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Genome-wide location analysis of the stress-activated MAP kinase Hog1 in yeast

Amparo Pascual-Ahuir [a], Kevin Struhl [b], Markus Proft [a,*]

[a] *Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica de Valencia, 46022 Valencia, Spain*
[b] *Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Boston, MA 02115, USA*

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Abstract

MAP kinase signal transduction pathways play a critical role in eukaryotic cells to unleash complex transcriptional programs to properly adapt to changing environments. The MAP kinase Hog1 upon activation is physically recruited to the chromatin of osmostress responsive genes. This allowed us to use *in vivo* chromatin immunoprecipitation in combination with microarrays (ChIP–Chip) to identify the transcriptional targets of Hog1 at the genomic scale. The ChIP–Chip method described here revealed that the stress-activated MAP kinase gets recruited to most of the osmoinducible genes. Interestingly Hog1 associates with both the 5′ upstream and the 3′ downstream sequences of stress genes. We confirmed by targeted ChIP at several stress genes that the MAP kinase crosslinks all over the transcribed regions in all cases tested. Taken together the genome-wide location analysis reported here is a powerful approach to determine the genomic binding patterns of an activated MAP kinase and will be of great interest to analyze other SAPKs under different environmental conditions.

Keywords: MAP kinase; Chromatin immunoprecipitation; ChIP–Chip; Hog1; Transcription; Elongation

1. Introduction

Mitogen-activated protein (MAP) kinases are key signaling molecules of eukaryotic cells to orchestrate adaptive responses to a wide variety of stress conditions. MAP kinase signaling pathways are evolutionarily conserved from yeast to humans [1]. One of the most important physiological functions of MAP kinases is the transcriptional activation of a large number of stress-regulated genes in the nucleus. The role of MAP kinases in stimulating gene expression goes far beyond the simple modulation of specific transcription factor activity. It has become clear, mainly from the work in the yeast model, that MAP kinases regulate transcription directly at the chromosome forming part of transcription complexes [2]. Their functions involve physical recruitment to the chromatin, phosphorylation of specific transcription factors, RNA polymerase II recruitment, chromatin modification, and transcript elongation. Whether MAP kinases of higher eukaryotes play similar roles as structural adaptors and essential subunits of transcriptional activator complexes remains to be confirmed experimentally. However, the stable interaction of mammalian MAP kinases with many specific transcription factors (reviewed in [2]) and the conservation of MAP kinase functions during evolution suggest that direct transcriptional activation in the chromatin context might be a general feature of MAP kinases.

Yeast cells have served as a powerful model to decipher the molecular mechanisms of MAP kinase cascade signaling and the functions of activated MAP kinases. One of the best studied signal transduction pathways in *Saccharomyces cerevisiae* is the high osmolarity glycerol (HOG) MAP kinase pathway [3]. Hyperosmotic stress leads to the rapid activation of its terminal MAP kinase Hog1 which is directly involved in an unexpectedly great number of

* Corresponding author. Fax: +34 96 3877859.
E-mail address: mproft@ibmcp.upv.es (M. Proft).

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adaptive processes. In a pre-transcriptional fast response Hog1 counteracts acute ion imbalances by directly phosphorylating the Na^+/H^+ antiporter Nha1 and the K^+ channel Tok1, thus enabling the cell to respond at the level of transcription [4]. Hog1 also inhibits cell cycle progression during stress by directly targeting the CDK inhibitor protein Sic1 [5] and may regulate translation efficiency by phosphorylating the Rck2 kinase [6,7]. Once activated, Hog1 is rapidly translocated to the nucleus [8] where it activates the complex transcriptional program upon hyperosmotic stress. Genomic expression profiling experiments revealed that Hog1 is necessary for the proper induction of the vast majority of >150 stress defense genes [9,10].

