Molecular and Cellular Biology

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Mol. Cell. Biol. 2013, 33(11):2228. DOI:
10.1128/MCB.00240-13.
Published Ahead of Print 25 March 2013.

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Deciphering Dynamic Dose Responses of Natural Promoters and Single *cis* Elements upon Osmotic and Oxidative Stress in Yeast

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Fine-tuned activation of gene expression in response to stress is the result of dynamic interactions of transcription factors with specific promoter binding sites. In the study described here we used a time-resolved luciferase reporter assay in living *Saccharomyces cerevisiae* yeast cells to gain insights into how osmotic and oxidative stress signals modulate gene expression in a dose-sensitive manner. Specifically, the dose-response behavior of four different natural promoters (*GRE2*, *CTT1*, *SOD2*, and *CCP1*) reveals differences in their sensitivity and dynamics in response to different salt and oxidative stimuli. Characteristic dose-response profiles were also obtained for artificial promoters driven by only one type of stress-regulated consensus element, such as the cyclic AMP-responsive element, stress response element, or AP-1 site. Oxidative and osmotic stress signals activate these elements separately and with different sensitivities through different signaling molecules. Combination of stress-activated *cis* elements does not, in general, enhance the absolute expression levels; however, specific combinations can increase the inducibility of the promoter in response to different stress doses. Finally, we show that the stress tolerance of the cell critically modulates the dynamics of its transcriptional response in the case of oxidative stress.

Environmental stress causes rapid changes in the gene expression program of a cell in order for it to adapt and survive. The yeast *Saccharomyces cerevisiae* has served as a powerful model to describe transcriptional adaptation both at the genomic scale and at the gene-specific scale. Upon most stresses, activated expression of defense genes occurs rapidly and transiently. Genomic profiling has revealed that hundreds of genes are responsive to osmotic and oxidative stress in yeast (1–5). Some of the upregulated gene functions, the so-called general stress response genes, are not specific for the particular stress, while other defense genes are exclusively activated by a specific stress (1, 6). The general stress response is executed by two homologous zinc finger activators, Msn2 and Msn4, which bind to a conserved stress response element (STRE), 5′-CCCCT-3′, located in the promoters of the responsive genes (7, 8). Under normal growth conditions, both activators are largely sequestered in the cytosol with the help of the TOR signaling pathway (9). Upon stress, Msn2 and Msn4 are hyperphosphorylated and relocalize to the nucleus in a process controlled by the cyclic AMP (cAMP)-dependent protein kinase A (10).

Osmotic stress is specifically sensed by the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase signaling pathway (11). Once activated by phosphorylation and transiently translocated to the nucleus, its terminal MAP kinase, Hog1, executes the transcriptional program specific for osmostress adaptation (12, 13). Hog1 activates transcription via several specific transcription factors (TFs; Sko1, Hot1, Smp1, Msn2, and Rtg3) which are directly contacted and phosphorylated by the kinase (14–20). The detailed study of Hot1- and Sko1-modulated transcription revealed that Hog1 plays multiple functions in the stimulation of gene expression. The MAP kinase can directly recruit RNA polymerase II in the case of Hot1-regulated genes (14). Furthermore, Hog1 is directly or indirectly involved in the recruitment of general coactivator complexes, such as the Rpd3 histone deacetylase, the SAGA coactivator, the SWI/SNF chromatin remodeling complex, and Mediator (21–23). The differential recruitment of transcriptional coactivator complexes might provide a mechanism to shape gene expression in a gene- and/or stress-dependent manner. Indeed, it was reported that upon severe osmotic shock the SAGA function is more important than the Mediator function (23). The Sko1 repressor/activator, together with Hot1, is one of the principle transcription factors involved in the yeast osmostress response (24, 25). The Sko1 basic leucine zipper protein recognizes cAMP-responsive element (CRE) sequences [5′-T(G/T)ACGT(A/C)A-3′] in stress-regulated promoters (26, 27). Binding of Sko1 to CRE sites under normal growth conditions does not confer activation because of the presence of the Ssn6-Tup1 corepressor complex (17, 28). The Sko1 activator function is rapidly unmasked upon osmostress by recruitment of activated Hog1, which leads to subsequent stimulation of gene expression (22, 28).

Oxidative stress also triggers a major transcriptional response in yeast. Two main transcriptional activators, Yap1 and Skn7, have been implicated in this adaptive response (29–31). Although many oxidative stress-responsive defense genes are regulated by both factors, it seems that Yap1 and Skn7 respond to different oxidative stimuli (32, 33). The Yap1 basic leucine zipper protein preferentially binds to AP-1 sequences (5′-TTACTAA-3′) in promoters of antioxidant genes (34). Yap1 transcriptional activity is modulated by its regulated import into the nucleus upon oxidative stress (35, 36). Hydrogen peroxide stress provokes specific intramolecular disulfide bonds in the Yap1 protein, which allow its nuclear accumulation and transcriptional activation (35, 37, 38).

Osmotic and oxidative stresses are physiologically connected. Osmotic stress causes overproduction of reactive oxygen species

Received 1 March 2013 Returned for modification 12 March 2013
Accepted 18 March 2013
Published ahead of print 25 March 2013
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doi:10.1128/MCB.00240-13

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(ROS), many antioxidant functions are highly inducible upon osmotic stress, and external addition of antioxidants rescues the sensitivity of yeast cells to osmotic stress (3, 4, 39). Additionally, an induction of the osmostress-sensing HOG pathway by some oxidative treatments has been reported (40). Therefore, it is important to understand to what level signaling pathways are shared upon both types of stresses.

The application of global location analysis has demonstrated that upon osmostress the transcriptional program is executed by the dynamic and complex binding of many transcription factors to their target promoters in the genome (2, 24). Considerable redundancy has been revealed; thus, in many cases of stress-activated defense genes, the binding of more than one transcriptional activator can be observed. Also, it seems that the transcription factors responding to osmostress regulate each other's expression in a hierarchical manner, which could be an additional way to create dynamic responses at different sets of target genes (2). Therefore, it is important to understand how fine-tuned gene expression is created in response to different stress stimuli, which makes it necessary to quantitatively compare the sensitivities of differentially regulated genes. This can be achieved by monitoring over an exhaustive range of stress conditions the gene expression output, which describes the dose-response behavior of a regulatory system (41). Here we apply such methods to different stress-regulated elements and investigate how gene expression is activated upon both osmotic and oxidative stimuli upon increasing stress doses in a time-resolved fashion in living yeast. We record the dose-response behavior of complete promoters and isolated upstream activating sequences. By covering the whole range of activating stress doses, we can define and compare the sensitivity, inducibility, and maximal activity for different regulatory elements. We show that natural stress-responsive promoters have different sensitivities to osmotic and oxidative stress, which are recapitulated by CRE-, STRE-, and AP-1-modulated gene expression.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study were wild-type strain BY4741 (*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and strains with the mutant alleles *sko1*::*KanMX4*, *hog1*::*KanMX4*, *ctt1*::*KanMX4*, *sod2*::*KanMX4*, *skn7*::*KanMX4*, and *yap1*::*KanMX4* (42, 43). A mutant with the double deletion *msn2*::*NAT msn4*::*KAN* in the BY4741 strain background was a gift from E. de Nadal (Barcelona, Spain). Yeast strains containing the indicated luciferase fusion genes were grown at 28°C in synthetic dextrose (SD) medium lacking histidine (0.67% yeast nitrogen base, 2% glucose, 50 mM succinic acid, pH 5.5, 0.1 g/liter leucine, 0.1 g/liter methionine, 0.025 g/liter uracil). For the luciferase assays, the cells were grown overnight to exponential growth phase and then preincubated with luciferin, as indicated below.

