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CHROMATIN REMODELING AT THE

SACCHAROMYCES CEREVISIAE PHO5 PROMOTER

by

ELIZABETH S. HASWELL

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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This thesis is dedicated to my family, for everything they have given me

and to Greg Jensen, my favorite person in the whole world

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CHROMATIN REMODELING AT THE SACCHAROMYCES CEREVISIAE PHO5 PROMOTER

Elizabeth S. Haswell

Erin K. O'Shea, Ph.D.

In order to initiate gene expression, transcriptional activators must gain access to chromatinized promoter DNA. Here we describe biochemical and genetic investigations into the process of chromatin remodeling at the *Saccharomyces cerevisiae PHO5* promoter. In response to phosphate starvation, *PHO5* is transcriptionally induced and its promoter chromatin structure undergoes a dramatic change. We developed and characterized an in vitro system in which partially purified *PHO5* minichromosomes undergo promoter chromatin remodeling. Several hallmarks of the *PHO5* chromatin rearrangements observed *in vivo* were reproduced in this system. The ATP-dependent chromatin remodeling complex SWI/SNF was sufficient, but not required, for Pho4p- and ATP-dependent chromatin remodeling in vitro. Mutants carrying null or conditional alleles of several candidate chromatin-modifying activities were assessed for their ability to induce *PHO5* expression in vivo. Our data support a model wherein SWI/SNF and the histone acetyltransferase gene Gcn5p act in a redundant manner to potentiate *PHO5* transcription.

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INTRODUCTION

The phosphate starvation response in yeast

How cells sense the availability of nutrients in their environment and how they adjust to and survive in conditions of deprivation are basic questions in biology. Our laboratory studies how the model organism *Saccharomyces cerevisiae* responds to starvation for inorganic phosphate. We hope to describe in molecular detail the complete cellular response to changes in extracellular levels of phosphate.

In response to phosphate starvation, budding yeast alter gene expression, protein localization, and protein activity; upon longer starvation, cell division and growth rate are arrested (for reviews of this regulatory system, see (1-3)). When starved for phosphate, yeast increase the expression and membrane localization of a high affinity phosphate transporter (4), which increases phosphate availability. In addition, yeast respond to a phosphate-poor environment by inducing the expression of an array of protein phosphatases which help the cell scavenge inorganic phosphate by cleaving phosphate from organic molecules. Acid phosphatase activity is increased approximately 50-fold by phosphate starvation. Most of this activity is provided by the Pho5 protein, an acid phosphatase associated with the cell wall. Other cellular phosphatases include Pho3p, also an acid phosphatase, and Pho8p, a vacuolar alkaline phosphatase.

PHO3 expression is constitutive, and *PHO8* is weakly regulated by phosphate availability. However, *PHO5* transcription is strongly regulated by environmental phosphate levels (5). The signal transduction pathway that regulates *PHO5* expression has been a topic of study for over 25 years. In the mid-1970's Oshima and colleagues identified the major regulatory genes required for *PHO5* induction in low P_i, *PHO2*, *PHO4*, and *PHO81*; and those required for repression in high P_i, *PHO80*, *PHO85*, and *PHO86* (6, 7).

In recent years, much progress has been made in elucidating the molecular details of the regulatory pathway connecting these genes to *PHO5* expression. *PHO4* encodes a basic helix-loop-helix transcriptional activator (8, 9) and *PHO2* encodes a homeodomain-

containing DNA-binding protein (10, 11); they induce *PHO5* expression in low phosphate through two genomic response elements in the *PHO5* promoter, termed UASp1 and UASp2 (for **u**pstream **a**ctivating **s**equence)(12). In high phosphate, a cyclin/CDK complex encoded by *PHO80* and *PHO85* phosphorylates Pho4p (13). Phosphorylation inhibits Pho4p nuclear import, activates Pho4p nuclear export, and prevents its interaction with Pho2p (14-17). The kinase activity of the Pho80p/Pho85p complex is in turn regulated by a cyclin/CDK inhibitor encoded by *PHO81* (18); upon phosphate starvation *PHO81* actively inhibits the Pho80p/Pho85p complex, allowing Pho4p to be active. How the CDK inhibition function of Pho81p is regulated is a topic of current investigation.

PHO5 expression is also regulated by the chromatin structure of its promoter (reviewed in (19, 20)). The results of several in vivo 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 (using H4 under glucose-repressible control) causes the loss of nucleosome positioning on the *PHO5* promoter and results in partial derepression of *PHO5* transcription (21). UASp1 and UASp2 are not required for this effect (22). In vivo footprinting studies demonstrate that a Pho4p mutant lacking its activation domain binds DNA when its recognition element is in a nucleosome-free region, but not when the element is packaged into a nucleosome (23). The presence of a hyper-stable nucleosome on the *PHO5* promoter prevents full induction, while the presence of pBR sequence allows derepression of *PHO5* (24). Taken together, these data provide strong evidence that packaging of Pho4p binding sites and the TATA box into chromatin is important for the repression of *PHO5* expression, and that accessibility of the promoter sequence to Pho4p and to TATA-binding protein (TBP) must be significantly altered for transcription to occur.

Thus, an alteration in the chromatin structure of its promoter is likely to be an important regulatory event in *PHO5* transcription. The *PHO5* promoter as a useful system in which to study chromatin remodeling during transcription will be discussed further after

the following general introduction to the composition and regulation of chromatin structure. The focus is on the structure, function, and regulation of chromatin in the yeast *Saccharomyces cerevisiae*, particularly in relationship to the nuclear process of transcription.

Chromatin

The packaging of genomic DNA into the nucleoprotein complex called chromatin serves to compact, organize, and regulate the accessibility of the eukaryotic genome. The repeating structural unit of chromatin is the nucleosome (reviewed in (25, 26). In a nucleosome core particle, 146 base pairs of DNA is wrapped approximately 1.7 times around a hydrophobic core formed by the histone octamer. The octamer consists of two copies of each of four histones (H2A, H2B, H3, and H4) and has a tripartite structure; a central $(H3\text{-}H4)_2$ tetramer is flanked by two (H2A-H2B) dimers. Core particles are connected by varying lengths of linker DNA depending on the organism and stage of development to form the recognized "beads on a string" structure as revealed by electron microscopy. Structural studies demonstrate that each histone dimer contacts the DNA helix through arginines inserted into the minor groove and through side-chain contacts with the phosphodiester backbone (27).

Histones. The sequence and structure of histones are highly evolutionarily conserved (28). Each of the four core histones can be divided into two domains: the ordered histone folds and the unstructured N-terminal tails. The histone folds are a protein structural motif found in a family of transcription factors that includes components of the general transcription machinery and gene-specific transcriptional regulators as well as the core histones (29-31). This helical domain mediates both the interactions between histone dimers and those between histones and DNA. The unstructured N-terminal tails are 15-30 amino acids long and extremely basic. The histone tails extend out from the nucleosome core through pores formed by the DNA helix. In vitro experiments indicate that the histone

tails do not contribute significantly to the stability or assembly of the core nucleosome, but rather facilitate the condensation of high order fibers (see (32) and references therein). The crystal structure of the nucleosome core supports a role for histone tails in internucleosomal contact, as the H4 tail of one nucleosome contacts the H2A of a neighboring nucleosome (27, 33).

Most organisms have highly repeated histone genes, a situation that severely complicates genetic analyses of histone function. However, *S. cerevisiae* has only two copies of each histone gene and studies in this organism have implicated histones in both activation and repression of gene expression (reviewed in (34)). Altered histone gene dosage leads to defects in both gene activation and repression (21, 22, 35, 36). Mutations in the globular domain of either H3 of H4, believed to destabilize the interaction between the $(H3\text{-}H4)_2$ tetramer and the (H2A-H2B) dimers (37), cause constitutive expression of the *PHO5* gene, and mutations in the N-terminal tail of H3 allow hyperactivation of *GAL1* (38) (39). In contrast, *PHO5* expression is prevented by deletion of the invariant sites of acetylation in the H4 N-terminus (40), and deletion of the H2A N-terminal tail prevents induction of *SUC2* gene (41).

Though the essential nature of chromatin is a repetitive and invariant one, its structure is regulated in a variety of ways, which in turn can affect the transcriptional potential of underlying genes. For example, telomeric chromatin, called heterochromatin, is capable of repressing gene expression. Heterochromatin's special properties can be traced to the assembly of a specialized structure containing the specific non-histone proteins Rap1p, Sir3p and Sir4p (reviewed in (42)). As this example illustrates, the non-histone components of chromatin can confer a specific transcriptional potential. Histones themselves may participate directly in regulation. Two histone variants have been identified in yeast. Cse4p, an H3 variant, is an essential component of centromeres (43), and Htz1p is an H3 variant of unknown function (44). Another potential venue for the local regulation of histone function is the covalent modification of the histone tails by acetylation,

phosphorylation methylation, ADP-ribosylation, ubiquitination, and glycosylation (45). Histone acetylation has been particularly well studied in this regard and will be discussed in further detail below.

Higher order structure. In higher eukaryotes, linker histones and other non-histone proteins facilitate further compaction of nucleosomal DNA (28). In *S. cerevisiae*, however, it is as yet unresolved whether a linker histone exists. Yeast chromatin has short linker regions between nucleosomes, which may preclude the binding of a linker histone. In general, yeast chromatin appears to be more flexible, transcriptionally active, DNase I sensitive, hyperacetylated, and less condensed than that of other organisms (summarized in (46)). On the other hand, sequencing of the *Saccharomyces* genome uncovered the gene *HHO1*, which is predicted to encode a protein homologous to histone H1 of higher eukaryotes (47, 48). Although no defects in growth, mating, or sporulation are found in an *hho1* null mutant, Hho1p behaves like a linker histone in vitro (49), is localized to the nucleus, and has a moderate effect on the expression of a *CYC1*-driven reporter gene (48).

How to combat chromatin-mediated repression

The packaging of genes into chromatin has a well-documented repressive effect on their transcriptional activity. In vivo, depletion of histones leads to an increase in basal gene expression in *S. cerevisiae* (35). In several systems a nucleosome has been demonstrated to prevent factor binding (50, 51). A large number of biochemical studies have demonstrated that in vitro transcription is repressed from templates that are assembled into nucleosomes, and that this can be overcome by pre-binding activators and general transcription factors (reviewed in (52). In summary, both in vivo and in vitro experiments indicate that the packaging of DNA into chromatin represses transcription by preventing access to the DNA by gene-specific and general transcriptional activators.

How does the cell achieve the necessary genomic compaction while allowing expression of appropriate genes? At least one answer has come from the discovery of two

classes of activities that directly enhance the accessibility of DNA in chromatin. These classes of activities are: 1) ATP-dependent chromatin remodeling complexes, which alter histone-DNA contacts in an ATP-dependent manner; and 2) histone acetyltransferases, which directly modify the histone tails. In recent years, significant advances have been made in the identification and characterization of both types of activities. Below is a description of these two classes of activities, and how they are thought to function. The complexes in each class specific to *S. cerevisiae* will be described in further detail in Chapters 2 and 3.

ATP-dependent chromatin remodeling complexes

The founding member of this class of activities is the SWI/SNF complex, which was identified in *S. cerevisiae* in a confluence of genetic and biochemical studies (reviewed in (53, 54)). Since this initial discovery, other activities capable of remodeling chromatin in an ATP-dependent manner have been identified in a wide array of organisms, indicating that their function is well conserved and important (55). All complexes in this class contain a helicase-like ATPase from the SNF2 subfamily of ATPases (56), but otherwise differ in subunit composition, size, and performance in various biochemical assays. It is likely that these complexes have different physiological functions as well; clear genetic evidence for a role in transcriptional regulation only exists for yeast and human SWI/SNF. A regulatory role in chromatin homeostasis during transcription, condensation, repair, or recombination remains an open possibility for most members of this class.

The exact nature of the changes in histone/DNA contacts caused by ATP-dependent chromatin remodeling complexes is unknown (see below). It is thus important to note that, as a result, "chromatin remodeling" simply describes activity in one or more in vitro assays. An example is the most commonly used assay, mononucleosome disruption. DNA that is rotationally positioned within a nucleosome is particularly sensitive to digestion with DNase I at 10 base pair intervals, where the helix curves away from the histone core. As defined by this assay, chromatin remodeling results in the disappearance

of this digestion pattern and the appearance of a pattern similar to that obtained with naked DNA. Other assays include increased accessibility to restriction enzymes or other nucleases, and the enhancement of transcription factor binding to mononucleosomes.

SWI/SNF-like. A second complex capable of in vitro chromatin remodeling that is highly homologous to SWI/SNF was isolated from yeast (57), and another identified in *Drosophila* (BRM), (58). Related complexes, termed BAFs, are found in human cells (59, 60). There is evidence for cell-type specific BAFs, each of which potentiates transcriptional activation in a particular promoter context (59, 61). The ATPase in each complex is a member of the SNF2 subfamily of helicase-like ATPases, and contains a conserved amino acid motif found in many proteins implicated in chromatin-based processes termed the bromodomain (56). Complexes in this subgroup are all large (1-2 MD) and have as many as 15 subunits. In vivo evidence suggests that SWI/SNF, the *Drosophila* BRM complex, and the BAF complexes function to increase transcription by opposing the repressive effects of chromatin (reviewed in (54)). Complexes in this subfamily have similar ATP-dependent activity profiles in vitro: they are capable of altering the rotational phasing of DNA in a mononucleosome; enhancing the binding of promoter-specific and general transcription factors to mononucleosomes and arrays, and facilitating activated transcription in vitro (62, 63).

ISW1-containing. Three distinct activities have been purified from *Drosophila* embryo extract: NURF (64, 65), CHRAC (66), and ACF (67). All of these complexes contain a SNF2L subfamily member termed ISWI (**i**mitation **sw**itch), a gene first identified on the basis of its similarity to yeast SWI2 (68). ISWI-containing complexes are distinguished by the presence of a domain not found in other members of the SNF2 family, the SANT domain (69). Intriguingly, outside of the shared ISWI subunit, these three complexes have distinct compositions and differ significantly in their ability to perform in various in vitro assays. NURF and ACF facilitate factor binding, chromatin remodeling, transcription, and nucleosome spacing (64, 67, 70). CHRAC enhances restriction enzyme

accessibility of nucleosomal arrays, spaces nucleosomes (66), and is capable of facilitating in vitro replication of SV40 DNA (71). A human ISWI-containing complex, RSF (remodeling and spacing factor) was purified from human cell extracts on the basis of its facilitation of in vitro transcription (72). Remodeling complexes in this class are around 0.5 MD and contain 2-4 subunits.

SWI/SNF-like versus ISWI-containing. SWI/SNF-like complexes and ISW1-containing complexes appear to function through significantly different mechanisms (see (73) and references therein). Only SWI/SNF-like complexes are able to alter the 10 base pair DNaseI digestion pattern of a mononucleosome, an effect thought to indicate a disruption of the rotational positioning of DNA. Furthermore, the ATPase activity of SWI/SNF is stimulated equally well by DNA and nucleosomal DNA (74), while NURF's ATPase activity is stimulated only by nucleosomes. NURF appears to act through histone tails, as it interacts with them in vitro, and cannot remodel nucleosomes that have been proteolysed to remove the histone tails (73). On the other hand, SWI/SNF and RSC are able to remodel mononucleosomes without tails but their catalytic activity is compromised by removal of the tails in nucleosomal arrays (75).

NRD/NURD/Mi-2. The human NURD, NRD, NuRD complexes (76-78), and a similar activity in *Xenopus* (79), contain the ATPase CHD4/Mi-2 and are capable of ATP-dependent chromatin remodeling. These complexes are linked to transcriptional repression, due to the fact that they contain the histone deacetylase HDAC, and are capable of deacetylating nucleosomal histones (see below for further discussion of histone acetylation/deacetylation). These results have prompted speculation that the CHD4-mediated chromatin remodeling activity increases the accessibility of the histone tails to HDAC in an ATP-dependent manner. Indeed, a modest enhancement of histone deacetylation activity by NRD in the presence of ATP was observed (76). These complexes are also linked to DNA methylation (80, 81), and may function in the developmentally regulated repression of certain chromatin domains (82).

Mechanism of action. ATP hydrolysis is an important aspect of the mechanism by which these complexes act. The ATPase activity contributed by Swi2p and Sth1p is absolutely required for the function of SWI/SNF and RSC, respectively (74, 83, 84). In addition, in several cases the subunit containing ATPase activity is capable of carrying out many of the functions attributed to the full complex, though at reduced efficiency. For example, ISWI is capable of carrying out nucleosome remodeling and chromatin assembly in the absence of other subunits of NURF, CHRAC, or ACF (85). In addition, BRG1 and hBRM, the ATPases in human BAF complexes, are capable of remodeling mononucleosomes and nucleosomal arrays alone, although the addition of other subunits increases their activity (86).

ATP-dependent remodeling complexes may act by lowering the transition state between two stable nucleosomal structures. A stable, presumably activated nucleosomal intermediate is observed after treatment with SWI/SNF or RSC, even after removal of ATP (87) or the remodeling complex (88-91). It is worth noting, however, that persistently remodeled chromatin is not observed on nucleosomal arrays, suggesting that the generation of a permanently altered state may be an artifact of experiments with isolated mononucleosomes (91, 92). A model wherein ATP-dependent chromatin remodeling complexes function to facilitate interconversion between different chromatin states can explain data implicating NURD and RSC in transcriptional repression (77, 93); these complexes might facilitate the binding of sequence-specific repressors or histone deacetylases to their targets within chromatin.

Recent evidence suggests that ATP-dependent chromatin remodeling complexes alter histone/DNA contacts by facilitating histone sliding. NURF (94), ISWI and CHRAC (95), and SWI/SNF (96) appear to catalyze the movement of histone octamers along the DNA. In the case of SWI/SNF-mediated histone migration, a four-way junction in the DNA can restrict movement, presumably by providing a barrier to sliding (96). Histone sliding could be effected through "looping," the creation of a small bulge or loop which is

propagated around the histone core, in a manner reminiscent of the movement of the small viral SP6 polymerase through a nucleosome (97, 98). It has been suggested that chromatin remodeling complexes bind to the linker region of DNA, and then translocate around the nucleosome (99). Though in vitro helicase activity has not been reported in this family of proteins, electron spectroscopic imaging reveals DNA looping or peeling off nucleosomal histones in the presence of SWI/SNF (100).

Acetyltransferases and deacetylases

The second class of activity that is postulated to affect transcription factor access to chromatin is histone acetyltransferases (HATs). The acetylation of invariant lysine residues within the N-terminal tails of all four core histones is associated with transcriptional activation, chromatin assembly, silencing, and recombination ((101), reviewed in (102)). Specific histone tail lysines are acetylated in connection with particular cellular processes. An illustration of this phenomenon is the different site specificities of cytoplasmic and nuclear HATs. Cytoplasmic HATs are involved in new chromatin assembly during DNA replication and acetylate a different subset of H3 and H4 tail lysines than nuclear HATs involved in transcriptional regulation and silencing.

Hyperacetylation of the N-terminal tails of histones has been long associated with transcriptional activity (28, 103). However, direct evidence that histone acetylation can modulate transcriptional activity was only recently gained upon the biochemical purification of the first HAT from *Tetrahymena*, p55 (104). p55 was found to be highly similar (40% identical) to the yeast co-activator Gcn5p. As previous studies had characterized Gcn5p as a transcriptional coactivator required for the expression of a subset of genes in yeast ((105) and references therein), this discovery provided a critical link between histone acetylation and gene regulation. A large number of HATs has now been identified in many organisms; the major families are described below.

Like Gcn5p, these more recently described HATs had been previously implicated in the process of transcriptional activation, and many were known to interact directly with

sequence specific activators. It is now clear that HAT activity is recruited to particular promoters where their function is required (reviewed in (106). Further evidence to support the model that histone acetylation regulates transcription in the context of chromatin is the discovery that histone deacetylases are encoded by genes previously identified as transcriptional corepressors (82, 107).

GCN5 family. Yeast Gcn5p is found in three complexes in vivo: SAGA (1.8 MD), ADA (0.8 MD), and an unnamed complex of approximately 200 KD (108-110). Only in the context of these complexes can Gcn5p acetylate nucleosomal histones in vitro; recombinant Gcn5p will acetylate only free H3 and H4 (111, 112). HGcn5, a human homologue of Gcn5p, has a similar free histone acetylation profile (113). p300/CBP Associating Factor (PCAF), a transcriptional coactivator for nuclear receptors that competes with E1A for association with p300/CBP, can acetylate both free and nucleosomal histones (113). PCAF has homology to Gcn5p in its carboxy terminus and is found in large complex containing several human counterparts of SAGA components (114, 115).

Mammalian coactivators. A large number of mammalian coactivators have been discovered to possess histone acetyltransferase activity. p300 and CBP, functional homologues proposed to integrate a variety of cellular signals by virtue of their association with DNA-binding transcriptional activators, are histone acetyltransferases capable of acetylating free H3 and H4 in vitro (116). SRC-1 (117), ACTR (118), and TIF2/GRIP1, all co-activators that bind steroid receptors directly in a ligand dependent manner, also have in vitro HAT activity. ACTR and SRC-1 are both capable of binding PCAF and p300/CBP in a multimeric complex.

MYST family. This well-conserved family of HATs was named after its founding members *MOZ*, *YBF2/SAS3*, *SAS2*, and Tip60 (119), genes linked to silencing and dosage compensation. Another member, *MOF*, is required for the X chromosome-specific acetylation of histone H4 in *Drosophila*, a prerequisite for dosage compensation (120, 121). The yeast *MOF* homologue, *ESA1*, is an essential gene, and provides the

HAT activity of the NuA4 complex (122-124). A homologous complex is found in Tetrahymena (125). Yeast Sas2p and Sas3p are involved in silencing at the mating type loci and the telomeres of *S. cerevisiae* (119); Sas3p has in vitro HAT activity (126).

Mechanism. Precisely how HATs affect transcriptional activation is still under investigation. There is a tight correlation between the HAT activity of various mutant versions of recombinant Gcn5p, and the ability of the same mutants to acetylate promoter regions and support transcription in vivo (111, 112). These data provide direct evidence that histone acetylation at a particular promoter affects its activity.

In vitro studies of reconstituted mononucleosomes and nucleosomal arrays have suggested several models to explain the effect of histone acetylation on transcriptional activity (see (45, 127)). One model is that acetylation of a lysine residue neutralizes its positive charge, decreasing the affinity of histone tails for DNA, and allowing transcriptional activators increased access to DNA. Both histone acetylation and removal of the histone tails facilitate binding by sequence-specific factors to in vitro assembled nucleosomes (for example, see (128)). An alternative model is suggested by in vitro experiments demonstrating that histone acetylation affects condensed fiber formation in nucleosomal arrays. Further support for a role for acetylation in regulating higher order chromatin structure comes from the crystal structure of the nucleosome core particle, which indicates that the histone tails may make substantial internucleosomal contacts (27, 33).

These data are correlative, however, and it is important to note that acetylation of histone tails is not always positively correlated with active transcription. Heterochromatin is a region of the genome that is relatively transcriptionally silent. In both yeast and *Drosophila*, heterochromatin is enriched in a specifically acetylated version of histone H4 (129, 130). Therefore, histone acetylation may serve to generate a signal that is recognized by factors involved in a variety of cellular processes, rather than mediate structural changes in the nucleosome or chromatin fiber (131). Histone tails interact with the yeast corepressor Tup1p in a manner that is affected by acetylation (132), and the silencing

proteins Sir3p and Sir4p interact only with acetylatable H3 and H4 N-termini in vitro (133). In addition, bromodomains, conserved protein motifs that are found in some SNF2 ATPases and in all HATs identified to date, bind acetylated lysines in the tail of H4 (134) and H3 and H4 peptides (135).

Thus, HATs may affect gene expression by acetylating histones and thereby altering chromatin structure or providing a binding surface for specific regulatory factors. Another possibility is that proteins with HAT activity affect transcription by acetylating non-histone proteins. For example, p300/CBP can acetylate the tumor suppressor p53, thereby regulating its DNA-binding activity (136). Further, as described above, transcriptional activation by nuclear hormone receptors appears to require the recruitment of three different HATs (ACTR, PCAF, and p300/CBP), implying that these three HATs have diverse and additive effects on activation. The modification of various lysines in the tail domains of all four core histones, in combination with the acetylation of other transcription factors, may regulate transcription through a complex array of signals.

Other activities

Though ATP-dependent chromatin remodeling complexes and histone acetyltransferases clearly serve widespread and highly conserved roles in the regulation of gene expression, it is rapidly becoming clear that they are not the only types of chromatin-specific factors involved in transcription. Transcription elongation through chromatin templates is facilitated by a mammalian complex termed FACT (137). FACT comprises a homologue of yeast SPT16, and an HMG protein (138). ARC/DRIP is a chromatin-specific co-activator from mammalian cells that interacts with SREBP, VP16, NF-KB, and several nuclear receptors to mediate transcription from chromatin templates (139, 140). ARC/DRIP is related to several other recently identified activities including CRSP, TRAP, and NAT/SMCC and to the yeast SRB/mediator complex (reviewed in (141)). Yeast mediator and the novel CCR4-NOT complex (142, 143) may also have chromatin-specific functions in gene expression ((34) and references therein) (142, 143).

PHO5 as a model system to study chromatin remodeling

Much recent progress has been made in understanding how the repressive effects of chromatin are dealt with in order to allow transcription. In particular, many cellular activities with chromatin-specific functions in gene expression have been identified. However, the specific mechanisms by which these factors function remain mysterious. Some progress may be gained through the study of the chromatin rearrangements that accompany the phosphate-regulated induction of the yeast gene *PHO5*.

The chromatin rearrangements that occur at the *PHO5* promoter upon transcriptional induction are dramatic and well characterized. *PHO5* is a nonessential gene, and the signal transduction pathway regulating its expression has been genetically defined, allowing *PHO5* induction to be easily manipulated in the laboratory. Furthermore, *S. cerevisiae* is a model organism wherein both biochemical and genetic analyses are easily performed. These traits combine to recommend *PHO5* as a system in which to investigate the regulation of chromatin rearrangements during transcription. This system has the potential to answer several outstanding questions in this field: Do sequence-specific transcriptional activators act before or after chromatin modifying activities? What is the nature of the mechanism by which chromatin modifying activities potentiate the process of transcription? And how do different kinds of chromatin modifying activities work together at a particular promoter to facilitate transcription?

This thesis describes the use of biochemical and genetic approaches designed to determine if activities in addition to Pho2p and Pho4p are required to mediate *PHO5* chromatin remodeling. Chapter 1 describes the reconstitution of the *S. cerevisiae PHO5* chromatin transition in vitro, and discusses its potential use in the identification of components required for the chromatin rearrangement at the *PHO5* promoter. Both in vitro and in vivo methods were employed to analyze the contribution of the remodeling complex SWI/SNF to *PHO5* remodeling, as described in Chapter 2. Chapter 3 reports the results of

a directed genetic investigation of factors required for *PHO5* induction in response to phosphate starvation. The Appendix describes a brief description of the interactions between Pho2p and Pho4p.

CHAPTER ONE

AN IN VITRO SYSTEM RECAPITULATES CHROMATIN REMODELING AT THE YEAST PHO5 PROMOTER

Introduction

The *Saccharomyces cerevisiae* gene *PHO5* is a well-characterized system in which to study regulated gene expression. *PHO5* encodes a secreted acid phosphatase, whose transcription is regulated in response to environmental phosphate levels (2). When phosphate is plentiful, *PHO5* expression is repressed; when phosphate is limiting, *PHO5* expression is induced. Activation of *PHO5* transcription requires two transcription factors, a basic helix-loop-helix protein termed Pho4p, and a homeodomain protein named Pho2p (12). In vitro, Pho2p enhances the binding of Pho4p to two regulatory sequences in the *PHO5* promoter, UASp1 and UASp2 (144, 145) see Appendix).

