INTERPLAY BETWEEN PROMOTER OCCUPANCY AND CHROMATIN REMODELING REQUIREMENTS IN TRANSACTIVATION OF THE *S. cerevisiae PHO5* GENE

A Dissertation

by

ARCHANA DHASARATHY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Genetics

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December 2004

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ABSTRACT

Interplay Between Promoter Occupancy and Chromatin Remodeling Requirements in Transactivation of the *S. cerevisiae PHO5* Gene.

(December 2004)

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In higher eukaryotes, DNA is packaged with histones and other proteins into chromatin. While this is important in the control of unwanted gene expression, chromatin also serves as a barrier to many vital functions in the cell. Therefore, cells have evolved many different types of chromatin remodeling enzymes to contend with this inhibitory structure and enable gene expression and other functions. The *Saccharomyces cerevisiae PHO5* gene is triggered in response to phosphate starvation. In this study, I evaluate the chromatin remodeling requirements of this gene with respect to the multisubunit complexes SWI/SNF and SAGA. I show, for the first time, physical recruitment of SWI/SNF to the *PHO5* promoter. I also demonstrate the role of promoter occupancy in influencing requirements for chromatin remodeling enzymes. Further, I describe various interactions between these two complexes at the *PHO5* promoter. This study presents evidence for the first instance of excess recruitment of an ATP-dependent remodeler potentially compensating for the lack of a histone acetyltransferase.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

-Sir William Bragg,

Nobel Prize winner in Physics, 1915

ACKNOWLEDGEMENTS

G.B Stern once said, “Silent gratitude isn't much use to anyone”. It is only befitting, therefore, for me to acknowledge the inestimable help and support of various people throughout the course of my graduate career. First of all, I would like to thank my advisor, Mike Kladde, for endless hours of scientific discussion and for all his encouragement and support. I would like to acknowledge my committee members, Dr. Dorothy Shippen, Dr. David Peterson and Dr. Yiwei Jiang, for all their helpful comments and suggestions. In particular, I owe an immense debt of gratitude to Dr. Dorothy Shippen and Dr. Geoffrey Kapler who gave me a tremendous amount of encouragement through many a rough phase.

I would like to thank past and present members of the Kladde lab: Chris Carvin, Walter Jessen, Laurie Friesenhahn, April Risinger, Alison Pirozzo, Megan Reynolds, Dan Neef, Jessica Kilgore and Scott Hoose for their help in lab and for being such terrific people to work with. I would like to thank Chris Carvin in particular for always being there for me through thick and thin! I’d like to express my gratitude to Angela Domitrovich, Libin Wang, Toni-Ann Mistretta and Lisa Forrester for their endless advice and support. I owe a debt of appreciation to Kelly Vaughan, Sarah Aubert and the Dwyer-Aubert clan, for being my family away from home. My thanks to Jyothi Jayaram, Sam Hill, Christine Gray, Upali Nanda, Mario Pennella, Seema Endley, Vijay Kovvali, Ritu Pathak, Rachel Idol, Jonathan Lindner and Bonny Butler for their friendship and support. My thanks are also due to members of the Polymenis, Bryk and Shippen labs, and to Dr. Suma Datta, Shawn Williamson, Dr. Max Summers and Dr. Mary Bryk, for

help with equipment and techniques. Other members of the Biochemistry and Biophysics Department are acknowledged as being wonderful colleagues. I would like to thank my cousin Dr. Srini Rajagopalan for being incredibly supportive and for his horrible puns. I would like to acknowledge my family for always being there for me. There are countless others who I have not named, but who mean so much to me: thank you for being there. Last but not least, I would like to pay an immense debt of gratitude to my parents, Dr. Geetha Dhasarathy and Mr. Sampath Dhasarathy, for being my best source of support, encouragement and inspiration. I would never have made it this far without you.

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CHAPTER I

INTRODUCTION

SCOPE

The primary scope of this dissertation is to explore the function of chromatin remodeling enzymes in transcriptional regulation using the *PHO5* gene from the budding yeast *Saccharomyces cerevisiae* as a model system. I present evidence for a major role of promoter occupancy in dictating requirements for chromatin remodeling activities in transcriptional activation of *PHO5*. Next, I discuss a possible explanation for the 'functional redundancy' of chromatin remodeling complexes SAGA and SWI/SNF.

The first part of the introduction provides background information on the field of chromatin structure and its role in repressing gene expression. I then discuss the salient features of many chromatin-remodeling enzymes that are recruited to effect transcriptional activation. The last part introduces the *S. cerevisiae* repressible acid phosphatase gene *PHO5* model system for the study of transcriptional regulation.

SIGNIFICANCE

The delicate threads of DNA that encode the 'blueprint of life' are carefully organized in the eukaryotic cell into a complex with histone and other proteins, called chromatin (Figure 1-1). While this solves the problem of packaging a 2 m-long stretch of DNA into a 5 μm nucleus, it is also a hindrance to many important biological processes in the cell.

This dissertation follows the style and format of Cell.

Hence, eukaryotes have evolved a variety of chromatin remodeling enzyme complexes that contend with this barrier to make DNA more accessible. Remodeling enzymes are highly conserved from yeast to humans. These multisubunit remodeling machines are recruited to specific regions in the genome by *trans*activator proteins that bind site-specifically to DNA and activate transcription and other biological processes.

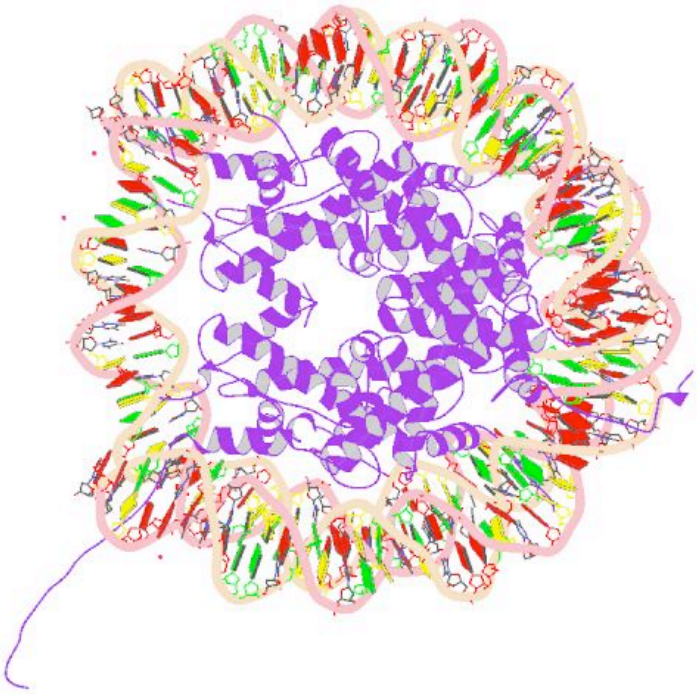

Figure 1-1. X-ray structure of the nucleosome core particle at 2.8Å resolution.

Ribbon diagram of the crystal structure of 146 bp of DNA wrapped around the histone core octamer to form the nucleosome core particle (Luger et al., 1997). Random coils protruding from the nucleosome represent the N-terminal tails of histones.

Aberrations in chromatin modifying and remodeling proteins can cause improper gene expression leading to diseases like cancer. In fact, a number of translocation events in cancer result from the fusion of genes encoding chromatin-remodeling complexes. For

instance, a type of acute leukemia involves the chromosomal translocation of genes coding for the human mixed lineage leukemia (MLL) and the CREB-binding protein (CBP) (Cairns, 2001). Also, truncations or deletions of the human Snf5/IniI component of human SWI/SNF are linked to malignant tumor formation (Versteege et al., 1998). Understanding the fundamental processes of chromatin remodeling at a basic level in yeast will help further studies in higher eukaryotes including humans. This dissertation will focus on the primary question of what dictates requirements for chromatin remodelers, with particular emphasis on the role of promoter occupancy.

CHROMATIN REMODELING: A MEANS TO AN END

The fundamental repeating unit of eukaryotic chromosomes, the nucleosome, is comprised of an octamer of histones (a dimer each of histone H3 and H4, surrounded by H2A and H2B dimers on the periphery) wrapped by 1.65 turns or 145-147 bp of a left-handed superhelix of DNA (Luger et al., 1997). This packaging of DNA serves not only to constrain the DNA within the confines of the nucleus, but also allows a broad range of changes in structure related to many biological processes like DNA replication, repair, gene expression and silencing. *Trans*activators bind to nucleosomes with greatly decreased affinity, especially at internal locations removed from the point of DNA exit/entry on the histone octamer surface (Pina et al., 1990; Owen-Hughes and Workman, 1994; Vettese-Dadey et al., 1994; Xu et al., 1998). At the yeast *PHO5* promoter, access of Pho4 to one of its DNA binding sites, UASp2, is blocked by virtue of its location at the center of a nucleosome (Venter et al., 1994). Thus, these studies indicate that nucleosomes can interfere with access of activators to their cognate binding sites.

How does the transcriptional machinery gain access to DNA? The term "chromatin remodeling" has been used to describe changes in nucleosomal structure as judged by increased accessibility to nucleases like DNase I or micrococcal nuclease (MNase), which demonstrated alterations in basic repeats of DNA. Eukaryotic cells bring about these structural changes by employing several highly conserved multisubunit, high-molecular weight complexes. These complexes, designated coactivators or remodelers, comprise two general classes. The first class utilizes the energy derived from ATP hydrolysis to disrupt nucleosomes (Jones and Kadonaga, 2000; Wolffe and Guschin, 2000), mostly by causing superhelical torsion in DNA. The second class consists of those complexes that post-translationally modify histones (Strahl and Allis, 2000) by acetylation, phosphorylation, methylation, ubiquitylation, sumoylation or ribosylation.

ATP-DEPENDENT CHROMATIN-REMODELING MACHINES

There are four main types of the first class of remodelers (Table 1-1), i.e. the Swi2/Snf2 subfamily, based on their adenosine triphosphatase (ATPase) domains: ATPases of the Swi2 group (SWI/SNF and RSC) contain bromodomains; ISWI-like ATPases (ACF, CHRAC, NURF, RSF) exhibit SANT and SLIDE motifs (putative DNA-binding domains); a split ATPase domain characterizes INO80.com and related enzyme complexes; and CHD-type enzymes, such as Mi-2, contain chromodomains (which interact with various chromatin components) and PHD fingers (a zinc finger-like motif). These four groups can also be distinguished by their biochemical properties and mechanisms of nucleosome remodeling (Langst and Becker, 2004). Swi2 ATPase activity is mainly stimulated by free DNA, whereas the CHD ATPase Mi-2 needs the presence of

nucleosomal (but not free) DNA to be induced to activity. ISWI hydrolyses ATP significantly in the presence of free DNA but is maximally stimulated by nucleosomes. Unlike Swi2 or Mi-2, ISWI requires the presence of the 20 amino acid- N-terminal 'tail' domain of histone H4 for full stimulation (Clapier et al., 2001; Hamiche et al., 2001; Clapier et al., 2002).

Table 1-1. Types of ATP-dependent chromatin remodeling complexes.

Type of ATPase	*Substrate*	*Domain(s)*
Swi2 (e.g. SWI/SNF, RSC)	Mainly free DNA	Bromodomains
ISWI-like (e.g. ACF, CHRAC, NURF, RSC)	Free DNA, but maximal stimulation by nucleosomal DNA	SANT and SLIDE domains
CHD-type (e.g. Mi-2/NuRD)	Nucleosomal (but not free) DNA	Chromodomains and PHD fingers
Ino80 (e.g. INO80.com)	Free/ nucleosomal DNA	Split ATPase domain, TELY and GTIE motifs

The members of the SWI/SNF complex were identified in screens for growth on sucrose (hence the term, Sucrose non-fermenters), while a second screen identified several genes involved in repression of the yeast *HO* gene, involved in mating-type switching (Switching-defective) (Winston and Carlson, 1992). Subsequently, this complex has been purified biochemically from many sources (Cairns et al., 1994; Peterson et al., 1994). The yeast SWI/SNF complex is known to contain 11 subunits. Using tyrosine iodination, Peterson and colleagues recently determined the relative stoichiometry of SWI/SNF subunits (Smith et al., 2003). While one copy each of 6 of the 11 subunits (Swi2, Swi1, Snf5, Swp73, Arp7 and Arp9) exists in the complex, the other

five are present in multiple copies (two copies of Swi3, Swp82, Snf6 and Snf11, as well as three copies of Swp29). Thus, SWI/SNF is predicted to have an apparent molecular mass of only 1.15 MDa (Smith et al., 2003)in contrast to the ~1.8 MDa predicted earlier.

INO80.com is another ATP-dependent remodeler that has been implicated in the activation of many genes. It is comprised of 12 known subunits, including actin (Act1) and the actin-related proteins Arp4, Arp5 and Arp8. Apart from the ATPase subunit, Ino80, it also contains the essential Rvb1 and Rvb2 proteins, which are related to the bacterial RuvB9 Holliday junction DNA helicase (Shen et al., 2000).

How do ATP-dependent remodelers work? While many models like 'twisting' and 'looping' have been invoked to explain catalyzed nucleosome mobility and sliding, one group (Havas et al., 2000) showed that ATP-dependent remodeling enzymes are able to generate negative superhelical torsion in DNA and chromatin. It has also been suggested that nucleosomes can be completely displaced from their template DNA by ATP-dependent remodeling enzymes (Lorch et al., 1999). The most current model suggested by Cairns and colleagues is the DNA pumping/translocation model (more similar to bulge migration) (personal communication).

The bromodomain of the Swi2/Snf2 ATPases can bind acetylated lysine residues (Shen et al., 2000) in histone N-terminal tails *in vitro* (Dhalluin et al., 1999; Jacobson et al., 2000; Owen et al., 2000) and is required for the stable anchoring of SWI/SNF (Hassan et al., 2001b; Hassan et al., 2002) to chromatin templates *in vitro*. Two other subunits, Swi3 and Snf5/Ini1 (Phelan et al., 1999; Geng et al., 2001) have also been shown to be important for remodeling activity of the SWI/SNF complex. Snf5 and Swi1 have been implicated in activator interactions as well (Prochasson et al., 2003). Besides

transcription, ATP-dependent remodeling machines have been implicated in DNA replication (Collins et al., 2002), repair and the maintenance of genome integrity (Hara and Sancar, 2002; Nilsen et al., 2002; Gaillard et al., 2003). SWI/SNF has also been shown to be involved in repression of genes (Sudarsanam et al., 2000; Martens and Winston, 2002) and in silencing at the telomeres and ribosomal DNA (Dror and Winston, 2004).

HISTONE-MODIFYING COMPLEXES

Histones undergo a vast number of post-translational modifications, including acetylation and methylation of lysines and arginines, phosphorylation of serines and threonines, ubiquitylation and sumoylation of lysines, as well as ADP-ribosylation (Peterson and Laniel, 2004). The biological significance of a number of these modifications has been studied in great detail, and many more examples of modifications have been identified by mass spectrometry in mammalian core histones (Zhang et al., 2003). Among this group of coactivators, one main class called histone acetyltransferases (HATs) can acetylate core histones and specific lysines in the N-terminal tails of nucleosomal as well as free histones (Grant et al., 1997; Eberharter et al., 1999). Four distinct multiprotein assemblies exist in yeast: Ada, NuA3, NuA4, and SAGA. The fact that several regulatory proteins like Gcn5, PCAF, p300 and CBP (Brown et al., 2000) all possess intrinsic histone acetyltransferase (HAT) activity suggested a role for histone acetylation in transcriptional activation. Gcn5 in particular has been extensively studied and has been shown to play a role in transcription (Gregory et al., 1998b; Kuo and Allis, 1998; Wang et al., 1998; Krebs et al., 1999; Syntichaki et al., 2000). It is the yeast

homolog of the first nuclear histone acetyltransferase to be identified from *Tetrahymena* (Brownell and Allis, 1995; Brownell et al., 1996). It is part of the SAGA complex (Spt-Ada-Gcn5 acetyltransferase), which is ~1.8 MDa in size and is composed of at least 14 subunits (Brown et al., 2000).

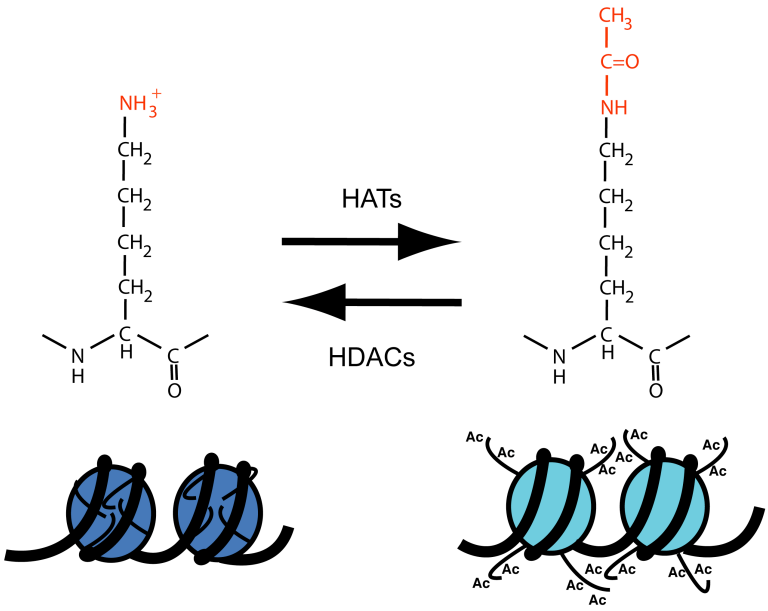

Figure 1-2. Equilibrium between acetylation and deacetylation.

Acetylation of histone tails by HATs neutralizes the charge conferred by the lysine residues and loosens the contact between DNA and histone tails. This change is reversible, and is accomplished by means of histone deacetylases (HDACs).

Apart from Gcn5, there are three main classes of SAGA components: the Ada proteins (Ada1, Ada2, Ada3 and Ada5/Spt20); the Spt proteins (Spt3, Spt7, Spt8 and Spt20/Ada5) and finally, a subset of TBP-associated factors (TAFs) ($TAF_{II}20/17$, $TAF_{II}25/23$, $TAF_{II}60$, $TAF_{II}68/61$ and $TAF_{II}90$) (Brown et al., 2000). Ada1 (Sterner et al., 1999), Spt7 (Gansheroff et al., 1995; Wu et al., 2004) and Spt20 (Roberts and

Winston, 1996; Wu et al., 2004) are vital to the integrity of the SAGA complex, while Spt3 and Spt8 are important in TBP interactions (Eisenmann et al., 1992; Eisenmann et al., 1994). SAGA also contains the protein Tra1 (Grant et al., 1998), which is thought to interact with transcription activators, thus enabling recruitment to genomic loci (Brown et al., 2001).

How does acetylation help remodel chromatin? It has been suggested that acetylation by the p300 HAT helps to transfer a H2A/H2B dimer to a histone chaperone, thus altering nucleosome structure (Ito et al., 2000). Further, in the absence of activator, histone acetylation stabilizes binding of SWI/SNF *in vitro* (Hassan et al., 2001b). Histone acetylation also interferes with higher-order folding (Tse et al., 1998), thus enhancing transcriptional activation. Further, histone acetylation neutralizes the positive charge on the histone tails (Figure 1-2), thus decreasing their affinity for DNA (Hong et al., 1993). Many silencing proteins like Sir2, Sir4 and Tup1 have also been shown to bind to hypoacetylated histone tails (reviewed in Kuo and Allis, 1998).

Another HAT complex, NuA4 (nucleosomal acetyltransferase histone H4) contains Esa1, the only known essential HAT in yeast (Smith et al., 1998) and Tra1 (Allard et al., 1999), which has been shown to interact with activators and thus facilitate HAT recruitment (Brown et al., 2001). Recent work by Côté and colleagues showed the existence of a smaller version of NuA4, the "piccolo NuA4 complex" (Boudreault et al., 2003) comprising just 3 (Esa1, Epl1, and Yng2) of the 13 stably associated proteins of NuA4. Of the known HATs, only the SAGA and NuA4 complexes, which preferentially acetylate histones H3 and H4, respectively, have been implicated in the regulation of transcriptional initiation.

ACTIVATOR-DEPENDENT RECRUITMENT OF REMODELERS

The classic "chicken and egg" story of whether global acetylation occurred before or after activator binding stood unresolved for a long time. Recent evidence indicates that the activation domains of the "acidic" class of activators can interact directly and specifically with a subset of purified chromatin remodelers; SWI/SNF, NuA4, and SAGA, as well as the Srb/mediator subcomplex of the RNA polymerase II holoenzyme (Figure 1-3), but not with the Ada or NuA3 complexes (Peterson and Logie, 2000; Fry and Peterson, 2001).

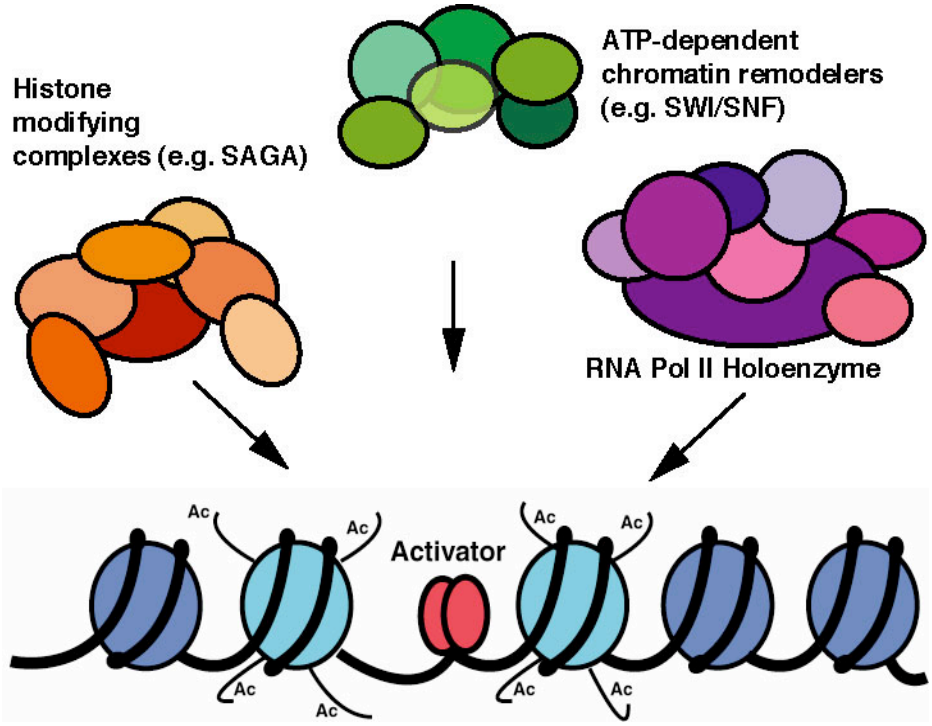

Figure 1-3. Recruitment theory of transcriptional activation.

