The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast

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The *atf1*⁺ gene of *Schizosaccharomyces pombe* encodes a bZIP transcription factor with strong homology to the mammalian factor ATF-2. ATF-2 is regulated through phosphorylation in mammalian cells by the stress-activated mitogen-activated protein (MAP) kinases SAPK/JNK and p38. We show here that the fission yeast Atf1 factor is also regulated by a stress-activated kinase, Sty1. The Sty1 kinase is stimulated by a variety of different stress conditions including osmotic and oxidative stress and heat shock. Deletion of the *atf1*⁺ gene results in many, but not all, of the phenotypes associated with loss of Sty1, including sensitivity to environmental stress and inability to undergo sexual conjugation. Furthermore, we identify a number of target genes that are induced rapidly in a manner dependent upon both the Sty1 kinase and the Atf1 transcription factor. These genes include *gpd1*⁺, which is important for the response of cells to osmotic stress, the catalase gene *λ* important for cells to combat oxidative stress, and *pyp2*⁺, which encodes a tyrosine-specific MAP kinase phosphatase. Induction of Pyp2 by Atf1 is direct in that it does not require de novo protein synthesis and results in a negative feedback loop that serves to control signaling through the Sty1/Wis1 pathway. We show that Atf1 associates stably and is phosphorylated by the Sty1 kinase in vitro. Taken together, these results indicate that the interaction between Atf1 and Sty1 is direct. These findings highlight a remarkable level of conservation in transcriptional control by stress-activated MAP kinase pathways between fission yeast and mammalian cells.

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The mitogen-activated protein kinase (MAPK) signaling pathways are critical for the response of cells to changes in their external environment (for review, see Marshall 1994; Herskowitz 1995; Wasiewicz and Cooper 1995; Treisman 1996). They serve to transduce signals generated at the cell surface to the nucleus, where changes in the gene expression pattern of the cell occur. The responses that follow can be varied, including alterations in the proliferation or differentiation state of the cell or changes in protective mechanisms that serve to combat different stress conditions. At the heart of such signaling pathways lies the MAPK module, a kinase cascade that culminates in the activation of a MAPK through phosphorylation by a MAPK kinase (MAPKK), which itself is phosphorylated and activated by a MAPKK kinase (MAPKKK). Such MAPK cascades are evolutionarily conserved, having been identified and characterized in numerous organisms ranging from yeast to mammals (Am-

merer 1994; Herskowitz 1995; Wasiewicz and Cooper 1995). In addition, it is clear that multiple independently acting cascades can function in any one cell. The most striking example of such multiplicity can be found in the budding yeast *Saccharomyces cerevisiae*, where at least five distinct pathways have been identified that serve to control very different physiological processes such as sporulation, mating, cell wall biosynthesis and response to osmotic stress (Ammerer 1994; Herskowitz 1995; Levin and Errede 1995). In mammalian cells three distinct pathways have been identified, although there are clear indications that more pathways do exist. The best-characterized pathway activates the extra-cellular-signal-regulated kinases ERK1 and ERK2 in response to a variety of growth factors and mitogens and has been shown to be involved in the control of cell proliferation and differentiation (Marshall 1994). The physiological function of the other two pathways that activate the MAPKs JNK/SAPK and p38 kinase is less well understood. Both pathways are activated by a variety of stress conditions (e.g., osmotic stress and UV radiation) as well as inflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor) and consequently have been implicated

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in playing a role in the response of cells to stress and in T-cell activation and inflammation (Davies 1994; Waskiewicz and Cooper 1995). The p38 kinase is related highly to the HOG1 kinase of *S. cerevisiae* (57% identity; Han et al. 1994; Freshney et al. 1994; Lee et al. 1994; Rouse et al. 1994), which functions in the response of cells to high extracellular osmolarity; thus, deletion of HOG1 results in cells sensitive to osmotic stress (Brewster et al. 1993). The conservation that is evident from the structural similarity between these stress-activated MAPKs is strikingly clear from the finding that HOG1 can be replaced functionally by p38 (Han et al. 1994).

A Hog1 homolog from *Schizosaccharomyces pombe*, Sty1, has been described recently (Millar et al. 1995), and is also known as Spc1 (Shiozaki and Russell 1995) and Phh1 (Kato et al. 1996). It is phosphorylated and activated by the Wis1 MAPKK. This pathway is involved intimately in the stress response of the cell; it is activated by a variety of stress conditions including osmotic stress, and deletion of either *wis1* or *sty1* results in increased sensitivity to heat and high osmotic conditions (Warbrick and Fantes 1991; Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996; Kato et al. 1996). However, the pathway has additional physiological roles. Both *wis1* or *sty1* deletion mutants are profoundly sterile, indicating that signaling through this pathway is required for one or more steps in the sexual differentiation pathway. In addition, the Sty1/Wis1 pathway plays a role in the control of mitotic initiation, because cells deleted for *wis1* or *sty1* are considerably longer at cell division than wild-type cells, and mutations in the pathway display strong genetic interactions with mutations in other genes such as *cdc25⁺* that result in mitotic defects (Millar et al. 1992, 1995; Shiozaki and Russell 1995). However, how the Sty1 kinase mediates these different activities is at present unknown and will depend absolutely upon the identification of relevant Sty1 phosphorylation targets.

Because the activation of MAPK pathways results in changes in the transcriptional pattern of the cell, a key step in understanding their function is the characterization of transcription factors whose activity is modulated by MAPK phosphorylation. In mammalian cells, a number of factors now have been identified (for review, see Treisman 1996). Elk-1, a member of the TCF family of ETS domain proteins that synergizes with the serum response factor (SRF) to mediate activation of certain immediate early genes such as the *c-fos* gene, was demonstrated to be regulated through phosphorylation by ERK1/2 (Gille et al. 1992; Marais et al. 1993). More recently, however, it has been shown to be a target of JNK/SAPK and p38 (Gille et al. 1995; Whitmarsh et al. 1995; Zinck et al. 1995), indicating that all three mammalian pathways can converge at a common factor and presumably, therefore, common promoters. Other targets show a greater degree of specificity. The cJun factor is regulated by JNK/SAPK (Hibi et al. 1993; Derijard et al. 1994; Kyriakis et al. 1994) but not by ERK1/2 or p38, whereas ATF-2 is phosphorylated and regulated by both JNK/SAPK (Gupta et al. 1995; Livingstone et al. 1995; van

Dam et al. 1995) and p38 (Raingeaud et al. 1995). In all three cases phosphorylation results in an increase in the activation potential of the factor, although the mechanisms that underlie these increases are not understood.

