A role for noncoding transcription in activation of the yeast *PHO5* gene

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Noncoding, or intergenic, transcription by RNA polymerase II (RNAPII) is remarkably widespread in eukaryotic organisms, but the effects of such transcription remain poorly understood. Here we show that noncoding transcription plays a role in activation, but not repression, of the *Saccharomyces cerevisiae PHO5* gene. Histone eviction from the *PHO5* promoter during activation occurs with normal kinetics even in the absence of the *PHO5* TATA box, showing that transcription of the gene itself is not required for promoter remodeling. Nevertheless, we find that mutations that impair transcript elongation by RNAPII affect the kinetics of histone eviction from the *PHO5* promoter. Most dramatically, inactivation of RNAPII itself abolishes eviction completely. Under repressing conditions, an ≈2.4-kb noncoding exosome-degraded transcript is detected that originates near the *PHO5* termination site and is transcribed in the antisense direction. Abrogation of this transcript delays chromatin remodeling and subsequent RNAPII recruitment to *PHO5* upon activation. We propose that noncoding transcription through positioned nucleosomes can enhance chromatin plasticity so that chromatin remodeling and activation of traversed genes occur in a timely manner.

elongation | intergenic transcription | RNA polymerase II

In addition to transcribing all protein-encoding genes, RNA polymerase II (RNAPII) also transcribes a large group of less known and poorly understood untranslated RNAs. Recent genome-wide studies in several species reveal that such noncoding transcription is much more extensive than previously thought and that it occurs across intergenic regions, introns, and exons (see, for example, refs. 1 and 2). Recently, genome-wide studies in yeast have identified many cases of intergenic transcripts associated with promoters (3–5), raising the question of whether and how intergenic transcription across a promoter is used as a means of regulating that gene's transcription.

During our studies on elongation and RNA processing factors in yeast, we discovered an intergenic transcript across the *PHO5* promoter. This finding led us to investigate whether noncoding transcription might play a role in regulating this gene. *PHO5* encodes an acid phosphatase that is regulated by phosphate availability (6). In high phosphate, four positioned nucleosomes are associated with the *PHO5* promoter region (7). During phosphate starvation, the Pho4 activator translocates to the nucleus (8) and binds to *PHO5* upstream activation sequences (UASp1 and UASp2) along with the Pho2 activator (9–11). This leads to eviction of the four positioned nucleosomes, making a 600-bp region effectively fully accessible (7, 12–14). Promoter remodeling is facilitated by, although not always absolutely dependent on, several transcription factor complexes including SAGA, Swi/Snf complex, INO80, and the Asf1 chaperone (14–18). High phosphate causes Pho4 accumulation in the cytoplasm, nucleosome reassembly on the promoter, and transcriptional repression of the gene.

Here we show that intergenic transcription plays a role in the kinetics of *PHO5* promoter remodeling.

Results

An Intergenic Transcript Across the *PHO5* Promoter. We initially noticed the appearance of an additional *PHO5* RNA species during our characterization of transcription in *rrp6* mutants (J.P.U., unpublished data), which lack a functional nuclear RNA exosome and accumulate intergenic transcripts (ref. 4 and references therein). However, detection of this transcript is possible even in wild-type cells in which noncoding RNAs are otherwise unstable. It is ≈2.4 kb in size and is observed only in cells grown in high-phosphate (repressing) conditions (Fig. 1*A*).

Using strand- and promoter-specific RT-PCR on total RNA from wild-type and *rrp6* cells (Fig. 1*B*), we deduced that intergenic transcription is antisense relative to *PHO5* mRNA.

A series of probes across the *PHO5* locus was hybridized with RNA isolated from wild type and *rrp6*, respectively. All probes that spanned the ≈2.4-kb region from 950 bp upstream of the *PHO5* transcription start site to the 3′ end of the *PHO5* ORF hybridized to the intergenic transcript, but the transcript was not detected with flanking probes 1 and 7 (Fig. 1*C*).

A strain carrying a temperature-sensitive allele of the largest subunit of RNAPII (*rpb1-1*) (19) was used to establish that the intergenic transcript is produced by RNAPII and that RNAPII can indeed be detected in the upstream *PHO5* promoter by ChIP, even in repressing conditions [supporting information (SI) Fig. 6].