The mechanisms by which Hog1 stimulates transcription are surprisingly complex. The MAP kinase interacts with and phosphorylates at least three structurally unrelated specific transcription factors: Sko1, Hot1, and Smp1 [11–13]. These DNA binding molecules have been shown to be required for the targeted recruitment of Hog1 to the chromatin at some osmoresponsive promoters [11,14]. Once present at the chromatin structure of inducible genes, Hog1 can stimulate transcription by distinct mechanisms. The kinase modulates the transcription factor output by stimulating the entry of chromatin modifying complexes. This has been described for the histone acetyl transferase (HAT) complex SAGA or the chromatin remodeling complex SWI/SNF in the case of the Sko1 repressor/activator switch upon stress [14]. Hog1 recruits directly the histone deacetylase Rpd3 to osmostress regulated promoters [15]. Finally the MAP kinase seems to recruit the RNA polymerase II machinery directly to Hot1 regulated stress genes [16]. The events leading to stimulated transcription upon activation of Hog1 are depicted in Fig. 1. Taken together, the activated MAP kinase Hog1 forms an intimate complex of the chromatin at various stress regulated promoter regions.

Here, we describe a general methodology for analyzing the transcriptional targets of Hog1 that can be applied to other MAP kinase pathways. We determined the genome-wide binding pattern of Hog1 upon hyperosmotic stress by the combination of chromatin immunoprecipitation and microarray hybridization (ChIP–Chip). The ChIP–Chip method is a powerful approach to identify the physical target sequences on a genomic scale for DNA-binding proteins and proteins that get recruited indirectly to chromatin. We used formaldehyde crosslink, chromatin immunoprecipitation, and hybridization of the co-precipitated DNA fragments to intergenic regions microarrays to identify the sequences bound by Hog1. The method reported here confirms the targeted recruitment of a MAP kinase to stress responsive promoters and reveals new findings about Hog1. We show that Hog1 recruitment strongly correlates with osmoinducibility of the gene. Furthermore we found that in many cases Hog1 is present at the 3′ downstream region of a stress regulated gene. Targeted ChIP analysis of some osmoresponsive genes confirmed Hog1 occupancy at the whole transcribed region and therefore a role of the MAP kinase in the process of transcript elongation [17].

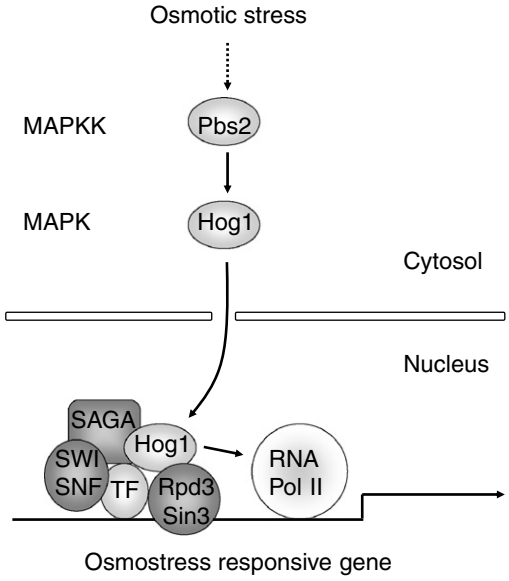

Fig. 1. Overview of the mechanisms of transcriptional activation by the stress-activated Hog1 MAP kinase. Hog1 is rapidly activated by phosphorylation in response to hyperosmotic stress and concentrates in the nucleus. The MAP kinase is recruited to stress responsive promoters by specific transcription factors (TF) like Sko1 or Hog1. Note that signaling events upstream of the MAP kinase kinase Pbs2 are not included. Hog1 recruits directly the histone deacetylase Rpd3/Sin3 to stress responsive promoters. Additionally Hog1 stimulates the entry of the chromatin remodeling complex SWI/SNF and the histone acetyl transferase complex SAGA. Finally, Hog1 is able to directly recruit RNA polymerase II holoenzyme to activate transcription in response to osmotic stress.

Therefore the ChIP–Chip method described here successfully identified the genes directly up-regulated by a stimulated MAP kinase at the genomic scale and gave invaluable insights into the mechanisms of transcriptional activation.