A central question concerning the conservation of the MAPK signaling pathways is whether it is restricted to the MAPK module or whether it extends downstream of the pathway to common transcription factors and target genes that are regulated by the pathways. Structural homologs of ATF-2 and cJun have been identified in *S. pombe*. The *atf1⁺* gene encodes a factor containing a bZip domain at its carboxyl terminus that is related closely to the bZip domain of ATF-2 (Takeda et al. 1995). Furthermore, it has a DNA-binding specificity that is similar to ATF-2 (TG/TACGTC/AC/A) and can activate transcription from a reporter containing ATF sites within its promoter. Although disruption of the *atf1⁺* gene is not lethal, it does result in rapid cell death in stationary phase as well as failure in the initiation of the sexual differentiation pathway. Here we show that Atf1 is downstream of the Wis1/Sty1 pathway and that the expression of multiple targets shown previously to be dependent upon Sty1 and/or Wis1 is also dependent upon Atf1. One such target is *pyp2⁺*, which encodes a tyrosine-specific phosphatase that dephosphorylates and hence inactivates Sty1. Therefore, through the Atf1 factor, a negative feedback loop operates to regulate the extent of signaling through this pathway. Furthermore, we show that the link between Sty1 and Atf1 is direct; Atf1 binds specifically to, and is phosphorylated by, the Sty1 kinase. These results demonstrate remarkable structural and functional conservation in the stress response between mammalian cells and fission yeast.

Results

Disruption of either atf1⁺ or wis1⁺ results in sensitivity to osmotic stress and suppression of uncontrolled meiosis driven by mutation in pat1⁺

We had shown previously that disruption of the *atf1⁺* gene resulted in sterility and loss of viability in stationary phase (Takeda et al. 1995). These properties are shared by mutants disrupted in either *sty1⁺* (Millar et al. 1995; Shiozaki and Russell 1995; Kato et al. 1996) or *wis1⁺* (Warbrick and Fantes 1991), indicating the possibility that the Atf1 factor might be a downstream target of the Wis1/Sty1 signaling pathway. Activation of this pathway can be triggered by a variety of conditions, including an increase in osmolarity, heat shock, UV radiation, oxidative stress, and the addition of DNA damaging agents such as bleomycin (Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996; Kato et al. 1996). This suggests that the pathway is vital for the protective response of cells to such stress conditions, supported by the finding that disruption of *sty1⁺* or *wis1⁺* results in hypersensitivity to high extracellular osmotic conditions. If Atf1 is an essential downstream target of the pathway, then its absence should also result in stress hypersensitivity. As shown in Figure 1A, this

Figure 1. Overlapping phenotypes caused by loss of Atf1 or components of the Sty1/Wis1 signaling pathway. (A) Atf1 is required for resistance to osmotic stress. Wild-type (HM123), $\Delta wis1$ (JM544) and $\Delta atf1$ (NT146) cells were grown on YPD plates and then streaked onto either YPD medium alone or YPD containing 0.5 M NaCl and incubated at 30°C for 3 days. (B) Deletion of *wis1* or *sty1* rescues the *pat1-114* phenotype. *pat1-114* (JM836) or $\Delta wis1$ *pat1-114* (JM1266) or $\Delta sty1$ *pat1-114* (JM1267) cells were plated and streaked on rich medium at either 25°C (left) or 33°C (right).

was found to be the case for osmotic stress. Unlike wild-type cells that proliferate normally on medium containing 0.5 M NaCl, both $\Delta atf1$ and $\Delta wis1$ cells fail to do so. An identical result was observed on medium containing 1.2 M sorbitol (data not shown).

Conversely, it might be expected that phenotypes ascribed to loss of Atf1 would also be seen in $\Delta wis1$ or $\Delta sty1$ cells. We had shown previously that the $\Delta pat1$ phenotype could be suppressed by *atf1*⁺ inactivation (Takeda et al. 1995). The *pat1*⁺ gene encodes a kinase that negatively regulates entry into meiosis. Strains carrying a temperature-sensitive allele of *pat1*⁺ can initiate meiosis at the restrictive temperature in haploid cells (Iino and Yamamoto 1985a); as a result of this meiotic induction, cells fail to grow at 33°C (Iino and Yamamoto 1985b). However, as shown in Figure 1B, both *pat1-114* $\Delta wis1$ and *pat1-114* $\Delta sty1$ cells grew well at this temperature on rich medium, indicating that mutation of *wis1*⁺ and *sty1*⁺, like mutation of *atf1*⁺, suppresses the phenotype that results from Pat1 inactivation. We also showed previously that suppression of the *pat1-114* phenotype by $\Delta atf1$ was influenced by cAMP levels (Takeda

et al. 1995); suppression on minimal medium was obtained in the presence of moderate to high levels but not on low levels of cAMP and caffeine. Similar observations were made with *pat1-114* $\Delta sty1$ cells (data not shown).

Mutations in either *sty1* or *wis1* result in a delay in the timing of mitotic initiation (Warbrick and Fantes 1991; Millar et al. 1995; Shiozaki and Russell 1995). This phenotype is not shared by $\Delta atf1$ cells. As the results in Table 1 demonstrate, unlike $\Delta sty1$ or $\Delta wis1$ cells, $\Delta atf1$ cells display no defect in the timing of mitotic initiation, their cell size at division being close to that seen with wild-type cells. Furthermore, mutants in either *sty1* or *wis1* are synthetically lethal with *cdc25-22* cells that express a temperature-sensitive mutant of the Cdc25 mitotic inducer. No such synthetic interaction was observed between $\Delta atf1$ and *cdc25-22* (Table 1).

Transcription from an ATF-site-dependent reporter is regulated by osmotic stress

We have described previously a reporter gene containing three tandem copies of the E4 ATF-binding site upstream of the basic promoter of the fission yeast *p25* gene (Takeda et al. 1995). Expression of this reporter was shown to be dependent upon the Atf1 factor. Since the results described above indicated that Atf1 could be a downstream target of the Sty1/Wis1 pathway, we asked whether expression of the reporter could be stimulated by an increase in osmolarity. As shown in Figure 2A, a time-dependent 2.5- to 3-fold increase was observed following treatment with 1.2 M sorbitol; expression from a control reporter that lacked the ATF sites was unaffected. This increase was abolished in $\Delta wis1$ cells and enhanced in $\Delta pyp1$ cells (Fig. 2B). Pyp1, like Pyp2, is a tyrosine-specific phosphatase that can inhibit the Sty1 MAPK pathway (Millar et al. 1995; Shiozaki and Russell 1995; Kato et al. 1996). Tyrosine phosphorylation of Sty1 kinase is increased in $\Delta pyp1$ cells. The results show that the expression of the reporter can be controlled by signaling through the Sty1 pathway.

