Coordinated Gene Regulation in the Initial Phase of Salt Stress Adaptation*

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Results: Vacuolar H+ -ATPase and glycerol biosynthesis affect the efficient coordination of transcriptional activation and repression upon osmostress.

Conclusion: Intracellular ion homeostasis, osmolyte production, and MAP kinase function modulate the dynamics of a transcriptional stress response.

Significance: The results underline the importance of cell physiology as a modulator of gene regulation.

Stress triggers complex transcriptional responses, which include both gene activation and repression. We used time-resolved reporter assays in living yeast cells to gain insights into the coordination of positive and negative control of gene expression upon salt stress. We found that the repression of “housekeeping” genes coincides with the transient activation of defense genes and that the timing of this expression pattern depends on the severity of the stress. Moreover, we identified mutants that caused an alteration in the kinetics of this transcriptional control. Loss of function of the vacuolar H+ -ATPase (vma1) or a defect in the biosynthesis of the osmolyte glycerol (gdpl) caused a prolonged repression of housekeeping genes and a delay in gene activation at inducible loci. Both mutants have a defect in the relocation of RNA polymerase II complexes at stress defense genes. Accordingly salt-activated transcription is delayed and less efficient upon partially respiratory growth conditions in which glycerol production is significantly reduced. Furthermore, the loss of Hog1 MAP kinase function aggravates the loss of RNA polymerase II from housekeeping loci, which apparently do not accumulate at inducible genes. Additionally the Def1 RNA polymerase II degradation factor, but not a high pool of nuclear polymerase II complexes, is needed for efficient stress-induced gene activation. The data presented here indicate that the finely tuned transcriptional control upon salt stress is dependent on physiological functions of the cell, such as the intracellular ion balance, the protective accumulation of osmolyte molecules, and the RNA polymerase II turnover.

* This work was supported by Ministerio de Economía y Competitividad Grant BFU2011-23326 (to M. P.).
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4 The abbreviations used are: HOG, high osmolarity glycerol; PolII, polymerase II.
important physiological aspect to consider is that the elevated intracellular ionic force itself interferes with transcriptional adaptation. Salt stress causes the dissociation of many DNA bound proteins such as transcription factors or RNA polymerase complexes from their chromosomal association sites. The activation of ion transporters at the plasma membrane has been identified as a mechanism to overcome this transcriptional barrier (21). Additionally, the intracellular sequestration of toxic ions in the vacuole is yet another determinant of salt tolerance in yeast, because stress-activated vacuolar H^+-ATPase function is necessary for the recovery from osmotic shock in a process parallel to the HOG pathway (22).

The physiology of the cell critically conditions the efficiency and timing of the transcriptional response. It has been shown that severe osmotic stress leads to an increasing delay in the induction of stress defense genes. This effect can be explained by a delay in HOG pathway signaling, a later nuclear translocation of Hog1, and the increasing dissociation of DNA associated protein complexes from the genome (21, 23–25). Accordingly an important function of Hog1 is to enable rapid transcriptional activation under conditions, which generally inhibit gene expression. This function triggers a general relocation of active transcription complexes from highly expressed genes to stress-induced loci (26, 27). Perturbations of the transcriptional response by changes in the cells physiology are not restricted to osmotic stress. It has been recently shown that also oxidative stress, at threshold levels, interferes with efficient transcriptional activation and, more importantly, that mutants in reactive oxygen species detoxification systems show much less dynamic transcriptional responses (28). Thus, the dynamic of a gene expression response to stress is to a great part dependent on cell physiology. Additionally, the tolerance of a yeast cell is determined by its history and, for example, the previous exposure to stress can modulate the next wave of transcriptional adaptation. In this process both the accumulation of defense proteins and the establishment of transcriptional memory in the experienced cells has been implicated (29, 30). It is clear from these results that a transcriptional stress response can only be fully understood in the physiological context of the cell. The processes, however, which determine a specific transcriptional profile, remain in most cases unexplored. Here we apply time-elapsed reporter studies to identify physiological determinants of the positive and negative gene regulation upon osmotic shock. We find that intracellular glycerol production and vacuolar H^+-ATPase activity modulate the timing and efficiency of the transcriptional response and that the turnover of RNA PolII rather than its absolute levels might be required for efficient activation of gene expression upon salt shock.

EXPERIMENTAL PROCEDURES

Yeast Strains and Procedures Conditions—Saccharomyces cerevisiae strains used in this study were: wild type BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and the mutant alleles kha1::KanMX4, mnd38::KanMX4, plo89::KanMX4, tok1::KanMX4, trk2::KanMX4, hog1::KanMX4, gpd1::KanMX4, nha1::KanMX4, nhx1::KanMX4, trk1::KanMX4, vma1::KanMX4, vma2::KanMX4, vma8::KanMX4, vnx1::KanMX4, pmc1::KanMX4, vam3::KanMX4, vam6::KanMX4, def1::KanMX4, and iwr1::KanMX4 (31); wild type W303-1A (MATa; ade2-1; ura3-1; his3-11,15; leu2-3,112; trp1) and its derivatives RPB3-3HA, RPB3-3HA hog1::KanMX4, RPB3-3HA gpd1::KanMX4 (this study), RPB3-3HA vma1::KanMX4 (this study), and ena1-4::HIS3. Yeast cultures were grown in yeast extract peptone containing 2% dextrose (YPD) or 2% galactose (YPGal) with or without the indicated supplementation of NaCl or KCl. Synthetic growth medium contained 0.67% yeast nitrogen base, 50 mM succinic acid, pH 5.5, and 2% dextrose (SD) or 2% galactose (SGal). According to the auxotrophies of each strain, methionine (10 mg/liter), histidine (10 mg/liter), leucine (10 mg/liter), or uracil (25 mg/liter) were added.

