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Different Mechanisms Confer Gradual Control and Memory at Nutrient- and Stress-Regulated Genes in Yeast

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Cells respond to environmental stimuli by fine-tuned regulation of gene expression. Here we investigated the dose-dependent modulation of gene expression at high temporal resolution in response to nutrient and stress signals in yeast. The *GAL1* activity in cell populations is modulated in a well-defined range of galactose concentrations, correlating with a dynamic change of histone remodeling and RNA polymerase II (RNAPII) association. This behavior is the result of a heterogeneous induction delay caused by decreasing inducer concentrations across the population. Chromatin remodeling appears to be the basis for the dynamic *GAL1* expression, because mutants with impaired histone dynamics show severely truncated dose-response profiles. In contrast, the *GRE2* promoter operates like a rapid off/on switch in response to increasing osmotic stress, with almost constant expression rates and exclusively temporal regulation of histone remodeling and RNAPII occupancy. The Gal3 inducer and the Hog1 mitogen-activated protein (MAP) kinase seem to determine the different dose-response strategies at the two promoters. Accordingly, *GAL1* becomes highly sensitive and dose independent if previously stimulated because of residual Gal3 levels, whereas *GRE2* expression diminishes upon repeated stimulation due to acquired stress resistance. Our analysis reveals important differences in the way dynamic signals create dose-sensitive gene expression outputs.

Cells continuously adapt their protein composition to changing environmental conditions. The regulation of gene expression is one of the fundamental mechanisms to adjust the global protein repertoire of the cell in order to maintain cell function and integrity in response to environmental challenges. Budding yeast is a powerful model to unravel the modes of transcriptional adaptation at the levels both of specific genes and of the whole organism (1, 2). Additionally, the basic structure of the signaling cascades responding to environmental perturbations is conserved from yeast to humans. It implies the alteration of core kinase activities, which modulate the expression of defense genes through a range of specific transcription factors. Extensive knowledge which precisely describes the molecular machinery and its global impact on gene expression in response to many types of stress has accumulated (3–7). However, the vast majority of these studies are performed with harsh environmental insults and therefore saturating stimulation. As a consequence, only very limited information or approaches are available to understand how cells adapt their gene expression programs to small or gradual changes in their environment.

It is assumed that cells have acquired mechanisms that ensure a transcriptional response which is finely adjusted according to the strength of the stress or stimulation. However, the nature of the signaling molecules which confer gradual transcription outputs remains to be determined in most cases. Fine-tuning of gene expression responses can occur with different purposes, and the generation of a graded response can be achieved at different stages along the signal transduction path. For example, a linear response to mating pheromone has been described for the yeast mating mitogen-activated protein (MAP) kinase cascade (8). Additionally, specific transcriptional activators such as yeast Msn2 or Crz1 and mammalian NF-κB transmit linear signals to their cognate promoters by modulating their nuclear accumulation (9–12).

The same signal transduction pathway might have to distinguish related signals that originated from different stressors. This has been very recently described for yeast Msn2, a transcription factor responding to general stress and capable of filtering different stress inputs to generate graded gene expression outputs (13). Furthermore, among the often numerous genes activated in response to a given stress, the cell has to impose different sensitivities to guarantee an equilibrated adaptive response. Here, chromatin structure has been implied in modulating the threshold of gene activation in the yeast phosphate response (14), and different natural promoters and *cis* regulatory elements confer characteristic dose-sensitive expression profiles upon osmotic and oxidative stress (15).

Here we investigated the mechanisms that confer a gradual and dose-sensitive gene expression for two types of environmental cues: (i) the availability of a specific carbon and energy source and (ii) cytotoxic stress. We used two very well defined model genes, the nutrient-regulated *GAL1* and stress-regulated *GRE2* genes.

The expression of the yeast *GAL* genes is specifically upregulated by the presence of galactose in the growth medium via the transcriptional activator Gal4 (16). Gal4 is already bound at its target promoters under noninducing conditions (without galactose), but its activation domain is inhibited by direct binding of the Gal80 repressor protein (17, 18). Upon growth in glucose-

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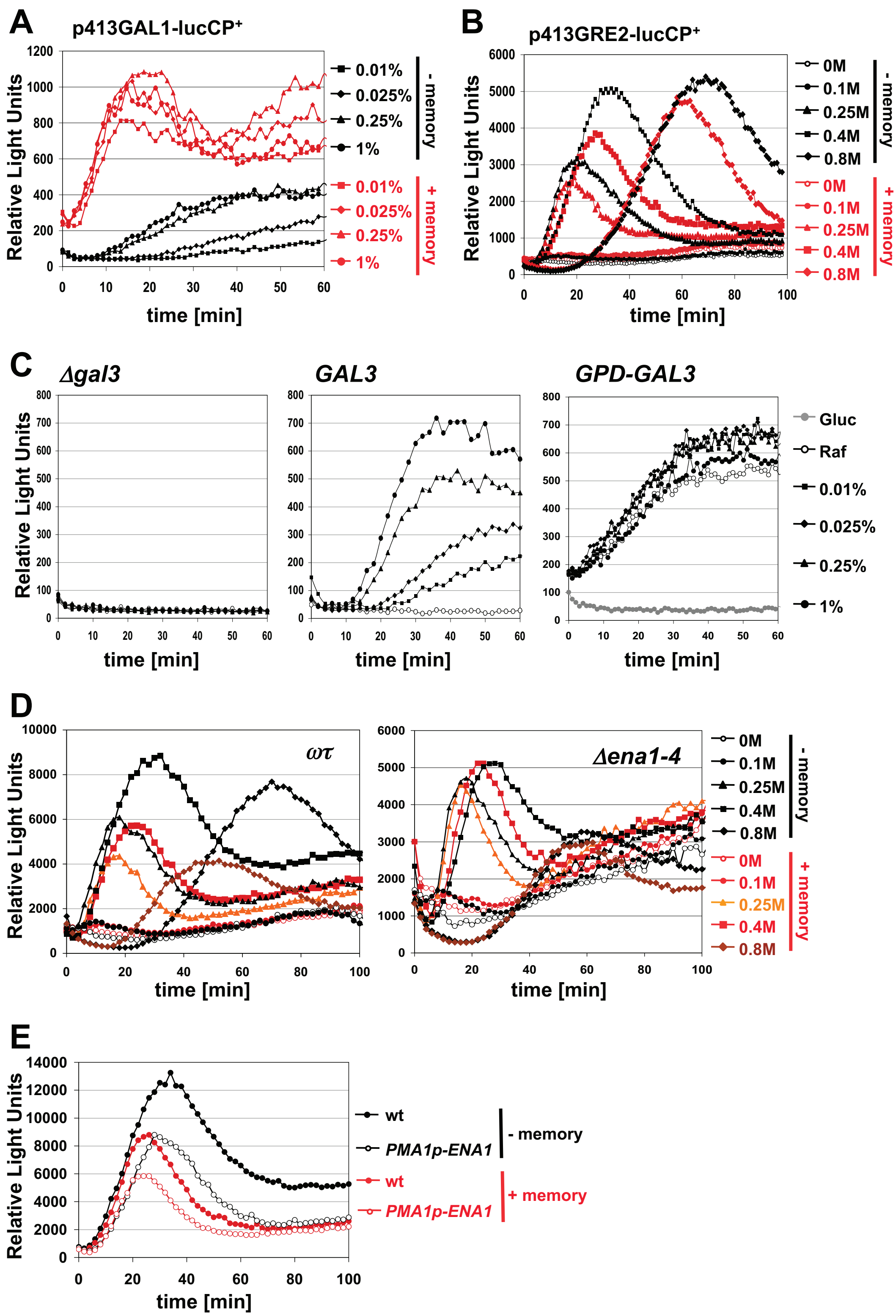


FIG 1 Comparison of transcriptional memory at the *GAL1* and *GRE2* genes. (A) *GAL1* expression is efficiently sensitized upon subsequent galactose induction. A *GAL1-lucCP*$^+$ reporter gene was used in live-cell luciferase assays to determine the expression rates in raffinose-containing minimal medium after supplementation with the indicated concentrations of galactose. Naive cells (− memory) were stimulated just once with galactose, while transcriptional memory (+ memory) was achieved by a previous galactose induction, as explained in Materials and Methods. (B) The stress-induced *GRE2* expression decreases and is not sensitized by repeated stimulation. A *GRE2-lucCP*$^+$ reporter gene was used in live-cell luciferase assays to determine the expression rates in glucose-containing minimal medium after supplementation with NaCl. Cells were pretreated with 0.7 M NaCl (+ memory) or not pretreated (− memory) before the induction with the indicated NaCl doses. (C) Effect of Gal3 levels on the dose response of *GAL1*. A *GAL1-lucCP*$^+$ reporter gene was used in live-cell luciferase

limiting and galactose-containing medium, *GAL* gene expression is induced with the help of the Gal3 inducer. Upon stimulation, Gal3 binds galactose and ATP, interrupts Gal80 inhibition of Gal4, and permits transcriptional activation (19–21). Gal4 additionally recruits chromatin-modifying complexes and mediator (22–27) in order to efficiently induce *GAL* gene expression.

