MOLECULAR AND CELLULAR BIOLOGY, Apr. 1999, p. 2817–2827 Vol. 19, No. 4
0270-7306/99/$04.00+0
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An In Vitro System Recapitulates Chromatin Remodeling at the *PHO5* Promoter

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Received 14 December 1998/Accepted 16 December 1998

The *Saccharomyces cerevisiae* gene *PHO5* is an excellent system with which to study regulated changes in chromatin structure. The *PHO5* promoter is packaged into four positioned nucleosomes under repressing conditions; upon induction, the structure of these nucleosomes is altered such that the promoter DNA becomes accessible to nucleases. We report here the development and characterization of an in vitro system in which partially purified *PHO5* minichromosomes undergo promoter chromatin remodeling. Several hallmarks of the *PHO5* chromatin transition in vivo were reproduced in this system. Chromatin remodeling of *PHO5* minichromosomes required the transcription factors Pho4 and Pho2, was localized to the promoter region of *PHO5*, and was independent of the chromatin-remodeling complex Swi-Snf. In vitro chromatin remodeling also required the addition of fractionated nuclear extract and hydrolyzable ATP. This in vitro system should serve as a useful tool for identifying the components required for this reaction and for elucidating the mechanism by which the *PHO5* promoter chromatin structure is changed.

The packaging of eukaryotic DNA into nucleosomes presents a barrier to cellular processes that require specific contacts with DNA. During transcription, sequence-specific DNA binding proteins and the basal transcription apparatus must recognize and bind to appropriate promoter elements. Biochemical and genetic analyses demonstrate that the packaging of DNA into nucleosomes inhibits its stable association with transcription factors (2, 29, 37, 57). A number of cellular activities capable of facilitating factor binding to chromatin have been identified (3). These activities are thought to function by directly modifying chromatin structure.

The *Saccharomyces cerevisiae* gene *PHO5* is a well-characterized system with which to study regulated gene expression. *PHO5* encodes a secreted acid phosphatase whose transcription is regulated in response to environmental phosphate levels (for a review, see reference 32). When phosphate is plentiful, *PHO5* expression is repressed; when phosphate is limiting, *PHO5* expression is induced. Activation of *PHO5* transcription requires two transcription factors: Pho4, a basic helix-loop-helix protein, and Pho2, a homeodomain protein (58). In vitro, Pho2 enhances the binding of Pho4 to two regulatory sequences in the *PHO5* promoter, UASp1 and UASp2 (4, 5).

When transcription of *PHO5* is activated, its promoter undergoes a dramatic change in chromatin structure (for a review, see reference 51). When yeast cells are grown in high-phosphate medium, two pairs of positioned nucleosomes flank a DNase I-hypersensitive site, which contains UASp1 (Fig. 1, $+P_i$). UASp2 and the TATA box are packaged into nucleosomes -2 and -1, respectively. In vivo footprinting experiments indicate that Pho4 does not bind the *PHO5* promoter under repressing conditions (57). When environmental phosphate is limiting, the positioned nucleosomes no longer protect the *PHO5* promoter, and Pho4 binds to UASp1 and UASp2 (Fig. 1, $-P_i$). In vivo footprinting of Pho2 at the *PHO5* promoter has not been performed, but in vitro experiments indicate that Pho2 binds to this region in coordination with Pho4 (4, 5). The process by which the four positioned nucleosomes become undetectable and the *PHO5* promoter is rendered sensitive to nucleases is termed the chromatin transition.

The mechanism by which *PHO5* chromatin structure is changed during induction is unknown. However, a number of in vivo studies have provided some clues. The *PHO5* chromatin transition is independent of transcription and DNA replication, as the loss of nucleosome positioning is unaffected by deletion of the *PHO5* TATA box (18) and occurs when cell division is prevented (44). The Pho4 transcriptional activation domain is required for the *PHO5* chromatin transition (52) but is not required for binding to naked DNA (19). If the activation domain is dispensable for binding to chromatin as well as to naked DNA, it may be required for interaction with a chromatin-remodeling activity or may be capable of changing chromatin structure itself.

It remains to be determined if factors besides Pho4 and Pho2 are required for *PHO5* chromatin rearrangement. Several activities known to modify chromatin structure have been identified in yeast, and a few have been tested for a role in *PHO5* induction. Loss-of-function mutations in several components of the ATP-dependent chromatin-remodeling complex Swi-Snf do not affect induction of acid phosphatase activity (10, 20, 45) or changes in *PHO5* chromatin structure (20). *PHO5* mRNA levels in high- and low-phosphate media are unaffected by mutations in the histone acetylase gene *GCN5* (40), although *gcn5* mutants have an unusual *PHO5* promoter chromatin structure under partially inducing conditions (21). The *PHO5* chromatin transition may involve the RNA polymerase holoenzyme, as artificial recruitment of the holoenzyme to *PHO5* results in a promoter that is constitutively nuclease sensitive (20).

The reconstitution of chromatin rearrangement in vitro has allowed the isolation of several chromatin-remodeling activities from *Drosophila* embryo (22, 54, 56) and HeLa cell (35) extracts. It has been difficult, however, to obtain genetic evidence that these activities are involved in the transcriptional regulation of specific genes in vivo. In contrast, studies employing *S. cerevisiae* have a singular advantage in that this organism

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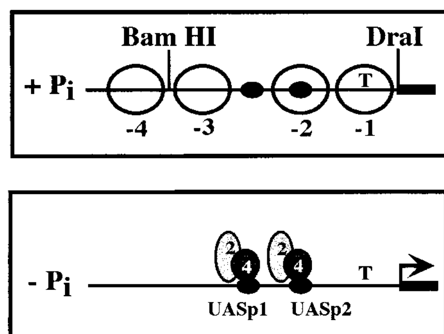

FIG. 1. Chromatin structure of the *PHO5* promoter from yeast grown in high- and low-phosphate media. Open circles, positioned nucleosomes; dark ovals, identified upstream activating sequences (UASs); T, location of the TATA box.

is easily manipulated in both biochemical and genetic experiments. This allows any result obtained in vitro to be rapidly tested for relevance in vivo. We describe here the reconstitution of the *S. cerevisiae PHO5* chromatin transition in vitro. We propose to use this biochemical system to identify the components required for the chromatin rearrangement at the *PHO5* promoter and to elucidate the mechanism by which it occurs.

MATERIALS AND METHODS

Plasmid construction. pTA-PHO5 was constructed in two steps. First, a *Bam*HI-to-*Spe*I fragment from pACD5 (62), containing *PHO5* sequence from nucleotide −542 to 1466, was inserted into pBluescript II KS to create pBSPHO5. Next, a 1.5-kb *Eco*RI fragment consisting of the *TRP1/ARS1* locus was released from pTA-R (41) and inserted into the *Eco*RI site of pBSPHO5 such that *PHO5* and *TRP1* are transcribed in opposite directions, creating plasmid pTA-PHO5. To obtain pTA-p1p2, the hexanucleotide Pho4 binding sites at UASp1 (CACGTT) and UASp2 (CACGTG) in pTA-PHO5 were replaced precisely with a *Spe*I site (ACTAGT) and a *Bam*HI site (GGATCC), respectively. In pTA-PHO5-ATGΔ, the sequence AATGTT containing the translational start site was replaced with the sequence AGATCT, creating a *Bgl*II restriction site. Bacterial replication and selection sequences were removed from all minichromosome constructs by digestion with *Not*I, and the minichromosome circles were self-ligated before introduction into yeast. pRSPHO4 was constructed by inserting a *Bam*HI-to-*Hin*dIII fragment of pACD4 (62) containing the *PHO4* promoter and open reading frame into pRS426.

Strains. *S. cerevisiae* YS18 (47) was used in all experiments. Northern analysis was performed with strains EY0244 (wild type), EY0168 (*pho3*Δ *pho5*Δ), and EY0168 harboring pTA-PHO5. For indirect end labeling, EY0255 (*pho2*Δ *pho4*Δ *pho80*Δ) harboring pTA-PHO5 was used. For analysis of chromatin remodeling of episomal *PHO5* in vivo, we used strains EY0246 (*pho3*Δ *pho5*Δ *pho4*Δ *pho80*Δ) and EY0243 (*pho3*Δ *pho5*Δ *pho80*Δ), harboring either pTA-PHO5-ATGΔ or both pTA-PHO5-ATGΔ and pRSPHO4. For minichromosome purification and nuclear extract preparation, either EY0255 or EY0579 (*pho2*Δ *pho4*Δ *pho80*Δ *snf6*Δ) was used. To make EY0579, *SNF6* was disrupted in a diploid by two-step gene replacement with pEY110 (16). After sporulation, *snf6*Δ haploid strains were identified by Southern blotting.

Northern blotting. Cell cultures were grown in medium lacking inorganic phosphate for 6 h as described previously (25), and total RNA was prepared as described previously (12). RNA was quantitated, and 20 μg of each sample was loaded on 6.7% formaldehyde–1.5% agarose gels and run in 1× E buffer (20 mM MOPS [morpholinepropanesulfonic acid] [pH 7.0], 5 mM Na acetate, 0.5 mM EDTA). The RNA was blotted to nylon and probed as described for Southern blotting below.

