Reconstitution of Nucleosome Positioning, Remodeling, Histone Acetylation, and Transcriptional Activation on the PHO5 Promoter

Andrea R. Terrell†, Sriwan Wongwisansri§¶, John L. Pilon§, and Paul J. Laybourn‡***

From the §Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523-1870, the ¶School of Medicine, Washington University, St. Louis, Missouri 63110, and ‡Proligo, Boulder, Colorado 80301

The PHO5 gene promoter is an important model for the study of gene regulation in the context of chromatin. Upon PHO5 activation the chromatin structure is reconfigured, but the mechanism of this transition remains unclear. Using templates reconstituted into chromatin with purified recombinant yeast core histones, we have investigated the mechanism of chromatin structure reconfiguration on the PHO5 promoter, a prerequisite for transcriptional activation. Footprinting analyses show that intrinsic properties of the promoter DNA are sufficient for translational nucleosome positioning, which approximates that seen in vivo. We have found that both Pho4p and Pho2p can bind their cognate sites on chromatin-assembled templates without the aid of histone-modifying or nucleosome-remodeling factors. However, nucleosome remodeling by these transcriptional activators requires an ATP-dependent activity in a yeast nuclear extract fraction. Finally, transcriptional activation on chromatin templates requires acetyl-CoA in addition to these other activities and cofactors. The addition of acetyl-CoA results in significant core histone acetylation. These findings indicate that transcriptional activation requires Pho4p, Pho2p, nucleosome remodeling, and nucleosome acetylation. Furthermore, we find that DNA binding, nucleosome remodeling, and transcriptional activation are separable steps, facilitating biochemical analysis of the PHO5 regulatory mechanism.

Chromatin functions to compact and organize DNA in the nucleus of eukaryotic cells in a manner that allows regulated access to genes for transcription and DNA replication. The role of nucleosomes in transcriptional regulation has become a major area of study. From in vitro studies, it is clear that nucleosomes can repress transcription by RNA polymerase II (1–3). Further confirmation that nucleosomes play a prominent role in gene regulation came from studies showing that histone H4 depletion in yeast cells results in nucleosome loss and transcription derepression of several RNA polymerase II-transcribed genes (4, 5). Barring artificial loss, nucleosomes must be reconfigured prior to transcriptional activation of these genes. Recently, many yeast activities that remodel chromatin have been identified, including the SWI/SNF, INO80, ISW1, ISW2, and RSC complexes (reviewed in Ref. 6). All of these activities contain a DNA- or chromatin-dependent ATPase subunit required for remodeling. In addition, many transcriptional activators recruit histone acetyltransferase activities, which in yeast include the ADA, Spt-Ada-Gcn5 acetyltransferase (SAGA), NuA3, and NuA4 complexes (7, 8).

The chromatin structure of the yeast PHO5 promoter regulates RNA polymerase II transcription of the PHO5 gene, which encodes the major, secreted acid phosphatase in yeast (9). Under repressive conditions (adequate phosphate) the PHO5 promoter is bound in an array of positioned nucleosomes (10). Activation of PHO5 through phosphate starvation is accompanied by a loss or reconfiguration of four nucleosomes from the promoter region (11). Activation is initiated through a signal transduction pathway that ultimately results in dephosphorylation of the transcription factor Pho4p, allowing its transport into the nucleus where it can bind to two upstream activation sequences (UAS)1 on the PHO5 promoter, UASp1 and UASp2 (12–15). Pho2p, a second transcription factor involved in PHO5 activation, binds cooperatively with Pho4p in vitro at both UASs (16). Pho4p and Pho2p must physically interact in vivo for transcription activation to occur (17). This interaction can occur regardless of phosphate concentration but does require the presence of DNA (18). Both transcription factors are required for full chromatin remodeling and activation of the PHO5 promoter (19). However, Pho4p is the primary trigger for activation, because Pho2p appears to be constitutively expressed and active. In addition, overexpression of PHO4 in a pho2 null strain is sufficient for the full chromatin transition and partial transcriptional activation (15).

Chromatin remodeling on the PHO5 promoter occurs in the absence of replication and does not require transcription (20, 21). These findings suggest that chromatin remodeling and activation of transcription can occur independently. Gcn5p, the catalytic subunit of ADA and Spt-Ada-Gcn5 acetyltransferase (HAT) complexes is required for full PHO5 derepression in a Δpho80 strain or a Δrdp3 strain and full activation in the absence of one of the UASs (22, 23). Furthermore, the rates of PHO5 chromatin remodeling and transcriptional activation are significantly delayed (2- to 3-fold) in a Δgcn5 strain (24). Interestingly, this delay appears to be specific for chromatin structure rather than for activator (Pho4p). Esa1p, the catalytic subunit of NuA4, is indicated to be involved in maintaining the

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** A recipient of a Junior Faculty Research Award from the American Cancer Society. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, MRB Bldg., Rm. 231, Colorado State University, Fort Collins, Colorado 80523-1870. Tel.: 970-491-5100; Fax: 970-491-0494; E-mail: laybourn@lamar.colostate.edu.

