Regulation of Schizosaccharomyces pombe Atf1 Protein Levels by Sty1-mediated Phosphorylation and Heterodimerization with Pcr1

The Atf1 transcription factor plays a vital role in the ability of Schizosaccharomyces pombe cells to respond to various stress conditions. It regulates the expression of many genes in a stress-dependent manner, and its function is dependent upon the stress-activated MAPK, Sty1/Spc1. Moreover, Atf1 is directly phosphorylated by Sty1. Here we have investigated the role of such phosphorylation. Atf1 protein accumulates following stress, and this accumulation is lost in a strain defective in the Sty1 signaling pathway. In addition, accumulation of a mutant Atf1 protein that can no longer be phosphorylated is lost. Measurement of the half-life of Atf1 demonstrates that changes in Atf1 stability are responsible for this accumulation. Atf1 stability is also regulated by its heterodimeric partner, Pcr1. Similarly, Pcr1 levels are regulated by Atf1. Thus multiple pathways exist that ensure that Atf1 levels are appropriately regulated. Phosphorylation of Atf1 is important for cells to mount a robust response to H₂O₂ stress, because the Atf1 phospho-mutant displays sensitivity to this stress, and induction of gene expression is lower than that observed in wild-type cells. Surprisingly, however, loss of Atf1 phosphorylation does not lead to the complete loss of stress-activated expression of Atf1 target genes. Accordingly, the Atf1 phospho-mutant does not display the same overall stress sensitivities as the atf1 deletion mutant. Taken together, these data suggest that Sty1 phosphorylation of Atf1 is not required for activation of Atf1 per se but rather for modulating its stability.

Eukaryotic cells have developed response mechanisms to combat the harmful effects of a variety of stress conditions. In the majority of cases, such responses involve changes in the gene expression pattern of the cell, leading to increased levels and activities of proteins that have stress-protective functions. Central to these responses are the sensing and signaling pathways that communicate with the nucleus and facilitate necessary changes in gene expression. Of particular importance are the pathways collectively known as MAPK pathways (1). The best characterized of these is a mammalian pathway that activates 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 (2, 3). Another subset of the MAPK pathways within a cell, the stress-activated protein kinases (SAPKs), responds to stress. In mammalian cells, two such pathways exist that lead to the activation of the JNK and p38 kinases (2, 3). A number of transcription factors are phosphorylated in response to SAPK activation, examples being c-Jun, which is regulated by JNK, and ATF2, which can be regulated by both JNK and p38 (4–8).

In Schizosaccharomyces pombe, a single member of the SAPK family called Sty1/Spc1/Phh1 (hereafter referred to as Sty1) has been identified (9–11). The Sty1 MAPK stimulates gene expression via the Atf1 transcription factor, which is similar to the human ATF2 factor, and binds to closely related DNA sequences (12, 13). Thus, the transcription factor targets of the S. pombe pathway are closely related to a subset of those phosphorylated and regulated by SAPKs in mammalian cells. The Atf1 factor was identified independently through the S. pombe DNA-sequencing project, by a genetic screen for sterile mutants that demonstrated a defect in G₁ arrest following nitrogen starvation and through a genetic screen for genes that could suppress the mating defect of sty1– cells (12, 14, 15). Atf1 binds to its cognate site together with a second basic-leucine zipper (b-ZIP) containing protein Pcr1 (16); the heterodimer has a significantly higher binding affinity than either homodimer complex (17). Disruption of the atf1 gene results in a range of phenotypes, including defects in sexual differentiation and maintenance of viability in stationary phase and sensitivity to osmotic stress and oxidative stress (14, 15, 18–20). Thus Atf1 is crucial for fission yeast cells to respond normally to...
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TABLE 1
S. pombe strains used in this study

| Strain     | Genotype                        | Source               |
|------------|---------------------------------|----------------------|
| NJ2        | h’ ura4-D18 leu1-32 ade6-M210 his7-366 | C. Hoffman           |
| NJ74       | h’atf1-2HA6His:LEU2 ura4-D18 leu1-32 ade6-M216 | This study           |
| NJ72       | h’atf1-11M-2HA6His:LEU2 gad7::ura4- ura4-D18 leu1-32 ade6-M216 | Kanoh et al. (15)    |
| H222       | h’gad7::ura4- ura4-D18 leu1-32 ade6-M216 | Watanabe and Yamamoto (16) |
| JX25       | h’pcre::ura4- ura4-D18 leu1-32 ade6-M216 | This study           |
| NJ437      | h’atf1::kan’ pcr1::ura4- leu1-32 ade6-11M-2HA6His:LEU2 gad7::ura4- ura4-D18 | Gordon et al. (37)   |
| NJ55       | h’mt2-1 leu1-32 ura4-D18         | Takeda et al. (14)   |
| NJ28       | h’atf1::ura4- leu1-32 ade6-11M-2HA6His:LEU2 gad7::ura4- ura4-D18 | Lab stock            |
| H178       | h’sty1-1atf1::ura4- ura4-D18 leu1-32 ade6 his7-366 | K. Kitamura          |
| NJ34       | h’ sty1::ura4- ura4-D18          | Lab stock            |
| KS553      | h’wis1::his1’ ura4- ura4-D18 leu1-32 his7-102 | Shiozaki et al. (35) |
| KS2081     | h’wis1DD:12myc ura4-D18 leu1-32 |                     |
| NJ260      | h’atf1::kan’ leu1-32 ura4-D18 ade6-M210 his7-366 |                     |

a range of different stress conditions. Previous studies have shown that stress-induced activation of Sty1 results in its translocation to the nucleus whereby it binds to, and phosphorylates, Atf1 (21). This finding, together with the observation that the expression of Atf1 target genes requires Sty1 activation, strongly suggested that phosphorylation of Atf1 stimulates its activity. However, the mechanism by which this regulation might occur is not known.

In mammalian cells, regulation of ATF2 by JNK or p38-induced phosphorylation has been investigated in detail. Regulation appears to be exercised at a number of different levels. In the absence of stimulating conditions, ATF2 is transcriptionally inactive due to an intramolecular interaction between the DNA binding domain and the amino-terminal region. It has been suggested that this intramolecular inhibition is disrupted, and transcriptional activities restored, when ATF2 interacts with other proteins, such as E1A or c-Jun, or when it is phosphorylated by SAPKs (22). Phosphorylation has also been shown to increase the activity of the isolated transactivation domain of ATF2, although the mechanism involved remains obscure (6, 8, 23). Yet another mode of regulation involves ubiquitin-mediated degradation by the 26 S proteasome; phosphorylation of ATF2 by SAPKs appears to protect it from ubiquitination and subsequent degradation (24–26). In this study, we sought to investigate how Atf1 may be regulated through Sty1-mediated phosphorylation. Our results indicate that phosphorylation regulates the stability of Atf1 as does heterodimerization with its binding partner Pcr1. This regulation, however, appears to be only partly responsible for the role of Sty1 in stimulating Atf1-dependent gene expression suggesting that at least one other mode of control must exist that does not involve direct Atf1 phosphorylation.

