DISSERTATION

IN VIVO AND *IN VITRO* STUDIES OF THE ROLE OF CHROMATIN IN *PHO5* REGULATION

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SRIWAN WONGWISANSRI ENTITLED *IN VIVO* AND *IN VITRO* STUDIES OF THE ROLE OF CHROMATIN IN *PHO5* REGULATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IN VIVO AND *IN VITRO* STUDIES OF THE ROLE OF CHROMATIN IN *PHO5* REGULATION

The *Saccharomyces cerevisiae PHO5* gene has served as an excellent model to study transcription in the chromatin environment. Transcription of *PHO5* is regulated by phosphate availability through chromatin dynamics. Under activating conditions, positioned nucleosomes on the *PHO5* promoter are remodeled by the coordinated function of transcriptional activators Pho2p and Pho4p. This dissertation describes the use of yeast chromatin assembly system to investigate the mechanism of *PHO5* regulation. Several transcription-related activities were reconstituted on the chromatin template, including ATP-dependent chromatin remodeling and histone acetylation, which have been demonstrated to be required for activation of numerous genes. Transcriptional activators Pho2p and Pho4p by themselves were capable of binding their recognition sequences in the chromatin-assembled DNA. Chromatin reconfiguration on the *PHO5* promoter required Pho2p, Pho4p and the ATP-dependent activity in the nuclear extract. However, chromatin remodeling activity alone was not sufficient to bring about *PHO5* transcriptional activation. We showed that the missing activity was histone acetyltransferase. Transcriptional activator binding to chromatin, ATP-dependent

chromatin remodeling and histone acetylation were separable events but all were required to activate *in vitro* transcription of *PHO5*.

In vivo study on the role(s) of histone deacetylase Rpd3p in *PHO5* regulation suggested that Rpd3p functions on the *PHO5* promoter through controlling acetylation level of the histone tails. Rpd3p also has an indirect role in *PHO5* regulation through its effect on phosphate uptake. I have demonstrated that Rpd3p regulates the turn over rate of the phosphate transporter Pho84p, which functions upstream of *PHO5* in the *PHO* pathway. *RPD3* deletion resulted in early recycling of Pho84p, resulting in a phosphate uptake defect and accelerated activation of genes involved in phosphate accumulation.

Both *in vitro* and *in vivo* studies described in this dissertation have contributed significantly to the field of *PHO5* regulation. The yeast chromatin reconstitution system has proved to be a very useful tool to study mechanism of transcription regulation. Chromatin reconstitution on bead-bound DNA (described in chapter 5) should provide an additional tool to investigate further details in the mechanism of transcription regulation in a chromatin context.

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Chapter 1

Background and Hypotheses

Chromatin is the universal substrate for all activities in eukaryotic cells that require access to the DNA sequence. In addition to providing an effective way to organize the genome, chromatin structure also provides access in a timely fashion for DNA binding proteins during DNA transactions. Rearrangement of chromatin structure to allow accessibility has proved to be coordinately performed by a network of several proteins. The conserved nature of these proteins during evolution indicates their vital function. Several families of related proteins have been discovered and their functions are beginning to be understood.

Transcription occurs in the context of chromatin, a highly organized protein-DNA complex. This study focuses on clarifying the mechanisms underlying transcriptional regulation in the chromatin context using the *PHO5* gene from budding yeast, *Saccharomyces cerevisiae* as a model. The first chapter is dedicated to providing a background on chromatin, chromatin structure, reconstitution, and remodeling activities as well as the *PHO5* regulatory pathway and phosphate metabolism. A background on gene transcription in the eucaryotic cell is also included. Chapter two describes the yeast *in vitro* chromatin reconstitution system developed in the Laybourn laboratory, which will be published in *Methods in Enzymology*. There are two main projects described in

this dissertation. One is the reconstitution of several aspects of transcription-related activities *in vitro*, described in chapter three. This study successfully showed the following: reconstitution of chromatin, chromatin remodeling, histone acetylation and transcriptional activation on the *PHO5* promoter. The second project is designed to elucidate how chromatin modification participates in *PHO5* transcription. This study, described in chapter four, demonstrated that histone deacetylase (HDAC) Rpd3p functions in *PHO5* regulation both directly on the *PHO5* promoter and indirectly in an upstream event in the regulatory cascade. Chapter five is a summary of what we know about *PHO5* currently and what should be performed to unravel the mechanism of *PHO5* expression in further detail. The development of a solid phase chromatin reconstitution system is also included in this chapter.

1.1 Nucleosome and Chromatin Structure

Eucaryotic genomes are organized in a DNA-protein complex known as chromatin. The fundamental repeating unit is the nucleosome. Nucleosomes contain 147 DNA base pairs and a core histone octamer, which is composed of two of each core histone proteins (H2A, H2B H3 and H4) (100, 173). The histones are among the most highly conserved proteins. In addition to the four core histones, chromatin from higher eucaryotes also contains linker histone H1 (or histone H5), which binds to the nucleosomes and help stabilize more compact chromatin structures (42, 153). However, in yeast cells, the presence of a linker histone is still uncertain. Therefore, only the function of the four core histones will be included in this dissertation. The structure of each histone protein can be divided into two parts, a structurally well-defined region

containing histone fold motifs and the more diverse unstructured extensions (histone tails). The structured portions are mostly involved in formation of the nucleosome through histone-histone and histone-DNA interactions (101). The unstructured tails are primarily implicated in regulating higher order structures through interactions between nucleosomes. The tails also provide the site for posttranslational modification, which is important for regulating gene activity (102). The histone tails, which protrude from the nucleosome core particle, make contact with DNA, neighbor nucleosomes, and non histone proteins to form higher order structures. Long arrays of nucleosomes or "beads on string" fold into the 30 nm fiber and beyond to form what is observed as the chromatin filament during cell cycle interphase. Only the first two levels of chromatin structure, nucleosomes and the 30 nm fibers, have been significantly characterized. Chromatin structure plays an important role in several biological processes, such as replication, transcription, repair, *etc*. Of special interested in this study is the impact of chromatin structure on transcription.

1.2 The *PHO5* Gene and Phosphate Responsive Signal Transduction Pathway (The *PHO* Pathway)

The yeast, *Saccharomyces cerevisiae*, *PHO5* gene has served as an excellent model to study transcriptional regulation in a chromatin context based on the detailed understanding of the *in vivo* chromatin structure and dynamics during the transition between repressing and activating conditions (57). The structure and dynamics of chromatin on the *PHO5* promoter will be discussed in more detail in the next section. The *PHO5* gene encodes the major secreted acid phosphatase (Apase) that functions in

acquisition of phosphate by hydrolyzing the phosphoester bonds of organic phosphate compounds in the environment. Several genes controlling *PHO5* expression through the *PHO* regulatory cascade have been identified by genetic analyses (Figure and table 1.1).

1.2.1 Transcriptional Activators, Pho2p and Pho4p

Pho2p encodes a homeodomain DNA-binding protein required for activation of *PHO5* and several other genes (19, 181). Pho2p appears to interact with Pho4p and cooperatively bind the *PHO5* promoter and activate *PHO5* transcription (7, 8, 61, 140). In addition, Pho2p facilitates Pho4p binding on the *PHO5* promoter by unmasking the activation domain and DNA binding domain (140). Transcription of *PHO2* is phosphate independent but the interaction of Pho2p and Pho4p is regulated by phosphate availability (61). Recently, it has been shown that Pho2p phosphorylation is required to achieve full activation of *PHO5* (95). The potential phosphorylation site is not in the region that is required for Pho4p-interaction suggesting that phosphorylation of Pho2p may strengthen the formation of Pho4p-Pho2p complex on to the DNA, rather than the interaction with Pho4p directly. In addition, the phosphorylated site is in the domain required for Pho2p interaction with its partners in the activation of the *HO* gene and adenine biosynthesis genes suggesting that phosphorylated Pho2p probably is also required for activation of those genes (17). Recombinant cdc2/CDC28 kinase has been shown to interact with Pho2p *in vitro* through yeast two hybrid analyses and to be capable of phosphorylating recombinant Pho2p *in vitro* (95).

PHO4 encodes a basic-helix-loop-helix DNA-binding protein. Pho4p is the ultimate regulator of *PHO5* activation. In addition to *PHO5*, Pho4p also activates

transcription of several other *PHO* genes in the *PHO* regulatory cascade (122). Cyclin-CDK pair, Pho80p-Pho85p, regulates Pho4p activity. It has been shown that Pho4p phosphorylation at each of the five different serine residues provides different regulatory functions for Pho4p activity (68, 78). When phosphate is plentiful (repressing conditions), the Pho80p-Pho85p complex phorphorylates Pho4p resulting in cytoplasmic localization thus preventing *PHO5* activation. Msn5p, an importin-β-family receptor (in the presence of GTP-bound Ran), binds only to phosphorylated Pho4p and exports it to cytoplasm (70). During activation, Pho4p is imported into the nucleus by Pse1/Kap121, a member in importin-β-family receptor. Phosphorylation of Pho4p prevents its binding to Pse1 causing its detention in the cytoplasm (71). In summary, phosphorylation of Pho4p inhibits *PHO5* transcription by preventing its interaction with the transcription activator Pho2p and the nuclear import receptor Pse1p and promoting its binding to the nuclear export receptor Msn5p, thus localizing Pho4p to the cytoplasm.

1.2.2 Cyclin-CDK Complex Pho80p-Pho85p and CDK Inhibitor (CKI) Pho81p

The cyclin-CDK pair, Pho80p-Pho85p, regulates Pho4p activation of *PHO5* as described in the previous section. Pho80p was shown to have homology to yeast cyclin and Pho85p was found to be a protein kinase related to $p34^{cdc2/CDC28}$ (154, 161). In addition to Pho80p, Pho85 also partners with Pho80p-related cyclin, Pcl7. Functions of both cyclin/CDK complexes are inhibited in response to phosphate depletion (92). Furthermore, Pcl7 also interacts with phosphate regulated CDK inihibitor (CKI), Pho81p. Deletion of *PCL7*, however, has no effect on *PHO5* regulation (92). Pho81p directly interacts with Pho80p-Pho85p in both high and low Pi conditions but only inhibits kinase

activity when cells are depleted for phosphate (138). Pho81p contains six tandem repeats of the ankyrin motif homologous to the INK family of mammalian CKI. It has been proposed that Pho81p might inhibit kinase activity by its interaction with Pho80p through this ankyrin motif (123). However, a recent study showed that Pho81p inhibits kinase activity through a novel minimal domain, which is C-terminal to the ankyrin repeats (64). Pho81p was demonstrated to interact with Pho80p and the Pho80-Pho85 complex, but not Pho85p alone (138). Pho81 forms a stable complex with Pho80-Pho85 under high and low phosphate conditions but inhibits kinase activity only when phosphate level is low. This finding suggests that the function of Pho81-Pho80-Pho85 is modulated posttranslationally (138).

1.2.3 Phosphate Transporters, and Related Gene Products that Affect Transport Activity

The search for phosphate sensors by genetic analysis has revealed two novel phosphate transporters, Pho90p and Pho91p, in addition to the three previously identified transporters Pho84p (23), Pho87p (24) and Pho89p (104). Pho84p is a H^+-coupled high affinity phosphate transporter and the most studied among this group. Pho84p functions optimally in moderately acidic condition (pH 5.0) (150). Pho89p has been identified to be a Na^+-coupled high affinity phosphate transporter with an optimum pH of 9.0 (104). Even though Pho89p is considered to be a high affinity transporter (Km =3 μM), it functions poorly in the acidic environment of standard culture conditions (126). Kinetic experiments revealed Pho87p, Pho90p and Pho91p to be the low affinity phosphate transporters. Even though Pho84p plays a major role in phosphate transport, it has no

direct function in phosphate signaling (177). Deletion of *PHO86* has been shown to result in constitutive *PHO5* expression. Pho86p has been genetically identified to be involved in phosphate uptake (24). Recently, Pho86p was shown to function as an endoplasmic reticulum resident protein required for proper localization of Pho84p to the plasma membrane (90). Transcription of *PHO81*, *PHO84* and *PHO86* is regulated by Pho4p-activation.

In addition to the *PHO* gene products described above, there are other genes that differentially affect *PHO5* regulation. In the search for genes involved in the Pho81-dependent phosphate signaling pathway, five genes were found, *PHO84*, *PHO86*, *PHO23*, *PMA1*and *ACC1* (91). All these genes function upstream of Pho81p in the *PHO* pathway. *PHO23* encodes a subunit for histone deacetylase complex, Rpd3p (98). Rpd3p has been shown to function directly on the *PHO5* promoter by regulating the histone acetylation state (168). Genetic experiments showed that *PHO81* is epistatic to *PHO23*, suggesting that Pho23p, either by itself or in the Rpd3p complex, functions in *PHO5* regulation upstream of Pho81p. *ACC1* is involved in fatty acid biosynthesis. Its function in the *PHO* pathway is still unclear. *PMA1* encodes a plasma membrane ATPase that functions in maintaining the proton gradient across plasma membrane. *PMA1* disruption impairs proton pumping and causes a defect in phosphate uptake under standard conditions. Increasing the proton concentration in the environment (lower pH) rescues the uptake defect in *pma1* mutant cells. This finding suggests that an intact proton gradient is critical for phosphate uptake, which in turn, affects regulation of the *PHO* gene family.

1.3 Phosphate Metabolism in Budding Yeast *Saccharomyces cerevisiae.*

Phosphate is an essential nutrient required for numerous cell activities, including biosynthesis of nucleic acid, cellular metabolites, and energy transduction. Several gene products in the *PHO* family are involved in phosphate metabolism including phosphate transporters (Pho84p), ER resident protein that facilitates membrane localization of Pho84p (Pho86p), phosphatases (Pho5p, Pho8p, Pho10p and Pho12p) and transcription regulatory proteins (Pho2p, Pho4p).

Phosphatase enzymes in budding yeast consist of acid and alkaline phosphatases, classified according to their optimum pH. Acid phosphatases, mostly expressed from *PHO5* and slightly from *PHO10* and *PHO12*, are responsible for scavenging phosphate from extracellular organic compounds in the acidic conditions. Expression of all three genes is phosphate repressible. The other acid phosphatase expressed by *PHO3* appears to be regulated by a different pathway. An alkaline phosphatase, encoded by *PHO8*, functions inside the cell vacuole and cytoplasm to release phosphate from internal phosphate compounds.

In *Saccharomyces cerevisiae*, there are at least five genes, Pho84p, Pho87p, Pho89p, Pho90p and Pho91p, that have been genetically identified to be involved in phosphate transport into the cell (177). Of all the identified transporters; Pho84p plays the major role in phosphate uptake. Those transporters can be categorized into low and high affinity phosphate transport systems. The low affinity transport system, with an estimated Km of 1 mM for phosphate appears to be expressed constitutively (119, 150). The high affinity transport system with a Km of approximately 1-15 μm is induced by Pi depletion. The extracellular phosphate concentration is signaled through *PHO* regulatory

genes but the underlying mechanism is still unclear. Pho84p function is dramatically influenced by the proton gradient across the plasma membrane. Phosphate transport activity is very low under high phosphate condition and independent of growth phase. In contrast, phosphate transport activity does change with cell growth phase under conditions of phosphate limitation (105). The phosphate uptake rate increased during exponential growth. However, when the extracellular phosphate concentration is reduced to near the Km of high affinity transporter, the rate of phosphate uptake decreases. This decrease in activity results from both a reduction of membrane assembled Pho84p and *PHO84* transcripts. The expression profile of Pho84p tightly correlates with a narrow range of extracellular phosphate concentration (10 ~ 100 μm) (105). The intracellular phosphate concentration in cells grown in low Pi conditions does not change significantly even at high growth rates indicating that the extracellular phosphate controls *PHO84* gene expression. However, the polyphosphate pool, another source for intracellular phosphate, is dramatically decreased after prolonged growth in low Pi. This finding suggests that the polyphosphate pool serves in phosphate storage and is used by cells meet their needs when phosphate is limiting. Phosphate and carbon sources act in concert to regulate the phosphate transport system as shown by glucose exhaustion resulting in transporter inactivation (105). In the search for the mechanism underlying phosphate sensing, Wykoff and O'Shea suggest that Pho84p is unlikely to be required for phosphate sensing and that the phosphate sensor should be inside cell since overexpression of another phosphate transporter can suppress constitutive expression of *PHO5* in a *PHO84* disrupted strain (177). Phosphate metabolism also involves intracellular biosynthesis of polyphosphate, a polymer of up to hundreds of phosphate

residues linked by high-energy phosphoanhydride bonds. A whole genome expression analysis searching for genes involved in Pi acquisition and storage have identified several novel genes that function in biosynthesis of polyphosphate, e.g., *PHM1* to *PHM5* (122). Deletion of *PHM1* to *PHM4* resulted in the loss of detectable polyphosphate, suggesting a role in polyphosphate biosynthesis. *PHM5* disruption led to longer chains of polyphosphate indicating that Phm5p is a polyphosphatase. At least one or two Pho4p binding sequences are found on the promoters of these novel *PHO*-regulated genes. Transcriptional regulation of *PHM1* to *PHM4* is also under control of the *PHO* pathway (122).

1.4 Reconstitution of Chromatin *In Vitro*: Yeast vs. *Drosophila* Systems

In vitro chromatin reconstitution systems have been established in the last two decades to provide biochemically-defined tools for studying the mechanisms underlying the numerous DNA activities in the chromatin environment, especially transcription regulation. The first *in vitro* chromatin assembly system was established using *Xenopus laevis* oocyte extract (48). With this system, a key observation was made that chromatin assembly requires ATP hydrolysis to provide regular nucleosome spacing. Subsequently, an *in vitro* assembly system was also developed from Hela cells (6) and *Drosophila* embryo extracts (12, 72). These systems also contain ATP-dependent nucleosome assembly activity. Additionally, nucleosome assembly can be mediated from a variety of salts (salt dialysis) or polyanions such as polyglutamate and RNA. The resulting chromatin prepared from salts or polyanions, however, yield irregularly-spaced rather than evenly-spaced nucleosomal arrays when assembled onto non-repetitive DNA.

Unlike salt–mediated chromatin assembly, reconstitutions with crude extracts yield regularly spaced chromatin, thus providing an efficient way to produce a good quality chromatin for subsequent study.

Drosophila embryo extract was fractionated and the assembly factors were purified and characterized (22, 66). One purified factor, called ACF (ATP-utilizing chromatin assembly and remodeling factor), consists of two subunits, Acf1 and ISWI. ISWI contains ATPase activity and is also a component of the other *Drosophila* chromatin remodeling complexes, NURF and CHRAC. ACF also exhibits nucleosome sliding and transcriptional activation activities on chromatin templates (66). More discussion of ACF on chromatin remodeling activity and the other complexes containing ISWI is in the following section.

Biochemical experiments have led to the identification of several histone binding proteins, which include nucleoplasmin, N1/N2, chromatin assembly factor-1 (CAF-1), nucleosome assembly protein-1 (NAP-1) and Spt6. A number of studies suggest that nucleoplasmin and NAP-1 preferentially bind histone H2A and H2B, while CAF-1, N1/N2 and Spt6 preferentially interact with H3 and H4. These proteins act as chaperones to mediate core histone transfer to DNA during nucleosome assembly.

Purified recombinant assembly proteins from *Drosophila* are available. The recombinant proteins efficiently assemble DNA into nucleosomal arrays as shown by micrococcal nuclease digestion. The efficiency of assembly is relatively high as shown by one dimensional (supercoiling assay) and two dimensional topological assays. A detailed description of micrococcal nuclease digestion, supercoiling and two dimensional topological assays can be found in Chapter two.

