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Transcriptional Repression by the Pho4 Transcription Factor Controls the Timing of *SNZ1* Expression▽†

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Nutrient-sensing kinases play important roles for the yeast *Saccharomyces cerevisiae* to adapt to new nutrient conditions when the nutrient status changes. Our previous global gene expression analysis revealed that the Pho85 kinase, one of the yeast nutrient-sensing kinases, is involved in the changes in gene expression profiles when yeast cells undergo a diauxic shift. We also found that the stationary phase-specific genes *SNZ1* and *SNO1*, whch share a common promoter, are not properly induced when Pho85 is absent. To examine the role of the kinase in *SNZ1*/*SNO1* regulation, we analyzed their expression during the growth of various yeast mutants, including those affecting Pho85 function or lacking the Pho4 transcription factor, an in vivo substrate of Pho85, and tested Pho4 binding by chromatin immunoprecipitation. Pho4 exhibits temporal binding to the *SNZ1*/*SNO1* promoter to down-regulate the promoter activity, and a Δ*pho4* mutation advances the timing of *SNZ1*/*SNO1* expression. *SNZ2*, another member of the *SNZ*/*SNO* family, is expressed at an earlier growth stage than *SNZ1*, and Pho4 does not affect this timing, although Pho85 is required for *SNZ2* expression. Thus, Pho4 appears to regulate the different timing of the expression of the *SNZ*/*SNO* family members. Pho4 binding to the *SNZ1*/*SNO1* promoter is accompanied by alterations in chromatin structure, and Rpd3 histone deacetylase is required for the proper timing of *SNZ1*/*SNO1* expression, while Asf1 histone chaperone is indispensable for their expression. These results imply that Pho4 plays positive and negative roles in transcriptional regulation, with both cases involving structural changes in its target chromatin.

The budding yeast *Saccharomyces cerevisiae* changes its gene expression profiles upon alterations in nutrient status to adapt to new conditions. Nutrient-sensing kinases play important roles in this adaptation process. Cyclic AMP-dependent kinase and Snf1 regulate gene expression, cell growth, and carbohydrate metabolism in response to glucose availability, Tor kinases regulate protein synthesis and autophagy responding to nitrogen availability, and Pho85 kinase regulates the *PHO* system responding to environmental phosphate (P_i) (4, 34). The Pho4 transcription factor activates the transcription of the *PHO* genes involved in the *PHO* system in response to P_i limitation in the medium (20). Under high-P_i conditions where the yeast cells do not need to express the *PHO* genes, the Pho85 kinase-Pho80 cyclin complex phosphorylates Pho4 to facilitate its exclusion from the nucleus, resulting in repression of the *PHO* genes (7, 8). When the P_i concentration in the medium becomes low, Pho85-Pho80 is inactivated by Pho81, a Cdk inhibitor, resulting in the accumulation of Pho4 in the nucleus to enable activation of the *PHO* genes. Pho85 also facilitates the degradation of Gcn4, a transcription factor that activates genes involved in amino acid metabolism under amino acid-starvation conditions, when sufficient amounts of the nutrients are available (2, 13). Based on a global gene expression analysis, we previously reported that Pho85 kinase was involved in the changes in gene expression responding to a diauxic shift (18). When yeast cells undergo a diauxic shift, they shift their metabolism from fermentation to respiration, and accordingly, the genes involved in mitochondrial function, gluconeogenesis, and storage carbohydrate synthesis are induced, while those involved in glycolysis are repressed (5). Pho85 function is required for yeast cells to carry out these changes properly (18). Thus, Pho85 functions in responding to alterations in the environmental nutrient conditions that are more general than those previously considered (18).

SNZ1 was discovered as a gene expressed in the stationary phase, and *SNO1*, which is adjacent to *SNZ1*, is regulated coordinately with the neighboring gene through a common promoter (3, 21). In the absence of Pho85, these genes were not induced properly at the late growth stage (18). When we looked at the *SNZ1*/*SNO1* promoter sequence, we found a putative Pho4-binding sequence (CACGTT) at −380 (taking the A of ATG of *SNZ1* as +1) in addition to three possible Gcn4-binding sites. Gcn4 is required for the activation of *SNZ1*/*SNO1* under amino acid depletion conditions (17). The involvement of Pho85 in the alterations of gene expression accompanying a diauxic shift and the presence of a prospective Pho4-binding site in the *SNZ1* promoter prompted us to test

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whether Pho85-Pho4 is involved in the regulation of *SNZ1*. We analyzed in detail the effects of various mutations that affect the functions of Pho85 and Pho4 and the chromatin structure on *SNZ*/*SNO* expression and timing during yeast growth. Here we report that Pho4 binds to the *SNZ1*/*SNO1* promoter in a P_i-dependent manner to repress *SNZ1*/*SNO1* to ensure the appropriate timing of their expression. We also demonstrate that Pho4 binding is accompanied by alterations in the chromatin structure at the *SNZ1* promoter and that the Rpd3 histone deacetylase (HDAC) is involved in the regulation of the timing of *SNZ1*/*SNO1* expression.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this work are listed in Table S1 in the supplemental material. To disrupt the *PHO81* locus, the BglII (+472)-XhoI (+2164) fragment, in which the XbaI (+1194)-to-BamHI (+1764) region had been replaced by a *URA3* fragment, was used, and successful disruption was confirmed by PCR using MN289/MN290 primers. MFY409 (Δ*cyc8*::*URA3*) was constructed by using pJS22 plasmid (26). *Escherichia coli* strain DH5α was used as a host for plasmids. Media for *E. coli* growth and rich medium (YPAD) for yeast were prepared as described previously (24, 25). Low-P_i medium was prepared by using a yeast nitrogen base without P_i (Q-Biogene) instead of the normal yeast nitrogen base in synthetic dextrose (SD) medium and was supplemented with 0.2 μM sodium P_i. For the incubation under low-P_i conditions, yeast cells were grown in YPAD medium for 6 or 24 h, collected by centrifugation, and resuspended in the same volume of low-P_i medium followed by incubation for an additional 6 h. For incubation under high-P_i conditions for 30 h, sodium P_i buffer was added after 24 h to reach a final concentration of 2 mM. To analyze the effect of 3-aminotriazole (3-AT), yeast cells grown to mid-log phase (A_{600} = 0.5) were collected, resuspended in SD medium lacking histidine and supplemented with 100 mM 3-AT, and incubated at 30°C for 1 h before proceeding to RNA or chromatin isolation.