1 The abbreviations used are: UAS, upstream activation sequence; MNase, micrococcal nuclease; HAT, histone acetyltransferase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; WCE, whole cell extract; NE, nuclear extract.
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H4 acetylation state on the PHO5 promoter (23). SWI/SNF is not required for activation of PHO5, because the PHO5 chromatin transition is unaffected and acid phosphatase levels achieve 70% of wild type levels under activating conditions in strains carrying an SNF2 disruption (25). A requirement for RSC has not been tested, and ISW1 and ISW2 do not seem to have a role in PHO5 regulation (26). However, INO80 has been reported to be required for full PHO5 activation (27, 28). Although the trans-acting factors and cis-acting sequences required for chromatin structure modulation and transcription activation of PHO5 have been extensively studied, the detailed mechanisms of this process are not yet understood.

Using minichromosome templates isolated from yeast cells, remodeling on PHO5 promoter was shown to require Pho4p, Pho2p, ATP, and fractionated nuclear extract, further elucidating the mechanism of remodeling (29). However, to study the fine details of chromatin structure modulation, a fully defined chromatin template was required. Here we describe the reconstitution of chromatin templates with purified, recombinant yeast core histones possessing many important aspects of the repressed PHO5 promoter. Based on footprinting results, we conclude that sequence-dependent intrinsic properties of the DNA can produce translational positioning of nucleosomes on the PHO5 promoter that approximates that seen in vivo. Consistent with nucleosome positioning, reconstitution of nucleosomes on the PHO5 promoter strongly repressed transcription. Both Pho4p and Pho2p can bind their cognate sites on chromatin-assembled templates without the aid of remodeling factors. However, nucleosome remodeling by these transcriptional activators requires an ATP-dependent activity in a yeast nuclear extract fraction. Finally, transcriptional activation on chromatin templates requires acetyl-CoA in addition to these other activities and cofactors. These findings indicate that transcriptional activation requires Pho4p, Pho2p, nucleosome remodeling, and nucleosome acetylation. Furthermore, DNA binding, nucleosome remodeling, and transcriptional activation are separable steps, in concordance with the ability to separate nucleosome remodeling and transcriptional activation in vivo (15, 20, 21). Finally, our results suggest that Pho4p and Pho2p binding to UASp2 in nucleosome −2 occurs prior to nucleosome remodeling or acetylation, which then function subsequent to activator binding.

EXPERIMENTAL PROCEDURES

Plasmids—The pPHO5-G-less plasmid was produced by subcloning the 526-bp BamHI to Apol fragment (−542 to −16) of pMH313 (5) and a DNA fragment containing no guanine bases in the RNA-like strand into pUC19. In this construct, three guanines were changed to cytosines in the RNA-like strand at positions −24, −22, and −17 relative to the ATG. The plasmid pMH313-G-less was produced by inserting a 100-bp DNA fragment containing no guanine residues in the RNA-like strand into the Apol and BamHI sites. The same three guanines were changed to cytosines between the RNA start sites and the ATG.

Nucleosomal Template Reconstitution—Reconstituent yeast core histones, purified as described previously (30), were provided by the Luger laboratory. Yeast core histones and recombinant yeast Nap1p were combined at a total core histones to Nap1p ratio of 1:1. After acetylation, the mixture was dialyzed for 1, 2, and 4 min. Free DNA (3 μg) was incubated under the same conditions except that micrococal nuclease was added to 0.35 unit/ml and digested for 1 and 4 min. Aliquots of 100 μl were transferred to tubes containing 12 μl of 0.5 mM EDTA, 5 μl of 2.5 mg/ml proteinase K (Sigma) and 1 μl of 2 x 37 °C for 10 min. DNA was desalted, precipitated, washed, and dried. The DNA was resuspended in H2O, and the concentration was determined by measuring absorbance (A260). 75 nmol of a 32P-end-labeled primer was added to 250 ng of DNA. The sequence of the primers used were 5′-CCACGTTGTAGTGGCAAG-3′ (mn2), 5′-ATGAGGAAAGAAGGATGGTATA-3′ (m58), and 5′-GGAAAGAATGAAACG-3′ (mn5). The primers bind where indicated in Fig. 1A. Multiple-round primer extension reactions were carried out in 50-μl reactions containing 0.1 mM dNTPs, 1.5 mM MgCl2, 10 mM HEPES-KOH, pH 8.4, and 50 mM KCl. Five cycles of primer extension were done. Extension products were extracted, precipitated, resuspended in formamide loading buffer, and resolved on a 6% sequencing gel. The gel was dried and exposed to a PhosphorImager screen (Amerham Biosciences). One-dimensional Topological Analysis—To analyze the degree of reconstitution, 5 μg of DNA was relaxed with 10 units of topoisomerase I (MBI Fermentas) in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 1 mM EDTA, and 30 μg/ml BSA for 40 min at 37 °C in a 100-μl reaction volume. Following initial relaxation, 10 units of topoisomerase II was added to each reaction and 0.1 μg of relaxed DNA, 3 mM MgCl2, and octamer-Nap1p complex in reaction buffer in a 19-μl reaction volume. Reconstitution was allowed to proceed for 2 h at 30 °C. The reaction was stopped by the addition of 100 μl of STOP (20 mM EDTA, 0.1% SDS, 200 mM NaCl, and 0.25 mg/ml glycerol) and 12.5 μg of proteinase K and incubated at 37 °C for 20 min. The DNA was extracted and precipitated as before. The DNA was divided and run on two 1% TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA)-agarose gels, one of which contained 1.8 μg/ml chloroquine, and visualized with ethidium bromide.