GRE2 is a prototypical gene involved in the hyperosmotic and oxidative stress defense, which includes the stimulated gene expression encompassing hundreds of different cellular functions (5, 28). Its promoter is bound by the specific transcription factor Sko1 in a complex with the general corepressor Cyc8-Tup1 under normal noninducing growth conditions (29). Upon hyperosmotic stress, transcriptional activation of *GRE2* is rapidly achieved by the association of Sko1 with the stress-activated MAP kinase Hog1, which switches Sko1 from repression to activation by multiple phosphorylation and the additional recruitment of chromatin modifiers and the mediator complex (30). As a result, *GRE2* gene expression is very fast and transiently activated, as commonly observed for transcriptional stress responses in yeast.

Additionally, the cell's history can modulate the transcriptional response at specific genes. Transcriptional memory has been described for several inducible yeast genes, including *GAL1*. Here, a previous galactose induction facilitates the transcriptional response to the second galactose exposure. Different mechanisms have been proposed to establish transcriptional memory at the *GAL* genes, including the tethering of actively transcribed *GAL1* to the nuclear envelope via the histone variant Htz1, prolonged chromatin remodeling via Swi/Snf, or the inheritance of signaling compounds such as the Gal1 and Gal3 inducers (31–34).

The general architectures of the *GAL1* and *GRE2* regulons are very similar and involve a switch of a promoter-bound transcription factor from an inactive (or repressed) state to an active state by the direct association with a specifically activated inducer. Here, we identify important differences in how both systems respond to gradual or repeated stimulation. This was possible by the high temporal resolution and quantification of gene expression using destabilized luciferase reporters for the two types of genes. This allowed us to accurately define the dynamic range of gene regulation and identify the molecules which modulate the characteristic dose-response pattern for *GAL1* and *GRE2* during nutrient sensing or acute osmotic stress adaptation.

MATERIALS AND METHODS

Yeast strains. The *Saccharomyces cerevisiae* strains used in this study were wild-type (wt) BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and strains carrying the mutant alleles *gal3*::*KanMX4*, *gcn5*::*KanMX4*, *snf2*::*KanMX4*, *gal11*::*KanMX4*, and *htz1*::*KanMX4* (35). Yeast strains expressing chromosomally tagged TAP fusion proteins were BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) with *GAL4-TAP-His3MX* and *GAL3-TAP-His3MX* (36). Yeast strains expressing chromosomally tagged hemagglutinin (HA) fusion proteins were W303-1A (*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) with *3×HA-HOG1, 3×HA-SKO1* (30), and *3×HA-RPB3* (37). Yeast strains expressing firefly luciferase were MMY116-2C (*MATα leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15*) without *GAL1-Fluc* and MMY116-2C with *GAL1-FLuc* integrated in the *URA3* locus (a gift from A. Mazo-Vargas). DBY746 (*MATα ura3-52 trp1-289 his3-Δ1 leu2-3,112*) and its *ena1-4*::*LEU2* derivative were used to study the effect of the *ENA* gene dose on the *GRE2* transcriptional memory.

Plasmid constructions. Single-copy reporter fusions with a destabilized luciferase gene (*lucCP*$^+$) were constructed as described previously (38). The upstream regulatory sequences of *GRE2* (nucleotides −940 to −7), *GAL1* (nucleotides −450 to −1) (38), *CTT1* (nucleotides −983 to −10), *SOD2* (nucleotides −977 to −16) (15), *ALD6* (nucleotides −785 to −2), and *HOR2* (nucleotides −948 to −33) (this study) were used. An integrative version of the *GAL1-lucCP*$^+$ reporter fusion was constructed by insertion of the *lucCP*$^+$ gene into the pAG306GAL1-ccdB Gateway destination vector (39), which was integrated into the *URA3* locus of yeast wild-type strain W303-1A. An integrative version of the *GRE2-lucCP*$^+$ reporter fusion was constructed by insertion of the *lucCP*$^+$ gene before the *KanMX* marker in the pUG6 plasmid. The *lucCP*$^+$ *KanMX*-containing cassette was PCR amplified and fused to the *GRE2* promoter in the genomes of yeast wild-type strains BY4741 and W303-1A. Multicopy integration plasmid pRS406-GAL1pr-Fluc was built by swapping *MET25* with the *GAL1* promoter in pRS406-MET25-Fluc and integrating into strain MMY116-2C (40). Both the plasmid and integrated strains were gifts from A. Mazo-Vargas. For constitutive or induced overexpression of *GAL3* under the control of the *TDH3* or *GAL1* promoter, the entire *GAL3* gene was inserted in the Gateway destination vectors pAG416GPD-ccdB and pAG416GAL1-ccdB (39). For constitutive overexpression of *ENA1* under the control of the *PMA1* promoter, the plasmid pRS699-ENA1 (a gift from J. M. Mulet, Valencia, Spain) was used.

Live-cell luciferase assays. Yeast strains containing the indicated luciferase fusion genes were grown at 28°C in synthetic dextrose (SD) or synthetic raffinose (SRaff) 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) to exponential growth phase. Culture aliquots were then incubated with 0.5 mM luciferin (Sigma) on a roller at 28°C for 90 min. The cells were then transferred in 100-μl aliquots in white 96-well plates (Nunc) with or without the indicated concentrations of NaCl, menadione, or galactose supplied from appropriate stock solutions. The light emission was then continuously recorded in a GloMax microplate luminometer (Promega) in three biological replicates. Data were processed with Microsoft Excel software. For representation of the relative light units of each reporter gene, we normalized the raw data for the number of cells in each assay. The maximal synthesis rate (V_{max}) and the maximal luciferase activity (A_{max}) were calculated as described previously (38).

ChIP. Chromatin immunoprecipitation (ChIP) was performed essentially as described previously (41). For the immunoprecipitation of HA fusion proteins, a mouse monoclonal anti-HA antibody (12CA5; Roche) was used in combination with Dynabeads protein A (Invitrogen). For the immunoprecipitation of TAP fusion proteins, pan-mouse IgG Dynabeads (Invitrogen) were used. For the immunoprecipitation of histone H3, a polyclonal anti-H3 antibody (ab1791; Abcam) was used in combination with Dynabeads protein A (Invitrogen). Quantitative PCR (qPCR) analyses at the indicated chromosomal loci were performed in real time using

assays to determine the expression rates in raffinose-containing minimal medium after supplementation with the indicated concentrations of galactose. Cells were grown in synthetic glucose medium before induction was started in synthetic raffinose medium containing the indicated galactose concentrations. Δ*gal3* mutants containing plasmid-carried *GAL3* under constitutive control (*GPD-GAL3*) or the empty vector (Δ*gal3*) were compared with wild-type cells containing the empty vector (*GAL3*). Constitutive overexpression of *GAL3* leads to *GAL1* induction by raffinose; therefore, an additional control in glucose-containing SD medium is included in the last panel. (D) The decrease of *GRE2* expression upon repeated NaCl induction depends on a functional *ENA* gene cluster. The indicated strains (DBY746 background) were compared for *GRE2-lucCP*$^+$ expression under conditions identical to those for panel B. (E) Transient activation of *GRE2* depends on the Ena1 levels. Yeast wild-type cells (BY4741) were assayed for *GRE2-lucCP*$^+$ expression in the presence of constitutive *ENA1* overexpression (*PMA1p-ENA1*) or the empty plasmid (wt) upon exposure to 0.4 M NaCl. Cells were pretreated (+ memory) or not (− memory) with 0.7 M NaCl. For all panels, the mean values for three independent biological replicas are shown for each galactose or NaCl concentration (standard deviation, <15%).