EXPERIMENTAL PROCEDURES

Yeast Strains and General Methods—S. pombe strains used are listed in Table 1. Yeast media and general experimental methods were as described (27). For the dilution assays, exponentially growing cells at a concentration of 1 × 10^6 cells/ml were diluted 5-fold a total of four times, and 7.5 μl of each dilution, including the starting dilution of 1 × 10^6 cells/ml, was plated on yeast extract plates containing various stress-inducing agents.

For all procedures requiring harvesting of cells, mild centrifugation (2 min, 580 relative centrifugal force) was used. This procedure did not lead to activation of Sty1 (data not shown). A previous study has also shown that mild centrifugation for 2 min at 800 relative centrifugal force did not lead to a difference in the profile of gene expression compared with that obtained from cells isolated by filtration (28), consistent with our finding that mild centrifugation does not activate the stress-activated MAPK pathway in fission yeast. Cycloheximide (Sigma C8459) was added to cultures at a final concentration of 100 μg/ml to inhibit protein synthesis.

Creation of an atf1-11M Allele and Integration—The atf1 cDNA was cloned into the pREP81 vector (29) with two hemagglutinin epitopes and six histidine residues placed at the carboxyl terminus of the open reading frame (ORF). Point mutations were introduced into pREP81(atf1−) by a PCR-based method. Serines at positions 2, 4, 140, 152, 172, 226, and 438 and threonine at position 77 were replaced by alanine, and threonines at positions 204, 216, and 249 were replaced by isoleucine (pREP81(atf1-11M)). For atf1−, a 1.4-kb EcoRI fragment, which encompassed the carboxyl-terminal region of the atf1 cDNA and the nmt1 terminator region, was subcloned into the YIplac128 vector (30). The vector was linearized with Mss1, and the resulting fragment was transformed into a wild-type strain (NJ2). Stable leu− clones were selected, and integration and copy number were determined by PCR and Southern blotting, respectively. This strain expressing the tagged version of Atf1 was indistinguishable from one expressing untagged Atf1. For atf1-11M, a 1.2-kb fragment upstream of the atf1 coding region was PCR-amplified, ligated to the atf1-11M ORF, and inserted into YIplac128 with the nmt1 terminator region cloned downstream. The plasmid was digested at a unique Stu site and transformed into the S. pombe atf1/gad7 disruptant (JX305) (15). Integrated clones were assessed as above.

Protein Extract Preparation and Western Blot Analysis—Native S. pombe cell extracts were prepared by glass bead lysis in the following buffer (50 mM Tris-CI pH 7.5, 0.15 mM KCl, 10 mM MgCl₂, 20 mM β-glycerophosphate, 5 mM EDTA, 0.1 mM sodium vanadate, 1 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride and protein inhibitor Complete™ (Roche Applied Science). Before lysis, cells were washed once in stop buffer (150 ml of NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN₃, pH 8).

Total cell extracts were resolved by SDS-PAGE and transferred electrophoretically to Immobilon-P™ (Millipore) or nitrocellulose. For the analysis of Atf1, 8% gels were prepared. Pcr1 was detected after extracts had been separated by 15%
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SDS-PAGE. Polyclonal anti-Atf1 and anti-Pcr1 antisera were obtained by immunizing rabbits with GST-Atf1 or GST-Pcr1. Anti-Atf1 and Anti-Pcr1 antisera were purified using antigen-spotted membranes and used at dilutions of 1:750 and 1:500, respectively. Anti-tubulin antiserum was obtained from Sigma (T5168) and used at a dilution of 1 in 1000, Mts4 antiserum (49) was used at a dilution of 1 in 5000, and a monoclonal anti-HA antibody (12CA5) was obtained from Roche Applied Science was used at a dilution of 1 in 1000. Detection was performed using a peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences Pharmacia) and chemiluminescence visualization (ECL+, Amersham Biosciences) was used according to the manufacturer’s instructions. Immunoprecipitations were carried out using an anti-hemagglutinin matrix (Roche Applied Science) according to the manufacturer’s instructions. Phosphatase analysis was carried out using a phosphatase (New England Biolabs) for 30 min at 30 °C. Quantification of Western blot signals was performed using the Chemi Genius Bioimaging system (Syngene) and the Chemi genius gel documentation and analysis system.

Denatured *S. pombe* extracts were prepared using the following method. A volume corresponding to $A_{400 \text{ nm}}$ 0.5–3.0 of cells was harvested and resuspended by vortexing in 1 ml of 0.3 M NaOH. 150 μl of 55% (v/v) trichloroacetic acid was added; the mixture was vortexed and incubated on ice for 10 min. The cells were pelleted at 4 °C for 10 min at 14,000 rpm. The supernatant was removed by aspiration, and the cells were spun briefly for a second time to remove remaining trichloroacetic acid. The pellet was resuspended in 75 μl of SDS gel-loading buffer per $A_{400 \text{ nm}}$ of cells (SDS gel loading buffer: 50 mM Tris-Cl (pH 6.8), 2% SDS (w/w), 0.1% bromphenol blue, 10% (v/v) glycerol, 10 mM dithiothreitol). Proteins were denatured for 10 min at 100 °C. Samples were centrifuged briefly, before loading onto SDS-PAGE gels.

Two-dimensional Gel Electrophoresis—Total cellular protein was prepared from NJ2, NJ71, and NJ72. 14 mg of whole cell protein was used in immunoprecipitation reactions. After the final wash the matrix was resuspended in two-dimensional gel sample buffer (8 M urea, 2% CHAPS, 0.002% bromphenol blue) and subjected to isoelectric focusing using 7-cm strips pH 4–7 (Amersham Biosciences) and the IPGphor (Amersham Biosciences) according to the manufacturer’s instructions. Isoelectric focusing steps were as follows: rehydration for 16 h; 5000 V for 1 h, 4000 V for 1 h 30 min, and then 5000 V for 2 h. The second dimension was separated by SDS-PAGE on an 8% gel.

