Stress induction of *HSP30*, the plasma membrane heat shock protein gene of *Saccharomyces cerevisiae*, appears not to use known stress-regulated transcription factors

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More than one transcription factor contributes to the *Saccharomyces cerevisiae* heat shock response. Many genes are induced through the activation of heat shock factor (Hsf1), a protein that is constitutively bound to heat shock promoter elements (HSEs). Other genes are switched on by Msn2/Msn4-dependent activation of a quite separate promoter element (the stress response element, STRE). While Hsf1 directs gene activation mainly in response to heat stress, STRE-directed transcription is stimulated not only by heat but also by several other stresses, starvation included. *HSP30*, encoding the plasma membrane heat shock protein, is shown in this study to be activated by several stresses. It is most strongly induced with heat shock, ethanol and weak organic acid exposure. The *HSP30* promoter has no good agreement to the HSE consensus and its stress activation is unaffected by a mutation (*hsf1-m3*) that causes defective heat shock activation of Hsf1-dependent genes. Activation of *HSP30* occurs with some, but not all, STRE-inducing stresses and is largely unaffected either by loss of the Msn2/Msn4 transcription factors or with mutation of all STRE-like consensus sequences of the promoter. Stress activation of *HSP30* appears therefore to involve as yet unidentified components of the yeast transcriptional apparatus.

Keywords: yeast, heat shock gene, stress activation, stress response

INTRODUCTION

Two quite distinct promoter sequences can direct heat shock activation of a *Saccharomyces cerevisiae* gene. These are the Heat Shock Element (HSE) and the general Stress Responsive Element (STRE) (reviewed by Ruis & Schüller, 1995; Mager & De Kruijff, 1995; Piper, 1997; Morano *et al.*, 1998). HSEs are the binding sites for heat shock factor (Hsf1), a transcriptional transactivator that directs gene induction in response to heat stress and chemical agents such as arsenite that cause the intracellular accumulation of abnormal proteins. Recently it was demonstrated that it is a depletion of the 'free'

Hsp70 chaperone pool in the cells of higher eukaryotes that acts as the signal for Hsf1 activation, such depletion occurring as Hsp70 associates with the partially heat-denatured proteins in heat shocked cells (Morimoto *et al.*, 1996).

Hsf1-independent but stress-activated yeast promoter elements were originally identified in the *CTT1* and *DDR2* genes (Belazzi *et al.*, 1991; Marchler *et al.*, 1993; Kobayashi & McEntee, 1993; Schüller *et al.*, 1994). Activation of these STRE sequences (consensus AGGGG or CCCCT) requires the Msn2 and Msn4 trans-activators, proteins that bind STRE sequences *in vitro* (Martinez-Pastor *et al.*, 1996). STREs, in contrast to HSEs, are activated not just by heat shock but also by a diverse range of other stress conditions including osmo-stress, oxidative stress, nitrogen starvation and exposure to weak organic acids, low external pH or ethanol (Marchler *et al.*, 1993; Schüller *et al.*, 1994; Ruis & Schüller, 1995; Martinez-Pastor *et al.*, 1996). STREs

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Abbreviations: HSE, heat shock element; PKA, protein kinase A; PDSE, post-diauxic shift element; STRE, stress response element.

also differ from HSEs in that their basal activity and heat induction are very strongly influenced by the levels of protein kinase A (PKA) activity (Marchler *et al.*, 1993).

HSE-mediated and STRE-mediated gene inductions probably serve different functions. Hsf1/HSE-mediated expression appears to primarily direct gene expression that increases the capacity for *growth* at high temperatures (37–39 °C), not resistance to more severe stress (Ruis & Schüller, 1995). Thus the *hsf1-m3* mutation, which causes loss of the heat-inducibility of HSE sequences and loss of high temperature growth, remarkably has no effect on the acquisition of tolerance to extreme temperatures (50 °C). In addition, tolerance to extreme temperatures can be induced by treatments which cause no HSE induction, such as osmotic dehydration or exposure to weak organic acids (Piper, 1993). In contrast, STRE-mediated transcription has all the properties of a system, activated by diverse types of sublethal stress, whereby cells are reprogrammed for the *survival* of more severe stress, such as desiccation, prolonged starvation, freezing, etc. (Ruis & Schüller, 1995). Consistent with this the *msn2 msn4* mutant that is defective in the transcription activators needed for STRE action exhibits hypersensitivity to several stresses (Martinez-Pastor *et al.*, 1996). There appears to be a good correlation between tolerances to these extreme stresses in *S. cerevisiae* and the levels of STRE-regulated gene products (Ruis & Schüller, 1995; Mager & De Kruijff, 1995; Piper, 1997). In general, the expression of STRE-regulated genes is minimal in cells in rapid glucose growth, being activated under growth-limiting conditions through a number of signal transduction pathways. The high-osmolarity-activated HOG (high osmolarity glycerol) pathway is one stress sensing pathway that influences STRE activity (Schüller *et al.*, 1994). Another very important influence is PKA activity, this being inversely related to the levels of several stress tolerances in yeast (Belazzi *et al.*, 1991; Marchler *et al.*, 1993; Schüller *et al.*, 1994) including thermotolerance (Piper, 1993). A lowering of PKA activity slows growth, increases basal STRE activity and increases the responsiveness of STRE sequences to heat stress (Marchler *et al.*, 1993).