Plasmids—The plasmid p413-GRE2-lucCP^+ for the expression of destabilized luciferase under control of the GRE2 promoter is described in Ref. 32. The p413-TDH3-lucCP^+ construct expressing destabilized luciferase under control of the constitutive TDH3 promoter was obtained by cloning the lucCP^+ gene into the Gateway destination vector pAG413-GPD-ccdB (HIS3; CEN) (33).

Reverse Transcriptase Assays—Total RNA was isolated by acid phenol extraction from yeast cells grown in the indicated condition. Total RNA samples were DNaseI-digested and purified with the RNeasy Mini kit (Qiagen). A total of 5 μg of RNA was converted into DNA using the Superscript III first strand cDNA synthesis kit (Invitrogen). The amount of DNA was quantified with the indicated gene specific primers by quantitative PCR in real time using the EvaGreen qPCR Master Mix (Biotium) on an Applied Biosystems 7500 sequence detection system. The ACT1 gene expression was used as a reference. The expression level was determined in triplicate from three independent cDNA samples.

Chromatin Immunoprecipitation—ChIP was performed essentially as described previously (34). Quantitative PCR analyses were performed in real time using the EvaGreen qPCR Master Mix (Biotium) on an Applied Biosystems 7500 sequence detection system. The following genomic regions were assayed: GRE2 (−301/−121), STL1 (−485/−292), TDH3 (−133/+14), and RPL2B (−159/−14). The POL1 (+1796/+1996) coding sequence was used as an internal control. All ChIP experiments were performed at three independent chromatin samples.

Continuous Growth Assays—for sensitivity assays in continuous growth, fresh overnight precultures of the yeast wild type strain (BY4741) in YPD or YPGal medium were diluted in triplicate in the same medium with or without the indicated concentration of NaCl in multiwell plates to the same optical density. Growth was then constantly monitored under the indicated salt stress conditions in a Bioscreen C system (Thermo) for the indicated times. The growth curves were processed in Microsoft Excel, and the half-maximal cell density was calculated for each growth condition. The time to reach half-maximal cell density (t_{50}) under each stress condition was compared with the t_{50} under nonstress conditions. This ratio was taken as an indicator of the relative growth efficiency.

Live Cell Luciferase Assays—Yeast cells transformed with the destabilized luciferase reporter genes GRE2-lucCP^+ or TDH3-lucCP^+ were grown to mid-log phase in SD medium. Culture aliquots were then incubated for 90 min with 0.5 mM luciferin...
(Sigma) at 28 °C. 120-μl aliquots of cells were then distributed in white 96-well plates (Nunc) and the indicated concentrations of NaCl added from appropriate stock solutions. The light emission was immediately and continuously quantified in a GloMax microplate luminometer (Promega) in three biological replicates. The data were processed in Microsoft Excel software.

RESULTS

Time-resolved Quantification of Positive and Negative Gene Regulation upon Salt Stress—Salt stress activates a complex transcriptional response in yeast cells, which involves both activation at defense genes and repression at “housekeeping” genes. To study this adaptive response in a truly kinetic manner, we used live cell reporter assays based on the expression of destabilized luciferase (lucCP+). We chose to compare the expression patterns in a time-resolved manner for the highly inducible GRE2 gene and the glycolytic TDH3 gene. GRE2 encodes a stress-inducible methylglyoxal reductase, whose expression is repressed upon normal growth conditions. GRE2 serves here as a prototypical stress-induced locus. TDH3 encodes a glyceraldehyde-3-phosphate dehydrogenase and is one of the most highly expressed genes in yeast upon normal growth. TDH3 is chosen here as a typical housekeeping gene.

The expression profiles of both genes were determined upon different salt (NaCl) doses. As expected, GRE2 expression was highly and transiently activated upon all stress treatments (Fig. 1A). Increasing salt concentrations provoked a successive delay in gene induction with the result that the GRE2 expression peak continuously shifted to later time points with greater salt doses. The TDH3 gene showed an opposite behavior. Here the initial high expression levels were transiently repressed, and the duration of low expression was continuously prolonged upon higher stress doses. Positive and negative control of gene expression appeared to be coordinated in a way that the highest GRE2 expression coincided with the lowest levels of TDH3. Consequently, the release from repression at the TDH3 gene occurs...
Loss of transcriptional activity at the housekeeping gene, however, occurred immediately upon salt shock and did not seem to correlate with the onset of transcription at the inducible locus. In fact, upon all stress treatments and in a certain time window, which increases upon harsher stress conditions, gene expression is absent at both the inducible and the housekeeping gene (Fig. 1B).

