Fission Yeast MAP Kinase Sty1 Is Recruited to Stress-induced Genes*

The stress-induced expression of many fission yeast genes is dependent upon the Sty1 mitogen-activated protein kinase (MAPK) and Atf1 transcription factor. Atf1 is phosphorylated by Sty1 yet this phosphorylation is not required for stress-induced gene expression, suggesting another mechanism exists whereby Sty1 activates transcription. Here we show that Sty1 associates with Atf1-dependent genes and is recruited to both their promoters and coding regions. This occurs in response to various stress conditions coincident with the kinetics of the activation of Sty1. Association with promoters is not a consequence of increased nuclear accumulation of Sty1 nor does it require the phosphorylation of Atf1. However, recruitment is completely abolished in a mutant lacking Sty1 kinase activity. Both Atf1 and its binding partner Pcr1 are required for association of Sty1 with Atf1-dependent promoters, suggesting that this heterodimer must be intact for optimal recruitment of the MAPK. However, many Atf1-dependent genes are still expressed in a pcr1Δ mutant but with significantly delayed kinetics, thus explaining for the relatively mild stress sensitivity displayed by pcr1Δ. Consistent with this delay, Sty1 and Atf1 cannot be detected at these promoters in this condition, suggesting that their association with chromatin is weak or transient in the absence of Pcr1.

All cells sense and react to changes in their environment. Single-celled organisms are especially vulnerable to such events and must respond to alterations in pH, osmolarity, and temperature as well as contending with potentially harmful toxins. One of the major ways in which cells respond to such changes is by radically altering their program of gene expression, leading to increased levels of proteins with stress protectant functions and a decrease in non-essential activities; this enables resources to be concentrated on adaptation to stress and the repair of damaged macromolecules (reviewed in Ref. 1).

Stress-activated MAP kinase pathways (SAPKs) are central in all eukaryotes to the facilitation of gene expression changes that occur in response to certain environmental cues. The mammalian SAPKs, p38 and JNK, respond to diverse stresses and signals and as a result phosphorylate a number of targets including the ATF2 transcription factor (2–6). In fission yeast, the Sty1 MAPK (also known as Spc1/Phh1, hereafter referred to as Sty1), is closely related to p38 and like its mammalian counterpart, responds to a variety of stimuli including osmotic, oxidative, and heat stress (7–9). Upon exposure to stressors, Sty1 is activated by its MAPK kinase (Wis1), via phosphorylation at Thr171 and Tyr173 and consequently translocates to the nucleus (7, 10). Activated Sty1 phosphorylates a number of targets including the bZIP transcription factor Atf1, the fission yeast homologue of mammalian ATF2 (11, 12). Both Sty1 and Atf1 are required for many of the stress-induced transcriptional changes that occur upon stress; a large subset of these occur in response to several different stresses and as a result have been termed the core environmental stress response or CESR (13).

Atf1 binds to its cognate site as a dimer with a second bZIP protein, Pcr1 (14). This heterodimer binds to DNA with much greater affinity than either homodimer (15, 16). However, whereas cells lacking atf1 are sensitive to a variety of insults, the stress phenotypes displayed by a pcr1Δ deletant are much less severe, suggesting a less crucial role in regulating gene expression (15, 17, 18).

Many signaling kinases, including SAPKs, modulate gene expression through regulatory phosphorylation of their target transcription factors or other chromatin-bound proteins (19). Previously we have addressed the function of Atf1 phosphorylation by Sty1. Surprisingly, phosphorylation is not required for stress-induced activation of Atf1 target genes but rather serves to positively regulate the stability of the Atf1 protein. Accord-
inger, the Atf1–11M phoso-mutant protein, which lacks intact MAPK sites, displays decreased stability compared with its wild type counterpart and accumulates to a lesser extent upon stress. Nevertheless, there is still robust activation of Atf1-dependent gene expression. Consistent with this, atf1–11M does not share the stress sensitivities displayed by an atf1 deletion mutant (18).

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Recruitment of Sty1 to Stress-induced Genes

These results posed a dilemma: Atf1 is phosphorylated by Sty1; its role in stress-induced activation of gene expression is Sty1-dependent, yet phosphorylation of Atf1 by Sty1 does not appear to be critical for transcriptional activation per se. Thus, what is the essential role of Sty1 in stress-induced gene expression?

Clues may come from studies of Hog1, the budding yeast homologue of Sty1, which is primarily responsible for mediating the osmotic stress response in this yeast (reviewed in Ref. 20). Hog1 can modulate transcription by phosphorylating its target transcription factors, thereby affecting their ability to activate gene expression (21, 22). However, utilizing the chromatin immunoprecipitation technique (ChIP), which allows the mapping of DNA-protein interactions, Hog1 was shown to associate closely with target promoters upon exposure to increased osmolarity but not in response to other stressors such as heat and ethanol (23). The kinase is targeted and anchored to promoters by various translation factors such as Hot1. Once there, Hog1 serves to recruit key components of the general transcriptional machinery such as RNA pol II (24). Indeed, tethering of Hog1 to an artificial promoter by use of a lexA fusion system is sufficient to drive gene expression in a stress-dependent manner (24). In addition, the kinase can affect transcription by directing the association of chromatin-remodeling enzymes, such as the Rpd3 histone deacetylase, to osmoreponsive promoters (25). These findings raised the possibility that the kinase might play a structural role during transcription in addition to functions that require its catalytic activity. Recent studies have shown that Hog1 not only regulates transcriptional initiation but also affects the elongation cycle. The kinase can associate with the ORF as well as the terminator region of osmoreponsive genes where it appears to act as a component of the RNA pol II elongation complex (26).