Plasmid constructions. Single-copy reporter fusions with destabilized luciferase (lucCP^{+}) were constructed as described in reference 41. The upstream regulatory sequences of *GRE2* (nucleotides −940 to −7), *CTT1* (nucleotides −983 to −10), *SOD2* (nucleotides −977 to −16), and *CCP1* (nucleotides −976 to −5) were amplified by PCR and inserted (SacI/SmaI) into the lucCP^{+} expression vector p413-lucCP+ (41). For the assay of specific *cis*-regulatory elements, synthetic oligonucleotides containing three repetitions of STRE, CRE, or AP-1 sequences spaced by 8 to 9 nucleotides were inserted into the BspEI site of plasmid p413CYC1Δ-lucCP^{+} (41). The sequences used were the following (the consensus sequence for each TF binding site is underlined): for STRE, 5′-CCGGCGATATCAGCCCCTGGAAAAAGCCCCTGCGCAAAGCCCCT-3′; for CRE, 5′-CCGGCGATATCATTACGTAATAGAATACATTACGTAATCGCGATCATTACGTAAT-3′; and for the AP-1 element, 5′-CCGGCATCGATCTTACTAAGCGCGAAATTAGTAACCGGCTAATTACTAAGT-3′. The correct single insertion of each element was confirmed by DNA sequencing. Combinations of regulatory elements were created by additional insertion of the oligonucleotides into the single restored BspEI site after the initial insertion.

Real-time measurement of luciferase activity. Aliquots of exponentially growing yeast cells expressing the destabilized luciferase gene under the control of the indicated natural and synthetic promoters were incubated at 28°C for 90 min with 0.5 mM luciferin (Sigma). The cultures were then transferred in 100-μl aliquots in white 96-well plates (Nunc). NaCl, hydrogen peroxide, or menadione was added from appropriate stock solutions. The light emission was then recorded continuously in a GloMax microplate luminometer (Promega) in three biological replicates. Data were processed in Microsoft Excel software. For representations of the light units produced by each reporter fusion during stress induction, the raw data were normalized for the number of cells in each assay. For the determination of the half-maximal (50%) effective concentration (EC_{50}), the maximal luciferase activity for each stress dose (A_{max}) was plotted against the stimulus concentration on a logarithmic scale. The curves were fitted in the linear range using Microsoft Excel, and then the stressor concentration which caused the half-maximal luciferase activity for each type of stress was calculated.

Quantitative analysis of yeast growth. For sensitivity assays in continuous growth, fresh overnight precultures of the indicated strains in SD medium were diluted in triplicate in multiwell plates to the same initial optical density. Growth was then continuously monitored in a Bioscreen C system (Thermo) for 72 h in SD medium without or with 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, or 2 mM hydrogen peroxide. The growth curves were processed in Microsoft Excel, and the half-maximal cell density was calculated for each strain. The time to reach half-maximal cell density (t_{50}) under each stress condition was compared to the t_{50} under nonstress conditions. This ratio was taken as an indicator of the relative growth efficiency.

RESULTS

The dose-response behavior of natural promoters upon osmotic and oxidative stress. Osmotic and oxidative stresses are harmful conditions for yeast cells and trigger transcriptional programs which include the activation of hundreds of defense genes. We compared the activation patterns of several stress-responsive promoters in yeast in a time-resolved fashion with the help of a destabilized luciferase assay (41). This assay makes use of a very short-lived version of luciferase which is rapidly degraded with the help of specific protein and mRNA degradation motifs. As the assay can be performed in small aliquots of living yeast cultures, the method is suitable to quantitate transient gene expression simultaneously under many different environmental conditions and continuously in real time. We aimed at defining common and distinct features of the dynamic and dose-dependent regulation of different yeast promoters. We chose to study the upstream control regions of the *GRE2*, *CTT1*, *SOD2*, and *CCP1* genes, all of which show strong inducibility upon osmotic and/or oxidative stress (7, 44–46). We focused on three different stress treatments: oxidative stress caused by hydrogen peroxide or menadione and osmotic stress caused by NaCl.

In order to obtain the entire dose-response behavior for all four promoters, we applied wide ranges of stress doses to single-copy luciferase reporters in living yeast cultures. The results are depicted in Fig. 1. We found that hydrogen peroxide caused a concentration-dependent increase of gene expression over a concentration range from 25 μM to approximately 500 μM, as indicated by the different maximal luciferase activities caused by each

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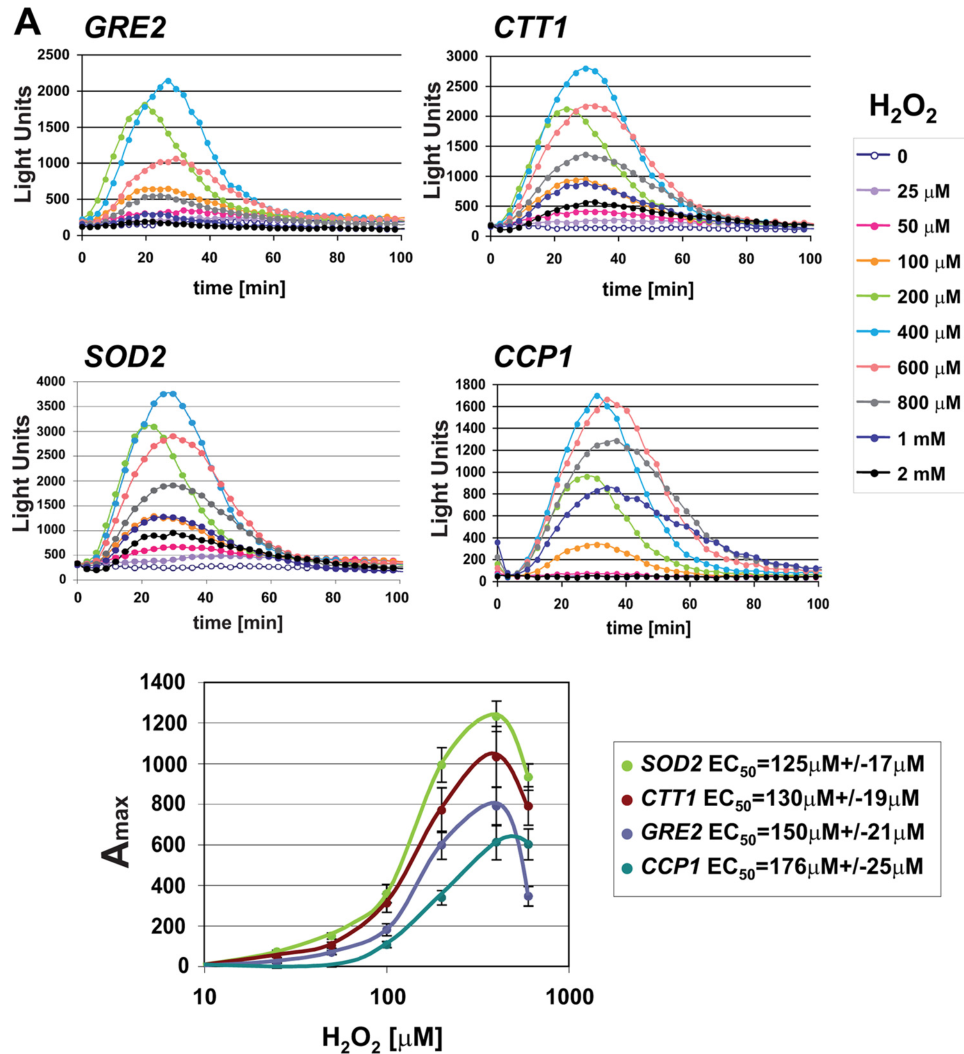

FIG 1 Comparison of the dose-response behavior of four yeast promoters upon oxidative and osmotic stress. The upstream control regions of the *GRE2*, *CTT1*, *SOD2*, and *CCP1* genes were analyzed by reporter fusions with destabilized luciferase in yeast wild-type strain BY4741. Liquid culture aliquots in glucose-containing minimal medium were preincubated with luciferin. At time zero, the indicated stress treatments were started and light emission was continuously recorded for the indicated times. The dose-response curves obtained for the various promoter-luciferase fusions are depicted in the upper panels for hydrogen peroxide (A), menadione (B), and NaCl (C) stress. The maximal steady-state activity was calculated for each promoter fusion for each stress dose, normalized for the cell number in the assay, and plotted against the stress dose (bottom). The dose-response curves were obtained for three independent culture aliquots and had an error of $<$15%. The EC_{50} was calculated for each stress type and promoter, as indicated in Materials and Methods.

dose within this range (Fig. 1A). This defines the dynamic range of H_2O_2 stress for all four promoter-luciferase fusions, for each of which it was similar but not identical. The *CTT1*- and *SOD2*-luciferase fusions appeared to respond to low hydrogen peroxide concentrations in a more sensitive manner than the *GRE2*- or *CCP1*-luciferase fusion. Accordingly, the H_2O_2 concentration required to stimulate gene expression to half-maximal activity (EC_{50}) is lower in the case of *CTT1* and *SOD2* than in the case of *GRE2* and *CCP1*.