Site-specific DNA-binding activators recruit chromatin remodeling complexes that modify histones or histone acetyltransferases (HATs) like SAGA and ATP-dependent chromatin remodeling machines (e.g. SWI/SNF) as well as the RNAP II holoenzyme in order to activate gene expression.

This interaction is sensitive to point mutations in the activation domain that eliminate activation function *in vivo*, and it appears functionally important because it leads to stimulation of transcription from chromatin templates *in vitro* (Utley et al., 1998; Ikeda et al., 1999). The current model, therefore, is that chromatin remodelers (which do not bind DNA appreciably on their own) act subsequent to activator binding (Ryan et al., 1998). Support for this model is found in recent studies, including our own, demonstrating that activators are able to bind their promoters *in vivo* in the absence of Swi2/Snf2, Gcn5, or Esa1, apparently recruiting these chromatin remodelers to the promoters upon which they act (reviewed in Fry and Peterson, 2001).

VARIABILITY IN REQUIREMENT FOR REMODELERS

Although a lot of light has been shed on the mechanism of recruitment of coactivators to promoters, the intriguing question of why promoters vary in their requirements for chromatin remodelers is essentially unanswered. Our lack of understanding of what delineates coactivator dependencies of a promoter is underscored if we consider the expression of the *PHO5* and *PHO8* genes, which code for acid and alkaline phosphatase, respectively. Despite being regulated by the same signaling pathway and the same transcriptional activator Pho4 (Figure 1-4) (Lenburg and O'Shea, 1996; Oshima, 1997), these genes show different dependencies for chromatin-remodeling coactivators.

The *PHO8* promoter demonstrates an absolute requirement for Gcn5 and Swi2 both under submaximal and maximally inducing conditions (Gregory et al., 1998b). However, both Gcn5 and Swi2 are dispensable as far as transcription at *PHO5* is concerned;

(Gaudreau et al., 1997; Gregory et al., 1998b; Haswell and O'Shea, 1999) although the deletion of *GCN5* or histone tails affects chromatin remodeling thus delaying *PHO5* induction (Barbaric et al., 2001). Ino80 has been shown to be required for full activation of *PHO5* and SWI/SNF has been implicated either by itself or in association with Htz1, the histone H2A variant (Santisteban et al., 2000; Steger et al., 2003). These results evoke the interesting question: Why do promoters under control of the same signal transduction pathways and the same transcriptional activators exhibit such strikingly disparate requirements for coactivators?

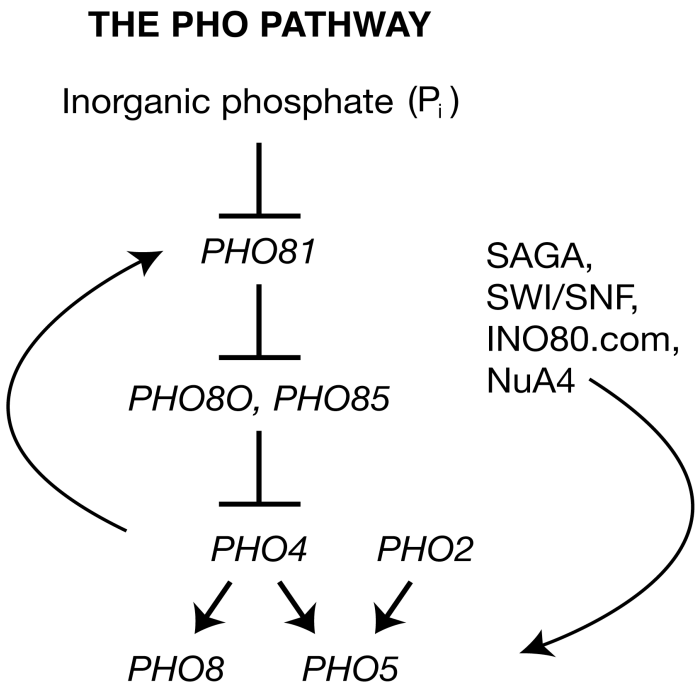

Figure 1-4. The PHO pathway.
In the yeast *S. cerevisiae*, the same signal transduction cascade regulates the repressible phosphatase genes *PHO5* and *PHO8*. The transactivator Pho4 is phosphorylated by the cyclin/cyclin-dependent kinase (CDK) pair Pho80-Pho85 and localized to the cytoplasm thus repressing PHO genes under conditions of high concentrations of extracellular phosphate (P_i). When yeast cells are starved for P_i, the cyclin/CDK pair is inhibited by Pho81 leading to Pho4 nuclear localization and PHO gene activation.

Possible reasons include, a) specificity of activators with respect to recruitment of chromatin remodeling complexes and b) promoter architecture. With respect to specificity of activators, some poly-glutamine activators exhibit a binding preference for NuA4 (Nourani et al., 2004) but not for SWI/SNF (Neely et al., 1999; Yudkovsky et al., 1999). On the other hand, acidic activators interact with most remodeling activities including NuA4, SAGA and SWI/SNF (reviewed in Fry and Peterson, 2001; Hassan et al., 2001a).

Recent findings provide some clues to help us answer this fundamental question. Expression of *PHO5*, while independent of SWI/SNF or SAGA under fully activating conditions, becomes highly dependent on these remodelers when the promoters are weakened by mutations in the DNA-binding sites for the activators (Burns and Peterson, 1997b). Further, the insertion of additional activator binding sites in a promoter, or overexpression of transcriptional activators, suppresses chromatin-remodeling defects (Laurent and Carlson, 1992; Gregory et al., 1998b). Additionally, maximum activation of *PHO5* requires the presence of the DNA-binding protein Pho2 (Barbaric et al., 1998), which binds cooperatively with Pho4, while Pho2 does not influence *PHO8* significantly (Oshima, 1997; Münsterkötter et al., 2000).

Another set of genes, *GAL1-10* and *GAL7*, express the proteins needed to utilize galactose (Johnston, 1987). In the poised or repressed state, the *GAL* genes are inactive by virtue of positioned nucleosomes that are placed over the TATA (*GAL1-10, GAL7*) as well as the major *GAL1* transcriptional start site. Galactose-stimulated induction causes Gal4-dependent nucleosome disruption and activation. Note that unlike Pho4, Gal4 is bound upstream of the GAL genes in a variety of non-glucose carbon sources without

causing chromatin remodeling, due to its association with the repressor protein Gal80 (Nogi et al., 1984; Giniger et al., 1985), which interacts directly with and inhibits the activation domain of Gal4 (Lue et al., 1987; Wu et al., 1996). Another important consideration is that unlike *PHO5*, all four UASg sites are accessible at *GAL1*, being located in a hypersensitive region. Expression from *GAL1* occurs at almost wild-type levels in *swi2/snf2, gcn5* or *ada* mutants (Marcus et al., 1994; Burns and Peterson, 1997a; Gaudreau et al., 1997). However, it is interesting to note that transcription is decreased in a *swi2*Δ strain containing a truncated version of the *GAL1* promoter containing only two low-affinity Gal4 binding sites (Marcus et al., 1994; Burns and Peterson, 1997a; Gaudreau et al., 1997). This SWI/SNF dependence was overcome by placing the low-affinity sites in a nucleosome-free region or replacing them with high-affinity sites (Burns and Peterson, 1997a).

Further, it was shown that while cells lacking either *SWI2* or *GCN5* did not differ much from wild type with respect to *GAL1* activity, cells where both coactivators were inactivated showed a much greater defect in transcription (Biggar and Crabtree, 1999). This suggested that Gal4 recruited both coactivators. Further, the differences in SWI/SNF dependence between the full-length and truncated promoters can be explained if, at the full-length promoter, Gal4 recruits more remodelers. On the other hand, at the truncated promoter with only two binding sites, the recruitment capabilities of Gal4 are impaired. This could explain why the truncated promoter is defective in transcriptional activation if either of the complexes is deleted (Biggar and Crabtree, 1999). Further support for these studies comes from a series of elegant experiments (Tanaka, 1996) showing that promoter occupancy could be modulated by changing a) the number of promoter binding

sites of an activator and/ or cooperative binding with another activator and b) the number of activation domains of an activator.

Thus, from these observations, we came up with a model whereby levels of activator binding to a promoter (i.e. fractional occupancy) would influence its requirements for chromatin remodeling. This premise raises key questions regarding chromatin-remodeling dependency. First, are individual chromatin remodelers needed throughout the time course of activation, or are they needed only at early time points, when promoter occupancy is low due to decreased factor concentration? Secondly, does enhanced promoter occupancy suppress the requirement for chromatin remodeling coactivators? The answers to each of the above considerations are critically important for understanding how activator binding translates to the coactivator dependence of various promoters, and forms the basis for the series of experiments described herewith.

THE PHENOMENON OF FUNCTIONAL REDUNDANCY

SWI/SNF and SAGA are both involved in the expression of a number of promoters (Pollard and Peterson, 1997). While deletions of the catalytic subunits of one or the other complex does not affect the expression of many genes, combined deletions of major SWI/SNF and SAGA subunits is lethal (Roberts and Winston, 1997; Sterner et al., 1999; Sudarsanam et al., 1999). It was also demonstrated that inactivation of SWI/SNF in *gcn5* or *ada* mutants caused loss of gene expression at many loci (Biggar and Crabtree, 1999). This suggests that there is some degree of redundancy between them, although they may have different mechanismss by which they activate transcription.

There has also been a lot of evidence to suggest the interdependence of SWI/SNF

and SAGA remodeling complexes in transcription. For instance, at the yeast *HO* promoter, ordered recruitment of first SWI/SNF and then SAGA was observed (Cosma et al., 1999; Krebs et al., 1999) in mitosis, suggesting that SWI/SNF was needed to allow HATs access to chromatin. On the other hand, HATs can acetylate nucleosomal arrays and effect transcription without prior addition of SWI/SNF (Utley et al., 1998; Ikeda et al., 1999). At the IFN-β promoter, the activator NF-κB recruits Gcn5 prior to SWI/SNF (Agalioti et al., 2000). However, it is interesting to note that SWI/SNF binding does not require prior acetylation if multiple NF-κB binding sites are present, suggesting that increased occupancy of the activator can stably recruit sufficient levels of SWI/SNF to activate transcription. Bromodomains, which are found in many chromatin remodeling complexes are thought to bind to acetylated lysine residues (Dhalluin et al., 1999; Ornaghi et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000). The bromodomain found in Gcn5 is not required *in vivo* for Gcn5-mediated histone acetylation but was shown to be indispensable for Swi2-dependent nucleosome remodeling and subsequent transcriptional activation. The Gcn5 bromodomain has also been shown to stabilize SWI/SNF association at a reporter gene *in vivo* (Syntichaki et al., 2000).

The observation that SWI/SNF and SAGA are so different in their composition and yet behave in a redundant manner genetically calls for some speculation. Are they 'redundant' because they both serve a common goal: that of remodeling chromatin to allow transcription? If so, a simple hypothesis follows that if one complex is not available to the cell, then more of the other might possibly be recruited in order that levels of gene expression remain constant. If they are involved in competing for the same activator

surface, then loss of one complex should result in increased recruitment of the other. On the other hand, if they are interdependent (i.e. one is needed for the other to associate with the promoter), it is hard to see how they could be redundant unless a large amount of the complex is recruited in the absence of the other. Thus, a lot remains to be understood with regard to how these two complexes interact functionally.

PHO5 AS A MODEL GENE

The yeast *S. cerevisiae* responds to variations in inorganic phosphate (P_i) levels in its environment by inducing around 22 genes which all work to scavenge P_i (Ogawa et al., 2000). This complex set of structural genes, encoding among others acid and alkaline phosphatases, constitutes the PHO system. Most of the acid phosphatases are secreted to the periplasmic space and require an acidic pH optimum for activity, including *PHO3, PHO5*, *PHO11* and *PHO12*. The enzymatic activity of these acid phosphatases can be measured quantitatively using 4-paranitrophenyl phosphate in a standard phosphatase assay and qualitatively on a plate with □-naphthyl-phosphate. The *PHO5* gene product accounts for more than 90% of this activity. The alkaline phosphatase *PHO8* is localized to the vacuole and is active at an alkaline pH optimum (Vogel and Hinnen, 1990). Both *PHO5* and *PHO8* have been well studied, and while regulated by the same signal transduction pathway and by a similar set of genes, their expressed activity levels are quite different. *PHO8* expression is about 10-times weaker than *PHO5* (Münsterkötter et al., 2000). Thanks to the incisive analysis of *PHO5* by many groups, it remains an ideal model for the study of transcriptional activation and chromatin remodeling.

PHO5 has two main transcriptional activators, Pho4 and Pho2, while Pho4 alone effects *PHO8* activation (Barbaric et al., 1992). The bHLH protein Pho4 binds cooperatively with Pho2, a homeodomain protein, to its two main binding sites, UASp1 and UASp2.

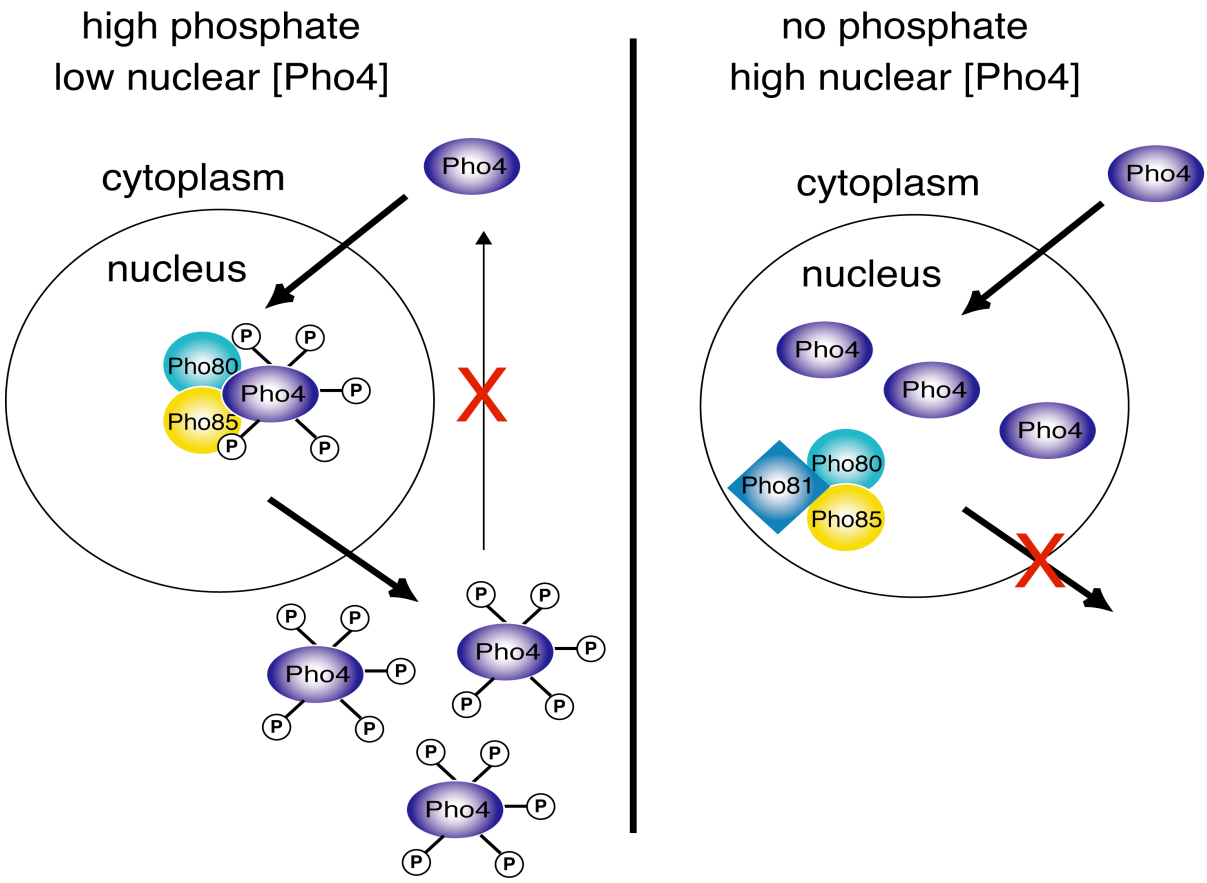

Figure 1-5. Phosphorylation of Pho4 regulates its nucleo-cytoplasmic localization.

The Pho80-Pho85 cyclin/cyclin-dependent kinase regulates Pho4 nuclear import by phosphorylation. This causes Pho4 to be exported from the nucleus via the Msn5 nuclear exporter and its import via Pse1/Kap121 is inhibited. In activating conditions, Pho81 inactivates Pho80-Pho85 thus allowing for constitutive nuclear import. Reproduced with permission from Christopher D. Carvin.

In conditions of high P_i, the PHO cluster is repressed due to phosphorylation of Pho4 by the cyclin/cyclin-dependent kinase pair Pho80-Pho85 (Kaffman et al., 1994). Although Pho4 is constitutively expressed (Lemire et al., 1985; Yoshida et al., 1989a), the phosphorylation of five residues serves to hinder transcription by Pho4 in several ways (Figure 1-5). First, it increases interaction with Msn5, the sole Pho4 nuclear exporter (Kaffman et al., 1998a; Komeili and O'Shea, 1999). Secondly, it prevents interaction with Pse1, the nuclear importer for Pho4 (Kaffman et al., 1998b; Komeili and O'Shea, 1999). Finally, phosphorylation prevents Pho4 interaction with Pho2 (Komeili and O'Shea, 1999), which is a homeodomain factor needed for cooperative binding of Pho4 to the *PHO5* promoter (Barbaric et al., 1996; Barbaric et al., 1998; Komeili and O'Shea, 1999).

Upon P_i starvation, the cyclin-dependent kinase inhibitor Pho81 inactivates Pho80-Pho85, thus allowing Pho4 entry into the nucleus and full activation of *PHO5* (Schneider et al., 1994). It is important to note that Pho4 regulates *PHO81* expression (Yoshida et al., 1989b; Creasy et al., 1993). This provides a positive feedback loop during PHO activation. Both the *PHO5* and *PHO8* promoter structures have been well characterized by the Hörz group (Almer and Hörz, 1986; Almer et al., 1986; Barbaric et al., 1992). Under repressive conditions of growth in high P_i, the *PHO5* promoter is organized into a series of five positioned nucleosomes (Figure 1-6) that constrain their TATA elements, and thereby block promoter association of the basal transcription complex $TF_{II}D$ (Bergman and Kramer, 1983; Almer et al., 1986; Fascher et al., 1990; Barbaric et al., 1992). Pho2 binding sites flank UASp1 and UASp2, the two Pho4 binding sites. UASp1 is contained in a hypersensitive site, which is accessible in high P_i (Almer

and Hörz, 1986; Fascher et al., 1990; Carvin et al., 2003a; Carvin et al., 2003b). UASp2 and the TATA box are located in nucleosome -2 and –1 respectively, and are inaccessible in repressed conditions. However, recent work suggests that UASp2 is accessible to Pho4 in repressed conditions as well (Adkins et al., 2004). Depletion of yeast nucleosomes *in vivo* leads to derepression of *PHO5* transcription even in the presence of P_i (Han and Grunstein, 1988; Han et al., 1988; Gregory et al., 1999a; Wyrick et al., 1999).

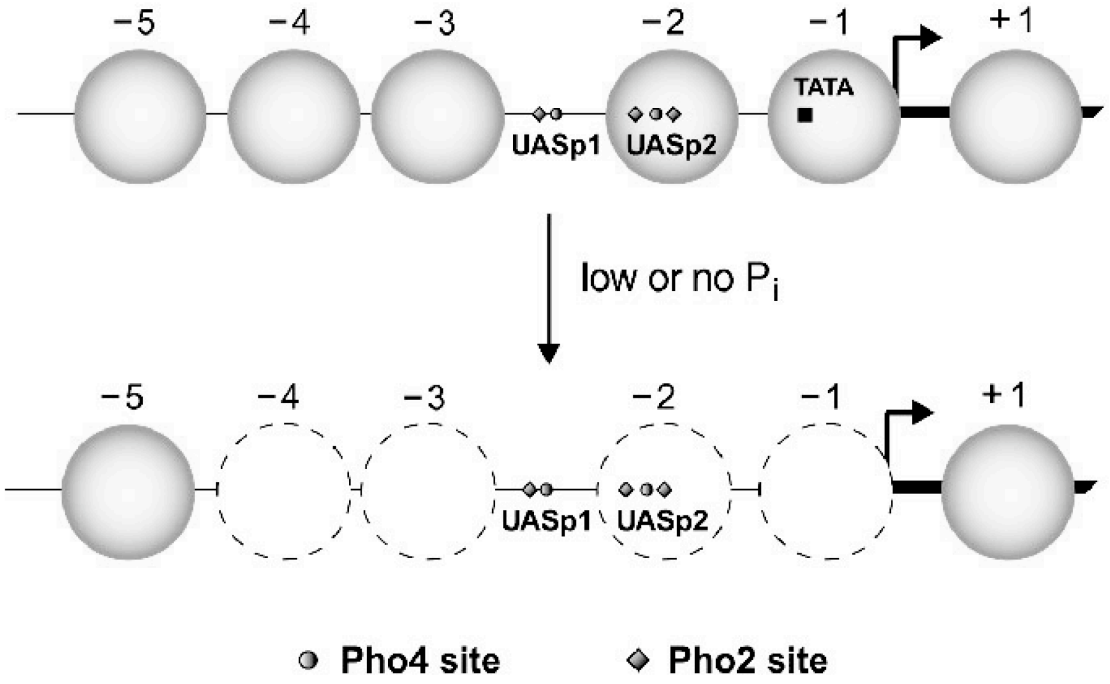

Figure 1-6. Nucleosome transitions on *PHO5* activation.