RNA Analysis—10-μg samples of total RNA isolated at the time points indicated in the figures were denatured with formaldehyde, separated on a 1% agarose gel, and transferred to a Hybond-N+ membrane (Amersham Biosciences). Probes for RNA-DNA hybridization were PCR-generated fragments internal to the gene concerned and labeled with $32P$ by use of a DNA Megaprinte labeling kit (Amersham Biosciences). *atf1* and *atf1-11M* mRNA levels were quantified relative to the *hmgl* transcript using the Chemi genius system.

Chromatin Immunoprecipitation Assays—The chromatin immunoprecipitation procedure is based on the methods described elsewhere (31, 32) with some modifications. All steps were performed on ice except when indicated. 100 ml of yeast cells $A_{950}$ = 0.6 were cross-linked for 10 min with 1% formaldehyde at 24 °C. After addition of 125 mM glycine and incubation for 5 min at 24 °C, cells were chilled on ice, washed with ice-cold dH2O, and suspended in 400 μl of lysis buffer (50 mM Heps-KOH, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 20 mM β-glycerophosphate, 50 mM NaF, 0.1 mM Na3VO4, 0.5 mM sodium pyrophosphate, Complete™ protease inhibitor EDTA free (Roche Applied Science), 1 mM EDTA). Crude extracts were prepared by 3–4 pulses (15 s at level 5) of bead beating in a FastPrep FP120 Ribolyzer (Thermo Savant, Bio 101, Inc., Vista, CA) until 70–80% of cells were broken. Extracts were sonicated four times for 10 s (level 5) using a Soniprep 150 (Sanyo) until chromatin was sheared to an average size of 500 bp and subsequently cleared of insoluble cell debris by short centrifugation at full speed. 5 μl of the whole cell extract was saved as an INPUT control. Immunoprecipitation was performed for 3–4 h with Dynal protein A-coated magnetic beads, which were previously incubated overnight with anti Atf1 antiserum. Precipitates were washed three times with 1 ml of lysis buffer, 1 ml of lysis buffer plus salt (like lysis buffer, except 500 mM NaCl), and 1 ml of wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA), respectively. Precipitates were eluted by 10 min of incubation at 65 °C in 40 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). Eluates were incubated at 65 °C in a total volume of 150 μl (100 μl for INPUT control) elution buffer containing RNase (0.1 mg/ml) for overnight, then doubled in volume with TE buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 mg/ml glycogen, and 0.5 mg/ml protease K and incubated for 2 h at 37 °C. Immunoprecipitated DNA was purified using the Qiagen PCR purification kit. We used standard PCR (data not shown) as well as real-time PCR, to quantify for each immunoprecipitate the relative amount of DNA corresponding to stress-induced (*gpd1* and *hsp9*) or stress-independent (*act1*) over the stress-independent promoter (*cdc2*). The ORF of the stress-independent *pol1* gene was used as an additional background control. Primers used relative to the transcription start codon corresponded to −214 to −193 and −168 to −149 for the *cdc2* promoter; −402 to −380 and −360 to −340 for the *gpd1* promoter; −264 to −245 and −220 to −199 for the *hsp9* promoter; −113 to −85 and −61 to −34 for the *act1* promoter; and +2520 to +2543 and +2567 to +2588 for the *pol1* ORF. For real-time PCR we set up 12.5-μl reactions containing 0.5 μl of purified DNA from a particular immunoprecipitate, 1 μl of 5 μM 5′-oligonucleotide primer, 1 μl of 5 μM 3′-oligonucleotide primer, 6.25 μl of SYBR Green PCR master mix (Applied Biosystems), and 3.75 μl of dH2O. Reactions were analyzed using an ABI 7900 thermal cycler according to the manufacturer’s instructions. We performed three independent PCR reactions and calculated the mean “threshold cycle number” (or $C_t$ value). The -fold enrichment of the stress-inducible promoters relative to stress-independent promoters for a particular sample was calculated using the following formula: -fold enrichment = $2^{C_t\text{INPUT}} - C_t\text{IP}/2^{C_t\text{INPUT}} - C_t\text{background}$, where $C_t$ IP is the $C_t$ value for the immunoprecipitate (*gpd1*, *hsp9*, *act1*, and *pol1*), and $C_t$ background is the $C_t$ value for the background control (*cdc2*).
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**RESULTS**

**Atf1 Protein Accumulates following Stress**—The Atf1 protein possesses eleven potential MAPK sites, namely a serine or threonine residue immediately followed by a proline (Fig. 1A). We first examined the levels of Atf1 protein before and after two different types of stress, namely oxidative stress (H\(_2\)O\(_2\)) and osmotic stress (sorbitol) to determine whether, by analogy to the mammalian ATF2 factor, phosphorylation modulated Atf1 stability (24–26). In wild-type cells the level of Atf1 was significantly increased following stress imposition (Fig. 1B). In addition to increased levels, the mobility of the Atf1 protein was retarded; this has been shown previously to be due to phosphorylation (12, 16). These results are consistent with the possibility that stability of Atf1 is linked to its phosphorylation state. Interestingly, the extent and kinetics of phosphorylation appear to be dependent on the particular type of stress applied. To directly test whether phosphorylation affects stability, we constructed a mutant atf1 gene containing single point mutations in all potential MAPK phosphoacceptor sites (atf1-11M). This gene was integrated into the atf1 locus so that atf1 mRNA expression was under the control of the endogenous promoter. Following the imposition of stress, neither retardation nor significant accumulation of the mutant atf1-11M protein was observed (Fig. 1B). The results are consistent with a lack of phosphorylation of the mutant protein and a dependence on direct phosphorylation for Atf1 accumulation.

There was a slight reduction in the amount of mRNA produced for the atf1-11M transcript compared with wild-type (Fig. 1C), but the levels did not seem to drop sufficiently enough to explain the dramatic decrease that we observed in the level of atf1-11M protein. A slight increase in the amount of atf1-11M protein over time was observed (Fig. 1B, compare levels at \(t = 0\) to \(t = 30\)) and was probably due to a modest increase in the atf1 mRNA levels that we observed upon stress (Fig. 1C) (12, 33). Previously, we have analyzed the global changes in gene expression in response to stress and found that, in H\(_2\)O\(_2\) stress, the levels of atf1 mRNA are induced 2.3- and 2.7-fold at 15 and 60 min of stress, respectively; in response to sorbitol stress the levels of atf1 mRNA are induced 2.6-fold at 15 min and have returned to basal levels by 60 min (34). These modest increases are in agreement with our Northern blot analysis (Fig. 1C).