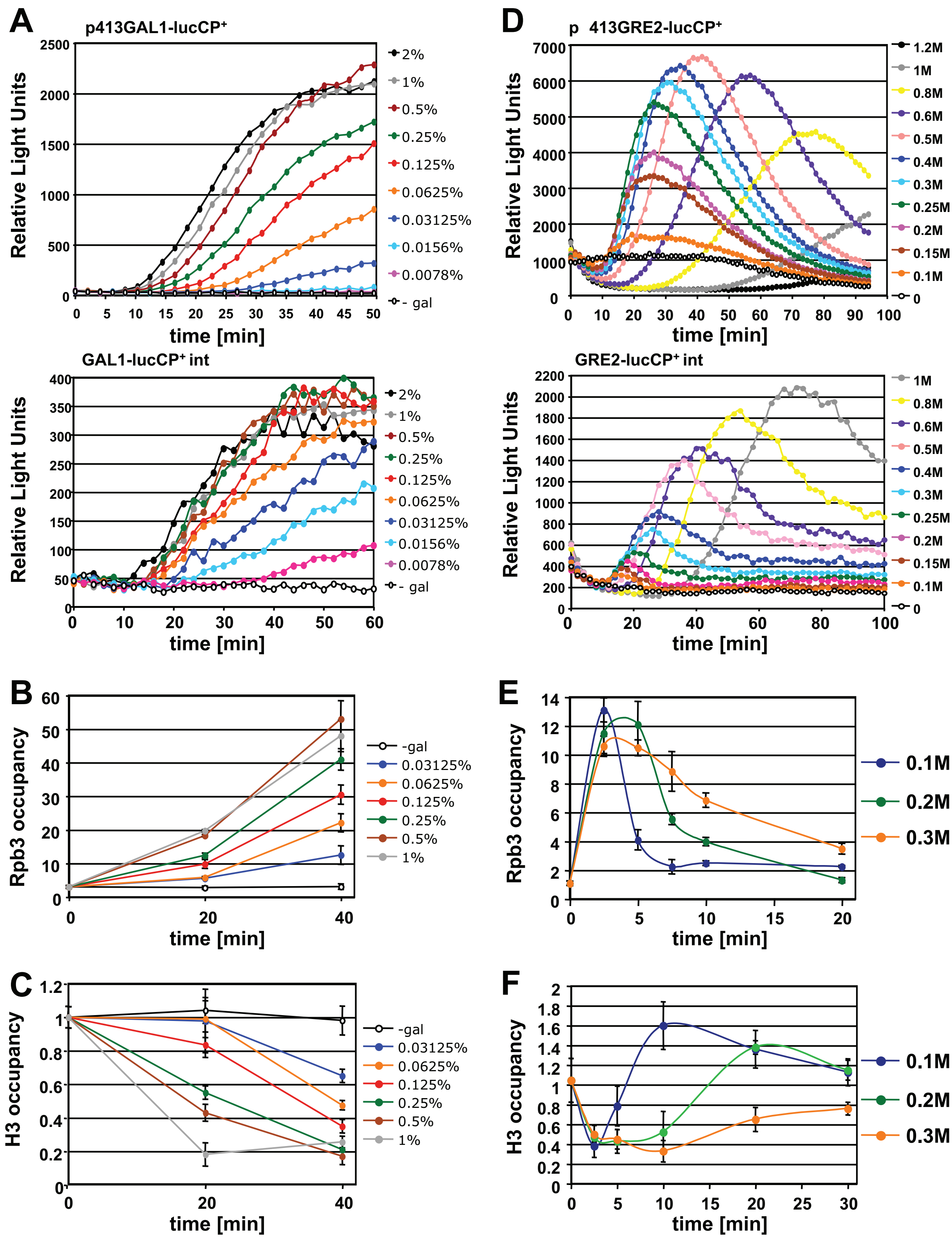


FIG 2 Comparison of the gradual gene expression, RNAPII occupancy, and histone remodeling at the *GAL1* and *GRE2* genes. (A) The expression of a *GAL1-lucCP*$^+$ reporter gene is dynamically modulated at the level of the synthesis rate. A live-cell luciferase assay was used to determine the expression rates in raffinose-containing minimal medium after supplementation with the indicated concentrations of galactose. *GAL1*-luciferase fusions were expressed from centromeric plasmids (upper panel) or after integration in the genome (lower panel). Data for the upper panel are from reference 38. The mean values for three independent biological replicas are shown for each galactose concentration (standard deviation, <15%). (B) Gradual association of RNAPII with the *GAL1* promoter is modulated by the galactose inducer concentration. ChIP of Rpb3-HA-expressing cells was used to determine the RNAPII density at the *GAL1*

an Applied Biosystems 7500 sequence detector and Fast EvaGreen Mastermix for qPCR (Biotium). All occupancy data are presented as fold IP over the *POL1* coding sequence (+1796/+1996) internal control. Each ChIP was performed in triplicate with three different chromatin samples. All primer sequences used for ChIP are available upon request.

Transcriptional memory experiments. For memory experiments at *GAL1*, cells containing the *GAL1-lucCP*$^{+}$ reporter gene were grown overnight in synthetic raffinose (SRaff) medium lacking histidine to exponential growth phase. A first round of induction was then performed for 2 h with 2% galactose, while naive cells remained in SRaff medium. Both cell cultures were then precipitated, washed once with water, and then incubated in fresh SD medium for 1 h. Finally, cells were changed to SRaff medium containing 0.5 mM luciferin for 90 min before starting the next induction with the indicated galactose concentrations and continuous measurement of luciferase activity. For the memory experiment under gradual Gal3 expression levels, the duration of the first round of galactose induction was reduced to 30 min.

For memory experiments at *GRE2*, cells containing the *GRE2-lucCP*$^{+}$ reporter gene were grown overnight in synthetic glucose (SD) medium lacking histidine to exponential growth phase. A first salt shock was applied by treatment with 0.7 M NaCl for 1 h, while naive cells remained in SD medium. We confirmed that those salt stress conditions did not cause any decrease in cell viability. Both cell cultures were then precipitated, washed once with water, and then incubated in fresh SD medium with 0.5 mM luciferin for 90 min. The indicated NaCl doses were then applied to aliquots of both cultures and the luciferase activity continuously measured.

Single-cell time-lapse luminescence microscopy. Cells with an integrated *GAL1-FLuc* reporter gene were grown over night in synthetic raffinose (SRaff) medium. We sonicated our yeast in a Diagenode Bioruptor UCD-200 sonicator for 30 s at medium intensity to obtain single-cell suspensions before loading them onto microfluidic plates (CellAsic). Bioluminescence imaging of yeast cells was performed with a DV Elite microscope equipped with UltimateFocus, an Evolve EMCCD camera, and a 60×/1.25-numerical-aperture (NA) phase oil objective lens. Cells were grown for 60 min at 30°C in SRaff medium at pH 3.8 with 200 μM beetle D-luciferin before switching to the SRaff-plus-galactose version of the same medium. We imaged cells every 4 min and processed raw data using same protocols as before (40). Briefly, cell segmentation was done in CellStat (MATLAB plug-in [42]), and single-cell gene expression was fit to an exponential curve using the induction model described previously (40).

RESULTS

Comparison of transcriptional memory at the nutrient-controlled *GAL1* and stress-induced *GRE2* genes. Transcriptional memory effects in yeast have been found predominantly at nutrient-regulated genes. We therefore sought to compare how the dose-dependent gene expression was modulated after a previous stimulation at nutrient- versus stress-regulated loci. We chose two prototypical yeast genes which represent the two classes of regulation, *GAL1* and *GRE2*. To determine the dose-response behavior of both genes, we applied reporter fusions with destabilized luciferase, which allow monitoring of gene expression in real time in the living yeast cell with a wide range of stimulus concentrations. We first investigated how the dose-sensitive induction of *GAL1* was modulated by repeated galactose exposure. The expression profile from *GAL1*-luciferase reporters in naive yeast cells was compared with that in cells that previously experienced a galactose induction. As shown in Fig. 1A, without prestimulus, *GAL1* responded with a gradual increase in reporter activity over the dynamic range of inducer concentrations. After previous stimulation, however, we observed a much faster response to galactose and an increased synthesis rate of the *GAL1*-luciferase reporter. Importantly, in the second response to galactose, even the lowest inducer concentration (0.01%) was able to activate *GAL1* gene expression to the maximal synthesis rate. We concluded that the *GAL1* dose response largely changed during the establishment of transcriptional memory and that the gene expression profile switched from a dose-sensitive graded mode to a dose-insensitive but highly responsive mode. These dynamic changes in dose-responsive *GAL1* stimulation are faithfully reproduced by the centromeric luciferase reporters used throughout this study compared to chromosomally integrated reporter genes (data not shown).

We next tested whether the dose-response behavior of the stress-activated *GRE2* gene changed upon repeated activation. Memory experiments were performed with the *GRE2*-luciferase real-time reporter and multiple NaCl induction. We observed in this case (Fig. 1B) that the induction of *GRE2* expression was neither faster nor more efficient nor more sensitive at low stress doses in the second round of stimulation. Irrespective of previous gene induction, *GRE2* expression always occurred at the same time and with remarkably similar synthesis rates. The only difference we observed was that cells which responded to salt stress for the second time aborted the induced *GRE2* expression a few minutes earlier than naive cells. Thus, it seemed that *GRE2* induction is not further enhanced or sensitized by previous stress treatment and that it instead reduced the amplitude of the transcriptional burst at *GRE2* in the second round of activation.

Next we wanted to gain insights into the mechanisms which sensitized *GAL1* gene expression to dose-independent maximal induction rates after previous induction. It has been previously reported that the transcriptional memory at *GAL1* was dominantly regulated by signaling molecules such as the Gal3 inducer (33). Thus, we manipulated the Gal3 levels and tested its impact on the dose-dependent induction profile of *GAL1*. As depicted in Fig. 1C, constitutive overexpression of *GAL3* led to a highly efficient *GAL1*-luciferase expression independently of the galactose

promoter in synthetic raffinose medium before and after induction with the indicated galactose concentrations. The mean values for three independent biological replicas are shown with the corresponding standard deviation. (C) Nucleosome remodeling is gradually stimulated by increasing galactose inducer concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the *GAL1* promoter in synthetic raffinose medium before and after induction with the indicated galactose concentrations. The mean values for three independent biological replicas are shown with the corresponding standard deviation. (D) Dynamic dose-response profile of a *GRE2-lucCP*$^{+}$ reporter gene upon NaCl stress. A live-cell luciferase assay was used to determine the expression rates in glucose-containing minimal medium after supplementation with the indicated concentrations of NaCl. *GRE2*-luciferase fusions were expressed from centromeric plasmids (upper panel) or after integration in the genome (lower panel). The mean values for three independent biological replicas are shown for each salt concentration (standard deviation, <15%). (E) Modulation of RNAPII recruitment at *GRE2* over the dynamic range of NaCl concentrations. ChIP of Rpb3-HA-expressing cells was used to determine the RNAPII density at the *GRE2* promoter in synthetic glucose medium before and after induction with the indicated NaCl concentrations. The mean values for three independent biological replicas are shown with the corresponding standard deviation. (F) The duration of transient nucleosome remodeling at *GRE2* is stimulated by increasing NaCl concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the *GRE2* promoter in synthetic glucose medium before and after induction with the indicated NaCl concentrations. The mean values for three independent biological replicas are shown with the corresponding standard deviations.

inducer concentration. Therefore, high *GAL3* levels can mimic the enhanced sensitivity of *GAL1* expression acquired during repeated galactose induction. The Gal3 inducer level is therefore a key element in changing the dose-response behavior at the *GAL1* gene during memory.