In high P_i conditions, the *PHO5* promoter is constrained by five positioned nucleosomes, which block binding of *trans*-activators to UASp2 and the TATA box. Upon activation in low phosphate, four nucleosomes are remodeled. Work from our lab indicates remodeling of nucleosome –5 as well.

Thus, chromatin remodeling is required for full activation, as well as for recruitment of the transcription machinery. However, it occurs prior to and independent

of transcription, since mutation of the TATA box hinders transcription, but does not prevent chromatin remodeling (Fascher et al., 1993). Previous work had identified remodeling of four nucleosomes upon P_i starvation; however work in our laboratory has shown that a fifth nucleosome is remodeled as well (unpublished data).

What are the chromatin-remodeling requirements at *PHO5* and *PHO8*? While full levels of *PHO5* activation have been shown to be independent of SWI/SNF and SAGA, *PHO8* expression is strictly dependent on both of these remodelers (Gregory et al., 1999b). The INO80.com ATP-dependent remodeling complex and the NuA4 HAT complex have also been implicated in *PHO5* activation (Steger et al., 2003; Nourani et al., 2004). The Asf1 histone chaperone has been recently shown to be needed for *PHO5* transactivation, but its loss apparently does not interfere with Pho4 binding to UASp2 (Adkins et al., 2004). Recent work from our lab showed that the histone methyltransferase Set1 negatively regulated *PHO5* expression (Carvin and Kladde, 2004), contrary to prior work that histone methylation is mainly associated with transcriptionally active chromatin. The Bdf1 protein has been shown to affect the kinetics of *PHO5* expression. While it does not appear to be essential for full activation of *PHO5*, a strict requirement for Bdf1 is imposed by a decrease in TATA accessibility. This possibly arises due to the interaction of $TF_{II}D$-bound Bdf1 with acetylated histone tails (Martinez-Campa et al., 2004). The mitotic activation of *PHO5* is also dependent on Swi2 and Gcn5 (Neef and Kladde, 2003). Thus, there is interplay of multifarious factors at the *PHO5* promoter enabling rapid activation.

In conclusion, the PHO system is an ideal system for the study of transcriptional regulation and chromatin structure. In this dissertation, I present evidence that the

coactivators Gcn5 and Swi2/Snf2 are needed for full activation when lower levels of Pho4 are present at the *PHO5* promoter. Thus, at greater levels of binding, there is a decreased requirement for one or the other of these remodelers. I also demonstrate for the first time at a yeast locus, the recruitment of a histone acetyltransferase (Gcn5) prior to an ATP-dependent remodeler (Swi2). This is also the first demonstration of the physical presence of Swi2 at the yeast *PHO5* promoter.

I also demonstrate that Pho4 binding at the *PHO5* promoter is delayed in both *gcn5*Δ and *swi2*Δ cells with respect to wild type. This delay in Pho4 binding at the promoter demonstrates for the first time a mechanistic basis for the kinetic delay in *PHO5* induction described previously (Barbaric et al., 2001; Neef and Kladde, 2003). Swi2 and Gcn5 have been shown to be partially redundant. In a yeast strain lacking Gcn5, we unexpectedly find recruitment of twice as much Swi2. This is the first evidence for an ATP-dependent remodeler potentially compensating for the lack of a histone acetyltransferase subunit to help achieve wild type levels of gene expression. The increased recruitment of Swi2 only occurs at increased levels of promoter-bound Pho4, hence the "delay" in activation in a strain deleted for *GCN5*. On the other hand, we can detect no difference in Gcn5 recruitment in a *swi2*Δ strain relative to wild type. The decreased amount of promoter-bound Pho4 might be responsible for this lower Gcn5 recruitment. Further, we find that there is very little SWI/SNF at the *PHO5* promoter in an *spt20*Δ strain. The highly reduced activity (about one third of wild type activity) of an *spt20*Δ strain compared to a wild type strain suggests that Pho4 occupancy is also decreased in this strain. I discuss several possible models to account for interplay of the SWI/SNF and SAGA remodeling complexes at the *PHO5* promoter.

CHAPTER II

PROMOTER OCCUPANCY IS A MAJOR DETERMINANT OF CHROMATIN-REMODELING ENZYME REQUIREMENTS

OVERVIEW

Chromatin creates transcriptional barriers that are overcome by coactivator activities such as the histone acetyltransferase Gcn5 and the chromatin remodeler SWI/SNF. The factors defining the differential requirements for chromatin remodeling activities in the transcription of various promoters remain elusive. Here, we show that transactivation of *Saccharomyces cerevisiae PHO5*, which does not require Gcn5 or SWI/SNF under fully inducing conditions, is highly dependent on both coactivators at reduced nuclear concentrations of Pho4 and thus lower levels of promoter-bound Pho4 transactivator. Conversely, physiological increases in Pho4 nuclear concentration and binding at *PHO5* suppress the need for both Gcn5 and SWI/SNF, suggesting that coactivator redundancy is established at high binding site occupancy. Consistent with this, using chromatin immunoprecipitation, we demonstrate that Gcn5 and SWI/SNF are recruited directly to *PHO5* and other strongly transcribed promoters, including *GAL1-10*, *RPL19B*, *RPS22B*, *PYK1,* and *EFT2*, which are thought to be independent of either coactivator. These results show that: a) binding site occupancy plays a crucial role in defining the extent to which transcription requires individual chromatin remodeling enzymes; b) Gcn5 and SWI/SNF associate with many more genomic targets than previously appreciated; and c) Gcn5 can be temporally recruited before SWI/SNF to yeast promoters.

INTRODUCTION

The incorporation of regulatory elements into nucleosomes interferes with their function by obstructing their accessibility to trans-acting factors (Owen-Hughes and Workman, 1994). Several highly conserved multisubunit complexes, termed coactivators or chromatin remodelers, act in concert with site-specific activators to help the transcriptional apparatus contend with chromatin structure (Narlikar et al., 2002). One class of coactivators contains an ATPase subunit (e.g. Swi2/Snf2 of SWI/SNF) that uses the energy derived from ATP hydrolysis to disrupt histone-DNA interactions (Vignali et al., 2000; Lusser and Kadonaga, 2003). A second class of coactivators post-translationally modifies specific amino acid residues of the basic core histone proteins, e.g. acetylates lysines in the histone amino termini (Howe et al., 1999). The SAGA (Spt-Ada-Gcn5 acetyltransferase) and NuA4 (nucleosomal acetyltransferase histone H4) complexes, which primarily acetylate histones H3 and H4 via their respective catalytic subunits Gcn5 and Esa1 (Brownell et al., 1996; Grant et al., 1997; Smith et al., 1998), regulate transcriptional initiation (Howe et al., 1999; Jenuwein and Allis, 2001). Gcn5 and SWI/SNF are partially redundant, performing independent but overlapping functions during transcriptional activation (Pollard and Peterson, 1997; Roberts and Winston, 1997; Ryan et al., 1998; Biggar and Crabtree, 1999; Sudarsanam et al., 1999; Wallberg et al., 2000; Stafford and Morse, 2001).

Although distinct programs of recruitment of chromatin remodelers and other multiprotein complexes have been reported for various promoters (Cosma et al., 1999; Agalioti et al., 2000; Soutoglou and Talianidis, 2002), common themes have emerged. Each transcriptional program is generally initiated by one or more site-specific activator

proteins that access metazoan enhancers or upstream activating sequences (UASs) in yeast. Activation domains then mediate the high-affinity interaction and hence temporal 'recruitment' of specific chromatin modifiers and remodelers, which do not bind DNA with specificity (Fry and Peterson, 2001; Hassan et al., 2001a). At the yeast *HO* promoter, for example, SWI/SNF is directly recruited prior to SAGA (Cosma et al., 1999). In contrast, the opposite order with HAT complexes being recruited before SWI/SNF has been shown at various promoters in human cells (Neely et al., 2002); however, this order has only been inferred in yeast at the *PHO8* promoter (Reinke et al., 2001). Histone acetylation by either the SAGA or NuA4 complexes can enhance the retention of specific coactivator complexes via their bromodomain modules (Syntichaki et al., 2000; Hassan et al., 2001a; Hassan et al., 2001b). Ultimately, changes in chromatin structure or remodeling facilitate assembly of the transcription preinitiation complex (PIC) onto the core promoter (Kingston and Narlikar, 1999; Agalioti et al., 2000; Lomvardas and Thanos, 2002).

Although much is known about how coactivators are recruited, why promoters vary in their requirements for chromatin modifiers and remodelers is unresolved. Given the central role of the activation domain in coactivator recruitment, it seems likely that various activator subclasses would exhibit distinct coactivator specificities. Indeed, activators with glutamine-rich activation domains, for example, interact directly with purified yeast NuA4 (Nourani et al., 2004) but not with SWI/SNF (Neely et al., 1999; Yudkovsky et al., 1999). Acidic activators, however, have been shown to interact with a similar subset of chromatin-associated activities, including yeast NuA4, SAGA, and SWI/SNF as well as their human counterparts (Fry and Peterson, 2001; Hassan et al.,

2001a). Thus, at least *in vitro*, acidic activators do not appear to show distinct interaction preferences among this subset of coactivators. This is consistent with *in vivo* studies suggesting that a variety of natural and chimeric activators are able to recruit overlapping sets of coactivators (Pollard and Peterson, 1998; Ryan et al., 1998; Biggar and Crabtree, 1999; Sudarsanam et al., 1999; Wallberg et al., 2000; Stafford and Morse, 2001; Cheng et al., 2002).

A few recent studies suggest that promoter architecture, the relative location of cis-regulatory sequences with respect to nucleosomes, orchestrates a specific coactivator recruitment program and hence requirements for individual coactivator complexes (Ryan et al., 1998; Ryan et al., 2000; Stafford and Morse, 2001; Lomvardas and Thanos, 2002). Thus, in some cases, promoters with a nucleosomal TATA (yeast *SUC2* and *PHO8*, human IFN-□) require Gcn5 and SWI/SNF for activation (Hirschhorn et al., 1992; Gregory et al., 1999b; Agalioti et al., 2000). This dependence is alleviated at other promoters where TATA is either naturally accessible or is exposed artificially (Ryan et al., 2000; Stafford and Morse, 2001; Lomvardas and Thanos, 2002). However, the well-studied *GAL1* and *PHO5* promoters at which TATA is occluded by nucleosomes, require neither SWI/SNF nor Gcn5 under fully activating conditions (Peterson and Herskowitz, 1992; Gaudreau et al., 1997; Gregory et al., 1998b; Dudley et al., 1999; Bhaumik and Green, 2001; Neef and Kladde, 2003). Interestingly, a prerequisite for both SWI/SNF and Gcn5 is imposed on *GAL1* and *PHO5* activation in mitosis (Krebs et al., 2000; Neef and Kladde, 2003), possibly because the chromatin architecture is condensed. However, many promoters have an absolute requirement for these coactivators in interphase, indicating that additional factors must play a role in determining a promoter's need for specific

chromatin modifiers and remodelers.

While *PHO5* induction does not require these coactivators in strict genetic terms, we and others have shown that both Gcn5 and SWI/SNF are needed to achieve full rates of initial promoter activation (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). Further, under fully activating conditions of P_i starvation, *PHO5* expression depends on Gcn5 when the promoter is weakened by mutations in either of the two UASs (Gregory et al., 1998a). Lastly, basal expression of *PHO5* in high-P_i medium, which is due in part to low levels of Pho4 transactivator binding (Carvin et al., 2003a), is highly dependent upon Gcn5 and SWI/SNF (Neef and Kladde, 2003). These observations are consistent with the hypothesis that *PHO5* promoter activity requires these remodelers when low levels of activator are associated with the promoter.

Testing this hypothesis, here we show that *PHO5* transactivation is strongly reduced in the absence of either Gcn5 or SWI/SNF at low levels of UAS-bound Pho4. By contrast, the requirement for either remodeler is alleviated when Pho4 binding site occupancy is increased, suggesting that functional redundancy is established at promoters with robust activator interactions. Thus, we also find significant recruitment of Gcn5 and SWI/SNF to several promoters known to exhibit strong activator binding and transcription at which they are currently thought not to function. These results define a critical role for promoter occupancy in determining the extent to which transactivation depends on specific chromatin modifiers and remodelers. Moreover, our data suggest that Gcn5 and SWI/SNF have many genomic targets and support a model in which high levels of promoter-bound activator drive the genetic redundancy that is observed between various coactivators.

MATERIALS AND METHODS

Yeast media, growth conditions, and rAPase activity assays

Defined, P_i-free medium (pH 5.5) was prepared as described (Neef and Kladde, 2003), except that it was supplemented with complete synthetic mix (CSM, Bio101). All starter cultures were grown in this medium with KH_2PO_4 (P_i) added back to 13.4 mM. For time courses, cells were washed and transferred to activating medium without P_i. For dose responses, cells were washed with defined, P_i-free medium with CSM and transferred for 12 h to the same medium containing the indicated concentrations of P_i and KCl to bring the potassium ion concentration to 13.4 mM. rAPase activity was assayed as previously described (Neef and Kladde, 2003).

For the doxycycline dose response study, the strains MRY3260 (wild type) and MRY3348 (*swi2*) were grown in defined CSM-LYS medium (Bio101) and reseeded in the same medium the next day. These strains contain the $Pho4^{SA1234PA6}$ constitutively nuclear variant of Pho4 (Komeili and O'Shea, 1999), expressed under the *tetO*$_7$ promoter and activated by a variant of the tet-on system (Belli et al., 1998). Thus we are able to vary *PHO5* expression by simply varying the doxycycline concentration in the medium. The strains were split into 8X 50ml conical tubes (15ml of culture each) and the appropriate concentrations of doxycycline were added to each tube. The cultures were subsequently aliquoted into 2 tubes of 5ml each. 50% ethanol carrier was added to the tube with the 0 g/ml doxycycline concentration. The cultures were grown for a period of 14 h and harvested for the rAPase assay as before.

Localization of Pho4 by GFP fluorescence

For GFP studies, the strains DNY2049, DNY2232 and MRY2985 (containing Pho4 tagged with GFP) were grown in medium with 13.4, 0.2, or 0 mM P_i for 12 h. Cells (1 mL) were sonicated, washed with 1X phosphate-buffered saline (PBS) and fixed with 70% ethanol for 20 min. Cells were washed again with 1X PBS, resuspended in 10 μL of 1 μg/mL DAPI, and incubated at room temperature for 12 min. The cells were then washed with PBS and 2 μL were placed on a slide for viewing with a Zeiss Axiovert 135 with a 100x Plan-Apochromat oil-immersion objective (Carl Zeiss MicroImaging). Representative cell images were collected using Zeiss Axiovision version 3.1.

ChIP analysis

Cultures (50 mL) were grown at 30°C with shaking for the indicated times and then were fixed at room temperature for 15 min by adding formaldehyde to a 1% final concentration. Cross-linking was quenched by addition of glycine to a final concentration of 125 mM and incubating for 5 min at RT. Cells were centrifuged at 3300Xg for 5 min at 4°C and washed twice with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), resuspended in 0.5 mL ice-cold lysis buffer [50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1% Triton-X 100, 0.1% deoxycholate, protease inhibitor cocktail (Roche 1 836 153), and 1 mM PMSF]. One mL of ice-cold, acid-washed glass beads (425-600 μm) were then added for lysing cells in a mini-bead beater 8 (Biospec Products) with two 1-min pulses at maximum speed separated by 5 min on ice. The supernatants were recovered after centrifugation at 1500 X g for 3 min and then sonicated with a Virsonic 100 sonicator (3X 25 sec at setting 4). Prior to IP, sonication efficiency and

chromatin yield were assessed by incubating 30 μL of sonicated whole cell lysate at 65°C overnight to reverse cross-links. DNA was then treated with proteinase K, purified by Wizard PCR preps resin (Promega), eluted in 0.1X TE, and analyzed for concentration as well as shearing to approximately 500 bp.

Each IP reaction (500 μL) contained equal amounts of fixed, sheared chromatin, 1M NaCl, and 2 μL of A-14 anti-myc rabbit polyclonal antibody (200 μg/mL; Santa Cruz Biotechnology), and was incubated overnight at 4ºC. The next day, protein A sepharose beads were washed with PBS and lysis buffer as required, and 40 μL of the washed protein A bead slurry was added to the IP reaction and incubated on a nutator for 1 hr at 4ºC. The pellets were washed to remove non-specifically adsorbed proteins and DNA with: 1 mL lysis buffer twice; 1 mL lysis buffer plus 0.5 M $NaCl_f$; 1 mL wash buffer; and twice with 1 mL TE. DNA-protein complexes were eluted in elution buffer, TES (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) by incubating twice at 65°C for 10 min. Cross-links of the immunoselected DNA-protein complexes were reversed and the DNA purified as described above. Analysis of immunoselected DNA fragments was performed by PCR of the indicated regions by radiolabeling for 25 cycles of amplification in reactions containing: 0.2 μL of [α^{32}P]-dCTP (10 mCi/mL); 0.2 mM_f dATP, dGTP, and dTTP; and 0.1mM_f dCTP. Radiolabeled PCR products were electrophoresed on a 4% nondenaturing polyacrylamide gel at 150 V for 3 h, and visualized by a Storm 860 phosphorimager (Molecular Dynamics), and quantified by ImageQuant software. For *PHO5* sequences, a single primer pair [(ADO236; CATGTAAGCGGACGTC; –456 to –441 relative to the *PHO5* ATG translation start) and (LFO740; GCCTTGCCAAGTAA-GGTGAC; –173 to –154)] was used to amplify the endogenous UASs of the *PHO5*

promoter as well as a negative control *PHO5* promoter region (*pho5* ΔUASs) by quantitative, competitive PCR. This negative control contains *PHO5* sequences from –1537 to +9 with two 50-bp deletions (encompassing UASp1 and UASp2 from −401 to −352 and −258 to −209, respectively), and was integrated either by gene replacement of (strains ADY2459 and ADY2461) or loop in at (strains ADY2695, ADY2701, ADY2719, ADY2727, ADY2915, ADY2921, and ADY2923) the *CAN1* locus (Carvin et al. 2003). Likewise, LFO644 (GGAAATGTAAAGAGCCCC; –547 to –530) and LFO645 (TTGAAGGTTTGTGGGG; –270 to –255) were used to simultaneously amplify the endogenous UAS_G region of the *GAL1-10* promoter and a negative control *GAL1-10* promoter. This negative control comprises the entire *GAL1-10* intergenic region (–698 to +36 relative to the *GAL1* ATG) with a deletion of all four Gal4 sites (UAS_G, –453 to –336), which was integrated by loop in at *CAN1*. Primers used for amplification of various yeast promoters (*PYK1*, *RPL19B*, *RPS22B*, and *EFT2*) were previously described (Reid et al., 2000).

Western blotting

Western blots were performed using standard techniques. Wild-type (MRY3049), *gcn5*Δ (MRY3055) and *swi2*Δ (MRY3053) yeast strains containing FLAG-tagged Pho4, along with an untagged strain (ADY3035), were grown in 100mL of medium with or without P_i to an OD_{600} of ~1 and centrifuged at 3300 X *g* for 5 min at 4°C. The cell pellet was washed with 1 mL ice-cold 50 mM Tris-HCl (pH 8.0), resuspended in 500 µL of ice-cold lysis buffer, and lysed by vortexing twice for 1 min (30 sec on ice) after addition of ice-cold 0.3 g glass beads (425-600 µm). Cell debris was pelleted by centrifugation at

5000 X *g* for 5 min at 4°C and the supernatant transferred to a new tube. Total protein was quantified using the BCA assay kit (Pierce) and 70 μg protein was analyzed per lane on a 10% SDS-PAGE (37.5:1 acrylamide:bis-acrylamide) gel. After transfer to PVDF membrane (Amersham Pharmacia), the blot was incubated overnight with rabbit anti-FLAG antibody (Sigma, F-742) and then with HRP conjugated anti-rabbit IgG (Amersham Pharmacia). Protein was detected with the ECL PLUS kit (Amersham Pharmacia) and visualized using a Storm 860 phosphorimager. The blot was re-probed with mouse monoclonal anti-yeast Pgk1 (3-phosphoglycerate kinase) antibody (Molecular Probes, 22C5-D8) followed by HRP-conjugated anti-mouse IgG.

RESULTS

SWI/SNF physically associates with the PHO5 *promoter upon transactivation prior to Gcn5*

Promoters differ vastly in their requirements for SWI/SNF and Gcn5 (Holstege et al., 1998; Biggar and Crabtree, 1999; Sudarsanam et al., 2000). With respect to *PHO5*, prior reports have shown that, after extended times under fully activating conditions (P_i starvation), there is no major effect on *PHO5* transcription in either *swi2*Δ or *gcn5*Δ strains (Gaudreau et al., 1997; Gregory et al., 1998b; Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). However, the kinetics of *PHO5* induction is strongly dependent on Gcn5 and SWI/SNF (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). Ada2 has been shown to be recruited to the *PHO5* promoter by Pho4 as a component of SAGA (Barbaric et al., 2003), however, direct recruitment of SWI/SNF has not been demonstrated.