To support the above conclusions, we examined Atf1 protein levels in different mutant backgrounds defective, or containing a constitutively active, Sty1 kinase pathway. Wis1 is an MAPK kinase that phosphorylates and thus activates Sty1. In both sty\(1\alpha\) and wis\(1\Delta\) cells, the level of Atf1 in the absence of stress was lower than in wild-type cells (Fig. 1D). Furthermore, no increase in levels and no retardation of mobility were observed following H\(_2\)O\(_2\) stress. In contrast, however, in wis\(1\)-DD cells, which have a constitutively active allele of the MAPK kinase wis1 (35), Atf1 levels were high in the absence of stress and a significant proportion had a retarded mobility. The levels did not increase further following stress. Thus all the results described above show a tight correlation between the phosphorylation of Atf1 and its level in the cell.
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**A**

|        | aff1 | aff1-11M | aff1 | aff1-11M |
|--------|------|----------|------|----------|
| H₂O₂   | -    | -        | +    | +        |
| α      |      |          |      |          |
| 75kD   |      |          |      |          |

**B**

|        | wt   | aff1 | aff1-11M |
|--------|------|------|----------|
| 75kD   |      |      |          |

**C**

|        | aff1-11M | aff1-11M |
|--------|----------|----------|
| - phosphatase | + phosphatase | -/+ phosphatase |
| 75kD    |          |          |

α-Atf1

**FIGURE 2.** Atf1 is phosphorylated under basal as well as stress conditions but not when its MAPK sites are mutated. A, the aff1-11M protein does not appear to be phosphorylated in the absence or presence of stress. The Atf1 protein was immunoprecipitated from aff1-HA or aff1-11M-HA cells in the absence or presence of 1 mM H₂O₂ (for 1 h), and half of each sample was treated with α-phosphatase. The status of Atf1 was analyzed by Western blotting. B, analysis of the Atf1 and aff1-11M proteins by two-dimensional gel electrophoresis. Atf1 protein was immunoprecipitated using an anti-HA antibody from either aff1-HA, aff1-11M-HA, or untagged cells (wt) that had been stressed with 1 mM H₂O₂ for 1 h. The precipitated proteins were separated by isoelectric focusing over the pH range 4–7. The second dimension the proteins were analyzed by Western blotting. C, aff1-11M protein was isolated from cells that had been stressed as in B. Half of the precipitated protein was treated with α-phosphatase. Half of the phosphorylated fraction was mixed with unphosphatased aff1-11M protein and analyzed by two-dimensional gel electrophoresis (as described in B) as were the unmixed fractions of aff1-11M. The arrows indicate the aff1-11M protein.

**Atf1 Is Phosphorylated under Basal and Stress Conditions but Not When Its MAPK Sites Are Mutated**—Although all the potential MAPK sites in Atf1 had been mutated, it was important to examine the phosphorylation status of aff1-11M, because it was possible that Sty1 could still phosphorylate a site that did not conform to the MAPK consensus motif. A previous study has shown that the mammalian homologue of Sty1, p38α, can phosphorylate one of its targets, TAB1, at a serine-alanine site that does not fit the MAPK consensus motif (36). Firstly, we stressed aff1-HA and aff1-11M-HA cells and analyzed the Atf1 protein from these and unstressed control samples. The immunoprecipitated protein fractions were split into two, and one-half of each sample was treated with α-phosphatase to remove the phosphate groups and analyzed by Western blotting (Fig. 2A). Interestingly, the Atf1 protein growing in rich media without stress appeared to be basal phosphorylated. Upon stress, as expected, Atf1 became hyperphosphorylated. In contrast, the aff1-11M protein did not appear to be phosphorylated, either in the presence or absence of stress. To confirm these findings, the immunoprecipitates prepared from stressed cells were subjected to two-dimensional gel electrophoresis. The Atf1 protein from stressed cells comprises several isoforms of approximately the same molecular mass consistent with multiple phosphorylated forms of Atf1. For aff1-11M, however, there was only one spot consistent with one isoform (Fig. 2B). This isoform corresponded to the unphosphorylated species, because upon mixing phosphatased with non-phosphatased aff1-11M, there was still only one spot observed by two-dimensional gel analysis (Fig. 2C). We have also analyzed wild-type Atf1 protein isolated from a sty1Δ mutant. In this case, the Atf1 protein exists as a single isoform when analyzed by two-dimensional gel electrophoresis. Furthermore, there was only one isoform both in the presence or absence of stress. These isoforms focused at the same pI. Moreover, these isoforms were carried out at the same pI as aff1-11M protein isolated from cells either in the absence of presence of stress (supplemental Fig. S1). Taken together, these data are consistent with aff1-11M not being a phospho-protein.

**Atf1 Is Stabilized upon Stress through Phosphorylation**—The increase in Atf1 levels following stress could be due to an increase in aff1 gene expression, an increase in mRNA stability, an increase in translation of aff1 mRNA, modulation of Atf1 protein stability, or a combination of various mechanisms. To investigate whether protein stability plays a role in this increase we first examined the half-life of Atf1 protein with or without stress. A plasmid containing a wild-type copy of the aff1 gene under the control of the thiamine-repressible nmt41 promoter was transformed into aff1Δ cells. This results in expression of Atf1 that is comparable to wild type (supplemental Fig. S2). Transcription of aff1 and synthesis of Atf1 protein were repressed by the addition of thiamine and cycloheximide, respectively, and 2 h later H₂O₂ was added to half of the culture. The levels of Atf1 protein were followed by Western blotting. In the absence of stress, the half-life of Atf1 was ~1.1 h (Fig. 3, A and B). Following H₂O₂ treatment however, a clear and significant increase (~6-fold) in the half-life of Atf1 was seen.

The levels of many regulatory proteins, including ATF2, are regulated by ubiquitin-dependent degradation. To determine whether Atf1 is also degraded via a proteasome-mediated mechanism, Atf1 levels were examined in a mutant (mts2-1) that had a temperature-sensitive mutation in the mts2 gene, which encodes a subunit of the 26 S proteasome (37). Even at the permissive temperature of 25 °C and under unstressed conditions, significantly higher levels of Atf1 were present (Fig. 3C). Proteasome-mediated degradation in this mutant at the permissive temperature is ~50% that of wild-type cells (38). The stabilization of Atf1 was further exacerbated at the higher temperature of 32 °C. Atf1 was also stabilized in another proteasome mutant, mts3-1 (data not shown). The pattern of Atf1 phosphorylation upon stress did not change in the mts2-1 mutant (supplemental Fig. S3). These data suggest that Atf1 turnover is mediated by the 26 S proteasome.