Together, the above results suggest that RNAPII actively transcribes across the uninduced *PHO5* gene and its promoter, producing an unstable, noncoding, antisense RNA.

Intergenic Transcription Is Not Important for *PHO5* Repression. Because some previous studies of *PHO5* chromatin structure and activation were conducted with versions of the *PHO5* gene that did not contain the region in which the intergenic transcript originates (20, 21), an effect of intergenic transcription on the capability of the *PHO5* gene to be turned on or off was not expected to be absolute. Indeed, it seemed reasonable to expect that the effect, if any, might be reminiscent of that observed upon mutation of the histone acetyltransferase *GCN5*, which affects the kinetics, but not the final level, of activated *PHO5* transcription (15).

We first examined whether transcription in general is required to establish or maintain histones at the repressed *PHO5* promoter. If not, a role for the intergenic transcript (representing only a fraction of general transcription) would be highly unlikely. For this purpose, wild-type and *rpb1-1* cells were grown in high phosphate at 23°C and then shifted to 37°C. Comparison of histone H3 levels by ChIP assays revealed no significant difference in histone density at the promoter between wild type and *rpb1-1* cells, indicating that ongoing transcription is not required

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Abbreviations: RNAPII, RNA polymerase II; 6AU, 6-azauracil; YPD, yeast extract/peptone/dextrose.

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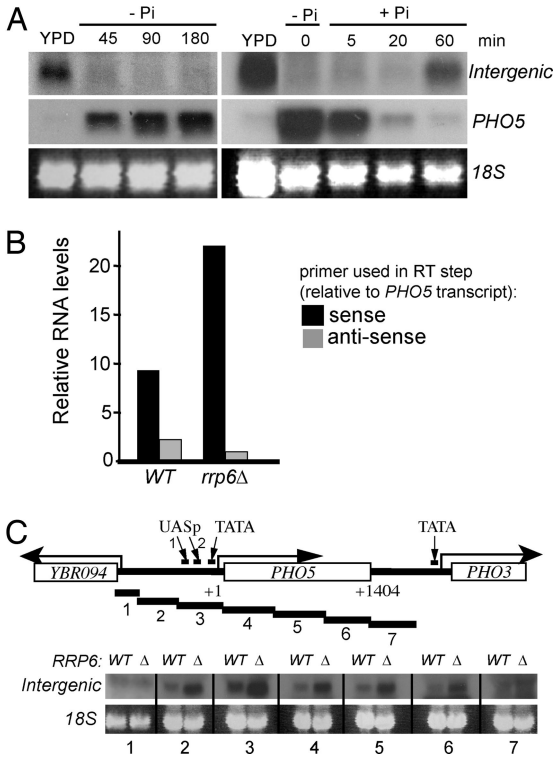

Fig. 1. An intergenic transcript is detected across the repressed *PHO5* promoter. (*A*) Northern blots of total RNA hybridized with *PHO5* promoter-specific probe (intergenic) and an ORF probe (*PHO5*). (*Left*) *PHO5* induction. (*Right*) *PHO5* shutoff. 18S is shown as loading control. (*B*) Two-step reverse transcriptase, real-time PCR was performed on total RNA from wild-type (WT) or *rrp6* cells grown in YPD using *PHO5* promoter-specific primers (across UASp2; see *C Upper*). The initial reverse transcriptase step was performed with a primer that was either complementary to (antisense) or on the same strand as (sense) the *PHO5* transcript. *y* axis indicates relative RNA levels (arbitrary units). The result shown is representative of two independent experiments. (*C Upper*) Schematic drawing of the *PHO5* region (+1 indicates the first base of the ORF; +1,404 indicates the last) and the probes (numbered) used to detect the intergenic transcript. The positions of UASp1 and -2 and the TATA box are also indicated. (*C Lower*) Northern blots on wild-type and *rrp6* RNA from cells grown in YPD using the probes indicated by numbers below. The intergenic transcript is detected with probes 2–6 (and stronger in *rrp6*).