We next applied menadione as an alternative inducer of intracellular oxidation and quantified the gene expression changes that it caused at the same promoters. A dose-sensitive response was observed at the four stress-regulated promoters at menadione concentrations ranging from 20 μM to approximately 120 μM (Fig. 1B). Menadione appeared to be a significantly less efficient inducer of gene expression than hydrogen peroxide at the four promoters studied here and caused only roughly one-third of the induction observed for H_2O_2. Similar to what we observed for hydrogen peroxide, the *CTT1*- and *SOD2*-driven luciferase reporters responded more efficiently to low menadione concentrations and, hence, were characterized by small EC_{50}s. Gene expression driven by the *GRE2* promoter was less sensitive to low menadione concentrations and needed higher drug concentrations to reach saturation. In the case of the *CCP1* gene, we observed a dose-response behavior for low menadione stimuli similar to that of *GRE2*, which was, however, more rapidly saturated at higher menadione concentrations, resulting in a low EC_{50}.

We finally quantified the dose responses of the four stress genes to osmotic stress provoked by NaCl (Fig. 1C). With the exception of the *CCP1*-luciferase fusion, which was not activated by any NaCl concentration, we observed dose-sensitive luciferase activities at salt concentrations ranging from 100 mM to approximately

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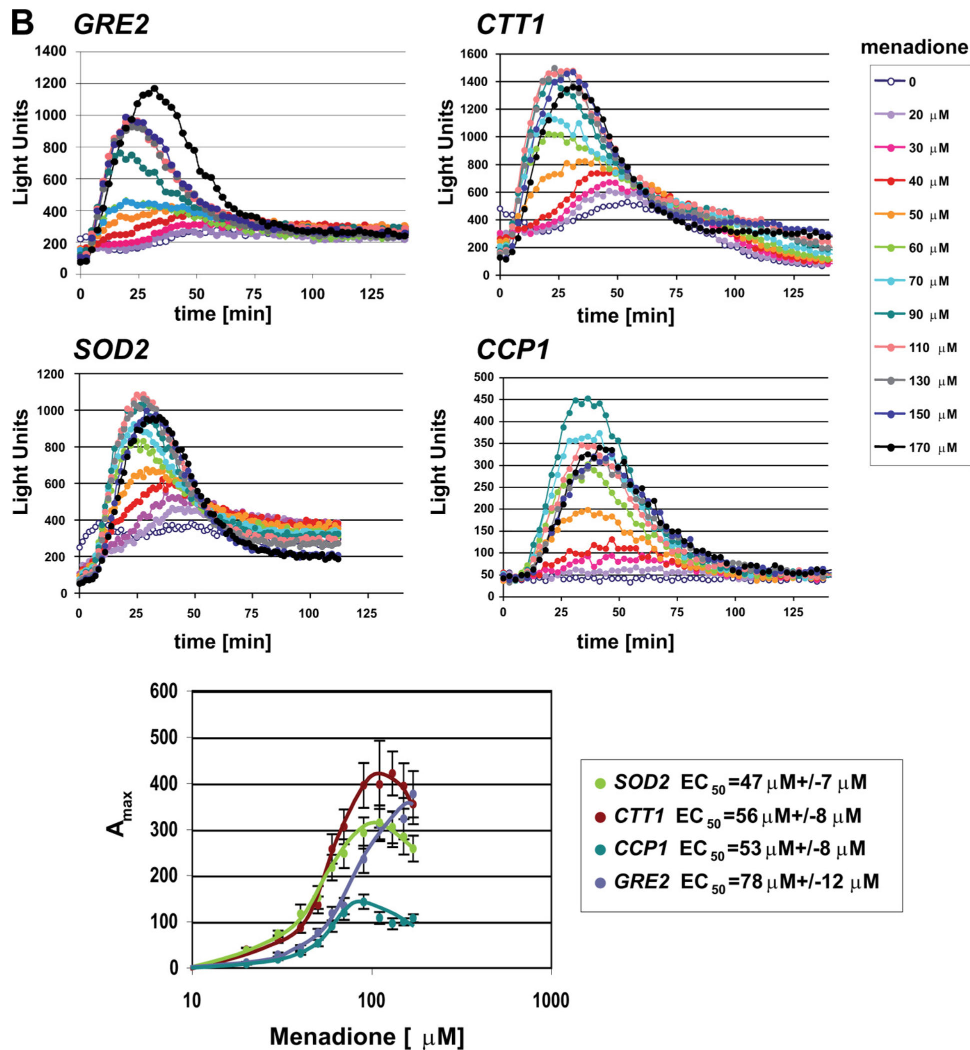

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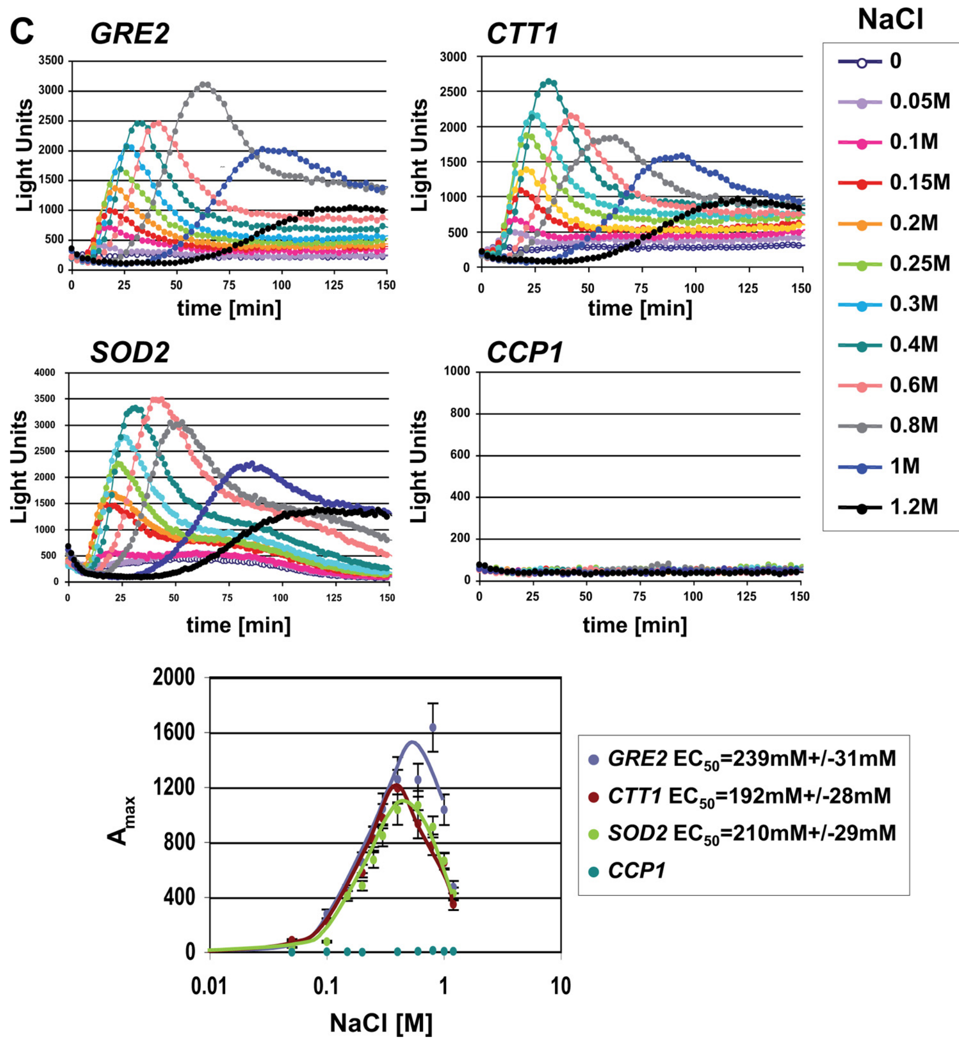
500 mM for *CTT1* and *SOD2* and up to 800 mM for *GRE2*. The sensitivity to low-NaCl stimuli was almost identical for the *GRE2*-, *CTT1*-, and *SOD2*-driven reporters; however, *GRE2* showed a prolonged dynamic range with higher salt concentrations. This was reflected by the apparently lower saturation concentrations of NaCl for *CTT1* and *SOD2* than *GRE2*.

