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The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*

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Deletion of the bacterial two-component response regulator homologue Skn7 results in sensitivity of yeast to oxidizing agents indicating that Skn7 is involved in the response to this type of stress. Here we demonstrate that following oxidative stress, Skn7 regulates the induction of two genes: *TRX2*, encoding thioredoxin, and a gene encoding thioredoxin reductase. *TRX2* is already known to be induced by oxidative stress dependent on the Yap1 protein, an AP1-like transcription factor responsible for the induction of gene expression in response to various stresses. The thioredoxin reductase gene has not previously been shown to be activated by oxidative stress and, significantly, we find that it too is regulated by Yap1. The control of at least *TRX2* by Skn7 is a direct mechanism as Skn7 binds to the *TRX2* gene promoter *in vitro*. This shows Skn7 to be a transcription factor, at present the only such eukaryotic two-component signalling protein. Our data further suggest that Skn7 and Yap1 co-operate on the *TRX2* promoter, to induce transcription in response to oxidative stress.

Keywords: oxidative stress/transcription/two-component/Yap1/yeast

Introduction

Reactive oxygen species (ROS) are responsible for a wide range of intracellular damage to DNA, proteins and cellular structures (for reviews see Ruis and Schuller, 1995; Moradas-Ferreira *et al.*, 1996). Free radicals are a normal by-product of respiring cells and are also produced by a wide range of different environmental chemicals. Hence cells have developed mechanisms to respond to this type of stress, termed the oxidative stress response (OSR). Although a number of the cellular proteins functioning in the OSR have been identified in eukaryotes, little is known regarding the signal transduction pathways that detect and respond to oxidative stress. Much of our knowledge in this field has come from work in the budding yeast *Saccharomyces cerevisiae*. In yeast, overexpression of two transcription factors, Yap1 and Yap2, was found to confer resistance to a wide range of drugs and oxidizing agents (Moradas-Ferreira *et al.*, 1996). Yap1 and Yap2 are members of the c-jun family of proteins which constitute part of the AP1 transcription factor of higher eukaryotes. Both Yap1 and Yap2 contain a basic leucine zipper domain (bZIP) adjacent to a DNA binding domain located at the N-terminus of the proteins. Null mutants of both *YAP1* and *YAP2* result in sensitivity of *S.cerevisiae* to the oxidizing agent hydrogen peroxide (Stephen *et al.*, 1995). Further, Yap1 binds directly to the promoter, and regulates the expression of the *TRX2* gene (Kuge and Jones, 1994) encoding thioredoxin which acts in the oxidative stress response to reduce protein disulfides. Yap1 also regulates *GSH1*, encoding glutamyl cysteine synthetase, in response to hydrogen peroxide. The connection between Yap1 and Yap2, and AP1 of higher cells is further strengthened by the observation that the AP1 is itself regulated by the oxidative stress status of the cell (Moradas-Ferreira *et al.*, 1996). Other work in *S.cerevisiae* has also demonstrated that there are distinct signalling pathways to hydrogen peroxide and superoxides, although the transcription factor(s) and signalling pathway for the superoxide response have not been characterized (Collinson and Dawes, 1992; Jamieson, 1992; Flattery-O'Brian *et al.*, 1993; Stephen *et al.*, 1995). In particular, the *GSH1* gene is induced by both hydrogen peroxide and menadione, a superoxide-generating drug (Stephen *et al.*, 1995). Although *YAP1* mutants abolish the hydrogen peroxide-induced expression of *GSH1* they have little effect on menadione-induced expression (Stephen *et al.*, 1995). Thus yeast mounts a complex defence to oxidative stress involving several different transcription factors and signalling pathways.

Recently it was shown that disruption of the *SKN7* gene [also known as *POS9* (Krems *et al.*, 1996) and *BRY1* (Morgan *et al.*, 1995b)] resulted in yeast cells becoming sensitive to hydrogen peroxide (Krems *et al.*, 1996). *SKN7* encodes a potential transcription factor which has been implicated in the regulation of cell wall biosynthesis and the cell cycle (Brown *et al.*, 1993, 1994; Morgan *et al.*, 1995b). Overexpression of *SKN7* suppresses the cell wall defect associated with mutation of the *KRE9* gene (Brown *et al.*, 1993) and additionally, the growth defect associated with deletion of *PKC1* (Brown *et al.*, 1994). Furthermore, deletion of the *SKN7* gene was found to be synthetically lethal in a *pkc1*Δ background (Brown *et al.*, 1994; Morgan *et al.*, 1995b). As the *PKC1* MAP kinase pathway is involved in cell wall biosynthesis (reviewed in Herskowitz, 1995; Igual *et al.*, 1996), this supports the notion that *SKN7* might play a role in the expression of cell wall genes. In addition, overexpression of Skn7 suppresses the lethality

associated with loss of the G_1 transcription factors SBF and MBF, suggesting a cell-cycle role (Morgan *et al.*, 1995b). These transcription factors recognize the sequences CACGAAAA (an SCB element) and ACGCGT (an MCB element) respectively, in the promoters of genes they regulate. These include the G_1 cyclin and DNA synthesis genes (for reviews see Johnston, 1992; Koch and Nasmyth, 1994). The lethality associated with loss of SBF and MBF has been shown to be due to the absence of G_1 cyclin expression. High copy *SKN7* was shown to restore G_1 cyclin expression through the MCB and SCB elements present in the cyclin promoters (Morgan *et al.*, 1995b). *SKN7* does not appear to bind directly to MCB and SCB elements (Morgan *et al.*, 1995b) and is more likely regulating an MCB/SCB binding factor other than MBF and SBF. The sequence of *SKN7* revealed homology to the DNA binding domain of heat shock factor (HSF1) (Brown *et al.*, 1993; Morgan *et al.*, 1995b) hence it is possible that *SKN7* binds to a sequence related to the HSE elements that HSF1 recognizes.

The Skn7 protein contains a potential receiver domain found in the two-component signal transduction family of proteins in prokaryotes (Brown *et al.*, 1993; Morgan *et al.*, 1995b). In bacteria, these signal transduction systems are a common method of detecting and responding to the environment (for reviews see Bourret *et al.*, 1991; Parkinson, 1993). Generally, the first component, a homodimer histidine kinase present in the cell membrane, detects the signal, and phosphorylates a conserved histidine residue on its partner. This phosphate is then transferred to a conserved aspartic acid residue within the 120 amino acid receiver domain of the second component, the response regulator protein. Response regulator proteins are generally transcription factors which are activated by the phosphorylation. In eukaryotes only a few potential two-component signal transduction proteins have been identified (reviewed in Morgan *et al.*, 1995a). In no case, including Skn7, have genes regulated by these systems been identified. In *S.cerevisiae*, genetic screens have identified one histidine kinase, Sln1, and two response regulator proteins, Ssk1 and Skn7. Sln1 and Ssk1 were shown to act in the regulation of the response of yeast to osmolarity by regulating the Hog1 MAP kinase pathway (Maeda *et al.*, 1994, 1995). However, deletion of *SKN7* does not result in any osmolarity defect (B.A.Morgan, unpublished observations). The only stress defect known for *skn7* mutants is in oxidative stress as mentioned above, although the role of Skn7 in the OSR remains unclear. Recently Krems *et al.* (1996) proposed that the receiver domain was essential for the ability of Skn7 to confer cellular resistance to oxidizing agents suggesting, perhaps, that an upstream histidine kinase could be important for the activity of Skn7 in the OSR.