When transcription of *PHO5* is activated, its promoter undergoes a dramatic change in chromatin structure (19, 146). When yeast 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 Pho4p does not bind the *PHO5* promoter under repressing conditions (50). When environmental phosphate is limiting, the positioned nucleosomes no longer protect the *PHO5* promoter, and Pho4p binds to UASp1 and UASp2 (Fig. 1, $-P_i$). In vivo footprinting of Pho2p at the *PHO5* promoter has not been performed, but in vitro experiments indicate that Pho2p binds to this region in coordination with Pho4p (144, 145) see Appendix). 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 (148), and occurs when cell division is prevented (147). The Pho4p transcriptional activation domain is required for the *PHO5* chromatin transition (23), but is not required for binding to naked

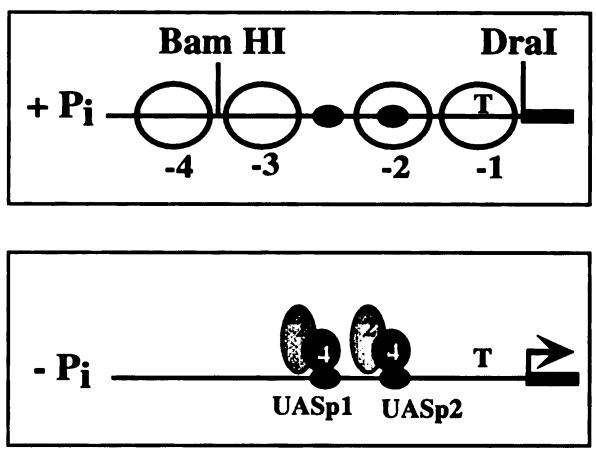


Figure 1. Chromatin structure of *PHO5* in high and low phosphate.

Positioned nucleosomes are indicated by open circles, and dark ovals represent identified upstream activating sequences (UASs). T denotes the location of the TATA box.

DNA (148). If the activation domain of Pho4p 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 Pho4p and Pho2p 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 (149-151) or changes in *PHO5* chromatin structure (149). *PHO5* mRNA levels in high and low phosphate are unaffected by mutations in the histone acetylase gene *GCN5*, although *gcn5* mutants have unusual *PHO5* promoter chromatin structure under partially inducing conditions (109, 152). 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 (149).

The reconstitution of chromatin rearrangement in vitro has allowed the isolation of several chromatin remodeling activities from *Drosophila* embryo (64, 66, 67) and Hela cell extracts (137). 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 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 (153) containing *PHO5* sequence from nucleotides -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 (154) 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 Pho4p 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 remaining sequences self-ligated before introduction into yeast. pRSPHO4 was constructed by inserting a Bam HI to Hind III fragment of pACD4 (153) containing the *PHO4* promoter and open reading frame into pRS426.

Strains. *S. cerevisiae* strain YS18 (10) was used in all experiments. Northern analysis was performed with strains EY0244 (WT), EY0168 (*pho3Δ pho5Δ*), and EY0168 harboring pTA-PHO5. For indirect end-labeling, EY0255 (*pho2Δ pho4Δ pho80Δ*) harboring pTA-PHO5 was used. For analysis of chromatin remodeling of episomal *PHO5* in vivo, we used strains EY0246 (*pho3Δ pho5Δ pho4Δ pho80Δ*) and EY0243 (*pho3Δ pho5Δ pho80Δ*), either harboring pTA-PHO5-ATGΔ or both pTA-PHO5-ATGΔ and pRSPHO4. For minichromosome purification and nuclear extract preparation, EY0255 or was used.

Northern Blots. Cell cultures were grown in media lacking inorganic phosphate for 6 hours as described previously (16), and total RNA was prepared as in (155). RNA was quantitated and 20 μg of each sample was loaded on 6.7% formaldehyde, 1.5% agarose gels and run in 1X E buffer (20 mM MOPS pH 7.0, 5 mM NaOAc, 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 according to the first steps of the procedure described by Simpson and colleagues (156, 157), with some modification. EY0255 cells harboring pTA-PHO5 or pTA-p1p2 were grown in 9 liters of synthetic media 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. Cells were washed once with 1 M sorbitol, and resuspended in 500 ml lyticase buffer (1.2 M sorbitol, 50 mM Tris-Cl pH 8.0, 5 mM β-mercaptoethanol). Spheroplasting was performed with 1 μg recombinant lyticase (158) per gram wet cell weight for approximately 30 min at 30°C (monitored until 90% of the cells lysed when vortexed in water, as observed microscopically). All subsequent manipulations were at 0-4°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 Ficoll buffer (18% Ficoll, 20 mM MOPS-NaOH pH 6.8, 1 mM $MgCl_2$, 1 mM PMSF) and dounced by hand (Wheaton 40 ml Dounce), 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 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 1-2 h in 9 ml elution buffer (200 mM NaCl, 5 mM $MgCl_2$, 10 mM PIPES-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 split between two 35 ml 0.4 M-1 M sucrose gradients in 25 x 89 mm QuickSeal tubes,, made in elution buffer supplemented to a final NaCl concentration of 250 mM. 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-stained agarose gels. Minichromosome-containing fractions were pooled, concentrated 10-fold on Centri-Prep concentrators (10,000 MWCO) that had been pre-blocked with insulin, and stored at -80°C in aliquots.

We estimate the average yield of this procedure to be approximately 40% based on Southern blot analysis comparing minichromosome DNA in whole cells and in the final fraction. The greatest loss occurred at the nuclear elution step, where 50-80% of the *PHO5* minichromosomes were recovered in the eluate. The final *PHO5* minichromosome fraction contained approximately 1 µg/ml minichromosomal DNA, 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 0.5X TBE at 4 V per cm for 4 h. Gels were prepared as described (159) and blotted to Hybond N+ membrane (Amersham) overnight in 20X SSC. Prehybridization and hybridization with random prime labeled probes were performed in Rapid Hyb Buffer (Amersham). Typically, restriction fragments of 100-300 base pairs embedded in low melting-point agarose were random prime labeled overnight at room temperature.

Micrococcal nuclease digestion (MNase) 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 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). 200 µl aliquots were placed in tubes containing 0.1-100 U MNase (Worthington, 15u/µl in water). 200 µl of buffer B (buffer A + 0.15% NP-40) were added to each tube, and reactions were incubated at 37°C for 5 min. Reactions were stopped with one tenth volume of 5% SDS, 250 mM EDTA. DNA was purified by digestion with 200

μg/ml proteinase K (final concentration) at 37°C for two hours, followed by phenol: chloroform extraction, RNase treatment, and ethanol precipitation. Samples were resuspended in the appropriate volume (10-20 μl) of water. For indirect end-labeling of minichromosomes, one tenth 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 a Ngo MI to Bgl II fragment of the *TRP1* gene, and a 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* loci 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, either harboring pTA-PHO5-ATGΔ or both pTA-PHO5-ATGΔ and pRSPHO4, were cultured, spheroplasted, treated with MNase, and Southern blotted as described above. Probes for analysis of nucleosome -2 and nucleosome +1 correspond to Cla I to Bst EII (probe A, Fig. 2) and Dra I to Sal I (probe B, 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 rPho4p. *Escherichia coli* strain BL21 harboring a T7-PHO4 expression vector (13) was grown in 3 liters of LB supplemented with 50 μg/ml carbenicillin to an A_{600} of 0.4. IPTG was added to 0.4 mM and the culture was grown 2 hours at 37°C. Cells were harvested, washed with B(0.1) (10% glycerol, 20 mM PIPES-NaOH pH 7.3, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, 0.1 M NaCl), and resuspended in 40 ml 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 DNase I, clarified through a 0.22 μm filter, and loaded onto a 10 ml SP-Sepharose High Performance (Pharmacia) column. rPho4p was eluted with a linear gradient from 100 to

1000 mM NaCl. Fractions containing rPho4p were pooled, adjusted to the conductivity of B(0.1) by dilution with B(0), loaded onto a BioScale S5 column (BioRad) and eluted as before. This procedure yielded approximately 15 mg rPho4p, which appeared as a single band in Coomassie-stained SDS-PAGE.

Preparation of S(0.3) extract. Nuclear extract was prepared from EY0255 as in (160), with the following modifications. Recombinant lyticase was used for spheroplasting, and lysis was performed with a hand-held glass dounce, 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, 0.1M KOAc, 1 µg/ml pepstatin A, 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). Fractions containing protein were pooled and concentrated 10-fold with Centricon concentrators (10,000 MWCO).

In vitro chromatin remodeling. 10 µl of minichromosomes (approximately 10 ng DNA in 250 mM NaCl, 5 mM $MgCl_2$, 10 mM PIPES-NaOH pH 7.3, 0.5 mM EGTA) were incubated in 50 µl reactions containing 12 mM HEPES-NaOH pH 7.5, 6 mM $MgCl_2$, 1 mM DTT, 5% glycerol, and 0.5 mM $CaCl_2$. 2 µg/ml poly(dC)poly(dG) and 0.1 mg/ml BSA were also added as non-specific competitors. After addition of rPho4p (90 nM), Pho2p (approximately 20 nM), and 1 µg of S(0.3), reactions were incubated at room temperature for 15 min. An ATP regeneration mix was added (final concentrations: 0.2 µg/ml creatine kinase in 10 mM glycine pH 8, 30 mM creatine phosphate, and 0.5 mM ATP or AMP-PMP) and reactions were incubated at 30°C for 30 min. Reactions were split and digested with either 0.1 or 0.05 U MNase for 5 min at 37°C. Digestion was stopped with one tenth volume stop solution (5% SDS, 250 mM EDTA). DNA was purified by overnight treatment with 200 µg/ml proteinase K 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 (161), retroviral integration (154), centromere function (162), and chromatin structure (163, 164). We modified a 1.5 kb *TRP1/ARS1* circle by inserting the *PHO5* promoter and open reading frame to form pTA-PHO5 (Fig. 2A). The *PHO5* promoter fragment 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 (165, 166).

When yeast cells are grown in media lacking inorganic phosphate, transcription of *PHO5* is induced (167). We therefore tested if phosphate starvation induces transcription of episomal *PHO5*. Wild type, *pho5Δ*, or *pho5Δ* strains carrying pTA-*PHO5* were grown in media lacking inorganic phosphate for 6 hours. Total RNA was isolated from these cultures, and Northern analysis was performed (Fig. 2B). *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 twenty 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 four-fold difference was reproducibly observed, as early as 3 hours and as late as 12 hours after transfer to media lacking phosphate. These data suggest that a factor necessary for *PHO5* transcription is limiting under these conditions, preventing

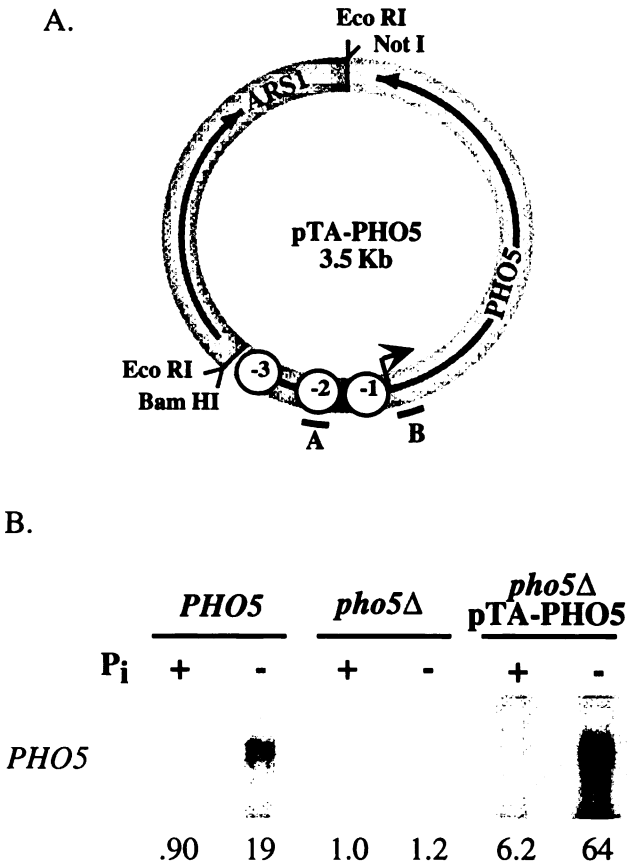


Figure 2. Construction and expression from pTA-PHO5.

(A) 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 indicate direction of transcription of *PHO5* and *TRP1*. Open circles show the locations of three positioned nucleosomes on the *PHO5* promoter. Bars represent the sequence from which probes A and B were derived. (B) Northern analysis of chromosomal and episomal *PHO5* expression in response to phosphate starvation. *PHO5*+, *pho5*Δ, or *pho5*Δ strains harboring pTA-PHO5 were grown for six hours in media either containing (+) or lacking (-) inorganic phosphate. *PHO5* transcript levels were quantified and normalized to the *ACT1* signal.

expression of all 20 copies of pTA-PHO5 in each cell. The limiting factor could be Pho4p, Pho2p, 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 (168, 169). We used this technique to compare the chromatin structure of chromosomal and episomal *PHO5* (Fig. 3) 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. 3A, sample 3), that correspond to those mapped previously (146). Three similarly positioned nucleosomes were detected on the *PHO5* promoter on pTA-PHO5 in vivo (compare sample 3 and sample 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.

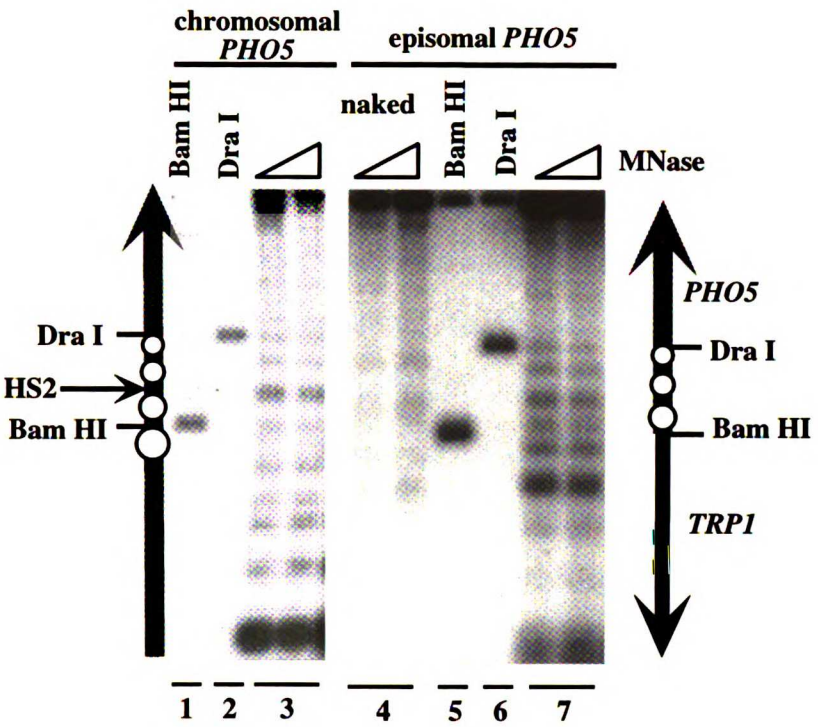
There is a detectable difference between the chromatin structure of chromosomal and episomal copies of *PHO5*. The nuclease hypersensitive site (HS2), visible on the chromosomal copy of *PHO5*, is not apparent on pTA-PHO5 (Fig. 3A). This may be explained by the observation that nucleosome -3 appeared slightly shifted in position towards nucleosome-2. However, nucleosomes -1 and -2 (incorporating the TATA box and UASp2) appeared correctly positioned, thereby reproducing the appropriate repressed state. Micrococcal nuclease digestion followed by indirect end-labeling thus indicates that the *PHO5* promoter on pTA-PHO5 is incorporated into positioned nucleosome 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

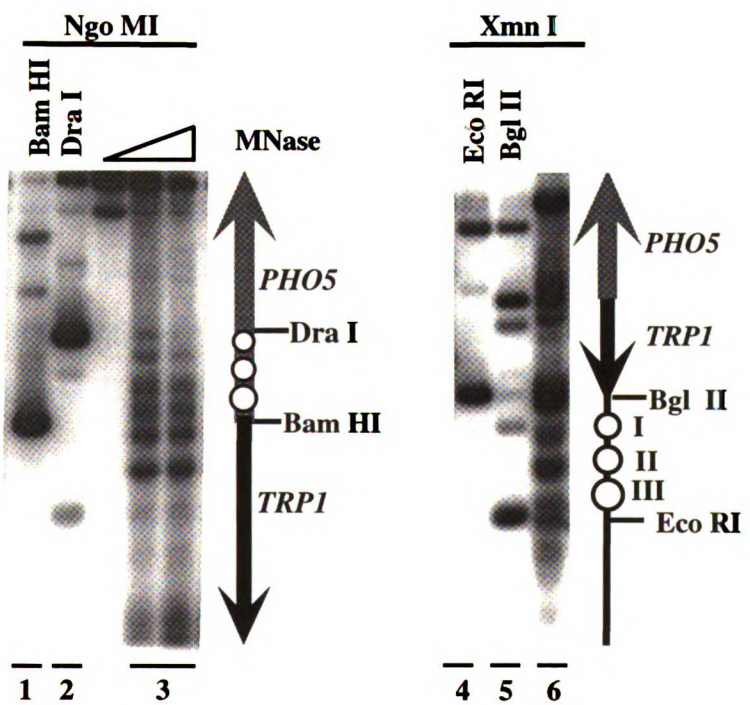
Figure 3. Nucleosomes on the *PHO5* promoter have similar position on the chromosome or on pTA-PHO5, and are not changed upon *PHO5* minichromosome preparation.

(A) Spheroplasts were treated with micrococcal nuclease (MNase), and the DNA was purified. For size standards, untreated DNA was digested with Bam HI (samples 1 and 5) or Dra I (samples 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 episomal *PHO5*, samples were digested with Ngo MI, and the probe used was derived from a Ngo MI to Bgl II fragment from *TRP1*. (B) Partially purified *PHO5* minichromosomes were treated with MNase and the DNA purified. Samples digested with Ngo MI were probed with a Ngo MI to Bgl II fragment from *TRP1*; samples digested with Xmn I were probed with a 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* sequence, respectively. The location of the hypersensitive site HS2 on chromosomal *PHO5* is indicated with a black arrow.

A.


chromosomal
PHO5
episomal PHO5
naked
Bam HI
Dra I
Bam HI
Dra I
MNase
Dra I
HS2
Bam HI
PHO5
Dra I
Bam HI
TRP1
1 2 3 4 5 6 7

B.


Ngo MI
Xmn I
Bam HI
Dra I
MNase
Eco RI
Bgl II
PHO5
Dra I
Bam HI
TRP1
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I
II
III
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1 2 3 4 5 6

by digestion with micrococcal nuclease, followed by Southern blotting. This assay has been previously employed for analysis of the *PHO5* chromatin transition (24, 146, 147). 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 structure of the *PHO5* promoter on pTA-PHO5-ATGΔ in a *pho4Δ*, a *PHO4*$^+$, or a *PHO4*$^+$ strain carrying a high copy 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. High-levels of Pho5p have been reported to inhibit cell growth, due to disruption of the normal function of the secretory pathway (170). All strains lacked chromosomal *PHO5*, and all were also *pho80Δ*, which causes constitutive expression of *PHO5* (167).

In a strain lacking Pho4p, the sequence underlying nucleosome -2 produced a pattern of bands, indicating that it is protected from MNase digestion (Fig. 4A, 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. 2), 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 Pho4p, we assayed in vivo remodeling of pTA-PHO5-ATGΔ in a *PHO4*$^+$ strain carrying the high copy plasmid pRSPHO4. As shown in Figure 4A, sample 3, remodeling of nucleosome -2 was observed under these conditions. This suggests that the concentrations of Pho4p in the nucleus under inducing conditions are 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

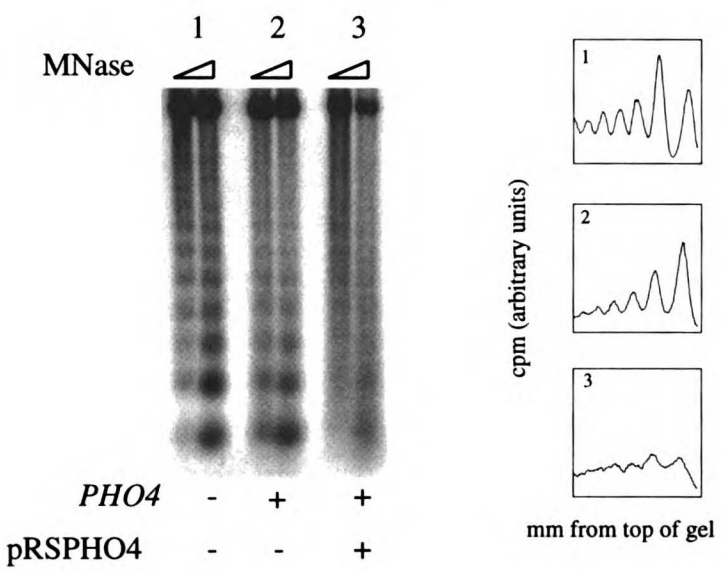
number drops to approximately five. Thus, the drop in template number may also allow remodeling of a higher proportion of the templates in each cell.

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. 4, panels A and B). The small amount of in vivo remodeling that is apparent at nucleosome +1 was also observed at another nucleosome in the *PHO5* ORF, 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 Pho4p 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, its transcription is regulated by environmental phosphate levels, and 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 (156, 157). Our goals were to remove genomic DNA and cellular debris in a manner gentle enough to leave minichromosomal chromatin intact.

PHO5 minichromosomes were prepared from a *pho2Δ pho4Δ* strain to prevent contamination of the chromatin template with the Pho4p and Pho2p transcription factors. Cells harboring pTA-PHO5 were spheroplasted with lyticase (158) and lysis was performed with a hand-held dounce. 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

A



B

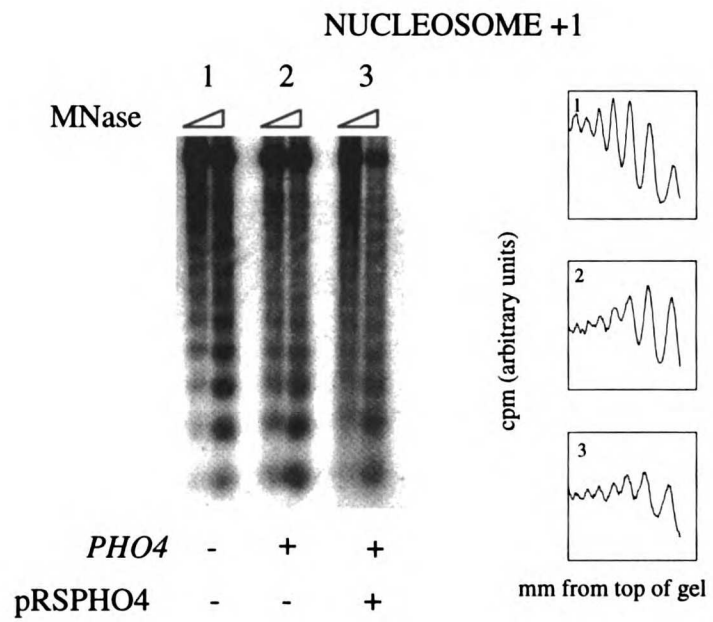


Figure 4. In vivo chromatin remodeling of episomal *PHO5*.

Spheroplasts from the indicated strains were treated with MNase, and the DNA was purified and Southern blotted. (A) The blot was probed with probe A. Data in each sample was quantified and the distance from the top of the gel was graphed against the signal density. (B) The blot shown in (A) was stripped and reprobed with probe B.

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 3B, sample 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 (171) and after purification (163). We therefore tested if positioned nucleosomes are present at these positions on purified *PHO5* minichromosomes. As shown in Fig 3B, sample 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 rPho4p and rPho2p, hydrolyzable ATP, and a fraction of nuclear extract. The scheme of our in vitro chromatin remodeling experiments is outlined in Figure 5A. *PHO5* minichromosomes were mixed with transcription factors and nuclear extract in a binding reaction. A source of energy was then added and the remodeling reaction was allowed to proceed. Reactions were split, and each half digested with one of two concentrations of micrococcal nuclease. DNA in each sample was purified, 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 Pho4p and Pho2p (172). We therefore tested if these transcription factors are sufficient to support in vitro chromatin remodeling of *PHO5* minichromosomes.

As shown in Fig. 5B, sample 1, recombinant Pho4p and Pho2p were not sufficient for remodeling of nucleosome -2 in vitro.

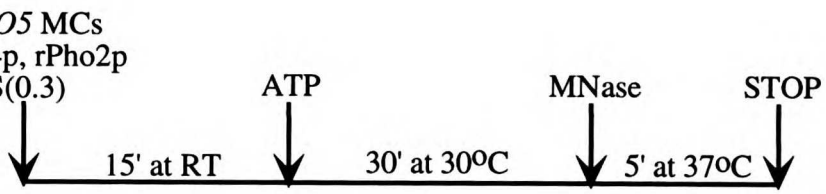
The inability of rPho2p and rPho4p to remodel chromatin in vitro suggested that remodeling of *PHO5* promoter chromatin requires an additional activity. We therefore tested if a fraction of *S. cerevisiae* nuclear extract, termed S(0.3), could provide a chromatin remodeling activity. Addition of S(0.3) had no effect in our assay (Fig. 5B, sample 2). By analogy with previously identified chromatin modifying complexes, such an activity might require either ATP or acetyl CoA. When we tested this possibility by incubating rPho4p and rPho2p, 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) were 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 rPho4p-dependent remodeling of *PHO5* minichromosome promoter chromatin, a non-hydrolyzable ATP analog was included in the regeneration system in place of ATP (Fig. 6). Whereas remodeling is observed when ATP is added (sample 2), there is no change in *PHO5* promoter chromatin structure when ATP is omitted (sample 1) or when an equivalent amount of AMP-PMP is substituted (sample 3). Reactions containing acetyl CoA as an energy source showed no remodeling under these conditions (data not shown). Thus, the transcription factors rPho2p and rPho4p, 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 Pho2p, Pho4p, and an ATP-dependent activity can remodel *PHO5* promoter chromatin.

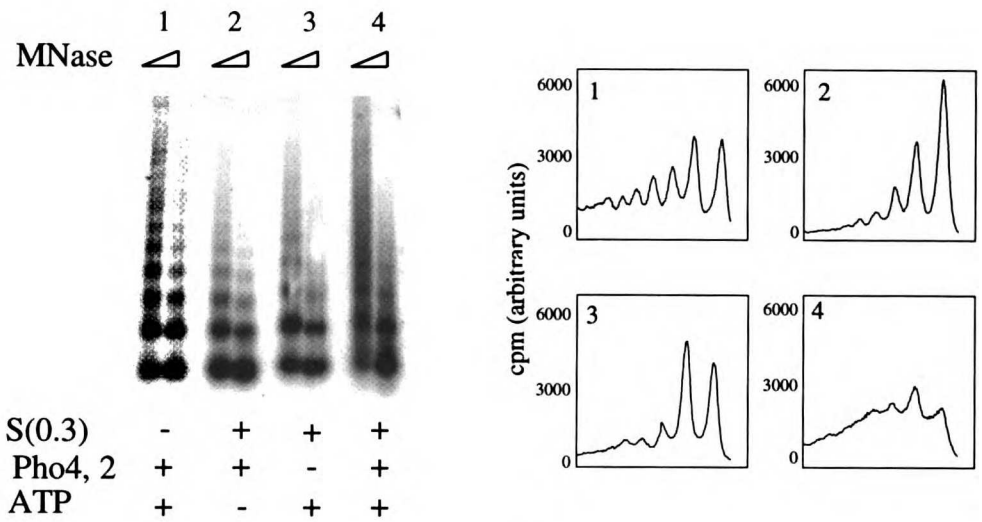
Figure 5. In vitro chromatin remodeling of *PHO5* minichromosomes requires rPho2p, rPho4p, S(0.3), and ATP, and is predominantly localized to the *PHO5* promoter.

(A) A schematic of the in vitro remodeling reaction. (B) Reactions were assembled and incubated as in (A), then split and digested with micrococcal nuclease. Samples were purified, electrophoresed, and transferred to nylon. Southern blotting was performed with probe A, and data graphed as in Fig. 5 (C) The blot shown in (B) was stripped and reprobed with probe B.

A.