Micrococcal Nuclease Digestion of Reconstituted Templates—Two micrograms of DNA, either naked or reconstituted chromatin (chromatin: DNA, w/w ratio of 1:1), was digested in a 100-μl reaction volume in reconstitution buffer and 5 mM CaCl2. Naked DNA was digested with 0.002, 0.004, 0.008, 0.016, and 0.024 unit of MNase, and chromatin was digested with 0.005, 0.02, 0.05, 0.10, and 0.20 unit of MNase at 37 °C for 10 min. DNA was purified as before and resolved on a 10-cm 1% agarose-TBE gel. The gel was stained with ethidium bromide and digitally scanned.

Pho4p Purification—Purification of Pho4p (expression vector pTR7) from E. coli was adapted from Kaffman et al. (31) and is as follows. Pho4p was expressed in BL21(DE3) Escherichia coli with 1 mM isopropyl-1-thio-β-d-galactopyranoside at an A600 of 0.7 for 3 h at 30 °C in 1 liter of LB broth containing 100 μg/ml ampicillin. The cells were washed once in 30 ml of RB0.1 buffer (20 mM Tris-OAc, pH 7.9, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM β-mercaptoethanol, and 100 mM potassium acetate), resuspended in 15 ml of RB0.1, and the cell suspension was adjusted to 1,000 rpm for 20 min at 4 °C in a Sorvall SS34 rotor. The lysate was loaded on a 0.7 ml 10% to 30% (w/v) linear DEAE-Sepharose FF (Amerham Biosciences) column equilibrated in RB0.1 column, and the protein was washed with RB0.1. Proteins were eluted with RB1.0 (same as RB0.1, but with 1 mM potassium acetate), and the peak Pho4p protein fractions were determined by SDS-PAGE and Coomassie Blue staining. The peak fractions were pooled and dialyzed into RB0.3 for 6 h at 4 °C then loaded at 0.4 ml/min onto a 5-ml SP-Sepharose FF (Amerham Biosciences) column equilibrated in RB0.3. The column was washed with RB0.3, then the proteins were eluted with RB1.0. Peak fractions were determined as described before. The Pho4p Sepharose pool was dialyzed against RB1.0 then loaded onto a Mono-Q column equilibrated in the same buffer. The column was washed with RB0.1, and proteins were eluted with an RB0.1 to RB1.0 linear gradient at 0.5 ml/min over 20 column volumes. Peak fractions were determined as described above, and protein concentrations were quantitated using the Coomassie Plus Protein Assay kit (Pierce) and stored in liquid nitrogen.

Pho2p Purification—Purification of Pho2p was adapted from Brazas and Stillman (32). The Pho2-His expression vector (M2025, a gift from Stillman et al.) was adapted from Kaffman’s laboratory was adapted from Kaffman et al. (31) and is as follows. Pho2p was expressed in BL21(pLysS) and Stillman (32). The Pho2-His expression vector (M2025, a gift from Stillman) was transformed into BL21(pLysS) and lysozyme was added to 1 mg/ml, and the cells were combined at a total core histones to Nap1p ratio of 1:8 (w/w). Acetylated core histones, purified as described previously (30), were provided by the Luger laboratory. Protein Assay kit (Pierce) and stored in liquid nitrogen.
were incubated on ice for 15 min then sonicated. Igepal (Sigma) was added to 0.1%, and the lysate was clarified by centrifugation at 12,000 × g for 30 min at 4 °C. The lysate was applied to a 1 ml nickel-nitriilotriacetic acid-agarose column (Qiagen) equilibrated in His binding buffer. The column was washed with His binding buffer followed by a wash buffer plus 60 mM imidazole. Pho2p was eluted with a linear 60 to 600 mM imidazole gradient in His binding buffer. The peak fractions were determined by Coomassie-stained SDS-PAGE gel and dialyzed for 4 h at 4 °C against storage buffer (20 mM HEPES-KOH, pH 7.9, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 100 mM potassium acetate, 1 mM FMPS, 10 mM β-mercaptoethanol, and 1 mM benzamidine). Protein concentrations were determined using the BCA Protein Assay kit (Pierce).