A Genetic Screen for the Identification of Physiological Determinants That Modulate the Coordinated Activation and Repression of Gene Expression upon Salt Stress—Having created a quantitative tool for the detection of the transient loss of gene expression upon salt stress, we next wanted to identify physiological functions that altered the coordinated transcriptional control upon salt stress. We tested whether defects in the intracellular ion homeostasis or glycerol production affected the timing of the transcriptional response at the TDH3 housekeeping gene. We therefore expressed the TDH3-lucCP reporter in mutants of the major intracellular Na⁺, K⁺, and H⁺ transporters located at the plasma membrane or the vacuolar, mitochondrial, Golgi, or endosomal membranes and in a gpd1 mutant deficient in salt-induced glycerol biosynthesis. Additionally we used a mutant strain deficient in the activity of the Hog1 MAP kinase responsible for the transcriptional activation of osmostress-inducible genes. As shown in Fig. 2A, the transient loss of TDH3-lucCP⁺ expression upon salt shock is very reproducible and unaffected in many of the yeast mutants studied here, including a mutant in the major stress-inducible cytoplasmatic Na⁺ extrusion system ena1–4. However, specific mutant strains were identified with an altered pattern of regulation. The gpd1 and vma1 (subunit of the vacuolar H⁺-ATPase) mutant strains showed an increased loss of the housekeeping gene expression (Fig. 2B). In the case of the gpd1 strain, we additionally observed a delay in the recovery of reporter activity after the salt shock. We also identified mutant strains with an opposite phenotype, which is an apparently less pronounced decrease of TDH3-lucCP⁺ expression during salt stress. The hog1 and trk1 (major K⁺ importer at the plasma membrane) mutant strains showed this behavior. Additionally, the hog1 mutant recovered TDH3-lucCP⁺ expression much more inef-
ficiently after salt exposure (Fig. 2B). The same mutant strains were next analyzed for their effect on the gene induction kinetics upon salt stress.

Defects in the Timing of Positive Gene Regulation upon NaCl Stress in the gpd1 and vma1 Mutants—Loss of Vma1 or Gpd1 function caused a more severe loss of gene expression at a housekeeping gene during salt stress. We addressed the question of whether the same mutations also caused a different pattern of gene activation at stress defense genes. Therefore we determined the dose-response profiles of the inducible GRE2 gene by the use of the live cell luciferase reporter assay in comparison with wild type. As shown in Fig. 3A, the vma1 and gpd1 mutants activated the GRE2-lucCP+ reporter in a less efficient manner. Specifically we detected for the vma1 mutant a reduction in the induced synthesis rates and induction folds and an increasing delay in gene activation clearly detectable at >0.4 M NaCl. Loss of Gpd1 function caused a general delay of GRE2 activation at any NaCl concentration tested accompanied by a reduction of synthesis rates and induction folds (Fig. 3B). We additionally measured the dose-dependent activation of the
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GRE2-luciferase reporter in a log1 deletion strain (data not shown) and expectedly found its activation more severely reduced (Fig. 3B). Finally, we also examined a trk1 mutant strain for the dynamic pattern of GRE2 induction, which in this case was indistinguishable from wild type cells (Fig. 3B). Thus, although the trk1 mutant showed an apparent alteration in the loss of gene expression at a housekeeping locus, it was not affected in the positive control of gene expression upon salt stress.

We additionally included other vacuolar mutants to test their effect on the dynamic of gene activation upon salt stress using the GRE2-luciferase reporter. Deletion of different subunits of the vacuolar H\(^+\)-ATPase such as Vma2 (Fig. 3C) or Vma8 (data not shown) again showed a strong decrease of GRE2 activation. Mutants affected in general vacuolar morphogenesis (vam3 and vam6) showed a significant but weaker reduction of reporter activation, whereas deletion of specific vacuolar cation transporters Vnx1 (Na\(^+\)) or Pmc1 (Ca\(^{2+}\)) did not alter the GRE2 induction profile (shown for vam6 and vnx1 in Fig. 3C). These results correlated very well with the sensitivity of the different vacuolar mutant strains upon high salt stress (Fig. 3D).

We next wanted to confirm by direct transcriptional assays that the lack of Gpd1 or Vma1 function negatively affected the timely induction of salt-inducible defense genes. We therefore measured the mRNA production at two highly inducible genes, GRE2 and CTT1, by RT-PCR. As shown in Fig. 4, the gpd1 and vma1 mutants showed a less efficient and delayed induction of transcription at both stress-activated genes. We concluded that both the efficient production of osmolytes such as glycerol and the vacuolar ion homeostasis are determinants of the timing of transcriptional induction upon salt stress.

Environmental Conditions That Interfere with Glycerol Accumulation Cause Important Gene Induction Defects upon Salt Stress—According to the previous results, an impairment of glycerol accumulation interfered with the timely and efficient up-regulation of defense genes upon salt shock. We next wanted to know to what extent growth conditions, which disfavor an efficient glycerol accumulation, would affect transcriptional activation. Glycerol is the main osmolyte produced by Saccharomyces cerevisiae upon hyperosmotic stress, and its biosynthesis depends on precursors from glycolysis. Therefore we reasoned that growth on only partially fermentable carbon sources could decrease the glycolytic flux and hence affect glycerol production. We confirmed this by comparing the intracellular glycerol accumulation of yeast cells grown in galactose or glucose upon NaCl shock. As shown in Fig. 5A, galactose growth reduced glycerol accumulation of the cells by ~60%. Accordingly, yeast cells grown with galactose as the carbon source were less tolerant to salt stress and showed a greatly decreased growth efficiency under NaCl stress conditions (Fig. 5B). We then measured the dose-regulated transcriptional activation of the GRE2 gene upon glucose and galactose growth by the real time luciferase assay (Fig. 5C). GRE2 induction occurred with an increasing delay toward higher salt stress conditions in galactose medium. Additionally the quantitative analysis of the GRE2 activation efficiency (Fig. 5D) revealed that galactose grown cells reached half-maximal GRE2 activation at much lower NaCl concentrations (186 mM) as compared with glucose grown cells (257 mM). Activated gene expression at GRE2 is dynamically increased within a broad NaCl concentration range (0.1–0.8 M) in glucose, but in a much more limited range (0.1–0.3 M) in galactose. These data demonstrate that the capacity of the yeast cell to counteract osmoticstress by glycerol accumulation is critically modulated by the carbon source, which in turn determines the efficiency and kinetics of transcriptional activation.