Preparation of *PHO5* minichromosomes. *PHO5* minichromosomes were prepared by the first steps of the procedure described by Simpson and colleagues (13, 42), with some modification. EY0255 or EY0579 cells harboring pTA-PHO5 or pTA-p1p2 were grown in 9 liters of synthetic medium to an A_{600} of 1.0. Cells were pelleted, washed with water, and incubated at 30°C for 30 min in a freshly prepared solution of 0.7 M β-mercaptoethanol–20 mM EDTA. The cells were washed once with 1 M sorbitol and resuspended in 500 ml of lyticase buffer (1.2 M sorbitol, 50 mM Tris-Cl [pH 8.0], 5 mM β-mercaptoethanol). Spheroplasting was performed with 1 ml of crude recombinant lyticase (46) per g (wet weight) of cells for 30 min at 30°C. All subsequent manipulations were at 0 to 4°C. A swinging-bucket rotor (Sorvall HB-6) was used in all centrifugation steps unless otherwise noted. The spheroplast pellet was washed two times with 1 M sorbitol and then thoroughly resuspended in 240 ml of Ficoll buffer (18% Ficoll, 20 mM MOPS-NaOH [pH 6.8], 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and Dounce homogenized by hand (Wheaton 40-ml Dounce homogenizer), 10 times with the loose pestle and 5 times with the tight pestle. The lysate was layered over an equal volume of glycerol-Ficoll buffer (20% glycerol, 7% Ficoll, 20 mM MOPS-NaOH [pH 6.8], 1 mM $MgCl_2$, 1 mM PMSF) and spun at 11.5 krpm for 30 min. The pellet was resuspended in 20 ml of Ficoll buffer and centrifuged at 4.5 krpm for 15 min. The supernatant was transferred to a fresh chilled tube, and nuclei were collected by centrifugation at 11.5 krpm for 25 min. Pelleted nuclei were flash frozen at −80°C, thawed on ice, and incubated for 1 to 2 h in 9 ml of elution buffer {200 mM NaCl, 5 mM $MgCl_2$, 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)]-NaOH [pH 7.3], 0.5 mM EGTA, 5 mM β-mercaptoethanol, 1 mM PMSF}. Nuclei were pelleted by centrifugation at 11.5 krpm for 10 min, and the eluate was split between two 35-ml 0.4 to 1 M sucrose gradients made in elution buffer supplemented to a final NaCl concentration of 250 mM. The gradients were spun at 45 krpm for 80 min in a VTi50 rotor, braked to 10 krpm, and then allowed to coast to a stop. DNA was purified from 50 μl of each 1-ml fraction and assayed on ethidium bromide-stained agarose gels. Minichromosome-containing fractions were pooled, concentrated 10-fold on Centri-Prep concentrators that had been preblocked with insulin, and stored at −80°C in aliquots.

The average yield from this procedure was approximately 40%. The greatest loss occurred at the nuclear elution step, where 50 to 80% of the *PHO5* minichromosomes were recovered in the eluate. The final *PHO5* minichromosome fraction contained approximately 1 μg of minichromosomal DNA per ml in a final volume of approximately 0.75 ml. This fraction contained a significant amount of cellular RNA but was free of genomic DNA.

Southern blotting. Samples were loaded on 1.2% agarose gels and run in 0.5× Tris-borate-EDTA at 4 V per cm for 4 h. Gels were prepared as described previously (43) and blotted to nylon membranes (Amersham) overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization and hybridization with random-prime-labeled probes were performed in Rapid Hyb Buffer (Amersham). Typically, restriction fragments of 100 to 300 bp embedded in low-melting-point agarose were random prime labeled overnight at room temperature.

Micrococcal nuclease digestion and indirect end labeling of chromosomal or episomal *PHO5* in vivo. Cells collected from 500 ml of cell culture grown to an A_{600} of 1.0 were spheroplasted as in the minichromosome preparation, washed three times with 1 M sorbitol, and resuspended in 2 ml of digestion buffer A (1 M sorbitol, 50 mM NaCl, 10 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol). Aliquots of 200 μl were placed in tubes containing 0.1 to 100 U of micrococcal nuclease (Worthington). Two hundred microliters of buffer B (buffer A plus 0.15% Nonidet P-40) was added to each tube, and the reaction mixtures were incubated at 37°C for 5 min. Reactions were stopped with 1/10 volume of 5% sodium dodecyl sulfate (SDS)–250 mM EDTA. DNA was purified by digestion with 200 μg of proteinase K per ml at 37°C for 2 h, followed by phenol-chloroform extraction, RNase treatment, and ethanol precipitation. For indirect end labeling of minichromosomes, 1/10 of each sample was digested with *Ngo*MI or *Xmn*I; genomic *PHO5* DNA was digested with *Stu*I. The Southern blot probes for analysis of episomal *PHO5* and *TRP1* nucleosome positioning were derived from an *Ngo*MI-to-*Bgl*II fragment of the *TRP1* gene and an *Xmn*I-to-*Sca*I fragment of *PHO5*, respectively. As pTA-PHO5 is maintained in high copy, hybridization of this probe to the chromosomal *TRP1* or *PHO5* locus did not interfere with analyses of minichromosomal chromatin structure. For analysis of chromosomal *PHO5*, the probe was derived from a *Stu*I-to-*Apa*I fragment of the *PHO5* upstream region.

Chromatin remodeling in vivo. EY0246 and EY0243, harboring either pTA-PHO5-ATGΔ or both pTA-PHO5-ATGΔ and pRSPHO4, were cultured, spheroplasted, treated with micrococcal nuclease, and Southern blotted as described above. Probes for analysis of nucleosome −2 and nucleosome +1 correspond to *Cla*I-to-*Bst*EII (probe A in Fig. 2) and *Dra*I-to-*Sal*I (probe B in Fig. 2) fragments of pBSPHO5, respectively. Southern blots were analyzed by phosphor screen autoradiography, and quantitative area analysis was performed with ImageQuant software.

Preparation of recombinant Pho4. *Escherichia coli* BL21 harboring a T7-PHO4 expression vector (24) was grown in 3 liters of L broth supplemented with 50 μg of carbenicillin per ml to an A_{600} of 0.4. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.4 mM, and the culture was grown for 2 h at 37°C. Cells were harvested, washed with B(0.1) (10% glycerol, 20 mM PIPES-NaOH [pH 7.3], 1 mM dithiothreitol, 1 mM PMSF, 1 μg of pepstatin A per ml, 0.1 M NaCl), and resuspended in 40 ml of B(0.1). Cells were lysed on ice by sonication and centrifuged at 16 krpm for 20 min in a Sorvall SS-34 rotor at 4°C. The lysate was treated with 10 U of DNase I, clarified through a 0.22-μm-pore-size filter, and loaded onto a 10-ml SP-Sepharose High Performance (Pharmacia) column. Pho4 was eluted with a linear gradient from 100 to 1,000 mM NaCl. Fractions containing Pho4 were pooled, adjusted to the conductivity of B(0.1) by dilution with B(0) [equivalent to B(0.1) except containing 0 M NaCl], loaded onto a BioScale S5 column (Bio-Rad), and eluted as before. This procedure yielded approximately 15 mg of Pho4, which appeared as a single band in SDS-polyacrylamide gel electrophoresis with Coomassie blue staining.

Preparation of S(0.3) extract. Nuclear extract was prepared from EY0255 or EY0579 as described previously (33), with the following modifications. Recombinant lyticase was used for spheroplasting, and lysis was performed with a

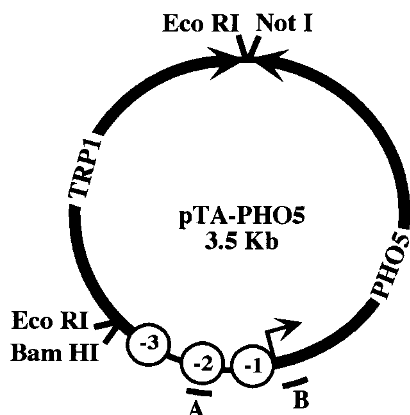

FIG. 2. Map of pTA-PHO5. Bacterial selection and replication sequences were removed, and a 3.5-kb circle containing *PHO5* and the *TRP1/ARS1* locus were religated to form pTA-PHO5. Black arrows, directions of transcription of *PHO5* and *TRP1*; open circles, locations of three positioned nucleosomes on the *PHO5* promoter; bars, sequences from which probes A and B were derived.

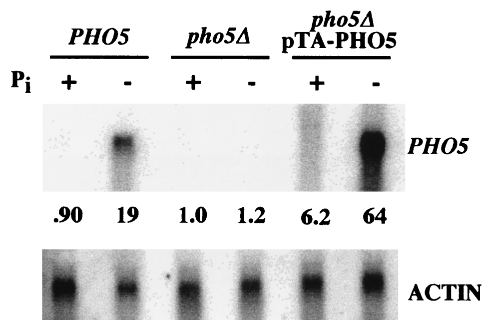

FIG. 3. Northern analysis of chromosomal and episomal *PHO5* expression in response to phosphate starvation. A $PHO5^+$ strain, a *pho5*Δ strain, and a *pho5*Δ strain harboring pTA-PHO5 were grown for 6 h in medium either containing (+) or lacking (−) inorganic phosphate (P_i). *PHO5* transcript levels were quantified and normalized to the actin signal.

hand-held glass Dounce homogenizer, as described above. The final protein pellet was resuspended in S(0.1) (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 1 mM EDTA, 0.1 M K acetate, 1 μg of pepstatin A per ml, 1 mM PMSF). For fractionation, 5 mg of nuclear extract was applied to a 1-ml SP-Sepharose Fast Flow (Pharmacia) column, washed with S(0.1), and step eluted with S(0.3). [S(0.3) is equivalent to S(0.1) except that it contains 0.3 M K acetate.] Fractions containing protein were pooled and concentrated 10-fold with Centricon concentrators.