Here we have identified a direct role for Skn7 in the OSR through the induction of *TRX2* and also a gene encoding thioredoxin reductase. The Skn7 protein binds directly to the *TRX2* promoter and co-operates with the Yap1 protein to induce gene expression. This is the first example of a gene known to be directly regulated by a response regulator protein in eukaryotes. However, in contrast to Krems *et al.* (1996) we find that signalling through the receiver domain of Skn7 appears to have no role in the OSR.

Results

skn7Δ strains are sensitive to a range of oxidizing agents

*skn7*Δ strains are sensitive to hydrogen peroxide (Krems *et al.*, 1996). We have confirmed this result and find that *skn7*Δ strains are sensitive to a range of oxidizing agents including *tetra*-butyl hydrogen peroxide, cadmium and menadione, but not significantly sensitive to diamide (data not shown). Thus the Skn7 protein is required for the cellular response to a variety of free radicals. Deletion of the *YAP1* gene also results in sensitivity of yeast cells to many of these agents (Kuge and Jones, 1994). The sensitivity of *yap1*Δ strains has been shown to be due to the role of the Yap1 protein in the induction of several genes which function in the OSR. Significantly, deletion of the *SKN7* gene does not enhance the sensitivity of a *yap1*Δ strain to diamide, hydrogen peroxide, cadmium or menadione (Krems *et al.*, 1996; our unpublished observations) suggesting that *SKN7* and *YAP1* are epistatic, functioning in the same pathway. The basis for the sensitivity of the *skn7*Δ strain to oxidizing agents is unknown but a likely explanation, based on the epistasis of *SKN7* and *YAP1*, is that Skn7 affects the expression of a set of genes also regulated by Yap1 in response to oxidative stress.

Skn7 is required for the induction of thioredoxin and thioredoxin reductase gene expression by the OSR

Expression of the *TRX2* gene is induced in response to several oxidizing agents including hydrogen peroxide; this induction is under the control of the Yap1 protein which binds the *TRX2* promoter (Kuge and Jones, 1994). To examine the role of Skn7 in *TRX2* gene expression, various yeast strains were treated with hydrogen peroxide and total RNA was isolated and probed with a *TRX2*-specific gene probe (Figure 1A). In *yap1*Δ strains the induction of *TRX2* is much reduced, although some residual induction remains as observed previously (Kuge and Jones, 1994). Significantly, deletion of the *SKN7* gene mimics the deletion of the *YAP1* gene. *TRX2* induction is almost abolished but once again residual induction is observed. The *skn7*Δ*yap1*Δ double mutant is no more defective in *TRX2* induction than either single mutant, consistent with the genetic epistasis described above. The residual induction of *TRX2* that occurs even in the *skn7*Δ*yap1*Δ double mutant suggests the existence of a further minor induction mechanism. However, the important result is that *skn7*Δ and *yap1*Δ mutants appear to have identical phenotypes with respect to *TRX2* induction, consistent with their functioning in the same pathway.

Next, the expression of a gene encoding thioredoxin reductase (Chae *et al.*, 1994) was tested. No name has yet been assigned to this gene and we will refer to it as *TRR1*. Thioredoxin reductase recycles the oxidized form of thioredoxin to the reduced form to scavenge more ROS, suggesting that *TRR1* expression may also be induced by ROS. In addition, the *TRR1* promoter contains a potential Yap1 binding site, identical to the SV40 AP-1 site (Figure

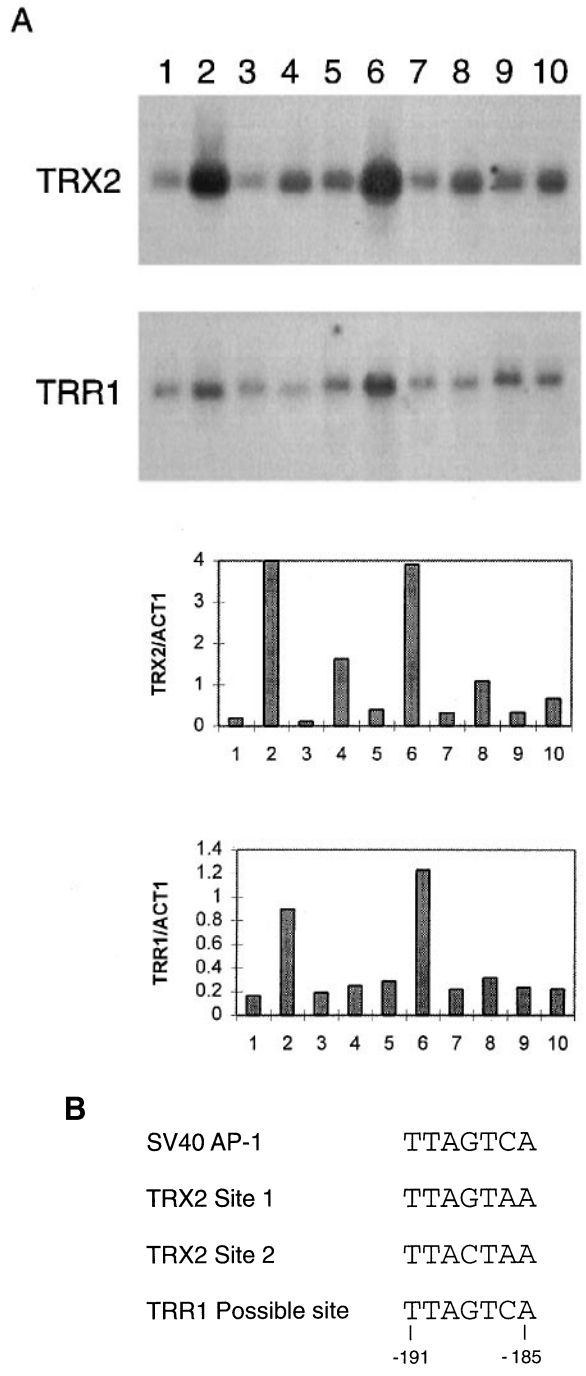

Fig. 1. Skn7 and Yap1 regulate the expression of *TRX2* and *TRR1* in response to hydrogen peroxide. (**A**) Northern blot analysis of RNA isolated from different mid-log yeast strains prior to (lanes 1, 3, 5, 7 and 9) and following treatment with 1 mM hydrogen peroxide for 1 h at 25°C (lanes 2, 4, 6, 8 and 10). Strains used were W303-1a (lanes 1 and 2), *skn7*Δ (lanes 3 and 4), W303-1b (lanes 5 and 6), *yap1*Δ (lanes 7 and 8) and *skn7*Δ*yap1*Δ (lanes 9 and 10). The panels below the data show quantitation of the RNA levels relative to the actin transcript. (**B**) Comparison of potential Yap1 binding sites (Kuge and Jones, 1994) with a possible Yap1 binding site in the *TRR1* promoter.

