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The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE)

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The *MSN2* and *MSN4* genes encode homologous and functionally redundant Cys_2His_2 zinc finger proteins. A disruption of both *MSN2* and *MSN4* genes results in a higher sensitivity to different stresses, including carbon source starvation, heat shock and severe osmotic and oxidative stresses. We show that *MSN2* and *MSN4* are required for activation of several yeast genes such as *CTT1*, *DDR2* and *HSP12*, whose induction is mediated through stress-response elements (STREs). Msn2p and Msn4p are important factors for the stress-induced activation of STRE dependent promoters and bind specifically to STRE-containing oligonucleotides. Our results suggest that *MSN2* and *MSN4* encode a DNA-binding component of the stress responsive system and it is likely that they act as positive transcription factors.

Keywords: heat-shock/stress/transcriptional regulation/yeast/zinc finger proteins

Introduction

In nature, environmental factors like temperature and nutrient availability are continuously changing. It is an intrinsic property of living organisms to sense and properly respond to environmental changes and specific responses have been found in all cells studied so far. The cellular machinery against threatening conditions (e.g. high temperature, oxidative or osmotic stress) includes several lines of defence. The first line consists of protective low molecular weight components (e.g. trehalose) and proteins (repair systems, chaperones) which are necessary for immediate survival. The rapid primary response also initiates the activation of signal transduction systems, which then trigger secondary events like activation of pre-existing enzyme activities and transcriptional induction of genes encoding factors with protective functions. A particularly well studied example of stress response is the series of events triggered by heat shock. When cells are exposed to high temperatures they respond with the production of a set of proteins called heat shock proteins, or HSPs. The regulation of the heat shock response includes transcriptional, translational and post-translational mechanisms (Lindquist and Craig, 1988; Parsell and Lindquist, 1994; Wu *et al.*, 1994). *HSP* genes contain one or more repeats of the heat shock response element (HSE) which is a binding site for the heat shock transcription factor (HSF). The transcriptional regulation of the stress genes has been studied extensively in yeast and other organisms (for review see Bienz and Pelham, 1987; Morimoto *et al.*, 1992; Mager and Moradas Ferreira, 1993; Piper, 1993; Mager and De Kruijff, 1995). In the case of the yeast *Saccharomyces cerevisiae*, other well characterized stress-response systems include the transcriptional induction of *TRX2* (Kuge and Jones, 1994) and *GSH1* (Wu and Moye-Rowley, 1994) by the transcription factor Yap1p in response to oxidative stress and the metal induced transcription of *CUP1* by the copper specific transcriptional factor Ace1p (Thiele, 1992).

Yeast cells exposed to mild stress develop tolerance not only against higher doses of the same stress (induced stress resistance), but also against stress caused by other agents (cross protection). Therefore, an integrating mechanism should exist which is sensing and responding to different forms of stress. A regulatory element responsive to a variety of stress conditions has been initially identified as a HSF-independent heat shock element in the promoters of *CTT1* (Wieser *et al.*, 1991) and *DDR2* (Kobayashi and McEntee, 1991). This element, which has the consensus core sequence AGGGG, has been designated stress response element (STRE) and is able to mediate transcription induced by various forms of stress (Kobayashi and McEntee, 1993; Marchler *et al.*, 1993; Schüller *et al.*, 1994; Ruis and Schüller, 1995). In accordance with its role in general stress resistance, STRE has been found to control stress inducible transcription of genes with protective functions. The list of genes controlled via STRE includes *CTT1*, *DDR2*, *HSP12*, *TPS2*, *GSY2* and *GPH1* (Ruis and Schüller, 1995). The mechanisms which sense and mediate different forms of stress through STREs are largely unknown. Two signalling pathways have been identified: a MAP kinase cascade, the HOG pathway, has been shown to mediate osmotic stress via STRE (Schüller *et al.*, 1994) and the RAS–cAMP pathway, which negatively regulates this element (Marchler *et al.*, 1993). The transcription factor(s) that recognize STREs have been elusive.

The *MSN2* and *MSN4* genes were initially identified as multicopy suppressors of the invertase defect in a Snf1p protein kinase mutant (Estruch and Carlson, 1993). They are functionally related and encode homologous transcriptional activators that contain two Cys_2His_2 zinc fingers at the C terminus and share similarity over the entire protein length. The large class of Cys_2His_2 zinc finger proteins includes the yeast Mig1p repressor, the mammalian Wilm's