It appears that an individual yeast heat shock gene can be under HSE control, under STRE control, or controlled by both the HSE and STRE. In addition it may be subject to other influences (e.g. *UBI4* which has an element causing higher expression levels in respiratory as compared to fermentative cultures; Watt & Piper, 1997). Studies on different yeast heat shock gene promoters have indicated that the heat induction of a few is regulated by the HSE alone. These promoters include *SSA4*, encoding a form of Hsp70 (Boorstein & Craig, 1990a), and *HSP82*, encoding the heat-inducible Hsp90 (McDaniel *et al.*, 1989). Others are under the control of STRE (not HSE) sequences; sometimes multiple synergistically acting STREs as in *HSP12* (Varela *et al.*, 1995). Heat shock genes of yet a third category (e.g. *SSA3*; Boorstein & Craig, 1990b) seem to be regulated by both the HSE- and PKA-responsive

sequences. In these latter promoters HSEs and STREs may operate independently although *SSA3* contains a STRE-like element [the post-diauxic shift element (PDSE)] that seems to be dependent on a downstream HSE for its activity (Boorstein & Craig, 1990b). Whether a heat shock gene is under HSE or STRE control in turn influences its stress induction pattern. Thus STRE-regulated genes (e.g. *HSP12*) are induced by several stresses and low PKA activity. In contrast the HSE-regulated *SSA4* appears to be induced primarily by heat shock alone. However, not all STRE-like promoter sequences are identical, since the different STREs controlling the genes for subunits of the trehalose synthase complex do not all direct the same patterns of regulation (Winderickx *et al.*, 1996).

In this study we attempted to identify whether *HSP30*, the gene encoding the integral plasma membrane heat shock protein of *S. cerevisiae* (Panaretou & Piper, 1992; Régnacq & Boucherie, 1992; Piper *et al.*, 1994, 1997), is under HSE or STRE control. To our surprise we obtained data that indicate *HSP30* is not under the control of either of these elements, suggesting that a third system for the activation of genes by heat shock must exist in yeast.

METHODS

Yeast strains, yeast culture and stress exposure. The yeast strains used for this study are listed in Table 1. Yeast growth, media recipes, DNA transformation procedures, stress exposure protocols, RNA extraction and the probing of Northern blots were exactly as reported previously (Watt & Piper, 1997; Piper *et al.*, 1997, 1998). The starvation of cultures for nitrogen was according to the procedure of Marchler *et al.* (1993).

Site-directed mutagenesis of *HSP30* promoter sequences.

Plasmid YIp30 was constructed by ligation of the 2.4 kb *NdeI* fragment of pUC19HSP30 (Régnacq & Boucherie, 1992) into *NdeI*-cleaved YIpac204 (Geitz & Sugino, 1988). YIp30 contains the entire *HSP30* ORF and 963 bp of upstream DNA, together with a unique *EcoRV* site within the *TRP1* gene of the vector. Mutagenesis of the *HSP30* promoter of YIp30 employed the oligonucleotide primers shown in Table 2, each consensus sequence being altered in turn by repeated use of the Stratagene QuickChange site-directed mutagenesis kit. After mutant promoter sequences had been confirmed by DNA sequence analysis, the YIp30-derived plasmids bearing multiple promoter mutations were digested (100 ng) with *EcoRV* then used to transform strain KT3 (Table 1) to tryptophan prototrophy. Correct integration of promoter mutant *hsp30* alleles at the *trp1* locus was confirmed by Southern blotting, *TRP1*⁺ transformants that were genomic insertions of single copies of the plasmid being used for further studies. KT3 transformed with YIp30 derivatives are designated by a suffix to the strain name (e.g. KT3-YIp30 is KT3 transformed with unmutated YIp30; KT3-STRE1(M) is KT3 transformed with YIp30 containing the STRE1(M) mutation in Table 2; KT3-STRE1(M)-PDSE1–6(M) is KT3 transformed with YIp30 containing all the mutations in Table 2; while KT3-PDSE1–6(M) has all six of the PDSE consensus sequences mutated as in Table 2).