Electrophoretic Mobility Shift Assays—For the competition EMSA both wild type and mutant probes were 24-bp double-stranded oligonucleotides. The EMSA gel showing Pho4p and Pho2p ternary complex formation was run with the indicated amounts of Pho4p and Pho2p, (Fig. 2B), 0.4 ng of labeled probe, and 100 ng of poly(dI-dC) in 15 µL of transcription buffer. The sequences of the mutant oligonucleotides were 5′-TTGCTCTGGCGGGACTCCGGGCA-3′ and 5′-TGGTCCCTGGGAGTTAATTGAATAG-3′. The sequences of the wild type probes were 5′-TTGATTAAAAGAGTTAATTGAATAG-3′ and 5′-TGATTCGCGGAGTTAATTGAATAG-3′. The sequences of the wild type probes were labeled using α-[32P]dATP. Reactions were 12 µl and contained 50 ng of labeled probe, 225 ng of Pho2p complex, 12 µl containing 25 ng of Pho4p complex, 37 ng of both wild type and mutant probes were 24-bp double-stranded oligonucleotides. Protein concentrations were determined using the BCA Protein Assay kit (Pierce).

To verify reconstitution, chromatin (a gift from Dr. B. Meyhack) and DNA was digested at 30 °C for 30 min. The reaction mixture (100 µl) contained 50 mM Tris-HCl, pH 8.0, 5% glycerol, 0.1 mM EDTA, 50 mM KCI, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate, 0.2 µCi of [3H]labeled acetyl-CoA, and 4.5 µM ATP. After incubation, the proteins were resolved by SDSPAGE (15%) acrylamide; 30:0.8, acrylamide:bis). The gels were Coomassie Blue-stained, dried, and Western blotted using an 8% polyacrylamide urea sequencing gel at 20 V/cm. The gel was dried and exposed to a PhosphorImager screen.

RESULTS

Primert Extension Footprinting Analyses of Nucleosome Positioning on the PHO5 Promoter—Nucleosomes are positioned on the PHO5 promoter in vivo. To determine chromatin structure on the reconstituted PHO5 promoter, we used micrococcal nuclease digestion in conjunction with multiround primer extension analysis to footprint the nucleosomes (34, 35). The primers used for this analysis are shown in Fig. 1A.

Nucleosome −1—We observed cleavage protection over the TATA-box and RNA start sites (Fig. 1, B and C). The protection occurred between approximately +7 and −123 bp, relative to the ATG (translational start codon) and corresponds to nucleosome −1. At the downstream end of nucleosome −1, strong protection ends at +7, with slight protection seen at +10 (Fig. 1B). No cleavage protection or enhancement is seen from +19 to +55. However, there is some ambiguity as to where protection ends due to lack of cleavage between +10 and +19 on both free DNA and chromatin samples. The upstream edge of nucleosome −1 was inferred from the protection at −123 (Fig. 1C). From −129 through −150 there are several sites of equal or stronger cleavage, defining the linker region between nucleosome −1 and −2. The lack of cleavage between −123 and −129 on both free DNA and the chromatin precludes defining the downstream edge of nucleosome −1 with greater precision. Thus, we place nucleosome −1 between +7 (=5) bp to −123 (−9) bp.

The clear footprint observed with primer m58 and primer m55 indicates a strong translational setting for nucleosome −1. In addition, the length of DNA associated with nucleosome −1 protection is very close to the 145 bp normally associated with a core particle. This translational setting places both the initiation region and the TATA element within nucleosome −1, −30 bp from each edge. Note the cleavage protection over 5 nt of the TATA
element, indicating a lack of accessibility for TATA-binding protein (TBP) binding.

A comparison of nuclease cleavage patterns on chromatin and free DNA templates in the region from 19 to 55 and 150 to 129 indicates a complete lack of protection. This pattern is consistent with these regions comprising stretches of linker DNA 30 bp long between nucleosomes 1 and 1 and nucleosomes 1 and 2.

Nucleosome –2—Protection from micrococcal nuclease cleavage is seen from –150 (±10 bp) through –311 (±6 bp), corresponding to nucleosome –2 (Fig. 1C and D). The nuclease protection is nearly complete (Fig. 1C, near the top), which is unusual for a nucleosome. Placing the downstream edge of this nucleosome at around –150 is based upon the clear protection through this site (Fig. 1C). Setting the upstream edge at around –311 is based on the protection from –289 to –311 and the lack of protection seen from –317 to –370 (see Fig. 1D). The slightly greater than 145-bp area of protection generally associated with a nucleosome core particle suggests multiple translational or rotational positions. As seen in vivo, nucleosome –2 contains UASp2 (the Pho4p binding site) near the nucleosome dyad and a Pho2p binding site closer to the upstream edge.

Enlarged Linker—An important feature of the repressed

**FIG. 1.** Micrococcal nuclease cleavage protection pattern on the reconstituted PHO5 templates: nucleosome –1, nucleosome –2, and the enlarged linker DNA. A, the primer mn5 binds to the lower DNA strand and extends downstream. The primers ml58 and mn2 bind the top DNA strand and extend upstream. The location of the recognition sites for the restriction enzymes used to generate probes are indicated below the promoter. The promoter elements are indicated above the promoter, open boxes for Pho2p binding sites, filled circles for Pho4p binding sites, a filled box for the TATA element, and bent arrows for the RNA start sites. The map units indicated below the figure are in base pairs with ATG designated as +1. B–D, multiround primer extension was carried out as described under “Experimental Procedures” with primer mn5 (B), primer ml58 (C), and primer mn2 (D). G, A, T, and C refer to the ddNTP included in the Sanger sequencing reactions resolved in these four gel tracks. Lane U is undigested DNA, lane D is digested free DNA, and lane C is digested chromatin. To the left of each figure are indicated locations in base pairs relative to the start codon (ATG) discussed in the text. To the right of each figure is a schematic map of the PHO5 promoter indicating the locations of promoter elements and the inferred location of the nucleosomes. The bent arrows indicate the RNA start sites.
**PHO5 Chromatin Remodeling in Vitro**