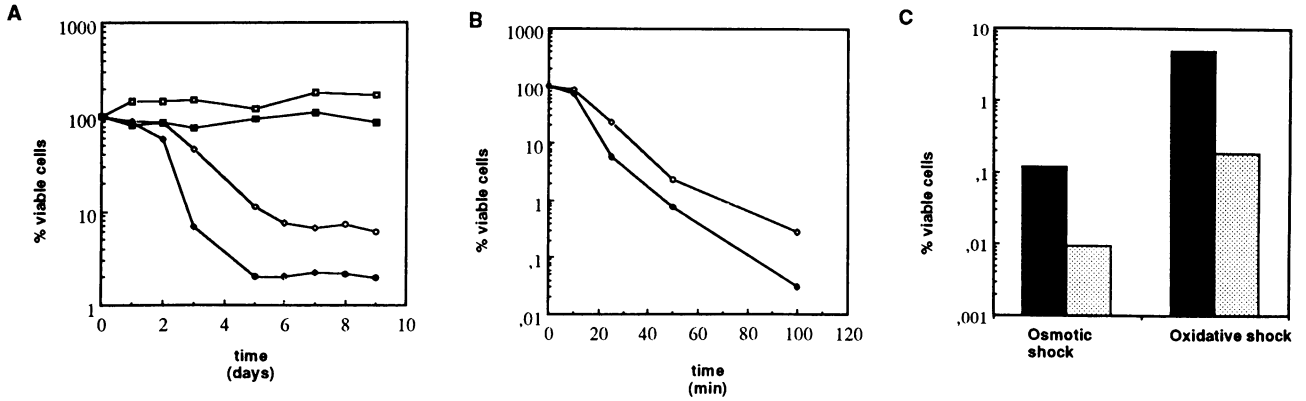

Fig. 1. Stress sensitivity of the *msn2 msn4* double mutant strain. (**A**) Resistance to carbon source starvation. Yeast cells from an overnight culture of W303-1A (open symbols) or *msn2 msn4* double mutant strain (filled symbols) were inoculated in YPD and grown until saturation (squares) or maintained in exponential growth during 2 days (circles). The cells were then washed and resuspended in synthetic medium without glucose (S) to an OD_{600} of 0.3. (**B**) Thermotolerance. Yeast cells from an overnight culture of W303-1A (open symbols) or *msn2 msn4* double mutant strain (filled symbols) were inoculated into SD and maintained in exponential growth for 24 h at 30°C. At an OD_{600} of 0.3, cells were aliquoted and transferred to a water bath at 45°C. (**C**) Survival after exposure to osmotic or oxidative stress. For the osmotic stress, overnight cultures were diluted with YPD to an OD_{600} of 0.1 and grown to an OD_{600} of 1.0. The cultures were then diluted to an OD_{600} of 0.2 in YPD 3 M NaCl and incubated at 30°C for 7 h. For the oxidative stress, overnight cultures were inoculated on YPD and grown to an OD_{600} of 0.3. Hydrogen peroxide was added to a final concentration of 5 mM, and incubated for 1 h with vigorous agitation at 30°C. W303-1A (closed bars) and *msn2 msn4* double mutant (stippled bars). In all the experiments viability was measured by plating the appropriate dilution in duplicate onto YPD plates. Colony forming units were counted after 2–3 days of incubation at 30°C.

tumour proteins and the early growth response (Egr1) factor of which a crystal structure is available (Pavletich and Pabo, 1991).

In this study we show that a defect in the *MSN2* and *MSN4* genes causes increased sensitivity to a variety of stress conditions. The observation, in gel retardation experiments, that both Msn2p and Msn4p can specifically bind to STRE sequences indicates their potential role as binding factors of the stress response element. Consistent with this, induction of STRE-regulated genes has been found to be defective in *msn2 msn4* mutants.

Results

The msn2 msn4 double mutant shows pleiotropic stress sensitivity

In the course of the investigation into the physiological role of *MSN2* and *MSN4* we observed that a double mutant shows reduced viability during the adaptation from glucose to another carbon source (e.g. raffinose; data not shown). We then investigated the role of *MSN2* and *MSN4* in the resistance to carbon source starvation. Figure 1A shows that a *msn2 msn4* mutant strain showed a significantly higher loss of viability than wild type when exponentially growing cells were shifted to media without any carbon source, whereas no difference was observed in stationary phase cells.

We further analysed cell survival after severe temperature, osmotic and oxidative stresses to distinguish whether the sensitive phenotype shown by the mutant is specific for starvation conditions or is part of a more general defect to stress resistance. Thermotolerance was analysed by measuring viability of exponentially growing cells after heat shock at 45°C. Figure 1B shows that the *msn2 msn4* strain exhibited a 4- to 5-fold lower survival rate than the wild type strain. The difference in viability between wild type and mutant strains was higher when the heat shock was applied in the absence of glucose (data not shown). The same effect has been reported for the *hsp30* mutant of *Neurospora crassa* (Plesofsky-Vig and Brambl, 1995). Preconditioning of cells for 1 h at 37°C (induced thermotolerance) increased the resistance to heat shock in both wild type and mutant cells, but again the rate of killing was higher in the *msn2 msn4* strain (data not shown). Survival of exponentially growing *msn2 msn4* cells after exposure to severe osmotic (7 h in 3 M NaCl) or oxidative (1 h in presence of 5 mM H_2O_2) stress was also dramatically impaired (Figure 1C). Pre-treatment with 0.3 M NaCl for 1 h or exposure to heat shock (37°C, 1 h) improved cell survival in high osmolarity, although the differences in viability between wild type and mutant strains remained. The stress sensitivity of the double mutant is restricted to severe stress. Under moderate stress conditions (progressive withdrawal of glucose by entering into stationary phase, 37°C, 0.8 M NaCl or 1 mM H_2O_2) we could not detect any difference in survival (starvation) or growth (other stress conditions) between the wild type and mutant strains (data not shown). From these results we conclude that the absence of *MSN2* and *MSN4* impairs the cellular response to different types of stress.

MSN2 and MSN4 are required for stress-dependent transcription of several stress-induced genes

It has been shown that the Msn2 and Msn4 proteins function as transcriptional activators (Estruch and Carlson, 1993). Therefore, it is reasonable to assume that the role of Msn2p and Msn4p in the stress response is the transcriptional activation of one or more genes involved in the protective response to different types of stress. We compared the transcript levels of different genes that have been involved in the stress response in the wild type and *msn2 msn4* double mutant strains after carbon source starvation (Figure 2). Among the genes tested, *HSP12*, *HSP26*, *CTT1* and *DDR2* showed defective induction in the *msn2 msn4* double mutant. Others, including *SSA3*, *UBI4* and *SSA1*, were induced at the same level in wild type and mutant strains (Figure 2, data not shown). The

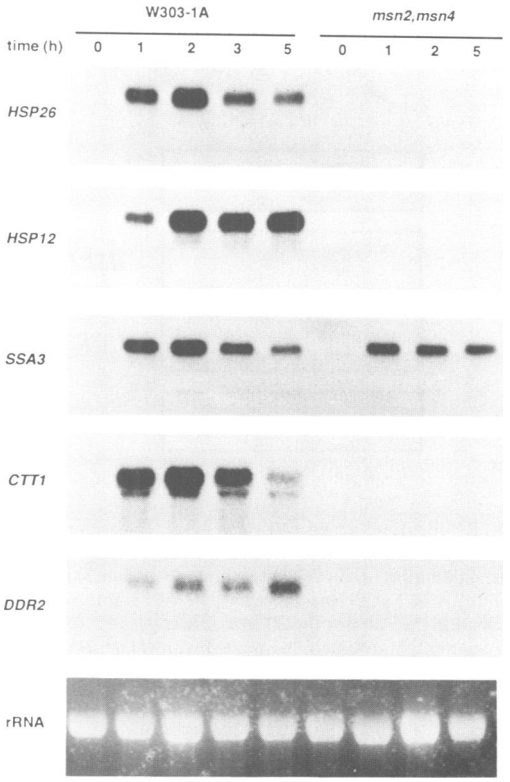

Fig. 2. Effect of the *msn2 msn4* double mutation on the activation of stress-inducible genes by carbon source starvation. Total RNA was prepared from wild type W303-1A or *msn2 msn4* double mutant strain after incubation at indicated times on S medium. 4 μg of each sample were fractionated on 1.6% agarose gels and analysed by Northern blot hybridization. The probes used are described in Materials and methods. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

