Nutrient-Regulated Antisense and Intragenic RNAs Modulate a Signal Transduction Pathway in Yeast

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The budding yeast *Saccharomyces cerevisiae* alters its gene expression profile in response to a change in nutrient availability. The PHO system is a well-studied case in the transcriptional regulation responding to nutritional changes in which a set of genes (PHO genes) is expressed to activate inorganic phosphate (Pi) metabolism for adaptation to Pi starvation. Pi starvation triggers an inhibition of Pho85 kinase, leading to migration of unphosphorylated Pho4 transcriptional activator into the nucleus and enabling expression of PHO genes. When Pi is sufficient, the Pho85 kinase phosphorylates Pho4, thereby excluding it from the nucleus and resulting in repression (i.e., lack of transcription) of PHO genes. The Pho85 kinase has a role in various cellular functions other than regulation of the PHO system in that Pho85 monitors whether environmental conditions are adequate for cell growth and represses inadequate (untimely) responses in these cellular processes. In contrast, Pho4 appears to activate some genes involved in stress response and is required for G1 arrest caused by DNA damage. These facts suggest the antagonistic function of these two players on a more general scale when yeast cells must cope with stress conditions. To explore general involvement of Pho4 in stress response, we tried to identify Pho4-dependent genes by a genome-wide mapping of Pho4 and Rpo21 binding (Rpo21 being the largest subunit of RNA polymerase II) using a yeast tiling array. In the course of this study, we found Pi- and Pho4-regulated intragenic and antisense RNAs that could modulate the Pi signal transduction pathway. Low-Pi signal is transmitted via certain inositol polyphosphate (IP) species (IP7) that are synthesized by Vip1 IP6 kinase. We have shown that Pho4 activates the transcription of antisense and intragenic RNAs in the KCS1 locus to down-regulate the Kcs1 activity, another IP6 kinase, by producing truncated Kcs1 protein via hybrid formation with the KCS1 mRNA and translation of the intragenic RNA, thereby enabling Vip1 to utilize more IP6 to synthesize IP7 functioning in low-Pi signaling. Because Kcs1 also can phosphorylate these IP7 species to synthesize IP8, reduction in Kcs1 activity can ensure accumulation of the IP7 species, leading to further stimulation of low-Pi signaling (i.e., forming a positive feedback loop). We also report that genes apparently not involved in the PHO system are regulated by Pho4 either dependent upon or independent of the Pi conditions, and many of the latter genes are involved in stress response. In *S. cerevisiae*, a large-scale cDNA analysis and mapping of RNA polymerase II binding using a high-resolution tiling array have identified a large number of antisense RNA species whose functions are yet to be clarified. Here we have shown that nutrient-regulated antisense and intragenic RNAs as well as direct regulation of structural gene transcription function in the response to nutrient availability. Our findings also imply that Pho4 is present in the nucleus even under high-Pi conditions to activate or repress transcription, which challenges our current understanding of Pho4 regulation.

Introduction

When environmental conditions change, the budding yeast *Saccharomyces cerevisiae*, like other microorganisms, makes a decision about growth, cell division, and which responses to elicit in a coordinated fashion. Starvation for nutrients, alterations in temperature or salt concentration, and the presence of toxic agents are critical stresses for yeast cells and elicit signals that evoke cellular responses favoring survival under the new conditions. Nutrient status is probably the most important condition that must be accurately and rapidly sensed and responded to in order to ensure cell survival. In this process, nutrient-sensing kinases including cикlic-adenosine-monophosphate-dependent kinase, Snf1, Tor, and Pho85 kinases play important roles in regulation at levels ranging from transcription to the activity of individual enzymes [1,2]. Transcriptional regulation is the most fundamental process in the nutritional response, and DNA-binding transcription factors and genes under their control are extensively characterized by conventional genetic and biochemical analyses and, more recently, expression profiling via DNA microarray and chromatin immunoprecipitation

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Abbreviations: AS RNA, antisense RNA; ChIP, chromatin immunoprecipitation; DIG, digoxigenin; IP, inositol polyphosphate; ncRNA, noncoding RNA; PI, inorganic phosphate

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Author Summary

How does a microorganism adapt to changes in its environment? Phosphate metabolism in the budding yeast *Saccharomyces cerevisiae* serves as a model for investigating mechanisms involved in physiological adaptation. The nutrient inorganic phosphate (Pi) is essential for building nucleic acids and phospholipids; when yeast cells are deprived of Pi, genes required for scavenging the nutrient are activated. This activation is mediated by the Pho4 transcription factor through its migration into or out of nucleus. The Pi-starvation (low-Pi) signal is transmitted by a class of inositol polyphosphate (IP) species, IP₇, which is synthesized by one of two IP₆ kinases, Vip1 or Kcs1. However, the IP₇ made primarily by Vip1 is key in the signaling pathway. Here we report that under Pi starvation Pho4 binds within the coding sequence of KCS1 to activate transcription of both intragenic and antisense RNAs, resulting in the production of a truncated Kcs1 protein and the likely down-regulation of Kcs1 activity. Consequently Vip1 can produce more IP₇ to enhance the low-Pi signaling and thus form a positive feedback loop. We have also demonstrated that Pho4 regulates, both positively and negatively, transcription of genes apparently uninvolved in cellular response to Pi starvation and that it sometimes does so independently of Pi conditions. These findings reveal mechanisms that go beyond the currently held model of Pho4 regulation.

Nutrient-Responsive Antisense and Intragenic RNAs

This system serves as a model for investigating mechanisms involved in physiological adaptation. The nutrient inorganic phosphate (Pi) is essential for building nucleic acids and phospholipids; when yeast cells are deprived of Pi, genes required for scavenging the nutrient are activated. This activation is mediated by the Pho4 transcription factor through its migration into or out of nucleus. The Pi-starvation (low-Pi) signal is transmitted by a class of inositol polyphosphate (IP) species, IP₇, which is synthesized by one of two IP₆ kinases, Vip1 or Kcs1. However, the IP₇ made primarily by Vip1 is key in the signaling pathway. Here we report that under Pi starvation Pho4 binds within the coding sequence of KCS1 to activate transcription of both intragenic and antisense RNAs, resulting in the production of a truncated Kcs1 protein and the likely down-regulation of Kcs1 activity. Consequently Vip1 can produce more IP₇ to enhance the low-Pi signaling and thus form a positive feedback loop. We have also demonstrated that Pho4 regulates, both positively and negatively, transcription of genes apparently uninvolved in cellular response to Pi starvation and that it sometimes does so independently of Pi conditions. These findings reveal mechanisms that go beyond the currently held model of Pho4 regulation.