We then tested how glycerol production and vacuolar ATPase activity contributed to the resistance to NaCl stress. As expected, growth of a gpd1 mutant strain was progressively delayed with increasing salt concentrations (Fig. 5E). A weaker sensitivity phenotype was observed for the vma1 mutant, in which a significant growth delay was only observed upon severe NaCl stress. However, in galactose medium, in which glycerol accumulation is impaired, the loss of Vma1 function led to a very strong salt sensitivity (Fig. 5E). These data indicated that both glycerol production and vacuolar ion sequestration might independently contribute to salt resistance of yeast cells.

Loss of Gpd1 and Vma1 Function Causes Defects in the Distribution of RNA PolII Complexes during Salt Stress—Activated gene expression upon salt shock seemed to be delayed and to occur with reduced efficiency in gpd1 or vma1 mutant strains. We wanted to find out whether both mutations affected the redistribution of RNA polymerase II complexes from housekeeping to stress-induced genes. Therefore, we followed the association of RNA PolII at both types of genes during the first minutes of NaCl stress by in vivo ChIP. As shown in Fig. 6A, we
Galactose growth reduces glycerol accumulation, growth efficiency, and the dynamics of transcriptional activation upon salt stress. A, measurement of intracellular glycerol accumulation in yeast wild type (BY4741). Cells were grown to mid-log phase with glucose or galactose as the carbon source and then subjected to osmotic stress by the addition of 0.8 mM NaCl. The glycerol content of the cells was determined in triplicate at the indicated time points. Error bars are S.D. B, growth kinetics upon salt stress of yeast wild type cells (BY4741) with glucose or galactose as the carbon source. The growth curves are the media of three independent yeast cultures for each condition. The error is S.D. C, comparison of the dose-response profile for the GRE2 reporter gene were grown in glucose or galactose containing minimal medium and then treated with the indicated NaCl concentrations. The expression of the luciferase reporter was measured continuously in the living cell. The time needed to reach maximal reporter expression is indicated for each carbon source. The stressor concentration to reach 50% of the maximal reporter gene activity (EC50) is indicated for both growth conditions. The data shown are the media of three independent yeast cultures for each condition. The error is S.D. D, the maximal reporter activity was calculated for the glucose or galactose grown cells and plotted against the NaCl concentration. The maximal luciferase activity was adjusted to 100 for each carbon source. The stressor concentration to reach 50% of the maximal reporter gene activity (EC50) is indicated for both growth conditions. Error bars are S.D. E, growth kinetics of gpd1 and vma1 mutant strains compared with wild type. Left panel, growth upon NaCl stress is compared in wild type and gpd1 mutant in glucose media; middle and right panels, growth upon NaCl stress is compared in wild type and vma1 mutant in glucose and galactose media. The data shown are the media of three independent yeast cultures for each condition. The error is S.D. OD, optical density.
confirmed that the delayed gene induction at the salt-inducible GRE2 and STL1 loci previously observed in a gpd1 mutant strain was due to a delayed and less efficient recruitment of RNA PolII at the respective promoter regions. Further inspection of RNA PolII occupancy at two highly expressed genes (TDH3 and RPL2B; Fig. 6B) revealed that indeed the loss of gene expression upon salt stress observed in yeast wild type cells was accompanied by a transient physical loss of polymerase complexes from these gene promoters. In the first instances of salt stress, loss of PolII from housekeeping promoters is comparable in wild type and gpd1 mutants. However, in gpd1 mutant cells, PolII reassociation occurs with a clear delay. We then extended the ChIP analysis of PolII redistribution to the vma1 mutant. In this case, we also observed a delayed, but not reduced, accumulation of RNA PolII at the inducible GRE2 and STL1 promoters (Fig. 6C). However, at housekeeping genes, the vma1 mutant did not show a more pronounced loss of RNA PolII (Fig. 6D). These data demonstrated that cells lacking Gpd1 or Vma1 function have difficulties in rapidly mounting transcriptional initiation at stress defense genes. Additionally it seems that the delayed RNA PolII association with inducible promoters is not the consequence of a slower dissociation of polymerase from housekeeping genes.

