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Chromatin potentiates transcription

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Chromatin isolated from the chromosomal locus of the *PHO5* gene of yeast in a transcriptionally repressed state was transcribed with 12 pure proteins (80 polypeptides): RNA polymerase II, six general transcription factors, TFIIS, the Pho4 gene activator protein, and the SAGA, SWI/SNF, and Mediator complexes. Contrary to expectation, a nucleosome occluding the TATA box and transcription start sites did not impede transcription but rather, enhanced it: the level of chromatin transcription was at least sevenfold greater than that of naked DNA, and chromatin gave patterns of transcription start sites closely similar to those occurring in vivo, whereas naked DNA gave many aberrant transcripts. Both histone acetylation and trimethylation of H3K4 (H3K4me3) were important for chromatin transcription. The nucleosome, long known to serve as a general gene repressor, thus also performs an important positive role in transcription.

RNA polymerase II | PHO5 | *Saccharomyces cerevisiae*

Assembly of purified histones on promoter DNA interferes with the initiation of transcription by RNA polymerase II and general transcription factors (GTFs) in vitro (1) and in vivo (2). Factors that relieve inhibition by histones have been identified by transcribing chromatin reconstituted with purified histones and genetic analysis. These factors include ATP-dependent chromatin remodelers (3, 4), histone-modifying enzymes (5–7), FACT (8), and TFIIS (9). Although informative, these studies are incomplete, because chromatin reconstituted with purified histones differs from chromatin assembled in vivo. Reconstituted chromatin lacks the patterns of histone modification, histone variants, and nonhistone proteins shown to play important roles in transcription in vivo. Nucleosome positioning, also important for transcription in vivo, cannot be accurately reconstituted in vitro. We have, therefore, investigated chromatin assembled in vivo as a template for transcription in vitro.

Results

PHO5 chromatin in the transcriptionally repressed state was excised from yeast chromosomes in circular form by recombination and purified by affinity chromatography as described (Fig. S1 *A* and *B*) (10, 11). *PHO5* chromatin isolated in this way was indistinguishable from chromatin at the chromosomal locus on the basis of digestion with specific and nonspecific endonucleases (Fig. S1 *C* and *D*) (10, 12). Because the chromatin was derived from a single copy gene, very small quantities were obtained: on the order of 10 fmol/L cell culture. At this low level, transcription cannot be detected directly by radioisotope incorporation or fluorescence, and therefore, we turned to RT-PCR. Two sets of primers were used: one to amplify the region downstream of the transcription start sites (TSSs) and detect all transcripts ("downstream" primer pair) and one to amplify any signal from cryptic transcripts originating upstream and reading through the promoter ("upstream" primer pair) (Fig. 1*A* and Fig. S2*A*). Subtraction of the upstream signal from the downstream signal revealed the level of promoter-specific transcription. This procedure was validated by controls: upstream and downstream primer pairs amplified their target sequences at the same rate (Fig. S2 *B* and *C*), and primer pairs were specific as shown by RT-PCR with mRNA extracted from cells grown under conditions of *PHO5* activation or repression (Fig. S2*D*) and RT-PCR with synthetic *PHO5* RNA (Fig. S2*E*). As an internal control against variation of reverse transcription efficiency and variable recovery of RNA after purification, lacI RNA was added to all samples as an internal standard, reverse-transcribed, and used for normalization (*Materials and Methods* and Fig. S3 *A* and *B*). To further reduce any aberrant signal from read-through transcription of the circular template, the template was linearized by cleavage at an NcoI site in the nucleosome-free 3′ UTR (Figs. S1*A* and S3*A*) (cutting efficiency was 94.0 ± 1.2% after 1 h at room temperature).

Transcription of Repressed Chromatin. Promoter-specific transcription of naked DNA extracted from *PHO5* chromatin circles by pol II and GTFs (Fig. S4) was detectable by the RT-PCR procedure (Fig. 1*B* and Fig. S5). In contrast, virtually no transcripts were produced from the native, repressed chromatin circles (Fig. 1*C* and Figs. S3*C* and S5). Addition of five proteins—Pho4, Mediator, TFIIS, SAGA, and SWI/SNF (Fig. S4)—elicited transcription from the chromatin circles (2.05 ± 0.24% transcripts per templates) (Fig. 1*C* and Figs. S3*C* and S5). Remarkably, the level of transcription from chromatin circles was greater than that from naked DNA under the same conditions (Fig. 1*D* and Figs. S3*E* and S5). SAGA could not be replaced by TFIID, consistent with SAGA-dependent transcription of *PHO5* in vivo (Fig. S6 *A* and *B*) (13). SWI/SNF could be replaced by RSC (Fig. S6*C*) (14), whereas addition of the histone chaperones Nap1 and Asf1 was without effect. The promoter specificity of transcription was confirmed by the use of a mutant chromatin template, in which the 24-bp *PHO5* core promoter sequence containing the TATA box was replaced by an unrelated sequence (12); virtually no specific transcripts were obtained (Fig. 1*C* and Fig. S3*C*).