In vitro chromatin remodeling. Ten microliters of minichromosomes (approximately 10 ng of DNA in 250 mM NaCl–5 mM $MgCl_2$–10 mM PIPES-NaOH [pH 7.3]–0.5 mM EGTA) was incubated in 50-μl reaction mixtures containing 12 mM HEPES-NaOH (pH 7.5), 6 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, and 0.5 mM $CaCl_2$; 2 μg of poly(dC)-poly(dG) per ml and 0.1 mg of bovine serum albumin per ml were also added as nonspecific competitors. After addition of Pho4 (90 nM), Pho2 (approximately 20 nM), and 1 μg of S(0.3), the reaction mixtures were incubated at room temperature for 15 min. An ATP regeneration mix (final concentrations of 0.2 μg of creatine kinase per ml in 10 mM glycine [pH 8], 30 mM creatine phosphate, and 0.5 mM ATP or adenylyl imidi-diphosphate [AMP-PMP] was added, and reaction mixtures were incubated at 30°C for 30 min. The reaction mixtures were split and digested with either 0.1 or 0.05 U of micrococcal nuclease for 5 min at 37°C. Digestion was stopped with 1/10 volume of 5% SDS–250 mM EDTA. DNA was purified by overnight treatment with 200 μg of proteinase K per ml at 37°C, two phenol extractions, chloroform extraction, and ethanol precipitation. Samples were analyzed by Southern blotting as described above and probed as described for in vivo chromatin remodeling.

RESULTS

Episomal *PHO5* is transcribed in response to phosphate starvation. Minichromosomes, or circular plasmids packaged into chromatin, have been purified from *S. cerevisiae* for the analyses of transcription (48), retroviral integration (41), centromere function (27), and chromatin structure (7, 53). We modified a 1.5-kb *TRP1/ARS1* circle by inserting the *PHO5* promoter and open reading frame to form pTA-PHO5 (Fig. 2). The *PHO5* promoter fragment that we inserted includes sequence that is packaged into nucleosomes −1 through −3. This sequence is sufficient for phosphate-regulated transcription of *PHO5* from a plasmid and for appropriate positioning of the three promoter nucleosomes (6, 18).

When yeast cells are grown in medium lacking inorganic phosphate, transcription of *PHO5* is induced (36). We therefore tested if phosphate starvation induces transcription of episomal *PHO5*. A wild-type strain, a *pho5*Δ strain, and a *pho5*Δ strain carrying pTA-PHO5 were grown in medium lacking inorganic phosphate for 6 h. Total RNA was isolated from these cultures, and Northern analysis was performed (Fig. 3). *PHO5* transcript levels were quantified and normalized to actin transcript levels. Transcription of chromosomal *PHO5* increased approximately 20-fold upon starvation for phosphate, whereas transcription from episomal *PHO5* was induced approximately 10-fold. Therefore, chromosomal *PHO5* and episomal *PHO5* were regulated by environmental phosphate levels to approximately the same degree.

pTA-PHO5 was maintained at approximately 20 copies per cell (data not shown). If starvation for phosphate causes induction of every copy of pTA-PHO5 to the same extent as the chromosomal copy, a strain harboring pTA-PHO5 should express 20 times as much transcript as a wild-type strain. However, induced levels of *PHO5* transcript from pTA-PHO5 were 3.4-fold higher than those measured for the chromosomal copy of *PHO5*. A three- to fourfold difference was reproducibly observed, as early as 3 h and as late as 12 h after transfer to medium lacking phosphate. These data suggest that some factor necessary for *PHO5* transcription is limiting under these conditions, and expression of all 20 copies of pTA-PHO5 in each cell is prevented. The limiting factor could be Pho4, Pho2, a putative chromatin-remodeling activity, or components of the general transcription machinery.

Episomal *PHO5* has correctly positioned nucleosomes under repressing conditions. Micrococcal nuclease digestion followed by indirect end labeling is used as an assay for positioned nucleosomes in vivo (34, 61). We used this technique to compare the chromatin structure of chromosomal *PHO5* with that of episomal *PHO5* (Fig. 4). Spheroplasts with an intact chromosomal *PHO5* locus harboring pTA-PHO5 were treated with micrococcal nuclease, and indirect end labeling was performed. Analysis of chromosomal *PHO5* revealed four positioned nucleosomes (Fig. 4A, lane 3), which correspond to those mapped previously (1). Three similarly positioned nucleosomes were detected on the *PHO5* promoter on pTA-PHO5 in vivo (compare lanes 3 and 7). The sequence upstream of nucleosome −3 on pTA-PHO5 (starting at the *Bam*HI site) is the start of the *TRP1* gene, which had a noticeably different pattern than the corresponding region of the chromosomal *PHO5* gene.

There is a detectable difference between the chromatin structures of chromosomal and episomal copies of *PHO5*. The nuclease-hypersensitive site (HS2), visible on the chromosomal copy of *PHO5*, is not apparent on pTA-PHO5 (Fig. 4A). This may be explained by the observation that nucleosome −3 appeared to be slightly shifted in position towards nucleosome −2. However, nucleosomes −1 and −2 (incorporating the TATA box and UASp2) appeared to be correctly positioned, thereby reproducing the appropriate repressed state. Micro-

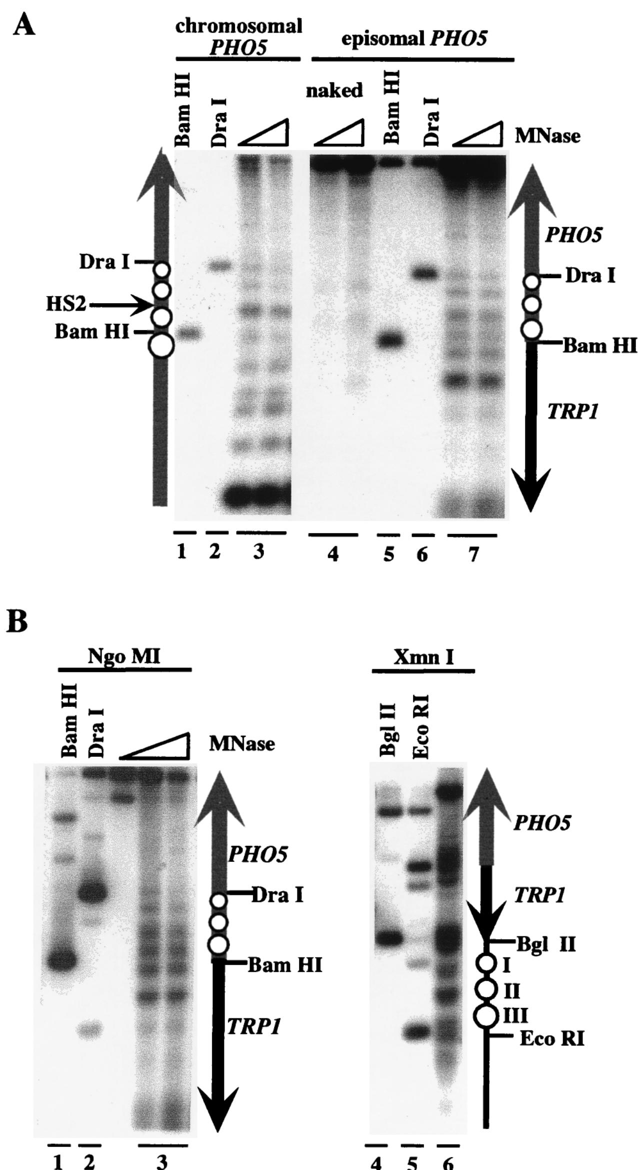

FIG. 4. Nucleosomes on the *PHO5* promoter have similar positions on the chromosome and on pTA-PHO5 and are not changed upon *PHO5* minichromosome preparation. (A) Spheroplasts were treated with micrococcal nuclease (MNase), and the DNA was purified. For size standards, untreated DNA was digested with *Bam*HI (lanes 1 and 5) or *Dra*I (lanes 2 and 6). For analysis of chromosomal *PHO5*, samples were digested with *Stu*I, Southern blotted, and hybridized to a probe derived from a *Stu*I-to-*Apa*I fragment of the *PHO5* upstream region. For analysis of episomal *PHO5*, samples were digested with *Ngo*MI, and the probe used was derived from an *Ngo*MI-to-*Bgl*II fragment from *TRP1*. (B) Partially purified *PHO5* minichromosomes were treated with MNase, and the DNA was purified. Samples digested with *Ngo*MI were probed with an *Ngo*MI-to-*Bgl*II fragment from *TRP1*; samples digested with *Xmn*I were probed with an *Xmn*I-to-*Sca*I fragment from *PHO5*. The schematics show inferred locations of nucleosomes (open circles) on *PHO5* and pTA-PHO5. The grey and black arrows represent *PHO5* and *TRP1* sequences, respectively. The location of the hypersensitive site HS2 on chromosomal *PHO5* is indicated with an arrow.

coccal nuclease digestion followed by indirect end labeling thus indicates that the *PHO5* promoter on pTA-PHO5 is incorporated into positioned nucleosomes with positioning that is very similar to that observed on chromosomal *PHO5*.

pTA-PHO5 is remodeled in vivo. To test if the promoter chromatin of episomal *PHO5* could be remodeled in vivo, we analyzed changes in chromatin structure by digestion with micrococcal nuclease followed by Southern blotting. This assay has been previously employed for analysis of the *PHO5* chromatin transition (1, 44, 50). To analyze changes in chromatin structure at the *PHO5* promoter, we used a probe derived from the *PHO5* sequence packaged into nucleosome -2 (Fig. 2, probe A). A pattern of nucleosomal bands implies that *PHO5* promoter sequence complementary to probe A is packaged into nucleosome -2 and is thereby protected from digestion. The disappearance of these bands implies that nucleosome -2 no longer protects the underlying DNA.

For this experiment, we compared the chromatin structures of the *PHO5* promoter on pTA-PHO5-ATGΔ in a *pho4*Δ strain, a *PHO4*$^+$ strain, and a *PHO4*$^+$ strain carrying a high-copy-number plasmid expressing *Pho4* (pRSPHO4). pTA-PHO5-ATGΔ is a derivative of pTA-PHO5 in which the *PHO5* ATG was replaced with a restriction site. This derivative was used to prevent production of Pho5, a secreted acid phosphatase, as high-level *PHO5* expression inhibits cell growth by disrupting the normal function of the secretory pathway (28). All strains lacked chromosomal *PHO5*, and all were also *pho80*Δ, which causes constitutive expression of *PHO5* (36).