FIGURE 6. The function of Gpd1 and Vma1 is important for the fast redistribution of RNA PolII from housekeeping to stress-inducible genes. ChIP analysis of Rpb3 in the indicated strain backgrounds. The cells were grown to mid-log phase and then subjected to a brief osmotic shock (0.4 M NaCl). RNA PolII occupancy was quantified by Rpb3-HA ChIP at the indicated gene promoters and normalized to the POL1 control region. Occupancy levels before stress were arbitrarily set to 1. The data shown are mean values from three independent chromatin samples. The error bars are S.D. A, comparison of Rpb3 association with the inducible GRE2 and STL1 promoters in wild type yeast (W303-1A) and the gpd1 deletion mutant. B, comparison of Rpb3 association with the TDH3 and RPL2B housekeeping gene promoters in wild type yeast (W303-1A) and the gpd1 deletion mutant. C, comparison of Rpb3 association with the inducible GRE2 and STL1 promoters in wild type yeast (W303-1A) and the vma1 deletion mutant. D, comparison of Rpb3 association with the TDH3 and RPL2B housekeeping gene promoters in wild type yeast (W303-1A) and the vma1 deletion mutant.
Dissociation of RNA PolII from Housekeeping Genes Occurs Independently of Its Recruitment at Inducible Genes—We have shown above that activated transcription upon salt stress occurs somehow coordinated with very low expression levels at housekeeping genes. We wanted to know whether the induced recruitment of the transcription machinery at stress genes caused its loss from highly expressed genes. To this end, we took advantage of the \textit{hog1} mutant strain, which is known to be defective in the activated recruitment of RNA PolII at the vast majority of osmostress-inducible genes (26, 27). This defect was confirmed for the \textit{GRE2} gene in response to NaCl stress (Fig. 7A). However, the same initial dissociation of RNA PolII was observed for the \textit{hog1} mutant at the \textit{TDH3} and \textit{RPL2B} genes (Fig. 7, B and C). Moreover, reassociation of the transcription machinery was severely delayed in the \textit{hog1} strain. These data indicated that the loss of RNA PolIII complexes from housekeeping genes was independent of the stimulated transcriptional initiation at stress-inducible genes. Loss of transcriptional activity at housekeeping genes might therefore be the direct result of the salt stress, as previously described for NaCl shock (21). We thus used a less severe salt treatment by KCl to assay the association kinetics of RNA PolII by ChIP. KCl caused a similar transient association of the transcription machinery at \textit{GRE2}, which again was completely dependent on Hog1 function. In this case, the dissociation of RNA PolIII was less pronounced at \textit{RPL2B} and not observed at \textit{TDH3}, whereas \textit{hog1} mutants showed a substantial and prolonged loss of transcription complexes at both housekeeping genes also upon KCl stress. These data show that the dissociation of RNA PolII complexes occurs dependent on the severity of cationic stress but independently of Hog1 function. The MAP kinase function in turn is essential for the reassociation of transcription complexes at housekeeping genes.

Effects of RNA PolII Turnover and Nuclear Import on the Coordinated Gene Expression upon Salt Stress—So far we have determined that physiological processes such as the intracellular ion homeostasis and osmolyte biosynthesis affect the kinetics and efficiency of the transcriptional response to salt stress. Given that the luciferase reporter applied here allow monitoring of dynamic gene expression changes in a very sensitive manner, we investigated how the recycling and nuclear abundance of RNA PolII complexes affected positive and negative regulation upon salt stress. We considered two mutant strains: \textit{def1} and \textit{iwr1}. Def1 marks arrested RNA PolII elongation complexes for proteasomal degradation (35). As shown in Fig. 8A, we observed a slightly decreased decay of the housekeeping lucif-
expression during the adaptation to high salt stress. Biological processes to critically affect the dynamics of gene activation. Taken together, we report here that a reduced pool of RNA PolII does not affect the stress gene was completely normal (Fig. 8).

FIGURE 8. Def1, but not iwr1, is important for efficient GRE2 induction upon salt stress. Yeast wild type (BY4741) and the def1 and iwr1 mutant strains expressing the TDH3-lucCP and GRE2-lucCP reporter gene were subjected to salt stress by the addition of 0.4 M NaCl. The expression of the luciferase reporters was measured continuously in the living cell. The data shown are mean values from six independent experiments and represent the relative light emission (log2 ratio) normalized for the mock treated cells of the same genetic background. A, comparison of the housekeeping (GPD) and stress-inducible (GRE2) luciferase reporter in wild type and the def1 mutant. B, comparison of the housekeeping (TDH3) and stress-inducible (GRE2) luciferase reporter in wild type and the iwr1 mutant. Significance values (*, p < 0.05; **, p < 0.002) were obtained with Student’s t test for the indicated intervals. Rel., relative.

erase reporter in a def1 mutant. Additionally we detected an apparently slower recovery of gene expression after salt shock in this mutant. The most prominent defect, however, was manifested in the def1 mutant for the activated GRE2 expression (Fig. 8A), which was reduced to less than 40%.

Iwr1 is an RNA PolII nuclear import factor, and its mutation causes a reduction of active PolII transcription complexes in the nucleus (36, 37). As depicted in Fig. 8B, the iwr1 mutant strain showed an increased loss and delayed recovery of housekeeping gene activity upon salt shock, a phenotype, which resembled the gpd1 mutant lacking proper osmolyte production. However, in this case the efficient induction of the GRE2 stress gene was completely normal (Fig. 8B). These data suggested that a reduced pool of RNA PolII does not affect the transient activation of stress genes but might be limiting for reactivating housekeeping genes after stress adaptation. Furthermore, it is possible that removal and turnover of nonproductive RNA PolII complexes during salt shock is necessary for efficient gene activation. Taken together, we report here physiological processes to critically affect the dynamics of gene expression during the adaptation to high salt stress.

DISCUSSION

In this work, we identify physiological functions that determine the dynamics of an adaptive transcriptional response in the case of salt stress. This scenario is chosen here because adaptation to salinity stress requires a considerable modification in gene expression, but the stress itself imposes a serious hurdle for efficient transcription. Therefore this type of adverse environmental condition is a good model to improve our understanding of the interplay between the physiological state of the cell and its ability to trigger an adequate activation of defense genes. It is well known that an increasing hyperosmotic challenge causes a delayed transcriptional response of yeast cells (38). Some molecular aspects of how high salinity interferes with the activation of gene expression have been revealed recently. Accordingly, hyperosmolarity slows down signal transduction through the MAP kinase pathway because of molecular crowding, delays the nuclear accumulation of activated Hog1 MAP kinase, and generally interferes with the association of specific and general transcription factors to chromosomal DNA (21, 23–25). Thus, yeast cells must have mechanisms to overcome the general inhibition of transcription under salt stress conditions, which can be described now in detail with the data presented here (Fig. 9).