**FIG. 2.** Strong repression of PHO5 transcription by reconstituted nucleosomes. Plasmid templates, containing both UASs and the core promoter were reconstituted. A, one-dimensional topological analysis. Plasmid DNA was reconstituted at the core histone to DNA ratio (w/w) indicated at the top, purified, and resolved on 1% agarose gels in the absence (−Chl) or presence (+Chl) of chloroquine. Lanes S and R are supercoiled and relaxed markers, respectively. In the right margin, the positions of relaxed (IR) and supercoiled (Is) closed circular DNA are indicated. B, micrococcal nuclease digestion analysis was used to verify that complete nucleosomes with uniform spacing were formed. Lane 1 contains undigested DNA. Free DNA (lanes 2–4) and reconstituted chromatin (lanes 5–7) were digested with increasing concentrations of nuclease, and the products were resolved on an agarose gel. The digestion products corresponding to a mono-, di-, and trinucleosome are indicated as such in the right margin. Lanes M, 100-bp DNA size markers. C, free DNA (lane 1) and chromatin templates reconstituted at the core histone to DNA ratio (w/w) indicated at the top (lanes 2–9) were transcribed using yeast whole cell extract. The PHO5 transcripts are indicated at the left of the figure. These results were found to be reproducible through several repetitions of this experiment.

**PHO5** promoter chromatin structure is the nucleosome-free region between nucleosomes −2 and −3, which includes UASp1 (the distal Pho4p binding site). Nucleosome protection beginning from approximately −370 indicates that nucleosome −3 is positioned upstream of UASp1 (Fig. 1D). In addition, we detect a region lacking protection between approximately −366 and −317 or around 50 bp in length. This linker is larger than the −30-bp length of the other linkers, corresponding to the situation in vivo (11). Previously, this enlarged linker (referred to as HS2) was mapped to a region from approximately −410 to −315 in vivo by indirect end-labeling and restriction endonuclease cleavage analysis (11). The micrococcal nuclease protection suggests that nucleosome −3 is located farther downstream on the reconstituted templates than in vivo. However, what is most important is that an enlarged linker is formed that leaves the Pho4p binding site in UASp1 accessible.

**FIG. 3.** Analysis of purified Pho4p and Pho2p proteins. A, Coomassie Blue-stained gel (12%) of purified Pho4p and Pho2p. Marker sizes, in kDa, are indicated to the left of the gel. B, EMSA showing Pho4p and Pho2p form a ternary complex on UASp1 of the PHO5 promoter. The arrows indicate the Pho4p-DNA, the Pho2p-DNA complex, and the Pho4p-Pho2p-DNA complexes. All lanes contain 0.4 ng of probe and 100 ng of poly(dI-dC) competitor. The amounts of each protein in nanograms are indicated below. C, competition EMSA demonstrating Pho2p binding specificity. Lanes 1 and 2 contain an additional 100 ng of poly(dI-dC), lanes 3–8 contain an additional 25, 50, 100, 200, 300, and 500 ng of unlabeled wild type probe, respectively. Lanes 9–14 contain an additional 25, 50, 100, 200, 300, and 500 ng of unlabeled mutant probe, respectively.

The PHO5 Promoter Is Strongly Repressed by Nucleosome Formation in Vitro—We have reconstituted plasmids with a PHO5 promoter construct containing 526 bp of the promoter upstream of a G-less cassette (pPHO5-Gless). Nucleosomes were formed at the ratios (w/w) of core histones to DNA indicated in Fig. 2. At the histone to DNA ratio of 1.0, nucleosomes were formed at physiological density as determined by topological assay (Fig. 2A). Micrococcal nuclease analysis verified that complete nucleosomes were formed with uniform spacing of −160 to 170 bp (Fig. 2B). Transcription from the chromatin templates was strongly repressed at core histone to DNA ratios of 0.43 and 0.57 and essentially completely repressed at a ratio of 1.0 (Fig. 2C).

Pho4p and Pho2p Bind to Both Free and Reconstituted Chromatin DNA—Pho4p and Pho2p are both required for the chromatin transition and transcriptional activation of PHO5 upon phosphate depletion (15). Therefore, it was necessary to purify both proteins to study the mechanism of chromatin structure transition and transcriptional activation in vitro (Fig. 3A). Both proteins bind a 106-bp UASp1 DNA probe (TthIII to MfeI, Fig. [image reference]).
A dashed line (Lanes 2)

lines indicate cleavages reduced by Pho2p, and 90, 180, and 360 ng of Pho2p, respectively.