We performed chromatin immunoprecipitation (ChIP) experiments on 13myc-tagged strains (Table 2-1) to assay for Swi2 and Gcn5 association at *PHO5*. ChIP analysis of the *PHO5* UAS region (Figure 2-1A) was performed at various times after shifting the cells to medium that lacks P_i. The two strains were assayed internally for repressible acid phosphatase (rAPase) activity and exhibit similar induction profiles (Figure 2-1B). Since these strains are also deleted for *PHO3*, coding for constitutive acid phosphatase, the measured activities essentially reflect *PHO5* expression (Komeili and O'Shea, 1999; Neef and Kladde, 2003). The immunoprecipitated DNA was analyzed by quantitative, competitive PCR (Figure 2-1C, left panel). As can be seen in Figure 2-1C (right), both SWI/SNF and Gcn5 are enriched over time at the endogenous *PHO5* promoter compared to the internal negative control locus, *pho5* Δ UASs, lacking both UASp1 and UASp2. Similar P_i-starvation dependent enrichments for these coactivators compared to another negative control region in the *WHI4* ORF are also observed (data not shown). While Gcn5 is significantly enriched at the promoter at about 4 h P_i starvation, SWI/SNF is not recruited until later. This is the first demonstration of the physical presence of Gcn5 and Swi2 (or any SWI/SNF subunit) at *PHO5*. Since Ada2 was previously shown to associate with the *PHO5* promoter as a part of SAGA (Barbaric et al., 2003), it is likely that Gcn5 association is also occurring via SAGA. Recruitment of Gcn5 before SWI/SNF agrees with what was shown previously by ChIP at the human interferon-β (IFN-β) promoter (Agalioti et al., 2000), but is opposite of that at the yeast *HO* promoter (Cosma et al., 1999; Krebs et al., 1999). Further, the two complexes do not achieve maximal binding until about 12 h after P_i deprivation.

Table 2-1. ***S. cerevisiae strains used in this study.***

Strain	*Genotype* [a, b]
ADY2459	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *GCN5-13myc-kanMX4 can1*Δ::*pho5*^{pro} ΔUASs-*LEU2*
ADY2461	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *SWI2-13myc-kanMX4 can1*Δ::*pho5*^{pro} ΔUASs-*LEU2*
ADY2695	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *3myc-PHO4 swi2*Δ::*kanMX4 CAN1*:*pho5*^{pro} ΔUASs-*LEU2*
ADY2701	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *CAN1*:*pho5*^{pro} ΔUASs-*LEU2*
ADY2719	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *3myc-PHO4 gcn5*Δ::*kanMX4 CAN1*:*pho5*^{pro} ΔUASs-*LEU2*
ADY2727	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *3myc-PHO4 CAN1*:*pho5*^{pro} ΔUASs-*LEU2*
ADY2915	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *CAN1*:*gal1-10*^{pro} ΔUAS$_G$-*LEU2*
ADY2921	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *GCN5-13myc-kanMX4 CAN1*:*gal1-10*^{pro} ΔUAS$_G$-*LEU2*
ADY2923	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *SWI2-13myc-kanMX4 CAN1*:*gal1-10*^{pro} ΔUAS$_G$-*LEU2*
ADY3035	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R [pRS426 *GPD*^{pro}-*PHO4-URA3*]
DNY2049	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *pho4*Δ::*kanMX4 bar1*Δ::R-*URA3*-R *can1*Δ::*PHO4-GFP-K. lactis LEU2*
DNY2232	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *pho4*Δ::*kanMX4 bar1*Δ::R-*URA3*-R *gcn5*Δ::*kanMX*4 *can1*Δ::*PHO4-GFP-K. lactis LEU2*
MRY2985	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *swi2*Δ::*kanMX*4 [pRS316 *PHO4-GFP-URA3*]
MRY3049	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R [pRS416 *TEF1*^{pro}-*FLAG-PHO4-URA3*]
MRY3053	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *swi2*Δ::*kanMX4* [pRS416 *TEF1*^{pro}-*FLAG-PHO4-URA3*]
MRY3055	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *gcn5*Δ::*kanMX4* [pRS416 *TEF1*^{pro}-*FLAG-PHO4-URA3*]
MRY3260	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R KL-URA3:*tetO*$_7$ ^{pro} PHO4^{SA1234PA6} *ho* :: new superactivator
MRY3348	*MAT***a** *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pho3*Δ::R *swi2*Δ::*kanMX4* KL-URA3:*tetO*$_7$ ^{pro} PHO4^{SA1234PA6} *ho* :: new superactivator

[a] The superscript 'pro' indicates promoter.
[b] R is a *Zygosaccharomyces rouxii* recombinase site that remains after intramolecular recombination (Roca et al., 1992).

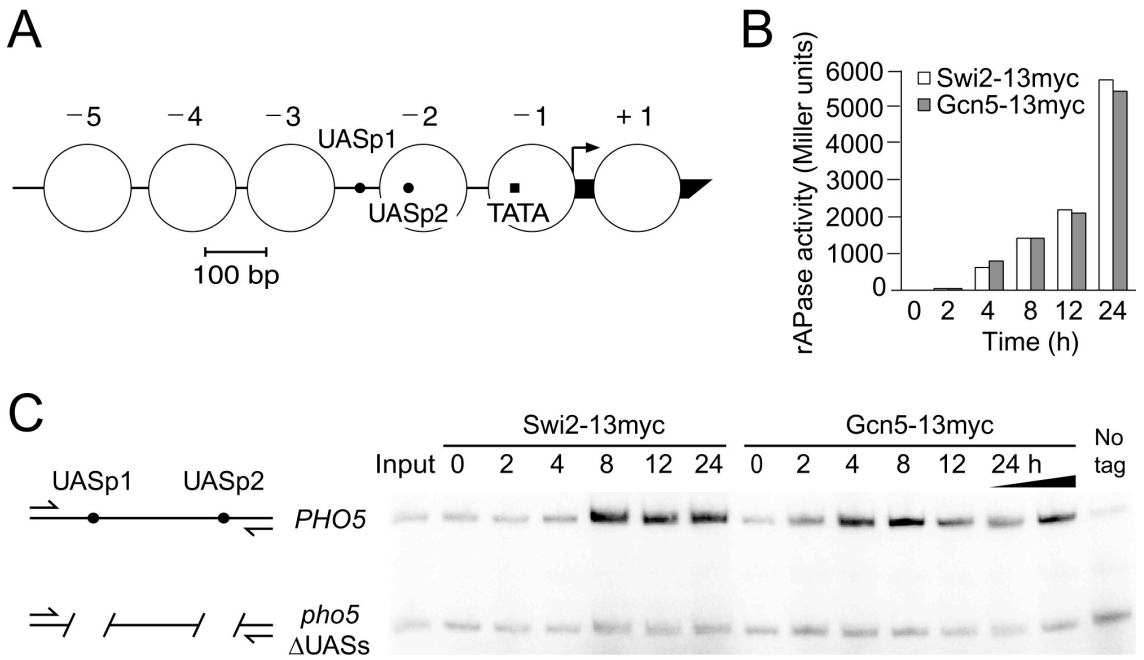

Figure 2-1. SWI/SNF associates with the *PHO5* promoter subsequent to Gcn5 recruitment.

(*A*) The *PHO5* promoter. Filled circles, UASp1 and UASp2; filled square, TATA element; large open circles, positioned nucleosomes _5 to +1(Almer and Hörz, 1986). (*B*) Time course of *PHO5* activation following transfer from high- to no-P_i medium. Swi2-13myc (ADY2459) and Gcn5-13myc (ADY2461) cells were harvested at the indicated times and assayed for rAPase activity. (*C*) Internal aliquots of cells from the Swi2- and Gcn5-13myc cultures assayed in *B* were subjected to ChIP analysis (see Materials and methods) at the indicated times of induction. DNA immunoselected with anti-myc antibody from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5* UASs) sequences by quantitative, competitive PCR as depicted in the panel at left. The negative control promoter has deletions of both UASp1 and UASp2 (filled circles) and thus is unable to bind Pho4 (Carvin et al., 2003). A wild-type strain (ADY2701) was starved of P_i for 24 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 24-h culture of Gcn5-13myc cells following cross-linking and was diluted 1:500 for PCR amplification. The ramp indicates inclusion of twice as much immunoselected DNA in the PCR amplification and demonstrates linearity. The ratio of *PHO5* to negative control (*pho5* UASs) product indicates the relative enrichment of each coactivator at the promoter.

This correlates with the time when *gcn5* and *swi2* cells reach wild-type levels of *PHO5* activation, i.e. when the kinetic delay in activation is completed (Barbaric et al.,

2001; Barbaric et al., 2003; Neef and Kladde, 2003).

Maximal association of Pho4 at PHO5 requires many hours following P_i starvation

Since Pho4, the principal PHO transcriptional activator, recruits chromatin remodeling complexes via its acidic activation domain (Neely et al., 2002; Barbaric et al., 2003), we examined the time course of Pho4 binding at the *PHO5* promoter following P_i starvation. A 3myc-Pho4 strain was used that is not defective in *PHO5* activation kinetics compared to the wild type. The time course of rAPase activity induced in no-P_i medium is shown in Figure 2-2A. In Figure 2-2B, ChIP analysis of internal aliquots of cells revealed that Pho4 binding is not detected before P_i starvation, and is maximal at about 12-14 h after withdrawing P_i. These results demonstrate that, in accord with the recruitment paradigm, Pho4 binding increases over time and precedes the recruitment of Gcn5 and Swi2. However, as transport of Pho4 from the nucleus to cytoplasm is complete by 1 h at very low or no P_i (Kaffman et al., 1998b; Barbaric et al., 2001), it is surprising that 12-14 h are required for Pho4 binding to peak at the *PHO5* promoter.

PHO5 *activation requires chromatin remodelers at low levels of Pho4 binding site occupancy*

Increased association of Pho4 with the *PHO5* promoter during activation is likely due in part to further occupancy of the low-affinity UASp1 as Pho4 accumulates in the nucleus. Additional interaction of Pho4 with the high-affinity UASp2, which is essential for *PHO5* activation (Venter et al., 1994), is believed to require disruption of nucleosome -2 (Svaren and Hörz, 1997). In support of this, the absence of the activity of various remodeling enzymes leads to modest (SWI/SNF, INO80.com)(Steger et al., 2003) or

severe (NuA4) (Nourani et al., 2004) decreases in Pho4 binding and promoter induction. In contrast, recent evidence suggests that Pho4 interacts with UASp2 in the absence of remodeled chromatin (Terrell et al., 2002; Adkins et al., 2004).

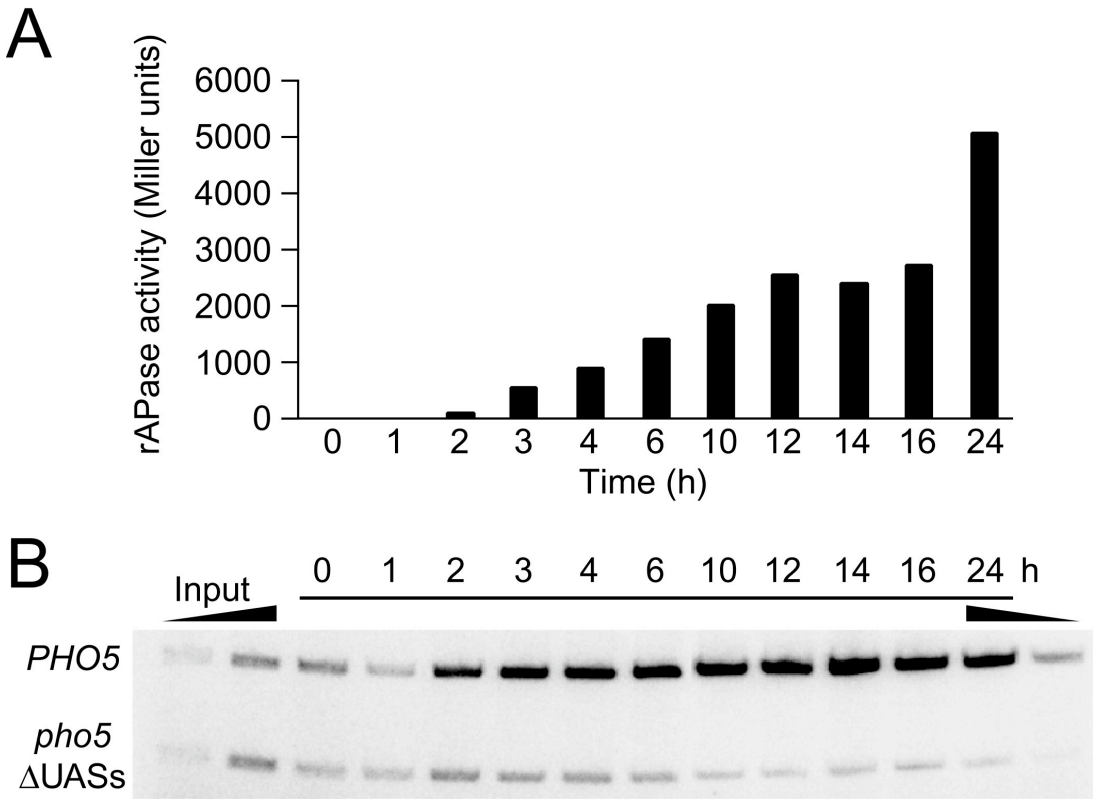

Figure 2-2. Pho4 binding at *PHO5* increases for many hours after P_i starvation.

After shifting strain ADY2727 (3myc-Pho4) from high- to no-P_i medium, cells were internally assayed at the indicated times for (*A*) rAPase activity and (*B*) 3myc-Pho4 binding by ChIP analysis. The first two lanes contain non-immunoselected (input) samples, whereas immunoselected DNA was assayed in all other lanes. Quantitative, competitive PCR amplification of the endogenous *PHO5* and negative control (*pho5* □UASs) promoters was performed as in Figure 2-1C. The PCR amplifications in the first and last lanes contained 2-fold less input DNA (taken from the 24-h culture and diluted 1:500) or immunoselected DNA.

Our ChIP results, showing that recruitment of Pho4, Gcn5, and Swi2 all peak at

around 12 h after *PHO5* induction, are most consistent with a model in which nucleosome -2 is disrupted over time to enable access of Pho4. Moreover, we hypothesized that the combined action of distinct classes of coactivator complexes might be required to achieve full induction at lower amounts of Pho4 site occupancy when, on average, only UASp1 rather than UASp2 is occupied. Conversely, high-level association of Pho4 and hence recruitment of coactivators might suppress the need for a particular chromatin-associated complex.

To test this hypothesis, we regulated the nuclear concentration of Pho4 to effect varying steady-state levels of UAS occupancy at the *PHO5* promoter in wild type, *gcn5*Δ and *swi2*Δ cells. The nucleo-cytoplasmic distribution of Pho4 is controlled through its phosphorylation by the Pho80-Pho85 cyclin-CDK (O'Neill et al., 1996; Kaffman et al., 1998a; Kaffman et al., 1998b; Komeili and O'Shea, 1999). Under conditions of high P_i availability, phosphorylation blocks nuclear import and promotes nuclear export, leading to cytoplasmic localization of Pho4. When P_i is limiting, *PHO4* expression is not affected (Legrain et al., 1986), Pho80-Pho85 activity is inhibited and unphosphorylated Pho4 accumulates in the nucleus. Thus, the most physiologically relevant way to regulate nuclear levels of Pho4 is to grow cells in the presence of various concentrations of P_i (Toh-e et al., 1973), leading to varying degrees of Pho80-Pho85 activity and redistribution of Pho4 between the nucleus and cytoplasm. We first tested and found that the nuclear level of Pho4-GFP increased in a graded manner across the population of cells at successively lower concentrations of P_i (Figure 2-3). This confirms previous results (Springer et al., 2003), ruling out the alternative scenario of an all-or-none binary

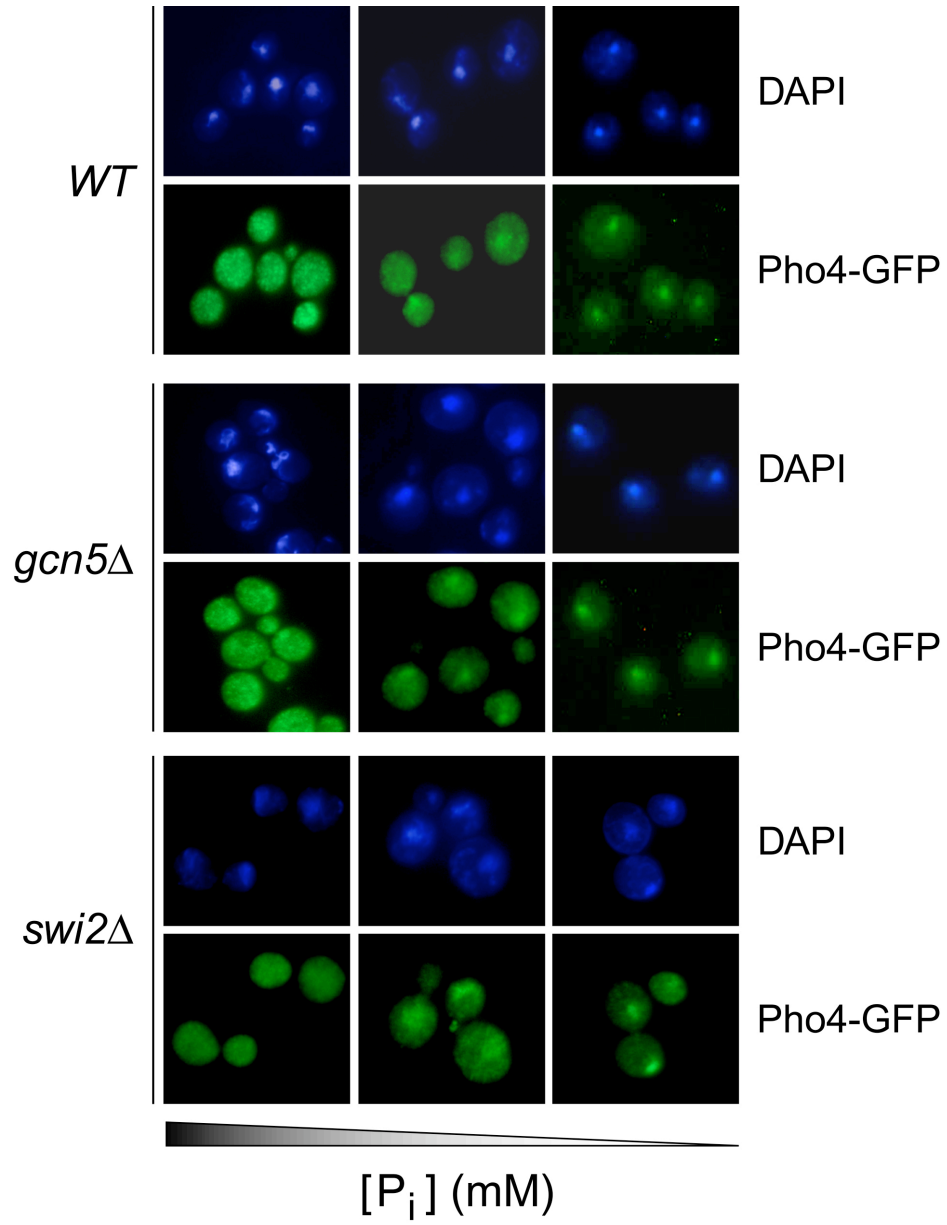

Figure 2-3. Pho4 nuclear concentration increases in a graded manner with the severity of P_i deprivation in the absence or presence of Gcn5 and SWI/SNF.

Wild-type (*WT*; DNY2049), *gcn5*Δ (DNY2232) and *swi2*Δ (MRY2985) strains were grown for 12 h in defined minimal medium containing either 13.4 mM (left), 0.2 mM (middle), or 0 mM (right) P_i. Cells were washed with PBS and either stained with DAPI to visualize nuclear DNA or visualized directly for Pho4-GFP fluorescence. Note the similar levels of fluorescence in *WT*, *gcn5*Δ and *swi2*Δ cells and the increasing nuclear focus of Pho4-GFP as the P_i concentration decreases from left to right.

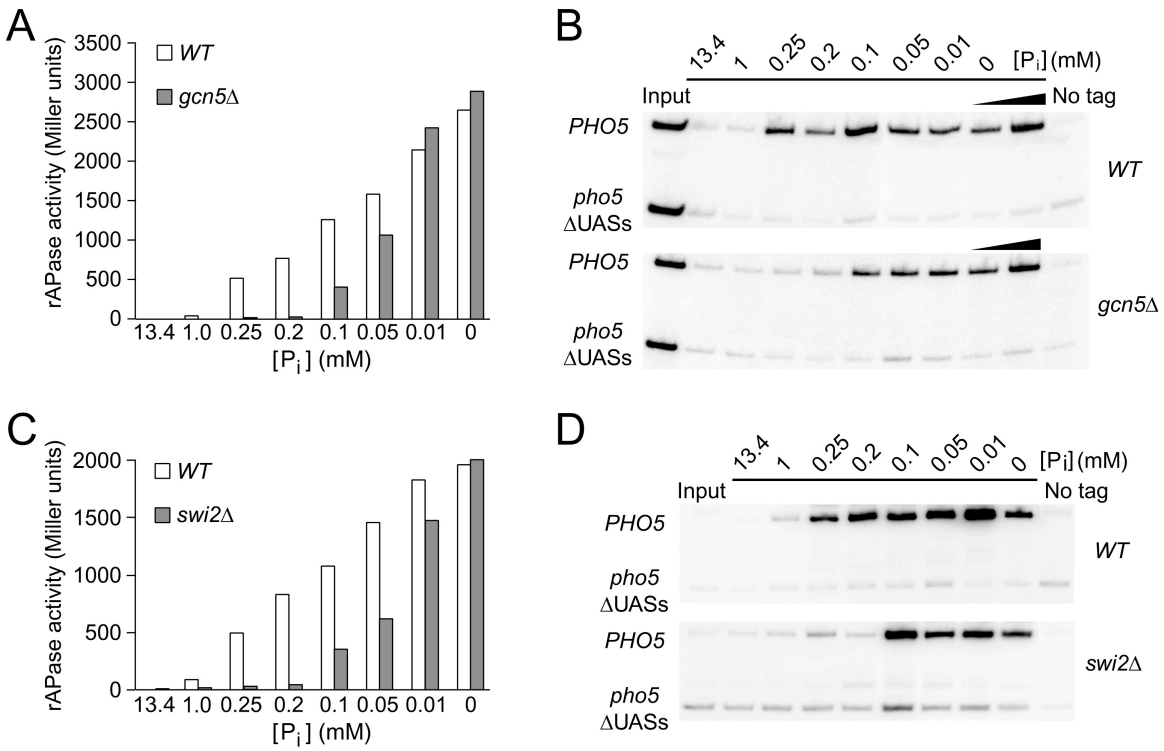

Figure 2-4. *PHO5* activation is markedly dependent on Gcn5 and SWI/SNF at low nuclear concentrations of Pho4 and decreased binding site occupancy.