An immediate consequence of salt stress is the instantaneous loss of gene expression from highly transcribed housekeeping genes. This rapid inhibition is not dependent on the stress dose and occurs similarly at the beginning of different NaCl exposures (Fig. 1); however, the salt dose determines how long the housekeeping functions remain untranscribed. Loss of housekeeping gene expression is caused by the dissociation of RNA PolII complexes from their promoters (Figs. 6 and 7). It is important to note that this loss of transcription complexes is not the cause of their reallocation at stress-inducible promoters as has been suggested recently (26), because a hog1 mutant with largely absent transcriptional initiation at stress-induced loci shows an increased and prolonged loss of PolII complexes from housekeeping genes. Thus, the immediate, and most likely passive, loss of transcriptional activity upon salt challenge seems to lead to a lag phase of adaptation in which apparently neither constitutive nor stress-inducible genes are expressed (Fig. 1). This phase becomes continuously longer with increasing salt doses. Previous work has identified the Hog1-dependent activation of plasma membrane transporters such as Nha1 to be
Indeed, glycerol accumulation occurs immediately after salt stress causes a rapid loss of transcription complexes and gene expression activity at housekeeping genes, which is followed by a lag phase of low transcriptional activity in general. The length of the lag phase is determined by different processes such as the biosynthesis of osmolytes (glycerol) or the intracellular ion distribution (vacuolar ATPase). Additionally, Def1 might play a role in the disassembly of RNA PolII at housekeeping genes and the assembly of active transcription complexes at stress-induced genes. The lag phase is followed by the transient gene expression burst at activated genes, whereas housekeeping genes remain completely inactive. Finally, the decline in activated expression at defense genes sets the mark for the subsequent recovery of RNA PolII complexes and expression at housekeeping genes.

Our results show that glycerol accumulation in the first minutes upon salt stress exposure determines the onset and efficiency of transcriptional activation. Impairment of glycerol biosynthesis causes a prolonged loss of housekeeping gene expression and a delayed activation at defense genes. Because the protective effect of glycerol is shown here to occur before the first wave of stress defense gene expression, we have to evoke mechanisms that do not rely on de novo protein synthesis and therefore are different from the well described transcriptional control of glycerol synthesis enzymes Gpd1 or Gpp2. Indeed, glycerol accumulation occurs immediately after salt exposure independently of de novo enzyme synthesis (20, 39, 40). Additionally, nontranscriptional mechanisms act on glycolytic enzyme activities such as Pfk2 (41) and on glycolytic transporters such as Fps1 (42) to facilitate glycerol biosynthesis and intracellular accumulation upon osmotic shock. Thus, during the lag phase before the activation of gene expression, glycolytic intermediates are temporarily directed toward glycerol production, which in turn facilitates an efficient transcriptional adaptation. In this scenario, we expect any decrease in glycolytic flux to cause a poor glycerol accumulation and a delayed transcriptional response. This is shown here by simply switching yeast cells from glucose to galactose metabolism, which is known to reduce the flux through glycolysis and to shift the metabolism partially toward respiration (43). The consequences for osmotic adaptation are shown in Fig. 5 and include a notable reduction in intracellular glycerol accumulation in accordance with previous work (44), a decrease in growth efficiency, and a much less dynamic and efficient activation of defense gene expression upon salt stress. Thus, we describe an example in which the physiological state of the cell, determined in this case by its mode of carbohydrate metabolism, is an important modulator of its transcriptional stress defense.

Apart from osmolyte production, the intracellular ion homeostasis also plays an important role in determining the transcriptional response to salt stress. According to our results, a lack of vacuolar ATPase activity delays the initiation of transcription at inducible genes upon salt stress (Figs. 3, 4, and 6). The proton translocating V-ATPase acidifies the vacuolar lumen and thus energizes the import of cations such as Na\(^+\) by other vacuolar transporters (45). Indeed, the function of the vacuolar ATPase is important for the survival of yeast cells upon severe salt stress (22, 46). Moreover, the fragmentation of vacuoles upon ionic stress might be an adaptive mechanism to ensure an enhanced sequestration of toxic Na\(^+\) ions upon salt stress (47). Of note, whereas the V-ATPase activity is salt-inducible, it seems to be important for proper ion homeostasis also under normal growth conditions, because vma mutants show elevated HOG pathway activation even in the absence of salt stress (22). These observations fit with a model in which in the initial phase of salt stress adaptation, both the biosynthesis of glycerol and the sequestration of toxic ions in the vacuoles are important and fast mechanisms to decrease the excess of Na\(^+\) in the cytosol and nucleus. Any interference with this pre-
transcriptional adaptation will result in a delay of the relatively slower transcriptional response (Fig. 9).