B of each lane. The reactions run with the same primer as was used in the footprinting extension reactions. The ddNTP used for termination is indicated at the top indicating sites of protection by Pho2p, and the lines indicate the areas of protection by Pho4p. The G, A, T, and C lanes are dideoxy sequencing reactions run with the same primer as was used in the footprinting extension reactions. The ddNTP used for termination is indicated at the top of each lane. B and C, graphical representation of the propensity for cleavage on (B) free DNA (lanes 10 and 11) and (C) chromatin (lanes 21 and 22). A dashed line was used for digestions without Pho2p or Pho4p, and a solid line was used for digestions with both Pho2p and Pho4p. Asterisks indicate cleavages reduced by Pho2p, and lines indicate regions of cleavage reduced by Pho4p. The location of previously defined Pho2p and Pho4p binding sites are shown schematically below the graphs (rectangles for Pho2p sites and ovals for Pho4p sites).

Fig. 4. Primer extension footprinting of Pho4p and Pho2p binding on free and chromatin assembled PHO5 promoter DNA. A, lanes 1–11 contain free DNA; lanes 12–22 contain chromatin. Lanes 1 and 12 are undigested samples (U). Lanes 11 and 22 contain no Pho2p or Pho4p (N). Lanes 2–4 contain 250, 500, and 1000 ng of Pho4p; lanes 5–7 contain 90, 180, and 360 ng of Pho2p; lanes 8–10 contain 500 ng of Pho4p and 90, 180, and 360 ng of Pho2p, respectively. Lanes 13–21 contain identical amounts and combinations of Pho4p and Pho2p as lanes 2–10. The stars indicate sites of protection by Pho2p, and the lines indicate the areas of protection by Pho4p. The G, A, T, and C lanes are dideoxy sequencing reactions run with the same primer as was used in the footprinting extension reactions. The ddNTP used for termination is indicated at the top of each lane. B and C, graphical representation of the propensity for cleavage on (B) free DNA (lanes 10 and 11) and (C) chromatin (lanes 21 and 22). A dashed line was used for digestions without Pho2p or Pho4p, and a solid line was used for digestions with both Pho2p and Pho4p. Asterisks indicate cleavages reduced by Pho2p, and lines indicate regions of cleavage reduced by Pho4p. The location of previously defined Pho2p and Pho4p binding sites are shown schematically below the graphs (rectangles for Pho2p sites and ovals for Pho4p sites).

A 0.3 μL Nuclear Extract Fraction Remodels the PHO5 Chromatin Structure in a Pho2p-, Pho4p-, and ATP-dependent Manner—Chromatin templates were incubated with micrococcal nuclease, and the DNA fragments were resolved by agarose gel electrophoresis and detection through Southern blot analysis. The chromatin structure over the promoter region was determined using the MfeI to BstEII probe encompassing nucleosome —2 (Fig. 1A). Pho4 and Pho2p do not remodel chromatin on their own (lanes 10, 11, and 12, Fig. 5A, and the corresponding tracing in Fig. 5B). A nuclear extract fraction from wild type yeast cells was shown to remodel the PHO5 chromatin structure of isolated minichromosomes (29). On our defined chromatin templates, we found that nucleosome remodeling was mediated by this nuclear extract fraction and that it, too, was dependent on the presence of Pho4p, Pho2p, and ATP (compare lanes 7–9 with lanes 13–15, Fig. 5A, and the
corresponding tracings in Fig. 5B). To ascertain the range of the remodeling that occurs in the transcribed region, these blots were stripped and hybridized with the Apol to BamHI (110 bp) probe (Fig. 1A) complementary to DNA in nucleosome +1. Much less remodeling occurs over the transcribed region (compare lanes 13–15 with lanes 16–18, Fig. 5A, and the corresponding tracings in Fig. 5B).

Acetyl-CoA Is Required for Activation of Transcription—The NE fraction containing chromatin remodeling activity does not support transcription, so a WCE from a pho4Δ was used. The addition of Pho4p, Pho2p, and the 0.3 α SP NE fraction, sufficient for chromatin remodeling, to transcription reactions containing reconstituted chromatin did not result in transcriptional activation (results not shown). It is important to note that the formation of nucleosomes does not render the DNA irreversibly transcriptionally incompetent, because transcription from the reconstituted template can be restored by proteinase K digestion and organic extraction of the DNA (data not shown). To test whether histone acetylation was required in addition to nucleosome remodeling, acetyl-CoA was added to the transcription reactions. Addition of Pho4p activates transcription approximately 3- to 4-fold on free DNA (Fig. 6, compare lanes 2–6 with lanes 7–11). Acetyl-CoA had no stimulatory effect and even had a slight inhibitory effect on transcription from free DNA templates at higher levels (Fig. 6, lanes 1–11). Interestingly, on chromatin the addition of acetyl-CoA was able to fulfill the additional requirement needed to relieve transcriptional repression and to allow transcriptional activation from the chromatin templates (Fig. 6, lanes 13–23). Pho4p activated transcription further on chromatin (lanes 19–23), but the stimulatory effect of acetyl-CoA was not dependent on Pho4p (lanes 14–18). Pho4p was not able to activate transcription from chromatin without acetyl-CoA (data not shown).

