Oxidative stress induces heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription

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Metallothioneins (MTs) are a class of low-molecular-weight, cysteine-rich metal-binding proteins that function in metal detoxification and oxidative stress protection. We demonstrate that transcription of the *Saccharomyces cerevisiae* MT gene *CUP1* is strongly activated by the superoxide anion generator menadione. This activation is exacerbated in a strain lacking the gene encoding Cu, Zn superoxide dismutase (*SOD1*). *CUP1* transcriptional activation by oxidative stress is dependent on a functional *CUP1* promoter heat shock element (HSE) and the carboxy-terminal *trans*-activation domain of heat shock transcription factor (HSF). Furthermore, protection against oxidative stress conferred by *CUP1* in a *sod1Δ* strain requires HSF-mediated *CUP1* transcription. Although in response to heat, HSF-mediated *CUP1* transcription and HSF phosphorylation are transient, both *CUP1* gene expression and HSF phosphorylation are sustained in response to oxidative stress. Moreover, the patterns of tryptic phosphopeptides resolved from HSF derived from cells subjected to heat shock or oxidative stress are distinct. These results demonstrate that transcription of the *S. cerevisiae* metallothionein gene under conditions of oxidative stress is mediated by HSF and that in response to distinct activation stimuli, HSF is differentially phosphorylated in a manner that parallels metallothionein gene transcription.

[**Key Words:** Metallothionein; heat shock factor; oxidative stress; superoxide dismutase; transcription; menadione]

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Stress conditions, including heat shock, oxidative stress, osmotic stress, and toxic metals, are deleterious to normal cellular function. To survive these and other environmental and physiological stresses, all organisms possess specialized defense mechanisms to protect themselves from stress. Aerobic organisms are continuously exposed to oxygen, which renders them prone to damage generated by oxygen-derived free radicals. Oxidative stress is largely mediated by reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), which are intermediates of oxygen reduction generated by metal ion-catalyzed redox reactions, metabolism of chemicals, and normal physiological activities, including respiration and inflammatory responses to infection (Halliwell and Gutteridge 1984). The free radicals generated by these mechanisms cause severe damage to critical cellular macromolecules, including nucleic acids, proteins, and lipids (Halliwell 1994). Oxidative damage has been strongly correlated with aging and a number of diseases

including Parkinson's disease, Lou Gehrig's disease (amyotrophic lateral sclerosis), rheumatoid arthritis, and cancer (Halliwell and Gutteridge 1984; Brown 1995). Therefore, free radical levels must be carefully monitored under both physiological conditions and when generated by environmental stress.

Cells employ a number of defense mechanisms to sense and respond appropriately to oxidative stress. Enzymes such as superoxide dismutases and catalases play critical roles in oxidative stress protection through catalyzing the conversion of ROS to less harmful products (Beyer et al. 1991; Halliwell 1994). As has been demonstrated in yeast, cells lacking a functional gene encoding Cu, Zn superoxide dismutase are highly sensitive to dioxygen and redox-cycling drugs, fail to grow on respiratory carbon sources, and exhibit increased spontaneous mutagenesis rates and a number of other phenotypes during aerobic growth (Gralla and Valentine 1991). Free radical scavenging activities are also exacerbated by small antioxidant molecules, including glutathione, thioredoxin, and ascorbic acid (Halliwell 1994). In addition to the prevention of oxidative damage, repair mechanisms are employed by cells to remove or repair dam-

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aged cellular components, including exo- and endonucleases for DNA-damage repair, proteolytic enzymes for degradation of severely damaged proteins, and phospholipases, glutathione peroxidase/transferase/reductase for degradation and repair of damaged lipids (Davies et al. 1990).

To appropriately mount an oxidative stress defense, cells must harbor oxidative stress sensors. A number of antioxidant responses in bacteria have been elegantly studied, and it has been established that ROS are directly sensed by key regulatory molecules that activate the expression of genes encoding antioxidant proteins at the level of transcription (Storz et al. 1990; Demple and Amabile-Cuevas 1991; Farr and Kogoma 1991). Distinct defense mechanisms are involved in H_2O_2 and $\text{O}_2^{\cdot-}$ detoxification in *Escherichia coli* through the *OxyR* and *SoxRS* regulons, respectively. *OxyR* directly senses oxidative stress to activate the expression of H_2O_2 -inducible genes, including those encoding catalase and alkyl hydroperoxide reductase (Storz et al. 1990). The *SoxRS* regulon is controlled in a two-stage process: An iron-sulfur protein *SoxR* is activated by increases in intracellular superoxide anion levels and triggers transcription of the *soxS* gene. The *soxS* protein in turn induces transcription of other genes of the regulon, including those encoding Mn superoxide dismutase, the DNA repair endonuclease IV, and glucose-6-phosphate dehydrogenase (Demple and Amabile-Cuevas 1991; Farr and Kogoma 1991; Wu and Weiss 1992).

One important class of eukaryotic stress responsive proteins are metallothioneins (MTs): small, cysteine-rich metal-binding proteins whose biosynthesis is induced by a variety of environmental and physiological stresses (Kägi 1991). MTs have a central role in metal detoxification, and correspondingly, MT genes are transcriptionally activated by metals (Thiele 1992; O'Halloran 1993). Furthermore, MTs are able to protect cells from the toxicity of a number of xenobiotics, including chemotherapeutic alkylating agents, and deleterious effects of radiation and chemicals that act as free radical generators (Kägi 1991). It has been shown recently that mouse MT protects cells against the cytotoxic and DNA-damaging effects of nitric oxide (Schwarz et al. 1995). Consistent with a role in oxidative stress protection, MT transcription is also induced by a variety of chemicals that generate ROS or that induce the inflammatory response (Kägi 1991; Dalton et al. 1994). However, the sensors of oxidative stress and the corresponding transcription factors that activate mammalian MT gene expression under these conditions have not yet been identified.