This close association between a protein kinase involved in signal transduction and genes under its control is likely to be a widespread phenomenon. Other budding yeast protein kinases subsequently found to interact directly with chromatin include Tor1, Sch9, Snf1, subunits of PKA and the MAP kinases Fus3 and Kss1 (27–29), whereas in higher eukaryotes, p38 and extracellular signal-regulated kinase (ERK) have been reported to associate with specific promoters during myogenic and adipogenic differentiation, respectively (30, 31).

We reasoned that the essential role of Sty1 in activating stress-induced gene expression in fission yeast might involve direct association with stress-dependent genes and phosphorylation of chromatin-bound targets other than Atf1. Here we show that Sty1 is indeed recruited to promoters and ORFs of Atf1-dependent genes. Moreover, we have carried out a detailed analysis of the requirements for such association and in particular, have examined the role of the Atf1/Pcr1 heterodimer in this recruitment.

**EXPERIMENTAL PROCEDURES**

Yeast Strains and General Methods—Schizosaccharomyces pombe strains used in this study are listed in Table 1. Yeast media and general experimental methods were as described (32).

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**TABLE 1**

*S. pombe* strains used in this study

| Strain | genotype | Integration cassette | Source |
|--------|----------|----------------------|--------|
| NJ2    | h− ura4-D18 leu1−32 ade6-M210 his7−366 | C. Hoffman |
| NJ23   | h− 972 | Lab stock |
| NJ42   | h− pcr1::ura4 ura4-D18 | This study |
| NJ260  | h− atf1::kan’ leu1−32 ura4-D18 ade6-M210 his7−366 | Ref. 18 |
| NJ294  | h− sty1::kan’ leu1−32 ura4-D18 ade6-M210 his7−366 | This study |
| NJ675  | h− sty1::HA6Hiscar4 leu1−32 ura4-D18 ade6-M216 his7−366 | This study |
| NJ711  | h− sty1−12myc4 ura1−11 His3−HA6Hist LEI12 leu1−32 ura4-D18 | Ref. 10 |
| NJ746  | h− sty1−12myc4 ura1−11 His3−HA6Hist LEI12 leu1−32 ura4-D18 | This study |
| NJ752  | h− sty1−12myc4 ura1−11 His3−HA6Hist LEI12 leu1−32 ura4-D18 | This study |
| NJ757  | h− sty1::kan’ ura4-D18 | This study |
| NJ760  | h− sty1::kan’ leu1::mtnt41sty1−3Pcar4 ura4-D18 (sty1-PK) | This study |
| NJ765  | h− sty1::kan’ leu1::mtnt41sty1K49R-3Pcar4 ura4-D18 (sty1d-PK) | This study |
| NJ917  | h− sty1−12myc4 ura4-D18 His3−HA6Hist LEI12 Pcr1::kan’ | This study |
| NJ922  | h− sty1−12myc4 ura1−11 His3−HA6Hist LEI12 leu1−32 ura4-D18 (sty1d-PK) | This study |
| NJ959  | h− sty1−12myc4 ura1−11 His3−HA6Hist LEI12 leu1−32 ura4-D18 | This study |
| NJ961  | h− sty1−12myc4 caf1::nat’ leu1−32 ura4-D18 | Lab stock |
| CW139  | h− ura4-D18 | |

To construct the sty1 kinase-dead Pk-tagged strain (NJ765) and its control strain (NJ760), first an exact deletion of sty1 was generated in a leu1−1 ura4-D18 strain (CW139) using standard procedures (33); oligonucleotide sequences are available upon request. The sty1 and sty1 kinase-dead (K49R) coding sequences were amplified from plasmids by PCR using primers Sty1_BamHI_fwd and Sty1_nostop_re_v2 (oligonucleotide sequences available upon request). The amplified 1-kb PCR product was digested with Xmal and BamHI and subcloned into previously SmaI/BamHI cut plasmid pREP41Pk (34). The obtained plasmids (pREP41sty1-Pk and pREP41sty1Kd-Pk) were digested with SacI/PstI, the resulting 3-kb band isolated and subsequently subcloned into the pINTA vector (35), which had been prepared by digestion with SacI and PstI, giving rise to plasmids pINTAsty1-Pk and pINTAsty1Kd-Pk. The integration cassettes from plasmids pINTAsty1-Pk and pINTAsty1Kd-Pk were obtained by a NotI digest, subsequently isolated and used for transformation of strain NJ757. Ura+/leu−colonies were selected and the integration events checked by genomic PCR and sequencing. The strains were verified by assessing cell length and stress sensitivity with the sty1-Pk (NJ760) and sty1Kd-Pk (NJ765) behaving as wild type and the sty1Δ mutant, respectively. These cells were grown in minimal media to ensure full induction from the mtnt41 promoter.