Addition of Acetyl-CoA to the Yeast Whole Cell Extract Results in Core Histone Acetylation on Chromatin Templates—Acetyl-CoA stimulates transcription from chromatin, but not from free DNA, suggesting that this cofactor is functioning through histone acetylation. To directly determine that the core histones are acetylated in our transcription reactions, we added [14C]acetyl-CoA. The primary acetyl group acceptors were histones H2A and H4, which represents an acetylation pattern similar to that of NuA4 (Fig. 7A). Even in the presence of WCE, no other protein was detectably labeled. In addition, the level and pattern of the HAT activity is independent of transcriptional repression and to allow transcriptional activation from chromatin without acetyl-CoA (data not shown).

Reconstitution of the Repressed State—The Horz and Bergman laboratories independently identified an array of at least four translationally positioned nucleosomes on the repressed PHO5 promoter (11, 37). Although extensive mutant searches have been conducted, to date no mutation has been found that disrupts nucleosome positioning. Therefore, we hypothesized that nucleosomes are positioned primarily through intrinsic
properties of the promoter DNA sequence. To test this hypothesis, we determined the micrococcal nuclease cleavage pattern on chromatin and free DNA templates to define the nucleosome positions. We observed positioning of a nucleosome over the TATA-box and RNA start sites (nucleosome −1) and another nucleosome over the downstream Pho2p binding site and UASp2 (nucleosome −2). Moreover, we found that an enlarged linker region between nucleosomes −2 and −3 containing UASp1 is established on our reconstituted templates. Thus, the nucleosome positioning on our reconstituted chromatin templates is consistent with that seen in vivo.

The reconstituted chromatin templates were formed using purified yeast core histones and Nap1p, so no other DNA-binding proteins are present. Hence, the nucleosome positioning on our reconstituted templates is directed primarily by sequence-dependent intrinsic properties of the DNA. From the results of a series of deletion experiments, Fascher et al. (21) concluded that intrinsic properties of the promoter DNA make “an essential contribution to the chromatin organization at the PHO5 promoter.” Therefore, the mechanism of nucleosome positioning in vivo is likely to be much the same as that driving the positioning on our reconstituted templates. Nucleosomes reconstituted on tandem repeats of the sea urchin 5 S rRNA gene formed arrays of positioned nucleosomes (38). In addition, a 200-bp fragment from the Drosophila Adh promoter will translationally position a single nucleosome correctly in vitro (39). However, the PHO5 promoter and the murine mammary tumor virus long terminal repeat promoter are the only single-copy gene promoters thus far determined to contain an array of nucleosomes that are translationally positioned primarily through histone-DNA interactions (40).

There is one minor but interesting difference between the nucleosome positioning of the reconstituted and in vivo chromatin. Although the endpoints of the enlarged linker have not been mapped to the base pair in vivo, the evidence available suggests that nucleosome −3 is positioned farther upstream in vivo than in vitro (11). In vivo, the enlarged linker was mapped to approximately −410 through −340 (70 bp) by restriction endonuclease accessibility and low resolution indirect end labeling. A closer analysis of a higher resolution indirect end-labeling experiment (11) suggests boundaries of −393 to −313 (70–80 bp). The enlarged linker on our reconstituted templates is formed from approximately −366 to −317 (50–60 bp). Therefore, the downstream end point of the enlarged linker on our reconstituted templates corresponds well with that seen in vivo. However, nucleosome −3 produces clear protection through −370. Therefore, the location of nucleosome −3 appears to be shifted 20–30 bp downstream in vitro. Similarly, on partially purified minichromosomes, nucleosome −3 is located farther downstream, suggesting that the positioning factor is being lost during preparation (29). This positioning factor is absent on our reconstituted templates, as well.
Pho4p is exported from the nucleus and is not bound to UASp1 under repressing conditions (12, 31). In addition, the PHO5 chromatin structure, as analyzed by low resolution indirect end labeling in pho2 and in pho4 cells, appears to be the same as that of wild type cells (15). Finally, deletion of both the Pho2p and Pho4p binding sites at UASp1 does not significantly affect the PHO5 promoter chromatin structure (21). However, a 20- or 30-bp downstream shift of nucleosome −3 would not have been resolved in these studies. Therefore, although Pho4p is unlikely to be involved in positioning nucleosome −3 farther upstream, such a role for Pho2p or another, unidentified protein cannot be excluded at this time.

We have succeeded in reconstituting many aspects of the in vivo chromatin structure on the repressed PHO5 promoter. Nucleosome formation has long been known to repress transcription in vitro (42, 43). However, the strong repression of transcription from the PHO5 promoter on our chromatin templates at sub-saturating densities of nucleosomes suggests that the PHO5 core promoter has a high affinity for nucleosome formation, positioning a nucleosome over the core promoter. Primer extension footprint analysis indicates this is the case.