The MT gene *CUP1* in the baker's yeast *Saccharomyces cerevisiae* is critical for copper detoxification and is transcriptionally activated by the copper metalloregulatory transcription factor *ACE1* (Thiele 1992; O'Halloran 1993). Recent experiments have demonstrated that expression of the *CUP1*-encoded MT, or mammalian MTs, suppresses a number of oxidative stress-induced growth defects of yeast strains lacking Cu, Zn superoxide dismutase (Tamai et al. 1993). These observations demonstrate that both yeast and mammalian MT proteins are

an important line of defense against oxidative stress. Consistent with a role in oxidative stress protection, the yeast *CUP1* gene is transcriptionally activated when cells are grown in the presence of high oxygen tensions or during respiration (Tamai et al. 1993). Here we demonstrate that menadione (vitamin K_3), a pro-oxidant that generates $\text{O}_2^{\cdot-}$ through redox cycling (Thor et al. 1982; Chaput et al. 1983), potentially activates *CUP1* transcription and this transcription is mediated by the heat shock factor (HSF). We show that a single functional copy of the *CUP1* gene protects cells from menadione toxicity in an HSF-dependent manner. Furthermore, both the duration and pattern of HSF phosphorylation, and the duration of *CUP1* transcription in response to heat and oxidative stress are distinct. Taken together, these results establish that HSF has a critical role in eukaryotic oxidative stress protection, and suggest that heat and oxidative stress signals are differentially communicated to HSF to activate gene transcription.

Results

Superoxide radical generation induces CUP1 transcription

In previous work we demonstrated that both the *S. cerevisiae* MT protein encoded by the *CUP1* gene and mammalian MTs protect yeast cells bearing a deletion of the gene encoding Cu, Zn superoxide dismutase (*SOD1*) from oxidative stress (Tamai et al. 1993). Therefore, these and other data demonstrate that MTs are an important line of oxidative stress protection (Thornalley and Vasak 1985; Felix et al. 1993; Schwarz et al. 1995). The yeast *CUP1* gene is transcriptionally activated when cells are grown in high oxygen concentrations or during enforced respiration, two conditions known to generate oxidative stress (Tamai et al. 1993). To ascertain whether yeast cells activate *CUP1* gene transcription in response to a direct oxidative stress, we exposed cells to menadione, a derivative of vitamin K_3 that generates superoxide anion through redox cycling (Hassan and Fridovich 1979; Chaput et al. 1983). As shown by primer extension analysis in Figure 1, treatment of *S. cerevisiae* cells with increasing concentrations of menadione results in a large induction of *CUP1* mRNA occurring at a threshold level of menadione ($400\ \mu\text{M}$), with maximum induction (~ 50 -fold) at $500\ \mu\text{M}$ menadione. The magnitude of the response to menadione decreases at higher concentrations, with decreased cell viability (data not shown), presumably attributable to free radical-mediated macromolecular damage at these concentrations of menadione (Chaput et al. 1983; Gralla and Valentine 1991; Halliwell 1994).

HSF mediates CUP1 activation during oxidative stress

The activation of *CUP1* transcription during glucose starvation, in which respiration is exacerbated, is dependent on the *S. cerevisiae* HSF (Tamai et al. 1994). However, glucose starvation has widespread physiological

Figure 1. *CUP1* transcription is induced by the superoxide generator menadione. Primer extension analysis of *CUP1* mRNA expressed in response to increasing concentrations of menadione. *S. cerevisiae* strain MCY1093 (containing multiple copies of the *CUP1* gene) was grown to log phase at 30°C and treated with the indicated concentrations of menadione for 70 min. Total RNA was extracted and *CUP1* mRNA levels were analyzed by oligonucleotide primer extension reactions. For glucose starvation induction, cells were grown to log phase in the presence of 2% glucose and switched to either 2% glucose or 0.05% glucose for 3 hr. The control mRNA primer extension products derived from *ADHI* mRNA and the *CUP1* mRNA primer extension products are indicated.

and regulatory effects in yeast cells (Johnston and Carlson 1992). To test whether HSF may be critical for the activation of *CUP1* expression in response to a direct oxidative stress, we assessed the ability of a *CUP1* gene harboring a nonfunctional heat shock element (HSE) to be activated in response to menadione administration. This HSE (HSE-M) contains two point mutations in conserved residues that were shown previously to abolish HSF binding in vitro and heat shock-induced *CUP1* transcriptional induction in vivo (Tamai et al. 1994). As demonstrated in Figure 2, menadione elicits a robust induction of *CUP1* mRNA levels for the wild-type promoter, whereas the *CUP1* HSE-M promoter is not activated at any concentration of menadione tested. Therefore, a functional interaction between HSF and the *CUP1* HSE is required for the activation of *CUP1* expression in response to oxidative stress.

The *S. cerevisiae* HSF harbors a number of functionally distinct regions identified by DNA binding, in vivo *trans*-activation, and in vitro biochemical and structural

studies (Sorger and Nelson 1989; Nieto-Sotelo et al. 1990; Sorger 1990; Jakobsen and Pelham 1991; Bonner et al. 1992; Flick et al. 1994). *S. cerevisiae* HSF contains two transcriptional activation domains, an amino-terminal element required for the transient response to heat shock and a carboxy-terminal region shown previously to be critical for the sustained response to heat shock and for the activation of *CUP1* transcription in response to both heat shock and glucose starvation (Nieto-Sotelo et al. 1990; Sorger 1990; Tamai et al. 1994). To ascertain whether the carboxy-terminal activation domain of HSF is important for *CUP1* induction in response to menadione, *CUP1* expression was compared in the wild-type strain MCY1093 and an isogenic derivative HSF(1–583) that lacks this activation domain. HSF(1–583), which is truncated at codon 583, is expressed at equally abundant levels as wild-type HSF protein (Tamai et al. 1994; data not shown). The data in Figure 3 show that wild-type cells gave rise to the large induction similar to that observed in Figure 1; however, cells harboring the HSF(1–583) allele induced *CUP1* mRNA levels only twofold. Therefore, the carboxy-terminal activation domain, contained within HSF residues 583–833, is essential for the potent activation of *CUP1* expression in response to menadione administration.