**ChIP Assays**—The ChIP procedure is based on methods described elsewhere (18) with modifications: in the case of Sty1-myC ChIPs, cultures were cross-linked for 30 min with 1% formaldehyde at 24 °C. Immunoprecipitation was performed with 15 μL of Dynal protein A-coated magnetic beads, which were previously incubated overnight with 4–5 μg of anti-Atf1 polyclonal antibody, anti-Pcr1 rabbit polyclonal antibody (18), anti-c-myc (A-14)X rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-HA (12CA5, CRUK), or mouse anti-V5 TAG (Pk) antibody (Serotec), respectively. We used standard PCR as well as real-time PCR to quantify the relative amount of DNA corresponding to stress-induced genes isolated from each immunoprecipitate. For standard PCR, gpd1 was compared...
with the stress independent promoters, hmg1 and cdc2. Primers used are denoted relative to translation start codons and corresponded to −315 to −294 and −148 to −129 for the cdc2 promoter, −494 to −475 and −275 to −256 for the gpd1 promoter, and −423 to −404 and −149 to −130 for the hmg1 promoter. For real time PCR quantifications, the relative enrichments of gpd1, hsp9, ctt1, and pka1 were measured over a stress-independent promoter (cdc2). The relative enrichments of stress-independent genes (cdc2 ORF, pol1 ORF) were measured as negative controls. Primers used are denoted relative to translation start codons and corresponded to −214 to −192 and −177 to −144 for the cdc2 promoter, +471 to +498 and +518 to +541 for cdc2 ORF, −446 to −421 and −388 to −363 for the ctt1 promoter, +1379 to +1399 and +1423 to +1439 for the ctt1 ORF, −402 to −380 and −360 to −340 for gpd1 promoter, +612 to +628 and +646 to +665 for gpd1 ORF, +1213 to +1241 and +1266 to +1286 for gpd1 3′-untranslated region, −264 to −245 and −220 to −199 for the hsp9 promoter, −652 to −630 and −608 to −588 for the pka1 promoter, +622 to +639 and +664 to +684 for the pka1 ORF, +1615 to +1636 and +1657 to +1681 for the pka1 3′-untranslated region, +2520 to +2543 and +2567 to +2588 for pol1 ORF. In addition, the following primers were used for the pka1 promoter (−143 to −121 and −101 to −75); there was no enrichment of Sty1 to these sequences (these data were not shown as part of Fig. 7). A fraction of each IP was assayed by Western blotting to ensure that equal quantities of Sty1 were being isolated from the samples before and after stress.

Western Blotting—Atf1 and Pcr1 were detected as described (18). Activated Sty1 was detected using the anti-phospho-p38 antiserum (GenWay). Other antibodies used were anti-HA antiserum (Genway). Other antibodies used were anti-HA antisera, mouse anti-myc (Cancer Research UK, 9E10) were used to detect Sty1 and Atf1 for their expression (13). The primer pairs used to amplify the promoters and control regions in this study are indicated in Fig. 1 along with their locations relative to the start codons. To assess whether Sty1 could associate with chromatin, we examined whether Sty1 was recruited to the gpd1 promoter upon osmotic stress using a ChIP assay analyzed by standard PCR. These experiments were performed using a strain in which the sty1 gene has been fused at its C terminus with 6 histidine residues and 2 copies of the HA epitope. This strain has previously been verified as behaving as wild type (7). Samples were collected during 1 h of osmotic stress; association of Sty1 with the gpd1 promoter was assessed by an increase in the ratio of the PCR product generated by primers specific for the gpd1 promoter compared with the control products that were amplified by primers recognizing the non-stress-specific promoters, cdc2 and hmg1. Sty1 recruitment was evident after 5 min of stress but had receded after an hour (Fig. 2A). This association with the promoter matched the kinetics of Sty1 activation that was assessed by Western blotting of Sty1 using antibodies that recognize the phosphorylated activation site. Sty1 phosphorylation was observed at 5 min but had almost disappeared by 60 min (Fig. 2B). This recruitment is consistent with the kinetics of gpd1 transcript accumulation (Ref. 13 and Fig. 10C). We observed significant amounts of gpd1 mRNA at 15 min, which had reduced considerably by 60 min. These data show that Sty1 is recruited to chromatin of an Atf1-dependent gene upon osmotic stress and that the kinetics of Sty1 activation match those of its association with the gpd1 target gene and those of transcript accumulation. This is consistent with the essential role of Sty1 in transcriptional activation requiring its direct recruitment to stress-induced genes.

**Recruitment of Sty1 to Stress-induced Genes**

**RESULTS**

**Sty1 Is Recruited to the gpd1 Promoter upon Osmotic Stress with Kinetics That Are Coincident with Those of Its Own Activation**

We first sought to examine whether Sty1 could associate with the promoters of stress-induced genes. Previously we have used ChIP to demonstrate that Atf1 is bound to the promoters of three stress-induced genes that form part of the CERS, namely gpd1, hsp9, and ctt1 (18). These three genes encode proteins with important stress protectant functions and they depend upon both Sty1 and Atf1 for their expression (13). The primer pairs used to amplify the promoters and control regions in this study are indicated in Fig. 1 along with their locations relative to the start codons. To assess whether Sty1 could associate with chromatin, we examined whether Sty1 was recruited to the gpd1 promoter upon osmotic stress using a ChIP assay analyzed by standard PCR. These experiments were performed using a strain in which the sty1 gene has been fused at its C terminus with 6 histidine residues and 2 copies of the HA epitope. This strain has previously been verified as behaving as wild type (7). Samples were collected during 1 h of osmotic stress; association of Sty1 with the gpd1 promoter was assessed by an increase in the ratio of the PCR product generated by primers specific for the gpd1 promoter compared with the control products that were amplified by primers recognizing the non-stress-specific promoters, cdc2 and hmg1. Sty1 recruitment was evident after 5 min of stress but had receded after an hour (Fig. 2A). This association with the promoter matched the kinetics of Sty1 activation that was assessed by Western blotting of Sty1 using antibodies that recognize the phosphorylated activation site. Sty1 phosphorylation was observed at 5 min but had almost disappeared by 60 min (Fig. 2B). This recruitment is consistent with the kinetics of gpd1 transcript accumulation (Ref. 13 and Fig. 10C). We observed significant amounts of gpd1 mRNA at 15 min, which had reduced considerably by 60 min. These data show that Sty1 is recruited to chromatin of an Atf1-dependent gene upon osmotic stress and that the kinetics of Sty1 activation match those of its association with the gpd1 target gene and those of transcript accumulation. This is consistent with the essential role of Sty1 in transcriptional activation requiring its direct recruitment to stress-induced genes.
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**FIGURE 1.** The locations of primer pairs used in the ChIP analysis. A, the stress-induced genes, *gpd1*, *hsp9*, and *ctt1*, are dependent upon Sty1 and Atf1 for their induction. B, *pka1* is a stress-induced Sty1-dependent, Atf1-independent gene. C, *pol1*, *cdc2*, and *hmg1* are stress and Sty1/Atf1-independent genes and are used as negative controls. The numbers refer to the starting point of each primer relative to the atg (designated as 1). For example, for *gpd1*, the amplicons −402 to −340, +612 to +665, and +1213 to +1286 represent promoter, ORF, and terminator regions, respectively. For *ctt1*, the amplicons −446 to −363 and +1379 to +1439 represent promoter and ORF regions, respectively. The amplicons analyzed by real-time PCR assays are shown as solid black bars. Those used in standard PCR (Fig. 2) are shown as open bars.