A yeast *Saccharomyces cerevisiae* chromatin assembly system has also been established in the Laybourn lab. Given that yeast is an excellent organism for molecular genetic and biochemical studies a yeast chromatin reconstitution system was seen to be very useful. The yeast chromatin assembly system developed in the Laybourn lab is ATP-independent. The yeast system is composed of yeast NAP1p and core histones. Details of the yeast chromatin reconstitution system are described in Chapter two.

1.5 Chromatin Remodeling Activities

The last decade of study on the mechanism of transcription regulation in the chromatin environment has led to the discovery of a number of regulatory complexes that are thought to function in opening chromatin structure. Reconfiguration of chromatin structure has proved to be an independent process as the chromatin mobilization is observed in the absence of transcription (60, 152) and replication (137). Even though chromatin remodeling activity is often linked with transcriptional activation, increasing evidence points to a role in gene repression, as well (39, 160). Additionally, chromatin mobilization has been observed to function not only in transcription but also in DNA replication (3, 43), recombination (87, 135) and repair (162).

The chromatin remodeling complexes have been categorized into two classes according to how they bring about changes in chromatin structure; ATP-dependent chromatin remodeling complexes and histone modifying enzymes. The first class appears to alter chromatin structure by utilizing energy from ATP hydrolysis while the latter class covalently modifies core histones, mostly on the N-terminal tails. Studies to date have shed light on several aspects of these remodeling activities including their mechanism of

action and the interdependence of their activities in making DNA more accessible to transcription activators and machinery.

1.5.1 ATP-Utilizing Complexes

ATP-dependent nucleosome remodeling complexes have been categorized into four classes according to the subunit containing ATPase activity. These are Swi2/Snf2-related factors, ISWI/SNF2L containing complexes, CHD1/Xenopus Mi-2 ATPases, and the Ino80 complex (Table 1.2). All these remodeling factors, except the Ino80 complex, have been extensively investigated. The individual ATPase subunit from each group confers almost full function as a chromatin remodeler in the absence of the other subunits. However, the interacting subunits can modulate function of the ATPase subunit both quantitatively and qualitatively. These other subunits function in regulation, targeting the complex to a specific promoter and assembly into functional complexes.

1.5.1.1 Swi/Snf and Related Complexes

The yeast SWI/SNF complex was the first identified and is the best characterized complex in this group. *Swi* mutations cause a defect in *HO* gene expression. The *HO* gene is required for mating type switching, hence the name Swi. Snf mutations were identified from *SUC2* expression defects, rendering cells to be sucrose non-fermentators, thus the name Snf. Subsequently, both Swi and Snf genes were found to be the same. The other yeast Swi/Snf-related complex is RSC. RSC contains several subunits that are homologous to Swi/Snf subunits, including Sth1 ATPase. RSC, however, is found to be more abundant in cells than Swi/Snf and several subunits are essential for viability, while

all the Swi/Snf subunits are dispensable. Homologues of Swi2/Snf2 ATPase are found in *Drosophila* (Brahma ATPase) and human (BRG1 and BRM ATPase). The ATPase subunits from each complex share high sequence homology in the ATPase domain and contain at least one "bromodomain" motif. This domain was found to bind acetylated lysine residues in the histone proteins, thus mediating interaction of ATP-utilizing complex and nucleosomes.

Genetic screens have identified several other subunits in the Swi/Snf complex and also provided a connection between chromatin and Swi/Snf function (84, 132, 157). Further study with the biochemically purified complex corroborated the Swi/Snf function in chromatin remodeling and revealed its DNA-dependent ATPase activity (25, 29, 127). Most of subunits in the Swi/Snf complex have homologues in the other organism. Interestingly, one group of subunits, called Arps (actin–related proteins), is found in all identified Swi/Snf homologue including yeast RSC, *Drosophila* Bhrama and human Swi/Snf. The Arp-like proteins may provide a link between remodeling and actin binding in nuclear structure (167).

Even though chromatin remodeling activity has been associated with gene activation, the result from genome wide analysis of Swi/Snf function exhibited Swi/Snf requirement in both gene activation and repression. Only a small number of genes require Swi/Snf for transcription activation (147). During the M phase of the cell cycle, the expression of several genes has been shown to require Swi/Snf activity while interphase transcription is Swi/Snf independent. This finding suggests that chromatin condensation renders gene activation Swi/Snf-dependent (80, 113). A few studies have shown that the transcription repressors or activators also determined the requirement for

Swi/Snf. Transcription of H2A and H2B is repressed by Hir corepressor and activated by Swi/Snf. Deletion of *HIR* partially derepressed transcription of H2Aand H2B, further activation is then Swi/Snf-independent (34). On the contrary, activation of *HIS3* by Gcn4p is Swi/Snf-independent. A mutation that weakens Gcn4 activity makes *HIS3* activation Swi/Snf-dependent (112). The number of newly identified remodeling complexes suggests a functional redundancy between chromatin remodeling factors.

The mechanism of counteraction of nucleosome repression by Swi/Snf complex has been described as "sliding", which alters nucleosome position in *cis* (174). The other chromatin remodeling mechanism has been described as in trans, which involves an octamer transfer to acceptor DNA (99). All ATPase subunits and some other subunits in the Sw/Snf family contain a protein motif called the 'bromodomain', which binds to acetylated lysines in the N-termini of histones. Bromodomain binding is non-specific. Recruitment of the Swi/Snf complex to a promoter appears to be through interaction with sequence-specific DNA binding proteins (114, 115, 180). Binding through the bromodomain is likely required for persistent interaction of ATP-dependent chromatin remodeling factor with nucleosomes after initial recruitment by gene-specific transcription activators (58).

1.5.1.2 ISWI ATPase/SNF2L Complexes

The ISWI ATPase family members are also members of the SNF2-ATPase superfamily, a large conserved group of ATPase/helicases. The founding member in this group is *Drosophila* ISWI, which was identified and cloned based on its homology to Brahma ATPase, the Swi/Snf homologue in *Drosophila* (37). Despite a certain degree

sequence homology, ISWI and Swi/Snf ATPase can not replace each other functionally (37). Three chromatin assembly and remodeling complexes from *Drosophila* containing ISWI have been identified; ACF (ATP-utilizing chromatin assembly and remodeling factor), NURF (nucleosome remodeling factor) and CHRAC (chromatin accessibility complex). Originally, ACF was identified from a search for chromatin assembly factors and was found later to function in chromatin remodeling-dependent transcriptional activation. ACF functions in facilitating the regular spacing of nucleosomes and in transcription activator-mediated nucleosomal array disruption (66). Analysis of the mechanism of ACF-mediated chromatin assembly suggests that ACF tracks along the DNA like a DNA helicase and assembles nucleosomes processively (45). CHRAC also facilitates formation of ordered nucleosomal arrays and increases accessibility to restriction enzymes (165). Unlike ACF and CHRAC, which function in nucleosome assembly, NURF catalyzes nucleosome disruption. NURF facilitates transcription factor-mediated disruption of ordered nucleosomal arrays, increasing access to binding sites for transcription factors leading to transcription initiation (108, 109).

ISWI homologues in yeast, yIsw1 and yIsw2, were identified based on sequence homology to *Drosophila* ISWI (158). Purified yIsw1 and yIsw2 complexes contain nucleosome-stimulated ATPase activity and nucleosome spacing *in vitro*. yIsw1 also possesses transcription factor-stimulated nucleosome disruption activity. *In vivo* experiments have shown that the Isw2 complex is required for transcription repression of early meiotic genes during mitotic growth. This Isw2 function is Ume6p-dependent and results in chromatin inaccessibility near the Ume6p binding site (49). Genome wide analysis has revealed parallel functions for the Isw2 and Sin3/Rpd3 histone deacetylase

complexes (39). Using immobilized chromatin templates, the interaction between the yIsw2 complex and DNA or nucleosomal arrays was demonstrated. The association occurred in an ATP-independent manner suggesting that ATP is required in the step after Isw2 complex binding (47).

Several ISWI containing complexes are also found in *Xenopus laevis* (xACF/CHRAC) and human (hSNF2L and hSNF2H) (53, 111). hSNF2H was found in two complexes, RSF (remodeling and spacing factor) and hACF, a human homologue of *Drosophila* ACF that also contains chromatin remodeling and assembly activities. The ISWI ATPases do not contain a bromodomain, but rather contain two SANT (SWI3, ADA2, N-CoR and TFIIIB B") domains. The SANT domain is also present in other chromatin-related proteins, including yeast Swi3p, Rsc8p, *Drosophila* ISWI, yeast and human and yeast Ada2 (HAT complex). The SANT domain is essential for the function of Swi3p, Ada2p and Rsc8p and has been shown to function in histone N-terminal tail interaction (18). ATPase activity from ISWI is preferentially stimulated by nucleosomes while Swi/Snf ATPase activity is stimulated equally well by either DNA or nucleosomes. The mode of action in nucleosome mobilization by ISWI containing activities is likely to be less drastic than what was observed from Swi/Snf complexes, especially RSC, since "octamer transfer" is not seen by any ISWI complexes. Rather, the mechanism of remodeling involves "nucleosome sliding" in *cis* (88).

Nucleosome remodeling by ISWI has been shown to facilitate *in vitro* transcription (94, 107), chromatin assembly and regular nucleosome spacing (66, 94, 165), as well as T-antigen-dependent replication (3). The histone H4 N-terminal tail has been shown to be required for stimulation of ISWI ATPase activity and CHRAC-induced

chromatin remodeling (27). Chromatin remodeling by human Swi/Snf can occur in the tailless nucleosome, although with lower efficiency (55). Like Swi/Snf, ISWI containing complexes have been shown to function in both transcription activation and repression (33, 49, 73). Interestingly, recent findings suggest a redundant role for Isw1p and Isw2p with Chd1p, a Mi-2 ATPase homologue, in transcriptional termination (2).

1.5.1.3 CHD Family or Mi-2 Group

The CHD/Mi-2 family members contain both chromatin remodeling and histone deacetylase activities. The best-known protein in this group is human NURD (nuclesome remodeling and deacetylase). The NURD complex contains the CHD3/4 ATPase, whose signature motif is a chromodomain. Subunits include HDAC 1 and 2 (histone deacetylase 1 and 2), often associated with gene repression (54, 76). An Mi-2/CHD–related complex identified in *Xenopus* egg extract also displays both chromatin remodeling and deacetylase activities (170). Like ISWI, Mi-2 ATPase activity is preferentially stimulated by nucleosomes. Both human NURD and *Xenopus* Mi-2 complexes have been found to contain MBD3 (182) and MBD3-like (169) subunits, respectively. MBD3 is a methyl-CpG binding protein. Therefore, the presence of MBD and MBD-like subunits in human NURD and *Xenopus* Mi-2 complexes, respectively, suggests that they might be responsible for directing the complex to methylated DNA domains. Mi-2/CHD ATPase remodeling activity then allows access for the HDAC to deacetylate local histones, leading to more compacted chromatin and transcriptional repression.

The *CHD* family member identified in yeast is *CHD1*, which is highly conserved from yeast to human (146, 176). *In vivo* experiments showed that mutations of *CHD1*, *ISW1* and *ISW2* exhibit a synthetic lethal phenotype in cells under environmental stresses. Synthetic lethality was also observed between *SWI/SNF* and *CHD1* mutations (156). These findings suggest genetic interaction among these proteins. Recently, Chd1p was also shown to be involved with transcription elongation (82, 83, 143).

1.5.1.4 Ino80p Complex

This novel complex was identified in budding yeast by the ability of an *INO80* mutation to confer transcriptional defects in several genes (36). Mutation of *INO80* also resulted in increased sensitivity to DNA damage, suggesting a role for this complex in DNA repair (141). The biochemically purified Ino80p complex has been shown to contain Rvb1 and Rvb2 helicases, not found in any other ATP-dependent chromatin remodeling complex (141). Rvb1 and 2 were demonstrated to bind TBP both *in vivo* and *in vitro*. A possible role of Rvb2 in TBP recruitment to target promoters has been suggested (124). Recently, two labs have obtained more details of how Ino80p might be regulated and recruited to a target promoter *in vivo*. (Discussed further in section **1.8 Chromatin and *PHO5* Transcription.**)

1.5.2 Histone Modifying Complexes (covalent modifications)

Posttranslational modification of nucleosomal histones has been linked to gene regulation. Several transcription-related histone modifications have been identified in organisms from yeast to human. These include acetylation, phosphorylation, methylation,

and ubiquitination (Table 1.3). These modifications modulate the function of transcription-related proteins, as well.

1.5.2.1 Acetylation vs. Deacteylation (HAT vs. HDAC)

Even though transcription is not always correlated with increasing histone acetylation (31), most genes still required HAT activity for gene expression. Gene-specific HAT and HDAC functions occur through recruitment by sequence specific DNA-binding proteins. Their functions can also be non-specific, providing global histone acetylation status to certain domains in the genome. Recently, a protein complex containing both HAT and HDAC activities has been identified. Its role is probably irrelevant to gene-specific recruitment but it might be involved in maintaining the balance of the two enzyme activities and regulating chromatin compaction more globally (178). Supporting this idea, global regulation of acetylation *in vivo* has been observed (168).

Several mechanisms have been proposed for how histone acetylation might contribute to transcription activation. Histone tails containing multiple lysine residues provide multiple positive charges that neutralize the negatively charged DNA backbone. Charge neutralization leads to more compaction and stability of the chromatin fiber. Acetylation, however, reduces the positive charge, which probably loosens the chromatin compaction. This facilitates the access of DNA binding proteins for their recognition site (5, 139). Another contribution is suggested by the identification and characterization of a bromodomain in several chromatin remodeling activities (Gcn5p from SAGA, Swi2p from Swi/Snf and Sth1p from RSC) and a transcription factor (TAF_{II}250 from TFIID). A bromodomain binds to acetylated lysine residues in nucleosome tails in a sequence-

independent manner. Histone acetylation mediated by yeast HATs recruits and prolongs binding of chromatin remodeling activity to the target promoter (52, 58, 59). Even though interaction through a bromodomain is non-specific, targeted recruitment of remodeling factors containing bromodomains can be modulated by gene specific transcriptional activators. The third possibility involves a "histone code", the pattern of combinatorial modification on histone tails that might be specifically recognized by variable transcription–related factors (159).

The reversible acetylation of lysine residues at the N-termini of core histones has been closely linked to transcriptional activation. Histone acetylation presumably weakens the interaction between nucleosomes, thus loosening the higher order structure and facilitating the access of the transcription machinery to the DNA (102). In yeast *Saccharomyces cerevisiae*, several histone acetyltransferases (HAT) complexes have been identified. There are two main classes of HATs, type A (nuclear) and type B (cytoplasmic). Transcription-related HATs are generally type A. Type A HATs identified in yeast include ADA, NuA3, NuA4, SAGA (50) and SLIK (SAGA-like)(129). These HATs are composed of multiple subunits, forming a high molecular weight complex with one subunit containing histone acetyltransferase activity. *GCN5* encodes the histone acetyltransferase (HAT) subunit of SAGA, ADA and SLIK complexes. Esa1p and Sas3p are the HAT subunits of NuA4p and NuA3 complexes, respectively. Gcn5p was discovered through its homology to *Tetrahymena* p55 protein, which was found to acetylate histones from an in-gel HAT assay. Gcn5p had been previously identified as a transcription co-activator, further confirming the idea that histone acetylation is associated with transcription activation. The subsequent finding that a

number of transcription activators contain HAT activity (21), together with the discovery of histone deacetylase activity associated with transcriptional repressors (151) have provided strong evidence that the histone acetylation status, mediated by HAT and HDAC complexes, regulates gene activity.

HAT activity has been found in several transcription-related proteins from yeast to human. These include TBP associated factors for RNA polymerase II (TAF_{II}) such as human TAF_{II} 250, yeast ($yTaf_{II}$ 130p/145p), and *Drosophila* ($dTAF_{II}$ 230) (110). Transcription by RNA polymerase III also confers histone acetylation activity from human TFIIIC (63, 85). The elongator complex associated with the C-terminus of the large subunit of RNA polymerase II contains Elp3p HAT, as well.

Unlike ATP-dependent chromatin remodeling activity (for example, Swi/Snf and ISWI complexes), multiple subunits in each HAT complexes contribute to complex activity and sometimes play an even more critical role than the HAT subunit. For instance, several genes have been shown to be Gcn5-independent but SAGA-dependent for expression (9, 93). Numerous genetic and biochemical studies have shown that the function of SAGA in transcription activation involves more than just histone acetylation (50, 62, 145). The additional activities include facilitating TBP recruitment by Spt3 (89) and complex assembly by Spt7and Spt20 (145).

The activity of the HAT complexes is modulated by subunit components, transcription activators (136) and the other histone modification such as phosphorylation (26, 97) and methylation (183).

There are three main classes of histone deacetylases (HDAC) identified in yeast. Rpd3p is the founding member of class I HDACs, initially identified as a transcription

repressor. Rpd3p was found to contain histone deacetylase activity after biochemical characterization of its mammalian homologue HDAC1 (151). Class I HDACs also includes Hos1 and Hos2p. Hos2p was found in a protein complex with Set3p called Set3C, which is an analogue to the mammalian deacetylase complex HDAC3/SMRT (128). Unlike other HDACs, Hos2p, and its associated Set3p, were found to be required for gene expression *in vivo* (171). Class II HDACs includes yeast Hda1p. Class III HDACs include Sir2p and have an interesting activity and regulation pattern.

Yeast Rpd3 was found in a large complex containing Sin3p co-repressor, Pho23p and Sap30p (74, 98). Rpd3p represses transcription in a gene specific manner through recruitment by DNA binding protein Ume6p, resulting in localized deacetylation of nucleosome on the target promoter (69). Histone deacetylation resulted in stabilization of chromatin and less accessibility for DNA binding gene activators and coactivators. Consistent with this, Rpd3-mediated repression is associated with reduced occupancy of Swi/Snf, SAGA and TBP on the target promoters (32). So far, Ume6p is the only known sequence-specific DNA binding protein that recruits Rpd3p to a target promoter. Genome wide analyses of Rpd3p binding showed that Rpd3p recruitment to most loci is Um6p-independent. Additionally, Rpd3p binds globally to non-promoter sequences. This finding suggests the existence of a novel DNA binding protein that recruits Rpd3p to the targeted regions (86). Genome wide association of Rpd3p has been suggested to occur through Isw2p (39). Rpd3p is found to localize to the promoter region of highly inducible genes. This localization suggests that Rpd3p–mediated gene repression required additional factors to accomplish its task. These factors could be the rest of the repressor complex (Sin3, Sap30 and Pho23) and/or posttranslational modification of

Rpd3p protein itself (86). Results from microarray deacetylation maps have shown sites in the genome, mostly URS element and promoter sequence, controlled by the individual HDACs (133). This study also confirms the finding that most Rpd3p recruitment happens through a novel, Ume6p-independent mechanism. Histone acetylation status on most promoters is controlled by either Rpd3p or Hda1p. Some promoters are affected by both HDACs. In addition, Hda1p has been found to function in the subtelomeric region called the HAST domain. Tup1p has been shown to recruit Hda1p to deacetylate histones on *ENA1* promoter. Like Rpd3p, this study showed that most Hda1p recruitment occurs through a novel mechanism, which is Tup1p-independent. Finally, Hos1, 2 and 3 were shown to function on ribosomal DNA and ribosomal protein coding genes (133).