Taken together, our real-time measurements reveal differences in the dose-sensitive modulation of gene expression conferred by different yeast promoters. *GRE2*, *CTT1*, and *SOD2* are activated by both oxidative and osmotic stresses; however, their sensitivities differ for different stress treatments. The *CTT1* and *SOD2* promoters seem to confer more sensitive responses to hydrogen peroxide or menadione stress, while the *GRE2* promoter confers a more dynamic dose response upon salt stress. These differences can be visualized when we compare the maximal activity for the different promoters upon each stress type (Fig. 2). *GRE2* is preferentially activated by salt stress over oxidative stress, while *CTT1* and *SOD2* are equally activated by both stimuli. *CCP1*, on the other hand, is specifically activated by oxidative stimuli over salt stress. We next wanted to know whether the different dose responses of natural promoters could be the result of the different sensitivities of single *cis* elements present in stress-responsive control regions.

Analysis of the dose response of single *cis* elements (CRE, STRE, and AP-1 sites) upon osmotic and oxidative stress. Several specific transcription factors (TFs) are known to trigger gene expression changes upon osmotic and oxidative stress. We focused on three different classes of well-documented TF-DNA interactions relevant for stress responses: the cAMP-responsive element (CRE) recognized by Sko1 (17, 47), the general stress-responsive element (STRE) bound by the Msn2 and Msn4 activators (7), and the AP-1 element targeted by the Yap family of activators (34). We aimed at identifying the dose-sensitive regulation of gene expression conferred by each type of *cis* element upon stress. Triple repetitions of each element were placed upstream of a nonregulated core promoter to drive the expression of destabilized luciferase and to allow the real-time measurement of the reporter activity. We quantified the luciferase reporter activity driven by the artificial promoters under the exhaustive stress conditions (H_2O_2, menadione, NaCl) established as described above to obtain the dose response conferred by each element upon the various stress stimuli (Fig. 3A). In general, we noticed that the insertion of just



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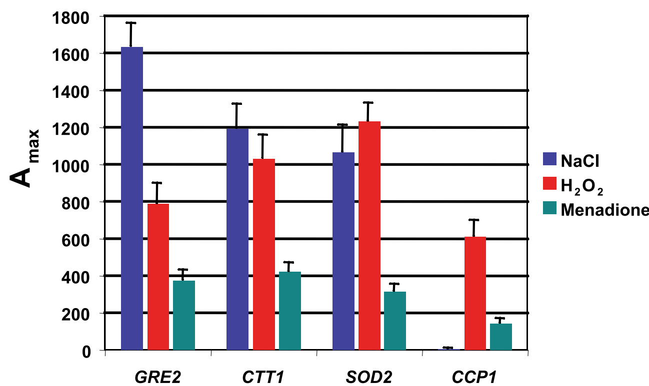

FIG 2 Maximal activation pattern of the *GRE2*, *CTT1*, *SOD2*, and *CCP1* promoters upon osmotic and oxidative stress. The optimal induction level (which was the maximal light emission in the real-time luciferase assay) for each promoter was obtained from the dose-response experiments whose results are shown in Fig. 1 for NaCl, hydrogen peroxide, and menadione treatment. The data presented are mean values derived from three biological replicates.

one type of *cis* element in the luciferase expression system was sufficient to reach maximal induction levels which were comparable to those obtained with full promoter regions, at least upon stress at the optimal dose for each type of element. This indicated that the elements were functional in the artificial promoter context created here and therefore allowed the comparison of the dose-sensitive performance of the three *cis* elements.

The CRE site conferred the efficient and sensitive induction of luciferase activity upon all three types of stress tested here (Fig. 3A). In contrast, the AP-1 site triggered activation of gene expression very efficiently upon hydrogen peroxide stress, moderately upon menadione treatment, and not at all upon salt stress. Introduction of the canonical STRE site significantly raised the initial uninduced reporter activity, which was only moderately activated by hydrogen peroxide or NaCl. To compare the performance of the different types of TF binding sites in a quantitative manner, we plotted the fold induction observed for each element under each stress treatment against the stress dose (Fig. 3B). Additionally, we could compare the maximal induction observed for each element across the three types of stimulus (Fig. 3C). We show that hydrogen peroxide triggered the most dynamic expression changes

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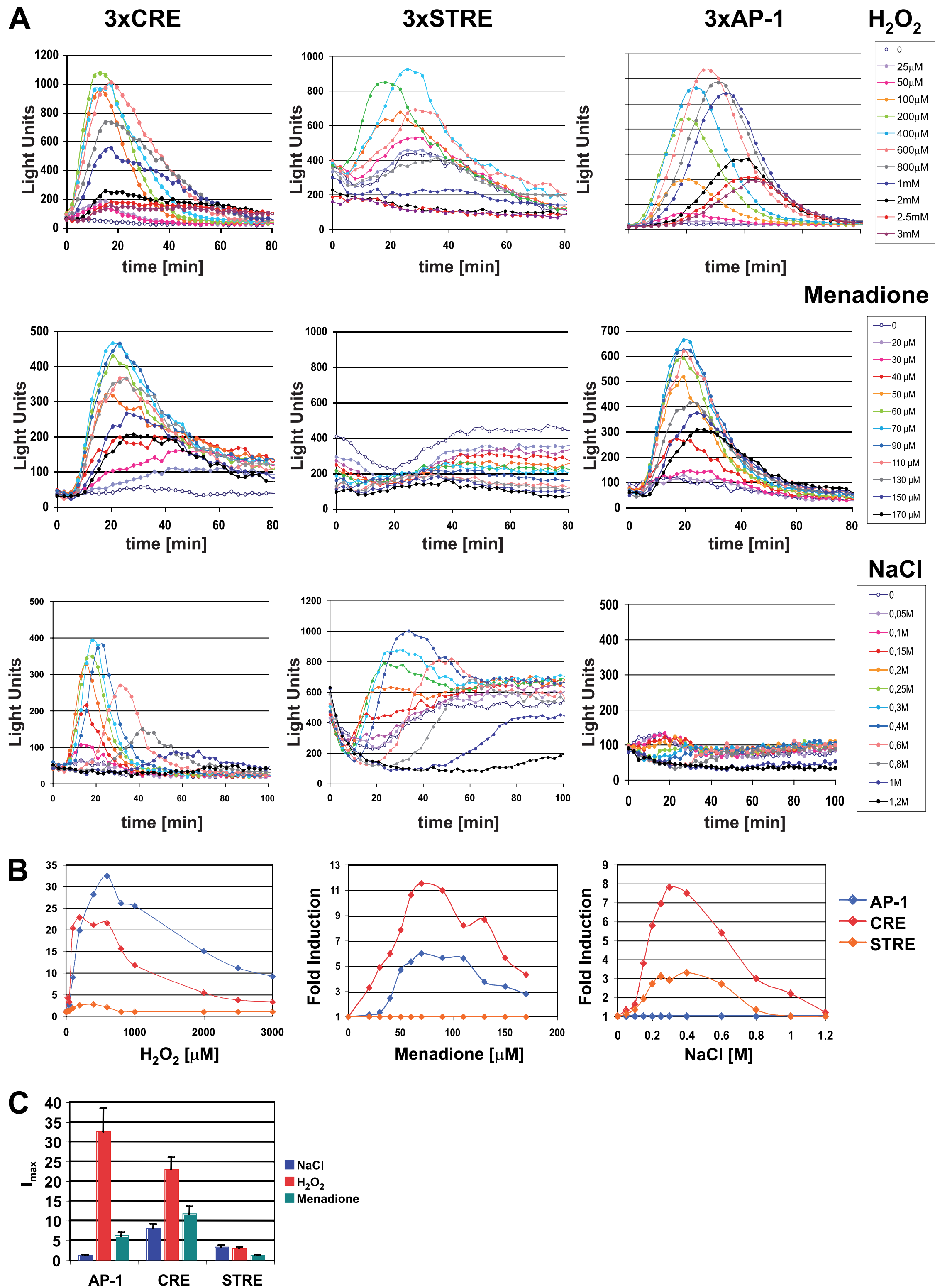