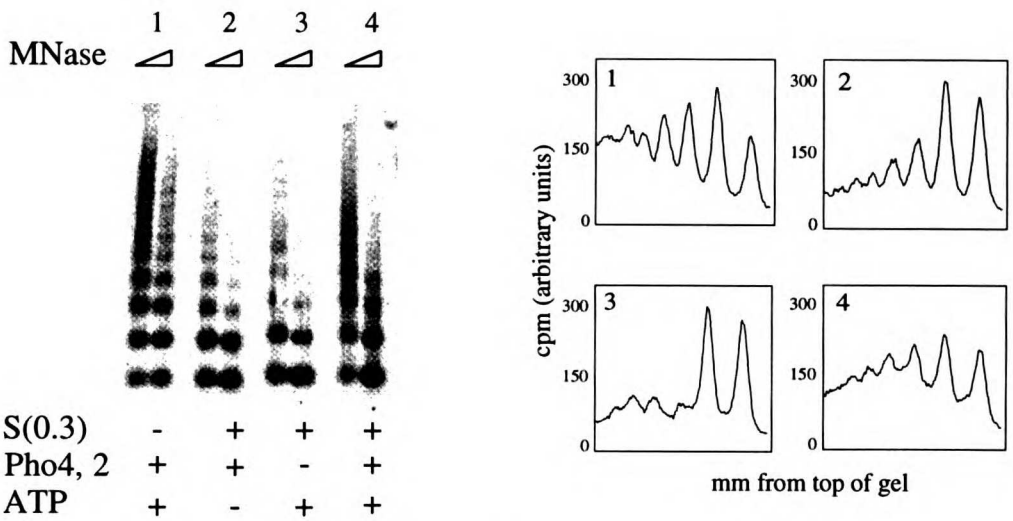


B.



NUCLEOSOME -2

C.



NUCLEOSOME +1

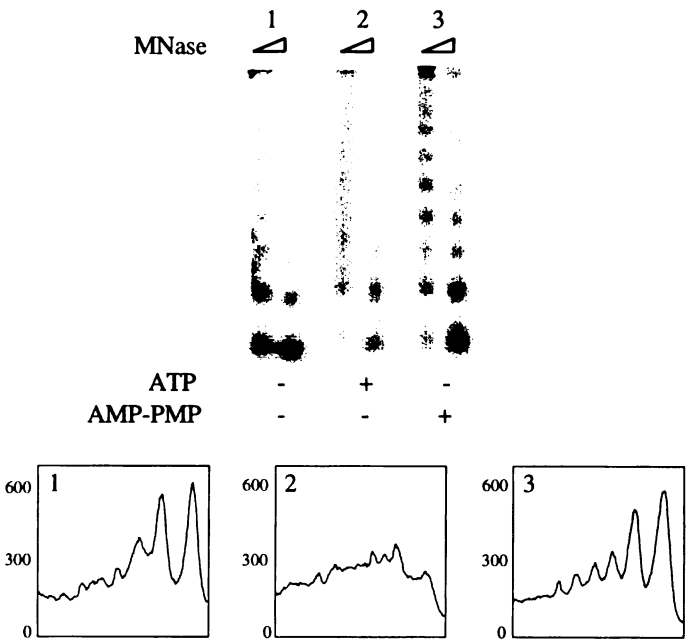


Figure 6. Hydrolyzable ATP is required for in vitro chromatin remodeling of *PHO5* minichromosomes.

Either buffer (sample 1), ATP (sample 2), or AMP-PMP (sample 3) was included in chromatin remodeling reactions with *PHO5* minichromosomes, rPho4p and rPho2p, and S(0.3). Samples were analyzed as described for Fig. 5.

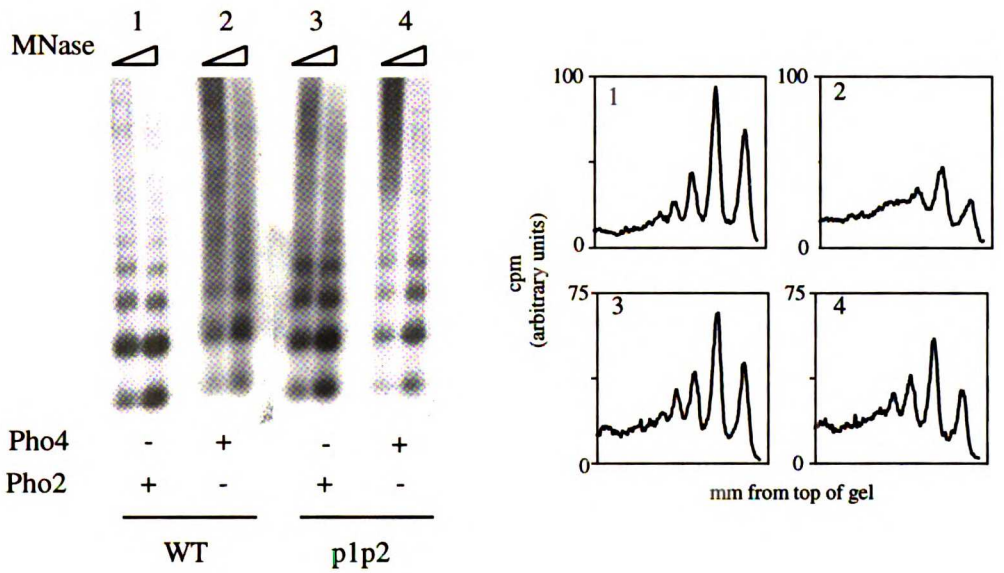


Figure 7. In vitro chromatin remodeling of *PHO5* minichromosomes in the absence of rPho2p requires rPho4p, UASp1 and UASp2.

Wild-type (WT) *PHO5* minichromosomes were tested for chromatin remodeling in the presence of either rPho2p (sample 1) or rPho4p (sample 2). Minichromosomes lacking the Pho4p binding sites at UASp1 and UASp2 (p1p2) were purified and tested in parallel as shown in samples 3 and 4. S(0.3) and ATP were included in all reactions.

S(0.3)-, ATP-, and rPho4p-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 (146). We tested if this was true of the remodeling observed in vitro by stripping and reprobing the Southern blot shown in Figure 5B with a sequence underlying nucleosome +1. When analyzed with this probe, all samples produced a largely nucleosomal pattern (Fig. 5C). Thus, the dramatic change in chromatin structure observed in the presence of rPho4p and rPho2p, S(0.3), and ATP at the *PHO5* promoter does not extend significantly into the *PHO5* open reading frame.

rPho4p can partially remodel *PHO5* chromatin in the absence of rPho2p. Overexpression of Pho4p can partially suppress the *PHO5* expression defect of a *pho2Δ* strain (172). We therefore tested if rPho4p was capable of supporting chromatin remodeling of *PHO5* minichromosomes in vitro without rPho2p. As indicated in Figure 7, rPho4p is capable of supporting partial remodeling of *PHO5* minichromosomes in the presence of S(0.3) and ATP without rPho2p (sample 2). However, when rPho2p is included in the remodeling reaction and rPho4p is left out, no remodeling is observed (sample 1). These results demonstrate that the transcription factor rPho4p is required for S(0.3)- and ATP-dependent chromatin remodeling of *PHO5* minichromosomes.

rPho4p-dependent chromatin remodeling without rPho2p is incomplete, and a nucleosomal pattern is visible (Fig 7. sample 1). When rPho2p is included in the reaction with rPho4p, S(0.3), and ATP, remodeling is more complete (see Fig. 5). Analyses of the DNA-binding and in vivo transcriptional activation properties of a version of Pho4p unable to interact with Pho2p suggest that Pho2p may affect the function of Pho4p in two ways: by modulating its ability to bind DNA, and by enhancing its ability to activate transcription (145, 173). By analogy, rPho2p may facilitate chromatin remodeling in our in vitro system by enhancing the binding of rPho4p to *PHO5* minichromosomes, or by enhancing the ability of Pho4p to support chromatin remodeling in a manner independent of DNA

binding. When higher concentrations of rPho4p were used, remodeling was complete in the absence of rPho2p; however, the relevance of remodeling at these concentrations is not clear (data not shown). Importantly, at the rPho4p concentrations used in this report, rPho2p is required for complete remodeling of *PHO5* minichromosomes in vitro, consistent with its role in vivo.

rPho4p-dependent chromatin remodeling requires UASp1 and UASp2. Deletion of a 26 bp region encompassing the Pho4p binding site in UASp1 has no effect on the assembly of repressive *PHO5* chromatin structure, but prevents Pho4p binding (50), and the chromatin transition (165) in vivo. These observations indicate that binding of Pho4p 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 rPho4p-dependent remodeling observed in the absence of rPho2p requires specific binding by rPho4p to UASp1 and UASp2. A version of pTA-PHO5, pTA-p1p2, was constructed in which the two Pho4p binding sites in UASp1 and UASp2 were replaced precisely with restriction sites. 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 in the presence of rPho4p (Fig. 7, sample 2), no rPho4p-dependent remodeling of p1p2 minichromosomes was observed (Fig. 7, sample 4). This result demonstrates that one or both of the Pho4p binding sites in UASp1 and UASp2 is required for rPho4p-dependent chromatin remodeling of *PHO5* minichromosomes in vitro.

Unexpectedly, p1p2 minichromosomes were remodeled when rPho2p was included in the remodeling reaction with rPho4p, 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 rPho2p to its site in UASp1 (which is intact in the p1p2 minichromosomes) can stabilize rPho4p

DNA binding. In contrast, the 26 bp deletion analyzed in previous studies partially destroys this Pho2p binding site, which may account for the completely unremodeled state of this mutant promoter in vivo.

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 rPho2p and rPho4p, a fraction of *S. cerevisiae* nuclear extract, and hydrolyzable ATP. In vitro chromatin remodeling of *PHO5* minichromosomes was localized to the *PHO5* promoter, and required specific binding by rPho4p to UASp1 and UASp2. 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 structure of the *PHO5* promoter under repressing and inducing conditions has been characterized (19). In high phosphate 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 (174). UASp1 and UASp2 are not required for this effect. This data suggests 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 Pho4p mutant lacking the activation domain binds UASp2 when it is in the hypersensitive site, but not when it is packaged into nucleosome –2 (23). This implies that nucleosome -2 presents a barrier to Pho4p 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 above reasons, 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 non-histone 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. 5B) 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. 5C) 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 rPho2p or rPho4p. 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 rPho2p or rPho4p. 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 non-specific 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 produce 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 (175). 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 Pho4p in *PHO5* chromatin remodeling. The activation domain of Pho4p is required for the *PHO5* chromatin transition in vivo. A version of Pho4p that lacks the activation domain is capable of binding to UASp1, but not to UASp2, under inducing conditions in vivo (23). When this Pho4p mutant is expressed in place of full-length Pho4p, 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 Pho4p 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, rPho4p and rPho2p cannot by themselves remodel *PHO5* minichromosomes

in our in vitro remodeling system (Fig. 5B, sample 1). It is therefore likely that an activity in addition to these transcription factors is required to change *PHO5* promoter chromatin structure in vivo.

If Pho4p 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 (149), a tantalizing result given recent evidence that the holoenzyme may associate with SWI/SNF (176) but see (57). Although the *PHO5* chromatin transition does not require SWI/SNF (149), another ATP-dependent chromatin remodeling complex may be associated with the holoenzyme, or chromatin remodeling may be a function intrinsic to the holoenzyme itself. It is also possible that the activation domain of Pho4p interacts directly with a chromatin remodeling activity. Substantial evidence supports a model in whereby yeast transcriptional regulators recruit histone acetylating (177, 178) and deacetylating (179) 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 Pho4p.

In the models above, Pho4p first binds DNA, and secondly mediates chromatin remodeling in a second step. In contrast, it is possible that chromatin remodeling must occur before Pho4p can bind to its recognition sites in the *PHO5* promoter. According to this model, a non-specific chromatin remodeling activity acts constitutively, in an ATP-dependent manner, to make chromatin more accessible. Subsequently, binding of Pho4p and Pho2p to the *PHO5* promoter prevents nucleosome positioning and stabilizes a nuclease sensitive state. A requirement for the Pho4p activation domain may be explained if it is necessary for stable association with *PHO5* promoter chromatin.

Identity of the remodeling activity in 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 (56). 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 (63). 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. Components of SWI/SNF are required for transcription of a small number of regulated genes, and none identified to date are essential (53, 54). It has been established that SWI/SNF is not required for the *PHO5* chromatin transition in vivo (149-151). RSC was identified on the basis of its homology to SWI/SNF and contains several essential subunits (57, 180). It is not known if RSC is required for *PHO5* expression.

Thus, the activity contained in S(0.3) could be RSC or SWI/SNF. 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 currently underway.

Reconstitution of the *PHO5* chromatin transition. We describe here the reconstitution of *PHO5* chromatin remodeling in vitro. The *PHO5* chromatin transition is 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 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 Pho2p and Pho4p are not sufficient to change *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 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.

CHAPTER TWO

A GENETIC AND BIOCHEMICAL ANALYSIS OF THE CONTRIBUTION OF SWI/SNF TO PHO5 CHROMATIN REMODELING

Introduction

One approach to the investigation of chromatin remodeling during transcription is to study *in vitro* chromatin rearrangements that are well characterized *in vivo*. Biochemical analysis of such a system can be used to identify components of the chromatin remodeling activity, and to elucidate the mechanism of the reaction. If a genetically malleable organism is used for *in vitro* studies, results from biochemical studies can be readily tested for *in vivo* relevance. In our study of the process of chromatin remodeling of the *PHO5* promoter in *S. cerevisiae*, we have been able to take advantage of both the genetic and biochemical approaches available for work in this organism.

The *PHO5* gene, which encodes a secreted acid phosphatase, is repressed when yeast are grown in high phosphate conditions and is induced upon phosphate starvation. Under repressing conditions, two pairs of positioned nucleosomes flank an 80 base pair hypersensitive site in the *PHO5* promoter. Upon activation of the gene, these four nucleosomes no longer protect the DNA from digestion with nucleases. Two transcription factors are required for *PHO5* expression: Pho4p, a helix-loop-helix protein with an acidic activation domain, and Pho2p, a homeodomain protein. Pho4p and Pho2p bind to two elements in the promoter, UASp1 and UASp2, that are required for *PHO5* expression (144).

The minimal requirements for the alteration in *PHO5* chromatin structure in vivo upon phosphate starvation are not known. The chromatin transition requires Pho2p and Pho4p (172), as well as the Pho4p binding sites in UASp1 and UASp2 (11). Additionally, the DNA-binding domain of Pho4p does not suffice to remodel chromatin in low phosphate (20), implying that the activation domain is required for this process. Previous studies have demonstrated that the generation of active chromatin at the *PHO5* promoter is independent of active transcription (165) or replication (147), ruling out the possibility that these cell processes are required for the chromatin transition.

We have established an *in vitro* system capable of *PHO5* chromatin remodeling in order to identify and characterize factors required for this process (181). Minichromosomes carrying the *PHO5* promoter and gene are purified from yeast as a source of chromatin template. Remodeling of the *PHO5* promoter *in vitro* occurs only in the presence of ATP, Pho4p and Pho2p, and a fraction derived from yeast nuclear extract termed S(0.3). Remodeling *in vitro* recapitulates several hallmarks of the physiological *PHO5* chromatin rearrangements, including restriction to the promoter region, and requirements for Pho2p, Pho4p, and the Pho4p binding sites.

These results demonstrate that Pho2p and Pho4p are required but are not sufficient for chromatin remodeling, and suggest that an ATP-dependent factor contributed by the S(0.3) is required for *PHO5* minichromosome promoter chromatin remodeling. It is possible that the *PHO5* minichromosomes or the recombinant versions of Pho4p and Pho2p do not display all the functions of their in vivo counterparts, and in vivo Pho2p and Pho4p are sufficient. However, as the *PHO5* minichromosomes contain wild type *PHO5* promoter sequence, and were assembled inside the cell, they have appropriately positioned nucleosomes, native histones, and any non-histone proteins required for remodeling. Recombinant Pho4p binds DNA ((182), see Appendix), facilitates in vitro transcription (183), and is capable of regulated interactions with several proteins important for its function, including Pse1p, Msn5p, and Pho2p (15-17). Similarly, recombinant Pho2p can interact and bind cooperatively to DNA with Pho4p (17, 144, 145), and Swi5p (184, 185). We therefore consider it unlikely that the recombinant versions of Pho2p and Pho4p used in our studies are defective in a chromatin remodeling function.

An attractive candidate for the activity found in our S(0.3) extract is the ATP-dependent chromatin remodeling complex SWI/SNF. The *SWI2/SNF2*, *SWI1*, *SWI3*, *SNF5*, and *SNF6* genes were first identified as an overlapping set of genes required for expression of the mating-type switching endonuclease *HO*, derepression of the *SUC2* gene in the appropriate sugar conditions, and expression from Ty elements (reviewed in (53,

54). These genes are required for expression of a diverse set of genes in yeast, including *AHD2*, *GAL1*, and *INO1*. *SWI/SNF* genes mediate transcriptional activation by endogenous and heterologous DNA transcriptional regulators, including yeast Gal4p, the *Drosophila* activators bicoid and fushi tarazu, and mammalian gluccocorticoid receptor (186-188).

Evidence that the SWI/SNF genes function to counteract the repressive effects of chromatin came initially from the identification of suppressors of *swi* mutants. *SIN1*, an HMG-like protein presumed to be a non-histone chromatin component, and *SIN2* encodes histone H3 (reviewed in (53). The chromatin rearrangements that accompany *SUC2* derepression require the *SWI2*, *SWI1*, and *SNF5* genes (189-191), further linking SWI/SNF genes to chromatin remodeling during transcriptional activation.

Several experiments prompted the suggestion that the SWI/SNF genes act in a complex (reviewed in (192). First, a null mutation in each gene causes the same phenotype as deletion of several genes. Second, the ability to activate transcription when tethered to a promoter, to associate in vitro with GR, and the stability of certain SWI/SNF gene products is dependent on the presence of the other gene products. This prediction was confirmed upon identification and characterization of a 2MD complex containing the five SWI/SNF genes (193, 194). The yeast SWI/SNF complex contains 11 subunits: Swi2p, a helicase-like ATPase; Swi1p; Swi3p; Snf5p; Snf6p; Snf11p, Swp29; Swp73; Swp82; Arp7 and Arp9 (reviewed in (55)). Purified yeast SWI/SNF is capable of altering the repeating pattern of DNase I digestion of a mononucleosome or nucleosomal array in vitro (195), facilitating binding of transcriptional regulators (195, 196), and of potentiating activated transcription (197).

SWI/SNF is not required for expression of all genes, and is not very abundant ((57), but see(176)), implying that it is recruited to particular promoters. This association is likely to be mediated through other factors, as SWI/SNF binds DNA only with low affinity and without sequence specificity (198). One way in which specific promoter

recruitment may occur is through association with specific chromatin structures. SWI/SNF contains an actin-related component that may mediate an association with the nuclear architecture (199-201), and associates with the chromatin component SIN1 (202). There is also evidence that SWI/SNF is recruited through interactions with sequence-specific transcriptional activators (203). Transcriptional activators that contain an acidic activation domain can recruit SWI/SNF from a yeast extract independent of activator interactions with RNA polymerase(203, 204). Acidic activation domains bind directly to purified SWI/SNF complex, and this interaction can stimulate in vitro transcription from nucleosomal templates (197).

These results introduce a paradox. Transcription factor binding is required to recruit SWI/SNF to a promoter, and in vivo data supports a role for SWI/SNF at a step subsequent to factor binding; SWI/SNF can act after factor binding in vivo (205) and facilitate transcriptional elongation (206). On the other hand, biochemical and genetic studies suggest that SWI/SNF functions to facilitate transcription factor binding. In vitro, yeast SWI/SNF has been shown to facilitate the binding of activators (91, 195, 196) and human SWI/SNF facilitates the binding of TATA-binding protein (207). Adding more binding sites or locating the binding sites at the edge of nucleosomes can render a previously SWI/SNF-dependent gene independent (208). SWI/SNF binding is also somewhat redundant with cooperative binding (196). The actual case may be a combination between these two scenarios; chromatin immunoprecipitation experiments at the *HO* promoter suggest that a "nucleating transcription factor," Swi5p, binds and recruits SWI/SNF, which in turn facilitates the binding of a second activator, SBF (209).

Though it was originally reported that the production of acid phosphatase activity in response to phosphate starvation was defective in SWI/SNF mutants (210), more recent studies in our laboratory and that of others contradict this result (149-151), and have further shown that *PHO5* chromatin remodeling is unchanged by mutations in *SWI2* (149). However, these results do not rule out a possible role of SWI/SNF in *PHO5*

minichromosome remodeling in vitro, and also leave the possibility that *PHO5* expression is affected in a more subtle way by SWI/SNF. This chapter describes further investigations into the role SWI/SNF may play in *PHO5* chromatin remodeling in vivo and in vitro.

Materials and Methods

Strains and media. *S. cerevisiae* strain YS18 (10) was used in all experiments. For nuclear extract preparation, either EY0255 (*pho2Δ pho4Δ pho80Δ*) or EY0579 (*pho2Δ pho4Δ pho80Δ snf6Δ*) was used. EY0255 and EY0579 harboring pTA-PHO5 were used in minichromosome purification, as described in Chapter 1. To make EY0579, the two-step gene replacement vector pEY110 (211) (EB0307) was integrated into EY0244 (wild type haploid). This strain was mated to EY0254 (*pho2Δ pho80Δ*) and excisants were selected by growth on 5-FOA. After sporulation, *pho2Δ pho8Δ snf6Δ* haploid strains were identified by Southern blotting, mated to EY0245 (*pho4Δ*), sporulated, and *pho2Δpho4Δ pho80Δ snf6Δ* spores identified. For *PHO5* mRNA production analyses, strains EY0244, EY0197 (*snf6Δ*), and EY0710 (*swi2Δ/SWI2*) were used. To make EY0197, *SNF6* was disrupted in EY0244 by one-step gene replacement using pSNF6d (EB0335) (150). To make EY0710, *SWI2* was disrupted in EY0258 (wild type, diploid) by one-step gene replacement using pSWI2d (EB0197) (150), and *swi2Δ/SWI2* heterozygotes identified by southern analysis. The diploid was then transformed with pRS-SWI2 (EB1098) or pRS-6.3 (EB1099) (212) and sporulated, to obtain *swi2Δ* cells covered by wild type or temperature sensitive versions of SWI2, respectively.

Synthetic media lacking inorganic phosphate, SD-P_i, was made as described in (213) except that 1.5 g/L KCl is added in place of potassium phosphate monobasic.

In vitro chromatin remodeling. *PHO5* minichromosome and transcription factor preparations were performed as described in Chapter 1. For chromatin remodeling reactions, 10 µl of minichromosomes (approximately 10 ng DNA in 250 mM NaCl, 5 mM

$MgCl_2$, 10 mM PIPES pH 7.3, 0.5 mM EGTA) were incubated in 50 µl reactions containing 2 mg/ml dCdG, 0.1 mg/ml BSA, 12 mM HEPES pH 7.5, 6 mM $MgCl_2$, 1 mM DTT, 5% glycerol, and 0.5 mM $CaCl_2$. After addition of 90 nM rPho4p or Pho4p DNA binding domain peptide, 20 nM rPho2p, and approximately 1 nM SWI/SNF complex (a generous gift of Craig Peterson), reactions were incubated at room temperature for 15 min. An ATP regeneration mix (final concentrations in a volume of 8.5 µl: 10 ng creatine kinase in 10 mM glycine pH 8, 30 mM creatine phosphate, and 10 mM ATP) was added, and reactions were incubated at 30°C for 30 min. Reactions were split in two, and digested with one of two different concentrations of micrococcal nuclease (Worthington) at 37°C for 5 min. Digestion was stopped with one tenth volume .25M EDTA/5% SDS, and DNA was purified by overnight treatment with 200 µg/µl proteinase K(final volume) at 37°C, followed by phenol extractions, chloroform extraction, and ethanol precipitation.

Southern Blotting. Samples were loaded on 1.2% agarose gels and run in 0.5X TBE at 4 V/cm for 4 h. Gels were prepared for blotting as described (159) and blotted to nylon membrane (Amersham) overnight in 20X SSC. Prehybridization and hybridization with random prime labeled probes were performed in Rapid Hyb Buffer (Amersham). Typically, fragments of 100-300 base pairs embedded in low melting-point agarose were random prime labeled overnight at room temperature with a kit (Gibco BRL). Probes for analysis of nucleosome -2 and the ORF corresponded to a Cla I/Bst EII fragment and an Xmn I to Mfe I fragment of *PHO5*, respectively.

***PHO5* mRNA Northern analysis.** Cultures were grown to OD_{600} of approximately 1.0 in YEPD. Cells were washed twice with sterile distilled water, then diluted to an OD_{600} of 0.5 in SD $-P_i$. At appropriate intervals, 25 ml of cell culture was removed, the cells pelleted, washed with water, and then flash-frozen in N_2 (l) and stored at –20°C. RNA was prepared essentially as in (214). 15 µg total cellular RNA per sample was loaded onto a 1.5% formadehyde/agarose gel and run for 4 hours at 115 V. Gel was blotted to Nylon in 20X SSC. Blots were hybridized as for Southerns above. Probes

were random prime labeled using 1 kb PCR products from the *PHO5* and *ACTIN* open reading frames.

Enzyme activity assays. Liquid acid phosphatase (APase) enzyme activity assays were performed as described previously (14), except that cultures were grown for 6 or 16 hours in synthetic media lacking inorganic phosphate before quantitation of APase activity. β-gal enzyme activity assays were performed as in (215). Cells were grown in SD media lacking uracil to mid-log phase, then transferred to SD-P_i lacking uracil for the indicated time periods.

Results

***PHO5* chromatin remodeling in vitro does not require the SWI/SNF complex.** In vitro remodeling of *PHO5* minichromosome promoter chromatin required S(0.3) and ATP. The yeast chromatin remodeling factor SWI/SNF contains an ATPase, which is required for its function in vivo (74, 84) and in vitro (195). We therefore considered the possibility 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* (149-151), it was of interest to determine if *PHO5* minichromosome remodeling requires this complex in vitro. Western blots of fractionated nuclear extract indicated that several components of SWI/SNF were primarily located in fractions other than S(0.3) (data not shown). To test more directly if SWI/SNF was responsible for the activity in the S(0.3) we chose to evaluate the chromatin remodeling activity in a S(0.3) extract lacking SWI/SNF activity.

The Snf6 protein is a component of the SWI/SNF complex, and is required for the structural integrity of the complex during purification (194). 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 *snf6Δ* strains. In vitro remodeling with components derived from a *snf6Δ* strain occurred to the same extent as those derived from *SNF6*$^+$ cells

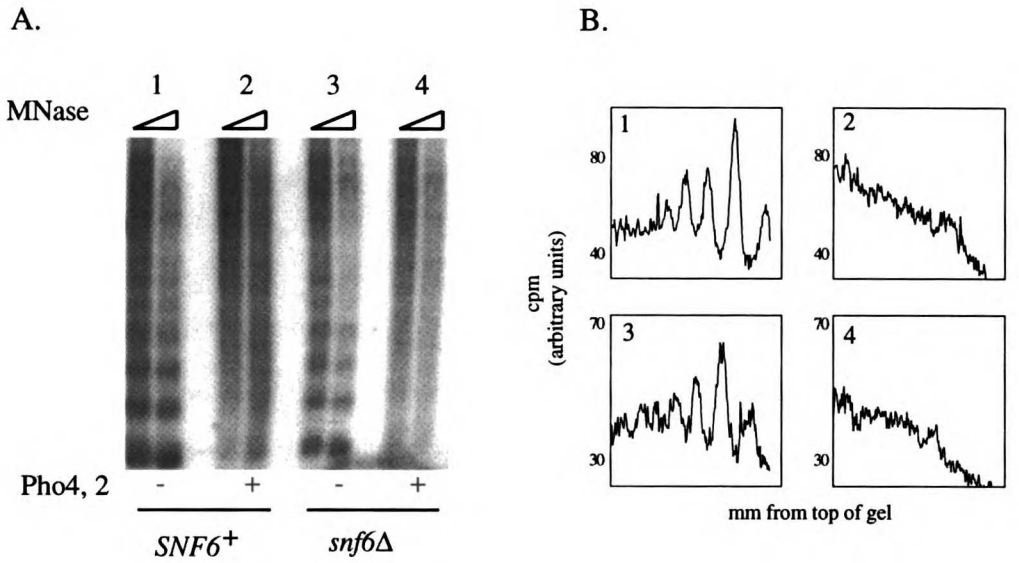


Figure 8. SWI/SNF is not required for in vitro chromatin remodeling of *PHO5* minichromosomes.

