

The Stress-Activated Hog1 Kinase Is a Selective Transcriptional Elongation Factor for Genes Responding to Osmotic Stress

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Summary

Regulation of gene expression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. Exposure of yeast to high osmolarity results in activation of the SAPK Hog1, which associates with transcription factors bound at target promoters and stimulates transcriptional initiation. Unexpectedly, activated Hog1 also associates with elongating Pol II and components of the elongation complex. Hog1 is selectively recruited to the entire coding region of osmotic stress genes, but not to constitutively expressed genes. Selective association of Hog1 with the transcribed region of osmoresponsive genes is determined by the 3' untranslated region (3' UTR). Lastly, Hog1 is important for the amount of the RNA polymerase II (Pol II) elongation complex and of mRNA produced from genes containing osmoresponsive coding regions. Thus, in addition to its various functions during transcriptional initiation, Hog1 behaves as a transcriptional elongation factor that is selective for genes induced upon osmotic stress.

Introduction

Adaptation to environmental stress requires changes in many aspects of the cell biology. In eukaryotic cells, SAPKs play an essential role for proper cell adaptation to extracellular stimuli (Kyriakis and Avruch, 2001). Exposure of cells to high osmolarity results in rapid activation of a conserved family of SAPKs, which includes the mammalian p38 and the yeast Hog1 (de Nadal et al., 2002; Sheikh-Hamad and Gustin, 2004). In *S. cerevisiae*, osmotic stress results in rapid activation of the SAPK Hog1, which elicits the program for cell adaptation that includes modulation of gene expression, translation, and cell cycle progression. The role of the SAPK in stress adaptation is illustrated by the fact that mutation of the

HOG1 gene results in cells unable to mount appropriate stress responses, and thus, *hog1Δ* cells are osmosensitive (de Nadal et al., 2002; Hohmann, 2002; Westfall et al., 2004).

Control of gene expression in response to stress is a major adaptive response controlled by SAPKs (Kyriakis and Avruch, 2001). Genome-wide transcriptional studies reveal that in response to osmotic stress, the Hog1 MAPK controls a rapid transcriptional response that involves a large number of genes required for cellular adaptation (Posas et al., 2000; Rep et al., 2000). There is not a uniform mechanism by which SAPKs, and MAP kinases in general, modulate gene expression. SAPKs can modify gene regulation by direct phosphorylation of transcription factors, both activators and repressors, modulate factors involved in chromatin remodeling and structure as well as Pol II recruitment (Kyriakis and Avruch, 2001; de Nadal et al., 2002; Alepuz et al., 2003; de Nadal et al., 2004).

In yeast, Hog1 regulates transcriptional induction by several mechanisms. Initially, the SAPK makes the cells competent to respond transcriptionally by phosphorylating and activating at least two proteins that reduce the ion concentration in the nucleus (Proft and Struhl, 2004). After this initial, pretranscriptional response, Hog1 directly phosphorylates and modifies the transcriptional properties of several DNA binding proteins (i.e., Sko1 and Smp1) (Proft et al., 2001; Proft and Struhl, 2002; de Nadal et al., 2003). Interestingly, Hog1 MAPK is also directly recruited to osmoresponsive promoters by specific transcription factors, such as Msn2/4, Hot1, or Sko1 (Alepuz et al., 2001; Proft and Struhl, 2002). This binding seems to be important to stimulate Pol II recruitment (Alepuz et al., 2003). In addition, the presence of Hog1 at promoters is also important to recruit the Rpd3 histone deacetylase complex and, thus, promote the modification of the chromatin at the promoters to properly initiate transcription (de Nadal et al., 2004). Thus, apart from the role of Hog1 in the modification of transcription factors, its specific chromatin association to stress-responsive promoters has shown a new dimension to the regulation of transcription initiation by signaling kinases.

Eukaryotic transcription is a complex process responsible for the production of a pre-mRNA molecule that is subjected to several modifications (5' capping, splicing, 3' end cleavage, and polyadenylation) before being transported to the cytoplasm. Thus, the process of transcription by Pol II involves multiple processes in the so-called transcription cycle and includes preinitiation, initiation, promoter clearance, elongation, and termination (Proudfoot, 2004; Sims et al., 2004; Aguilera, 2005; Buratowski, 2005). The elongation stage of transcription is actually a central process that coordinates multiple steps in mRNA biogenesis and maturation, and several complexes travel together with the elongating Pol II throughout the coding region after initiation of transcription at the promoters. Capping, splicing, 3' end processing, surveillance, and export of mRNA are modulated through interactions with the Pol II transcription

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elongation complex (TEC). Elongation factors are defined by their ability to associate with or affect the activity of the TEC, and they can affect the elongation rate or processivity of Pol II or mRNA maturation (Sims et al., 2004; Mason and Struhl, 2005). It has become clear that the elongation stage of transcription is a dynamic and highly regulated stage of the transcription cycle (Sims et al., 2004). However, an elongation factor that selectively associates with a particular class of genes has not been described.

In this work, we show that the Hog1 MAPK interacts with elongating Pol II and with general components of the TEC. The SAPK selectively travels with elongating Pol II through stress-responsive genes, and this selectivity is mediated by the 3' UTRs of osmoresponsive genes. Lastly, association of Hog1 with the TEC is important for the full transcriptional output of osmo-responsive coding regions in response to stress. Thus, the Hog1 SAPK does not only function at the level of transcriptional initiation, but it also acts as a selective elongation factor for genes induced by osmotic stress.

Results

Hog1 Interacts with the Rpb1 Subunit of the Pol II

To dissect the role of Hog1 in transcriptional regulation, we attempted to identify the subunit(s) of the intact Pol II complex that directly interacts with Hog1 by using a photocrosslinking label transfer strategy (Brown et al., 2001). GST-Hog1 was conjugated with the Sulfo-SBED photocrosslinking agent and incubated with yeast Pol II purified from a TAP-Rpb9 tagged strain (Rani et al., 2004). Rpb1 was unambiguously identified as a target, because it is clearly separated from other Pol II subunits and visualized by an antibody (8WG16) against this specific subunit (Figure 1A). In addition, a second lower-migrating protein from the Pol II complex, likely to be Rpb2, may be interacting directly with Hog1. Because interaction with other subunits could be masked by background signals, we cannot completely exclude that Hog1 interacts with some other subunits of the polymerase.

As phosphorylation of Rpb1 is a key step in the transcriptional process (Sims et al., 2004), we analyzed whether the Hog1 SAPK can associate with Rpb1 irrespective of its phosphorylation status. We expressed GST-Hog1 in yeast cells and performed GST pull-down experiments with glutathione-Sepharose beads. The presence of precipitated Rpb1 was probed with specific antibodies against total (8WG16) or phosphorylated Rpb1 (H14 or H5). As shown previously, coprecipitation of Hog1 with Pol II is more efficient from yeast extracts of cells treated with osmostress (Alepuz et al., 2003). In addition, Hog1 interacts with Pol II phosphorylated at serine 2 or serine 5 of the C-terminal domain in yeast cell extracts (Figure 1B). Correspondingly, *in vitro* binding experiments using GST-Hog1 expressed and purified from *E. coli* together with yeast purified Tap-Rpb3 complex treated with alkaline phosphatase show that Hog1 interacts more efficiently with phosphorylated Rpb1 (Figure 1C). Taken together, our data indicate that the interaction of Hog1 is more efficient with phosphorylated Pol II.