**FIGURE 2.** Sty1 is recruited to the *gpd1* promoter upon osmotic stress in a manner coincident with its own activation. A, ChIP assays showing recruitment of Sty1–6His2HA to the *gpd1* promoter upon osmotic stress. Samples were prepared from *sty1–6His2HA* cells treated with 1 M sorbitol for the time points indicated (in minutes). The DNA recovered from the IP was assayed by PCR using primers specific to the *gpd1*, *hmg1*, and *cdc2* promoters, the latter two of which are not induced upon stress. The control lanes show DNA amplified from two different amounts of whole cell extracts (WCE) prior to performing the IP. B, the kinetics of Sty1 activation were assayed by Western blotting of protein extracts prepared from the cells exposed to osmotic stress in A. The blots were probed with antibodies against the activation site of the p38 MAP kinase (anti-phospho-p38), which recognize the activation site of Sty1 when dually phosphorylated upon Thr\(^{171}\) and Tyr\(^{173}\). Total levels of Sty1 were assessed by re-probing the blot with anti-HA antiserum.

***Sty1 Is Recruited to Atf1-dependent Promoters upon a Variety of Stress Conditions***—Next we assessed whether Sty1 could associate with promoters under different types of stress other than osmotic stress. For these assays we used a strain where the *sty1* gene had been C-terminal fused with 12 copies of the myc epitope at its native locus such that the expression of *sty1-myc* is under the control of the *sty1* promoter. In terms of phenotype and gene induction this strain behaves as wild type (Refs. 10 and 38 and data not shown). The *sty1-myc* cells were isolated in the presence and absence of stress and ChIP assays were performed in triplicate. The DNA recovered from the IPs was analyzed by quantitative real-time PCR. Recruitment was determined by comparing the enrichment in the immunoprecipitates of regions corresponding to the *gpd1*, *ctt1*, and *hsp9* promoters compared with the *cdc2* control promoter, which is not expected to be bound by Sty1 (~fold over *cdc2* promoter). In addition we measured recruitment to regions of DNA that would not be expected to be bound by Sty1 namely the *cdc2* ORF and *pol1* ORF (we have previously shown that Atf1 is not bound to these control regions (18)). We found that Sty1 is recruited to the *gpd1*, *ctt1*, and *hsp9* promoters upon sorbitol-induced osmotic stress, confirming the result observed with the standard PCR assay. Upon stress we observed between 3- and 7.5-fold enrichment of these promoters in the Sty1-containing IPs compared with either the *sty1-myc* strain in the absence of stress or to an untagged control (wild type) (Fig. 3A).

The lack of recruitment of Sty1 to promoters in the absence of stress is consistent with the predominantly cytoplasmic localization of Sty1 under basal conditions (10). We also monitored the amount of Sty1 that was recovered in either the absence or presence of stress for these and all other assays in this study and found that equal amounts of Sty1 were precipitated in both conditions (data not shown).

The Hog1 MAP kinase has been shown to be recruited to promoters only upon osmotic stress and not during exposure to heat, ethanol, or sorbic acid (23). As Sty1 is required for gene expression upon exposure to a variety of stressors (13), we examined whether it was also recruited to the CESR promoters upon other stress conditions. As shown in Fig. 3, B and C, Sty1 was recruited to the *gpd1*, *hsp9*, and *ctt1* promoters upon treatment of cells with oxidative stress as well as upon heat shock. Taken together, these data indicate that Sty1 is recruited to a number of Atf1-dependent promoters in response to different stress conditions, consistent with the critical role of Sty1 in controlling transcription upon exposure to a variety of stressors (13, 39).

***Nuclear Localization of Sty1 Is Not Sufficient to Induce Promoter Recruitment in the Absence of Sty1 Activation***—The findings described above are consistent with Sty1 activation being a prerequisite for promoter binding. The simplest way to test this hypothesis would be to assess Sty1 promoter recruitment in a mutant where its activation was abolished such as the *wis1Δ* mutant. However, in this instance Sty1 would be constitutively cytoplasmic as activation by Wis1 is required for its translocation into the nucleus (10). To resolve this matter we made use of a mutation in the *caf1* gene (also known as *hba1*), which encodes a Ran-binding protein that plays a role in controlling the localization of Sty1. Loss of this gene results in constitutive
accumulation of Sty1 in the nucleus (40). Sty1 is not phospho-
rylated under basal conditions in the caf1Δ mutant (Ref. 40 and
data not shown), and the levels of Sty1 do not change upon
stress in this mutant background (data not shown). As Wis1 is
predominantly cytoplasmic, the caf1Δ mutation results in the
separation of most of the Sty1 MAPK from its upstream
MAPKK.

We examined promoter recruitment of Sty1 in a caf1Δ
mutant and did not observe any significant promoter associa-
tion upon either osmotic (Fig. 4A) or oxidative stress (Fig. 4B).
We performed indirect immunofluorescence to check that
Sty1-myc was indeed constitutively localized in the nucleus and
found that Sty1 displayed a nuclear localization in 80% of sty1-
mvc caf1Δ cells under basal conditions (Fig. 4C), which is in
good agreement with the original study (40). Upon oxidative
stress, there was a only a very slight recruitment of the kinase to
the CESR promoters presumably because the fraction of Sty1
that remains cytoplasmic in the caf1Δ mutant can still be acti-
ved; alternatively this could be due to the activation of con-
stitutively nuclear Sty1 by the fraction of Wis1 that shuttles in
and out of the nucleus (41). We conclude that Sty1 recruitment
to promoters does not arise simply as a consequence of its
increased nuclear accumulation that occurs upon stress but
rather that Sty1 needs to be activated to associate with
promoters.