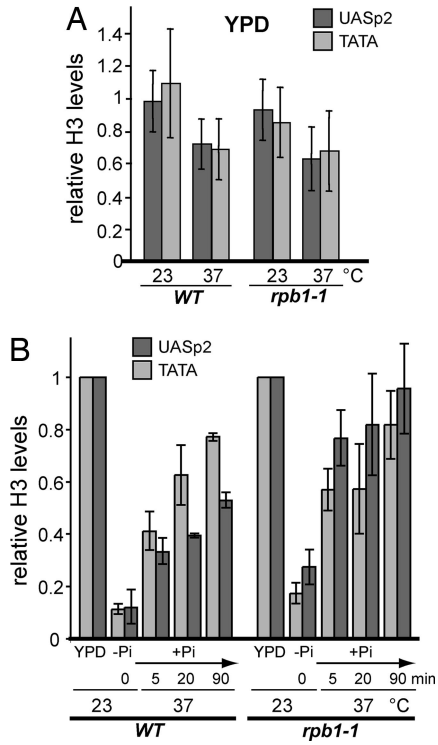

Fig. 2. RNAPII activity is not required to maintain or establish repressed promoter chromatin. (*A*) Relative histone H3 occupancy (H3C ChIP) at the *PHO5* promoter in WT and *rpb1-1* cells grown in YPD at 23°C or 37°C, as indicated. *y* axis indicates relative histone H3 levels (arbitrary units). (*B*) Relative histone H3 density (H3C ChIP) at the *PHO5* promoter in WT and *rpb1-1* cells during the establishment of phosphate repression. Measurements from cells grown in high phosphate (YPD), in the absence of phosphate (−Pi, time = 0), and at different times after shifting to 37°C and into phosphate-containing medium (+Pi, different time points). Density in YPD was set to 1, and all other values are expressed relative to that.

to maintain histones at the promoter during repression (Fig. 2*A*). We also found that general RNAPII transcription is not required for establishment of repression (Fig. 2*B*). In this experiment, wild-type and *rpb1-1* cells were grown at 23°C in phosphate-free medium and then shifted to 37°C for 30 min before adding phosphate. H3C ChIP analysis showed that, upon adding phosphate, histones were rapidly deposited onto the *PHO5* promoter also in the transcription-defective *rpb1-1* cells (Fig. 2*B*), indicating that transcription is not required for a normal rate of promoter closing either.

Transcription Is Required for Normal Kinetics of Promoter Remodeling. We next asked whether general transcription might somehow facilitate histone eviction from the *PHO5* promoter during activation. Wild-type and *rpb1-1* cells were grown at the permissive temperature and then shifted to 37°C for 30 min before phosphate starvation. By 6 h of induction, wild-type cells had ≈80% fewer histones at the promoter than in high phosphate (Fig. 3*A*). In sharp contrast, histones were not evicted at all upon phosphate starvation in the *rpb1-1* strain, suggesting that transcription might indeed play a role in *PHO5* promoter opening.

To check that the inability to remodel in *rpb1-1* was not simply due to a defect in PHO signaling, we monitored Pho4 localization. Pho4 translocates to the nucleus upon successful signal transduction (8). Using GFP-tagged Pho4, we found that at 37°C the majority of Pho4 is in fact found in the nucleus by 1.5 h of phosphate starvation in both wild-type and *rpb1-1* cells (Fig. 3*B*). Furthermore, the PHO induction cascade was bypassed altogether by using a *PHO5* promoter derivative (*PHO5v33*), where the Pho4 binding sites are replaced by Gal4 binding sites (15). This promoter responds to the addition of galactose in a manner virtually identical to the response of the normal *PHO5* promoter during phosphate starvation (ref. 15 and references therein). Also using this construct, histone eviction was negligible at the restrictive temperature in *rpb1-1*, whereas histone density was reduced to very low levels in wild type (Fig. 3*C*).

The TATA Box Is Not Required for Normal Kinetics of Promoter Remodeling. Previous experiments by Hörz and coworkers (22) with promoter derivatives lacking a TATA box have argued against transcription of the *PHO5* gene being an absolute requirement for its chromatin remodeling, but to address the possibility that it might affect the kinetics of chromatin remodeling we mutated the *PHO5* TATA box at its native genomic site from TATATAA to **CCTAGGA**, asking whether this mutation affected histone eviction from the promoter during activation. RNAPII ChIP showed that, as expected, polymerase recruitment to the TATA-less promoter in response to activation was virtually abolished as a consequence of this mutation, but the kinetics of histone eviction during *PHO5* induction was largely