Wild-type (*WT*, ADY2727), *gcn5*Δ (ADY2719) and *swi2*Δ (ADY2695) strains containing 3myc-Pho4 were grown 12 h in medium with the indicated concentrations of P_i and were internally assayed for (*A*, *C*) rAPase activity and (*B*, *D*) 3myc-Pho4 binding by ChIP. In the ChIP analyses, the first lane contains non-immunoselected 0 mM-P_i sample (input), whereas immunoselected DNA was assayed in all other lanes. Quantitative, competitive PCR amplification of the endogenous *PHO5* and negative control (*pho5* ΔUASs) promoters was done as in Figure 2-1C. The ramp in *B* indicates the addition of 2-fold more immunoselected 0 mM-P_i sample to the PCR amplification. The untagged (no tag) specificity control was a wild-type strain (ADY2701) that was starved of P_i for 12 h, and carried in parallel through all experimental steps. Identical results were obtained when cells were grown for 16 h at the same concentrations of P_i, demonstrating that the concentration of P_i in the medium was not significantly altered.

response where the fraction of cells with nuclear-localized Pho4 increases as the concentration of P_i is decreased. Loss of Gcn5 and SWI/SNF could indirectly lead to a shift in the *PHO5* response to P_i deprivation by affecting Pho4 protein levels. This

seemed unlikely, however, as there were no apparent differences in fluorescent intensity of Pho4-GFP between wild type, *gcn5*Δ and *swi2*Δ strains (Figure 2-3). Furthermore, levels of rAPase activity are the same in wild type versus coactivator mutant cells at 0.01 mM and 0 mM P_i (Figure 2-4A, C). Nevertheless, we directly determined by western blotting that levels of a fully active FLAG-tagged version of Pho4 are unaffected in wild type, *gcn5*Δ and *swi2*Δ strains in medium containing or lacking P_i (Figure 2-5). We conclude that the degree of Pho4 binding site occupancy at *PHO5* is a crucial determinant of the promoter's need for the chromatin remodelers Gcn5 and SWI/SNF in activation; ranging from essentially complete dependence to independence at low and high levels of promoter occupancy, respectively.

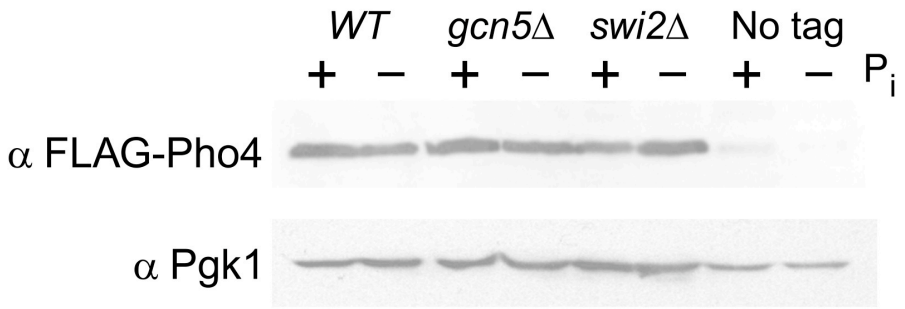

Figure 2-5. Deletion of *GCN5* or *SWI2/SNF2* does not affect Pho4 protein levels.

Wild-type (*WT*; MRY3049), *gcn5*Δ (MRY3055) and *swi2*Δ (MRY3053) strains expressing FLAG-*PHO4* or a strain expressing untagged (no tag) *PHO4* (ADY3035) were grown for 12 h in the presence (+) or absence (–) of P_i before whole cell extracts were isolated. Equivalent amounts of total protein were analyzed by immunoblotting and probing with anti-FLAG antibody (top). The blot was reprobed with monoclonal anti-yeast Pgk1 antibody (bottom) to provide a loading control.

Moreover, Gcn5 and SWI/SNF are required for maximal association of Pho4 with the

PHO5 promoter at intermediate concentrations of P_i. This likely reflects the necessity for Gcn5 and SWI/SNF activity in exposing the high-affinity UASp2 in nucleosome -2 (see Summary and conclusions).

Doxycycline-regulated expression of a Pho4 variant also demonstrates a requirement for chromatin remodelers

While our experiments thus far indicated a clear dependence on chromatin-remodeling coactivators at low levels of Pho4 binding, there were concerns that differential rates of phosphate depletion might cause variation between experiments and from strain to strain. Further, positive feedback regulation on *PHO81,* an upstream repressor of *PHO80/PHO85,* might produce indirect effects on *PHO5* regulation (Ogawa et al., 1995). To circumvent these concerns, we developed a system in which *PHO5* expression is tightly regulated by the ectopic ligand doxycycline, in yeast (Figure 2-6) (Belli et al., 1998) and is no longer under P_i control. In brief, this 'tet-on' system consists of the yeast repressor Ssn6 fused to the wild-type tet repressor (*tetR*) from *E.coli* that binds to seven *tetO* sites ($tetO_7$) in the *absence* of doxycycline, thus minimizing basal expression. In the same cell, a fusion protein between the potent transactivation domain, VP16, and a *tetR* variant (*tetR'*), binds the $tetO_7$ operators in the *presence* of the antibiotic. Both tet fusions are constitutively expressed and the $tetO_7$ promoter replaces the endogenous *PHO4* promoter. This promoter has been reported to express graded concentrations of several factors uniformly across all cells in a population (Kringstein et al., 1998; Rossi et al., 2000; Becskei et al., 2001).

This $tetO_7$ promoter was used to drive expression of the constitutively nuclear Pho4

variant, Pho4^{SA2134PA6} (Komeili and O'Shea, 1999), which has serine to alanine changes at SP sites 1-4 and a proline to alanine change at SP6. These changes prevent phosphorylation of the variant by Pho80-Pho85 in high phosphate medium, and hence its usual export to the cytoplasm. This variant is fully functional for *PHO5* activation (Komeili and O'Shea, 1999), and hence it is expected to elicit full chromatin remodeling.

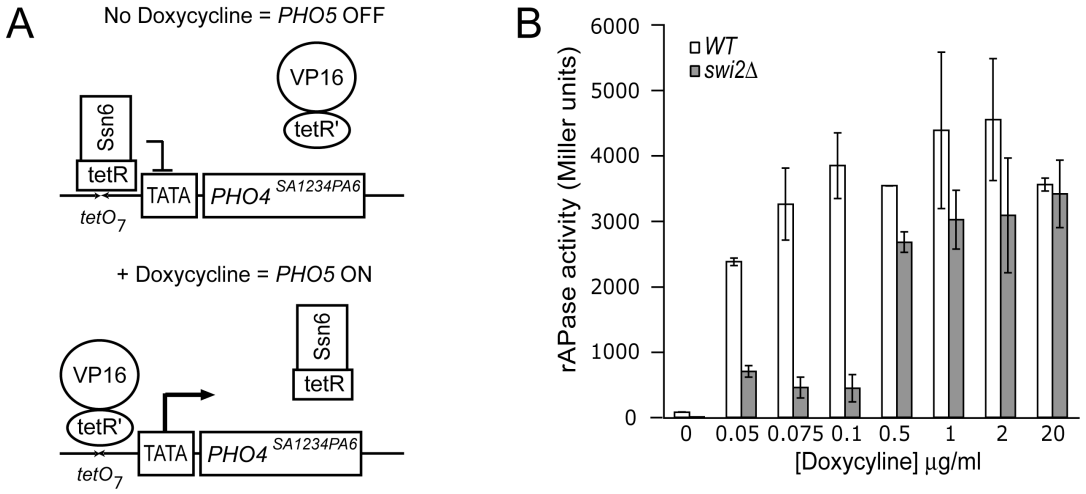

Figure 2-6. A doxycycline-regulated Pho4 variant demonstrates Swi2 dependence in *PHO5* activation.

(*A*) Pho4^{SA1234PA6}, a constitutively nuclear variant of Pho4 {Komeili, 1999 #267}, is expressed under the *tetO*$_7$ promoter at the endogenous *PHO4* locus and activated by a variant of the dual tet-on system {Belli, 1998 #227}. (*B*) The strains MRY3260 (wild type) and MRY3348 (*swi2*□) containing the variant mentioned in (*A*) were grown in defined CSM-LYS medium (Bio101) and reseeded in the same medium the next day, and the indicated concentrations of doxycycline were added to each tube. Ethanol carrier (50%) was added to the tube with the 0 □g/ml doxycycline concentration. The cultures were grown for a period of 14 h and harvested for the rAPase assay as before.

The strains MRY3260 (wild type) and MRY3348 (*swi2*□) were grown in defined CSM-LYS medium (Bio101) and reseeded in the same medium the next day. These

strains contain the Pho4^{SA1234PA6} constitutively nuclear variant of Pho4 (Komeili and O'Shea, 1999), expressed under a modified *tetO*$_7$ promoter system (Figure 2-6A and)(Belli et al., 1998). Thus we are able to vary *PHO5* expression by simply varying the doxycycline concentration in the medium. As seen in Figure 2-6B, while the wild-type strain requires merely 0.05 g/ml of doxycycline to activate reasonable levels of *PHO5*, the *swi2* strain requires ten times that amount (0.5 g/ml) before it can approach wild-type levels at the same concentration. This demonstrates that the increased dependence on remodelers that we see in no-P_i media for *swi2* strains also applies for a system that is ectopically regulated.

Gcn5 and SWI/SNF associate with additional strongly transcribed promoters

Previous microarray studies suggested that deletion of *GCN5* or *SWI2*/*SNF2* affects the transcript levels of less than 5% of yeast genes (Holstege et al., 1998; Sudarsanam et al., 2000). However, we have shown that both Gcn5 and SWI/SNF associate with the *PHO5* promoter under conditions of high transactivator binding when neither remodeler is required for transcriptional activity. Thus, we hypothesized that Gcn5 and SWI/SNF are also recruited to other highly transcribed promoters that do not require their activities for transcription. The *GAL1-10* promoter, for example, has an upstream regulatory region (UAS_G) that contains four binding sites (two high and two low affinity) for the strong acidic activator Gal4. Although Gal4 is not an abundant activator protein, high-level occupancy of UAS_G occurs due to DNA-binding cooperativity (Giniger et al., 1985). Previous studies have shown that Gal4 recruits Gcn5 as a component of the SAGA complex to the *GAL1-10* UAS_G (Bhaumik and Green, 2001; Larschan and Winston,

2001), but physical association of SWI/SNF has not been demonstrated. Deletion of genes coding for Gcn5 or SWI/SNF subunits only modestly affect *GAL1* expression (Peterson and Herskowitz, 1992; Dudley et al., 1999; Sudarsanam and Winston, 2000; Bhaumik and Green, 2001).

As above, we performed ChIP in strains expressing Swi2-13myc and Gcn5-13myc that, in addition to the normal genomic *GAL1-10* locus, also contain a negative control locus, *gal1-10* ΔUAS$_G$. This control comprises the entire *GAL1-10* promoter with UAS$_G$ (all four Gal4 sites) deleted. Figure 2-7A demonstrates that both SWI/SNF and Gcn5 are recruited to the endogenous *GAL1-10* UAS$_G$ in galactose medium when the promoter is transcriptionally active, but not in repressive glucose medium. Strong recruitment of both coactivators lends further support to work suggesting that Gcn5 and SWI/SNF perform partially redundant functions at the *GAL1* promoter (Roberts and Winston, 1997; Pollard and Peterson, 1998; Biggar and Crabtree, 1999). Moreover, *GAL1* expression becomes strongly dependent on Gcn5 and SWI/SNF after deleting the two high-affinity Gal4 sites of UAS$_G$ (Marcus et al., 1994; Burns and Peterson, 1997b; Gaudreau et al., 1997), consistent with our working model that high levels of activator binding establish functional redundancy.

We further hypothesized that promoters regulated by the abundant general regulatory factors Abf1 and Rap1, which thus strongly associate at target genes (Morse, 2000), would thus recruit significant levels of multiple coactivators. In doing so, our model predicts that functional redundancy might be established whereby the need for a single, specific remodeling activity is alleviated. Strong Abf1 and Rap1 targets include the RNA pol II-transcribed promoters of genes coding for ribosomal proteins, additional

aspects of protein synthesis and glycolytic enzymes (Reid et al., 2000; Lieb et al., 2001). Rap1 target genes are generally transcribed at extremely high rates during growth in rich medium, averaging 45 mRNAs per hour as compared to 7 mRNAs per hour for all yeast genes, accounting for an estimated 37% of total RNA pol II-derived transcripts (Holstege et al., 1998).

To test this hypothesis, we used ChIP analysis to assay for the association of Gcn5 and SWI/SNF at representative Rap1 targets for which transcript levels are unaffected in *gcn5*Δ or *swi2*Δ strains (Holstege et al., 1998; Sudarsanam et al., 2000), including *RPL19B* (ribosomal protein), *EFT2* (protein synthesis), and *PYK1* (glycolysis). As predicted, each of these Rap1 target genes shows significant association with Gcn5 and SWI/SNF relative to the *gal1-10* ΔUAS_G negative control. Both coactivators are also recruited to the *RPS22B* promoter, which is a target for Abf1 but not Rap1 (Reid et al., 2000; Lieb et al., 2001). This is consistent with studies showing that the activation domains of Abf1 and Rap1 are interchangeable and that both factors can function with non-ribosomal gene core promoters (Buchman and Kornberg, 1990; Goncalves et al., 1996; Cheng et al., 2002). Thus, we conclude that Gcn5 and SWI/SNF associate with a variety of heavily transcribed promoters. Interestingly, at any given locus in Figure 2-7 (also at *PHO5*; Figure 2-1), the levels of enrichment of Gcn5 and SWI/SNF relative to the negative control are similar; suggesting that activator binding level determines their recruitment frequency. As cross-linking or immunoprecipitation efficiency may be different for these coactivators, these data indicate that similar relative levels of both are recruited, not necessarily the same absolute levels.

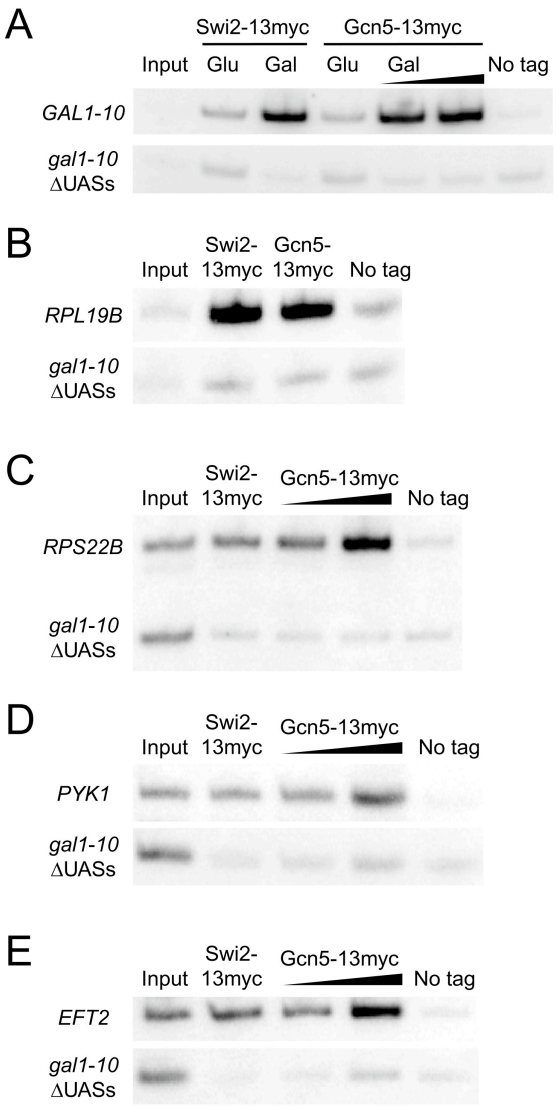

Figure 2-7. SWI/SNF and Gcn5 are recruited to strongly transcribed promoters that do not require them for transcription.

Gcn5-13myc (ADY2921) and Swi2-13myc (ADY2923) strains were grown in parallel with an untagged strain (ADY2915). Cells were initially grown in YPD, washed and resuspended in either YPD or YPG medium for another 6 h. Non-immunoselected (lane 1, input) and immunoselected (all other lanes) DNA was analyzed by PCR using primers for the (*A*) *GAL1-10*, (*B*) *RPL19B*, (*C*) *RPS22B*, (*D*) *PYK1* and (*D*) *EFT2* promoters as well as a negative control, mutated promoter (*gal1* UASs). In *A*, quantitative, competitive PCR analysis of *GAL1* using a single PCR primer pair amplifying both *GAL1* and the control locus was performed as for *PHO5* in Figure 2-1C. In *B*-*C*, analysis of only the YPD-grown, Gcn5-13myc and Swi2-13myc samples is shown and the experimental and *gal1*UASs loci were assayed in the same PCR amplification by including a second pair of primers. For simplicity, the low-level signal at the endogenous *GAL1* locus is not presented in *B*-*C*.

DISCUSSION

We find that the extent to which *PHO5* induction requires activity of the Gcn5 HAT and SWI/SNF remodeler is strongly related to the nuclear concentration of the acidic activator Pho4 and thus levels of promoter-bound transactivator. Consistent with this, maximal Pho4 binding at the *PHO5* promoter requires ~12 h of P_i deprivation (Figure 2-2), corresponding well with the kinetic delay in *PHO5* activation exhibited by single *gcn5* and *swi2*/*snf2* mutants (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). This suggests that each remodeling enzyme is primarily needed when Pho4 binding is limiting. Indeed, *PHO5* activation is markedly dependent on both Gcn5 and SWI/SNF at low steady-state promoter occupancy (Figure 2-4). Conversely, high nuclear levels of Pho4 lead to marked increases in Pho4 binding and promoter activation in the absence of either Gcn5 or SWI/SNF. Our data suggest, therefore, that the necessity for a specific remodeler can be circumvented by driving promoter occupancy, establishing functional redundancy through increased recruitment of coactivators that normally associate with the promoter. In support of this model, Gcn5 and SWI/SNF are strongly recruited to *PHO5* when Pho4 occupancy is high and to representative promoters that are robustly occupied by the abundant transactivators Abf1 and Rap1 (Figure 2-7).

Association of Pho4 with PHO5 *UASp2 requires chromatin modifiers and remodelers*

When yeast cells are deprived of P_i, Pho4 is imported into the nucleus and activates genes within the PHO cluster (Lenburg and O'Shea, 1996). At the *PHO5* promoter, Pho4 is first thought to bind cooperatively with the non-acidic activator Pho2 at the accessible, low affinity UASp1 (CACGTT), and then at the high-affinity UASp2

(CACGTG), located in nucleosome -2 in the repressed promoter (Svaren and Hörz, 1997). The absolute correlation of *PHO5* induction with chromatin disruption has led to the widely accepted view that binding of Pho4 to UASp2 requires remodeling of nucleosome -2. Consistent with this view, several chromatin modifiers and remodelers are recruited to *PHO5*, including SAGA (Figure 2-1; (Barbaric et al., 2003), NuA4 (Nourani et al., 2004), INO80.com (Steger et al., 2003), and SWI/SNF (Figure 2-1). This is the first demonstration that SWI/SNF is brought directly to the activated *PHO5* promoter and, moreover, is preceded by association of Gcn5, likely in SAGA (Figure 2-1). Recruitment of HATs prior to SWI/SNF also occurs in human cells (Neely et al., 2002). Importantly, our data demonstrate that, in yeast, Gcn5 and SWI/SNF can be recruited in the opposite order of that observed at mitotically-induced genes (Cosma et al., 1999; Krebs et al., 2000).

Our results provide further evidence that efficient association of Pho4 with UASp2 requires chromatin remodeling. The resolution of ChIP analysis precludes assignment of the relative amounts of Pho4 bound to UASp1 vs. UASp2, as they are only 103 bp apart. However, the increase in Pho4 binding is initially modest and then increases dramatically due to cooperative binding (Figure 2-4). These results are clearly consistent with initial, limited Pho4 binding at the low affinity UASp1 followed by a large cooperative increase in binding upon exposure of the high-affinity UASp2, as we observed previously (Carvin et al., 2003a). Furthermore, in the absence of Esa1 HAT activity, *PHO5* chromatin remodeling and activation under P_i-free conditions is severely deficient and Pho4 binding achieves only about 10% of wild-type levels (Nourani et al., 2004). The need for Esa1 can be overcome by Pho4 overexpression. By contrast, we and

others find that the loss of Gcn5 HAT or SWI/SNF remodeler activity can be fully suppressed by wild-type levels of Pho4 expression (Gaudreau et al., 1997; Gregory et al., 1998b; Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). These results suggest a greater need for acetylation by Esa1 in NuA4 than by Gcn5 in SAGA for *PHO5* induction in medium lacking P_i (Nourani et al., 2004). However, a striking finding of our work is that, even in the presence of a wild-type copy of *ESA1*, *PHO5* induction requires Gcn5 and SWI/SNF at intermediate P_i concentrations (0.25-0.2 mM; Figure 2-4). Thus, we find that in the absence of efficient chromatin remodeling, increased activator concentration is again required to achieve high levels of activator binding.

