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The Skn7 Response Regulator of *Saccharomyces cerevisiae* Interacts with Hsf1 In Vivo and Is Required for the Induction of Heat Shock Genes by Oxidative Stress

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Submitted January 21, 2000; Revised May 1, 2000; Accepted May 12, 2000

Monitoring Editor: Elizabeth Craig

INTRODUCTION

Cells must survive challenges from the environment with regard to heat, UV radiation, and heavy metals as well as tolerate the endogenous generation of reactive oxygen intermediates during respiration. Oxygen, in the form of superoxide anion (O_2^-), hydroxyl ion (OH^-), and hydrogen peroxide (H_2O_2), causes damage to nucleic acids, cell membranes, and proteins (reviewed by Halliwell, 1994). Yeast, in common with all other organisms, has evolved protective mechanisms to survive in the presence of these by-products of aerobic metabolism and can mount distinct adaptive responses to different sources of oxidative stress (Jamieson, 1992; Ruis and Schüller, 1995). For example, the Cu,Zn-linked superoxide dismutase, encoded by the *SOD1* gene, detoxifies superoxide anion to hydrogen peroxide; catalase, encoded by the cytosolic *CTT1* gene, can catalyze the breakdown of hydrogen peroxide. Other free radical scavengers in the cell include glutathione, ascorbic acid, and thioredoxin. The *SKN7* gene was initially isolated as a multicopy suppressor of a *kre9Δ* mutation, which results in defective cell wall biosynthesis (Brown et al., 1993). The *SKN7* gene was also cloned as *POS9* in a screen for mutants resulting in sensitivity to hydrogen peroxide (Krems et al., 1995), suggesting a role for *SKN7* in the yeast oxidative stress response. The oxidative stress induction of the small antioxidant molecule thioredoxin, encoded by the *TRX2* gene, was subsequently shown to be regulated by *SKN7* and the yeast AP-1 homologue *YAP1*, both of which bind to distinct sites within the *TRX2* promoter (Kuge and Jones, 1994; Morgan et al., 1997).

Of the many proteins that are induced under adverse environmental conditions, perhaps the best understood are the heat shock proteins (Lindquist and Craig, 1988; Mager and De Kruijff, 1995). The major heat shock proteins have
been classified according to their molecular weight: Hsp104, the Hsp90 and Hsp70 families, Hsp60, Hsp26, and Hsp12 (Craig, 1992; Mager and Ferreira, 1993). The Hsp70 family of heat shock proteins act as molecular chaperones; this family contains at least five heat-inducible isoforms and others that are expressed constitutively at high levels (Craig, 1992; Rassow et al., 1997). Their principal role includes the transport and folding of polypeptides and the solubilization of denatured proteins (Craig et al., 1994). Hsp104 is thought to protect the cell during exposure to lethal heat shock and is required for cross-protection against a variety of stress conditions (Sanchez et al., 1992), although its exact function remains unclear.

The induction of these genes in response to heat shock is mediated by Heat Shock Factor, encoded by the essential HSFL gene (Sorger and Pelham, 1988). The Hsf1 protein binds to heat shock elements (HSEs) consisting of tandem inverted repeats of the sequence AGAAn (where n is any nucleotide) found in the promoters of many heat shock genes (Fernandes et al., 1995). In Saccharomyces cerevisiae, Hsf1 binds HSEs as a trimeric complex: constitutively to high-affinity sites (Gross et al., 1990) and inducibly to low-affinity sites (Giardina and Lis, 1995; Erkine et al., 1999). The protein undergoes extensive phosphorylation on serine and threonine residues upon heat shock. This posttranslational modification has been correlated with its transcriptional activation (Sorger and Pelham, 1988; Sorger, 1990). However, evidence has also been presented to suggest that hyperphosphorylation of serine residues located between the transcriptional and C-terminal activation domains is involved in the deactivation of Hsf1 (Ho and Jakobsen, 1994). In addition to HSEs, a number of stress-responsive genes also contain stress-responsive elements (STREs) within their promoters, through which the Zn-finger transcription factors Msn2 and Msn4 can activate stress gene expression in response to a variety of stress conditions independent of Hsf1 (Schüller et al., 1994; Martínez-Pastor et al., 1996).

In addition to its role in the heat shock response, Hsf1 has also been shown to protect the cell against heavy metals, such as copper and cadmium, through its activation of the copper metallothionein gene CUP1 (Silar et al., 1991; Sewell et al., 1995). Hsf1 becomes phosphorylated in response to the superoxide generator menadione. This modification correlates with transcriptional activation of CUP1 by oxidative stress (Liu and Thiele, 1996). Hence, Hsf1 also plays a critical role in the cell’s defense against oxidative stress. Because our previous work had established a role for the Skn7 response regulator in oxidative stress protection (Morgan et al., 1997), it seemed possible that Skn7 and Hsf1 share overlapping functions.

The Skn7 protein contains a region with a high degree of homology to the receiver domain of bacterial two-component systems, a class of proteins involved in signal transduction in bacteria and lower eukaryotes (Stock et al., 1989; Parkinson, 1993). Thus, a membrane-bound sensor histidine kinase can phosphorylate a conserved aspartate residue within the receiver domain of its cognate response regulator. This phospho-aspartate form of the response regulator can then carry out a function appropriate to the incoming signal, usually the transcriptional activation of a specific set of genes. A conserved aspartate, residue D427 in Skn7, has been shown to be required for the function of the protein in cell wall biosynthesis (Brown et al., 1993) and the activation of G1 cyclin expression (Morgan et al., 1995). However, phosphorylation of this residue does not appear necessary for the role of SKN7 in the oxidative stress response (Morgan et al., 1997).

The Skn7 protein also contains a C-terminal glutamine-rich region consistent with a possible role in transcriptional activation (Brown et al., 1993; Morgan et al., 1995). Toward the N terminus there is a region of extensive homology to the helix-turn-helix DNA-binding domain of Hsf1 (Figure 1). This domain is separated from the receiver motif by a region of coiled-coil structure, again similar to the leucine zipper domain of the yeast Hsf1. Given the degree of conservation in the structures of both the DNA-binding domain and the leucine zipper region of the Skn7 and Hsf1 proteins (Figure 1), it was of interest to determine whether Skn7 interacted with Hsf1 and to establish the significance of these interactions in the yeast stress response.

