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Protein Kinase A Regulates Constitutive Expression of Small Heat-Shock Genes in an Msn2/4p-Independent and Hsf1p-Dependent Manner in *Saccharomyces cerevisiae*

Scott B. Ferguson,* Erik S. Anderson,* Robyn B. Harshaw,* Tim Thate,† Nancy L. Craig† and Hillary C. M. Nelson*,[1]

**Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6059 and †Howard Hughes Medical Institute and the Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185*

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ABSTRACT

Hsf1p, the heat-shock transcription factor from *Saccharomyces cerevisiae*, has a low level of constitutive transcriptional activity and is kept in this state through negative regulation. In an effort to understand this negative regulation, we developed a novel genetic selection that detects altered expression from the *HSP26* promoter. Using this reporter strain, we identified mutations and dosage compensators in the Ras/cAMP signaling pathway that decrease cAMP levels and increase expression from the *HSP26* promoter. In yeast, low cAMP levels reduce the catalytic activity of the cAMP-dependent kinase PKA. Previous studies had proposed that the stress response transcription factors Msn2p/4p, but not Hsf1p, are repressed by PKA. However, we found that reduction or elimination of PKA activity strongly derepresses transcription of the small heat-shock genes *HSP26* and *HSP12,* even in the absence of *MSN2/4*. In a strain deleted for *MSN2/4* and the PKA catalytic subunits, expression of *HSP12* and *HSP26* depends on *HSF1* expression. Our findings indicate that Hsf1p functions downstream of PKA and suggest that PKA might be involved in negative regulation of Hsf1p activity. These results represent a major change in our understanding of how PKA signaling influences the heat-shock response and heat-shock protein expression.

THE heat-shock response is a highly conserved physiological response to severe changes in environmental conditions. It is characterized by an increase in the expression of heat-shock proteins (HSPs), which maintain protein homeostasis by alleviating protein misfolding defects and aggregation, thus protecting the cell from damage. In the yeast *Saccharomyces cerevisiae*, two response elements regulate heat-shock gene expression: the heat-shock element (HSE), which is bound by the heat-shock transcription factor Hsf1p (Sorger and Pelham 1988; Wiederrecht *et al.* 1988), and the stress response element (STRE), which is bound by the partially redundant transcription factors Msn2p and Msn4p (Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996). These two binding sites are distributed differentially among different heat-shock genes, causing Hsf1p and Msn2p/4p to have distinct contributions to the heat-induced expression of these genes (Treger *et al.* 1998; Boy-Marcotte *et al.* 1999; Amoros and Estruch 2001; Grably *et al.* 2002).

The heat-shock transcription factor is the primary transcriptional regulator for the eukaryotic heat-shock response (Jolly and Morimoto 2000; Pirkkala *et al.* 2001). In all eukaryotes, HSF binds HSEs in the promoters of most HSPs and strongly activates heat-shock gene expression in response to heat and other environmental stresses. In metazoans, multiple isoforms of HSF regulate tolerance to stresses, and they are also involved in developmental programs, including eye lens development and gametogenesis (Christians *et al.* 2003). The isoforms have unique and synergistic functions, with HSF1 as the predominant isoform for heat-induced HSP expression. In yeast, a single essential gene, *HSF1*, responds to various stresses and must therefore integrate diverse stimuli and elicit an appropriate transcriptional response (Sorger and Pelham 1988; Wiederrecht *et al.* 1988).

HSF activity must be tightly controlled to avoid inappropriate expression of heat-shock proteins (Nollen and Morimoto 2002). Thus, HSF is negatively regulated, kept in an inactive or low activity conformation prior to stress. In *S. cerevisiae*, a low level of Hsf1p activity is essential to maintain constitutive expression of HSPs necessary for normal cellular processes. Under normal growth conditions, Hsf1p is bound to strong HSEs (Jakobsen and Pelham 1988). Following heat shock, the occupancy of Hsf1p increases at all types of HSEs (Giardina and Lis 1995; Erkine *et al.* 1999; Hahn *et*

[1]*Corresponding author:* Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, 813 Stellar-Chance, 422 Curie Blvd., Philadelphia, PA 19104-6059.
E-mail: hnelson@mail.med.upenn.edu

al. 2004), and its transcriptional activity increases dramatically (Sorger and Pelham 1988). The phosphorylation state of Hsf1p also changes in response to stress, with some sites responsible for activation and others for attenuation of activity (Sorger and Pelham 1988; Høj and Jakobsen 1994; Hashikawa and Sakurai 2004). The constitutive and heat-induced phosphorylation of Hsf1p has been studied, but the signaling pathways that are responsible for the negative regulation and heat-induced activation have yet to be elucidated.

The Msn2p/4p transcription factors are not conserved from yeast to metazoans and are not required for viability. Under nonstress conditions, Msn2p/4p are localized to the cytoplasm; however, following stress they move into the nucleus where they drive the expression of their target genes (Schmitt and McEntee 1996; Gorner *et al.* 1998; Beck and Hall 1999). These genes include some of the same heat-shock genes regulated by Hsf1p, as well as genes involved in antioxidant and carbohydrate metabolism (Gasch *et al.* 2000; Causton *et al.* 2001). The control of Msn2p/4p seems to be primarily through negative regulation, with the Ras/cAMP pathway playing a major role (Gorner *et al.* 1998; Smith *et al.* 1998). As with Hsf1p, phosphorylation plays a critical role in the activity of Msn2p (Chi *et al.* 2001).

In this work, we have undertaken a novel genetic approach in an attempt to understand the signaling pathways that negatively regulate the expression of heat-shock genes in the absence of stress. For our first genetic selection, we have chosen the *HSP26* promoter, which has been well characterized (Susek and Lindquist 1989; Susek and Lindquist 1990; Chen and Pederson 1993) and whose heat-induced expression is known to be regulated by Hsf1p and Msn2p/4p (Boy-Marcotte *et al.* 1999; Amoros and Estruch 2001). To identify regulators of *HSP* expression, we used a Tn7-insertional mutagenesis approach (Bachman *et al.* 2002; Uhl *et al.* 2003), which allowed us to efficiently identify loss-of-function alleles in two genes, *CDC25* and *RAM1*. In a complementary approach, we used an overexpression library to identify two dosage compensators, *PDE2* and *MSI1*.

These four genes, which are known components of the RAS/cAMP signaling cascade, dramatically increase the constitutive expression of *HSP26* when they are either mutated or overexpressed. This increase of *HSP26* expression is preserved even in an *msn2/4*Δ strain. The Ras/cAMP pathway regulates the activity of protein kinase A (PKA), a serine/threonine kinase that plays a role in stress resistance and nutrient signaling and specifically represses the activity of Msn2p (Broach 1991; Thevelein and de Winde 1999). Of the heat-shock genes we tested, reduction in the level of PKA activity strongly induces the Msn2p/4p-independent expression of the genes encoding two small heat-shock proteins, *HSP26* and *HSP12*, and modestly induces *HSP104*. In contrast, the expression of the larger heat-shock proteins *SSA3, SSA4*, and *HSP82* was unaffected by loss of PKA activity. By using a conditional allele of *HSF1*, we have shown that the increased expression of the small heat-shock genes observed in the absence of PKA is dependent on Hsf1p. Our results reveal that PKA plays a role in the negative regulation of Hsf1p-dependent transcription in addition to its already established role in repressing Msn2p/4p. In contrast to the role of PKA in the regulation of Msn2p/4p activity, which affects all STRE-containing genes, the role of PKA in the regulation of Hsf1p activity affects only a subset of HSE-containing genes. In addition, we have shown that Hsf1p is unlikely to be a direct target of PKA, as deletion of PKA causes an increase in Hsf1p phosphorylation.