GRE2 expression was modulated by repeated stimulation in a manner opposite to that for *GAL1*, as the second wave of transient gene expression was shorter than the initial one in this case. We hypothesized that the accumulation of defense proteins in the first round of stimulation could prepare the cells for the second salt shock and thereby permit an efficient adaptation with a diminished transcriptional response. We considered two physiological adaptations as most relevant for the tolerance to salt stress: the accumulation of the osmolyte glycerol and the enhanced extrusion of Na^+. Both processes can be blocked by single deletions in key structural genes, such as in the *gpd1* and *ena1-4* mutants, respectively. We repeated the memory experiments in those specific mutant strains. The *gpd1* cells were indistinguishable from the wild type (data not shown), but we detected important differences for the *ena1-4* mutant, which lacks all copies of the Ena Na^+ extrusion ATPase. As shown in Fig. 1D, in this mutant the *GRE2-lucCP*$^+$ reporter responded almost equally during the first and second exposures to NaCl stress. Finally, we altered the *ENA1* expression levels by the use of an additional copy of the gene under the control of the constitutive *PMA1* promoter. The effect of increasing *ENA1* expression was a subsequent decrease of the *GRE2* expression peak (Fig. 1E), thus providing additional evidence that the gradual *GRE2* expression depended on the amount of the Ena1 Na^+ pump. Taken together, these experiments show that important differences exist in the responses of differentially regulated genes to previous exposure. *GAL1* gene expression is highly sensitized to previous exposure, which leads to a dose-independent activation likely driven by the Gal3 inducer. The transient *GRE2* expression is not sensitized to previous exposure and seems to be modulated principally by the physiology of the cell, which dictates the amplitude of the transcriptional response at this gene.

Dose-dependent expression of *GAL1* in cell populations corresponds with gradual histone remodeling and RNAPII association. The pattern of the gradual response of *GAL1* to increasing galactose concentrations was recorded with real-time luciferase reporters, which were expressed from centromeric plasmids or integrated into the yeast genome. The *GAL1*-luciferase expression was stimulated to a detectable level with a minimal galactose concentration of approximately 0.02% for plasmid expression or 0.01% for genomic expression. Increasing stimulus concentrations provoked a continuous increase of the reporter activity until a threshold concentration of 0.5% was reached (Fig. 2A). Greater galactose concentrations did not further increase the reporter activity; however, they slightly decreased the lag time between stimulation and response. Since *GAL1* transcript levels are actively repressed in the absence of galactose, we interpreted the *GAL1*-luciferase expression data as the actual mRNA synthesis rates which are dynamically modulated in a stimulus dependent manner. Galactose concentrations from 0.02% to 0.5% result in a gradual activation of *GAL1* promoter activity. We next addressed whether this dynamic behavior was attributable to a galactose-dependent regulation of RNA polymerase II (RNAPII) association at *GAL1*. We performed *in vivo* ChIP experiments to quantify the association of the RNAPII subunit Rpb3 with the *GAL1* promoter within the range of galactose concentrations which apparently cause graded transcription outputs. As shown in Fig. 2B, RNAPII recruitment is slow and inefficient at low threshold concentrations (0.03% galactose) and is continuously faster and more efficient until an upper threshold concentration of 0.5% galactose is reached. As a result, we can correlate the dynamic behavior observed with the *GAL1p*-driven luciferase expression system with the gradual association of the transcription machinery at the *GAL1* promoter.

We then wanted to know whether the gradual increase of *GAL1* promoter activity was accompanied by graded chromatin remodeling. Therefore, we determined the histone H3 density at *GAL1* by ChIP over the same range of galactose concentrations. As depicted in Fig. 2C, we found that the speed and efficiency of nucleosome remodeling at *GAL1* are gradually increased in the dynamic range of galactose concentrations. Thus, at the nutrient-regulated *GAL1* gene, an ample range of inducer concentrations is transduced to a graded remodeling of its nucleosomal promoter structure and the dynamic entry of the transcription machinery.

Dose-dependent expression of *GRE2* relies on the temporal regulation of histone remodeling and RNAPII association at constant synthesis rates. We next extended our analysis of graded gene regulation to the transiently activated *GRE2* gene. We first determined the complete dose-response profile in response to NaCl stress *in vivo* by the use of plasmid-borne or integrated *GRE2*-luciferase reporters. As shown in Fig. 2D, *GRE2* responded with characteristic and transient activation profiles in an NaCl concentration range from 0.1 M to 1 M. Moderate salt concentrations (0.15 to 0.4 M) induced *GRE2* always at the same time and with almost identical synthesis rates. However, in the same dynamic range of salt concentrations, a gradual increase of the stimulus (NaCl) provoked gradually increasing maximal reporter activities. This apparently was achieved by continuously prolonging the time during which *GRE2-lucCP*$^+$ remained actively expressed at maximal synthesis rates. In summary, in contrast to the gradual regulation of synthesis rates seen in the case of *GAL1*, the *GRE2*-luciferase reporter was dynamically regulated temporally while maintaining constant gene expression rates.

We then wanted to determine whether this particular dose-response profile correlated with transcriptional events. Thus, we directly measured the association of RNAPII and histone H3 by ChIP at *GRE2* at NaCl concentrations which cause dynamic changes in the maximal expression. We found that RNAPII association with the *GRE2* promoter occurred very rapidly at low salt concentrations (0.1 to 0.3 M) (Fig. 2E). Clearly, increasing salt concentrations did not stimulate the absolute RNAPII levels but continuously increased the time during which the RNA polymerase remained associated with *GRE2*. These data correlated well with the rapid loss of histone H3 from the *GRE2* promoter region, which was continuously prolonged, but not more efficient, in response to stimulation by increasing NaCl shock (Fig. 2F). Taking these observations together, we find that the differential expression of *GRE2* caused by increased stress doses is achieved mainly by regulating the time during which the promoter remains actively transcribed with practically constant synthesis rates, RNAPII occupancy, and histone eviction.

We then wanted to determine whether this temporal pattern of dose response was general for stress-responsive genes. Therefore, we quantified the dose-sensitive expression patterns of two more osmotic-stress-inducible natural promoters, *HOR2* and *ALD6*. The respective fusions with destabilized luciferase were suitable for determination of the entire dose-response profiles for both genes upon NaCl stress (Fig. 3A). The comparison of the stimulus-dependent modulation of both the maximal expression and the synthesis rates revealed almost identical patterns for *GRE2*, *ALD6*, and *HOR2* (Fig. 3B). At all three genes the maximal synthesis rate was reached with low stress doses (0.2 M NaCl), while the maximal expression further increased until 0.4 M NaCl due to prolonged activation of the respective fusion genes. Finally we tested the dose-response behavior of two additional genes, *SOD2* and *CTT1*, in response to a different type of environmental cue such as oxidative stress (Fig. 3C). As depicted in Fig. 3D, the two genes showed very similar patterns of maximal gene expression and synthesis rates upon increasing stimulation with menadione compared to the previous patterns obtained for salt stress. Thus, the temporal modulation of gene activity in response to increasing stress doses might be a general feature for stress-responsive genes in yeast.

Increasing galactose stimuli gradually decrease the lag phase and cell-to-cell variability to activate *GAL1* gene expression. We investigated the dose-sensitive response of *GAL1* at the level of single cells. *GAL1* gene expression has been reported to occur in a bimodal fashion, especially at lower galactose concentrations (43–46). Therefore, we wanted to test to what degree bimodality was the source of the gradual *GAL1* regulation. We performed time-elapsed induction studies and recorded the traces of *GAL1*-luciferase-expressing single cells upon stimulation with different galactose concentrations. As shown in Fig. 4, a high galactose stimulus (0.5%) leads to a fast and homogeneous induction throughout the cell population, while lower galactose concentrations increase the lag phase and the heterogeneity of gene induction. However, even very low inducer concentrations (0.02%) activated *GAL1* expression in most of the cells over time, and the slope of *GAL1* induction was largely unaffected by the inducer concentration (Fig. 4d and e). Thus, the gradual decrease of *GAL1* expression in a cell population is mostly the result of a heterogeneous induction delay caused by suboptimal inducer concentrations.