Genome-Wide Location Analysis Reveals Binding of Stress-Activated Hog1 to Promoter and 3' Regions of Osmoresponsive Genes

We performed genome-wide location analysis to map the chromosomal regions to which Hog1 is recruited upon stress. Input and immunoprecipitated samples from HA-Hog1 expressing yeast cells subjected to a brief hyperosmotic shock were amplified, Cy5 and Cy3 labeled, and hybridized with microarrays containing almost all yeast intergenic regions. As expected for a protein that is indirectly recruited to chromatin, relative binding values for Hog1 are generally low with median log ratios of 3:5 for the best signals. We therefore considered those 72 that showed at least 1.5-fold enrichment in at least half of the datasets. Despite the apparently relaxed definition of a Hog1 target site, 87% (63 of the 72 top ranking interactions) of the identified intergenic regions correspond to genes that are >3-fold upregulated upon hyperosmotic stress, as identified by transcriptional profiling experiments (Posas et al., 2000; Rep et al., 2000). Among these genes, we identified *STL1*, *GRE2*, *CTT1*, *GPD1*, and *TSL1*, which are well-characterized osmoresponsive genes. Expression of many (35) of these stress genes is activated greater than 10-fold. Unexpectedly, the Hog1-interacting regions of osmoresponsive genes correspond either to the promoter (35 cases), or the 3' downstream region (28 cases), or both (ten cases). Taken together, this observation suggests stress-activated Hog1 SAPK is both recruited to promoters and 3' downstream regions of genes whose expression is induced upon osmotic shock.

Hog1 Associates with the Coding and 3' Regions of Osmoresponsive Genes

Hog1 is recruited to osmostress promoters through its association with specific DNA binding transcription factors to induce formation of the preinitiation complex (Alepuz et al., 2001, 2003; Proft and Struhl, 2002). The genomic location analysis above and our observation that Hog1 interacts more efficiently with phosphorylated Rpb1 prompted us to directly determine whether Hog1 association is restricted to the promoter regions or also occurred at the coding regions where phosphorylated Pol II is present. We utilized chromatin immunoprecipitation to follow the binding of several components of the transcription machinery to various regions of six osmostress-activated genes (*STL1*, *GRE2*, *CTT1*, *FAA1*, *GPD1*, and *TSL1*) before and after the addition of NaCl. All six genes are highly induced upon osmostress in a Hog1-dependent manner, although, their expression is driven by distinct sets of specific transcription factors. Thus, *GRE2* and *FAA1* are bound by the Sko1 transcription factor (Rep et al., 2001; Proft et al., 2005), *GPD1* is mainly regulated by Hot1 (Rep et al., 1999), and *CTT1* and *TSL1* are regulated by the Msn2,4 activators (Rep et al., 1999; Tamas et al., 2000). All six genes tested by standard ChIP assays ranked among the top 100 intergenic regions bound by Hog1 in our genome-wide location assay.

As expected, TBP and Pol II association was induced by osmostress at all six loci, and we detected constitutive binding of Sko1 at the *STL1*, *GRE2*, and *FAA1* promoters and stress-induced binding of the Hot1 activator

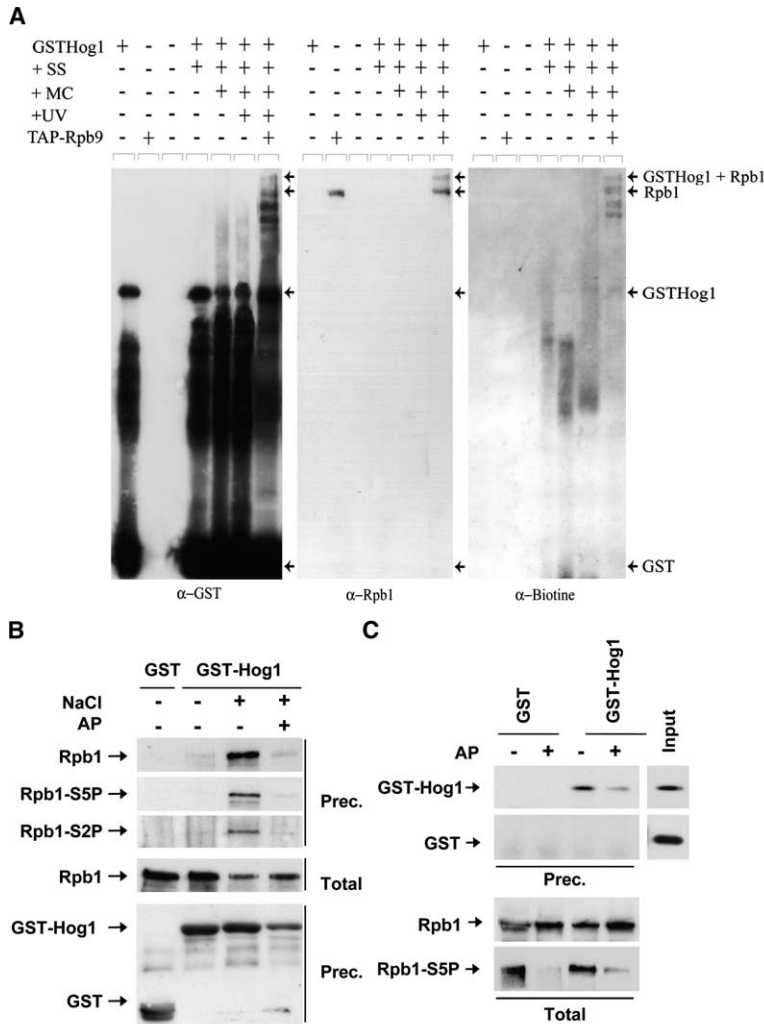


Figure 1. Hog1 Interacts with the Rpb1 Subunit of Pol II

(A) Hog1 photocrosslinks with Rpb1. GST-Hog1 was conjugated with sulfo-SBED (SS), purified (MC), and incubated with purified Rpb9 complex, and the mixture was exposed to UV light. Western blot using anti-GST (α -GST), anti-Rpb1 (8WG16), or streptavidin-HPR (α -biotin) to follow sulfo-SBED containing proteins.

(B) Hog1 coprecipitates in vivo with phosphorylated Rpb1. Yeast cells containing GST or GST-Hog1 were subjected (+) or not (-) to a brief osmotic stress (0.4 M NaCl, 10 min), and GST proteins were pulled down from yeast extracts treated (+) or not (-) with alkaline phosphatase (AP). Immunoblot using anti-Rpb1 (8WG16), antiphosphorylated Ser5 Rpb1 (H14), or antiphosphorylated Ser2 Rpb1 (H5). Total extract represents <20% of total input protein (middle). The amount of precipitated GST proteins was detected by using anti-GST (bottom).