1B). Northern blot analyses revealed that the *TRR1* gene is indeed induced by hydrogen peroxide and that this induction is regulated by the *YAP1* gene (Figure 1A). Furthermore, like *TRX2* expression, Yap1 appears to act principally on the induced levels of *TRR1* and not the basal levels which were reduced by some 30% in each

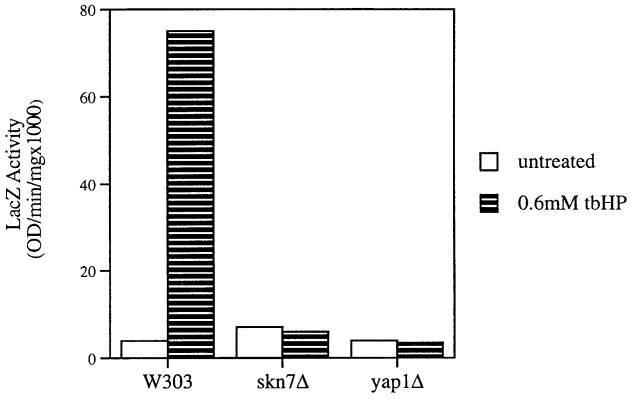

Fig. 2. Skn7 is required for induction by *tetra*-butyl hydrogen peroxide of LacZ fused to the *TRX2* promoter. Mid-log cultures of W303-1a, *skn7*Δ and *yap1*Δ strains, transformed with the TRXLACZ plasmid (Kuge and Jones, 1994), were treated with *tetra*-butyl hydrogen peroxide for 1 h. β-galactosidase assays were performed on the untreated and treated cultures.

case. The induction of *TRR1* gene expression was not as pronounced as *TRX2*, however, but there was a very slight residual induction in a *yap1*Δ strain. *SKN7* is also required for the induction of the *TRR1* gene and, in agreement with the data for *TRX2*, *skn7*Δ and *yap1*Δ strains appear identical (Figure 1A). Again the *skn7*Δ*yap1*Δ double mutant shows no significant additional loss of *TRR1* expression as would be expected if these proteins were acting independently to regulate *TRR1* gene expression.

As mentioned above, both the *skn7*Δ and *yap1*Δ strains are more sensitive than wild type strains to *tetra*-butyl hydrogen peroxide. A *TRX2* promoter–LacZ reporter construct has been used previously to confirm that *TRX2* expression is induced in response to this treatment (Kuge and Jones, 1994). We therefore used this construct to provide independent evidence for the role of Skn7 in *TRX2* expression. The reporter plasmid was introduced into *skn7*Δ and *yap1*Δ strains and, as expected for the latter strain, there was no induction of LacZ activity following treatment with *tetra*-butyl hydrogen peroxide relative to reporter expression in a wild type background (Figure 2). Consistent with the dual function of Yap1 and Skn7 in control of *TRX2* expression, the *skn7*Δ strain showed a similar absence of induction of β-galactosidase following oxidative stress.

Diamide, a thiol oxidant (Plummer *et al.*, 1981), also induces *TRX2* gene expression and this expression is Yap1 dependent (Kuge and Jones, 1994). We confirmed these data but found that the effect of the *SKN7* gene on *TRX2* expression following diamide treatment was less marked, although some loss of induction clearly did occur (Figure 3). A similar result was obtained with the *TRR1* gene. The double *skn7*Δ *yap1*Δ strain showed a loss of induction of *TRX2* and *TRR1* similar to that observed with *yap1*Δ alone. These data might partly explain why *skn7*Δ strains show no significant sensitivity to diamide (see above) whereas *yap1*Δ strains are sensitive (Kuge and Jones, 1994) and indicates some separation of Skn7 and Yap1 function.

Skn7 binds the TRX2 promoter

The simplest explanation for the data described above is that the Skn7 protein binds directly the *TRX2* promoter

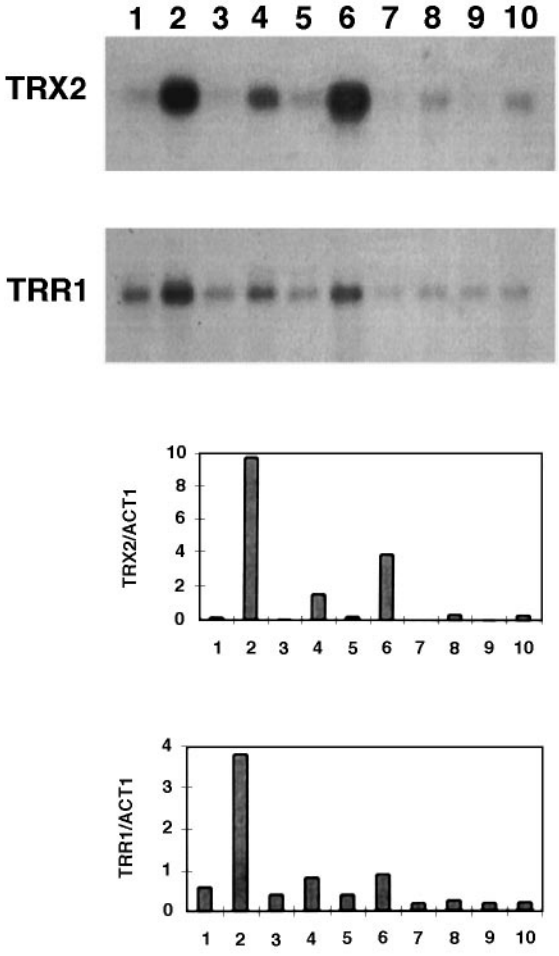

Fig. 3. Skn7 and Yap1 regulate the expression of *TRX2* and *TRR1* in response to diamide. Northern blot analysis of RNA isolated from different mid-log yeast strains prior to (lanes 1, 3, 5, 7 and 9) and following treatment with 1.5 mM diamide for 1 h at 25°C (lanes 2, 4, 6, 8 and 10). Strains used were W303-1a (lanes 1 and 2), *skn7*Δ (lanes 3 and 4), W303-1b (lanes 5 and 6), *yap1*Δ (lanes 7 and 8) and *skn7*Δ*yap1*Δ (lanes 9 and 10). The panels below the data show quantitation of the RNA levels relative to the actin transcript.

in order to regulate transcription. To examine this, crude extracts were prepared from a variety of strains and then incubated with a DNA fragment from the *TRX2* promoter consisting of the entire upstream region from the potential TATA to the stop codon of the immediate upstream gene. When these protein–DNA complexes were analysed by electrophoretic mobility shift assays (EMSAs) two bands were found to be sensitive to the presence of the Skn7 protein (Figure 4A). The most highly retarded band, termed band 1, was present only in wild type extracts and not in the *skn7*Δ, *yap1*Δ or the *skn7*Δ*yap1*Δ double mutant extracts, suggesting that band 1 is dependent on both the Skn7 and Yap1 proteins. In contrast, a faster-migrating band, termed band 2, was present in the wild type and *yap1*Δ extracts, but not the *skn7*Δ or *skn7*Δ*yap1*Δ extracts, suggesting that band 2 is dependent on the presence of Skn7 and not Yap1. The most straightforward interpretation of these data is that band 1 contains both Skn7 and Yap1 whilst band 2 contains only Skn7. In agreement with this suggestion, when the *skn7*Δ*yap1*Δ strain was transformed with a CEN plasmid carrying only the *SKN7*