We present evidence here for both genetic and direct biochemical interaction between Hsf1 and the Skn7 response regulator. Furthermore, we show that a protein other than Heat Shock Factor, Skn7, can bind to HSEs in vitro, is localized to the nucleus under normal and oxidative stress growth conditions, and is required for the full induction of heat shock genes in response to oxidative stress.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions**

The yeast strains used were as follows: W303-1a (a ade2-1 trp1-1 can1-100 leu2-3112 his3-11 ura3-52); W303-1a skn7Δ (a ade2-1 trp1-1 can1-100 leu2-3112 his3-11 ura3 skn7Δ::HIS5); MYY290, a ura3 derivative of strain AH216 (a leu2 his3 phoC phoE); MY385 (a leu2 his3 ura3 phoC phoE hisf1::HIS3) (Smith and Yaffe, 1991); and DR20-2b, which was obtained as a haploid HIS+ spore clone from a cross of MY385 and W303 skn7Δ. Minimal and rich media for yeast propagation have been described previously (Sherman et al., 1996).

**β-Galactosidase Assays**

The vector pZJHSE2-137 (a gift from E. Craig, University of Wisconsin, Madison, WI) containing a region of the SSA1 promoter responsible for heat shock activation of HSP70 cloned into the 2μ-based lacZ fusion plasmid pLG60 was transformed into W303-1a and the isogenic skn7Δ strain. Transformants were grown to midlog phase in selective medium at 30°C and harvested before or after the addition of t-butyl hydrogen peroxide to 0.6 mM for 1 h. For the heat shock experiment, cells were initially grown in selective minimal medium at 25°C, and cells were harvested before and 1 h after the culture was shifted to a 37°C water bath. Cell extracts were prepared as described previously (Guarente and Mason, 1983). Units of activity are given as the change in OD420 per minute per milligram of total protein. Values represent the average of duplicate samples in two independent experiments.

**Hydrogen Peroxide Sensitivity Assays**

Strains were tested for sensitivity to hydrogen peroxide by taking a suspension of cells in water and making a single streak of the suspension from the edge to the center of the plate, which contained a disk of 3MM paper onto which was placed 0.5–2 μl of t-butyl hydrogen peroxide (Sigma Chemical, St. Louis, MO). The t-butyl hydrogen peroxide was allowed to diffuse freely throughout the agar, and the extent of growth inhibition from the center point of the plate was taken as a measure of the sensitivity of a given strain to oxidative stress.
Thermotolerance and Viability Assays

Midlog-phase cultures were grown in rich glucose medium (YPD) at 25°C, and an aliquot was shifted to a test tube placed in a 51°C water bath. Samples were taken at regular intervals, diluted into ice-cold YPD, and immediately plated onto YPD agar to assess cell viability. Survival was determined after 3 days of growth at 30°C and expressed as percentage viability compared with cells that were maintained at 25°C throughout the experiment. Cell counts were performed in duplicate, and values correspond to the average of two experiments.

Plasmid Constructions

CEN SKN7 and D427N-SKN7 plasmids were constructed by inserting a 3.8-kilobase (kb) XbaI-SacI fragment of pBAM1 or pBAM2 (Morgan et al., 1997), which contains the entire SKN7 coding and promoter regions, into the multiple cloning site of YCplac111 (Gietz and Sugino, 1988). YexH is a modified derivative of the 2μ-based galactose-inducible expression plasmid pEMBLYe4 (Murray, 1987) in which a six-histidine tag sequence was inserted upstream of the multiple cloning site to allow the N-terminal tagging of inserted genes. YexH-SKN7 was constructed by inserting a 2-kb BamHI fragment containing the SKN7 coding region into the BamHI cloning site of the vector, which allows galactose-inducible expression of the 6His-tagged protein. Plasmid p426GAG (PGAL1-GST, URA3, 2μ) is based on the high-copy-number yeast vector pRS426 (Christianson et al., 1992) and was used to generate a GST-tagged Hsf1 as follows. A PCR strategy was used to generate a 2.5-kb XhoI–EcoRI fragment from plasmid pAKS80 (gift from A. Sewell, University of Utah, Salt Lake City, UT) that contained the entire HSF1 genomic sequence. This fragment was inserted into XhoI–EcoRI-digested plasmid p426-GAG to produce pGST-HSF1. The plasmid pGAL-HA-SKN7 was obtained by inserting a 2-kb BamHI fragment of the SKN7 gene from YexH-SKN7 into plasmid YCP16 (Foreman and Davis, 1994). The integrating epitope-tagged form of SKN7 was generated by inserting the 2-kb BamHI SKN7 fragment into the unique BamHI site of the integrating vector pRS306-Myc, which is based on the vector pRS306 (Christianson et al., 1992) into which has been inserted six copies of the Myc epitope tag (a gift from D. Fesquet, NIMR, London, UK). The SKN7-GFP plasmid was made by inserting a PCR-generated XhoI-ended SKN7 fragment into the unique XhoI site of plasmid pRS416-sGFP-Nuf2T (a gift from the P. Silver laboratory, Harvard University, Boston, MA).

Fluorescence Microscopy

GFP was detected in unfixed cells with a Nikon (Garden City, NY) Optiphot-2 equipped with a MicroMax charge-coupled device camera (Princeton Instruments, Princeton, NJ). Images were taken at 100× magnification and converted to Photoshop version 4.0 format.
RNA Analysis

Northern hybridization was performed as described previously (White et al., 1986). In all cases, probes for hybridization to heat shock genes used in this study were derived from PCR amplification of an internal fragment of the coding sequence of the gene, either from genomic DNA or from plasmids containing the gene of interest. The internal control used for mRNA quantitation in hydrogen peroxide- and heat shock-treated cells was ACT1, the abundance of which was found to be relatively insensitive to these conditions.