Taken together, the above results indicated that the phosphorylation of Atf1 resulted in increased accumulation of the Atf1 protein due, in part at least, to increased stability. To confirm these observations we examined the role of the MAPK phosphorylation sites upon Atf1 stability. A plasmid containing the aff1-11M cDNA under the control of the thiamine-repressible nmt41 promoter was transformed into aff1Δ cells. The levels of Atf1 protein following thiamine and cycloheximide treatment were compared with those of cells containing a wild-type copy of the aff1 cDNA. As shown (Fig. 4), aff1-11M is considerably less stable than the wild-type Atf1 protein. Following the addition of thiamine and cycloheximide, aff1-11M levels decreased at a significantly higher rate than wild-type Atf1 protein. This experiment was performed in the absence of stress.
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The Atf1 protein is protected from degradation upon stress. A, stress-induced delay in the degradation of Atf1. The atf1Δ cells harboring the pREP41 atf1Δ plasmid with the atf1Δ CDS under the control of the nmt41 promoter were grown in minimal medium (MM) without thiamine to mid-log phase. Thiamine (THI) and cycloheximide (CHX) were added to repress the expression of the atf1Δ gene and protein synthesis, respectively. After 2 h, H2O2 (6 mM) was added to half the culture. Protein extracts were prepared and probed with anti-Atf1 antiserum and anti-tubulin antibody as a loading control. The asterisk indicates a nonspecific band that is recognized by the anti-Atf1 antiserum. B, the relative densities of the Atf1 bands were calculated using the relevant loading control sample for each time point. C, Atf1 accumulates in a mutant defective in 26 S proteasome activity. Total cell extracts of wild-type or mts2-1 cells growing in YE at 25 °C or 32 °C were prepared and subjected to Western blot analysis using an anti-Atf1 antiserum or anti-Mts4 antibody as a loading control.

suggesting that the phosphorylation of Atf1 by Sty1 is important for regulating the stability of Atf1 under basal as well as stress conditions. Consistent with these findings, Atf1 stability was also greatly reduced in stl1-Δ mutant cells (compare Atf1 protein in Fig. 5B, left-hand panel with Fig. 5A). There was no increase in stability of Atf1 upon stress in cells lacking Sty1 MAPK activity (Fig. 5B, right-hand panel). Collectively, examination of the atf1Δ N1 mutant protein and stability of Atf1 in a stl1-Δ mutant demonstrates that the phosphorylation of Atf1 by Sty1 at MAPK sites is responsible for regulating the stability of Atf1 in both the absence and presence of stress.

Atf1 Stability Is Regulated through Heterodimerization with Pcr1—During the course of this study, a second regulatory pathway that controls the level of Atf1 protein was observed. The level of Atf1 protein in cells deleted for pcr1Δ was reduced compared with wild-type cells (Fig. 6). The Atf1 protein was still phosphorylated in the absence of Pcr1 as indicated by a reduction in its mobility suggesting that heterodimerization is not a requirement for phosphorylation of Atf1. Similarly, the levels of Pcr1 protein were low in cells deleted for atf1Δ (Fig. 6).

These results suggest that the two heterodimerization partners can influence each other’s level perhaps through a mechanism of mutual protection from degradation. This would ensure preferential accumulation of heterodimers in the cell and limit the possible imbalanced accumulation of one protein over the other, which could lead to the formation of homodimers.

To study this further, atf1Δ and atf1Δ pcr1Δ cells were transformed with the plasmid containing a wild-type copy of the atf1Δ gene under the control of the thiamine-repressible nmt41 promoter and grown in the absence of thiamine. Following the addition of thiamine and cycloheximide, Atf1 protein levels were followed over time (Fig. 5C). It is clear from these data that Atf1 is degraded more rapidly in the absence of Pcr1. This is the case both in the absence or presence of stress. However, upon H2O2 treatment, there was a slight increase in stability of Atf1 compared with the pcr1Δ mutant in the absence of stress, which is presumably due to phosphorylation of Atf1. However, the Atf1 protein is still less stable in the pcr1Δ mutant under stress than in the wild-type background in the absence of stress (compare Fig. 5C, right-hand panel to Fig. 5A).

We have demonstrated that Atf1 protein levels are controlled by phosphorylation and by interaction with Pcr1. There is a modest up-regulation of atf1Δ gene expression following the activation of Sty1 by a number of different stress conditions (Fig. 7A) (12, 33, 34). However, there was strong up-regulation of pcr1Δ gene expression; very strong activation of pcr1Δ transcription was seen following treatment with sorbitol, CaCl2 (Fig. 7A), or H2O2 (Fig. 7A) (33). From our previous microarray study, the levels of pcr1Δ mRNA were found to increase 5- and 8.4-fold, upon treatment for 15 min with H2O2 and sorbitol, respectively (34). The transcription of gpx1Δ is shown as a positive control; its expression has been shown previously to be...
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**FIGURE 5.** The stability of Atf1 is regulated by the MAPK Sty1 and by its heterodimerization partner Pcr1. Cells were grown as in Fig. 3. In all experiments in this figure the cells are deleted for the *atf1* gene but contain the pREP41*atf1*⁺ plasmid. A, *atf1*Δ mutant cells harboring the pREP41*atf1*⁺ plasmid were used as the control sample. At t = 0, cells were treated with cycloheximide and thiamine and samples were analyzed for Atf1 by Western blotting. In the right-hand panel, 6 mM H₂O₂ was added 10 min after the addition of thiamine and cycloheximide; B, the experiment was repeated using a *sty1-1* mutant strain in the absence (left-hand panel) or presence of 6 mM H₂O₂ (right-hand panel). The stress was added 10 min after the addition of cycloheximide and thiamine. C, the experiments in B were repeated using the *pcr1Δ* mutant.

**FIGURE 6.** Pcr1 regulates Atf1 levels, and Atf1 regulates Pcr1 levels. The levels of Atf1 and Pcr1 are low in *pcr1Δ* and *atf1Δ* cells, respectively. Exponentially growing cultures of wild-type, *atf1Δ*, or *pcr1Δ* cells growing in YE were stressed by the addition of 6 mM H₂O₂ (upper panels) or 1 M sorbitol (lower panels) for the times indicated. Protein extracts were probed with anti-Atf1 antibody, anti-Pcr1 antibody, or anti-tubulin antibody as a loading control.