Table 1. $\Delta atf1$ cells do not have a cell size at division defect

Strain	Temperature (°C)	Cell size at division (μm)
wt	30	14.2 ± 0.3
<i>cdc25-22</i>	26	19.3 ± 0.3
<i>wis1::ura4</i>	30	23.4 ± 1.3
<i>sty1::ura4</i>	30	23.2 ± 1.6
<i>atf1::ura4</i>	30	13.1 ± 1.6
<i>cdc25-22 wis1::ura4</i>	26	cdc ⁻
<i>cdc25-22 sty1::ura4</i>	26	cdc ⁻
<i>cdc25-22 atf1::ura4</i>	26	18.1 ± 1.6

Loss of *atf1* does not delay the timing of mitosis.

*Cell size measurements of septated cells grown in liquid synthetic minimal medium (EMM). (cdc) Cell division cycle phenotype. Measurements were the mean of 30 individual determinations (±S.D.).

Figure 2. Atf1 activity is regulated by environmental stress. (A) Wild-type cells containing either a promoter-less *lacZ* reporter (pLacZ; stippled bars) or a *lacZ* reporter fused to an Atf1-dependent promoter (pATFLacZ; solid bars) (Takeda et al. 1995) were assayed for β -galactosidase activity following growth in minimal medium containing 1.2 M sorbitol for the indicated times. (B) Wild-type (HM123), $\Delta wis1$ (JM544), or $\Delta pyp1$ (NT132) cells containing a *lacZ* reporter fused to an ATF-dependent promoter (Takeda et al. 1995) were grown for 3 hr in minimal medium (stippled bars) or minimal medium containing 1.2 M sorbitol (solid bars), and subsequently assayed for β -galactosidase activity.

Atf1 is required for the induction of pyp2 and mediates feedback inhibition of the Sty1 MAPK in response to osmotic stress

Previous results demonstrated that transcription of the *pyp2*⁺ gene encoding a tyrosine-specific phosphatase was induced by increased extracellular osmolarity in a Sty1- and Wis1-dependent manner (Millar et al. 1995). Furthermore, this induction results in the initiation of a negative feedback loop; the newly synthesized Pyp2 dephosphorylates, and hence inactivates, the Sty1 kinase, resulting in transient tyrosine phosphorylation of Sty1 following its activation. In support for such a regulatory pathway, we show in Figure 3A that the loss of Sty1-associated phosphotyrosine following prolonged exposure of cells to osmotic stress is dependent on protein synthesis. When cells are pretreated with cycloheximide and then challenged with 0.9 M KCl, tyrosine phosphorylation of Sty1 remains high for up to 3 hr. Importantly, however, such treatment did not prevent the induction of *pyp2* mRNA; indeed, elevated levels of *pyp2*⁺ transcription persisted over the time course of the experiment, whereas in wild-type cells, induction was transient (Fig. 3B). These results strongly suggest that the induction of *pyp2*⁺ mRNA by activation of the Sty1 MAP kinase occurs via a pre-existing transcription factor acting on the *pyp2*⁺ promoter.

We considered Atf1 to be a strong candidate for such a factor. Not only was there an overlap in phenotypes that derive from mutations in *atf1*⁺ or in the Sty1/Wis1 pathway, but the activity of Atf1 as measured by the reporter assay appeared to be regulated by the Sty1 kinase. Therefore, we asked whether the induction of *pyp2*⁺ transcription by stress required Atf1. Log-phase

cultures of wild-type cells or $\Delta atf1$ cells were treated with 0.9 M KCl and the expression of *pyp2* was observed over time. As shown in Figure 4A, the large stimulation of *pyp2* mRNA expression was completely lost in $\Delta atf1$ cells. Reintroduction of the *atf1*⁺ gene expressed from the thiamine-repressible *nmt1* promoter efficiently reversed this loss. Similar results were observed when wild-type or $\Delta atf1$ cells were shocked with 1.2 M sorbitol, 1 mM H₂O₂, or elevated temperature (data not shown). All of these conditions stimulate the Sty1 pathway (Degols et al. 1996; Kato et al. 1996; M.G. Wilkinson and J.B.A. Millar, unpubl.).

Because expression of *pyp2* is dependent upon Atf1 and because the Pyp2 phosphatase has been shown to be responsible for feedback inhibition of the Sty1 kinase, then it should follow that in $\Delta atf1$ cells activation of Sty1 is prolonged upon exposure to osmotic stress. To test this prediction, either wild-type cells or $\Delta atf1$ cells bearing an epitope-tagged version of Sty1 were challenged with 0.9 M KCl and the phosphotyrosine content on the Sty1 protein was assessed. As shown in Figure 4B, unlike wild-type cells where tyrosine phosphorylation is maximal between 15 and 30 min after exposure but then declines, phosphorylation on Sty1 remains high in $\Delta atf1$ cells. These results show that Atf1 mediates feedback inhibition of the Sty1 kinase in response to osmotic stress.

Atf1 is required for the induction of gpd1 and catalase mRNAs in response to environmental stress

The expression of *gpd1*⁺, a gene that encodes glycerol-3-phosphate dehydrogenase required for glycerol biosyn-

Figure 3. Feedback inhibition of Sty1 kinase requires protein synthesis. (A) Dephosphorylation of Sty1 in response to environmental stress requires de novo protein synthesis. Log phase cultures of wild-type cells bearing an epitope-tagged version of the Sty1 kinase [pREP41-sty1(HA-6His)] were grown in minimal medium lacking leucine and thiamine either in the presence (+ Cyclohex.) or absence of (Control) of 0.1 mg/ml cycloheximide for 2 hr and then incubated for the times indicated in the same medium containing 0.9 M KCl. Sty1 was isolated by Ni^{2+} -NTA affinity precipitation and probed by Western blot for the presence of phosphotyrosine (α -PTyr) or Hemagglutinin epitope tag (α -HA). (B) Inhibition of protein synthesis inhibits disappearance but not induction of Pyp2 mRNA. Log phase cultures of wild-type cells were grown in minimal medium either in the presence (+ Cyclohex.) or absence of (Control) of 0.1 mg/ml cycloheximide for 2 hr and then incubated for the times indicated in the same medium containing 0.9 M KCl. The level of *pyp2* mRNA was assessed by Northern blot analysis using a *pyp2* specific probe. Ethidium staining of ribosomal RNA was used to illustrate approximate equal loading in each lane.