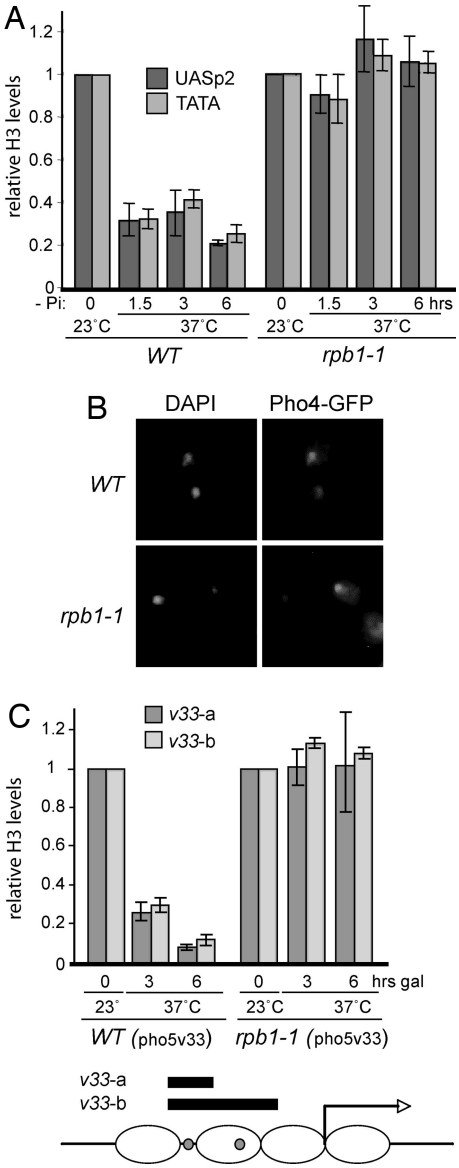

Fig. 3. *PHO5* promoter remodeling is abolished when RNAPII is inactivated. (*A*) Relative histone H3 density (H3C ChIP) in the *PHO5* promoter in WT and *rpb1-1* cells during phosphate starvation at 37°C. (*B*) WT and *rpb1-1* cells expressing a Pho4-GFP fusion protein were grown in high phosphate and then transferred to medium lacking phosphate at 37°C. GFP and DAPI fluorescence are shown. (*C*) Relative histone H3 density (H3C ChIP) in the indicated regions of the galactose-regulated *PHO5v33* promoter in WT and *rpb1-1* cells during galactose induction at 37°C. In both *A* and *C*, density at time = 0 (23°C) was set to 1 and all other values are expressed relative to that. The schematic in *C* indicates location of PCR products used to measure histone H3 density in the *PHO5v33* promoter. Small gray spheres indicate the position of Gal4 binding sites (replacing UASp1 and 2).

unaffected by this defect in polymerase recruitment (SI Fig. 7), confirming and extending the conclusion from previous studies of TATA-less *PHO5* plasmid constructs (22): RNAPII recruitment to the *PHO5* promoter is not required for normal histone eviction.

Defects in Transcriptional Elongation Affect the Kinetics of Promoter Remodeling at *PHO5*. To further test the idea that intergenic transcription helps condition the *PHO5* promoter for remodeling, we specifically targeted the elongating form of RNAPII using strains or conditions causing defects in this process, namely