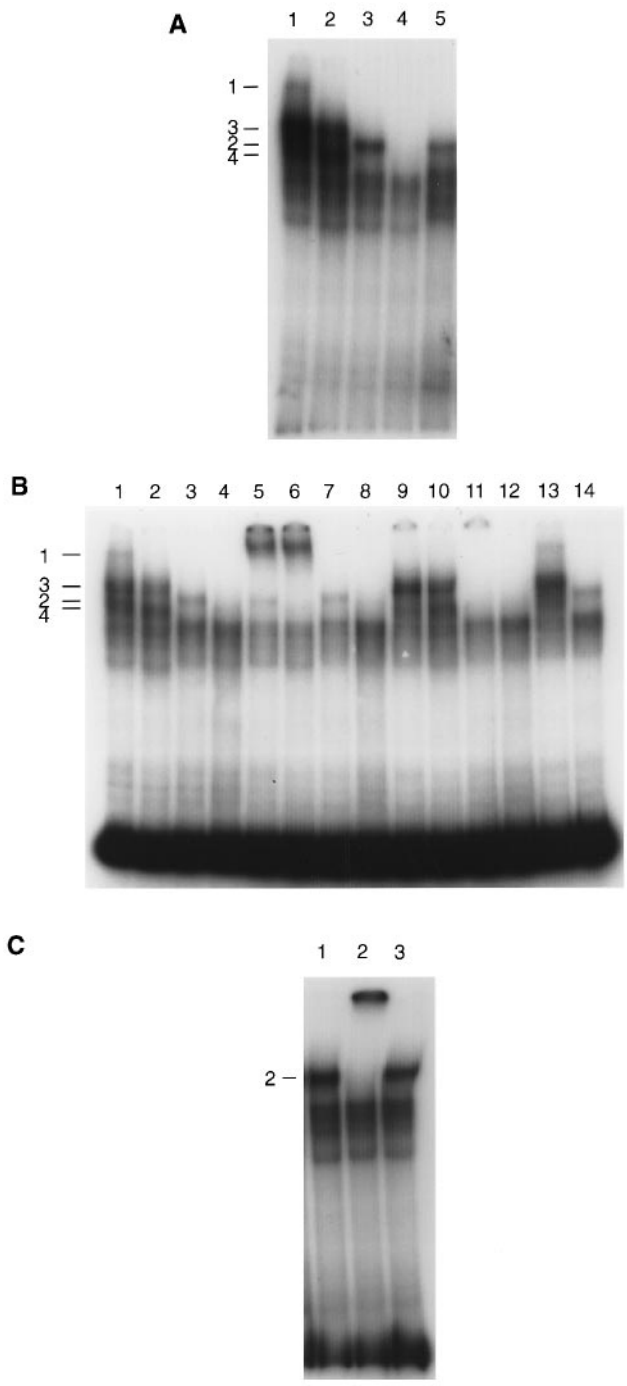

Fig. 4. Skn7 and Yap1 bind the *TRX2* promoter directly. (**A**) Crude extracts prepared from W303-1a (lane 1), *skn7*Δ (lane 2), *yap1*Δ (lane 3), *skn7*Δ*yap1*Δ (lane 4) and *skn7*Δ*yap1*Δ transformed with pBAM1 (lane 5) were analysed by EMSA using the probe TRXΔ1 (see Figure 5 and Materials and methods). Bands which are sensitive to the presence or absence of the Skn7 and Yap1 proteins are indicated as 1–4. (**B**) The probe TRXΔ1 was mixed with crude extracts prepared from W303-1a (lane 1), *skn7*Δ (lane 2), *yap1*Δ (lane 3) and *skn7*Δ*yap1*Δ (lane 4), and analysed by EMSA. In lanes 5–8 the same crude extracts also contained the Yap1 polyclonal antiserum while in lanes 9–12 the crude extracts contained the Skn7 polyclonal antiserum. Tracks 13 and 14 are the W303-1a and *yap1*Δ extracts incubated with preimmune serum at the same dilution as the polyclonal antisera. (**C**) Using the probe TRXΔ1, the *yap1*Δ crude extract was analysed by EMSA. In lane 1 no antiserum was added, in lane 2 the Skn7 polyclonal antiserum, and in lane 3 the preimmune serum at the same dilution as the Skn7 polyclonal antiserum.

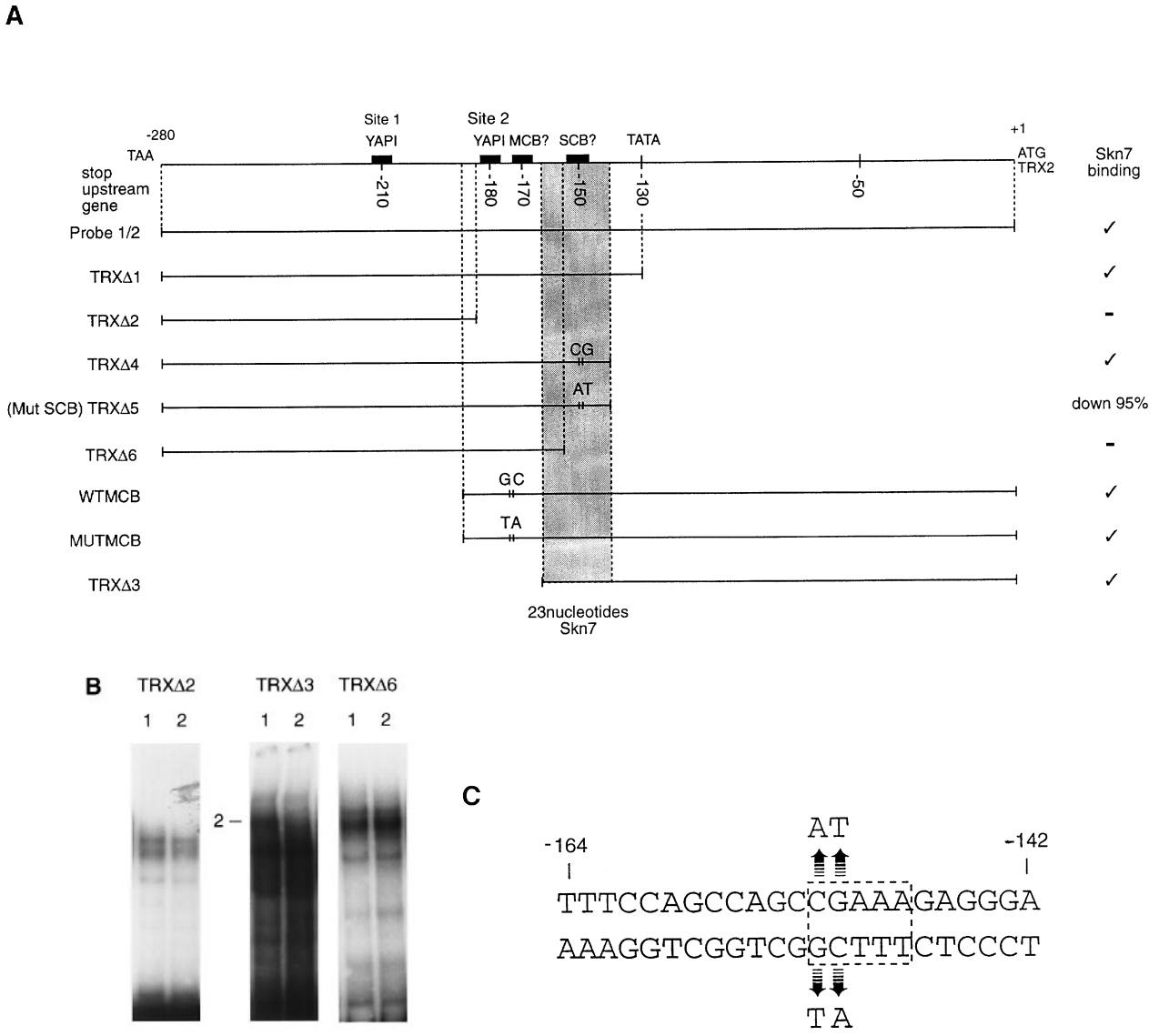

Fig. 5. Skn7 binding region in the *TRX2* promoter. (**A**) Schematic diagram of the *TRX2* promoter and the probes used to identify the Skn7 binding. Near match MCB and SCB elements are indicated. Probes are described in Materials and methods. (**B**) To map the Skn7 binding site the probes TRXΔ2, TRXΔ3 and TRXΔ6 were mixed with crude extracts prepared from *yap1*Δ (lane 1) and *skn7*Δ*yap1*Δ strains (lane 2), and analysed by EMSA. Band 2, containing the Skn7 protein, could only be detected with the TRXΔ3 probe (lane 1). (**C**) DNA sequence of the 23 nucleotide region of the *TRX2* promoter to which Skn7 binds. The potential SCB element and the double point mutation of this sequence in the TRXΔ5 probe is indicated.

gene and the crude extracts were analysed by EMSA, only band 2 reappeared (Figure 4A).