(A) S(0.3) extract and minichromosomes were purified from *SNF6+* (samples 1 and 2) or *snf6Δ* (samples 3 and 4) strains, and assayed for chromatin remodeling in the presence and absence of rPho2p and rPho4p. An ATP-regeneration mix was added to all samples. (B) Densitometric traces of the lanes in (A).

(Fig. 8, compare samples 2 and 4), demonstrating that Snf6p is not required for remodeling in vitro. We can therefore infer that the 2 MD SWI/SNF complex is not required for *PHO5* chromatin remodeling in vitro.

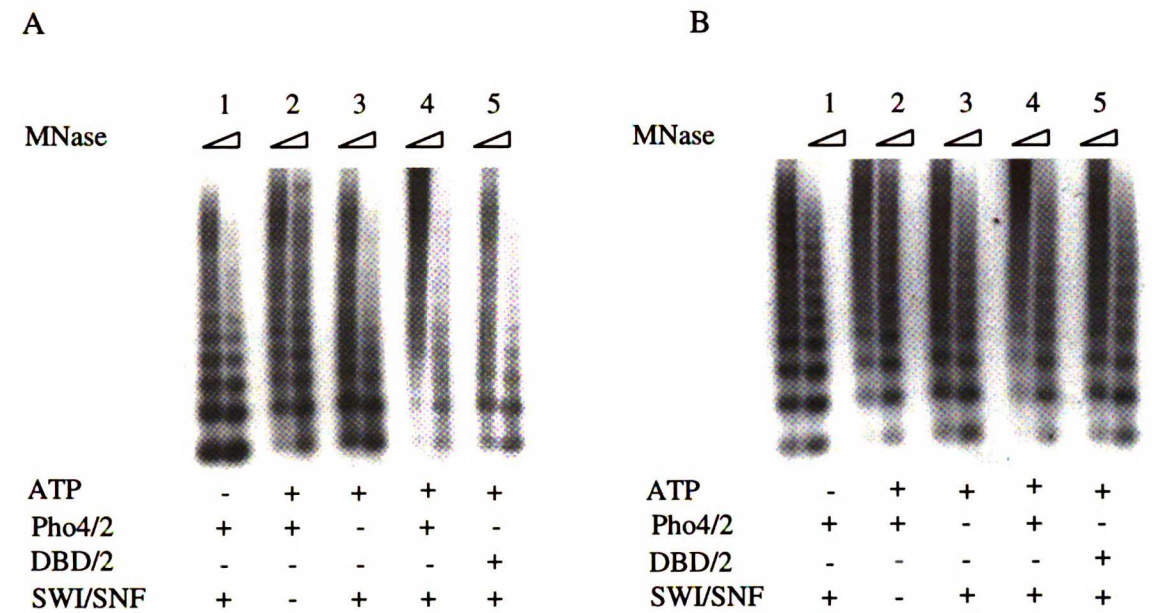
Purified SWI/SNF can remodel *PHO5* minichromosomes in vitro in a rPho2p-, rPho4p- and ATP-dependent manner. Although SWI/SNF is not required, we wished to determine whether SWI/SNF is sufficient to remodel chromatin in our in vitro system. Purified SWI/SNF, a kind gift of Craig Peterson, was incubated with *PHO5* minichromosomes and an ATP-regeneration system in the presence or absence of the transcription factors rPho2p and rPho4p. After the remodeling reaction, chromatin was digested with micrococcal nuclease and the DNA was purified. Samples were separated by agarose gel electrophoresis, transferred to nylon membrane, and hybridized to a ^{32}P-labeled probe derived from *PHO5* promoter sequence.

Much to our surprise, rPho4p-dependent remodeling occurred in the presence of purified SWI/SNF and ATP (Fig. 9A, C). This SWI/SNF-dependent chromatin remodeling activity resembled the in vivo chromatin transition in that the remodeling activity was predominantly restricted to the *PHO5* promoter region (Fig. 9B, D). We conclude that SWI/SNF, and an activity distinct from SWI/SNF contained in the S(0.3) fraction, are functionally indistinguishable in our in vitro chromatin remodeling system. It is also notable that little remodeling occurred when the Pho4p DNA binding domain alone was used in place of the full-length protein. This result suggests that DNA binding is not sufficient for in vitro chromatin remodeling and is consistent with the requirement for the Pho4p activation domain observed in vivo (23).

***SNF6* is required for rapid induction of *PHO5* upon starvation for phosphate.** As SWI/SNF appeared to be sufficient but not required for *PHO5* minichromosome remodeling in vitro, we then chose to investigate more carefully a possible role for SWI/SNF in the *PHO5* chromatin transition in vivo. To do so, we analyzed the kinetics of *PHO5* mRNA production after cells were transferred into

Figure 9. SWI/SNF-dependent chromatin remodeling of *PHO5* minichromosomes in vitro.

In vitro remodeling reactions were performed and samples processed as described in Materials and Methods. In (A), a Southern blot was probed with radiolabeled sequence derived from *PHO5* promoter sequence packaged into nucleosome -2. Chromatin remodeling of *PHO5* minichromosomes occurs in the presence of SWI/SNF, rPho2p, full-length rPho4p, and ATP. Little remodeling is seen when the Pho4p DNA-binding domain is added in place of full-length rPho4p. (B) The blot shown in (A) was stripped and reprobed with sequence corresponding to *PHO5* sequence approximately 1 Kb from its ATG. Much less remodeling is seen in lane 4 when this probe is used. This suggests that most SWI-SNF-dependent chromatin remodeling is localized to the *PHO5* promoter region of the minichromosomes. (C) and (D) are quantitative line analyses of (A) and (B).


A
MNase
1 2 3 4 5
ATP - + + + +
Pho4/2 + + - + -
DBD/2 - - - - +
SWI/SNF + - + + +
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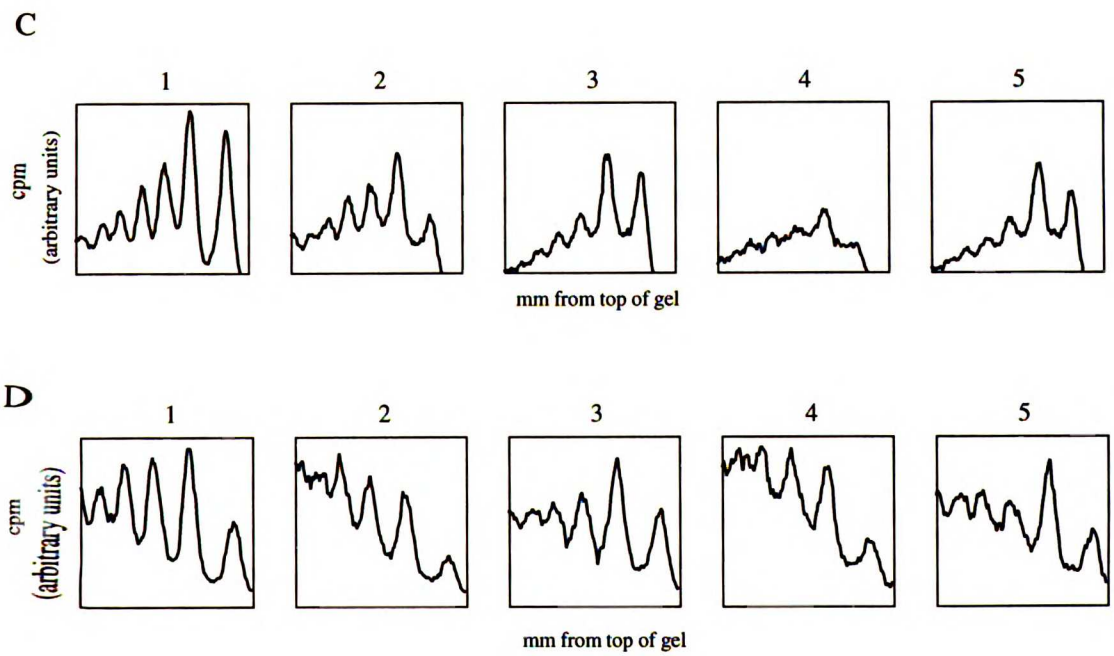
C
1 2 3 4 5
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phosphate-free media. Cell grown to mid-log phase in rich media were washed several times with water and then transferred to synthetic media lacking inorganic phosphate, SD-P_i. Aliquots of the cell culture were removed at various timepoints and total cellular RNA prepared. *PHO5* transcripts detected by Northern analysis were normalized to levels of *ACT1* mRNA present in the same samples.

PHO5 transcripts are detected within 30 minutes in wild type cells after transfer to SD-P_i (Fig. 10A). These results are consistent with those described previously (5, 216). In contrast, *PHO5* mRNA is not detected until 2 hours of phosphate starvation in a *snf6Δ* strain. The level of *PHO5* mRNA in the *snf6Δ* strain , normalized to the *ACT1* level, is approaching that of the wild type strain by three hours after induction. These data indicate that Snf6p is required for rapid derepression of *PHO5* expression upon phosphate starvation; but is not required for *PHO5* expression at later timepoints.

***SWI2* is required for rapid induction of *PHO5* upon starvation for phosphate.** Swi2p, the catalytic subunit of SWI/SNF, is required for SWI/SNF chromatin remodeling function (74, 84). To further test the role of SWI/SNF in *PHO5* expression, we looked at *PHO5* mRNA production in a *swi2Δ* strain harboring either a wild type or a temperature sensitive allele of *SWI2* on a multicopy plasmid (212). The results, shown in Figure 10B, are similar to those obtained with *snf6Δ* cells. *PHO5* mRNA is not induced until 1 hour after induction in the *swi2ts* strain, while a 4-fold increase is visible by 30 minutes in the strain harboring the wild type *SWI2* expression plasmid. The observed delay in induction in the *swi2ts* strain cannot be attributed to a slower growth rate, as both the wild type and the temperature sensitive strain grew at the same rate, even after 90 minutes of incubation at 37°C.

Long-term induction of *PHO5* does not require *SWI2*. The observation that components of the SWI/SNF complex are required for a rapid induction of *PHO5* transcript in response to phosphate limitation appears to conflict with previous reports that acid phosphatase activity levels under repressing and inducing condition are unaffected by

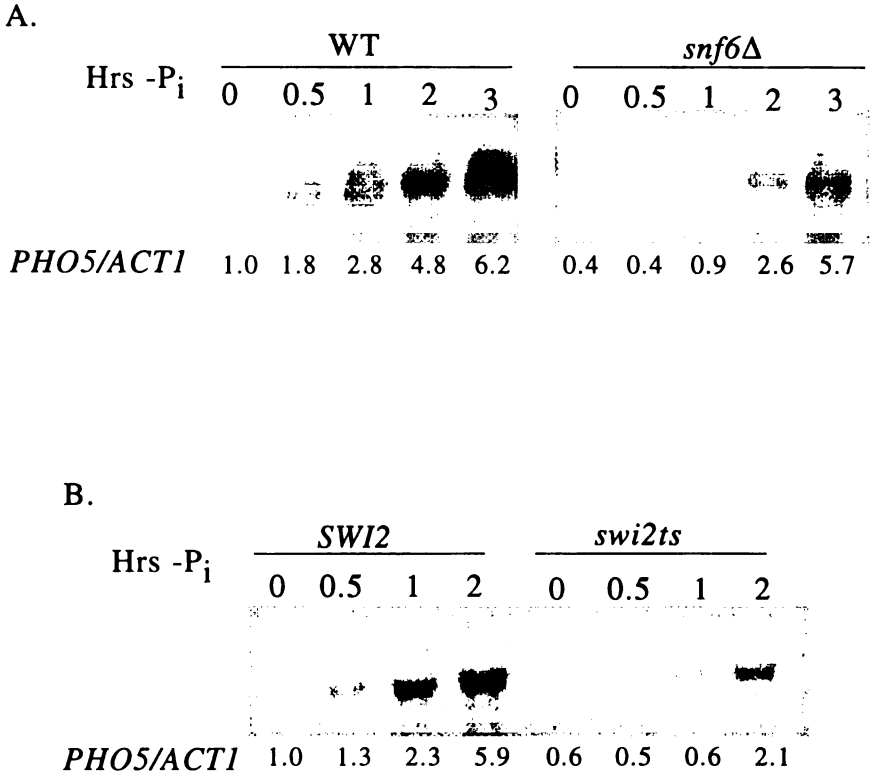


Figure 10. Northern analysis of endogenous *PHO5* induction in *snf6*Δ and *swi2ts* strains.

(A) Wild type and *snf6*Δ strains were transferred to SD-P_i and aliquots of the culture taken at 0, 30, 60 and 90 minutes. Total cellular RNA was prepared and *PHO5* transcript level in each sample quantified and normalized to the *ACT1* transcript level in that sample. (B) *swi2*Δ cells harboring a plasmid expressing either wild type *SWI2* or a *swi2ts* were analyzed as in (A) except that cultures were grown at 25°C to mid-log, then grown at 37°C for one hour before transfer to SD-P_i and further incubation at 37°C for the indicated times.

deletion of SWI genes. We therefore investigated the production of acid phosphatase activity (APase) in our wild type and *swi2ts* strains after longer periods of phosphate starvation. Cultures were grown in rich media at 25°C to mid-log phase, then transferred to 37°C for one hour. Cells were washed and transferred to pre-warmed synthetic media, either SD-P_i or SD +P_i, and grown for 6 or 16 hours at 37°C. A colorimetric acidphosphatase assay was used to quantify *PHO5* expression under these conditions, as described in (14).

The results are shown in Table 1. In a wild type strain, APase activity is induced approximately18-fold and 27-fold, after 6 hours and 16 of starvation, respectively. In the swi2ts strain, induction at 6 hours was 10-fold, significantly lower than wild type, and consistent with the delay observed at the early timepoints. However, after 16 hours of phosphate starvation, the level of APase activity is not significantly different between the two strains.

	Acid Phosphatase Units[a]			
Time	6 hrs		16 hrs	
Phosphate	+	-	+	-
WT	0.12	2.1	0.13	3.5
fold induction	18		27	
swi2 ts	0.14	1.5	0.14	3.2
fold induction	10		23	

[a] $U = \frac{OD_{420}}{OD_{600}}$

Table 1. Induction of APase activity in WT and *swi2ts* strains.

Induction of expression from UASp1-driven LacZ reporter is delayed. We considered that the observed defect in *PHO5* induction could result from a defect in the phosphate-sensitive signal transduction pathway that regulates Pho4p nuclear

localization. To test this, we analyzed the rate at which Pho4p was localized to the nucleus after phosphate starvation, in order to determine if the defect in induction could be attributed to this step. Pho4p is localized to the nucleus at the same rate in WT and in *snf6Δ* strains (data not shown), indicating that the delay in *PHO5* transcript production observed in *snf6Δ* strains is not due to a defect in phosphate starvation signal perception or transduction.

We attempted to further delineate the step in *PHO5* induction that requires SWI/SNF for rapid response. Although SWI/SNF is clearly implicated in chromatin remodeling, the possibility remains that it is generally required for rapid initiation or elongation of transcripts. Reports that yeast SWI/SNF is found in the RNAP holoenzyme (176), though controversial (57), suggest a more general role for this factor in transcription. We reasoned that expression from a promoter driven by multimerized Pho4p binding sites might not require chromatin remodeling. If induction of this reporter gene was not delayed in a SWI/SNF mutant, we could attribute the SWI/SNF-dependent delay to a defect in chromatin remodeling. We compared the rate of induction of a LacZ reporter plasmid driven by multimerized UASp1 (145) in a WT and a *snf6Δ* strain.

	β-gal units				
min in –Pi media	0	30	60	120	180
WT	5.5	24.5	41.4	198.7	294.2
snf6Δ	2.7	4.8	3.1	10.2	20.9

[a] $U = \frac{1000 \times (OD_{420})}{\text{time} \times \text{vol} \times OD_{600}}$

Table 2. Induction of a UASp1 reporter in wild type and *snf6Δ* strains.

Cells were grown in SP-P_i as described for Figure 10. At the indicated time points aliquots of the cultures were removed and β-gal enzyme activity assays performed. The

results, presented in Table 2, indicate that induction of the UASp1 reporter is delayed in a *snf6Δ* strain compared to wild type. In a wild type strain, we observed increased levels of β-gal activity after 30 minutes of starvation for phosphate. In a *snf6Δ* strain, however, a significant induction was not observed until 2 hours after transfer to SD-P_i. Thus, two possible interpretations of the data remain. Snf6p may be required for general rapid induction of transcription in response to phosphate starvation, in a manner unrelated to the chromatin state of the particular promoter in question. Alternatively, Snf6p may be required to oppose a repressive chromatin environment on the UASp1 reporter as well as on the chromosomal *PHO5* promoter.

Discussion

We present here an analysis of the contribution of the chromatin remodeling complex SWI/SNF to *PHO5* promoter chromatin remodeling, both in vivo and in vitro. Using *PHO5* minichromosomes and a nuclear extract fraction derived from wild type and mutant strains, we show that Snf6p, an integral component of SWI/SNF is not required for chromatin remodeling in vitro (Fig. 8). We were thus surprised to find that purified SWI/SNF is capable of remodeling *PHO5* minichromosomes in vitro in a rPho4p-, rPho2p-, and ATP-dependent manner (Fig. 9). This remodeling was restricted to the *PHO5* promoter region and behaved identically to the activity present in S(0.3) extract.

These results prompted a more detailed analysis of the requirement for SWI/SNF in *PHO5* induction in vivo. Interestingly, *SWI2* and *SNF6* are required for rapid induction of *PHO5* in vivo (Fig. 10), and the kinetic defect observed in these mutants occurs at a step subsequent to Pho4p nuclear localization. We were unable to determine whether the rate-limiting step is in chromatin remodeling or in another aspect of transcription, as it is also seen in the induction of Pho4p-driven reporter plasmids without characterized chromatin structure over the promoter (Table 2). We were able to reproduce previous results indicating that *swi-* mutants do not have significantly altered levels of acid phosphatase

activity after long-term phosphate starvation (Table 1), indicating that the defect we observe in the absence of SWI/SNF activity can be eventually overcome.

Redundancy in vitro. One explanation for these observations is that both SWI/SNF and another, unidentified factor are capable of remodeling *PHO5* chromatin. According to this model, SWI/SNF can remodel *PHO5* promoter chromatin in vitro but is not required because the second activity can compensate. Functional redundancy between two or more ATP-dependent chromatin remodeling machines would explain why *swi*⁻ or *snf*⁻ mutants are eventually capable of inducing *PHO5*. SWI/SNF may be required for the immediate response, and the second factor can only take over after some delay.

If this is the case, Pho4p may recruit SWI/SNF to the *PHO5* promoter. As described in the introduction, SWI/SNF may be recruited to specific promoters by acidic activation domains in vivo (197, 203, 204). This model for SWI/SNF action is consistent with our data and previously published data on the requirements for *PHO5* remodeling. If the Pho4p DNA binding domain alone is expressed in yeast in place of full-length Pho4p, no chromatin remodeling is seen (23). Thus, an activation domain appears to be required for remodeling *in vivo*; this is also seen in our *in vitro* remodeling reactions. However, this function is not specific to Pho4p, as the VP16 activation domain can substitute when fused to the Pho4p DNA-binding domain (23), and Gal4p can completely replace Pho4p if UASp1 is replaced with a Gal4p binding site (152). Perhaps the ability to interact with remodeling complexes is a common property of acidic activation domains, allowing several activators and several remodeling activities to function at the *PHO5* promoter. Figure 11A depicts this model schematically.

Missing the specific factor. A second interpretation of the data presented in this chapter is that a single, unidentified activity is required to remodel *PHO5* chromatin in vivo, but the in vitro system doesn't reproduce the in vivo situation accurately. It is possible that the in vitro system lacks a specificity factor that would make only the S(0.3) extract activity required in vivo but several activities able to function in vitro. For example,

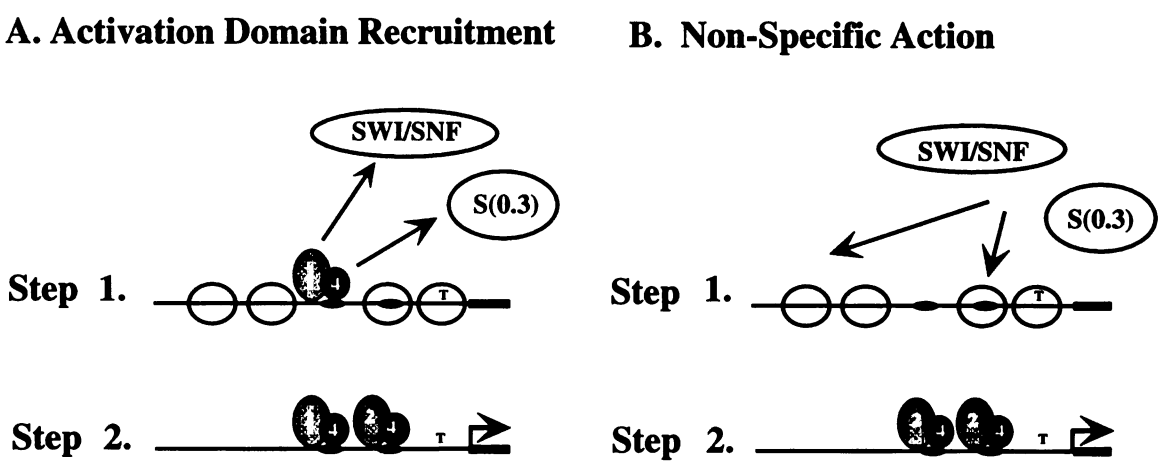


Figure 11. Two models to explain the sufficiency of SWI/SNF to remodel *PHO5* chromatin in vitro.

(A) The Pho4p and Pho2p transcriptional activators are able to bind the nucleosome-free region of the *PHO5* promoter in the absence of remodeling activities. The activation domain of Pho4 then recruits either the SWI/SNF complex or the complex contained in the S(0.3) fraction. These complexes remodel chromatin and allow occupation of the nucleosomal Pho4 site. Stable occupation of these sites allows recruitment of the RNA polymerase holoenzyme and activates transcription initiation. (B) Constitutive activity of either the SWI/SNF complex or the activity in the S(0.3) fraction at the *PHO5* promoter allows Pho4p and Pho2p to bind the promoter. The binding of Pho4p and Pho2p stabilizes a conformation of the promoter that is accessible to RNA polymerase, and transcriptional activation ensues.

chromatin remodeling in the presence of a specific repressor might require a specific remodeling complex. If such a protein were removed during template purification, chromatin remodeling would no longer be specific. Although we have taken considerable care to duplicate the physiological reaction *in vitro*, there are many ways in which such important components might be omitted from the reaction. In this case, the effect of SWI/SNF mutants on *PHO5* induction is easily explained as a general defect in initiating activated transcription, particularly as we saw a defect in induction from UASp1 reporter plasmids, which we would not expect to require chromatin remodeling.

Our result that SWI/SNF remodels chromatin in vitro would be explained by a second model of action illustrated in Figure 11B, wherein ATP-dependent remodeling machines are not recruited to the *PHO5* promoter, but constitutively modify all histone/DNA contacts (70). The resulting generalized loss of nucleosome stability might facilitate DNA binding by Pho4p, and as a result create an open chromatin conformation. The insufficiency of the DNA-binding domain of Pho4p to remodel chromatin might be explained if Pho4p's ability to bind chromatin is enhanced through interaction with Pho2p, as this requires a different domain of Pho4p (145). Improved binding of Pho4p to chromatinized UASp2 might facilitate the stabilization of an open conformation created by the ATP-dependent activities.

In summary, the genetic and biochemical analyses presented here do not present a clear picture of the role SWI/SNF plays in *PHO5* expression. Further investigations must be undertaken to identify the source of the delay in *PHO5* induction in the absence of *SWI2* and *SNF6*. A search for activities that are functionally redundant with SWI/SNF might focus on the identification and characterization of the activities present in the S(0.3) extract. A genetic approach to identify gene products required for *PHO5* expression may provide information as well.

CHAPTER THREE

GENETIC REQUIREMENTS FOR PHO5 INDUCTION

Introduction

The packaging of genomic DNA into chromatin compromises access by sequence-specific transcriptional activators (52). A dramatic indication of this phenomenon is observed at certain promoters, where a gross change in chromatin structure accompanies transcriptional activation. One example of this kind of promoter is the yeast *PHO5* gene (146, 217). We can infer from biochemical experiments that an activity in addition to the transcription factors Pho2p and Pho4p are required for the observed rearrangements of *PHO5* promoter chromatin (218).

Several previously characterized chromatin remodeling activities have been tested for defects in *PHO5* induction in vivo (109, 149-152), but these experiments have not definitively identified a single required activity. To extend these studies, we chose the simple and straightforward approach of reverse genetics to identify chromatin remodeling activities likely to be involved in the *PHO5* chromatin transition. *PHO5* expression was characterized in strains bearing null mutations in candidate activities. We tested two families of candidate genes, helicase-like ATPases from the SNF2 family, and histone acetyltransferases.

We have previously shown that a partially purified activity, termed S(0.3) and the chromatin remodeling activity SWI/SNF are capable of remodeling *PHO5* minichromosome promoter chromatin in an ATP- and transcription factor-dependent manner (181, 218). Therefore, one type of activity likely to be involved in *PHO5* chromatin transition in vivo is an ATP-dependent chromatin remodeling complex. Whether SWI/SNF is directly involved in the regulation of *PHO5* expression is not clear (see Chapter 2). However, a number of other ATP-dependent activities capable of modifying chromatin structure have been identified in S. cerevisiae, all of which are candidate activities for a role in *PHO5* chromatin remodeling. Each of these activities contains (or is) a member of the SNF2 family of proteins, which contain a helicase-like ATPase motif.

Members of this family are generally implicated in DNA-dependent processes such as transcriptional activation and repression, repair, and recombination (56).

RSC (remodels the structure of chromatin), a 1 MD complex estimated to be approximately 10 times more abundant than SWISNF, was purified from yeast extract on the basis of its similarity to SWI/SNF (57). Several of the approximately 15 components have related counterparts in SWI/SNF, including the ATPase Sth1p (strongly related to Swi2p/Snf2p), Sfh1p (Snf5), Swh3p/Rsc8p (Swi3), Rsc6p/Swp73p (180) (219-221). Unlike their counterparts in SWI/SNF, several of these gene products are essential for viability. RSC may exist in two forms within the cell, distinguished by their inclusion of Rsc3p (57), and Rsc1p or Rsc2p (222).

Though its biochemical properties are well studied, the precise physiological role of RSC remains to be determined. Data linking RSC to cell cycle control include the cell-cycle dependent phosphorylation of Sfh1p and the observation that conditional alleles of Sfh1p and Sth1p causes arrest in G2/M (83, 220). These cell cycle phenotypes may indicate a role for RSC in regulating specific chromatin structures during mitosis, as RSC mutants have centromere chromatin that shows increased accessibility to nucleases (223).

There is also evidence to suggest that RSC functions in transcriptional activation or repression. Sth1p is required for the expression of several meiotic genes (224), and non-essential RSC subunits display genetic interactions with components of the SAGA and SWI/SNF complexes (222). It was recently reported that depletion of two RSC components, Swh3 and Sth1p derepresses the *CHA1* gene (93). However, this derepression is activator independent and the *CHA1* gene is located very near the heterochromatic yeast mating type loci (225), suggesting that the effects may be indirect through silent chromatin rather than indicating a direct role for RSC in the repression.

In *S. cerevisiae* there are two members of the ISWI family of ATPases, named after the Drosophila gene that is found in NURF, CHRAC, and ACF. *ISW1* is found in a four subunit complex and is capable of ATP-dependent nucleosome array disruption, facilitation

of restriction enzyme accessibility, and nucleosome spacing. *ISW2* is in a complex with one other protein and can space nucleosomal arrays (226). In addition, *ISW2* is required for sporulation, implying that it may play a role in the expression of sporulation-specific genes, or in other chromatin-related events that take place during meiosis.(227). A clear role for these factors in the regulation of chromatin structure during transcriptional induction remains to be demonstrated.