DNA manipulation. Standard *E. coli* and yeast genetic methods and DNA manipulations were as described previously (24, 25). The primers used in this work are listed in Table S2 in the supplemental material. To construct the *PHO4*-tagged MFY376 strain, a fragment containing *PHO4* tagged with His_6 and $Flag_3$ tags was amplified using primers Pho4-Flag-F and -R and the pUG6H3Flag plasmid (10) as the template, followed by transformation. Successful replacement and production of tagged Pho4 were confirmed by PCR and an immunoblot analysis with an anti-Flag antibody, respectively (data not shown). To construct the yeast strain with a deletion of *ASF1*, *HDA1*, *RPD3*, or *SIN3*, the adaptamer-mediated PCR method was employed to prepare the DNA fragments for transformation (22). The target fragments for each locus were amplified with the appropriate primer pairs (listed in Table S2 in the supplemental material) to place the forward tag sequence (GGAATTCCAGCTGACCACC) and the reverse tag sequence (GATCCCCGGGAATTGCCATG) at the 5′ and 3′ termini, respectively. Two partially overlapping fragments of the *URA3* gene from *Kluyveromyces lactis* (Kl*URA3*) were synthesized by PCR with primer set MN464 and MN345 and primer set MN344 and MN465 to place the forward tag and the reverse tag at the 5′ and 3′ termini, respectively. The target and the Kl*URA3* fragments with the corresponding tags were combined and annealed through the tag and were then subjected to PCR to prepare fusion fragments with the 5′ and 3′ portions of Kl*URA3*, respectively. The two PCR fragments were combined and used to transform yeast. Successful disruption was confirmed by PCR. A reporter plasmid bearing the *SNZ1* promoter and *lacZ* was constructed as follows. The promoter fragment (−970 to +30) was cloned by PCR using the MN449/MN450 primer pair to incorporate BamHI and BglII sites at the 5′ and 3′ termini, respectively, and was incorporated into the pMF811 plasmid (19). To mutagenize the putative Pho4-binding sequence in the *SNZ1* promoter, a QuikChange II site-directed mutagenesis kit (Stratagene), the MN459/MN460 primer pair, and the *SNZ1* promoter fragment were used. Successful mutagenesis was confirmed by DNA sequencing. To replace the chromosomal *SNZ1* locus with the mutant *SNZ1* lacking the putative Pho4-binding site, the DNA fragments for transformation were prepared by the adaptamer-mediated PCR method (22) as described above. The resulting Ura-positive transformants were transferred to medium containing 5-fluoroorotic acid to select Ura-negative colonies. Loss of the Kl*URA3* marker and successful replacement were confirmed by PCR and DNA sequencing, respectively.

Chromatin immunoprecipitation (ChIP) was carried out essentially as described previously (9). Briefly, yeast cells producing Flag-tagged Pho4 were cultivated in high- or low-P_i medium at 30°C to an A_{600} of 1.0 to 1.2, and the proteins were then cross-linked to DNA by adding formaldehyde. For time course experiments, cells were grown in YPAD medium at 30°C at an initial A_{600} of 0.05, and at 12, 18, 24, and 36 h, portions of the culture were removed and the proteins were cross-linked. After an incubation at 4°C for 12 h, the cells were disrupted and centrifuged to obtain crude extracts. An anti-Flag monoclonal antibody (Sigma) was added to the extracts to precipitate the cross-linked material, and then the cross-links were reversed by incubation at 65°C. The precipitated DNA was analyzed by PCR using gene-specific primers (MN1023/MN1025, MN1186/MN1187, MN1178/MN1179, and MN972/MN973 for *SNZ1*, *SNZ2*, *SNO2*, and *PHO5*, respectively).

Analytical methods. RNA was isolated from yeast cells grown in YPAD medium for 12, 18, 24, and 36 h by use of a RiboPure yeast RNA extraction kit (Ambion). Northern analysis was carried out as described previously (18). The β-galactosidase assay was described previously (19). Chromatin was isolated from yeast cells grown in YPAD medium at 30°C for 24 to 27 h, as described previously (27), and was digested with micrococcal nuclease (MNase) (0.1 or 0.2 units/μl) in a 200-μl reaction mixture at 37°C for 10 min. The digested chromatin was then treated with RNase, digested with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. For high-resolution mapping by primer extension, a TCGACTTTCCGGACATTGTACTGTGGGT primer covering −495 to −461 (taking the A of ATG of the *SNZ1* open reading frame [ORF] as +1) was end labeled with ^{32}P. To analyze the chromatin structure of the fully activated *SNZ1* promoter, 3-AT was added to the W303 culture at an A_{600} of 1.0, and the culture was incubated at 30°C for 1 h before the chromatin preparation. The chromatin and the purified DNA samples were digested with MNase and purified as described above. The digested DNA was then mixed with the labeled primer and subjected to a primer extension reaction as described previously (16). The products were separated on a 6% polyacrylamide–50% urea denaturing gel, and the gel was exposed to X-ray film. The autoradiogram was scanned with an Epson ES-10000G scanner, and the TIFF image thus generated was analyzed using MultiGauge software (Fujifilm, Japan).

RESULTS

Pho85 is required for *SNZ*/*SNO* gene expression. In the course of our previous microarray study (18), we noticed that *SNZ1* and *SNO1* were not properly induced during the late growth stage when Pho85 was absent (Fig. 1A). The expression of *SNZ2* and *SNO2*, the other members of the *SNZ*/*SNO* family, was also affected by a Δ*pho85* mutation, with a lesser effect on *SNZ2* (Fig. 1B). *SNZ1* has a prospective Pho4-binding site at −380 (CACGTT), whereas *SNZ2* and *SNO2* lack the binding sequences in their promoters, but two are present in the *SNO2* ORF at +271 and +492 (CTGCAC). In the absence of Pho85, Pho4 accumulates in the nucleus to activate the *PHO* genes regardless of the P_i condition (7, 8). Under this condition, if Pho4 binds to the *SNZ1* promoter, then the observed defect in the induction of *SNZ1*/*SNO1* in a Δ*pho85* mutant suggests repression by Pho4, which is generally considered to be a transcriptional activator. Therefore, we first used Northern analysis to examine whether the expression of the *SNZ* and *SNO* genes required Pho85. We detected *SNZ1* expression in the wild-type (wt) cells grown for 30 h under high-P_i conditions (Fig. 1C, lane 1). When the P_i concentration in the medium was low, *SNZ1* expression was reduced to some extent (lane 2), suggesting P_i-dependent expression of *SNZ1*. In the cells grown for 12 h, that is, to the mid-log phase, *SNZ1* expression was barely detectable under both sets of P_i conditions (lanes 1 and 2). In the absence of Pho85, *SNZ1* expression was diminished (lane 3), which coincided with the results of the GeneChip analysis (Fig. 1A). Similarly, in the absence of Pho80, a cyclin partner of Pho85 kinase for the phosphorylation of Pho4 in vivo, *SNZ1* expression was also diminished (lane 4). The introduction of a Δ*pho4* mutation to the Δ*pho85* mutant restored the *SNZ1* expression, but not to the wt level (lane 6),