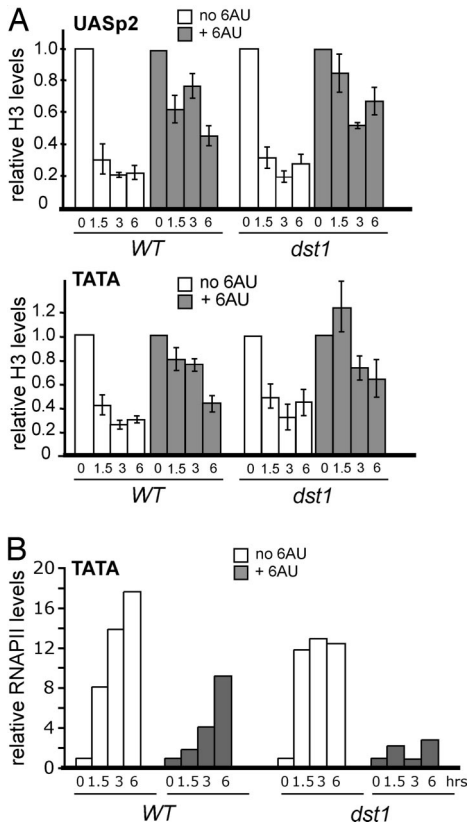

Fig. 4. *PHO5* promoter remodeling is delayed when RNAPII elongation is impaired. (*A*) Relative histone H3 density (H3C ChIP) in the indicated regions of the *PHO5* promoter (UASp2 and TATA) in WT and *dst1* cells during phosphate starvation, with or without 6AU treatment to inhibit RNAPII transcript elongation. (*B*) As in *A*, but RNAPII recruitment to the TATA box (4H8 ChIP). Density at time = 0 was set to 1, and other values are expressed relative to that.

rpb2-10 (carrying an elongation-impairing mutation in the Rpb2 subunit) or *dst1*Δ (lacking the gene encoding TFIIS) strains and the elongation inhibitor 6-azauracil (6AU). Although, as expected, the results were much less dramatic than upon complete disruption of RNAPII transcription, the effect on *PHO5* promoter histone eviction of these phenotypically innocuous mutations and 6AU was nevertheless significant and highly reproducible. H3C ChIP revealed that 6AU treatment led to a slower loss of histones (Fig. 4*A*). Significantly, RNAPII recruitment to the *PHO5* TATA box was also both delayed and decreased under these elongation-prohibiting conditions, this effect being more dramatic in *dst1*Δ cells (Fig. 4*B*). Similar results were also obtained here by using the Gal-responsive *PHO5* promoter derivative (*PHO5v33*) used in Fig. 3*C* (data not shown).

Similarly, in the *rpb2-10* strain (SI Fig. 8), an almost 2-fold-higher level of histones remained at the promoter (both at UASp2 and TATA) after 1.5 h of phosphate starvation compared with wild type. Only at later time points did the level of histone eviction reach wild-type levels, and there was an accompanying delay in transcriptional activation of *PHO5*, as revealed by delayed RNAPII recruitment to the *PHO5* TATA box.

Together, these data further support the idea that efficient transcript elongation across the *PHO5* gene under repressing conditions is required for its rapid activation.

Deletion of the 3′ End of the *PHO5* ORF Affects Histone Eviction from the Promoter. We finally sought to more specifically block intergenic transcription across the *PHO5* promoter, to investigate whether it is relevant for *PHO5* promoter remodeling. Because

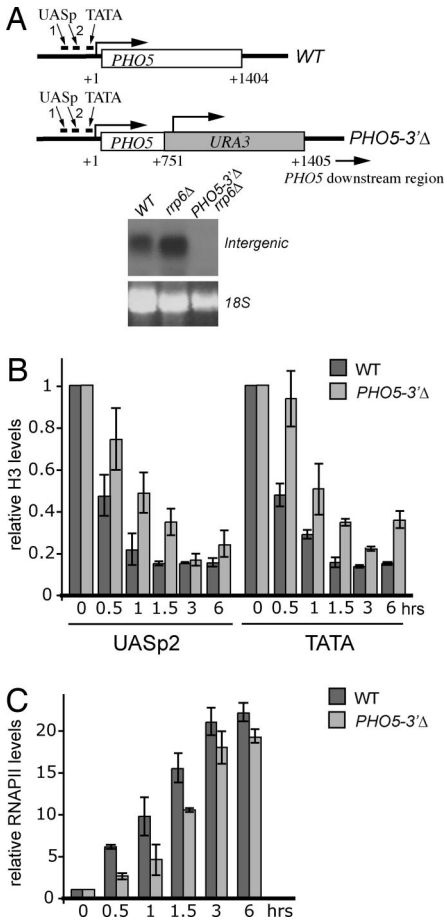

Fig. 5. Abolishing the intergenic transcript leads to slower *PHO5* promoter remodeling and RNAPII recruitment to the *PHO5* TATA box. (*A Upper*) Schematic of the *PHO5* locus in WT and *PHO5-3′Δ*. *URA3* replaces *PHO5* +751–1,404 (to end of gene). (*A Lower*) Northern blot (using probe 3 from Fig. 1*C*) showing absence of intergenic transcription in the *rrp6* version of the strain is shown. Note that only *RRP6* cells were used in *B* and *C* and other experiments addressing the functional consequences of intergenic transcription. (*B*) Relative histone H3 occupancy (H3C ChIP) in the indicated regions of the *PHO5* promoter in WT and *PHO5-3′Δ* cells during the course of phosphate starvation. (*C*) As in *B*, but RNAPII recruitment to the TATA box (4H8 ChIP). Density at time = 0 was set to 1, and other values are expressed relative to that. Histone density in the two strains at time = 0 was similar.