Class III HDAC, Sir2 mediates gene silencing at telomeres, mating type loci and ribosomal RNA genes. There is a correlation between the function of Rpd3p and Sir2p protein near telomeric region. Even though HDACs are generally considered a transcriptional repressor, genome-wide analysis has shown that deletion of Rpd3p resulted in 40 % repression of endogeneous gene within 20 kb of a telomere. This effect from Rpd3p deletion may be an indirect result of histone overexpression since Rpd3p represses histone gene expression. Rpd3p also activates genes in telomeric regions, which are generally repressed by Sir proteins, through deacetylation of lysine 12 of histone H4. Since acetylation of these lysine residues is essential for Sir3p interaction and gene silencing, deacetylation could disrupt Sir3p binding and lead to activation (16).

HDAC1 and 2 are classified as class I HDACs and are the mammalian homologues of Rpd3p. These enzymes are found in two distinct mammalian complexes, NURD and mammalian Sin3. Mammalian Sin3 contains several components that

resemble yeast Rpd3, suggesting a similar function for both complexes. The more interesting complex is NURD, which consists of a diverse group of proteins with different activities. These include Mi-2 ATPase, HDAC 1 and 2, RbAp 46 and 48, MBD3and MTA2. Mi-2 is an ATP-dependent chromatin remodeling activity in the Swi2/Snf2 superfamily and possesses nucleosome stimulated ATPase. Histone deacetylation is associated with decreased gene activity as histone hypoacetylation is often found in the silenced loci of heterochromatin. Interestingly, ATP increased histone deacetylase activity of NURD, suggesting that the remodeling activity of Mi-2 increased access for the deacetylase in the NURD complex (155). The presence of both chromatin remodeling and histone deacetylase activities in the same complex suggests that remodeling activities are involved in gene repression, as well. Consistent with the role of the NURD complex in gene repression, MBD3 methyl CpG binding protein involved with gene silencing is also present in the complex.

1.5.2.2 Phosphorylation

Transcription-related histone phosphorylation has been found on histone H1 and H3 so far. *In vitro* experiments suggested that histone phosphorylation and acetylation occur in an orderly fashion (26, 97). This led to the identification of histone kinase Snf1p in budding yeast (96). Phosphorylation of histone H3 serine 10 by Snf1p is required for subsequent Gcn5p-mediated acetylation at the *INO1* promoter and transcriptional activation (96). Phosphorylation of linker histone H1 affects transcription through neutralization of a "charge patch" facilitating its dissociation from chromatin (35).

Histone phosphorylation may also have a role in DNA repair through modification of histone H2A in budding yeast and histone variant H2A.X in mammals (65).

1.5.2.3 Methylation

Recently, the role of histone methylation in transcription regulation has garnered as much interest as histone acetylation. This is due to identification of several histone methyltransferases (HMT) and their combinatorial function with the other histone modifications (acetylation, phosphorylation) in regulating gene activity.

In higher eucaryotes, lysine K4 and K9 of histone H3 are the most common methylated residues. While methylated K9 H3 is implicated in gene silencing, methylated K4 H3 is asscociated with transcriptionally active genes (183). However, in budding yeast, methylation of H3 K4 is implicated in gene silencing (148). The HMT that is responsible for metylation of H3 K9 has not yet been reported in budding yeast. These different findings probably reflect that the gene silencing process has evolved further in higher eucaryote.

In mammalian cells, methylation of H3 K9 is carried out by SUV39H (131). Heterochromatin protein-1 (HP1), which marks heterochromatic regions in the nucleus, has been found to preferentially bind methylated H3 K9 through its chromodomain (also found in ATP-dependent chromatin remodeling Mi-2 group) (118). Histone H3 K4 methylation is mediated by human Set9, which is homologous to budding yeast protein, Set1p. An interesting aspect of how histone methylation contributes to gene activity was revealed through a study by Nishioka *et al.* (121). Methylation of H3 K4 mediated by Set9 facilitates transcription through its ability to prevent the binding of NURD and

methylating of gene silencing- associated H3 K9, which is implicated in marking genes for silencing. Therefore H3 K4 methylation counteracts gene silencing by preventing recruitment of these activities. This is consistent with the previous studies by Rea *et al.* indicating that phosphorylation of H3 serine 10 and acetylation of H3 K9, associated with upregulation, interfere with SUV39H –mediated H3 K9 methylation (131).

Regulation of methylation of H3 K4 and K79 in budding yeast demonstrated the interplay between modifications of different types on the same histone (*cis* regulation) and on different histones (*trans* regulation). Yeast Set1p mediates H3 K4 methylation and contains a SET domain. Yeast Dot1p is an HMT that has no SET domain and mediates K79 methylation on histone H3 (41, 116, 163). Both methylations require the preceding H2B ubiquitination by Rad6p and the function of both methylations is associated with gene silencing (for further details see the next section).

However, a genome wide study in budding yeast showed H3 K4 methylation correlates well with gene activation. The transcriptionally inactive regions are actually undermethylated (15). A hypothesis that reconciles the conflicting roles of H3 K4 methylation suggests that silent regions are probably marked by low levels of H3 K79 methylation. This low level of H3 K79 methylation probably allows binding of silencing proteins (Sir proteins) in the silent chromosome region while high levels of H3 K79 metylation on transcriptionally active genes blocks binding of Sir proteins (164). Deletion of *DOT1* results in low levels of H3 K79 methylation throughout the genome and probably permits promiscuous binding of Sir proteins genome wide. This, in turn, lead to reduction of Sir protein at silent regions, resulting in their up-regulation.

The relationship between histone acetylation and methylation has been demonstrated through *in vivo* experiments in gene activation of the estrogen receptor pS2 promoter. During estrogen stimulation, histone H3 was acetylated by CBP at K18 followed by K23. Acetylation of both lysines provides a better binding site on chromatin for arginine methyltransferase CARM1, and methylation takes place at R17. These events lead to transcriptional activation on the pS2 promoter, and indicate the interplay between modifications leading to gene activation (30). Histone methylation occurs prior to acetylation, as well. Methylation of histone H4 R3 facilitates p300-mediated acetylation and transcriptional activation by a nuclear hormone receptor (172).

1.5.2.4 Ubiquitination

Ubiquitin is well known for its function in tagging short-lived proteins for degradation by the proteasome system. However, histone ubiquitination is also an important posttranslational modification implicated in the regulation of several genes. It has been shown that histone H2B K123 is ubiquitin conjugated by Rad6p, an E2 ubiquitin-conjugating enzyme (134). This ubiquitination of H2B is an essential prerequisite for histone H3 methylation at K4 and K79 (20, 148). The order of modification is a "one way" regulation in which H2B ubiquitination precedes H3 methylation at both lysine residues. Briggs *et al.*, suggest that H2B ubiquitination function as a master switch of gene silencing (20).

There are a few proposed mechanisms for how combinations of histone modifications regulate gene activity. First, it has been proposed that histone modifications establish ordered recruitment of transcriptional activators and co-

activators. Earlier recruitments modify histones in such a way that benefits the subsequent recruitment of DNA binding proteins. Second, histone modification establishes transcription to be either "on" or "off" by providing recognition sites for either positive regulators or negative regulators. For example, an inactive stage is characterized by deacetylation of K14 H3 prior to methylation of K9H3 and an active stage is characterized by phosphorylation of lysine 10 prior to acetylation of K14 (14).

1.6 RNA Polymerase II Transcription

Eucaryotic RNA polymerase II holoenzyme is a multi subunit complex composed of RNA polymerase II (RNA Pol II) and the general transcription factors (GTF), including TFIIA, B, D (TBP and TAF_{II} proteins), E, F and H. RNA Pol II itself is capable of synthesizing RNA from a DNA template but requires the GTFs to recognize a promoter. Basal transcription on free DNA templates requires only the RNA Pol II holoenzyme. Transcription on chromatin templates needs gene-specific transcriptional activators and co-activators to counteract nucleosome repression. The topics discussed in the following sections focus on transcription in the chromatin environment.

1.6.1 Transcription Initiation

Transcription initiation requires the function of numerous factors. Transcriptional activators and co-activators bind the enhancer and recruit the general transcription machinery. A preinitiation complex may be assembled in a stepwise fashion. For basal transcription *in vitro*, preinitiation complex formation may start with promoter recognition by TFIID, consisting of TBP (TATA-binding protein) and several

TAF_{II}s proteins (TBP-associated factors). TBP binds at its recognition sequence, the TATA box, present in the promoter sequence of most genes, and forms the docking platform for the assembly of the other GTFs. TFIIA and B are then recruited, followed by TFIIF and RNA Pol II, positioning RNA Pol II over the start site. TFIIE and H are then recruited to Pol II holoenzyme-DNA complex, resulting in promoter melting and transcription initiation.

In vivo transcription occurs in a chromatin environment. Several transcription activators and co-activators have been identified as key players in counteracting gene repression by nucleosomes and establishing the transcriptionally competent promoter. Generally, transcription initiation begins with targeting of gene specific DNA-binding proteins to the promoter. Transcription co-activators have no direct contact with DNA but interact with transcription activators or other machinery via protein-protein interaction and exert their activities on the local chromatin. Transcription co-activators include those that are the components of the general transcription machinery and mediator complex (part of RNA Pol II holoenzyme) as well as separate proteins and complexes. Activators and co-activators coordinately function to increase the accessibility of the promoter in chromatin for binding by the transcriptional machinery. Both *in vivo* and *in vitro* studies have shown that the order of recruitment for activators and co-activators is gene specific (40, 44). Using *HO* gene expression, a well-ordered recruitment of transcription activators and co-activators was demonstrated. Transcription of *HO* starts with the binding of Swi5p activator to its binding site in late mitosis, subsequently recruiting the Swi/Snf complex to the promoter. Swi/Snf is required to "open" chromatin for the SAGA complex to acetylate histone in the promoter region.

Swi/Snf and SAGA remain bound to *HO* promoter and facilitate binding of heterodimeric activator Swi4/Swi6t leading to *HO* transcriptional activation (28). The order of recruitment can be the other way around, as seen for *IFN-β* gene expression. After enhanceosome formation (assembly of several activators over the nucleosome free region called enhancer), Gcn5p is recruited to acetylate nucleosomes and followed by recruitment of CBP-Pol II holoenzyme complex. Nucleosome acetylation subsequently recruits Swi/Snf complex resulting in chromatin remodeling that facilitates access for the TFIID to initiate transcription (1). In addition to histone acetylation and ATP dependent chromatin remodeling activity, histone phosphorylation was demonstated to function in sequential manner in promoting transcription initiation. Lo *et al.* purified H3 serine-10 kinase from budding yeast and demonstrated that its kinase activity is required for Gcn5p-dependent histone acetylation in *INO1* expression (96).

The phase in cell cycle also affects the requirement of particular genes for co-activators. Krebs *et al.* showed that Swi/Sni ATP dependent chromatin remodeling activity is required for Gcn5-mediated acetylation and gene expression on *GAL1* promoter during mitotic gene expression (80). However, interphase expression of *GAL1* was shown to be Swi/Snf independent.

1.6.2 Transcription Elongation

Most studies on transcriptional regulation have focused on initiation. Recently, interest in transcription elongation has dramatically increased as several elongation-related factors have been identified. Transcription elongation has been shown to be coordinated by several proteins (125). Carboxyl-terminal domain (CTD) of the largest

subunit of RNA Pol II is a key player in the regulation of elongation. CTD is composed of multiple repeats of heptapeptide, $-Y_1S_2P_3T_4S_5P_6S_7-$, with the number of repeats increasing with organism complexity. Serine number 2 and 5 are subjected to phosphorylation. Prior to initiation, RNA Pol II contains a hypoacetylated CTD. After recruitment to the promoter, CTD is rapidly phosphorylated by the TFIIH CTD kinase activity, converting it to an active form for elongation.

The early phase of transcription elongation is associated with TFIIH-mediated hyperphosphorylation on serine 5 of the CTD, leading to recruitment of mRNA capping enzyme (77, 106). In this early phase of elongation, histone methyltransferase Set1p is also recruited to the serine 5 phosphorylated CTD by one of the elongation-related complexes, Paf1, resulting in trimethylation of H3 K4 (81). Set1p-mediated trimethylation of H3 K4 is found in 5' region of genes and suggested to be a "marker" for transcribed genes (117). H3 K4 trimethylation is suggested to be involved with pre-mRNA 3' processing as Set1p is also found in a complex with Swd2p, a component of the cleavage-polyadenylation factor that facilitates poly A addition at the 3' end of mRNA. The next phase of elongation occurs when phosphorylation at serine 5 is reduced while phosphorylation at Serine 2 is increased by Ctk1 kinase activity. The Pol II machinery "takes off ", leaving behind transcription factors and mediators. Phosphorylated serine 2 recruits histone methyltransferase Set2p leading to methylation on H3 K36 (83). Unlike tri-methylation at H3 K4 which is enriched at 5' end of transcribed genes (117), Set2p-mediated H3 K36 is found throughout the coding region (83). Set2p by itself interacts directly with the serine 2 phosphorylated CTD RNA Pol II and other elongation factors (Paf1p complex, Chd1p etc.) in the coding regions of several

genes (83). Therefore, it is likely that Set2p "tags along" with RNA Pol II, together with elongation factors, during elongation. Processings of pre-mRNA (5'capping, splicing and polyadenylation) occurs concomittently with RNA Pol II transcription (130). These mRNA processing factors have been shown to interact with elongation factors and RNA Pol II itself (130). Chd1p, interacts with several elongation factors including Set2p and is found to be involved with transcription termination (2).

1.7 Chromatin Structure and Dynamics on the *PHO5* Promoter

A positioned nucleosomal array on the *PHO5* promoter prevents transcription *in vivo* (57, 149). Transcription on the *PHO5* promoter is regulated by phosphate signaling through the *PHO* pathway, which leads to a transition in chromatin structure that renders it nuclease sensitive (4). Upon activation during phosphate depletion, four positioned nucleosomes on the *PHO5* promoter are remodeled allowing the transcription machinery to bind and initiate transcription (Figure 1.2). The details of *PHO5* chromatin fluidity are discussed in the following section.

Transcription activators, Pho4p and Pho2p, are required to generate the active chromatin structure on the *PHO5* promoter (7, 38). The *PHO5* promoter contains two binding sites for Pho4p, the upstream activating sequences p1 and p2 (UASp1 and UASp2). Upstream to both Pho4p binding sites are recognition sequences for Pho2p (8). The UASp1 site is located in an enlarged linker (the DNA between nucleosomes) between nucleosome -2 and -3 and is constitutively available for binding. The UASp2 binding site is in nucleosome -2. The TATA-box and RNA start sites are in nucleosome -1. Mutations in Pho4p that disrupt interaction with Pho2p also disrupt Pho4p function in

PHO5 activation through UASp1 indicating that interaction between these two activators is required for activation through UASp1 (7). Results from mutational analysis of the Pho2p binding site also suggest that Pho2p is required for Pho4p binding at the UASp1, but not at UASp2. Overexpression of Pho4p partially activates *PHO5* transcription in the yeast strain carrying a *pho2* gene disruption and UASp1 deletion (13, 38). These results imply that Pho4p can bind at UASp2 in nucleosome –2 even in the absence of Pho2p, but probably with lower affinity.

In addition to gene-specific transcription activators, numerous studies have focused on identifying the activity that mobilizes nucleosomes on the *PHO5* promoter during gene activation. These include ATP-dependent remodeling and histone acetyltransferase (HAT) activities. In 1990, Vidal and Gaber showed that acid phosphatase activity in an *RPD3* deletion strain is up-regulated under repressing conditions (166). That finding suggested that histone acetylation is involved in *PHO5* regulation since Rpd3p was later found to contain histone deacetylase activity (151). About the same time, a number of remodeling activities, both ATP-dependent and covalent modifiers, were identified in several organisms and appeared to have functions related to transcriptional regulation. Some of them were novel proteins and some were already identified to be transcription activators or associated with RNA polymerase II holoenzyme. A few were investigated for their role in *PHO5* regulation. Two proteins were Gcn5p, a subunit of SAGA complex that contains histone acetyltransferase activity, and Snf2p, a subunit of the Swi/Snf ATP-dependent remodeling complex. Both activities seemed to be dispensable for the full induction of *PHO5* under activating condition (46, 51). However, a study by the Hörz lab revealed a function for Gcn5p in the kinetics of

chromatin remodeling and transcription of *PHO5*. Disruption of *GCN5* has no effect on the steady state level of activation, but delays the initial gene activation after sensing phosphate depletion by lowering the rate of remodeling (10). This delay from *GCN5* disruption is also observed in high phosphate conditions (from yeast strain lacking *PHO80* and *PHO4* expression is galactose dependent therefore no signal transduction through the *PHO* pathway) suggesting that Gcn5p is not recruited through the phosphate signaling pathway. Global histone acetylation/deacetylation has been demonstrated over a 4.25 kb locus including the *PHO5* promoter and coding sequence (168). Histone acetyltranferases Gcn5p and Esa1p as well as deacetylases, Rpd3p and Hda1p, coordinately regulate the pattern of histone acetylation over this region. Since there is no evidence of physical interaction between Pho4p and Gcn5p and *PHO5* induction did not increase acetylation of H3 K9 (168), it is unlikely that Gcn5p is recruited to *PHO5* in a gene specific manner. Therefore, it is likely that Gcn5p-mediated transcription activation on the *PHO5* promoter results from non-specific recruitment, not through phosphate signaling. In other words, it is not Pho4p-mediated. However, Barbaric *et al.* showed that SAGA recruitment to *PHO5* promoter is Pho4p-dependent (9). Even though Gcn5p is the HAT subunit in the SAGA complex, it is also found in the other HAT complexes such as ADA. In addition, while the Gcn5p subunit of SAGA is not required, Spt3, 7 and 20 as well as Ada2 have been shown to be required for *PHO5* expression. Therefore Pho4p-dependent, SAGA-mediated *PHO5* expression is likely to require the other subunits rather than Gcn5p. The mode of function for these subunits involves chromatin remodeling (Gcn5p and Ada2p), TBP recruitment (Spt3p) and assembly of the SAGA complex (Spt7p and Spt20p). Spt7p subunit has also been identified by Nishimura *et al.*

to have a function in *PHO5*, *PHO8* and *PHO84* expression (120). Since no physical interaction of Spt7p on the *PHO5* promoter has been demonstrated, the effect of *SPT7* disruption is probably through a combination of indirect effects through interfering with *PHO84* expression as well as disintegration of the SAGA complex.

Most studies on *PHO5* expression have been performed using asynchronous cell cultures and transcription was induced under phosphate starvation through the phosphate signaling pathway. A recent study on cell cycle-regulated gene expression demonstrated several *PHO* genes that are up-regulated during mitosis despite phosphate abundance (repressing condition). This mitotic induction is shown to be under the control of Pho4p, Pho2p and Pho81p. Interestingly, the requirement for remodeling activity of the Gcn5p containing and Swi/Snf complexes, which are dispensable in asynchronous cells under phosphate starvation, are essential in mitotic induction (113). It is likely that *PHO5* induction from phosphate depletion in asynchronous cells and cell cycle regulation in synchronized culture are controlled through the same signal transduction pathway (the *PHO* pathway). The requirement for the remodeling activities in the mitotic induction of *PHO5* is probably explained by the global requirement of Gcn5p and Swi/Snf complexes for mitotic gene expression (80). It is also likely that intracellular phosphate, either in the form of polyphosphate or free phosphate, is the key transcription repressor of *PHO5* expression in mitotic phase, and that transcription occurs to ensure sufficient phosphate throughout cell cycle progression.