Atf1 protein following stress is matched by an increase in its partner Pcr1 through transcriptional regulation, thus preserving a balance between the two factors.

**Phenotype of atf1-11M Cells**—A strong correlation between the phosphorylation of Atf1 and the activation of Sty1 and Atf1-dependent transcription has been shown previously (12, 13). However, the importance of Atf1 phosphorylation in stress responses has not been directly demonstrated. We compared the stress sensitivity of cells lacking *atf1* to cells having the *atf1-11M* allele in place of the wild-type gene. Whereas *atf1Δ* cells were unable to grow on plates containing 2.4 M sorbitol, 0.9 M KCl, or 0.2 M CaCl₂, *atf1-11M* mutant cells grew normally (Fig. 8A). Thus, although protein levels are significantly lower, sufficient protein must be present to offer protection against these osmotic and salt stress conditions. Interestingly, the *atf1-11M* mutant grew slower than wild-type cells on 1 mM H₂O₂, but its phenotype was still considerably milder than that of cells completely lacking Atf1. These results also suggest that the phosphorylation of Atf1 is dispensable for adaptation of growth under osmotic or salt stress conditions.

Next we examined the survival response to acute salt stress (1 M CaCl₂, Fig. 8B). At this concentration of CaCl₂, 50% of wild-type cells remain viable after 10 h. In contrast, only 0.4% of *atf1Δ* cells are viable. In the case of *atf1-11M* cells, the pattern of survival was similar to that seen with wild-type cells. Therefore, neither acute nor adaptive response to osmotic stress and salt stress appears to be defective in the mutant cells.

A different picture emerged when we tested the sensitivity of the mutant cells to high levels of H₂O₂. Both Pap1 and Atf1 are required for the oxidative stress response with Pap1 being critical for an adaptive response to low levels of H₂O₂ stress and Atf1 being required to respond normally to an acute level of these stresses (20). *atf1-11M* mutant cells were significantly more resistant to very high levels of H₂O₂ as compared with *atf1Δ* cells, although they were clearly more sensitive than wild-type cells (Fig. 8C). Thus the inability to phosphorylate Atf1 does have a detrimental effect on an acute H₂O₂ response.

**Stress-induced Transcription Still Occurs in the atf1-11M Mutant**—We also compared the stress-induced expression of a number of Atf1-target genes in wild-type and *atf1-11M* mutant cells. Treatment of wild-type cells with 1.2 M sorbitol, 0.25 M CaCl₂, or 6 mM H₂O₂ resulted in significant transcriptional activation of all of the *Atf1* target genes we tested (Fig. 9A). In response to sorbitol, activation of target gene expression was activated by Sty1 (39). The increased transcription of *pcr1* was lost in strains deleted for *atf1* and *wis1* and was constitutively high in the strain that contained the constitutively active *wis1-DD* allele (Fig. 7B). Thus *pcr1* expression was dependent upon the Sty1 pathway (Fig. 7) and the Atf1 transcription factor (Fig. 7B). This suggests the possibility that the accumulation of
very similar in wild-type and atf1-11M cells. However, activation in response to CaCl2 treatment was not identical; the activation of both gpd1 and gpx1 was lower in the mutant cells, although hsp9 expression was unaffected. Finally, in response to H2O2 treatment, the activation of all three target genes was significantly reduced in the mutant background, although clear activation did still occur. Given that gene expression is activated by stress in the atf1-11M mutant, it is unlikely that the atf1-11M allele results in a constitutively activated version of Atf1.

These results shown in Fig. 9A contrast starkly with the complete loss of activation of all three target genes when either the atf1 gene or the sty1 gene is deleted (10, 12, 13, 40). Thus lack of Atf1 phosphorylation does have an adverse effect on Atf1-mediated gene expression dependent upon the nature of stress signal and the nature of the target gene itself. However, in no case have we seen complete loss of induced expression, which indicates that Sty1 must also regulate Atf1-dependent activity by a mechanism that does not involve its direct phosphorylation.

The activation of transcription in the atf1-11M mutant under osmotic and salt stress could be due to redundancy whereby other b-ZIP transcription factors might be replacing the mutant Atf1 protein at stress-dependent promoters. Therefore, we decided to test whether the atf1-11M protein was still capable of binding to the promoters of Atf1-dependent genes. This was analyzed by chromatin immunoprecipitation using antiserum against Atf1 (Fig. 9B). We examined the association of the Atf1 and atf1-11M proteins with the promoters of two Atf1-dependent genes, namely gpd1 and hsp9, both of which contain a consensus Atf1 binding site. We observed a strong enrichment, in the Atf1 and atf1-11M immunoprecipitates, of the regions corresponding to the gpd1 and hsp9 promoters compared with the cdc2 control promoter, which is not expected to be bound by Atf1. This enrichment was not observed in immunoprecipitates prepared from atf1Δ cells. As a further control, we analyzed the Atf1-independent promoter, act1. We did not observe binding of Atf1 or atf1-11M to this promoter. These data clearly demonstrate that Sty1-dependent phosphorylation of Atf1 is not required for this transcription factor to bind to its target promoters.

Although atf1-11M is able to bind to promoters and activate transcription, the atf1-11M mutant still displays sensitivity to H2O2. The overall levels of induction of Atf1-dependent genes seemed to be lower under this stress (Fig. 9A). The phenotype could be a direct result of loss of Atf1 phosphorylation or alternatively as a consequence of reduced levels of Atf1 protein. If the latter explanation was true, then increasing the amount of atf1-11M protein in the cell might be expected to rescue the growth defect. Indeed, overexpression of atf1-11M rescued the growth defect to the same extent, because overexpression of wild-type atf1+ consistent with the sensitivity of the atf1-11M mutant to H2O2 was due to loss of protein rather than as a direct loss of phosphorylation (Fig. 9C).