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
BJ2168	<i>a leu2-3,112 trp1 ura3-52 prb1-1122 pep4-3 prc407 gal2</i>	Piper <i>et al.</i> (1997)
KT3	BJ2168 Δ <i>hsp30::URA3</i>	Piper <i>et al.</i> (1997)
W303-1A	<i>a ade2 can1 his3 leu2 trp1 ura3</i>	Martinez-Pastor <i>et al.</i> (1996)
<i>msn2 msn4</i>	W303-1A <i>msn2-Δ3::HIS3 msn4-1::TRP1</i>	Martinez-Pastor <i>et al.</i> (1996)
MYY290	<i>a leu2 his3 ura3</i>	Smith & Yaffe (1991)
MYY335	MYY290 <i>hsf1-m3</i>	Smith & Yaffe (1991)
JC482	<i>α ura3 leu2 his4</i>	Belazzi <i>et al.</i> (1991)
JC302-26B	JC482 <i>ras2::LEU2</i>	Belazzi <i>et al.</i> (1991)
JC303-79	JC482 <i>ras2::LEU2 sra1.13</i>	Belazzi <i>et al.</i> (1991)

Table 2. Primers used for site-directed mutagenesis of the *HSP30* gene promoter within plasmid Ylp30

Primer	Sequence*	Location†
STRE1-M	GCCGTATTCTTCTT GCG CGTAAGAAAGTTG	–803 to –774
PDSE1-M	GCAGGGTGCCGAAT TCGCT TGGAAACCGC	–675 to –647
PDSE2-M	CCTTGCGTC AGCGA GCGCGATTTTGTGG	–600 to –572
PDSE3-M	CGACTCTTATTCCGA AGCGA TCCGCCACATCC	–430 to –399
PDSE4-M	CTTCGTTATTTTCATC AGCGCT CTCTGCTATGGCAAGG	–366 to –330
PDSE5-M	GCATCTCATCGAAAACCTTTCCTC AGCGAG ATTGGCCAAAG	–314 to –275
PDSE6-M	GTTAGAAAGTATAATATC GGTCGCTCG CCTCATTACAAGTTG	–256 to –215

* Each of these primers was used, together with a primer of exactly complementary sequence (not listed), in the QuickChange procedure for site-directed mutagenesis. Nucleotide changes from the wild-type *HSP30* promoter sequence are underlined and in bold.

† Annealing position relative to the A of the ATG start codon as +1.

RESULTS

Stresses that lead to strong induction of Hsp30 mRNA

Previous work has shown that Hsp30 mRNA is induced during entry to stationary phase under conditions where glucose becomes limiting (Régnacq & Boucherie, 1992; Riou *et al.*, 1997); also that Hsp30 protein is induced in purified plasma membranes by glucose limitation, heat shock and exposure to either ethanol or sorbic acid (Panaretou & Piper, 1992; Régnacq & Boucherie, 1992; Piper *et al.*, 1994, 1997). The effects of different stresses on Hsp30 mRNA levels are shown in Fig. 1. *HSP30* mRNA is detectable even during exponential growth on glucose (Fig. 1, lane 1), but is further induced by heat shock and ethanol (12- and 7-fold increases over basal levels, respectively, in this experiment). Different kinds of severe osmotic stress (2 M sorbitol, 1 M NaCl or 1 M KCl for 1 h) cause only a small induction (two- to threefold). Moderate induction is also seen with oxidative stress (0.4 mM hydrogen peroxide, 1 mM menadione or 1 mM cadmium chloride for 1 h; Fig. 1). Cold shock or more moderate osmotic stresses (0.5 M NaCl or 0.5 M KCl for 1 h) do not cause induction (Fig. 1). In other experiments (see below) we found that weak

organic acid stress was an even stronger inducer of *HSP30* than heat or ethanol treatment.

Both actin (*ACT1*) and *PDA1* transcript levels were used to control for RNA loading in Fig. 1. The latter mRNA has been suggested as a superior loading control for Northern blots compared to actin mRNA, since the levels of actin mRNA are not constant in cells (Wenzel *et al.*, 1995). In general we have found *PDA1* mRNA to be a satisfactory loading control, although its levels increase slightly under conditions of severe osmotic stress (Fig. 1).

Since heat, ethanol and weak acid exposure are the strongest inducers of Hsp30 mRNA, these stress regimes and carbon or nitrogen starvation were used in most subsequent experiments.