Numerous studies have focused on identifying the role of Swi/Snf ATP dependent chromatin remodeling activity on *PHO5* expression. Disruption of Swi/Snf activity is also claimed to cause a kinetic delay in *PHO5* transcription and is required for Gcn5p-

mediated *PHO5* activation in late mitosis during cell cycle progression (10, 80). The evidence so far suggests that Swi/Snf activity is only required for *PHO5* gene activity in a condensed chromatin of the mitotic phase. The remodeling activity that is required for *PHO5* induction during interphase is the Swi/Snf paralogue, the Ino80p complex. A genetic screen identified Ino80p through a requirement in phospholipid biosynthesis gene regulation. Deletion of *INO80* resulted in decreased gene activation in a number of unrelated genes, including *PHO5* (36). An Ino80p complex was purified from budding yeast that contains 12 protein subunits. This biochemically purified protein complex is able to remodel chromatin and activate transcription *in vitro* (141). A chromatin immunoprecipitation (ChIP) experiment has shown that Ino80p is recruited to the *PHO5* promoters in a Pho4p-dependent manner suggesting physical association between Pho4p and the Ino80 complex. Interestingly, Pho4p-dependent recruitment of Ino80p is also observed on *PHO84*, the high affinity phosphate transporter gene. The Swi/Snf complex is also recruited to both *PHO5* and *PHO84* promoters but recruitment is more prominent on the *PHO84* promoter. This again emphasizes the small role that Swi/Snf complex plays on the *PHO5* promoter. Recent studies have identified small molecules that probably regulate chromatin remodeling activity of the Swi/Snf, ISWI and Ino80 complexes (142, 144). Inosital polyphosphate molecules such as IP_4, IP_5 and IP_6 appear to regulate Swi/Snf, ISWI, and Ino80p differently. Mutations that disrupt kinase activity in ARG82/IPK2, required for production of inositol polyphosphate IP_4 and IP_5, resulted in a decrease in chromatin remodeling activity and transcription of *PHO5* (144). Deletion of *ARG82* also reduces chromatin remodeling on the *PHO84* promoter. Further studies on the physiological regulation of inositol polyphosphate metabolite production

will clarify the connection between the signal transduction pathway and chromatin remodeling activities that control transcription of *PHO5* and *PHO84*.

Another class of ATP-dependent chromatin remodeling factors, Isw1p and Isw2p, was also investigated for a role in *PHO5* regulation. Numerous studies have shown that yeast Isw1 and Isw2 are likely to be involved with chromatin remodeling that lead to transcriptional repression (39, 49). *In vivo* studies have shown that neither Isw1p nor Isw2p regulate the chromatin structure and expression of *PHO5* (75, 144).

1.8 Hypotheses

It is now widely accepted that chromatin structure plays an important role in all activities involving DNA. In the Laybourn lab, we are interested in identifying the mechanisms of transcriptional regulation in the chromatin environment. One of the gene models we use is *PHO5* from the budding yeast *Saccharomyces cerevisiae.*

This dissertation consists of two major projects. One project is the reconstitution of transcriptional regulation on the *PHO5* promoter. The other project is the clarification of the direct and potential indirect functions of histone deacetylase Rpd3p in the regulation of *PHO5* transcription. For the first project, it was hypothesized that transcription-related activities, which include chromatin assembly, remodeling, histone acetylation and transcription activation, could be recreated *in vitro.* The experiments proposed to accomplish the first project began with reconstitution of chromatin *in vitro* from purified components with the yeast assembly system on the *PHO5* promoter. The reconstituted chromatin template was used to investigate the requirement for transcription-related activities, which include ATP-dependent chromatin remodeling and

histone acetylation. This system allows the transcription activation process to be dissected to detail, so the mechanism of transcription regulation can be clarified.

The second project proposed to investigate the role of Rpd3p, a histone deacetylase, in *PHO5* regulation. It was hypothesized that Rpd3p functions in *PHO5* expression. This function could be either indirect through the activity of the gene regulatory factor Pho4p or direct through the histone acetylation state on the *PHO5* promoter. The methods used to accomplish this project include an acid phosphatase enzyme activity assay, a reverse transcriptase real time PCR, a phosphate uptake assay, and a fluorescence microscopy.

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Figure 1.1. Regulatory cascade and phosphate sensing in the *PHO* pathway. Heavy arrows indicate production of gene product. Dashed lines indicate regulatory interaction, arrow for positive and flat ends for negative. *PHO5* encodes the secreted acid phosphatase (Pho5p), which functions outside cell to scavenge phosphate from extracellular source. *PHO2* and *PHO4* encode transcription activators (Pho2p and Pho4p) that regulate activation of *PHO5*, *PHO81*, *PHO84* and *PHO86* through phosphate availability. *PHO80/PHO85* encode cyclin (Pho80p) and cyclin dependent kinase (CDK, Pho85p), respectively. Pho80p/Pho85p inactivate Pho4p activity through phosphorylation. *PHO81* encodes a CDK inhibitor that inhibits kinase activity of Pho85p. Phosphate depletion initiates signaling in the *PHO* pathway through Pho81p activity. In the absence of phosphate, CDK inhibitor Pho81p inhibits kinase activity of Pho85p resulting in localization of an active Pho4p in the nucleus. Pho2p and Pho4p then activate genes (*PHO5*, *PHO81*, *PHO84* and *PHO86*) that are required for phosphate acquisition. When cells have enough phosphate, Pho81p is inhibited and Pho4p is phosphaorylated by Pho85p resulting in re-localization to the cytoplasm thus reducing expression of *PHO5*, *PHO81*, *PHO84* and *PHO86*.

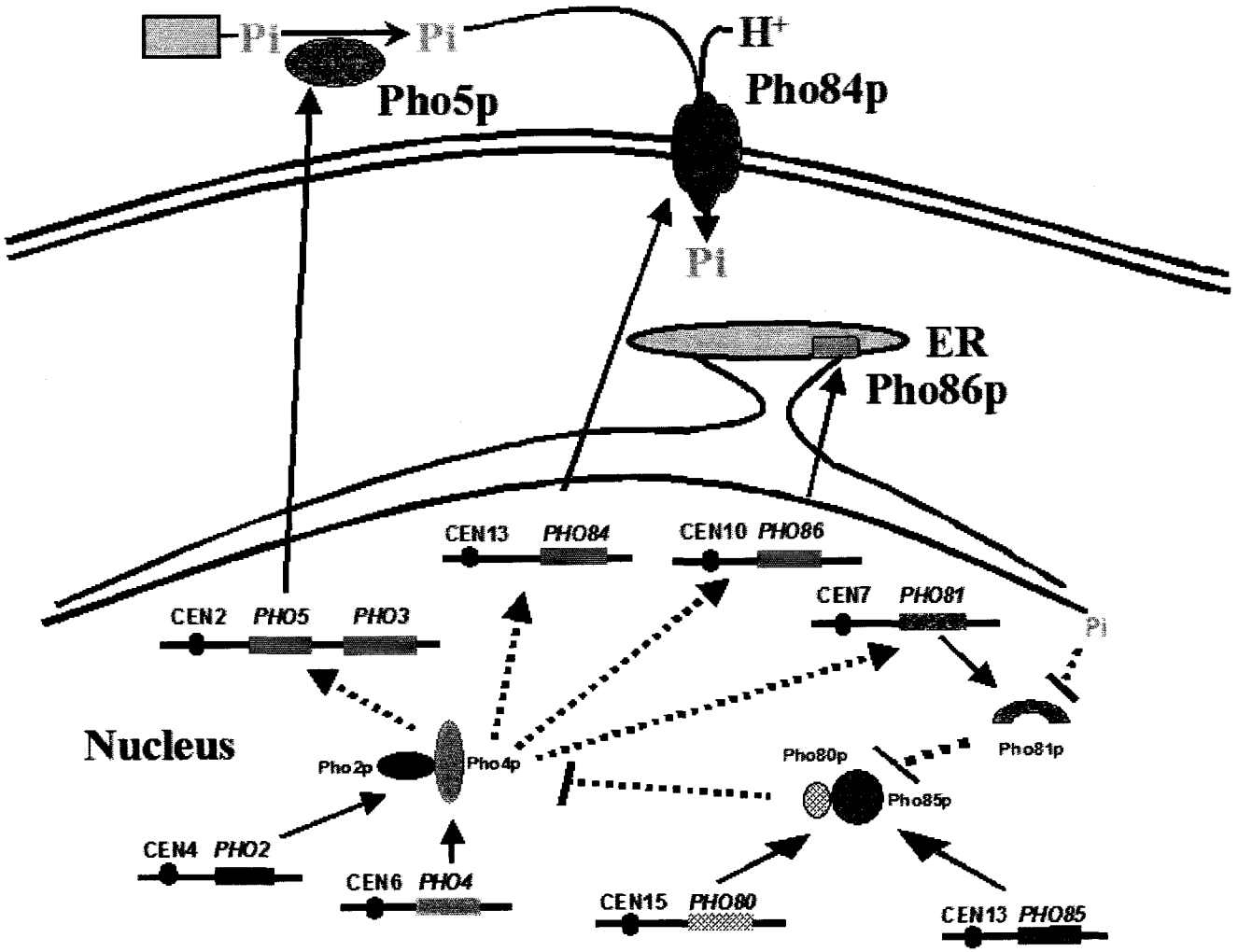

Figure 1.1 Regulatory cascade and phosphate sensing in the *PHO* pathway.

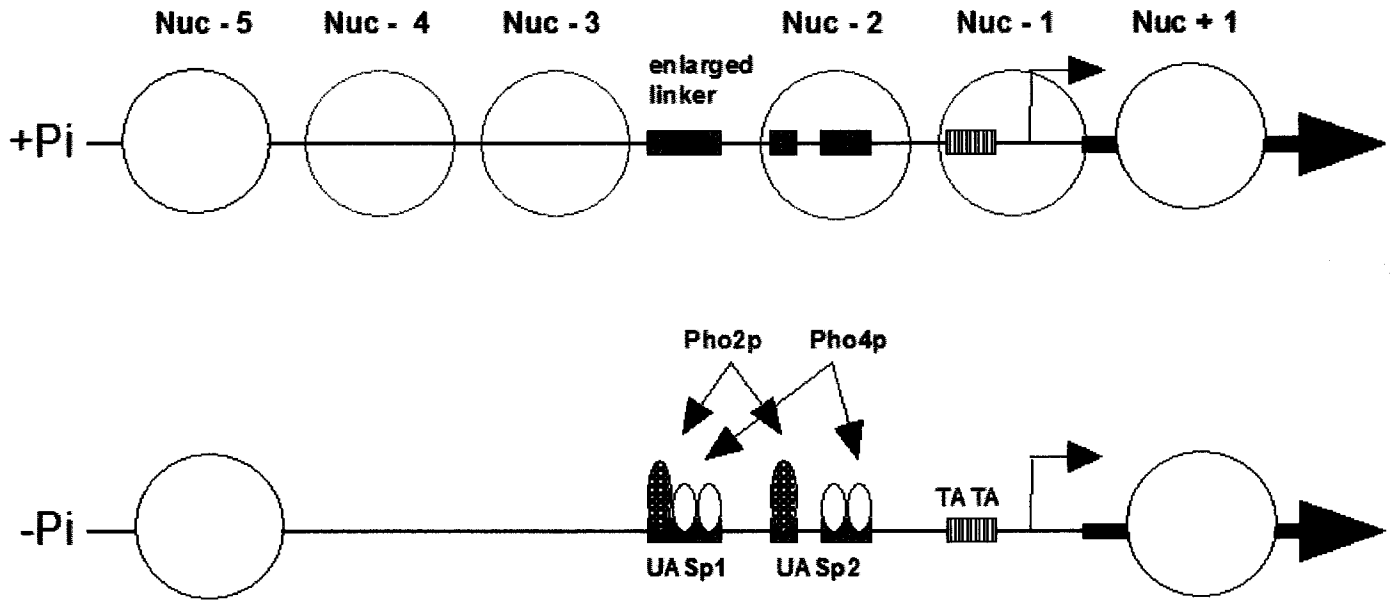

Figure 1.2. Nucleosome position and remodeling during transcriptional activation. Circles represent the approximate location of nucleosomes. The boxes and small circles represent the binding sites for transcriptional activators Pho2p, Pho4p (UASp1 and UASp2) and TBP (TATA box). At high phosphate (+Pi), the *PHO5* promoter is containing within a nucleosome array including an enlarge linker between nucleosome –2 and –3 and its expression is repressed. However, under activating conditions (-Pi), the positioned nucleosomes are no longer detected on the *PHO5* promoter. Nucleosomes are reconfigured by coordinated function of chromatin remodeling activities *e.g.* Swi/Snf , Ino80p and Gcn5. The detailed mechanism of remodeling remained to be determined.

Table 1.1. List of *PHO* genes in the *PHO* pathway and their function

PHO2	encodes the transcriptional activator Pho2p that functions in the regulation of *PHO5* and also in the other genes e.g. *HIS4*, *ADE.*
PHO3	encodes acid phosphatase enzyme Pho3p. Gene expression is repressed by *PHO5* activation
PHO4	encodes the transcriptional activator Pho4p. Activity is regulated through phosphorylation by Pho85p and localization.
PHO5	encodes the major secreted acid phosphatase Pho5p. Gene expression is induced by phosphate depletion.
PHO80	encodes cyclin Pho80p that function together with Pho85p to regulate Pho4p activity.
PHO81	encodes the CDK inhibitor Pho81p that functions upstream of Pho80p/Pho85p. Its activity is regulated by phosphate.
PHO84	encodes the H^+- coupled, high affinity phosphate transporter Pho84p. Gene expression is phosphate dependent
PHO85	encodes cyclin dependent kinase (CDK) Pho85p.
PHO86	encodes the ER resident protein Pho86p and facilitate the ER exit of Pho84p. Gene expression is phosphate dependent.

Table 1.2. Classification of ATP-dependent chromatin remodeling complexes. * The name of the complex and its subunit containing ATPase activity (in bracket) are listed. (Current reviews are in (11, 103, 111))

Swi/Snf group	Yeast Swi/Snf (Swi2/Snf2)[¥] and RSC (Sth1) complexes
	Drosophila Brahma (Brm) complex
	Human Swi/Snf (hBRG1 or hBRM)
ISWI group	*Drosophila* ACF, CHRAC and NURF (dISWI) complexes
	Yeast Isw1 (Isw1p) and Isw2 (Isw2p) complexes
	Xenopus ACF/CHRAC (xISWI)
	Human RSF, hACF and hCHRAC (hSNF2h) complexes
CHD/Mi-2 group	Human NURD (CHD3/4) complex
	Xenopus Mi-2 (xCHD) complex
	Yeast Chd1p
INO80 group	Yeast INO80 (Ino80p)[¥] complex

* A multitude of novel complexes have been discovered in many organisms. Described here in this dissertation represent some of these complexes in each category.

[¥] Function in *PHO5* regulation.

The ATPase subunit from each group contains a signature motif that is unique to individual group, bromodomain in Swi/Snf group, SANT domain in ISWI group and chromodomain in CHD/Mi-2 group. *INO80* and its human (*hINO80*) and *Drosophila* (*dINO80*) homologues contain two conserved regions, the TELY motif at the amino terminus and the GTIE motif at the carboxy terminus. No SANT domain was identified in INO80 ATPase, even though identification was based-on sequence homology to ISWI ATPase.

Table 1.3 Classification of histone modifying activities described in this dissertation. Current reviews are in (14, 56, 67, 79, 111, 159, 164, 179).

Acetylation		
	HAT*	Yeast ADA (Gcn5p), SAGA (Gcn5p), NuA3 (Sas3p), NuA4 (Esa1p) and SLIK (Gcn5p) complexes
		Human TAF_{II} 250, Yeast TAF_{II} 130p/145p and *Drosophila* $dTAF_{II}$ 250
	HDAC[§]	Class I: Yeast Rpd3p, Hos1p and Hos2p, Mammalian HDAC1 and 2
		Class II: Hda1p,
		Class III: Sir2p
		Class IV: Hos3p
Phosphorylation		Yeast Snf1p
Methylation[¥]		Mammalian SUV39H (H3-K9), human Set9 (H3-K4)
		Yeast Set1p (H3-K4), Set2p (H3-K36), Set3p and Dot1p (H3-K79)
Ubiquitination[£]		Yeast Rad6p (H2B-K123)

* List of yeast nuclear HAT complexes with subunit containing HAT activity (in bracket) and TAF_{II}s containing HAT activity

[§] List of proteins with HDAC activity from yeast and mammalian cells

[¥] List of histone methyltransferase (HMT) activity and the target histones and lysine residues (in bracket)

[£] Yeast Rad6p is E2 enzyme. Reference for E3 enzyme is in (175)

Chapter 2

Reconstitution of Yeast Chromatin Using yNAP1

This Chapter is written in the form of a manuscript to be submitted for publication in *Methods in Enzymology.* The following text and references have been re-formatted to maintain consistency with the rest of the dissertation.

2.1 Introduction

The eukaryotic genome is maintained in a DNA-protein complex known as chromatin. The fundamental unit is the nucleosome, in which the core histone octamer, $(H3\text{-}H4\text{-}H2A\text{-}H2B)_2$, has DNA wound around it in 1.65 turns (5). Chromatin structure plays an important role in several biological processes, including transcription, through its function in organizing DNA and its ability to block access to the DNA. Transcription can occur in the chromatin environment through the help of accessory transcription co-activators that function to open chromatin structure, providing access to the transcription machinery.

The yeast, *Saccharomyces cerevisiae*, *PHO5* gene is used as a model to study transcription regulation in a chromatin context based on the detailed understanding of the *in vivo* chromatin structure and dynamics during the transition between activated and repressed conditions (1, 2). Yeast cells have proven to be a powerful experimental organism for investigating the role of chromatin structure in transcription regulation. The largest impediment to biochemical experiments was the lack of a chromatin reconstitution system. In late 1992, no one had developed a procedure for the purification of large quantities of yeast core histones or a method for reconstitution of yeast core histones into nucleosomal templates. In fact, the prevailing attitude was that native yeast core histones could not be purified, without using methods like acid extraction, and that purified yeast core histones could not be reconstituted into nucleosomes. Since then, although it presented many difficulties, we succeeded in purifying yeast core histones in quantities (milligrams) sufficient for biochemical and biophysical analyses (7). In addition, we have developed a chromatin reconstitution

system wholly derived from yeast. Through the development of this system we have been able to answer several important biological questions.

2.2 Purification of Native Yeast Core Histones

The procedure for purification of yeast core histones was built on the procedures for purification from calf thymus, chicken erythrocytes, and *Drosophila* embryos. The major steps are preparation of nuclei, solublizing chromatin fragments by digestion with micrococcal nuclease digestion, fractionation of the chromatin fragments from the bulk of the cellular components, and purification of the core histones from the genomic DNA by hydroxylapatite chromatography. There are two major differences between the yeast procedure and the original procedures. First, yeast cells have tough cell walls that must be removed prior to nuclei preparation. Second, sucrose gradient fractionation was not effective for fractionation of the chromatin fragments and was replaced by gel filtration chromatography. Another important difference is the addition of ribonuclease A (RNAase A) to the micrococcal nuclease digestion. RNAase A breaks up the abundant ribonuclear particles in yeast nuclei, thus preventing them from contaminating the chromatin peak of the gel filtration column. Due to the high cost of using the commercially available "Lyticase" (Sigma) in the large quantities required for spheroplasting hundreds of grams of yeast cells, we have opted to use recombinant endoglucanase.