**DISCUSSION**

In this study we aimed to investigate the effect of phosphoryla-
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...ation upon Atf1. An allele was constructed that contained mutations in all the potential MAPK phosphorylation sites; as a result the characteristic electrophoresis mobility alteration of Atf1 that is due to phosphorylation was lost. Nevertheless, major loss of Atf1 function was not observed. In contrast to *atf1Δ* cells, the *atf1-11M* mutant was not particularly sensitive to a wide range of stress conditions and could still activate expression of a number of Atf1 target genes in a stress-dependent manner. Furthermore, the *atf1-11M* protein was still able to bind to its b-ZIP partner Pcr1.6 These results present a dilemma: Atf1 is phosphorylated by Sty1, and its ability to activate gene expression is Sty1-dependent; yet, direct phosphorylation per se appears not to be crucial. Further studies will be required to solve this dilemma. Hog1, the budding yeast homologue of Sty1, has been found to be intimately associated with osmotic stress promoters. The kinase is recruited through a variety of transcription factors, including Hot1 (41). Once there, Hog1 can recruit further components of the transcriptional machinery, including RNA polymerase II (42, 43). The recruitment of RNA polymerase II does not depend on Hot1 phosphorylation but does require the kinase activity of Hog1. Interestingly, a strain containing a mutant version of Hot1 with all five MAPK sites mutated was still able to induce expression of the *STL1* gene upon stress, which is reminiscent of our observations with *atf1-11M*. This does not seem to be a universal finding, however, as mutation of all four Hog1 sites in the *Smp1* transcription factor abolished transcriptional activation of a stress-inducible reporter construct (44). Furthermore, the phosphorylation of Atf2, the mammalian homologue of Atf1, appears to be absolutely critical for its function, because a mouse lacking the two MAPK phosphorylation sites displays the same lethal phenotype as an Atf2 null mutant.7 It will be interesting to determine whether the essential role of Sty1 in transcriptional regulation involves its association with, and the recruitment of transcriptional regulators to, the promoters of target genes. One further possibility is that Atf1 is present in unstressed cells as a complex with other factors that act to repress its activity. Phosphorylation of Atf1 or these factors by Sty1 may be sufficient to disrupt the complex resulting in derepression. In this regard, Hog1 has been found to phosphorylate the Sko1 transcriptional repressor. This converts Sko1 into a transcriptional activator with the subsequent recruitment of other transcriptional factors (45).

One clear phosphorylation-dependent effect that we did observe was an increase in the stability of Atf1 following activation of the Sty1 pathway. Following stress induction, Atf1 protein accumulates; this is lost in a *sty1Δ* or *wis1Δ* background or when the potential Atf1 phosphorylation sites are mutated. Thus the increase in Atf1 levels is clearly dependent upon direct Atf1 phosphorylation. The major reason for the accumulation appears to be due to an increase in the half-life of the protein. Although the underlying molecular mechanism remains unknown, it must be mediated through an altered sensitivity to degradation, because Atf1 is stabilized in mutants that are defective in proteasome subunits.

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6 C. Wilkinson and N. Jones, unpublished data.

7 W. Breitwieser and N. Jones, unpublished data.
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Atf1 exists as a phospho-protein even in unstressed conditions (Fig. 2A) (12, 15). Our data indicate that the basal phosphorylation appears to be at one or more of the consensus MAPK sites, because we did not observe a mobility shift when treating the atf1-11M protein isolated from unstressed cells with phosphatase. Analysis of the levels of atf1-11M protein in whole cell extracts along with the stability assay suggests that this basal phosphorylation serves to stabilize the Atf1 protein even in the absence of stress. Presumably the protein is subjected to continual turnover, as indicated by the stabilization observed in proteasome mutants even in the absence of stress. The basal phosphorylation permits a certain degree of stability, which is increased by hyperphosphorylation upon stress. The varying levels of phosphorylation could serve to determine the extent to which the ubiquitination machinery is able to interact with Atf1. In most cases where proteasomal substrates are phosphorylated, the modification acts to promote the interaction with an E3 ubiquitin ligase and thus degradation (46). In the case of Atf1, phosphorylation has the opposite effect and results in stabilization. Similarly, phosphorylation of the replication factor Cdc6 prevents its association with the anaphase-promoting complex thus rendering Cdc6 immune from degradation (47). Future work will aim to identify the ubiquitination machinery responsible for targeting Atf1 for degradation and to elucidate how phosphorylation prevents this from occurring. It will be interesting to see whether the function of Sty1 phosphorylation identified here, namely protection from degradation, extends to its other substrates.

This study also demonstrates that Atf1 levels are tightly coordinated with the levels of its partner protein Pcr1. These two proteins preferentially heterodimerize and bind to each other in the absence and presence of stress (data not shown). In the absence of Pcr1, Atf1 levels decrease due to an increased rate of degradation. Similarly, in the absence of Atf1, Pcr1 levels decrease. Thus there appears to be an interplay that results in cross-protection between the two partner proteins that would ensure that they are both present in the cell at similar stoichiometric levels. Given the higher efficiency of heterodimerization over either Atf1 or Pcr1 homodimerization, such regulation would result in the presence of heterodimers only. Although the mechanism that ensures such coordination is unknown, the situation is very reminiscent of that for Mat-a2 and Mat-a1, which upon dimerization protect each other from ubiquitination and degradation; thus the complex is significantly more stable than either monomer (48). pcr1 gene expression is also under the control of Atf1 and is activated following stress. We suggest that this will lead to higher levels of Pcr1 protein synthesis, which could lead to a reinforcement of the increase in Atf1 levels due to phosphorylation-dependent stabilization. Given the dependence of Atf1 levels on Pcr1 and vice versa, it would make little sense to increase the level of one partner in the absence of the other. When activation of the Sty1 pathway is lost, pcr1 transcription decreases leading to a decrease in Pcr1 protein levels and as a consequence, Atf1 protein levels. In addition, transcription of atf1 is reduced and phosphorylation of Atf1 is lost, which would lead to an increased degradation rate. Thus through a combination of these mechanisms the levels of the Atf1/Pcr1 heterodimer can be strictly and sensitively controlled.

The regulation of atf1 mRNA is also complex and regulated by the Sty1 kinase (Fig. 7) (12, 33). We have shown here that Sty1, as well as Atf1, also controls pcr1 mRNA levels (Fig. 7). Under oxidative stress, a further level of regulation is mediated by the Csx1 protein, which binds to atf1 mRNA and stabilizes it (33). Sty1 is also required for the stabilization of the atf1 and pcr1 mRNAs upon exposure to H$_2$O$_2$. It is not clear, however, why these mRNAs should become destabilized specifically upon stress. Furthermore, this effect seems to be confined to oxidative stress, but it does add yet another layer of regulation by Sty1 onto an already complex regulatory network controlling the levels of Atf1 and Pcr1.

Why does the atf1-11M mutant display sensitivity to H$_2$O$_2$ and not other stresses? It is possible that this is due to the fact that there are more than twice as many genes that require Atf1 for 3-fold or more increased transcription upon H$_2$O$_2$ as, for example, upon sorbitol stress (34). It is possible therefore, that in the case of atf1-11M, the reduced amount of protein has a more significant effect in cases where the pool of activated genes is particularly high. The fact that mild overexpression of atf1-11M rescues the atf1-11M defect on H$_2$O$_2$ is consistent with this hypothesis.