MATERIALS AND METHODS

Plasmid construction: The integrative plasmid pHN3102 contains 2888 bp upstream of the *HSP26* start codon fused to the *HIS3* selectable marker open reading frame. The *HSP26* promoter sequences were generated by PCR amplification from the *S. cerevisiae* strain W303-1A using primers SF135 (GGGGTACCTAAGCATCAAAGAAGGTGCG) and SF136 (ATTCCTCGAGTTGTTTAGTTTGTTTGTTTGCTTTTTTG GATACC), which add *Kpn*I and *Xho*I restriction sites to the 5′ and 3′ ends of the fragment, respectively. The cloning process introduces a 4-bp change in the *HSP26* promoter just upstream of the start from CAAATTAAC ATG to CAA**c**T**cg**A**g** ATG. The *HIS3* open reading frame, as well as 221 bp 3′ of the stop codon, were amplified from the pRS403 plasmid (Sikorski and Hieter 1989) using primers SF137 (ATTCCTC GAGATGACAGAGCAGAAAGCC) and SF138 (ATTCGCGGC CGCTTTCACACCGCATAGATCCG), which add *Xho*I and *Not*I sites to the 5′ and 3′ ends of the fragment, respectively. The *HSP26* promoter and *HIS3* ORF PCR fragments were cloned into the *URA3*-marked integrative plasmid pRS406 (Sikorski and Hieter 1989). The λTRP cDNA library was obtained from ATCC. Standard protocols were used to convert the phage library to the pTRP plasmid library, which expresses cDNAs from a *GAL1* promoter (Elledge *et al.* 1991; Ramer *et al.* 1992).

Yeast strains and media: Strains were grown on either YPDA rich media (YPD supplemented with 40 mg/ml adenine) or synthetic complete media (SC) using as carbon sources either 2% dextrose (SC Dex) or a 2% galactose/2% raffinose mixture (SC Gal/Raf; Ausubel *et al.* 2004). When necessary, aminoglycosides were used at the following concentrations: 200 μg/ml Geneticin G418 (Invitrogen, Carlsbad, CA) and 100 μg/ml ClonNAT (Werner BioAgents, Jena, Germany). For growth comparisons of a given strain on SC medium containing 3-amino-1,2,4-triazole (3-AT), equal numbers of cells were plated on both nonselective and selective media. The minimal inhibitory concentration of 3-AT for the wild-type P_{HSP26}-*HIS3* reporter strain is ~7 mm, as seen in Figure 1. This concentration was used in Figure 7 to demonstrate the weak phenotype of *MSI1*, while 30 mm was used as a more stringent selection for other experiments.

Yeast strains, listed in Table 1, are all derived from W303-1A (*MAT*a *ade2- 1 trp1-1 can1-100 leu2,3-112 his3-11,15 ura3-1*). The *HIS3* conditional reporter strains were generated by integration of *Bam*HI-linearized pHN3102 into a diploid W303-1 strain, followed by subsequent sporulation to obtain strains YHN801 and YHN802, and into the *msn2*Δ *msn4*Δ strain YHN-963 to obtain strain YHN966. The proper integration of the

TABLE 1

Strains used in this study

Strain	Relevant genotype[a]
YHN801	*MAT*α *HSP26::*P_{HSP26}*-HIS3,URA3*
YHN802	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3*
YHN917	*MAT***a**/α ρ^0 cir^0
YHN932	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 ram1-doh1::Tn7*
YHN934	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 ram1-doh3::Tn7*
YHN941	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 ram1-doh8::Tn7*
YHN946	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 cdc25-doh11::Tn7*
YHN947	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 cdc25-doh12::Tn7*
YHN963	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX*
YHN966	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX*
YHN1005	*MAT*α *ram1*Δ*::kanMX*
YHN1007	*MAT*α *cdc25*Δ*::kanMX*
YHN1077	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX ram1*Δ*::kanMX*
YHN1078	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX cdc25*Δ*::kanMX*
YHN1082	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP*
YHN1083	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk2*Δ*::loxP*
YHN1084	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk3*Δ*::loxP*
YHN1085	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk2*Δ*::loxP*
YHN1086	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk3*Δ*::loxP*
YHN1087	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk2*Δ*::loxP tpk3*Δ*::loxP*
YHN1090	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX* + pRS424GAL1[b]
YHN1091	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX* + P*GAL1-PDE2-TRP1*
YHN1092	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX* + P*GAL1-MSI1-TRP1*
YHN1114	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk2*Δ*::loxP tpk3*Δ*::loxP*
YHN1116	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk2*Δ*::loxP tpk3*Δ*::loxP*
YHN1126	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 ram1*Δ*::kanMX*
YHN1128	*MAT***a** *HSP26::*P_{HSP26}*-HIS3,URA3 cdc25*Δ*::kanMX*
YHN1172	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX* P_{tetO_2}*-HSF1, adh*$_P$*-tetR-VP16, natMX4 adh*$_P$*-tetR′-SSN6::LEU2*
YHN1173	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk2*Δ*::loxP tpk3*Δ*::loxP* P_{tetO_2}*-HSF1, adh*$_P$*-tetR-VP16, natMX4 adh*$_P$*-tetR′-SSN6::LEU2*
YHN1189	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX HSF1-13Myc::TRP1*
YHN1201	*MAT***a** *msn2*Δ*::loxP msn4*Δ*::kanMX tpk1*Δ*::loxP tpk2*Δ*::loxP tpk3*Δ*::loxP HSF1-13Myc::TRP1*

[a] All strains are derivatives of W303-1 and were constructed for this study.
[b] Funk *et al.* (2002).

reporter was confirmed by PCR. Gene knockouts were performed as previously described (Guldener *et al.* 1996; Goldstein and McCusker 1999). For strains where serial disruptions using the *kanMX4* cassette was necessary, the marker was excised using cre-*loxP* recombination (Guldener *et al.* 1996). All gene knockouts were confirmed by PCR analysis. In addition, we confirmed the *msn2*Δ *msn4*Δ alleles by showing a complete absence for the heat-induced expression of *CTT1*, a gene whose heat-induced expression is completely dependent on *MSN2* and *MSN4* (data not shown; Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996).

The diploid ρ^0 cir^0 strain YHN917 was created from Y842 by standard protocols (Fox *et al.* 1991; Tsalik and Gartenberg 1998). Loss of the 2μ plasmid was confirmed by the absence of a PCR product for the 2μ-encoded *REP1* gene. Loss of mitochondrial DNA was confirmed by an inability to utilize glycerol as the sole carbon source and a lack of mitochondrial staining with Hoechst 33258.

The strain YHN1172 was created to allow tetracyline-repressible expression of *HSF1*. First, the native *HSF1* promoter from YHN963 was replaced with a tetracycline operator cassette containing the repressor binding site ($tetO_2$) and the TetR-VP16 tTA transactivator (Belli *et al.* 1998a,b). Then, the *TetR′-SSN6* repressor from pCM245 was integrated at the *leu2* locus by linearization with *Cla*I (Belli *et al.* 1998b), creating a wild-type *LEU2* allele. This dual repression system allows *HSF1* transcription to be strongly repressed by the addition of doxycycline to the growth media at a concentration of 20 μg/ml.