Figure 2. *CUP1* transcriptional induction by menadione requires the *CUP1* HSE (HSE_{CUP1}). Primer extension analysis of *CUP1* mRNA expressed from *CUP1* HSE-WT and *CUP1* HSE-M genes in response to menadione. Isogenic strains DTY3 [*CUP1* HSE-WT] and DTY176 [*CUP1* HSE-M] were grown to log phase at 30°C and treated with the indicated concentrations of menadione for 95 min. Total RNA was isolated, and *CUP1* mRNA was detected by oligonucleotide primer extension assays. The mRNA primer extension products derived from *ADHI* mRNA and the *CUP1* mRNA are indicated. These strains contain a single chromosomal copy of the *CUP1* gene.

Figure 3. *CUP1* transcriptional induction by menadione requires an HSF carboxy-terminal transcription activation domain. Primer extension analysis of *CUP1* mRNA expressed from strains harboring either the wild-type *HSF* gene or the *HSF(1–583)* allele in response to menadione administration. Isogenic strains MCY1093 (*HSF*) and DTY179 [*HSF(1–583)*] were grown to log phase at 30°C and treated with menadione at the indicated concentrations for 60 min. Total RNA was isolated and subjected to oligonucleotide primer extension assays using *ADHI* mRNA as an internal control.

The HSF carboxy-terminal activation domain has been demonstrated previously to be dispensable for heat shock-activated transcription of two members of the *HSP70* gene, *SSA1* and *SSA3* (Tamai et al. 1994). Furthermore, although the *SSA1*, *SSA3* and *HSC82*, *HSP82* genes—members of the *hsp70* and *hsp90* family, respectively—are highly induced in response to heat shock (Slater and Craig 1987; McDaniel et al. 1989; Boorstein and Craig 1990; data not shown), mRNA levels from these genes are not induced, or induced only two- to threefold, in response to menadione concentrations up to 750 μ M (data not shown). Taken together, these experiments demonstrate that both the HSF-binding site within the *CUP1* promoter and the HSF carboxy-terminal *trans*-activation domain are required for the specific, potent oxidative stress-responsive activation of *CUP1* transcription. We demonstrated previously that the HSF-dependent glucose starvation activation of *CUP1* transcription requires the *SNF1* gene, encoding a serine–threonine protein kinase, and the *SNF4* gene, encoding a *SNF1* cofactor (Celenza and Carlson 1989; Tamai et al. 1994). Presumably, this is because glucose starvation generates oxidative stress through forced respiration,

whereas *SNF1* is essential for the activation of genes encoding key respiratory components (Johnston and Carlson 1992). According to this hypothesis, *SNF1* and *SNF4* would be dispensable for oxidative stress generated by means other than respiration. As predicted, *CUP1* activation through menadione-mediated superoxide production, which is independent of respiration, does not require *SNF1* and *SNF4* (data not shown).

CUP1 expression protects cells from oxidative stress

The observation that the *CUP1*-encoded MT provides a line of defense against oxidative stress (Tamai et al. 1993) suggests that the HSF-dependent activation of *CUP1* transcription is a critical step in this defense mechanism. To test this prediction, we first ascertained whether the MT protein is synthesized in yeast cells exposed to menadione. The data presented in Figure 4 demonstrate clearly that *CUP1*-encoded MT protein levels are rapidly induced in response to 200 μ M menadione, whereas cells incubated in the absence of menadione exhibit low, steady-state levels of MT. It should be noted that in the SC–Cys–Met medium used for the [35 S]cysteine pulse-labeling experiments, menadione is a more potent inducer of *CUP1* mRNA and protein expression because of the lack of exogenous cysteine and methionine, which provide abundant sources of antioxidant activity. This result was verified by *CUP1* mRNA analysis in SC and SC–Cys–Met media by primer extension experiments (data not shown).

Genetic experiments were conducted to determine whether the *CUP1* gene protects cells from menadione-induced oxidative stress, and if this protection requires HSF-dependent *CUP1* transcription. Isogenic yeast strains harboring wild-type *CUP1* and *SOD1* genes, or single or double deletions were first compared for their level of menadione resistance. The data in Figure 5 (left) show that although yeast cells lacking the *SOD1* gene

Figure 4. MT protein is rapidly expressed in response to oxidative stress. [35 S]cysteine-labeled whole-cell extracts were prepared from MCY1093 cultures treated with 0 or 200 μ M menadione for the indicated times and fractionated on a 25% non-denaturing polyacrylamide gel followed by fluorography. The MT protein (*CUP1*) is indicated by a bracket.

Figure 5. Protection of yeast cells from oxidative stress requires a functional *CUP1* HSE. (Left) *CUP1* protects *sod1Δ* cells from menadione-induced oxidative stress. Isogenic wild-type, *cup1Δ sod1Δ*, and *sod1Δ cup1Δ* strains were streaked onto synthetic complete agar with increasing concentrations of menadione. (Right) A functional *CUP1* HSE is required for oxidative stress protection by *CUP1*. A *sod1Δ cup1Δ* strain transformed with pRS315 (Vector), pRS315CUP1–HSEWT (HSE-WT), or pRS315CUP1–HSEM (HSE-M) was streaked to SC agar containing menadione at the indicated concentrations.