Changes in the gene expression pattern upon salt stress are profound and aim to equip the cell with a different protein composition, which is more compatible with the changing environment of the cells. Although transcription initiation complexes have to form at many stress defense loci simultaneously, this process does not seem to depend on an especially high pool of active RNA PolII complexes. We show here that an RNA PolII import-deficient cell (iwr1) is not affected in the timely induction of osmostress-responsive genes. This could indicate that the amount of active PolII complexes in the nucleus is not a limiting factor for a massive gene activation at stress genes. Probably most transcription complexes can be derived from the transiently repressed housekeeping loci. Interestingly, we find that a lack of RNA PolII turnover in a def1 mutant strongly interferes with the efficient activation of a stress gene upon NaCl stress. Def1 plays a crucial role in the targeting, ubiquitilation, and degradation of stalled RNA PolII elongation complexes (35, 48). This phenomenon has been discovered upon treatment with DNA damaging agents or irradiation, which causes transcriptional arrest. However, ubiquitilation of RNA PolII also occurs upon reduction of its elongation or promoter escape rate in the absence of physical DNA lesions (49). Salt stress could in fact provoke very inefficient transcriptional elongation because it generally interferes with the association of DNA bound proteins in vivo (21). Thus, salt stress could also activate the Def1-mediated RNA PolII removal from unproductive sites in the genome to allow the efficient activation at inducible loci. Our preliminary data shown here might therefore stimulate research on the broader implication of RNA PolII turnover in stress responses. Taken together, our work demonstrates that the dynamics and efficiency of stress-induced gene activation is not only conditioned by the directly implicated transcription factors and signal transducers but also by many other physiological adaptations.

Acknowledgments—We thank Francisco Estruch for helpful discussions and the gift of the def1 and iwr1 yeast deletion strains. We thank Ramón Serrano for the gift of the ena1–4 deletion strain.

REFERENCES
1. de Nadal, E., Ammerer, G., and Posas, F. (2011) Controlling gene expression in response to stress. Nat. Rev. Genet. 12, 833–845
2. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11, 4241–4257
3. Gasch, A. P., and Werner-Washburne, M. (2002) The genomics of yeast responses to environmental stress and starvation. Funct. Integr. Genomics 2, 181–192
4. Saito, H., and Posas, F. (2012) Response to hyperosmotic stress. Genetics 192, 289–318
5. de Nadal, E., and Posas, F. (2010) Multilayered control of gene expression by stress-activated protein kinases. EMBO J. 29, 4–13
6. Hohmann, S. (2009) Control of high osmolarity signalling in the yeast Saccharomyces cerevisiae. FEBS Lett. 583, 4025–4029
7. de Nadal, E., Casadóme, L., and Posas, F. (2003) Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. Mol. Cell. Biol. 23, 229–237
8. Martínez-Montañés, F., Pascual-Ahuir, A., and Profi, M. (2010) Toward a genomic view of the gene expression program regulated by osmostress in yeast. OMICS 14, 619–627
9. Profi, M., Pascual-Ahuir, A., de Nadal, E., Ariño, J., Serrano, R., and Posas, F. (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J. 20, 1123–1133
10. Proft, M., and Serrano, R. (1999) Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in Saccharomyces cerevisiae: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol. Cell. Biol. 19, 537–546
11. Rep, M., Reiser, V., Gartner, U., Thevelein, J. M., Hohmann, S., Ammerer, G., and Ruis, H. (1999) Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. Mol. Cell. Biol. 19, 5474–5485
12. Ruiz-Roig, C., Noriega, N., Duch, A., Posas, F., and de Nadal, E. (2012) The Hog1 SAK complex controls the Rtg1/Rtg3 transcriptional complex activity by multiple regulatory mechanisms. Mol. Biol. Cell 23, 4286–4296
13. Westfall, P. J., Patterson, J. C., Chen, R. E., and Thorer, J. (2008) Stress resistance and signal fidelity independent of nuclear MAPK function. Proc. Natl. Acad. Sci. U.S.A. 105, 12121–12127
14. Ariño, J., Aydar, E., Druhl, S., Ganser, D., Jorrín, J., Kahm, M., Krause, F., Petrezselyová, S., Yenush, L., Zimmermannová, O., van Heusden, G. P., Kschisch, M., Ludwig, J., Palmer, C., Ramos, J., and Sychrová, H. (2014) Systems biology of monovalent cation homeostasis in yeast: the translucen contribution. Adv Microb Physiol 64, 1–63
15. Hohmann, S. (2002) Osmotic adaptation in yeast: control of the yeast osmoysyte system. Int. Rev. Cytol. 215, 149–187
16. Hohmann, S., Kranz, M., and Nordlander, B. (2007) Yeast osmoregulation. Methods Enzymol. 428, 29–45
17. Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. (1994) GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. 14, 4135–4144
18. Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., and Adler, L. (1997) The two isoenzymes for yeast NAD+-dependent glycerol-3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmo-adaptation and redox regulation. EMBO J. 16, 2179–2187
19. Norbeck, J., Pählman, A. K., Akhtar, N., Blomberg, A., and Adler, L. (1996) Purification and characterization of two isoenzymes of α-glycerol-3-phosphatase from Saccharomyces cerevisiae: identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 271, 13875–13881
20. Klipp, E., Nordlander, B., Krüger, R., Genenmark, P., and Hohmann, S. (2005) Integrative model of the response of yeast to osmotic shock. Nat Biotechnol 23, 975–982
21. Profi, M., and Struhl, K. (2004) MAP kinase-mediated stress relief that precedes and regulates the timing of transcriptional induction. Cell 118, 351–361
22. Li, S. C., Diakov, T. T., Rizzo, J. M., and Kane, P. M. (2012) Vacular H+-ATPase works in parallel with the HOG pathway to adapt Saccharomyces cerevisiae cells to osmotic stress. Eukaryot. Cell 11, 282–291
23. Babazadeh, R., Adiels, C. B., Smedh, M., Petelenz-Kurdziel, E., Goksör, M., and Hohmann, S. (2013) Osmostress-induced cell volume loss delays yeast Hog1 signaling by limiting diffusion processes and by Hog1-specific effects. PLoS One 8, e80991
24. Miermont, A., Waharte, F., Hu, S., McClean, M. N., Bottani, S., Léon, S., and Hersen, P. (2013) Severe osmotic compression triggers a slowdown of intracellular signaling, which can be explained by molecular crowding. Proc. Natl. Acad. Sci. U.S.A. 110, 5725–5730
25. Van Wuytswinkel, O., Reiser, V., Siderius, M., Kelders, M. C., Ammerer, G., Ruis, H., and Mager, W. H. (2000) Response of Saccharomyces cerevisiae to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway. Mol. Microbiol. 37, 382–397
26. Cook, K. E., and O’Shea, E. K. (2012) Hog1 controls global reallocation of RNA Pol II upon osmotic shock in Saccharomyces cerevisiae. G3 2, 1129–1136
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27. Nadal-Ribelles, M., Conde, N., Flores, O., González-Vallinas, I., Eyras, E., Orozco, M., de Nadal, E., and Posas, F. (2012) Hog1 bypasses stress-mediated down-regulation of transcription by RNA polymerase II redistribution and chromatin remodeling. Genome Biol. 13, R106