To demonstrate that Skn7 binds directly to the *TRX2* promoter, an EMSA was performed as above except that a polyclonal antibody raised against the Skn7 protein was included (see Materials and methods). EMSA revealed that bands 1 and 2 were clearly supershifted by the Skn7 antibody (Figure 4B). To confirm this, the experiment was repeated using only *yap1*Δ extracts and longer autoradiograph exposures (Figure 4C). The antibody supershifted band 2 completely and the preimmune serum was without effect. Thus Skn7 must bind directly to the *TRX2* promoter.

The Yap1 protein also binds directly to the *TRX2* promoter at two sites termed site 1 and site 2 (Kuge and Jones, 1994; Figure 5). In our EMSA two bands in addition to band 1, bands 3 and 4, are sensitive to the presence of the *YAP1* gene (Figure 4). To determine whether all these Yap1-dependent bands contain the Yap1 protein, various crude extracts were mixed with a polyclonal antibody against Yap1 and the effect on the bands examined. The wild type extracts clearly show a prominent supershifted band and essentially recreate the pattern obtained from *yap1*Δ extracts (Figure 4B). Hence all the Yap1-dependent bands contain the Yap1 protein. In agreement with the suggestion above that bands 1, 3 and 4, but not band 2, contain the Yap1 protein, only band 2 appears not to be supershifted by the Yap1 antibody. This has been confirmed by protracting the EMSA electrophoresis step to ensure complete separation of band 1 from the supershifted Yap1 band (data not shown). Hence band 1 contains at least the Skn7 and Yap1 proteins, band 2 contains Skn7, and bands 3 and 4 contain Yap1. Another important conclusion from these data is that significant binding of Skn7 and Yap1 proteins can be detected in the absence of oxidative stress

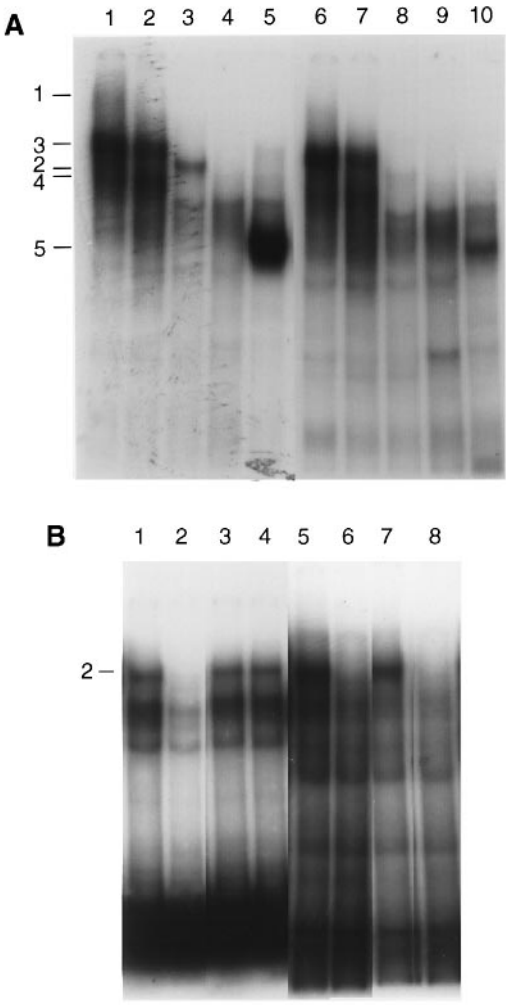

Fig. 6. The potential SCB and MCB elements are not important for Skn7 binding to the *TRX2* promoter. (**A**) Crude extracts from strains W303-1a (lanes 1 and 6), *skn7*Δ (lanes 2 and 7), *yap1*Δ (lanes 3 and 8), *skn7*Δ*yap1*Δ (lanes 4 and 9) and *skn7*Δ*yap1*Δ transformed with a C-terminal truncation derivative of Skn7 (lanes 5 and 10, band 5), were analysed by EMSA. Extracts were mixed with the probe TRXΔ4 (wild type sequence; lanes 1–5) and with the probe TRXΔ5 (mutant SCB; lanes 6–10) respectively. (**B**) The role of the potential SCB and MCB elements was further examined in lanes 1–4 (SCB) and lanes 5–8 (MCB). In lanes 1–4 *yap1*Δ extracts were mixed with the TRXΔ1 probe and excess unlabelled competitor DNA as follows: no competitor (lane 1), TRXΔ1 unlabelled DNA (lane 2), unlabelled SCB containing fragment from the *HO* promoter (lane 3) and unlabelled lambda phage DNA (lane 4). In lanes 5–8 *yap1*Δ (lanes 5 and 7) and *skn7*Δ*yap1*Δ (lanes 6 and 8) crude extracts were mixed with the WTMCB probe (wild type MCB; lanes 5 and 6) and with the MUTMCB probe (mutant MCB; lanes 7 and 8). Band 2 containing Skn7 is indicated. Note that the MUTMCB probe was labelled with approximately half the efficiency of the WTMCB probe explaining the apparent overall lower binding with this probe.

and, furthermore, that Skn7 can bind in the absence of the *YAP1* gene and vice versa.

To map the *TRX2* promoter site responsible for Skn7-specific binding, a series of overlapping probes for EMSA was constructed from the upstream region of the gene (Figure 5A). The probes TRXΔ1 (Figure 4A), TRXΔ3 (Figure 5B), TRXΔ4 (Figure 6A), WTMCB (Figure 6B) and MUTMCB (Figure 6B), containing the Skn7 binding site, gave a single band which was not detectable in *skn7*Δ extracts. In contrast, no Skn7-sensitive band could be