(*sod1Δ*) grow on plates containing 20 μ M menadione, cells harboring deletions in both the *SOD1* and *CUP1* genes (*sod1Δ cup1Δ*) fail to grow in the presence of 20 μ M menadione. These observations establish that the *CUP1* gene provides an important line of defense against oxidative stress toxicity. We then tested whether the HSF-dependent transcriptional activation of *CUP1* is critical for the oxidative stress protection conferred by *CUP1*. A strain harboring deletions of both the *SOD1* and *CUP1* genes (*sod1Δ cup1Δ*) was transformed with a single-copy plasmid containing either the wild-type *CUP1* gene or a *CUP1* gene with the nonfunctional HSE (*CUP1*–HSE-M). The transformant containing the wild-type *CUP1* gene restored menadione resistance to that of the *sod1Δ* strain, whereas the same strain containing the *CUP1*–HSE-M gene was indistinguishable from the *sod1Δ cup1Δ* strain (Fig. 5, right). These results demonstrate that the oxidative stress protection provided by *CUP1* in a *sod1Δ* background requires the presence of a functional HSE, and that HSF-dependent transcription of the *CUP1* gene is critical for MT-mediated resistance to oxidative stress.

CUP1 expression is hypersensitive in strains compromised for oxidative stress protection

Menadione is an efficient generator of superoxide anion through intracellular redox reactions (Hassan and Fridovich 1979; Thor et al. 1982; Chaput et al. 1983). Work by Gralla and Valentine has established that the Cu, Zn superoxide dismutase gene is a major line of oxidative stress protection in yeast cells and that yeast lacking a functional *SOD1* gene is exquisitely sensitive to menadione and other redox cycling drugs (Gralla and Valentine 1991). To test the hypothesis that HSF-dependent *CUP1* transcription is responding to oxidative stress and that the threshold response to increasing menadione concentrations (Fig. 1) is caused by other operational oxidative stress protection systems, including the Cu, Zn superoxide dismutase, we compared *CUP1* gene activation in isogenic wild-type and *sod1Δ* strains. Primer extension analysis showed the typical threshold response

for menadione-responsive *CUP1* gene activation in the wild-type strain (Fig. 6). In contrast to the wild type, we observed a menadione dose-dependent activation of *CUP1* gene expression in the *sod1Δ* strain, which is compromised severely in the enzymatic disproportionation of superoxide anion (Gralla and Valentine 1991). Furthermore, as expected, the menadione concentration that gives rise to maximal *CUP1* expression is correspondingly reduced in this genetic background. The increased basal levels of *CUP1* mRNA observed in the *sod1Δ* strain are consistent with the observation that *sod1Δ* cells are oxidatively stressed under standard growth conditions (Gralla and Valentine 1991). These data strongly suggest that the HSF-dependent transcriptional activation of *CUP1* by menadione occurs in response to either superoxide radical generation or to other ROS derived from the superoxide anion. We also observed that 8-hydroxy-2'-deoxyguanosine levels, an index of free radical-mediated oxidative damage (Fraga et al. 1990), correlated with *CUP1* transcription (X. Liu, J. Dykens, and D.J. Thiele, unpubl.), consistent with the notion that menadione induces *CUP1* transcription through the generation of free radicals.

Oxidative stress induces HSF phosphorylation

The *S. cerevisiae* HSF has been shown to be inducibly phosphorylated in response to heat shock stress, a post-translational modification that has been proposed to have an important role in heat stress signaling (Sorger and Pelham 1988; Sorger 1990). To understand the mechanism of action of HSF in response to distinct stress conditions, we ascertained whether HSF is inducibly phosphorylated under conditions of oxidative stress. Cells were grown at 23°C (non-heat-shock conditions) to log-phase and pulse-labeled with [32 P]inorganic phosphate in the presence of increasing concentrations of menadione. Parallel cultures were used to prepare total cellular RNA from which the levels of *CUP1* mRNA were assessed by primer extension analysis (Fig. 7). Autoradiography of HSF immunoprecipitated from extracts prepared from these cells, and resolved by SDS–PAGE, demonstrated

Figure 6. Yeast cells defective in superoxide radical detoxification hyperactivate *CUP1* gene expression in response to menadione. (A) Primer extension analysis of *CUP1* mRNA expressed from wild-type and isogenic *sod1Δ* strains in response to increasing concentrations of menadione. Isogenic strains DTY3 (wild-type) and DTY116 (*sod1Δ*) were grown to log phase at 30°C and treated with menadione at the indicated concentrations for 90 min. Total RNA was extracted, and *CUP1* and *ADHI* (control) mRNA levels were detected by oligonucleotide primer extension analysis. (B) Quantitative analysis of *CUP1* mRNA levels expressed in the wild-type and *sod1Δ* strains as a function of menadione concentration, as determined by PhosphorImager analysis. Relative *CUP1* mRNA levels, normalized to *ADHI* mRNA levels, are shown on the y-axis and menadione concentration in micromolar concentrations is shown on the x axis. *CUP1* mRNA levels are from the wild-type (■) and *sod1Δ* (○) strains, respectively.

that HSF is phosphorylated in response to menadione in a manner that parallels *CUP1* mRNA expression. Western blotting demonstrated that HSF steady-state levels in each lane in these experiments were similar (Fig. 7). As observed in Figure 1, at high concentrations of menadione, which are cytotoxic (Thor et al. 1982; Chaput et al. 1983; Gralla and Valentine 1991), both HSF phosphorylation and *CUP1* mRNA levels are reduced. Therefore, HSF is inducibly phosphorylated in response to both heat shock and oxidative stress activation conditions.

To investigate the relationship between HSF phosphorylation and *CUP1* transcriptional activation, we compared the kinetics of HSF phosphorylation and *CUP1* gene expression in response to menadione and heat shock. The data in Figure 8 demonstrate that in response to both stresses, HSF is rapidly inducibly phosphorylated, and this phosphorylation parallels the rapid increase in *CUP1* mRNA levels. Furthermore, in response to menadione, HSF phosphorylation is sustained over at least 2 hr, in parallel with *CUP1* mRNA levels (Fig. 8, left). However, in response to heat shock stress both the phosphorylation of HSF and *CUP1* gene expression are transient (Fig. 8, right). The sustained and transient nature of HSF phosphorylation in response to these two stresses was confirmed by phosphorImager quantitation normalized by densitometry scanning of the x-ray film from the Western blot (data not shown). Therefore, these results demonstrate that HSF is phosphorylated

rapidly in response to both heat and oxidative stress, but the duration of HSF phosphorylation correlates with the duration of *CUP1* mRNA expression—sustained in response to oxidative stress and transient in response to heat stress.

