthermore, we found that AZC treatment activates the HSE regulon as strongly as, if not more strongly than, does temperature upshift. We have also shown that a mutation in heat shock factor affects induction of the HSE-containing genes *HSP42* and *SSA4* in response to AZC treatment. Critically, the mutation alters the induction of these genes in the same way in response to either AZC treatment or temperature upshift. Thus, AZC treatment strongly and selectively induces the HSE regulon by the same mechanism as does temperature upshift, namely by activating heat shock factor.

Misfolded Proteins Are Competent to Mediate Selective Activation of Heat Shock Factor in Response to Heat Shock—Based on the above arguments, we conclude that the misfolding of a fraction of cellular protein in the absence of temperature change mimics heat shock in selectively and strongly activating heat shock factor. Therefore, misfolded proteins elicit the appropriate cellular response and do so sufficiently strongly and selectively for them to be intermediates in the cellular response to heat shock. Given that misfolded proteins are known to accumulate in heat-shocked cells (7), they are competent to mediate at least part of the cellular response to heat shock.

Unfortunately, it is not yet known if misfolded proteins are kinetically competent to be intermediates in the heat shock response, *i.e.* that misfolded proteins accumulate sufficiently rapidly upon heat shock and that misfolded proteins cause activation of heat shock factor sufficiently quickly. Although activation of heat shock factor is slow in response to AZC treatment, it is likely that equilibration of the analog into the cellular proline pool prior to incorporation into protein is slow. The issue of kinetic competence remains unresolved.

If misfolded proteins are intermediates in the cellular response to heat shock, then heat shock factor must be very sensitive to protein misfolding in cytoplasmic space. Temperature upshift is a very mild environmental change and is unlikely to cause extensive protein misfolding. In addition, AZC treatment at concentrations sufficient to activate heat shock factor does not appear to cause widespread protein dysfunction: 1) AZC treatment almost exclusively affects the expression of a small and discrete subset of genes that are also induced by temperature upshift; 2) AZC-arrested cells are viable (15); 3) AZC arrest is reversible (15); and 4) AZC-arrested cells are responsive to subsequent treatments, *e.g.* heat shocks (15) and rapamycin.[4] Indeed, neither AZC treatment nor temperature upshift strongly activates the endoplasmic reticulum unfolded protein response, even though both treatments should misfold proteins throughout the cell, including those in the endoplasmic reticulum.

The fraction of protein containing AZC (when cells are treated with a concentration of the analog just sufficient to activate heat shock factor) should constitute an upper limit for the fraction of cellular protein whose misfolding is just sufficient to activate heat shock factor. We are attempting to determine this number.

The STRE Regulon Is Relatively Insensitive to Protein Misfolding—Although AZC treatment profoundly activates the HSE regulon and genes whose expression is dependent thereon (*e.g.* the ribosomal protein genes), it weakly, at best, induces the STRE regulon. This possibility is supported by our observation that AZC does not lead to the accumulation of glycogen and trehalose, an STRE regulon-dependent phenomenon (15), nor does it significantly activate expression of *STRE-lacZ* reporter constructs.[2] However, AZC incorporation into cellular protein does not appear to affect the activability of the STRE regulon (15). Rather, AZC treatment simply fails to strongly activate this regulon. The primary signal for activation of the STRE regulon by heat shock may be the misfolding of cellular protein, but with the STRE regulon requiring higher levels of protein misfolding than those sufficient to activate heat shock factor (and inhibit proliferation). Indeed, the time course of activation of *CTT1* expression by temperature upshift parallels that of the heat shock factor-dependent transcripts, consistent with the notion of a common trigger. Alternatively, the STRE pathway may primarily respond to heat-induced oxidative stress or some other stress that coincides with protein misfolding upon heat shock (2, 5). It is clear that Msn2 and Msn4 contribute to the induction of *HSP12* by AZC treatment. Given that the STRE regulon is activated by multiple stresses to the cell, it is possible that any partial activation of the regulon in response to analog treatment is caused by an indirect mechanism, *e.g.* because of proliferation arrest. The mechanism by which the STRE regulon is activated by heat shocks remains elusive.

How Do Cells Sense Heat Shocks?—Thermally misfolded protein likely triggers activation of heat shock factor in response to heat shocks. The sensor for activation of the STRE regulon upon heat shock remains unclear. However, misfolded protein is not the sole sensor of heat shock in yeast. The cell integrity pathway, which is required for acquired thermotolerance and for maintenance of the cell surface, is activated by heat shocks, including temperature upshift (28). This pathway is not activated by protein misfolding (15). Rather, the cell integrity pathway is activated upon heat shock by thermal stress to the cell surface (28) detected by the plasma membrane sensors Hcs77 (29) and Mid2 (30). In summary, misfolded protein is competent to be an intermediate in the cellular response to heat shock, but it is clearly not the only mechanism by which a cell can detect thermal stress.

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