Notably, transcription of chromatin required addition of carrier plasmid DNA (Fig. S3*D*) or carrier rat liver chromatin. Carrier nucleic acid might serve as a histone acceptor (15, 16) or a trap for adventitiously bound proteins contaminating the *PHO5* promoter chromatin and interfering with transcription.

As an additional test of significance, the TSSs of the transcripts from naked DNA and chromatin were mapped by 5′ RACE followed by deep sequencing. For comparison with TSSs of *PHO5* transcripts in vivo, the same procedure was applied to total RNA extracted from a *pho80Δ* strain, in which *PHO5*

Significance

The nucleosome, the unit of coiling DNA in chromatin, has long been known to interfere with the initiation of transcription in vitro. Nevertheless, we find that chromatin isolated from yeast is a better template for transcription than the corresponding naked DNA in vitro. Transcription of chromatin requires an additional 20 proteins beyond those required for the transcription of naked DNA.

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The authors declare no conflict of interest.

Data deposition: The 5′ RLM (RNA ligase mediated)-RACE sequence data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE93669).

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Fig. 1. Chromatin transcription with purified proteins. (*A*) Diagram of *PHO5* gene and promoter chromatin in the transcriptionally repressed state (17). Nucleosomes (orange ovals) previously numbered *N* – 3, *N* – 2, and *N* – 1 are renumbered *N* – 2, *N* – 1, and *N* + 1 for consistency with the literature for all eukaryotic genes. UASp1 and UASp2, binding sites for the Pho4 activator protein, are indicated by black circles, and the TATA box is indicated by a blue circle. TSSs are indicated by red arrows. Quantitation of *PHO5* transcripts was performed by reverse transcription (RT primer shown by the black arrow) followed by qPCR with the use of two primer sets (Fig. S2): a downstream primer pair to amplify the region downstream of the TSSs in vivo (18) (amplicon 85 bp) and an upstream primer pair to amplify the region that includes TSSs (amplicon 81 bp). As explained in the text, the result for the upstream primer pair was subtracted from that for the downstream pair to obtain the level of promoter-dependent ("specific") transcription. (*B*) Repressed *PHO5* chromatin (right bar) or an equivalent amount of naked DNA (left bar) purified from repressed chromatin was transcribed with pol II and six GTFs at 1× concentration (*Materials and Methods*). Transcription levels were normalized to the value obtained from the naked DNA reaction (percentage; $n = 3$). (*C*) Repressed *PHO5* chromatin was transcribed with pol II, GTFs, TFIIS, Pho4, Mediator, SAGA, and SWI/SNF in the combinations indicated below the bars at 1× concentration. TATAm denotes a mutant chromatin template, in which a 24-bp core promoter sequence containing the TATA box was replaced by an unrelated sequence (12). Absolute amounts of specific transcripts were determined with the use of synthetic *PHO5* RNA as a reference. The activity is presented as the mean number of transcripts per template (percentage) ± SE. (*D*) Transcription reactions were performed with equal amounts of chromatin or naked DNA templates and the complete set of transcription proteins at the levels indicated on the abscissa. Chromatin to DNA transcription ratio (± SE) is plotted against the level of transcription proteins ($n = 8$).

transcription is constitutively activated (17). Most TSSs in vivo were located within a 20-bp initiation region 50–70 bp downstream of the TATA box (Fig. 2), consistent with a previous report (18). Our transcripts from chromatin in vitro exhibited a closely similar pattern, whereas the TSSs of naked DNA transcripts differed, especially by the occurrence of a prominent initiation site upstream of the TATA box (Fig. 2 and Fig. S2*A*).