Differential HSF phosphorylation during heat and oxidative stress

The activation of *CUP1* gene transcription by HSF exhibits distinct properties in response to heat or oxidative stress. Although the activation of *CUP1* expression is rapid in response to each stress, both HSF phosphorylation and *CUP1* expression are transient in response to heat but sustained in response to oxidative stress. These observations suggest that the two stresses may communicate by distinct mechanisms to activate the HSF protein. Because protein phosphorylation is a widely used mechanism to transduce signals to proteins in general (Hunter 1995) and is known to modulate the activity of several transcription factors (Hunter and Karin 1992), we compared the tryptic phosphopeptide pattern of HSF immunoprecipitated from cells exposed to heat shock or oxidative stress by two-dimensional mapping (Fig. 9). The tryptic phosphopeptide analysis of HSF immunoprecipitated from control cells (23°C) showed that HSF has a low basal level of phosphorylation. Heat shock, for either 15 or 60 min, induced the phosphorylation of sev-

Figure 7. Menadione induces HSF phosphorylation in a dose-dependent manner. *S. cerevisiae* strain MCY1093 was metabolically labeled with [32 P]phosphoric acid in the absence (C) or presence of the indicated concentrations of menadione or subjected to heat shock (HS) at 39°C for 20 min. HSF protein was immunoprecipitated with anti-HSF polyclonal antiserum from whole-cell extracts and fractionated on 6% SDS-PAGE, followed by electroblotting to nitrocellulose membrane. Phosphorylated HSF was detected by autoradiography, and HSF protein steady-state levels were detected by immunoblotting. Total RNA was prepared from unlabeled cells treated under the same conditions in a parallel culture. *CUP1* and *ADHI* (control) mRNA levels were detected by oligonucleotide primer extension analysis. In low phosphate medium the effective concentrations of menadione are shifted to lower levels.

eral tryptic peptides not apparent in the control sample. Furthermore, no obvious difference was observed between the HSF tryptic phosphopeptide pattern from cells subjected to transient (15 min) or sustained (60 min) heat shock. However, treatment of cells with menadione resulted in the appearance of several tryptic phosphopeptides not found in either the control sample or HSF immunoprecipitated from heat-shocked cells. Phosphoamino acid analysis demonstrated that HSF is inducibly phosphorylated during heat shock or menadione treatment predominantly on serine residues (data not shown). These results strongly suggest that HSF is differentially phosphorylated in response to heat and oxidative stress, conditions that activate MT gene transcription with distinct characteristics.

Discussion

In this report we have demonstrated that the *S. cerevisiae* MT gene *CUP1*, which protects yeast cells from oxidative stress, is transcriptionally activated in response to the potent superoxide anion generator mena-

dione. This activation requires HSF and a *CUP1* promoter HSE. Interestingly, although menadione potently activates *CUP1* expression, we detected little menadione activation of transcription of two members of the *S. cerevisiae* *hsp70* gene family, *SSA1* and *SSA3*, and the two members of the *hsp90* family, *HSP82* and *HSC82*, even though HSF binds to HSEs within the promoters of these genes (Slater and Craig 1987; McDaniel et al. 1989; Boorstein and Craig 1990). This differential gene activation in response to menadione may be a function of unique interactions HSF makes with the nonconsensus HSE found within the *CUP1* promoter. Alternatively, HSF may engage in promoter-specific interactions with additional transcriptional regulatory molecules. It has been demonstrated recently that *S. cerevisiae* HSE elements are not functionally equivalent with respect to both HSF binding and transcriptional activation under control and heat shock conditions (Young and Craig 1993; Bonner et al. 1994; Giardina and Lis 1995). Similarly, it was found that binding of HSF to an HSE is insufficient to activate the *hsp70* gene in murine erythroleukemia cells under heat shock conditions (Hensold et al. 1990). Studies of the mouse *hsp70* gene have also demonstrated that although menadione activates HSF binding to the *hsp70* promoter in vitro, neither *hsp70* mRNA nor protein accumulate under these conditions (Bruce et al. 1993). Furthermore, although salicylate was shown to activate HSF binding to the human *hsp70* gene HSEs in vivo, this condition does not induce *hsp70* gene transcription (Jurivich et al. 1992). Taken together, these observations suggest that a subset of HSF-responsive genes may be activated under conditions other than heat shock and that activation of these genes is dependent on promoter context or additional regulatory steps subsequent to DNA binding.

In prokaryotic organisms, distinct regulatory molecules sense elevations in superoxide anion, hydrogen peroxide, and heat shock, although some degree of overlap in target gene expression has been observed in response to these stresses (Storz et al. 1990; Farr and Kogoma 1991; Lindquist 1992). We observed potent activation of *CUP1* expression in response to menadione in this study and in response to high oxygen or enforced respiration previously (Tamai et al. 1993, 1994). Because each of these conditions is known to generate superoxide anion, this strongly supports the notion that *CUP1* activation occurs in response to superoxide radical or a metabolic derivative. Furthermore, the hypersensitivity of *CUP1* transcription to menadione in an *sod1Δ* strain is consistent with ROS serving as the signal in gene activation, although we cannot eliminate the possibility that the actual signal may be macromolecular damage caused by the ROS generated under these conditions. However, *CUP1* transcriptional activation is not observed in the presence of any concentration of hydrogen peroxide used (data not shown). This suggests that *CUP1* transcriptional activation is not a consequence of damage by ROS in general and, furthermore, that peroxide and superoxide species, as in prokaryotes (Dempfle and Amabile-Cuevas 1991; Farr and Kogoma 1991; Storz and

Figure 8. Kinetic analysis of HSF phosphorylation and *CUP1* transcription in response to heat shock or oxidative stress. *S. cerevisiae* strain MCY1093 was grown as described in the legend to Fig. 7 and incubated in the presence of either 400 μ M menadione (left) or heat-shocked at 39°C (right) for the indicated times. HSF phosphorylation and steady-state levels of *CUP1* and *ADHI* mRNA were determined as described in the legend to Fig. 7.