2. Method

Here we describe the experimental steps to analyze the *in vivo* association of the Hog1 MAP kinase with promoter sequences in the whole yeast genome by ChIP–Chip. They essentially include: (1) formaldehyde-induced protein–DNA and protein–protein crosslinking of osmotically stressed yeast cultures; (2) ChIP: preparation of sheared chromatin, immunoprecipitation with specific antibodies, reversal of the crosslinks, protease treatment, and purification of co-precipitated genomic DNA fragments; (3) amplification by linker ligation mediated PCR and fluorescent labelling of DNA; (4) hybridization to intergenic regions microarrays; and (5) data analysis. An overview of the experimental steps of the ChIP–Chip method is given in Fig. 2.

2.1. Crosslinking and chromatin immunoprecipitation (ChIP) of Hog1

The ChIP procedure is based on the method described by [18] with the following specifications. A yeast strain

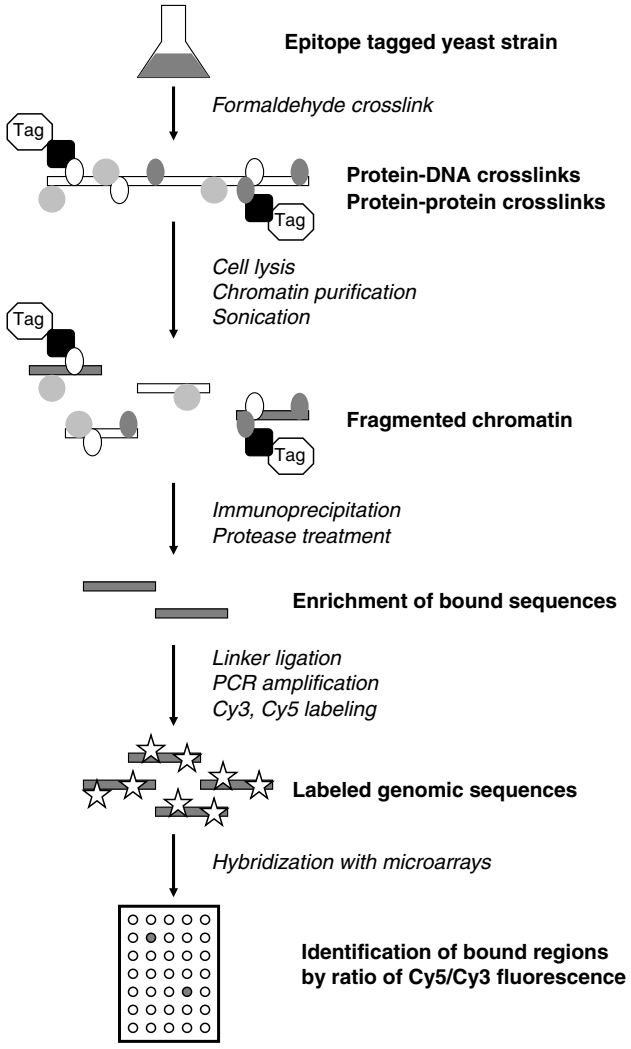

Fig. 2. Overview of the experimental steps of the ChIP–Chip method.

expressing a fully functional HA epitope tagged Hog1 from the chromosomal locus was used for ChIP. Chromatin association of stress-activated Hog1 MAP kinase is very transient and peaks around 5 min after exposure of yeast cells to 0.4 M NaCl as measured by ChIP at the *GRE2* and *STL1* promoters. Therefore it is critical to crosslink and harvest the cells during the first minutes of adaptation to osmotic stress.