In a strain lacking Pho4, the sequence underlying nucleosome -2 produced a pattern of bands, indicating that it is protected from micrococcal nuclease digestion (Fig. 5A, sample 1). In a *PHO4*$^+$ strain, minimal remodeling of the episomal *PHO5* promoter was observed (sample 2). This is consistent with the results of Northern analysis (Fig. 3) and supports the hypothesis that a factor required for *PHO5* expression is limiting in the cell, allowing only a subset of the pTA-PHO5 templates to be transcribed. To test if a limiting factor was Pho4, we assayed in vivo remodeling of pTA-PHO5-ATGΔ in a *PHO4*$^+$ strain carrying the high-copy-number plasmid pRSPHO4. As shown in Fig. 5A, sample 3, remodeling of nucleosome -2 was observed under these conditions. This suggests that the concentration of Pho4 in the nucleus under inducing conditions is insufficient to support chromatin remodeling and activation of transcription of the majority of the copies of episomal *PHO5*. It should be noted that in the strain carrying pRSPHO4, the pTA-PHO5-ATGΔ copy number drops to approximately five. Thus, the drop in template number may also allow remodeling of a higher proportion of the templates in each cell.

To test if this remodeling was localized to the promoter region of episomal *PHO5*, the blot was stripped and reprobed with a probe derived from nucleosome $+1$ (Fig. 2, probe B). This sequence is mostly nucleosomal, even under conditions that allowed remodeling of nucleosome -2 (compare Fig. 5A and B). The small amount of in vivo remodeling that is apparent at nucleosome $+1$ was also observed at another nucleosome in the *PHO5* open reading frame, as well as at a nucleosome in the *TRP1* gene (data not shown). Thus, episomal *PHO5* chromatin is remodeled in vivo, when high enough levels of Pho4 are present, and this remodeling is predominantly restricted to the promoter region. For the purposes of this report, an increase in micrococcal nuclease sensitivity at nucleosome -2 defines chromatin remodeling in our in vitro system (see Discussion).

Preparation of *PHO5* minichromosomes with intact chromatin structure. With evidence that the *PHO5* promoter on pTA-PHO5 has correctly positioned nucleosomes, that its transcription is regulated by environmental phosphate levels, and that it can be remodeled in vivo, we developed a procedure to prepare *PHO5* minichromosomes for in vitro study. Our protocol was based on the first steps of a purification procedure developed by Simpson and colleagues (13, 42). Our goals were to re-

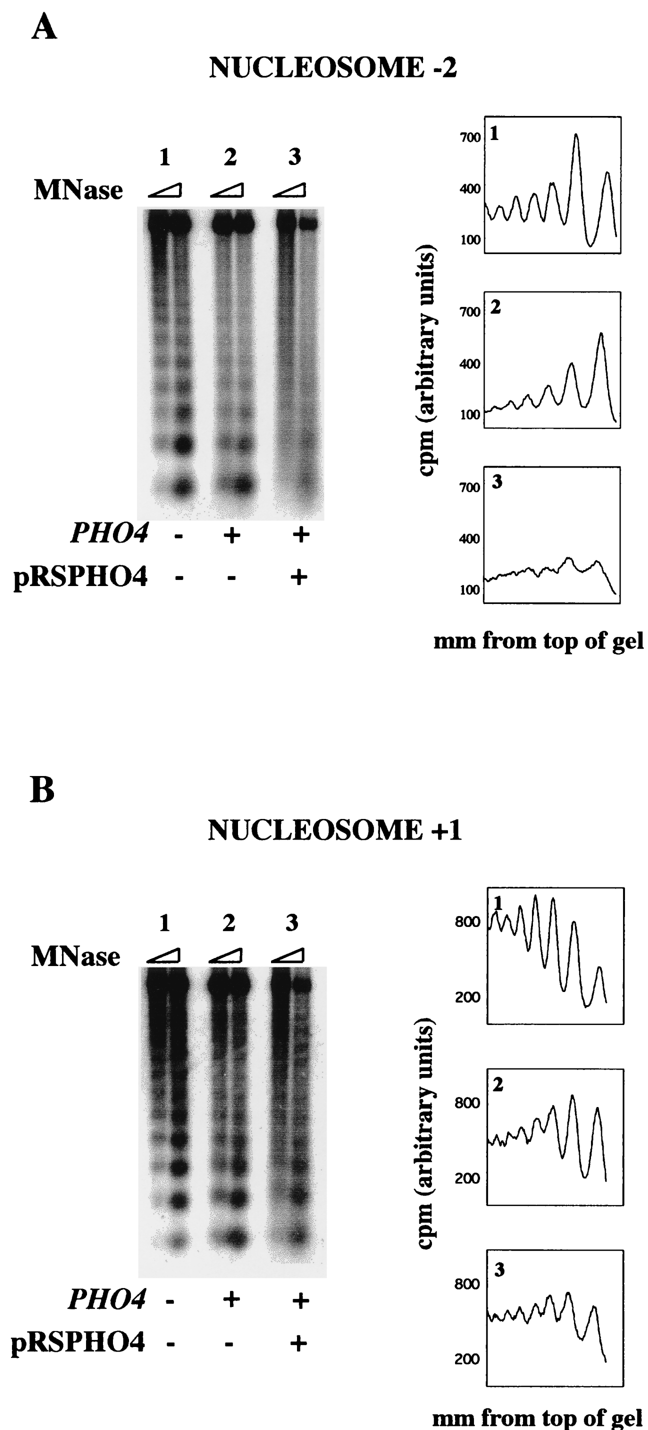

FIG. 5. In vivo chromatin remodeling of episomal *PHO5*. Spheroplasts from the indicated strains were treated with micrococcal nuclease (MNase), and the DNA was purified and Southern blotted. (A) The blot was probed with probe A (Fig. 2). Data from each sample were quantified, and the distance from the top of the gel was graphed against the signal density. (B) The blot shown in panel A was stripped and reprobed with probe B (Fig. 2).

move genomic DNA and cellular debris in a manner gentle enough to leave minichromosomal chromatin intact.

PHO5 minichromosomes were prepared from a *pho2*Δ *pho4*Δ strain to prevent contamination of the chromatin template with the Pho4 and Pho2 transcription factors. Cells harboring pTA-PHO5 were spheroplasted with lyticase (46), and lysis was performed with a hand-held Dounce homogenizer. Nuclei were purified away from cell debris and other organelles by spinning through a glycerol cushion, and unlysed spheroplasts and whole cells were removed by differential centrifugation. Minichromosomes were eluted from the purified nuclei, presumably by diffusing through fissures in the nuclear envelope created by flash freezing. The resulting eluate was further purified on a linear sucrose gradient, and fractions containing minichromosomes were pooled and concentrated.

We tested if the chromatin structure of *PHO5* minichromosomes changed during their preparation by digesting *PHO5* minichromosomes with micrococcal nuclease in vitro and then analyzing nucleosome positioning by indirect end labeling. As shown in Fig. 4B, lane 3, the digestion pattern observed with *PHO5* minichromosomes was unchanged from that observed on pTA-PHO5 in vivo.

Three positioned nucleosomes (named I, II, and III) are positioned on the *TRP1/ARS1* circle, both in vivo (63) and after purification (53). We therefore tested if positioned nucleosomes are present at these positions on purified *PHO5* minichromosomes. As shown in Fig. 4B, lane 6, three appropriately positioned nucleosomes were detected on the *TRP1/ARS1* sequence. These data indicate that partially purified *PHO5* minichromosomes contain correctly positioned nucleosomes, both over the *PHO5* promoter and on the *TRP1/ARS1* sequence, and are therefore appropriate chromatin templates for biochemical analysis of *PHO5* chromatin remodeling.

In vitro remodeling of *PHO5* minichromosomes requires Pho4 and Pho2, hydrolyzable ATP, and a fraction of nuclear extract. The scheme of our in vitro chromatin-remodeling experiments is outlined in Fig. 6A. *PHO5* minichromosomes were mixed with transcription factors and nuclear extract in a reaction mixture. A source of energy was then added, and the remodeling reaction was allowed to proceed. Reaction mixtures were split, digested with micrococcal nuclease, transferred to nylon, and probed with sequence corresponding to nucleosome −2 as for analysis of in vivo remodeling.

The chromatin transition and transcriptional activation of *PHO5* in vivo require the transcription factors Pho4 and Pho2 (17). We therefore tested if these transcription factors are sufficient to support in vitro chromatin remodeling of *PHO5* minichromosomes. As shown in Fig. 6B, sample 1, recombinant Pho4 and Pho2 were not sufficient for remodeling of nucleosome −2 in vitro.

The inability of Pho2 and Pho4 to remodel chromatin in vitro suggested that remodeling of *PHO5* promoter chromatin requires an additional activity. We therefore tested if a fraction of *S. cerevisiae* nuclear extract, termed S(0.3), could provide a chromatin-remodeling activity. Addition of S(0.3) had no effect in our assay (Fig. 6B, sample 2). By analogy with previously identified chromatin-modifying complexes, such an activity might require either ATP or acetyl coenzyme A. When we tested this possibility by incubating Pho4 and Pho2, S(0.3), and an ATP regeneration system with *PHO5* minichromosomes, chromatin remodeling was observed (sample 4). Under these conditions, chromatin remodeling was also observed when a probe derived from nucleosome −3 or a probe derived from all three positioned nucleosomes (−1 through −3) was used (data not shown). These data indicate that the *PHO5* promoter nucleosomes are remodeled in our in vitro system.