Two other members of the SNF2 family of proteins in *S. cerevisiae* have been implicated in transcriptional regulation. Yeast Chd1p is a member of a protein family that contains a highly conserved motif called the chromodomain and a DNA binding domain as well as the ATPase/helicase domain common to all members of the SNF2 family (228). There are some indications that Chd1p may play a role in the regulation of transcription. Purified yeast Chd1p is capable of modifying the DNase digestion pattern of a mononucleosome; and DNA array analysis indicates that this gene is required for expression of a number of genes (229). In addition, yeast *CHD1* displays synthetic genetic interactions with several members of the SWI/SNF complex (182, 230). Another SNF2 family member, *INO80*, was identified in a screen for inositol auxotrophs. Ino80p is required for full induction of a number of reporter genes, and is found in a large molecular weight complex in yeast extracts (230).

There is also evidence that promoter *PHO5* induction involves histone acetylation. Both Rpd3p and Sin3p, components of a histone deacetylase complex (231, 232) are required for repression and for full activation of *PHO5* (233, 234), implying that histone deacetylation activity is required to maintain a repressive chromatin structure at the *PHO5* promoter. If so, it may be inferred that histone acetylation is required for *PHO5* induction as well. Indeed, there is indirect evidence for this model: the histone H4 N-terminal residues 4-23, which contain the invariant acetylatable lysine residues, are required for full induction of *PHO5* (40).

The histone acetyltransferase Gcn5p may play a role as well. Deletion of the cyclin *PHO80* prevents phosphorylation of Pho4p and allows constitutive expression of *PHO5* in high phosphate. Under these conditions, a *gcn5Δ* strain exhibits unusual *PHO5* promoter chromatin structure. Further, a crippled *PHO5* promoter lacking one of the UAS elements requires GCN5 for expression in response to phosphate starvation (152). Nevertheless, in a *PHO80+* strain, wild type *PHO5* mRNA levels in high and low phosphate are unaffected by deletion of *GCN5* (109, 152).

Multiple complexes containing Gcn5p have been purified from yeast (108-110); a large 1.8 MD complex, termed SAGA and a smaller 0.8 MD complex termed ADA have been well characterized. SAGA contains a large number of polypeptides, which can be divided into at least four subgroups: the Ada subgroup (Ada2p, Ada3p, Gcn5p) which are essential for acetyltransferase activity; the SPT subgroup (Spt3p, Spt8p, Spt20/Ada5p, and Spt7p) which interact with TBP and are critical for structural integrity of the complex; a set of histone-like TAFs (TAF_{II} 60, 17, and 61) and other TAFs (TAF_{II} 90 and 25) which appear to be required for interaction with chromatin (235); and Tra1p, a homologue of mammalian TRRAP, a c-Myc –associated transcriptional cofactor (236, 237). The ADA complex also contains Gcn5p, Ada2p and Ada3p. ADA does not appear to be a subcomplex of SAGA, however. The Ahc1 protein is found in and required for the stability of ADA but is not part of SAGA and does not affect its stability (238). Both SAGA and ADA can potentiate activated transcription from in vitro assembled nucleosome arrays (239), but only SAGA is capable of directly binding acidic activators (177).

A number of other complexes with HAT activity have been identified and are implicated in transcriptional regulation. The NuA4 complex is approximately 1.3 MD and preferentially acetylates histone H4 (124). The catalytic subunit of NuA4 is Esa1p, an essential acetyltransferase from the MYST family (122-124). The NuA4 complex shares many in vitro characteristics with SAGA: it is capable of interacting with acidic activation domains and potentiating transcriptional activation (177). *SAS3* was identified as an

enhancer of the silencing defects observed in sir1 mutants (119), and encodes another member of the MYST family with in vitro HAT activity (126). The elongator complex, a recently described complex which associates with the phosphorylated CTD of RNAP holoenzyme, contains a HAT that is essential for its function (240, 241).

The results presented in Chapter 2 suggested that SWI/SNF plays a role in *PHO5* chromatin remodeling and induction, but that it is not absolutely required. Furthermore, in vitro chromatin remodeling of *PHO5* minichromosomes required ATP (Chapter 1). We therefore considered that a second ATP-dependent chromatin remodeling activity might play a role in *PHO5* induction. We chose to assess the role in *PHO5* induction played by 7 members of the SNF2 family of helicase-like ATPases most similar to Snf2p, including Sth1p, Isw1p, Isw2p, Chd1p, Ino80p, and two putative ORFs, YDR334W and YFR038W. We also tested the *PHO5* expression requirements for Gcn5p and Sas3p, two histone acetyltransferases known to be present in complexes capable of acetylating nucleosomes. These gene products, the complexes they are found in, and associated activities are listed in Table 3.

Table 3. Chromatin remodeling and modifying activities employed in this study

NAME	COMPLEX	SIZE	FUNCTION
SNF2/SWI2	SWI/SNF	2MD	Required for expression from a subset of yeast promoters (Winston, 1992); Nucleosome disruption, facilitates factor binding (Cairns, 1998); Facilitates in vitro transcription (Neely, 1999); Histone sliding (Whitehouse, 1999)
STH1	RSC	1MD	Arrest in G2/M transition (Du, 1998); Nucleosome disruption (Cairns, 1996).
ISW1	ISW1		Nucleosome disruption, spacing, nuclease digestion (Tsukiyama, 1999)
ISW2	ISW2		Nucleosome spacing (Tsukiyama, 1999)
CHD1	Maybe		Synthetic lethal with SWI/SNF components, mononucleosome disruption (Tran, 2000)
INO80	Maybe		Required for maximal expression from several inducible UAS elements (Ebbert, 1999)
YDR334W			None known
YFR038W			None known
GCN5	SAGA	1.8	Required for expression from a subset of yeast promoters (Grant, 1998); Promoter-proximal acetylation of nucleosomal histones H3 and H4 (Kuo, 1996); Directed by transcriptional activators (Utley, 1998); Facilitates in vitro transcription (Steger, 1998).
SAS3			Regulator of telomeric silencing (Reifsnyder, 1996); Acetylation H2A, H3, and H4 (Takechi, 1999)
RPD3	SIN3		Required for repression of a subset of yeast promoters (Vidal, 1991); Promoter-proximal deacetylation of nucleosomal histone H3 and recruited by gene-specific repressors (Kadosh, 1997)

Materials and Methods

Media and strains. Growth and phosphate starvation media were used as described in Chapter 2. *S. cerevisiae* strain YS18 (10) was used in all experiments. *S. cerevisiae* strains and plasmids used in this study are summarized in Table 2.

To generate strains EY0646, EY0636, and EY0638, standard gene replacement techniques were used (242). EY0258 (wild type, diploid) was transformed with pT7-isw1 cut with Bam HI, pBS-isw2 cut with Pst I and Hind III, or pAJ731 cut with Bam HI. After sporulation, correct integrants were identified by southern blot analysis. Similarly, EY0657 was generated by transforming EY0258 with pDJ63 cut with Eco RI and Xba I. For analysis of *STH1* function, EY0258 was transformed with p137-1-10 and, after sporulation, *sth1Δ* cells harboring p137-1-10 were selected.

pINO80dv was constructed as follows. A 1200 bp fusion PCR product, consisting of 500 base pairs of upstream and 450 base pairs of downstream *INO80* sequence joined with a Bam HI site, was inserted into pBluescript. Then 1700 base pair Bam HI/Bgl II fragment of the *HIS3* gene from EB0098 was inserted into the Bam HI site of this vector. EY0258 was transformed with an Ngo MI and Sal I fragment of this construct and a correct integrant, EY0632, identified by Southern blot. pINO80 was generated by amplifying two fragments (one containing sequence from bp –480 to the Xba I site at bp 2554; the other containing sequence from the Xba site to bp 4470) and inserting them, connected at the Xba I site, into the Bam HI and Eco RI sites of pRS316. EY0632 was transformed with pRSINO80 and spores requiring pRSINO80 for viability were selected for analysis of *INO80* function.

p334dv was created by amplifying two fragments, containing sequence from bp (–511 to –1(and bp (6240 to 6829) of YDR334W. These PCR fragments were inserted into the Xba I and Hind III sites of pRS306. A Bam HI/Bgl II fragment of *TRP1* from EB0103 was inserted into the Bam HI site of this construct to make p334dv. To create EY0660, EY0258 was transformed with a Sal I/Not I digestion of p334dv and correct

spores identified by genomic PCR. Similarly, pGCN5dv was created by amplifying and fusing sequence from bp (-500 to –1) and bp (1321 to 1830) of *GCN5*. The fusion PCR product was inserted into pRS306; this construct was cut with Bam HI to integrate into EY0258. A correct spore was identified by Southern blot and this strain named EY0716.

EY0662, EY0686, EY0730,and EY0770 were generated by standard strain construction techniques (242); mating the appropriate single null strains, selecting diploids, sporulating, and identifying spores with the desired segregating traits. To generate strains EY0717 and EY0720, we employed PCR-based gene disruption (the templates were a kind gift of Sean O'Rourke). *SAS3* was disrupted in EY0258 with a PCR product containing *C. glabrata HIS3* and *RPD3* with a PCR product containing *K. lactis URA3*. EY0662, EY0686, EY0730,and EY0770 were generated by standard strain construction techniques (242); mating the appropriate single null strains, selecting diploids, sporulating, and identifying spores with the desired segretated traits.

Temperature sensitive alleles. A Hind III/Bam HI fragment containing the *INO80* promoter and open reading frame from pRSINO80 was transferred to pRS315 to create pRSINO80B. pRSINO80B was subjected to in vitro hydroxylamine mutatgenesis as in (243). EY0632 harboring pRSINO80 was transformed with the mutant library, and a transformant capable of growth on 5-FOA at 25°C but inviable at 37°C was identified. The responsible plasmid was rescued and named pRSINO80ts.

A temperature sensitive allele of YFR038W was generated as follows. First, YFR038W sequence from 2 to 1100 was amplified from yeast genomic DNA by PCR and inserted into the Hind III/Xho I sites of pPW66R. This construct was cut with Bcl I (in the middle of the YFR038W sequence) and integrated into the genome of EY0258, replacing the YFR038W promoter with the *CUP1* promoter and fusing a heat-inducible degradation signal derived from DHFR to the N-terminus of the YFR038W open reading frame. After sporulation, temperature sensitive spores were selected and tested for appropriate integration by genomic PCR.

Northern analysis and enzyme activity assays. Northern analysis, acid phosphatase assays, and β-gal activity assays were performed as described in Chapter 2.

Table 4. *S. cerevisiae* strains used in this study

Strain	Genotype[a]
EY0244	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR*
EY0646	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR isw1Δ:: URA3*
EY0636	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR isw2Δ:: LEU2*
EY0638	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR chd1Δ:: HIS3*
EY0662	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR isw1Δ:: URA3 isw2Δ:: LEU2 chd1Δ:: HIS3*
EY0660	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR YDR334WΔ:: TRP1*
EY0686	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR isw1Δ :: URA3 isw2Δ:: LEU2 chd1Δ:: HIS3 YDR334WΔ:: TRP1*
EY0632	*MAT A/α his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR ino80Δ/INO80*
EY0657	*MAT A/α his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR sth1Δ/STH1*
EY0683	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR YDR038W ts degron*
EY0716	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR gcn5Δ::LEU2*
EY0717	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR sas3Δ::cg HIS3*
EY0720	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR rpd3Δ:: kl URA*
EY0730	*MAT A his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR gcn5Δ::LEU2 rpd3Δ::URA3*
EY0770	*MAT A/α his3-11, 15 leu2-3, 112 trp1Δ99 ura3Δ-432 canR gcn5Δ swi2Δ/SWI2*

[a] all strains used are derived from YS18 (Senstag, 1987).

Table 5. Plasmids used in this study

Plasmid	Description	Source/Reference
pT7-isw1	isw1:: URA3 gene replacement vector	Toshio Tsukiyama
pBS-isw2	isw2:: LEU2 gene replacement vector	Toshio Tsukiyama
PAJ731	chd1:: HIS3 gene replacement vector	Hien Tran
p334dv	YDR334W:: TRP1 gene replacement vector	This study
pINO80dv	ino80:: HIS3 gene replacement vector	This study
pRSINO80	*INO80* in pRS315	This study
PRSINO80B	*INO80* in pRS316	This study
pRSino80ts	*ino80ts* in pRS315	This study
pDJ63	Sth1-D2::HIS3 in pUC19 gene replacement vector	Du, 1998
p137-1-10	Sth1-3ts in pRS314	Du, 1998
p38tsdegron	YFR038::URA3 ts degron integration vector	This study
pSWI2d	swi2::HIS3 gene replacement vector	Schneider, 1995
pRS-SWI2	*SWI2* in pRS315	Biggar, 1999
pRS-6.3	*swi2ts* in pRS315	Biggar, 1999
pGCN5dv	gcn5::LEU2 gene replacement vector	This study

Results

***ISW1*, *ISW2*, *CHD1*, and YDR334W are dispensable for *PHO5* induction.** Four of the SNF2 family members selected for analysis are not essential for viability: *ISW1*, *ISW2*, *CHD1*, and the putative YDR334W (226, 229), data not shown). Yeast strains carrying null mutations in each of these four genes were generated and then tested for their ability to induce *PHO5* expression. Cultures were grown to mid-log phase in rich media, then washed several times with water and transferred to media lacking inorganic phosphate. Samples of this culture were taken at the indicated time points and total cellular RNA isolated. These samples were then analyzed by Northern for production of *PHO5* mRNA, the levels quantified and normalized to the levels of *ACTIN* mRNA in the same sample. The results, shown in Figure 12A, demonstrate that deletion of *ISW1*, *ISW2*, *CHD1*, or YDR334W does not significantly affect the rate at which *PHO5* expression is induced, or the approximate level of *PHO5* mRNA produced at each time point.

An *isw1Δ isw2Δ chd1Δ* YDR334WΔ strain cannot induce *PHO5* expression. The results shown in Figure 12A suggest that *ISW1*, *ISW2*, *CHD1*, and YDR334W do not play a role in *PHO5* induction. Alternatively, one or more of these genes might be involved, but functionally redundant. *ISW1*, *ISW2*, and *CHD1* are synthetically sensitive to environmental stresses such as high temperature (226); *ISW1* and YDR334W are synthetically temperature sensitive (data not shown), indicating that these gene products may be involved in a common cellular process. To test if these activities have overlapping or redundant functions, a triple mutant (*isw1Δ isw2Δ chd1Δ*) and a quadruple mutant (*isw1Δ isw2Δ chd1Δ YDR334WΔ*) were constructed and tested for *PHO5* mRNA production under inducing conditions. Interestingly, the triple mutant showed severely reduced levels of *PHO5* mRNA compared to the wild type strain (approximately a 2-fold induction, and the quadruple mutant did not significantly induce *PHO5* in the time provided (Figure 12B). It is notable that the *isw1Δ isw2Δ chd1Δ*

YDR334WΔ strain exhibited temperature sensitive growth and grew very slowly even at 25°C (data not shown).

***STH1, INO80*, and YFR038W do not affect *PHO5* expression.** *STH1* was previously determined to be required for cell viability (57). Though *INO80* was reported to be non-essential (230), we found that this gene is essential in our strain background. We also found that YFR038W is essential for cell growth (data not shown, Figure 13A). To test if these essential SNF2 paralogues are required for the induction of *PHO5* expression upon phosphate starvation, we obtained or generated temperature sensitive alleles of each gene. Temperature sensitive alleles of *INO80* and YFR038W were generated as described in Materials and Methods. A temperature sensitive allele of *STH1* was provided by Du and Laurent (83). Strains carrying conditional alleles of YFR038W, *INO80*, and *STH1* as the only allele were tested for growth on solid media at 25°C and 37°C. These strains grew well at 25°C but no growth was observed at 37°C (Figure 13A).

Strains carrying these temperature sensitive alleles were assayed by Northern analysis for their ability to induce *PHO5* transcription at the nonpermissive temperature. Cultures were grown to mid-log phase at 25°C, then transferred to 37°C for an hour before washing and transfer to SD –Pi. Interestingly, none of these genes are defective in *PHO5* mRNA production at the non-permissive temperature (Figure 13B and C). Ebbert et al. reported that *INO80* is required for maximal induced expression from a UASp1-driven reporter (230). We believe that this defect in expression is specific to multicopy plasmids, as we do not see a defect in induction of acid phosphatase activity in our strain or in the *ino80Δ* or *ino80ts* strain used by Ebbert et al. (data not shown). The data presented inFigures 12 and 13 demonstrate that deletion of any of the 7 SNF2 family members we tested does not significantly affect *PHO5* induction kinetics.

***GCN5*, but not *SAS3*, is required for rapid induction of *PHO5*.** We tested two histone acetyltransferases for effects on *PHO5* expression, *GCN5* and *SAS3*. Null mutants in each gene were generated and their ability to produce *PHO5* mRNA in

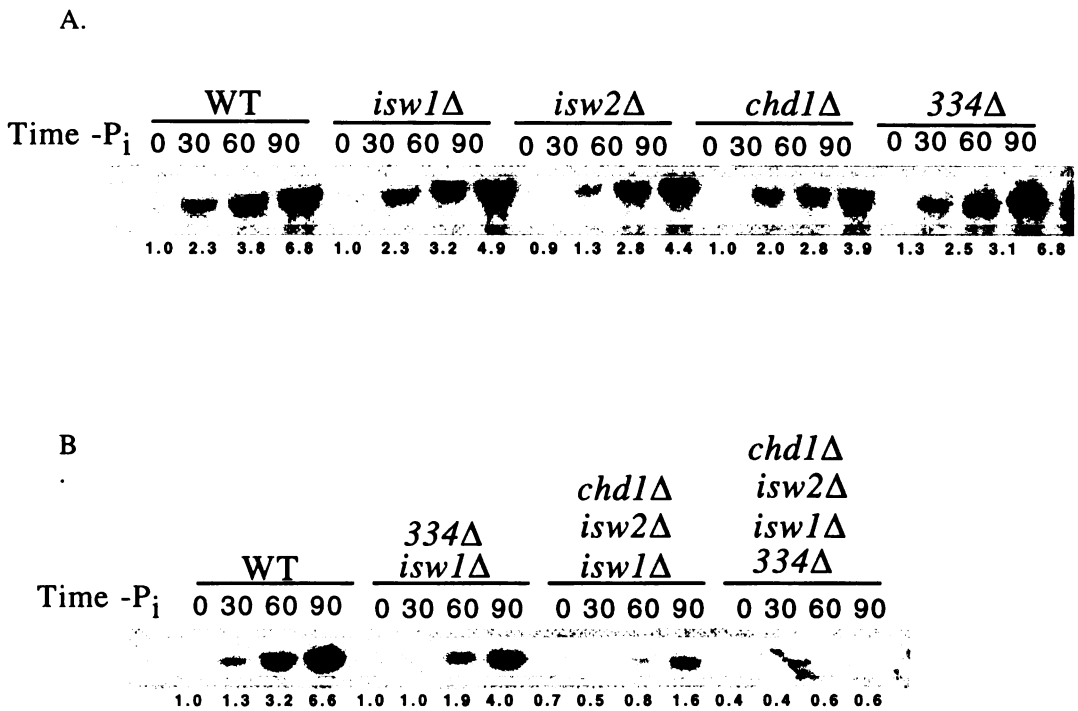


Figure 12. Contribution of *ISW1, ISW2, CHD1* and YDR334W to *PHO5* induction.

(A) Wild type, *isw1*Δ, *isw2*Δ, *chd1*Δ, and YDR334WΔ strains were transferred to SD-P_i and aliquots of the culture taken at 0, 30, 60 and 90 minutes. Total cellular RNA was prepared and *PHO5* transcript level in each sample quantified and normalized to the *ACT1* transcript level in that sample. (B) Wild type, and mutant strains were analyzed as in (A) except that cultures were grown at 25°C for the duration of the experiment.

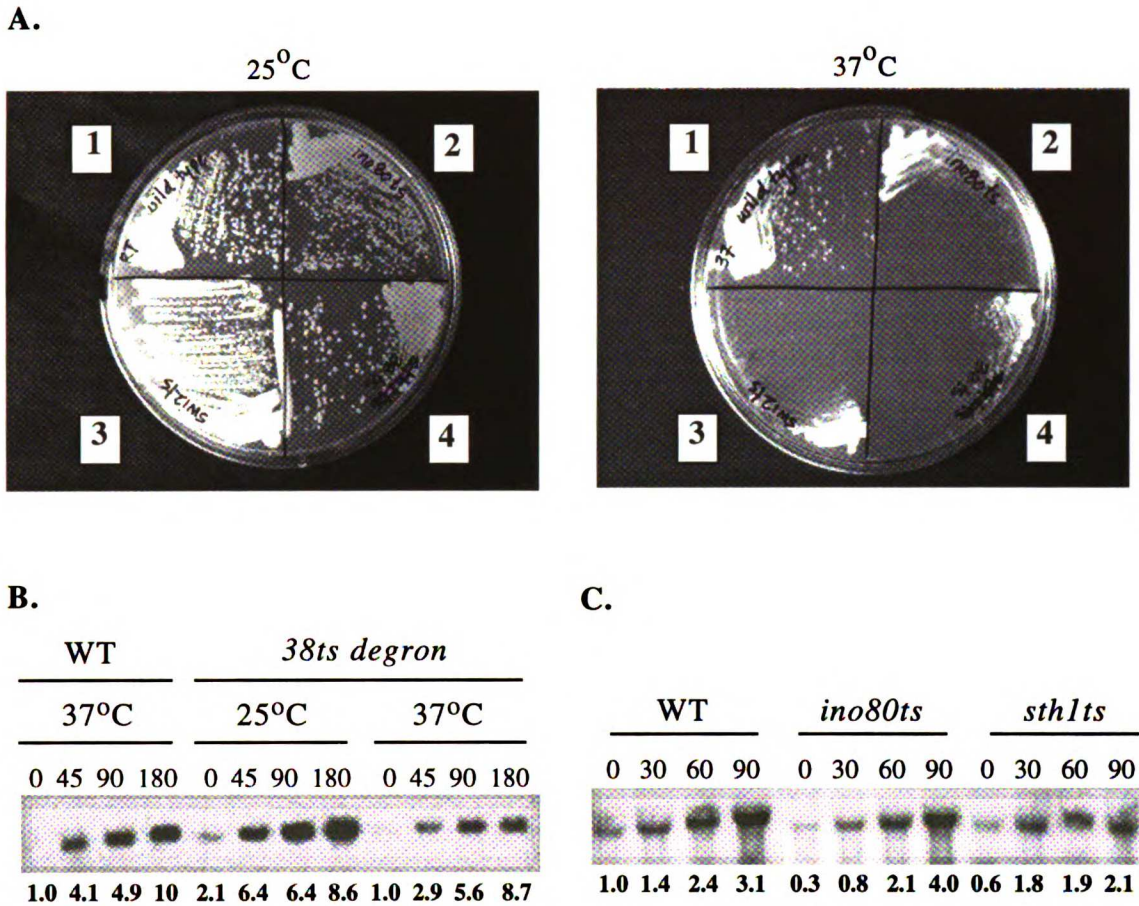


Figure 13. Contribution of *INO80*, *STH1*, and YFR038W to *PHO5* induction. (A) Wild type (1), i*no80ts* (2), YFR038W ts degron (3), and swi2*ts* (4) strains were incubated on YEPD plates at 25°C and 37°C. (B) Wild type and YFR038W ts degron strains were grown at 25°C to mid-log phase, then either incubated at 37°C for one hour before transfer to SD-P_i and further incubation at 37°C for the indicated times, or left at 25°C. (C) Wild type, *ino80ts*, *sth1ts* strains were treated as in (B).

response to starvation of inorganic phosphate assayed by Northern analysis as described above (Fig. 14A). Surprisingly, a *gcn5Δ* strain exhibits a delay in the production of *PHO5* mRNA. This effect is specific to *GCN5*, as the *sas3Δ* mutant strain induced *PHO5* expression normally. These data suggest that acetylation by a Gcn5p-containing complex is required for the rapid induction of *PHO5*.

***RPD3* does not oppose *GCN5* action in *PHO5* induction.** Several previous reports have demonstrated that the histone deacetylase gene *RPD3* is required for repression of *PHO5* (233, 234). We therefore hypothesized that the effects of Gcn5p and Rpd3p on the acetylation state of the *PHO5* promoter might oppose each other, as they do during the process of sporulation (244). If this were the case, we might expect that deletion of *RPD3* would suppress the *PHO5* induction defect of a *gcn5Δ* strain. To test this, we constructed a *rpd3Δ gcn5Δ* mutant and assayed *PHO5* mRNA production in response to phosphate starvation. The results, shown in Figure 14B, indicate that deletion of *RPD3* does not alleviate the defect of a *gcn5Δ* strain. The reverse is true; *PHO5* transcript levels are significantly lower in the *rpd3Δ gcn5Δ* than in the *gcn5Δ* strain. It was previously reported that *PHO5* induction is delayed in the absence of Rpd3p (233). Although we did not observe a significant delay in *PHO5* expression in our *rpd3Δ* strain, the fact that removing Rpd3p in a *gcn5Δ* background enhances rather than allays this defect suggests that Rpd3p may play a positive role in *PHO5* activation.

***SWI2* and *GCN5* have overlapping functions in *PHO5* induction.** We have demonstrated that both *gcn5Δ* and *swi2 ts* strains exhibit a delay in *PHO5* mRNA production upon phosphate starvation. However, as *PHO5* expression was not eliminated in either strain, we considered the possibility that SWI/SNF and Gcn5p act at *PHO5* in partially redundant manner. To test this hypothesis, we constructed a strain carrying null mutations in both *SWI2* and *GCN5*, harboring a *swi2ts* allele on a multicopy plasmid. Cultures were treated as for Fig 13 and Northern analysis performed.

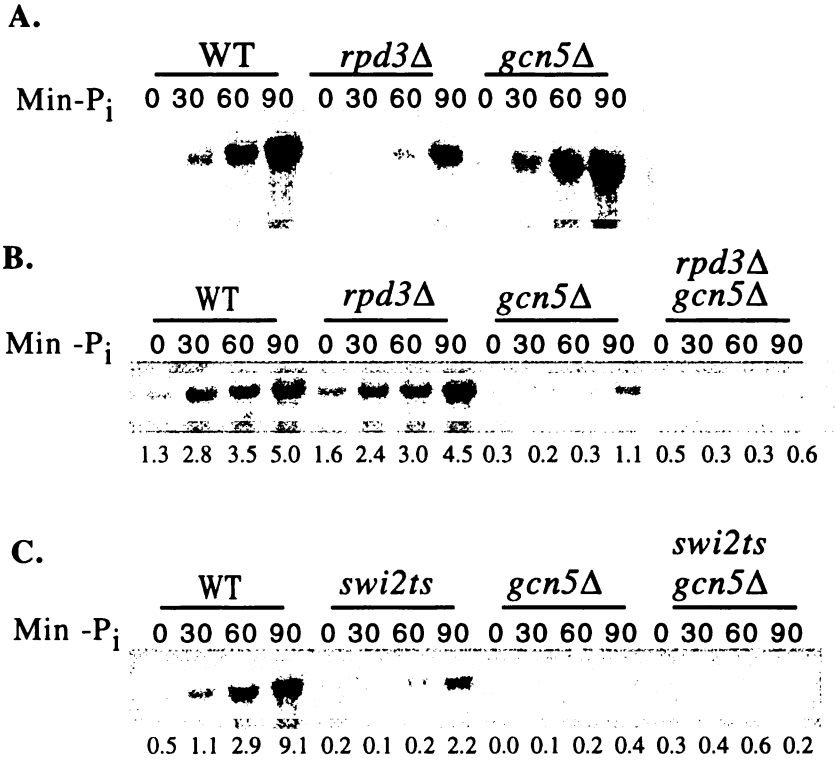


Figure 14. Contribution of *GCN5, SAS3, RPD3* and *SWI2* to *PHO5* induction.