Yeast cells (MAP51 = W303-1A with *3xHA-HOG1*) were grown to $A_{600} = 0.8$ in 100 ml YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Cells were treated for 5 min with 0.4 M NaCl (from a 5 M stock). Formaldehyde was added to the culture to a final concentration of 1% (from a 37% stock, Merck, p.a. quality). Crosslinking reactions were carried out for 20 min at room temperature with occasional swirling and stopped by the addition of 15 ml 3 M glycine (prepared freshly) to each 100 ml of original culture. Cells were incubated for another 5 min at room temperature. Cells were centrifuged in 50 ml Falcon tubes in a tabletop centrifuge (3000 rpm, 3 min) and then washed twice with 40 ml ice-cold TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) and once with 10 ml FA-lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF [added freshly from a 100 mM stock in isopropanol]). Crosslinked cells were resuspended in 1 ml ice-cold FA-lysis buffer supplemented with 0.5% SDS and transferred to a 2 ml screw cap vial (Biospec Products). Approximately 1 ml of Zirconia/Silica beads (Biospec Products) were added and cells were disrupted for 10 min (5 rounds of 2 min each with 2 min on ice in between) at maximal speed at 4 °C in a Mini Bead Beater 8 (Biospec Products). The lysate was collected into a fresh 1.5 ml Eppendorf tube and centrifuged for 1 min at 4 °C in a minicentrifuge at 13,000 rpm. High molecular chromatin fragments are insoluble at this point. The supernatant was discarded and the pellet solubilized in 1 ml ice-cold FA-lysis buffer. After centrifugation (same settings) the chromatin containing pellet was resuspended in 1 ml cold FA-lysis buffer. The samples were then sonicated three times for 30 s at 4 °C (Branson S450A, medium sonicator settings: output 50%, needle 5). Sonication should result in the fragmentation of the chromatin into soluble pieces of an average size of 400 bp. Samples were finally centrifuged for 10 min at 4 °C in a minicentrifuge at 13,000 rpm. Soluble total chromatin preparations (supernatant) were transferred to clean 1.5 ml Eppendorf tubes and stored at −80 °C for up to several months.

To immunoprecipitate HA-Hog1 crosslinked chromatin fragments 500 μl of total chromatin samples were incubated with 5 μl of anti-HA antiserum (mouse monoclonal 12CA5) for 30 min on a roller at room temperature. In the meantime Protein A–Sepharose CL-4B (Pharmacia) beads were washed twice with FA-lysis buffer. Beads were finally prepared as a 50% slurry in FA-lysis buffer. Twenty microliters of Protein A–Sepharose beads were added to each sample and the mixture incubated for another 60 min on a roller. The beads were pelleted for 30 s at 2000 rpm, the supernatant was discarded and the beads washed for 5 min each time on a roller with 1 ml of the following buffers: 2× with FA-lysis buffer, 2× with FA-lysis buffer supplemented with 0.5 M NaCl, 1× with buffer B (10 mM Tris–HCl, pH 8, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), 1× with TE (10 mM Tris–HCl, pH 8, 1 mM EDTA). To elute the chromatin fragments from the beads the samples were resuspended in 250 μl of buffer C (50 mM Tris–HCl pH 7.5, 10 mM EDTA, 1% SDS) and incubated at 65 °C with occasional mixing for 10 min. The samples were then centrifuged (2000 rpm, 30 s in a minicentrifuge) and the eluted material (supernatant) transferred into a fresh 1.5 ml Eppendorf tube. After addition of 250 μl of TE buffer and 20 μl Pronase solution (Roche, 20 mg/ml) the samples were incubated over night at 65 °C. At this point the formaldehyde induced crosslinks are reversed. Equally 50 μl of total input chromatin samples (before immunoprecipitation) was decrosslinked.

Finally genomic DNA fragments were purified by addition of 50 μl of 4 M LiCl and extraction with 500 μl phenol/

chloroform/isoamylalcohol (25:24:1). Samples were once more extracted with 500 μl chloroform/isoamylalcohol (24:1). DNA was precipitated after addition of 1 ml ethanol and 20 μg glycogen (Roche) as a carrier over night at −20 °C. DNA fragments were pelleted in a minicentrifuge at 13,000 rpm for 10 min, dried, resuspended in 100 μl TE buffer and stored at −20 °C.