ChIP-on-Chip Analysis Revealed Novel PHO-Type Genes

To analyze the Pho4 binding sites in the yeast genome, we used two kinds of oligo-DNA arrays, an Affymetrix high-density oligo-DNA array harboring 25-mer oligonucleotides with 4-nucleotide spatial resolution (high-resolution [HR] chip) and an Agilent yeast whole genome 44K array that had 60-mer oligos with ca. 270-nucleotide spatial resolution (low-resolution [LR] chip). The complete datasets of the HR chip analysis are found in the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE13350. Analysis with the HR and LR arrays revealed that Pho4 bound
to 51 and 57 genes, respectively, under low- but not high-Pi conditions (Table S2). Thirty-five genes were common in the two analyses, and all but two of these (KCS1 and SHE9) had a prospective Pho4 binding sequence (CACGTG/T or CTGCAC) in their upstream regions. For the two exceptions, a binding sequence was present within the ORF (Table S2). Among the 35 genes, 16 had already been reported as PHO genes, that is, genes regulated by Pho4 in a Pi-dependent manner, by genetic, biochemical, and microarray analyses [30,31]. Our results demonstrated that Pho4 actually binds to the upstream regions of these genes depending on Pi conditions. In addition to the known PHO genes, our analyses identified 19 genes as possible novel PHO-type genes. Among them, eight genes, PST1, MNN1, HOM3, HOR7, PTK2, CBF1, SUR1, and GLN1, showed the expected pattern of Rpo21-binding (Table S2). Representative results for PHO8 and PST1 are shown in Figure 1A: Pho4 binding to the upstream regions of these two genes depending upon Pi condition is demonstrated by the two different ChIP-on-chip analyses (Figure 1A), Pi- and Pho4-dependent transcription by Rpo21 binding (Figure 1A) and by northern analysis (Figure 1B), and a Pi-dependent in vivo binding of Pho4 to their promoter regions by gene-specific PCR of a chromatin-immunoprecipitated (ChIPed) DNA fragments dependent on Pi conditions analyzed by gene-specific PCR. Total DNA in the whole cell extract (input) or ChIPed DNA fragments prepared from the wt cells grown under high- or low-Pi conditions were amplified by PCR for 25 or 30 cycles using primer pairs specific to the promoter regions of the genes designated on the left-hand side of the panel. The AIR2 ORF is the negative control for Pho4 binding. doi:10.1371/journal.pbio.0060326.g001

Figure 1. Phosphate Condition-Dependent Pho4 Binding and Pho4-Dependent Expression of Genes

(A) Localization of Pho4 around the PHO8 and PST1 loci. Schematic representations of the regions of these genes (adopted from the results of pattern matching analysis provided by Saccharomyces Genome Database [SGD], http://www.yeastgenome.org/ATContents.shtml) showing prospective Pho4 binding sites (X) are at the top. The next two rows show Pho4 localization to these regions in the wild-type (WT) cells grown under high (H)- or low (L)-phosphate (Pi) conditions analyzed with high- and low-resolution (HR and LR) chips. Localization of Rpo21, the largest subunit of RNA polymerase II, to these regions in the WT or a Δpho4 mutant analyzed with HR chip is shown in the bottom two rows. Yeast cells containing Pho4-Flag × 3 or Rpo21-Flag × 3 were processed for chromatin immunoprecipitation (ChIP) as described in Methods, and enrichment in the immunoprecipitated fraction relative to whole genome DNA is shown in each panel. Blue bars above and below the x-axis represent genes on the Watson and Crick strands, respectively. The y-axis scales are log2(signal ratio) and normalized ratio for HR and LR chips, respectively. In HR chip panels, brown bars represent signals that are judged as significant binding, whereas gray bars are not. The normalized ratio values of the peaks are shown in the LR chip panels. (B) Northern analysis of genes showing Pho4- and Pi-dependent expression. Total RNA was isolated from the wt, Δpho4, or Δpho85 cells grown under high (H)- or low (L)-Pi conditions, subjected to northern blot analysis, and probed with a digoxigenin (DIG)-labeled probe for each gene as designated. (C) Enrichment of promoter regions in the chromatin-immunoprecipitated (ChIPed) DNA fragments dependent on Pi conditions analyzed by gene-specific PCR. Total DNA in the whole cell extract (input) or ChIPed DNA fragments prepared from the wt cells grown under high- or low-Pi conditions were amplified by PCR for 25 or 30 cycles using primer pairs specific to the promoter regions of the genes designated on the left-hand side of the panel. The AIR2 ORF is the negative control for Pho4 binding.
The upstream region of CDC19 did not play a major role in transcriptional regulation of CDC19.

**Pho4 Can Either Activate or Repress Gene Expression Independent of Phosphate Conditions**

ChIP-on-chip analyses with the HR and LR arrays revealed that 140 and 30 genes, respectively, showed Pi-independent binding of Pho4, and among them, nine genes (URA3, MUC1, CIS3, ILV3, PDC1, YPS3, YLR137W, HPF1, and ASN1) were commonly detected. Each of these nine genes has the prospective Pho4 binding site in its promoter or ORF (Table S3). We focused on ILV3, ASN1, CIS3, and YPS3 for further analysis. Analyses of the binding profiles of Pho4 to these genes by ChIP-on-chip analysis using the two different platforms are shown in Figure 2A. Although the binding profiles of Rpo21 in the wild-type (wt) or Δpho4 mutant exhibited some inconsistency with those expected from Pho4 binding profiles, northern analysis demonstrated Pi-independent but Pho4-dependent expression, either a decrease (ILV3 and ASN1) or an increase (CIS3 and YPS3) in the absence of Pho4 (Figure 2B). Gene-specific PCR demonstrated that Pho4 bound to the upstream region of these genes in vivo irrespective of Pi conditions (Figure 2C). These results raise possibilities that Pho4 can bind to a promoter under high-Pi conditions and that Pho4 binding can lead to transcriptional activation (ASN1 and ILV3) or repression.

**Figure 2.** Phosphate-Independent Localization of Pho4 to the Intergenic Region or ORF of Yeast Genes

(A) Localization of Pho4 around the ILV3, ASN1, CIS3, and YPS3 loci. Prospective Pho4 binding sites (X) in each region are shown in the schematic representation of the regions containing these genes at the top. Next two rows show Pho4 localization to these regions in the wild-type (WT) cells grown under high (H) or low (L)-phosphate (Pi) conditions analyzed with high- and low-resolution (HR and LR) chips. The bottom two rows show Rpo21 localization to these regions analyzed with an HR chip.

(B) Northern analysis of genes showing Pi-independent but Pho4-dependent expression, as in the legend to Figure 1B. Pho4 dependency was either positive (ILV3 and ASN1) or negative (CIS3 and YPS3) for expression. ACT1 is the loading control.

(C) Enrichment of promoter regions in the ChIPed DNA fragments independent of Pi conditions analyzed by gene-specific PCR. Conditions for PCR are as described in the legend to Figure 1.

(D) A schematic representation of the ASN1 promoter showing Pho4 and Gcn4 binding sites (AAGCTG and TGACTC, respectively) and activities of the wt and mutant (ASN1mut; lacking Pho4 binding site) promoters represented by β-galactosidase activity. The wt, Δpho85, Δpho4, or Δgcn4 cells harboring respective reporter plasmids were grown to the mid-log phase under high-Pi conditions before the preparation of cell extracts. The values are an average of three different assays, each of which contains measurements with three independent clones.