(A) Wild type, *gcn5*Δ, and *sas3*Δ strains were transferred to SD-P_i and aliquots of the culture taken at 0, 30, 60 and 90 minutes. Total cellular RNA was prepared and *PHO5* transcript level in each sample quantified and normalized to the *ACT1* transcript level in that sample. (B) Wild type and mutant strains were analyzed as in (A). (C) Wild type, *gcn5*Δ, and *swi2*Δ or *swi2*Δ*gcn5*Δ strains harboring a plasmid expressing a *swi2ts* allele were analyzed as in (A) except that cultures were grown at 25°C to mid-log, then grown at 37°C for one hour before transfer to SD-P_i and further incubation at 37°C for the indicated times.

The results are presented in Figure 14C. *PHO5* mRNA levels are first detectable by 30 minutes in a wild type strain. In *gcn5Δ* or *swi2-ts* strains grown at the non-permissive temperature, *PHO5* transcript levels are detectable at 2 hours. In the *gcn5Δ swi2-ts* strain, no *PHO5* expression is detected during the course of the experiment. These results suggest that SWI/SNF and Gcn5p may act in a redundant manner to mediate *PHO5* gene expression (see Discussion).

Long Term Induction. As described in Chapter 2, *SWI2* is required for a rapid response to phosphate starvation, but not for production of acid phosphatase (APase) activity in the long term (Fig. 10). We therefore tested long-term induction of *PHO5* activity in a *gcn5Δ* strain, in the presence and absence of Swi2p function. Acid phosphatase activities were performed on cell samples 6 and 16 hours after transfer to SD-P_i or SD +P_i. Interestingly, *gcn5Δ* cells induce lower than wild type levels of APase after 6 hours of induction, but reach wild type levels by 16 hours (Table 6). This pattern is reminiscent of that seen in a swi2ts strain, and indicates that both Swi2p and Gcn5p are required for the rapid response but are not required for long term induction of *PHO5* transcripts.

	Acid Phosphatase Units			
Time	6 hrs		16 hrs	
Phosphate	+	-	+	-
WT	0.12	2.1	0.13	3.5
fold induction	18		27	
gcn5Δ	0.15	0.94	0.10	2.2
fold induction	6.3		22	
swi2 ts gcn5 Δ	0.17	0.66	0.13	0.99
fold induction	3.8		7.6	

Table 6. Contribution of Gcn5p and Swi2p to the induction of APase activity.

When Swi2p activity is removed in a *gcn5Δ* background, APase activity is induced only 8-fold after 16 hours of starvation, compared to 27-fold in a wild type strain. This significant decrease in APase activity supports a model wherein Swi2p and Gcn5p act independently to mediate rapid induction of *PHO5* expression, but act redundantly at later timepoints.

Discussion

We have investigated the role seven members of the SNF2 family of helicase-like ATPases, including *STH1, INO80, ISW1, ISW2, CHD1,* YDR334W, YFR038W may play in the induction of *PHO5*. It is worth noting that four of the seven family members we tested display some type of chromatin remodeling activity in vitro. Only *STH1* and *INO80* have been linked to the expression of specific genes. Our experiments indicate that *PHO5* chromatin remodeling does not require these gene products or the complexes in which they putatively function (Fig. 12 and 13); although these activities may be involved, they are not required. We also assessed the contribution of two yeast HATs to *PHO5* induction in vivo. We found that *SAS3* does not affect the kinetics or levels of *PHO5* mRNA production, but that *gcn5Δ* mutants display a delay in *PHO5* mRNA production (Fig. 14).

The effect of slow growth on *PHO5* induction. A defect in the kinetics of induction was observed in combinations between the nonessential mutants. We noticed that the level of this defect correlates precisely with the growth rate of these strains; an *isw1Δ isw2Δ chd1Δ* strain was relatively slow at inducing *PHO5*, while a *isw1Δ isw2Δ chd1Δ YDR334WΔ* strain did not produce detectable levels of *PHO5* mRNA in during the course of the experiment (Fig. 3 B). One model to explain this data is that induction of *PHO5* is sensitive to slow growth. However, *PHO5* chromatin remodeling can occur in the absence of cell division or DNA replication (147). Furthermore, a strain harboring a conditional allele of Sth1p, which fails to divide at all upon transfer to 37°C (83), shows no

defect in *PHO5* induction. We favor the interpretation that *isw1Δ isw2Δ chd1Δ YDR334WΔ* are generally sick and as a result do not respond to phosphate starvation. We have observed that *PHO5* induction in response to phosphate starvation will not proceed when cells are starved for amino acids or treated with alpha factor (data not shown). However, we cannot rule out the possibility that Isw1p, Isw2p, Chd1p and the putative protein encoded by YDR334W all contribute additively to *PHO5* chromatin remodeling or another aspect of transcriptional induction.

Slow growth does not always results in a defect in *PHO5* induction. The temperature sensitive YFR038W ts degron and sth1ts strains are slow growing at room temperature and stop cell division almost immediately upon transfer to 37°C. *PHO5* induction is unaffected in these strains. As both Swi2p and Gcn5p are already implicated in the regulation of transcription initiation, we believe that the observed defect in *PHO5* induction in *swi2ts* and *gcn5Δ* strains is specific. However, further experimentation is required to resolve this issue.

The SWI/SNF complex and Gcn5p have redundant functions. Several recent reports have suggested that ATP-dependent chromatin remodeling complexes and histone acetyltransferase complexes have partially overlapping functions in the regulation of gene expression (109, 212, 245, 246). Mutations in components of the ATP-dependent chromatin remodeling complex SWI/SNF are synthetically lethal (or synthetically sick, depending on the strain background) with mutations in the histone acetyltransferase Gcn5p. While expression of *HO* and *SUC2* requires both *GCN5* and the SWI/SNF complex, other genes, such as *GAL1* and *ACT1*, only require SWI/SNF in the absence of *GCN5*, and vice versa. Still other genes, such as *TUB2,* are expressed independent of either factor, though it remains to be seen whether this category includes any inducible genes. We show here that *PHO5* can be placed in the second category. Gcn5p and Swi2p are required for rapid induction of *PHO5*. When Swi2p is inactivated in a *gcn5Δ* background, we observed a severe defect in *PHO5* transcript production (Fig. 3C).

How exactly SWI/SNF and Gcn5p might cooperate to facilitate transcription in the context of chromatin is unknown. SWI/SNF might act first, to make the histone tails available for acetylation by Gcn5p. This covalent modification might then stabilize an open, or accessible chromatin state (62). This model is supported by the results of chromatin immunoprecipitation experiments with the HO promoter. To activate HO expression, the transcriptional activator Swi5p binds first, followed by SWI/SNF. Binding by SWI/SNF is required for binding by SAGA (209) (209, 247). On the other hand, acetylases may act first, providing a signal that is recognized by SWI/SNF. Hyperacetylated regions of the genome may act as multi-nucleosomal signals for SWI/SNF activity; or acetylation of histones may simply facilitate SWI/SNF action.

Long term induction. In a strain lacking both Swi2p and Gcn5p, no *PHO5* mRNA is detected within 3 hours after transfer to medium lacking inorganic phosphate. A low level of induced acid phosphatase activity is detected after several hours of starvation, and by 16 hours a 7-fold induction of this activity is detected. What might be mediating *PHO5* expression at the longer time points? Perhaps Pho4p and Pho2p, or a non-specific ATP-dependent chromatin remodeling complex or a HAT can remodel *PHO5* chromatin, given enough time. Another possibility is that DNA replication, although not required for *PHO5* chromatin remodeling, provides a window of opportunity wherein Pho4p can direct the assembly of a preinitiation complex on recently duplicated DNA that has not yet been fully assembled into nucleosomes.

CONCLUSION

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 (50-52, 248). A number of cellular activities capable of facilitating factor binding to chromatin have been identified (249). Though these activities are thought to function by directly modifying chromatin structure, the mechanism by which they function is still under investigation.

In my thesis work, I have attempted to determine how transcription takes place in the context of chromatin. I describe here biochemical and genetic investigations into the chromatin remodeling process that occurs at the yeast *PHO5* promoter upon induction in response to starvation for inorganic phosphate. I developed a system in which *PHO5* chromatin remodeling is recapitulated in vitro. Pho4p and Pho2p are not sufficient to remodel chromatin in this system, implying that at least one other factor in addition to Pho2p and Pho4p is required for the *PHO5* chromatin transition in vivo.

A candidate for this activity is the ATP-dependent chromatin remodeling complex SWI/SNF. I analyzed the contribution of the ATP-dependent chromatin remodeling complex SWI/SNF to *PHO5* chromatin remodeling in vitro, and conclude that SWI/SNF is sufficient but not required for *PHO5* minichromosome chromatin remodeling in the in vitro system. SWI/SNF also plays a complicated role in *PHO5* expression in vivo. Several of its subunits are required for rapid induction upon phosphate starvation; however, SWI/SNF is not absolutely required for *PHO5* expression, as transcript levels in a *swi*- mutant eventually approach those of a wild type strain after extended induction.

A similar pattern of *PHO5* mRNA production is observed in a strain carrying a null allele of the histone acetyltransferase gene *GCN5*. Strikingly, when Swi2p function is removed in a *gcn5*Δ background, a severe defect in the production of *PHO5* mRNA is

observed, and only a modest level of *PHO5* mRNA is produced, even after 16 hours of phosphate starvation. These data support a model wherein SWI/SNF and Gcn5p act in a redundant manner to mediate chromatin remodeling at the *PHO5* promoter during the rapid response to starvation for inorganic phosphate. Below, we further discuss this model in the context of the results presented here and two recent reports from other laboratories.

***PHO5* versus *PHO8*.** Both the SWI/SNF and SAGA complexes are required for chromatin rearrangements at the *PHO8* promoter. *PHO8* encodes an alkaline phosphatase, and its expression is weakly regulated by phosphate through two Pho4p binding sites, one high affinity and one low affinity (250). These sites are presumed to be constitutively accessible for Pho4p binding, as they are located in linker regions between positioned nucleosomes. Pho2p is not required for *PHO8* expression. It was recently reported that remodeling of promoter chromatin and induction of *PHO8* is dependent on both SWI/SNF and SAGA (251). In *swi2* mutants, no change is observed in the promoter chromatin structure of *PHO8* upon starvation, while a modest alteration occurs in *gcn5*Δ mutants. It is intriguing that *PHO5* and *PHO8* show different requirements for chromatin-specific coactivators, though both are dependent on Pho4p for expression. We propose that Pho4p recruits SWI/SNF and SAGA to both the *PHO5* and *PHO8* promoters, but that these two activities are redundant at *PHO5*, while they are each required at *PHO8*.

What makes an activity SWI/SNF dependent? One thing is clear: dependence on SWI/SNF is not an absolute. Several laboratories have reported that a SWI/SNF-independent promoter can be made SWI/SNF-dependent by destabilizing the interaction between an activator and its DNA binding site, either by employing low-affinity DNA-binding sites or by placing them in a nucleosome (149, 208, 212). Furthermore, SWI/SNF activity is partially redundant with cooperative binding between transcriptional activators (196). Thus, *PHO5* may be SWI/SNF-independent because the high-affinity Pho4p binding site in the large hypersensitive region of the *PHO5* promoter is more easily accessed than its counterpart in the *PHO8* promoter, or because Pho2p and Pho4p bind

cooperatively at *PHO5* but not at *PHO8*. Alternatively, SWI/SNF may be required for chromatin remodeling at a step after Pho4p binding, as Pho4p is still able to bind to its key regulatory element in *PHO8* even in *swi* and *gcn5* mutants. There is some in vivo evidence that SWI/SNF can activate transcription at a step after activator binding, such as assembly of the pre-initiation complex, or promoter clearance (251). If SWI/SNF is required at *PHO8* and *PHO5* at a step after activator binding, the number of Pho4p binding sites or the presence of Pho2p should not matter.

***SPT7* versus *GCN5*.** In a second recent report, Nishimura and colleagues demonstrate that *spt7* mutants are unable to induce *PHO5* expression in response to phosphate limitation (252). *SPT7* was first identified as a gene that reduces expression from Ty elements (253). It has subsequently been identified as a component of the SAGA complex (108). This result, in combination with our data that Gcn5p, the catalytic subunit of SAGA, is required for rapid induction of *PHO5*, suggest that the SAGA complex is involved in *PHO5* expression.

A comparison of our results (Chapter 3) and those reported by Nishimura et. al. reveals that *PHO5* expression is more affected by mutations in *SPT7* than in *GCN5*. It has been previously observed that mutations in *SPT7* have more severe transcriptional defects than a null allele of *GCN5*((254) and references therein). SAGA may thus have functions in addition to HAT activity that affect transcription in vivo, such as forming a physical bridge between activators and the general transcription apparatus. The partial defect in *PHO5* expression in a gcn5Δ strain that we describe here could be explained if the bridging function rather than the HAT activity of SAGA is what is required for *PHO5* induction.

Future Directions. We favor a model wherein SWI/SNF and SAGA act in a redundant fashion to remodel *PHO5* chromatin upon induction in low phosphate. However, direct evidence for this model is lacking. Chromatin immunoprecipitation experiments could be used to test if SWI/SNF and SAGA are localized to the *PHO5* promoter under inducing conditions. A direct interaction between Pho4p and components

of SWI/SNF and SAGA would also support this model. Whether or not SWI/SNF and SAGA can be placed at the *PHO5* promoter, it will be of interest to determine why they are each required for *PHO8* but not for *PHO5* promoter chromatin remodeling. Many experiments to identify cis- or trans-acting elements which contribute to SWI/SNF dependence can be envisioned, such as the construction of chimeric *PHO5/PHO8* promoters and in vivo analysis of their expression in *swi-* and *saga-* backgrounds. Finally, we cannot discount the possibility that an unidentified chromatin remodeling activity, not included in our group of candidates, is required for *PHO5* expression. An unbiased genetic approach may identify novel remodeling activities, components of chromatin, or coactivator components that collaborate with Pho2p and Pho4p to activate transcription of the *PHO5* gene.

APPENDIX

Introduction

The *Saccharomyces cerevisiae* acid phosphatase gene *PHO5* is regulated by the availability of phosphate in the environment. The *PHO5* promoter contains two upstream activating elements required for phosphate response, UASp1 and UASp2. *PHO4* and *PHO2*, first identified as genes required for the induction of acid phosphatase activity (255), encode transcription factors which act through these cis elements to activate transcription of *PHO5*.

Pho4p is a 312 amino acid protein containing a basic helix-loop helix (bHLH) DNA binding and dimerization domain in the C-terminus and an activation domain in the N-terminus. The DNA binding and dimerization domain specifically recognizes and binds to the sequence CACGTG/T in UASp1 and UASp2 both in vitro (148) and in vivo under conditions of phosphate limitation (12). Pho4p contains a strong acidic activation domain; residues 75 to 99 are essential and sufficient to confer both transcriptional activation and chromatin remodeling activities (256). Pho4p is also required for expression of *PHO8*, *PHO10*, *PHO11*, *PHO81* and *PHO84* (reviewed in (1)).

Pho2p is a 560 amino acid DNA-binding protein containing a homeobox (257). It was originally reported that Pho2p bound to a site in between UASp1 and Uasp2 (12); however deletion of this region has no effect of PHO5 expression levels (258). Instead, Pho2p appear to bind to A/T-rich sequences in the *PHO5*, *PHO81*, and *PHO84* promoters (259). Pho2p is also required for the expression of several genes unrelated to phosphate regulation, including *HO* (260), *HIS4* (261), *TRP4* (262), and several *ADE* genes (263). Pho2p binds cooperatively with Swi5p at the *HO* promoter (184, 264). There is evidence that Pho2p performs a similar function in concert with Pho4p at the *PHO5* promoter. Over-expression of Pho4p suppresses the *PHO5*-uninducible phenotype of a *pho2* mutation. A reporter gene construct driven by UASp1 requires Pho2p for induced expression in low phosphate (265). Furthermore, two hybrid experiments point to a phosphate-dependent interaction between Pho2p and Pho4p (266). We describe here a

brief analysis of cooperative interactions between Pho2p and Pho4p both in solution and on *PHO5* promoter DNA.

Materials and Methods

Preparation of recombinant proteins. rPho4p was prepared as described in Chapter 1. Pho2p was purified from yeast. Yeast strain Y57 (*pho2Δ pho4Δ pho80Δ)* harboring a plasmid containing *PHO2* fused to two polyoma tags at the N-terminus, expressed from the GPD-promoter (EB0284). Whole cell extract was prepared from cells grown to an OD_{600} of about 0.5, then pelleted, washed, and resuspended in 1 mL/g pellet HSB (10% glycerol, 2mM Hepes pH 7.5, 1 mM EDTA, 0.5% NP-40, 0.4 M KOAc, 80 mM β-glycerophosphate, 10 mM NaF, 10 nM caliculyn A, 1mM PMSF, and 2 mM DTT). An equal volume of glass beads was added and the solution placed in a multi-vortexer for 30 minutes at 4°C. The resulting extract was clarified by centrifugation and stored at -80°C at a concentration of approximately 20 mg/ml.

To purify Py_2Pho2p, 5 ml of whole cell extract was incubated for 2 hours with 500 µl of Protein G sepharose beads pre-conjugated to α-polyoma antibodies. The beads were pelleted and washed three times with HSB. Tagged Pho2p was eluted for 30 minutes at 25°C in10% glycerol, 20 mM HEPES, 0.1 M KOAc, 0.1% N-octylglucoside, 1 mM DTT, 1mM PMSF, and 1 mM polyoma peptide (of sequence EYMPME).

Non-denaturing gel electrophoresis. UASp1, UASp2 or UASp1* probes were made by cutting pCP1G-, p73-2, or pFP2- with Hind III, labeling with Klenow and ^{32}-P labeled dCTP, and cutting a second time with EcoRI. The resulting 100 bp Hind III/Eco RI fragment, labeled at one end, was isolated on a 4% preparative gel and eluted in TE overnight at 25°C. 100,000 cpm of probe was incubated with varying concentrations of rPho4p (from 3 to 300 nM) and 1-5 µL Py_2Pho2p in 50 mM HEPES pH 7.6, 10 mM MgOAc, 5 mM EDTA, 10% glycerol, 100 µg/mL BSA, 2.5 mM DTT and 20 µg/ml dCdG

in a total volume of 25µl at 25°C for 20 minutes. Reactions were run at 200 V on 6% non-denaturing acylamide gels at 4°C.

Co-immunoprecipitations. In 200 µl volume, 500 µg of WCE containing Py2 Pho2p or 5 µl purified Py2Pho2p were mixed with 20 µl α-polyoma beads and 300 ng rPho4p and incubated for 1 hr at 4, spin. Beads were washed with HSB, boiled for 3 minutes in 20 µl sample loading buffer. Western analysis was performed with α-Pho4p polyclonal antibodies and α-polyoma antibodies.

Quantitative DNaseI Footprinting. Quantitative DNase I footprint analysis was performed essentially as described in (215). Probes and reactions were prepared as for band shift assays (see above), except that reaction volumes were 200 µl and the buffer included 90 mM K glutamate. Approximately 0.1 nM probe DNA was used. After incubation at 25°C for 20 minutes, 1 ul of 0.1 M $CaCl_2$ and approximately 1 ng of DNase I (in 2 ul) were added. After a 2 minute incubation on ice, the reactions were stopped with ice-cold ethanol and NaOAc and transferred to dry ice for 15 minutes. DNA was precipitated, resuspended in loading buffer, boiled 10 minutes with vigorous vortexing, then loaded on a 8% acrylamide TBE gel and run at 35 W for 3 hours. Protection by rPho4p was quantified by phosphorimager analysis. K_A was calculated as described in (267).

Results and Discussion

Pho2p and Pho4p interact in vitro. We performed co-immunoprecipitation experiments to test if Pho2p and Pho4p interact in solution. Whole cell extract was prepared from a yeast strain overexpressing polyoma-tagged Pho2p (Py_2Pho2p), and incubated with recombinant Pho4p. Py_2Pho2p is efficiently immunoprecipitated with agarose beads covalently linked to α-polyoma antibodies (Figure 15A, compare lanes 3 and 4 with 7 and 8). rPho4p is detected in the immunoprecipitate only when Py_2Pho2p is also

added, indicating that Py_2Pho2p and Pho4p interact in solution (Fig. 15A, compare lanes 2 and 4).

To determine if the interaction between Py_2Pho2p and rPho4p is direct, immunoprecipitation reactions were performed with purified Py_2Pho2p. As shown in Fig. 15B, rPho4p and purified Py_2Pho2p coimmunoprecipitate, even in the presence of the DNA intercalating agent ethidium bromide. These data suggest that Pho2p and Pho4p interact in solution, and that this interaction does not require other proteins or DNA. Others report that amino acids 200-247 of Pho4p are required for interaction with Pho2p (266), for cooperative binding in vitro, and for activation of transcription from UASp1 (145).

Py_2Pho2p and rPho4p bind cooperatively to UASp1 but not UASp2. That Py_2Pho2p and rPho4p interact directly suggested that they may affect each other's function. One obvious possibility is that the interaction between these two transcriptional activators enhances their ability to bind DNA. To test if Py_2Pho2p and rPho4p bind DNA in a cooperative manner, non-denaturing gel electrophoresis experiments were performed with *PHO5* sequence containing UASp1 or UASp2 (Fig 16B). Incubating a labeled UASp1 probe with rPho4p produces a two species; (I) naked probe, and (II), a slower-migrating complex that is likely to be rPho4p bound to DNA (Fig 16B). When Py_2Pho2p is included in the reaction, a third species is detected (III). This species may correspond to a ternary complex between DNA, Py_2Pho2p, and rPho4p, and indicate that both proteins occupy UASp1 at the same time. Furthermore, the presence of Py_2Pho2p enhances binding by rPho4p (Fig. 16B). A ternary complex is not observed when labeled UASp2 probe is incubated with Py_2Pho2p and rPho4p (Fig. 16B), suggesting that Py_2Pho2p does not bind to this sequence. The presence of Py_2Pho2p does cause a modest enhancement of rPho4p binding to UASp2; however, this is also observed when a mock preparation of Py_2Pho2p is added (data not shown). While this work was underway, a report was published describing two major in vitro binding sites for Pho2p at the *PHO5* promoter, one

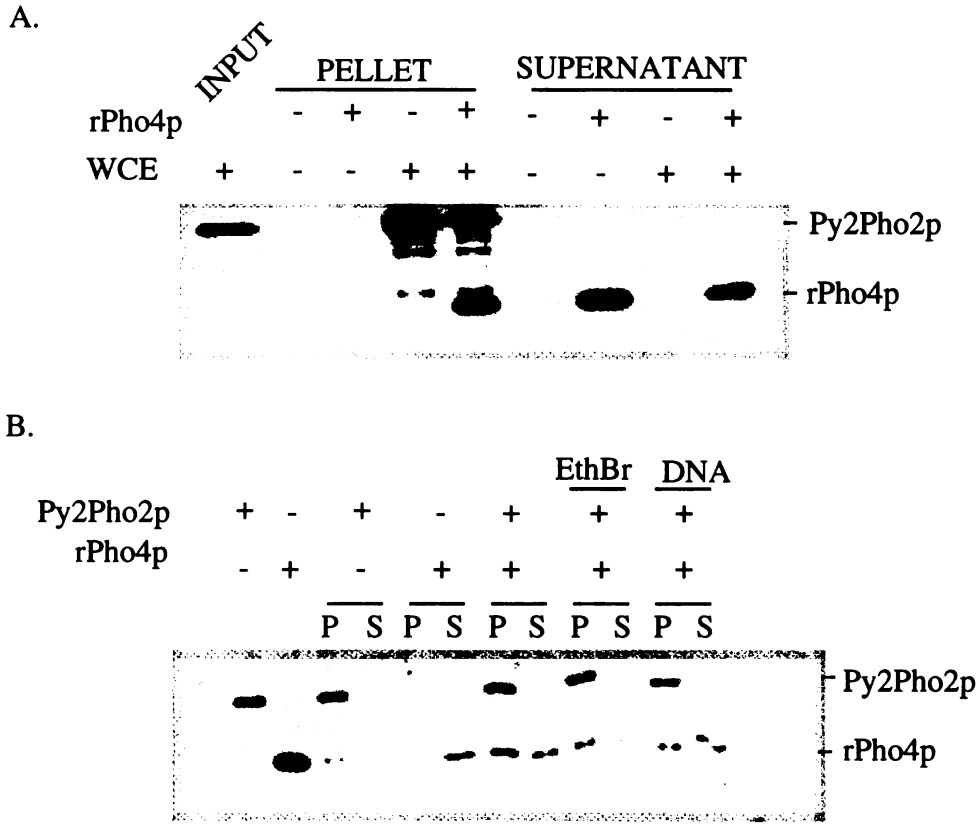


Figure 15. Py2Pho2p and rPho4p co-immunoprecipitate.

(A) A whole cell extract containing Py2Pho2p was incubated with or without rPho4p and immunoprecipitated with Protein G Sepharose bead pre-conjugated to α–polyoma antibodies. Pellet and supernatant fractions were separated by PAGE and transferred to nylon membrane. Western analysis was performed with α-polyoma and α-Pho4 antibodies. (B) Immunoprecipitation reactions and Western analysis were preformed as in (A) except that affinity-purified Py2Pho2p was used instead of extract.

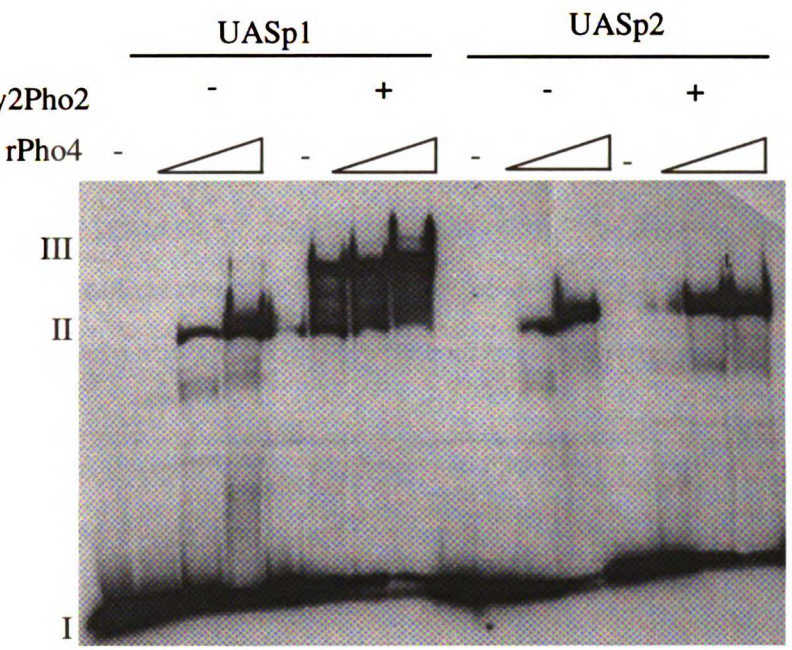
Figure 16. Py_2Pho2p and rPho4p bind cooperatively to UASp1, but not UASp2 or UASp1*.

(A) *PHO5* promoter sequence in probes used for bandshift or Dnase I footprinting. (B) and (C) Various concentrations of rPho4p (ranging from 3 to 90 nM) were incubated with Py_2Pho2p and labeled UASp1, UASp2, or UASp1* probes. After incubation at 25°C, the reactions were subjected to non-denaturing gel electrophoresis.