**Sty1 Kinase Activity Is Required for Its Recruitment**—As activa-
tion of Sty1 is a prerequisite for recruitment to promoters, we
Recruitment of Sty1 to Stress-induced Genes

Speculated that its own catalytic activity would also be required. To address this issue, we generated a strain that expressed a version of Sty1 that has a mutation in the predicted ATP binding site resulting in a so-called “kinase-dead” form of the enzyme (K49R). This mutated version of the enzyme (K49R). This mutated version of Sty1 kinase-dead to stress-induced promoters. Assays were carried out as described in the legend to Fig. 3A using samples prepared from sty1/Pk and sty1kd-Pk cells that had been exposed to 1 m sorbitol or 2 m H2O2 for 15 min. Cells grown without stress were used as a control (indicated by −). B, Western blot to examine the activation of Sty1 in the sty1 kinase-dead (sty1kd-Pk) strain compared with the sty1-Pk strain. Whole cell extracts were prepared before or after 1 m sorbitol stress. Sty1 activation was assessed using anti-phospho-p38 antiserum. The loading was assessed using anti-Pk antiserum.

**FIGURE 5. Sty1 kinase activity is required for its recruitment to stress-responsive promoters.** A, ChIP assays to assess the recruitment of Sty1 and Sty1 kinase-dead to stress-induced promoters. Assays were carried out as described in the legend to Fig. 3A using samples prepared from sty1/Pk and sty1kd-Pk cells that had been exposed to 1 m sorbitol or 2 m H2O2 for 15 min. Cells grown without stress were used as a control (indicated by −). B, Western blot to examine the activation of Sty1 in the sty1 kinase-dead (sty1kd-Pk) strain compared with the sty1-Pk strain. Whole cell extracts were prepared before or after 1 m sorbitol stress. Sty1 activation was assessed using anti-phospho-p38 antiserum. The loading was assessed using anti-Pk antiserum.

Sty1 kinase activity for its association with promoters led us to examine whether phosphorylation of Atf1 was the critical event mediating the recruitment. Sty1 phosphorylates Atf1, and this results in the modulation of stability of Atf1. However, loss of this phosphorylation through mutation of the 11 potential MAPK sites in Atf1 does not abolish stress-induced transcription of Atf1 target genes. Accordingly, this mutated version of Atf1, Atf1–11M, is still bound to Atf1-dependent promoters (15). We asked whether Sty1 is recruited to promoters in the absence and presence of stress. This is not affected by a lack of Atf1 phosphorylation.

Taken together, these data indicate that phosphorylation of Atf1 is not the critical event in recruitment of Sty1 to stress-dependent promoters. Moreover, it is consistent with our previous study that demonstrated that transcription is still activated upon stress in the atf1–11M mutant (18).

**Sty1 Is Not Recruited to an Atf1-independent Gene**—The genes that we have assessed above, namely gpd1, hsp9, and ctt1, all require Atf1 for their expression. However, in our previous analysis of stress-induced transcription by whole genome profiling (13), we identified a group of stress-induced CESR genes that are dependent upon Sty1 but not Atf1 (see Fig. 5, group 3 genes of Chen et al. (13)). We asked whether Sty1 was recruited to the promoter of one of these Sty1-dependent/Atf1-independent genes: namely pka1. The expression data for pka1 are shown in Fig. 7A and the requirement for Sty1 but not Atf1 is evident.

The primer pairs used for this ChIP analysis are indicated in Fig. 1A. Sty1 was not recruited to the pka1 promoter (or to the body of the gene) upon either osmotic or oxidative stress (Fig. 7B). These data suggest that Sty1 controls pka1 and probably other Atf1 independent genes by a mechanism that does not involve intimate association with promoters.
Sty1 Recruitment Is Abolished in Cells Lacking Either Atf1 or Pcr1—The recruitment of Sty1 to Atf1-dependent genes but not to one whose expression is independent of this factor suggested that Atf1 is required for association of the kinase with chromatin, even though phosphorylation of Atf1 is not. We tested this by examining Sty1 recruitment in an atf1Δ mutant. As shown, there was no association of Sty1 with the promoters of Atf1-dependent CESR genes in atf1Δ upon exposure to either osmotic or oxidative stress (Fig. 8, A and B, respectively). These data are consistent with Atf1 acting as a recruiting factor for Sty1. It is possible, however, that recruitment is determined by Pcr1 and that in the absence of Atf1, Pcr1 can no longer bind to promoters as it preferentially forms heterodimers with Atf1 rather than homodimers. To address this possibility, we examined whether Pcr1 could still bind to the CESR promoters in the atf1Δ mutant. As shown in Fig. 9B, we were unable to detect binding of Pcr1 to hsp9, ctt1, and gpd1 promoters in the atf1Δ mutant either upon osmotic or oxidative stress.