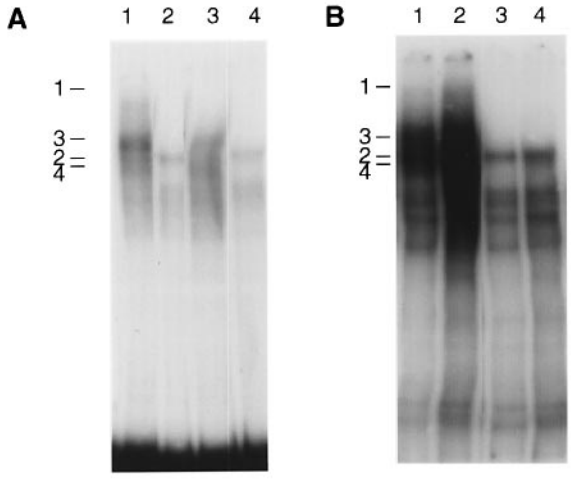

Fig. 7. The effect of H_2O_2 and diamide on Skn7 and Yap1 binding to the *TRX2* promoter. (**A**) The TRXΔ1 probe was mixed with crude extracts from W303-1a (lanes 1 and 3) and a *yap1*Δ strain (lanes 2 and 4) isolated prior to (lanes 1 and 2) and following treatment with mM H_2O_2 for 1 h at 25°C (lanes 3 and 4). (**B**) The TRXΔ1 probe was mixed with crude extracts from W303-1a (lanes 1 and 2) and a *yap1*Δ strain (lanes 3 and 4) isolated prior to (lanes 1 and 3) and following treatment with 1.5 mM diamide for 1 h at 25°C (lanes 2 and 4).

detected with probes TRXΔ2 and TRXΔ6 (Figure 5B). As indicated, Skn7 binds within a 23 nucleotide region between the Yap1 binding site 2 (Kuge and Jones, 1994) and the potential TATA sequence (Figure 5A and C). The potential DNA binding domain of Skn7 has homology to HSF1 and, whilst the 23 nucleotide sequence does not contain a complete HSE element, there is some limited homology to one (data not shown). Within the 23 nucleotides is also some limited homology to an SCB element (Figure 5C). Mutation of the CG to TA within this lowers Skn7 binding to this region 20-fold (Figure 6A). However, the addition of a large molar excess of a fragment from the *HO* gene promoter containing SCB elements did not compete with Skn7 binding (Figure 6B). Thus, although the CG nucleotides are important for Skn7 binding, this sequence is unlikely to constitute a functional SCB element.

There is also one potential MCB element in the *TRX2* promoter (Figure 5A). To test whether this sequence had any role in Skn7 binding, the essential CG core was mutated to TA and a fragment with the mutation tested in EMSA. There was no effect on Skn7 binding (Figure 6B). Thus neither of the potential MCB or SCB sites appear to be relevant for Skn7 activation of *TRX2*.

Next, we determined whether any significant changes in binding to the *TRX2* promoter occurred following treatment of various strains with hydrogen peroxide. Extracts were prepared from the cells at a time when maximal induction of the *TRX2* and *TRR1* genes had been observed and mixed with a probe from the *TRX2* promoter. No obvious effect on the binding of the Yap1 or Skn7 proteins was observed under these conditions (Figure 7A). This implies that, at least *in vitro*, DNA binding *per se* is not the major regulatory step in the induction of gene expression and that another mechanism is involved. In addition, no new bands were evident, suggesting that the induction does not involve the binding of a new protein to the *TRX2* promoter.

Kuge and Jones (1994) demonstrated that Yap 1 binding *in vitro* is induced significantly by diamide. To test whether Skn7 responds in a similar fashion, crude extracts were prepared from various strains treated with 1.5 mM diamide and analysed for the binding of Yap1 and Skn7. Like Kuge and Jones (1994) we observed induction of Yap1 binding following diamide treatment (Figure 7B, lanes 1 and 2). However, in contrast no significant induction of Skn7 binding occurred (Figure 7B, lanes 3 and 4).

Signalling through the receiver domain of Skn7 plays no role in the OSR

Recently it was suggested that the receiver domain of Skn7 was necessary for the resistance of yeast cells to oxidative stress (Krems *et al.*, 1996). Hence we tested whether the receiver domain of Skn7 was required for the induction of *TRX2* following oxidative stress. In Skn7, Asp427 is the phosphorylable amino acid (Brown *et al.*, 1994; Morgan *et al.*, 1995b) and previously we demonstrated that mutation of this residue to an asparagine residue blocked the ability of Skn7 to rescue the temperature sensitivity of a *swi4*^{ts} *swi6*Δ strain (Morgan *et al.*, 1995b). Hence CEN plasmids containing either the wild type *SKN7* gene or with a D427N mutation were introduced into the *skn7*Δ strain and the sensitivity to hydrogen peroxide was tested (Figure 8A). Both plasmids clearly behave identically; they fully complemented the sensitivity of the *skn7*Δ strain to hydrogen peroxide. Next, the same strains were grown in minimal medium and treated with 1 mM hydrogen peroxide for 1 h. Northern hybridization revealed that Skn7^{D427N} behaves identically to wild type and *TRX2* expression is induced normally (Figure 8B). Finally, the CEN plasmids were introduced into the *skn7*Δ*yap1*Δ strain. When crude extracts of these strains were characterized by EMSA the wild type and D427N version of Skn7 demonstrated similar binding abilities to a *TRX2* promoter fragment (Figure 8C). Thus signalling by means of the receiver domain has no role in the induction of *TRX2* in response to oxidative stress or on the binding of Skn7 to the *TRX2* promoter.

The receiver domain as a whole, however, is essential for the response to oxidative stress. A C-terminal truncation of Skn7, missing amino acids 353–623 containing the receiver domain and the glutamine-rich region, was isolated by transposon mutagenesis (Morgan *et al.*, 1996). This construct was unable to rescue the sensitivity of *skn7*Δ to hydrogen peroxide (data not shown). Nevertheless this truncated protein, which contains the domain with homology to the HSF1 DNA binding domain, was still able to bind the *TRX2* promoter (band 5 in Figures 6A and 8C). In summary, the receiver domain may play a structural role that is necessary for the oxidative stress response but phosphorylation of Asp427 seems unimportant.

Discussion

The Yap1 transcription factor is required for the OSR through the induction of gene expression (reviewed in Moradas-Ferreira *et al.*, 1996). Deletion of the *SKN7* gene also results in the increased sensitivity of yeast to oxidative stress. Since response regulator proteins like Skn7 in bacteria are transcription factors, it seemed likely that Skn7, in addition to Yap1, was also required for the induction of gene expression. This is indeed the case: two genes normally induced by oxidative stress, *TRX2* and *TRR1*, require the *SKN7* gene for induction. This is a direct mechanism as Skn7 was shown to bind at least the *TRX2* promoter. This is the first gene that Skn7 has been shown to regulate directly and is the first example of a direct gene target for any eukaryotic two-component signal transduction protein.

The binding of Skn7 to a specific region within the *TRX2* promoter also, of course, confirms that it is a transcription factor. Despite response regulators being almost entirely transcription factors in prokaryotes, the

A

Strains	Zone of inhibition (mm)
W303-1a	11
*skn7*Δ + YCplac33	19.5
*skn7*Δ + pBAM1	11
*skn7*Δ + pBAM2	11