HSF1 was tagged with 13 tandem copies of the Myc epitope to enable efficient immunoprecipitation and detection of the Hsf1 protein. The 13Myc tagging cassette was amplified from the pFA6a-13Myc-TRP1 plasmid (Longtine *et al.* 1998) and integrated at the 3′ end of the *HSF1* open reading frame in strains YHN963 and YHN1114 to generate strains YHN1189 and YHN1201, respectively.

***In vitro* Tn7 transposition mutagenesis:** The Tn7 donor plasmid pNB3 was a generous gift from Nurjana Bachman and Jef Boeke. This plasmid contains a mini-Tn7 transposon marked with *LEU2*, as well as the *Escherichia coli* πAN7 origin and kanamycin resistance marker (Bachman *et al.* 2002). Target DNA was prepared from the diploid ρ^0 cir^0 strain YHN917. The absence of mitochondrial and 2μ plasmid DNA prevents the transposon library from being heavily biased by these abundant sequences, which can frequently yield false positives. The genomic DNA was isolated by a CsCl banding protocol and then sonicated to yield fragments averaging 3–5 kb. The *in vitro* transposition reaction was performed as previously described (Bachman *et al.* 2002).

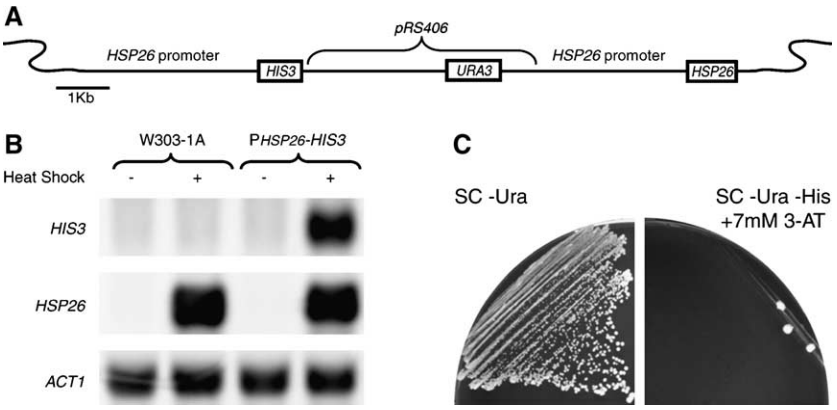

FIGURE 1.—Creation of P_{HSP26}-*HIS3* reporter strain. (A) The *HIS3* auxotrophic marker was fused to the *HSP26* promoter and integrated in tandem with the native *HSP26* gene. The targeted *HSP26* locus on chromosome II is diagrammed. (B) Total RNA was isolated from the wild-type strain (W303-1A) and the P_{HSP26}-*HIS3* reporter strain (YHN802) that were either maintained at 30° or heat-shocked at 37° for 15 min. Northern blots were probed with *HIS3* and *HSP26* probes, with *ACT1* as a loading control. (C) The wild-type P_{HSP26}-*HIS3* reporter strain (YHN802) was struck on nonselective SC Dex −Ura medium or selective SC Dex −Ura −His medium containing 7 mM 3-AT.

The reporter strain YHN802 was transformed with the mutagenized genomic DNA using the TRAFO protocol (GIETZ and WOODS 2002) and plated onto SC Dex lacking leucine and uracil. After 3 days of incubation at 30°, the mutagenized strains were replica plated onto 2% agar (to reduce background) and then onto SC Dex media lacking uracil, leucine, and histidine and supplemented with 30 mM 3-AT. These plates were incubated at 30° for an additional 3–5 days.

Candidate strains were crossed with the α-derivative of the reporter strain, YHN801, and sporulated. Dissected tetrads were tested for cosegregation of the Leu+ and His+ phenotypes. To excise the transposons as plasmids, genomic DNA from the candidate strains was digested with *Bam*HI (to rescue only the right arm of the transposon) or *Nde*I or *Sph*I (to rescue both arms of the transposon), self-ligated, and transformed into *E. coli*. Rescued plasmids were sequenced and the identity of the insertion site was determined using the BLAST tool on the Saccharomyces Genome Database website (CHERRY *et al.* 1998; BALL *et al.* 2000).

RNA preparation and Northern blot analysis: Total RNA was isolated from yeast cells using the hot acidic phenol method (AUSUBEL *et al.* 2004). RNA samples were fractionated on a 1% agarose denaturing gel containing formaldehyde and transferred by vacuum blotting onto ZetaProbe nylon membrane (Bio-Rad, Richmond, CA). Biotin-labeled DNA probes were generated by incorporation of Biotin-16-dUTP nucleotide (Roche, Indianapolis) into PCR products from cloned fragments of the *HSP12*, *HSP26*, *SSA3*, *SSA4*, *HSP82*, and *HSP104* ORFs. For hybridization and detection, we used the North2South chemiluminescent detection kit (Pierce, Rockford, IL) and a Fluorchem 8800 cooled CCD detection system (Alpha Innotech).

Phosphorylation analysis: Myc-tagged strains were grown to midlog phase and harvested by centrifugation, and the pellets were frozen in liquid N_2 and stored at −70° for subsequent analysis. Pellets were resuspended in FA lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) along with a yeast-specific protease inhibitor cocktail and a serine/threonine phosphatase inhibitor cocktail. The resuspended pellets were boiled for 10 min, and then the cells were disrupted with zirconia-silica beads in a Mini-Bead-Beater-8 (Biospec Products). The extracts were clarified by centrifugation and then incubated with agitation with 25 μl of anti-Myc(9E10) antibody conjugated agarose beads (Santa Cruz Biotechnology) for 90 min at room temperature. The beads were washed three times with FA lysis buffer plus protease inhibitors. Half of the beads were then incubated for 30 min at 37° with 25 units of calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA). The beads were then boiled in sample buffer and resolved on a 7.5% SDS-PAGE. Hsf1p was detected by Western blotting of the immunoprecipitates using an anti-Myc(9E10) antibody-HRP conjugate (Santa Cruz Biotechnology).

RESULTS

Isolation of mutations that derepress expression from the *HSP26* promoter: The Hsf1 protein is negatively regulated and is thereby kept in a low activity state prior to stress (SORGER and PELHAM 1988; WIEDERRECHT *et al.* 1988). In an effort to understand the mechanism underlying this negative regulation, we have designed a genetic selection based on the promoter of the *HSP26* gene fused to the *HIS3* reporter gene. The *HSP26* promoter was selected for the screen because of its low level of expression in cells grown at 30°, as well as its robust expression following heat shock (CHEN and PEDERSON 1993; AMOROS and ESTRUCH 2001). The *HIS3* gene encodes imidazolegylcerol-phosphate dehydratase (IGP dehydratase). The *HIS3* gene was selected as a reporter because one can compensate for any background expression with the IGP dehydratase competitive inhibitor 3-AT (HORECKA and SPRAGUE 2000). Approximately 3 kb of the *HSP26* promoter was cloned upstream of the *HIS3* open reading frame and integrated into W303-1A derivatives in tandem with the native *HSP26* gene at the *HSP26* genomic locus (Figure 1A).