thesis, is induced by osmotic stress in a Sty1- (M.G. Wilkinson and J.B.A. Millar, unpubl.) and Wis1-dependent (Aiba et al. 1995) manner. We predicted, therefore, that expression of *gpd1*⁺ would also require Atf1. That this is indeed the case is demonstrated in Figure 5A. Treatment of wild-type cells by the addition of 0.9 M KCl resulted in a modest increase in *gpd1* mRNA levels; the increase was ~3- to 5-fold, which corresponds closely to previous observations (Aiba et al. 1995). It is striking that although the stimulation by stress is small in comparison to *pyp2*⁺, deletion of *wis1*⁺ or *atf1*⁺ results in an almost complete loss in expression (Fig. 5A). This indicated that the uninduced levels of *gpd1*⁺ were also dependent upon the Sty1 pathway and the Atf1 factor.

The catalase gene in fission yeast is induced by osmotic and oxidative stress and by UV radiation (Nakagawa et al. 1995). Since the same signals activate the Sty1 pathway, we asked whether this factor was involved in catalase expression. As shown in Figure 5B, the

induction of catalase mRNA following the addition of 1.2 M sorbitol was lost in Δ *atf1* cells. Therefore, with respect to the expression of *pyp2*⁺, *gpd1*⁺, and catalase, there is a similar dependence on Atf1 and the stress-activated pathway.

As shown previously (Takeda et al. 1995) and in Figure 6A, using the E4 ATF site as a probe, Atf1-dependent complexes can be detected readily by the electrophoretic mobility shift assay (EMSA) following incubation of the probe with whole-cell extracts from wild-type cells. The complex is not seen with extracts from Δ *atf1* cells and is competed efficiently with unlabeled probe but considerably less well with a probe that has point mutations

Figure 4. The Atf1 transcription factor is required for induction of *pyp2* feedback inhibition of the Sty1 kinase in response to environmental stress. (A) Induction of the Pyp2 MAP kinase phosphatase requires Atf1. Log phase cultures of either wild-type cells (PR109) bearing the empty vector pREP81 (WT), Δ *atf1* cells (NT146) bearing pREP81 (Δ *atf1*) or Δ *atf1* cells bearing pREP81-atf1 (Δ *atf1* + pREP81-atf1) were grown in minimal medium lacking leucine and thiamine and then incubated for the times indicated (in minutes) in the same medium containing 0.9 M KCl. The level of *pyp2* mRNA was assessed by Northern blot analysis using a *pyp2* specific probe. Ethidium staining of ribosomal RNA was used to illustrate approximate equal loading in each lane. (B) Feedback inhibition of Sty1 phosphorylation requires Atf1. Wild-type cells or cells deleted for Atf1 (Δ *atf1*) were transformed with an epitope-tagged version of Sty1 and grown in minimal medium lacking leucine and thiamine at 30°C. They were then incubated in the same medium containing 0.9 M KCl for the times indicated. The Sty1 protein was extracted using Ni^{2+} -NTA agarose beads from the cellular lysates and probed by Western blot for the presence of either phosphotyrosine (α -PTyr) or hemagglutinin tag (α -HA).

Figure 5. The Atf1 factor is required for induction of *gpd1* and catalase mRNAs in response to osmotic stress. (A) Log phase cultures of either wild-type cells (PR109) or $\Delta atf1$ cells (NT146) were grown in rich medium and then incubated for the times indicated (in hours) in the same medium containing 0.9 M KCl. The level of *gpd1* mRNA was assessed by Northern blot analysis using a *gpd1* specific probe. (B) The level of catalase mRNA was assessed by Northern blot analysis using a catalase specific probe as described above, except that osmotic stress was induced with 1.2 M sorbitol.

within the ATF binding sequence (Takeda et al. 1995). Furthermore, there was no significant difference in complex formation with extracts from sorbitol-treated cells, suggesting that activation of Atf1 does not entail an increase in its DNA binding activity (Fig. 6A). Examination of the sequences upstream of the *pyp2*⁺, *gpd1*⁺, and catalase transcriptional start sites revealed the presence of multiple potential Atf1 binding sites. In the case of the *gpd1*⁺ gene, three potential sites are evident at positions -420 to -413 (TTACGTAA), -371 to -364 (TGACGTTT), and -324 to -317 (TTACGTCA) relative to the translation initiation codon. To test whether this upstream region of *gpd1*⁺ could bind to Atf1, EMSA was carried out using a probe extending from -459 to -248. As shown in Figure 6B, a slow migrating complex was detected with wild-type extracts that was missing with extracts from $\Delta atf1$ cells. Again, no difference in the degree of complex formation was seen with extracts from sorbitol-treated cells. These results indicate that Atf1 can bind to sequences upstream of the *gpd1*⁺ transcriptional start site, suggesting that the regulation of *gpd1*⁺ expression by Atf1 is direct.

Atf1 is phosphorylated in vitro by Sty1 kinase

Taken together, the results described above strongly indicate that the activity of Atf1 is regulated by the Sty1/Wis1 signaling pathway. We next addressed whether the

link between the pathway and Atf1 was direct. Log-phase cultures containing an HA-epitope and His₆-tagged Sty1 expressed ectopically from a thiamine-repressible *nmt1* promoter were subjected to osmotic stress, and the Sty1 protein was immunoprecipitated at various times thereafter. Kinase assays were performed with the immunocomplexes using GST-Atf1 as a substrate. Atf1 was found to be an excellent Sty1 substrate (Fig. 7A). Efficient phosphorylation was obtained within 15 min of stress treatment; at later time points there was a gradual decrease, in keeping with the transient nature of Sty1 induction. The presence of multiple phosphorylated species was due to breakdown of the GST-Atf1 fusion protein (data not shown). There was no phosphorylation with immunocomplexes isolated from cells treated in the presence of thiamine; under these conditions no Sty1 protein was synthesized as judged by Western analysis (Fig. 7B).