(C) In vitro interaction of Hog1 with Rpb1 decreases after dephosphorylation of Rpb1. TAP-Rpb3 purified from yeast and treated (+) or not (-) with AP was incubated with GST, and GST-Hog1 purified from *E. coli*, and the samples were analyzed as in (B). Quantification of four independent experiments showed that binding of Hog1 to Rpb1 was reduced to 40% by AP treatment.

to the *STL1* promoter (Figures 2A and 2B and data not shown). Also, as expected, the transcription factors (Sko1 and Hot1) associate exclusively with their target DNA sites in the promoters, TBP association is detected only at the TATA box regions, and Pol II associates with the entire coding region. Importantly, Hog1 is recruited at all six stress genes not only at the promoter but also over the entire transcribed regions upon hyperosmotic shock (Figures 2A and 2B and data not shown). This suggests a possible role of Hog1 in the process of transcript elongation in addition to the regulation of transcription initiation.

In contrast to its presence at the coding regions of all osmoreponsive genes tested, Hog1 is not detectably associated with the coding regions of two constitutively expressed loci (*ADH1*, *ACT1*) under osmotic stress conditions (Figure 2C). Importantly, Pol II association at the *ADH1* and *ACT1* coding regions occurs at comparable levels to those observed at the coding regions of the stress-inducible genes. This result indicates that Hog1 association with coding regions occurs specifically at genes activated by hyperosmotic stress.

We then analyzed the kinetics of Hog1 and Pol II association at the *STL1* coding region upon the addition of NaCl. As shown in Figure 2D, the initial recruitment (first

5 min after osmotic stress) of Hog1 and Pol II at the *STL1* ORF is similar. However, Pol II association is observed for more than 20 min after salt treatment, which correlates with RNA expression (Northern blot; data not shown), whereas the binding of Hog1 is strongly reduced after 10 min. Therefore, binding of Hog1 to the coding region is restricted temporally, which might suggest a role for Hog1 at early steps of the elongation process.

Hog1 Interacts with Components of the Pol II Transcript Elongation Complex

A number of complexes (e.g., Spt4-Spt5, FACT, THO, and Paf1) associate with Pol II in vitro and travel with elongating Pol II throughout the coding region in vivo (Krogan et al., 2002, 2003; Pokholok et al., 2002; Strasser et al., 2002; Mason and Struhl, 2003; Sims et al., 2004). We therefore tested whether Hog1 is part of a larger complex containing these elongation factors by performing GST pull-down experiments in extracts from osmotically stressed cells expressing GST-Hog1 and TAP-tagged versions of Spt4, TFIIIS, Paf1, and Thp1. In all cases, GST-Hog1, but not the GST control, coprecipitates the TAP-tagged elongation factor (Figure 3 and Figure S1 available in the Supplemental Data with this article online). This interaction also occurs in the

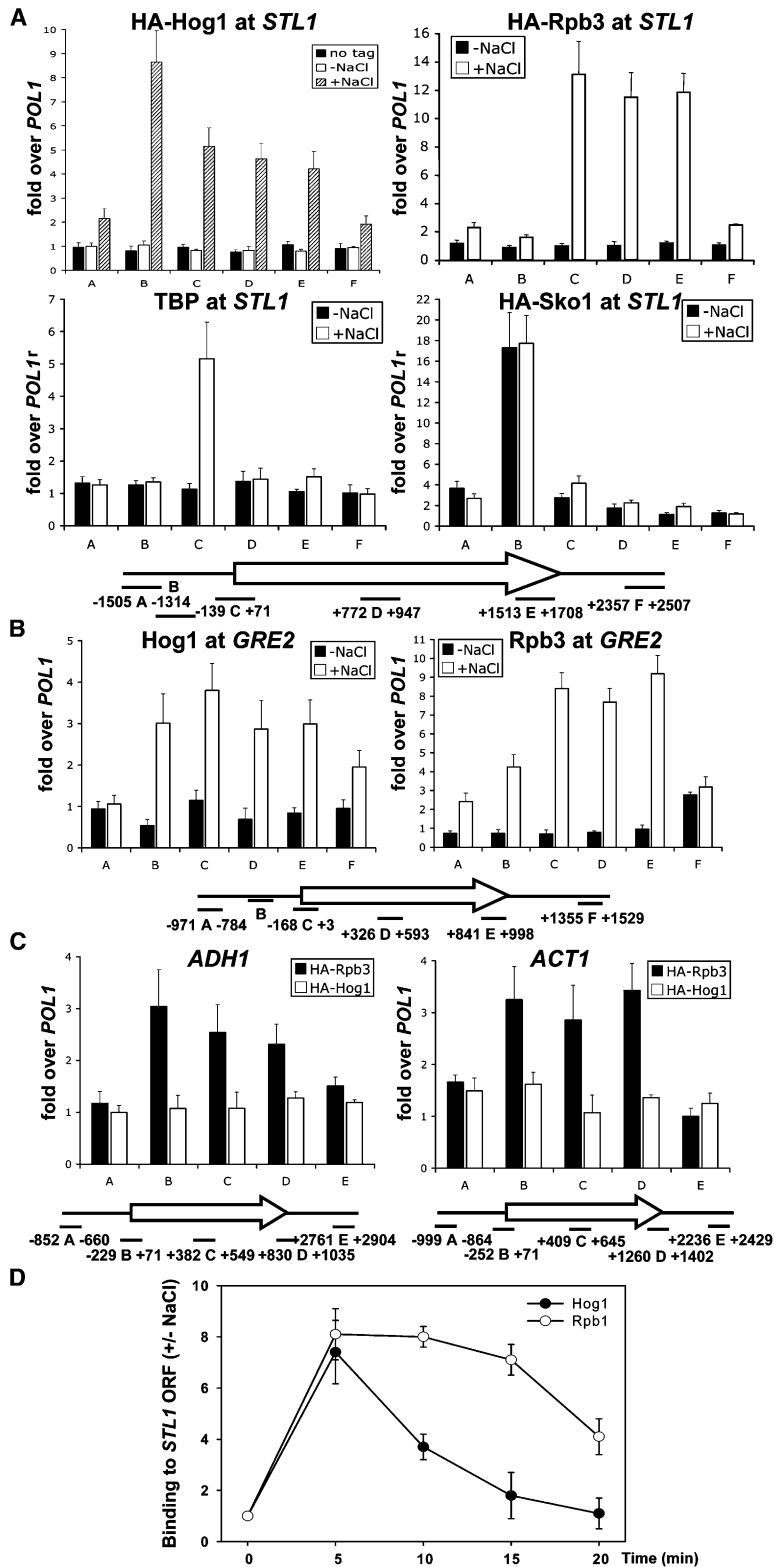


Figure 2. Hog1 Associates with Both Promoter and ORF of Osmoresponsive Genes but Is Not Recruited To Constitutively Expressed Genes

(A) Hog1 is recruited to the promoter and ORF regions of the osmoresponsive gene *STL1* in response to stress. Hog1, Rpb3, TBP, and Sko1 association in vivo with the indicated regions of the *STL1* and *GRE2* loci in wild-type (W303-1A; no tag), MAP51 (HA-Hog1), MAP37 (HA-Sko1), and W303-1A HA-Rpb3 (HA-Rpb3) that were or were not treated for 5 min with 0.4 M NaCl. Standard deviations are shown.