HSP12, *CTT1* and *DDR2* genes have been shown to be regulated via STREs. *HSP26* also contains STRE-like sequences in its promoter (Mager and De Kruijff, 1995; Ruis and Schüller, 1995). In order to obtain more direct evidence that the STREs are indeed the targets of Msn2p and Msn4p we performed a more detailed analysis of the known STRE-regulated genes using different kinds of stresses (Figure 3). RNA from wild type and mutant cells heat shocked at 37°C (Figure 3A), or exposed to 0.4 M NaCl, 10 mM sorbic acid or 7% ethanol (Figure 3B), was prepared and subjected to Northern blot analysis. Expression of *CTT1*, *HSP12* and *DDR2* induced by heat shock, high salt concentration, sorbic acid or ethanol is abolished or severely reduced in the *msn2 msn4* double mutant.

MSN2 and MSN4 are involved in the stress-induced expression driven by STREs

Since mutation of *MSN2* and *MSN4* impairs the expression of STRE-regulated genes, we tested whether the stress-induced expression conferred by the STRE to a heterologous *LEU2–lacZ* gene was also affected in the *msn2 msn4* double mutant. Strain GG18 (Marchler *et al.*, 1993), carrying a single chromosomally integrated STRE–*LEU2–lacZ* reporter gene (pCTT1-18 7x), was deleted for both *MSN2* and *MSN4* genes, resulting in strain GG18-msn2msn4. Logarithmic cells of wild type and mutant were exposed to different stress conditions and β-galactosidase activity was determined (Figure 4A). In the mutant the basal level of transcription was reduced by a factor of 10. Stress induction of transcription by heat shock, low pH, sorbic acid and high ethanol concentrations was completely abolished in the double mutant. For osmotic and oxidative stresses as well as derepression (growth on YPE), low levels of transcription were observed. However, the factor of induction was comparable with the wild type response. Induction by osmotic stress detected in the *msn2 msn4* double mutant was HOG pathway dependent (Figure 4B).

Overexpression of MSN2 and MSN4 genes improves resistance to starvation and thermal stresses

If absence of expression of STRE regulated genes could be the reason for the stress-sensitive phenotype observed in the *msn2 msn4* double mutant, constitutive expression of these genes might result in increased stress resistance. We investigated whether the overexpression of either of the gene products rendered the cells more resistant to stress. The coding regions of *MSN2* or *MSN4* were fused to the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) promoter (Schena *et al.*, 1991) and the resulting plasmids (pG3MSN2 and pG3MSN4) were used to transform the wild type strain W303-1A. We then compared the resistance of the Msn2p- and Msn4p-overexpressing cells with respect to carbon source starvation and heat shock sensitivity with that of the control wild type strain. Figure 5 shows that there was an enhancement of the resistance to starvation (Figure 5A) and thermotolerance (Figure 5B) in the pG3MSN2 and pG3MSN4 transformants compared with the cells transformed with the vector pG3. However, growth on SD medium was negatively affected by the overexpression of the *MSN2* gene and to a lesser extent by the overexpression of the *MSN4* gene (data not shown). The resistance to both thermal and nutritional stresses was also improved by increasing the *MSN2* and *MSN4* gene dosages under the control of their respective promoters using the high copy number plasmids pEL32 and pEL45 (Estruch and Carlson, 1993). However, the survival rate in this case was lower compared with cells containing the pG3-based plasmids (results not shown). The growth defect might be a consequence of the inappropriate activation of defence mechanisms under non-stress conditions. Figure 6 shows that cells transformed with pG3MSN2 express *CTT1* and *HSP26* even in non-stress conditions whereas no transcript is detected in control cells. In Msn4p-overexpressing cells a weak induction of *CTT1* was observed. Consistent with a role of these factors in the regulation via STREs we found that overexpression of Msn2p and Msn4p activates the STRE–*lacZ* reporter in non-stress conditions (Table I). However, the relatively low level of activity, compared with levels detected under stress conditions, suggests that regulation of the Msn2p and Msn4p function includes post-translational mechanisms. These results are consistent with the constitutive expression of the *MSN2* gene that we have observed. The same low amounts of *MSN2* RNA were detected in stressed and non-stressed cells (data not shown).