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(CIS3 and YPS3). The former hypothesis challenges the current model of Pho4 regulation in which, under high-Pi conditions, Pho4 is excluded from the nucleus through phosphorylation by Pho85-Pho80 [5]. Therefore, we further analyzed whether the activity of the ASN1 promoter was dependent on Pho4 under high-Pi conditions by measuring reporter activity in cells grown in a high-Pi medium. The wt promoter was active under high-Pi conditions (Figure 2D), and its activity level decreased to almost 50% when a prospective Pho4 binding site (at −451 with A of ATG as +1) in the promoter was mutated (ASN1/mut). In the absence of Pho85 where Pho4 became active, the activity of the wt promoter was stimulated by 1.3-fold compared to that in the wt strain, but the ASN1/mut promoter showed a level of the activity similar to that observed in the wt strain. In the absence of Pho4 (Δpho4), the activity of the wt ASN1 promoter decreased to 50% whereas that of the mutant promoter showed a similar level of the activity in the wt cells. Gcn4 activates ASN1 under amino-acid-starvation conditions [32], and in the absence of Gcn4 (Δgen4), the promoter activity was decreased drastically (Figure 2D). These results further demonstrated that Pho4 can activate ASN1 regardless of Pi conditions, which requires the Pho4 binding sequences in its promoter.

Phosphate- and Pho4-Regulated Antisense and Intragenic RNAs in the KCS1 Locus

Our ChIP-on-chip analyses using two different platforms demonstrated the binding of Pho4 within the KCS1 and SHE9 ORFs depending on Pi conditions (Figure 3A and Table S2). Because, to our knowledge, gene transcription mediated by the binding of a yeast transcription factor within an ORF is very rare with only three precedents [33–35], we further analyzed the regulation of KCS1 by Pho4. Whereas gene-specific PCR using a primer set specific to the KCS1 promoter (−908 to +70) failed to detect an enrichment of the Pho4-bound fragment (Figure 3B, top panel), the ORF-specific primer (+36 to +1102) could detect an enrichment of the Pho4-bound fragment prepared only from cells grown in low-Pi medium (Figure 3B, second panel from the top). In the absence of Pho85 where Pho4 became active, Pho4 binding to the ORF was detected under both high- and low-Pi conditions (Figure 3B, bottom panel) whereas that to the upstream region was not (Figure 3B, second panel from the bottom). These results demonstrated that Pho4 binding within the KCS1 ORF depends on Pi conditions. We also analyzed Rpo21 binding by ChIP-on-chip and found that Rpo21 was localized to the KCS1 locus in Pi- and Pho4-dependent manners (Figure 3A, bottom two panels). This result indicated the presence of Pi- and Pho4-dependent transcription in the KCS1 locus. To examine whether binding of Pho4 within the ORF could direct KCS1 mRNA transcription, initiation of transcription within the ORF (intragenic RNA), or synthesis of AS RNA, we carried out northern analysis using RNA probes specific to the sense or antisense strands of KCS1 (Figure 3C). With a RNA probe hybridizing with the KCS1 mRNA (Figure 3E), a transcript of ca. 3,200 nucleotides (nt), approximately the size of the KCS1 ORF (3,150 nt; Figure 3E), was detected, which was not dependent on either Pho4 or Pi conditions (Figure 3C, top panel, lanes 1 to 4, designated by upper arrow). Judging from its size, this RNA species is highly likely to be the KCS1 mRNA, because this band was not detected in Δkcs1 cells (unpublished data). In the absence of Pho85, a transcript with a smaller size (ca. 2,600 nt) was detected (Figure 3C, top panel, lanes 5 and 6, designated by lower arrow), together with the KCS1 mRNA and short transcripts ranging from 2,300 to 1,800 nt in length (lanes 2, 5, and 6, designated by a vertical bar). These short sense transcripts were also detected in the wt cells under low-Pi conditions, though weakly (lane 2), but not observed in a Δpho4 (lanes 3 and 4) or Δpho4 Δpho85 double mutant (lane 7). These results indicated that Pho4 binding within the KCS1 ORF can activate the transcription of RNAs shorter than the KCS1 mRNA in both Pi- and Pho4-dependent manners. It should be noted that the KCS1 mRNA level appeared reduced when these short RNA species were abundant in Δpho85 cells (lanes 5 and 6). On the other hand, an AS RNA probe covering from −715 to +295 of the KCS1 gene (Figure 3E) could detect a short transcript of ca. 500 nt, which was dependent on both Pho4 and Pi conditions (Figure 3C, middle panel, lanes 1 to 7), whereas that covering from −715 to −228 failed to do so (unpublished data). Therefore, the AS RNA was highly likely to be encoded between +295 and −228. The presence of AS RNA in PHO genes is reported in PHO5 [14] and PHO84 [15], and in both cases, RR6 affects the stability of the AS RNA. We tested the effect of a Δrrp6 mutation and found that the mutation also stabilized the AS RNA in the KCS1 locus while not significantly affecting the amount of sense RNAs (Figure 3D).

To examine whether the 2,600 nt transcript is a processing product and to confirm the presence of the AS RNA, we determined transcriptional start points of the sense and AS RNAs in the Δpho85 strain by the 5′ rapid amplification of cDNA ends (RACE) method and found that the sense RNAs started mainly at −14 and +537 and that the AS transcription started at +235 (Figure 3E). The sense RNA starting at −14 is highly likely to be the KCS1 mRNA, because the transcription start points of the sense RNA in the wt were also mapped mainly to this point (unpublished data). The one starting at +537 can be ca. 2,600 nt in length when transcribed through the ORF, the size of which coincides well with the estimated size of the short transcript detected by northern analysis (Figure 3C, top panel, designated by lower arrow). This result supported the conclusion that the 2,600 nt RNA is not a processing product but is transcribed from within the KCS1 coding sequence.

Three prospective Pho4 binding sites (at +406, +1127, and +1193) are present in the 5′ half of the KCS1 ORF (Figure 3E), and the one closest to the 5′ end is sandwiched by the transcription start points of the intragenic (+537) and AS RNAs (+235), which suggests a possibility that binding of Pho4 to the +406 site activates both of the 2,600 nt sense and AS RNAs. We constructed the KCS1 mutant (KCS1mut) in which three prospective Pho4 binding sites were mutated while keeping the amino acid sequence intact and analyzed the wt or KCS1mut in a low copy (YCp) plasmid in Δkcs1 cells. We found that the KCS1 mRNA was normally produced (Figure 4A, top panel, lanes 1 to 4), whereas the AS and intragenic RNAs were produced from wt KCS1 but not from KCS1mut under low-Pi conditions (second panel, lanes 2 and 4). Both the AS and truncated sense RNAs were synthesized from wt KCS1 in Δkcs1 Δpho85 cells (lanes 5 and 6) whereas KCS1mut produced RNA of the wt size but not the intragenic or AS RNAs (lanes 7 and 8). These results indicated that the
Figure 3. Pi- and Pho4-Dependent Antisense and Intragenic RNA Species in the KCS1 Locus

(A) The positions of possible Pho4 binding sites within the KCS1 ORF (X) and Pi-dependent localization of Pho4 within the KCS1 ORF detected by HR- and LR-chip analyses are shown in the top two panels. In this case, the results using an HR chip bearing chromosome 3, 4, 5, and 6 sequences are shown for better resolution. Pi- and Pho4-dependent localization of Rpo21 to the KCS1 locus in the wt and Δpho4 strains under high- or low-Pi conditions using the HR chip is shown in the bottom two panels. The slight difference in the data processing programs between the chromosome 3–6 chip and the whole genome chip caused the different appearance of the figures as compared to Figures 1A and 2A.