The greater efficiency of chromatin than naked DNA as a template for transcription became apparent when transcription proteins were limiting. At a high concentration of all proteins,

Fig. 2. Determination of TSSs by 5′ RACE. *PHO5* TSSs in vivo were determined with total RNA extracted from a *pho80Δ* strain, in which *PHO5* is constitutively activated (17). *PHO5* TSSs in vitro were determined with products of transcription reactions performed with repressed (*pho4Δ*) *PHO5* chromatin or naked DNA and transcription proteins at 1× concentration (*Materials and Methods*). The data are normalized to the total number of mapped reads per experiment and displayed as reads per thousand. The region of the promoter containing the TATA box is shaded light blue, and the region within which initiation occurs in vivo is shaded yellow.

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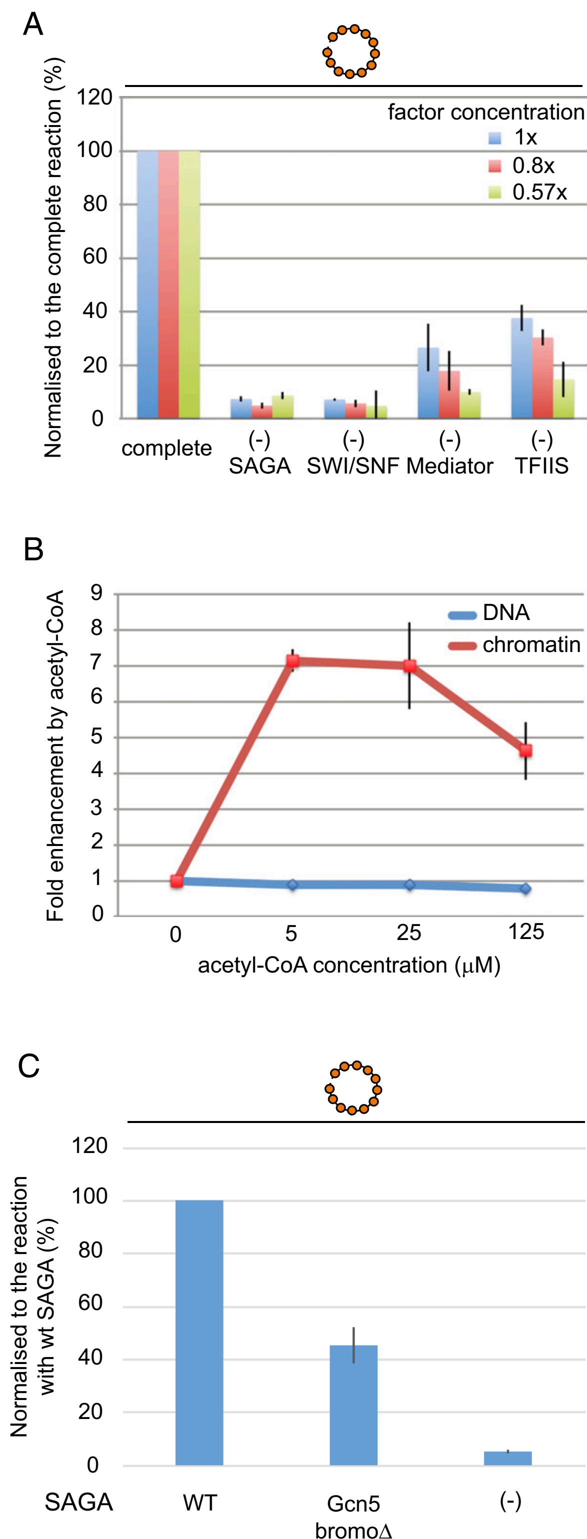

Fig. 3. Dependence of chromatin transcription on protein factors and acetyl-CoA. (*A*) Repressed *PHO5* chromatin was transcribed as in Fig. 1*C* with transcription proteins at 1×, 0.8×, and 0.57× concentrations, except for the omission of SAGA, SWI/SNF, Mediator, and TFIIS as indicated. Transcription levels were normalized to the value obtained from the complete reaction (percentage). (*B*) As in *A* with acetyl-CoA at the concentrations indicated. The ratios of transcription levels with and without acetyl-CoA are plotted on the ordinate. (*C*) Effect of gcn5 bromoΔ SAGA on chromatin transcription. Repressed (*pho4Δ*) *PHO5* chromatin was transcribed as in Fig. 1*C* with transcription proteins at 1× concentration, except that gcn5 bromoΔ SAGA was substituted for WT SAGA where indicated. Transcription levels were normalized to the value obtained from the reaction with WT SAGA (percentage).

the transcription of chromatin was twofold greater than that of naked DNA (Fig. 1*D* and Figs. S3*E* and S5), whereas transcription of chromatin was about sevenfold greater than that of naked DNA when the level of transcription proteins was reduced about 1.75-fold (Fig. 1*D* and Figs. S3*E* and S5). Similar results were obtained when transcription from chromatin was compared with that from naked DNA in the presence of "mock chromatin" (the fraction resulting from the same chromatin purification procedure performed on the yeast strain lacking sites for the R recombinase) (Fig. S7). The possibility that the enhanced transcription of chromatin was caused by contaminants in the solution was thereby excluded. These observations raise the possibility of near-total dependence of specific transcription on chromatin structure at lower levels of transcription proteins in vivo (5 molecules pol II per *PHO5* gene in vivo compared with 500 molecules per gene in vitro).