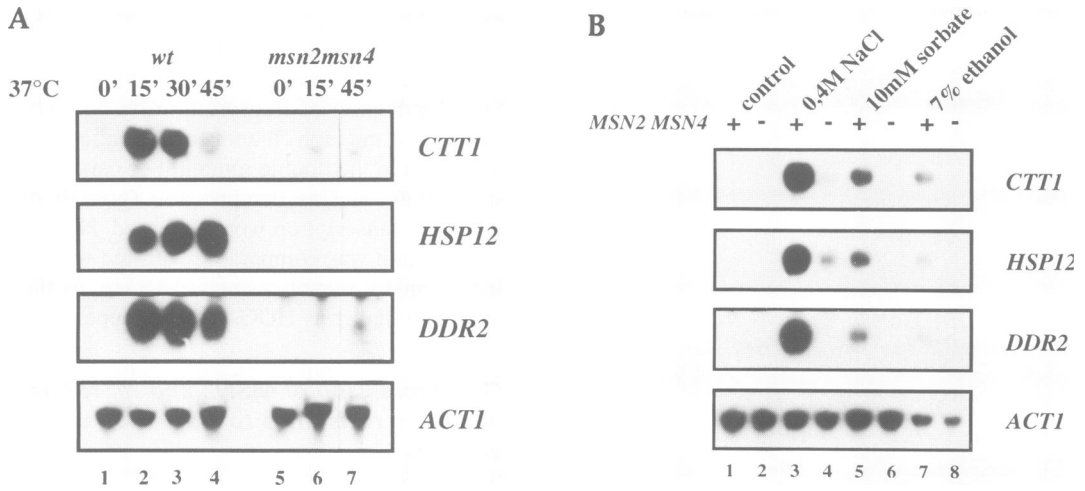

Fig. 3. Effect of the *msn2 msn4* double mutation on the stress-induced expression of STRE-regulated genes. (**A**) Heat shock. Logarithmic cultures of strain GG18 (lanes 1, 2, 3 and 4) and GG18msn2msn4 (lanes 5, 6 and 7) grown on YPD at 25°C were transferred to a water bath at 37°C and aliquots harvested at the indicated times. (**B**) Treatment with different stress conditions. Cultures of strains GG18 (lanes 1, 3, 5 and 7) and GG18msn2msn4 (lanes 2, 4, 6 and 8) growing logarithmically on YPD were treated with 0.4 M NaCl (lanes 3 and 4), 10 mM sorbic acid (lanes 5 and 6) or 7% ethanol (lanes 7 and 8) by adding concentrated solutions and further incubating the cultures for 20 min. Lanes 1 and 2 represent untreated control. RNA was prepared, 30 μg of each sample was fractionated on 1.2% agarose gels, blotted and probed with *CTT1*, *HSP12*, *DDR2* and *ACT1* fragments.

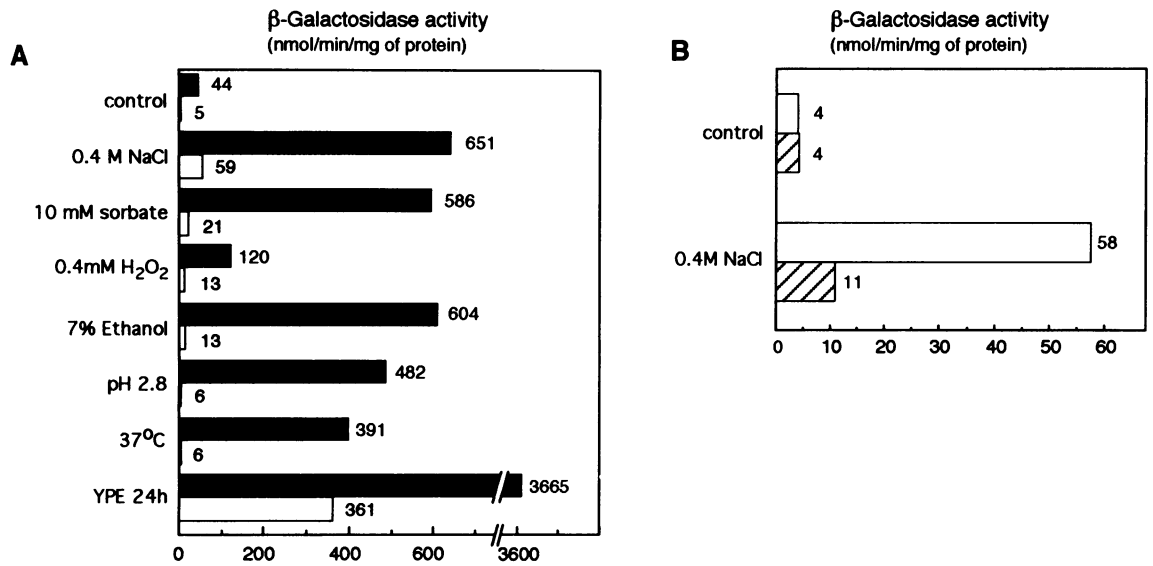

Fig. 4. Effect of the *msn2 msn4* double mutation on the expression of a STRE-driven *LEU2–lacZ* reporter gene. (**A**) Logarithmic cultures of strain GG18 (closed bars) and GG18msn2msn4 (open bars) were subjected to various stress conditions. (**B**) Osmotic induction in GG18msn2msn4 (open bars) and GG18msn2msn4pbs2 (hatched bars) strains. Cells were grown on YPD, subjected to stress conditions for 1 h and β-galactosidase activity assayed in crude extracts. Data presented are the mean of at least three independent experiments.

Msn2 and Msn4 proteins bind in vitro to the STRE

Taken together, our data show clearly that *MSN2* and *MSN4* are required for STRE-mediated induction of transcription by different stresses. Sequence comparisons and computer assisted molecular modelling support the idea that Msn2p and Msn4p can exert their effect through specific binding to STRE. Predictions are based on the zinc fingers II and III from Egr1, for which a crystal structure, together with bound DNA, is available (Pavletich and Pabo, 1991). Finger II of Egr1 binds to the nucleotide sequence [3′]GGT[5′]. In the crystal structure, residues Arg46 and His49 make specific contacts to GC pairs. In the multiple alignment they are found conserved in the sequences of Msn2p, Msn4p, Mig1p and Egr1p (Figure 7). Thr52, being responsible for the 5′-TA pair specificity of Egr1p Finger II, is replaced by an arginine residue in Msn2p, Msn4p and Mig1p. In accordance with established recognition codes for Cys_2His_2 zinc fingers (Choo *et al.*, 1994), we predict that the zinc fingers I of Msn2p, Msn4p and Mig1p will bind specifically to the nucleotide sequence [3′]GGG[5′].