(B) Demonstration of Pi-dependent enrichment of the KCS1 ORF fragment but not the 5' upstream region by gene-specific PCR. Total DNA in the whole cell extract (input) or ChIPed DNA fragments prepared from the wt or Δpho85 cells grown under high- or low-Pi conditions were subjected to PCR using the primer set specific to the 5' upstream region or ORF of KCS1 as designated. The conditions for PCR are the same as those described in the legend to Figure 1.

(C) Northern analysis of KCS1 sense and AS RNA expression in the wt and various pho mutant strains as designated under high (H)- or low (L)-Pi conditions using strand-specific DIG-RNA probes. For detection of KCS1 sense transcripts, an RNA probe specific to the 3' region of KCS1 was employed (see Figure 3E). The arrows on the left-hand side of the panel indicate the positions of the KCS1 sense (the top panel) and AS transcripts (the middle panel) in the wt and Δpho85 strains.
generation of the 2,600 nt intragenic as well as AS RNAs are activated by Pho4, which requires the presence of at least one of the prospective Pho4 binding sites. Short sense transcripts around 2,000 nt (Figure 4A, lanes 2, 5, and 6, designated by a vertical bar) decreased to below detectable levels with KCS1/mut (lanes 7 and 8), which suggested that these transcripts are also dependent on Pho4.

**Antisense RNA Can Cause Production of Truncated Kcs1 Protein**

Antisense RNA functions in cis to inhibit sense RNA transcription by transcriptional collision as reported in the IME4 case [16] and in trans to form a hybrid with sense RNA to inhibit its function [9,10]. To reveal the role of the KCS1 AS RNA that is regulated by Pho4 and Pi conditions, we first asked whether the AS RNA could affect the synthesis of the Kcs1 protein by immunoblotting. Kcs1 protein whose C-terminus was tagged with the c-myc epitope was produced from the wt KCS1 or KCS1/mut gene in a YCp plasmid (Figure 4A, second panel from the bottom). Kcs1 protein of the wt size (1,143 amino acids including the myc tag) was detected in all cases (lanes 1 to 8), and Δkcs1 Δpho85 cells harboring the wt KCS1 plasmid produced a truncated protein together with the normal one (Figure 4A, second panel from the bottom, lanes 5 and 6). The band observed between 83 and 62 kDa markers was nonspecific staining with anti-myc antibody because it was also detected in the absence of Kcs1-myc protein in the extract (unpublished data). Because both the AS and truncated sense RNAs were produced in Δkcs1 Δpho85 cells, the AS RNA hybridizing to the 5' region of the KCS1 mRNA might inhibit normal translation initiation or the truncated sense RNA might be translated, either of which could use the in-frame initiation codon at +676 (Figure 3E) to produce truncated Kcs1 protein composed of 825 amino acids plus 95 amino acids from the c-myc tag. To examine whether the AS RNA could act in trans, for example, by hybridizing to the 5' region of the KCS1 mRNA, we constructed a plasmid in which the AS RNA was produced from the GAL1 promoter by placing a KCS1 fragment covering +295 to −950 downstream of the promoter and introduced it into a Δkcs1 strain harboring the KCS1/mut gene, so that the AS RNA was provided only in trans and the short sense transcripts including the 2,600 nt intragenic RNA were not produced (Figure 4A, top panel, lane 7). The truncated Kcs1 protein was observed only when transcription of the AS RNA was induced from the plasmid in galactose medium (Figure 4B, second panel from the bottom, lanes 9 and 10). As expected, the short sense transcripts were not detected when the AS RNA was overproduced in trans (Figure 4B, top panel). These results indicated that the AS RNA can act in trans (i.e., inhibition of the normal translation initiation, possibly by hybrid formation with the KCS1 mRNA). To test hybrid formation, we tried to detect the presence of the double-stranded (ds) RNA in the total RNA sample by RNase protection analysis using single-stranded RNA-specific RNase, followed by reverse transcription (RT) and PCR amplification of the protected fragments. Reverse transcription was carried out using either sense- or antisense-strand-specific primers, hybridizing to from +243 to +293 and from −16 to +5 (sense and AS in Figure 4C, respectively). As shown in Figure 4C, dsRNA of ca. 250 bp in length protected from RNase digestion was detected in Δpho85 cells in which both sense and AS RNAs were present (Figure 4C, second panel from the top, lanes 13, 14, 17, and 18). When the RNA sample was not digested with RNase, the sense strand was successfully amplified, whereas the AS strand was not (lanes 16 and 20), indicating that the KCS1 mRNA was present but the AS RNA was not in the RNA sample tested. In Δpho85 cells in which both sense and AS RNAs were present (Figure 4C, lane 14), dsRNA of ca. 260 bp in length protected from RNase digestion was detected (Figure 4C, second panel from the top, lanes 13, 14, 17, and 18). The Δkcs1 mutant cells do not produce the KCS1 sense or AS RNAs, and accordingly the sense or AS primer failed to synthesize cDNA (Figure 4C, bottom panel). When KCS1/mut was expressed in Δkcs1 cells under low-Pi conditions, the sense RNA was produced but not the AS (Figure 4A, lane 4; Figure 4C, the middle panel, lanes 16 and 20), and therefore dsRNA was not detected (lanes 13, 14, 17, and 18). Reciprocally, when the AS RNA was expressed in Δkcs1 cells, only the AS was detected (Figure 4C, second panel from the bottom, lanes 16 and 20), and the protected dsRNA fragments were, if any, below the detectable level (lanes 13, 14, 17, and 18). To confirm that the protected dsRNA was specifically amplified, we carried out the RT reaction using primers hybridizing upstream of the transcription start points of the sense or AS RNAs (at −14 and +235, respectively, in Figure 3E, and those designated by asterisks in Figure 4C). The RNA samples prepared from the strains producing the KCS1 mRNA (wt, Δpho85, and Δkcs1 + KCS1/mut) could generate cDNA of ca. 430 bp in length when amplified with the sense*AS primer pair only in the absence of RNase digestion (Figure 4C, lanes 21 and 23). Similarly, the AS*sense primer pair could synthesize cDNA when the RNA samples containing the AS RNA were not digested by RNase (lanes 24 and 26). These results indicated that the protected dsRNA fragment was specifically transcribed and amplified by the RT-PCR reaction and therefore strongly suggested that the AS RNA can form a hybrid with the KCS1 mRNA in vivo. Such a hybrid may inhibit normal translation initiation, leading to the generation of the truncated Kcs1 protein.
Figure 4. Requirement of Pho4 Binding Sites for the Expression of the KCS1 Intragenic and AS RNAs and for the Production of the Truncated Kcs1 Protein

(A) Expression of the KCS1 sense and AS RNAs from the wt or mutant (Mut; lacking the three Pho4 binding sites) KCS1 gene in a YCp plasmid in Dkcs1 or Dkcs1Dpho85 strain under high (H)- or low (L)-Pi conditions was analyzed by northern blotting with strand-specific RNA probes. Positions of the KCS1 sense (top panel) and AS RNA (second panel from the top) are designated by arrows and a vertical bar on the left-hand side of the panels. The positions of RNA size markers (nt) are on the right. ACT1 is the loading control. The Kcs1 protein tagged with the c-myc epitope at its C terminus was detected by western blotting using anti-myc antibody. The full-length and truncated Kcs1 proteins are designated by two arrows on the left-hand side of the second panel from the bottom. The positions of marker proteins are on the right. Actin is the loading control for western analysis. The faint band between 83 and 62 kDa markers observed in all lanes is nonspecific staining of yeast protein by the anti-myc antibody.