Pol II, GTFs, SAGA, and SWI/SNF were required at all concentrations of the transcription proteins (Fig. 3*A* and Fig. S6*D*). The dependence on SAGA and SWI/SNF reproduces the requirement for these factors for *PHO5* transcription in vivo (13, 19). SWI/SNF also stimulated transcription of naked DNA (Fig. S6*E*), perhaps through its Snf6 subunit, shown to stimulate transcription in vivo independently of the rest of the SWI/SNF complex (20). The effect of Mediator was concentration-dependent, increasing from 5- to 10-fold on 2-fold dilution of the other proteins; the effect of TFIIS increased from about three- to sixfold on twofold dilution (Fig. 3*A*). TFIIS has also been implicated in the initiation of transcription in vivo (21). A stimulatory effect of Pho4 on transcription was observed when the concentration of SAGA was decreased (Fig. S6*F*), suggesting a Pho4–SAGA interaction.

The importance of SAGA for chromatin transcription could be, at least in part, because of its histone acetyltransferase activity. Indeed, chromatin transcription was strongly dependent on the concentration of acetyl-CoA in the reaction, with an optimum at about 5 μM (Fig. 3*B*). In contrast, there was no effect of acetyl-CoA on transcription of naked DNA. The requirement of acetyl-CoA for chromatin transcription may reflect an interaction with nucleosomes, because bromodomains in the Gcn5 and Spt7 subunits of SAGA bind acetylated H3/H4 lysine tails (22). *PHO5* promoter chromatin is acetylated on H4 in the repressed state (Fig. S1*E*) and further acetylated on H3 by SAGA on activation in vivo (23). Support for a role of acetylated histone tails in the recruitment of transcription proteins came from the use of a mutant SAGA complex lacking the Gcn5 bromodomain (Fig. S4 and Table S1). Replacement of WT SAGA with the mutant diminished transcription of *PHO5* chromatin (Fig. 3*C*).

Transcription of Activated Chromatin. Previous studies have shown the remodeling of *PHO5* promoter chromatin on transcriptional activation (17). A nucleosome covering a Pho4 binding site and one farther upstream ($N - 1$ and $N - 2$ in Fig. 4*A*) are largely removed (12) (Fig. 4*A* and Fig. S1 *C* and *D*), whereas the nucleosome covering the TSSs ($N + 1$) is removed from only 40% of promoters and shifted downstream in the remainder, exposing the TATA box but still covering the TSSs (Fig. 4*A*) (12, 24). Chromatin circles isolated from the *PHO5* gene in the activated state retain this remodeled chromatin structure (Fig. S1 *C* and *D*) (12). We expected that activated *PHO5* circles would be transcribed even more efficiently than the repressed circles, with a loss of requirement for the histone-modifying and remodeling factors SAGA, acetyl-CoA, and SWI/SNF complex. We were, therefore, surprised to find transcription of activated chromatin at about the same level as or even slightly less than that of repressed chromatin