2.3 β-1,3-Glucanase Production

This protocol is based on one sent to us by the Schekman lab (UC, Berkeley) in 1992.

2.3.1 *E. coli* Transformation and Assay Yeast Cell Growth

On day 1, transform DH5α *E. coli* cells with the glucanase expression vector (pUV5-G1S, a gift from S-H. Shen, National Research Council of Canada) (8). At the same time, or on day 2 start a culture of yeast cells for use as assay yeast cells. These cells need to be grown to a density between 1 to 4 OD_{600} (a density of 1 to 2 is best). There is a clear decrease in how easily cells will spheroplast with the culture density. After harvesting the assay cells by centrifugation, wash them with water and resuspend them in 50 mM Tris-HCl, pH 7.4 to around 20 OD_{600}, and store them at 4 °C for up to a week. The cold shock buffer, 0.5 mM $MgSO_4$, can be made up ahead of time and chilled to 4 °C.

2.3.2 Cell Growth and Induction

On day 2, pick two single colonies from the pUV5-G1S transformation into two 5 ml LB/AMP (Luria Broth, 10 g Triptone, 5 g yeast extract, 5 g NaCl per liter containing 100 μg/ml ampicillin) cultures and grow them overnight in a shaker at 37 °C. On day 3, inoculate a 100 ml culture of LB/AMP with one of the 5 ml cultures and grow for 2 h shaking at 37 °C. Then, inoculate five 1liter cultures of LB/AMP with 20 ml of the 100 ml culture and grow them in a shaker at 37 °C to an OD_{600} of 0.5 (about 4 h). At this point, add IPTG to 0.4 mM to induce expression. Continue shaking at 37 °C for 5 h. Harvest cells by centrifugation at 5 k rpm, 5 min, in a GS3 rotor or equivalent and freeze and store the bacterial pellets at –80 °C.

2.3.3 Osmotic or Cold Shocking

All the steps in osmotic or cold shocking are done at room temperature unless otherwise stated. Osmotic or cold shocking extracts periplasmic proteins from *E. coli* without lysing the cells. On day 4, thaw cells to room temperature and wash cells by resuspending them in 1 liter 25 mM Tris-HCl, pH 7.4 and centrifuging them as before. Resuspend the cells in 1/50 of the original culture volume (100 ml) 25 mM Tris-HCl, pH 7.4. Measure the volume with a graduated cylinder and add EDTA to 2 mM. Add an equal volume of 40% sucrose, 25 mM Tris-HCl, pH 7.4 and mix the cells gently for *exactly* 20 min. Spin out the cells in a GSA rotor at 7,500 rpm for 10 min. Pour off all of the supernatant. Removal of as close to all of the sucrose buffer as possible is important. Place cell pellets on ice and resuspend them in 1/50 of original volume (100 ml) cold shock buffer (0.5 mM $MgSO_4$). Mix the cells gently for 20 min on ice. Spin out cells in an SS34 rotor at 4 °C, 10 k rpm for 10 min. The supernatant contains the glucanase activity.

2.3.4 Storage and Further Purification

We have found it most convenient to store the crude periplasmic proteins containing substantial gluconase activity by adding $(NH_4)_2SO_4$ to 90% saturation and storing at 4 °C. The Schekman laboratory suggested an optional purification by CM Sepharose chromatography. For this purification, dialyze the supernatant against 50 mM NaOAc, pH 5.0. Batch bind the crude extract to CM Sepharose resin pre-equilibrated in 50 mM NaOAc, pH 5.0. Pour the resin into a short, wide column and

wash the column with 2 to 3 column volumes 50 mM NaOAc, pH 5.0. Elute the glucanase activity with 150 mM NaCl, in 50 mM NaOAc, pH 5.0.

2.3.5 Lyticase/Glucanase Assay

In order to determine how much glucanase to add to obtain complete spheroplasting, it is important to assay the activity of your preparation. Make fresh 2X Assay Buffer containing 100 mM Tris-HCl, pH 7.4, 80 mM β-mercaptoethanol. Centrifuge 1 ml glucanase/$(NH_4)_2SO_4$ solution at 14 k rpm, 10 min, 4 °C in an Eppendorf tube, pour off the supernatant and resuspend the protein pellet in 1 ml 2X Assay Buffer. In each assay reaction, place 1 ml 2X Assay Buffer, 0.92 ml H_2O, and 0.08 ml assay cells (to a final OD_{600} of 0.8 to 0.9). Then add 0 to 50 µl Lyticase or glucanase. Incubate the reactions at 30 °C for 30 min while mixing (yeast cells settle quickly). Vortex the reaction and quickly measure OD_{600}. One unit of glucanase activity is defined as a 10% decrease in OD_{600} in 30 min at 30 °C. The window of linearity of this assay is very narrow, in the range of a 20% to 60% decrease. Measurements obtained with a change of less than a 20% will tend to produce high units/ml measurements. Measurements obtained with a change greater than 60% will tend to produce low unit/ml measurements.

2.4 Yeast Core Histone Purification

We have purified core histones from 100 g to 1 kg of yeast cells. The procedure does not work well for less than 100 g due to large losses during cell lysis. We have found it best to start with at least 250 g of yeast cells. Therefore, the protocol provided

here is based upon that amount of starting material. We have not found that use of protease deficient yeast strains has made an appreciable difference in the quality or the yield of core histones obtained. However, we have found the use of yeast cells harvested in logarithmic growth phase to be critical, as cells become much more resistant to spheroplasting in late log and stationary growth phases.

2.4.1 Solutions (components indicated with an * are added just prior to use)

Prespheroplasting buffer (2.5 liters) contains 100 mM Tris-HCl, pH 7.9 and 60 mM β-Mercaptoethanol (β-ME) and is warmed to 37 °C to help thaw yeast cell pellets. Spheroplasting Buffer (1.25 liters at 30 °C) contains 0.7 M Sorbitol (2 M Sorbitol Stock, filtered through Whatman 1 paper, stored at 4 °C), 0.75% yeast extract 1.5% Peptone (5X yeast extract and Peptone stock or 5% and 10%, respectively), 10 mM Tris-HCl, pH 7.4 (2 M stock), and 10 mM β-ME. YPD + 1 M Sorbitol (1.25 liters, 30 °C) consists of 1% yeast extract, 2% Peptone, 2% dextrose, 1 M sorbitol, 10 mM Tris-HCl, pH 6.8, *1 mM PMSF, *2 mM Benzamidine, and *2 mM $Na_2S_2O_5$. Sorbitol buffer (2.5 liter, 4 °C) contains 1 M sorbitol, 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, *1 mM PMSF, *2 mM Benzamidine, and *2 mM $Na_2S_2O_5$. Lysis Buffer (125 ml, 4 °C) is made up of 18 % Ficoll 400, 20 mM KH_2PO_4, 0.25 mM EDTA (0.5 M, pH 8.0 stock), 0.25 mM EGTA (0.5 M, pH 8.0 stock), 0.5 mM spermidine (50 mM stock), 0.15 mM spermine (50 mM stock), and adjusted to pH 6.8 with KOH. Then reducing agents and protease inhibitors are added to *3 mM DTT, *2 mM , *2 mM $Na_2S_2O_5$, *1 mM PMSF, *2 μM Pepstatin A, *0.6 μM Leupeptin, and *2 X 10^{-4} % Chymostatin. Nuclei Storage Buffer (125 ml, 4 °C) contains 100 mM Tris-OAc, pH 7.9, 50 mM KOAc, 20% glycerol, 2 mM EDTA,

*3 mM DTT, *2 mM , *2 mM $Na_2S_2O_5$, *1 mM PMSF, *2 μM Pepstatin A, *0.6 μM Leupeptin, and *2 X 10^{-4} % Chymostatin. A separate batch of nuclei storage buffer (3 liters, 4°C) is made that contains only the subset of reducing agent and protease inhibitors *3 mM DTT, *2 mM , *2 mM $Na_2S_2O_5$, and *1 mM PMSF. Hydroxylapatite chromatography buffers (4 °C, 0.22 μm filtered) are Buffer A (1.5 liters), containing 80 mM Na_2HPO_4, pH 6.8 and10% glycerol, and Buffer B (1 liter) containing 80 mM Na_2HPO_4, pH 6.8, 2.5 M NaCl, and 10% glycerol. Both Buffer A and B contain *0.1 mM PMSF, *2 mM $Na_2S_2O_5$, and *1 mM DTT.

2.4.2 Nuclei Preparation

Thaw 250 grams (wet weight, approx. 2 x 10^{12} cells) of yeast cells (stored at -80°C as pellets) in 2500 ml (10 ml/g) prespheroplasting buffer pre-warmed to 37 °C (to help thaw yeast pellets). Save a 1 ml sample as an untreated control while monitoring the OD change during spheroplasting. Harvest cells in GS3 (or JA-10) rotor at 7 k rpm, 4 °C, 10 min. Resuspend cells in 1250 ml Spheroplasting Buffer (5 ml/g) at 30 °C. Add 2 million units of recombinant β-1,3-glucanase and incubate at 30°C while stirring. Spheroplasting is monitored by change in OD_{600} (dilute cells to less than 1 OD_{600} in dH_2O, usually around 200- to 500-fold). OD_{600} is measured every 15 to 20 min and should be reduced to 30% to 40% of the initial OD_{600}. However, it is not advisable to let the spheroplasting continue for more than 60 min. If OD has not changed enough in 30 minutes add more endoglucanase. Spin out cells at 7 k rpm, 4 °C for 10 min. in a GS3 rotor. Resuspend cells in 5 ml/g cells YPD + 1 M Sorbitol (1.25 liters, 30 °C).

Cells should be noticeably more difficult to resuspend due to spheroplasting (will tend to clump). Spin out cells at 7 k rpm, 4 °C for 10 min. in a GS3 rotor. Resuspend cells in 5 ml/g cells 1 M Sorbitol (1.25 liters, 4 °C). Spin out cells at 7 k rpm, 4 °C, 10 min. in a JA-10 rotor. Repeat the 1 M Sorbitol wash. Resuspend cells in at least 1 ml/g cells Lysis buffer (250 ml, 4 °C). Pass total volume through the Yamato homogenizer three times while turning at 1 k rpm at 4 °C. Then check pH and make sure it has not dropped. Low pH < 6.0 seems to promote clumping of organelles and this will drastically lower yield. If pH has dropped, adjust the pH to around 7.0. Complete lysis and the lack of organelle clumping can be checked microscopically. The organelles should be streaming between lysed cells. Centrifuge lysed cells at 6.5 k rpm, 4 °C, 10 min in a GSA rotor. Note: If your volume is less than 100 ml it works best to do these spins in an SS-34 (or JA-20) rotor. Transfer supernatants, everything that will pour including looser components of the pellets, to new bottles and recentrifuge at 6.5 k rpm. Transfer supernatants as before to new bottles, spin at 6.5 k rpm, 4 °C, 10 min in a GSA rotor. Pour off supernatants and recentrifuge as before. Combine supernatants and spin at 5.5 k rpm, 4 °C, 10 min in a GSA rotor. Pour off supernatants and recentrifuge as before two or three more times, depending on the amount of cell debris remaining in the supernatants. Under the microscope, the first two pellets will be mostly unlysed cells. The subsequent pellets should be mostly cell debris. The nuclei are visible in all the pellets as small round dots. After the 5 low speed spins the supernatant should be cloudy and essentially devoid of intact cells. Pellet nuclei by spinning at 12.5 k rpm, 4 °C, 30 minutes in a GSA rotor. The nuclear pellet is mostly white and the supernatant should be clear. Check the pellet and supernatant under a microscope. The pellet

should be mostly nuclei with very few cells. If the supernatant contains a large number of nuclei, recentrifuge it at 12.5 k rpm. Good preparations will have a nuclei yield of 40-100g, however around 15-20g is fine. If the yield is large (>70g), split it into two aliquots. Resuspend the nuclei (pellets) in 0.5 ml/g cells (125 ml) Nuclei Storage Buffer. Flash freeze the nuclei in liquid nitrogen and store at -80 °C.

2.4.3 Chromatin Digestion and Nuclei Extraction

Thaw nuclei and pellet at 12.5 k rpm, 4 °C, 20 min in a GSA rotor. Resuspend nuclei in 50 ml nuclei storage buffer per aliquot. For a test micrococcal nuclease digestion, remove 1 ml of nuclei to an Eppendorf tube, warm to 37 °C, 10 min. Add 3 µl 1 M $CaCl_2$, 8 µl 10 units/ml micrococcal nuclease (Worthington; 13.6 µl 0.08 Sigma Units/ml), and incubate at 37 °C. Remove 100 µl aliquots at 0', 1', 2', 5', 10', 15', 20', 30' into tubes containing 50 µl protease digestion buffer (20 mM EDTA, pH 8, 200 mM NaCl, 1% SDS, 0.25 mg/ml glycogen) and 1 µl 10 mg/ml RNAase A (Worthington) and incubate at 37 °C, 5 min. To each aliquot add 30 µl dH_2O, 20 µl 10% SDS, and mix. Then add 40 µl 5 M NaCl, mix, and 200 µl $CHCl_3$/ isoamyl alcohol (24/1), mix, and centrifuge 5 min. Transfer the upper, aqueous layer to new tubes and add 600 µl Ethanol, invert, and centrifuge 15 min. Wash the pellets with 800 µl 75% ethanol. Dry the pellets in a SpeedVac and resuspend pellets in 12 µl TE. Load samples onto a 1.5% agarose gel (TBE); 3 µl of 0' through 30', 10 µl of 1' through 10'. Run the gel until the bromophenol blue is approximately 0.75 of total length of gel. Stain gel with ethidium bromide or SYBR Gold and photograph or scan.

Conduct a preparative micrococcal nuclease digestion of the rest of the nuclei by adding 1 M $CaCl_2$ and 200 units/ml micrococcal nuclease (Worthington, or 500 units/ml Sigma) to 3 mM and 7 units/ ml (Worthington, or 0.08 units/ ml Sigma), respectively. Add 50 µl RNAase A (10 mg/ml, Worthington) and incubate the digestion at 37 °C for the time determined in the test digestion to produce chromatin fragments corresponding primarily to 10 nucleosome length (1600 to 1700 bp). Stop digestion by adding 0.5 M EDTA to 30 mM, and mix. Spin out nuclei at 16 k rpm, 4 °C, 15 min in SS-34 rotor. Save pellets for further extraction. Second and even third extractions can be done by resuspending in 60 ml 0.2 mM EDTA and centrifuging down the nuclei as before. The chromatin is cleanest and at the highest concentration in the first extraction, however.

2.4.4 Sephacryl S-300 Gel Filtration Chromatography (run at 4 °C)

Equilibrate column (1 liter Pharmacia Sephacryl S-300 resin in a XK 50/60 column) with Nuclei Storage buffer. Run column at 5 ml/min, absorbance units full scale (AUFS) of 2 at 280 nm, and chart speed set at 0.15 cm/min. This column is run isocratically, but both intakes are put in buffer and it is run at 50% B. Inject 50 ml nuclear extract onto column and run at 5 ml/min. The chromatin will be found in the void volume (excluded peak). The chromatin peak in identified by running 100 µl of column fractions on a 1% agarose gel (stained with ethidium bromide or SYBR Gold) and on an 18% SDS-polyacrylamide gel (stained with Coomassie Brilliant blue). The DNA is purified by adding 100 µl protease digestion buffer (20 mM EDTA, pH 8, 200 mM NaCl, 1% SDS, 0.25 mg/ml glycogen), 5 µl 2.5 mg/ml proteinase K, and incubating

at 37 °C, 15 min. To this is added 200 µl 0.6 M NaOAc, 400 µl $CHCl_3$. The mixture is vortexed and centrifuged 5 min. The DNA is then ethanol precipitated from the aqueous phase, washed and resuspended in 10 µl agarose gel sample buffer. A judgment must be made in deciding which fractions contain most of the chromatin, but little of the higher molecular weight contaminating proteins. This is usually the first half of the excluded peak and perhaps one fraction past the fraction with the greatest absorbance. In the excluded peak, the large amount of DNA often causes these lanes of the protein gel to run somewhat blurry. Note: chromatin cannot be frozen for storage.

2.4.5 Hydroxylapatite Chromatography (run at 4 °C)

Check the chromatin concentration of the pooled S-300 peak by measuring the OD_{260}. This is best done by diluting the chromatin 1/20 (50 µl) to 1/50 (20 µl) fold into 1 ml 1M NaOH. Yield for the first extraction is typically approximately 20-40 mg chromatin in about 250 ml.

Pour a hydroxylapatite (Bio-Rad Macro-Prep Ceramic Hydroxyapatite Type I, C#158-2000) column using 1 ml resin per 2 mg chromatin. An approximately 30 ml column is usually required. Best results have been obtained using a XK 50/30 column with two adjustable adapters. A short, fat column is preferable. The resin should be loaded with chromatin to capacity. If after the sample is loaded, you have more than half the column remianing white you have used too much resin. Note: HT gel defining is important. Pack and equilibrate the column with 3 to 4 column volumes (CV) at 0.3 M NaCl. Set the $AUFS_{280}$ at 0.5, the flow rate at 3.0 ml/min, and the chart speed at 0.1 cm/min.

Load the gel filtration column chromatin peak pool onto the HT column at about 2 ml/min. It is often easiest to use the P1 peristaltic pump to load the sample. Wash the column at 3.0 ml/min with a 0.3 M to 0.8 M NaCl gradient over 5 CV, then hold at 0.8 M NaCl for 2 to 4 column volumes. Continue to wash at 0.8 M NaCl until the absorbance has returned to baseline.

Elute the core histones by stepping to 2.5 M NaCl at 3.0 ml/min for 5 CV while collecting 4 ml fractions. The core histones should come off within the first 0.5 to 0.7 CV. The core histone peak fractions are identified by running 50 µl of each fraction on 18% SDS-PAGE. The peak is usually contained within 4 to 5 fractions with a sharp leading edge containing the purest fractions and a somewhat broader trailing edge. Some contaminants will elute with the core histones and many of them will elute after the core histones. Peak fractions will have a concentration of 0.5 to 1 mg/ml as determined by A_{280} (ext. coefficient 0.42mg/ml Abs U^{-1}). If fractions are less than 95% pure, run them next to core histones of a known concentration to estimate their concentration. For an example of purified yeast core histones see Pilon et al (7)

2.5 Expression and Purification of Recombinant Yeast Nap1p

2.5.1 Expression

Late afternoon day 1, transform plasmid (pGEX-NAP1) into BL21 (DE3) E. coli cells. First thing in the morning day 2, pick six colonies into each of six 5 ml cultures of LB/ampicillin (12.5 µl 40 mg/ml ampicillin). Grow cultures at 37 °C while shaking until turbid (approx 2 hrs). Pour two of each 5 ml culture into each of three 100 ml LB/ ampicillin cultures in 250 ml flasks (250 µl 40 mg/ml ampicillin). Grow 100 ml cultures

at 37 °C shaking until turbid (approx. 2 hrs). Poured 100 ml cultures into each of three 1 liter of LB/ ampicillin cultures in Fernbach flasks (2.5 ml 40 mg/ml ampicillin). Grow until the cultures reach an OD_{600} of around 0.5 (approx. 2 hrs). Add IPTG to 0.4 mM (0.4 ml 1M per liter) to induce expression. Grow cells another three hours at 37°C. Harvest cells in GS3 rotor (500 ml bottles) at 8 krpm, 5 min, 4 °C. Pour off supernatants (discard) and freeze pellets at –80 °C overnight.