Reconstitution of Transcriptional Activation—We began to study the mechanism of PHO5 activation by adding back various activities and cofactors to determine their effects on the transcription level from our well-characterized chromatin template. We have found that recombinant Pho4p and Pho2p can bind to both sets of PHO5 promoter regulatory elements (UASps) on free DNA and assembled into chromatin. This finding reiterates the importance of regulation of Pho4p function through phosphorylation and nuclear localization (12, 31). Differences in nucleosome protection patterns indicate that these transcription factors bind their cognate sites and interact with each other differently on free DNA versus chromatin. For example, Pho4p produces enhanced cleavages downstream of the UASps in free DNA but protects these regions in chromatin. In addition, although Pho2p cleavage protection at both binding sites is enhanced by Pho4p, the effect at the site upstream of UASp2 is much more apparent on chromatin than on free DNA. These findings are consistent with an important role for Pho4p-Pho2p interactions in PHO5 activation and indicate that chromatin structure participates in these interactions.

In our system, Pho4p is sufficient for transcription activation on naked DNA, presumably through increased recruitment of the general transcriptional factors and RNA polymerase II. Pho4p physically interacts with TFIIB, TFIIEβ, and the TATA-binding protein (TBP) (18). However, Pho4p alone could not counteract nucleosome repression.

We have shown that Pho4p and Pho2p can bind to the promoter in the presence of nucleosomes. Their ability to bind both in the enlarged linker and on nucleosome −2 suggests that their binding is not ordered but, rather, that they can occupy both UASps simultaneously.

The presence of Mg2+ in the transcription buffer might promote compaction of the templates (44). However, although compaction of the DNA may occur thereby preventing core promoter access by the general transcription machinery, it does not prevent all access to the promoter, because the transcription factors Pho4p and Pho2p have access to the DNA at the same Mg2+ concentrations used for in vitro transcription experiments (Fig. 4).

Pho4p and Pho2p binding is not sufficient for nucleosome remodeling. In the presence of a nucleotide extract fraction and ATP, these transcriptional activators can remodel nucleosomes −2. Whether Pho4p and Pho2p function in remodeling through recruitment or nucleosome restructuring or sliding remains to be determined. It is clear that remodeling is not dependent on histone acetylation, because it occurs without the addition of acetyl-CoA. Finally, transcriptional activation is still not observed (data not shown). Thus, remodeling is not sufficient, and there is another barrier to transcription.

Core histone tail acetylation, which primarily affects chromatin compaction, is a potential additional requirement for transcriptional activation (45). To test this idea, we added acetyl-CoA to the reactions and found that nucleosomal repression was relieved. Furthermore, we determined the core histones, particularly H4, were highly acetylated under these conditions (Fig. 7). The level of HAT activity in our WCE is quite high and was not dependent on recruitment. Consistent with this result, the histone acetylation state on the PHO5 promoter in vivo has not been found to change significantly between the repressed and activated states (23). Because transcription is dependent on the presence of acetyl-CoA and only the core histones are detectably acetylated, core histone acetylation is likely to be a prerequisite for transcription from the chromatin-assembled PHO5 promoter. A requirement for acetylation of other substrates after transcriptional initiation cannot be ruled out. However, the lack of acetyl-CoA stimulation of transcription from free DNA templates argues against this idea.

The core histone acetylation specificity in the yeast WCE resembles that of the NuA4 complex (46). This acetylation pattern suggests that this complex is the most abundant or active HAT activity in the extract and may be primarily responsible for counteracting chromatin repression of basal and activated transcription in the presence of acetyl-CoA. Supporting this idea, we find that addition of NuA4, but none of the other three complexes, stimulated transcription further (results not shown).

Although we have not shown directly that transcriptional activation requires chromatin remodeling, the simplest interpretation of our results is that maximal activation from the reconstituted PHO5 promoter requires Pho4p, Pho2p, remodeling activity, and histone acetylation. Our results suggest that the role of histone acetylation and nucleosome remodeling lie downstream of Pho4p and Pho2p binding. Possibilities include allowing recruitment of the basal transcriptional machinery, initiation, and elongation. Current experimentation in our laboratory is designed to differentiate between these possibilities.

The events in PHO5 regulation are separable. The nucleosome positioning and the transcriptionally repressed state can be reproduced with core histones and PHO5 promoter DNA alone. Pho4p and Pho2p binding can occur without remodeling. Remodeling appears to be dependent on binding of these factors but is not dependent on histone acetylation. In fact, we have tested our yeast nuclear extract fraction (NE(S0.3)) and found it to be devoid of HAT activity (data not shown). Finally, acetylation is not dependent on either Pho4p binding or ATP-dependent remodeling. The ability to separate these events greatly facilitates their mechanistic dissection. The system we have designed to study chromatin remodeling is unique in that it uses only purified components for reconstitution. This allows the study of PHO5 transcriptional regulation in a well-defined environment. In addition, our experimental system facilitates combined transcription, nucleosome remodeling, and histone acetylation studies, giving a more complete picture of the processes of PHO5 transcriptional activation.

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Reconstitution of Nucleosome Positioning, Remodeling, Histone Acetylation, and Transcriptional Activation on the PHO5 Promoter
Andrea R. Terrell, Sriwan Wongwisansri, John L. Pilon and Paul J. Laybourn

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