abrogation of RNAPII transcriptional initiation can be achieved only by removing the TATA box or initiator, we initially looked for potential TATA boxes near the end of the *PHO5* ORF. No potential TATA boxes inside the *PHO5* ORF were detected by sequence searching, but in the region immediately downstream from it, two fairly conserved cryptic sites were found. Three different approaches were then pursued to block intergenic transcription. First, we attempted to block usage of the potential TATA sites, either by inserting a *URA3* marker right after the *PHO5* STOP codon or by replacing the entire downstream region between *PHO5* and *PHO3* with a bidirectional terminator sequence (normally found between *FBA1* and *YKL061W*), which has no discernable TATA sequences. Neither of these approaches halted intergenic transcription, nor did they affect *PHO5* promoter activation (data not shown). This suggests that intergenic transcription is initiated by (non-TATA) sequence elements near the 3′ end of the *PHO5* ORF itself. Second, we inserted the bidirectional terminator at a position 500 bp into the *PHO5* ORF, hoping to thereby block intergenic transcription through the *PHO5* promoter region. Interestingly, although this approach resulted in the disappearance of a stable, detectable intergenic transcript in wild-type cells, a slightly longer intergenic transcript could still be detected across the *PHO5* promoter in *rrp6* cells (SI Fig. 9), supporting the idea that RNAPII continued to transcribe through the *PHO5* promoter region (SI Fig. 10). Accordingly, insertion of the terminator at this position also failed to affect *PHO5* promoter activation (data not shown).

Given that the above approaches did not halt intergenic transcription, the region containing the intergenic transcription start site region was deleted by substitution with a *URA3* marker (*PHO5*-3′Δ) orientated in the sense direction, in the hope that any potential transcription through the *PHO5* promoter from the inserted marker gene itself, as well as from initiation region(s) downstream from the site of insertion, could be abrogated (the marker replaced *PHO5* ORF sequence from +751 to +1,404; Fig. 5*A Upper*). The intergenic transcript was indeed absent from wild-type and, importantly, also from *rrp6*Δ cells carrying this modification (Fig. 5*A Lower* and data not shown), although RNAPII ChIP analysis suggested that transcription through the promoter had still not been reduced to background levels (SI Fig. 10), pointing to some "sporadic," noncoding transcription still occurring across the locus.

Nevertheless, this specific block clearly led to slower *PHO5* promoter remodeling, with H3C ChIP revealing a significant delay in histone loss compared with wild-type cells (Fig. 5*B*). Thus, considering that four nucleosomes are being evicted from the *PHO5* promoter during remodeling (13, 14), the wild-type cell population lost nucleosomes at a rate of ≈2.2 nucleosomes per hour, whereas cells lacking the intergenic transcript did so at a rate of ≈1.1 nucleosomes per hour. A corresponding delay in RNAPII recruitment to the *PHO5* regulatory region upon activation was also observed in the absence of the intergenic transcript (Fig. 5*C*). Conversely, we obtained further evidence that the intergenic transcript does not play a role in maintaining a closed promoter and repressing *PHO5* transcription under noninducing conditions (data not shown).

Taken together, these data support the idea that noncoding transcription through the *PHO5* promoter affects the speed of histone remodeling during transcriptional activation, but not repression, of the gene.

Discussion

Here we identified an intergenic RNAPII-generated transcript that initiates in the region around the end of the *PHO5* ORF and is transcribed in the antisense direction up to and across the *PHO5* promoter. This transcript was also recently independently identified by whole-genome microarray studies (3–5). By employing various mutations (and the compound 6AU) affecting RNAPII transcript elongation and generation of the intergenic transcript, we uncovered evidence for the idea that the kinetics of activation, but not repression, of *PHO5* is affected by intergenic transcription. Below we argue that transcription across the *PHO5* promoter somehow contributes positively to chromatin plasticity, enabling rapid nucleosome disassembly upon activation.