To determine whether expression from the P_{HSP26}-*HIS3* fusion mirrors that of the native *HSP26* gene, we compared the levels of constitutive and heat-induced mRNAs in the parent strain (W303-1A) and a version of the parent strain that contains the P_{HSP26}-*HIS3* reporter (YHN802). The constitutive levels of *HSP26* are undetectable in both strains, but are rapidly induced to comparable levels following a 15-min heat shock at 37°. The constitutive levels of *HIS3* are undetectable in both strains, but the *HIS3* level increases in the reporter strain (YHN802) following a 15-min heat shock at 37°, indicating that the P_{HSP26}-*HIS3* gene fusion is being regulated in a manner similar to that of *HSP26* (Figure 1B). We also determined the ability of the reporter strain to grow on selective media lacking histidine in the presence

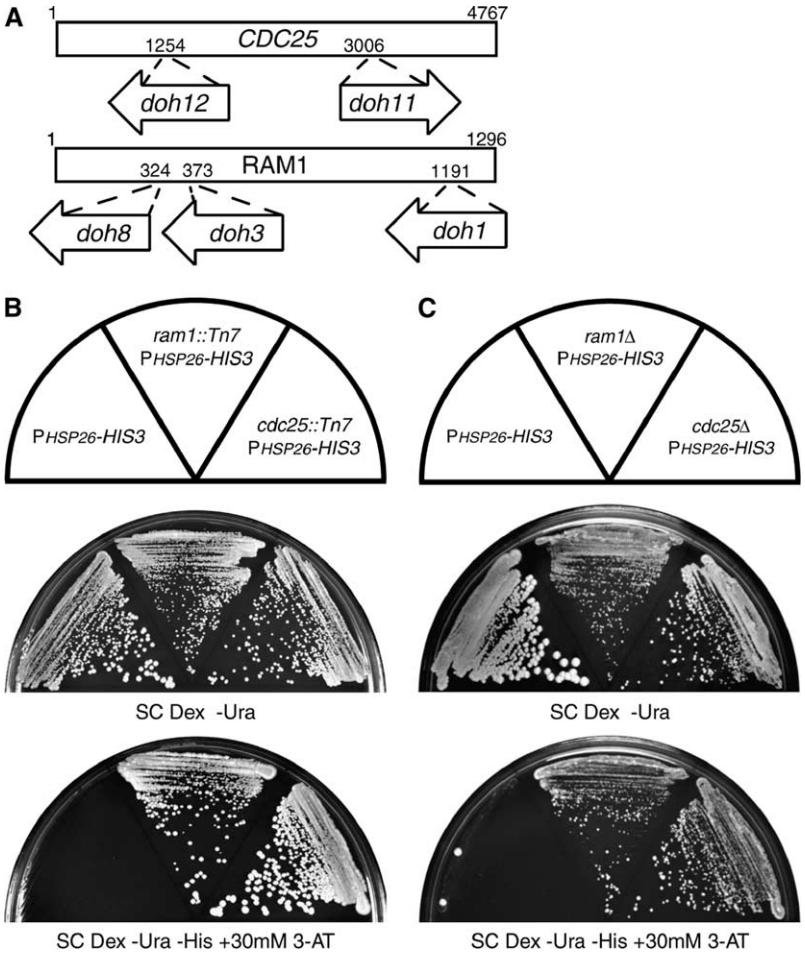

FIGURE 2.—Isolation of *doh* alleles. (A) Location of the transposition insertion alleles that disrupt the ORFs of *CDC25* and *RAM1* and exhibit a *doh* phenotype. (B) The wild-type reporter strain (YHN802), the reporter strain harboring the *doh8 ram1::Tn7* transposition allele (YHN941), and the reporter strain harboring the *doh12 cdc25::Tn7* transposition allele (YHN947) were struck on nonselective SC Dex −Ura medium or selective SC Dex −Ura −His medium containing 30 mM 3-AT. (C) The wild-type reporter strain (YHN802), the reporter strain with a *ram1*Δ allele (YHN1126), and the reporter strain with a *cdc25*Δ allele (YHN1128) were struck on nonselective SC Dex −Ura medium or selective SC Dex −Ura −His medium containing 30 mM 3-AT.

of varying concentrations of 3-AT. The reporter strain (YHN802) is unable to grow on media in the presence of 7 mM 3-AT (Figure 1C), confirming the low level of constitutive expression from the P_{HSP26}-*HIS3* promoter fusion. We used this reporter strain to identify mutants involved in the stress response that are able to grow on selective media in the absence of stress, thereby generating **d**erepression **o**f ***H****SP* expression (*doh*) strains that exhibit increased constitutive *HSP26* expression.

Mutations were generated using a Tn7-mediated mutagenesis protocol (BACHMAN *et al.* 2002). This approach uses a Tn7 transposase that lacks site selection specificity and therefore randomly integrates a mini-Tn7 transposon into sheared genomic *S. cerevisiae* DNA (STELLWAGEN and CRAIG 1997; BIERY *et al.* 2000). In addition to a *LEU2* selectable marker, the mini-Tn7 transposon contains *E. coli* sequences that facilitate direct rescue and identification of the integration site. Sheared yeast genomic DNA was combined *in vitro* with the mutant Tn7 transposase and the mini-Tn7 transposon to create a library of mutagenized genomic DNA that could be transformed into yeast.

Approximately 80,000 transformants of the P_{HSP26}-*HIS3* reporter strain (YHN802) were screened. After replica plating, we found 13 strains that grew on stringent selective media containing 30 mM 3-AT, greater than four times the minimal inhibitory concentration. As described below, five of these candidates (eventually) proved to have a *doh* phenotype. Two of the five strains demonstrated 2:2 meiotic cosegregation of the Leu+ transposon and His+ growth phenotypes following a backcross to the isogenic wild-type reporter strain. This suggests that a single causative chromosomal lesion was responsible for upregulated expression from the *HSP26* promoter. The mini-Tn7 was isolated from strains containing the *doh11* and *doh12* alleles (YHN946 and YHN947). Sequencing revealed independent integrations in the Ras guanine nucleotide exchange factor *CDC25* (Figure 2A). The reporter growth phenotype of the *cdc25* insertions could be suppressed by the presence of a plasmid-borne copy of *CDC25* (data not shown). Cdc25p catalyzes the exchange of guanine nucleotides on the two small GTPases, Ras1p and Ras2p, resulting in increased Ras signaling (BROEK *et al.* 1987; ROBINSON *et al.* 1987). Inactivation of Cdc25p would reduce the downstream signaling functions of the Ras proteins.

Three of the 13 potential *doh* strains were sterile. Because two of the *doh* candidates affected the Ras/cAMP pathway, we thought it might be possible that the sterile *doh* candidates were at the locus of the farnesyl-

transferase β-subunit *RAM1*. The Ram1 protein is responsible for conferring substrate specificity to the Ram1/2 farnesyltransferase holoenzyme. The Ram1/2 holoenzyme adds a 15-carbon farnesyl lipid moiety to the C-terminal CaaX box motif of substrate proteins, including Ras and **a**-factor mating pheromone, enabling them to localize to the membrane and elicit signaling functions (He *et al.* 1991). The inability to synthesize mature **a**-factor could explain the sterility of these potential *doh* candidates, as they were in a *MAT***a** strain. Isolation and sequencing of the mini-Tn7 transposons from strains carrying the *doh1, doh3*, and *doh8* alleles (YHN932, YHN934, and YHN941) showed that they contained independent insertions in *RAM1* (Figure 2A). Reintegration of the rescued mini-Tn7 plasmid into a naive reporter strain at the *RAM1* locus recapitulated the His+ growth phenotype (data not shown), supporting the idea that these disruptions in the *RAM1* ORF are responsible for upregulation of the *HSP26* promoter.