HSP30 appears not to be subject to Hsf1/HSE regulation

Several heat shock genes are activated by Hsf1 bound to HSE sequences (see Introduction). The HSE usually comprises at least three alternating adjacent repeats of the 5 bp motif nGAAn, each repeat comprising one half-turn of the DNA double helix (Sorger, 1991). However,

Fig. 1. Northern analysis of RNA from cells of strain BJ2168 subjected to different stresses. Hybridization was to radiolabelled probes specific for *HSP30*, *PDA1* or *ACT1* probes (the latter two being loading controls). RNA samples (each 20 µg) were from unstressed 30 °C control cells (lane 1) or from cells subjected to heat shock for 1 h at 39 °C (lane 2); cold shock for 3 h at 15 °C (lane 3); osmotic stress [either 2 M sorbitol (lane 4), 0.5 M NaCl (lane 5), 1 M NaCl (lane 6), 0.5 M KCl (lane 7) or 1 M KCl (lane 8)]; 6% (w/v) ethanol (lane 9); 0.4 mM hydrogen peroxide (lane 10); 1 mM menadione (lane 11); 1 mM cadmium chloride (lane 12) and 10 µM 4-nitroquinoline-*N*-oxide (lane 13). For samples 4–13 stress exposure was for 1 h at 30 °C. Both short (10 min; first row) and long (2 h; second row) exposures of the *HSP30* blot are shown.

Fig. 2. Nucleotide sequence of the *HSP30* 5'-noncoding region. The 4/5 bp agreements to STRE and PDSE consensus sequences are shown in bold, their orientation being indicated by an underlying arrow. The two closest agreements to the HSE consensus are indicated by dotted overlining and the two TATA sequences are boxed.

in at least one situation (the *CUP1* promoter) two alternating nGAAn repeats are sufficient for Hsf1 binding and in this instance Hsf1 interaction provides both heat and oxidant (menadione) inducibility (Liu & Thiele, 1996). Examination of the promoter region of *HSP30* (Fig. 2) did not reveal two or more juxtaposed nGAAn repeats that probably could constitute a functional HSE. In addition, heat induction of *HSP30* was unaffected by a mutation (*hsf1-m3*) that abolishes the heat inducibility of HSE sequences (see Fig. 5). *hsf1-m3* is a nonsense mutation within the *HSF1* gene that probably causes a considerable reduction in Hsf1 levels, since the production of functional Hsf1 in this mutant requires nonsense suppression during the translation of Hsf1 mRNA (Lindquist & Kim, 1996). This mutation, although allowing the basal HSE activity essential for yeast viability, results in a complete absence of the heat-inducibility of HSE sequences (Smith & Yaffe, 1991). From the apparent lack of a HSE in the *HSP30* promoter and absence of any influence of *hsf1-m3* we concluded that *HSP30* is unlikely to be subject to Hsf1/HSE regulation.

Mutagenesis of putative STRE and PDSE-like elements within the *HSP30* gene promoter

Further examination of the *HSP30* promoter (Fig. 2) also did not reveal any perfect match to the STRE consensus AGGGG (or CCCCT), even though this is a gene clearly induced by a number of different stresses (Fig. 1). There were, however, a number of 4/5 bp agreements to the STRE consensus, these being a CCCCCG sequence at

Fig. 3. Analysis of Hsp30 mRNA in wild-type BJ2168 (lanes 1, 6, 12 and 17) and untransformed KT3 cells (lane 11); also transformants KT3-YIp30 (lanes 2, 7, 13 and 18), KT3-STRE1(M) (lanes 3, 8, 14 and 19), KT3-STRE1(M)-PDSE1-6(M) (lanes 4, 9, 15 and 20) and KT3-PDSE1-6(M) (lanes 5, 10, 16 and 21). The RNA samples analysed were from either unstressed cells (lanes 1–5) or cells exposed for 1 h at 30 °C to nitrogen starvation (lanes 6–10), weak acid (sorbate) stress at pH 4.5 (lanes 11–16) or 7 % ethanol (lanes 17–21). In KT3 transformants the normal 1.3 kb Hsp30 mRNA (B) originates from the *HSP30* gene introduced on the vector, since this RNA is absent in untransformed KT3 cells which synthesize only a larger transcript (A) originating from the *hsp30::URA3* allele (lane 11). The latter transcript is not visible in uninduced cell samples (lanes 1–10). The blots were also probed with *PDA1* mRNA as a control for RNA loading.

–785 indicated as ‘STRE1’ in Fig. 2, as well as several AGGGA or TCCCT sequences. The latter are designated PDSE1–6 in Fig. 2 since they more closely correspond to the consensus of the PDSE or ‘post-diauxic shift’ element identified in the *SSA3* promoter by Boorstein & Craig (1990b). This *SSA3* PDSE element contains a similar motif (4/5 bp match) to the STRE consensus and it, like the STRE, is activated by low PKA activity levels. However, the PDSE appears not to be identical to the STRE since it is not under Msn2 Msn4 transcription factor control (Ruis & Schüller, 1995) and, at least in the *SSA3* promoter, appears to require a downstream HSE to cause heat activation (Boorstein & Craig, 1990b).