Protein Extraction and Pull-Down Experiments

Yeast cell breakage was achieved through repeated vortexing with glass beads for 5 × 30 s with 30-s rests on ice in breakage buffer (150 mM NaCl, 1% NP40, 50 mM Tris-HCI, pH 7.5, 1 mM EDTA, 10 mM NaF, 50 mM β-glycerol phosphate). At time of use, a protease inhibitor mixture was added to a final concentration of 100 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 50 μg/ml Nα-p-tosyl-L-lysine chloromethyl ketone, and 100 μg/ml t-1-tosylamide-2-phenylethyl chloromethyl. Cleared lysates were prepared by centrifugation for 20 min at 18,000 rpm (Beckman [Fullerton, CA] SS34 rotor), and 1 mg of whole cell extract was incubated at 4°C with 50 μl of a 50% suspension of glutathione–Sepharose beads (Pharmacia, Piscataway, NJ) equilibrated in breakage buffer in a total volume of 250 μl. The beads were then harvested and washed four times in breakage buffer containing 200 mM NaCl, followed by one wash in the same buffer with 50 mM NaCl, and finally resuspended in an equal volume of 2× SDS sample buffer. Coinmunoprecipitations with 9E10 mAb were performed by incubating 500 μg of cell extract with 2 μg of anti-myc mAb (Berkeley Antibody, Berkeley, CA) for 1 h, and precipitates were recovered by incubation with continuous mixing at 4°C for 1 h, and precipitates were recovered by incubation with continuous mixing at 4°C for 1 h, and precipitates were recovered by incubation with continuous mixing at 4°C for 1 hour, and precipitates were recovered by incubation with continuous mixing at 4°C for 1 min. Western analysis was performed in accordance with Western analysis was performed in accordance with Western analysis was performed in accordance with Western analysis was performed in accordance with Western analysis was performed in accordance with Western analysis was performed in accordance with manufacturers’ guidelines (Amersham), and membranes were exposed to X-ograph XB-200 film (Eastman Kodak, Rochester, NY) for between 30 s and 5 min.

For in vitro association assays, 1 mg of cell extract prepared as described above (with the omission of EDTA from the breakage buffer) was added to 200 μl of Ni2+-nitrilotriacetic acid (NTA) resin (50% slurry) equilibrated in breakage buffer. After incubation with mixing at 4°C for 1 h, the resin was washed four times in wash buffer (200 mM NaCl, 50 mM Tris-HCI, 1% NP40) and once in wash buffer containing 50 mM NaCl. Beads were then boiled for 2 min in 2× sample buffer, and the supernatant was subjected to SDS-PAGE as described above.

Purification of 6His-Skn7 and Mobility Shift Assays

A 2-kb BamHI fragment of the original SKN7 genomic clone in YEp24, which contains the entire SKN7 ORF (Morgan et al., 1995), was inserted into plasmid pQE-30 (Qiagen, Chatsworth, CA). Transformants DH-5α (minimum 2 l) was grown to OD600 = 0.5 at 37°C and then brought to 25°C by brief incubation on ice before induction by addition of isopropyl-1-thio-β-D-galactoside to 1 mM for 5 h at 25°C. Cells were harvested by centrifugation (5 min, 3000 rpm, GSA Sorval rotor, Kendro Laboratory Products, Newtown, CT), washed in cold distilled water, and resuspended in 2–5 ml of breakage buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 10% glycerol, 0.5% NP40). At the time of use, lysozyme was added at 1 mg/ml and PMSF at 1 mM. The cell suspension was incubated on ice for 30 min and then passed twice through a chilled French press chamber, and the clarified supernatant was then incubated with 5 ml of a 50% slurry of Ni2+-NTA resin, equilibrated in binding buffer (250 mM NaCl, 50 mM Tris-HCI, pH 7.5, 15 mM imidazole), and allowed to mix at 4°C for 1 h before the material was packed into a 5-ml column. After washing in binding buffer, tagged protein was eluted by a step gradient of binding buffer containing 50, 100, and 250 mM imidazole. Bradford protein assays (Bio-Rad, Richmond, CA) were carried out on 0.5-ml fractions, and DNA-binding activity was assayed by gel mobility shift assay.

Mobility shift assays have been described elsewhere (Lowndes et al., 1991). 6His-Skn7 protein was incubated with 0.5 mg (1 × 107 cpn) of 32P 5’-end-labeled double-stranded oligonucleotides of the following sequences: HSE2, 5’ tgcATTTTCCAAGGGCTTCCATGGC; MUT HSE, 5’ tgcATTTTCCAAAGGCTTCCATGGC. Binding reactions in 25 mM Tris-HCI, pH 7.5, 100 mM NaCl, 1 mM EDTA, 7 mM MgCl2, 10% glycerol, protease mixture as described above, and 1 μg of poly(dIdC) nonspecific competitor DNA were incubated at room temperature for 15 min and on ice for another 20 min. Protein–DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel (37.5:1) by electrophoresis at 200 V in 0.5× TBE buffer (89 mM tris base, 89 mM boric acid, 2 mM EDTA) for 2 h. Gels were dried overnight on X-Omat AR film at −20°C.

**RESULTS**

**Skn7Δ Cells Are Sensitive to Acute Heat Stress**

Deletion of the SKN7 gene does not confer a temperature-sensitive phenotype when cells are shifted from 25 to 37°C (Morgan et al., 1995). However, given the high degree of homology between the DNA-binding domains of Skn7 and Hsf1, we further investigated the effect of an skn7Δ mutation on cell viability under acute heat shock at 51°C. Cells deleted for the SKN7 gene were found to be some 10 times more sensitive to the lethal effects of acute heat stress than the isogenic wild-type strain (Figure 2). It has been reported that the main cause of cell death under these conditions is the generation of toxic intermediates of oxygen metabolism (Davidson et al., 1996). Because SKN7 has been shown to be required for cell survival under conditions of oxidative stress (Krems et al., 1996; Morgan et al., 1997), it was of interest to determine whether SKN7 has a role, together with HSF1, in the induction of heat shock gene expression in response to oxidative stress.