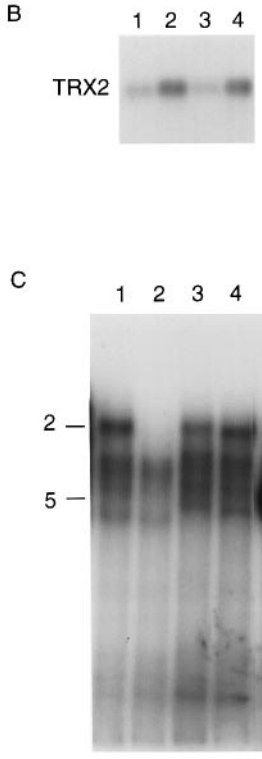

Fig. 8. The Asp427 residue in the response regulator domain of Skn7 is not required for the oxidative stress response. (**A**) 20 μl of a fresh overnight culture of the strains indicated were streaked radially on a YPD plate and allowed to dry. A filter disc saturated with hydrogen peroxide was placed in the centre of the plate. Following incubation at 30°C the extent of inhibition of growth of each strain from the disc was measured. pBAM1 contains wild type Skn7 and pBAM2 contains Skn7 Δ427N. (**B**) The *skn7*Δ strain transformed with pBAM1 or pBAM2 was grown to mid-log in selective media and then treated with 1 mM H_2O_2 for 1 h. RNA was extracted preincubation (0 time) and after the 1 h treatment (1 h). A Northern blot of these samples pBAM1 0 time (lane 1), pBAM1 1 h (lane 2), pBAM2 0 time (lane 3) and pBAM2 1 h (lane 4) was probed with a labelled *TRX2* fragment. (**C**) Using the TRXΔ1 probe crude extracts of the *yap1*Δ strain (lane 1), the untransformed *skn7*Δ*yap1*Δ strain (lane 2) and the *skn7*Δ*yap1*Δ strain transformed with pBAM1 (lane 3), pBAM2 (lane 4) and the C-terminal truncation derivative of Skn7 (lane 5) were analysed by EMSA.

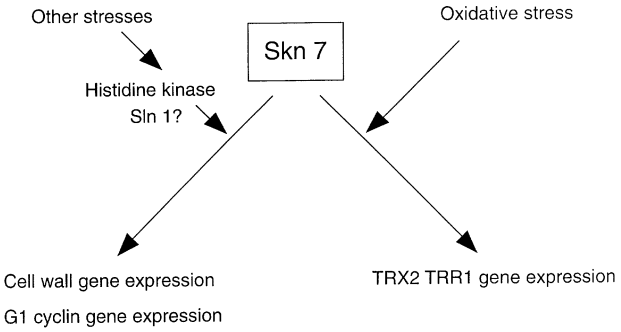

Fig. 9. Regulation of gene expression by Skn7. Two pathways are envisaged for the regulation of Skn7. In one pathway, a histidine kinase, potentially Sln1, regulates the activity of Skn7 on G_1 cyclin and cell wall gene expression in response to unidentified stress. In the other pathway, in response to oxidative stress, Skn7 is regulated independently of phosphorylation of Asp427 in the response regulator domain to activate *TRX2* and *TRR1* gene expression.

two-component systems so far characterized in eukaryotes lie upstream of MAP kinase pathways (Morgan *et al.*, 1995a) and appear to function in regulating MAPKKK activity. This is certainly true of the only other eukaryotic response regulator protein, Ssk1, which controls the Hog1 MAP kinase pathway in budding yeast (see Introduction). Thus our demonstration that Skn7 is a transcription factor makes it the only example known at present that functions as a classical prokaryotic response regulator.

The precise role of Skn7 in the regulation of *TRX2* and *TRR1* expression is unclear at present. The fact that binding of Yap1 is not significantly affected by the absence of Skn7 and vice versa suggests that no interaction is required for binding to occur. However, EMSA analysis revealed one band that clearly contained both the Yap1 and Skn7 proteins. Attempts to co-immunoprecipitate Skn7 with Yap1 failed to identify an interaction (unpublished observations). Moreover, Krems *et al.* (1996) could detect no association using the two-hybrid technique. Possibly the interaction of Yap1 and Skn7 may require prior binding to DNA, hence a direct contact between Yap1 and Skn7 cannot be excluded. There is indirect evidence that Skn7 may interact with at least one other transcription factor. Skn7 induces G_1 cyclin expression through SCB and MCB promoter elements and activates these elements in a reporter construct (Morgan *et al.*, 1995b). The basis of this activation is unclear but since Skn7 does not bind directly to MCBs and SCBs, it may recruit or activate an MCB/SCB binding factor. In spite of this, the near matches to these elements in the *TRX2* promoter seem irrelevant to induction of the gene.

Apart from Skn7 and Yap1 function in *TRX2* expression, other transcriptional events are also taking place as residual *TRX2* induction occurs even in the absence of both proteins. Nevertheless they are responsible for bulk induced levels of the gene. The dual role of Skn7 and Yap1 in control of *TRX2* is consistent with their genetic epistasis and also the Northern hybridization data on *TRX2* induction. In response to hydrogen peroxide, *skn7*Δ strains behave identically to *yap1*Δ strains, the *skn7*Δ*yap1*Δ double mutant strain behaving like the single deletion of either *SKN7* or *YAP1*. However, when the oxidizing agent used was diamide, deletion of the *SKN7* gene had a less deleterious effect than deletion of the *YAP1* gene for sensitivity to the agent and for both *TRX2* and *TRR1* induction. The double delete combination in this case behaves identically to the deletion of *YAP1* alone. Moreover, Yap1 binding increases detectably in response to diamide in contrast to Skn7. This increase in Yap1 binding occurs even in the absence of Skn7 (data not shown). Thus there must be some separation in function of the two proteins and some activation of Yap1 which is Skn7 independent. This is not observed with hydrogen peroxide treatment and indicates that Yap1 is regulated differently by these treatments.

The existence of the receiver domain in Skn7 suggests that it responds to phosphorylation from a histidine kinase. Asp427 is the residue within the receiver domain that would normally be phosphorylated by the histidine kinase (Bourret *et al.*, 1991; Parkinson, 1993). In general, a mutation of this aspartate to asparagine is used to test functionality of the receiver; the similarity of the two amino acids ensuring the structural integrity of the domain. We and others have used a D427N mutation to show that signalling to the receiver domain is essential for the function of Skn7 both in cell wall biosynthesis (Brown *et al.*, 1994) and in G_1 cyclin expression (Morgan *et al.*, 1995b). Thus the normal behaviour of Skn7 D427N in the OSR is surprising. Krems *et al.* (1996) used D427A and D427R mutations in examining the Skn7 response to oxidative stress and concluded that the receiver domain was essential. These particular mutations are rarely used in studying the phosphorylable aspartate residue and conceivably they might have disrupted the tertiary structure of the domain, producing the conflicting results. Certainly when we deleted the receiver domain, this destroyed the function of Skn7 in the OSR. In any event, our result with the D427N mutation indicates that the dephosphorylated form of Skn7 is active in the OSR, which does not, of course, necessarily preclude a role for a histidine kinase.

The only known histidine kinase in budding yeast is Sln1, with a role in the response to osmotic stress (Maeda *et al.*, 1994, 1995). The recent completion of the *S.cerevisiae* genomic sequence has revealed no other likely histidine kinases (B.A.Morgan, unpublished observations), implicating Sln1 in control of Skn7. Deletion of *SKN7* does not lead to osmotic sensitivity (B.A.Morgan, unpublished observations), although whether mutation of *SLN1* results in increased sensitivity to oxidative stress is not yet clear. Interestingly, Sln1 appears to be constitutively active, the kinase activity becoming inactivated in high osmolarity. Thus, its cognate response regulator, Ssk1, is activated when dephosphorylated. If Sln1 controlled Skn7 activity and responded to oxidative stress as it does to osmotic stress, this might lead to dephosphorylation of the Skn7 receiver domain by analogy with Ssk1. Since the Asp427 is not essential for regulation of OSR genes, this could direct Skn7 to these genes rather than cell wall or cyclin genes. Note, however, that Skn7 is also phosphorylated on serine and/or threonine residues (Brown *et al.*, 1994) so that control of the protein could involve alternative pathways without any histidine kinase involvement. Some form of modification of Skn7 seems to be required for its DNA binding activity, as bacterially produced Skn7 is unable to bind the *TRX2* promoter (data not shown).