Tartaglia 1992), may be differentially sensed by yeast cells. Consistent with this idea, previous studies have shown that pretreatment of *S. cerevisiae* cells with menadione or hydrogen peroxide protected cells from subsequent challenge with the homologous agent but did not confer cross resistance to the heterologous form of oxidative stress (Jamieson 1992; Flattery-O'Brien et al.

1993). The mammalian transcription factor NF- κ B is activated by hydrogen peroxide and cell lines overexpressing Cu, Zn superoxide dismutase, which produce increased levels of H_2O_2 , display increased NF- κ B activation by oxidative stress. In contrast, cell lines with reduced levels of H_2O_2 , through overexpression of catalase, have a severely dampened NF- κ B response to oxidative stress (Schmidt et al. 1995). Therefore, mammalian transcription factors also appear to be responsive to distinct ROS.

The *S. cerevisiae* HSF protein has been shown to harbor two transcriptional activation domains: an amino-terminal region required for transient activation of HSE-driven reporter genes and a carboxy-terminal domain required for a sustained activation in response to heat stress (Nieto-Sotelo et al. 1990; Sorger 1990). We have demonstrated that the activation of *CUP1* gene transcription in response to heat, glucose starvation, or direct oxidative stress requires the carboxy-terminal domain localized between HSF amino acids 583 and 833. In response to heat stress, *CUP1* transcription is transient (Tamai et al. 1994). However, in response to glucose starvation or oxidative stress, *CUP1* transcription is sustained for at least 2 hr. Although the precise biochemical mechanisms underlying the transient nature of the heat shock response is not known, it has been proposed that the *hsp70* gene products, elevated in response to heat stress, feed back to down-regulate the activity of HSF (Abravaya et al. 1992; Baler et al. 1992). Our observation that the *hsp70* or *hsp90* genes tested were activated only modestly during menadione exposure while they are robustly activated by heat stress could be consistent with the sustained nature of *CUP1* expression in response to menadione and the transient activation in response to

Figure 9. HSF is differentially phosphorylated in response to menadione and heat shock. 32 P-labeled HSF was purified by immunoprecipitation from cells incubated for 60 min under control conditions or heat-shocked for 15 or 60 min, or menadione treated (400 μ M) for 20 min. The purified HSF was subjected to trypsin digestion. Equal amounts of protein were used for all samples except for the 60-min heat shock, which was overloaded to reveal any potential differences in the phosphorylation pattern between the 15- and 60-min heat shock times. Phosphorylated tryptic peptides were resolved by electrophoresis and thin layer chromatography, and visualized by autoradiography. (Arrowheads) The sample origin on the TLC plates.

heat stress, in light of the *hsp70* controlling model. However, the observation that *hsp70* expression is insufficient to down-regulate HSF activity in mammalian cells (Rabindran et al. 1994) and our HSF phosphorylation data suggest that additional factors are likely to contribute to the modulation of the duration of the HSF-mediated transcriptional response.

An examination of the kinetics of HSF phosphorylation in vivo demonstrated that HSF is phosphorylated rapidly in response to both heat and oxidative stress. As has been observed previously for the yeast and higher eukaryotic HSF proteins in response to heat shock, this phosphorylation correlates with a dramatic reduction in the electrophoretic mobility of HSF on SDS PAGE (Sorger and Pelham 1988; Sarge et al. 1993). Because it has been observed that stresses such as heat shock result in rapid protein glycosylation, it is not known whether phosphorylation or other post-translational modifications completely account for the change in electrophoretic mobility (Jethmalani et al. 1994).

We observed two striking features of HSF phosphorylation in these experiments. First, HSF phosphorylation kinetically paralleled *CUP1* transcriptional activation. In response to heat stress, HSF was phosphorylated rapidly and declined to a low level of phosphorylation in concert with the transient nature of *CUP1* mRNA induction. In response to oxidative stress, HSF was also rapidly inducibly phosphorylated and, in parallel with *CUP1* expression, high-level HSF phosphorylation was maintained over a period of at least 2 hr in the continued presence of menadione. Although it has not yet been firmly established whether phosphorylation of the *S. cerevisiae* HSF is critical for transcriptional activation, these data, and the demonstration that phosphorylation has a key role in the activation of many transcription factors, strongly support this hypothesis (Hunter and Karin 1992). Second, two-dimensional resolution of tryptic phosphopeptides derived from HSF from control, heat-shocked, and menadione-treated cultures establishes that HSF is differentially phosphorylated under these three conditions. In cells grown under control conditions, these experiments and previous investigations indicate the presence of a low level of HSF phosphorylation (Sorger 1990). Whether this basal phosphorylation is important for the essential function of HSF under control growth conditions has not yet been established. In response to heat shock or oxidative stress conditions, several additional HSF tryptic phosphopeptides are re-

solved in addition to those observed in control cultures. Furthermore, the pattern of tryptic phosphopeptides observed from HSF derived from heat-shocked or menadione-treated cultures is distinct, indicating that steady-state HSF phosphorylation is distinct under these two conditions. This could be a consequence of differential phosphorylation or dephosphorylation of HSF under the two activation conditions. Whether these differences in the HSF phosphorylation state are caused by distinct sites of phosphorylation in the carboxy-terminal *trans*-activation domain is not known.