2.5.2 Cell Lysis

Resuspension Buffer (50 ml) contains 20% sucrose (w/v), 100 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 mM DTT, 1 mM PMSF, 1 mM NaS_2O_5, and 1 mM Benzamidine.

Resuspend cell pellets in 10 ml per liter resuspension buffer (30 ml total). Add lysozyme to 200 μg/ml (measure volume, add e.g. 8.8 mg/ 44 ml) and incubate on ice 20 min. Add Brij 58 to 0.1% (for example add 0.44 ml of a 10% stock solution). Add KCl to 75 mM (for example add 1.1 ml 3 M stock solution) and swirl gently to mix. You should see obvious cell lysis. Spin samples in a Beckman Ty70Ti rotor at 45k rpm for 90 min at 4 °C. Remove and save supernatants containing the GST-NAP1, freeze in liquid N_2 and store at –80 °C.

2.5.3 Purification

Dialysis Buffer (4 liters) consists of 25 mM Tris-HCl, pH 7.5, 10 % glycerol, 50 mM NaCl, 2 mM DTT, 0.2 mM PMSF, 1 mM Benzamidine, and 1 mM NaS_2O_5.

Thaw and dialyze the supernatant against 2 liters dialysis buffer twice for 1.5 hr each (save 0.5 ml sample). Measure the volume of the dialyzed sample (usually 40 to 45 ml) and add 538 ml 4 M $(NH4)_2SO_4$ per liter. Stir the sample in an ice/water bath for 20 min. Centrifuge at 15k rpm, 20 min, 4 °C in SS-34 rotor and save the supernatant. Measure the volume of the supernatant, (usually 60 to 65 ml), add $(NH4)_2SO_4$ to 65% saturation (857 ml/liter 4 M), and stir in ice/water bath for 15 min. Centrifuge the samples at 15k rpm, 20 min, 4 °C in SS-34 rotor. Discard the supernatants (save a 0.5 ml sample) and store pellets at –80 °C.

Resuspend pellets in 10 ml PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSF) at 4 °C. Dialyze the sample against 2 liters PBS + 1% Triton X-100 for two hours twice at 4 °C. Save two 0.5 ml samples at –80 °C.

Equilibrate Glutathione Sepharose 4B Resin (Pharmacia C# 17-0757-01). You will need 2 ml 50% slurry per 5 mg GST-protein. We usually obtain 2.5 μg GST-protein per ml of culture. We grew 3 liters so we expect around 7.5 mg of GST-NAP1. Therefore, we need 3 ml of a 50% slurry of the resin. To prepare the resin, remove 2.5 ml resin from the stock bottle (75 % slurry), spin down the resin at 500 xg in a clinical centrifuge, 5 min, and remove the supernatant. Wash resin with 18.8 ml PBS (no Triton X-100) at 4 °C by inverting, spinning down resin at 500 xg, 5 min, removing the supernatant, and resuspending the resin in 1.88 ml PBS to produce a 50% slurry.

Test affinity purification of GST-yNAP1 with glutathione resin by adding to 0.5 ml dialyzed extract to 150 μl resin (50% slurry), incubating on rotator at 4 °C for 60 min. Pellet resin at 500 xg, 5 min and remove supernatant (save as sample). Wash the

resin with 750 µl PBS (no Triton X-100, 10 bed volumes), centrifuging the suspension at 500 xg, 5 min and discarding the washes (repeat wash two more times). Elute with 75 µl elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0, 1 bed volume) while mixing on a rotator at room temperature for 10 min. Centrifuge resin at 500 xg, 5 min. Repeat elution two more times and pool eluates.

Run samples (first dialysis, first precipitation supernatant, second precipitation supernatant, second salt pellet resuspended in PBS, Glutathione Sepharose 4B input dialyzed vs PBS-Triton X-100, supernatant from incubation with resin, Glutathione resin after elution, and eluate pool), 1 and 5 µl of each, on 8% SDS-polyacrylamide gels and stain gels with Coomassie Brilliant Blue.

Affinity purify the rest of the GST-yNAP1 with glutathione resin by adding 3 ml or 50% slurry in PBS (1.5 ml into each of two 15 ml tubes) to rest of dialyzed sample. Incubate the resin on a rotator at 4 °C for 60 min. Pellet the resin at 500 xg 5 min, remove supernatant and save. Wash the resin (resuspend) with 15 ml PBS (no Triton-X100, 10 bed volumes; 7.5 ml/tube). Centrifuge down the resin at 500 xg, 5 min, discard washes. Repeat the wash two more times. Elute the GST-yNAP1 with 1.5 ml elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0, 1 bed volume; 0.75 ml/tube) while mixing on a rotator at room temperature for 10 min. Centrifuge down the resin at 500 xg, 5 min. Repeat the elution two more times, and pool eluates.

Further purify the GST-yNAP1 to separate it from a nuclease activity by mono Q column chromatography (run column at 0.5 ml/min, AUFS = 2.0, fraction size 1 ml). Dialyze eluant against 4 liters column buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5 mM PMSF. Column buffers contain 20

mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 1 mM $Na_2S_2O_5$, and either no NaCl (Buffer A) or 1M NaCl (Buffer B). Load onto a MonoQ column (1 ml) equilibrated with column buffer at 100 mM NaCl (10% B). Wash the column with 5 column volumes 0.1 M NaCl (5ml 10% B). Elute the GST-yNap1 with a 0.1 to 0.8 M NaCl linear gradient over 50 column volumes (50 ml 10% to 80% B). Determine the peak fractions by 8% PAGE-SDS followed by Coommassie Brilliant Blue staining. Dialyze the pooled peak fraction into 10 mM Hepes-KOH, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM β-glycerophosphate, 1 mM DTT, and 0.1 mM PMSF.

2.6 Reconstitution of Chromatin with Yeast Core Histones and Yeast Nap1p

Purified yeast core histones are diluted wtih 10 mM Hepes-KOH, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM β-glycerophosphate, 1 mM DTT, and 0.1 mM PMSF to 350 μg/ml. Yeast core histone and recombinant yNap1p (at 1 mg/ml) are combined at a ratio of 4:1 (w/w) and diluted 2 fold with 50 mM Hepes, pH 7.6, 0.1 mM EDTA, pH 8.0, and 10% glycerol. The core histones and Nap1p are incubated together on ice for 30 min. The appropriate amount of core histones and Nap1p are then combined with DNA in 10 mM Hepes, pH 7.6, 50 mM KCl, 5% glycerol, 1% PEG 8000. Reconstitution was allowed to proceed at 30°C for 4 h and reconstituted chromatin templates were used immediately or stored at 4°C (never frozen) for up to a week.

2.7 Topological Assay for the Degree of Chromatin Reconstitution

Topological assays are the best way to measure the degree of assembly of your chromatin onto plasmid templates. Topoisomerase I enzyme relieves torsional stress in DNA through single-strand breakage and rejoining reactions, and requires no energy input (ATP). We pre-treat the closed circular plasmid DNA in order to start with relaxed DNA in order to know that any supercoils in the DNA was the result of nucleosome formation. Each nucleosome formed constrains one negative supercoil in the DNA wrapping the core histone octamer and forms a compensating positive supercoil in the DNA linking the nucleosomes. The positive supercoils in the non nucleosomal DNA are subject to removal by the topoisomerase I. Upon extraction and purification, the DNA retains the negative supercoils constrained by the nucleosomes. The degree of supercoiling is assayed by electrophoretic mobility on native agarose gels.

2.7.1 One Dimensional Topological Analysis

To analyze the degree of reconstitution, 6 μg of plasmid DNA (for 10 assembly assay reactions) is relaxed with 3.5 U topoisomerase I (MBI Fermentas, or recombinant *Drosophila* topoisomerase I purified according to Hsieh et al. (3)) in 50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, and 30 μg/ml BSA for 1 h at 30 °C in a 100 μl reaction volume. Following the initial relaxation, 20 U more topoisomerase I is added and the DNA is combined with the core histone/yNAP1 complex at various ratios. Each reconstitution reaction contains 500 ng relaxed DNA and the appropriate amount octamer: Nap1p complex in 10 mM Hepes, pH 7.6, 50 mM KCl, 5.5 mM $MgCl_2$, 5% glycerol, 1% PEG 8000, and 140 ng BSA in a reaction volume

of 19 μl. Reconstitution was allowed to proceed for 4 hrs at 30°C. The reaction was stopped by the addition of 100 μl STOP (20 mM EDTA, 0.1% SDS, 200 mM NaCl, and 0.25 mg/ml glycogen) and 12.5 μg proteinase K, and incubated at 37°C for 20 min. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by ethanol precipitation. The samples are split in half and run on 1% TBE-agarose gels +/- chloroquine then stained with ethidium bromide or SYBR Gold. The optimal chloroquine concentration in the second gel must be titrated for the specific plasmid DNA used, but is usually in the 2 to 4 μg/ml range. By comparing the sample run in the absence and presence of chloroquine we obtain a clear measure of the degree of assembly (Figure 3.2A, Chapter3).

2.7.2 Two Dimensional Topological Assay

To quantitate the number of nucleosomes formed on the plasmid DNA we use two dimensional topological analysis based on a procedure described by Peck and Wang (6) as modified by Shimamura et al (9). The sample preparation is identical to that for 1 dimensional topological analysis. Load 750 ng of DNA purified from chromatin reconstituted in the presence of topoisomerase I on a 20 x 20 cm 1% TBE (1X) gel. Load the samples in the far left lanes with two empty lanes in between them. Typically, we can load two experimental samples, one topological marker and one fully relaxed DNA sample on a single gel (for examples see Pilon et al. (7) and Laybourn and Kadonaga (4)). The first sample is loaded into lane 2, the gel run 20 min at 160 Volts, then the next sample is loaded in lane 5, the gel run 20 min at 160 volts. Finally, the markers (supercoiled and relaxed, respectively) are loaded at the same time into lanes 8

and 10. The gel is electrophoresed in the absence of chloroquine for 16 h at 58 Volts. The gel is then equilibrated in chloroquine (the optimal concentration differs for each plasmid, but generally ranges from 2 to 4 μg) for 6 to 8 hours in 1X TBE at room temperature while protected from light. The gel is turned 90 degrees (often requires some trimming) such that the left side of the gel is now at the top, and electrophoresed for 16 h at 67 volts. Topological markers can be produced by mixing DNA extracted from partial assembly reactions (intermediate histone:DNA ratios) in the presence of topoisomerase I. Alternatively, markers can be made by mixing DNA combined with increasing amounts of ethidium bromide in the presence of topoisomerase I, followed by extraction with butanol and purified. Following electrophoresis, the DNA is visualized by ethidium bromide or SYBR Gold staining (Figure 2.1).

2.8 Micrococcal Nuclease Digestion Analysis of the Quality of the Reconstituted Chromatin

To assess the quality of the chromatin formed, as measured by evenness of nucleosome spacing, we use micrococcal nuclease digestion. The DNA fragments produced are purified by extraction and precipitation and resolved by native agarose gel electrophoresis. Micrococcal nuclease makes double stranded cleavages preferentially in the DNA linking nucleosomes. A limited digestion of the chromatin DNA produces the characteristic "ladder" of DNA fragments corresponding to mono-, di-, tri-, tetra-, etc. nucleosomes. Generally, we consider a seven or greater rung ladder to be acceptable. The fragments produced from assembly with yeast core histones and NAP1

are multiples of 160 to 170 bp. Fragments of less than 100 bp indicate incomplete assembly.

Assemble chromatin on 2.1 μg supercoiled DNA (no topoisomerase I or $MgCl_2$) in 210 μl. To the assembly reaction add 290 μl 10 mM Hepes, pH 7.6, 50 mM KCl, 1% PEG 8000, 5% glycerol, and 5mM $CaCl_2$ (freshly made). Warm the sample to 37 °C for 5 min, then add 15 μl 1:20 dilution of micrococcal nuclease stock (2 μl 500 U/μl micrococcal nuclease stock (Worthington) plus 38 μl 10 mM Hepes, pH 7.6, 50 mM KCl, 1% PEG 8000, 5% glycerol, and 5mM $CaCl_2$) and incubate samples at 37 °C. Remove 125 μl aliquots at 1, 2, 4, and 8 min into tubes containing 25 μl 10 mM Tris-HCl, pH 8.0 and 0.5 M EDTA. Add 200 μl chromatin STOP (see above), then add 15 μl 2.5 mg/ml proteinase K, incubate at 37 °C for 20 min. Add 300 μl phenol/ $CHCl_3$/IAA, vortex, spin 5 min. Transfer 350 μl of upper aqueous layer to new tubes containing 23.3 μl 4 M NH_4OAc, mix. Add 990 μl 100% ethanol, invert, centrifuge 15 min. Remove supernatant with a drawn pipette and dry pellets for 5 min on a 60 °C heating block or until ethanol is gone. Resuspend pellets in 15 μl dH_2O, heat at 60 °C for 2 min, vortex, spin down, then add 3 μl 6X loading buffer. Run samples on a 1.2 % agarose-TBE gel with 100 bp markers until the bromophenol blue dye has run to 0.8 the length of the gel. The gel is then stained with ethidium bromide or SYBR Gold, destained, and the image digitally scanned (Figure 3.2B, Chapter 3).

2.9 Sucrose Gradient Purification of Assembled Chromatin Templates

We have found it to be useful at times to purify our chromatin templates away from the GST-yNAP1. The most reliable method we have found is sucrose gradient

sedimentation. We have found sizing column chromatography to work, as well. However, sucrose gradient sedimentation has the added benefit of fractionating fully assembled chromatin from partially assembled DNA templates. We have found that trying to remove GST-yNAP1 using glutathione-agarose beads results in disassembly of the chromatin.

The only draw back to purification of the chromatin templates is a slight (2 to 3-fold) dilution. The effect of the dilution can be minimized by assembling a larger quantity of chromatin at a higher concentration. For example, we typically assemble 50 μg of DNA in a 0.6 to 1 ml volume.

We prepare two solution containing 5% or 30% sucrose, respectively, and 10 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 0.1 mM benzamidine. We form 13 ml 5% to 30% linear sucrose gradients in Beckman SW41 ultracentrifuge tubes. The assembled chromatin is layered onto top of a gradient and the gradient centrifuged at 40,000 rpm for 4 hr at 4 °C in a Beckman SW41 rotor. We collect 1 ml fractions from the bottom of the gradient. The peak is determined by DNA agarose gel (1%), and SDS-PAGE (18%) as shown in Figure 2.2. The concentration of the chromatin can be estimated by comparison with a known standard sample. Concentration determination using UV spectroscopy is generally unsatisfactory.

2.10 General Comments

Assembly of yeast chromatin using purified core histones and yNAP1 works efficiently and produces good quality chromatin in the absence of cofactors or additional assembly factors. Early on, we attempted to form chromatin with yeast core histones

using salt dialysis methods, but met with no success. For undetermined reasons, this approach results in insoluble aggregates at core histone to DNA ratios above 0.4 (w/w). We have shown that chromatin formed with purified yeast core histones and yeast NAP1 recapitulates the chromatin structure, including nucleosome positioning, on the *PHO5* promoter (10). We have also used these chromatin templates to investigate the mechanism of transcriptional activation on this promoter. We have found yNAP1 assembles nucleosomes as efficiently on linear fragments as on closed circular plasmids. Finally, we have found yNAP1 to assemble chromatin with recombinant yeast core histones or purified native yeast core histones with equivalent efficiency and quality. In fact, we have found yNAP1 works well with a broad range of core histones, recombinant or native, from yeast, *Drosophila*, and *Xenopus*.

2.11 Acknowledgements

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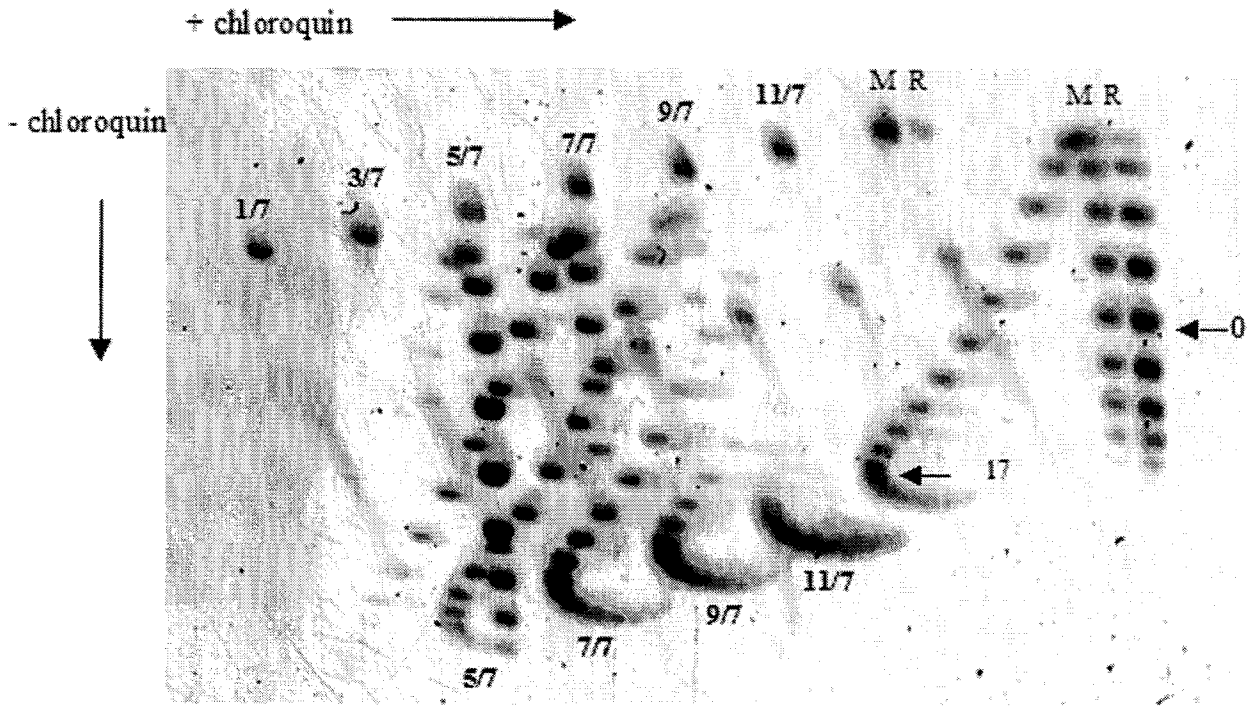

Figure 2.1. Two-dimensional Topological analysis. Plasmid DNA (350 ng) from chromatin reconstituted in the presence of topoisomerase I, purified and resolved on 1% agarose gel. The first dimension is in the absence of chloroquin. The second dimension is resolved in the presence of chloroquin (3.5 μM). Chromatin is reconstituted with different ratio of core histone to DNA (w/w); 1/7, 3/7, 5/7, 7/7, 9/7 and 11/7. Each samples, supercoiled markers (M) and relaxed markers (R) are indicated.