To test if ATP hydrolysis is required for S(0.3)- and Pho4-dependent remodeling of *PHO5* minichromosome promoter chromatin, a nonhydrolyzable ATP analog was included in the regeneration system in place of ATP (Fig. 7). Whereas remod-

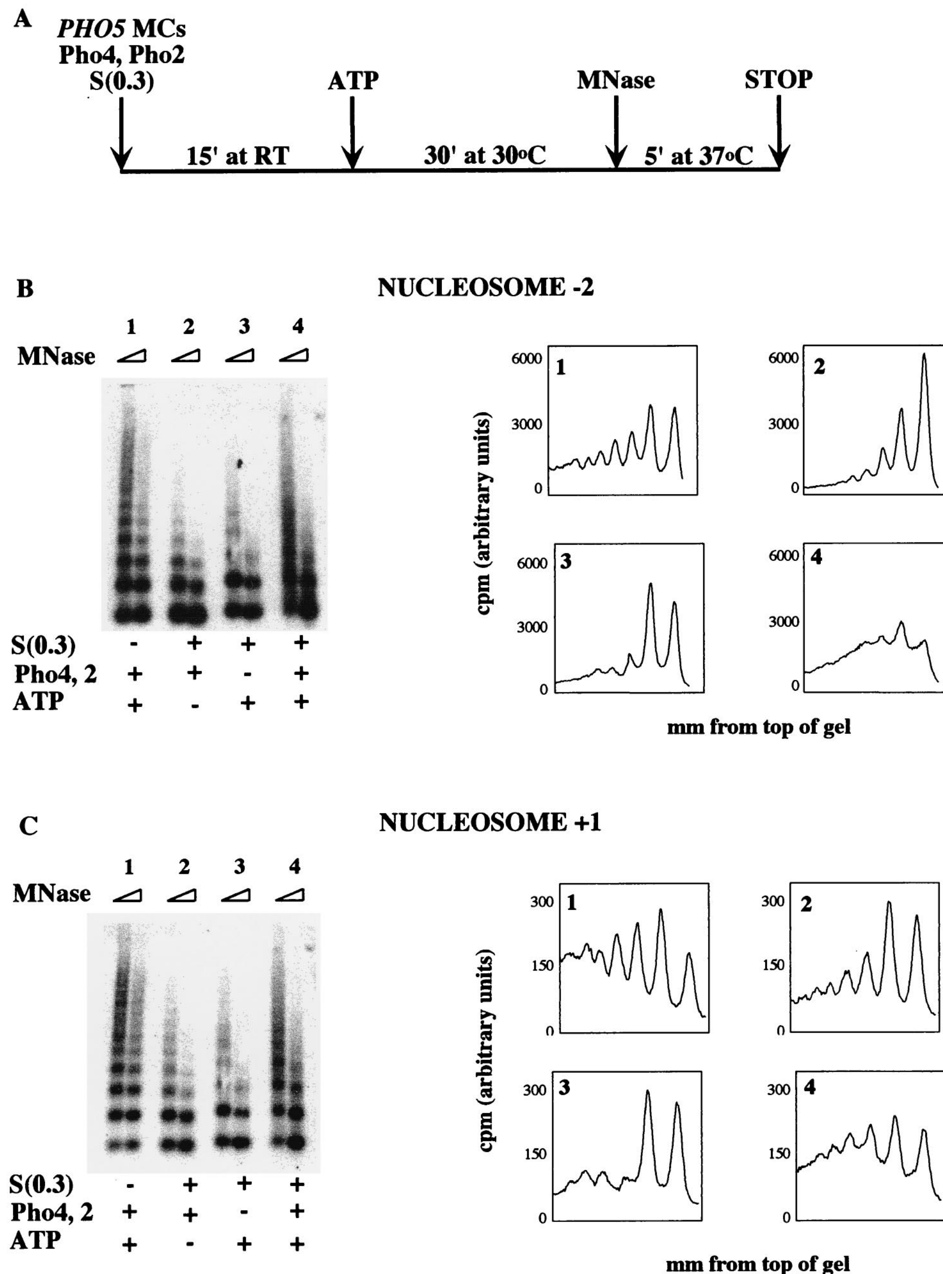

FIG. 6. In vitro chromatin remodeling of *PHO5* minichromosomes requires Pho2, Pho4, S(0.3), and ATP and is localized predominantly to the *PHO5* promoter. (A) Schematic of the in vitro remodeling reaction. MC, minichromosome; MNase, micrococcal nuclease. (B) Reaction mixtures were assembled and incubated as shown in panel A and then split and digested with micrococcal nuclease. Samples were purified, electrophoresed, and transferred to nylon. Southern blotting was performed with probe A, and data were graphed as in Fig. 5. (C) The blot shown in panel B was stripped and reprobed with probe B.

eling is observed when ATP is added (sample 2), there is no change in the *PHO5* promoter chromatin structure when ATP is omitted (sample 1) or when an equivalent amount of AMP-PMP is substituted (sample 3). Samples containing acetyl coenzyme A as an energy source showed no remodeling under these conditions (data not shown).

Thus, the transcription factors Pho2 and Pho4, S(0.3), and ATP hydrolysis were all necessary for in vitro chromatin remodeling of nucleosome −2. No remodeling was observed if any of these three components were withheld. These data imply that Pho2, Pho4, and an ATP-dependent activity can remodel *PHO5* promoter chromatin.

S(0.3)-, ATP-, and Pho4-dependent chromatin remodeling is restricted to the *PHO5* promoter region. In vivo, the loss of positioned nucleosomes in response to phosphate starvation is restricted to the four positioned nucleosomes on the *PHO5* promoter (1). We tested if this was true of the remodeling observed in vitro by stripping and reprobing the Southern blot shown in Fig. 6B with a sequence underlying nucleosome +1. When analyzed with this probe, all samples produced a largely

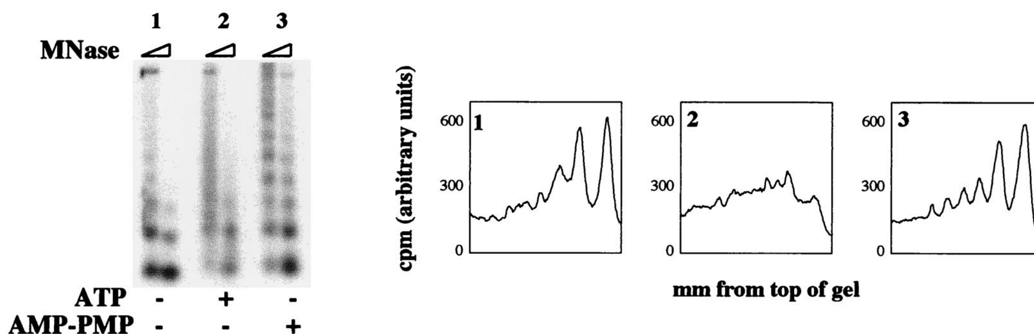

FIG. 7. Hydrolyzable ATP is required for in vitro chromatin remodeling of *PHO5* minichromosomes. Either buffer (sample 1), ATP (sample 2), or AMP-PMP (sample 3) was included in chromatin-remodeling reactions with *PHO5* minichromosomes, Pho4 and Pho2, and S(0.3). Samples were analyzed as described for Fig. 6. MNase, micrococcal nuclease.

nucleosomal pattern (Fig. 6C). Thus, the dramatic change in chromatin structure observed in the presence of Pho4 and Pho2, S(0.3), and ATP at the *PHO5* promoter does not extend significantly into the *PHO5* open reading frame.

Pho4 can partially remodel *PHO5* chromatin in the absence of Pho2. Overexpression of Pho4 can partially suppress the *PHO5* expression defect of a *pho2*Δ strain (17). We therefore tested if Pho4 was capable of supporting chromatin remodeling of *PHO5* minichromosomes in vitro without Pho2. As indicated in Fig. 8, Pho4 is capable of supporting partial remodeling of *PHO5* minichromosomes in the presence of S(0.3) and ATP without Pho2 (sample 2). However, when Pho2 is included in the remodeling reaction mixture and Pho4 is left out, no remodeling is observed (sample 1). These results demonstrate that the transcription factor Pho4 is required for S(0.3)- and ATP-dependent chromatin remodeling of *PHO5* minichromosomes.

Pho4-dependent chromatin remodeling without Pho2 is incomplete, and a nucleosomal pattern is visible (Fig. 8, sample 1). When Pho2 is included in the reaction with Pho4, S(0.3), and ATP, remodeling is more complete (Fig. 6, sample 4). Analyses of the DNA binding and in vivo transcriptional activation properties of a version of Pho4 unable to interact with Pho2 suggest that Pho2 may affect the function of Pho4 in two ways: by modulating its ability to bind DNA and by enhancing its ability to activate transcription (4). By analogy, Pho2 may facilitate chromatin remodeling in our in vitro system by enhancing the binding of Pho4 to *PHO5* minichromosomes or by enhancing the ability of Pho4 to support chromatin remodeling in a manner independent of DNA binding. When higher concentrations of Pho4 were used, remodeling was complete in the absence of Pho2 (data not shown); however, the relevance of remodeling at these concentrations is not clear. Importantly, at the Pho4 concentrations used in this study, Pho2 is required for complete remodeling of *PHO5* minichromosomes in vitro, consistent with its role in vivo.