The five independent mini-Tn7 insertions in *CDC25* and *RAM1* upregulate expression from the *HSP26* promoter, but it is unclear whether these insertions are hypomorphic or complete loss-of-function alleles. To test this, we deleted the complete open reading frames of *CDC25* or *RAM1* by PCR-generated allele replacement. Previous studies had indicated that *RAM1* is not an essential gene, but that *CDC25* appeared to be essential, at least in the SP1 and BY4730 strain backgrounds (Broek *et al.* 1987; He *et al.* 1991; Giaever *et al.* 2002). In our W303-1A strain background, we were able to obtain viable haploid strains of both null mutants (YHN1005 and YHN1007). Recent studies have also found that *cdc25*Δ is viable in the W303-1A strain background, presumably because of the presence of a functional copy of *SDC25*, which has homology to *CDC25* (Folch-Mallol *et al.* 2004). We crossed the *ram1*Δ and *cdc25*Δ alleles into the wild-type reporter strain (YHN802) and found that the *cdc25*Δ and *ram1*Δ alleles behaved similarly to the *cdc25::Tn7* and *ram1::Tn7* alleles with respect to activation of the P_{HSP26}-*HIS3* reporter (Figure 2C). These results strongly suggest that the *doh* phenotype observed in the transposon insertion alleles is due to partial or complete loss of Cdc25p or Ram1p function.

Upregulation of the *HSP26* promoter is maintained in the absence of *MSN2/4*: It is well established that the Ras/cAMP pathway regulates the *HSP26* promoter by modulating the nuclear localization of the partially redundant Msn2 and Msn4 transcription factors (Gorner *et al.* 1998; Smith *et al.* 1998). To determine whether the effect we observe is due to enhanced Msn2p/4p activity at the *HSP26* promoter, we generated an *msn2*Δ *msn4*Δ version of the reporter strain called YHN966. Similar to the wild-type reporter strain (YHN802), this new reporter strain had undetectable levels of *HIS3* or *HSP26* mRNA at 30° and showed the same inability to grow on 3-AT plates (data not shown). The *cdc25*Δ and *ram1*Δ alleles were crossed into the *msn2/4*Δ reporter strain and resulted in increased expression from the *HSP26* promoter, as determined by growth of the reporter strain on selective medium (Figure 3A). In addition, we found that the *cdc25*Δ and *ram1*Δ alleles upregulated the expression of *HSP26* (Figure 3B). These results suggest that the *doh* phenotype is independent of Msn2p/4p.

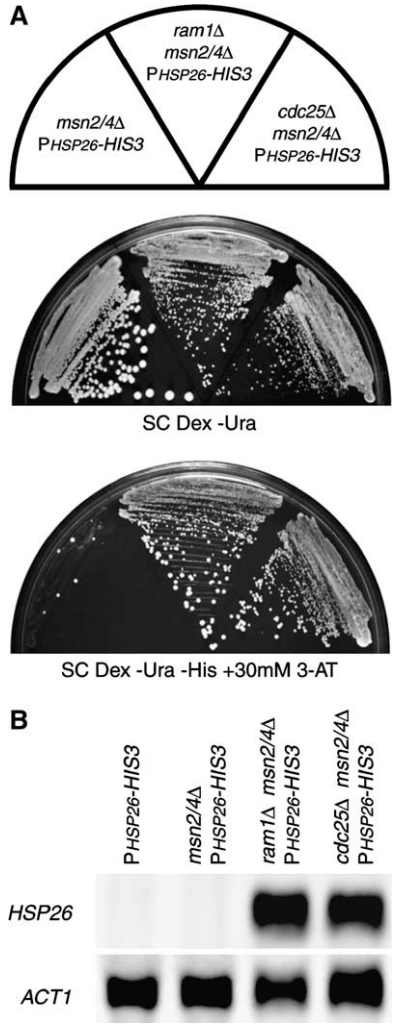

Figure 3.—*MSN2* and *MSN4* are not required for *doh* phenotypes. (A) The *msn2/4*Δ reporter strain (YHN966), the *msn2/4*Δ reporter strain with the *ram1*Δ allele (YHN1077), and the *msn2/4*Δ reporter strain with the *cdc25*Δ allele (YHN1078) were struck on nonselective SC Dex −Ura medium or selective SC Dex −Ura −His medium containing 30 mm 3-AT. (B) A Northern blot of total RNA isolated from the indicated strains grown at 30° was probed with a *HSP26*-specific probe, with *ACT1* as a loading control.

High-level expression of *PDE2* or *MSI1* activates expression from the *HSP26* promoter in an Msn2p/4p-independent manner: In a complementary approach to the mini-Tn7 disruption, we searched for genes whose overexpression led to an increase in activity from the P_{HSP26}-*HIS3* reporter in the absence of Msn2p/4p. We used a cDNA library whose inserts are driven by the *GAL1* promoter (Elledge *et al.* 1991; Ramer *et al.* 1992)

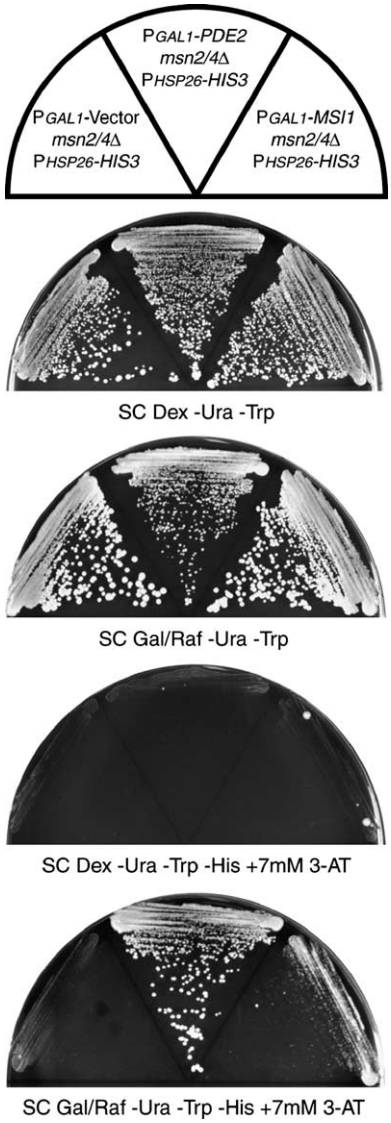

FIGURE 4.—Overexpression of *PDE2* or *MSI1* activates the P*HSP26-HIS3* reporter. The *msn2/4*Δ reporter strain with an empty vector (YHN1090), the *msn2/4*Δ reporter strain with the P*GAL1-PDE2* plasmid (YHN1091), and the *msn2/4*Δ reporter strain with the P*GAL1-MSI1* plasmid (YHN1092) were struck on nonselective SC Dex −Ura −Trp medium, nonselective SC Gal/Raf −Ura −Trp medium, selective SC Dex −Ura −Trp −His medium containing 7 mM 3-AT, and selective SC Gal/Raf −Ura −Trp −His medium containing 7 mM 3-AT.