High activator binding site occupancy confers functional redundancy for coactivator complexes

Yeast genes have been classified into three major groups with respect to their need for Gcn5 and SWI/SNF; those requiring both, either, or neither activity, suggesting that the remodelers have overlapping but independent functions (Holstege et al., 1998; Biggar and Crabtree, 1999; Sudarsanam and Winston, 2000). Taken together, our results with the *PHO5* system support a model for these differential dependencies whereby the time-averaged level of activator binding, or promoter occupancy, dictates the probability that sufficient coactivators are recruited to effect chromatin remodeling and transcriptional induction. We propose that when UASp1 is primarily occupied (rather than UASp2) at intermediate P_i concentrations, the activities of SAGA and SWI/SNF, and perhaps INO80.com (Steger et al., 2003) and NuA4 (Nourani et al., 2004), are prerequisites for *PHO5* promoter induction. Thus, Gcn5 and SWI/SNF function is

required at low levels of activator binding, which probably reflects conditions of P_i depletion in natural environments that are not devoid of P_i.

Increasing the nuclear level of Pho4 at successively lower concentrations of P_i (Figure 2-3) provides an effective means to mount a response of the appropriate magnitude (Figure 2-4). Under extreme conditions of no P_i, a robust level of activator binding drives recruitment of multiple remodeling activities by simple chemical principles, ensuring chromatin disruption and transcription. In good agreement with this model, we have observed a strong correlation between the extent of promoter occupancy and chromatin disruption using a galactose-regulated allele of *PHO4* (our unpublished data). Moreover, loss of Gcn5 and SWI/SNF activity delay chromatin remodeling and activation of *PHO5* following P_i starvation (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). However, at higher levels of Pho4 binding, sufficient amounts of chromatin modifiers and remodelers are recruited to suppress the need for a specific activity (i.e. the transcriptional defects of single *gcn5* and *swi*/*snf* mutants are not observed). In such cases, Gcn5 and SWI/SNF appear to be fully redundant.

We also utilized an ectopic system to complement the results we observed in P_i -depleted medium. To avoid concerns like positive feedback regulation of *PHO81* potentially producing effects on *PHO5* regulation, and possible differential rates of intracellular P_i depletion, we used a modified doxycycline-regulatable tet-on system. As seen in Figure 2-6B, while the wild-type strain requires merely 0.05 g/ml of doxycycline to activate reasonable levels of *PHO5*, the *swi2* strain requires ten times that amount (0.5 g/ml) before it can approach wild-type levels at the same concentration. This demonstrates that the increased dependence on remodelers that we see in no-P_i media for

*swi2*Δ strains also applies for a system that is ectopically regulated. We observed similar results for a *gcn5*Δ strain (data not shown).

Possible global roles for coactivators

We show substantial recruitment of Gcn5 and Swi2/Snf2 to *GAL1*, genes involved in protein synthesis (*RPL19B*, *RPS22B*, and *EFT2*), and a glycolytic promoter (*PYK1*) (Figure 2-7), supporting the view that these coactivators have widespread roles in transcription (Biggar and Crabtree, 1999). Recruitment occurs despite observations that transcript levels of each of these genes are unaffected in *gcn5* or *swi2*/*snf2* mutants (Holstege et al., 1998; Sudarsanam et al., 2000). Esa1 is also recruited to *PHO5* (Nourani et al., 2004) and ribosomal promoters (Reid et al., 2004). It was previously inferred that the abundant ribosomal promoter activators Abf1 and Rap1 could recruit SAGA to natural and foreign core promoters (Cheng et al., 2002). Our finding that Gcn5 directly associates with *RPL19B* and *RPS22B* is consistent with this conclusion and shows that SWI/SNF is directly recruited as well.

Why might chromatin modifiers and remodelers be recruited to such strongly transcribed promoters? Robust recruitment and retention of coactivators may ensure that an active chromatin configuration is established at critical promoters following deposition of nascent chromatin in S phase. Alternatively, increasing evidence suggests that chromatin remodelers are continuously required because there is a rapid, dynamic equilibrium between active and repressive chromatin structures (Biggar and Crabtree, 1999; Sudarsanam et al., 1999; Boeger et al., 2003). Intriguingly, Gcn5 and SWI/SNF are both enriched to similar levels at each promoter that we analyzed (Figs. 2-1 and 2-7).

This strongly suggests that, in line with our working model, the ‘recruitment potential’ of bound upstream activator(s) determines the quantity of complexes that are recruited to a given promoter. Furthermore, by our model, changes in activator concentration, activator DNA-binding domain/binding site affinity, and binding cooperativity are all expected to modulate the transcriptional requirements for individual chromatin modifiers and remodelers at various promoters. Additionally, weakening an activation domain renders reporters with either nucleosomal or non-nucleosomal TATA elements more dependent on activation by Gcn5 and SWI/SNF (Stafford and Morse, 2001), presumably due to a decreased ability to recruit coactivators. We propose that each of these factors must be evaluated to fully delimit the coactivator requirements of a given promoter.

CHAPTER III

INTERACTIONS BETWEEN CHROMATIN REMODELING COMPLEXES SAGA AND SWI/SNF AT THE BUDDING YEAST *PHO5* PROMOTER

OVERVIEW

Previous studies have suggested the partial functional redundancy of the Gcn5 and Swi2 subunits of the chromatin remodelers SAGA and SWI/SNF, respectively. In fact, at *PHO5*, it has been observed that deleting either *SWI2* or *GCN5* still allows wild-type levels of activation after several hours of P_i starvation. While at early times of induction both Swi2 and Gcn5 are required for *PHO5* activation, they are not needed at later times. Here, we demonstrate that Pho4 binding at the *PHO5* promoter is delayed in both *gcn5*Δ and *swi2*Δ cells with respect to wild type. While in the *gcn5*Δ strains, Pho4 levels at the promoter approach that of wild type at later times of P_i starvation, binding is more impaired in *swi2*Δ strains. This delay in Pho4 binding at the promoter demonstrates for the first time a mechanistic basis for the kinetic delay in *PHO5* induction described previously (Barbaric et al., 2001; Neef and Kladde, 2003).

We also find an increased level of SWI/SNF recruitment in a *gcn5*Δ strain, at later times of P_i starvation. This is the first demonstration of an excess of a recruited ATP-dependent remodeler possibly compensating for the lack of a HAT subunit. On the other hand, in a *swi2*Δ strain, there is no significant increase in the level of Gcn5 association at the promoter, possibly due to decreased Pho4 binding. Further, there is very little SWI/SNF recruitment in an *spt20*Δ strain, which contains only a partially formed SAGA complex. This suggests possible physical interactions of SWI/SNF with the SAGA

complex, although recent evidence indicates that SWI/SNF is recruited independently of SAGA (Natarajan et al., 1999). On the other hand, the decreased activity of an *spt20*Δ strain might simply be due to low Pho4 binding at the *PHO5* promoter, leading to reduced recruitment of SWI/SNF. This would demonstrate a role for promoter occupancy in defining physical and/or functional interactions of coactivator complexes at promoters. In this chapter, I discuss the various interactions and possible interpretations of our data.

INTRODUCTION

Eukaryotic organisms establish programs of gene control by means of chromatin, the complex of DNA with histones and other proteins that serves as a barrier to many biological functions (Owen-Hughes and Workman, 1994). Chromatin is not static in nature, but is a highly dynamic structure, compacting and unfolding in the cell in response to external signals. The multisubunit complexes SWI/SNF and SAGA facilitate transcription by remodeling the structure of chromatin, thus enabling access of transactivators and the basal transcriptional machinery to DNA (Lee et al., 1993; Juan et al., 1994; Vettese-Dadey et al., 1996). While SAGA effects disruption of higher-order chromatin structure by acetylation of lysine residues of histone tails (Tse et al., 1998), SWI/SNF performs the same task by utilizing the energy derived from ATP-hydrolysis to generate superhelical torsion in DNA (Havas et al., 2000). It has been observed that SAGA and SWI/SNF target many common genes for activation of transcription (Pollard and Peterson, 1997). Evidence for the partial functional redundancy of SWI/SNF and SAGA came from observations that deletion of many SAGA subunits is lethal in combination with those of SWI/SNF components (Roberts and Winston, 1997; Sterner et

al., 1999). Further, conditional inactivation of the ATPase subunit of SWI/SNF in a *gcn5*Δ strain causes loss of expression of many genes, demonstrating that they act in independent but partially redundant pathways (Biggar and Crabtree, 1999). A mechanistic interpretation of this data is rendered difficult, as the loss of both Gcn5 and Swi2 function is synthetically lethal in most genetic backgrounds.

Several studies have demonstrated the interdependence of SWI/SNF and SAGA. At the yeast *HO* promoter, for instance, SWI/SNF is recruited prior to SAGA (Cosma et al., 1999; Krebs et al., 2000). However, evidence from other labs indicated that HATs can acetylate nucleosomal arrays and effect transcription *without* prior SWI/SNF recruitment (Utley et al., 1998; Ikeda et al., 1999). Further, several groups observed the opposite phenomenon, i.e. acetylation of histones prior to SWI/SNF recruitment. Our own studies (see Chapter II, Figure 2-1) showed that, at the yeast *PHO5* promoter, there is substantial recruitment of Gcn5 prior to SWI/SNF. Recent work demonstrated that the activator NF-κB is the first to arrive at the interferon-β (IFN-β) promoter, ~2 h post-infection. The GCN5/PCAF HAT complex is recruited next (3 h post-infection), while SWI/SNF does not arrive at the promoter until about 6 h after infection (Agalioti et al., 2000). It is of interest to note that SWI/SNF recruitment proceeds without histone acetylation if multiple copies of NF-κB binding sites are available at the promoter (Agalioti et al., 2000), suggesting a role for increased factor levels, i.e. promoter occupancy, in facilitating recruitment. This could also mean that activators with high affinity for SWI/SNF would bypass the need for histone acetylation by achieving sufficient recruitment of SWI/SNF. The yeast activator Gcn4 has been shown interact specifically with SWI/SNF, independently of SAGA and mediator (Natarajan et al., 1999). It is

interesting to note that higher GST–Gcn4p concentrations were needed for specific binding of SWI/SNF subunits in yeast extracts than were needed for binding of SAGA or SRB/mediator subunits. This could imply one of two possibilities; a) the activator Gcn4 has a higher affinity for SAGA and mediator than for SWI/SNF, or b) that the levels of SAGA in the cell are higher on average compared to SWI/SNF.

The yeast *PHO5* promoter has been studied extensively to dissect the relationship between chromatin remodeling and transcriptional activators. While early studies revealed that deletion of *SWI2* and *GCN5* had little or no effect on full activation of *PHO5* (Gaudreau et al., 1997; Gregory et al., 1998b; Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003), it was later found that *gcn5*Δ strains show a delay in induction (Barbaric et al., 2001; Neef and Kladde, 2003) as do *swi2*Δ strains (Neef and Kladde, 2003). Deletion of *ADA2* had the same effect as deletion of *GCN5* on *PHO5* expression. However, loss of other SAGA subunits, most significantly Spt20 and Spt7, were found to decrease *PHO5* expression, and to a lesser extent, Spt3 (Barbaric et al., 2003). Studies presented in Chapter II have revealed the importance of promoter occupancy in determining requirements for chromatin-remodeling complexes at *PHO5*. We also showed that both the Gcn5 and Swi2/Snf2 subunits are recruited to *PHO5* subsequent to activator binding in a P_i-starvation dependent manner (compare Figure 2 -1 to 2-2). At initial points in P_i starvation, there is less promoter occupancy of Pho4, the main *PHO5* transactivator. However, at later times, Pho4 levels at the promoter approach wild-type levels in the mutant *gcn5*Δ but not *swi2*Δ strains, although the level of Pho4 is unaffected by loss of either coactivator (Figure 2-5). The intriguing question of why *PHO5* activation in a *gcn5*Δ or *swi2*Δ strain attains wild-type levels after many hours of

P_i starvation still remains. While there have been many reports suggesting partial functional redundancy of SWI/SNF and SAGA, the effects of deleting either *GCN5* or *SWI2* on the recruitment of SWI/SNF and SAGA have not been investigated. We tested how deletion of SAGA subunit Gcn5 affected recruitment of SWI/SNF. We show here that in a *gcn5*Δ strain, there is twice as much SWI/SNF recruited to the *PHO5* promoter compared to a wild-type strain after prolonged P_i starvation. On the other hand, Gcn5 recruitment to the *PHO5* promoter is unaffected in a *swi2*Δ strain. Further, we show that this increased SWI/SNF recruitment is barely detectable in strains lacking the Spt20 subunit of SAGA. Deletion of *GCN5* does not result in loss of the entire SAGA complex (Sterner et al., 1999; Wu et al., 2004), but loss of *SPT20* leads to a severe reduction in the number of SAGA subunits, although partial complexes still assemble (Wu et al., 2004). However, these partial complexes do not contain Gcn5, the main HAT subunit; Tra1, which interacts with activators (Grant et al., 1998); or Spt3, which is needed for TBP-interaction (Eisenmann et al., 1992; Roberts and Winston, 1997; Sterner et al., 1999; Lee et al., 2000). This lack of SWI/SNF binding could be merely due to decreased Pho4 binding at the promoter, since rAPase activity does not achieve wild type levels.

Thus, although SWI/SNF and SAGA have been shown to be partially redundant, this is the first instance where enhanced recruitment of an ATP-dependent remodeler potentially compensates for the lack of a HAT subunit. Our data are noteworthy because SWI/SNF does not appear to require the presence of Gcn5 to bind at later points in the time course of phosphate starvation, Gcn5 is needed earlier, when activator levels are low at the promoter. This correlates well with earlier studies that showed SWI/SNF recruitment occurred independent of Gcn5 provided there was sufficient activator present

(Natarajan et al., 1999; Agalioti et al., 2000; Dilworth et al., 2000). We also find that in *swi2*Δ strains, the level of Gcn5 recruitment is unchanged relative to wild-type cells. However, the rAPase activity approaches wild-type levels after many hours of P_i starvation. The lower levels of Pho4 binding in *swi2*Δ strains compared to *gcn5*Δ strains might explain why there is no increase in Gcn5 recruitment. Our finding that Pho4 binding is delayed at the *PHO5* promoter in *gcn5*Δ and *swi2*Δ cells relative to wild type demonstrated the mechanistic basis for the kinetic delay in activation observed previously.

MATERIALS AND METHODS

Yeast media, growth conditions, and rAPase activity assays

Defined, P_i-free medium (pH 5.5) was prepared as described (Neef and Kladde, 2003), except that it was supplemented with complete synthetic mix (CSM, Bio101). All starter cultures were grown in this medium with KH_2PO_4 (P_i) added back to 13.4 mM. For time courses, cells were washed and transferred to activating medium without P_i. rAPase activity was assayed as previously described (Neef and Kladde, 2003).

ChIP analysis

All yeast strains used in this study are listed in Table 3-1. ChIP was performed as described in Chapter II. Cultures (50 mL) were grown at 30°C with shaking for the indicated times and then were fixed at room temperature for 15 min by adding formaldehyde to a 1% final concentration. Cross-linking was quenched by addition of glycine to a final concentration of 125 mM and incubating for 5 min at room temperature.

Table 3-1. *S. cerevisiae* strains.

Strain	*Genotype* [a, b]
MRY3207	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *gcn5*□::*kanMX4 SWI2-13myc-kanMX4 can1*□::*pho5*^{pro} □UASs-*LEU2*
MRY3223	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *SWI2-13myc-kanMX4 can1*□::*pho5*^{pro} □UASs-*LEU2*
ADY2695	*MAT***a** *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *3myc-PHO4 swi2*□::*kanMX4 CAN1*:*pho5*^{pro} □UASs-*LEU2*
ADY2700	*MAT***a** *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *3myc-PHO4 CAN1*:*pho5*^{pro} □UASs-*LEU2*
ADY2701	*MAT***a** *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *CAN1*:*pho5*^{pro} □UASs-*LEU2*
ADY2719	*MAT***a** *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *3myc-PHO4 gcn5*□::*kanMX4 CAN1*:*pho5*^{pro} □UASs-*LEU2*
ADY2727	*MAT***a** *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *3myc-PHO4 CAN1*:*pho5*^{pro} □UASs-*LEU2*
MRY3219	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *SWI2-13myc-kanMX4 can1*□::*pho5*^{pro} □UASs-*LEU2*
ADY3384	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *SWI2-13myc-kanMX4 can1*□::*pho5*^{pro} □UASs-*LEU2 spt20::R-URA3-R*
ADY3398	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *GCN5-13myc-kanMX4 CAN1*:*pho5*^{pro} □UASs-*LEU2*
ADY3399	*MAT*□ *leu2*□*0 lys2*□*0 ura3*□*0 pho3*□::R *swi2*□::*kanMX4 GCN5-13myc-kanMX4 CAN1*:*pho5*^{pro} □UASs-*LEU2*

[a] The superscript 'pro' indicates promoter.
[b] R is a *Zygosaccharomyces rouxii* recombinase site that remains after intramolecular recombination (Roca et al., 1992).

Radiolabeled PCR products were electrophoresed on a 5% nondenaturing polyacrylamide gel at 5 W for 3 h, and visualized by a Storm 860 phosphorimager (Molecular Dynamics), and quantified by ImageQuant software. For *PHO5* sequences, a single primer pair [(ADO236; CATGTAAGCGGACGTC; –456 to –441 relative to the *PHO5* ATG translation start) and (LFO740; GCCTTGCCAAGTAA-GGTGAC; –173 to –154)] was used to amplify the endogenous UASs of the *PHO5* promoter as well as a negative control *PHO5* promoter region (*pho5* □UASs) by quantitative, competitive PCR. This negative control contains *PHO5* sequences from –1537 to +9 with two 50-bp deletions

(encompassing UASp1 and UASp2 from −401 to −352 and −258 to −209, respectively), and was integrated either by gene replacement of (strains MRY3207 and MRY3223) or loop in at (strains ADY2695, ADY2701, ADY2719, ADY2727, ADY2915, ADY2921, and ADY2923) the *CAN1* locus (Carvin et al., 2003a). To quantitate binding at the promoter, the ratio of the *PHO5* promoter PCR product to that of the control band was determined.

RESULTS

Pho4 association at the PHO5 *promoter is delayed in* gcn5Δ *strains*

Many eukaryotic genes have differential requirements for the chromatin-remodeling complexes SWI/SNF and SAGA (Holstege et al., 1998; Biggar and Crabtree, 1999; Sudarsanam et al., 2000). The induction of the *Saccharomyces cerevisiae PHO5* gene approaches wild-type levels in either *swi2*Δ or *gcn5*Δ strains (Gaudreau et al., 1997; Gregory et al., 1998b; Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). However, the kinetics of *PHO5* induction are strongly dependent on Gcn5 and SWI/SNF (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). We have also shown that levels of Pho4 at the promoter influence requirements for these remodelers (see Chapter II). To evaluate the reason for the kinetic delay in *PHO5* induction in these mutant strains, we determined levels of Pho4 binding at the *PHO5* UASs at various times following starvation for P_i. Wild-type and *gcn5*Δ strains containing 3myc-tagged Pho4 were initially grown in CSM +P_i medium and then transferred to CSM no-P_i medium. Aliquots of cells were periodically removed and assayed for repressible acid phosphatase (rAPase) activity as previously described (Neef

and Kladde, 2003) and for ChIP analysis of 3myc-Pho4 binding using the A-14 anti-myc antibody.

The rAPase activities reveal that at early times of induction, activation of the *PHO5* promoter is delayed in the *gcn5*Δ strain compared to the wild type (Figure 3-1A) as previously reported (Barbaric et al., 2001; Barbaric et al., 2003; Neef and Kladde, 2003). As seen in Figure 3-1 B and C, Pho4 is observed at the promoter within 2 h of P_i starvation in the wild-type cells but is detectable only around 4 h in the mutant *gcn5*Δ strain. It also takes longer to achieve near wild-type levels of Pho4 binding at the promoter.

Pho4 association at the PHO5 *promoter is delayed in* swi2Δ *strains and does not equal wild-type levels*

Strains deleted for *SWI2* coding for the ATPase subunit of the SWI/SNF complex also exhibit a kinetic delay in *PHO5* induction with respect to wild-type cells (Neef and Kladde, 2003). We wanted to assess whether a similar delay in Pho4 binding occurred in *swi2*Δ relative to wild-type cells. As seen for the *gcn5*Δ strain, the rAPase activity revealed that there was a delay in *PHO5* induction in the *swi2*Δ strain compared to the wild type. ChIP analysis at the *PHO5* promoter of internal aliquots of cells used in Figure 3-2A revealed that Pho4 binding is delayed in *swi2*Δ strains with respect to the wild-type cells, requiring nearly 6 h for significant Pho4 binding to be detected. It is noteworthy that the *swi2*Δ strain fails to achieve wild-type levels of binding, even after several hours of P_i starvation, levels of bound Pho4 are well below that of wild type. This is very different from what is seen with the *gcn5*Δ strain, and indicates a greater role for

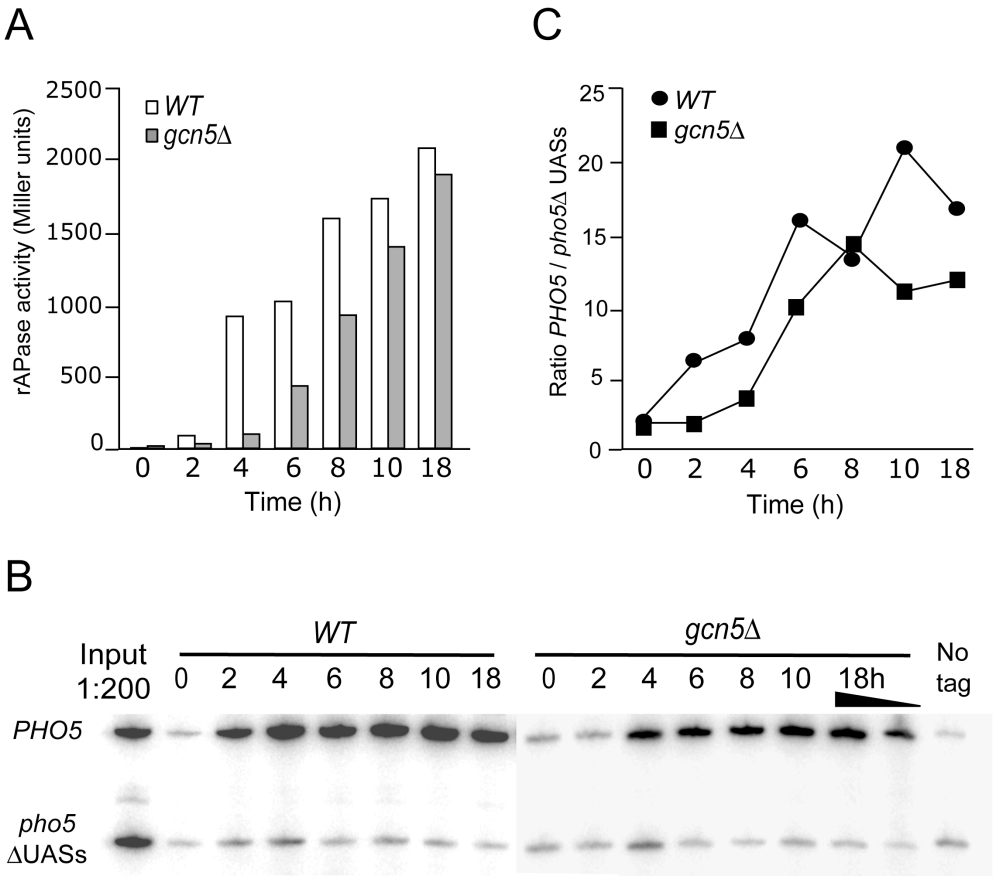

Figure 3-1. Pho4 binding at the *PHO5* promoter is delayed in a *gcn5*Δ strain.