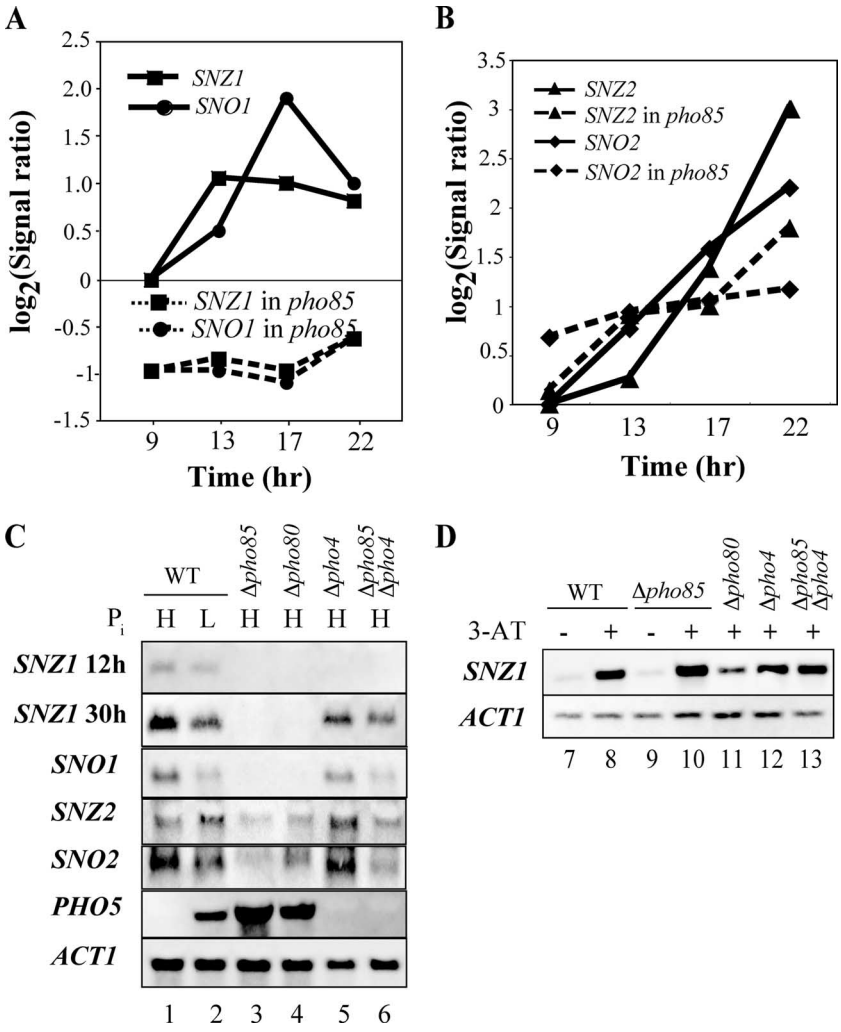

FIG. 1. Requirement of Pho85 for *SNZ*/*SNO* gene expression. (A) Time course of *SNZ1*/*SNO1* expression as determined by GeneChip analysis (based on data from reference 18). The extents of *SNZ1* (squares) and *SNO1* (circles) expression are shown as $\log_2$(signal ratio) values. Solid and broken lines designate expression in the wt and a Δ*pho85* mutant, respectively. (B) Time course of *SNZ2*/*SNO2* expression by GeneChip analysis (18). *SNZ2* (triangles) and *SNO2* (diamonds) expression levels in the wt and a Δ*pho85* mutant are shown as described for panel A. (C) Northern analysis of *SNZ1*, *SNO1*, *SNZ2*, and *SNO2* expression in the wt and various *pho* mutants and under high (H)- or low (L)-P_i conditions. Total RNA was extracted from the yeast cells (designated at the top of the panel) grown for 12 (*SNZ1* only) or 30 h and was subjected to Northern analysis as described in Materials and Methods, and then the blot was hybridized with the respective digoxigenin-labeled probe. To analyze *PHO5* expression, yeast cells were incubated in high- or low-P_i medium for 5 h before RNA isolation. *ACT1* was detected with RNA prepared from a 12-h culture. (D) Effect of 3-AT on *SNZ1* expression. Yeast strains, as designated at the top of the panel, were grown to mid-log phase, resuspended in SD medium lacking histidine and with (+) or without (−) 100 mM 3-AT, and then incubated for 1 h before RNA isolation.

while a Δ*pho4* mutation alone did not affect *SNZ1* expression significantly (lane 5). Similarly, *SNO1* expression was diminished in the absence of either Pho85 or Pho80 (lanes 3 and 4) but was not affected significantly by the absence of Pho4 (lane 5). A Δ*pho4* Δ*pho85* double mutation, however, failed to restore *SNO1* expression to the wt level (lane 6). *SNZ2* and *SNO2* also required Pho85 for their expression (Fig. 1C, lane 3) but were not as dependent on Pho80 as *SNZ1* and *SNO1* (lane 4). A loss of Pho4 did not appear to affect *SNZ2*/*SNO2* expression (lane 5), and again the introduction of a Δ*pho4* mutation to the Δ*pho85* mutant could restore their expression to some extent but not to the wt level (lane 6). *PHO5* is a typical *PHO* gene and is shown as a control that is activated by Pho4 under low-P_i conditions and inactivated by Pho85-Pho80 under high-P_i conditions, as demonstrated in Fig. 1C.

These results revealed that Pho85 is required for *SNZ1*/*SNO1* expression at a late growth stage (30 h) and suggested that Pho4 inhibits *SNZ1*/*SNO1* expression in the absence of either member of the Pho85-Pho80 complex. Notably, the fact that *SNZ1* expression failed to restore the wt level in a Δ*pho85* Δ*pho4* double mutant suggests that Pho85 may have a different function than the inactivation of Pho4 in the regulation of *SNZ1*. Pho85 is also required for the expression of the other *SNZ*/*SNO* genes tested, but their dependence on Pho80 or Pho4 is different from that of *SNZ1*.

SNZ1 expression is responsive to amino acid starvation (17).

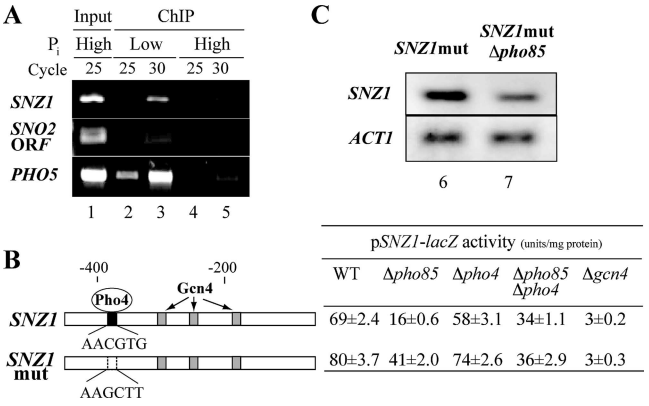

FIG. 2. Pho4 binding to the *SNZ1* promoter and involvement of the Pho4-binding site in transcriptional regulation of *SNZ1*. (A) In vivo Pho4 binding to the *SNZ1* promoter and the *SNO2* ORF demonstrated by ChIP followed by gene-specific PCR. Total DNA in the extract (Input) and the immunoprecipitated chromatin fragment prepared from the wt (MFY376) cells grown under low- or high-P_i conditions were subjected to PCR with either the *SNZ1* or *PHO5* promoter-specific or *SNO2* ORF-specific primers, and the DNA was amplified by 25 or 30 cycles of the reaction. (B) A schematic representation of *SNZ1* and its mutant (*SNZ1*mut) promoters, showing the Pho4-binding site (black box) with the wt (AACGTG) or mutant (AAGCTT) sequences and the three Gcn4-binding sites (shaded boxes). The activities of the wt and *SNZ1*mut promoters, represented by β-galactosidase activity data, are shown on the right side. The wt and various mutant strains harboring the reporter plasmid were grown in high-P_i medium for 30 h prior to the assay of the reporter activity. The values represent averages and standard errors of the results of three independent assays. (C) Northern analysis of chromosomal *SNZ1* expression in the *SNZ1*mut and *SNZ1*mut Δ*pho85* double mutant. Total RNA was isolated from cells grown for 30 h in YPAD medium and was subjected to Northern analysis as described in Materials and Methods. *ACT1* is a loading control.