and transformed the library into the *msn2/4*Δ reporter strain (YHN966). We isolated four clones whose *doh* phenotype was dependent on galactose as the carbon source. Retransformation of the plasmids into the YHN966 reporter strain confirmed the *doh* growth phenotype. Three of the clones were independent isolates of *PDE2* and gave a robust phenotype, while the sole clone of *MSI1* gave a weaker phenotype (Figure 4). *PDE2* encodes the high-affinity cAMP phosphodiesterase, which antagonizes the Ras/cAMP signaling pathway by directly degrading cAMP (Sass *et al.* 1986). *MSI1* was originally identified as a multicopy suppressor of *IRA1*, the GTPase-activating protein that negatively regulates Ras signaling. Overexpression of *MSI1* can suppress an activated Ras/cAMP pathway and decrease levels of cAMP through a complex mechanism that is not yet fully understood (Ruggieri *et al.* 1989; Johnston *et al.* 2001). These results support the idea that the *doh* phenotype is Msn2p/4p independent and is due to alterations in the Ras/cAMP pathway.

Deletion of PKA induces expression from the *HSP26* and *HSP12* promoters: We have isolated several factors that repress Ras/cAMP activity and result in upregulation of expression from the *HSP26* promoter in an Msn2p/4p-independent manner. In yeast, the primary effector of the Ras/cAMP pathway is PKA (Broach 1991; Thevelein and de Winde 1999). In *S. cerevisiae*, PKA consists of the inhibitory regulatory subunit Bcy1p, which responds to cAMP levels, and three partially redundant catalytic subunits, Tpk1p, Tpk2p, and Tpk3p. To test the role of PKA kinase activity in the regulation of *HSP26*, we deleted each of the three redundant catalytic subunits of yeast PKA in an *msn2/4*Δ reporter strain. Deletion of individual or pairs of *TPK* subunits (strains YHN1082–1087; Table 1) had no effect on the ability of the reporter strain to grow on selective media (data not shown). Deletion of all three *TPK* subunits is lethal in most strains, including ours, but a concomitant deletion of *MSN2/4* can suppress this lethality (Smith *et al.* 1998). We deleted all three *TPK* subunits in the *msn2/4*Δ reporter strain, creating strain YHN1116. Deletion of the PKA catalytic subunits caused an increase in expression of the reporter, as seen by the ability of the strain to grow on selective medium (Figure 5). These results suggest that each of the Tpk catalytic subunits is redundant for the *doh* phenotype. PKA is known to repress the activity of Msn2p/4p; however, our results suggest that the *doh* phenotype must represent a different mechanism of PKA regulatory activity.

Our results show that loss of PKA activity increases the expression of the P_{HSP26}-*HIS3* reporter. To confirm that *HSP26* expression was increased in the *msn2/4*Δ *tpk1/2/3*Δ strain, we examined levels of *HSP26* by Northern blot analysis. In comparison to the *TPK1/2/3* strain, the *tpk1/2/3*Δ strain had dramatically increased expression for *HSP26* (Figure 6). To determine whether this phenotype was specific to the *HSP26* promoter, we also examined the effect of deletion of PKA on the expression of several different heat-shock promoters in an *msn2/4*Δ background. *HSP12* showed the same dramatic increase as *HSP26*. However, expression of *HSP104* was increased only very slightly, while expression of *SSA3, SSA4*, and *HSP82* was unaffected (Figure 6). In contrast to PKA regulation of Msn2p/4p, which involves subcellular localization and therefore does not differentiate between different STRE-containing promoters (Gorner *et al.* 1998; Smith *et al.* 1998), PKA regulation of this Msn2p/4p-independent mechanism shows promoter selectivity.

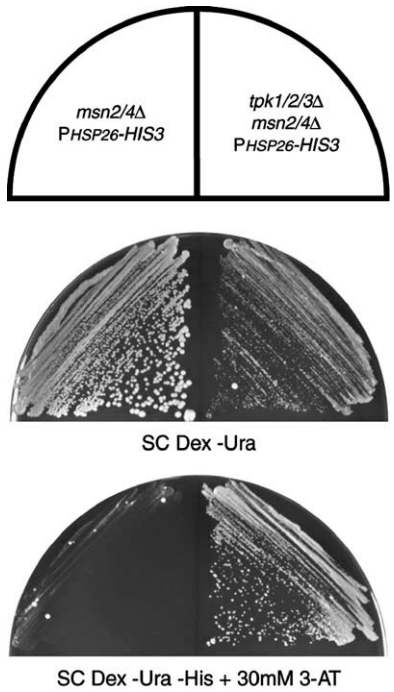

FIGURE 5.—Loss of PKA activates the P_{HSP26}-*HIS3* reporter. The *msn2/4*Δ reporter strain (YHN966) and the *msn2/4*Δ reporter strain with the *tpk1/2/3*Δ alleles (YHN1116) were struck on nonselective SC Dex −Ura medium or selective SC Dex −Ura −His medium containing 30 mM 3-AT.

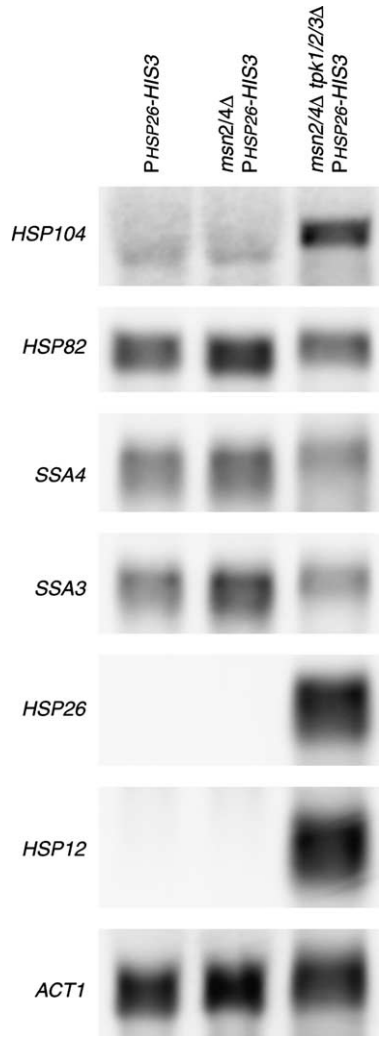

FIGURE 6.—Loss of PKA specifically derepresses small heat-shock gene expression. (A) Total RNA was isolated from the wild-type reporter strain (YHN802), the *msn2/4*Δ reporter strain (YHN966), and the *msn2/4*Δ reporter strain with the *tpk1/2/3*Δ alleles (YHN1116). The Northern blot was probed with *HSP12*-, *HSP26*-, *SSA3*-, *SSA4*-, *HSP82*-, or *HSP104*-specific probes, with *ACT1* as a loading control.