(B) Heterologous KCS1 AS RNA expression and production of the truncated Kcs1 protein. A Δkcs1 strain harboring KCS1mut-myctag in a YCp vector and pGAL1-AS RNA in a YEp vector or vector alone was analyzed for KCS1 sense and AS RNA expression by northern blotting as designated. AS RNA expression was induced by incubating the transformants in galactose medium for 6 h (Gal) or repressed in glucose medium (Glc). Production of Kcs1-myctag protein was detected as described above.

(C) An RNA hybrid formation of the KCS1 mRNA with the AS RNA detected by RNase protection assay and RT-PCR. A schematic representation of the KCS1 gene of interest is shown at the top. The arrows on the bar designate the major transcription start points of the mRNA (at -14 and of the AS RNA +235). The boxes below the bar indicate the positions of the strand-specific primers used for the detection of dsRNA: the blank boxes are sense-strand-specific primers (sense, +243 to +223, and sense*, +415 to +395), and the black ones are primers for the AS strand (AS, /C0 16 to +5, and AS*, /C0 68 to /C0 48). Total RNA was isolated from the wt, Dpho85, Δkcs1, Δkcs1 harboring KCS1mut, or Δkcs1 expressing the AS RNA under the TDH3 promoter, grown in high-Pi medium, except that Δkcs1 harboring KCS1mut was in low-Pi medium. About 10 μg of each RNA sample was incubated with (+) or without (−) RNase ONE. For negative control (−RNA), total RNA was replaced by yeast tRNA. The digestion products were then used as templates for first-strand cDNA synthesis with strand-specific primers as designated, and the cDNA products were subjected to PCR amplification with the appropriate reverse primer. The sense-strand-specific (sense) primer, from +243 to +223; antisense-strand-specific (AS), from /C0 16 to +5; sense*, from +415 to +395; and AS*, from /C0 68 to /C0 48. For lanes 14, 16, 18, 20, 23, and 26, one-tenth of the amount of the cDNA template was used for PCR compared to those in the other lanes. The PCR products were analyzed by electrophoresis on a 5% polyacrylamide gel, and 10-fold dilutions of the PCR products were loaded in lanes 16, 20, 23, and 26. The positions of DNA size markers are shown on the right-hand side of each panel.

(D) Pho4-directed and Pi-dependent transcription within the KCS1 ORF. The N-terminally truncated wt or mutant (Mut) KCS1 ORF (+105 to +3150) placed in a YCp plasmid was introduced into Δkcs1 or Δkcs1Δpho4 strain, and transcription of sense RNA under high (H)- or low (L)-Pi conditions was detected by northern blotting using a strand-specific probe (top panel). The positions of RNA size markers are shown on the right and ACT1 is the loading control.

(E) The intragenic RNA also can encode the truncated Kcs1-myc protein. The truncated wt or Mut KCS1 ORF (+105 to +3150) tagged with c-myc at its C
Intragenic RNA Can Produce the Truncated Kcs1 Protein

Although we demonstrated that the truncated Kcs1 protein could be generated independently of the short sense RNAs, it is still possible that the truncated protein is translated from the 2,600 nt intragenic RNA. To test this possibility, we constructed plasmids harboring N-terminally truncated KCS1 or KCS1mut ORF fragments (+105 to +3150) and introduced them into Δkcs1 or Δkcs1 Δpho4 mutants. Because these KCS1 fragments lack the KCS1 promoter, the plasmids are unable to produce the full-length KCS1 mRNA. With a strand-specific RNA probe (Figure 3E), we could detect Pi- and Pho4-dependent transcripts of ca. 2,600 nt in length (Figure 4D, lanes 27, 28, 31, and 32). This transcript was not observed in the KCS1mut (lanes 29, 30, 33, and 34), indicating that the presence of the Pho4 binding sites is required for the production of this RNA species. Thus Pho4 could activate transcription from downstream of its binding site in the KCS1 ORF. Although this downstream transcription was plasmid-borne, the observed similarity in the size of transcript and its regulation strongly indicate that the 2,600 nt transcript is actually transcribed in the chromosomal KCS1 locus in a Pho4-dependent fashion and not a processing (or limited degradation) product. This plasmid-derived transcript could produce protein that had a size similar to that of the truncated Kcs1 protein (Figure 4E, lanes 36 and 39), indicating that the 2,600 nt intragenic RNA can encode the truncated Kcs1 protein. Taken together, these results indicated that Pho4 binding within the KCS1 ORF provokes transcription of both of the AS and the 2,600 nt intragenic RNAs, which may lead to production of the truncated Kcs1 protein by alteration of translation initiation through the formation of a hybrid with the KCS1 mRNA and by translation of the 2,600 nt intragenic transcript.

Pho4 Modulates Phosphate-Signaling Pathway via Antisense and Intragenic RNAs

What is the biological relevance of Pi- and Pho4-dependent production of the AS and intragenic RNAs and consequently of the truncated Kcs1 protein? KCS1 codes for inositol hexakisphosphate (IP6) kinase synthesizing 5-diphospho-myo-inositol pentaphosphate (5-PP-IP5) [36]. The same substrate is used by another yeast IP6 kinase, Vip1, to synthesize IP7 isomers, 4- or 6-PP-IP5, that function in Pi signaling in the PHO system [37,38]. Therefore, it is conceivable that a decrease in the Kcs1 activity can supply more substrate for Vip1, thereby enhancing Pi signaling. The fact that overproduction of Kcs1 reduces the extent of PHO5 derepression whereas a deletion of KCS1 derepresses PHO5 under high-Pi conditions [39] supports this model. Although the levels of normal Kcs1 protein did not appear to be altered significantly in the presence of the AS RNA and 2,600 nt intragenic transcript (Figure 4A), the formation of a hybrid RNA could affect the normal level of the KCS1 mRNA, which may cause a slight difference in the level of the Kcs1 protein not detectable by western analysis. In addition, the presence of the truncated Kcs1 may perturb normal function of Kcs1. To test these hypotheses, we analyzed the effects of the AS RNA and the intragenic sense transcript on PHO84 and PHO5 expression by northern analysis (Figure 5). PHO84 responds more quickly to a change in Pi conditions than PHO5 [40]. In the wt cells, PHO5 and PHO84 were expressed only under low-Pi conditions (Figure 5, lanes 1 and 2) but not in the absence of Pho4 (lanes 5 and 6). The two genes were expressed under high-Pi conditions in Δkcs1 cells (Figure 5, lanes 7 and 8) as reported [39], which was suppressed by the wt KCS1 in a YCp plasmid (lanes 9 and 10). This result indicated that the plasmid-borne Kcs1 is functional. KCS1mut that produced neither the AS RNA nor the intragenic transcript (Figure 4A) showed a decreased expression level of the two genes under low-Pi conditions (Figure 5, lane 12), suggesting that the low-Pi signal was not transmitted sufficiently to activate Pho4. On the other hand, overexpression of the AS RNA in the wt cells resulted in a significant derepression of the two genes under high-Pi conditions (lanes 1 and 13), suggesting that the low-Pi signal was transmitted to activate Pho4 under high-Pi conditions in this case. This stimulatory function of the AS RNA was dependent on the presence of Vip1 IP6 kinase (lane 19) that functions in low-Pi signal transmission [38]. This result further supported the conclusion that the AS RNA functions in the low-Pi signal transduction pathway. We also overproduced a truncated Kcs1 protein (dKcs1, +670 to +3150) in the wt cells, which caused derepression of PHO84, albeit weakly, and barely detectable expression of PHO5 under high-Pi conditions (lane 15). The different expression levels of PHO84 and PHO5 can be attributed to different responsiveness of the two genes against the change in the environmental Pi level [40]. We also assayed the activity of acid phosphatase encoded by PHO5 in the strains with a combination of various plasmids as tested in northern analysis and found that the levels of the enzyme activities correlated with the mRNA level (unpublished data). These results indicated that the presence or absence of the AS RNA and the 2,600 nt intragenic RNA cause altered regulation of PHO5 and PHO84 responding to Pi conditions, and therefore it is likely that the Kcs1 activity was modulated by the RNAs, the truncated Kcs1 protein, or both. The apparently weak effect on Pi signaling of dKcs1 compared to that of the AS RNA suggests that the truncated protein is not solely responsible for the stimulation of the low-Pi signal. The AS RNA could play a certain role in this stimulation process, possibly through modulation of the KCS1 mRNA and protein levels. Thus, Pho4 appears to enhance low-Pi signaling by expressing the AS and the intragenic RNAs from within the KCS1 ORF, thereby constituting the positive feedback loop in the Pi signaling pathway.