Finger III of Egr1p binds to the nucleotide sequence [3′]GCG[5′]. The residues Arg74, Glu77 and Arg80 from the crystal structure, found to determine sequence specificity, are conserved in Finger II of Mig1p, which we predict will bind to the same nucleotide sequence. The respective residues in Msn2p and Msn4p are arginine, asparagine and glutamine. In accordance with recognition

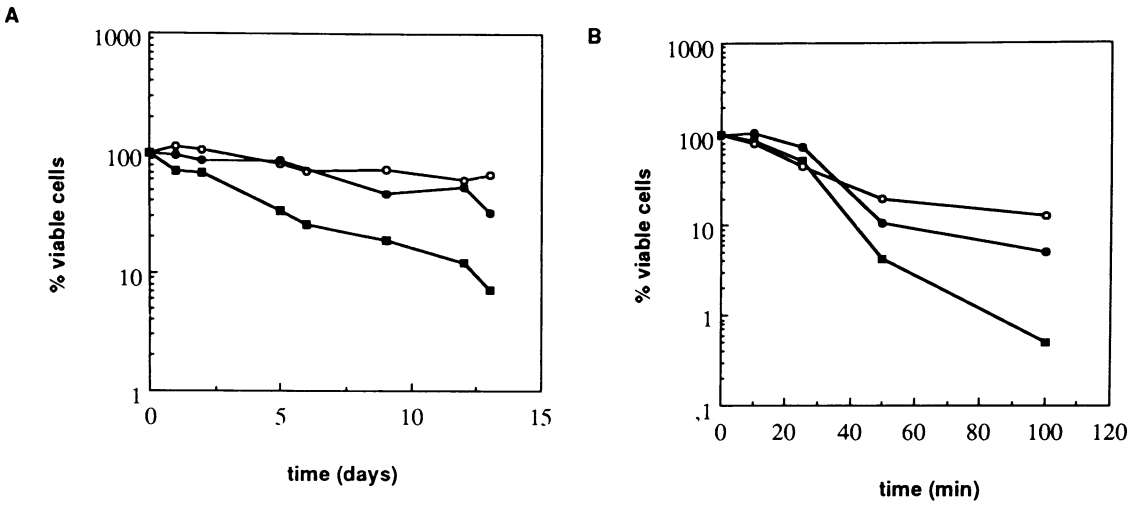

Fig. 5. Resistance to carbon source starvation (**A**) and thermotolerance (**B**) in strains overexpressing the *MSN2* or the *MSN4* genes. Overnight cultures of W303-1A transformed with pG3 (filled squares), pG3MSN2 (open circles) or pG3MSN4 (filled circles) were inoculated in SD–Trp and maintained in exponential growth during 24 h. Then they were either transferred to S–Trp medium at an OD_{600} of 0.3 (A) or shifted to 45°C (B) and viability measured at the indicated times.

codes we predict specific binding with respect to the nucleotide sequence [3′]GAN[5′] for Msn2/Msn4 Finger II. Results from homology modelling of the Msn2/STRE-complex (data not shown) indicate specificity for an AT pair at the 5′-position of the nucleotide sequence recognised by Msn2p and Msn4p. However, selection by the respective glutamine might not be that strong and permit some variation. From the data we infer that the two zinc fingers of Msn2p and Msn4p bind to the STRE sequence with high specificity and that the binding occurs in a manner very similar to that observed in the crystal structure of Egr1p.

To check experimentally whether Msn2p and Msn4p bind to the STRE in a specific manner, we synthesized full length Msn2p *in vitro* from plasmid pMM1. The attempts to obtain Msn4p in the same way were unsuccessful and therefore we expressed a GST fusion of the DNA binding domain of Msn4p in *Escherichia coli* (see Materials and methods). The proteins were used for gel shift assays with a labelled oligonucleotide that included the sequence of a functional STRE from the *HSP12* promoter (base pairs –221 to –241; Varela *et al.*, 1995). Figure 8 shows that both Msn2p and Msn4–GSTp are able to bind to this oligonucleotide (lanes 2 and 8). Similar results were obtained using a Msn2–GST fusion protein (results not shown). The specific nature of this interaction was determined by showing a reduction in binding in the presence of an excess of the same, unlabelled, oligonucleotide (lanes 3 and 9). To show that Msn2 and Msn4 proteins recognize the AGGGG sequence, we introduced a single base pair change, generating the mutated sequence AGTGG. This oligonucleotide cannot compete with the STRE sequence (lanes 4 and 10). This indicates that Msn2p and Msn4p are unable to bind to the mutated sequence. Competition experiments further supported the specific recognition of the STRE sequences by Msn2p and Msn4–GSTp. Oligonucleotides with similar elements from the promoters of the *CTT1* (lanes 5 and 11) and *DDR2* genes (lanes 6 and 12) competed effectively, but an oligonucleotide with the Mig1p binding site (lanes 7 and 13) is unable to compete for binding.

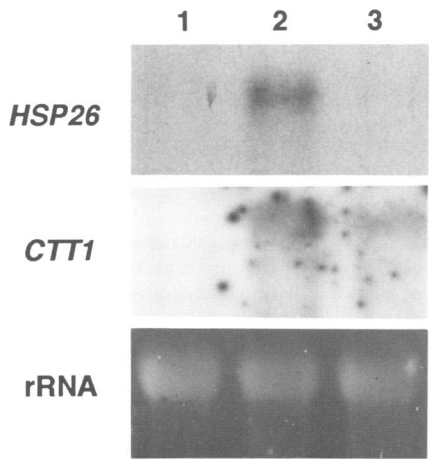

Fig. 6. Constitutive expression of the stress induced genes in cell overexpressing Msn2p or Msn4p. Wild type cells transformed with pG3 (1), pG3MSN2 (2) or pG3MSN4 (3) were used. Total RNA was isolated from exponentially growing cells on SD–trp medium and analysed by Northern blot hybridization using *HSP26* or *CTT1* as probes. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

Table I. Effect of Msn2p and Msn4p overexpression on transcriptional induction of STRE-LEU2–lacZ reporter gene

Plasmid	β-Galactosidase activity[a] (nmol/min/mg protein)
pG3	22.9
pG3MSN2	85
pG3MSN4	44

[a]Mean for three independent transformants

Discussion

The induction of a variety of genes in response to environmental stresses is considered a protective mechanism against the harmful effects that some conditions can have on cells. This response provides higher resistance to subsequent insults of the same stress (induced stress