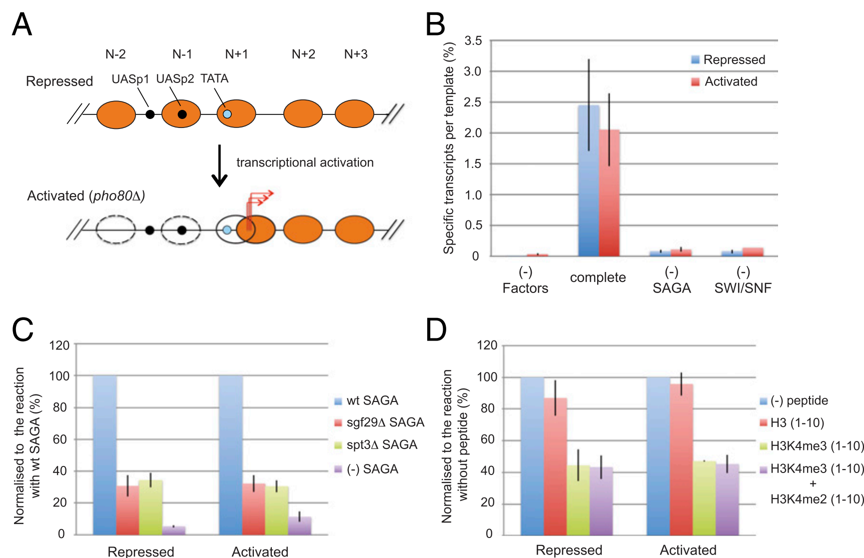

Fig. 4. Involvement of H3K4me3 in chromatin transcription. (*A*) Diagram of *PHO5* gene and promoter chromatin in the transcriptionally repressed and activated states (12), with labeling as in Fig. 1*A*. (*B*) Repressed (*pho4Δ*) or activated (*pho80Δ*) *PHO5* chromatin was transcribed as in Fig. 1*C* with transcription proteins at 1× concentration, except for the omission of SAGA, SWI/SNF, or all transcription proteins as indicated. Activity is presented as in Fig. 1*C* ($n = 7$). (*C*) Effect of sgf29Δ and spt3Δ SAGA on chromatin transcription. Repressed (*pho4Δ*) or activated (*pho80Δ*) *PHO5* chromatin was transcribed as in Fig. 1*C* with transcription proteins at 1× concentration, except that sgf29Δ or spt3Δ SAGA was substituted for WT SAGA where indicated. Transcription levels were normalized to the value obtained from the reaction with WT SAGA (percentage). (*D*) Effect of histone H3 peptides on chromatin transcription. Repressed (*pho4Δ*) or activated (*pho80Δ*) *PHO5* chromatin was transcribed as in Fig. 1*C* with transcription proteins at 1× concentration with the addition of histone H3 peptides (1–10) with or without methylation at K4 as indicated (40 μM final concentration). Transcription levels were normalized to the value obtained from the reaction without H3 peptide (percentage).

(Fig. 4*B*). Transcription of activated *PHO5* chromatin remained dependent on the SWI/SNF and SAGA complexes (Fig. 4*B*).

The increase in transcription of chromatin relative to that of DNA, observed on reduction in concentration of transcription proteins (Fig. 1*D* and Figs. S3*E* and S5), points to an interaction of the proteins with chromatin. The Sgf29 subunit of SAGA binds di- or trimethylated H3K4 (25), and trimethylation of H3K4 (H3K4me3) is found at the promoters of both repressed and activated *PHO5* circles in vitro (Fig. S1 *E* and *F*) as previously reported in vivo (26). Support for a role of H3K4me3 in chromatin transcription came from the use of a mutant SAGA complex lacking Sgf29 (Fig. S4 and Table S1). Replacement of WT SAGA with the mutant diminished transcription of *PHO5* chromatin in both repressed and activated states (Fig. 4*C*).

Additional support for a role of H3K4me3 in chromatin transcription came from competition with H3 peptides (H3 residues 1–10). Addition of the H3 peptide containing K4me3 diminished transcription, whereas addition of peptides with no modification had no significant effect (Fig. 4*D*). Addition of a dimethylated H3K4 peptide produced no greater effect (Fig. 4*D*). The inhibition of transcription by H3K4me3 peptide increased with increasing concentration of peptide, reaching a plateau at about 20 μM. As mentioned, the enhancement of transcription by chromatin points to transcription protein–chromatin interaction, and the involvement of H3K4me3 suggests that a target of this interaction is the nucleosome.

The essential role of SAGA after chromatin remodeling (Fig. 4*B*) may reflect a role as a component of the transcription preinitiation complex (PIC). The Spt3 and Spt8 subunits of SAGA have been shown to interact with TBP, and these interactions are important for transcription in vivo (13, 27). Support for a role of SAGA in the PIC came from the use of a mutant SAGA complex lacking Spt3 (Fig. S4 and Table S1). Replacement of WT SAGA with the mutant diminished transcription of *PHO5* chromatin in both repressed and activated states (Fig. 4*C*). The requirement for both Spt3 and Sgf29, even after chromatin remodeling (Fig. 4*C*), points to the persistence of SAGA in the PIC.