If Atf1 alone was determining Sty1 recruitment, it might be possible to observe association of Sty1 with promoters in the absence of Pcr1 and thus we assessed whether we could detect Sty1 recruitment in a pcr1Δ mutant. As shown, Sty1 was not recruited to the promoters of the three CESR genes in the absence of Pcr1, either upon osmotic stress (Fig. 8A) or oxidative stress (Fig. 8B). This was true at both early and late time points (15 and 60 min). Given the dependence of Pcr1 binding upon Atf1 (Fig. 9B), we asked whether the converse was true by examining the binding of Atf1 to promoters in the absence of Pcr1. As shown in Fig. 9A, Atf1 was no longer associated with the CESR promoters in the pcr1Δ mutant upon either osmotic or oxidative stress. We have previously shown that the levels of Atf1 are regulated by Pcr1 and vice versa (18). Even though...
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FIGURE 8. Sty1 is not recruited to stress-responsive promoters in the absence of either Atf1 or Pcr1. A, samples were processed for ChIP assays as described in the legend to Fig. 3A. The following strains were used: sty1-myc, sty1-myc atf1Δ, and sty1-myc pcr1Δ. The stress applied was 1 M sorbitol for 15 and 60 min. B, samples of the strains described in A were processed for ChIP analysis. Cells were treated with 2 mM H2O2 for 15 and 60 min.

There is less Atf1 protein in a pcr1Δ mutant, there is still more than in the atf1−11M mutant (Fig. 9C) and we observed binding of both Sty1 and Pcr1 in the latter (Fig. 6). Thus we conclude that the lack of binding of Atf1 and Sty1 to promoters in pcr1Δ is due to loss of Pcr1 and Atf1 at promoters and not caused by reduced levels of Atf1. Taking all the above data together, we conclude that Sty1 cannot be recruited to chromatin in the absence of either Atf1 or Pcr1. Given the interdependence of binding between Atf1 and Pcr1, it was not possible to differentiate between recruitment being dictated by the intact Atf1-Pcr1 heterodimer or by one of these factors alone.

Some Atf1-dependent Genes Are Still Expressed in a pcr1Δ Mutant—The dependence of Sty1 recruitment upon Pcr1 presented an interesting dilemma: cells lacking Pcr1 grow in a variety of conditions that are stressful enough to render atf1−11M and sty1Δ mutants inviable (15, 17, 18). These observations suggested that in the absence of Pcr1, cells are still able to produce sufficient mRNA from genes encoding stress protectant functions to ensure survival. Surprisingly, this transcription appears to be independent of the stable recruitment of both Sty1 and Atf1 to promoters (Figs. 8 and 9B). To address this issue, we performed genome-wide expression analyses of the response to osmotic and oxidative stress in a pcr1Δ mutant and compared the findings to our previous data acquired from wild type and atf1Δ cells (13). For this analysis, we exposed pcr1Δ cells to sorbitol and H2O2 and collected samples at 15 and 60 min after stress. The resulting changes in gene expression were analyzed by DNA microarray hybridization. Clearly many of the changes in gene expression that are dependent upon Atf1 (according to a previous study (13)) also require Pcr1. This is illustrated by the average expression patterns of Atf1-dependent genes as shown in Fig. 10A. However, the average increase in expression of these genes is higher in pcr1Δ than in atf1Δ upon both osmotic and oxidative stress consistent with the less severe phenotype displayed by the pcr1Δ mutant.

FIGURE 9. Atf1 is not detected at promoters in the absence of Pcr1 and vice versa. A, samples were processed for ChIP assays as described in the legend to Fig. 3A. The following strains were used: sty1-myc, sty1-myc atf1Δ, and sty1-myc pcr1Δ. The stress applied was 1 M sorbitol for 15 and 60 min. The DNA bound to Atf1 was recovered after immunoprecipitation reactions using antiserum raised against Atf1. B, samples of the strains described in A were processed for ChIP analysis. Cells were treated with 2 mM H2O2 for 15 and 60 min. The DNA bound to Pcr1 was recovered after immunoprecipitation reactions using antiserum raised against Pcr1. C, Western blot to examine the levels of Atf1 protein in pcr1Δ compared with the atf1−11M mutant. The loading was assessed using anti-tubulin antiserum.
genes whose expression is absolutely dependent upon both Atf1 and Pcr1 upon stress (e.g. group 2 genes in Fig. 10A). The genes that fall into these two categories are listed in Table 2.

The two groups of genes cannot be distinguished on the basis of associated GO terms and both groups contain a mixture of well defined stress protectant genes and uncharacterized genes. The CERS genes examined with respect to Sty1 recruitment, namely gpd1, hsp9, and ctt1, are all dependent upon both Atf1 and Pcr1 for optimal transcription, but upon sorbitol stress in particular, display a considerable delay in their expression. We confirmed their patterns of expression by Northern blotting. As shown in Fig. 10C, the pcr1Δ mutant displayed a greatly reduced level of expression for each of the three genes upon exposure to H2O2 but considerable induction of all three genes after 60 min of sorbitol stress. It is likely that some of the remaining expression observed upon H2O2 stress is regulated by Pap1 (42). However, this transcription factor is only activated by oxidative stress (43) and thus cannot explain the delayed transcription observed upon osmotic stress. As described earlier, we cannot detect the binding of either Atf1 or Sty1 to promoters in the absence of

### Table 2

| Gene name | Annotation* |
|-----------|-------------|
| Group 1   |             |
| vip1      | Protein with rrm RNA recognition motif |
| msp2      | Protein kinase inhibitor |
| SPAC9E9.04| Homologue of Bap31 |
| hsp9      | Heat shock protein |
| gpd1      | Glycerol-3-phosphate dehydrogenase |
| SPCP31B10.06| Protein containing a C2 domain |
| SPAC2F28.05| Protein with aldo-keto reductase family |
| SPCC38.12 | Member of the subtilisin N-terminal region |
| SPBC21C3.19| Protein of unknown function |
| SPBC11C11.06c| Protein of unknown function |
| git5      | Heterotrimetric G protein β subunit Git5, Git5, Gpb1 |
| SPAC4D7.02c| Member of the glycerophosphoryl diester phosphodiesterase family |
| pyp2      | Protein-tyrosine phosphatase |
| SPCC757.03c| Hypothetical protein |
| gpd1      | Glycerol-3-phosphate dehydrogenase |
| hsp9      | Heat shock protein |
| ctt1      | Protein containing an α,β-trehalose-phosphate synthase |
| rds1      | Stress response protein |
| SPAC9G12.09| Protein containing an aldo-keto reductase family |
| gpx1      | Glutathione peroxidase Gpx1 |
| mfp2      | Protein kinase inhibitor |
| mrf1;etr1 | Protein of unknown function |
| grx1      | Protein containing a short chain dehydrogenase domain |
| SPCC1398.12| Protein of unknown function |
| gpd1      | Glycerol-3-phosphate dehydrogenase |
| hsp9      | Heat shock protein |
| ctt1      | Protein containing an α,β-trehalose-phosphate synthase |
| gpd1      | Glycerol-3-phosphate dehydrogenase |