We have shown that phosphorylation regulates the stability of Atf1, but could it still play a role in transcriptional regulation? Certainly it does not seem to be critical for either DNA binding or activation of transcription per se, but our analysis does not exclude that it may play other roles. Intriguingly, the pattern of phosphorylation of Atf1 varies with both time and type of stress (Fig. 1B) (20). Thus it may be that the differentially phosphorylated isoforms of Atf1 have different functions in addition to their role in stabilization. Future work will aim to address this intriguing possibility.

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REFERENCES

1. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
2. Waskiewicz, A. J., and Cooper, J. A. (1995) Curr. Opin. Cell Biol. 7, 798–805
3. Kyrakis, J. M., and Avruch, J. (1996) BioEssays 18, 567–577
4. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
5. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
6. Livingstone, C., Patel, G., and Jones, N. (1995) EMBO J. 14, 1785–1797
7. Rangreaud, J., Gupta, S., Rogers, J. S., Dickenson, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
8. van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P. (1995) EMBO J. 14, 1798–1811
9. Shiozaki, K., and Russell, P. (1995) Nature 378, 739–743
10. Millar, J. B., Buck, V., and Wilkinson, M. G. (1995) Genes Dev. 9, 2117–2130
11. Kato, T., Jr., Okazaki, K., Murakami, H., Stettler, S., Fantes, P. A., and Okayama, H. (1996) FEBS Lett. 387, 207–212
12. Shiozaki, K., and Russell, P. (1996) Genes Dev. 10, 2276–2288
Regulation of S. pombe Atf1 Protein Levels

13. Wilkinson, M. G., Samuels, M., Takeda, T., Toone, W. M., Shieh, J. C., Toda, T., Millar, J. B., and Jones, N. (1996) Genes Dev. 10, 2289–2301
14. Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995) EMBO J. 14, 6193–6208
15. Kanoh, J., Watanabe, Y., Ohsugi, M., Iino, Y., and Yamamoto, M. (1996) Genes Cells 1, 391–408
16. Watanabe, Y., and Yamamoto, M. (1996) Mol. Cell. Biol. 16, 704–711
17. Kon, N., Krawchuk, M. D., Warren, B. G., Smith, G. R., and Wahls, W. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13765–13770
18. Degols, G., and Russell, P. (1997) Mol. Cell. Biol. 17, 3356–3363
19. Nguyen, A. N., Lee, A., Place, W., and Shiozaki, K. (2000) Mol. Biol. Cell 11, 1169–1181
20. Quinn, J., Findlay, V. J., Dawson, K., Millar, J. B., Jones, N., Morgan, B. A., and Toone, W. M. (2002) Mol. Biol. Cell 13, 805–816
21. Gaits, F., Degols, G., Shiozaki, K., and Russell, P. (1998) Genes Dev. 12, 1464–1473
22. Li, X. Y., and Green, M. R. (1996) Genes Dev. 10, 517–527
23. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Science 267, 389–393
24. Firestein, R., and Feuerstein, N. (1998) J. Biol. Chem. 273, 5892–5902
25. Fuchs, S. Y., and Ronai, Z. (1999) Mol. Cell. Biol. 19, 3289–3298
26. Fuchs, S. Y., Tappin, I., and Ronai, Z. (2000) J. Biol. Chem. 275, 12560–12564
27. Moreno, S., Klar, A., and Nurse, P. (1991) Methods Enzymol. 194, 795–823
28. Lyne, R., Burns, G., Mata, J., Penkett, C. J., Rustici, G., Chen, D., Langford, C., Vetrin, D., and Bähler, J. (2003) BMC Genomics 4, 27
29. Basi, G., Schmid, E., and Maundrell, K. (1993) Gene (Amst.) 123, 131–136
30. Gietz, R. D., and Sugino, A. (1987) Gene (Amst.) 74, 527–534
31. Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. (1999) Methods Mol. Biol. 119, 469–479
32. Tanaka, T., Kanpp, D., and Nasmyth, K. (1997) Cell 90, 649–660
33. Rodriguez-Gabriel, M. A., Burns, G., McDonald, W. H., Martin, V., Yates, J. R., 3rd, Bähler, J., and Russell, P. (2003) EMBO J. 22, 6256–6266
34. Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bähler, J. (2003) Mol. Biol. Cell 14, 214–229
35. Shiozaki, K., Shiozaki, M., and Russell, P. (1998) Mol. Biol. Cell 9, 1339–1349
36. Cheung, P. C., Campbell, D. G., Nebreda, A. R., and Cohen, P. (2003) EMBO J. 22, 5793–5805
37. Gordon, C., McGurk, G., Dillon, P., Rosen, C., and Hastie, N. D. (1993) Nature 366, 355–357
38. Seeger, M., Gordon, C., Ferrell, K. and Dubiel, W. (1996) J. Mol. Biol. 263, 423–431
39. Yamada, K., Nakagawa, C. W., and Mutoh, N. (1999) Yeast 15, 1125–1132
40. Degols, G., Shiozaki, K., and Russell, P. (1996) Mol. Cell. Biol. 16, 2870–2877
41. Alepuz, P. M., Jovanovic, A., Reiser, V., and Ammerer, G. (2001) Mol. Cell 7, 767–777
42. Alepuz, P. M., de Nadal, E., Zapater, M., Ammerer, G., and Posas, F. (2003) EMBO J. 10, 2433–2442
43. de Nadal, E., Zapater, M., Alepuz, P. M., Sumoy, L., Mas, G., and Posas, F. (2004) Nature 427, 370–374
44. de Nadal, E., Casadome, L., and Posas, F. (2003) Mol. Cell. Biol. 23, 229–237
45. Proft, M., and Struhl, K. (2002) Mol. Cell 9, 1307–1317
46. Tyers, M., and Jorgensen, P. (2000) Curr. Opin. Genet. Dev. 10, 54–64
47. Mailand, N., and Diffley, J. F. X. (2005) Cell 122, 915–926
48. Johnson, P. R., Swanson, R., Rakhilina, L., and Hochstrasser, M. (1998) Cell 94, 217–227
49. Wilkinson, C. R., Wallace, M., Seeger, M., Dubiel, W., and Gordon, C. (1997) J. Biol. Chem. 272, 25768–25777