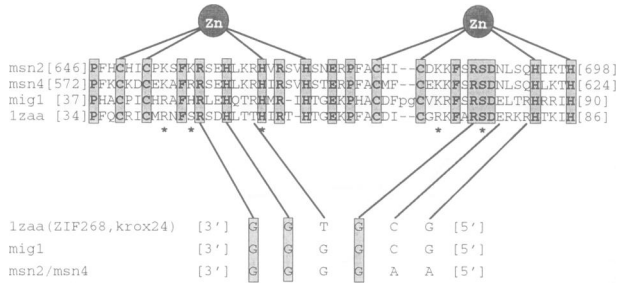

Fig. 7. Prediction of the DNA sequence recognised by Msn2 and Msn4 zinc fingers. Multiple sequence alignments showing the two zinc fingers of Msn2p, Msn4p and Mig1p compared with the sequence of two zinc fingers (II and III) from the early growth response protein Egr1 (krox24, 1zaa) of mouse. A crystal structure of Egr1 is available under the PDB-entry code 1zaa. The numbers given for 1zaa refer to the numbering in the PDB-entry. Completely conserved positions in the alignment are shaded grey. The coordination of the two zinc ions is indicated. Amino acid residues that contact the backbone phosphate in the crystal structure of the Egr1–DNA complex are marked by an asterisk. Arg46, His49 and Arg74 are found to make specific hydrogen bonding interactions to GC base pairs (shaded grey). Their overall conservation suggest similar contacts for the zinc fingers of Mig1p, Msn2p and Msn4p. Thr52, being responsible for recognition of a TA base pair in Egr1, is replaced by arginine in the other three proteins. We predict, in analogy to the other arginines, specificity for a GC base pair at the respective position. Finger III of Egr1 and Finger II of Mig1 are identical with respect to their DNA-contacting residues. Apparently their DNA-binding specificities are the same. In Msn2p and Msn4p, Glu77 is replaced by an asparagine, while Arg80 is replaced by a glutamine. In a computer-generated three-dimensional model of the Msn2–STRE-complex, we could demonstrate that the asparagine and the glutamine are capable of making direct hydrogen-bonding interactions to AT base pairs at the respective positions of the STRE (data not shown). The specificity for AT base pairs of asparagine in the 'middle' position of this type of zinc finger is well known (Choo *et al.*, 1994). The selection of AT base pairs at the 5′ position of the STREs through the respective glutamine might not be that strong and permit some variation.

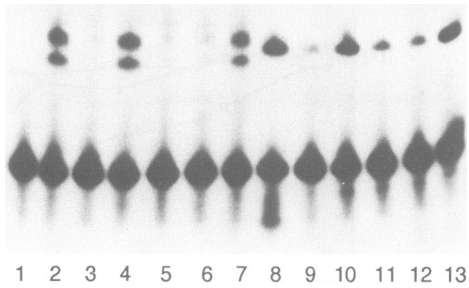

Fig. 8. The zinc finger regions of Msn2p and Msn4p bind to oligonucleotides containing STRE sequences. Gel retardation was performed with a ^{32}P-end labelled oligonucleotide derived from the *HSP12* promoter (nucleotides –241 to –221, HSP12–STRE). The proteins used were *in vitro* synthesized Msn2p (lanes 2–7) or an affinity-purified GST fusion containing the DNA binding domain of Msn4p (lanes 8–13). Mock-translated reticulocyte lysate was added in lane 1. Unlabelled oligonucleotides were used in a 200-fold excess as competitors: HSP12–STRE (lanes 3 and 9), mutated HSP12-STRE (lanes 4 and 10), CTT1–18 (lanes 5 and 11), DDR2 (lanes 6 and 12) and MIG1bs (lanes 7 and 13). See Materials and methods for details.

resistance) and, additionally, induces increased general stress resistance (cross protection). It has been shown that the HSF, through the HSE, is required for transcriptional activation of HSPs in response to heat and some other stress conditions (Morimoto *et al.*, 1992). Moreover, in yeast, several genes have been identified that do not contain HSEs whose transcription is induced by heat and other stress signals. Some of them are activated through STRE, a *cis* regulatory sequence that mediates response to many forms of stress (Ruis and Schüller, 1995; Varela *et al.*, 1995). It has been hypothesized that an UAS element of this type may function as a component integrating the signal that induces general stress resistance. This would imply that mutants lacking the factor(s) that bind to STREs would show an impaired resistance to different stress conditions.

In the course of the analysis of *msn2 msn4* mutants, increased sensitivity to carbon source starvation was observed. Further experiments showed that this defect is part of a pleiotropic sensitivity to stress factors. Resistance to unrelated stress types such as heat, oxidative and osmotic stress is reduced significantly in the double mutant. This increased sensitivity is restricted to severe acute stress and is not observed in stationary phase cells. All these phenotypes could be caused by absence of STRE-dependent transcription. In wild type cells, genes activated through this element are minimally expressed in logarithmic cells growing on glucose but are rapidly inducible by stress conditions, with their products likely to provide a first line of defence. Consistent with this physiological role we observed an improved general stress resistance and constitutive expression of STRE regulated genes under non-stress conditions in cells overexpressing Msn2p or Msn4p. On the other hand, activation of defence mechanisms independent of *MSN2* and *MSN4* would contribute to the high level of resistance observed in stationary phase cells. The apparent absence of a growth defect exhibited by the *msn2 msn4* double mutant under mild stress conditions suggests the existence of STRE independent mechanism(s) that allow growth under unfavourable conditions after adaptation (Miralles and Serrano, 1995).