* Annotations are based on the S. pombe Gene data base.
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Sty1 with ChIP analysis was 500 bp we chose to examine association of induced genes. As the average size of DNA produced during the whether Sty1 also associates with the coding regions of stress-transcriptional elongation complex (26). Thus we examined budding yeast MAPK Hog1 is also associated with the ORF of dead mutant of Sty1 that was not recruited to the promoters of version of Sty1 for the analysis. As a control, we used the kinase-analysis. This problem was prevented by using the Pk-tagged strain we also observed higher levels of nonspecific enrichment of detectable binding of Atf1 in transcriptional initiation complexes. Here we have examined the recruitment of Sty1 to promoters using three different epitope-tagged versions of the kinase, assaying the binding using both real-time and standard gel-based PCR as a read-out of association, and performing the analysis with a variety of primer pairs to identify the genes in question. We conclude that Sty1 is indeed recruited to stress-dependent promoters.

Pcr1 (Figs. 8 and 9). Thus the Atf1-dependent expression in \( pcr1/ \) must be driven either by the binding of Atf1 and Sty1 at levels that are sufficiently low and/or transient enough to evade detection by the ChIP assays or alternatively by other factors whose expression depends upon Atf1 and Sty1. Taken together, these data demonstrate that whereas optimal stress-induced gene expression requires both Atf1 and Pcr1, significant but delayed induction of many Atf1-dependent genes still occurs in cells lacking Pcr1, particularly upon osmotic stress. These findings provide an explanation for the less severe stress phenotypes displayed by \( pcr1/ \) compared with \( atf1/ \).

Sty1 is recruited to the Open Reading Frames of Stress-induced Genes—In addition to its recruitment to promoters, the budding yeast MAPK Hog1 is also associated with the ORF of stress-induced genes where it interacts with components of the transcriptional elongation complex (26). Thus we examined whether Sty1 also associates with the coding regions of stress-induced genes. As the average size of DNA produced during the ChIP analysis was 500 bp we chose to examine association of Sty1 with \( gpd1/ \) and \( ctt1/ \) as these genes are 1158 and 1539 bp in length, respectively. Primer pairs could therefore be designed sufficiently far apart so as not to amplify both promoter and ORF from the same piece of DNA (primer pairs shown in Fig. 1). During initial experiments using myc-tagged Sty1, we observed recruitment of the kinase to both the ORF and terminator regions of \( gpd1/ \) upon osmotic and oxidative stress, although the-fold increase of binding over the control regions was considerably less than we had observed for the promoter regions (data not shown). However, using the untagged control strain we also observed higher levels of nonspecific enrichment of these regions than we had observed during the promoter analysis. This problem was prevented by using the Pk-tagged version of Sty1 for the analysis. As a control, we used the kinase-dead mutant of Sty1 that was not recruited to the promoters of stress-dependent genes (Fig. 5). Upon both osmotic and oxidative stress, Sty1 was recruited to the ORF and terminator region of the \( gpd1/ \) gene (Fig. 11) but not to the ORFs of the constitutively expressed \( cdc2/ \) or \( pol1/ \) genes. Interestingly, we did not observe recruitment to the \( ctt1/ \) ORF, whereas above we showed that Sty1 is associated with the promoter of this gene. The reason for this is not clear but we can eliminate primer design as an explanation as the oligonucleotides in question do amplify this region from whole cell extract (data not shown).

We conclude that Sty1 can be recruited not only to promoters but also to the body of at least some stress-induced genes as well as their terminator regions. This suggests that the role of SAPKs in transcriptional elongation control may be evolutionarily conserved.

DISCUSSION

Sty1 Is Stably Associated with Atf1-dependent Stress-responsive Promoters—Many stress-induced transcriptional events in fission yeast depend upon the Sty1 MAPK and its target transcription factor Atf1 (13). However, in contrast to a number of transcription factors that are regulated by MAPKs, the phosphorylation of Atf1 by Sty1 is not required for robust induction of gene expression (18). We postulated that the essential role of Sty1 in transcriptional activation might be a more direct one necessitating its recruitment to chromatin where it could either phosphorylate other targets and/or act as a structural adaptor in transcriptional initiation complexes. Here we have examined the recruitment of Sty1 to promoters using three different epitope-tagged versions of the kinase, assaying the binding using both real-time and standard gel-based PCR as a read-out of association, and performing the analysis with a variety of primer pairs to identify the genes in question. We conclude that Sty1 is indeed recruited to stress-dependent promoters.

Recruitment of Sty1 to Chromatin by Atf1/Pcr1—Sty1 interacts with Atf1 (11, 12), the latter being a good candidate for the factor that recruits Sty1 to chromatin. However, given the loss of detectable binding of Atf1 in \( pcr1/ \) and vice versa, it is hard to assess the contribution of Pcr1 to this process as it is not possible to study Sty1 recruitment in a situation where only one of the two bZIP proteins is bound to DNA. Thus Sty1 recruitment could be mediated exclusively through either Atf1 or Pcr1, or through the heterodimer. Our in vivo binding data confirms previous in vitro assays that demonstrate that Atf1 and Pcr1 bind avidly to DNA as a heterodimer but poorly as homodimers (16). It also confirms genetic data demonstrating that the Atf1-Pcr1 heterodimer (rather than either corresponding homodimer) is required for activation of the \( ade6/ \) meiotic hot spot for recombination (15).