FIG 3 Comparison of the dose-response behavior of single *cis*-regulatory elements upon osmotic and oxidative stress. Destabilized luciferase genes driven by single regulatory elements (CRE, STRE, or AP-1) were used in yeast wild-type cells. The dose-sensitive activation of each element was monitored for hydrogen peroxide, menadione, and NaCl stress in the living cell. (A) The dose-response curves are depicted for each element and type of stress. The represented data are the mean values obtained from three independent culture aliquots, with the error being $<15\%$. (B) Comparison of the dose-sensitive induction profiles for AP-1, STRE, and CRE. The maximal fold induction is plotted against the stress dose for the indicated stress treatments. (C) Comparison of the maximal fold induction (I_{max}) for AP-1, STRE, and CRE upon the indicated stresses.

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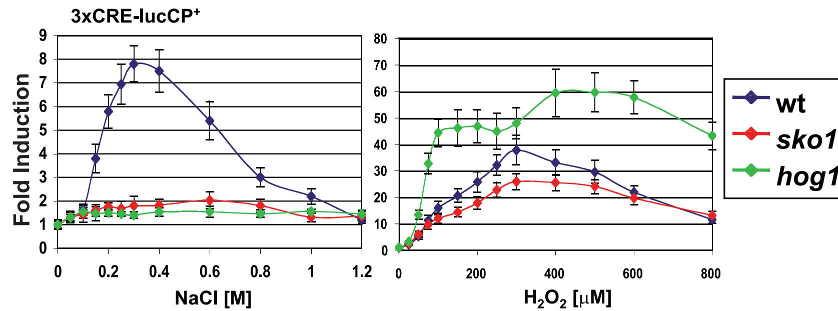

FIG 4 Activated gene expression from CRE sites occurs exclusively through Hog1 and Sko1 upon osmotic stress and independently of both factors upon oxidative stress. A CRE-driven luciferase reporter was used in yeast wild-type (wt) and *hog1* and *sko1* mutant cells. The dose-response curves upon NaCl and hydrogen peroxide stress were recorded for each strain background. The maximal fold induction is plotted against the stress dose for the indicated stress treatments to compare the CRE-driven luciferase performance for each strain. Data presented are the mean values from three biological replicates.

through the CRE and AP-1 sites and triggered expression changes to a minor degree through the STRE site. Oxidative stress provoked by menadione appeared to activate gene expression via CRE in a more sensitive way than via AP-1. Salt stress, in turn, induced the reporter gene activity mainly through CRE and moderately through STRE.

In summary, these data (i) confirm that AP-1 sites trigger specific signaling upon oxidative stress and show that they are the most efficiently induced by hydrogen peroxide, (ii) show that CRE sites can activate gene expression efficiently upon both salt and diverse oxidative stress treatments, and (iii) demonstrate that STRE sites confer only limited transcriptional activation upon salt and hydrogen peroxide stress. We next wanted to gain insights into the role of specific transcription factors and their role in the stress-specific activation of the *cis* elements investigated here.

Activation of CRE depends on Hog1 and Sko1 upon salt stress but not upon oxidative stress. Stress-activated transcription through CRE sites via the Sko1 repressor/activator, which is directly regulated by the Hog1 MAP kinase in response to osmotic stress, has been reported (16, 17, 47). We addressed the question whether Sko1 and Hog1 were the only factors responsible for the upregulation of gene expression from CRE sites upon osmotic and oxidative stimuli. We therefore expressed the CRE-regulated luciferase reporter in mutant strains lacking Sko1 or Hog1 function and quantified the dose-response behavior upon NaCl and hydrogen peroxide stresses. We confirmed that CRE-mediated activation depends on Sko1 and Hog1 upon salt stress (Fig. 4, left). However, upon stimulation with hydrogen peroxide, we observed a similar dose response in the wild-type and *sko1* mutant strains (Fig. 4, right). Loss of Hog1 function led to hyperactivation of the CRE-driven luciferase gene and to approximately 3-fold increased induction levels at low peroxide concentrations. These results identify the Sko1 and Hog1 regulators to be the exclusive activators through CRE upon osmotic stress, while both factors do not participate in activation through CRE upon oxidative stress.

Msn2 and Msn4 confer basal and stress-induced gene expression from STRE sites which is only partially affected by Hog1 upon osmostress. The transcriptional activators Msn2 and Msn4 are known to stimulate gene expression from STREs in response to general stress (7). We tested their contribution to NaCl- and H_2O_2-induced transcription by the use of the STRE-driven luciferase reporter in living yeast cells. First, we quantified the complete dose-response performance upon both types of stress by comparing the wild type and the *msn2 msn4* mutant (data not shown). Interestingly, we found the most pronounced loss of reporter gene activity in the double mutant under basal growth conditions prior to stress. In Fig. 5, representative induction profiles are shown for both stresses. Loss of Msn2 and Msn4 function results in an approximately 5-fold decrease of the uninduced expression level. While in wild-type cells STRE-regulated luciferase activity transiently increased (2- to 3-fold upon both NaCl and H_2O_2 shock), the *msn2 msn4* mutant maintained the reduced reporter activity throughout the stress treatment. We concluded that the Msn2 and Msn4 factors are important not only for stress-induced transcription but also for normal levels of basal gene expression from STRE sites.

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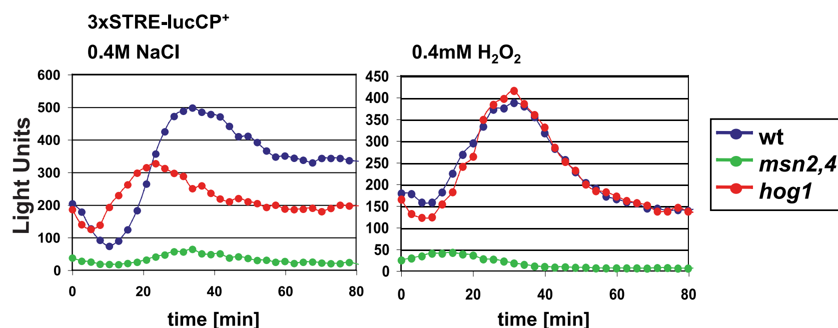

FIG 5 Msn2 and Msn4 sustain basal and stress-induced gene expression from STRE sites. A STRE-driven luciferase reporter was used in yeast wild-type and *msn2 msn4* and *hog1* mutant strains. The reporter activity was assayed in living cells treated with the indicated stress doses. The data presented are the mean values obtained from three independent culture aliquots, with the error being <15%.