At the molecular level, the analysis of expression of STRE regulated genes, such as *CTT1*, *HSP12* and *DDR2* confirmed that stress induced activation of these genes is defective in the *msn2 msn4* mutant. The transcriptional induction of *SSA3* under carbon source starvation in the mutant strain might be explained by STRE independent mechanism(s). Two types of regulatory element function in the *SSA3* promoter: the PDS element (T/AAGGGA; Boorstein and Craig, 1990) which is induced late in the yeast growth curve after the diauxic shift, resembles in sequence the STRE and is regulated by cAMP and the HSE which is able to mediate heat and glucose starvation induction (Tamai *et al.*, 1994). The defects in expression shown by the STRE-regulated genes in the *msn2 msn4* mutant are observed in all the stress conditions tested, suggesting a general role of *MSN2* and *MSN4* in the activation through STRE. This role is in agreement with the general stress tolerance defect shown by the *msn2 msn4* mutant. The *MSN2* and *MSN4* genes apparently have partly redundant functions in the stress response. We have found defects in tolerance and in STRE-dependent transcription in the *msn2* single mutant (results not shown)

but in all the cases the phenotype of the *msn2 msn4* double mutant was more severe than that of either mutant alone.

Two other lines of evidence support the idea that *MSN2* and *MSN4* are involved in the stress response through STRE. First, we have shown that transcriptional activation of a *LEU2–lacZ* reporter gene which is regulated via STREs is dramatically affected in the *msn2 msn4* double mutant. The basal activity of this reporter gene is reduced by a factor of 10 and induction is completely abolished in the case of heat shock, low pH, sorbate (at pH 6) and high ethanol concentration. From the pattern of transcription observed with the STRE reporter gene, one might ask whether the conditions which induce the molecular mechanisms that activate Msn2p and Msn4p belong to one or several pathways. Heat shock, low pH and high alcohol concentrations might have the common effect of denaturing proteins and therefore it is tempting to speculate that this effect might be the upstream event which activates these factors. Induction by a factor that resembles the wild type situation is still observed in oxidative (H_2O_2) and osmotically stressed cells and in cells grown on rich media with ethanol as a carbon source (derepression). Among the different MAP kinase pathways that have been described in yeast (Ammerer, 1994; Herskowitz, 1995; Ruis and Schüller 1995) only the HOG pathway affects transcription via STREs (Brewster *et al.*, 1993; Schüller *et al.*, 1994; Kamada *et al.*, 1995) and specifically transmits activation of STRE-dependent genes by osmotic stress. The situation in *S.cerevisiae* differs from the one in mammalian cells where MAP kinase pathways are activated by various types of stress (Han *et al.*, 1994; Kyriakis *et al.*, 1994; Rouse *et al.*, 1994). Our results reveal that osmotic induction dependent on the HOG pathway occurs in the *msn2 msn4* double mutant, suggesting that there must be several pathways that activate transcription via STREs and that additional factors may bind to the same element. The Ras–cAMP pathway negatively regulates STREs (Wieser *et al.*, 1991; Marchler *et al.*, 1993), however, the genetic relationship between *MSN2, MSN4* and protein kinase A remains to be elucidated.

Finally, evidence that Msn2p and Msn4p directly mediate STRE-dependent transcription was obtained by showing that these proteins bind to STRE sequences. According to the rules of DNA sequence recognition by Cys_2His_2 zinc fingers which are theoretically and experimentally well established (Choo and Klug, 1994), Msn2p and Msn4p specifically bind the sequence NAGGGG. This hypothesis was further strengthened by computer assisted molecular modelling. Using *in vitro* translated Msn2p and purified Msn2 and Msn4 proteins expressed in *E.coli* as GST fusions, we have shown that indeed both proteins are able to bind specifically to oligonucleotides that include STREs sequences. No binding was observed with a mutated oligonucleotide or with an oligonucleotide containing the Mig1 binding site, contrary to what it had been suggested (Estruch and Carlson, 1993).

MSN2 and *MSN4* were initially cloned as multicopy suppressors of a $snf1^{ts}$ mutant. However, the Snf1p protein kinase is apparently not involved in STRE regulation, suggested by the fact that stress induced activation of STREs is also observed in *snf1* mutants (C.Schüller and H.Ruis, unpublished observation). Why then were these genes cloned in a screen for *snf1* suppressors? We have observed that in yeast a complete absence of usable carbon source causes a metabolic arrest that greatly delays the ability to use raffinose or galactose as a carbon source (M.Martinez-Pastor and F.Estruch, unpublished results). We suggest that, during this crisis, activation of stress response genes improves resistance and that this effect would be facilitated by the overexpression of *MSN2* and *MSN4* genes.

Taken together these data underline our earlier hypothesis on the role of STREs in the establishment of general stress resistance via induction of transcription of several genes in response to various stress conditions. The STRE apparently is the integration point of parallel pathways which function through a set of transcription factors. The identification of specific transcription factors binding to STREs opens the avenue to a more detailed analysis of the pathways that transmit the different types of stress signals to this element.

Materials and methods

Yeast strains, media and genetic methods

Gene disruptions were produced in *Saccharomyces cerevisiae* strains W303-1A (MATa, *SUC2, ade2, can1, his3, leu2, trp1, ura3*) (Thomas and Rothstein, 1989) and GG18 (MATa, *leu2, ura3, his3, trp1, ade8, cta1-2, CTT1-18/7x-LEU2-lacZ*) (Marchler *et al.*, 1993). The *MSN2* and *MSN4* gene disruptions (*msn2-Δ3::HIS3* and *msn4-1::TRP1*) have already been described (Estruch and Carlson, 1993) and were introduced into W303-1A or GG18 to replace the wild type alleles (Rothstein, 1983), generating the mutant strains Wmsn2msn4 or GG18msn2msn4, respectively. The *pbs2::LEU2* gene disruption was introduced into GG18msn2msn4 strain as described in Schüller *et al.*, 1994. Standard methods were used for genetic analysis and transformation (Rose *et al.*, 1990).

Rich medium (YPD) is 1% (w/v) yeast extract, 2% bactopeptone and 2% dextrose. In experiments involving the strain GG18 and its derivatives, 2% meat peptone was used instead of bactopeptone. In YPE dextrose was substituted by 3% ethanol. Synthetic growth medium (S) is 0.67% yeast nitrogen base without amino acids (Difco) supplemented with adenine (20 mg/l), uracil (20 mg/l), tryptophan (20 mg/l), histidine (20 mg/l), leucine (30 mg/l) and lysine (30 mg/l) and 2% dextrose (SD) or 2% raffinose (SRaf). In the experiments with yeast transformants either uracil or tryptophan was omitted from the synthetic medium for plasmid maintenance. In experiments involving gene expression measurements the culture and stress conditions were applied as described in Schüller *et al.* (1994).