SAGA, SWI/SNF, or mediator mutants cause severely reduced dose responses of *GAL1*. The expression of the *GAL1* gene is finely tuned depending on the galactose availability, and we have shown above that this regulation involves the graded modulation of promoter activity and nucleosome eviction in a cell population. We next wanted to know how impaired nucleosome remodeling affected the dynamic adaptation of *GAL1* promoter activity to changing inducer concentrations. Therefore, we determined the induction profile of the *GAL1*-luciferase reporter gene in response to a wide range of galactose concentrations in mutants with defects in various coactivator complexes. We included in this study the *gcn5* (SAGA histone acetyltransferase), *snf2* (SWI/SNF chromatin-remodeling complex), and *gal11* (mediator complex) mutants, with mutations in genes previously identified as important for full *GAL1* transcriptional activation via Gal4 (22–27), and additionally the *htz1* mutant in the histone variant H2AZ. The comparison of the dose-response profiles obtained for all mutant strains (Fig. 5A) revealed that loss of SAGA or SWI/SNF function significantly reduced the dynamic range of luciferase synthesis rates driven by the *GAL1* promoter (Fig. 5B). While wild-type cells continuously increase the expression rate until a galactose concentration of 0.5% is reached, *gcn5* and *snf2* mutants have truncated dose responses. Both mutants reach a maximal synthesis rate at very low inducer concentrations, which cannot be further increased. The *gal11* and *htz1* mediator mutants revealed an even stronger reduction in the dynamic gene expression at *GAL1*. To attribute the observed loss of dynamic *GAL1* promoter activity in response to gradual increment of inducer to impaired chromatin remodeling, we next compared the changes in histone H3 occupancy among the different mutant strains. As shown in Fig. 5C, loss of SAGA, SWI/SNF, or mediator function impaired the efficient and dose-dependent histone eviction from the *GAL1* promoter. Thus, a correlation exists between efficient nucleosome removal and the gradual adaptation of *GAL1* promoter activity, which relies on the activity of the coactivator complexes investigated here. In the absence of histone variant H2AZ, we still observed efficient histone H3 remodeling at *GAL1* (Fig. 5B). Therefore, the defect of *htz1* mutants in the galactose-dependent modulation of *GAL1* gene expression is likely caused by effects other than impaired nucleosome eviction.

We next applied the same exhaustive analysis of dose-response profiling at the stress-regulated *GRE2* gene. An NaCl gradient was applied to the same set of mutants, and their dose-dependent *GRE2* expression profiles were determined (Fig. 6A). As the *GRE2* gene expression is dynamically regulated in response to increasing salt stress via modulation of its maximal expression level, we chose this parameter to identify alterations in the dose-dependent behavior of this gene. As shown in Fig. 6B, the loss of SWI/SNF activity did not affect the dose-response profile of *GRE2*. Mutations in SAGA or H2AZ caused a general reduction in the maximal *GRE2*-luciferase activities; however, a continuous increase in reporter activity was still observed in the dynamic inducer range (0.1 to 0.5 M NaCl). Loss of mediator function caused a very poor expression of *GRE2* at any salt concentration. Importantly, and different from the case for the *GAL1* gene, SWI/SNF activity is dispensable for the efficient adaptation of *GRE2* activity to increased stimulation by salt. Also, in the absence of SAGA, the absolute expression levels of *GRE2* decreased; however, the gradual increase in the maximal expression following the NaCl gradient was maintained. In conclusion, chromatin modifiers such as SWI/SNF or SAGA have distinct roles in the establishment of specific dose responses, exemplified here for the *GAL1* and *GRE2* genes.

Gradual association of Gal3 and temporally regulated recruitment of Hog1 recapitulate the different dose-response behaviors of *GAL1* and *GRE2*. The regulation of the dose-dependent expression of *GAL1* and *GRE2* depends on different mechanisms. We next investigated the signaling compounds which were responsible to establish a specific dose-response pattern at the two genes. We first focused at the specific transcription factors Gal4 and Sko1, which bind directly to the *GAL1* or *GRE2* promoter regions and confer galactose- or salt-induced transcriptional activation. We found that Gal4 binding to *GAL1* was generally stimulated by galactose but independently of the concentration tested (Fig. 7A). Sko1 binding to *GRE2* was slightly increased by low salt doses (Fig. 7B) but did not correlate with the increasing *GRE2* promoter activity observed before in this range of salt stimuli. Therefore, the differential binding of the direct transcriptional activator Gal4 or Sko1 was not a mechanism to establish the dynamic dose responses at the *GAL1* or *GRE2* gene. We then determined the association of a second class of regulators, the Gal3 inducer and the Hog1 MAP kinase. Both signaling molecules are imported into the nucleus upon stimulation, associate with the promoter regions via Gal4 or Sko1, and are required to trigger the transcriptional switch from repression to activation. As shown in

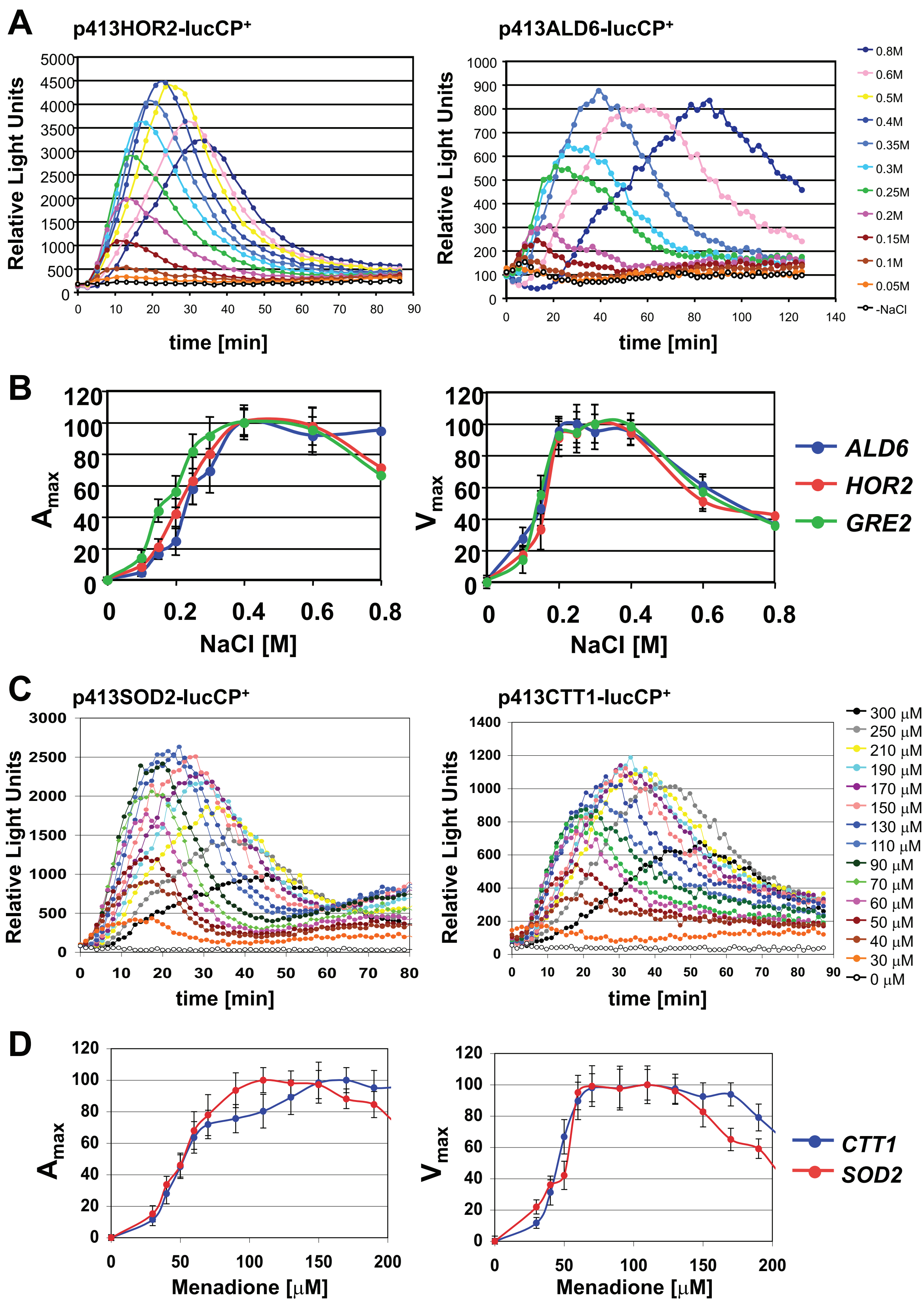


FIG 3 Dose-sensitive modulation of salt and oxidative stress-regulated yeast genes. (A) Live-cell reporter fusions with destabilized luciferase were used to determine the dose-response profiles of the *HOR2* and *ALD6* genes in response to NaCl stress. The mean values for three independent biological replicas are shown for each salt concentration (standard deviation, <15%). (B) Maximal expression levels (A_{max}) and synthesis rates (V_{max}) of the *ALD6*, *HOR2*, and *GRE2* genes upon NaCl stress. Error bars indicate standard deviations. For each gene, the highest value for A_{max} or V_{max} was adjusted to 100. (C) Live-cell reporter fusions with destabilized luciferase were used to determine the dose-response profiles of the *SOD2* and *CTT1* genes in response to menadione stress. The mean values for three independent biological replicas are shown for each menadione concentration (standard deviation, <15%). (D) Maximal expression levels (A_{max}) and synthesis rates (V_{max}) of the *SOD2* and *CTT1* genes upon menadione stress. Error bars indicate standard deviations. For each gene, the highest value for A_{max} or V_{max} was adjusted to 100.