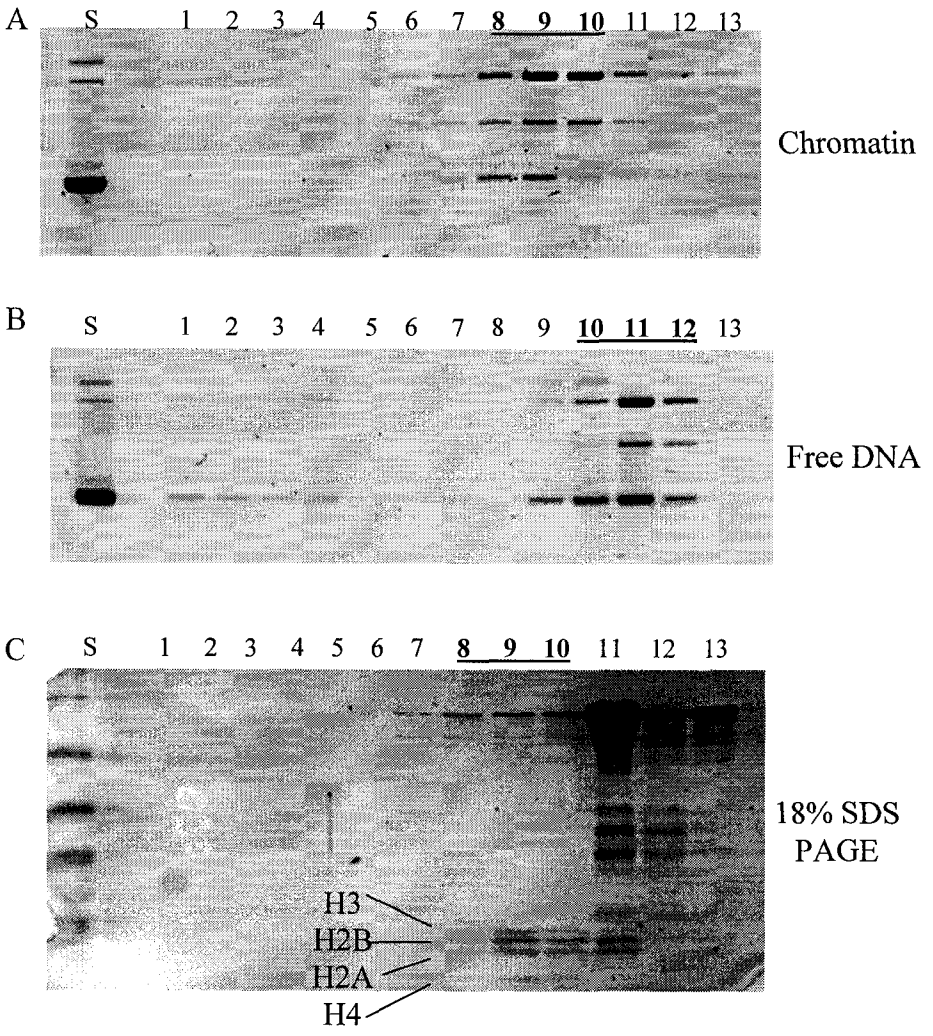

Figure 2.2 Sucrose Gradient ultracentrifugation of the assemble chromatin on plasmid DNA pPHO5 G-less. S = Supercoiling DNA. Number 1 to 13 is the fraction containing DNA and proteins. After chromatin assembly and ultracentrifugation, one hundred micro liter from 1 ml fraction is deproteinized, phenol/chloroform and ethanol precipitation. The resulting DNA is dissolved in water and run on 1% gel electrophoresis. Fifty microliter is TCA precipitated and run on SDS–PAGE 18%.

Chapter 3

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

This chapter was published in *Journal of Biological Chemistry.* The following text is identical to that published in the journal. The references and figure numbers have been re-formatted to maintain consistency with the rest of the dissertation. For preparation of this manuscript, I contributed the experiments for Figure 3.2A, B and C, Figure 3.6 and Figure 3.7A and B. I participated in the writing of Materials and Methods for Figure 7 and assisted in proofreading the manuscript. The reference for this chapter is

Terrell, A. R., S. Wongwisansri, J. L. Pilon, and P. J. Laybourn. 2002. Reconstitution of nucleosome positioning, remodeling, histone acetylation, and transcriptional activation on the PHO5 promoter. J Biol Chem **277:**31038-47.

3.1 Summary

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

3.2 Introduction

Chromatin functions to compact and organize DNA in the nucleus of eukaryotic cells in a manner that allows regulated access to genes for transcription and DNA replication. The role of nucleosomes in transcriptional regulation has become a major

area of study. From *in vitro* studies, it is clear that nucleosomes can repress transcription by RNA polymerase II (27, 28, 32). Further confirmation that nucleosomes play a prominent role in gene regulation came from studies showing that histone H4 depletion in yeast cells results in nucleosome loss and transcription derepression of several RNA polymerase II transcribed genes (16, 17). Barring artificial loss, nucleosomes must be reconfigured prior to transcriptional activation of these genes. Recently, many yeast activities that remodel chromatin have been identified, including the SWI/SNF, INO80, ISW1, ISW2, and RSC complexes (reviewed in (21)). All of these activities contain a DNA- or chromatin-dependent ATPase subunit required for remodeling. In addition, many transcriptional activators recruit histone acetyltransferase activities, which in yeast include the ADA, SAGA, NuA3, and NuA4 complexes (15, 34).

The chromatin structure of the yeast *PHO5* promoter regulates RNA polymerase II transcription of the *PHO5* gene, which encodes the major, secreted acid phosphatase in yeast (4). Under repressive conditions (adequate phosphate) the *PHO5* promoter is bound in an array of positioned nucleosomes (2). Activation of *PHO5* through phosphate starvation is accompanied by a loss or reconfiguration of four nucleosomes from the promoter region (3).

Activation is initiated through a signal transduction pathway that ultimately results in dephosphorylation of the transcription factor Pho4p, allowing its transport into the nucleus where it can bind to two upstream activation sequences on the *PHO5* promoter, UASp1 and UASp2 (11, 25, 30, 42). Pho2p, a second transcription factor involved in *PHO5* activation, binds cooperatively with Pho4p *in vitro* at both UASs (5).

Pho4p and Pho2p must physically interact *in vivo* for transcription activation to occur (37). This interaction can occur regardless of phosphate concentration but does require the presence of DNA (29). Both transcription factors are required for full chromatin remodeling and activation of the *PHO5* promoter (46). However, Pho4p is the primary trigger for activation, as Pho2p appears to be constitutively expressed and active. In addition, overexpression of *PHO4* in a *pho2* null strain is sufficient for the full chromatin transition and partial transcriptional activation (11).

Chromatin remodeling on the *PHO5* promoter occurs in the absence of replication and does not require transcription (12, 36). These findings suggest that chromatin remodeling and activation of transcription can occur independently. Gcn5p, the catalytic subunit of ADA and SAGA HAT complexes is required for full *PHO5* derepression in a Δ*pho80* strain or a Δ*rpd3* strain and full activation in the absence of one of the UASs (14, 43). Further, the rates of *PHO5* chromatin remodeling and transcriptional activation are significantly delayed (2-3-fold) in a Δ*gcn5* strain (6). Interestingly, this delay appears to be chromatin structure rather than activator (Pho4p) specific. Esa1p, the catalytic subunit of NuA4, is indicated to be involved in maintaining the H4 acetylation state on the *PHO5* promoter (43). Swi/Snf is not required for activation of *PHO5,* as the *PHO5* chromatin transition is unaffected and acid phosphatase levels achieve 70% of wild type levels under activating conditions in strains carrying a *SNF2* disruption (13). A requirement for RSC has not been tested and ISW1 and ISW2 do not seem to have a role in *PHO5* regulation (26). However, *INO80* has been reported to be required for full *PHO5* activation (9, 38). Although the *trans*-acting factors and *cis*-acting sequences required for chromatin structure modulation and

transcription activation of *PHO5* have been extensively studied, the detailed mechanisms of this process are not yet understood.

Using minichromosome templates isolated from yeast cells, remodeling on *PHO5* promoter was shown to require Pho4p, Pho2p, ATP, and fractionated nuclear extract, further elucidating the mechanism of remodeling (20). However, to study the fine details of chromatin structure modulation, a fully defined chromatin template was required. Here we describe the reconstitution of chromatin templates with purified, recombinant yeast core histones possessing many important aspects of the repressed *PHO5* promoter. Based on footprinting results, we conclude that sequence-dependent intrinsic properties of the DNA can produce translational positioning of nucleosomes on the *PHO5* promoter that approximates that seen *in vivo*. Consistent with nucleosome positioning, reconstitution of nucleosomes on the *PHO5* promoter strongly repressed transcription. Both Pho4p and Pho2p can bind their cognate sites on chromatin-assembled templates without the aid of remodeling factors. However, nucleosome remodeling by these transcriptional activators requires an ATP-dependent activity in a yeast nuclear extract fraction. Finally, transcriptional activation on chromatin templates requires acetyl CoA in addition to these other activities and cofactors. These findings indicate that transcriptional activation requires Pho4p, Pho2p, nucleosome remodeling, and nucleosome acetylation. Further, DNA binding, nucleosome remodeling, and transcriptional activation are separable steps, in concordance with the ability to separate nucleosome remodeling and transcriptional activation *in vivo* (11, 12, 36). Finally, our results suggest that Pho4p and Pho2p binding to UASp2 in nucleosome –2 occurs prior

to nucleosome remodeling or acetylation, which then function subsequent to activator binding.

3.3 Experimental Procedures

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

3.3.2 Nucleosomal Template Reconstitution-- Recombinant yeast core histones, purified as described (44) were provided by the Luger laboratory. Yeast core histones and recombinant yeast Nap1p were combined at a total core histones to Nap1p ratio of 1:8 (w/w). Acetylated bovine serum albumin (New England Biolabs) was added to 100 μg/ml, Igepal (Sigma) was added to 0.02 %, and the mixture was dialyzed for 16 h at 4 °C against 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 120 mM NaCl, 0.2 mM PMSF, 0.5 mM DTT, and 0.02 % Igepal. Following dialysis glycerol was added to 5% and the complex was stored at 4°C. For chromatin reconstitution, the core histones and Nap1p were pre-incubated at 37°C for 15 min. Then the appropriate amount of core histones and Nap1p were combined with DNA under the same conditions. Reconstitution was

allowed to proceed at 30°C for 45 min and reconstituted chromatin templates were stored at 4°C.

3.3.3 Nucleosome Footprinting by Micrococcal Nuclease Digestion of Reconstituted Chromatin and Multi-Round Primer Extension-- Reconstituted chromatin (3 μg DNA) was digested at 37°C in 200 μl at 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, and 4 mM $CaCl_2$. Micrococcal nuclease (Worthington) was added to 1.0 unit/ml and digested for 2 and 4 minutes. Free DNA (3μg) was incubated under the same conditions except that micrococcal nuclease was added to 0.35 U/ml and digested for 1 and 2 minutes. Aliquots of 100 μl were transferred to tubes containing 12 μl 0.5 M EDTA, 5μl of 2.5 mg/ml proteinase K (Sigma) and incubated at 37 °C for 10 min. The DNA fragments were extracted, precipitated, washed, and dried. The DNA was resuspended in H_20 and the concentration determined by A_{260}. Seventy-five nmoles of a ^{32}P end-labeled primer was added to 250 ng of DNA. The sequence of the primers used were

5'-CCACGTGTGAGTGCCAAG-3' (mn2),

5'-ATGAGGAAAGGAGAGTAGGGTGGTATA-3' (ml58), and

5'-GAATTGTCGAAATGAAACG-3' (mn5). The primers bind where indicated in Figure 3.1A. Multiple-round primer extension reactions were carried out in 50 μl reactions containing 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 10 mM HEPES-KOH pH 8.4, and 50 mM KCl. Five cycles of primer extension were done. Extension products were extracted, precipitated, resuspended in formamide loading buffer, and resolved on a 6% sequencing gel. The gel was dried and exposed to a PhosphorImager screen.

3.3.4 One Dimensional Topological Analysis-- To analyze the degree of reconstitution, 5 μg of DNA was relaxed with 10 U Topoisomerase I (MBI Fermentas) in 50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, and 30 μg/ml BSA for 40 min at 37 °C in a 100 μl reaction. Following initial relaxation 10 U more of Topoisomerase I was added. Reconstitution reactions contained 500 ng of relaxed DNA, 3 mM $MgCl_2$ and octamer:Nap1p complex in reconstitution buffer in a 19 μl reaction volume. Reconstitution was allowed to proceed for 2 hrs at 30°C. The reaction was stopped by the addition of 100 μl of STOP (20 mM EDTA, 0.1% SDS, 200 mM NaCl, and 0.25 mg/ml glycogen) and 12.5 μg of proteinase K, and incubated at 37°C for 20 min. The DNA was extracted and precipitation as before. The DNA was divided and run on two 1% TBE-agarose gels, one of which contained 1.8 μg/ml chloroquine, and visualized with ethidium bromide.

3.3.5 Micrococcal Nuclease Digestion of Reconstituted Templates-- Two micrograms of DNA, either naked or reconstituted chromatin (CH: DNA w/w ratio of 1:1), was digested in a 100 μl reaction volume in reconstitution buffer and 5 mM $CaCl_2$. Naked DNA was digested with 0.002, 0.004, 0.008, 0.016, and 0.024 U MNase, and chromatin was digested with 0.005, 0.02, 0.05, 0.10, and 0.20 U MNase at 37 ˚C for 10 min. DNA was purified as before and resolved on a 10 cm 1 % agarose-TBE gel. The gel was stained with ethidium bromide and digitally scanned.

3.3.6 Pho4p Purification-- Purification of Pho4p (expression vector T7-PHO4, a gift from the O'Shea lab) was adapted from Kaffman *et al.* (24) and is as follows. Pho4p was expressed in BL21 DE3 *E. coli* with 1 mM IPTG at an OD_{600} of 0.7 for 3 hours at 30°C in 1 l of LB broth containing 100 μg/ml ampicillin. The cells were washed once in 30 ml RB0.1 buffer (20 mM Tris-OAc pH 7.9, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM β-mercaptoethanol, and 100 mM K acetate), resuspended in 5 ml RB0.1, sonicated, and the debris pelleted at 10,000 rpm for 20 minutes at 4°C in a Sorvall SS-34 rotor. The lysate was loaded at 0.7 ml/min onto a 10 ml DEAE Sepharose FF (Pharmacia) column equilibrated in RB0.1 column and the column was washed with RB0.1. Proteins were eluted with RB1.0 (same as RB0.1, but with 1 M K acetate) and the peak Pho4p protein fractions were determined by SDS-PAGE and Coomassie staining. The peak fractions were pooled and dialyzed into RB0.3 for six hours at 4°C then loaded at 0.4 ml/min onto a 5 ml SP Sepharose FF (Pharmacia) column equilibrated in RB0.3. The column was washed with RB0.3 then the proteins were eluted with RB1.0. Peak fractions were determined as before. The Pho4p SP Sepharose pool was dialyzed against RB0.1 then loaded onto a Mono-Q column equilibrated in the same buffer. The column was washed with RB0.1 and proteins were eluted with a RB0.1 to RB1.0 linear gradient at 0.5 ml/min over 20 column volumes. Peak fractions were determined as described above, protein concentrations were quantitated using the Coomassie Plus Protein Assay Kit (Pierce), and stored in liquid nitrogen.

3.3.7 Pho2p Purification-- Purification of Pho2p was adapted from Brazas and Stillman (8). The Pho2-His expression vector (M2025, a gift from David Stillman) was

transformed into BL21 pLysS *E. coli*. Two hundred fifty ml of LB was supplemented with 34 μg/ml chloroamphenicol and 100 μg/ml ampicillin. Pho2-His was induced with 1 mM IPTG at an OD_{600} of 0.8 for three hours at 30°C. Cells were washed once in His binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole, and 1 mM PMSF). Cells were pelleted and resuspended in 10 ml His binding buffer. Lysozyme was added to 1 mg/ml and the cells were incubated on ice for 15 min then sonicated. Igepal (Sigma) was added to 0.1% and the lysate was clarified by centrifugation at 12,000 X g for 30 min at 4°C. The lysate was applied to a 1 ml Ni^{+}-NTA-Agarose column (Qiagen) equilibrated in His binding buffer. The column was washed with His binding buffer followed by His binding buffer plus 60 mM imidazole. Pho2-His was eluted with a linear 60 mM to 600 mM imidazole gradient in His binding buffer. The peak fractions were determined by Coomassie-stained SDS-PAGE gel and dialyzed for 4 hr at 4°C against storage buffer (20 mM HEPES-KOH pH 7.9, 10 % glycerol, 0.5 mM EDTA, 0.5 mM DTT, 100 mM K acetate, 1 mM PMSF, 10 mM β-mercaptoethanol, and 1 mM benzamidine). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce).

3.3.8 Electrophoretic Mobility Shift Assays (EMSA)-- For the competition EMSA both wild type and mutant probes were 24 bp double stranded oligomers. EMSA gel showing Pho4p and Pho2p ternary complex formation run with the indicated amounts of Pho4p and Pho2p (Figure 3.3B) and 0.4 ng of labeled probe and 100 ng poly dIdC in 15μl of transcription buffer. The sequences of the mutant oligos were 5'-TTGCCTACGCCGGCGACTCCGCGA-3' and

5'-TGATTCGCGGAGTCGCCGGCGTAG-3'. The sequences of the wild type probes were 5'-TGATTAAAAGAGTTAATTGAATAG-3' and 5'-TTGCCTATTCAATTAACTCTTTTA-3'. Oligos were annealed and all probes were labeled with Klenow using α^{32}P-dATP. Reactions were 12 µl and contained 50 ng labeled probe, 225 ng Pho2p competitor as described in the Figure 3.3 legend, and EMSA binding buffer (12 % glycerol, 20 mM Tris-Cl pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and 100 µg/mL BSA). Reactions proceeded on ice for 15 min with labeled probe then an additional 15 min with competitor. Polyacrylamide gels were 5% acrylamide (40:1 acrylamide:bis) in 0.5 X TBE. EMSA gels were run at 30 mA for 1.5 hrs (24 bp UASp1 probe, Figure 3.3B) or 2.5 hrs (106 bp UASp2 probe, Figure 3.3C).

3.3.9 Pho4p and Pho2p Footprinting by Deoxyribonuclease I Digestion and Multi-Round Primer Extension-- To verify reconstitution, chromatin templates were digested with MNase and analyzed on a 1.2% TBE-agarose gel for production of a ~170 bp ladder. Naked or reconstituted chromatin DNA (500 ng), 100 ng of poly dI/dC, purified Pho4p and Pho2p (amounts indicated in Figure 3.4 legend), 1% PEG 3350, 50 mM HEPES-KOH pH 7.6, 100 mM potassium glutamate, 5 mM EDTA, 10 mM magnesium acetate, 2.5 mM DTT, and 10% glycerol in a 20 µl reaction volume were incubated at 30°C for 15 min. DNaseI (Worthington) was added (2 U/ml for naked DNA, 16 U/ml for chromatin) and DNA was digested at 30 °C for 2 min. The reaction was stopped and the DNA was purified as described above (one dimensional topological analysis). Twenty cycles of primer extension was conducted in 50 µl 50 mM KCl, 10 mM Hepes-KOH pH 8.4, 0.75 mM $MgCl_2$, 0.05 mM dATP, dTTP, dGTP, and dCTP, 125 ng DNA,

1 U *Taq* polymerase, and 0.1 pmol γ^{32}P-ATP end labeled primer (5'-ATTTGGCATGTGCGATCTCTT-3'). DNA products were extracted and precipitation. Products were resuspended in TBE-formamide dye and electrophoresed on a 5% polyacrylamide-urea gel next to dideoxy sequence generated using the same primer and template. The gel was then dried and visualized using a PhosphorImager screen.