To determine whether Pho85 is involved in this process, we analyzed *SNZ1* expression in the presence of 3-AT in the wt and various *pho* mutants (Fig. 1D). *SNZ1* was induced regardless of the presence or absence of Pho85 when the cells were treated with 3-AT (Fig. 1D, lanes 8 and 10), and a Δ*pho4* mutation did not affect the induction (lanes 12 and 13). These results indicated that Pho85 is not required for *SNZ1* expression in response to amino acid starvation and suggested that the Pho85 requirement is elicited specifically in the cells at the late growth stage. However, Pho85 does not appear to have a general effect on the genes whose expression is induced specifically in the late growth phase, since the loss of Pho85 does not cause reduced expression of *SPG1* and *NGR1*, which are both induced at the stationary phase (12, 18).

Pho4 binds to the *SNZ1* promoter to inhibit *SNZ1* expression. To study whether Pho4 is directly involved in the transcriptional regulation of *SNZ1*, we analyzed in vivo Pho4 binding to the *SNZ1* promoter by ChIP from cells grown under either high- or low-P_i conditions. Pho4 is known to bind to the *PHO5* promoter in a P_i-dependent fashion, as confirmed by our ChIP experiment (Fig. 2A, lanes 2 to 5). The *SNZ1* promoter fragment was also enriched in the chromatin fragments prepared from cells grown under low-P_i conditions (Fig. 2A, lane 3), indicating that Pho4 binds to the *SNZ1* promoter in vivo in a P_i-dependent manner. We also analyzed the in vivo binding of Pho4 to the *SNZ2*/*SNO2* region. The *SNZ2* promoter fragment (the intergenic region of *SNZ2*/*SNO2*) was not enriched (data not shown), whereas Pho4 bound weakly to the *SNO2* ORF under low-P_i conditions (Fig. 2A, lane 3).

Next we examined whether the prospective Pho4-binding site in the *SNZ1* promoter was functioning in the transcriptional regulation of *SNZ1* by two methods, a reporter assay and Northern analysis. After growth for 30 h, the wt promoter fused to *lacZ* showed a reduced level of activity in a Δ*pho85* mutant (about 23% of the wt level); activity was restored to about 50% of the wt level by introducing a Δ*pho4* mutation (Fig. 2B). A Δ*pho4* mutation alone did not affect significantly the activity level (Fig. 2B). A deletion of Gcn4 almost abolished the promoter activity, indicating that Gcn4 is required for *SNZ1* activation. When the sequence of the prospective Pho4-binding site (AACGTG) was altered to AAGCTT (*SNZ1*mut), the promoter activity in a Δ*pho85* mutant was reduced to about 50% of the wt level, and this was barely affected by the introduction of a Δ*pho4* mutation to the mutant (Fig. 2B). This activity level was almost equal to that of the wt promoter observed in a Δ*pho85* Δ*pho4* double mutant, suggesting that this binding site is required for Pho4 to exert its inhibitory function. This notion was further strengthened by Northern analysis of the expression of the chromosomal *SNZ1*mut in the wt (MFY365) cells or in Δ*pho85* mutant (MFY366) cells in which the wt *SNZ1* promoter sequence had been replaced by the mutant promoter sequence lacking the Pho4-binding site (Fig. 2C). *SNZ1*mut was expressed in the absence of Pho85, but not fully (Fig. 2C, lane 7), indicating that Pho4 binding is required for *SNZ1* repression in the absence of Pho85. Taken together, these results suggested that Pho4, when activated in the absence of Pho85 and bound to the *SNZ1* promoter, functions to inhibit *SNZ1* expression, while Gcn4 plays a major role in the activation of *SNZ1*. Since the mRNA level and the promoter activity of *SNZ1* were not restored to the wt level in the double mutant or with the *SNZ1*mut promoter, Pho85 seems to exert its effect through an additional, unknown mechanism to regulate *SNZ1* expression.

Pho4 functions to delay the timing of *SNZ1* expression. We next assessed the biological relevance of the inhibition of *SNZ1* by Pho4. One idea is that Pho4 may prevent the untimely expression of *SNZ1*, since *SNZ1* is expressed specifically in the late growth (postdiauxic) stage to stationary phase (21). If this hypothesis is correct, then in the absence of Pho4, *SNZ1* could be transcribed in an earlier growth stage. To test this idea, we analyzed periodical *SNZ1* expression from 12 h to 36 h in the wt, Δ*pho4*, *SNZ1*mut, Δ*pho81*, and Δ*pho85* strains (Fig. 3A, five rows from the top). In the wt cells, *SNZ1* expression became apparent at 24 h and was maximal at 36 h (Fig. 3A, left panel). On the other hand, when Pho4 was absent, *SNZ1* expression was detected at 18 h and increased along with the incubation period (24 and 36 h). A similar *SNZ1* expression profile was observed for the *SNZ1*mut strain. In a Δ*pho81* mutant in which the low-P_i signal was not transmitted to inactivate Pho85, and in which Pho4 was therefore inactive, advanced expression of *SNZ1* was also observed. In the absence of Pho85, *SNZ1* expression was not detected throughout the incubation period. Taken together, these results suggested that *SNZ1* expression at the late growth stage is regulated by Pho85-Pho4, depending on the P_i conditions. *SNO1* expression is coregulated with *SNZ1* (21), as observed with the wt panel