Discussion

As long known (1, 2) and recapitulated here (Fig. 1*B* and Fig. S5), nucleosomes inhibit the initiation of transcription by RNA polymerase II and GTFs. It is commonly assumed that chromatin remodeling relieves this inhibition by the removal of nucleosomes and exposure of naked DNA for transcription. On this basis, the most that we could hope for in the transcription of chromatin in vitro would be a level of transcription comparable with that obtained with naked DNA. It was, therefore, surprising that a level almost an order of magnitude greater was achieved. Because transcription from chromatin relative to naked DNA increases as the concentration of transcription proteins is reduced and because the levels of transcription proteins are much lower in vivo (see above), it is likely that chromatin is not only stimulatory but required for transcription in vivo.

Several lines of evidence suggest that interaction of transcription proteins with nucleosomes is responsible for the stimulation of transcription: an inverse relationship of stimulation with transcription protein concentration points to direct transcription protein–nucleosome interaction, and the H3K4me3 and histone tail acetylation, maximal at the promoter region (Fig. S1 *E* and *F*), play important roles in the recruitment (Figs. 3*C* and 4 *C* and *D*). It remains to be determined whether nonhistone components of promoter chromatin contribute to the potentiation of transcription. MS of purified *PHO5* chromatin circles has thus far revealed only histones (11), but proteins present in one copy or a small number of copies on the circles may have escaped detection by this analysis.

Our finding of the stimulation of transcription by chromatin should not be conflated with the relief of repression by histone modifications, such as acetylation and ubiquitylation (5–7, 28). These modifications relieve repression by recruiting remodelers (5, 7) and histone chaperones (6) or by chromatin decondensation (28). Relief of repression represents the removal of a negative effect, not the presence of a positive one. Only the comparison reported here, of naturally assembled chromatin with the corresponding naked DNA, could reveal a positive role of the nucleosome.

Our findings are in keeping with other evidence for interaction of transcription proteins with nucleosomes. The YEATS domain of Tfg3 (Taf14), a subunit of TFIIF (Fig. S4), binds to acetylated H3K9 (29). Mediator binds to nucleosomes (30) and the histone H4 tail (31). Bromodomains in SWI/SNF and RSC contribute to the binding of these complexes to acetylated nucleosomes in vitro (7, 22). RSC is persistently associated with the +1 nucleosome of transcriptionally active genes in vivo (32). Deletion of RSC resulted in a genome-wide decrease in occupancy of a nucleosome at the +1 position and a decrease in gene expression (32), pointing to the importance of the nucleosome for transcription.

As mentioned above, activation of the *PHO5* gene is accompanied by movement of the +1 nucleosome downstream, exposing the TATA box but still covering the TSSs (Fig. 4*A*). Mutation of the TATA box makes *PHO5* expression dependent on acetylatable lysine residues in the histone H4 N-terminal region and on the bromodomain factor Bdf1, a TFIID-associated protein (33). Apparently, in the absence of a key promoter element, promoter nucleosomes may even play a predominant role in the assembly of the PIC. Many yeast genes, including so-called "housekeeping" genes, resemble the activated *PHO5* gene, with a nucleosome-free region followed by a +1 nucleosome covering the TSSs. Despite the absence of well-defined TATA and initiator elements, the TSSs of these genes are almost always about 10–15 bp inside the upstream border of the +1 nucleosome (34). The findings reported here for *PHO5* may, therefore, apply generally: promoter chromatin potentiates transcription.