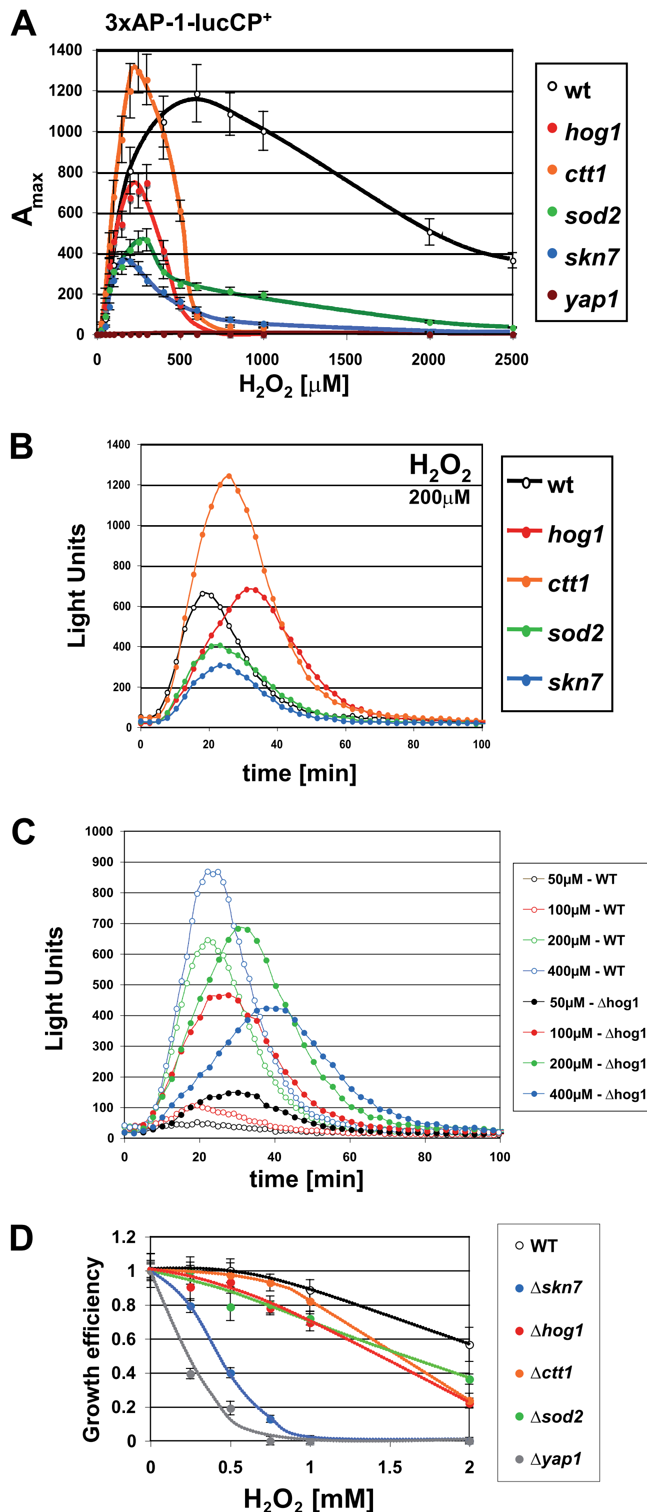

FIG 6 Cell physiology modulates the dose response of stress-activated gene expression. An AP-1-driven luciferase reporter was used in the indicated yeast strains, and its activation profiles were recorded upon growth of the living cell with hydrogen peroxide. (A) Yap1 is indispensable for H_2O_2-induced gene expression from AP-1 sites, while other mutants affected in oxidative stress signaling or defense show altered dose-response profiles. The maximal reporter activity was measured in triplicate upon each stress treatment and was plotted against the hydrogen peroxide concentration for each strain. (B) Snapshot of the AP-1-induced luciferase reporter activity in the indicated strains upon a mild oxidative stress (200 μM H_2O_2). The live cell luciferase profiles were obtained for three independent culture aliquots, and the error was <15%. (C) H_2O_2-induced gene expression is both positively and negatively affected by the loss of Hog1 function. The live cell AP-1-induced luciferase profiles were obtained in wild-type and *hog1* mutant cells at the indicated H_2O_2 concentrations. Data were derived from three independent culture aliquots, and the error was <15%. (D) Growth efficiency of the wild type and the different mutant strains upon hydrogen peroxide stress. Data were obtained from three independent culture aliquots for each strain.

We next wanted to distinguish the effect of Msn2 and Msn4 from that of indirect regulators, such as the Hog1 MAP kinase. Hog1 has been reported to act through STREs upon osmotic stress (48). We recorded the dose-response behavior of an STRE-dependent luciferase reporter in a *hog1* mutant and found no effect on the basal or the hydrogen peroxide-induced expression levels and a partial reduction of induction upon NaCl stress (Fig. 5; shown for representative stress doses).

The dynamic activation from AP-1 sites mediated by Yap1 upon oxidative stress is critically dependent on cell physiology. We next investigated the factors which modulate transcriptional activation from AP-1 sites upon oxidative stress. AP-1 sequences are targeted by the Yap family of transcriptional activators, and Yap1 has been identified to be one of the major transcription factors acting upon oxidative stress (30). We confirmed the pivotal role of the Yap1 protein in H_2O_2- and menadione-induced transcription from AP-1 sites (Fig. 6A and data not shown). When we tested other regulatory mutants for AP-1-activated gene expression, we found reduced reporter activity for the *skn7* mutant lacking a second specific transcription factor involved in the response to oxidative stress. To assess the function of Skn7 in the Yap1-mediated transcriptional response, we compared the dose-response behavior of AP-1-driven gene expression in *skn7* mutant cells with that in the wild type (Fig. 6A). We found that AP-1-regulated luciferase expression was impaired for high H_2O_2 concentrations, while for low peroxide doses (<200 μM), *skn7* mutants reached maximal reporter activities comparable to those for the wild type. The dose-response profile for the *skn7* mutant showed saturation at stress doses much lower than those for the wild type. We concluded that Skn7 is not directly involved in transcription from AP-1 sites, as it appeared to be normal for weak stress conditions. We assumed, rather, that a greater stress susceptibility in *skn7* mutants caused a truncated dose response at higher peroxide concentrations.

We wanted to gain more insights into how general stress sensitivity could change the dose-sensitive transcriptional response. Therefore, we investigated AP-1-stimulated gene expression in two additional mutant strains with mutations in genes affecting enzymatic activities required for efficient detoxification of oxidative stress: *sod2* (superoxide dismutase) and *ctt1* (catalase). In the case of *sod2*, we found a dose-response profile for hydrogen peroxide stress reduced similarly to that for the *skn7* mutant. Cells lacking Ctt1 showed even higher activation of the AP-1-induced luciferase reporter at low peroxide concentrations; however, activation upon higher stress doses was severely reduced compared to that in the wild type. These data suggested that the stress sensitivity of a cell critically modulates the dynamics of the dose-sensitive transcriptional response. In order to see how an increased sensitivity to peroxide stress affects the transcriptional induction profile, we focused on the AP-1-induced luciferase profiles *in vivo* in

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different mutant backgrounds upon a moderate H_2O_2 insult. As depicted in Fig. 6B, we observed overactivation of the AP-1-driven reporter in the *ctt1* mutant strain upon treatment with 200 μM H_2O_2. Compared to the length of activation in the wild type, this overactivation was produced for a longer time, during which the reporter remained actively expressed. These data indicate that under these specific stress conditions, a wild-type cell can efficiently reduce the oxidative insult within a few minutes, while *ctt1* mutants more slowly detoxify the oxidative stress and, hence, show an enlarged activation profile for the AP-1 site. Other mutants, such as *hog1*, *sod2*, or *skn7* mutants, showed a different phenotype under the same stress conditions. They showed a decreased efficiency of activation of AP-1-induced luciferase which resulted in a reduced maximal activity of the reporter. A possible explanation for this is that while a stress dose of 200 μM H_2O_2 is too low to interfere with efficient transcriptional activation in the wild type, in sensitive mutant backgrounds, the same stress dose can already provoke a general decrease in the efficiency of activated gene expression.