2.2. PCR amplification and labeling of co-purified and total DNA fragments

The purified genomic DNA fragments (coprecipitated with Hog1 in the IP samples and random in the total samples before immunoprecipitation) have to be amplified before labeling and hybridization to the microarrays. We used the following linker ligation mediated PCR procedure. 40 μl of IP'd DNA (undiluted, rest of the samples should be stored at −20 °C for sequence specific ChIP analysis) or total DNA (1:8 diluted in H_2O) was treated with T4 DNA polymerase for 30 min at 12 °C in a PCR machine without the heated lid option. Reactions contained: 40 μl DNA, 11 μl T4 DNA polymerase buffer (New England Biolabs), 0.5 μl BSA (10 mg/ml; New England Biolabs), 0.5 μl dNTP mix (20 mM each), 0.5 μl T4 DNA polymerase (New England Biolabs), and 57.8 μl ddH_2O. DNA samples were then cleaned up using the Qiagen PCR purification kit (#28004) and eluted in 30 μl of ddH_2O.

Remaining single stranded extensions in the DNA fragments were removed by the following nuclease treatment: 30 μl of DNA solution was mixed with 4 μl of 10×Mung Bean Nuclease buffer (New England Biolabs), 4 μl of ddH_2O, and 2 μl of Mung Bean Nuclease (10 units/μl; New England Biolabs). Reactions were carried out for 60 min at 37 °C. Products were again cleaned up with the Qiagen PCR purification kit (#28004) and eluted in 25 μl of ddH_2O.

In the next step the blunt ended DNA fragments were ligated to unidirectional linkers to allow for the subsequent PCR amplification. Linkers were prepared as follows: 375 μl of 40 μM oligonucleotide 1 (GCGGTGACCCGG GAGATCTGAATTC) and 375 μl of 40 μM oligonucleotide 2 (GAATTCAGATC) were mixed and 250 μl of 1 M Tris–HCl, pH 7.9 was added. Samples were heated in 100 μl aliquots for 5 min at 95 °C and then transferred to a 70 °C heating block which was allowed to cool down slowly to 25 °C. Double stranded linkers were then incubated at 4 °C over night and finally stored at −20 °C. Linker ligation reactions contained: 25 μl DNA solution (see above), 13 μl ddH_2O, 5 μl 10×ligase buffer, 6.7 μl annealed linkers (15 μM, see above), 0.5 μl T4 DNA ligase (New England Biolabs). Ligation reactions were carried out at 16 °C over night. Samples were cleaned up using the Qiagen PCR purification kit (#28004) and eluted in 25 μl of ddH_2O.

Linker-mediated PCR were performed as follows: 4 μl of 10×Taq polymerase buffer (New England Biolabs), 4.75 μl ddH_2O, 5 μl dNTP mix with dUTP (Clontech hybridization solution kit), 1.25 μl of 40 μM oligonucleotide 1 were added to the DNA solution (25 μl from above). The following steps were set up in the PCR program: 2 min 55 °C, 5 min 72 °C, 2 min 94 °C, 35 cycles of 30 s 94 °C–30 s 54 °C– 1 min 72 °C, 4 min 72 °C, hold at 4 °C. During the first step 10 μl of polymerase mix (8 μl ddH_2O, 1 μl 10×Taq reaction buffer (New England Biolabs), 1 μl Taq polymerase [5 units/μl; Perkin Elmer, regular Taq polymerase], 0.1 μl Pfu Turbo [2.5 units/μl; Stratagene]) was added to each tube. The successful amplification of the genomic DNA fragments was checked by running 5 μl of each reaction on a 1.5% agarose gel. A smear at fragment sizes from 200 to 600 bp should be clearly visible.

Amplified DNA samples were precipitated by adding 5.5 μl of 3 M NaOAc (pH 5.2) and 150 μl of ice-cold ethanol and incubating at −20 °C for 60 min. DNA was pelleted by centrifugation at 13,000 rpm in a minicentrifuge at 4 °C for 20 min. The pellet was washed with 70% ethanol, air dried, and resuspended in 10 μl fluorescent labeling buffer (Clontech). Fluorescent dyes Cy3 and Cy5 (Clontech fluorescent labeling kit) were dissolved in 45 μl DMSO. Dye stocks can be kept at −20 °C for several months in the dark. Ten microliter of the dissolved dye (Cy5 for IP samples, Cy3 for total samples) was added to the DNA solutions and incubated at room temperature in the dark for 60 min with occasional mixing. Finally 2 μl of 3 M NaOAc (pH 5.2) and 50 μl of ethanol were added and the DNA precipitated for 2 h at −20 °C. DNA was pelleted by centrifugation at 13,000 rpm in a minicentrifuge at 4 °C for 20 min, washed with 70% ethanol, air dried, and resuspended in 100 μl of ddH_2O. The labeled probes were purified using NucleoSpin extraction columns (Clontech) according to the instructions of the manufacturer. Probes were eluted twice with 50 μl NE buffer. Labeled and purified probes can be stored in the dark for several months at −20 °C.