Plasmid constructions

The Msn2–GSTp fusion was produced in *E.coli* transformed with pZf32–GEX3x. To construct this plasmid, the C-terminal *Eco*RI fragment of *MSN2* (Estruch and Carlson, 1993) was fused to the glutathione *S*-transferase gene (GST) in plasmid pGEX-3x (Pharmacia). pG3MSN2 was constructed by subcloning the *Bgl*II (position –8 to the ATG codon) – *Hin*dIII (position +68 from the stop codon) fragment into the *Bam*HI site of pG-3 (Schena *et al.*, 1991). pG3MSN4 contains the *Hin*dII–*Eco*RI fragment containing the *MSN4* sequence (Estruch and Carlson, 1993) in the *Bam*HI site of pG-3. To construct pMM1, the *Bgl*II–*Hin*dIII fragment containing the *MSN2* open reading frame was cloned into the polylinker of Bluescript SK vector (Stratagene). Plasmids pZfh45–GEX3x, pEL32 and pEL45 have been described in Estruch and Carlson (1993).

Viability measurements

Viability was measured by plating the appropriate dilution of cells on YPD plates, in duplicate, and expressed as a percentage of the initial colony-forming units, measured before the stress shock. The viability experiments were repeated at least three times, yielding similar results. We have observed that resistance to stress of cells grown on minimal medium was repeatedly higher compared with cells grown on rich medium (compare Figures 1A and 5A). This result suggests that growth

on minimal medium involves exposition to mild stress conditions and results in the development of a certain degree of basal tolerance against severe stress.

Preparation and Northern analysis of RNA

After being subjected to different stress conditions, cells were harvested, washed with RNase-free water and frozen at –70°C. Cells were broken by vortexing with glass beads in LETS buffer [0.1 M LiCl, 0.01 M Na_2–EDTA, 0.01 M Tris–HCl (pH 7.4), 0.2% SDS] and one volume of LETS saturated phenol. After two extractions with phenol:chloroform:isoamylic alcohol, the RNA was precipitated with one volume of 5 M lithium chloride or with two volumes 80% ethanol and stored at –70°C for at least 2 h. The precipitate was washed with 70% ethanol and resuspended in RNase-free water. The amount of RNA was quantified by measuring the absorbance at 260 nm. RNA was fractionated on agarose gels containing formaldehyde (Maniatis *et al.*, 1982) and blotted on Hybond-N (Amersham) membrane.

The following gene probes were used: a 0.5 kb *Eco*RI *HSP12* cDNA fragment (Praekelt and Meacock, 1990), a 1.4 kb *Sph*I–*Bgl*II *HSP26* fragment obtained from plasmid pVZ26 (Susek and Lindquist, 1990), an *Eco*RV–*Bam*HI fragment of *SSA3* obtained from plasmid SSA3H (Werner-Washburne *et al.*, 1987), the 1.3+1.1 kb *Eco*RI fragments of *CTT1* from plasmid pRB322-5109 (Spevak *et al.*, 1983), a 1.5 kb *Hin*dIII fragment of *DDR2* from plasmid pBRA2 (McClanahan and McEntee, 1986) and a 0.9 kb *Hin*dIII–*Xho*I fragment of *ACT1* from plasmid pYA301 (Gallwitz and Sures, 1980).

Enzyme activities

β-Galactosidase activity of crude extracts prepared by breakage of yeast cells with glass beads was assayed using *o*-nitrophenyl-β-D-thiogalactoside as substrate (Miller, 1972; Rose and Botstein, 1983). Protein concentrations were assayed at 280 nm as described by Layne (1957).

Band shift assays

For *in vitro* synthesis of Msn2p, *Apa*I-linearized pMM1 DNA (5 μg) was transcribed with T3 polymerase. RNA synthesis and capping were carried out as described previously (Estruch and Carlson, 1990). RNA was translated with [^{35}S] methionine in a rabbit reticulocyte lysate (Promega) according to the instructions of the supplier. Expression of the Msn2–GST and Msn4–GST fusion proteins from plasmids pZf32–GEX3x and pZfh45–GEX3x respectively, in *E.coli*, and protein purification, were performed as described in Smith and Johnson (1988).

A synthetic double-stranded oligonucleotide containing *HSP12* sequences –221 to –241 (5′-CGA TTT TCC AGG GGC TGT A-3′) was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim). As competitors the same non-labelled oligonucleotide, an oligonucleotide derived from this containing a single base mutation in the STRE sequence (5′-CGA TTT TCC AGT GGC TGT A-3′), oligo CTT1-18 (containing CTT1 sequences from –368 to –356), oligo DDR2 (containing DDR2 sequences from –165 to –189) and oligo MIG1bs containing the Mig1p DNA binding site (5′-GGT AAA AAT GCG GGG AA-3′) were used. Binding reactions were carried out in 10 μl volumes containing buffer 30 mM Tris–HCl (pH 7.5), 90 mM KCl, 7 mM $MgCl_2$, 1 mM DTT, 10 mM $ZnSO_4$, 4% Ficoll and 1 μg poly (dI-dC) (Pharmacia). After addition of the protein (0.4 μg of fusion protein or 2 μl of reticulocyte lysate) and the non-labelled competitors (in a 200-fold excess), the reactions were incubated at room temperature for 15 min. Approximately 0.2 ng/lane of radiolabeled probe was added, and the reaction was incubated for an additional 15 min at room temperature. The reaction mixtures were loaded immediately onto 7% polyacrylamide gels (acrylamide to bisacrylamide weight ratio of 40:1), 4% glycerol in 0.5× TBE. Gels were pre-electrophoresed for 1 h at 20 mA in 0.5× TBE buffer at 4°C and, after loading of the samples, electrophoresed at 20 mA at 4°C until the bromophenol blue reached three quarters of the gel. The gels were then transferred to Whatman 3M, dried and autoradiographed.

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