Recently, a second ATF-like protein in fission yeast has been described to be encoded by the *pcr1*⁺ gene (Watanabe and Yamamoto 1996). Furthermore, it appears that Atf1 and Pcr1 can form heterodimers both in vitro and in vivo. Therefore, we tested whether Pcr1 was a substrate for Sty1 using the immunocomplex kinase assay. As shown in Figure 7C, no inducible phosphorylation was seen. In mammalian cells, the stress-activated kinases phosphorylate and activate members of both the

Figure 6. Atf1 binding activity does not change in response to osmotic stress. (A) EMSA was performed with the labeled E4 ATF probe described previously (Takeda et al. 1995) using extracts derived from wild-type (HM123) or $\Delta atf1$ (NT146) cells or from wild-type cells incubated in medium containing 1.2 M sorbitol. Where indicated, unlabeled competitor DNAs were added at a molar ratio of 100:1; the competitors were either the oligonucleotides E4ATF (wt) or E4M1 (m1) (Takeda et al. 1995). (B) EMSA was performed as above except that the probe used represented sequences -459 to -248 with respect to the translation initiation site of the *gpd1*⁺ gene.

Figure 7. Atf1 is phosphorylated by the Sty1 kinase in vitro. (A) Cells mutated for *sty1* (JM1144) were transformed with a pREP41 vector containing an HA-epitope and His₆-tagged version of Sty1. Expression of the modified Sty1 is regulated by the thiamine-repressible promoter present in this vector; expression is permissible in the absence but not the presence of thiamine. The transformed cells were grown in minimal medium either lacking or containing thiamine; KCl was added to a concentration of 0.9 M; and aliquots subsequently harvested at the times indicated. Extracts were prepared and Sty1 protein was immunoprecipitated from each extract using a hemagglutinin-specific antibody. The immune complexes were tested for kinase activity using GST-Atf1 fusion protein as a substrate. The kinase assays were carried out for 30 min at 30°C and the samples separated by SDS-PAGE and autoradiographed. The arrow indicates the position of the full-length fusion protein. The smaller species represent truncated products of the fusion protein that accumulate following expression in bacteria. (B) The immunoprecipitated complexes described above were assayed directly for the presence of Sty1 protein by Western analysis using the hemagglutinin-specific antibody. The position of HA-His₆-Sty1 is indicated by the arrow. Expression of the modified Sty1 protein is repressed in the presence of thiamine. (C) Transformed cells containing HA-His₆-Sty1 were grown in the absence of thiamine and aliquots harvested before (-) and 15 min after (+) the addition of 0.9 M KCl. Extracts were prepared, Sty1 protein immunoprecipitated, and kinase assays performed as described in Fig. 7A. In addition to GST-Atf1, GST-Pcr1 and GST-Pap1 were used as substrates. Only the GST-Atf1 fusion was phosphorylated. The arrows indicate the migration position of each of the full-length fusion proteins.

ATF family (ATF-2) and the AP-1 family (cJun, junD). The *pap1*⁺ gene of *S. pombe* encodes a transcription factor that has AP-1 DNA binding specificity (Toda et al. 1991). We tested whether the Pap1 protein was also a substrate for Sty1. As shown in Figure 7C, no phosphorylation was observed. These results highlight the specificity of the Sty1-dependent phosphorylation of Atf1.

The mammalian SAPKs, both SAPK/JNK and p38, form stable complexes with their substrates in vitro (Hibi et al. 1993; Derijard et al. 1994; Kyriakis et al. 1994; Gille et al. 1995; Raingeaud et al. 1995). Such complex formation is essential for phosphorylation to occur and provides at least one mechanism by which targets can be recognized and phosphorylated by specific members of the MAPK family. In order to investigate whether Sty1 could form a stable complex with Atf1, lysates from osmotically stressed cells expressing HA-His₆-Sty1 were incubated with GST-Atf1 protein prebound to glutathione-agarose beads. Following extensive washing, kinase assays were performed. As shown in Figure 8A,

the GST-Atf1 fusion was phosphorylated after preincubation in lysates from stressed cells but not from unstressed cells or cells that were grown in the presence of thiamine and therefore did not express the Sty1 protein. In addition, the kinase activity that binds to the GST-Atf1 beads is Wis1-dependent (data not shown). These results are consistent with Sty1 binding to its substrate Atf1 to form a stable complex in vitro.

A physical interaction between Sty1 and Atf1 was confirmed by two additional assays. The first used the two-hybrid assay (Fig. 8B). Sty1 was fused to the Gal4 DNA binding domain and Atf1 to a strong activation domain. Coexpression of the fusion proteins resulted in activation of the Gal4-driven reporter, whereas expression of either alone failed to do so. The second involved the incubation of GST-Atf1 with extracts from cells expressing epitope-tagged Sty1 followed by Western analysis of bound proteins. As shown in Figure 8C, Sty1 bound to GST-Atf1 but not to GST alone or a GST fusion containing the carboxy-terminal region of Atf1.

Figure 8. Interaction between Sty1 and Atf1. (A) *sty1* mutant cells (JM1144) containing HA-His₆-Sty1 as described in Fig. 7A were grown in minimal medium either lacking or containing thiamine to control Sty1 expression. KCl was added to a concentration of 0.9 M and aliquots harvested at the times indicated. Extracts were prepared and incubated with GST-Atf1 protein prebound to GSH-Sepharose beads for 2 hr at 4°C, as described in Materials and Methods. The beads were extensively washed, kinase reaction buffer added, and a kinase reaction assay allowed to proceed for 30 min at 30°C. The phosphorylation of the GST-Atf1 protein was assessed by SDS-PAGE and autoradiography. (B) Two-hybrid analysis of Atf1-Sty1 interaction. *S. cerevisiae* strain Y187 was transformed with: (1) pAS-CYH2 expressing the GAL4 DNA binding domain (BD-cont.) and pACTII-Atf1 containing the Atf1 sequence fused to a strong activation domain (Atf1-AD); or (2) pAS1-CYH2-Sty1 containing the GAL4 DNA binding domain fused to Sty1 (BD-Sty1) and pACTII containing the unfused activation domain (Cont-AD); or (3) pAS1-CYH2-Sty1 and pACTII-Atf1. Expression of a *lacZ* reporter containing GAL4 binding sites within its promoter was measured by assessing β-galactosidase activity in extracts as described in Materials and Methods. Measurements are expressed as the mean of three independent determinations (+s.d.). (C) Extracts of wild-type cells expressing HA-tagged Sty1 were incubated in the presence of 2 μg of GST, GST-Atf1 (a fusion containing full-length Atf1) or GST-Atf1(C-term) (a fusion containing the carboxy-terminal half of Atf1). After 30 min at 4°C, proteins were precipitated with glutathione beads, extensively washed, separated by SDS-PAGE, and probed for the presence of Sty1 using a monoclonal anti-HA antibody.