Induction of *HSP26* and *HSP12* in strains with compromised PKA function requires *HSF1* expression: The heat-inducible expression of *HSP12* and *HSP26* is known to be dependent on both Msn2p/4p and Hsf1p (BOY-MARCOTTE *et al.* 1999; AMOROS and ESTRUCH 2001; HAHN *et al.* 2004). We have shown that deletion of PKA increases expression of *HSP12* and *HSP26* independent of Msn2p/4p. To determine whether the PKA regulation of these genes is dependent on Hsf1p activity, we needed to construct a conditional allele of *HSF1*. Because *HSF1* is required for viability under all conditions, we used a regulatable expression system developed to study essential genes (BELLI *et al.* 1998a,b). We constructed a conditional promoter-replacement allele of *HSF1* using a tetracycline-regulatable dual expression system that represses *HSF1* transcription in the presence of doxycycline. To test the conditional allele of *HSF1*, it was integrated into an *msn2/4*Δ strain background to generate strain YHN1172. The growth of this strain is strongly inhibited on plates containing doxycycline, consistent with the essential role of *HSF1* (data not shown). In addition, the presence of doxycycline inhibited the heat-shock induction of HSE-containing genes, including *HSP104*, *SSA4*, and *HSP26* (Figure 7A) as well as *HSP82*, *SSA3*, and *HSP12* (data not shown). This supports the idea that Hsf1p levels had been depleted in the presence of doxycycline. Incubation of a *tpk1/2/3*Δ *msn2/4*Δ (YHN1114) strain with the wild-type *HSF1* promoter did not inhibit *HSP12* or *HSP26* expression (Figure 7B), indicating that doxycycline alone does not affect *HSP* expression.

To test the Hsf1p dependence of the *doh* phenotype, the *HSF1* conditional allele was created in an *msn2/4*Δ *tpk1/2/3*Δ strain background to generate strain YHN1173. The growth of this strain was also strongly inhibited by doxycycline (data not shown). Because of the *tpk1/2/3*Δ, YHN1173 has the expected high levels of *HSP26* and *HSP12* in the absence of doxycycline (Figure 7C). Incubation of YHN1173 with doxycycline resulted in a dramatic reduction of the *HSP26* and *HSP12* transcripts (Figure 7C). These results indicate that activation of small *HSP* expression by the absence of PKA activity requires *HSF1* expression.

Deletion of PKA induces hyperphosphorylation of Hsf1p: Increases in Hsf1p transcriptional activity from either heat shock or glucose starvation are associated

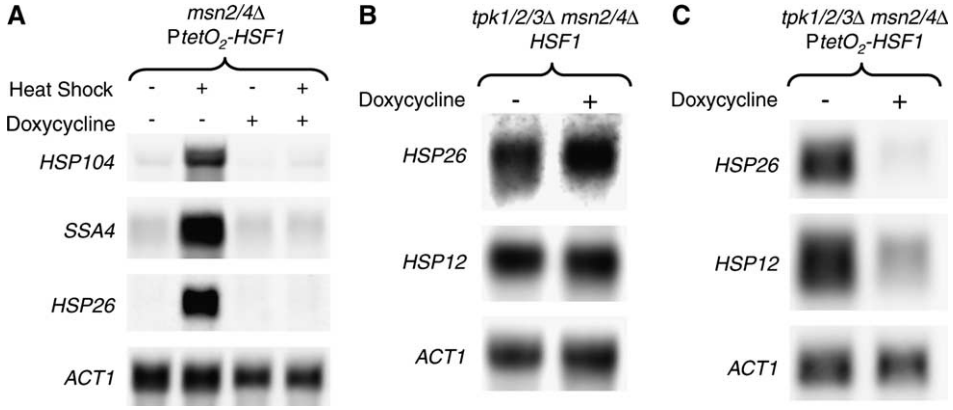

FIGURE 7.—*HSF1* is required for small *HSP* expression in strains lacking PKA activity. A tetracycline-regulatable dual expression system was used to create a conditional allele of *HSF1* in the *msn2/4*Δ and the *msn2/4*Δ *tpk1/2/3*Δ strain backgrounds, creating strains YHN1172 and YHN1173, respectively. (A) Total RNA was isolated from the tetracycline-repressible *HSF1* strain (YHN1172) that had been grown for 12 hr in minimal medium or minimal medium supplemented with 20 μg/ml of doxycycline (a tetracycline analog) and then either maintained at 30° or heat-shocked at 37° for 15 min. Northern blots were probed with *HSP104*, *SSA4*, and *HSP26* probes, with *ACT1* as a loading control. (B) Total RNA was isolated from an *msn2/4*Δ *tpk1/2/3*Δ strain with the wild-type *HSF1* promoter (YHN1114) that had been grown for 12 hr at 30° in YPD or YPD supplemented with 20 μg/ml of doxycycline. The Northern blots were probed with *HSP12*- and *HSP26*-specific probes, with *ACT1* as a loading control. (C) Total RNA was isolated from the *msn2/4*Δ *tpk1/2/3*Δ strain carrying the tetracycline-repressible allele of *HSF1* (YHN1173) that had been grown for 12 hr at 30° in minimal media or minimal media supplemented with 20 μg/ml of doxycycline. The Northern blots were probed with *HSP12*- and *HSP26*-specific probes, with *ACT1* as a loading control.

with hyperphosphorylation of the protein (SORGER and PELHAM 1988; SORGER 1990; JAKOBSEN and PELHAM 1991; HØJ and JAKOBSEN 1994; HAHN and THIELE 2004; HASHIKAWA and SAKURAI 2004). Hyperphosphorylation can be detected as a decrease in the electrophoretic mobility of Hsf1p and confirmed by loss of that altered mobility upon treatment with phosphatase. To determine whether deletion of PKA alters Hsf1p phosphorylation, we analyzed the effect of the *tpk1/2/3*Δ on the electrophoretic mobility of Hsf1p with and without phosphatase treatment. To facilitate this, we tagged the Hsf1p allele at the C terminus with 13 copies of the Myc epitope and generated strains with the genotypes *msn2/4*Δ *HSF1-13Myc* (YHN1189) and *msn2/4*Δ *tpk1/2/3*Δ *HSF1-13Myc* (YHN1201). The 13Myc tag does not disrupt Hsf1p function, as crossing the *HSF1-13Myc* allele into the P_{HSP26}-*HIS3* reporter background in the presence and absence of the *TPK* catalytic subunits gives the same phenotype as the wild-type *HSF1* allele with respect to growth on selective media (data not shown).

Myc-tagged Hsf1p was immunoprecipitated from strains YHN1189 and YHN1201 grown at 30° and compared to Myc-tagged Hsf1p immunoprecipitated from YHN1189 that had been heat-shocked at 40° for 30 min. As seen before, heat shock decreases the electrophoretic mobility of Hsf1p (SORGER and PELHAM 1988; SORGER 1990; HAHN and THIELE 2004; HASHIKAWA and SAKURAI 2004). However, deletion of the Tpk catalytic subunits also decreased the electrophoretic mobility of Hsf1p (Figure 8). To confirm that this altered mobility was due to an increase in phosphorylation, we treated the immunoprecipitated samples with phosphatase. The increase in electrophoretic mobility upon phosphatase treatment is consistent with the idea that PKA deletion, like heat shock, increases the phosphorylation state of Hsf1p. Further experiments will be needed to determine whether the increase in phosphorylation is responsible for the increase in Hsf1p's transcriptional activity and, if so, what kinase is responsible for the phosphorylation.