Several protein kinases have been identified that have an important role in response to stress, including the MAP kinases, protein kinase C, AMP kinase, and others (Corton et al. 1994; Kyriakis et al. 1994; Levin and Errede 1995). One mechanism by which a protein kinase senses changes in oxidative stress is illustrated in the FixL-J system in the nitrogen-fixing bacteria *Rhizobium meliloti* (Gilles-Gonzalez et al. 1994). FixL is a heme-binding, oxygen-sensing protein kinase that autophosphorylates at a histidine residue under low-oxygen tensions and that subsequently transfers the phosphate group to FixJ. The phosphorylated FixJ protein activates transcription of downstream target genes involved in nitrogen fixation. Whether yeast cells utilize oxidative stress-sensing protein kinases or other regulatory molecules in the activation of MT gene transcription is currently unknown. Further work on HSF will elucidate the importance of stress-dependent phosphorylation in gene activation and the mechanisms by which the oxidative stress signals are sensed and transmitted to HSF to activate expression of critical stress-responsive genes.

Materials and methods

Strains and growth conditions

The *S. cerevisiae* strains used in this study and the corresponding genotypes are listed in Table 1. For glucose starvation, menadione, or heat shock induction experiments, *S. cerevisiae* strains were grown overnight at 30°C or 23°C in synthetic complete medium (SC) minus the indicated nutrients. Basal levels of *CUP1* mRNA expression or responses to menadione were indistinguishable in cells grown at either of these temperatures (Tamai et al. 1994; data not shown). Overnight cultures were used to inoculate fresh cultures to an optical density at 650 nm (OD_{650}) of 0.4–0.5 and grown at the same temperature to early to mid-logarithmic phase (OD_{650} of 1.0–1.5). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature and resuspended in the same volume of fresh medium.

Table 1. *S. cerevisiae* strains used in this work

Strains	Genotype	Reference of source
DTY3	<i>MATα trp1-1 his leu2-3,112 gal1 ura3-50 cup1-HSE-WT</i>	Hamer et al. (1985)
DTY113	<i>MATα trp1-1 his leu2-3,112 gal1 ura3-50 cup1 Δ61</i>	Tamai et al. (1993)
DTY116	<i>MATα trp1-1 sod1Δ::TRP1 his leu2-3,112 gal1 ura3-50 cup1s</i>	Tamai et al. (1993)
DTY117	<i>MATα trp1-1 sod1Δ::TRP1 his leu2-3,112 gal1 ura3-50 cup1Δ::URA3</i>	Tamai et al. (1993)
MCY1093	<i>MATα his4-539 ura3-52 lys2-801</i>	Celenza and Carlson (1989)
DTY176	<i>MATα trp1-1 his leu2-3,112 gal1 ura3-50 cup1-HSE-M</i>	Tamai et al. (1994)
DTY179	<i>MATα his4-539 ura3-52 lys2-801 HSF(1–583)::URA3</i>	Tamai et al. (1994)

Glucose starvation induction was carried out as described previously (Tamai et al. 1994). For heat shock induction, cell cultures (5 ml/vol) in glass culture tubes were incubated at 39°C in a shaking (300 rpm) water bath for times indicated in the figure legends. For menadione induction, a freshly prepared stock solution of 50 mM menadione dissolved in 100% ethanol was added to final concentrations indicated in the figure legends, and cells were incubated at 23°C, 300 rpm, for the times indicated in the figure legends. The same volume of 100% ethanol was added to control cultures for menadione induction experiments. For menadione agar plates, the stock menadione solution prepared as above was added to autoclaved synthetic complete agar. Menadione plates were wrapped with aluminum foil to exclude light either during storage at 4°C or during yeast cell culturing at 23°C or 30°C. Plasmids pRS315CUP1-HSEWT and pRS315CUP1-HSEM were constructed by subcloning the 5.3-kb *SalI*-*PstI* *CUP1* fragments from pRS426HSEWT and pRS426HSEM (Tamai et al. 1994) to the corresponding sites of the yeast centromere plasmid pRS315 using standard molecular techniques (Ausubel et al. 1987). Yeast and *E. coli* strains were grown and maintained by standard techniques (Ausubel et al. 1987).

RNA isolation and analysis

Five-milliliter cultures were harvested at 3000 rpm, 4°C, for 5 min and washed once with 5 ml of sterile glass-distilled water and once with 1 ml of RNA extraction buffer. Total cellular RNA was prepared as described previously (Ausubel et al. 1987). Ten micrograms of each RNA sample was subjected to electrophoretic analysis on 1% borax-agarose gel after denaturation with formaldehyde and formamide to verify the quantity and integrity of RNA used in subsequent primer extension reactions (Tamai et al. 1994). *CUP1*, *ADH1*, *ACT1*, *SSA1*, *SSA3*, *HSP82*, and *HSC82* mRNA levels were detected by primer extension analysis with 20 µg of each RNA sample, using oligonucleotide primers specific for each, as described previously (Silar et al. 1991). All mRNA levels were quantitated using a PhosphorImager (Molecular Dynamics) and each experiment was repeated at least twice. In all experiments, *CUP1* mRNA levels were normalized to that of *ADH1* or *ACT1*.

³⁵S-Labeling and detection of the CUP1-encoded MT protein

S. cerevisiae cell cultures were grown overnight at 30°C in synthetic complete medium minus methionine and cysteine (SC-Met-Cys). The overnight cultures were used to inoculate fresh cultures in SC-Met-Cys to an OD₆₅₀ of 0.4–0.5 and grown to early to mid-logarithmic phase (OD₆₅₀ of 1.0–1.5). Cells were incubated at 30°C in the presence of 20 µCi/ml of [³⁵S]cysteine (ICN, 1.28 mCi/nmole, 12.8 µCi/µl) and 0 or 200 µM menadione for the times indicated in the legend to Fig. 4. Cells were harvested and lysed by vortexing at 4°C in the presence of equal volumes of acid-washed glass beads and buffer (10 mM Tris-HCl at pH 7.8, 10 mM PMSF, 1 mM dithiothreitol), and the cell extracts were saturated with CuSO₄ and subjected to electrophoresis on a 25% nondenaturing polyacrylamide gel. The gel was fluorographed with EN³HANCE (Amersham), dried under vacuum, and exposed to x-ray film at –80°C, as described (Zhou and Thiele 1993).