2.3. Hybridization with DNA microarrays

Prior to the hybridization to microarray slides the corresponding labeled probes (IP sample Cy5 labeled + total sample Cy3 labeled) were pooled and the volume reduced to 20 μl using Microcon 30 filter columns (Millipore). 2 μl of salmon sperm DNA (10 mg/ml) was added. Hybridization to the microarray slides was performed over night at 45 °C essentially as described by [19]. We used microarrays with a total of >6500 yeast intergenic sequences spotted in duplicate onto polylysine glass slides [20].

To determine the Hog1 bound genomic sequences we hybridized three microarray slides with three independent Cy5/Cy3 labeled IP/total probes. The slides were washed as described by [19] and immediately scanned on an Axon scanner. Data were analyzed by using the Axon GenePix 4.0 software and transferred to Microsoft Excel. From further analysis all spots with poor quality (flagged by GenePix) or no detectable fluorescence signals were excluded. The median fluorescence for Cy5 and Cy3 was background subtracted and set to the same overall intensities for both channels for each slide. The median ratio of Cy5/Cy3 was

calculated for each intergenic region and spots were ranked in descending order.

2.4. Statistical analysis of the ChIP–Chip data

By the use of 3 microarray slides spotted in duplicate hybridized to three independently generated probes we generated 6 data points for each Hog1-intergenic region (IR) interaction. As expected for a secondarily recruited signaling molecule the binding values of Hog1 are low. Best scores (median Cy5/Cy3 ratios of all datapoints) for positive interactions were not greater than 3. Therefore we defined a true Hog1-IR association by a median Cy5/Cy3 ratio of >1.5 in at least 3 of the 6 datasets. This procedure identified a total of 72 intergenic sequences.

Hog1 as the terminal MAP kinase of the osmotic stress-activated HOG pathway is responsible for most of the transcriptional activation during hyperosmolarity. Therefore we compared our genomewide location data for Hog1 with two expression profiles of the yeast genome after exposure to NaCl stress [9,10]. 87% (63 of the 72 top ranking interactions) of the identified intergenic regions corresponded to genes that are >3-fold upregulated upon hyperosmotic stress, as identified by transcriptional profiling experiments. Expression of many (35) of these stress genes was activated greater than 10 fold. Interestingly, the Hog1-interacting regions of osmoresponsive genes corresponded either to the promoter (35 cases), or the 3′ downstream region (28 cases), or both (10 cases).