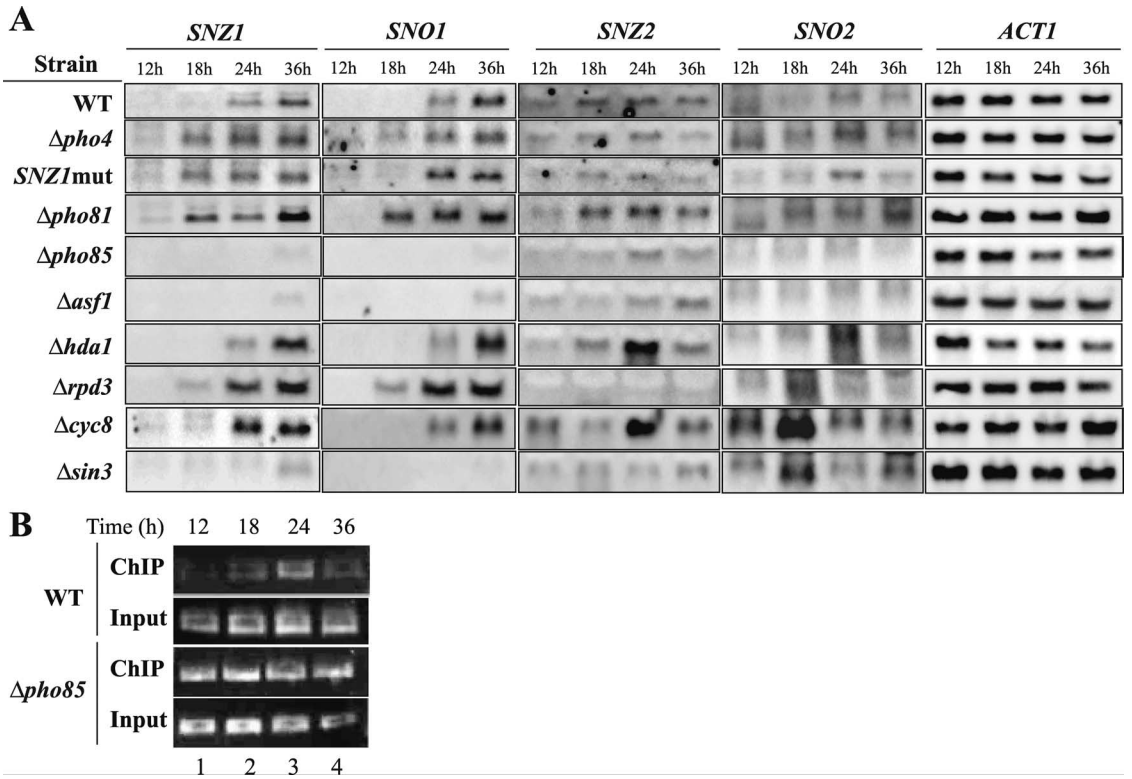

FIG. 3. (A) Northern analysis of the time course of *SNZ1*, *SNO1*, *SNZ2*, and *SNO2* gene expression in the wt and in various mutants affecting the *PHO* system or chromatin structure. Total RNA was isolated from cells grown in YPAD medium at 12, 18, 24, and 36 h and was subjected to Northern analysis as described in Materials and Methods. *ACT1* is shown as a loading control. (B) Temporal Pho4 binding to the *SNZ1* promoter in the wt (MFY376) and Δ*pho85* (MFY377) strains as demonstrated by ChIP and PCR. Total DNA in the extract (Input) and the immunoprecipitated chromatin fragments prepared from the wt and mutant cells at the designated times were subjected to PCR with the *SNZ1* promoter-specific primer and were amplified by 30 cycles of the reaction.

(Fig. 3A). The expression of *SNO1* was affected by these mutations in a manner similar to that seen with *SNZ1*, except that the absence of Pho4 slightly advanced the timing whereas that of the Pho4 binding site (*SNZ1*mut) did not (Fig. 3A, second panel from the left). Although it is a member of the *SNZ*/*SNO* family, *SNZ2* is expressed at the prediauxic stage (21). Its expression in the wt became detectable at 12 h, peaked at 18 and 24 h, and decreased at 36 h. We observed a similar profile in the mutant strains, except for Δ*pho85*, in which *SNZ2* expression was reduced (Fig. 3A, middle panel). The loss of Pho4 appears to have little effect on *SNZ2* expression in the time course experiment compared to the data shown in Fig. 1C. These results indicate that Pho4 does not play a major role in the regulation of the timing of *SNZ2* expression. *SNO2* is considered to be coregulated with *SNZ2* (21), but our results showed that the timing of expression of the two genes was different, in that *SNO2* peaked at 24 h with a barely detectable level at 18 h in the wt and *SNZ1*mut (Fig. 3A, second panel from the right). The absence of Pho4 appeared to increase the *SNO2* expression level, whereas that of Pho81 shifted the peak to 36 h. The absence of Pho85 abolished *SNO2* expression. These results revealed that *SNZ2* and *SNO2* are not coregulated and that Pho4 appears to inhibit *SNO2* expression but that expression of *SNZ2* is not inhibited to an appreciable extent (Fig. 1C and 3A). A control experiment with the *ACT1* probe revealed no significant differences in the amounts of loaded RNA (Fig. 3A, right panel). Thus, the P_i-dependent Pho4 binding appears to delay both *SNZ1* and *SNO1* expression until the appropriate growth stage, probably with less effect on the latter, and to inhibit *SNO2* expression.

To examine whether Pho4 binding to the *SNZ1* promoter is temporary or constitutive, we carried out a ChIP experiment with cell extracts prepared from cells grown for 12, 18, 24, and 36 h in YPAD medium. In the wt cells, an enrichment of the Pho4-bound fragment was observed at 24 h, and weak enrichment was observed at 18 and 36 h (Fig. 3B, lanes 2 to 4), indicating that Pho4 binding occurs at the late growth stage. In Δ*pho85* cells in which Pho4 is localized in the nucleus constitutively, Pho4 bound to the promoter throughout the growth stages (Fig. 3B).

Chromatin structure affects *SNZ1* expression and its timing. How can Pho4 exert an inhibitory function by binding to the *SNZ1* promoter? When Pho4 activates *PHO5*, Pho4 binding triggers alterations in the nucleosome positioning at the *PHO5* promoter and the Asf1 histone chaperone removes histones from the promoter region, thereby opening the TATA box (1, 32). We considered the possibility that similar chromatin alterations caused by Pho4 binding may lead to transcriptional repression of *SNZ1*. To examine this possibility, we first analyzed the effects of mutations that affect chromatin structure (Δ*asf1*, Δ*hda1*, Δ*rpd3*, and Δ*sin3*) and general repression (Δ*cyc8*) on the timing of *SNZ*/*SNO* gene expression. A pro-