Discussion

In this paper, we reported three novel findings derived from the ChIP-on-chip analyses of Pho4 and Rpo21 binding throughout the entire yeast genome: (i) the finding of novel PHO-type genes, (ii) the ability of Pho4 either to activate or to repress transcription independently of environmental Pi conditions, and (iii) the presence of Pi-regulated AS and
intragenic RNAs that modulate Pi signal transmission. We demonstrated that 18 genes that had not been classified previously as involved in the PHO system showed Pho4 binding in a Pi-dependent fashion (Table S2 and Figure 1), and at least four of them, viz., MNN1, CBF1, PST1, and PTK2, clearly showed Pho4 binding to their promoters in vivo dependent on Pi conditions and consequently transcription that was dependent on both Pi conditions and Pho4 (Figure 1B and 1C). Harbison et al. reported Pho4 binding profiles under low-Pi conditions [41], and their results share MNN1 and PTK2 out of our 18 novel PHO-type genes. Gonze et al. predicted AR09 and PST1 as PHO-type genes by computational analysis [42]. KC51 expression is reported to increase under Pi-limiting conditions [27] and in the absence of Pho85 by microarray analysis [43], probably because of the use of an oligo-DNA array bearing 3′-nested probes that detects Pi- and Pho4-dependent intragenic RNA. Cross-regulation of phosphate and sulfate metabolism has been suggested [44], and in this context, it is noteworthy that we found CBF1, which encodes a transcription factor that regulates MET genes under the control of Pi conditions and Pho4.

Pho4 and Stress Response in Yeast

Judging from the Gene Ontology terms of these 18 newly recognized PHO-type genes (Saccharomyces Genome Database [SGD], http://www.yeastgenome.org/), they apparently do not have any functional relationship to either Pi metabolism or Pi signaling and are not categorized in a specific functional group (Table S4). Their expression profiles by global analysis, however, showed some similarities in that 12 of them are induced by either nitrogen depletion or amino acid starvation [32,45] and 9 of them are induced in the stationary phase [45]. This raises the possibility that Pho4 is involved in the regulation of a certain set of genes that responds to these nutrient-limiting or stress conditions. Pho4 is also reported to activate the transcription of genes involved in G1 arrest caused by DNA damage [28]. Thus, Pho4 appears to activate the transcription of genes responding to various stress conditions. This notion implies possible cross talk between Pi starvation and other stress conditions, the requirement of function of some, if not all, of these 18 genes in the adaptation of yeast cells to Pi starvation, or both.

Pho4 Can Function Independent of Pi Conditions

The results in this paper imply that Pho4 is present in the nucleus even under high-Pi conditions to activate or repress transcription (Figure 2), an implication that challenges our current understanding of Pho4 regulation. If the current model were correct, then Pho4 should somehow avoid phosphorylation by Pho85, or if phosphorylated, then the modified Pho4 should have much less affinity to the Msn5 exportin to remain in the nucleus. Recently, Zappacosta et al. reported Pi-dependent phosphorylation of Pho4 at Ser242 and Ser243 by a kinase other than Pho85 [46]. Phosphorylation of these two sites, however, appears less dependent on Pi than that at those sites modified by Pho85 (i.e., Ser at 100, 114, 128, 152, and 223) [46]. We could imagine that, under high-Pi conditions, prior phosphorylation of the Ser242, Ser243, or both by this unknown kinase could prevent phosphorylation of the other Ser residues by Pho85, thereby decreasing the affinity of Pho4 for Msn5 while increasing the affinity to the target promoter, including ASN1. Alternatively, Pho4 modified at Ser242, Ser243, or both might have more affinity to a yet unknown factor than to the exportin, and the resulting complex might be recruited to the target promoter regardless of phosphorylation by Pho85.

The Pho4 transcription factor appeared to repress CIS3 and YPS3, both cell wall constituents. Expression of CIS3 is repressed by nitrogen starvation and in the stationary phase, implying that Pho4 can function as both an activator and a repressor under these stress conditions. The functioning of a yeast transcription factor as both an activator and a repressor has precedents (e.g., Rap1 and Abf1) [47,48]. Transcriptional repression by the two factors is often accompanied by silent chromatin structure. In a separate paper, we have reported that Pho4 negatively regulates the expression of SNZ1, a stationary phase-specific gene, and that this regulation is accompanied by alterations in chromatin structure evoked by Pho4 binding [49]. The mechanism underlying transcriptional repression of CIS3 and YPS3 by Pho4 is yet to be clarified, but we suppose that a similar mechanism with the SNZ1 may apply in these cases.

Biologically Functional Yeast Antisense and Intragenic RNAs

We demonstrated the presence of Pi-regulated AS RNA in the KC51 locus. A large-scale cDNA sequencing by Miura et al. revealed the presence of many AS RNA species [17], including an AS RNA in the KC51 locus transcribed from +293 to −43. Although its start point is different a little from our result by

Figure 5. Effect of KC51 and VIP1 on PHO84 and PHO5 Expression Analyzed by Northern Blotting

The wt, various mutants, or those harboring plasmids expressing KC51, KC51mut, AS RNA, or a truncated Kcs1 protein (dKcs1) were incubated for 5 h in high (H)- or low (L)-Pi media before isolation of total RNA as described in Methods. ACT1 is the loading control.