**SSA1-LacZ Induction by Hydrogen Peroxide Requires SKN7**

To investigate the potential role of the SKN7 gene in the induction of heat shock protein expression, we assessed the expression of an Hsp70-LacZ reporter construct in wild-type and skn7Δ cells. The SSA1 gene encodes a major isoform of the yeast Hsp70 protein that is abundant under nonstressed conditions and is strongly induced by heat shock (Craig, 1992). The plasmid pJZHSE2-137 contains an HSE, HSE2, from the SSA1 promoter fused to the β-galactosidase coding sequence (Slater and Craig, 1987; Park and Craig, 1989). The HSE2 sequence is responsible for the majority of both basal and heat shock–induced expression of SSA1 (Slater and Craig, 1987). β-Galactosidase assays were carried out on wild-type W303-1a and isogenic skn7Δ cells containing the reporter plasmid after treatment for 1 h with hydrogen peroxide.
Peroxide. In wild-type cells, an eightfold induction of \( \beta \)-galactosidase activity was observed 1 h after the addition of \( t \)-butyl hydrogen peroxide (Table 1). In the \( \text{skn7} \Delta \) strain, basal expression of HSE2-\( \text{LacZ} \) activity was reduced and induction in response to oxidative stress was abolished. However, induction of HSE2-\( \text{LacZ} \) activity in response to a temperature shift from 25 to 37°C was unaffected in the \( \text{skn7} \Delta \) strain, although the overall level of expression in the \( \text{skn7} \Delta \) cells was reduced. The decreased basal expression of HSE2-\( \text{LacZ} \) in the \( \text{skn7} \Delta \) strain extends previous results that indicated a similar reduction in basal expression levels of an \( \text{SSA1-LacZ} \) reporter construct in response to an HSE2 double point mutation (Park and Craig, 1989). Thus, HSE2 appears to be critical for both basal and stress-induced expression of the \( \text{SSA1} \) gene. It has been proposed that Yap1, which has been shown to interact with Skn7 at the \( \text{TRX2} \) promoter (Morgan et al., 1997), is also required for the induction of HSE2-\( \text{LacZ} \) activity in response to hydrogen peroxide (Stevens et al., 1995). However, in our genetic background, we found no evidence that yap1\( \Delta \) affects the hydrogen peroxide induction of the \( \text{SSA1} \) HSE2-\( \text{LacZ} \) reporter construct (D.C. Raitt, unpublished observations). Although dispensable for heat shock induction, our data suggest that Skn7 may be required for full induction of HSE-driven gene expression in response to free radical stress.

**Skn7 Can Specifically Bind the HSE2 Element from the \( \text{SSA1 Promoter} \)**

The DNA-binding domain of Skn7 is highly homologous to that of Hsf1 (Figure 1B). To determine whether Skn7 can recognize and bind specifically to the same recognition sequence as Hsf1, we performed electrophoretic mobility shift assays (EMSA) with *Escherichia coli*-expressed 6His-Skn7. The \( \text{SKN7} \) gene was inserted into plasmid pQE-30, and the 6His-Skn7 fusion protein was subsequently purified on an \( \text{Ni}^{2+} \)-NTA agarose affinity column (see MATERIALS AND METHODS). As demonstrated by EMSA, the 6His-Skn7 fusion protein binds specifically to the 26-base pair sequence encompassing the HSE2 region of the \( \text{SSA1} \) promoter (Figure 3). To confirm the presence of the 6His-Skn7 protein in the retarded complex, polyclonal antiserum to the protein was added to the band shift reaction mixture. The retarded complex formed with the HSE2 probe and the 6His-Skn7 protein was super-shifted by the anti-Skn7 antibody, whereas no effect was observed with the addition of preimmune serum at the same concentration.

To determine the specificity of this binding, competitive binding assays were performed with the native HSE2 oligonucleotide and a mutated probe, \( \text{MUT-HSE2} \), in which the G and C positions of the consensus HSE sequence AGAAnnT-TCn were changed to A and T, respectively. These mutations within the consensus have previously been shown to abolish binding of Hsf1 (Park and Craig, 1989). The native 26-mer HSE competes efficiently for the binding of Skn7 at a 10-fold molar excess (Figure 3). However, the mutated version of the HSE, \( \text{MUT-HSE2} \), does not compete for binding of the 6His-Skn7 protein at up to 100-fold molar excess. Similar results have been seen with an *E. coli*-expressed Skn7 derivative consisting of the DNA-binding domain alone fused in frame to GST and also with full-length 6His-Skn7 protein purified from yeast (our unpublished results). These results demonstrate that Skn7 binds to HSEs with a specificity similar to that of Hsf1.

**Induction of Heat Shock Gene Expression by Hydrogen Peroxide Requires Skn7**

We have previously shown that Skn7 cooperates with the yeast AP-1 homologue Yap1 in the oxidative stress induction of the \( \text{TRX2} \) gene (Morgan et al., 1997). Given the defect in hydrogen peroxide–mediated induction of HSE-\( \text{LacZ} \) expression in \( \text{skn7} \Delta \) cells relative to isogenic wild-type cells

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**Table 1. Induction of \( \text{SSA1-LacZ} \) in response to \( t \)-butyl hydrogen peroxide requires Skn7**

<table>
<thead>
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<th>Strain</th>
<th>Hydrogen peroxide (0.6 mM)</th>
<th>Heat shock</th>
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<td>25°C</td>
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<tr>
<td>( \text{W303-1a} )</td>
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<td>( \text{W303-1a skn7} \Delta )</td>
<td>0.04</td>
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</table>

Wild type and isogenic \( \text{skn7} \Delta \) cells containing pZJHSE2-137 were assayed for \( \beta \)-galactosidase levels before (–) and after (+) the addition of \( t \)-butyl hydrogen peroxide to 0.6 mM for 1 h. For the heat shock experiment, cells were grown at 25°C and transferred to 37°C for 1 h. \( \beta \)-Galactosidase activity is expressed as change in \( \text{OD}_{420} \) per minute per milligram of protein. Values are averages of duplicate samples from two independent experiments.
described above, we explored the possibility that Skn7 could have a role in the induction of heat shock genes in response to oxidative stress. Therefore, Northern analysis was carried out on a number of heat shock genes in W303-1a and W303-1a skn7Δ. In wild-type cells, the genes encoding HSP12, HSP26, and HSP104 were found to be strongly induced by t-butyl hydrogen peroxide (Figure 4A). However, the 5-fold induction of HSP12 in response to hydrogen peroxide was practically abolished by the skn7Δ deletion. Similarly, the 18-fold induction of HSP26 was reduced significantly in skn7Δ cells, and the 9-fold induction of HSP104 was again reduced by the skn7Δ mutation. These heat shock genes, therefore, appear to be dependent on the Skn7 response regulator for their full induction in response to hydrogen peroxide-mediated oxidative stress. Furthermore, evidence for a role of Skn7 in the oxidative stress induction of other heat shock proteins (SSA1 and HSP82) has recently been presented (Lee et al., 1999); however, in the W303-1a genetic background, we found no significant induction of any Hsp70 gene (SSA1, SSA2, SSA3, or SSA4) or of HSP82 in response to 0.6 mM t-butyl hydrogen peroxide (our unpublished results).