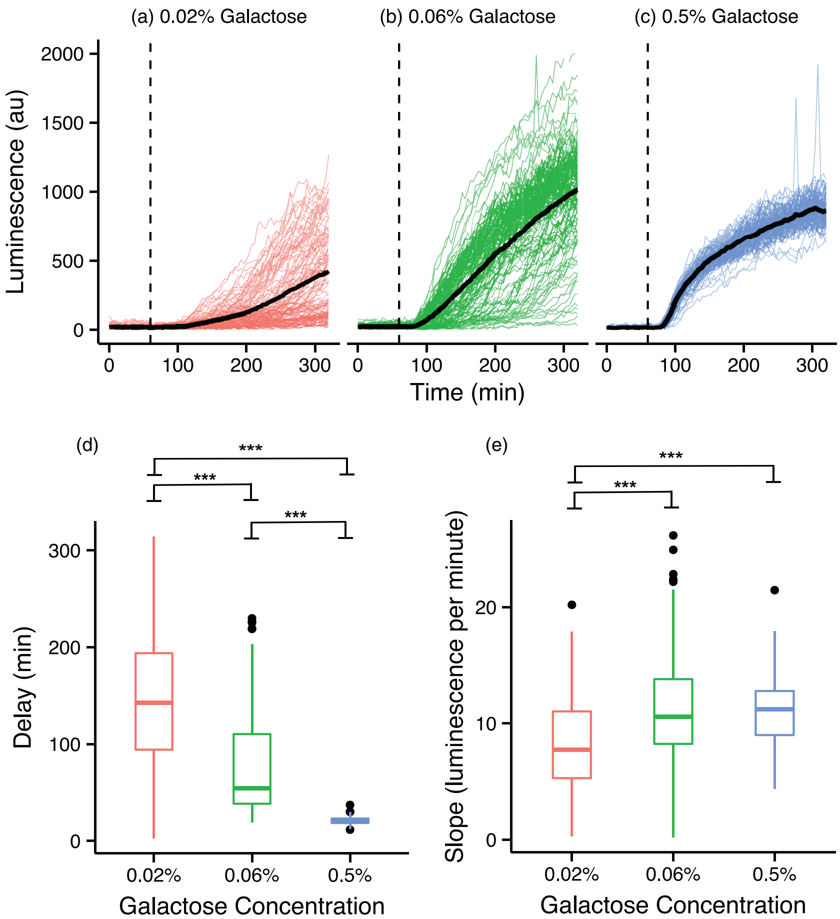


FIG 4 Graded dose response of *GAL1* expression results from a heterogeneous induction delay across the population. (a to c) Time-lapse luminescence microscopy was used to measure gene expression in single cells after induction (dashed line) in medium with 0.02, 0.06, and 0.5% galactose. Average gene expression is shown as black curve. (d and e) Distributions of the delay (d) and slope (e) of induction across different numbers of cells (n = 90, 136, and 97, respectively). Statistical analysis confirms that the delay becomes longer whereas the slope is not significantly different across all galactose concentrations (***, $P < 0.001$ by Student's t test).

Fig. 7A, Gal3 association with the *GAL1* promoter increases gradually with increasing galactose concentrations. The Gal3 inducer bound slowly and less efficiently with low galactose concentrations and faster and more efficiently with higher galactose stimulation. These data correlated with the dynamic nucleosome remodeling, entry of RNAPII, and modulation of promoter activity in the same range of galactose concentrations. Of note, Gal3 association with *GAL1* in response to high galactose concentrations was transient, although expression of *GAL1* occurs for longer times. However, our observation is in agreement with previous findings that report transient Gal3 association with *GAL* genes only in the early phase of galactose induction (47).

The recruitment over time of the Hog1 MAP kinase was finally determined for increasing salt stress at *GRE2*. As shown in Fig. 7B, Hog1 association was indistinguishable at early time points for the different NaCl doses tested; however, Hog1 remained bound for longer times with increasing stimulus. These data correlated with the temporal regulation of gene expression, nucleosome remodeling, and RNAP II association at *GRE2* observed before. In conclusion, the different patterns of dose-dependent gene expression activity at *GAL1* and *GRE2* are recapitulated by the specific association pattern of transcriptional inducer molecules such as Gal3 or Hog1.

We finally wanted to determine whether the Gal3 inducer level was the decisive factor for the sensitivity and efficiency of *GAL* gene expression. We therefore placed the *GAL3* gene under the control of the *GAL1* promoter and monitored the effect of a gradual activation of *GAL3* by limiting galactose concentrations on the second round of *GAL1*-luciferase expression. As shown in Fig. 7C, the gradual increase of *GAL3* preactivation mimicked the transition from slow and insensitive to fast and highly sensitive *GAL1* gene activation. These data indicate that the Gal3 inducer level is an important determinant of regulating the dose-sensitive *GAL* gene expression.

DISCUSSION

Cells execute transcriptional programs in response to many different environmental stimuli and threats. A single cell such as a yeast cell has acquired a multitude of gene expression responses triggered by external stimuli, which is well documented by extensive literature published over the past decades. Traditionally these environmental stress responses were investigated with severe insults, which activate the signaling pathways to a maximal level. However, the adaptation to subtle changes in the cell's environment might be of more physiological importance, and generally we expect that cells are able to adapt their transcriptional re-

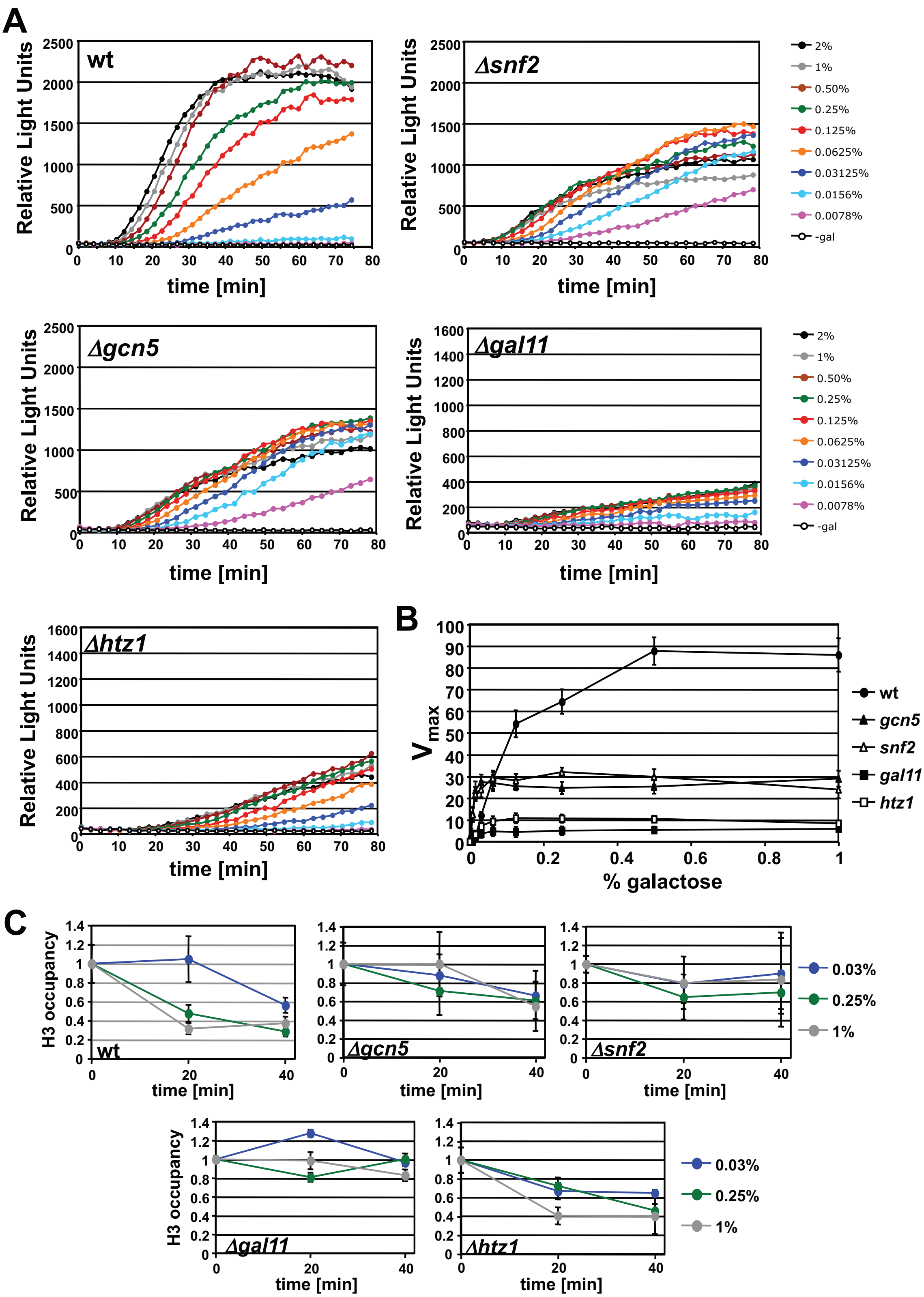


FIG 5 The efficiency of dose-sensitive regulation at *GAL1* depends on coactivator complexes and histone remodeling. (A) The *GAL1-lucCP*$^+$ reporter gene was used in live-cell luciferase assays in the indicated yeast strains to determine the expression rates in raffinose-containing minimal medium after supplementation with the indicated concentrations of galactose. The mean values for three independent biological replicas are shown for each galactose concentration (standard deviation, <15%). (B) Comparison of galactose-dependent modulation of *GAL1* synthesis rates. Data shown represent the mean values for the maximal synthesis rate for each galactose concentration determined in three independent biological replicates for the indicated yeast strains. Error bars indicate standard deviations. (C) Comparison of nucleosome remodeling at *GAL1* in response to increasing galactose inducer concentrations. ChIP of histone H3 was used to determine the nucleosome occupancy at the *GAL1* promoter in YP-raffinose medium before and after induction with the indicated galactose concentrations. The mean values for two independent biological replicas are shown with the corresponding standard deviation.