Pho4-dependent chromatin remodeling requires UASp1 and UASp2. Deletion of a 26-bp region encompassing the Pho4 binding site in UASp1 has no effect on the assembly of repressive *PHO5* chromatin structure, but it prevents Pho4 binding (57) and the chromatin transition (18) in vivo. These observations indicate that binding of Pho4 to the hypersensitive region is not required for nucleosome positioning yet is required for changes in chromatin structure upon induction. We wished to test if the Pho4-dependent remodeling observed in the absence of Pho2 requires specific binding by Pho4 to UASp1 and UASp2. A version of pTA-PHO5, pTA-p1p2, in which the two Pho4 binding sites in UASp1 and UASp2 were replaced precisely with restriction sites, was constructed. Wild-type and p1p2 minichromosomes were prepared in parallel and tested for remodeling in the in vitro system. Whereas the *PHO5* promoter of wild-type minichromosomes was partially remodeled

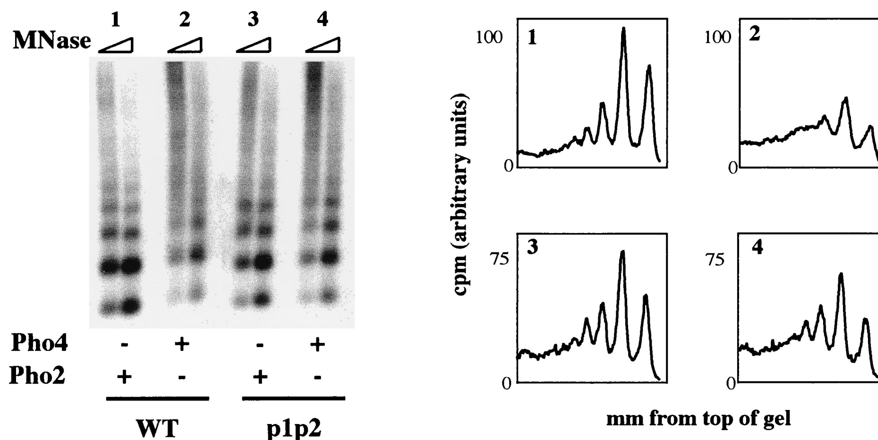

FIG. 8. In vitro chromatin remodeling of *PHO5* minichromosomes in the absence of Pho2 requires Pho4, UASp1, and UASp2. Wild-type (WT) *PHO5* minichromosomes were tested for chromatin remodeling in the presence of either Pho2 (sample 1) or Pho4 (sample 2). Minichromosomes lacking the Pho4 binding sites at UASp1 and UASp2 (p1p2) were purified and tested in parallel (samples 3 and 4). S(0.3) and ATP were included in all reaction mixtures. MNase, micrococcal nuclease.

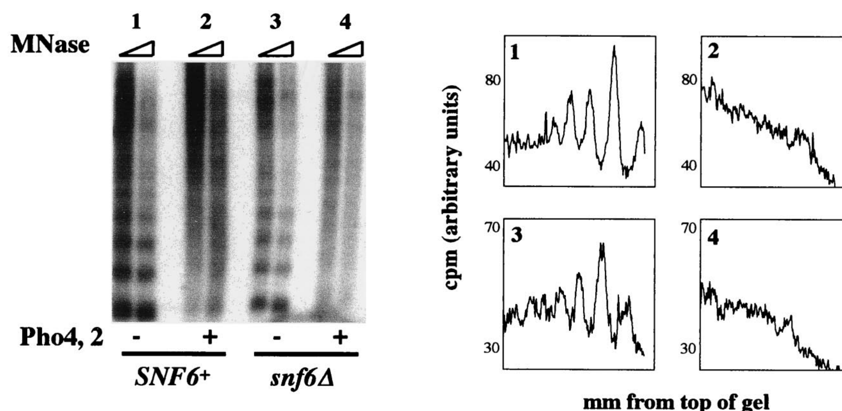

FIG. 9. Swi-Snf is not required for in vitro chromatin remodeling of *PHO5* minichromosomes. S(0.3) extract and minichromosomes were purified from a *SNF6*$^+$ (samples 1 and 2) or *snf6*Δ (samples 3 and 4) strain and assayed for chromatin remodeling in the presence and absence of Pho2 and Pho4. An ATP regeneration mix was added to all samples. MNase, micrococcal nuclease.

in the presence of Pho4 (Fig. 8, sample 2), no Pho4-dependent remodeling of p1p2 minichromosomes was observed (Fig. 8, sample 4). This result demonstrates that one or both of the Pho4 binding sites in UASp1 and UASp2 are required for Pho4-dependent chromatin remodeling of *PHO5* minichromosomes in vitro.

Unexpectedly, p1p2 minichromosomes were remodeled when Pho2 was included in the remodeling reaction with Pho4, S(0.3), and ATP (data not shown). Yeast strains harboring pTA-p1p2 express low levels of acid phosphatase activity upon phosphate starvation (data not shown), suggesting that the deletion of UASp1 and UASp2 is not sufficient to prevent transcription of episomal *PHO5*. It is possible that binding of Pho2 to its site in UASp1 (which is intact in the p1p2 minichromosomes) can stabilize Pho4 DNA binding. In contrast, the 26-bp deletion analyzed in previous studies partially destroys this Pho2 binding site, which may account for the completely unremodeled state of this mutant promoter in vivo.

***PHO5* chromatin remodeling in vitro does not require the Swi-Snf complex.** We reasoned that the factor(s) supplied by the S(0.3) extract might have ATPase activity, since ATP is required for in vitro remodeling. The yeast remodeling factor Swi-Snf contains an ATPase, which is required for its function (30). It was therefore possible that the putative ATP-dependent activity in S(0.3) was the Swi-Snf complex. As Swi-Snf is not required for *PHO5* chromatin remodeling in vivo (10, 20, 45), it was of interest to determine if *PHO5* minichromosome remodeling required this complex in vitro.

The Snf6 protein is a component of the Swi-Snf complex (9, 38). We tested the requirement for Swi-Snf in our in vitro system by preparing in parallel S(0.3) extract and *PHO5* minichromosomes from *SNF6*$^+$ and *snf6*Δ strains. In vitro remodeling with components derived from a *snf6*Δ strain occurred to the same extent as with those derived from *SNF6*$^+$ cells (Fig. 9, compare samples 2 and 4), demonstrating that *SNF6* is not required for remodeling in vitro. We can therefore infer that the 2-MDa Swi-Snf complex, which requires Snf6 for its structural integrity (38), is not required for *PHO5* chromatin remodeling in vitro.

DISCUSSION

We have developed an in vitro system in which *PHO5* minichromosomes undergo promoter chromatin remodeling. To obtain chromatin templates with positioned nucleosomes, an important feature of the repressed *PHO5* promoter in vivo, we purified minichromosomes carrying the *PHO5* gene from yeast. Chromatin remodeling in our system required the presence of the transcriptional activators Pho2 and Pho4, a fraction of *S. cerevisiae* nuclear extract, and hydrolyzable ATP.

In vitro chromatin remodeling of *PHO5* minichromosomes was localized to the *PHO5* promoter and did not require the Swi-Snf complex. Specific binding by Pho4 to UASp1 and UASp2 was required for Pho4-dependent chromatin remodeling. As these are also characteristics of the *PHO5* chromatin transition in vivo, we believe that this system will allow the identification of physiologically relevant chromatin-remodeling activities.

Choice of chromatin template. The chromatin structures of the *PHO5* promoter under repressing and inducing conditions have been characterized (51). In high-phosphate medium, four nucleosomes are positioned on the *PHO5* promoter such that UASp1 is in a hypersensitive region between nucleosomes −2 and −3, UASp2 is packaged into nucleosome −2, and the TATA box is packaged into nucleosome −1. These four nucleosomes lose their positioning upon *PHO5* induction and no longer protect the promoter from nuclease digestion (Fig. 1).

Many studies suggest that the stability and placement of positioned nucleosomes on the *PHO5* promoter are important for regulation of *PHO5* expression. In vivo depletion of histone H4 causes the disappearance of positioned nucleosomes from the *PHO5* promoter and weak expression under repressing conditions (26). UASp1 and UASp2 are not required for this effect. These data suggest that the presence of nucleosome −1, which packages promoter sequence including the TATA box, is required for appropriate repression of basal *PHO5* expression. Another experiment demonstrated that a Pho4 mutant lacking the activation domain binds UASp2 when it is in the hypersensitive site but not when it is packaged into nucleosome −2 (52). This implies that nucleosome −2 presents a barrier to Pho4 binding to UASp2 under repressing conditions. Both of these experiments suggest that the packaging of UASp2 and the TATA box into nucleosomes is an important characteristic of the repressed *PHO5* promoter.

For the reasons described above, we sought templates that have appropriately positioned nucleosomes for our in vitro study of *PHO5* remodeling. Reconstitution of *PHO5* promoter chromatin with purified histones and *Drosophila* embryo extracts did not produce templates with positioned nucleosomes (data not shown). We therefore chose to purify chromatin

templates from yeast cells. By using this strategy, we ensured that *PHO5* promoter chromatin would be assembled with native histones, appropriately acetylated or otherwise modified. Additionally, any nonhistone proteins required for nucleosome positioning would be present during chromatin assembly.

Definition of chromatin remodeling. To describe and characterize our in vitro system, we have defined chromatin remodeling of *PHO5* minichromosomes as an increase in micrococcal nuclease sensitivity at the DNA sequence packaged into nucleosome −2. Samples probed with sequence from nucleosome −2 (Fig. 6B) showed the same loss of nucleosomal bands as when they were probed with sequence from nucleosome −3 or sequence from all three nucleosomes (data not shown). In contrast, samples probed with sequence from nucleosome +1 (Fig. 6C) or +5 (data not shown) were mostly nucleosomal. Remodeling as defined here thus extends from nucleosome −1 to nucleosome −3, but it does not extend into the *PHO5* open reading frame.