PDR12, another gene strongly induced by weak organic acid stress (Piper *et al.*, 1998), also possesses several AGGGA sequences within its promoter (not shown). We therefore wondered if this might constitute a stress responsive element in *PDR12* and *HSP30*. At least two key residues within each of these 4/5 bp agreements to the STRE and PDSE consensus sequences of the *HSP30* promoter (Fig. 2) were therefore mutated on a *HSP30* gene present within the integrative vector YIp30 (see Methods). These mutations (Table 2) were introduced into YIp30 singly and in combination. After confirmation of the introduced changes by sequencing, the

plasmids bearing these promoter mutations were integrated into the defective *trp1* allele of the *hsp30::URA3* yeast strain KT3 (Table 1) by transformation to tryptophan prototrophy. KT3 does not synthesize a normal 1.3 kb Hsp30 mRNA since it has a *URA3* gene fragment insertional inactivation of the *HSP30* gene, at +18 within the *HSP30* ORF (Piper *et al.*, 1997). Thus normal-sized Hsp30 mRNA in KT3 transformed with YIp30-derived plasmids originates solely from the introduced *HSP30* allele (Fig. 3).

Exponential phase YEPD cultures of wild-type BJ2168, also transformants KT3-YIp30, KT3-STRE1(M), KT3-STRE1(M)-PDSE1-6(M) and KT3-PDSE1-6(M) (see Methods), were transferred to fresh YEPD and either placed for 1 h at 30 °C (nonstressed control cells), heat shocked for 1 h at 39 °C, or incubated for 1 h at 30 °C with either 7 % ethanol at pH 7 or 9 mM sorbic acid at pH 4.5. An identical set of cell samples was also incubated in nitrogen starvation medium. RNA was then prepared from these cultures and analysed by Northern blotting (Fig. 3). Remarkably, mutation of all the STRE and PDSE consensus sequences of the promoter [as in KT3-STRE1(M)-PDSE1-6(M)] had no dramatic effects on stress induction, although some minor effects cannot be ruled out. It caused less than twofold changes in *HSP30* induction in response to

Fig. 4. Comparison of the stress-induced expression of *HSP30* and *HSP12*. RNA samples (10 µg) analysed were from either unstressed BJ2168 cells (lane 1) or cells stressed with either 9 mM sorbic acid (1 h at 30 °C at pH 4.5) (lane 2), 7% ethanol (1 h at 30 °C at pH 6.5) (lane 3), nitrogen starvation (1 h at 30 °C in nitrogen starvation medium) (lane 4) or heat shock (1 h at 39 °C) (lane 5). Both short (first row) and long (second row) exposures of the *HSP30* blot are shown.

either sorbate at pH 4.5 (Fig. 3, lanes 11–16), 7% ethanol (Fig. 3, lanes 17–21) or a 39 °C heat shock (data not shown). Sorbate, ethanol and heat shock are all STRE-inducing stresses (Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996). Furthermore the wild-type and promoter mutant *HSP30* alleles were not induced by nitrogen starvation (Fig. 3, lanes 1–10). Indeed while performing this analysis we noticed that while *HSP30* is induced by glucose starvation, it is not induced by nitrogen starvation. The latter condition strongly induces STRE controlled genes (Marchler *et al.*, 1993). Since mutation of two nucleotides within each of the closest agreements to the STRE and PDSE did not significantly affect the stress induction of *HSP30*, we concluded that the strong sorbate, ethanol and heat shock inductions of this gene are largely independent of STRE- or PDSE-like promoter sequences. This conclusion is reinforced by studies of these inductions in transcription factor mutants, described below.

Comparison of stress-induced expression of *HSP30* and *HSP12*

The data in Fig. 3 indicate that the stress induction of *HSP30* is not due to the STRE-like sequences within the promoter. Moreover *HSP30* is not appreciably induced by nitrogen starvation (Fig. 3, lanes 6–10), a strong inducer of STRE-regulated genes. To show that our conditions of nitrogen starvation would indeed cause STRE induction, we also measured Hsp12 mRNA levels. *HSP12* is a good example of a STRE-regulated gene, its

promoter having five repeats of the AGGGG STRE motif that confer activation in response to osmostress, heat shock and transition to stationary phase (Varela *et al.*, 1995). Northern analysis of the changes to *HSP30* and *HSP12* transcripts in strain BJ2168 in response to weak acid stress, ethanol, nitrogen starvation and heat shock are shown in Fig. 4. Transcripts for both Hsp30 and Hsp12 are at low levels during exponential growth and appreciably induced in response to ethanol (Fig. 4, lanes 1 and 3). Hsp30 mRNA is strongly induced, and Hsp12 mRNA is moderately induced, by weak organic acid stress (Fig. 4, lane 2). Conversely, Hsp30 mRNA is less strongly induced by heat shock than Hsp12 mRNA. Most noteworthy, however, is the finding that Hsp30 mRNA is not induced under the conditions of nitrogen starvation that induce Hsp12 mRNA (Fig. 4, lane 4). Thus although these two heat shock genes share some similarities in their stress activations, there are also major differences that show that *HSP12* and *HSP30* are not subject to identical controls. *HSP12* is induced by both nitrogen starvation and moderate osmostress (Varela *et al.*, 1995), stresses that do not induce *HSP30* (Figs 1, 3 and 4). In addition there are differences in the magnitudes of the inductions of these two genes by sorbate and by heat (Fig. 4).