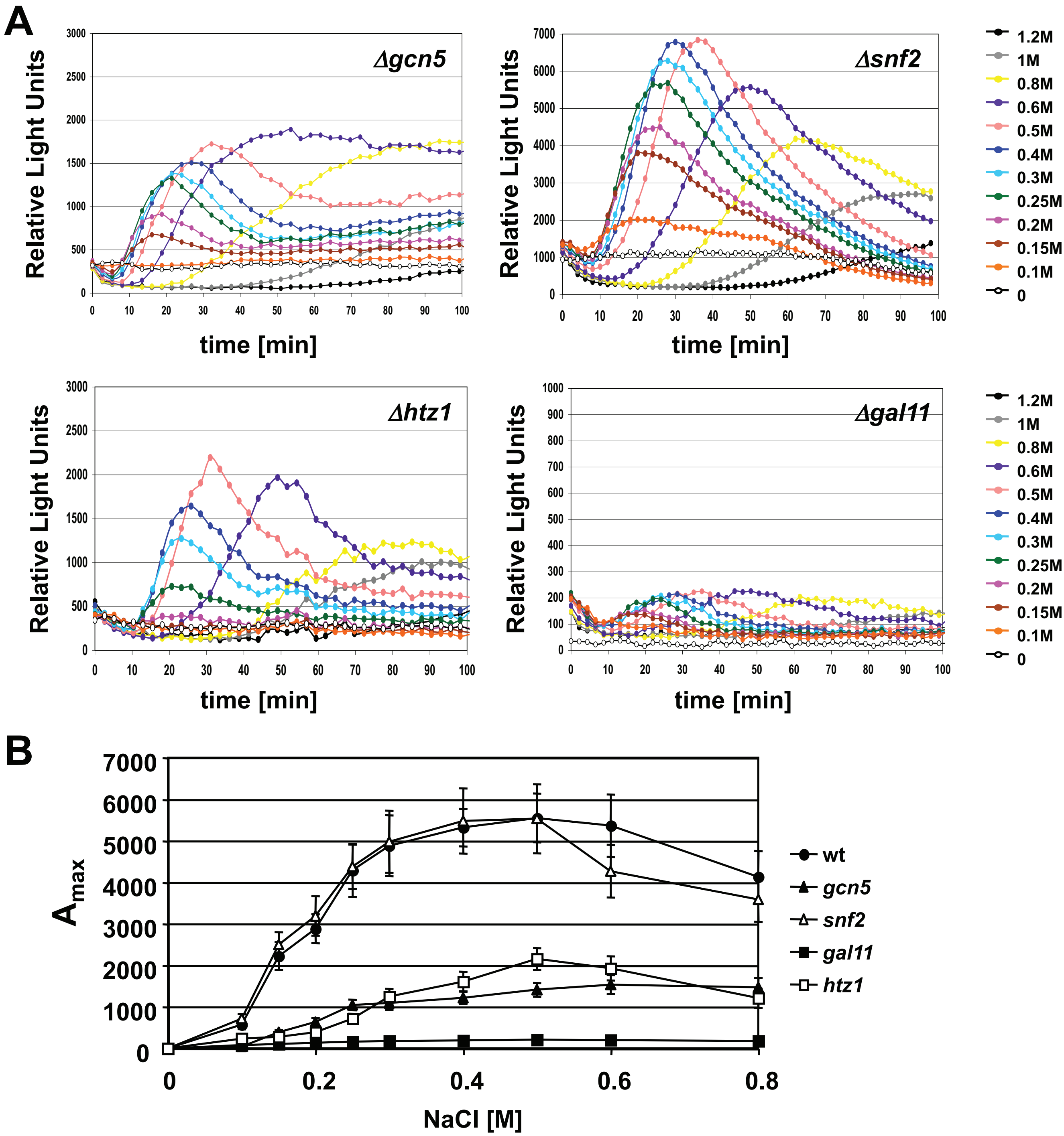
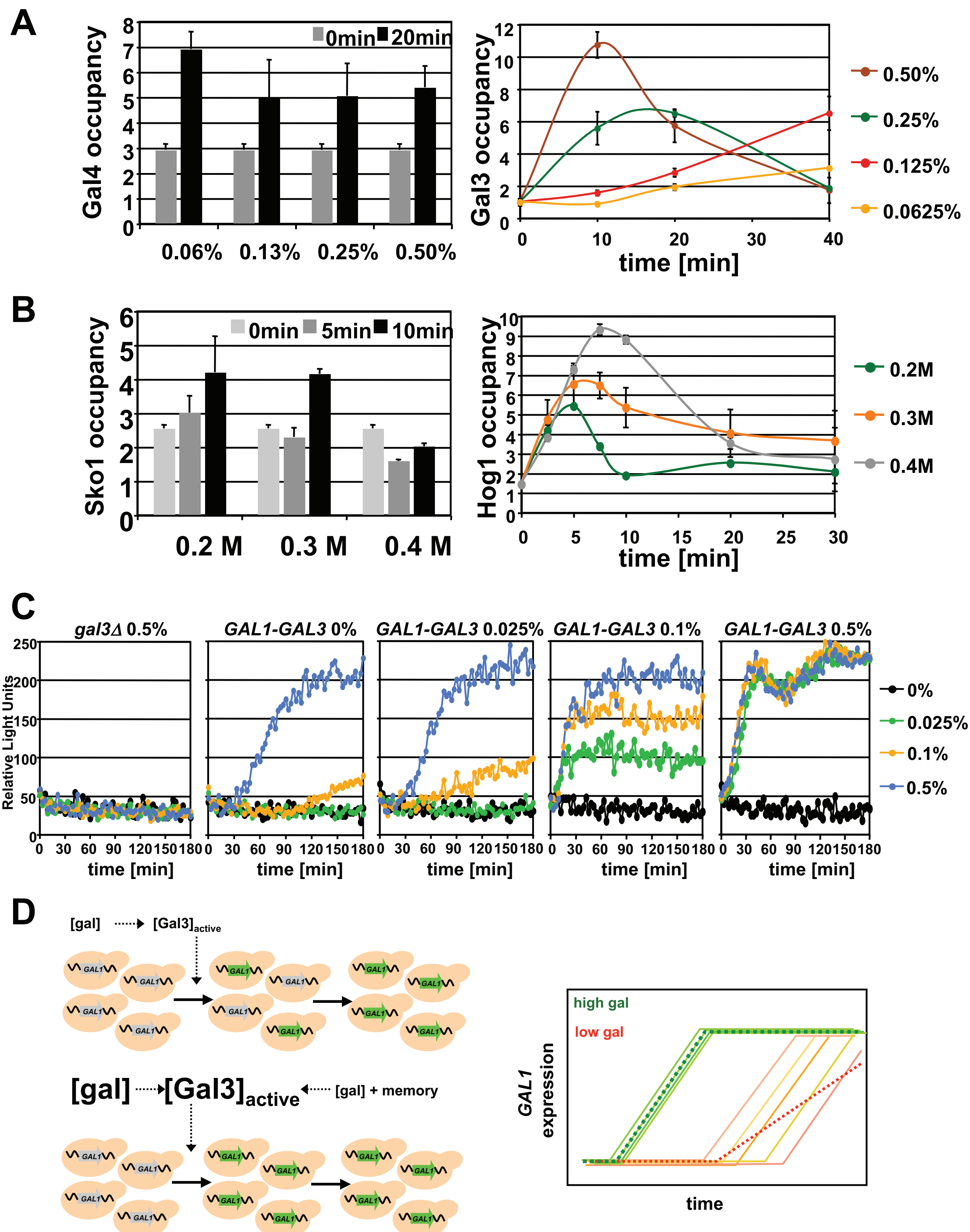


FIG 6 Function of coactivator complexes and histone H2AZ in the dose-sensitive regulation of *GRE2*. (A) The *GRE2-lucCP*$^{+}$ reporter gene was used in live-cell luciferase assays in the indicated mutant strains to determine the expression rates in glucose-containing minimal medium after supplementation with the indicated concentrations of NaCl. The mean values for three independent biological replicas are shown for each salt concentration (standard deviation, <15%). (B) Comparison of the NaCl-dependent modulation of *GRE2* gene expression. Data shown represent the mean values for the maximal expression rate for each salt concentration determined in three independent biological replicates. Error bars indicate standard deviations.

sponses gradually in response to the severity of the stress. It is largely unknown how gene regulatory systems adapt to these "suboptimal" signals, mostly because it is experimentally challenging to quantify the dynamic gene expression upon gradually changing stimulation. Here, the recent application of destabilized luciferase reporters for continuous live-cell measurements in yeast turned out to be especially useful (15, 38). This real-time survey of gene expression activity reveals the dynamic range of differentially regulated groups of genes. In the case of the two genes studied in detail in cell populations here, we find a graded


A
Gal4 occupancy
0min 20min
0.06%
0.13%
0.25%
0.50%
Gal3 occupancy
time [min]
0.50%
0.25%
0.125%
0.0625%
B
Sko1 occupancy
0min 5min 10min
0.2 M
0.3 M
0.4 M
Hog1 occupancy
time [min]
0.2M
0.3M
0.4M
C
gal3Δ 0.5%
GAL1-GAL3 0%
GAL1-GAL3 0.025%
GAL1-GAL3 0.1%
GAL1-GAL3 0.5%
Relative Light Units
time [min]
0%
0.025%
0.1%
0.5%
D
[gal] [Gal3]active
GAL1
[gal] [Gal3]active [gal] + memory
high gal
low gal
GAL1 expression
time

dose response in concentration ranges well below the stimuli normally used for these types of genes: *GAL1* expression gradually adapts from 0.01 to 0.5% galactose, while *GRE2* expression continuously increases from 100 to 400 mM NaCl. These specific stimulus concentrations that provoke gradual outputs might reflect evolutionary adaptation to the naturally occurring environmental changes. It is important to note that the sensitivity of gene expression to a common signal, such as a nutrient or a stress, can be different for specific responsive genes. Here, the chromatin structure and the combination of different *cis*-regulatory elements in promoters have been implicated in creating characteristic dose sensitivities of yeast genes (14, 15). In our present study, we reveal different strategies that ensure an appropriate transcriptional activation corresponding to subtle environmental changes.