To determine whether Skn7 also contributed to heat shock-induced expression, we performed Northern analysis of heat-shocked W303-1a and isogenic skn7Δ cells. The kinetics of induction of seven genes (HSP12, HSP26, SSA1, SSA3, SSA4, HSP82, and HSP104) were found to be virtually indistinguishable between wild-type and skn7Δ cells (Figure 4B; our unpublished results). Skn7, therefore, is specifically required for the oxidative stress induction of heat shock genes. This is in accord with our observations that skn7Δ cells show no increased sensitivity upon a temperature shift from 25 to 37°C compared with the isogenic wild-type strain (D.C. Raitt, unpublished observations).

**Genetic Interactions between SKN7 and HSF1**

To explore the possibility that SKN7 and HSF1 may interact in vivo, we took a genetic approach and determined whether deletion of the SKN7 gene combined with a conditional mutation in HSF1 would result in a synthetic phenotype. Although the gene encoding yeast heat shock factor is essential, an hsf1 temperature-sensitive allele, hsf1-m3, has been isolated (Smith and Yaffe, 1991). Thus, an skn7Δ derivative of W303-1a was crossed with the hsf1Δ strain MYY385, and an hsf1Δ spore clone containing the HIS3−marked skn7Δ deletion, strain DR20-2b, was selected for further study. Both the hsf1Δ strain and strain DR20-2b were then tested for growth at various temperatures. As expected, both strains grew at the permissive temperature of 25°C and neither grew at the restrictive temperature of 37°C (our unpublished results). However, at an intermediate temperature of 33°C, the hsf1Δ strain formed colonies but the double mutant, DR20-2b, failed to grow (Figure 5A). Because these strains were not isogenic, strain DR20-2b was transformed with a CEN version of the SKN7 gene. This restored growth at 33°C (Figure 5A), indicating that the increased temperature sensitivity of DR20-2b is due specifically to the deletion of the SKN7 gene rather than to genetic background effects. Thus, deletion of SKN7 exacerbates the growth defect of the hsf1Δ strain.

We then assessed whether overexpression of the SKN7 gene could suppress the growth defect associated with the hsf1Δ allele at higher temperatures. The hsf1Δ strain was transformed with the high-copy vector Yep24-SKN7 or the empty vector alone. The hsf1Δ cells expressing high levels of Skn7 displayed strong growth at a temperature of 35°C, whereas the hsf1Δ strain containing the empty vector alone could not form colonies at this temperature (Figure 5B). However, the high-copy expression of SKN7 failed to rescue the hsf1Δ strain at 37°C (our unpublished results). Given the pleiotropic nature of the hsf1-m3 allele, which causes defects in mitochondrial protein import, reduces heat shock gene induction in response to increased temperatures, and leads to specific defects in cell cycle progression (Smith and Yaffe, 1991), the absence of suppression at 37°C is perhaps not surprising. Although there is clearly some overlap between the functions of SKN7 and HSF1 in the cell, the Skn7 response regulator cannot completely substitute for Hsf1 and thus fails to rescue the hsf1-m3 mutation at 37°C.

Both HSF1 and SKN7 have been shown to play a role in the activation of stress-responsive gene expression under conditions of free radical stress (Krems et al., 1996; Liu and...
Figure 4. Skn7 is required for the induction of heat shock gene expression specifically in response to hydrogen peroxide. (A) Northern blot analysis of the effect of skn7Δ mutations on heat shock gene induction by oxidative stress. Total RNA was prepared from midlog-phase cultures of W303-1a and W303-1a skn7Δ grown at 30°C in YPD. Samples for RNA extraction were taken before (time 0) and at the times indicated after the addition of 0.6 mM t-butyl hydrogen peroxide. Northern blots were prepared as described (see MATERIALS AND METHODS) and hybridized to probes specific for HSP12, HSP26, and HSP104. (B) Skn7 is not required for the heat shock induction of heat shock gene expression. W303-1a and skn7Δ cells were grown to midlog phase at 25°C, and a portion of the culture was transferred to a 39°C water bath. Cells were harvested at the times indicated, and RNA was extracted for Northern hybridization with the probes specified in A. Quantitation of mRNA was by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) and was expressed relative to the ACT1 transcript, the abundance of which appeared to be unaffected by oxidative stress.
Thiele, 1996; Morgan et al., 1997). Therefore, we wished to establish whether they might somehow cooperate in the yeast oxidative stress response. We assessed the sensitivity to oxidative stress of the hsf1Δ, skn7Δ, and skn7Δ hsf1Δ strains by means of a standard plate assay (see MATERIALS AND METHODS). The hsf1Δ mutation alone caused a modest increase in hydrogen peroxide sensitivity relative to the wild-type strain MYY385 (Figure 6), and a more pronounced effect was observed with the skn7Δ mutation compared with its wild-type parent, W303-1a. However, the skn7Δ hsf1Δ strain showed an enhanced hypersensitivity to hydrogen peroxide stress relative to either the skn7Δ or the hsf1Δ strain alone (Figure 6). Introduction of SKN7 on a CEN plasmid reversed this increased sensitivity, restoring it to that of the hsf1Δ strain, indicating that the phenotype was not due to genetic background effects. Confirming this result, an isogenic strain containing the skn7Δ deletion in an hsf1Δ background was also found to be hypersensitive to oxidative stress relative to either single mutant alone (A.L. Johnson, unpublished results). The additive effect of these mutations on stress sensitivity suggests that Skn7 and Hsf1 might in some way cooperate in the response to free radical stress.

In a complementary approach, we found that the W303-1a skn7Δ cells containing a high-copy plasmid with multiple HSE inserts exhibited significantly enhanced hydrogen peroxide sensitivity (D.C. Raitt, unpublished results). By presenting the cell with multiple copies of the binding site for Hsf1, the protein may have been competed away from its native binding sites, thereby decreasing its overall activity in the cell. Thus, when Hsf1 activity is depleted, either through competition for binding sites or by mutation, the survival of skn7Δ cells under stress is further compromised. This observation further supports the proposal that both Skn7 and Hsf1 contribute to cell survival during oxidative stress, perhaps through a related or shared pathway.