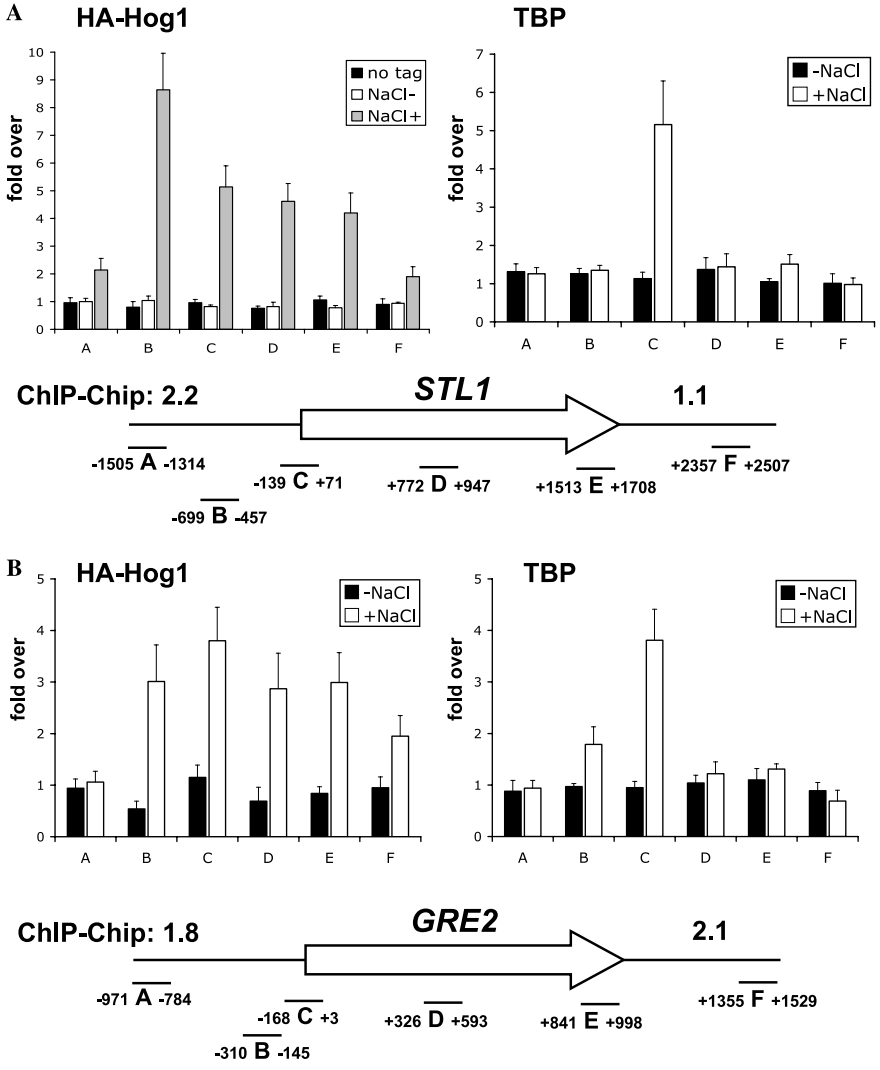

Fig. 3. *In vivo* recruitment of Hog1 and TBP to various stress-induced loci: (A) *STL1*, (B) *GRE2*, (C) *FAA1*, (D) *GPD1*. Cells were stressed or not with 0.4 M NaCl for 5 min. HA-Hog1 and TBP were immunoprecipitated with anti-HA or anti-TBP antibodies, respectively. Standard ChIP experiments are represented as the fold enrichment of the indicated DNA regions over an unbound control region (internal primers to the *POL1* ORF). Data are obtained by the analysis of three independent chromatin preparations. In the case of *STL1* an additional no-tag control is included (parental wild type strain exposed to salt stress). Exact locations of the primer pairs used in the quantitative PCR are depicted below each ChIP experiment. Binding values obtained by ChIP–Chip are given for the 5′ upstream and 3′ downstream region of each gene. They represent the mean Cy5/Cy3 ratio from 3 independent microarray hybridizations. Note that the *STL1* 3′ downstream region was not positively identified by ChIP–Chip. For *GPD1*, no ChIP–Chip data were generated for the promoter region (n.d.).