We used two other assays to analyze the change in chromatin structure on *PHO5* minichromosomes after in vitro remodeling: restriction enzyme accessibility and micrococcal nuclease digestion followed by indirect end labeling. The *Cla*I restriction site, located near UASp2 in the *PHO5* promoter, became more accessible in the presence of ATP and S(0.3) but was unaffected by the presence of Pho2 or Pho4. Loss of nucleosome positioning, as detected by the indirect end-labeling technique, was also observed in the presence of ATP and S(0.3) but did not require Pho2 or Pho4. Thus, the changes in chromatin structure that are detected by these assays occur in the absence of sequence-specific DNA binding proteins and may therefore be ascribed to nonspecific remodeling activities provided by the S(0.3). Thus, restriction enzyme accessibility and indirect end labeling were not useful assays for the identification of a *PHO5*-specific chromatin-remodeling activity.

The nature of the alteration in histone-DNA contacts that produces an increase in micrococcal nuclease sensitivity is not known. One interpretation is that the nucleosomes are removed from the promoter upon induction, and as a result the entire promoter becomes accessible to nuclease digestion. Proposed mechanisms for histone removal include nucleosome sliding, transfer of nucleosomes to an acceptor molecule, and disassembly of the histone octamer (49). Alternatively, the *PHO5* promoter may still be packaged into chromatin under inducing conditions but in such a way that the DNA is no longer protected from interaction with nucleases.

Role of Pho4 in *PHO5* chromatin remodeling. The activation domain of Pho4 is required for the *PHO5* chromatin transition in vivo. A version of Pho4 that lacks the activation domain is capable of binding to UASp1, but not to UASp2, under inducing conditions in vivo (52). When this Pho4 mutant is expressed in place of full-length Pho4, the *PHO5* promoter remains packaged into positioned nucleosomes, and there is no expression of *PHO5* under inducing conditions.

One explanation for these observations is that Pho4 directly remodels *PHO5* chromatin, and its activation domain is required for this activity. To date, however, no transcription factor has been shown to be sufficient for chromatin rearrangement in vitro. Furthermore, Pho4 and Pho2 cannot by themselves remodel *PHO5* minichromosomes in our in vitro remodeling system (Fig. 6B, sample 1). It is therefore likely that an activity in addition to these transcription factors is required to change the *PHO5* promoter chromatin structure in vivo.

If Pho4 itself does not remodel chromatin, it may recruit a remodeling factor to the *PHO5* promoter through its activation domain. Artificial recruitment of the RNA polymerase holoenzyme to the *PHO5* promoter causes constitutive *PHO5* expression and prevents the assembly of positioned nucleosomes (20), a tantalizing result given recent evidence that the holoenzyme may associate with Swi-Snf (59) (but see reference 11). Although the *PHO5* chromatin transition does not require Swi-Snf (20), another ATP-dependent chromatin-remodeling complex may be associated with the holoenzyme, or chromatin remodeling may be an function intrinsic to the holoenzyme itself. It is also possible that the activation domain of Pho4 interacts directly with a chromatin-remodeling activity. Substantial evidence supports a model whereby yeast transcriptional regulators recruit histone-acetylating (14, 55) and -deacetylating (23) complexes to the promoters they regulate. Similarly, an ATP-dependent chromatin-remodeling complex may be recruited to the *PHO5* promoter through interaction with the activation domain of Pho4.

In the models described above, Pho4 first binds DNA and then mediates chromatin remodeling in a second step. In contrast, it is possible that chromatin remodeling must occur before Pho4 can bind to its recognition sites in the *PHO5* promoter. According to this model, a nonspecific chromatin-remodeling activity acts constitutively, in an ATP-dependent manner, to make chromatin more accessible. Subsequently, binding of Pho4 and Pho2 to the *PHO5* promoter prevents nucleosome positioning and stabilizes a nuclease-sensitive state. A requirement for the Pho4 activation domain may be explained if it is necessary for stable association with *PHO5* promoter chromatin.

Identity of the remodeling activity in the S(0.3) extract. The activity contained in the S(0.3) extract may be a member of the rapidly growing family of ATP-dependent chromatin-remodeling machines, each of which contains a member of the Snf2 family of helicase-like ATPases (15). These complexes are capable of altering the DNase I digestion pattern of a mononucleosome, facilitating factor binding to sites within nucleosomes, and potentiating activation of transcription from chromatin templates (8). Members of this family thus appear to have in common the ability to modify histone-DNA contacts in an ATP-dependent manner.

Two remodeling complexes in this family have been defined in yeast: Swi-Snf and RSC (remodels the structure of chromatin). Components of Swi-Snf are required for transcription of a small number of regulated genes, and none identified to date are essential (39, 60). It has been established that Swi-Snf is not required for the *PHO5* chromatin transition in vivo (10, 20, 45), and we show here that it is not required for chromatin remodeling in vitro. RSC was identified on the basis of its homology to Swi-Snf and contains several essential subunits (11, 31). It is not known if RSC is required for *PHO5* expression.

Thus, the activity contained in the S(0.3) extract could be RSC or a subcomplex of either Swi-Snf or RSC. In addition to those that are contained in Swi-Snf and RSC, there are several other members of the Snf2 family in yeast, and these may be components of novel chromatin-remodeling machines that function in a similar manner. The S(0.3) extract might contain one of these putative activities or a completely novel type of ATP-dependent chromatin-remodeling activity, unrelated to Swi-Snf or RSC. Identification of this activity through conventional fractionation and reconstitution experiments is under way.

Reconstitution of the *PHO5* chromatin transition. We describe here the reconstitution of *PHO5* chromatin remodeling in vitro. The *PHO5* chromatin transition was studied in *S. cerevisiae*, a model organism that is amenable to both genetic and biochemical experiments. This allowed us to compare the in vivo *PHO5* chromatin transition with our in vitro remodeling

reaction, and it provides a way to confirm the physiological relevance of future in vitro results.

We used reagents that resembled their in vivo counterparts to assemble our in vitro chromatin-remodeling system. The template contained relevant sequences for *PHO5* expression and had appropriately positioned nucleosomes, the transcription factors used were those required in vivo for *PHO5* expression, and the source of remodeling activity was an *S. cerevisiae* extract. We used an in vitro assay to detect changes in minichromosomal chromatin structure that occur in vivo at the *PHO5* locus upon induction.

In vitro chromatin remodeling of *PHO5* minichromosomes recapitulates many hallmarks of the *PHO5* chromatin transition in vivo. In addition, we show that the transcription factors Pho2 and Pho4 are not sufficient to change the *PHO5* chromatin structure and that an additional ATP-dependent activity is required. This extends our knowledge of the *PHO5* chromatin transition and illustrates the power of our in vitro approach. We anticipate that our system will prove to be a useful tool to provide insight into the mechanism of the *PHO5* chromatin transition. We hope to use our in vitro system to identify the components required for *PHO5* chromatin remodeling and to characterize the alteration of histone-DNA contacts that occurs during the *PHO5* chromatin transition.

ACKNOWLEDGMENTS

We thank Mary Maxon for Pho2, Wolfram Horz for yeast strain YS18, Yasuji Oshima for pACD5 and pACD4, and Marian Carlson for pEY110. We also acknowledge Sandy Johnson, Keith Yamamoto, Joachim Li, Mary Maxon, and members of the O'Shea lab for insightful comments on the manuscript and Beverly Emerson for a particularly useful suggestion.

This work was supported by NIH grant GM51377 to E.K.O. and by an NSF Predoctoral Fellowship to E.S.H. E.K.O. is a Packard Foundation Fellow and an NSF Presidential Faculty Fellow.