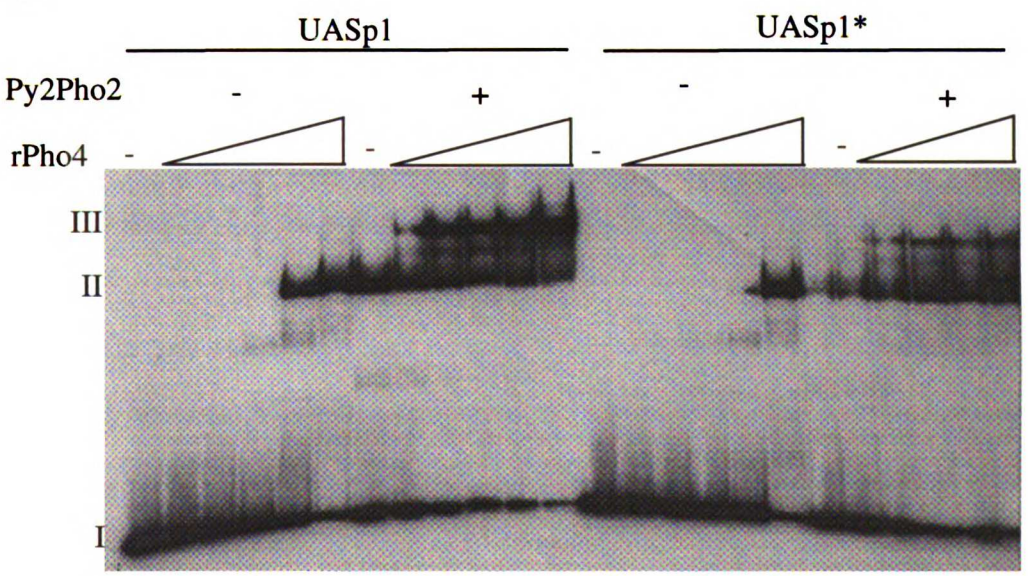
A.

UASp1	AGAAATATA**TATT**AAATTAGCACGTTTTCGCATAG
UASp1*	AGAAATATA**GCGC**AAATTAGCACGTTTTCGCATAG
UASp2	TTGGCACTCACACGTGGGGACTAGCACAG

B.



C.



at UASp1 called M2 (-385 to –358), and one at UASp2 called M3 (-320 to –291) (144). The M3 sequence was not included in our UASp2 probe. It is worth noting that Pho2p may affect Pho4p function at UASp1 and UASp2 in different ways. Several lines of evidence suggest that at UASp1, Pho2p binds cooperatively with Pho4p, while at UASp2 Pho2p acts to counteract the effects of a repression domain within Pho4p (145, 173). A similar role has been proposed for Pho2p at the *ADE* genes (268).

Py_2Pho2p enhances the affinity of rPho4p for UASp1 approximately 10-fold. DNase I footprinting was used to quantify the DNA binding cooperativity between rPho4p and Py_2Pho2p. Typical footprints are shown in Figure 17. The association constant (K_A) for rPho4p binding to UASp1 and UASp2 was calculated in the absence and presence of Py_2Pho2p. Data for two separate experiments under these conditions are displayed in Table 5. The presence of Py_2Pho2p enhances the binding of rPho4p to UASp1 by approximately 10-fold, and to UASp2 approximately 2-fold.

DNA PROBE	Py_2Pho2p	K_A
UASp1	-	3.6×10^8
	-	4.6×10^8
	+	3.8×10^9
	+	4.6×10^9
UASp2	-	7.3×10^8
	-	1.3×10^9
	+	1.7×10^9
	+	9.3×10^8
UASp1*	-	5.9×10^8
	+	1.5×10^9

Table 7. Association constants for rPho4p binding to DNA.

Cooperative binding requires A/T rich sequence in UASp1. The A/T-rich tract upstream of UASp1 was replaced with a G/C rich sequence (Fig 16A), creating probe UASp1*. Non-denaturing gel electrophoresis revealed that only a small amount of complex III is observed when UASp1* is used. This result implies that a ternary complex

between rPho4p, Py_2Pho2p, and UASp1* DNA does not form or is unstable. Furthermore, cooperative binding between rPho4p and Py_2Pho2p on UASp1* is not observed, either by band-shift analysis (Fig. 16C.) or by DNaseI Footprinting (Table 5).

Pho2p and Pho4p are both required for *PHO5* expression. The results presented here suggest that at least one level at which Pho2p functions to promote *PHO5* expression is at the level of Pho4p binding to UASp1. In addition, the A/T rich sequence bordering the Pho4p binding site at UASp1 is required for both cooperative binding and formation of a ternary complex, suggesting that this may be the sequence in the *PHO5* promoter that is recognized by Pho2p.

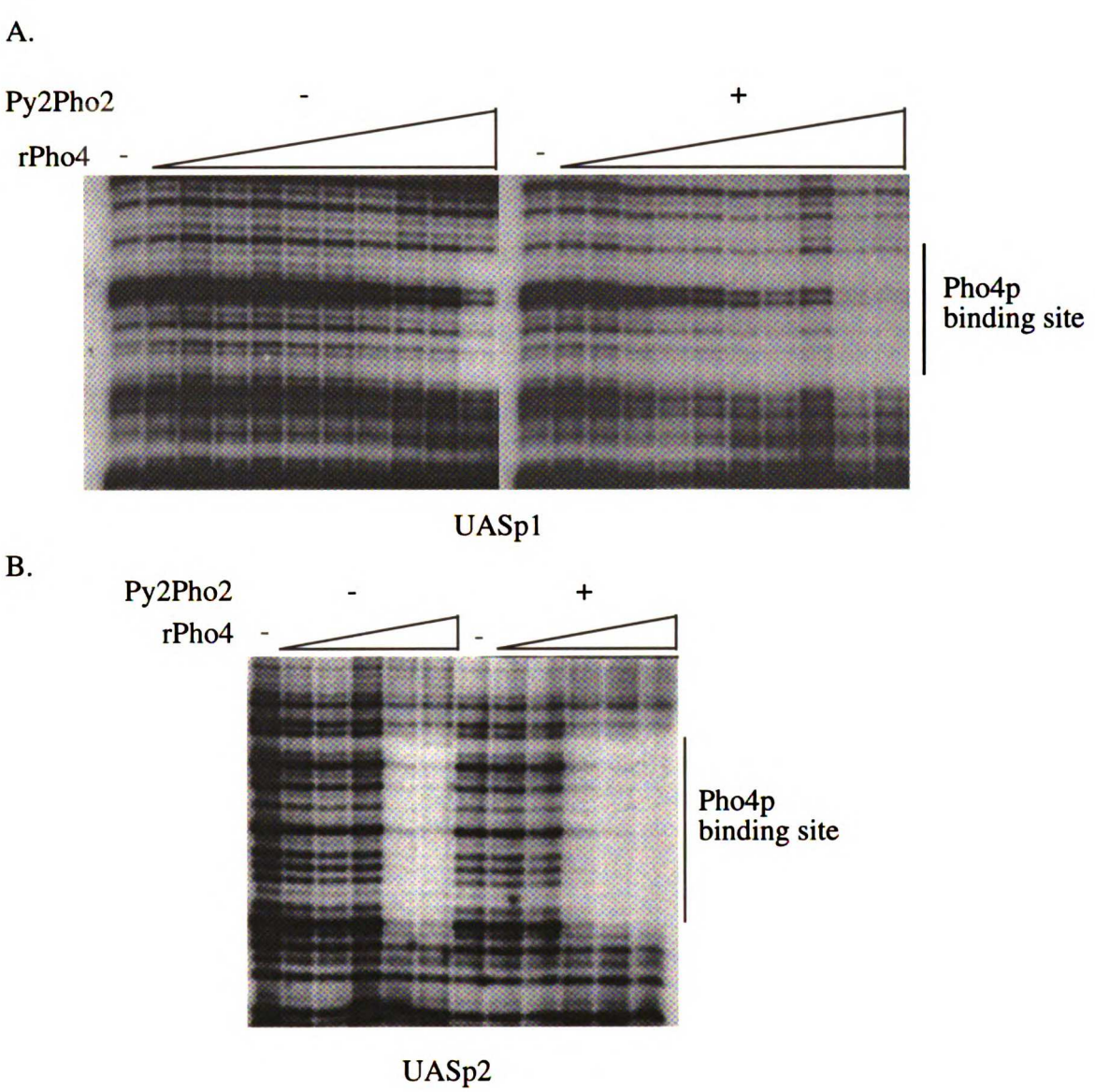


Figure 16. Py2Pho2p and rPho4p bind cooperatively to UASp1, but not UASp2 or UASp1*

Various amounts of rPho4p were incubated with Py2Pho2p and labeled UASp1, UASp2, or UASp1* and subjected to non-denaturing gel electrophoresis.

REFERENCES

1. Oshima, Y., N. Ogawa, and S. Harashima. 1996. *Regulation of phosphatase synthesis in Saccharomyces cerevisiae--a review.* Gene **179:**171-7.

2. Lenburg, M. E., and E. K. O'Shea. 1996. *Signaling phosphate starvation.* Trends Biochem. Sci. **21:**383-387.

3. Vogel, K., and A. Hinnen. 1990. *The yeast phosphatase system.* Molecular Microbiology **4:**2013-2017.

4. Lau, W. W., R. W. Howson, P. Malkus, R. Schekman, and E. K. O'Shea. 2000. *Pho86p, an endoplasmic reticulum (ER) resident protein in saccharomyces cerevisiae, is required for ER exit of the high-affinity phosphate transporter pho84p.* Proc Natl Acad Sci U S A **97:**1107-12.

5. Lemire, J. M., T. Willcocks, H. O. Halvorson, and K. A. Bostian. 1985. *Regulation of repressible acid phosphatase gene transcription in Saccharomyces cerevisiae.* Mol Cell Biol **5:**2131-41.

6. To, E. A., Y. Ueda, S. I. Kakimoto, and Y. Oshima. 1973. *Isolation and characterization of acid phosphatase mutants in Saccharomyces cerevisiae.* J Bacteriol **113:**727-38.

7. Ueda, Y., E. A. To, and Y. Oshima. 1975. *Isolation and characterization of recessive, constitutive mutations for repressible acid phosphatase synthesis in Saccharomyces cerevisiae.* J Bacteriol **122:**911-22.

8. Koren, R., J. LeVitre, and K. A. Bostian. 1986. *Isolation of the positive-acting regulatory gene PHO4 from Saccharomyces cerevisiae.* Gene **41:**271-80.

9. Berben, G., M. Legrain, V. Gilliquet, and F. Hilger. 1990. *The yeast regulatory gene PHO4 encodes a helix-loop-helix motif.* Yeast **6:**451-4.

10. 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.

11. Berben, G., M. Legrain, and F. Hilger. 1988. *Studies on the structure, expression and function of the yeast regulatory gene PHO2.* Gene **66:**307-12.

12. 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.

13. 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.

14. O'Neill, E. M., A. Kaffman, E. R. Jolly, and E. K. O'Shea. 1996. *Regulation of Pho4 nuclear localization by the Pho80-Pho85 cyclin-CDK complex.* Science **271:**209-212.

15. 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:**2673-83.

16. Kaffman, A., N. M. Rank, E. M. O'Neill, L. S. Huang, and E. K. O'Shea. 1998. *The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus.* Nature **396:**482-6.

17. Komeili, A., and E. K. O'Shea. 1999. *Roles of phosphorylation sites in regulating activity of the transcription factor Pho4.* Science **284:**977-80.

18. Schneider, K. R., R. L. Smith, and E. K. O'Shea. 1994. *Phosphate-regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81.* Science **266:**122-6.

19. Svaren, J., and W. Horz. 1997. *Transcription factors vs. nucleosomes: regulation of the PHO5 promoter in yeast.* Trends Biochem. Sci. **22:**93-97.

20. Svaren, J., and W. Horz. 1995. *Interplay between nucleosomes and transcription factors at the yeast PHO5 promoter.* Seminars in Cell Biology **6:**177-183.

21. Kim, U. J., M. Han, P. Kayne, and M. Grunstein. 1988. *Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae.* Embo J **7:**2211-9.

22. Han, M., and M. Grunstein. 1988. *Nucleosome loss activates yeast downstream promoters in vivo.* Cell **55:**1137-1145.

23. 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.

24. Straka, C., and W. Horz. 1991. *A functional role for nucleosomes in the repression of a yeast promoter.* EMBO J. **10:**361-368.

25. Kornberg, R. D., and Y. Lorch. 1999. *Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome.* Cell **98:**285-94.

26. Luger, K., and T. J. Richmond. 1998. *DNA binding within the nucleosome core.* Current Opinion in Structural Biology **8:**33-40.

27. Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond. 1997. *Crystal structure of the nucleosome core particle at 2.8 A resolution.* Nature **389:**251-60.

28. Wolffe, A. P. 1995. *Chromatin: Structure and Function.* Academic Press, London.

29. Burley, S. K., X. Xie, K. L. Clark, and F. Shu. 1997. *Histone-like transcription factors in eukaryotes.* Curr Opin Struct Biol **7:**94-102.

30. Arents, G., and E. N. Moudrianakis. 1995. *The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization.* Proc Natl Acad Sci U S A **92:**11170-4.

31. Arents, G., R. W. Burlingame, B. C. Wang, W. E. Love, and E. N. Moudrianakis. 1991. *The nucleosomal core histone octamer at 3.1 A resolution: a*

tripartite protein assembly and a left-handed superhelix. Proc Natl Acad Sci U S A **88:**10148-52.

32. Workman, J. L., and R. E. Kingston. 1998. *Alteration of nucleosome structure as a mechanism of transcriptional regulation.* Annu Rev Biochem **67:**545-79.

33. Luger, K., and T. J. Richmond. 1998. *The histone tails of the nucleosome.* Curr Opin Genet Dev **8:**140-6.

34. Perez-Martin, J. 1999. *Chromatin and transcription in Saccharomyces cerevisiae.* FEMS Microbiol Rev **23:**503-23.

35. Han, M., U. J. Kim, P. Kayne, and M. Grunstein. 1988. *Depletion of histone H4 and nucleosomes activates the PHO5 gene in Saccharomyces cerevisiae.* Embo J **7:**2221-8.

36. Clark-Adams, C. D., D. Norris, M. A. Osley, J. S. Fassler, and F. Winston. 1988. *Changes in histone gene dosage alter transcription in yeast.* Genes Dev **2:**150-9.

37. Kruger, W., C. L. Peterson, A. Sil, C. Coburn, G. Arents, et al. 1995. *Amino acid substitutions in the structured domains of histones H3 and H4 partially relieve the requirement of the yeast SWI/SNF complex for transcription.* Genes Dev **9:**2770-9.

38. Mann, R. K., and M. Grunstein. 1992. *Histone H3 N-terminal mutations allow hyperactivation of the yeast GAL1 gene in vivo.* Embo J **11:**3297-306.

39. Wechser, M. A., M. P. Kladde, J. A. Alfieri, and C. L. Peterson. 1997. *Effects of Sin- versions of histone H4 on yeast chromatin structure and function.* Embo J **16:**2086-95.

40. Durrin, L. K., R. K. Mann, P. S. Kayne, and M. Grunstein. 1991. *Yeast histone H4 N-terminal sequence is required for promoter activation in vivo.* Cell **65:**1023-31.

41. Hirschhorn, J. N., A. L. Bortvin, S. L. Ricupero-Hovasse, and F. Winston. 1995. *A new class of histone H2A mutations in Saccharomyces cerevisiae causes specific transcriptional defects in vivo.* Mol Cell Biol **15:**1999-2009.

42. Grunstein, M. 1997. *Molecular model for telomeric heterochromatin in yeast.* Curr Opin Cell Biol **9:**383-7.

43. Meluh, P. B., P. Yang, L. Glowczewski, D. Koshland, and M. M. Smith. 1998. *Cse4p is a component of the core centromere of Saccharomyces cerevisiae.* Cell **94:**607-13.

44. Smith, M. M., and M. S. Santisteban. 1998. *Genetic dissection of histone function.* Methods **15:**269-81.

45. Wolffe, A. P., and J. J. Hayes. 1999. *Chromatin disruption and modification.* Nucleic Acids Res **27:**711-20.

46. Grunstein, M. 1990. *Histone function in transcription.* Annu Rev Cell Biol **6:**643-78.

47. Landsman, D. 1996. *Histone H1 in Saccharomyces cerevisiae: a double mystery solved?* Trends Biochem Sci **21:**287-8.

48. Ushinsky, S. C., H. Bussey, A. A. Ahmed, Y. Wang, J. Friesen, et al. 1997. *Histone H1 in Saccharomyces cerevisiae.* Yeast **13:**151-61.

49. Patterton, H. G., C. C. Landel, D. Landsman, C. L. Peterson, and R. T. Simpson. 1998. *The biochemical and phenotypic characterization of Hho1p, the putative linker histone H1 of Saccharomyces cerevisiae.* J Biol Chem **273:**7268-76.

50. 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.

51. 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.

52. 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:**265-97.

53. Winston, F., and M. Carlson. 1992. *Yeast SNF/SWI transcriptional activators and the SPT/SIN connection.* Trends Genet. **8:**387-391.

54. Peterson, C. L., and J. W. Tamkun. 1995. *The SWI-SNF complex: a chromatin remodeling machine?* Trends Biochem. Sci. **20:**143-146.

55. Muchardt, C., and M. Yaniv. 1999. *ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job.* J Mol Biol **293:**187-98.

56. 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.

57. Cairns, B. R., Y. Lorch, Y. Li, M. Zhang, L. Lacomis, et al. 1996. *RSC, an essential, abundant chromatin-remodeling complex.* Cell **87:**1249-1260.

58. Dingwall, A. K., S. J. Beek, C. M. McCallum, J. W. Tamkun, G. V. Kalpana, et al. 1995. *The Drosophila snr1 and brm proteins are related to yeast SWI/SNF proteins and are components of a large protein complex.* Mol Biol Cell **6:**777-91.

59. Wang, W., Y. Xue, S. Zhou, A. Kuo, B. R. Cairns, et al. 1996. *Diversity and specialization of mammalian SWI/SNF complexes.* Genes Dev **10:**2117-30.

60. Kwon, H., A. N. Imbalzano, P. A. Khavari, R. E. Kingston, and M. R. Green. 1994. *Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex.* Nature **370:**477-81.

61. Armstrong, J. A., J. J. Bieker, and B. M. Emerson. 1998. *A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF in vitro.* Cell **95:**93-104.

62. Kingston, R. E., and G. J. Narlikar. 1999. *ATP-dependent remodeling and acetylation as regulators of chromatin fluidity.* Genes Dev **13:**2339-52.

63. Cairns, B. R. 1998. *Chromatin remodeling machines: Similar motors, ulterior motives.* Trends Biochem. Sci. **23:**20-25.

64. Tsukiyama, T., and C. Wu. 1995. *Purification and properties of an ATP-dependent nucleosome remodeling factor.* Cell **83:**1011-1020.

65. Tsukiyama, T., C. Daniel, J. Tamkun, and C. Wu. 1995. *ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor.* Cell **83:**1021-6.

66. Varga-Weiss, P. D., M. Wilm, E. Bonte, K. Dumas, M. Mann, et al. 1997. *Chromatin-remodeling factor CHRAC contains the ATPases ISWI and topoisomerase II.* Nature **388:**598-602.

67. 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:**145-55.

68. Elfring, L. K., R. Deuring, C. M. McCallum, C. L. Peterson, and J. W. Tamkun. 1994. *Identification and characterization of Drosophila relatives of the yeast transcriptional activator SNF2/SWI2.* Mol Cell Biol **14:**2225-34.

69. Aasland, R., A. F. Stewart, and T. Gibson. 1996. *The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB.* Trends Biochem Sci **21:**87-8.

70. Mizuguchi, G., T. Tsukiyama, J. Wisniewski, and C. Wu. 1997. *Role of nucleosome remodeling factor NURF in transcriptional activation of chromatin.* Mol. Cell **1:**141-150.

71. Alexiadis, V., P. D. Varga-Weisz, E. Bonte, P. B. Becker, and C. Gruss. 1998. *In vitro chromatin remodelling by chromatin accessibility complex (CHRAC) at the SV40 origin of DNA replication.* Embo J **17:**3428-38.

72. LeRoy, G., G. Orphanides, W. S. Lane, and D. Reinberg. 1998. *Requirement of RSF and FACT for transcription of chromatin templates in vitro.* Science **282:**1900-4.

73. Georgel, P. T., T. Tsukiyama, and C. Wu. 1997. *Role of histone tails in nucleosome remodeling by Drosophila NURF.* Embo J **16:**4717-26.

74. 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.

75. Logie, C., C. Tse, J. C. Hansen, and C. L. Peterson. 1999. *The core histone N-terminal domains are required for multiple rounds of catalytic chromatin remodeling by the SWI/SNF and RSC complexes.* Biochemistry **38:**2514-22.

76. Tong, J. K., C. A. Hassig, G. R. Schnitzler, R. E. Kingston, and S. L. Schreiber. 1998. *Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex.* Nature **395:**917-21.

77. Xue, Y., J. Wong, G. T. Moreno, M. K. Young, J. Cote, et al. 1998. *NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities.* Mol Cell **2:**851-61.

78. Zhang, Y., G. LeRoy, H. P. Seelig, W. S. Lane, and D. Reinberg. 1998. *The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities.* Cell **95:**279-89.

79. Wade, P. A., P. L. Jones, D. Vermaak, and A. P. Wolffe. 1998. *A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase.* Curr Biol **8:**843-6.

80. Wade, P. A., A. Gegonne, P. L. Jones, E. Ballestar, F. Aubry, et al. 1999. *Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation.* Nat Genet **23:**62-6.

81. Zhang, Y., H. H. Ng, H. Erdjument-Bromage, P. Tempst, A. Bird, et al. 1999. *Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation.* Genes Dev **13:**1924-35.

82. Knoepfler, P. S., and R. N. Eisenman. 1999. *Sin meets NuRD and other tails of repression.* Cell **99:**447-50.

83. Du, J., I. Nasir, B. K. Benton, M. P. Kladde, and B. C. Laurent. 1998. *Sth1p, a Saccharomyces cerevisiae Snf2p/Swi2p homolog, is an essential ATPase in RSC and differs from Snf/Swi in its interactions with histones and chromatin-associated proteins.* Genetics **150:**987-1005.

84. Richmond, E., and C. L. Peterson. 1996. *Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2.* Nucleic Acids Res **24:**3685-92.

85. Corona, D. F., G. Langst, C. R. Clapier, E. J. Bonte, S. Ferrari, et al. 1999. *ISWI is an ATP-dependent nucleosome remodeling factor.* Mol Cell **3:**239-45.

86. Phelan, M. L., S. Sif, G. J. Narlikar, and R. E. Kingston. 1999. *Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits.* Mol Cell **3:**247-53.

87. Imbalzano, A. N., G. R. Schnitzler, and R. E. Kingston. 1996. *Nucleosome disruption by human SWI/SNF is maintained in the absence of continued ATP hydrolysis.* J Biol Chem **271:**20726-33.

88. Lorch, Y., B. R. Cairns, M. Zhang, and R. D. Kornberg. 1998. *Activated RSC-nucleosome complex and persistently altered form of the nucleosome.* Cell **94:**29-34.

89. Schnitzler, G., S. Sif, and R. E. Kingston. 1998. *Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state.* Cell **94:**17-27.

90. Cote, J., C. L. Peterson, and J. L. Workman. 1998. *Perturbation of nucleosome core structure by the SWI/SNF complex persists after its detachment, enhancing subsequent transcription factor binding.* Proc Natl Acad Sci U S A **95:**4947-52.

91. Owen-Hughes, T., R. T. Utley, J. Cote, C. L. Peterson, and J. L. Workman. 1996. *Persistent site-specific remodeling of a nuclesome array by transient action of the SWI/SNF complex.* Science **273:**513-516.

92. Logie, C., and C. L. Peterson. 1997. *Catalytic activity of the yeast SWI/SNF complex on reconstituted nucleosome arrays.* Embo J **16:**6772-82.

93. Moreira, J. M., and S. Holmberg. 1999. *Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling complex RSC.* Embo J **18:**2836-44.

94. Hamiche, A., R. Sandaltzopoulos, D. A. Gdula, and C. Wu. 1999. *ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF.* Cell **97:**833-42.

95. Langst, G., E. J. Bonte, D. F. Corona, and P. B. Becker. 1999. *Nucleosome movement by CHRAC and ISWI without disruption or trans- displacement of the histone octamer.* Cell **97:**843-52.

96. Whitehouse, I., A. Flaus, B. R. Cairns, M. F. White, J. L. Workman, et al. 1999. *Nucleosome mobilization catalysed by the yeast SWI/SNF complex.* Nature **400:**784-7.

97. Studitsky, V. M., D. J. Clark, and G. Felsenfeld. 1994. *A histone octamer can step around a transcribing polymerase without leaving the template.* Cell **76:**371-82.

98. Bednar, J., V. M. Studitsky, S. A. Grigoryev, G. Felsenfeld, and C. L. Woodcock. 1999. *The nature of the nucleosomal barrier to transcription: direct observation of paused intermediates by electron cryomicroscopy.* Mol Cell **4:**377-86.

99. Pazin, M. J., and J. T. Kadonaga. 1997. *SWI2/SNF2 and related proteins: ATP-driven motors that disrupt protein- DNA interactions?* Cell **88:**737-40.

100. Bazett-Jones, D. P., J. Cote, C. C. Landel, C. L. Peterson, and J. L. Workman. 1999. *The SWI/SNF complex creates loop domains in DNA and polynucleosome arrays and can disrupt DNA-histone contacts within these domains.* Mol Cell Biol **19:**1470-8.

101. McMurry, M. T., and M. S. Krangel. 2000. *A role for histone acetylation in the developmental regulation of VDJ recombination.* Science **287:**495-8.

102. Grunstein, M. 1997. *Histone acetylation in chromatin structure and transcription.* Nature **389:**349-52.

103. Mizzen, C. A., and C. D. Allis. 1998. *Linking histone acetylation to transcriptional regulation.* C.M.L.S. **54:**6-20.

104. Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, et al. 1996. *Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation.* Cell **84:**843-51.

105. Candau, R., and S. L. Berger. 1996. *Structural and functional analysis of yeast putative adaptors. Evidence for an adaptor complex in vivo.* J Biol Chem **271:**5237-45.

106. Kuo, M. H., and C. D. Allis. 1998. *Roles of histone acetyltransferases and deacetylases in gene regulation.* Bioessays **20:**615-26.

107. Pazin, M. J., and J. T. Kadonaga. 1997. *What's up and down with histone deacetylation and transcription?* Cell **89:**325-8.

108. Grant, P. A., L. Duggan, J. Cote, S. M. Roberts, J. E. Brownell, et al. 1997. *Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex.* Genes Dev **11:**1640-50.

109. Pollard, K. J., and C. L. Peterson. 1997. *Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression.* Mol Cell Biol **17:**6212-22.

110. Saleh, A., V. Lang, R. Cook, and C. J. Brandl. 1997. *Identification of native complexes containing the yeast coactivator/repressor proteins NGG1/ADA3 and ADA2.* J Biol Chem **272:**5571-8.

111. Kuo, M. H., J. Zhou, P. Jambeck, M. E. Churchill, and C. D. Allis. 1998. *Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo.* Genes Dev **12:**627-39.

112. Wang, L., L. Liu, and S. L. Berger. 1998. *Critical residues for histone acetylation by Gcn5, functioning in Ada and SAGA complexes, are also required for transcriptional function in vivo.* Genes Dev **12:**640-53.

113. Blanco, J. C., S. Minucci, J. Lu, X. J. Yang, K. K. Walker, et al. 1998. *The histone acetylase PCAF is a nuclear receptor coactivator.* Genes Dev **12:**1638-51.

114. Ogryzko, V. V., T. Kotani, X. Zhang, R. L. Schlitz, T. Howard, et al. 1998. *Histone-like TAFs within the PCAF histone acetylase complex.* Cell **94:**35-44.

115. Vassilev, A., J. Yamauchi, T. Kotani, C. Prives, M. L. Avantaggiati, et al. 1998. *The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily.* Mol Cell **2:**869-75.

116. Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. *The transcriptional coactivators p300 and CBP are histone acetyltransferases.* Cell **87:**953-9.

117. Spencer, T. E., G. Jenster, M. M. Burcin, C. D. Allis, J. Zhou, et al. 1997. *Steroid receptor coactivator-1 is a histone acetyltransferase.* Nature **389:**194-8.

118. Chen, H., R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, et al. 1997. *Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300.* Cell **90:**569-80.

119. Reifsnyder, C., J. Lowell, A. Clarke, and L. Pillus. 1996. *Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases.* Nat Genet **14:**42-9.

120. Hilfiker, A., D. Hilfiker-Kleiner, A. Pannuti, and J. C. Lucchesi. 1997. *mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila.* Embo J **16:**2054-60.