³²P-Labeling and immunoprecipitation of HSF

Yeast cells were grown for 24 hr in 3/5 phosphate SC medium (in which the phosphate content is 3/5 that of normal SC) (Bos-

tian et al. 1983) at 23°C, harvested, and washed with sterile glass-distilled water. Cells were resuspended in the same volume of low-phosphate SC medium (phosphate content is 1/50 that of normal SC) and inoculated to low-phosphate SC medium to an initial OD₆₅₀ of 0.1–0.2 in a final volume of 5 ml. The cultures were grown for 8–10 hr at 23°C to OD₆₅₀ of 1.0–1.5, harvested, and resuspended in fresh low-phosphate SC medium. To each 5-ml culture 0.5 mCi [³²P]phosphoric acid was added, followed by the addition of menadione to the indicated concentration or heat shock treatment as described above. Subsequently, the 5-ml cultures were harvested and washed with 5 ml of sterile H₂O. Cells were pelleted and resuspended in 300 µl of SDS harvest buffer (0.5% SDS, 10 mM Tris-HCl at pH 7.4, 1 mM EDTA, 1 mM Na₃VO₄, and 20 mM NaF) with freshly added protease inhibitors (1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 0.5 µg/ml leupeptin), and 500 µl acid-washed glass beads. Cells were lysed by vortexing three times at top speed for 1 min each at 4°C, with 15-sec intervals of chilling on ice. SDS harvest buffer (400 µl) was added to the cell lysate, followed by centrifugation at 4°C for 5 min at 3000 rpm. The glass beads were washed with another 400 µl of SDS harvest buffer and centrifuged as above. The combined supernatant was heated at 100°C for 5 min and centrifuged at 4°C for 5 min at 12,000 rpm. The protein concentration of each extract was determined using Bio-Rad dye staining (Bradford assay). NP-40 was added to the extract to a final concentration of 1%, mixed, aliquoted, and frozen at –80°C. For immunoprecipitation 150 µg of cell extract was thawed on ice and 1 µl of polyclonal anti-HSF antiserum (gift from Dr. Peter Sorger, Massachusetts Institute of Technology, Cambridge) was added. The reactions were incubated on a rotating wheel for 2–4 hr at 4°C. Protein A-Sepharose beads incubated with 2% bovine serum albumin (BSA) for 1 hr and washed with SDS harvest buffer were resuspended in SDS harvest buffer to 50% suspension. Twenty microliters of the suspension was added to each immunoprecipitation reaction and incubated for another hour on the rotating wheel at 4°C. The extract was centrifuged at 12,000 rpm, 4°C, and the supernatant was discarded. The protein A-Sepharose beads were washed five times at 4°C, each with 1 ml of RIPA buffer (175 mM NaCl, 10 mM NaPO₄ at pH 7.0, 1% NP-40, 16% sodium deoxycholate, 0.1% SDS). Thirty microliters of SDS sample buffer (0.5 M Tris-HCl at pH 6.8, 10% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) was added, and the beads were heated at 100°C for 5 min. The samples were spun briefly in a microcentrifuge, and the supernatant was loaded on a 6% SDS-polyacrylamide gel and subjected to electrophoresis at 120 V until the bromophenol blue dye ran out of the gel. The proteins were transferred to nitrocellulose membrane by electroblotting for 2 hr at 250 mA, and the membrane was air-dried and exposed to Kodak Biomax film at –80°C with an intensifying screen. The membranes were subsequently immunoblotted with the polyclonal anti-HSF-antiserum to ascertain the steady-state levels of HSF protein. The exposure time required for autoradiographic detection of HSF phosphorylation was 12–48 hr. This does not interfere with immunodetection (ECL, Amersham), which requires exposure times of <5 sec. HSF phosphorylation levels were normalized by PhosphorImaging of the nitrocellulose membrane and densitometry scanning of the HSF bands detected by Western blotting using multiple exposure times to x-ray film.

Two-dimensional tryptic mapping of ³²P-labeled HSF

HSF was purified by immunoprecipitation from cells metabolically labeled with [³²P]phosphoric acid and electroblotted to nitrocellulose membranes as described above. The nitrocellu-

lose bands containing HSF were excised from the membrane and incubated in 20 mM chloramine T dissolved in 50 mM Tris-HCl (pH 8.3), 2 mM EDTA, to oxidize all thiols and then washed twice with water. This was followed by incubation in 0.5% polyvinylpyrrolidone 40 (PVP-40) dissolved in 100 mM acetic acid for 30 min at 37°C, washed with 1 ml H₂O five times, and 1 ml 50 mM ammonium bicarbonate twice. Two hundred microliters of 50 mM ammonium bicarbonate was added to the membrane, followed by addition of 15 µl of trypsin (1 mg/ml dissolved in 0.1 mM HCl). The membranes were incubated at 37°C for 3 hr after which another 15 µl of trypsin was added and incubated overnight at 37°C. The supernatant was lyophilized following addition of 700 µl of H₂O. The dried pellets were redissolved in 600 µl of H₂O and relyophilized. This was repeated with 400 µl of H₂O. The dried pellets were dissolved in pH 1.9 buffer and subjected to electrophoresis in the first dimension on TLC plates with pH 1.9 buffer and thin layer chromatography in the second dimension using isobutyric acid buffer as described (Boyle et al. 1991). The TLC plates were dried and exposed to a PhosphorImager screen.

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