(B) Hog1 associates with the *GRE2* promoter and coding region. Hog1 and Rpb3 association in vivo with the indicated regions of *GRE2*.

(C) Hog1 is not recruited to constitutively expressed genes. Hog1 and Rpb3 association in vivo with the indicated regions of *ADH1* and *ACT1*.

(D) Kinetics of occupancy of Hog1 and Rpb1 to the *STL1* ORF (+402 to +630) in cells that were stressed by 0.4 M NaCl for the indicated times.

Data represent the mean and standard deviation of three independent experiments.

presence of DNase I, and it does not occur with a truncated version of Hog1 that lacks the C-terminal domain (residues 265–435; Figure S1). These observations indicate that Hog1 interacts with larger Pol II-containing entities and not just the individual components. In addition, they strongly support the idea that Hog1 can indeed

interact and travel with the elongating Pol II machinery in vivo through the coding regions of the stress-responsive genes. These results do not address whether Hog1 directly contacts Pol II in vivo or whether Hog1 association occurs in the context of a macromolecular complex containing all or a subset of the elongation factors.

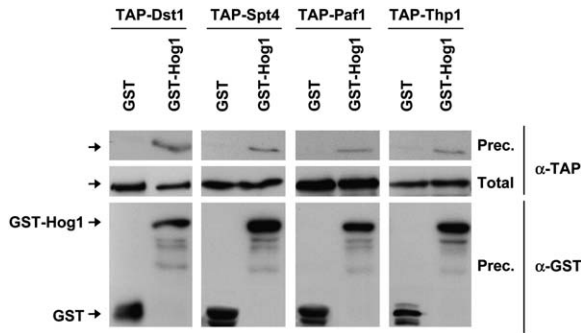


Figure 3. Hog1 Associates with the Elongating Polymerase Complex

TAP-tagged-Dst1, Spt4, Paf1, or Thp1 strains that express GST or GST-Hog1 were subjected to a brief osmotic shock (10 min, 0.4 M NaCl). GST proteins were pulled down by glutathione Sepharose 4B, and the presence of TAP proteins was probed by immunoblotting using anti-TAP (PAP, Sigma) (top). Total extract represents <20% of total input protein (middle). The amount of precipitated GST proteins was detected using anti-GST (bottom).

Elongation Factors Are Important for Transcription in Response to Osmotic Stress

The association of Hog1 with elongating Pol II suggested the possibility that the Pol II elongation machinery might be required for cells to respond to osmotic stress. Thus, we tested whether mutations of components involved in transcription elongation results in decreased osmotic stress gene expression and compromised cell adaptation to high osmolarity. Although deletion of several genes involved in transcriptional processing does not affect cell viability upon stress, strains lacking Spt4, TFIIS, Thp1, and Paf1 show compromised survival in the presence of high osmolarity (Figure 4A and Figure S2). Correspondingly, expression of osmoresponsive genes such as *STL1*, *CTT1*, and *GRE2* is significantly affected in these mutant strains (Figure 4B). Therefore, as is the case for promoter-associated factors (e.g., DNA binding activators and the Rpd3 histone deacetylase complex), several elongation factors are important for transcriptional activation in response to osmotic stress and the ability to grow under such conditions.

Hog1 Association with Coding Regions Is Important for mRNA Production of Osmoresponsive Genes upon Stress

To analyze the role of Hog1 within the Pol II elongation complex, we had to uncouple the processes of transcriptional initiation and elongation in response to hyperosmotic stress. Specifically, we measured RNA levels of several osmoresponsive genes (*STL1* and *CTT1*) and control genes (*ADH1* and *lacZ*) whose expression is driven by the LexA promoter and the LexA-VP16 activator in the presence or absence of osmotic stress (Figure 5A). The amount of *STL1* mRNA is 7-fold higher after 10 min of osmotic stress than in absence of stress, and this induction is dependent on Hog1 (Figure 5A) but independent of Hot1, the primary transcription factor that regulates the expression of the wild-type *STL1* gene (data not shown). The catalytic activity of Hog1 is required both for binding of Hog1 to the *STL1* coding region and for LexA-*STL1* mRNA production upon stress (Figure S3). Correspondingly, the catalytically inactive

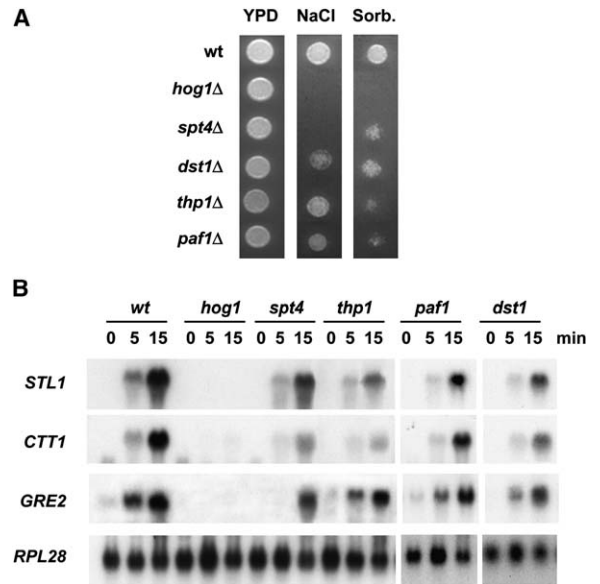


Figure 4. Mutations in Specific Elongation Factors Display Impaired Osmotic Stress Gene Expression

(A) Wild-type (BY4741) and the indicated mutant strains were spotted on YPD plates with or without 1.2 M NaCl or 2.4 M sorbitol. Growth was scored after 4 days.

(B) RNA levels in wild-type and mutant strains were grown to midlog phase in rich medium and then subjected to osmotic shock (0.4 M NaCl) for the indicated time.

Hog1 does not bind to the native *CTT1* or *TSL1* genes (Figure S5). Similarly, osmotic stress causes a rapid 10-fold increase in expression of *CTT1* mRNA that depends on Hog1. In marked contrast, no increase of mRNA production is observed for the control genes *ADH1* or *lacZ* (1.1-fold and 0.9-fold respectively).