Previous data concerning the function of Skn7 indicated that phosphorylation of the conserved aspartate (D427) within the receiver domain was not required for survival under conditions of oxidative stress (Morgan et al., 1997). Significantly, an episomal D427N version of SKN7 was also found to reverse the hypersensitivity of DR20-2b to hydrogen peroxide (Figure 6). Furthermore, the Skn7D427N mutant also restored HSF2-LacZ expression in skn7Δ cells (our unpublished results), suggesting that phosphorylation of D427 is not required for Skn7p function through binding HSEs. In contrast, Skn7D427N fails to activate CLN1 and CLN2 expression in a swi4Δ swi6Δ background (Morgan et al., 1995) or to rescue the cell wall assembly defect of the kre9Δ mutant (Brown et al., 1994). Hence, phosphorylation of the receiver domain, presumably by the SLN1-YPD1 phosphohistidine relay (Li et al., 1998; Posas et al., 1996), can direct the activation of Skn7 function to different target genes.

Skn7 and Hsf1 Interact Physically

To extend these data suggesting a genetic interaction between Skn7 and Hsf1, we undertook co-purification analysis to determine if these proteins interact physically. Thus, the SKN7 gene was placed under the control of the GAL1 promoter in plasmid YCP16 (Foreman and Davis, 1994) and fused in frame to the hemagglutinin (HA) epitope, and the HSF1 gene was fused in frame to the GST epitope in the high-copy expression plasmid p426-GAG. Cell extracts were prepared from galactose-induced cultures and incubated with GST-Sepharose beads (see MATERIALS AND METHODS). Immunoblot analysis indicated that Skn7-HA copurified with GST-Hsf1 on the GST-Sepharose beads (Figure 7A, lane 4). When the extracts were prepared from a strain containing the empty vector, p426-GAG, Skn7-HA was not detected in association with the beads (Figure 7A, lane 2). To confirm this result, we used extracts prepared from the same strain containing p426-GAG-HSF1 grown on glucose to repress synthesis of Skn7-HA from the GAL promoter. Skn7 was found not to associate with the GST-Sepharose beads.

Figure 5. Genetic interactions between SKN7 and HSF1. (A) Single-copy expression of SKN7 rescues the growth defect of DR20-2b (hsf1Δ skn7Δ) at 33°C. DR20-2b (hsf1Δ skn7Δ) was transformed with the CEN vector YCplac111 (vector) or with this vector containing the SKN7 gene (+ SKN7) and was incubated at permissive (25°C) and intermediate (33°C) temperatures on rich medium. (B) High-copy expression of SKN7 rescues the growth defect of the hsf1Δ strain, MYY385, at 35°C. The hsf1Δ strain MYY385 was transformed with the 2μ-based plasmid Yep24 containing the SKN7 gene or with the vector alone (vector) and was streaked onto selective medium and incubated at 35°C for 4 d. The wild-type strain MYY290 (HSF1) was included as a positive growth control.
To determine whether Hsf1 also copurifies with Skn7, the complementary experiment was undertaken with an Ni2+-NTA affinity matrix to purify 6His-tagged Skn7 from a galactose-induced cell extract. Subsequent Western analysis of the proteins associated with the nickel-bound Skn7 indicated that Hsf1 copurified with the 6His-tagged Skn7 protein (Figure 7C, lane 3). No Hsf1 was pulled down in an extract without 6His-Skn7 (Figure 7C, lane 2). These results are consistent with the copurification of Skn7 with Hsf1 and strongly suggest that they interact physically in vivo.

**Skn7 Can Interact with Itself In Vivo**

Because Hsf1 is known to form homotrimers through its leucine zipper, we determined whether Skn7 could also interact with itself, given that Skn7 contains a similar coiled-coil region (Figure 1). To address this question, communoprecipitations were carried out with extracts from cells containing integrated 6Myc-tagged Skn7 under control of its own promoter and with high-copy HA-tagged SKN7 under galactose-inducible expression. Western analysis with 12CA5 antibody revealed that precipitation with monoclonal 9E10 anti-myc antibody specifically coprecipitates HA-Skn7 (Figure 7D, lane 4) from a galactose-induced extract containing 6Myc-Skn7. In the absence of a Myc-tagged SKN7 gene, or with the use of a cell extract derived from glucose-grown cells expressing GAL-HA-SKN7, no such coprecipitation was apparent (Figure 7D, lanes 2 and 3). These data suggest that Skn7 can oligomerize in vivo and that this may be central to its function, because disruption of the leucine zipper motif in Skn7 significantly compromises its function (Alberts et al., 1998).

**Skn7p Is Localized to the Nucleus**

To determine whether regulated nuclear import and export is an important mechanism by which the activity of the Skn7 response regulator may be controlled, we constructed an SKN7-GFP fusion protein in the CEN plasmid pRS416-sGFP (a gift from P. Silver) and examined the localization of Skn7. Figure 8 (C) shows that Skn7-GFP colocalizes with the DAPI-stained nucleus (B) under nonstressed growth conditions. This localization was unaffected by the addition of t-buty1 hydrogen peroxide (our unpublished results). The nuclear localization of Skn7 is consistent with its role as a transcriptional regulator.

**DISCUSSION**

Both Skn7 and Hsf1 have previously been shown to play important roles in the cellular response to oxidative stress. In the case of the response regulator Skn7, it has been shown to cooperate with the yeast AP-1 homologue Yap1 at the TRX2 promoter and to specifically activate transcription of the thioredoxin and thioredoxin reductase genes in the presence of hydrogen peroxide (Morgan et al., 1997). Similarly, Hsf1-dependent activation of the CUP1 metallothionein gene was observed in yeast cells treated with the superoxide generator menadione (Liu and Thiele, 1996). Here, we provide evidence that Skn7 is required for efficient heat shock gene activation in response to hydrogen peroxide. We also show that Skn7 and Hsf1 interact physically and genetically and have identified target genes known to be regulated by Hsf1 that are also regulated by Skn7 in response to free radical stress.
purification of Hsf1 with 6His-tagged Skn7. Lane 1, immunoprecipitate with anti-Hsf1 antibody from 1 mg of whole cell extract of galactose-grown cells expressing pGAL-SKN7–6His; lane 2, Ni²⁺-NTA agarose beads plus 1 mg of cell extract that does not contain the 6His-Skn7 protein; lane 3, Ni²⁺-NTA agarose beads plus 1 mg of galactose-induced extract from cells expressing pGAL-SKN7–6His; lane 4, 20 μg of input galactose-induced extract. (D) Skn7p can interact with itself. Coimmunoprecipitations from cell extracts containing galactose-induced pGAL-Skn7-HA and 6Myc-Skn7 were performed with the use of 9E10 mAb followed by 12CA5 Western blot analysis. HA-Skn7p expression was under the control of the GAL promoter, and integrated 6Myc-Skn7 was under the control of its own promoter. Immunoprecipitations with 1.5 μg of 9E10 were as follows: lane 1, 20 μg of extract from galactose-grown cells; lane 2, immunoprecipitation of extract from glucose-grown cells; lane 3, immunoprecipitation of extract from galactose-grown cells that did not contain the Myc-tagged SKN7; lane 4, immunoprecipitation of extract from galactose-grown cells containing 6Myc-Skn7 and pGAL-Skn7-HA. Western blot analysis was carried out with 12CA5 mAb.