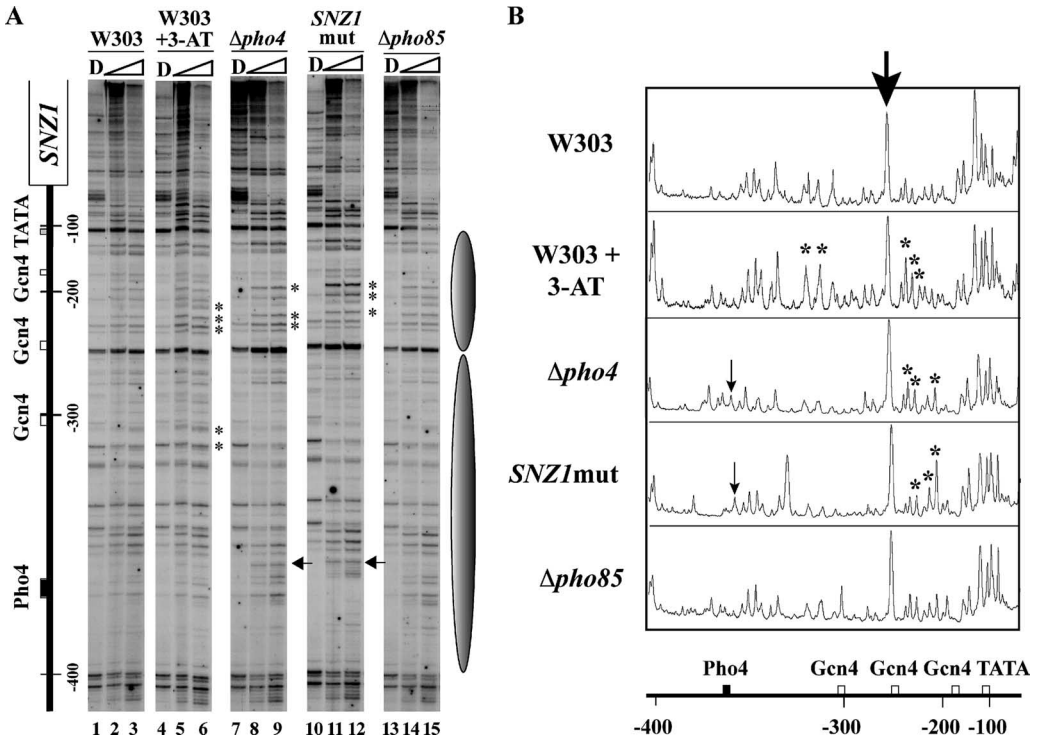

FIG. 4. A high-resolution analysis of chromatin structure in the *SNZ1* promoter region. (A) Chromatin DNA was isolated from the strains designated at the top of the panel, digested with MNase (0.1 or 0.2 units/μl) as described in Materials and Methods, and subjected to a primer extension reaction using a ^{32}P-labeled primer (−495 to −461). A schematic representation of the *SNZ1* promoter region, showing the TATA box and the Pho4- and Gcn4-binding sites, is on the left side of the panel. Numerals designate nucleotide distance, taking the A of ATG of the *SNZ1* ORF as +1. D, purified DNA digested with MNase. The arrows indicate enhanced MNase cleavage signals near the Pho4-binding site observed in the chromatin samples from Δ*pho4* and *SNZ1*mut strains. Asterisks are for those observed between the two downstream Gcn4-binding sites (at −255 and −180) and near the upstream Gcn4 site at −305 compared to those in the wt and Δ*pho85* strains. Possible nucleosome positions are designated by gray ellipses on the right-hand side of the panel. (B) A densitometric analysis of the gel images. The lanes containing the chromatin samples digested with 0.1 U/μl MNase were analyzed (lanes 2, 5, 8, 11, and 14 in Fig. 4A). The intensity of the bands was normalized with reference to the strongly cleaved band at −255 (bold arrow). The asterisks and arrows designate the peaks of the corresponding bands in panel A. A schematic representation of the *SNZ1* promoter is shown at the bottom.

teome analysis revealed a physical interaction between Pho4 and Sin3 forming the HDAC complex with Rpd3 (6). Repression by the Cyc8-Tup1 corepressor reportedly involves histone deacetylation (30), and the corepressor can interact with Rpd3 in vitro (33). Asf1 counteracts silencing by chromatin, and its absence diminished *SNZ1*, *SNO1*, and *SNO2* expression and significantly reduced *SNZ2* expression (Fig. 3A), indicating that Asf1 activity is required for *SNZ*/*SNO* expression. HDAC usually functions to repress transcription by stabilizing chromatin. The loss of Rpd3 HDAC advanced the timing of *SNZ1*/*SNO1* expression, whereas that of Hda1, another yeast HDAC, did not appear to affect the timing but increased the expression levels of the two genes at 36 h (Fig. 3A). This observation suggests the possibility that a specific HDAC activity is required to regulate the timing of *SNZ1*/*SNO1* expression. The absence of Cyc8 appeared to increase *SNZ1* expression at 24 h and to advance the timing slightly (Fig. 3A) but did not appear to affect *SNO1* expression (Fig. 3A). These results suggested the possibility that Cyc8 may also be involved in *SNZ1* repression at the late growth stage. Although Sin3 forms a complex with Rpd3, the effect of a Δ*sin3* mutation was completely different from that of Δ*rpd3*, i.e., a decrease in *SNZ1*/*SNO1* expression, as seen with Δ*asf1* (Fig. 3A). These mutations showed different effects on *SNZ2*/*SNO2*. The absence of Hda1 or Cyc8 made *SNZ2* expression peak at 24 h, whereas that of Rpd3 or Sin3 caused a decrease in *SNZ2* expression (Fig. 3A). With respect to *SNO2*, the absence of Hda1 increased its expression at 24 h whereas that of Rpd3, Cyc8, or Sin3 advanced its peak to 18 h (Fig. 3A). These results indicated that the chromatin structure is involved in the regulation of the *SNZ*/*SNO* genes by altering the timing and probably the expression levels of these genes, with different effects on the individual members.

Pho4 alters chromatin structure in the *SNZ1* promoter region. We next analyzed whether Pho4 binding affects the chromatin structure at the *SNZ1* promoter region by limited digestion of isolated nuclei with MNase followed by high-resolution mapping of MNase cleavage sites by primer extension analysis with a primer covering −495 to −461 (taking the A of ATG of the *SNZ1* ORF as +1) (Fig. 4). In naked DNA as well as in the chromatin samples in the tested strains, MNase cleaved strongly at around −400, −255, and −105 in the *SNZ1* promoter (Fig. 4A), and the spaces between each pair of bands were about 145 bp in length, which could accommodate a single nucleosome. Thus, we supposed that nucleosomes might be positioned in these promoter regions (Fig. 4A). Since the

cells from which the chromatin was isolated were grown for 24 to 27 h in YPAD medium, the *SNZ1* promoter in the wt should have been partially activated (Fig. 3A). When the *SNZ1* promoter was fully activated by the addition of 3-AT, the two regions encompassing the upstream Gcn4-binding site (at −305) and that sandwiched between the other two Gcn4-binding sites (at −255 and −180) became more susceptible to MNase digestion in the chromatin samples (Fig. 4A, lanes 5 and 6) compared to those in the wt (lanes 2 and 3) and Δ*pho85* (lanes 14 and 15) strains. Similarly, in the chromatin samples prepared from Δ*pho4* and *SNZ1*mut strains in which Pho4 was absent from the cell and Pho4 failed to bind to the promoter because of the loss of the binding site, respectively, the region between the two downstream Gcn4-binding sites (at −255 and −180) showed increased accessibility (Fig. 4A, lanes 8, 9, 11, and 12). These observations were confirmed by densitometric analysis of the gel image (Fig. 4B). Thus, in the absence of Pho4 binding, the chromatin structure of these regions became unstable, allowing proteins more access to the *SNZ1* promoter.

In the absence of either Pho4 (Δ*pho4*) or the Pho4-binding site (*SNZ1*mut), MNase cleaved a site adjacent to the Pho4-binding site (Fig. 4A and 4B), which was not observed in the wt or Δ*pho85* strain (Fig. 4A and 4B), indicating that the accessibility of MNase to this site is Pho4 dependent. This result revealed in vivo binding of Pho4 to the *SNZ1* promoter, which is in good agreement with ChIP analysis data for Pho4 binding (Fig. 2A).

DISCUSSION

SNZ1 and *SNO1* are expressed specifically in the postdiauxic to stationary phases (21). In this work, we have shown that Pho4 functions to repress or down-regulate the transcription of *SNZ1* so that *SNZ1* is appropriately expressed in this late growth stage. Three lines of evidence support this conclusion. (i) Northern analysis and a reporter assay demonstrated that Pho4 inhibits *SNZ1* expression in the absence of Pho85, which allows Pho4 to become active (Fig. 1 and 2). (ii) The ChIP analysis revealed that Pho4 binds to the *SNZ1* promoter in vivo in a P_i-dependent and transient fashion, and this binding is required for repression/downregulation of *SNZ1* (Fig. 2). (iii) The absence of Pho4 in the cell, Pho4 binding to the *SNZ1* promoter lacking the Pho4 binding site (*SNZ1*mut), or Pho81, which transmits low-P_i signal, advances the timing of *SNZ1* expression (Fig. 3A). *SNO1* is coregulated with *SNZ1* (3, 21), and its timing appears to be regulated similarly by Pho4 but in a weaker manner (Fig. 3A). We can imagine that a destabilized nucleosome in the absence of Pho4 could affect the bidirectional transcription of *SNZ1* and *SNO1* to different extents. *SNZ2* and *SNO2*, other members of the *SNZ*/*SNO* family, are expressed at an earlier growth stage than *SNZ1*/*SNO1*, that is, at the diauxic to postdiauxic stage (21). Pho4 does not appear to affect the timing of *SNZ2* expression, although it may affect the expression level to some extent (Fig. 3A). On the other hand, Pho4 binding within the *SNO2* ORF may play an inhibitory role in *SNO2* transcription and thus regulate its timing of expression (Fig. 2A and Fig. 3A). These observations showed that Pho4 binding is likely to contribute to the different timings of expression of the genes of the *SNZ*/*SNO* family, although we are still unable to exclude the possibility that the effect of Pho4 is indirect.