Importantly, whereas Hog1 binding upon stress occurs at both the promoter and coding regions of osmoresponsive genes (Figure 2), Hog1 association at the artificial LexA-*STL1* gene is observed only at the coding region upon stress and is not present at the promoter (Figure 5B). Conversely, in a strain carrying the *STL1::lacZ* reporter construct, binding of Hog1 is restricted to the promoter and does not occur at the coding region (Figure S4). Taken together, these observations indicate that the recruitment of Hog1 SAPK to the coding regions of stress-responsive genes is critical for proper mRNA production in response to osmotic stress.

Stress-Activated Hog1 Stimulates Pol II Density at Osmoresponsive Coding Regions

To understand the mechanism by which Hog1 stimulates mRNA production during the elongation process, we assessed Pol II association through the coding region of the LexA-*STL1* gene. In response to stress, Pol II density at the *STL1* coding region is ~2-fold higher when compared to the level in nonstressed cells (Figure 6A). In contrast, no differences in Pol II association are observed in a *hog1* strain. In addition, association of the Spt4 elongation factor is induced upon osmotic stress at the *STL1* coding region in a manner that depends on Hog1 (Figure 6B). Therefore, the presence of Hog1 at the coding regions increases the density of

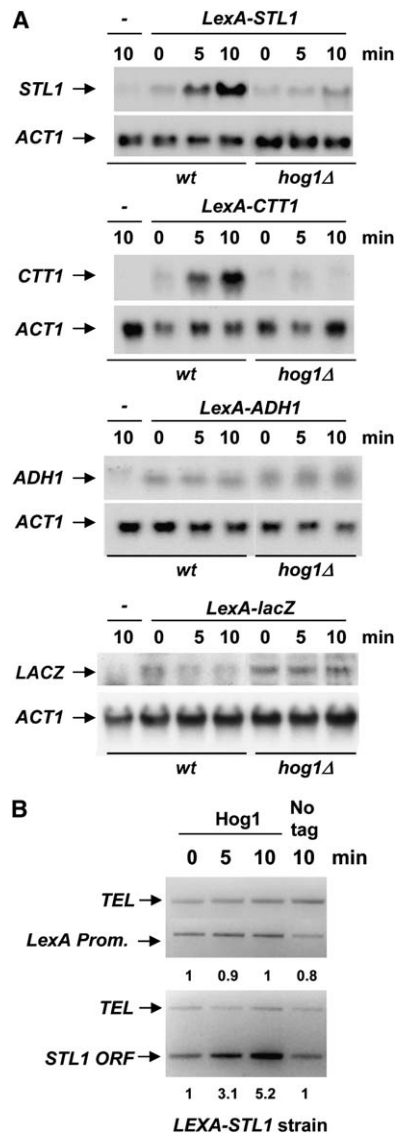


Figure 5. Hog1 Is Important for Induction of Osmoresponsive Genes in Response to Stress

(A) Hog1 increases *STL1* and *CTT1* mRNA production when expressed by the LexA-Vp16 activator. RNA levels in wild-type or *hog1Δ* cells lacking endogenous *STL1* or *CTT1* were transformed with a vector carrying *STL1*, *CTT1*, *ADH1*, or *lacZ* genes under the LexA promoter, or an empty vector as control (–) and with a plasmid containing the LexA binding domain fused to the VP16 transcriptional activator.

(B) Upon osmotic shock, Hog1 is recruited to the *STL1* ORF, but not to the promoter in the *LexA-STL1* locus. Strains described in (A) lacking endogenous *STL1* and with an integrated tagged Hog1-HA were treated with 0.4 M NaCl for the indicated times and assayed for the presence of Hog1 at the LexA promoter (Prom.) or *STL1* ORF. Quantification is depicted as fold binding over *TEL1*.

elongating Pol II and a Pol II elongation factor at stress-responsive genes.

Binding of Hog1 to Stress-Responsive Coding Regions Depends on the 3' UTR

Hog1 is recruited to stress-responsive coding regions in genes driven by native (Figure 2) and heterologous

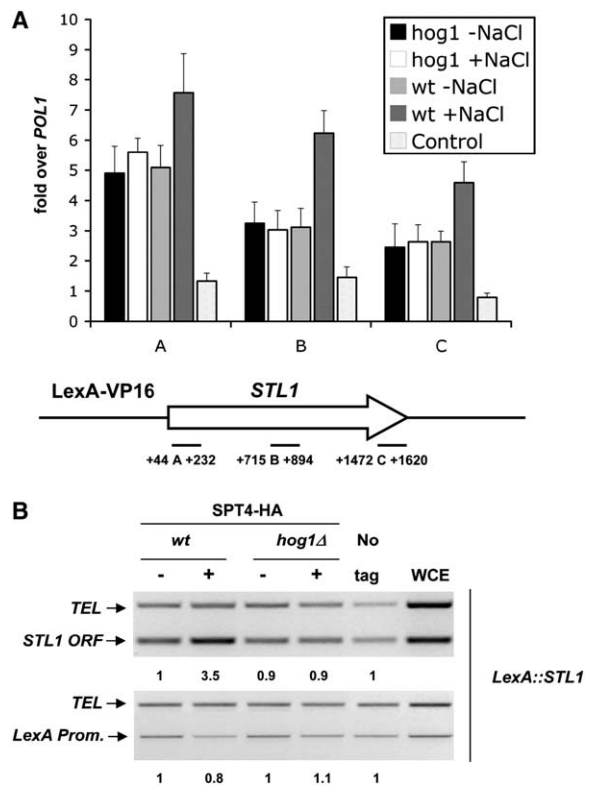


Figure 6. Increased Level of the Pol II Elongation Complex in Response to Hog1 Activation

(A) Association of Rpb1 in wild-type and or *hog1Δ* strains (both *stl1Δ*) containing the *LexA-STL1* construct or control cells lacking the LexA-VP16 plasmid.

(B) Association of Spt4-HA to the *LexA* promoter or *STL1* coding region of the *LexA-STL1* construct in wild-type or *hog1* strains (both *stl1Δ*) that were (+) or were not (–) exposed to hyperosmotic stress. Quantification is depicted as fold binding over *TEL1*.

Data represent the mean and standard deviation of three independent experiments.

(Figure S3) promoters. To identify the determinants within osmotically inducible coding regions required for Hog1 recruitment, we first compared binding of Hog1-HA to the *STL1* coding region in *LexA-STL1* plasmids that either contain or lack the 3' UTR of *STL1*. As shown in Figure 7A, binding of Hog1 to the *STL1* coding region is only observed when the 3' UTR region was present. In addition, the 3' UTR region is required for binding of Hog1 to *STL1* even in the presence of the wild-type promoter (Figure S6). We then replaced the 3' UTR of the uninducible *ADH1* gene by the 3' UTR of *STL1* and found that Hog1 is now recruited to the *ADH1* coding region (Figure 7B). Thus, the 3' UTR of *STL1* is necessary and sufficient for Hog1 binding to coding regions, but only under conditions of hyperosmotic stress.