HSP30 stress induction is largely unaffected by the loss of Msn2 and Msn4, two proteins needed for strong stress activation of the STRE

The Msn2 and Msn4 zinc finger proteins are transcriptional activators needed for the strong stress activation of several STRE-regulated genes, such as *CTT1*, *DDR2* and *HSP12* (Martinez-Pastor *et al.*, 1996). STRE activation is severely reduced in *msn2 msn4* mutant cells, cells that are also hypersensitive to several stresses. The responses of Hsp30 mRNA and Hsp12 mRNA to two STRE-inducing stresses, heat and sorbate, were studied in isogenic wild-type and *msn2 msn4* cells (Fig. 5). The strong sorbate induction of *HSP30* was not diminished in *msn2 msn4* cells, although the much weaker sorbate induction of *HSP12* was lost (Fig. 5, lanes 1–4). However, there did appear to be a slight reduction in the weaker heat induction of *HSP30* in *msn2 msn4* mutant cells, this being about 50% of wild-type (Fig. 5, lanes 1, 2, 5 and 6). These results are a further indication that much of the stress induction of *HSP30* is mainly independent of the STRE.

HSP30 stress induction is not appreciably controlled by levels of PKA activity

Lowering PKA activity increases STRE-mediated gene expression and the responsiveness of STREs to stress (see Introduction). We investigated basal and heat-shock-induced expression of *HSP30* in glucose-grown cultures of three strains (Belazzi *et al.*, 1991; Table 1) with either normal (JC482), constitutively high (JC303-79) or constitutively low (JC302-26B) PKA activity levels. Although low PKA activity causes elevated expression of STRE-regulated genes, it was not

Fig. 5. Comparison of the stress-induced expression of *HSP30* and *HSP12* in transcription factor mutants. RNA samples (10 µg) were from *msn2 msn4* cells (lanes 2, 4 and 6) and their isogenic wild-type W303-1A (lanes 1, 3 and 5); also the *hsf1-m3* mutant (lanes 8 and 10) and its isogenic wild-type parent MYY290 (lanes 7 and 9). Cells were either unstressed (lanes 1, 2, 7 and 8), weak-acid-stressed at pH 4.5 with 9 mM sorbate for 1 h at 30 °C (lanes 3 and 4) or heat shocked for 1 h at 39 °C (lanes 5, 6, 9 and 10). The blots were probed with *HSP30*, *HSP12* and *PDA1* sequences. Both short (first row) and long (second row) exposures of the *HSP30* blot are shown.

Fig. 6. Basal and heat-induced *HSP30* expression in strains with normal, constitutively high or constitutively low PKA activity. RNAs were from strains JC482 (lanes 1 and 2), JC303-79 (lanes 3 and 4) and JC302-26B (lanes 5 and 6), either in exponential growth on glucose (lanes 1, 3 and 5) or heat shocked for 1 h at 39 °C (lanes 2, 4 and 6).

associated with an increased basal level of *HSP30* expression (Fig. 6). PKA activity levels also did not cause major changes to the heat shock induction of this gene (Fig. 6).

DISCUSSION

When we commenced this study we suspected that, since *HSP30* is a heat shock gene, it would be controlled by either the HSE or the STRE, or perhaps be controlled by both of these promoter elements. Unexpectedly our data indicate that stress activation of this gene is due to

neither of these promoter elements. *HSP30* is induced by more than one stress, including several that also induce the STRE [heat shock, ethanol, sorbate exposure (Figs 1 and 3–6) and carbon starvation (Régnaq & Boucherie, 1992; Riou *et al.*, 1997)]. However, it is not induced by certain other STRE-inducing stresses (moderate osmotic stress, nitrogen starvation). Moreover the basal and stress-induced expressions of this gene, unlike those of the STRE and PDSE, appear to be largely independent of PKA activity (Fig. 6). Further evidence that *HSP30* is not STRE-regulated was obtained by comparing its stress induction with that of the well-characterized STRE-regulated gene *HSP12* (Figs 4 and 5).

There are other stress responsive promoter elements in yeast besides the HSE and STRE. One is the binding site for Yap1 protein homologues (consensus TTACTAA; Fernandes *et al.*, 1998). Several of the genes responsive to oxidative stress are under Yap1 control. However, the *HSP30* promoter does not have a perfect agreement to the Yap1 binding consensus and, while it is slightly induced by hydrogen peroxide (Fig. 1), shows a normal sorbate and ethanol induction in *yap1* mutant cells (data not shown). There are, however, a number of Yap1 homologues in yeast which have yet to be assigned a function (Fernandes *et al.*, 1998), as well as possible degenerate Yap1 recognition sequences in the *HSP30* promoter. We have therefore not yet excluded the possibility that the stress induction of *HSP30* is due to one of these Yap1-related factors.