(*A*) Time course of *PHO5* activation following transfer from high- to no-P_i medium. *WT* (ADY2727) and *gcn5*Δ (ADY2719) cells were harvested at the indicated times and assayed for rAPase activity. (*B*) Internal aliquots of cells from the *WT* and *gcn5*Δ cultures assayed in *A* were subjected to ChIP analysis (see Materials and methods) at the indicated times of induction. DNA immunoselected with anti-myc antibody against 3myc-tagged Pho4 from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5* ΔUASs) sequences by quantitative, competitive PCR. The negative control promoter has deletions of both UASp1 and UASp2 and thus is unable to bind Pho4 (Carvin et al., 2003). A wild-type strain (ADY2701) was starved of P_i for 18 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 18-h culture of *WT* 3myc-Pho4 cells following cross-linking and was diluted 1:200 for PCR amplification. The ramp indicates inclusion of half as much immunoselected DNA in the PCR amplification and demonstrates linearity. (*C*) Relative amounts of bound Pho4 as determined by ratios of the *PHO5* promoter PCR product to that of the *pho5* ΔUASs.

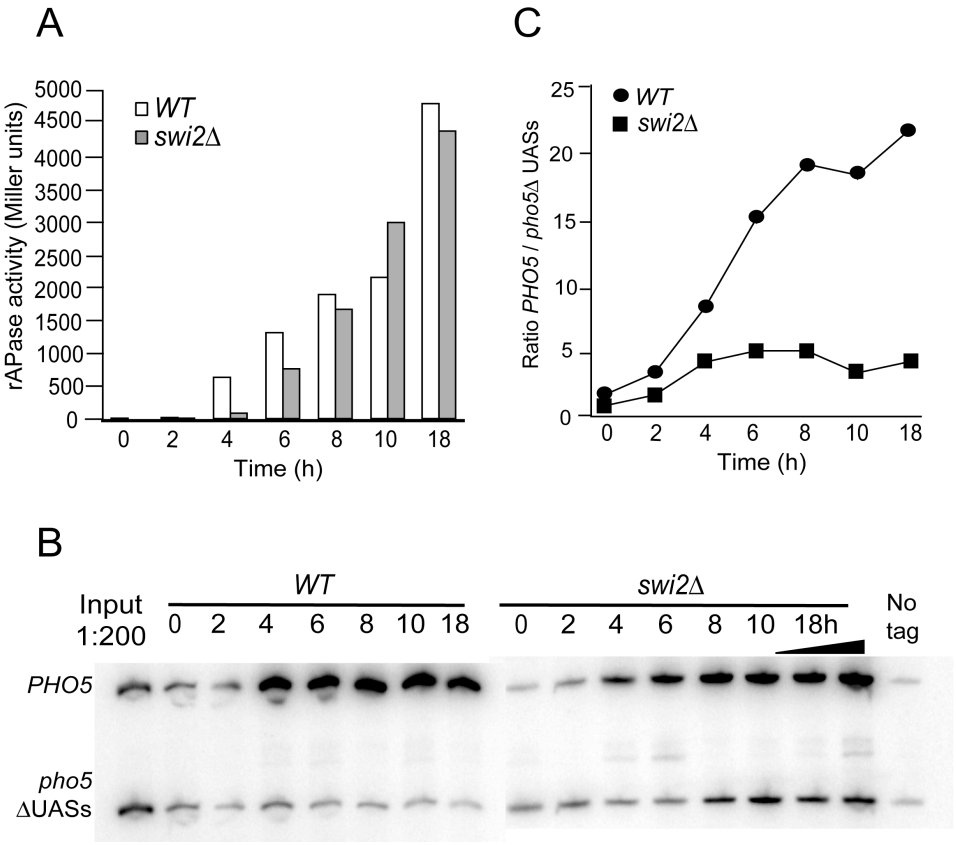

Figure 3-2. Pho4 binding at the *PHO5* promoter is delayed in a *swi2Δ* strain and does not achieve wild-type levels of binding.

(*A*) Time course of *PHO5* activation following transfer from high- to no-P_i medium. *WT* (ADY2727) and *swi2Δ* (ADY2695) cells were harvested at the indicated times and assayed for rAPase activity. (*B*) Internal aliquots of cells from the *WT* and *swi2Δ* cultures assayed in *A* were subjected to ChIP analysis (see Materials and methods) at the indicated times of induction. DNA immunoselected with anti-myc antibody against 3myc-tagged Pho4 from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5 Δ*UASs) sequences by quantitative, competitive PCR as in Figure 3-1. A wild-type strain (ADY2701) was starved of P_i for 18 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 18-h culture of *WT* 3myc-Pho4 cells following cross-linking and was diluted 1:200 for PCR amplification. The ramp indicates inclusion of twice as much immunoselected DNA in the PCR amplification and demonstrates linearity. (*C*) Relative amounts of bound Pho4 as determined by ratios of the *PHO5* promoter PCR product to that of the *pho5 Δ*UASs.

SWI/SNF in facilitating chromatin remodeling and activator binding at the *PHO5* promoter.

Increased amounts of SWI/SNF recruitment in a gcn5Δ *strain after many hours of P_i starvation*

Although the mutant strains *swi2*Δ and *gcn5*Δ are delayed in *PHO5* activation, they achieve wild-type levels of rAPase activity at later points in the time course (Barbaric et al., 2001; Neef and Kladde, 2003). Given the evidence for partial functional redundancy of SWI/SNF and SAGA complexes, we tested whether more SWI/SNF is recruited in a *gcn5*Δ strain to the *PHO5* promoter, thus bypassing the need for histone acetylation by SAGA. The strains MRY42 (*GCN5 SWI2*-13myc) and MRY26 (*gcn5*Δ *SWI2*-13myc) were grown in CSM high-P_i medium and then washed and transferred to CSM no-P_i medium. Aliquots of cells were removed at various times to assay for rAPase activity and for SWI/SNF recruitment by ChIP analysis. As seen before (Figure 3-1 A; (Barbaric et al., 2001; Neef and Kladde, 2003), we find that at early times in P_i starvation there is less rAPase activity in the *gcn5*Δ strain compared to the wild-type strain (Figure 3-3A). Also, at early times of P_i starvation there is less Swi2 associated at the *PHO5* promoter in the *gcn5*Δ strain compared to the wild-type strain (Figure 3-3B).

However, strikingly, at later time points, we see that there is almost *twice* as much Swi2 recruitment in the *gcn5*Δ strain compared to the wild-type strain (Figure 3-3C). This seemed indicative of two things: one, that Swi2 does not require the presence of Gcn5 inorder to be recruited to the *PHO5* promoter, provided that sufficient levels of Pho4 associate at the promoter at later times of induction (Figure 3-1 B and C). Secondly, the

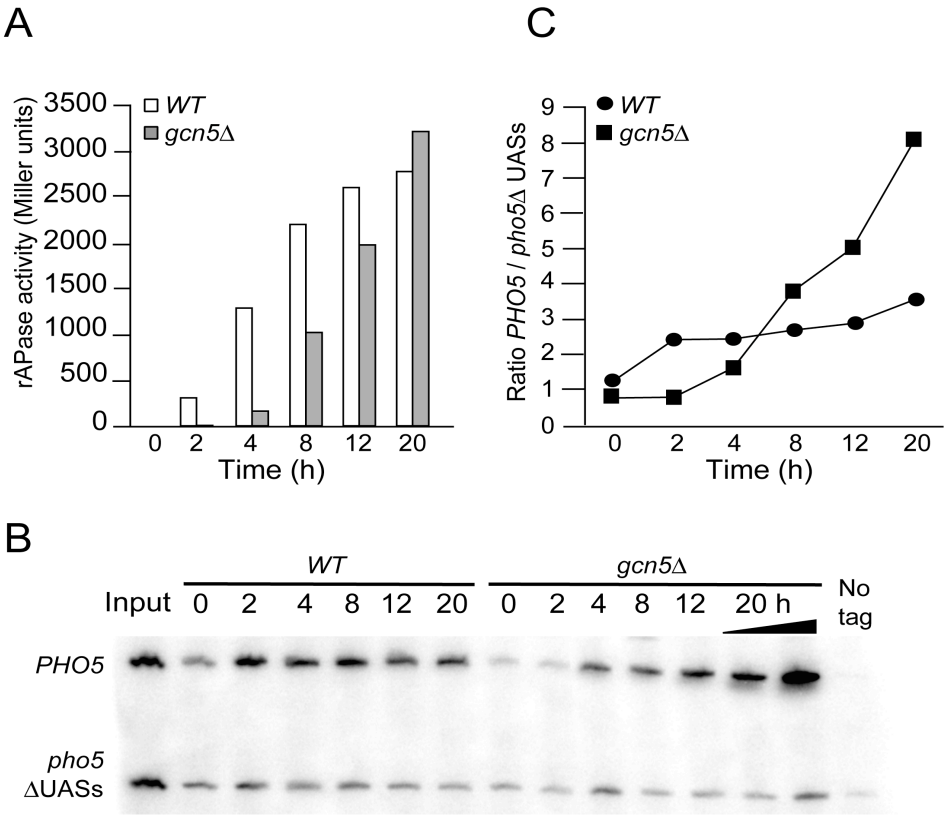

Figure 3-3. Increased recruitment of SWI/SNF in a *gcn5*Δ strain after many hours of P_i starvation.

(*A*) Time course of *PHO5* activation following transfer from high- to no-P_i medium. *WT* (MRY3223) and *gcn5*Δ (MRY3207) cells were harvested at the indicated times and assayed for rAPase activity. (*B*) Internal aliquots of cells from the *WT* and *swi2*Δ cultures assayed in *A* were subjected to ChIP analysis (see Materials and methods) at the indicated times of induction. DNA immunoselected with anti-myc antibody against Swi2-13myc from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5* ΔUASs) sequences by quantitative, competitive PCR as in Figure 3-1. A wild-type strain (ADY2701) was starved of P_i for 18 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 20 h culture of *WT* cells following cross-linking and was diluted 1:200 for PCR amplification. The ramp indicates inclusion of twice as much immunoselected DNA in the PCR amplification and demonstrates linearity. (*C*) The ratio of *PHO5* to negative control (*pho5* ΔUASs) product indicates the relative enrichment of each coactivator at the promoter.

increased recruitment of Swi2 in a *gcn5*Δ strain suggests a competition between SWI/SNF and SAGA for the Pho4 activator surface.

Loss of Swi2 binding at the PHO5 *promoter in an* spt20Δ *strain*

While deletion of Gcn5 does not cause any major perturbation to SAGA, deletion of Spt20 results in the formation of a partial complex that lacks many of the major subunits, including Tra1, Gcn5 and Spt3 (Wu et al., 2004). The activation domains of many acidic activators have been shown to interact with Tra1 (Brown et al., 2001). Therefore, such a partially formed SAGA complex is not expected to be recruited to the *PHO5* promoter in *spt20*Δ strains. In *gcn5*Δ cells, SAGA might potentially serve as a competitor for Swi2 to some extent. We reasoned that if SAGA were truly competing with SWI/SNF, then deletion of Spt20 should result in increased recruitment of Swi2 to the *PHO5* promoter as well. Hence we performed rAPase and ChIP assays in both wild-type and *spt20*Δ strains to detect Swi2-13myc association at the *PHO5* promoter. Surprisingly, we observed that in an *spt20*Δ strain, there is very low to undetectable amounts of Swi2 even after 14 h of P_i starvation (Figure 3-4). The wild type, in contrast, was enriched for the presence of SWI/SNF. Given that the rAPase levels are also low in an *spt20*Δ strain, it is quite possible that there is less Pho4 binding, which would account for the absence of Swi2 at the *PHO5* promoter. However, this remains to be tested. There is also the possibility of less Pho4 protein levels in the *spt20*Δ strains.

Gcn5 recruitment at the PHO5 *promoter is unaffected in a* swi2Δ *strain*

While *swi2*Δ strains have lower levels of Pho4 binding compared to wild-type strains (Figure 3-2 B and C), their ability to induce *PHO5* appears to be unaffected once a threshold level of activator binding is achieved. To discern the reason for the increase in rAPase activity after many hours of P_i starvation, we tested whether there is improved recruitment of Gcn5 in *swi2*Δ strains compared to the wild type, which might bypass the necessity for an active SWI/SNF complex. After growth in CSM no-P_i medium for a period of 20 h, we assayed wild-type and *swi2*Δ cells for rAPase activity. ChIP was performed as before from internal aliquots utilizing the A-14 anti-myc antibody against Gcn5-13myc. The rAPase activity is fairly unchanged in both strains, and we also find that Gcn5 was recruited to similar levels in both strains. This begs the question by what means are wild-type levels of rAPase activity achieved in a *swi2*Δ strain, if not increased recruitment of Gcn5? It is possible that there is increased recruitment of the other ATP-dependent remodeler known to be recruited to *PHO5*, INO80.com (Steger et al., 2003). Alternatively, at high concentrations of promoter-bound Pho4, there might be sufficient recruitment of the basal transcriptional machinery that might be sufficient to disrupt chromatin structure and allow wild-type levels of expression as suggested previously (Gaudreau et al., 1997).

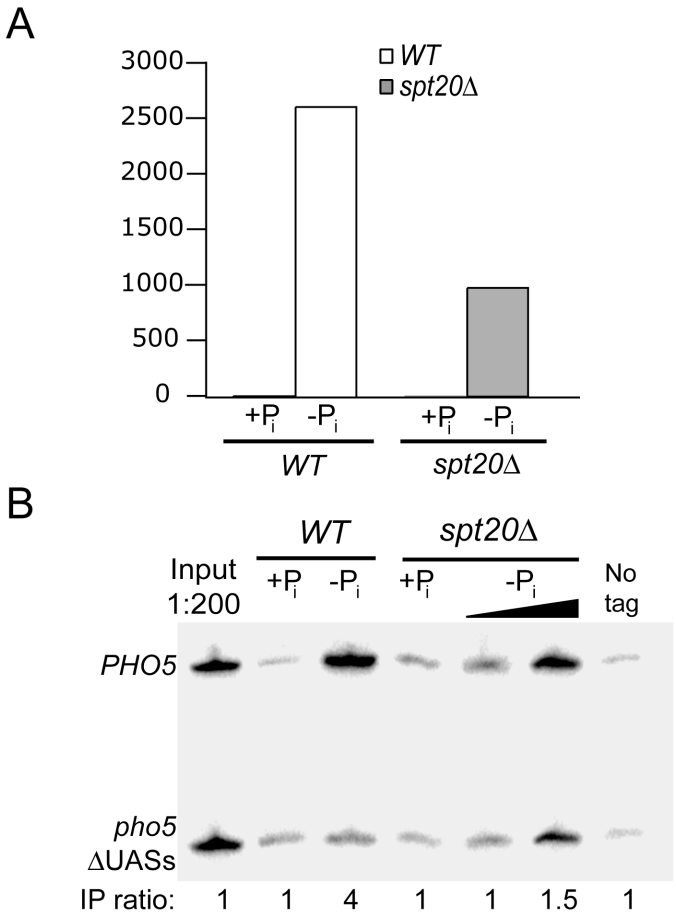

Figure 3-4. Loss of SWI/SNF binding at the *PHO5* promoter in an *spt20*Δ strain

(*A*) *PHO5* activation in high- and no-P_i medium. *WT* (MRY3219) and *spt20*Δ (ADY3384) cells were harvested after 18 h of growth in high- and no-P_i medium and assayed for rAPase activity. (*B*) Internal aliquots of cells from the *WT* and *spt20*Δ cultures assayed in *A* were subjected to ChIP analysis (see Materials and methods). DNA immunoselected with anti-myc antibody against Swi2-13myc from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5* ΔUASs) sequences by quantitative, competitive PCR as in Figure 3-1. A wild-type strain (ADY2701) was starved of P_i for 18 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 18 h culture of *WT* cells grown in $+P_i$ medium following cross-linking and was diluted 1:200 for PCR amplification. The ramp indicates inclusion of twice as much immunoselected DNA in the PCR amplification and demonstrates linearity.

DISCUSSION

In this study, we aim to further define the interplay between the chromatin-remodeling complexes SWI/SNF and SAGA at the *PHO5* promoter. While both complexes have been shown to be physically present at the *PHO5* promoter (Barbaric et al., 2003), deletion of one or the other subunits seemed not to have drastic effects on *PHO5* activation at high activator levels at the promoter (see Chapter II). An interesting question is how the cell compensates for the lack of one or the other subunit. While the presence of other chromatin-remodeling complexes has been demonstrated at *PHO5* (Steger et al., 2003; Nourani et al., 2004), we asked whether the lack of Gcn5 was compensated for by increased recruitment of Swi2. This hypothesis was based on the evidence from many labs that Swi2 and Gcn5 are functionally redundant (Biggar and Crabtree, 1999; Sudarsanam et al., 1999), although they are mechanistically different in their mode of action. We find that at early times of activation in a *gcn5*Δ strain (when Pho4 levels at the promoter are low), Swi2 recruitment is barely detected at the *PHO5* promoter (Figure 3-3). However, at later times of P_i starvation, when there is increased Pho4 binding at the promoter, SWI/SNF recruitment does not require Gcn5 activity. We also find that there is *twice as much* Swi2 recruited in a *gcn5*Δ strain compared to the wild type at later times of activation. This is suggestive of a competition between SAGA and SWI/SNF for interaction with the activator surface. On the other hand, deletion of *SPT20* (which eliminates many subunits from SAGA) leads to diminished SWI/SNF recruitment to *PHO5* even after several hours of starvation for P_i (Figure 3-4). This might be due to decreased Pho4 binding, but that remains to be tested. We also find that there is no change in Gcn5 recruitment to the *PHO5* promoter in a *swi2*Δ strain relative to the

wild type (Figure 3-5). Possibly, *swi2*Δ strains achieve wild-type levels of *PHO5* activity by recruitment of the INO80 complex, another ATP-dependent chromatin remodeler, or by some other means.

Pho4 binding at the PHO5 *promoter is delayed in* swi2Δ *and* gcn5Δ *strains*

At the yeast *PHO5* promoter, multiple complexes function together to effect nucleosome remodeling leading to gene expression in conditions of low P_i. Following the initial binding of the activators Pho4 and Pho2, a number of chromatin remodeling machines are recruited to the *PHO5* promoter, including SAGA, SWI/SNF, INO80.com and NuA4. Of these complexes, the effects of both SWI/SNF and SAGA on *PHO5* have been widely studied. Both *GCN5* (Barbaric et al., 2001; Neef and Kladde, 2003) and *SWI2* (Neef and Kladde, 2003) deletions influence the kinetics of induction and remodeling of *PHO5* at early times of activation, although they have little to no effect on full levels of activation at later times.

Of the two, Gcn5 is the first to be recruited to the *PHO5* promoter, while Swi2 is detected soon afterward (Figure 2-1). The reason for the kinetic delay in *PHO5* activation in *swi2*Δ or *gcn5*Δ strains is consistent with our observation that there is less Pho4 at the promoter during early times of P_i starvation (Figures 3-1 and 3-2). Of the two Pho4 binding sites at the *PHO5* promoter, one lies in the middle of nucleosome –2. Thus, only at sufficient activator concentrations would recruitment of chromatin remodelers be high enough that chromatin remodeling would expose the Pho4 binding site in nucleosome –2. Hence this data fits very well with our current knowledge of *PHO5* promoter architecture. An interesting observation is that while the levels of Pho4 at the promoter in

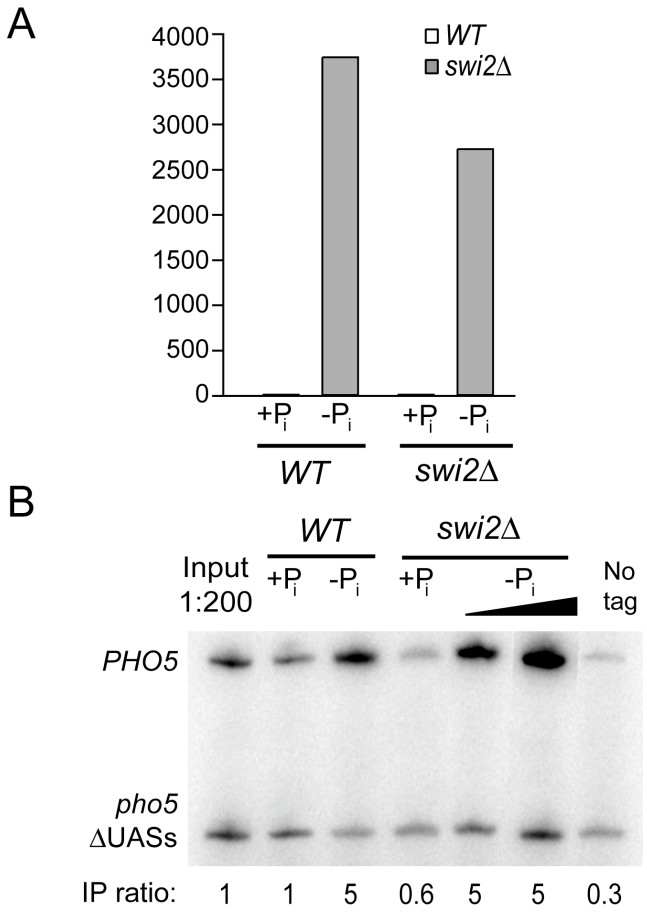

Figure 3-5. Gcn5 recruitment to the *PHO5* promoter is unchanged in a *swi2*Δ strain.