Taken together, these data suggest that the physiology of the cell can affect the transcriptional response in an important manner. Both positive and negative changes can be observed dependent on the stress dose. This was confirmed by comparing the *in vivo* activation profiles at low, medium, and high stress doses, for example, for the *hog1* mutant (Fig. 6C). The AP-1-induced luciferase reporter is clearly overactivated in the mutant at very low peroxide concentrations (50 to 100 μM), its activation is delayed but still as efficient as that of the wild type at moderate concentrations (200 μM,) and its activation is severely decreased at higher peroxide concentrations (400 μM).

We finally determined the tolerance to oxidative stress of the different mutant strains. We found that growth efficiency was most severely affected by hydrogen peroxide in the *yap1* and *skn7* mutants, followed by the *sod2* and *hog1* mutants, which had moderate sensitivity, and the *ctt1* mutant, which was the least sensitive of the mutant strains tested (Fig. 6D). These data in general confirm the observation that increased stress sensitivity shifts the dose-response profile of gene activation toward low stress doses. The *sod2* mutant is a notable exception, which might imply that there is no simple correlation between stress tolerance and the efficiency of stress-induced gene expression.

Combination of *cis* elements and their effect on the dose-response behavior upon oxidative stress. Many stress-responsive yeast promoters contain more than one type of upstream activation element. Combinations of different *cis* elements might be advantageous for the cell to yield higher activated expression levels and/or to create specific stress responses for specific subsets of defense genes. We addressed the question of how combinations of different TF binding sites modulate the overall dose response by the use of the real-time luciferase assay. We created reporter genes under the control of the combinations of CRE-STRE, AP-1–STRE, and AP-1–CRE and recorded their dose-response behavior in comparison with that of reporters activated by just one of the combined elements. We performed this study with H_2O_2 as the stimulus because each of the elements alone was responsive to this specific stress. When we plotted the absolute levels of expression of each construct against the stress dose, we did not observed in any of the cases an enhanced gene expression by any combination of CRE, STRE, or AP-1 sites (Fig. 7). Strikingly, each combination gave an intermediate dose-response expression profile compared

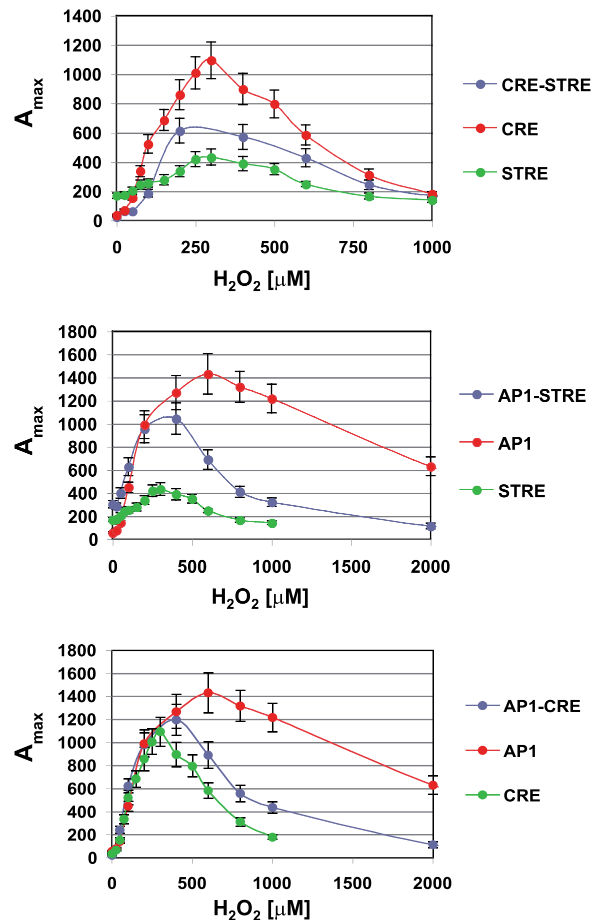

FIG 7 Combination of different stress-responsive *cis* elements does not enhance absolute expression levels. AP-1, CRE, and STRE sequences were combined to drive destabilized luciferase gene expression in yeast wild-type cells, and the luciferase activation profiles were recorded upon growing hydrogen peroxide stress. The maximal luciferase activity was determined in triplicate upon each stress treatment and was plotted against the hydrogen peroxide concentration for the indicated constructs.

to that of the respective single-element derivatives. This suggested that in the artificial promoters created here, each regulatory element activated transcription independently from the other without additive or synergistic effects. This effect was not caused by steric competition between neighboring sites, because the 3× CRE reporter showed significantly higher expression than the 1× CRE reporter (data not shown). However, we observed that in some instances the combination of *cis* elements enhanced the inducibility of the stress response. This was evident when we compared the induction profiles rather than the absolute expression profiles of a luciferase reporter controlled by AP-1 and CRE sites. The combination of both regulatory elements increased the induction profile upon oxidative stress by the combination of low uninduced expression levels (conferred by CRE) and high inducibility upon stress (data not shown).

DISCUSSION

In the present study, we measured how stress modulates gene expression over an exhaustive range in order to know the dynamic response of complex gene promoters and individual response el-

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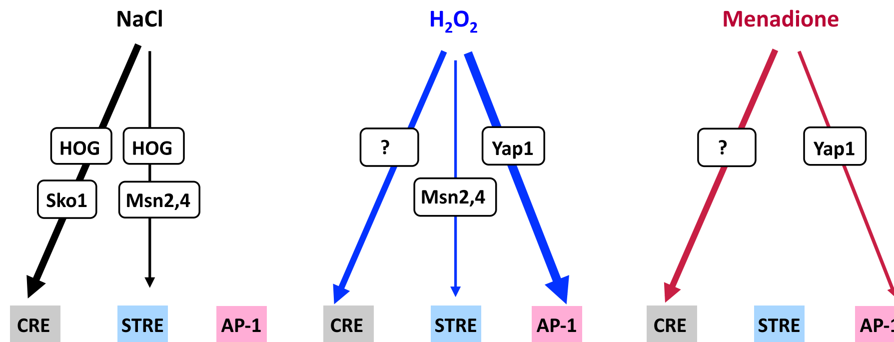

FIG 8 Model of differential gene expression upon osmotic and oxidative stress. The schematic representation summarizes the contribution of specific regulatory elements (CRE, STRE, and AP-1) to the induction of gene expression by the indicated stresses. The elements confer different inducibility upon the distinct stress treatments, as revealed by the dose responses reported here.

ements. The obtained dose-response profiles contain important biological information on the sensitivity of regulatory regions, the discrimination between different types of stress, the capacity of signal transducers and regulators to resolve dynamic stress doses, and the interference of the stress with transcriptional efficiency. It is important to note that the principle assay used here to measure dose-dependent gene expression is not a direct transcription assay but, rather, determines gene expression indirectly by the use of destabilized enzyme activity. However, all genes studied here are characterized by very low transcript levels under normal growth conditions but by the massive induction of transcription upon the relevant stress treatments. Therefore, the activation of luciferase activity in the reporter assays throughout the work is mainly a consequence of stimulated transcription at the diverse promoters and not due to regulation of transcript stability or translation. Additionally, we have confirmed for various promoter fusions that upon stress luciferase expression profiles generally correlate with the recruitment of PolII RNA at the corresponding endogenous genes (A. Rienzo, A. Pascual-Ahuir, and M. Proft, unpublished observations). We estimate that the luciferase reporter system detects gene expression with a delay of approximately 20 min with respect to the time of PolII binding at the promoter and approximately 15 min with respect to the time of mRNA production.