121. Smith, E. R., A. Pannuti, W. Gu, A. Steurnagel, R. G. Cook, et al. 2000. *The drosophila MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation.* Mol Cell Biol **20:**312-8.

122. Clarke, A. S., J. E. Lowell, S. J. Jacobson, and L. Pillus. 1999. *Esa1p is an essential histone acetyltransferase required for cell cycle progression.* Mol Cell Biol **19:**2515-26.

123. Smith, E. R., A. Eisen, W. Gu, M. Sattah, A. Pannuti, et al. 1998. *ESA1 is a histone acetyltransferase that is essential for growth in yeast.* Proc Natl Acad Sci U S A **95:**3561-5.

124. Allard, S., R. T. Utley, J. Savard, A. Clarke, P. Grant, et al. 1999. *NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p.* Embo J **18:**5108-19.

125. Ohba, R., D. J. Steger, J. E. Brownell, C. A. Mizzen, R. G. Cook, et al. 1999. *A novel H2A/H4 nucleosomal histone acetyltransferase in Tetrahymena thermophila.* Mol Cell Biol **19:**2061-8.

126. Takechi, S., and T. Nakayama. 1999. *Sas3 is a histone acetyltransferase and requires a zinc finger motif.* Biochem Biophys Res Commun **266:**405-10.

127. Fletcher, T. M., and J. C. Hansen. 1995. *Core histone tail domains mediate oligonucleosome folding and nucleosomal DNA organization through distinct molecular mechanisms.* J Biol Chem **270:**25359-62.

128. Lee, D. Y., J. J. Hayes, D. Pruss, and A. P. Wolffe. 1993. *A positive role for histone acetylation in transcription factor access to nucleosomal DNA.* Cell **72:**73-84.

129. Braunstein, M., A. B. Rose, S. G. Holmes, C. D. Allis, and J. R. Broach. 1993. *Transcriptional silencing in yeast is associated with reduced nucleosome acetylation.* Genes Dev **7:**592-604.

130. Turner, B. M., A. J. Birley, and J. Lavender. 1992. *Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei.* Cell **69:**375-84.

131. Winston, F., and C. D. Allis. 1999. *The bromodomain: a chromatin-targeting module?* Nat Struct Biol **6:**601-4.

132. Edmondson, D. G., M. M. Smith, and S. Y. Roth. 1996. *Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4.* Genes Dev **10:**1247-59.

133. Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, and M. Grunstein. 1995. *Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast.* Cell **80:**583-92.

134. Ornaghi, P., P. Ballario, A. M. Lena, A. Gonzalez, and P. Filetici. 1999. *The bromodomain of Gcn5p interacts in vitro with specific residues in the N terminus of histone H4.* J Mol Biol **287:**1-7.

135. Dhalluin, C., J. E. Carlson, L. Zeng, C. He, A. K. Aggarwal, et al. 1999. *Structure and ligand of a histone acetyltransferase bromodomain.* Nature **399:**491-6.

136. Gu, W., and R. G. Roeder. 1997. *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell **90:**595-606.

137. Orphanides, G., G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg. 1998. *FACT, a factor that facilitates transcript elongation through nucleosomes.* Cell **92:**105-16.

138. Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey, and D. Reinberg. 1999. *The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins.* Nature **400:**284-8.

139. Naar, A. M., P. A. Beaurang, S. Zhou, S. Abraham, W. Solomon, et al. 1999. *Composite co-activator ARC mediates chromatin-directed transcriptional activation.* Nature **398:**828-32.

140. Rachez, C., B. D. Lemon, Z. Suldan, V. Bromleigh, M. Gamble, et al. 1999. *Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex.* Nature **398:**824-8.

141. Wu, W. H., and M. Hampsey. 1999. *Transcription: Common cofactors and cooperative recruitment.* Curr Biol **9:**R606-9.

142. Liu, H. Y., V. Badarinarayana, D. C. Audino, J. Rappsilber, M. Mann, et al. 1998. *The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively.* Embo J **17:**1096-106.

143. Denis, C. L., M. P. Draper, H. Y. Liu, T. Malvar, R. C. Vallari, et al. 1994. *The yeast CCR4 protein is neither regulated by nor associated with the SPT6 and SPT10 proteins and forms a functionally distinct complex from that of the SNF/SWI transcription factors.* Genetics **138:**1005-13.

144. 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.

145. 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.

146. 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.

147. 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.

148. 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.

149. 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.

150. **Schneider, K.** 1995. Ph.D. Thesis. University of California, San Francisco.

151. 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:**2131-44.

152. Gregory, P. D., A. Schmid, M. Zavari, L. Lui, S. L. Berger, et al. 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.

153. Yoshida, K., Z. Kuromitsu, N. Ogawa, and Y. Oshima. 1989. *Mode of expression of the positive regulatory genes PHO2 and PHO4 of the phosphatase regulon in Saccharomyces cerevisiae.* Mol Gen Genet **217:**31-9.

154. Pryciak, P. M., A. Sil, and H. E. Varmus. 1992. *Retroviral integration into minichromosomes in vitro.* EMBO J. **11:**291-303.

155. 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.

156. Dean, A., D. S. Pederson, and R. T. Simpson. 1989. *Isolation of yeast plasmid chromatin.* Methods Enzymol. **170:**26-41.

157. Roth, S. Y., and R. T. Simpson. 1991. *Yeast minichromosomes.* Methods Cell Biol. **35:**289-314.

158. Scott, J. H., and R. Scheckman. 1980. *Lyticase: endoglucanase and protease activities that act together in yeast cell lysis.* J. Bacteriol. **142:**414-23.

159. 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.

160. 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.

161. 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.

162. Kingsbury, J., and D. Koshland. 1993. *Centromere function on minichromosomes isolated from budding yeast.* Mol. Biol. Cell **4:**859-70.

163. 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.

164. Bergman, L. W. 1986. *A DNA fragment containing the upstream activator sequence determines nucleosome positioning of the transcriptionally repressed PHO5 gene of Saccharomyces cereviesiae.* Mol. Cell. Biol. **6:**2298-2304.

165. 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.

166. 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.

167. 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.

168. Wu, C. 1980. *The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I.* Nature **286:**854-860.

169. 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.

170. 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.

171. 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. **221-232.**

172. 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.

173. Shao, D., C. L. Creasy, and L. W. Bergman. 1996. *Interaction of Saccharomyces cerevisiae Pho2 with Pho4 increases the accessibility of the activation domain of Pho4.* Mol Gen Genet **251:**358-64.

174. 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.

175. Steger, D. J., and J. L. Workman. 1996. *Remodeling chromatin structure for transcription: what happens to the histones?* Bioessays **18:**875-884.

176. Wilson, C. J., D. M. Chao, A. N. Imbalzano, G. R. Schnitzler, R. E. Kingston, et al. 1996. *RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling.* Cell **84:**235-44.

177. Utley, F. T., K. Ikeda, P. A. Grant, J. Cote, D. Steger, et al. 1998. *Transcriptional activators direct histone acetyltransferase complexes to nucleosomes.* Nature **394:**498-502.

178. Drysdale, C. M., B. M. Jackson, R. McVeigh, E. R. Klebanow, Y. Bai, et al. 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.

179. 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:**365-71.

180. 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.

181. Haswell, E. S., and E. K. O'Shea. 1998. *Specificity of ATP-dependent chromatin remodeling at the yeast PHO5 promoter.* Cold Spring Harb Symp Quant Biol **63:**563-7.

182. Haswell, E. S., and E. K. O'Shea. 1998. p. .

183. O'Shea. 1995. p. .

184. Brazas, R. M., L. T. Bhoite, M. D. Murphy, Y. Yu, Y. Chen, et al. 1995. *Determining the requirements for cooperative DNA binding by Swi5p and Pho2p (Grf10p/Bas2p) at the HO promoter.* J Biol Chem **270:**29151-61.

185. Bhoite, L. T., and D. J. Stillman. 1998. *Residues in the Swi5 zinc finger protein that mediate cooperative DNA binding with the Pho2 homeodomain protein.* Mol Cell Biol **18:**6436-46.

186. Peterson, C. L., and I. Herskowitz. 1992. *Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription.* Cell **68:**573-83.

187. Yoshinaga, S. K., C. L. Peterson, I. Herskowitz, and K. R. Yamamoto. 1992. *Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors.* Science **258:**1598-1604.

188. Laurent, B. C., and M. Carlson. 1992. *Yeast SNF2/SWI2, SNF5, and SNF6 proteins function coordinately with the gene-specific transcriptional activators GAL4 and Bicoid.* Genes Dev **6:**1707-15.

189. Hirschhorn, J. N., S. A. Brown, C. D. Clark, and F. Winston. 1992. *Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure.* Genes Dev **6:**2288-98.

190. Wu, L., and F. Winston. 1997. *Evidence that Snf-Swi controls chromatin structure over both the TATA and UAS regions of the SUC2 promoter in Saccharomyces cerevisiae.* Nucleic Acids Res **25:**4230-4.

191. Gavin, I. M., and R. T. Simpson. 1997. *Interplay of yeast global transcriptional regulators Ssn6p-Tup1p and Swi-Snf and their effect on chromatin structure.* Embo J **16:**6263-71.

192. LaMarco, K. 1994. *Dissecting a complex process.* Proc Natl Acad Sci U S A **91:**2886-7.

193. 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.

194. 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.

195. Cote, J., J. Quinn, J. L. Workman, and C. L. Peterson. 1994. *Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex.* Science **265:**53-60.

196. Utley, R. T., J. Cote, T. Owen-Hughes, and J. L. Workman. 1997. *SWI/SNF stimulates the formation of disparate activator-nucleosome complexes but is partially redundant with cooperative binding.* J Biol Chem **272:**12642-9.

197. Neely, K. E., A. H. Hassan, A. E. Wallberg, D. J. Steger, B. R. Cairns, et al. 1999. *Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays.* Mol Cell **4:**649-55.

198. Quinn, J., A. M. Fryberg, R. W. Ganster, M. C. Schmid, and C. L. Peterson. 1996. *DNA-binding properties of the yeast SWI/SNF complex.* Nature **379:**844-847.

199. Cairns, B. R., H. Erdjument-Bromage, P. Tempst, F. Winston, and R. D. Kornberg. 1998. *Two actin-related proteins are shared functional components of the chromatin-remodeling complexes RSC and SWI/SNF.* Mol Cell **2:**639-51.

200. Peterson, C. L., Y. Zhao, and B. T. Chait. 1998. *Subunits of the yeast SWI/SNF complex are members of the actin-related protein (ARP) family.* J Biol Chem **273:**23641-4.

201. Wade, P. A., and A. P. Wolffe. 1999. *Transcriptional regulation: SWItching circuitry.* Curr Biol **9:**R221-4.

202. Perez-Martin, J., and A. D. Johnson. 1998. *The C-terminal domain of Sin1 interacts with the SWI-SNF complex in yeast.* Mol Cell Biol **18:**4157-64.

203. Yudkovsky, N., C. Logie, S. Hahn, and C. L. Peterson. 1999. *Recruitment of the SWI/SNF chromatin remodeling complex by transcriptional activators.* Genes Dev **13:**2369-74.

204. Natarajan, K., B. M. Jackson, H. Zhou, F. Winston, and A. G. Hinnebusch. 1999. *Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/mediator.* Mol Cell **4:**657-64.

205. Ryan, M. P., R. Jones, and R. H. Morse. 1998. *SWI-SNF complex participation in transcriptional activation at a step subsequent to activator binding.* Mol Cell Biol **18:**1774-82.

206. Brown, S. A., and R. E. Kingston. 1997. *Disruption of downstream chromatin directed by a transcriptional activator.* Genes Dev **11:**3116-21.

207. Imbalzano, A. N., H. Kwon, M. R. Green, and R. E. Kingston. 1994. *Facilitated binding of TATA-binding protein to nucleosomal DNA.* Nature **370:**481-485.

208. Burns, L. G., and C. L. Peterson. 1997. *The yeast SWI-SNF complex facilitates binding of a transcriptional activator to nucleosomal sites in vivo.* Mol Cell Biol **17:**4811-9.

209. Cosma, M. P., T. Tanaka, and K. Nasmyth. 1999. *Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter.* Cell **97:**299-311.

210. Abrams, E., L. Neigeborn, and M. Carlson. 1986. *Molecular analysis of SNF2 and SNF5, genes required for expression of glucose-repressible genes in Saccharomyces cerevisiae.* Mol Cell Biol **6:**3643-51.

211. 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:**2544-53.

212. Biggar, S. R., and G. R. Crabtree. 1999. *Continuous and widespread roles for the Swi-Snf complex in transcription.* Embo J **18:**2254-64.

213. Difco Laboratories. 1998. *Difco manual,* 11th ed ed. Difco Laboratories Division of Becton Dickinson and Company, Sparks, MD.

214. Schmitt, M. E., T. A. Brown, and B. L. Trumpower. 1990. *A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae.* Nucleic Acids Research **18:**3091.

215. Ausubel, F. M. 1987. *Current protocols in molecular biology.* Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, New York.

216. Bostian, K. A., J. M. Lemire, and H. O. Halvorson. 1983. *Physiological control of repressible acid phosphatase gene transcripts in Saccharomyces cerevisiae.* Mol Cell Biol **3:**839-53.

217. Almer, A., and W. Horz. 1986. *Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast.* EMBO J. **5:**2681-2687.

218. Haswell, E. S., and E. K. O'Shea. 1999. *An in vitro system recapitulates chromatin remodeling at the PHO5 promoter.* Mol Cell Biol **19:**2817-27.

219. Tsuchiya, E., M. Uno, A. Kiguchi, K. Masuoka, Y. Kanemori, et al. 1992. *The Saccharomyces cerevisiae NPS1 gene, a novel CDC gene which encodes a 160 kDa nuclear protein involved in G2 phase control.* Embo J **11:**4017-26.

220. Cao, Y., B. R. Cairns, R. D. Kornberg, and B. C. Laurent. 1997. *Sfh1p, a component of a novel chromatin-remodeling complex, is required for cell cycle progression.* Mol Cell Biol **17:**3323-34.

221. Treich, I., and M. Carlson. 1997. *Interaction of a SWI3 homolog with STH1 provides evidence for a SWI/SNF-related complex with an essential function in saccharomyces cerevisiae.* Mol. Cell. Biol. **17:**1768-1775.

222. Cairns, B. R., A. Schlichter, H. Erdjument-Bromage, P. Tempst, R. D. Kornberg, et al. 1999. *Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains.* Mol Cell **4:**715-23.

223. Tsuchiya, E., T. Hosotani, and T. Miyakawa. 1998. *A mutation in NPS1/STH1, an essential gene encoding a component of a novel chromatin-remodeling complex RSC, alters the chromatin structure of Saccharomyces cerevisiae centromeres.* Nucleic Acids Res **26:**3286-92.

224. Yukawa, M., S. Katoh, T. Miyakawa, and E. Tsuchiya. 1999. *Nps1/Sth1p, a component of an essential chromatin-remodeling complex of Saccharomyces cerevisiae, is required for the maximal expression of early meiotic genes.* Genes Cells **4:**99-110.

225. Moreira, J. M., and S. Holmberg. 1998. *Nucleosome structure of the yeast CHA1 promoter: analysis of activation- dependent chromatin remodeling of an RNA-polymerase-II-transcribed gene in TBP and RNA pol II mutants defective in vivo in response to acidic activators.* Embo J **17:**6028-38.

226. Tsukiyama, T., J. Palmer, C. C. Landel, J. Shiloach, and C. Wu. 1999. *Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae.* Genes Dev **13:**686-97.

227. Trachtulcov, P., I. Janatov, S. D. Kohlwein, and J. Hasek. 2000. *Saccharomyces cerevisiae gene ISW2 encodes a microtubule-interacting protein required for premeiotic DNA replication.* Yeast **16:**35-47.

228. Woodage, T., M. A. Basrai, A. D. Baxevanis, P. Hieter, and F. S. Collins. 1997. *Characterization of the CHD family of proteins.* Proc Natl Acad Sci U S A **94:**11472-7.

229. Tran, H., D. Steger, and S. Johnson. 2000. p. .

230. Ebbert, R., A. Birkmann, and H. J. Schuller. 1999. *The product of the SNF2/SWI2 paralogue INO80 of Saccharomyces cerevisiae required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex.* Mol Microbiol **32:**741-51.

231. Rundlett, S. E., A. A. Carmen, R. Kobayashi, S. Bavykin, B. M. Turner, et al. 1996. *HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription.* Proc Natl Acad Sci U S A **93:**14503-8.

232. Kadosh, D., and K. Struhl. 1998. *Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo.* Genes Dev **12:**797-805.

233. Vidal, M., and R. F. Gaber. 1991. *RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae.* Mol Cell Biol **11:**6317-27.

234. Vidal, M., R. Strich, R. E. Esposito, and R. F. Gaber. 1991. *RPD1 (SIN3/UME4) is required for maximal activation and repression of diverse yeast genes.* Mol Cell Biol **11:**6306-16.

235. Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. J. Steger, J. C. Reese, et al. 1998. *A subset of TAF(II)s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation.* Cell **94:**45-53.

236. Saleh, A., D. Schieltz, N. Ting, S. B. McMahon, D. W. Litchfield, et al. 1998. *Tra1p is a component of the yeast Ada.Spt transcriptional regulatory complexes.* J Biol Chem **273:**26559-65.

237. Grant, P. A., D. Schieltz, M. G. Pray-Grant, J. R. Yates, 3rd, and J. L. Workman. 1998. *The ATM-related cofactor Tra1 is a component of the purified SAGA complex.* Mol Cell **2:**863-7.

238. Eberharter, A., D. E. Sterner, D. Schieltz, A. Hassan, J. R. Yates, 3rd, et al. 1999. *The ADA complex is a distinct histone acetyltransferase complex in Saccharomyces cerevisiae.* Mol Cell Biol **19:**6621-31.

239. Steger, D. J., A. Eberharter, S. John, P. A. Grant, and J. L. Workman. 1998. *Purified histone acetyltransferase complexes stimulate HIV-1 transcription from preassembled nucleosomal arrays.* Proc Natl Acad Sci U S A **95:**12924-9.

240. Otero, G., J. Fellows, Y. Li, T. de Bizemont, A. M. Dirac, et al. 1999. *Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation.* Mol Cell **3:**109-18.

241. Wittschieben, B. O., G. Otero, T. de Bizemont, J. Fellows, H. Erdjument-Bromage, et al. 1999. *A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme.* Mol Cell **4:**123-8.

242. Guthrie, C., and G. R. Fink. 1991. *Guide to yeast genetics and molecular biology.* Academic Press Inc., San Diego.

243. Guthrie, C., and G. R. Fink. 1991. *Guide to yeast genetics and molecular biology.* Academic Press, San Diego.

244. Burgess, S. M., M. Ajimura, and N. Kleckner. 1999. *GCN5-dependent histone H3 acetylation and RPD3-dependent histone H4 deacetylation have distinct, opposing effects on IME2 transcription, during meiosis and during vegetative growth, in budding yeast.* Proc Natl Acad Sci U S A **96:**6835-40.

245. Roberts, S. M., and F. Winston. 1997. *Essential functional interactions of SAGA, a Saccharomyces cerevisiae complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes.* Genetics **147:**451-65.

246. Sudarsanam, P., Y. Cao, L. Wu, B. C. Laurent, and F. Winston. 1999. *The nucleosome remodeling complex, Snf/Swi, is required for the maintenance of transcription in vivo and is partially redundant with the histone acetyltransferase, Gcn5.* Embo J **18:**3101-6.

247. Krebs, J. E., M. H. Kuo, C. D. Allis, and C. L. Peterson. 1999. *Cell cycle-regulated histone acetylation required for expression of the yeast HO gene.* Genes Dev **13:**1412-21.

248. Kornberg, R. D., and Y. Lorch. 1992. *Chromatin structure and transcription.* Ann. Rev. Cell Biol. **8:**563-87.

249. Armstrong, J. A., and B. M. Emerson. 1998. *Transcription of chromatin: these are complex times.* Curr. Opin. Genet. Dev. **8:**165-172.

250. Barbaric, S., K. D. Fascher, and W. Horz. 1992. *Activation of the weakly regulated PHO8 promoter in S. cerevisiae: chromatin transition and binding sites for the positive regulatory protein PHO4.* Nucleic Acids Res **20:**1031-8.

251. Gregory, P. D., A. Schmid, M. Zavari, M. Munsterkotter, and W. Horz. 1999. *Chromatin remodelling at the PHO8 promoter requires SWI-SNF and SAGA at a step subsequent to activator binding [In Process Citation].* Embo J **18:**6407-14.

252. Nishimura, K., K. Yasumura, K. Igarashi, S. Harashima, and Y. Kakinuma. 1999. *Transcription of some PHO genes in Saccharomyces cerevisiae is regulated by spt7p.* Yeast **15:**1711-7.

253. Winston, F., C. Dollard, E. A. Malone, J. Clare, J. G. Kapakos, et al. 1987. *Three genes are required for trans-activation of Ty transcription in yeast.* Genetics **115:**649-56.

254. Grant, P. A., D. E. Sterner, L. J. Duggan, J. L. Workman, and S. L. Berger. 1998. *The SAGA unfolds: convergence of transcription regulators in chromatin-modifying complexes.* Trends Cell Biol **8:**193-7.

255. Toh-E, A., and Y. Oshima. 1974. *Isolation and characterization of acid phosphatase mutants in Saccharomyces cerevisiae.* J. Bacteriol. **113:**727-38.

256. McAndrew, P. C., J. Svaren, S. R. Martin, W. Horz, and C. R. Goding. 1998. *Requirements for chromatin modulation and transcription activation by the Pho4 acidic activation domain.* Mol Cell Biol **18:**5818-27.

257. Burglin, T. R. 1988. *The yeast regulatory gene PHO2 encodes a homeo box [letter].* Cell **53:**339-40.

258. Rudolph, H., and A. Hnnen. 1987. *The yeast PHO5 promoter: phosphate-control elements and sequences mediating mRNA start-site selection.* Proc. Natl. Acad. Sci. USA **84:**1340-1344.

259. Magbanua, J. P., K. Fujisawa, N. Ogawa, and Y. Oshima. 1997. *The homeodomain protein Pho2p binds at an A/T-rich segment flanking the binding site of the basic-helix-loop-helix protein Pho4p in the yeast PHO promoters.* Yeast **13:**1299-308.

260. Brazas, R. M., and D. J. Stillman. 1993. *Identification and purification of a protein that binds DNA cooperatively with the yeast SWI5 protein [published erratum appears in Mol Cell Biol 1993 Nov;13(11):7200].* Mol Cell Biol **13:**5524-37.

261. Arndt, K. T., C. Styles, and G. R. Fink. 1987. *Multiple global regulators control HIS4 transcription in yeast.* Science **237:**874-80.

262. Braus, G., H. U. Mosch, K. Vogel, A. Hinnen, and R. Hutter. 1989. *Interpathway regulation of the TRP4 gene of yeast.* Embo J **8:**939-45.

263. Daignan-Fornier, B., and G. R. Fink. 1992. *Coregulation of purine and histidine biosynthesis by the transcriptional activators BAS1 and BAS2.* Proc Natl Acad Sci U S A **89:**6746-50.

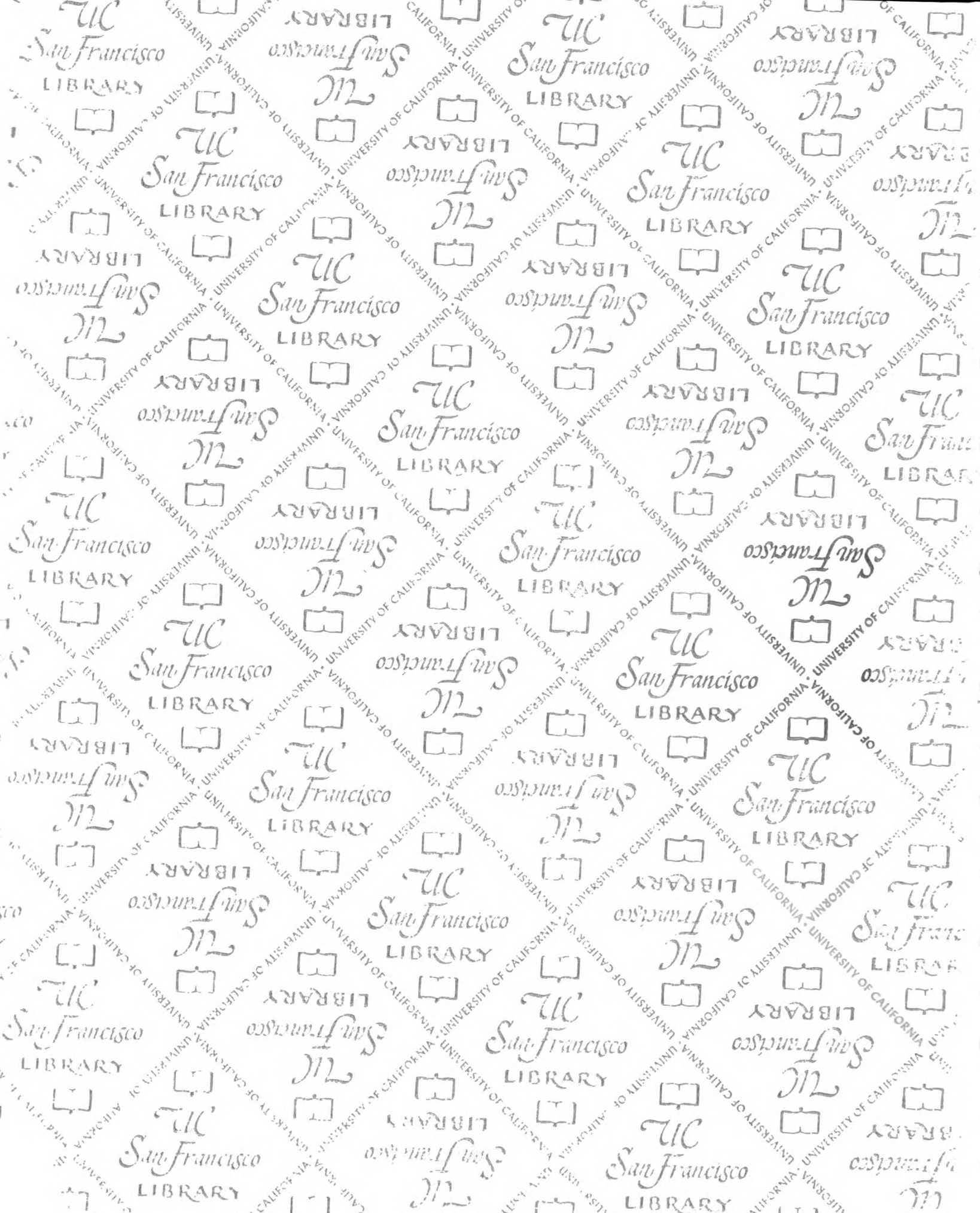
264. Brazas, R. M., and D. J. Stillman. 1993. *The Swi5 zinc-finger and Grf10 homeodomain proteins bind DNA cooperatively at the yeast HO promoter.* Proc Natl Acad Sci U S A **90:**11237-41.

265. Senstag, C., and A. Hinnen. 1988. *A 28-bp segment of the Saccharomyces cerevisiae PHO5 upstream activator sequnce confers phosphate control to the CYC1-lacZ gene fusion.* Gene **67:**223-228.

266. Hirst, K., F. Fisher, P. C. McAndrew, and C. R. Goding. 1994. *The transcription factor, the Cdk, its cyclin and their regulator: directing the transcriptional response to a nutritional signal.* Embo J **13:**5410-20.

267. Brenowitz, M., D. F. Senear, M. A. Shea, and G. K. Ackers. 1986. *Quantitative DNase footprint titration: a method for studying protein- DNA interactions.* Methods Enzymol **130:**132-81.

268. Zhang, F., M. Kirouac, N. Zhu, A. G. Hinnebusch, and R. J. Rolfes. 1997. *Evidence that complex formation by Bas1p and Bas2p (Pho2p) unmasks the activation function of Bas1p in an adenine-repressible step of ADE gene transcription.* Mol Cell Biol **17:**3272-83.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