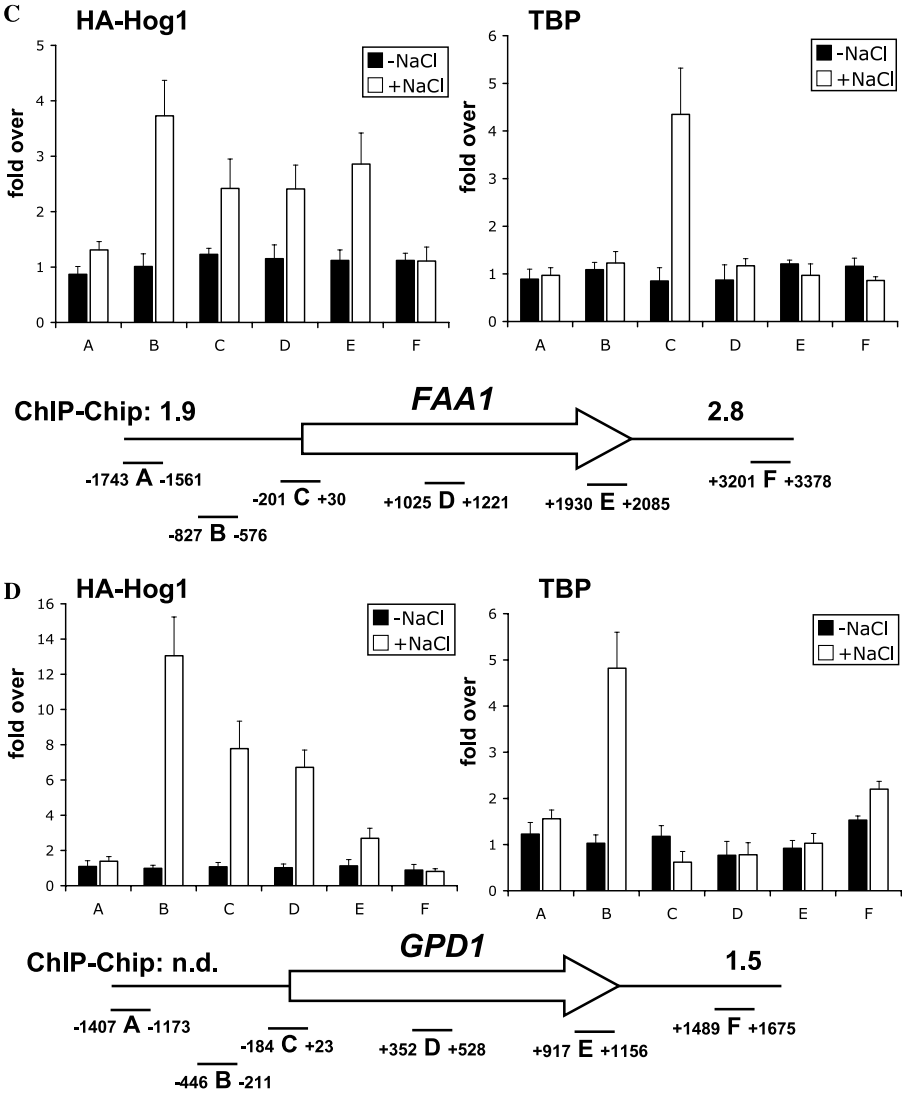

Fig. 3 (*continued*)

An alternative method to statistically analyze ChIP–Chip data is the Chipper algorithm [21]. This approach has the advantage that it does not require additional control experiments and attributes probability values (*p*-values) to each protein-IR interaction based on the inherent asymmetrical distribution of the ChIP–Chip data.

2.5. Verification of the microarray data by targeted ChIP experiments

We selected several stress-induced genes that were identified as Hog1 targets by the above described ChIP–Chip method to assess by standard ChIP how meaningful the obtained genomic data are. Specifically we tested whether association of the activated MAP kinase occurred not only in the respective promoter regions but extended to the whole transcribed region. We analyzed Hog1-HA and TBP (TATA binding protein) association at the *GRE2*, *STL1*, *FAA1*, and *GPD1* loci by standard ChIP as described by [22]. As depicted in Fig. 3, we confirmed Hog1 recruitment at all four loci. Moreover, Hog1 crosslinks all over the whole ORF regions in all cases, while TBP binds exclusively at the TATA box regions of the respective promoters. Taken together we confirm a possible role for Hog1 as a selective elongation factor during osmostress as was suggested by our genomic location analysis [17].

3. Conclusions

The methodology described here illustrates how the ChIP analysis can be used to identify the genome wide location of a stress-activated MAP kinase. We successfully applied this approach to the stress-activated MAP kinase Hog1. The genomic ChIP–Chip data identify the genes which are directly targeted and activated by the MAP kinase. Moreover, the Hog1 occupancy all over the transcribed regions of stress genes characterizes the MAP kinase as a transcription elongation factor. The technology presented here will

therefore be of great interest to describe the impact of other MAP kinases on transcription at the genomic scale.

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