REFERENCES

1. **Almer, A., H. Rudolph, A. Hinnen, and W. Horz.** 1986. Removal of positioned nucleosomes from the yeast *PHO5* promoter upon *PHO5* induction releases additional upstream activating DNA elements. EMBO J. **5:**2689–2696.
2. **Archer, T. K., P. Lefebre, R. G. Wolford, and G. L. Hager.** 1992. Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation. Science **255:**1573–1576.
3. **Armstrong, J. A., and B. M. Emerson.** 1998. Transcription of chromatin: these are complex times. Curr. Opin. Genet. Dev. **8:**165–172.
4. **Barbaric, S., M. Munsterkotter, C. Goding, and W. Horz.** 1998. Cooperative Pho2-Pho4 interactions at the *PHO5* promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. Mol. Cell. Biol. **18:**2629–2639.
5. **Barbaric, S., M. Munsterkotter, J. Svaren, and W. Horz.** 1996. The homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast *PHO5* promoter. Nucleic Acids Res. **24:**4479–4486.
6. **Bergman, L. W., and R. A. Kramer.** 1983. Modulation of chromatin structure associated with derepression of the acid phosphatase gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **258:**7223–7227.
7. **Bergman, L. W., M. C. Stranathan, and L. A. Preis.** 1986. Structure of the transcriptionally repressed phosphate-repressible acid phosphatase gene (*PHO5*) of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**38–46.
8. **Cairns, B. R.** 1998. Chromatin remodeling machines: similar motors, ulterior motives. Trends Biochem. Sci. **23:**20–25.
9. **Cairns, B. R., Y. J. Kim, M. H. Sayre, and B. C. Laurent.** 1994. A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. Proc. Natl. Acad. Sci. USA **91:**1950–1954.
10. **Cairns, B. R., R. S. Levinson, K. R. Yamamoto, and R. D. Kornberg.** 1996. Essential role of Swp73p in the function of yeast Swi/Snf complex. Genes Dev. **10:**2131–2144.
11. **Cairns, B. R., Y. Lorch, Y. Li, M. Zhang, L. Lacomis, H. Erdument-Bromage, P. Tempst, J. Du, B. Laurent, and R. Kornberg.** 1996. RSC, an essential, abundant chromatin-remodeling complex. Cell **87:**1249–1260.
12. **Cox, J. S., C. E. Shamu, and P. Walter.** 1993. Transcriptional induction of genes encoding endoplasmic reticulum resident protein requires a transmembrane protein kinase. Cell **73:**1197–1206.
13. **Dean, A., D. S. Pederson, and R. T. Simpson.** 1989. Isolation of yeast plasmid chromatin. Methods Enzymol. **170:**26–41.
14. **Drysdale, C. M., B. M. Jackson, R. McVeigh, E. R. Klebanow, Y. Bai, T. Kokubo, M. Swanson, Y. Nakatani, P. A. Weil, and A. G. Hinnebusch.** 1998. The Gcn4p activation domain interacts specifically in vitro with RNA polymerase II holoenzyme, TFIID, and the Adap-Gcn5p coactivator complex. Mol. Cell Biol. **18:**1711–1724.
15. **Eisen, J. A., K. S. Sweder, and P. C. Hanawalt.** 1995. Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Res. **23:**2715–2723.
16. **Estruch, F., and M. Carlson.** 1990. *SNF6* encodes a nuclear protein that is required for expression of many genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2544–2553.
17. **Fascher, K.-D., J. Schmitz, and W. Horz.** 1990. Role of *trans*-activating proteins in the generation of active chromatin at the *PHO5* promoter in *S. cerevisiae*. EMBO J. **9:**2523–2528.
18. **Fascher, K.-D., J. Schmitz, and W. Horz.** 1993. Structural and functional requirements for the chromatin transition at the *PHO5* promoter in *Saccharomyces cerevisiae* upon *PHO5* activation. J. Mol. Biol. **231:**658–667.
19. **Fisher, F., P.-S. Jayaraman, and C. R. Goding.** 1991. c-Myc and the yeast transcription factor Pho4 share a common CACGTG-binding motif. Oncogene **6:**1099–1104.
20. **Gaudreau, L., A. Schmid, D. Blaschke, M. Ptashne, and W. Horz.** 1997. RNA polymerase holoenzyme recruitment is sufficient to remodel chromatin at the yeast *PHO5* promoter. Cell **89:**55–62.
21. **Gregory, P. D., A. Schmid, M. Zavari, L. Lui, S. L. Berger, and W. Horz.** 1998. Absence of Gcn5 HAT activity defines a novel state in the opening of chromatin at the *PHO5* promoter in yeast. Mol. Cell **1:**495–505.
22. **Ito, T., M. Bulger, M. J. Pazin, R. Kobayashi, and J. T. Kadonaga.** 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell **90:**145–155.
23. **Kadosh, D., and K. Struhl.** 1997. Repression by Ume6 involves recruitment of a complex containing corepressor and Rpd3 histone deacetylase to target promoters. Cell **89:**365–371.
24. **Kaffman, A., I. Herskowitz, R. Tjian, and E. K. O'Shea.** 1994. Phosphorylation of the transcription factor Pho4 by a cyclin-CDK complex, Pho80-Pho85. Science **263:**1153–1156.
25. **Kaffman, A., N. M. Rank, and E. K. O'Shea.** 1998. Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev. **12:**2673–2683.
26. **Kim, U.-J., P. Kayne, and M. Grunstein.** 1988. Depletion of histone H4 and nucleosomes activates the *PHO5* gene in *Saccharomyces cerevisiae*. EMBO J. **7:**2221–2228.
27. **Kingsbury, J., and D. Koshland.** 1993. Centromere function on minichromosomes isolated from budding yeast. Mol. Biol. Cell **4:**859–870.
28. **Kleene, R., M. Janes, B. Meyhack, K. Pulfer, and A. Hinnen.** 1995. High-level expression of endogenous acid phosphatase inhibits growth and vectorial secretion in *Saccharomyces cerevisiae*. J. Cell. Biochem. **57:**238–250.
29. **Kornberg, R. D., and Y. Lorch.** 1992. Chromatin structure and transcription. Annu. Rev. Cell Biol. **8:**563–887.
30. **Laurent, B. C., I. Treich, and M. Carlson.** 1993. The yeast SNF2/SWI2-protein has DNA-stimulated ATPase activity required for transcriptional activation. Genes Dev. **7:**583–591.
31. **Laurent, B. C., X. Yang, and M. Carlson.** 1992. An essential *Saccharomyces cerevisiae* gene homologous to *SNF2* encodes a helicase-related protein in a new family. Mol. Cell. Biol. **12:**1893–1902.
32. **Lenburg, M. E., and E. K. O'Shea.** 1996. Signaling phosphate starvation. Trends Biochem. Sci. **21:**383–387.
33. **Lue, N. F., P. M. Flanagen, R. J. Kelleher, A. M. Edwards, and R. D. Kornberg.** 1991. RNA polymerase II transcription *in vitro*. Methods Enzymol. **194:**545–550.
34. **Nedospasov, S. A., A. N. Shakhov, and G. P. Georgiev.** 1989. Analysis of nucleosome positioning by indirect end-labeling and molecular cloning. Methods Enzymol. **170:**408–420.
35. **Orphanides, G., G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg.** 1988. FACT, a factor that facilitates transcript elongation through nucleosomes. Cell **92:**105–116.
36. **Oshima, Y.** 1982. Regulatory circuits for gene expression: the metabolism of galactose and phosphate, p. 159–180. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Paranjape, S. M., R. T. Kamakaka, and J. T. Kadonaga.** 1994. Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63:**265–297.
38. **Peterson, C. L., A. Dingwall, and M. P. Scott.** 1994. Five *SWI/SNF* gene products are components of a large multisubunit complex required for transcriptional enhancement. Proc. Natl. Acad. Sci. USA **91:**2905–2908.
39. **Peterson, C. L., and J. W. Tamkun.** 1995. The SWI-SNF complex: a chromatin remodeling machine. Trends Biochem. Sci. **20:**143–146.

40. **Pollard, K. J., and C. L. Peterson.** 1997. Role of *ADA/GCN5* products in antagonizing chromatin-mediated transcriptional repression. Mol. Cell. Biol. **17:**6212–6222.
41. **Pryciak, P. M., A. Sil, and H. E. Varmus.** 1992. Retroviral integration into minichromosomes *in vitro*. EMBO J. **11:**291–303.
42. **Roth, S. Y., and R. T. Simpson.** 1991. Yeast minichromosomes. Methods Cell Biol. **35:**289–314.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Schmid, A., K.-D. Fascher, and W. Horz.** 1992. Nucleosome disruption at the yeast *PHO5* promoter upon *PHO5* induction occurs in the absence of DNA replication. Cell **71:**853–864.
45. **Schneider, K.** 1995. Ph.D. thesis. University of California, San Francisco.
46. **Scott, J. H., and R. Scheckman.** 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. **142:**414–423.
47. **Senstag, C., and A. Hinnen.** 1987. The sequence of the *Saccharomyces cerevisiae* gene *PHO2* codes for a regulatory protein with unusual amino acid composition. Nucleic Acids Res. **15:**233–246.
48. **Silva, J., S. Zinker, and P. Gariglio.** 1987. Isolation and partial characterization of 2-microns yeast plasmid as a transcriptionally active minichromosome. FEBS Lett. **214:**71–74.
49. **Steger, D. J., and J. L. Workman.** 1996. Remodeling chromatin structure for transcription: what happens to the histones? Bioessays **18:**875–884.
50. **Straka, C., and W. Horz.** 1991. A functional role for nucleosomes in the repression of a yeast promoter. EMBO J. **10:**361–368.
51. **Svaren, J., and W. Horz.** 1997. Transcription factors vs. nucleosomes: regulation of the *PHO5* promoter in yeast. Trends Biochem. Sci. **22:**93–97.
52. **Svaren, J., J. Schmitz, and W. Horz.** 1994. The transactivation domain of Pho4 is required for nucleosome disruption at the *PHO5* promoter. EMBO J. **13:**4856–4862.
53. **Thoma, F., L. W. Bergman, and R. T. Simpson.** 1984. Nuclease digestion of circular *TRP1ARS1* chromatin reveals positioned nucleosomes separated by nuclease-sensitive regions. J. Mol. Biol. **177:**715–733.
54. **Tsukiyama, T., and C. Wu.** 1995. Purification and properties of an ATP-dependent nucleosome remodeling factor. Cell **83:**1011–1020.
55. **Utley, F. T., K. Ikeda, P. A. Grant, J. Cote, D. Steger, A. Eberharter, S. John, and J. Workman.** 1998. Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. Nature **394:**498–502.
56. **Varga-Weiss, P. D., M. Wilm, E. Bonte, K. Dumas, M. Mann, and P. B. Becker.** 1997. Chromatin-remodeling factor CHRAC contains the ATPases ISWI and topoisomerase II. Nature **388:**598–602.
57. **Venter, U., J. Svaren, J. Schmitz, A. Schmid, and W. Horz.** 1994. A nucleosome precludes binding of the transcription factor Pho4 *in vivo* to a critical target site in the *PHO5* promoter. EMBO J. **13:**4848–4855.
58. **Vogel, K., W. Horz, and A. Hinnen.** 1989. The two positively acting regulatory proteins Pho2 and Pho4 physically interact with *PHO5* upstream activation regions. Mol. Cell. Biol. **9:**2050–2057.
59. **Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston, and R. A. Young.** 1996. RNA polymerase II holoenzyme contains *SWI/SNF* regulators involved in chromatin remodeling. Cell **84:**235–244.
60. **Winston, F., and M. Carlson.** 1992. Yeast *SNF/SWI* transcriptional activators and the *SPT/SIN* connection. Trends Genet. **8:**387–391.
61. **Wu, C.** 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. Nature **286:**854–860.
62. **Yoshida, K., N. Ogawa, and Y. Oshima.** 1989. Function of the *PHO* regulatory genes for repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **217:**40–46.
63. **Zakian, V. A., and J. F. Scott.** 1982. Construction, replication, and chromatin structure of *TRP1 R1* circle, a multicopy synthetic plasmid derived from *Saccharomyces cerevisiae* chromosomal DNA. Mol. Cell. Biol. **2:**221–232.