Galactose induction at the *GAL1* gene is slow and inefficient at threshold concentrations. This might be due to the repressive chromatin structure at the *GAL1* promoter region, which has to be overcome by the activated Gal3 inducer. The initial repressed levels of Gal3 in combination with low galactose concentrations delay the transition of *GAL1* to the on state. Consequently, the time point of active gene expression becomes much more variable for individual cells at low galactose doses; however, it is important to note that even at the lowest inducer concentrations, all cells finally actively express *GAL1* with comparable induction kinetics. Therefore, the key determinants which explain a gradual galactose response are the signaling events that permit the first round of transcription (Fig. 7D). It is likely that with few Gal3 molecules present in a cell that has not metabolized galactose over a longer time, the rate-limiting step for efficient *GAL* gene transcription is a threshold concentration of active Gal3 bound at the Gal4 transcriptional activator. In this model, the grade of active Gal3 counteracting the Gal80-mediated repression and additional recruitment of SAGA and Swi/Snf coactivators would increasingly favor *GAL1* transcriptional initiation along with growing galactose concentrations. On the other hand, the fact that the expression of *GAL3* itself is activated by galactose makes the *GAL* system especially modulatable. An inducible sensor such as Gal3 allows adaptation of the sensitivity of the *GAL* gene activation to environmental needs in a way that yeast cells, for example, which frequently encounter galactose as an energy source, would respond more readily in the following round of stimulation (33, 34). Of note, transcriptional memory in yeast has been identified predominantly at genes responsive to nutritional stimuli (31, 48). Thus, gradually regulated promoter activity over a range of metabolite concentrations with the ability to modulate the sensitivity by specific inducible signal transducers might be a general scheme of nutrient-stimulated gene expression in yeast.

The adaptation of gene expression to different grades of cytotoxic stress seems to follow a different principle. Intuitively one might think that a gene product that functions in the detoxification of an acute stress has to be produced as soon as possible and, at least in the beginning of the stress defense, regardless of the strength of the insult. Such an "emergency" response is identified here in the case of the prototypical stress defense gene *GRE2*. At this gene, maximal levels of nucleosome eviction and preinitiation complex formation are observed almost immediately (experimentally at 2 min) after salt stress exposure. Importantly, and in contrast to the case for *GAL1*, low stress doses provoke maximal induction at *GRE2*. Our data suggest that activation of the HOG signaling pathway in the range of mild salt stress always triggers the same signal to its target promoters, which in all cases leads to full transcriptional activation in the first instances of adaptation. Only at salt concentrations above 0.4 M NaCl can a progressive delay in gene expression be observed, which can be explained by general inhibition of the transcription process and a slowdown of signal transduction at high osmolarity (49–51). Our results also indicate that the switch-like behavior is a general feature for stress-responsive promoters and not restricted to *GRE2* activation by salt stress. We therefore speculate that genes of acute stress responses might generally switch to active transcription easily and independently of the stress dose and that mainly the duration of the on state would be dictated by the strength of the stress. This regulatory mode can provide the cell the most efficient protection, as the absolute production of defense gene mRNAs continuously increases from very low stress levels to stress levels that actually start to inhibit gene expression in general (Fig. 3B). This also implies that the dynamic adaptation of gene expression to stress results not from gradual activation but from the timely shutdown of transcription, a process whose molecular basis is substantially unknown and therefore of special interest for future studies on stress regulation. In general, the osmotic and oxidative stress responses might be optimized to execute very rapid transcriptional activation, which cannot be further enhanced during transcriptional memory. In line with this assumption, the most notable effect of memory on the *GRE2* expression is a reduction in the amplitude during repeated salt stress. This reduction is produced by the accumulation of defense proteins such as the cation exporter Ena1 in the case of NaCl stress. Therefore, stress-induced genes might be

FIG 7 The Gal3 inducer and the Hog1 MAP kinase show different patterns of dose-dependent recruitment at target promoters. (A) Gal3 association with the *GAL1* promoter is gradually increased with galactose concentration. ChIP of Gal4-TAP- or Gal3-TAP-expressing yeast cells was used to determine the association of both factors with the *GAL1* promoter in synthetic raffinose medium after the indicated times of induction with different galactose concentrations. Left panel, Gal4 occupancy before and after 20 min of galactose induction; right panel, Gal3 occupancy before and after the indicated galactose induction. The mean values for three independent biological replicas are shown with the corresponding standard deviations. (B) Hog1 occupancy at the *GRE2* promoter is temporally regulated in response to increasing NaCl stimuli. ChIP of Sko1-HA- or Hog1-HA-expressing yeast cells was used to determine the association of both factors with the *GRE2* promoter in synthetic glucose medium after induction with the indicated concentrations of NaCl. Left panel, Sko1 binding to *GRE2*; right panel, Hog1 recruitment at the *GRE2* promoter upon increasing salt stress. The mean values for three independent biological replicas are shown with the corresponding standard deviations. (C) Gradual induction of Gal3 confers increasing sensitivity of *GAL1* expression. The memory experiment of Fig. 1A was modified by a brief first induction with limiting galactose concentrations (depicted above the graphs). A *gal3* mutant was either transformed with the empty vector (left panel) or transformed with single-copy *GAL1prom-GAL3* (other panels). The galactose concentrations used for the second induction are given at the right. (D) Model of the dose-dependent regulation of *GAL1* expression. Low galactose concentrations provoke slow and heterogenous promoter activation at the level of individual cells. The time needed for engagement of most of the cells in active transcription is long, while high galactose concentrations (or a low galactose stimulus after previous induction) provoke a fast and homogeneous transition to the on state. This regulation leads to a gradual reduction of *GAL1* expression levels in a cell population (depicted at the right). Shown is single-cell *GAL1* expression upon high and low galactose stimulation, with the dotted lines indicating the population average.

modulated predominantly by the cellular defense capacity, which determines the time needed to maintain maximal gene expression. It is worth noting that a positive memory effect has been reported recently for the yeast response to oxidative stress when the cells were previously treated with a mild dose of salt (52). Future work might therefore reveal the importance of acquired resistance versus transcriptional memory for different stress types and doses.

The nuclear expression of both *GAL1* and *GRE2* is modulated by signals which originate in the cytoplasm and are then sent to the chromosomal genes via signaling proteins Gal3 and Hog1, respectively. Galactose-bound Gal3 and phosphorylated Hog1 physically interact with their target genes through DNA-bound transcription factors. Here we show that the dynamics of Gal3 or Hog1 association during gradual stimulation faithfully reflect the grade of chromatin remodeling, RNAPII density, and transcriptional output of the regulated genes. Therefore, Gal3 and Hog1 are very likely to be responsible for the specific dose responses observed at their target genes. Importantly, Gal3 protein levels in the uninduced state are very low, which explains the need for high inducer concentrations to efficiently switch on transcription of *GAL* genes. In contrast, Hog1 protein levels are constitutively high (approximately 10-fold more abundant than uninduced Gal3 [36]) independently from the stress condition, thereby ensuring maximal signaling rates at low stress doses. Of note, the increasingly longer, but not more efficient, association of Hog1 with its *GRE2* target promoter reported here is in agreement with gradually longer phosphorylation of the MAP kinase upon increasing salt stimulation (53). An additional layer of regulation might affect the chromatin structure at stress- versus nutrient-regulated genes. In the case of *GAL1*, nucleosome remodeling seems to be more important to achieve efficient transcription, and accordingly, we find that the Swi/Snf and SAGA chromatin modifiers are crucial for the dynamic increase of *GAL1* activity. In the case of *GRE2*, nucleosome remodeling either might occur in a much easier fashion or might be less important for activated transcription. This notion is supported by our finding that Swi/Snf is completely dispensable for the dose-dependent *GRE2* regulation, and even in the absence of SAGA, the transcriptional output is gradually stimulated by increasing salt stress. Both coactivator complexes, however, have been shown to be recruited to the *GRE2* promoter upon salt shock (30). Taking our findings together, gradual stimulation of inducible yeast genes can be conferred by different principles, i.e., modulation of the time in the "on" state in the case of stress genes or gradual modulation of the transition to the "on" state in the case of nutrient-regulated genes. The efficiency of signal transduction is a key determinant for the type of response, and its reinforcement during memory provides a way to switch from one mode to another.

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