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5′-RACE analysis (Figure 3E), we think it highly likely that this AS RNA coincides with the Pi-regulated AS RNA that we have reported here. The Pi-regulated AS RNA in KCS1, however, did not appear to be coregulated with the KCS1 mRNA in the wt cells (Figure 3C), and this contrasts with the observation in higher eukaryotes that sense and AS pairs are frequently coregulated [50]. Although Miura et al. did not describe the regulation of the KCS1 AS RNA, they claim coregulation between the sense and AS RNA in the GAL10 locus. However, the fact that the fold induction of the AS RNA is much less compared to that of the GAL10 mRNA when cells are grown in galactose medium and that Gal4 dependency of the GAL10 AS RNA was not analyzed points out that more work is necessary to establish coregulation of the sense and AS RNA at the GAL10 locus.

With respect to biological function of ncRNA in yeast, a noncoding intergenic transcript (SRG1), originating from upstream of SER3 on the same strand and activated by Cha1 transcription factor in the presence of serine [13], inhibits the binding of activators to the SER3 upstream activating sequence and of TATA-binding protein to its TATA box, leading to repression of SER3 [12]. Yeast AS RNA has been reported in the IME4 locus, which is expressed only in the haploid state to inhibit the IME4 mRNA transcription by transcriptional collision and thereby determines cell fate (i.e., the entry into meiosis) [16]. The AS RNA at the PHO5 locus is constitutively expressed at a low level from ca. 1,400 bp downstream of the PHO5 TATA box through its promoter and is proposed to increase chromatin plasticity to enhance histone eviction upon a shift to low-Pi conditions [14]. Those in the PHO84 locus are suggested to recruit and/or stimulate Hda1 histone deacetylase for silencing of PHO84 in aging yeast cells [15]. Although these AS RNA species are found in PHO genes, they have not been reported to be regulated by environmental Pi conditions to facilitate the activation of PHO genes. The KCS1 case presents a different situation from them in that the AS and intragenic RNAs are activated by Pho4 in response to Pi starvation and may modulate the level of Kcs1 IP₆ kinase to enhance Pi signaling, thereby stimulating the activation of PHO genes. We observed a decrease in the KCS1 mRNA level when Pho4 binds to the KCS1 ORF under low-Pi conditions or in a Δpho85 mutant (Figure 3C, lanes 2, 5, and 6). This observation suggests a possibility that transcriptional elongation of KCS1 mRNA is inhibited directly by Pho4 binding within the ORF. However, the scenario may not be so simple, because the KCS1 mRNA transcription itself can interfere with Pho4 binding, as reported in the SER3/SRG1 case [12]. Alternatively, the AS RNA could cause transcriptional collision with the mRNA, and hybrid formation of the AS RNA with the mRNA could lead to degradation of the mRNA. Both of these events could lead to a reduction in the KCS1 mRNA level.

The stimulation of low-Pi signaling by Pho4-dependent intragenic and AS RNA represents an autoregulation (induction) or positive feedback loop responding to Pi limitation that can be envisioned as follows. Upon Pi limitation, the low-Pi signal is transmitted to Pho81, leading to inhibition of Pho85-Pho80 and thereby stimulating Pho4 migration into the nucleus [6]. Pho4 then activates transcription of the AS and intragenic RNAs in the KCS1 locus: the AS RNA could reduce the KCS1 mRNA level by hybrid formation and possible transcriptional collision, which can lead to stabilization of Pho4 binding, resulting in the production of more AS and intragenic RNAs and consequently of more truncated Kcs1 protein using the downstream ATG codon at +676. These events could lead to down-regulation of Kcs1 activity, enabling Vip1 IP₆ kinase to utilize more IP₂ to synthesize 4- or 6-PP-IP₅ functioning in low-Pi signaling. Because Kcs1 also can phosphorylate these IP₅ species to synthesize 4,5- or 5,6-PP₂-IP₅ (IP₇) [37], reduction of the Kcs1 level can ensure accumulation of the IP₇ species to further stimulate low-Pi signaling, leading to complete inhibition of Pho85-Pho80. When Pho becomes sufficient, this loop runs in an opposite way for efficient inactivation of Pho4 and consequent repression of PHO genes. Though putative Pho4 binding sequences are present at −464 and −154 in its promoter, VIPI expression was dependent on neither Pi condition nor Pho4 (unpublished data), as in the case of the KCS1 mRNA. Inositol polyphosphate (IP) plays an important role in intracellular signal transduction as second messengers. The absence of Kcs1 and Vip1 causes abnormal vacuolar function and cell morphology, respectively, suggesting that they bear important cellular function [37,51]. Therefore, it is reasonable that the genes involved in IP synthesis are not regulated directly by individual nutrients (in this case, Pi) but indirectly by AS and intragenic RNAs responding to the nutrient, so that signal transduction and normal cellular function are not easily perturbed by fluctuation in the status of an individual nutrient.

Positive feedback in the PHO system is also suggested to function in switching of Pi transporters [52], in which SpI2, activated by Pho4, down-regulates low-affinity Pi transporters, Pho87 and Pho90, whereas high-affinity Pi transporter Pho84 is activated by Pho4. When the intracellular Pi level increases via the high-affinity transporter, Pho4 is inactivated to switch the transporters.

**PHO System To Explore Intimately Wired Transcriptional Regulation System**

Our finding expands the role of the Pho4 transcription factor beyond the regulation of the PHO system. The current consensus view is that it is the master regulator of the genes involved in the system, in that Pho4 activates transcription of the structural genes composing the PHO system to coordinate cellular response to Pi starvation [3]. Our findings indicate that Pho4 can modulate the activity levels of the products of apparently non-PHO genes by activating antisense and intragenic RNA expression to stimulate low-Pi signal transduction. A Δpho85 deletion causes pleiotropic mutant phenotypes [1], some of which could be based on otherwise dormant transcriptional initiation, either intergenic or intragenic, or on the AS strand, caused by hypervactive Pho4. In fact, we have also found Pi- and Pho4-regulated AS RNA in the GTO3 locus and intragenic sense transcript in the SHE9 locus (unpublished data). GTO3 encodes an omega class glutathione-S-transferase having glutaredoxin activity, which is suggested to maintain an adequate redox state of specific target proteins, not in the general defense against oxidative stress [53]. SHE9, also known as MDM33, encodes a mitochondrial inner membrane protein functioning to maintain mitochondrial morphology [54]. Although, at present, we are unable to elucidate
whether the GTO3 AS RNA or the SHE9 intragenic transcript can affect the annotated function of the respective gene product, this line of work will lead us to uncover yet unknown protein functions in the cellular response to Pi starvation.

Regulation of gene expression and function by these nonconventional RNA species that are regulated by nutrient signals need not be restricted to the PHO system. Other inducible systems including GAL, glucose repression, and various stresses may well have these RNA species regulated by corresponding signals. High-throughput cDNA sequencing by Miura et al. and other works using microarrays [17–19] have revealed the presence of many intergenic, intragenic, and antisense RNAs in the yeast transcriptome. The finding of nutrient-regulated RNAs that are not coding annotated proteins adds more complexity to the intimately wired transcriptional regulation system by which yeast cells adapt to alterations in environmental conditions. High-resolution mapping of transcription factor and RNA polymerase II binding and very recent development of DNA–RNA hybridization techniques [55] will help to identify these regulatory RNAs. The yeast system, which can be manipulated by an array of genetic tools and for which there exists a substantial body of genetic information, will be the best resource to explore the complexity of the genetic network, including ncRNA species that function in responding to external signals.