DISCUSSION

All cells require the ability to carefully coordinate their gene expression to compensate for environmental perturbations. It is just as important to be able to repress the expression of HSPs in the absence of stress as it is to upregulate their expression in the presence of heat or other stresses. We have developed a genetic selection to identify genes that when mutated or overexpressed can increase constitutive (*i.e.*, nonstressed) expression from a heat-shock promoter. In this study, we used the *HSP26* promoter and found that the Ras signaling pathway, specifically the cAMP-dependent protein kinase

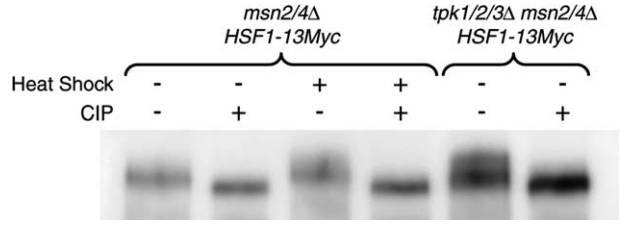

FIGURE 8.—Deletion of PKA increases Hsf1p phosphorylation. The phosphorylation state of Myc-tagged Hsf1 was analyzed in strains YHN1172 (*HSF1-13Myc::TRP1 msn2/4*Δ) and YHN1173 (*HSF1-13Myc::TRP1 msn2/4*Δ *tpk1/2/3*Δ). Yeast expressing Myc-tagged Hsf1 were grown at 30° (heat shock −) or heat-shocked at 40° for 30 min (heat shock +). Hsf1-13Myc was enriched by immunoprecipitation from cell extracts and split into two pools. These samples were incubated with and without calf intestinal alkaline phosphatase (CIP +/−) and resolved on a 7.5% SDS-PAGE, and Hsf1-13Myc was detected by Western blotting with Myc antibodies.

PKA, represses heat-shock gene expression through a new mechanism that is dependent on Hsf1p.

Previous studies of regulation of HSP expression had led to the suggestion that Ras signaling via PKA did not affect Hsf1p activity (Engelberg *et al.* 1994). This apparent misconception occurred because activated Ras alleles do not appear to further repress Hsf1p's already low level of constitutive activity. Additional studies had shown that the cAMP/PKA signaling pathway could regulate the activity of two other heat-inducible transcription factors, Msn2p and Msn4p. Msn2p and Msn4p bind to STRE sequences that are found in some, but not all, heat-shock promoters. PKA can regulate the cellular localization and thus activity of Msn2p (Gorner *et al.* 1998; Smith *et al.* 1998). This solidified the opinion that PKA regulation of heat-shock expression was not occurring through Hsf1p, but rather through Msn2p/4p. However, recent studies suggest that the regulation of heat-shock genes through the Ras/cAMP signaling pathway cannot be solely attributed to the Msn2/4 proteins (Boy-Marcotte *et al.* 1999; Grably *et al.* 2002; Aranda and del Olmo 2003; Versele *et al.* 2004). In support of these studies, we have now shown specifically that inactivation of PKA in an *msn2/4*Δ background can still increase heat-shock gene expression. Furthermore, we have used conditional expression of *HSF1* through a P_{tetO_2}-*HSF1* allele to show that the increased heat-shock gene expression in the absence of PKA is dependent on Hsf1p. These results represent a major change in our understanding of how PKA signaling influences the heat-shock response and heat-shock protein expression.

Hsf1p is required for the expression of only a subset of heat-shock genes in the absence of PKA. Deletion of PKA dramatically increases Hsf1-dependent expression at *HSP12* and *HSP26*, but has only a limited effect on *HSP104* and no detectable effect on *SSA3*, *SSA4*, or *HSP82*. Comparison of the promoter sequences of these six heat-shock genes does not immediately suggest a reason why *HSP12* and *HSP26* are regulated by PKA in a manner different from that of the other four genes. In addition, comparison of the constitutive HSE occupancies of all six genes (Hahn *et al.* 2004) does not show a correlation with PKA sensitivity. Because of the promoter selectivity, the influence of PKA on Hsf1p activity is unlikely to affect a global aspect of Hsf1p, such as its ability to bind HSEs or increase its transcriptional activity, but instead must affect a particular promoter-specific aspect of Hsf1p activity.

One possible explanation for why regulation of *HSP12* and *HSP26* is different from that of the other heat-shock genes is based on their functions as heat-shock proteins. The Hsp12 and Hsp26 proteins are part of a group called the small heat-shock proteins (sHsp's). Within the protein chaperone network, the sHsp's form a reservoir for nonnative refoldable proteins. In addition, they have specific roles in apoptosis, cytoskeletal organization, and formation of the eye lens (Haslbeck 2002). In contrast, the large heat-shock proteins, including the *SSA* family, *HSP82*, and *HSP104*, assist in the refolding of nonnative proteins and the disruption of aggregated proteins (Ellis 1990; Buchner 1996). Given the unique role of the small heat-shock proteins compared to their larger colleagues, it is not surprising that their expression is coregulated to some extent.

In addition to increasing the Hsf1p-dependent expression of some heat-shock genes, deletion of PKA also causes an increase in Hsf1p phosphorylation (Figure 8). Further analyses are needed to determine what sites are phosphorylated. From the crude SDS-PAGE analysis, it appears that the hyperphosphorylation pattern of Hsf1p differs whether it is activated by PKA deletion or heat shock. Given that Hsf1p phosphorylation increases upon deletion of PKA, it is unlikely that Hsf1p is a direct target of PKA phosphorylation. Instead, these data support a model where PKA represses an activating kinase under constitutive conditions.

It is also possible that the hyperphosphorylation of Hsf1p seen in the absence of PKA is not directly related to the increase in Hsf1p-dependent transcription. If this were the case, then a PKA deletion could affect Hsf1p transcriptional activity by activating an ancillary transcription factor that is normally repressed in the presence of PKA. During the preparation of this manuscript, a new publication reported that Srb9p, a component of the general RNA polymerase II transcription apparatus, is a direct target of PKA. Inactivation of PKA or deletion of *SRB9* caused an increase in expression at several promoters, including *HSP12* and *HSP26* (Chang *et al.* 2004). Expression at other heat-shock promoters was not studied, nor was the contribution of Hsf1p to this phenotype. We have deleted the *SRB9* gene in YHN966, the *msn2/4*Δ reporter strain, and found that it did not exhibit a strong growth phenotype on selective media (data not shown). Although this suggests that Srb9p is independent of the *doh* phenotype, further studies will be needed to confirm this hypothesis.

Recently, Hahn and Thiele (2004) have shown that the yeast AMPK homolog, Snf1p, functions as an activator of Hsf1p activity under glucose-limiting conditions. These conditions also decrease the production of cAMP and hence reduce the activity of PKA, which we have proposed to be a repressor of Hsf1p activity under normal growth conditions. Despite the complementary roles of Snf1p and PKA as activator and repressor under opposing conditions, their influence on heat-shock gene expression does not totally overlap. For example, Snf1p and PKA both influence Hsf1-dependent expression of *HSP26*, but only Snf1p can influence *SSA3* expression (Hahn and Thiele 2004). In addition, the Snf1 kinase appears to modify Hsf1p directly, while PKA is unlikely to directly phosphorylate Hsf1p *in vivo*. Therefore, these two glucose-regulated kinases can each affect heat-shock gene expression via Hsf1p in a unique

way. Further studies on the identification of the sites of phosphorylation, as well as the kinase(s) responsible for the PKA-dependent modifications, will help us further resolve this new twist in the complex regulation of the activity of the heat-shock transcription factor.

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