Materials and Methods

Transcription Assay. Naked DNA templates were purified from chromatin circles (yeast strains listed in Table S2) by phenol–chloroform extraction or the QIAquick PCR Purification Kit (QIAGEN) and resuspended in the same buffer as chromatin circles [40 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, 1 mM EDTA, 10% (vol/vol) glycerol, 5 mM DTT, 1× protease inhibitors]. Template chromatin or DNA circles were first linearized by cleavage at an NcoI site in the 3′ UTR for 60 min at room temperature [cut efficiency on chromatin checked by quantitative PCR (qPCR); 94.0 ± 1.2%] followed by the addition of carrier plasmid DNA (100 ng per reaction). To allow for histone acetylation and chromatin remodeling, 20-μL linearized templates were incubated with Pho4 activator (11.76 nM), SAGA (20 nM), and SWI/SNF (20 nM) in the presence of 1 mM ATP, 10 μM acetyl-CoA, 5 mM sodium butyrate, 8 mg/mL creatine kinase, and 10 mM phosphocreatine for 30 min at room temperature in a reaction volume of 25 μL. After histone acetylation and chromatin remodeling, purified pol II, GTFs, TFIIS, and Mediator were added to the reaction, and PIC formation was allowed to proceed for 10 min at room temperature. After PIC assembly, NTP, RNaseOUT (Invitrogen), and the reference lacI RNA were added to the reaction, and transcription was allowed to proceed for 30 min at 30 °C. Final reaction mixtures (50-μL reaction) contained 1 fmol chromatin or DNA templates (20 pM); 100 ng carrier plasmid (0.5 nM); Pho4 (5.88 nM); SAGA (10 nM); SWI/SNF (10 nM); pol II (8.3 nM); TBP (10.6 nM); IIB (12.5 nM); IIE (15 nM); IIF (8.3 nM); IIH (6.3 nM); IIS (12 nM); Mediator (10 nM); 40 mM Hepes-KOH, pH 7.5; 80 mM potassium acetate; 5 mM DTT; 7.5 mM magnesium acetate; 5% (vol/vol) glycerol; 2 mM ATP; 0.8 mM UTP, TTP, and GTP; 40 units RNaseOUT; 5 μM acetyl-CoA; 2.5 mM sodium butyrate; 4 mg/mL creatine kinase; 5 mM phosphocreatine; and 12.2 pg reference lacI RNA (2 pM). These concentrations of purified proteins were considered as 1× concentration. Reactions were terminated with the addition of buffer RLT containing guanidine (RNeasy Plus Micro; QIAGEN), and samples were spun through gDNA Eliminator Spin Columns to remove DNA. RNA was subsequently purified according to the manufacturer's instructions.

Reverse Transcription. Quantitation of in vitro *PHO5* transcripts was performed with RT-PCR using lacI RNA as a normalization reference. Because lacI RNA was spiked into the transcription reaction at the time of initiation, it was subjected to almost all of the experimental error introduced during transcription and the multistage process required to purify and process the RNA. Purified *PHO5* transcripts and lacI reference RNA were reverse-transcribed to cDNA in the same tube (QuantiTect Reverse Transcription Kit; QIAGEN) using the following gene-specific RT (reverse transcription) primers: *PHO5* RT primer (RT2: 5′-CCAAC-CATTTGCAG-3′) and lacI RT primer (RT3: 5′-AGCTTCCACAGC-3′). RT reaction was performed for 15 min at 42 °C followed by inactivation of reverse transcriptase for 3 min at 95 °C. First-strand cDNA was purified (Econospin Column; Epoch Life Science) and analyzed by qPCR as described below. All primers used for qPCR are listed in Table S3.

Quantitation of in Vitro Transcripts. After reverse transcription, *PHO5* transcripts were quantified with qPCR (ABI POWER SYBR Green PCR Master Mix) using an ABI 7900HT Fast Real-Time PCR System. For quantitation of *PHO5* in vitro transcripts, the following primers were used (300 nM final concentration): *PHO5* downstream primer pair (p14: 5′-CAAGCAAATTCGAGATTACCAA-3′, p16: 5′-AGGGAATGGTACCTGCATTG-3′), *PHO5* upstream primer pair (p21: 5′-AAGTC-GAGGTTAGTATGGCTTCA-3′, p22: 5′-CATTGGTAATCTCGAATTTGCTT-3′), and lacI primer pair (p5: 5′-TGGTGGTGTCGATGGTAGAA-3′, p6: 5′-TGGTCATCCAGCGGA-TAGT-3′). Amplification efficiency (E) of each primer pair was determined by replicate qPCR analysis of a dilution series prepared from reference *PHO5* and lacI DNA of known amounts using the equation $E = 10^{(-1/\text{slope})}$ (Fig. S2*B*) ($E_{\text{downstream}} = 1.93$; $E_{\text{upstream}} = 1.93$; $E_{\text{lacI}} = 1.93$). Importantly, when the difference in Ct (cycle threshold) value (ΔCt) between the upstream and downstream *PHO5* primer pairs was recorded with a dilution series of reference *PHO5* DNA of known amount, ΔCt values were nearly zero at all template concentrations tested (Fig. S2*C*). This control shows that the *PHO5* upstream and downstream primer pairs amplify their target sequences at exactly the same rate, enabling us to accurately calculate ΔCt between the upstream and downstream Ct values and thereby, accurately determine the relative amount of bona fide vs. cryptic transcription.