Complete dose responses allow quantitative comparison of the performance of promoters for specific stresses. Here we show that yeast promoters have characteristic activation patterns upon osmotic and oxidative stresses. The *GRE2* promoter is especially responsive to osmotic stress, where it is activated to expression levels higher than those in response to oxidative stress. Other promoters, such as *CTT1* or *SOD2*, seem to respond equally well to both stresses, while yet other promoters with an activation profile highly specific for oxidative stress exist, such as *CCP1*. These differences might reflect the specific contribution of the encoded enzyme activities to the adaptation to NaCl or oxidative stress. The Gre2 protein has methylglyoxal reductase activity and might therefore be involved in detoxifying this inhibitory compound, which accumulates upon osmotic and oxidative insult (49, 50). On the other hand, the Ctt1 and Sod2 enzymes degrade reactive oxygen species, which are directly generated by oxidative stress and only secondarily produced upon salt stress (39). The individual susceptibilities to different stresses might be conferred by specific combinations of upstream activating elements in promoters. Here we quantified the dose-dependent regulation of three promoter elements relevant to osmotic and oxidative responses. Of note, the insertion of CRE, AP-1, or STRE sequences in an artificial core promoter was sufficient to efficiently activate gene expression to similar absolute expression levels, at least under their optimal induction conditions. Therefore, we assume that all three elements were as functional in the reporters created here as natural promoters, which allows their functional comparison. The CRE site conferred efficient activation upon salt stress and oxidative stress. In contrast, AP-1 mediated oxidative stress signaling that was especially efficient upon hydrogen peroxide stress, while it was completely unresponsive to salt stress. The STRE sequence seemed to confer very limited activation upon salt and oxidative stress. Different combinations of these *cis* regulators might therefore create dose-response profiles specific for natural promoters (Fig. 8). Indeed, the search for TF binding sites in the promoter regions (1 to 500 bp upstream of the ATG codon) of the four genes investigated here revealed striking differences: *GRE2* contains 2 CRE sites and no AP-1 or STRE site, while *CTT1*, *SOD2*, and *CCP1* contain different combinations of AP-1 and STRE sites. Additional elements, such as Skn7 or Hot1 binding sites, not investigated here, might add to the more salt-responsive induction profile of *GRE2* versus that of *CTT1* or *SOD2*. In general, our results indicate that single stress-responsive promoter elements confer efficient transcriptional activation which cannot be enhanced by including other types of *cis* elements in the same promoter. We speculate that upon the appropriate stimulation, elements such as CRE or AP-1 activate transcription to maximal levels. Combination of *cis* elements in natural promoters might therefore be more important to define the range of stimuli able to activate a particular promoter than to increase absolute expression levels.

Determining the specific dose responses for DNA elements also allows one to know how the signaling pathways operate to modulate their activities upon different stresses. Here we show that salt and oxidative stresses trigger adaptive responses separately in yeast (Fig. 8). This conclusion is supported by several experimental findings: (i) transcriptional activation through AP-1 elements is specific for oxidative stress and does not occur upon stress caused by any NaCl concentration known to cause ROS accumulation, and (ii) although CREs or STREs stimulate gene expression in response to both osmotic and oxidative stimuli, only the osmotic stimulus is triggered by the HOG osmosensing path-

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way through the Sko1 or Msn2 and Msn4 transcription factors. This suggests that although it is well-known that osmotic stress causes oxidative damage, the ROS production caused by salt stress does not secondarily trigger a transcriptional response. Therefore, the induction of antioxidant systems by salt stress does not occur indirectly through ROS but, rather, is the product of independent signaling (osmotic or oxidative) to the respective antioxidant promoters.

The continuous measurement over a range of doses also allows comparison of the efficiencies with which different stresses stimulate gene expression. Here we show that different oxidative treatments, such as hydrogen peroxide and menadione treatments, have different impacts on the activation of both natural and artificial promoters. For example, in the case of activation through AP-1 sites, hydrogen peroxide is an approximately 5-fold better inducer of gene expression than menadione, while signaling through CRE sites seems to be less discriminative for menadione (Fig. 3). Activation of AP-1 by both oxidants is completely dependent on Yap1. Therefore, it is likely that hydrogen peroxide is a better inducer of Yap1 activity. The critical event in Yap1 induction upon oxidative stress is its oxidation in the cytosol and formation of specific disulfide bonds, which trigger nuclear import and gene activation (37, 38). Interestingly, it is known that different oxidants, such as hydrogen peroxide or diamide, provoke different activated forms of Yap1 (35, 51). This might have consequences for the transactivation properties of Yap1, as suggested here for hydrogen peroxide and menadione.

An important determinant that shapes gene expression responses is the inhibitory effect of the stress itself. Here we measured for three different stimuli how the expression of luciferase activity is modulated in response to gradually growing stress doses. As expected, we found in all cases that, within a certain range of concentrations, the maximal expression levels increased with the dose. The concentration ranges which provoked differential responses in the wild type were approximately 0.05 to 0.5 mM for hydrogen peroxide, 30 to 100 μM for menadione, and 100 to 500 mM for NaCl. For harsher stress conditions, we observed that the maximal expression levels declined for all treatments. A plausible explanation for this is that the stress itself interferes with efficient gene expression. In line with this interpretation, it has been demonstrated that salt stress already disturbs the association of proteins with chromosomal DNA at moderate concentrations and that higher NaCl concentrations delay and decrease the efficiency of transcriptional activation (52, 53). Thus, salt stress directly interferes with transcription at critical concentrations. Severe oxidative stress also seems to inhibit but not delay gene expression (Fig. 1). It remains to be seen whether elevated ROS levels directly interfere with the process of transcription. Here we demonstrate that the stress tolerance of a yeast cell determines its way to mount a transcriptional response to different stress doses. This is especially interesting in cases of moderately sensitive strains, such as *ctt1* or *hog1* mutants, upon hydrogen peroxide stress. The growth inhibition by oxidative stress of these mutants occurs for different reasons and is caused by the lack of a specific antioxidant enzyme in the case of *ctt1* or supposedly by the lower level of expression of several antioxidant genes in the case of *hog1*. Both mutants showed a hypersensitive phenotype regarding their dose-response profile, which means that they overactivated gene expression at very low stress doses, saturated this response very early with low doses, and severely diminished the response upon stronger oxidation (Fig. 6). It is likely that the reduced antioxidant capacity in *ctt1* and *hog1* mutants produces activation of the Yap1 peroxide sensor at lower H_2O_2 concentrations than in the wild type. On the other hand, inhibition of gene expression in general was accomplished much earlier with lower peroxide concentrations. These effects shift the optimal transcriptional response of sensitive mutants toward lower stressor concentrations. These experiments also have important practical implications. If we had performed a transcription assay of an inducible gene at a peroxide concentration of $>$0.5 mM, we might have come to the conclusion that Hog1 is an activator of the oxidative stress response, while the same experiment at $<$0.2 mM would have suggested a repressor role for Hog1 in oxidative stress signaling. However, our data indicate that Hog1 is not directly involved in transcriptional activation stimulated by oxidative stress. This is important to note, because activation of the HOG pathway has been reported to occur upon oxidative stress (40). Another example which highlights the importance of dose responses for the correct interpretation of biological systems comes from the comparison of the *skn7* and *yap1* mutants, defective in the two major transcriptional activators operating upon oxidative stress. Both mutants were highly sensitive to peroxide stress; however, only the lack of Yap1 completely abolished transcriptional activation from AP-1 sites upon H_2O_2 treatment, an expected result, given that Yap1 directly binds AP-1 sequences. A lack of Skn7 also dramatically decreased peroxide-activated gene expression controlled by AP-1; however, this happened only at high H_2O_2 concentrations, while the transcriptional response was unaffected at low stress doses. Therefore, Skn7 activity is not generally required for Yap1-mediated activation, and complete dose-response experiments are critical to differentiate the direct from indirect effects of regulators on stress-regulated gene expression.

As a conclusion, deciphering the dynamic response of gene expression yields important insights into the dose-sensitive modulation of promoters and specific promoter elements as well as their direct and indirect regulators. The application of time-resolved gene expression assays, as reported here, will certainly help us to understand how gene regulation is constantly adapted to changing environmental stress.

ACKNOWLEDGMENTS

This work was supported by the Ministerio de Economía y Competitividad (grant BFU2011-23326 to M.P.) and the Ministerio de Ciencia e Innovación (predoctoral FPI grant to A.R.).

We thank E. de Nadal for the kind gift of the Δ*msn2* Δ*msn4* mutant strain.

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