Discussion

SAPKs regulate the expression of specific classes of genes that permit the adaptation to environmental stress. In general, SAPKs function at the level of transcriptional initiation, and they mediate their effects by direct phosphorylation of transcriptional regulatory

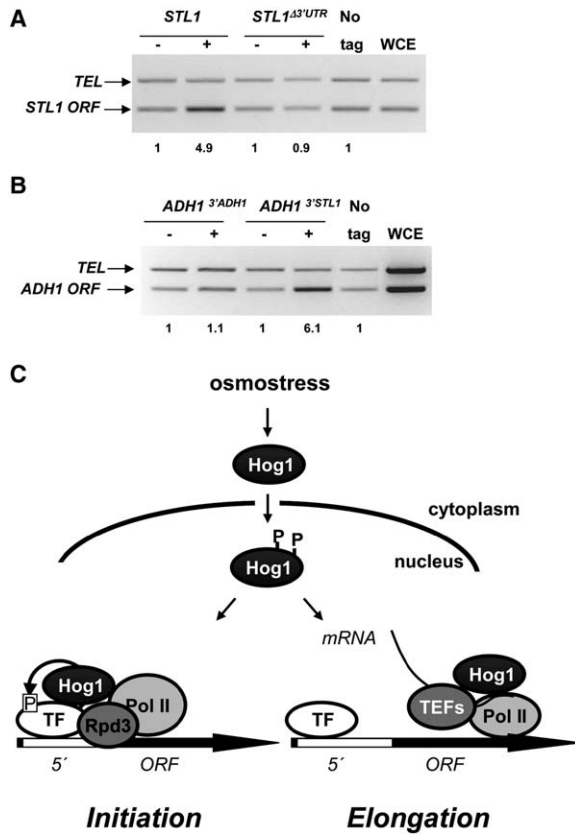


Figure 7. The *STL1* 3' UTR Is Necessary and Sufficient for Hog1 Association to Stress-Responsive Genes

(A) Removal of 3' UTR in *STL1* abolishes binding of Hog1 to *STL1* ORF. Strains containing the *LexA-STL1* vector or a plasmid containing a deletion of the 3' UTR (*LexA-STL1 Δ 3'UTR*) and the *LexA-Vp16* were analyzed for binding of Hog1 to the *STL1* ORF. Quantification is depicted as fold binding over *TEL1*.

(B) The presence of *STL1* 3' UTR region induces recruitment of Hog1 to the *ADH1* ORF. As in (A), Hog1-HA binding was analyzed to the *ADH1* ORF in plasmids containing *STL1* or *ADH1* 3' UTRs. Quantification is depicted as fold binding over *TEL1*.

(C) Schematic diagram depicting the role of Hog1 in the transcription cycle. Upon osmotic stress, Hog1 is activated and concentrates into the nucleus, where it regulates several aspects of the transcription cycle. There are several mechanisms by which Hog1 modulates transcription initiation: the direct regulation of transcription factor activity, direct stimulation of the recruitment of the Pol II at osmoresponsive promoters, recruitment of the Rpd3 histone deacetylase complex, and modification of chromatin. In addition, Hog1 is also involved in the process of elongation (see Discussion).

proteins. The yeast Hog1 SAPK, which regulates the expression of a large number of genes in response to hyperosmotic stress, directly phosphorylates and modifies the transcriptional properties of several DNA binding proteins (e.g., Hot1, Smp1, and Sko1). However, Hog1 plays multiple roles in the process of transcriptional initiation beyond modification of DNA binding regulatory proteins (Figure 7C). Hog1 associates with osmotic stress promoters, it can directly stimulate the recruitment of the Pol II to promoters, and it regulates initiation by the modification of chromatin through recruitment of the Rpd3 histone deacetylase complex. These multiple mechanisms mediated by Hog1 appear to be required for full expression of the osmoresponsive

gene program. It is possible that selective regulation of these mechanisms might underlie the response to different levels or types of hyperosmotic stress.

Here, we show that the role of Hog1 in the regulation of the transcription cycle is not limited to transcription initiation but rather extends to the process of transcriptional elongation (Figure 7C). Elongation factors are defined by their ability to affect the activity of or to associate with the transcriptional elongation complex (TEC), and they can affect elongation rate, Pol II processivity, or maturation of mRNA. Binding experiments and photocrosslinking analysis show that Hog1 is part of the Pol II TEC, interacting directly with phosphorylated Rpb1. Furthermore, active Hog1 travels with Pol II through the coding regions of osmoresponsive genes, and this feature is important for proper mRNA production. Therefore, we demonstrate that Hog1 fits with the description of a bona fide elongation factor, with the feature that its role in elongation is restricted to osmoresponsive genes.

Unbiased genome-wide location analysis shows that Hog1 is recruited to many (perhaps most of all) osmotic stress-inducible genes. Highly expressed constitutive genes, however, are not at all enriched. The specificity at the promoter level is very likely mediated by the interaction of Hog1 SAPK with a set of specific transcriptional activators, including Msn2-4, Hot1, and Sko1, as it has been shown explicitly for a few stress responsive promoters (Alepez et al., 2001; Prof and Struhl, 2002; Alepez et al., 2003). In addition to the binding to promoters, the ChIP-chip analysis has shown that the SAPK generally shows the same occupancy patterns as elongating Pol II at inducible transcribed regions. It is worth noting that constitutively expressed genes such as *ADH1* and *ACT1* do not recruit Hog1 in their transcribed regions, and their transcription is not affected upon stress as it happens with osmoresponsive genes.

By uncoupling Hog1-dependent transcriptional initiation from transcriptional elongation, we show that Hog1 at coding regions is important for induction of stress-responsive mRNAs. Furthermore, Hog1 is important for increased association of Pol II and the Spt4 elongation factor at coding regions, strongly suggesting that it directly affects the process of Pol II elongation. Very interestingly, even when driven by a constitutive *lexA-Vp16* activator, osmoinducible coding regions are regulated by stress in a Hog1-dependent manner, whereas stress-unrelated sequences were constitutively expressed independently of the presence of the SAPK. These experiments suggest that Hog1 can be recruited to transcribed regions independently of the promoter bound-specific transcription factors dedicated to osmotic stress adaptation. Actually, the recruitment of Hog1 in promoter regions does not guarantee the recruitment of Hog1 at the transcribed regions as observed in a chimeric gene that contains an osmoresponsive promoter and a nonstress responsive ORF (*STL1:lacZ*). It is worth noting that binding of Hog1 to *STL1* is abolished in the absence of the 3' UTR region of *STL1* and that the 3' UTR region of *STL1* confers Hog1 binding to a nonstress responsive ORF such as *ADH1*. We therefore propose that some feature of the 3' UTR regions of the osmoresponsive genes is required for the specific association of the SAPK with the TEC.