About 12 reactions (samples 1–12) were performed in each transcription assay. We first quantified relative *PHO5* expression levels between different samples using lacI as a normalization reference [comparative quantitation (35)]. The amount of *PHO5* transcripts in sample 1 (usually chromatin transcribed with a complete set of factors) detected by the downstream primer pair was set as one. Then, the relative amount of *PHO5* transcripts (R) detected by the upstream or downstream primer pair in a given sample (sample X; X = 1 – 12) was calculated using the efficiency corrected ΔΔCt method (35):

$$R = \frac{E_{PHO5}^{\Delta Ct PHO5}}{E_{lacI}^{\Delta Ct lacI}} = \frac{1.93^{\Delta Ct PHO5}}{1.93^{\Delta Ct lacI}}.$$

$$\text{For the upstream signal: } \Delta Ct_{PHO5} = Ct_{downstream}(\text{sample 1}) - Ct_{upstream}(\text{sample X}).$$

$$\text{For the downstream signal: } \Delta Ct_{PHO5} = Ct_{downstream}(\text{sample 1}) - Ct_{downstream}(\text{sample X}).$$

$$\Delta Ct_{lacI} = Ct_{lacI}(\text{sample 1}) - Ct_{lacI}(\text{sample X}).$$

After comparative quantitation, the absolute amount of *PHO5* transcripts was determined as follows. For each transcription assay, the absolute amount of *PHO5* transcripts in sample 1 was determined using synthetic *PHO5* RNA as a reference. Synthetic *PHO5* RNA was transcribed in vitro with T7 RNA polymerase using a T7 promoter–*PHO5* hybrid DNA as a template (transcribed from a known *PHO5* in vivo TSS), with the concentration measured by NanoDrop (Thermo Scientific). Serial dilutions of synthetic *PHO5* RNA in parallel with the *PHO5* transcripts from sample 1 were reverse-transcribed, and purified cDNA was analyzed by qPCR with the *PHO5* downstream primer pair. A standard curve of synthetic *PHO5* RNA dilution series was generated and used to determine the absolute amount of *PHO5* transcripts in sample 1 detected by the downstream primer pair. The absolute amount of *PHO5* transcripts in each sample (detected by the upstream or downstream primer pair) was subsequently calculated using the relative *PHO5* expression (R) determined by comparative quantitation described above. The amount of *PHO5* transcripts divided by the amount of DNA template gave the transcription efficiency [transcripts per template (percentage)]. Promoter-dependent transcription signal in each sample was calculated by subtracting the upstream signal from the downstream signal. RT-PCR results [transcripts per template (percentage)] are presented as the mean of several transcription experiments (n = 3–36) ± SEM.

5′ RACE. In vitro transcripts that contained γ and β phosphates at the 5′ end were first converted to 5′-monophosphorylated RNA using 5′ RNA polyphosphatase (Epicentre). This treatment allows TSSs from in vitro transcripts to be assayed by the T4 RNA ligase-mediated RNA adaptor tagging 5′ RACE strategy (FirstChoice RLM-RACE Kit, Ambion). In vivo total RNA was extracted from *pho80Δ* strains (ySH141), in which *PHO5* is constitutively activated (17); 5′ RACE reactions were performed according to the manufacturer's instructions (FirstChoice RLM-RACE Kit; Ambion). The following primers were used: 5′ RACE *PHO5*-specific outer primer (5′-TAGCCAGACTGACAGTAGGGTATC-3′) and 5′ RACE *PHO5*-specific inner primer (5′-ATTCTGCAGCATTTCACAACCTTCAGGCAAATC-3′).

Deep Sequencing. After nested PCR of 5′ RACE samples, DNA was prepared for next generation sequencing using an NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs), except that the end prep step was omitted; in the final purification step, 17 μL 0.1× TE buffer (10 mM Tris pH 8, 1 mM EDTA) was used to elute the DNA from the AMPure XP Beads, and 15 μL was reclaimed as the purified RACE library. DNA concentration was determined using a Qubit dsDNA HS Assay Kit (Life Technologies), and DNA integrity was determined by running an aliquot on a Bioanalyzer High Sensitivity DNA Chip (Agilent). The library was accurately quantified using a KAPA Library Quantification Kit (KAPA) and then, subjected to paired end sequencing on an Illumina MiSeq instrument. For analysis, paired ends were separated, and each end was treated as a single-end read. Reads that began precisely at the 5′ end with the RLM (RNA ligase mediated)-RACE adaptor (5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAA-3′) were identified, the adaptor was removed, and after trimming, only reads 25 bp or longer were retained. Reads were then mapped to the *Saccharomyces cerevisiae* genome (April 2011/sacCer3 assembly) with bowtie. The number of reads with identical 5′ ends was determined, and the 5′-most base was identified as the TSS. Data were normalized to the number of reads per base per thousands of aligned reads per sample and displayed using the UCSC Genome Browser.

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