hsf1Δ skn7Δ strain is decreased relative to that of the parental hsf1Δ strain. Second, the growth defect of the hsf1Δ strain is partially suppressed by high-copy expression of the SKN7 gene. That this suppression is partial indicates that Skn7 can fulfill some but not all of the functions of Hsf1 in the cell. This observation is not surprising given the pleiotropic nature of the hsf1-m3 mutation (Smith and Yaffe, 1991). Third, by reducing the activity of Hsf1 in skn7Δ cells through construction of the double mutant strain hsf1Δ skn7Δ, the sensitivity of skn7Δ cells to hydrogen peroxide is greatly enhanced. This hypersensitivity could be suppressed by ectopic expression of either SKN7 or SKN7Δ427N. These observations indicate that Skn7 and Hsf1 have overlapping functions in the stress response, presumably in the activation of particular stress-responsive genes.

Consistent with these observations, we have obtained several lines of evidence suggesting that Skn7 and Hsf1 interact physically. First, in extracts prepared from strains coexpressing Skn7-HA and GST-Hsf1, HA-tagged Skn7 copurified with GST-Hsf1 on glutathione-Sepharose beads. Physical

Figure 7. Hsf1 and Skn7 copurify. (A) Western blot analysis with 12CA5 mAb reveals that pGAL-Skn7-HA copurifies with GST-Hsf1. Lane 1, crude extract containing GST vector alone and pGAL-Skn7-HA; lane 2, GST pull-down from extract containing pGAL-Skn7-HA and GST vector alone; lane 3, crude extract containing pGAL-Skn7-HA and GST-Hsf1; lane 4, GST pull-down from cells containing pGAL-Skn7-HA and GST-Hsf1. (B) Lane 1, 20 μg of input protein (HA-Skn7); lane 2, GST pull-down of extract from cells containing empty GST vector and pGAL-Skn7-HA; lane 3, GST pull-down of extract from cells containing GST-Hsf1 vector and pGAL-Skn7-HA and grown in glucose; lane 4, GST pull-down of extract from galactose-grown cells containing pGAL-Skn7-HA and pGST-Hsf1. (C) Nickel-affinity co-purification of Hsf1 with 6His-tagged Skn7. Lane 1, immunoprecipitate with anti-Hsf1 antibody from 1 mg of whole cell extract of galactose-grown cells expressing pGAL-SKN7–6His; lane 2, Ni²⁺-NTA agarose beads plus 1 mg of cell extract that does not contain the 6His-Skn7 protein; lane 3, Ni²⁺-NTA agarose beads plus 1 mg of galactose-induced extract from cells expressing pGAL-SKN7–6His; lane 4, 20 μg of input galactose-induced extract. (D) Skn7p can interact with itself. Coimmunoprecipitations from cell extracts containing galactose-induced pGAL-Skn7-HA and 6Myc-Skn7 were performed with the use of 9E10 mAb followed by 12CA5 Western blot analysis. HA-Skn7p expression was under the control of the GAL promoter, and integrated 6Myc-Skn7 was under the control of its own promoter. Immunoprecipitations with 1.5 μg of 9E10 were as follows: lane 1, 20 μg of extract from galactose-grown cells; lane 2, immunoprecipitation of extract from glucose-grown cells; lane 3, immunoprecipitation of extract from galactose-grown cells that did not contain the Myc-tagged SKN7; lane 4, immunoprecipitation of extract from galactose-grown cells containing 6Myc-Skn7 and pGAL-Skn7-HA. Western blot analysis was carried out with 12CA5 mAb.

Figure 8. The Skn7 response regulator is localized in the nucleus. An SKN7-GFP fusion protein expressed from a CEN plasmid was visualized in cells from a midlog-phase culture (A; Nomarski image). The Skn7p-GFP fusion protein visualized by fluorescence (C) colocalizes with the DAPI signal of nuclear DNA (B).
interaction between these proteins was also shown with the use of a complementary pull-down assay; Hsf1 associated with 6His-tagged Skn7 on nickel–agarose beads. Previous work had suggested that Skn7 interacts with a number of proteins, including Yap1 (Morgan et al., 1997), Rho1 (Alberts et al., 1998), Snl1-Ypd1 (Li et al., 1998), and Mbp1 (Bouquin et al., 1999). In no case has coimmunoprecipitation been demonstrated with any of these proteins. Only a small proportion of total cellular Skn7 may associate with any of these proteins at one time, rendering coimmunoprecipitation studies difficult. Nonetheless, the copurification of Skn7 with Hsf1 described here argues strongly for a physical interaction.

**Skn7 Is Required to Activate Heat Shock Gene Expression Specifically in Response to Hydrogen Peroxide**

An implication of the interaction between Hsf1 and Skn7 is a role for Skn7 in regulating heat shock gene expression. Preliminary studies with the use of an SSA1-LacZ fusion construct indicated that Skn7 was required for HSE-mediated LacZ induction in response to hydrogen peroxide but was not required for the heat shock induction of the reporter (Table 1). Northern analysis of heat shock gene expression in wild-type and skn7Δ cells confirmed that SKN7 was not required for induction of these genes in response to heat shock. For example, when cells are shifted from 25 to 39°C, the heat shock induction of HSP12 in skn7Δ cells was identical to that in the isogenic wild-type strain. In contrast, the 5-fold induction of this gene by hydrogen peroxide in the wild type was largely abolished in the isogenic skn7Δ strain. The skn7Δ mutation also significantly reduced the 20-fold induction of HSP26 and the 9-fold induction of HSP104 by hydrogen peroxide without affecting their responses to heat shock activation (Figure 4). The residual induction of these genes in response to oxidative stress could be dependent on the other known activators of these genes, such as Hsf1 and the Msn2/Msn4 transcription factor. The latter can activate expression through upstream activation sequence elements unrelated to HSEs (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996).