Stimulation of RNA Pol II density by Hog1 at the *LexA-STL1* locus (about 2-fold; Figure 6) seems to be less pronounced than the increase of *STL1* mRNA upon stress (Figure 5). It is possible that measurements of Pol II density are not strictly linear with mRNA levels, because the experimental background in ChIP experiments is considerably higher than in RNA determinations. More interestingly, this result suggests the possibility that, apart from stimulating transcription upon recruitment to the TEC (as shown here), Hog1 MAPK may also affect mRNA processing or stability.

The Hog1 SAPK is part of the TEC during the initial phase of osmotic stress adaptation. Recruitment of Hog1 to ORFs of stress genes is initially identical to that of the Pol II. However, whereas the presence of Pol II occurs for long periods of time at the ORFs, the presence of the SAPK is restricted at the initial phase of the elongation. These data could suggest that the requirement of the SAPK in elongation could be important for the function of the TEC at the initial stages of elongation. One candidate for such a function could be the modification of the nucleosomes occupying the stress-responsive ORF regions during the initial rounds of transcription. In mammalian cells, p38 SAPK stimulates histone 3 phosphorylation indirectly via the histone kinases Msk1/2 (Soloaga et al., 2003). Alternatively, Hog1 might mediate the correct assembly of processing activities required for efficient mRNA production in response to stress. Here, the need of a rapid response to increase the survival rate upon hyperosmotic stress might require additional mechanisms that involve the presence of the SAPK. By analogy, other rapid responses might also utilize the signaling molecules to increase the rate of transcription and to permit a rapid adaptation to external stimuli.

Experimental Procedures

Yeast Strains and Plasmid DNAs

Yeast strains used in this study are the wild-type strain BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) and its derivatives containing chromosomally integrated *DST1-TAP*, *SPT4-TAP*, *PAF1-TAP*, and *THP1-TAP* or their corresponding deletions (a gift from Dr. M. Peter, ETH Zürich), YGM117 (*MATa stl1::Nat hot1::kanMX4*), YGM88 (*MATa stl1::KanMX4*), YGM89 (*MATa hog1::Nat stl1::kanMX4*), YGM103 (*MATa stl1::Nat HOG1-HA6-KAN*), YGM150 (*MATa adh1::Nat HOG1-HA6-KAN*), YGM143 (*MATa stl1::Nat SPT4-HA6-KAN*), and YGM144 (*MATa stl1::Nat SPT4-HA6-KAN hog1::LEU2*). The *RPB9-TAP* strain was kindly provided by Dr. S. Hahn. Derivatives from W303-1A (*leu2-3,112 ura3-1 his3-11 trp1-1 can1-100*) were YEN173 and YGM73. In these strains, the genomic locus of *HOG1* and *HOT1*, respectively, was tagged at the carboxyl terminus with a sequence encoding 6-HA epitope tag followed by the *S. pombe HIS3* as selectable marker. In strains PAY150 and PAY154, the genes encoding TBP and Rpb1 were similarly tagged but with 18-Myc epitope tags followed by *Kluyveromyces lactis TRP1* as selectable marker. For ChIP, the following W303-1A derivatives were used: MAP51 (3HA-*HOG1*, *ura3::loxP*), W303-1A HA-Rpb3 (3HA-*RPB3*, kind gift from Paul Mason), and MAP37 (3HA-*SKO1*, *ura3::KAN MX*).

The LexA binding domain fused to VP16 transcriptional activator is cloned into pDB20. The plasmids expressing different ORFs were created by PCR as follows: the LexA operators, (a fragment of 389 bp containing eight LexA operators) obtained from pSH18-34 (Steve Hanes and Roger Brent, unpublished), were cloned into pRS413. Then *STL1*, *CTT1*, and *ADH1* (entire coding regions plus 350–400 bp 3' of each gene) were introduced into this plasmid to create *LexA-STL1*, *LexA-CTT1*, and *LexA-ADH1*. The plasmid PEN159

contains a BamHI-PstI fragment of the eight colE1-overlapping LexA operators fused to *lacZ* derived from pSH18-18 (Estruch and Carlson, 1990) and cloned into pRS415. Plasmids in Figure S3 to monitor Hog1-HA by ChIP were PVR53, a YCplac111-based plasmid containing a fusion of *HOG1* and three tandem repeats of HA epitope (YPYDVPDYA) inserted before the *HOG1* stop codon, and the PVR53-K52S, K53N derivative, which is catalytically inactive (Alepez et al., 2001). Control plasmids used were YCplac111 (Gietz and Sugino, 1988). *STL1::lacZ* fusion used in Figure S4 was constructed by insertion of the –825/+4 nucleotides of the *STL1* gene into the integrative plasmid Yip358R (Alepez et al., 2003). Plasmid PGM68 (*LexA-STL1^{Δ3'UTR}*) was created by cloning a PCR fragment containing solely the ORF, from ATG to the stop codon, of the *STL1* gene into a pRS413 vector. Plasmids PGM64 and PGM66 (*LexA-ADH1^{ΔSTL1}* and *LexA-ADH1^{ΔSTL1}*, respectively) were constructed by cloning a 400 bp PCR fragment from the stop codon to 3' either corresponding to *STL1* or *ADH1* into a plasmid containing *ADH1* ORF (PGM63). More detailed information about the construction of all plasmids is available on request.

ChIP

ChIP was performed as described previously (Kuras and Struhl, 1999; Alepez et al., 2001). Yeast cultures were grown to early log phase (OD₆₀₀ = 0.6–1.0) before aliquots of the culture were exposed to osmotic stress treatment (0.4 M NaCl) for the time specified in the figure legends. For crosslinking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Primer mixes were adjusted for balanced signals. Quantitative PCR analysis of stress genes and constitutively expressed genes utilized the following primers (sequences are available upon request), with locations indicated by the distance from the respect ATG initiation codon: *STL1* (–1505/–1314 STR8829/8830; –698/–416 STR6480/6481; –139/+71 STR8831/8832; +772/+947 STR8833/8834; +1513/+1708 STR8835/8836; +2357/2507 STR8837/8838), *GRE2* (–971/–784 STR8905/8906; –310/–145 STR5459/5460; –168/+3 STR5557/5558; +326/+593 STR8907/8908; +841/+998 STR6825/6826; +1355/+1529 STR8909/8910), *FAA1* (–1743/–1561 STR6973/6974; –827/–576 STR6993/6994; –201/+30 STR8785/8786; +1025/+1221 STR8787/8788; +1930/+2085 STR8789/8790; +3201/+3378 STR8791/8792), *GPD1* (–1402/–1173 STR9070/9071; –446/–211 STR9072/9073; –184/+23 STR6135/6136; +352/+528 STR9074/9075; +917/+1156 STR7891/7892; +1489/+1675 STR9076/9077), *TSL1* (–1139/–963 STR9058/9059; –371/–147 STR9060/9061; –174/+20 STR9062/9063; +1307/+1529 STR9064/9065; +3067/+3238 STR9066/9067; +3587/+3753 STR9068/9069), *ACT1* (–999/–864 STR9128/9129; –252/+71 STR5274/5275; +409/+645 STR5457/5458; +1260/+1402 STR9130/9131; +2236/+2429 STR9132/9133), *ADH1* (–852/–660 STR9458/9459; –229/+71 STR5276/5277; +382/+549 STR9460/9461; +830/+1035 STR9462/9463; +2761/+2904 STR9464/9465), *STL1*(I), –372/–112; *STL1*(II), +402/+630; *STL1*(III), +1000/+1280; *STL1*(IV), +2060/+2334; and *TEL1* (region 490 bp right arm of chromosome VI). *LexA* Ops (–234/0; pSH18-34), *lacZ* ORF (+1950/+2250), and *ADH1* ORF (+720/+950). An internal fragment of the *POL1* gene (nucleotides 2499–2717) was used as a negative control. In Figure S4, the oligonucleotides used for amplifying *STL1* promoter included a forward primer in *STL1* (–130) and a reverse primer from *lacZ* (+84). Experiments were performed on three independent chromatin preparations, and quantitative PCR analysis was performed in real time by using an Applied Biosystems 7700 sequence detector. Immunoprecipitation efficiencies were calculated in triplicate by dividing the amount of PCR product in the immunoprecipitated sample by the amount of PCR product in the input sample. Data in Figures 2B, 3A, and 3B are presented as fold immunoprecipitation over the *POL1* coding sequence control.