(*A*) *PHO5* activation in high- and no-P_i medium. *WT* (MRY3219) and *spt20*Δ (ADY3384) cells were harvested after 18 h of growth in high- and no-P_i medium and assayed for rAPase activity. (*B*) Internal aliquots of cells from the *WT* and *spt20*Δ cultures assayed in *A* were subjected to ChIP analysis (see Materials and methods). DNA immunoselected with anti-myc antibody against Swi2-13myc from formaldehyde cross-linked chromatin (all lanes except lane 1) was analyzed for the presence of *PHO5* and negative control (*pho5* ΔUASs) sequences by quantitative, competitive PCR as in Figure 3-1. A wild-type strain (ADY2701) was starved of P_i for 18 h and carried in parallel through all steps to serve as an untagged (no tag) specificity control. Non-immunoselected DNA (input, lane 1) was obtained from the 18-h culture of *WT* cells grown in +P_i medium following cross-linking and was diluted 1:200 for PCR amplification. The ramp indicates inclusion of twice as much immunoselected DNA in the PCR amplification and demonstrates linearity.

the *gcn5*Δ strains approximate wild-type levels (although delayed), the *swi2*Δ strains exhibit a much lower level of binding, even after several hours of P_i starvation.

The question then remains, how do the *gcn5*Δ and *swi2*Δ strains compensate for the loss of the other subunit? Is the delay merely due to delayed activator binding due to the absence of one out of the four remodelers that are known to be recruited, or is there an alternative explanation? We sought to answer this question by determining SWI/SNF recruitment in a *gcn5*Δ strain.

Increased recruitment of SWI/SNF at the PHO5 *promoter in a* gcn5Δ *strain*

When we observe SWI/SNF recruitment for several hours after P_i starvation in wild-type and *gcn5*Δ cells, we find that there is twice as much recruitment of the ATP-dependent remodeler in a *gcn5*Δ strain. This additional recruitment potentially bypasses the need for Gcn5. This is the first demonstration of an ATP-dependent remodeler potentially compensating for the lack of a HAT complex. It is however worthwhile to determine whether there is truly a decrease in acetylation at the *PHO5* promoter, or whether there is additional recruitment of other HATs, for instance NuA4, which has already been shown to be recruited to *PHO5* (Nourani et al., 2004).

How can an ATP-dependent remodeler compensate for the loss of a HAT subunit? SWI/SNF has also been shown to function at many of the genes at which SAGA plays an important role (Pollard and Peterson, 1997). It has been demonstrated that deletion of many SWI/SNF subunits are lethal in combination with those of SAGA or the mediator complex (Roberts and Winston, 1997). Further, they are also lethal in combination with TATA-binding protein (TBP) mutants as well as the architectural

transcription factors Nhp6a and Nhp6b (Yu et al., 2000; Biswas et al., 2004). Previous work has demonstrated that SWI/SNF and SAGA both interact with TBP and the transcriptional machinery. SWI/SNF stimulates TBP and TFIIA binding to a nucleosomal TATA site *in vitro* (Imbalzano et al., 1994), while histone acetylation has been shown to facilitate TBP binding (Biswas et al., 2004). Perhaps SWI/SNF compensates for the lack of acetylation by increased TBP recruitment.

Is the increased SWI/SNF recruitment truly compensating for the lack of Gcn5? Unfortunately, the synthetic lethality of *swi2*Δ *gcn5*Δ strains precludes the simplest way to test this. A potential way to test this hypothesis would be to utilize partial loss-of-function mutants of SWI/SNF subunits, which do not impair the ATPase function, but only the recruitment. The increased recruitment of SWI/SNF in a *gcn5*Δ strain is suggestive of a competition between SWI/SNF and SAGA for interaction with the activator surface. However, prior work indicates that they are recruited independently of each other (Natarajan et al., 1999). Also, Gcn5 is recruited prior to Swi2 at *PHO5* (Figure 2-1). Interactions of SWI/SNF with Gcn4 *in vitro* were observed at higher GST–Gcn4 concentrations than were needed for SAGA binding (Natarajan et al., 1999). This suggests that: a) some proteins have higher affinity for SAGA than for SWI/SNF, or, alternatively, b) the levels of SAGA in the cell are higher as compared to SWI/SNF. If there were truly a competition between SWI/SNF and SAGA for interaction with the activator surface, we reasoned that in an *spt20*Δ strain, which contains only a partially formed SAGA complex (Wu et al., 2004), we should see a further increase in SWI/SNF recruitment.

SWI/SNF recruitment is undetectable in an spt20Δ *strain*

In a *gcn5*Δ strain, the integrity of the rest of the SAGA complex is still maintained (Wu et al., 2004). Hence, in this strain, SWI/SNF is still in somewhat of a competition with SAGA, since subunits in the complex like Tra1 can still interact with the activator. If there is competition between Swi2 and Gcn5, then in an *spt20*Δ strain, which results in a partial, but more minimal SAGA complex than a *gcn5*Δ strain (Wu et al., 2004), there should be much more SWI/SNF recruitment. To our surprise, we saw quite the contrary: *less* SWI/SNF was recruited to the *PHO5* promoter; being barely discernible by ChIP. This suggests that SWI/SNF needs some subunit in SAGA, but not Gcn5, in order to stably associate with the *PHO5* promoter. An alternative explanation is that there might be less association of Pho4 with the promoter, which might explain the inability to recruit as much SWI/SNF, however this remains to be determined.

How is SWI/SNF targeted to promoters? It has been shown that SWI/SNF can bind non-specifically to DNA and nucleosomes (Quinn et al., 1996; Côté et al., 1998), and specifically through interactions with activators such as VP16, Gcn4, Hap4, Gal4, Pho4 and Swi5 (Neely et al., 1999; Yudkovsky et al., 1999; Neely et al., 2002). Of the various subunits of the SWI/SNF complex, three have been shown to be involved in interactions with acidic activation domains: Tra1, the largest subunit that is found in both SAGA and NuA4 (Brown et al., 2001); Snf5, through its N-terminal domain; and Swi1 by virtue of its second quarter (Prochasson et al., 2003). It was found that deletion of the activator-interacting domains of these subunits left SWI/SNF ATPase function intact but impaired its recruitment. Individual deletions of each activator-interacting domain did not yield a *swi*⁻ phenotype, i.e. they did not display any major phenotypic changes such as

growth deficiencies on media lacking inositol or raffinose or containing sulfometuron methyl, which inhibits amino acid biosynthesis. However, deletion of both domains resulted in rather strong SWI/SNF phenotypes (Prochasson et al., 2003). Hence we can make a prediction that if SAGA is in direct competition with SWI/SNF for activator interaction surfaces, then in a *gcn5*Δ strain, mutations in either of these subunits should now reduce the association of SWI/SNF with the activator. It will be informative to test this model and determine the extent to which the single and double deletions affect *PHO5* activation. In other words, does partial impairment of SWI/SNF recruitment decrease the ability to bypass the loss of Gcn5? This will demonstrate that the additional recruitment of SWI/SNF in a *gcn5*Δ strain is required for activation of *PHO5* after prolonged P_i starvation.

Bromodomains are found in many chromatin remodeling complexes and have been implicated in binding to acetylated lysine residues (Dhalluin et al., 1999; Ornaghi et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000). The bromodomain found in Gcn5 was shown to be essential for Swi2-dependent nucleosome remodeling and transcriptional activation. The Gcn5 bromodomain has also been shown to stabilize SWI/SNF interaction at the promoter of an artificial gene *in vivo* (Syntichaki et al., 2000). It is worth investigating what role the Gcn5 bromodomain plays in SWI/SNF recruitment to the *PHO5* promoter.

CHAPTER IV

SUMMARY AND CONCLUSIONS

FACTORS DICTATING CHROMATIN-REMODELING REQUIREMENTS

Studies over the years have proposed various means by which cells regulate expression of their genes. In the past, the various processes from transcription to the formation of a polypeptide were traditionally viewed as subsequent steps in a linear pathway. All that has changed, and today we find an increasing amount of evidence for the inter-dependence of several biological events. While genetic studies link together proteins that function in pathways, biochemical data reveal physical evidence of protein-protein interactions. Thus, the formerly 'linear' path from gene to protein has several stages that are interconnected and this allows a finer degree of control at many steps along the route (Orphanides and Reinberg, 2002).

Chromatin is a key player in gene expression, mainly because it functions at the starting point. Once thought of as merely a neat solution to the packaging problem, it is now recognized as much more of a dynamic structure, playing a role in many vital cellular processes. Not all chromatin is created equal, and depending on the placement of eukaryotic genes in euchromatic or heterochromatic regions, they will be transcriptionally active, or not (Richards and Elgin, 2002). Even within the euchromatic regions, based on where the factor-binding sites are situated, the gene may or may not show increased dependence on chromatin-remodeling complexes. An intriguing question is that while activators have been shown to bind prior to recruitment of chromatin-

remodelers (Utley et al., 1998; Ikeda et al., 1999), how do the activators bind to DNA to begin with? The answer lies in two facets of transactivator binding.

First, the sequence that an activator recognizes might be in an exposed region of DNA, i.e. the UASp1 Pho4-binding site, to which the activator can bind since it is non-nucleosomal. Factors like the glucocorticoid receptor that bind only to a short DNA sequence, and only to one side of the double helix (in the major groove) can recognize their sequence even on placement in a nucleosome (reviewed in Urnov and Wolffe, 2001). Other activators like nuclear factor 1(NF1) bind to a longer DNA sequence and completely surround the double helix, thus requiring more assistance with unraveling chromatin (reviewed in Urnov and Wolffe, 2001). Our experiments in Chapter II demonstrate that levels of factor binding influence the requirements for chromatin-remodeling coactivators. Further, changes in activator concentration, activator DNA-binding domain/binding site affinity, and binding cooperativity are all expected to modulate the transcriptional requirements for individual chromatin modifiers and remodelers at various promoters. An interesting thought is that promoters of genes that need to be highly transcribed potentially have several strong activator binding sites, thus facilitating rapid expression. This is borne out by our evidence in Chapter II that shows strong SWI/SNF and SAGA recruitment to ribosomal and glycolytic pathway promoters (Figure 2-7).

A COMPETITION MODEL TO EXPLAIN FUNCTIONAL REDUNDANCY

A vast array of interactions exists at the *PHO5* promoter (Figure 4-1). First, there is competition for the UASp2 site between the nucleosome and Pho4/Pho2. Nucleosomes

are thought to alternate between a stable conformation and an intermediate, unstable form (Anderson et al., 2002; Li and Widom, 2004). Thus, activator-binding sites are constantly being exposed and protected, and a competition exists between a nucleosome and an activator for specific sites in DNA. Secondly, SWI/SNF and SAGA also collaborate to compete off the nucleosome. Chromatin remodelers appear to be of greater importance when activator-binding sites are nucleosomal. Therefore, at a promoter like *PHO5*, which recruits no less than four different remodeling complexes, activation (or at least the rate of activation) is affected if even one is absent.

One can thus envision the possibility of there being increased recruitment of one or the other complex in such a scenario, in order to achieve full rates of activation. Indeed this seems to be the case in a *gcn5*Δ strain, when at later time points of P_i starvation, increased recruitment of SWI/SNF (Figure 3-3) is observed. At early points, however, when activator binding is low, there seems to be less SWI/SNF recruitment compared to the wild-type cells. On the other hand, there does not seem to be any increased levels of Gcn5 in a *swi2*Δ strain relative to wild-type cells. One possibility is that this is due to less Pho4 binding at the promoter in *swi2*Δ cells. Also, we find that Gcn5 is recruited prior to Swi2 at *PHO5*, suggesting that perhaps Pho4 has greater affinity for SAGA compared to Swi2. This is clearly a possibility, as seen for Gcn4 *in vitro* (Natarajan et al., 1999). We cannot, however, rule out the possibility that there might simply be less SWI/SNF compared to SAGA in the cell. It would be of great interest to determine whether, in the absence of Gcn5, there is a) any change in acetylation at the *PHO5* promoter and b) increased recruitment of NuA4, the H4 HAT. Similarly, in the absence of Swi2, there is

little to no change in Gcn5 recruitment, but perhaps there is instead increased recruitment of other ATP-dependent remodeling complexes like INO80.com.

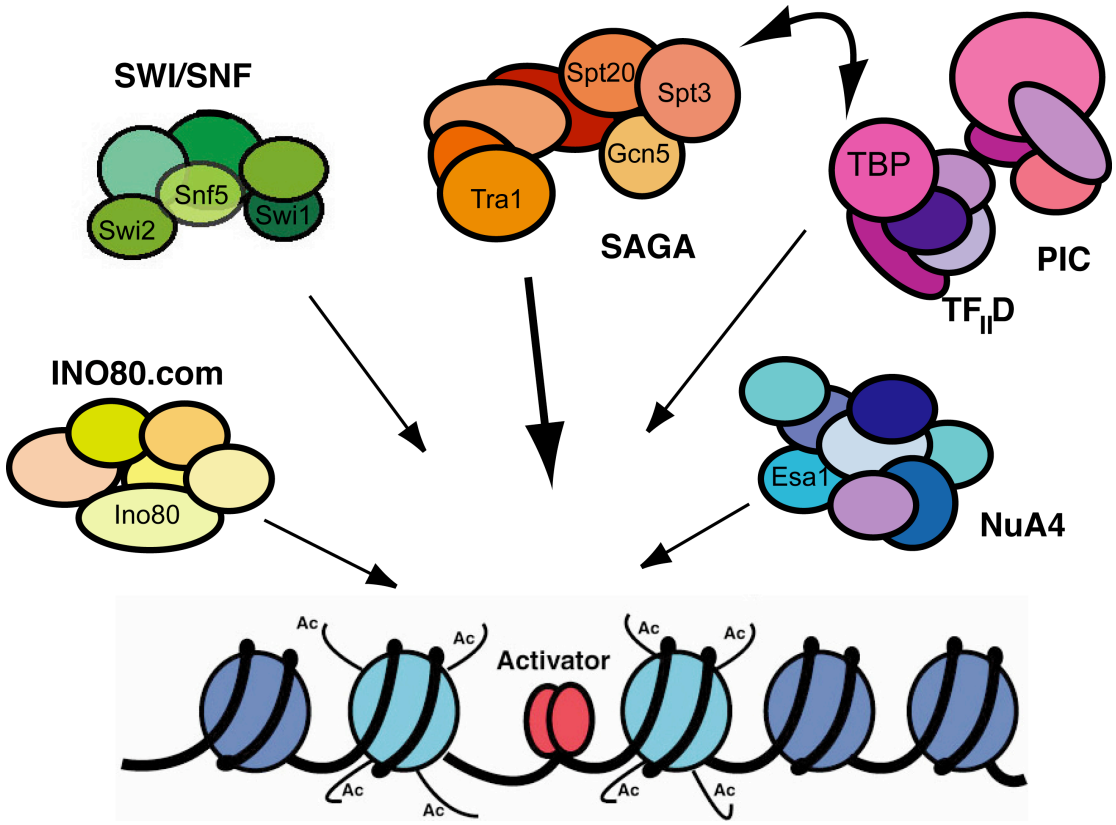

Figure 4-1. Multiple interactions exist at the *PHO5* promoter.

Upon P_i starvation, the principal *PHO5* activator, Pho4, is in competition with the nucleosome to bind at its UAS sites. SAGA and SWI/SNF collaborate to contend with the nucleosome, but also compete for interactions with the activator. Interactions between SAGA and SWI/SNF are unknown as yet. $TF_{II}D$ is recruited through interactions of TBP with Spt3 and other SAGA subunits.

Past work has demonstrated that acetylation of nucleosome templates by SAGA or NuA4 stabilizes SWI/SNF binding *in vitro* to nucleosomes after activator dissociation, and that acetylation is needed for stable SWI/SNF binding (Hassan et al., 2001b).

Further, Gcn5 activity stabilizes SWI/SNF binding to promoters *in vivo* (Syntichaki et al., 2000). The retinoic acid receptor/ retinoid X receptors (RAR/ RXR) are stimulated by ligands to bind to their target sites upstream of many RA-responsive genes. Using a chromatin template-based transcription system *in vitro*, it was demonstrated that effective transactivation by RAR/RXR required acetylation prior to hSWI/SNF *in vitro* (Dilworth et al., 2000). Transcriptional stimulation by the p300 HAT is also synergistic with hSWI/SNF activity. Interestingly, in the absence of histone acetylation hSWI/SNF does not effect ATP-dependent remodeling, unless it is added much before HeLa nuclear extracts, which are known to contain a number of activators (Dilworth et al., 2000). Thus, SWI/SNF can stimulate transcription from chromatin templates without acetylation, presumably it just requires the presence of high levels of activator binding (Neely et al., 1999; Agalioti et al., 2000). In complexes lacking Gcn5, although the SAGA complex is recruited to promoters by the principal activator-interacting subunit Tra1, due to the absence of the Gcn5 bromodomain, the complex probably does not associate with the promoter as well.

At the yeast *HO* gene, SWI/SNF is recruited before SAGA (Cosma et al., 1999). The association of TBP with the promoter induces DNA bending of ~30 to 80 degrees, causes distortion and unwinding of the DNA, which has been correlated with transcriptional activation (Wu et al., 2001). Interestingly, TBP binding is needed for nucleosome remodeling by SWI/SNF on an artificial *PHO5* promoter with a Gcn4 binding site placed between the two positioned nucleosomes (Lomvardas and Thanos, 2002). The converse, i.e. association of increased levels of SAGA containing Gcn5 with the promoter in the absence of SWI/SNF was clearly a possibility: many labs have demonstrated an order of recruitment where SAGA associates with the promoter much

before SWI/SNF (Agalioti et al., 2000). However, the level of Gcn5 recruitment remains the same in the *swi2*Δ strain, implying that the absence of SWI/SNF is compensated for perhaps by the presence of other remodeling complexes, INO80.com and NuA4. Alternatively, there is sufficient Pho4 that allows adequate recruitment of the transcriptional machinery and HMG group proteins like Nhp6a and Nhp6b to allow wild-type levels of activation. Other possibilities such as increased post-transcriptional levels of Pho4 might also play a role in achieving similar levels of *PHO5* expression.

To discern whether Gcn5 and Swi2 are truly functionally redundant, we will utilize partial loss of function mutants of Swi2 in conjunction with a *gcn5*Δ strain to test whether activity of *PHO5* is affected. Also, both Snf1 and Snf5 mutants are not that deleterious to the cell individually, but in combination they exhibit a synthetic phenotype (Prochasson et al., 2003). It will be of interest to see what effect they have on *PHO5* activity in a *gcn5*Δ strain. Another question would be whether this more SWI/SNF is recruited at other promoters in a *gcn5*Δ strain. If so, it would indicate a global phenomenon of redundancy, especially since SWI/SNF and SAGA have many common targets (Pollard and Peterson, 1997). If not, then perhaps it only occurs at promoters like *PHO5*, which has nucleosomal activator-binding sites.

How is *PHO5* transcription effected? In summary, we see that P_i starvation leads to recruitment of Pho4 and Pho2, followed by SAGA and then SWI/SNF. These complexes, and perhaps NuA4 and INO80.com as well, collaborate to prevent the nucleosomes from returning to their 'stable' conformation and thus occluding Pho4 occupancy. This is validated by the observation that there is a delay in Pho4 binding to the promoter in *gcn5*Δ and *swi2*Δ strains. The evidence that there is twice as much

SWI/SNF recruited in a *gcn5*Δ strain, but not in an *spt20*Δ strain can perhaps be explained by the hypothesis that SAGA competes with SWI/SNF for binding to the activator surface. However, in the *gcn5*Δ strain, the rest of the SAGA complex still collaborates with SWI/SNF in competing with the nucleosome. A simpler explanation stems from the observation that there is less rAPase activity in an *spt20*Δ strain. This is suggestive of less Pho4 promoter occupancy, which could explain the decreased amounts of SWI/SNF recruitment.

TBP recruitment and assembly of the basal transcriptional machinery has been shown to be sufficient for chromatin remodeling and transcriptional activation of *PHO5,* and chromatin remodeling was observed even in a strain deleted for *SWI2* (Gaudreau et al., 1997). TBP recruitment is potentially diminished in an *spt20*Δ strain since Spt3 and Spt8 are no longer part of the complex, which would explain the decreased *PHO5* expression in these strains (Wu et al., 2004). The other alternative is that there are interactions between SWI/SNF and some other subunit in the SAGA complex that enables better association with the promoter. However, prior work has suggested that SWI/SNF is recruited independently by activators, and does not associate with at least the SRB/mediator complex *in vitro* (Natarajan et al., 1999). Future experiments will be needed to provide more insight into the interplay of all these various complexes in expression of *PHO5*.

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