The requirement for Skn7 in hydrogen peroxide induction of HSP12 is intriguing given that induction of this gene by a variety of other stresses has been shown to be mediated through STRE sequences by Msn2/Msn4 (Martinez-Pastor et al., 1996) and, in response to osmotic shock, by the high osmolarity glycerol (HOG) MAPK pathway (Varela et al., 1995). We note, however, that hydrogen peroxide induction of this and other STRE-regulated genes does not appear to be affected significantly by mutations in Msn2 or Msn4 (Schüller et al., 1994). It appears likely, therefore, that regulation of HSP12 (and perhaps other STRE-regulated genes such as HSP26 and HSP104) in response to hydrogen peroxide–generated oxidative stress is mediated principally by Skn7 and Hsf1, whereas activation in response to other stress conditions is regulated through the STRE elements and downstream effectors of the HOG pathway. In this context, it is interesting that the HOG1 pathway is itself regulated by the SLN1 histidine kinase, which also seems to regulate the activity of the Skn7 response regulator (Li et al., 1998).

**Structural Homology between Skn7 and Hsf1**

With regard to the Skn7–Hsf1 interaction in the oxidative stress response, the structural similarities of the two proteins are of particular interest. We have shown through EMSA that Skn7 purified from yeast or E. coli can bind to a 26-base pair probe derived from the HSE2 regulatory region of the SSA1 promoter. This binding is of a similar specificity to that of Hsf1, insofar as mutation of the GAAnTTTC sequence to AAAnnTTT ablates binding of both Hsf1 and Skn7. Previously, we identified a regulatory site within the TRX2 promoter through which Skn7 can act (Morgan et al., 1997). The site contains the sequence CCGAAA in which mutation of the CG nucleotides to AT was found to reduce the binding of Skn7 by 20-fold. The common motif between this regulatory sequence and HSEs is the GAA triplet, three inverted repeats of which in the sequence AGAAn constitute a consensus HSE. Although the exact consensus binding site for Skn7 has not been established, the triplet GAA evidently represents a potential core recognition sequence.

In terms of sequence specificity of Skn7 recognition of HSEs versus that of Hsf1, there is one notable divergence between their otherwise highly conserved DNA-binding domains. This occurs at the last residue of the α3 sequence recognition helix of Hsf1 (Harrison et al., 1994), where the invariant M58 and G60 residues are replaced by K58 and D60, respectively, in the Skn7 protein (with residues numbered according to Figure 1). These substitutions may have a significant effect on DNA-binding specificity or the stability of Skn7 relative to Hsf1 because G60 of Hsf1 has been proposed to contact the DNA (Damberger et al., 1994). All other residues proposed to contact the DNA, however, are conserved between Skn7 and Hsf1.

The other region of structural homology between these two proteins lies between residues 222 and 303 of the Skn7 protein. This stretch contains five heptad repeats, with hydrophobic residues at positions 1 and 4 and polar residues elsewhere in the repeat units, characteristic of regions that form coiled-coil structures (Lups, 1996). Hsf1 contains six heptad repeats that have been shown to mediate trimerization of the protein through the formation of triple-stranded α-helical coiled coils (Sorger and Nelson, 1989; Peteranderl and Nelson, 1992; Rabindran et al., 1993). These coiled coils are also known to mediate heterodimerization and homodimerization, e.g., of the yeast GCN4 member of the bZIP transcription factor family (Harbury et al., 1993). We have demonstrated that Skn7, in common with Hsf1, can interact with itself in vivo and that these proteins can interact with each other. We have also shown that the Skn7 response regulator is constitutively localized to the nucleus and can bind to HSEs with a similar specificity to that of Hsf1. We are currently exploring the possibility that it is through the formation of heterodimers and/or heterotrimmers that Skn7 and Hsf1 can mediate the activation of heat shock genes, and possibly other sets of genes, in response to oxidative stress.

The markedly increased sensitivity to oxidative stress of an skn7Δ strain relative to either single mutant (Figure 6) supports such a cooperative association.

In summary, we have shown that Skn7 interacts with Hsf1 and can bind to the same consensus sequence as Hsf1. We have further shown that SKN7 is required for the induction of heat shock genes in response to t-butyl hydrogen peroxide, although it is not required for their
heat shock induction. In addition, we have demonstrated for the first time the nuclear localization of a response regulator in yeast, which was found to be independent of the presence or absence of oxidative stress. In the light of these data, we propose a model whereby Skn7 becomes activated in response to hydrogen peroxide stress and either by dimerization or through the formation of a heterotrimeric Skn7–Hsf1 complex binds to the promoters of stress-responsive genes. The actual mechanism by which either Skn7 or Hsf1 activates gene expression in response to stress remains unclear, although recent evidence suggests that Hsf1 can interact with TATA-binding protein (Mason and Lis, 1997) and with a phosphatase that may modulate the transcriptional activity of a subset of promoters, including CUP1, through interaction with the repressor region of Hsf1 (Lin and Lis, 1999). Hsf1 can also antagonize nucleosomal repression (Erkine et al., 1996). It is possible, therefore, that the transcription activation function of Hsf1 is enhanced by its interaction with Skn7 or that Skn7 itself potentiates the interactions with components of the basal transcription apparatus at heat shock core promoters in response to oxidative stress.

ACKNOWLEDGMENTS

We gratefully acknowledge the kind gifts of plasmid pZJHSE2-137 and anti-GST HSF antibodies from E. Craig and the gifts of strains, plasmids, and antibody reagents used in the initial stages of this work from S. Lindquist. We also thank A. Sewell for plasmid pAKS80. We thank A. Spanos, D. Fesquet, and W. Morgan for helpful advice and discussion. D.C.R. was supported by a European Community Network fellowship (contract number ERB-CHRXCT920248). Work performed in the laboratory of D.S.G. was supported by a grant from the National Institute of General Medical Sciences (GM45842).

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