We observed binding of Atf1 and Pcr1 to stress-responsive genes under basal conditions with just a modest increase in binding upon stress. In contrast, we do not see recruitment of Sty1 to chromatin until the cells are stressed consistent with its change in localization that occurs upon such conditions. Many stress-induced genes are transcribed at a low level in the absence of stress. Our data suggests that this basal transcription is dependent upon both Atf1 and Pcr1 (13) (Fig. 10, B and C) and that the stress-induced increase in transcription is mediated by the recruitment of Sty1 to these promoters. We hypothesize that Sty1 recruits additional transcriptional machinery and/or chromatin remodeling activities to mediate the dra-
matic up-regulation in expression of various stress-responsive genes.

Sty1 Kinase Activity Is Required for Its Recruitment to Chromatin—We made several observations suggesting that Sty1 kinase activity is required for its association with promoters. First, we found that the kinetics of Sty1 recruitment match those of its own activation (Fig. 2). Second, forced nuclear localization in the absence of stress-induced activation of Sty1 was not sufficient to result in the association of the kinase with promoters (Fig. 4); and finally the catalytically inactive form of Sty1 was not recruited to stress-responsive genes. These findings suggest that phosphorylation of a Sty1 target is necessary for optimal recruitment. However, this target is not Atf1 as recruitment still occurs in the atf1–11M mutant (Fig. 6). We also believe that this factor is unlikely to be Pcr1 as it does not appear to be a target for Sty1 phosphorylation (12). In budding yeast, Hog1 is required for the recruitment of a variety of factors to osmoresponsive genes including RNA pol II and mediator components although there is currently no evidence to suggest that it phosphorylates any of these factors (24, 25). A kinase-dead allele of Hog1 is still recruited to the GPD1 promoter but to a lesser extent than wild type. In contrast, recruitment to other genes, CTTL1, STLI, and HSP12 is abolished in the hog1 kinase-dead mutant (23). Our current work is focused on identifying the critical chromatin-bound target(s) for Sty1.

Many Atf1-dependent Genes Are Still Induced in a pcr1Δ Mutant—Both Atf1 and Pcr1 are required for binding to the meiotic hotspot created by a mutation in the ade6 gene (15) and for chromatin remodeling in this region (44). In contrast, our data shows that despite a requirement for the heterodimer to bind to stress-responsive genes, there are considerable differences in the transcription profiles of the atf1Δ and pcr1Δ mutants. Our results provide an explanation as to why pcr1Δ cells are not as sensitive to stress as atf1Δ cells (15, 18, 45); in a pcr1Δ mutant, there is still considerable expression of Atf1-dependent genes, albeit with delayed kinetics. These results raise the question as to what is controlling expression of Atf1-dependent genes in the pcr1Δ mutant, because neither Atf1 nor Pcr1 can be detected on the promoters (Figs. 8 and 9). We propose that Atf1 binds weakly and/or transiently to promoters as a homodimer or as a heterodimer with another factor and that the ChIP assay is not sensitive enough to detect such binding. In contrast, Pcr1 may not be able to bind to DNA at all as a homodimer in vivo or as a heterodimer with a factor other than Atf1 thus explaining why there is no significant transcription of Atf1-dependent genes in the atf1Δ mutant. Furthermore, the levels of Pcr1 are greatly reduced in the atf1Δ mutant (18). We have tried to detect the presence of homodimers of either Atf1 or Pcr1 by differentially epitope tagging the two copies of each protein in a diploid cell and looking for their co-immunoprecipitation but we failed to observe either homodimer.4

What distinguishes the two classes of Atf1-dependent genes such that one group absolutely requires Pcr1 for expression, whereas the other group can still be induced to some extent in the pcr1Δ mutant? One possibility is that the promoters of the genes have subtly different sequences representing the Atf1/Pcr1 binding site such that Atf1 homodimers, or heterodimers between Atf1 and another bZIP partner, can still bind to the promoters of genes in group 1 but not group 2. In this regard it is interesting to note that a family of CRE-related sites has been identified to which Atf1/Pcr1 can bind in vitro (46). Future work will characterize the binding sites for Atf1 in the different groups of genes that we have identified (Fig. 10) and determine whether they differ in their ability to bind Atf1 homodimers.

Sty1 Is Recruited to the ORFs of Stress-induced Genes—We also identified recruitment of Sty1 to the ORF of the gpd1Δ gene. It remains to be seen whether this association is due to its direct recruitment to these sequences or whether it is there as a consequence of its initial recruitment to promoters and subsequent progression into the body of the gene. For Hog1, its recruitment to ORFs has been uncoupled from promoter recruitment by virtue of constructing chimeric genes with different promoters. Indeed the 3′-untranslated region of the STL1 gene was shown to be important for this (26). The recruitment of MAP kinases to ORFs has not been reported in higher eukaryotes, and thus our observation that Sty1 is targeted to the coding regions of genes makes it more likely that this finding is conserved and raises the possibility that Sty1 may play a role in transcriptional elongation.

Budding and fission yeast are believed to have diverged more than 500 million years ago, yet the recruitment of MAP kinases to stress-activated promoters has been conserved. It remains to be seen whether this aspect of stress signaling is confined to single-celled eukaryotes or if it extends to higher eukaryotes but our results make it more likely that such a mechanism will be universally conserved. The Sty1 pathway is similar to higher eukaryotic stress-activated MAPK pathways and furthermore, both fission yeast and mammalian kinases are activated by a number of different signals, hence studying the role of Sty1 at promoters will hopefully provide a useful paradigm for understanding how p38 and JNK might operate.

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