It is possibly significant that the strongest stress inducers of *HSP30* (Figs 1 and 3–6) appear to be those treatments that will increase membrane fluidity. Elevated temperatures and ethanol both increase fluidity of the

phospholipid bilayer. Alkanols and organic acids such as sorbate have propensities to partition into the bilayer which increase as their hydrophobicity increases. It is possible therefore that *HSP30* is responding to a membrane-fluidity-sensing system, a system that stimulates a signal transduction pathway acting on this and probably other genes in a HSE- and STRE-independent manner. Part of the function of such a system may be to minimize the effects of stress on the membrane. *HSP30* encodes a highly hydrophobic integral membrane protein which, though detectable in exponentially growing yeast cells, increases dramatically in level in response to heat shock, ethanol and sorbate treatment (Panaretou & Piper, 1992; Régnacq & Boucherie, 1992; Piper *et al.*, 1994, 1997). There are preliminary indications that this Hsp30 protein may have several actions at the plasma membrane. Besides exerting a negative effect on stress-induced increases in plasma membrane H⁺-ATPase activity (Piper *et al.*, 1997), it also acts to reduce membrane fluidity at higher growth temperatures (K. Obuchi, unpublished results). These actions appear to be reducing the effects of sublethal stress, since Hsp30 null cells take longer to resume growth after a number of nonlethal stress exposures that cause temporary growth arrest (Piper *et al.*, 1997; K. Obuchi, unpublished results).

The sensor of the heat shock response in higher eukaryotic cells is widely thought to be Hsp70 (see Introduction). However, there is now increasing evidence that membrane composition can affect the sensing of heat stress, at least in microbes. Membrane lipid composition affects HSE activation by heat shock in yeast (Chatterjee *et al.*, 1997), as well as the heat shock responsiveness of the unicellular cyanobacterium *Synechocystis* PCC 6803 (Horváth *et al.*, 1998). Hsp30 null cells have higher membrane fluidity at high temperatures (K. Obuchi, unpublished results), but whether they display an altered stress response still remains to be determined.

One of the strongest inducers of *HSP30* appears to be weak organic acid stress (Figs 3–5). Water-soluble weak organic acid food preservatives such as sorbic acid appear to induce a discrete stress response in yeast that assists adaptation to growth in the presence of these compounds (Piper *et al.*, 1987, 1988). This response leads to strong induction of two plasma membrane proteins, one of which is Hsp30 (Piper *et al.*, 1997) and the other an ATP-binding cassette transporter (Pdr12; Piper *et al.*, 1998). Weak organic acid (sorbate) stress also induces the STRE, but this is not the promoter element by which this stress causes induction of *HSP30* (Figs 3–5). Instead an alternative weak-acid-responsive element appears to exist, a putative element that may be especially important in the adaptive response to weak organic acid stress. To identify this element, we are currently constructing deletions of the *HSP30* promoter, and also analysing weak-acid-sensitive mutants so as to identify the proteins involved in the sensing of weak acid stress and the signalling of this stress to the transcription apparatus.

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REFERENCES

- Belazzi, T., Wagner, A., Wieser, R., Schanz, M., Adam, G., Hartig, A. & Ruis, H. (1991). Negative regulation of transcription of the *Saccharomyces cerevisiae* catalase T (*CTT1*) gene by cAMP is mediated by a positive control element. *EMBO J* **10**, 585–592.
- Boorstein, W. R. & Craig, E. A. (1990a). Structure and regulation of the SSA4 HSP70 gene of *Saccharomyces cerevisiae*. *J Biol Chem* **265**, 18912–18921.
- Boorstein, W. R. & Craig, E. A. (1990b). Regulation of a yeast *HSP70* gene by a cAMP responsive transcriptional control element. *EMBO J* **9**, 2543–2553.
- Chatterjee, M. T., Khalawan, S. A. & Curran, B. P. G. (1997). Alterations in cellular lipids may be responsible for the transient nature of the yeast heat shock response. *Microbiology* **143**, 3063–3068.
- Fernandes, L., Rodrigues-Pousada, C. & Struhl, K. (1998). Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol Cell Biol* **17**, 6982–6993.
- Geitz, R. D. & Sugino, A. (1988). New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenised yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527–534.
- Horváth, I., Glatz, A., Varvasovszki, V. & 8 other authors (1998). Membrane physical state controls the signalling mechanism of the heat shock response of *Synechocystis* PCC 6803: identification of *hsp17* as a fluidity gene. *Proc Natl Acad Sci USA* **95**, 3513–3518.
- Kobayashi, N. & McEntee, K. (1993). Identification of cis and trans-components of a novel stress regulatory pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* **13**, 248–256.
- Lindquist, S. & Kim, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc Natl Acad Sci USA* **93**, 5301–5306.
- Liu, X.-D. & Thiele, D. J. (1996). Oxidative stress induces heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. *Genes Dev* **10**, 592–603.
- McDaniel, D., Caplan, A. J., Lee, M.-S., Adams, C. C., Fishel, B. R., Gross, D. S. & Garrard, W. T. (1989). Basal-level expression of the yeast *HSP82* gene requires a heat shock regulatory element. *Mol Cell Biol* **9**, 4789–4798.
- Mager, W. H. & De Kruijff, A. J. J. (1995). Stress-induced transcriptional activation. *Microbiol Rev* **59**, 506–531.
- Marchler, G., Schuller, C., Adam, G. & Ruis, H. (1993). A *Saccharomyces cerevisiae* UAS element controlled by kinase A activates transcription in response to a variety of stress conditions. *EMBO J* **12**, 1997–2003.
- Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2 and Msn4 are required for transcriptional induction through the stress-response element (STRE). *EMBO J* **15**, 2227–2235.
- Morano, K., Liu, P. & Thiele, D. (1998). Protein chaperones and the heat shock response of *Saccharomyces cerevisiae*. *Curr Opin Microbiol* **1**, 197–203.
- Morimoto, R. I., Kroeger, P. E. & Cotto, J. J. (1996). The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. In *Stress-Inducible Trans-*