Discussion

Our main conclusion is that the Atf1 transcription factor is regulated and directly phosphorylated by the Sty1 stress-activated kinase. As such, this demonstrates a remarkable degree of conservation between the function of stress-activated MAPK pathways in fission yeast and mammalian cells where the related ATF-2 factor is regulated by SAPK/JNK and p38 kinases. In addition, it represents the first biochemical demonstration in a yeast system of a direct link between a specific transcription factor and an MAPK signaling pathway.

The conclusion is supported by three lines of evidence: (1) There is considerable overlap in the phenotypes that result from the deletion of *atf1* or components of the pathway such as *sty1* or *wis1*. Thus in both cases the resulting mutant cells are sterile, defective in survival at stationary phase, hypersensitive to stress, and suppressed in the uncontrolled meiotic induction that normally accompanies loss of Pat1 kinase activity. (2) The expression of a number of target genes is regulated by both the stress-activated pathway and Atf1. These include the genes *pyp2*⁺, *gpd1*⁺, and catalase (this study) and *ste11*⁺ and *fbp1*⁺ (Takeda et al. 1995; Kato et al. 1996; Stettler et al. 1996). (3) Atf1 is a substrate for the Sty1 kinase in vitro. In addition, it can form a complex with Sty1 that is sufficiently stable to be detected by the two-hybrid interaction assay and to withstand stringent washing conditions. Such complex formation is a common feature of the mammalian stress-activated MAPKs and their physiologically relevant target proteins.

Regulation of Atf1

The mechanism by which phosphorylation of Atf1 by Sty1 facilitates Atf1-dependent transcription is presently unknown. An important step will be to identify the sites on Atf1 that are phosphorylated by Sty1. Preliminary experiments suggest that multiple phosphorylation sites exist and that the elimination of single sites is not sufficient to render the factor inactive (M. Samuels and N. Jones, unpubl.). In the case of the mammalian ATF-2 factor, regulation is mediated at the level of transcription activation; the factor has an activation domain that is stimulated by SAPK/JNK or p38 phosphorylation (Gupta et al. 1995; Livingstone et al. 1995). Current evidence would suggest that it is also the transactivation properties of Atf1 that are stimulated by Sty1 phosphorylation, as there is no evidence that either the degree of DNA binding of Atf1 or its specificity is altered following induction (Fig. 6B). Recent evidence suggests that Atf1 is present in cells as a heterodimer with another bZip protein Pcr1, and deletion of the *pcr1*⁺ gene results in a phenotype similar to that of *Δatf1* cells (Watanabe and Yamamoto 1996). This suggests that it is the heterodimer that is the subject of regulation by the stress-activated pathway. However, Pcr1 is not phosphorylated by Sty1 and so we suggest that it is the Atf1 component of the heterodimeric complex that is the target of regulation. It will be important in the future to determine the

individual activities associated with each of the subunits of the complex. Preliminary evidence indicates that the expression of only a subset of the Atf1-dependent target genes is also dependent upon Pcr1, suggesting differential roles for Atf1 homodimeric and Atf1/Pcr1 heterodimeric complexes (W.M. Toone and N. Jones, unpubl.).

Genes that are regulated by Atf1

A model for the different biological roles that have been attributed to Atf1 is presented in Figure 9. At least five different genes that are dependent upon the Atf1 factor have now been described (Takeda et al. 1995; this study). In three of these cases (*ste11*⁺, *gpd1*⁺, and *pyp2*⁺), activation by stress conditions in a Sty1/Wis1-dependent manner has been demonstrated. The known function of the products encoded by these genes helps to explain the defective phenotypes associated with deletion of *atf1*⁺. The sterile phenotype of Δ *atf1* cells is, at least partially, explained by the loss of expression of the *ste11*⁺ (Takeda et al. 1995) gene that encodes a transcription factor required for the expression of genes necessary for the sex-

ual differentiation pathway. However, it is likely that Atf1 plays more than one role in the regulation of this pathway, because ectopic expression of *Ste11* suppresses the conjugation defect of Δ *atf1* cells without an accompanying increase in spore formation (data not shown). Very similar observations were made in Δ *sty1* cells expressing *ste11*⁺ from the SV40 promoter (Kato et al. 1996). The additional role of Atf1, therefore, presumably involves the expression of another target required in meiosis.

It is important for cells to maintain intracellular osmolarity in response to changes in the extracellular water potential. One way to accomplish this is to adjust the internal concentrations of an osmolyte such as glycerol. The *gpd1*⁺ gene is involved in the synthesis of glycerol, and its expression has been shown to be important for the response of the cell to high osmolarity conditions. The dependence on Atf1 of its expression explains why Δ *atf1* cells are osmosensitive. The expression of *gpd1* is induced upon osmotic shock. The level of induction, however, is rather modest (three- to fivefold), because of a relatively high level of basal, uninduced expression; nevertheless, this basal expression is dependent upon Atf1 and Wis1. This may reflect a persistent basal level of signaling through the Sty1/Wis1 pathway that can be activated acutely upon imposition of stress.

Both the Δ *sty1* (Degols et al. 1996) and Δ *atf1* (data not shown) cells are mildly sensitive to oxidative stress. This phenotype is very likely explained by the requirement of the Sty1/Wis1 pathway and the Atf1 factor for the induced expression of the catalase gene following a variety of stress conditions. Catalase is a potent free-radical scavenger and plays an important role in the protection of cells against oxidant injury. Accordingly, the induction of catalase activity by H₂O₂ is a common feature of yeast and mammalian cells.

The *pyp2*⁺ gene is another important downstream target of the Sty1 kinase and the Atf1 factor and is responsible, at least in part, for feedback inhibition of the Sty1 pathway by the Pyp2 tyrosine-specific phosphatase. As a result, down-regulation of Sty1 kinase activity observed normally on prolonged exposure to osmotic stress is lost in Δ *atf1* cells. One caveat is that the Sty1 kinase is over-expressed in these experiments and that the stoichiometric quantity of MAP kinase phosphatase relative to MAP kinase may be important for the timing of inactivation. Nevertheless, we believe this provides the first full example of a feedback loop controlling an MAPK pathway to be described. Considerable attention has been focused on the mechanisms of MAPK pathway activation, whereas relatively little is known of the mechanisms by which the signal is attenuated, despite the fact that the duration of signal flow through the cascade may be critical for the ultimate cellular response. Indeed, more recent experiments have uncovered Pyp2- and Atf1-independent mechanisms operating to attenuate signaling through the Sty1 pathway that are yet uncharacterized (Degols et al. 1996; M.G. Wilkinson and J.B.A. Millar, unpubl.).