It is clear that Skn7 regulates at least two groups of genes, those requiring phosphorylation of Asp427, which

include cell wall and G_1 cyclin genes, and the OSR genes which do not require the phosphorylation (Figure 9). The role of Skn7 in regulation of this disparate group of genes remains obscure. The functional receiver domain argues that the protein transduces signals rather than acting as a non-specific transcription factor. The feature common to the genes mentioned above might be an involvement in stress. Apart from the OSR, *skn7*Δ strains are lethal in combination with *pkc1*Δ, the protein kinase C gene (Brown *et al.*, 1994; Morgan *et al.*, 1995). PKC1 regulates a MAP kinase pathway that responds to hypotonic stress (reviewed in Herskowitz, 1995) and that regulates the synthesis of cell wall genes (Igual *et al.*, 1996). There is also the Skn7 DNA binding domain which is related to that of the heat shock factor. Indeed we have recently found that *skn7*Δ strains are sensitive to specific heat stresses (D.Raitt, unpublished observations). Concerning the G_1 cyclin genes, certain stresses lead to a cell cycle delay in G_1 (Rowley *et al.*, 1993; unpublished observations) and we are currently exploring the possibility that Skn7-induced cyclin expression is necessary for recovery from this arrest.

Materials and methods

Strains and growth conditions

The strains of *S.cerevisiae* used in this study were as follows: W303-1a (**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3*), *skn7*Δ (**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 skn7::HIS3*), W303-1b (α *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3*), *yap1*Δ (α *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11 ura3 yap1::TRP1*), *skn7*Δ*yap1*Δ (α *ade2-1 trp1-1 can1-11 leu2-3,112 his3-11 ura3 skn7::HIS3 yap1::TRP1*).

Minimal and rich media used for yeast growth have been described previously (Sherman *et al.*, 1986). Chemicals used in the sensitivity assays were obtained from the following manufacturers: 1.76 M hydrogen peroxide from BDH, 7.7 M *tetra*-butyl hydrogen peroxide, diamide, menadione and cadmium sulfate from Sigma. The chemicals were used at the concentrations indicated in the relevant experiments.

Yeast techniques

The *skn7*Δ*yap1*Δ strain was constructed by disrupting the *SKN7* gene in the *yap1*Δ strain. This was performed by transforming a restriction fragment carrying a HIS3 insertion in the wild type *SKN7* gene into the his3$^-$ *yap1*Δ strain and selecting for his$^+$ (Morgan *et al.*, 1995b). Possible double mutants were then tested by PCR to confirm the disruption. The yeast transformations were performed by a derivation of the lithium acetate technique described previously (Schiestl and Gietz, 1989).

β-galactosidase assays were performed on mid-log phase cells as described previously (Guarente, 1983). Activities are given in OD units at 420 nm per min per mg of protein. Values represent the averages for duplicate samples in three independent experiments.

Plasmid constructs

An *Sst*I–*Xba*I fragment was isolated from plasmids pB-BRY1 and pB-BRY1 Asn427 respectively (Morgan *et al.*, 1995b) and ligated with the *URA3* CEN vector YCplac33 (Gietz and Sugino, 1988) digested with *Sst*I–*Xba*I to create plasmids pBAM1 (wild type *SKN7*) and pBAM2 (*skn7* D427N) respectively. The C-terminal truncation derivative of Skn7 was constructed by transposition of a derivative of the bacterial transposon Tn*1000* (Morgan *et al.*, 1996) into the plasmid YEp24/BRY1 (Morgan *et al.*, 1995b).

RNA analysis

RNA was extracted from yeast strains grown under described conditions, Northern blotted and probed with various gene-specific probes as described previously (Morgan *et al.*, 1995b). Equal amounts of RNA were loaded in all lanes. This was confirmed by probing all Northerns with the *ACT1* gene encoding actin. The probes for the *TRX2* and *TRR1* genes were obtained by PCR from the yeast genome using gene-specific oligonucleotides.

Oligonucleotides used for gel mobility shift assays

The DNA sequence of the oligonucleotides used were as follows: Shift 1, 5′ CATTATTGATGTGTTATTTAAAGATATCG 3′; Shift 2, 5′ CATAACTTGAGTGCCAGTGAAT 3′; TRXΔ1, 5′ TATACTGATATCCCTCTTTCGG 3′; TRXΔ2, 5′ GAAAAGAGCCACCTTGTAGG 3′; TRXΔ3, 5′ TTTCCAGCCAGCCGAAAGAGG 3′; TRXΔ4, 5′ TCCCTCTTTCGGCTGGCT 3′; TRXΔ5, 5′ TCCCTCTTTATGCTGGCTGGAAACTG 3′; TRXΔ6, 5′ GCTGGCTGGAAACTGAACGCGC 3′; WTMCB, 5′ TCTTTTCTTACTAAGCGCGTTCAGTTTCCAGCCAGCCG 3′; and MUTMCB, 5′ TCTTTTCTTACTAAGCTAGTTCAGTTTCCAGCCAGCCG 3′.

Probes were obtained by PCR using the combinations of these oligonucleotides described below. PCR was performed on the TRXLACZ plasmid described in Figure 2. The following oligonucleotides were used to obtain probes 1/2, oligonucleotides Shift 1 and Shift 2: TRXΔ1, oligonucleotides TRXΔ1 and Shift 2; TRXΔ2, oligonucleotides TRXΔ2 and Shift 2; TRXΔ3, oligonucleotides TRXΔ3 and Shift 1; TRXΔ4, oligonucleotides TRXΔ4 and Shift 2; TRXΔ5, oligonucleotides TRXΔ5 and Shift 2; TRXΔ6, oligonucleotides TRXΔ6 and Shift 2; WTMCB, oligonucleotides WTMCB and Shift 1; MUTMCB, oligonucleotides MUTMCB and Shift 1.

Protein extracts and gel mobility shift assays

Yeast cell extracts were prepared from mid-log cultures. Cell pellets were vortexed for 8×30 s with glass beads in 100–200 μl of 200 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 10% glycerol and protease inhibitor mix (PI mix-100 μg/ml phenylmethyl sulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 50 μg/ml TLCK and 100 μg/ml TPCK). After centrifugation, protein concentrations were determined and extracts were stored at –70°C. EMSAs of DNA–protein complexes were conducted as follows: 7 μg protein extract were incubated with 0.5 ng ^{32}P 5′-end-labelled DNA fragment in 10 μl of 25 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 7 mM $MgCl_2$, 10% glycerol, PI mix, 1 mg/ml 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 50 ng/ml poly(dI:dC) at room temperature for 15 min and on ice for a further 15 min. Competition experiments included a 50-fold molar excess of unlabelled competitor DNA over labelled probe. In EMSA supershift experiments, anti-Skn7 or anti-Yap1 polyclonal serum was added to binding reactions at a serum dilution of 10^{-2}. Reaction mixes were loaded directly onto a 4% (37.5:1) non-denaturing polyacrylamide gel and electrophoresed at 200 V in 0.6× TBE buffer at 7°C until free DNA reached the bottom of the gel. The gel was dried onto Whatman 3MM paper and autoradiographed.

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