Genome-Wide Location Analysis

Microarray preparation, amplification of total and immunoprecipitated DNA, fluorescent labeling, and microarray hybridization were performed as described previously (Moqtaderi and Struhl, 2004). Yeast strain MAP51 expressing a functional 3HA-epitope-tagged Hog1 was grown to exponential growth phase and briefly (5 min) treated with 0.4 M NaCl. Three independent chromatin samples were immunoprecipitated and the resulting DNA fragments amplified and labeled with Cy5 fluorescent dye (Amersham Biosciences).

Three independent total chromatin samples were similarly amplified and labeled with Cy3 fluorescent dye. Microarrays containing essentially all intergenic regions of the *Saccharomyces cerevisiae* genome spotted in duplicate were hybridized with a mixture of labeled fragments from immunoprecipitated and total chromatin samples. Slides were scanned on an Axon scanner, and data were analyzed with Axon GenePix 4.0 software. Raw data were then further analyzed in Microsoft Excel. Poor-quality or undetectable DNA spots were removed and the data normalized for equal background-subtracted median fluorescence of Cy3 and Cy5 over the entire arrays. Spots were ranked in descending order by their median Cy5: Cy3 ratios. Feature names were obtained from the *Saccharomyces* Genome Database (SGD). Positive interactions were defined by a median Cy5: Cy3 ratio of >1.5 in at least three of the six datasets. An enrichment of tRNA containing intergenic regions and centromeric regions was observed. Individual testing of two centromeres and six different tRNA genes by targeted ChIP revealed that those DNA sequences were slightly enriched independently of the presence of the HA antigen and therefore were identified as false positives. Centromeric regions and tRNA containing sequences were therefore removed from further analysis. DNA regions were then individually screened for the presence of an osmotic stress inducible ORF either upstream or downstream of the respective intergenic sequence.

GST Pull-Down Experiments

pGEX-4T Hog1 was expressed in *E. coli* DH5 and purified by using glutathione Sepharose 4B beads (Pharmacia) in buffer B as described (Bilsland-Marchesan et al., 2000). In vivo interaction between Hog1 and Rpb1 was determined by GST pull-down experiments. When necessary, cells were either subjected to stress (0.4 M NaCl, 5 min) or untreated. Three milligrams of yeast extracts in buffer A were treated when necessary with 1 μ l of alkaline phosphatase (20 U/ μ l; Roche) for 1 hr at 37°C and incubated with glutathione Sepharose 4B beads for 4 hr and then washed extensively. For in vitro binding assays, Rpb3-TAP protein was expressed and purified from yeast crude extracts in buffer A (50 mM Tris-HCl [pH 7.5], 15 mM EDTA, 15 mM EGTA, 0.1% Triton X-100, 150 mM NaCl, and 2 mM DTT plus antiproteases) by using rabbit IgG-Agarose beads (Sigma). Rpb3-TAP bound to beads was incubated with 1 μ g of GST or GST-Hog1 purified from *E. coli* overnight and then washed extensively in columns. When necessary, purified Rpb3-TAP was treated for 2 hr at 37°C with 1.5 μ l of alkaline phosphatase (20 U/ μ l; Roche). Interactions were analyzed with anti-GST antibody. The antibodies used to detect the different forms of Rpb1 were from Covance (8WG16 and H14). To analyze the association of Hog1 with components of the elongation complex, 1 μ g of yeast extract, from cells expressing specific TAP-tagged proteins, in buffer A (50 mM Tris-HCl [pH 7.5], 15 mM EDTA, 15 mM EGTA, 0.1% Triton X-100, 150 mM NaCl, and 2 mM DTT plus antiproteases and phosphatase inhibitors) was incubated with 50 μ l of glutathione Sepharose 4B beads overnight at 4°C. The beads were washed extensively with buffer A, resuspended in loading buffer, and resolved by SDS-PAGE. The antibody used to detect the TAP-tagged proteins was the PAP antibody from Sigma.

Photocrosslinking Assays

Photocrosslinking experiments were performed as described previously (Brown et al., 2001). Briefly, recombinant GST-Hog1, or GST, was incubated with ~30 molar equivalents of Sulfo-SBED (Pierce) for 60 min on ice. The excess of crosslinking reagent was removed from conjugated GST-Hog1 or GST by centrifugation in a Microcon column YM-3 (Millipore), 1.5 hr at 13000 \times g. Then, ~5 pmol of purified yeast TAP-Rpb9 complex (purification was performed as described in Rani et al. [2004]) was incubated with ~1 pmol of conjugated GST-Hog1 or GST, in IP buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, and 0.1% Tween-20). The mixtures were exposed to the UV light (λ 365 nm). Reactions were quenched with a final concentration of 2 mM N-ethylmaleimide (Sigma) in loading buffer without reducing agents. Reactions were separated on a 8% SDS-PAGE gel and transferred to PVDF. Membrane was probed with streptavidin-HRP, anti-GST, or anti-Rpb1 (8WG16).

Northern Blot Analysis

Yeast strains were grown to midlog phase in rich medium and then subjected to osmotic shock (0.4 M NaCl) for the indicated times. For

the expression analysis of genes under the LexA promoter, midlog phase cells were grown in minimal medium for plasmid selection at an optical density at 600 nm of 0.9–1.0. Total RNA and expression of specific genes were probed by using labeled PCR fragments containing the entire ORF of *STL1* (1.7 kbp), *CTT1* (1.7 kbp), *ADH1* (1.0 kbp), *lacZ* (3.2 kpb), and *ACT1* (1.4 kbp). Signals were quantified by using a Fujifilm BAS-5000 phosphorimager.

Supplemental Data

Supplemental Data include five figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/23/2/241/DC1/>.

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