Although to date many of the targets that we have

Figure 9. The stress activated Sty1 kinase pathway in fission yeast. This model depicts the interactions described in this paper and previous reports (Millar et al. 1995; Shiozaki and Russell 1995; Takeda et al. 1995; Degols et al. 1996; Kato et al. 1996; Stettler et al. 1996). Activation of Sty1 kinase by a signal transduction pathway triggered by numerous stress conditions results in the phosphorylation and activation of the transcription factor Atf1. Active Atf1 promotes the transcription of a number of target genes that are involved in the stress response of the cell (*gpd1*⁺, *fbp1*⁺, *catalase*), in the sexual differentiation pathway (*ste11*⁺) or in a negative feedback control loop (*pyp2*⁺).

identified as being Atf1-dependent are also dependent upon the Sty1/Wis1 pathway, there are likely to be additional targets regulated by the pathway but independent of Atf1. This conclusion comes from the observation that *Δatf1* cells do not display any cell size at division defect and show no synthetic interaction with *cdc25-22* cells; both phenotypes are associated with loss of Sty1 or Wis1. Therefore, the control of the initiation of mitosis by the Sty1 pathway cannot be explained by its control of Atf1 activity. Perhaps a different transcription factor controlled by Sty1 is involved.

Regulation of ATF activity by stress-activated MAPK pathways is conserved

Atf1 shows homology to the mammalian factor ATF-2 (Takeda et al. 1995). This homology is almost entirely restricted to the bZip domain; it would appear to be significant because it is greater than the sequence similarity seen between bZip domains of many of the different mammalian members of the ATF family. The activity of ATF-2 is regulated by the stress-activated MAPK pathways through the phosphorylation of critical threonine residues in the amino-terminal activation domain (Gupta et al. 1995; Livingstone et al. 1995). Our finding that Atf1 is regulated by the equivalent pathway in fission yeast highlights a remarkable level of conservation in the function of the stress-activated pathways. Although it has been clear for some time that the signaling pathways in yeast and mammalian cells show a degree of conservation, this is the first time that the conservation extends to a downstream transcription factor target. It would appear, therefore, that the Atf1 factor is both a structural and functional homolog of ATF-2, and, indeed, preliminary experiments indicate that ectopic expression of ATF-2 in *Δatf1* cells results in partial suppression of the defective mating phenotype (T. Takeda and N. Jones, unpubl.). It should be noted, however, that outside of the bZip domain there is no obvious homology between the two factors. In particular, features of the mammalian ATF-2 transactivation domain such as the zinc-finger and kinase interaction domain cannot be found in Atf1. This may mean that the two factors are regulated differently even though they both require the stress-sig-

naling pathway. On the other hand, it may mean that there are common features that are difficult to recognize until careful structure and function analyses have been carried out. Support for this possibility comes from a comparison of the ATF-2 and cJun factors. Outside of the binding domain, very little homology is apparent. Yet it is now clear that they both contain activation domains that are regulated in a similar fashion; they are dependent upon phosphorylation by stress-activated MAPKs and contain a region involved in stable complex formation with these kinases. Furthermore, ATF-2 has a Hob-1-like motif that has been shown to be an important component of the cJun activation domain, and mutations within this motif of ATF-2 cripple its activity (A. Clark and N. Jones, unpubl.). Thus ATF-2 and cJun clearly overlap at the functional level despite the fact that there is little similarity at the primary amino acid sequence level. We suggest that a similar situation may exist between ATF-2 and Atf1.

The situation appears to be quite different in the budding yeast *S. cerevisiae*. An element has been identified (STRE) that mediates the activation of many genes in response to a variety of stress conditions including osmotic and oxidative stress. This element, however, is not an ATF binding site, and two recently described factors that recognize and bind to it are zinc-finger-containing proteins rather than bZip proteins (Martinez-Pastor et al. 1996). One budding yeast bZip-containing factor that does play a significant role in stress response is the Yap1p activator, which mediates the induction of certain genes by oxidative stress (Ruis and Schuller 1995). However, this factor is homologous to the fission yeast factor Pap1 rather than Atf1; the DNA binding specificities of Pap1 and Atf1 are clearly different. Furthermore, the Sty1 homolog in budding yeast, Hog1p, is activated by osmotic stress only, and so it appears that the HOG pathway, unlike the Sty1 pathway in fission yeast or the JNK/SAPK or p38 pathways in mammalian cells, is not a general stress-signaling pathway. Therefore, with respect to the signaling pathways and the transcription factors that are involved in the stress response, it appears that fission yeast more closely resembles what has been observed in mammalian cells. As such it offers a useful model for studying these stress-related events.

Table 2. *Strains used in this study*

Strain no.	Genotype	Reference/source
HM 123	<i>h⁻leu-32</i>	Our stock
PR 109	<i>h⁻leu1-32ura4-D18</i>	P. Russell (The Scripps Research Institute, La Jolla, CA)
NT146	<i>h⁻leu1-32ura4-D18atf1::ura4⁺</i>	Takeda et al. 1995
JM544	<i>h⁻leu1-32ura4-D18wis1::ura4⁺</i>	Millar et al. 1995
JM1144	<i>h⁻sty1-1leu1-32ura4-D18</i>	Millar et al. 1995
SOP13	<i>h⁻leu1-32ura4-D18ade6-216pyp1::ura4⁺</i>	P. Russell (The Scripps Research Institute, La Jolla, CA)
JM 836	<i>h⁻leu1-32 ura4-D18 pat1-114</i>	This study
JM 1266	<i>h⁻leu1-32 ura4-D18 wis1::ura4⁺ pat1-114</i>	This study
JM 1267	<i>h⁻leu1-32 ura4-D18 sty1::urta4⁺ pat1-114</i>	This study
JM1352	<i>h⁻leu1-32ura4-D18ade6-M210cdc25-22</i>	This study

Materials and methods

Yeast strains, media, and general methods

The yeast strains used in this study are described in Table 2. Media and general experimental methods have been described elsewhere [Moreno et al. 1991; Alfa et al. 1993].