28. Dolf-Edo, L., Rienzo, A., Poveda-Huertes, D., Pascual-Ahuir, A., and Prof, M. (2013) Deciphering dynamic dose responses of natural promoters and single cis elements upon osmotic and oxidative stress in yeast. Mol. Cell Biol. 33, 2228–2240

29. Berry, D. B., Guan, Q., Hose, J., Haroon, S., Gebbia, M., Heisler, L. E., Nislow, C., Giaever, G., and Gasch, A. P. (2011) Multiple means to the same end: the genetic basis of acquired stress resistance in yeast. PLoS Genet. 7, e1002353

30. Guan, Q., Haroon, S., Bravo, D. G., Will, J. L., and Gasch, A. P. (2012) Cellular memory of acquired stress resistance in Saccharomyces cerevisiae. Genetics 192, 495–505

31. Wizneler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Banham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Fourny, F., Friend, S. H., Gentenal, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M’Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. I., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Véronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Philippens, P., Johnston, M., and Davis, R. W. (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285, 901–906

32. Rienzo, A., Pascual-Ahuir, A., and Prof, M. (2012) The use of a real-time luciferase assay to quantify gene expression dynamics in the living yeast cell. Yeast 29, 219–231

33. Alberti, S., Gitler, A. D., and Lindquist, S. (2007) A suite of Gateway cloning vectors for high-throughput genetic analysis in Saccharomyces cerevisiae. Protoc. Mol. Biol.

34. Aparicio, O., Geisberg, J. V., Sekinger, E., Yang, A., Moqtaderi, Z., and Thevelein, J. M. (2012) The use of a real-time luciferase assay to quantify gene expression dynamics in the living yeast cell. PLoS One 6, e20829

35. Rep, M., Albertyn, J., Thevelein, J. M., Prior, B. A., and Hohmann, S. (1999) Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in Saccharomyces cerevisiae. Microbiology 145, 715–727

36. Bourman, J., Kiewiet, J., Lindenbergh, A., van Eunen, K., Siderius, M., and Bakker, B. M. (2011) Metabolic regulation rather than de novo enzyme synthesis dominates the osmo-adaptation of yeast. Yeast 28, 43–53

37. Petelzen-Kurdziel, E., Kuehn, C., Nordlander, B., Klein, D., Hong, K. K., Jacobson, T., Dahl, P., Schaber, J., Nielsen, J., Hohmann, S., and Klipp, E. (2013) Quantitative analysis of glycerol accumulation, glycolysis and growth under hyper osmotic stress. PLoS Comput. Biol. 9, e1003084

38. Dihazi, H., Kessler, R., and Esrich, K. (2004) High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. J. Biol. Chem. 279, 23961–23968

39. Tamás, M. J., Luyten, K., Sutherland, F. C., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B. A., Kilian, S. G., Ramos, J., Gustafsson, L., Thevelein, J., and Hohmann, S. (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol. Microbiol. 31, 1087–1104

40. Ostergaard, S., Olsson, L., Johnston, M., and Nielsen, J. (2000) Increasing galactose consumption by Saccharomyces cerevisiae through metabolic engineering of the GAL gene regulatory network. Nat. Biotechnol. 18, 1283–1286

41. Rios, G., Ferrando, A., and Serrano, R. (1997) Mechanisms of salt tolerance conferred by overexpression of the HAL1 gene in Saccharomyces cerevisiae. Yeast 13, 515–528

42. Li, S. C., and Kane, P. M. (2009) The yeast lysosome-like vacuole: endpoint and crossroads. Biochim. Biophys. Acta 1793, 650–663

43. Hamilton, C. A., Taylor, G. J., and Good, A. G. (2002) Vacuolar H+-ATPase, but not mitochondrial F1F0-ATPase, is required for NaCl tolerance in Saccharomyces cerevisiae. FEMS Microbiol. Lett. 208, 227–232

44. Jacobson, T., Dahl, P., Schaber, J., Nielsen, J., Hohmann, S., and Klipp, E. (2003) Extra-cellular Ca2+ sensing contributes to excess Ca2+ accumulation and vacuolar fragmentation in a pmr1Delta mutant of S. cerevisiae. J. Cell Sci. 116, 1637–1646

45. Wilson, M. D., Harreman, M., Taschner, M., Reid, J., Walker, J., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (2013) Proteasome-mediated processing of Def1, a critical step in the cellular response to transcription stress. Cell 154, 983–995

46. Somesh, B. P., Reid, J., Liu, W. F., Søgaard, T. M., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (2005) Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest. Cell 121, 913–923