Pho85 is required for the expression of all of the *SNZ*/*SNO* genes tested (Fig. 1 and 3). The observations that the mRNA level and the promoter activity of *SNZ1* are not restored to the wt level in a Δ*pho4* Δ*pho85* double mutant and with the *SNZ1*mut promoter suggest that Pho85 is required to counteract an as-yet-unknown repressor other than Pho4 or to activate an unknown activator. This notion is more significant for *SNO1*, *SNZ2*, and *SNO2*, since expression of those genes was barely recovered in the double mutant, suggesting that Pho85 regulates those genes mainly through a different pathway than via Pho4. *SNZ1* and *SNO1* are required for yeast growth when the intracellular vitamin B_6 level is low, suggesting an involvement of these genes in the biosynthesis of the vitamin (23). *SNZ2* does not appear to be required for the vitamin B_6 pathway (23). On the other hand, *SNZ2* and *SNO2* are induced by thiamine depletion whereas *SNZ1* and *SNO1* are not (23). Although physical interactions among the Snz and Sno proteins have been reported (23), at present, we cannot provide a clear explanation of why the timing of the expression of these *SNZ*/*SNO* genes is differently regulated and how the difference is related to apparently distinct functions of the members of the *SNZ*/*SNO* gene family.

Since the binding of Pho4 to the *SNZ1* promoter is dependent on the P_i condition (Fig. 2A), although not as strongly as that of *PHO5*, the repression of *SNZ1* by Pho4 should be regulated by the P_i condition. In the late growth stage, the amount of P_i in the medium is usually limited, resulting in derepression of some *PHO* genes; specifically, *PHO84* and *PHO5*, which show a quick response to P_i limitation (31), are induced at 22 h of incubation, whereas the other *PHO* genes are still repressed (5, 18). Since the amount of nuclear Pho4 increases as the environmental P_i concentration decreases, this different response probably reflects the different affinities of binding of Pho4 to the specific promoters. Pho4 appears to bind transiently to the *SNZ1* promoter, peaking at 24 h (Fig. 3B), and we assume that this binding responds to the P_i limitation to prevent the inappropriate or untimely expression of *SNZ1*. Advanced *SNZ1* expression in a Δ*pho81* mutant, in which the low-P_i signal is not transmitted to inactivate Pho85-Pho80, supports the idea of *SNZ1* repression by P_i-responsive Pho4 binding (Fig. 3A). Upon entering the postdiauxic to stationary phase, an activator of *SNZ1*, possibly Gcn4, is induced sufficiently to overcome the repression by Pho4. Gcn4 is likely to play a major role in the activation process, since, in the absence of Gcn4, *SNZ1* promoter activity at 30 h was drastically decreased (Fig. 2B). We can imagine the possibility that Gcn4 may destabilize Pho4 binding, possibly by nucleosome replacement. In the absence of Pho85, too much Pho4 accumulates in the nucleus for the activator to relieve repression. Although the Pho85-Pcl5 Cdk-cyclin complex phosphorylates Gcn4 to trigger its degradation (2, 13), *PCL5* expression is drastically reduced in the postdiauxic stage (19), making the complex much less active, and therefore Gcn4 becomes much more stable, thus leading to the activation of the transcription factor at this growth stage. When fully activated by amino acid starvation (treatment with 3-AT), Gcn4 can overcome the repression of *SNZ1* caused by the presence of excess Pho4 in a Δ*pho85* mutant (Fig. 1D).

Our conclusion implies that Pho4 functions as both an activator and a repressor. Rap1 and Abf1 are also known to possess the two opposite functions. Rap1 activates genes encoding glycolytic enzymes and ribosomal proteins and maintains the silent chromatin structure at telomeres and the silent mating type locus (15, 28). Repression by Abf1 also involves the silent chromatin structure (14, 36). In the case of Pho4, activation (derepression) of *PHO5* requires alterations in nucleosome positioning at its promoter, which involves Pho4 binding and its activation domain (32). The Asf1 histone chaperone displaces histones for *PHO5* activation by Pho4 (1, 32). Our high-resolution mapping results indicated that a loss of Pho4 binding increases the accessibility of MNase to the *SNZ1* promoter region (Fig. 4). Since the Pho4-binding site at −380 may exist at the edge of a nucleosome (Fig. 4A), we suppose that Pho4 can stabilize an array of nucleosomes positioned in the *SNZ1* promoter, leading to the repression of *SNZ1*. In the absence of Pho4 binding, the nucleosome array becomes unstable, making Gcn4 more easily accessible to its binding sites, as represented by the increased MNase cleavage bands around −255 and −180. This scenario can account for the advanced activation of *SNZ1* in the Δ*pho4* and *SNZ1*mut strains (Fig. 3A). The loss of Asf1 abolished *SNZ1*/*SNO1* expression (Fig. 3A), indicating that Asf1 activity is required in the activation process of *SNZ1*/*SNO1*, probably by removing histones from the promoter region. Since Asf1 activity is also required for *SNZ2* expression, its participation does not appear to be specific to Pho4-involved regulation but is instead rather general.

The idea of involvement of chromatin structure in the transcriptional regulation of *SNZ1* is also supported by a microarray analysis demonstrating that histone H4 depletion activates *SNZ1*/*SNO1* (35). A proteome analysis revealed a physical interaction between Pho4 and Hht1 comprising histone H3 (11), and with Sin3 forming the HDAC complex with Rpd3 (6), suggesting the direct involvement of Pho4 in transcriptional repression through interactions with the nucleosome. In fact, we have shown that the absence of Rpd3, but not Hda1, advances the timing of *SNZ1*/*SNO1* expression (Fig. 3A), suggesting the possibility that the Rpd3-Sin3 HDAC complex is recruited to the *SNZ1* promoter through an interaction with Pho4. The absence of Sin3, however, abolished *SNZ1*/*SNO1* expression, which argues against this model. Since Sin3 functions as a scaffold protein interacting with a variety of factors to form heterogeneous HDAC complexes regulating global gene expression (29), we suppose that the loss of Sin3 causes a global effect that leads to rather indirect repression of *SNZ1*/*SNO1*. *SNZ2* expression is altered by the deletion of *ASF1*, *RPD3*, *HDA1*, *CYC8*, or *SIN3*, suggesting that the chromatin structure is also involved in *SNZ2* regulation. However, their effects on the expression level or timing are different from the results seen with *SNZ1*. This implies that a complex interplay of factors, including Pho4 and those that remodel chromatin structure, may be responsible for the different timing of expression among the *SNZ*/*SNO* gene family members.

The frequent appearance of the Pho4-binding sequence (C ACGTG/T and CTGCAC) in the intergenic regions of the yeast genome (ca. 2,800) suggests that Pho4 may be involved more generally in either positive or negative transcriptional regulation than is currently considered to be the case. A Pho4-binding analysis conducted on a genome-wide scale by ChIP-on-chip analysis, combined with a global analysis of the Pho4 effect on gene expression, should yield more insights into the broad functions of this transcription factor.