Intergenic Transcription and Its Consequences. Data published over the last few years indicate that intergenic transcription is much more widespread than had previously been expected. However, studies addressing the putative role(s) of such transcription are few (23–26). Previous data in yeast demonstrated a repressive role for intergenic transcription in the regulation of *SER3*, activated when serine is limiting (25). RNAPII generating this noncoding transcript (called *SRG1*) represses transcription by transcriptional interference, i.e., by inhibiting binding of activators to the *SER3* UAS, and of TBP to its TATA box. The situation at *PHO5* is fundamentally different from that at *SER3*/*SRG1*: in contrast to *SRG1*, the intergenic transcript across *PHO5* is transcribed at low levels. Second, the *SRG1*

transcript initiates upstream of *SER3*, and on the same strand, whereas the *PHO5* (antisense) intergenic transcript is initiated ≈1,400 bases downstream from the *PHO5* TATA box. Transcription across the *PHO5* promoter does not result in transcriptional interference, but instead seems to allow efficient histone eviction upon activation, promoting timely recruitment of RNAPII to the *PHO5* TATA box and transcription of the gene.

Possible Mechanism Underlying the Effect of *PHO5* Intergenic Transcription. Intergenic transcription could in theory affect histone–DNA interactions at *PHO5* in at least three different ways. First, the RNA transcript itself could facilitate more rapid activation, perhaps by acting as a histone acceptor/chaperone, as RNA has been shown to do *in vitro* (27). Second, physical movement of RNAPII through the promoter might increase the level of histone modification, such as acetylation and methylation, or increase insertion of the histone H2A variant Htz1. Finally, RNAPII movement through the region might cause temporary histone/nucleosome displacement, which could be required for or increase histone loss at the locus upon activation. Indeed, RNAPII-generated changes in chromatin integrity are well documented in a number of experimental systems (28–31).

If the presence of the RNA transcript itself were important, we reasoned that a higher level of the intergenic transcript near the *PHO5* locus might facilitate promoter remodeling. The *rrp6* stain allowed a test of these conditions because it has significantly higher levels of the intergenic transcript. In turn, nucleosomes might be evicted faster in *rrp6* cells than in wild type. However, we found that histone eviction in *rrp6* cells is similar to that of wild-type cells (data not shown). Likewise, expressing the intergenic transcript in trans from a plasmid failed to suppress the slower histone eviction and RNAPII TATA box recruitment observed in *PHO5-3′Δ* (data not shown). Finally, insertion of a terminator ≈500 bp into *PHO5* did not affect the kinetics of *PHO5* activation, although it resulted in a dramatic decrease of stable intergenic transcript. Together, these results argue against, but do not rule out, a positive role for the intergenic RNA transcript itself.

We also used the *rpb1-1* and *PHO5*-3′Δ strains to test whether transcription across the *PHO5* promoter affects the acetylation (H3-K9, H3-K27, H3-K18, H4-K5, and H4-K12) or methylation (H3-diMetK4, H3-diMetK36, and triMetK4) level of histones, or density of the histone variant Htz1, but ChIP assays failed to detect major changes in these histone characteristics (after normalizing to histone density) when transcription was inactivated (data not shown). Although these data in themselves do not rule out the possibility that RNAPII-mediated changes in histone modification play a role in *PHO5* histone eviction, they do argue against this possibility. The *PHO5*-3′Δ mutation also does not result in a measurable change in the accessibility of promoter chromatin to restriction enzymes (data not shown), suggesting that, as expected, nucleosome positioning is not dramatically altered by intergenic transcription.

We also considered the possibility that the result we obtained with *PHO5*-3′Δ might be due to loss of putative promoter–terminator contacts (“gene looping”), rather than loss of intergenic transcription. However, slower *PHO5* activation was not observed in strains where such looping would be disrupted because the *PHO5* terminator was removed (but intergenic transcription still occurred) (data not shown), arguing against a role for gene looping.