Materials and Methods

Yeast strains and molecular biology. Standard yeast genetics and media were used as described [56]. For phosphate-limited medium, Yeast Nitrogen Base (YNB) without phosphate (Q-Biogene) was used instead of normal YNB (SD medium) and was supplemented with 0.2 mM or 2 mM sodium phosphate to make low- and high-Pi media, respectively. The yeast strains used in this work are listed in Table S5. Yeast strains and molecular biology.

DNA manipulation. Standard Escherichia coli and yeast protocols were employed [56,57]. Plasmids and primers used in this work are listed in Tables S5 and S6, respectively. A Pho85–U3R3 fragment [25] was used to disrupt the PHO85 locus of the BY4741 (MFY371) strain, and successful disruption was confirmed by PCR and constitutive expression of acid phosphatase (unpublished data). To disrupt the PHO4 locus, Pho4Δ-F and -R primers were used to amplify the LEU2 marker having PHO4 sequences (from +1 to +100 and from +830 to 929 with A of ATG as +1) at its termini, and the resulting fragment was introduced into MFY371. Successful disruption was confirmed by PCR and failure to express PHO4. For disruption of the VPD1 and RRP6 loci, the adaptor- mediated PCR method was employed to prepare the DNA fragments for disruption [58]. The detailed methods are described in Text S1. Disruption of GCN4 is described elsewhere [49]. To construct PHO4-tagged strains, MFY376 and MFY377, a fragment containing PHO4 tagged with His × 6 and Flag × 3 was amplified using primers Pho4-Flag-F and -R and pUG6H3Flag plasmid as a template [59], followed by transformation of MFY371 and MFY373, respectively. Rpo21 fragments tagged with His × 6 and Flag × 3 (Rpo21-Flag-F and -R) were used to construct MFY378 and MFY379.

To mutagenize the prospective Pho1 binding site in the ASN1 promoter and in the KCSI ORF, a QuickChange II site-directed mutagenesis kit (Stratagene) and appropriate primers were used. The detailed methods are described in Text S1. Successful mutagenesis and the whole sequence of the mutant ASN1 promoter and of the mutant KCSI ORF were confirmed by DNA sequencing. The promoter fragment (–920 to –1 and ORF (–1 to +3143) of KCSI were amplified by PCR using MN1132/1133 and MN1134/1135 primers, respectively, so that EcoRI-NcoI and Ncol-Xhol fragments containing the respective sequences were generated. The two fragments were then ligated together (olig site and introduced into plasmid pS313 containing ptu2::3 × myc epitope and pMF1530 plasmid. The wt Ncol-BamHII (–1875) fragment had been replaced by the mutant fragment that lacked the three prospective Pho1 binding sites prior to incorporation of the EcoRI-Xhol KCSI fragment into pRS313 to generate the pMF1531 plasmid. To construct plasmids pMF1527 and pMF1529 producing the wt and mutant Kcsi protein tagged with six copies of the c-myc epitope at their C-termini, respectively, the EcoRI-Xhol fragment containing the 6 × myc sequence. Plasmids pMF1530 and pMF1560 overexpressing the KCSI AS RNA were constructed by ligating the KpnI-EcoRI (+291 to –920) fragment downstream of the TDH3 and GAL1 promoters in the pRS323 plasmid, respectively. Plasmid pMF1503 overproducing N-terminally truncated Kcsi protein (Δ683) was constructed by placing the NcoI-Xhol fragment (–676 to +3150) that had been cloned by PCR downstream of the TDH3 promoter in pRS326.

ChIP-on-chip analysis. Yeast cells producing tagged protein were cultivated in high- or low-Pi medium as described above to a cell density of A660 = 1.0–1.2, and chromatin immunoprecipitation was carried out. ChIPed DNA fragment was amplified by the 17 RNA polymerase-mediated method (T7RPM) followed by cDNA synthesis and the ligation-mediated PCR (LM-PCR) method for HR and LR analysis, respectively as described [41,61]. For T7RPM, ChIPed DNA (about 100 ng) was dephosphorylated in the reaction mixture (30 μl) containing 2 units of CIAP and 0.2 units of LAP at 37°C, followed by incubation at 50°C for 15 min each. DNA was purified using a MinElute Reaction CleanUp kit (Qiagen) and eluted from the column with 10 μl of elution buffer (10 mM Tris.HCl, 0.1 mM EDTA). The clean-up procedure except for the cleanup with an RNEasy Mini kit (Qiagen). One microliter of 100 μM 17 degenerate primer (GGATCTTAACTAGCTACTATAGAA-GCGACCGCNSNNSNNSNNSSNH) was added to the RNA product, which was incubated at 70°C for 10 min, then on ice for 2 min, followed by cDNA synthesis. About 500 ng of cDNA was subjected to a second round of in vitro transcription and subsequent cDNA synthesis, followed by labeling using an in vitro transcription labeling kit (Alphagene). Labeled cRNA was hybridized to Affymetrix high-density oligonucleotide arrays of S. cerevisiae whole genome (Watson strand) or of chromosomes 3, 4, 5, and 6, which were processed and analyzed as described [62]. For LM-PCR, phosphorylation of 5′ termini of ChIPed DNA fragments by T4 polynucleotide kinase and T4 polynucleotide kinase performed simultaneously by ligase of annealed linkers (MN974 and MN975) at 15°C for 16 h. The resulting fragments were amplified by PCR using MN974 primer, followed by PCR labeling with Cy3-dUTP and Cy5-dUTP for ChIPed and whole cell extract DNA, respectively. The data were analyzed with ChIP Analytics 3.0 software (Agilent).

Analytical methods. The procedures for RNA isolation, northern and immunoblot analyses, and assay for β-galactosidase were as described [25,43]. DNA probes were prepared by PCR using digoxigenin (DIG)-PCR labeling mix (Roche). RNA probes were prepared by transcription by T7 RNA polymerase-mediated method (T7RPM). Total RNA from yeast was recovered by precipitation in the presence of ethanol. First-strand cDNA was then synthesized using a primer specific to the sense or antisense strand, followed by PCR amplification after inactivation of
reverse transcriptase and addition of appropriate reverse primer. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel.

Supporting Information

Table S1. Accession Numbers of the Genes Described in This Article

Table S2. Genes That Bind Pho4 Dependent on Phosphate Conditions

Table S3. Genes That Bind Pho4 Independent of Phosphate Conditions

Table S4. Gene Ontology Terms and Expression Profile of Novel PHO-Type Genes

Table S5. Yeast Strains and Plasmids Used in This Work

Table S6. Primers Used in This Work

Acknowledgments

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Author contributions. MN and AT conceived and designed the experiments. MN and TK performed the experiments. MN, YK, KS, and TI analyzed the data. YK, KS, and AT contributed reagents/ materials/analysis tools. MN and AT wrote the paper.

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Table S6. Primers Used in This Work

Found at doi:10.1371/journal.pbio.0060326.s006 (48 KB XLS).

Text S1. Supporting Methods

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