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REFERENCES

1. **Adkins, M. W., S. R. Howar, and J. K. Tyler.** 2004. Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. Mol. Cell **14:**657–666.
2. **Bömeke, K., R. Pries, V. Korte, E. Scholz, B. Herzog, F. Schulze, and G. H. Braus.** 2006. Yeast Gcn4p stabilization is initiated by the dissociation of the nuclear Pho85p/Pcl5p complex. Mol. Biol. Cell **17:**2952–2962.
3. **Braun, E. L., E. K. Fuge, P. A. Padilla, and M. Werner-Washburne.** 1996. A stationary-phase gene in *Saccharomyces cerevisiae* is a member of a novel, highly conserved gene family. J. Bacteriol. **178:**6865–6872.
4. **Carroll, A. S., and E. K. O'Shea.** 2002. Pho85 and signaling environmental conditions. Trends Biochem. Sci. **27:**87–93.
5. **DeRisi, J. L., V. R. Iyer, and P. O. Brown.** 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:**680–686.
6. **Graumann, J., L. A. Dunipace, J. H. Seol, W. H. McDonald, J. R. Yates III, B. J. Wold, and R. J. Deshaies.** 2004. Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. Mol. Cell. Proteomics **3:**226–237.
7. **Kaffman, A., N. M. Rank, E. M. O'Neill, L. S. Huang, and E. K. O'Shea.** 1998. The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature **396:**482–486.
8. **Kaffman, A., N. M. Rank, and E. K. O'Shea.** 1998. Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev. **12:**2673–2683.
9. **Katou, Y., K. Kaneshiro, H. Aburatani, and K. Shirahige.** 2006. Genomic approach for the understanding of dynamic aspect of chromosome behavior. Methods Enzymol. **409:**389–410.
10. **Katou, Y., Y. Kanoh, M. Bando, H. Noguchi, H. Tanaka, T. Ashikari, K. Sugimoto, and K. Shirahige.** 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature **424:**1078–1083.
11. **Krogan, N. J., G. Cagney, H. Yu, G. Zhong, X. Guo, A. Ignatchenko, J. Li, S. Pu, N. Datta, A. P. Tikuisis, et al.** 2006. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature **440:**637–643.
12. **Martinez, M. J., S. Roy, A. B. Archuletta, P. D. Wentzell, S. S. Anna-Arriola, A. L. Rodriguez, A. D. Aragon, G. A. Quinones, C. Allen, and M. Werner-Washburne.** 2004. Genomic analysis of stationary-phase and exit in *Saccharomyces cerevisiae*: gene expression and identification of novel essential genes. Mol. Biol. Cell **15:**5295–5305.
13. **Meimoun, A., T. Holtzman, Z. Weissman, H. J. McBride, D. J. Stillman, G. R. Fink, and D. Kornitzer.** 2000. Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex. Mol. Biol. Cell **11:**915–927.
14. **Miyake, T., J. Reese, C. M. Loch, D. T. Auble, and R. Li.** 2004. Genome-wide analysis of ARS (autonomously replicating sequence) binding factor 1 (Abf1p)-mediated transcriptional regulation in *Saccharomyces cerevisiae*. J. Biol. Chem. **279:**34865–34872.
15. **Moretti, P., and D. Shore.** 2001. Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast. Mol. Cell. Biol. **21:**8082–8094.
16. **Morohashi, N., Y. Yamamoto, S. Kuwana, W. Morita, H. Shindo, A. P. Mitchell, and M. Shimizu.** 2006. Effect of sequence-directed nucleosome disruption on cell-type specific repression by α2/Mcm1 in the yeast genome. Eukaryot. Cell **5:**1925–1933.
17. **Natarajan, K., M. R. Meyer, B. M. Jackson, D. Slade, C. Roberts, A. G. Hinnebusch, and M. J. Marton.** 2001. Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol. Cell. Biol. **21:**4347–4368.
18. **Nishizawa, M., Y. Katou, K. Shirahige, and A. Toh-e.** 2004. Yeast Pho85 kinase is required for proper gene expression during the diauxic shift. Yeast **21:**903–918.
19. **Nishizawa, M., M. Tanabe, N. Yabuki, K. Kitada, and A. Toh-e.** 2001. Pho85 kinase, a yeast cyclin-dependent kinase, regulates the expression of *UGP1* encoding UDP-glucose pyrophosphorylase. Yeast **18:**239–249.
20. **Oshima, Y.** 1997. The phosphate system in *Saccharomyces cerevisiae*. Genes Genet. Syst. **72:**323–334.
21. **Padilla, P. A., E. K. Fuge, M. E. Crawford, A. Errett, and M. Werner-Washburne.** 1998. The highly conserved, coregulated *SNO* and *SNZ* gene

families in *Saccharomyces cerevisiae* respond to nutrient limitation. J. Bacteriol. **180:**5718–5726.
22. **Reid, R. J. D., M. Lisby, and R. Rothstein.** 2002. Cloning-free genome alterations in *Saccharomyces cerevisiae* using adaptamer-mediated PCR. Methods Enzymol. **350:**258–277.
23. **Rodríguez-Navarro, S., B. Llorente, M. T. Rodríguez-Manzaneque, A. Ramne, G. Uber, D. Marchesan, B. Dujon, E. Herrero, P. Sunnerhagen, and J. E. Perez-Ortin.** 2002. Functional analysis of yeast gene families involved in metabolism of vitamins B1 and B6. Yeast **19:**1261–1276.
24. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. A laboratory course manual. Cold Harbor Spring Laboratory Press, Cold Spring Harbor, NY.
25. **Sambrook, J., and D. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Schultz, J., and M. Carlson.** 1987. Molecular analysis of *SSN6*, a gene functionally related to the SNF1 protein kinase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**3637–3645.
27. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*. EMBO J. **10:**3033–3041.
28. **Shore, D.** 1994. RAP1: a protean regulator in yeast. Trends Genet. **10:**408–412.
29. **Silverstein, R. A., and K. Ekwall.** 2005. Sin3: a flexible regulator of global gene expression and genome stability. Curr. Genet. **47:**1–17.
30. **Smith, R. L., and A. D. Johnson.** 2000. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. Trends Biochem. Sci. **25:**325–330.
31. **Springer, M., D. D. Wykoff, N. Miller, and E. K. O'Shea.** 2003. Partially phosphorylated Pho4 activates transcription of a subset of phosphate-responsive genes. PLoS Biol. **1:**E28.
32. **Svaren, J., and W. Horz.** 1997. Transcription factors vs nucleosomes: regulation of the *PHO5* promoter in yeast. Trends Biochem. Sci. **22:**93–97.
33. **Waterborg, J. H.** 2000. Steady-state levels of histone acetylation in *Saccharomyces cerevisiae*. J. Biol. Chem. **275:**13007–13011.
34. **Wilson, W. A., and P. J. Roach.** 2002. Nutrient-regulated protein kinases in budding yeast. Cell **111:**155–158.
35. **Wyrick, J. J., F. C. Holstege, E. G. Jennings, H. C. Causton, D. Shore, M. Grunstein, E. S. Lander, and R. A. Young.** 1999. Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature **402:**418–421.
36. **Zou, Y., Q. Yu, and X. Bi.** 2006. Asymmetric positioning of nucleosomes and directional establishment of transcriptionally silent chromatin by *Saccharomyces cerevisiae* silencers. Mol. Cell. Biol. **26:**7806–7819.