criptional Responses, pp. 139–163. Edited by U. Feige, R. I. Morimoto, I. Yahara & B. S. Polla. Boston, MA: Birkhauser.

Panaretou, B. & Piper, P. W. (1992). The plasma membrane of yeast acquires a novel heat shock protein (Hsp30) and displays a decline in proton-pumping ATPase levels in response to heat shock and the entry to stationary phase. *Eur J Biochem* **206**, 635–640.

Piper, P. W. (1993). Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **11**, 339–356.

Piper, P. W. (1997). The yeast heat shock response. In *Yeast Stress Responses*, pp. 75–99. Edited by S. Hohmann & W. H. Mager. Austin, TX: R. G. Landes.

Piper, P. W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K., Praekelt, U. M., Meacock, P., Régnacq, M. & Boucherie, H. (1994). Induction of major heat shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. *Microbiology* **140**, 3031–3038.

Piper, P. W., Ortiz-Calderon, C., Holyoak, C., Coote, P. & Cole, M. (1997). Hsp30, the integral plasma membrane heat shock protein of yeast, is a stress-inducible regulator of plasma membrane H⁺-ATPase. *Cell Stress Chaperones* **2**, 12–24.

Piper, P. W., Mahé, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., Mühlbauer, M., Coote, P. & Kuchler, K. (1998). The Pdr12 ATP-binding cassette (ABC) transporter is required for development of weak organic acid resistance in yeast. *EMBO J* **17**, 4257–4265.

Régnacq, M. & Boucherie, H. (1992). Isolation and sequence of *HSP30*, a yeast heat-shock gene coding for a hydrophobic membrane protein. *Curr Genet* **23**, 435–442.

Riou, C., Nicaud, J. M., Barre, P. & Gaillardin, C. (1997). Stationary-

phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast* **13**, 903–915.

Ruis, H. & Schüller, C. (1995). Stress signalling in yeast. *BioEssays* **17**, 959–965.

Schüller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C. & Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J* **13**, 4382–4389.

Smith, B. J. & Yaffe, M. P. (1991). Uncoupling thermotolerance from the induction of heat shock proteins. *Proc Natl Acad Sci USA* **88**, 11091–11094.

Sorger, P. K. (1991). Heat shock factor and the heat shock response. *Cell* **65**, 363–365.

Varela, J. C. S., Praekelt, U. M., Meacock, P. A., Planta, R. J. & Mager, W. H. (1995). The *Saccharomyces cerevisiae* *HSP12* gene is activated by the high osmolarity glycerol pathway and negatively regulated by protein kinase A. *Mol Cell Biol* **5**, 6232–6245.

Watt, R. & Piper, P. W. (1997). *UBI4*, the polyubiquitin gene of *Saccharomyces cerevisiae*, is a heat shock gene that is also subject to catabolite derepression control. *Mol Gen Genet* **253**, 439–447.

Wenzel, T. J., Teunissen, A. W. R. H. & Steensma, H. Y. (1995). *PDA1* mRNA: a standard for quantitation of mRNA of *Saccharomyces cerevisiae* superior to *ACT1* mRNA. *Nucleic Acids Res* **23**, 883–884.

Winderickx, J., de Winde, J. H., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P. & Thevelein, J. M. (1996). Expression regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*. *Mol Gen Genet* **252**, 470–482.

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