Plasmids

To express various GST proteins, the plasmids pGST-Atf1, pGST-Atf1(C-term), pGST-Pcr1 and GST-Pap1 were constructed by inserting PCR-generated fragments containing full-length Atf1, Pcr1, or Pap1 or truncated Atf1 (amino acids 286–586) into the vector pGEX-KG [Guan and Dixon 1991]. The proteins were expressed and purified on glutathione-agarose beads as described previously [Guan and Dixon 1991]. For the two-hybrid interaction assay, full-length Sty1 and Atf1 sequences were cloned into the vectors pAS1-CYH2 and PACTII, respectively [Durfee et al. 1993].

RNA isolation and hybridization

The isolation of mRNA and subsequent Northern analysis was performed as described previously [Millar et al. 1995]. A 1-kb *EcoRV* fragment from pGEM-3Z-pyp2 was used to probe for *pyp2* mRNA [Millar et al. 1995], a 1.1-kb *EcoRI* fragment was used to probe for *gpd1* mRNA [Pidoux et al. 1990], and a 1.5-kb PCR-derived fragment covering the whole open reading frame was used to probe for catalase mRNA [Nakagawa et al. 1995].

Sty1 kinase assays

Δ Sty1 cells containing the plasmid pRep41 HA/6xHis sty1 [Millar et al. 1995] were grown in EMM containing 75 μ g/ml uracil, and where indicated, 2 μ M thiamine until they reached midlog phase. Then 40 ml of cells were mixed with either 17 ml fresh (Edinburgh minimal medium (EMM) or EMM containing 3 M KCl and incubated for the appropriate length of time. The cells were rapidly cooled and harvested 4°C. The resulting pellets were washed with ice-cold H₂O and resuspended with 40 μ l of lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 15% glycerol, 0.1% TritonX-100, 1 mM DTT, 5 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin, 10 g/ml leupeptin, 1 mM EGTA, 1 mM tetrasodium pyrophosphate, 100 mM β -glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate] and broken by vortexing with 1 ml of chilled acid-washed glass beads. The resulting supernatant was removed and assayed for protein concentration by use of the Bio-Rad Protein Assay Kit.

For the immunocomplex kinase assay, 170 μ g of lysate was incubated with 5 μ l of monoclonal anti-HA antibody for 1 hr at 4°C. Then 20 μ l of protein G-Sepharose (dry volume) was added for an additional 1 hr. The beads were collected, and washed 3 $\times$ with 400 μ l of lysis buffer. Then 2 μ g of GST-Atf1 protein was added in wash buffer. The buffer was removed and 20 μ l of kinase reaction buffer [20 mM HEPES (pH 7.5), 20 mM MgCl₂, 2 mM DTT, 20 μ M ATP, 5 μ Ci [γ -³²P] ATP] was added. The reaction was carried out for 30 min at 30°C and stopped by adding 5 μ l of 5 $\times$ Lammeli buffer and heated to 95°C for 5 min. Samples were then run on 10% PAGE gels, transferred onto PVDF membranes, and autoradiographed.

For the binding kinase assay, 300 μ g of lysate was incubated with 2 μ g of GST-Atf1 protein prebound to glutathione-Sepharose for 2 hr at 4°C. The beads were collected, washed 3 $\times$ with 400 μ l lysis buffer, and 20 μ l kinase reaction buffer [20 mM HEPES (pH 7.5), 20 mM MgCl₂, 2 mM DTT, 20 μ M ATP, 5 μ Ci [γ -³²P] ATP] was added. The reaction was for 30 min at 30°C and

was stopped by adding 5 μ l of 5 $\times$ Lammeli buffer and heated to 95°C for 5 min. Samples were then run on 10% PAGE gels, transferred onto PVDF membranes, and autoradiographed.

Western blot analysis

The PVDF membranes were blocked for 1 hr with 3% BSA containing 0.1% (vol/vol) Tween 20. Anti-HA antibody was added at 1/1000 dilution in blocking buffer for 1 hr and the blot was washed 3 $\times$ for 15 min with TBS plus 0.5% NP40. Anti-mouse HRP at 1/10000 was applied for 1 hr and the blot was washed 3 $\times$ for 15 min. ECL (Amersham) was performed to visualize the bound antibody.

Electrophoretic mobility shift assay

Cells were grown to mid-log phase, harvested, washed with 1 ml of H₂O, resuspended in 40 μ l of lysis buffer [25 mM HEPES (pH 7.6), 0.1 mM EDTA, 150 mM KCl, 0.1% TritonX100, 25% Glycerol, 1 M urea, 1 mM DTT, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM EGTA, 1 mM tetrasodium pyrophosphate, 100 mM β -glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate] and lysed as above. Then 30 μ g lysate was incubated in 20 μ l binding buffer containing 25 mM HEPES (pH 7.6), 34 mM KCl, 5 mM MgCl₂, and 2 μ g of poly[d(I-C)] for 10 min at room temperature, and then for 20 min with 1 ng ³²P-labeled probe. Reactions were run out on a 4% acrylamide gel in 0.5 M TBE, dried down, and exposed for autoradiography.

Two-hybrid interaction assay

The *atf1* gene was amplified by PCR using the 5' oligonucleotide 5'-ACATACCATGGCATCCCCGCTCCCGTCAATACCTTCCA-3' incorporating an *NcoI* site (shown italicized) and the 3' oligonucleotide 5'-ACGTCTCGAGAGATCAAAAA-CAGTCTAGTACCT-3' incorporating a *XhoI* site (shown italicized) to produce a 1734-bp fragment that was digested with *NcoI* and *XhoI* and ligated into the *NcoI* and *XhoI* sites of pACTII [Durfee et al. 1993] to form PACTII-atf1 expressing a fusion protein of the GAL4 activation domain with the *atf1* ORF under the control of the *adh* promoter. The plasmid pRep41HA/6xHis sty1 [Millar et al. 1995] was digested with *NdeI* and *BamHI* and a fragment containing the *sty1* ORF ligated into the *NdeI* and *BamHI* sites of pAS1-CYH2 [Durfee et al. 1993] to form pAS1-CYH2-Sty1. Expression of both fusion proteins in *S. cerevisiae* strain Y187 [Durfee et al. 1993] was verified by Western blot using a monoclonal antibody to the hemagglutinin tag (12CA5).

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