A Model to Explain the Effect of Intergenic and Sporadic Transcription. Considering the results described above, as well as previous data from others on the disruptive effect of RNAPII transcription through nucleosomes, we suggest that the actual movement of RNAPII through the *PHO5* promoter affects nucleosome eviction by somehow increasing the local rate of nucleosome exchange/turnover. In support of this idea, more generally inhibiting or abrogating transcription (6AU and in particular *rpb1-1*) within and outside the *PHO5* locus reduced nucleosome eviction to a much greater extent than specific abrogation of the intergenic transcript (*PHO5-3′Δ*). Indeed, it is possible that noncoding, sporadic transcription, i.e., random transcription originating from multiple spurious initiation sites and terminating at random, is as widespread as the intergenic transcription that gives rise to detectable transcripts. The concept of widespread, sporadic transcription may well be of substantial general importance in all eukaryotes, but it might play a particularly important role in maintaining chromatin plasticity in an active genome such as that of *Saccharomyces cerevisiae*.

The Kinetics of Gene Regulation. It is obvious that the speed at which a cell responds to a stimulus by activating a set of genes and repressing another is of pivotal importance to the fate of that cell. However, this aspect of gene regulation is often not appreciated. Instead, the amplitude of regulation or the absolute levels of expression are generally seen as the hallmarks of a regulated gene. Here we have shown that the rate of activation of the *PHO5* promoter is affected by intergenic transcription across it, whereas the final level of induction is not. Given that intergenic transcripts are often found to be expressed in a tissue- or time-dependent manner, the effect of noncoding transcription described here may be a general one, and one important consequence of it could be to increase the rate of chromatin remodeling and thereby the rate of gene induction, rather than to affect the final steady-state levels of expression. Experiments in higher cells suggest that intergenic transcription is generally extremely widespread. Our findings thus have important implications for the approaches that should be taken to study the effect of noncoding transcription also in metazoans.

Materials and Methods

Yeast Strains and Growth Conditions. Strains used are listed in *SI Text*. For *PHO5* activation, yeast strains were grown in yeast extract/peptone/dextrose (YPD; high-phosphate conditions) to mid-log phase, and then shifted to phosphate-free minimal media in a final concentration of typically 0.5×10^7 cells per milliliter. KH_2PO_4 was added to 10 mM final concentration to cultures in *PHO5* shutoff experiments. For galactose induction (*PHO5v33*), strains were grown in yeast extract/peptone medium containing 2% raffinose. Galactose was added to 2% final concentration, and cells were diluted to 0.5×10^7 cells per milliliter. *rpb1-1* and its wild-type counterpart were grown at 23°C as the permissive temperature and at 37°C as the restrictive temperature. Temperature shifts were performed 30 min before phosphate starvation, phosphate addition, or galactose induction. 6AU was added to a final concentration of 50 μg/ml, with cells made *URA3*$^+$ by transformation with a CEN plasmid, as required.

ChIP Assays and Real-Time PCR Analysis. ChIP assays were performed essentially as previously described (32). 4H8 (anti-Rpb1 antibody) was from Upstate Biotechnology (Lake Placid, NY), and the H3C antibody was a gift from Alain Verreault (Institute for Research in Immunology and Cancer, Montreal, QC, Canada). Coprecipitated DNA was analyzed in triplicate by quantitative PCR using the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences are available upon request. Values were normalized to inputs. All values expressed in bar graphs represent means ± SEM of at least two independent experiments and three independent ChIP assays.

Northern Blots. Total RNA was extracted by using the hot acid phenol method. RNA was separated on 1% formaldehyde agarose gels and blotted onto Nylon membranes. Blots were hybridized with PCR probes (primer sequences available upon

request) labeled by random primer labeling. Details are available upon request.

RT-PCR Analysis. Total RNA was extracted using RNeasy mini kits (Qiagen, Valencia, CA) followed by DNase treatment with the Turbo DNA-free kit (Ambion, Austin, TX). For asymmetric RT-PCR, 1 μg of RNA was incubated with one primer only, either sense or antisense, in a reaction using Multiscribe reverse transcriptase. Next, the DNA product was amplified by standard real-time PCRs using both sense and antisense primers and Absolute QPCR SYBR green reagents (Abgene, Rochester, NY). Values were normalized to those obtained in the *FBA1* gene (primer sequences available upon request).

Microscopy. Cells were prepared and visualized as previously described (15).

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