3.3.10 Whole Cell Extract Preparation-- Whole cell extracts (WCE) were prepared from yeast strains YS25 (*MATα; his3-11, 15; leu2-3,112; ura3Δ5; pho4::ura3Δ5; canR*) (a gift from Dr. B. Meyhack) and YS27 (*MATα; his3-11,15; leu2-3,112; ura3Δ5; pho4::ura3Δ5; pho2::ura3Δ5; canR*) (a gift from Dr. W. Hörz). The extracts were prepared according to the procedure of Woontner *et al.* (45).

3.3.11 Nuclear Extract Fraction Preparation of 0.3 M SP-Sepharose -- Yeast nuclear extracts (NE) were prepared and fractionated as described (20) using yeast strain YS18 (*MATα; his3-11,15; leu2-3,112; ura3Δ5; canR*) (a gift from Dr. B. Meyhack).

3.3.12 *In Vitro* Chromatin Remodeling-- One microgram of reconstituted chromatin was digested with micrococcal nuclease (MNase, Worthington) in a 100 μl reaction volume following a 30 min incubation at 30 ˚C with various components as indicated in the Figure 3.5 legend. Reactions contained 1 μg DNA, 6 mM $CaCl_2$, proteins, and ATP as described in the figure legend, and were brought to the final volume with reconstitution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 100 μg/ml BSA, 0.02 % Igepal). Naked DNA was digested with 0.004, 0.016, or 0.032 U MNase

and chromatin was digested with 0.05, 0.25, and 0.75 U micrococcal nuclease. Reactions were stopped by the addition of 100 µl stop (20 mM EDTA, 200 mM NaCl, 0.1% SDS, and 250 µg/ml glycogen) and 5 µl 2.5 mg/ml proteinase K, incubated at 37°C for 20 min, extracted with phenol/chloroform/isoamyl alcohol (IAA), extracted with chloroform/IAA, and precipitated with ethanol. Two hundred ng of DNA was run on a 20 cm 1.5% agarose gel in 1 X TBE, Southern blotted (Turboblotter, Schleicher and Schuell), and probed with either a 169 bp *Mfe* I to *BstE* II fragment representing nucleosome –2, or a 110 bp *Apo* I to *Bam* HI fragment representing nucleosome +1 (Figure 3.1A). Before reprobing, blots were stripped by boiling in 0.1 % SDS and cooling to room temperature.

3.3.13 *In Vitro* Transcription-- Transcription reactions contained naked or reconstituted DNA (500 ng), 100 µg of whole cell extract, 1% PEG 3350, 50 mM HEPES-KOH pH 7.6, 100 mM potassium glutamate, 5 mM EDTA, 10 mM magnesium acetate, 2.5 mM DTT, 10% glycerol, 2 mM ATP and CTP, 25 µM UTP, 0.25 U RNase Inhibitor (5'/3'), 30 mM phosphocreatine (Sigma), 0.042 U creatine kinase (Sigma), and 10 µCi ^{32}P-UTP in a 30 µl reaction volume. Reactions proceeded at 22°C for 30 min. RNase T1 buffer (120 µl 10 mM Tris-Cl pH 7.5, 300 mM NaCl, 5 mM EDTA) and 50 U RNase T1 (Boehringer Mannheim) were added and reactions were incubated at 22°C for 15 min. Eight µl 10% SDS, 17.5 µL 2.5 mg/ml Proteinase K (Sigma), and γ^{32}P-ATP end labeled recovery standard (approximately 1000 cpm/ reaction) were added and incubated at 37°C for 20 min. Fifteen µg of glycogen was added and nucleic acids were ethanol precipitated. The nucleic acid pellets were washed with 75% ethanol,

resuspended in TBE-Formamide dye, and electrophoresed on an 8% polyacrylamide urea sequencing gel at 20 V/cm. The gel was dried and exposed to a PhosphorImager screen.

3.3.14 Histone Acetyl Transferase (HAT) Assays--Two μg of core histones, free or assembled into chromatin, were incubated with yeast WCE from YS27 cell at 30 °C for 60 min. The reaction mixture (100 μl) contained 50 mM Tris-HCl, pH 8.0, 5% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 1 mM PMSF, 10 mM Na butarate, 0.2 μCi ^{14}C-acetyl CoA, and 4.5 μM acetyl CoA. After incubation, the proteins were resolved by SDS-PAGE (15% acrylamide 30:0.8 acrylamide:bis). The gels were coomassie stained, dried and exposed to a PhosphorImager screen. Alternatively, the incubations contained 0.5 μg core histones in chromatin and only unlabelled acetyl CoA. After SDS-PAGE, the proteins were transferred to a PVDF membrane and the acetylation state determined by Western blot using anti-unacetylated H4 antibody (Upstate Biotech). The same blotted membrane was Western blotted again using anti-acetylated H4 antibody (Upstate Biotech).

3.4 Results

3.4.1 Primer Extension Footprinting Analysis of Nucleosome Positioning on the *PHO5* Promoter— Nucleosomes are positioned on the *PHO5* promoter *in vivo*. To determine chromatin structure on the reconstituted *PHO5* promoter we used micrococcal nuclease digestion in conjunction with multi-round primer extension analysis to

footprint the nucleosomes (22, 35). The primers used for this analysis are shown in Figure 3.1A.

Nucleosome –1. We observed cleavage protection over the TATA-box and RNA start sites (Figure 3.1B and 3.1C). The protection occured between approximately +7 and -123 bp, relative to the ATG (translational start codon) and corresponds to nucleosome -1. At the downstream end of nucleosome –1, strong protection ends at +7, with slight protection seen at +10 (Figure 3.1B). No cleavage protection or enhancement is seen from +19 to +55. However, there is some ambiguity as to where protection ends due to lack of cleavage between +10 and +19 on both free DNA and chromatin samples. The upstream edge of nucleosome –1 was inferred from the protection at –123 (Figure 3.1C). From –129 through –150 there are several sites of equal or stronger cleavage, defining the linker region between nucleosome –1 and –2. The lack of cleavage between –123 and –129 on both free DNA and the chromatin precludes defining the downstream edge of nucleosome –1 with greater precision. Thus, we place nucleosome –1 between +7 (± 5) bp to –123 (± 9) bp.

The clear footprint observed with primer ml58 and primer mn5 indicates a strong translational setting for nucleosome -1. In addition, the length of DNA associated with nucleosome –1 protection is very close to the 145 bp normally associated with a core particle. This translational setting places both the initiation region and the TATA element within nucleosome -1, approximately 30 bp from each edge. Note the cleavage protection over 5 nt of the TATA element, indicating a lack of accessibility for TBP binding.

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

Nucleosome –2. Protection from micrococcal nuclease cleavage is seen from -150 (± 10 bp) through –311 (± 6 bp), corresponding to nucleosome -2 (Figures 3.1C and 3.1D). The nuclease protection is nearly complete (Figure 3.1C, near the top), which is unusual for a nucleosome. Placing the downstream edge of this nucleosome at around –150 is based upon the clear protection through this site (Figure 3.1C). Setting the upstream edge at around -311 is based on the protection from -289 to –311 and the lack of protection seen from –317 to –370 (see Figure 3.1D). The slightly greater than 145 bp area of protection generally associated with a nucleosome core particle suggests multiple translational or rotational positions. As seen *in vivo*, nucleosome -2 contains UASp2 (the Pho4p binding site) near the nucleosome dyad and a Pho2p binding site closer to the upstream edge.

Enlarged linker. An important feature of the repressed *PHO5* promoter chromatin structure is the nucleosome-free region between nucleosomes -2 and -3, which includes UASp1 (the distal Pho4p binding site). Nuclease protection beginning from approximately –370 indicates that nucleosome -3 is positioned upstream of UASp1 (Figure 3.1D). In addition, we detect a region lacking protection between approximately -366 and –317 or around 50 bp in length. This linker is larger than the approximately 30 bp length of the other linkers, corresponding to the situation *in vivo* (3). Previously, this

enlarged linker (referred to as HS2) was mapped to a region from approximately -410 to -315 *in vivo* by indirect end-labeling and restriction endonuclease cleavage analysis (3). The micrococcal nuclease protection suggests that nucleosome –3 is located farther downstream on the reconstituted templates than *in vivo*. However, what is most important is that an enlarged linker is formed that leaves the Pho4p binding site in UASp1 accessible.

3.4.2 The *PHO5* Promoter is Strongly Repressed by Nucleosome Formation *In Vitro*-- We have reconstituted plasmids with a *PHO5* promoter construct containing 526 bp of the promoter upstream of a G-less cassette (pPHO5-Gless). Nucleosomes were formed at the ratios (w/w) of core histones to DNA indicated in Figure 3.2. At the histone to DNA ratio of 1.0, nucleosomes were formed at physiological density as determined by topological assay (Figure 3.2A). Micrococcal nuclease analysis verified that complete nucleosomes were formed with uniform spacing of approximately 160 to 170 bp (Figure 3.2B). Transcription from the chromatin templates was strongly repressed at core histone to DNA ratios of 0.43 and 0.57 and essentially completely repressed at a ratio of 1.0 (Figure 3.2C).

3.4.3 Pho4p and Pho2p Bind to Both Free and Reconstituted Chromatin DNA-- Pho4p and Pho2p are both required for the chromatin transition and transcriptional activation of *PHO5* upon phosphate depletion (11). Therefore, it was necessary to purify both proteins to study the mechanism of chromatin structure transition and transcriptional activation *in vitro* (Figure 3.3A). Both proteins bind a 106 base pair

UASp1 DNA probe (*TthIII* II to *Mfe* I; Figure 3.1A) independently, and form a ternary complex when combined (Figure 3.3B) (5). Since Pho2p, a homeodomain protein, does not have well defined binding site, we tested the binding specificity of our purified Pho2p to UASp2. An unlabeled wild type probe, identical to the labeled probe (*Mfe* I to *BstE* II; Figure 3.1A), successfully competed for Pho2p binding (lanes 1 through 8, Figure 3.3C). However, a probe in which the order of the base pairs in the AT-rich region required for Pho2p binding had been randomized did not compete for Pho2p binding (lanes 9 through 14, Figure 3.3C).

Pho4p and Pho2p protect both naked DNA and chromatin from deoxyribonuclease I (DNase I) cleavage (Figures 3.4A, 3.4B, and 3.4C). Pho4p protected UASp1 and UASp2 on free DNA and chromatin (vertical lines, Figure 3.4A; horizontal lines on Figures 3.4B and 3.4C). Even though Pho2p can bind DNA in the absence of Pho4p in EMSAs (lanes 5 and 6, Figure 3.3B), the Pho2p protection (asterisks on Figures 3.4A, 3.4B, and 3.4C) was stronger with Pho4p present on both free DNA and chromatin. Protection is observed on chromatin DNA at the same Pho4p and Pho2p concentrations that produced protection on free DNA. However, protection on chromatin is more dependent on both proteins being present than protection on free DNA (particularly apparent in Figures 3.4B and 3.4C). Pho2p protected the binding site near UASp2 more strongly on chromatin than on free DNA. Pho4p protected a larger region on UASp1 on chromatin than seen on free DNA, but produced none of the adjacent heightened nuclease sensitivity. Increasing the concentration of the activator proteins past that shown did not result in more complete protection. These higher

concentrations caused reproducible, but nonspecific, alterations in the cleavage pattern, presumably due to aggregation of proteins on the DNA (results not shown).

3.4.4 A Nuclear Extract Fraction of 0.3 M SP Sepharose Remodels the *PHO5* Chromatin Structure in a Pho2p, Pho4p, and ATP-Dependent Manner-- Chromatin templates were incubated with micrococcal nuclease and the DNA fragments were resolved by agarose gel electrophoresis and detection through Southern blot analysis. The chromatin structure over the promoter region was determined using the *Mfe* I to *BstE* II probe encompassing nucleosome –2 (Figure 3.1A). Pho4 and Pho2p do not remodel chromatin on their own (lanes 10, 11 and 12 Figure 3.5A and the corresponding tracing in Figure 3.5B). A nuclear extract fraction from wild type yeast cells was shown to remodel the *PHO5* chromatin structure of isolated minichromosomes (20). On our defined chromatin templates, we found that nucleosome remodeling was mediated by this nuclear extract fraction and that it, too, was dependent on the presence of Pho4p, Pho2p, and ATP (compare lanes 7, 8 and 9 with lanes 13, 14, and 15 Figure 3.5A and the corresponding tracings in Figure 3.5B). To ascertain the range of the remodeling that occurs in the transcribed region, these blots were stripped and hybridized with the *Apo* I to *Bam* HI (110 bp) probe (Figure 3.1A) complementary to DNA in nucleosome +1. Much less remodeling occurs over the transcribed region (compare lanes 13, 14, and 15 with lanes 16, 17, and 18 Figure 3.5A and the corresponding tracings in Figure 3.5B).

3.4.5 Acetyl CoA is Required for Activation of Transcription-- The NE fraction containing chromatin remodeling activity does not support transcription, so a WCE from

a *pho4Δ* was used. The addition of Pho4p, Pho2p, and the 0.3 M SP NE fraction, sufficient for chromatin remodeling, to transcription reactions containing reconstituted chromatin did not result in transcriptional activation (results not shown). It is important to note that the formation of nucleosomes does not render the DNA irreversibly transcriptionally incompetent, as transcription from the reconstituted template can be restored by proteinase K digestion and organic extraction of the DNA (data not shown). To test whether histone acetylation was required in addition to nucleosome remodeling, acetyl CoA was added to the transcription reactions. Addition of Pho4p activates transcription approximately three- to four-fold on free DNA (Figure 3.6, compare lanes 2 through 6 with lanes 7 through 11). Acetyl CoA had no stimulatory effect and even had a slight inhibitory effect on transcription from free DNA templates at higher levels (Figure 3.6, lanes 1 through 11). Interestingly, on chromatin the addition of acetyl CoA was able to fulfill the additional requirement needed to relieve transcriptional repression and to allow transcriptional activation from the chromatin templates (Figure 3.6, lanes 13 through 23). Pho4p activated transcription further on chromatin (lanes 19 through 23), but the stimulatory effect of acetyl CoA was not dependent on Pho4p (lanes 14 through 18). Pho4p was not able to activate transcription from chromatin without acetyl CoA (data not shown).

3.4.6 Addition of Acetyl CoA to the Yeast Whole Cell Extract Results in Core Histone Acetylation on Chromatin Templates-- Acetyl CoA stimulates transcription from chromatin, but not from free DNA, suggesting that this cofactor is functioning through histone acetylation. To directly determine that the core histones are acetylated

in our transcription reactions we added ^{14}C-acetyl CoA. The primary acetyl group acceptors were histone H2A and H4, an acetylation pattern similar to that of NuA4 (Figure 3.7A). Even in the presence of WCE, no other protein was detectably labeled. In addition, the level and pattern of the HAT activity is independent of ATP-dependent remodeling activity and Pho4p activation (right hand 2 lanes, Figure 3.7A). Western blot analysis of core histone acetylation by the activity in our whole cell transcription extract was conducted, as well (Figure 3.7B). The results demonstrate that histone H4 is unacetylated in the absence of acetyl CoA. Interestingly, the H4 acetylation appears to be quantitative, since the histone H4 is no longer recognized by antibody directed against the unacetylated H4 tail. NuA4 has been shown to fully acetylate histone H4 (forms tetra acetylated H4) by transferring an acetyl group to the lysines at positions 5, 8, 12 and 16 (1). These antibodies were raised against an H4 amino terminal tail peptide, so it is conceivable that they would not recognize the fully acetylated histone H4 tail.

3.5 Discussion

3.5.1 Reconstitution of the Repressed State

The Hörz and Bergman laboratories independently identified an array of at least four translationally positioned nucleosomes on the repressed *PHO5* promoter (3, 7). Although extensive mutant searches have been conducted, to date no mutation has been found that disrupts nucleosome positioning. Therefore, we hypothesized that nucleosomes are positioned primarily through intrinsic properties of the promoter DNA sequence. To test this hypothesis, we determined the micrococcal nuclease cleavage pattern on chromatin and free DNA templates to define the nucleosome positions. We

observed positioning of a nucleosome over the TATA-box and RNA start sites (nucleosome -1) and another nucleosome over the downstream Pho2p binding site and UASp2 (nucleosome -2). Moreover, we found that an enlarged linker region between nucleosome -2 and -3 containing UASp1 is established on our reconstituted templates. Thus, the nucleosome positioning on our reconstituted chromatin templates is consistent with that seen *in vivo*.

The reconstituted chromatin templates were formed using purified yeast core histones and Nap1p, so no other DNA-binding proteins are present. Hence, and the nucleosome positioning on our reconstituted templates is directed primarily by sequence-dependent intrinsic properties of the DNA. From the results of a series of deletion experiments, Fascher *et al.* (12) concluded that intrinsic properties of the promoter DNA make "an essential contribution to the chromatin organization at the *PHO5* promoter." Therefore, the mechanism of nucleosome positioning *in vivo* is likely to be much the same as that driving the positioning on our reconstituted templates. Nucleosomes reconstituted on tandem repeats of the sea urchin 5S rRNA gene formed arrays of positioned nucleosomes (39). In addition, a 200 bp fragment from the *Drosophila Adh* promoter will translationally position a single nucleosome correctly *in vitro* (23). However, the *PHO5* promoter and the MMTV LTR promoter are the only single copy gene promoters thus far determined to contain an array of nucleosomes that are translationally positioned primarily through histone-DNA interactions (40).

There is one minor but interesting difference between the nucleosome positioning of the reconstituted and *in vivo* chromatin. While the endpoints of the enlarged linker have not been mapped to the base pair *in vivo*, the evidence available

suggests that nucleosome –3 is positioned farther upstream *in vivo* than *in vitro* (3). *In vivo,* the enlarged linker was mapped to approximately –410 through –340 (70 bp) by restriction endonuclease accessibility and low-resolution indirect end-labeling. A closer analysis of a higher-resolution indirect end-labeling experiment (3) suggests boundaries of –393 to –313 (70 to 80 bp). The enlarged linker on our reconstituted templates is formed from approximately –366 to –317 (50 to 60 bp). Therefore, the downstream endpoint of the enlarged linker on our reconstituted templates corresponds well with that seen *in vivo*. However, nucleosome –3 produces clear protection through –370. Therefore, the location of nucleosome –3 appears to be shifted 20 to 30 bp downstream *in vitro*. Similarly, on partially purified minichromosomes Nucleosome –3 is located farther downstream, suggesting that the positioning factor is being lost during preparation (20). This positioning factor is absent on our reconstituted templates, as well.

Pho4p is exported from the nucleus and is not bound to UASp1 under repressing conditions (Kaffman, 1994; O'Neill, 1996(41). In addition, the *PHO5* chromatin structure, as analyzed by low-resolution indirect end-labeling in *pho2* and in *pho4* cells, appears to be the same as that of wild type cells (11). Finally, deletion of both the Pho2p and Pho4p binding sites at UASp1 does not significantly affect the *PHO5* promoter chromatin structure (12). However, a 20 or 30 bp downstream shift of nucleosome –3 would not have been resolved in these studies. Therefore, while Pho4p is unlikely to be involved in positioning nucleosome –3 farther upstream, such a role for Pho2p or another, unidentified protein cannot be excluded at this time.

We have succeeded in reconstituting many aspects of the *in vivo* chromatin structure on the repressed *PHO5* promoter. Nucleosome formation has long been known to repress transcription *in vitro* (31, 33). However, the strong repression of transcription from the *PHO5* promoter on our chromatin templates at sub-saturating densities of nucleosomes suggests that the *PHO5* core promoter has a high affinity for nucleosome formation, positioning a nucleosome over the core promoter. Primer extension footprint analysis indicates this is the case.

3.5.2 Reconstitution of Transcriptional Activation

We began to study the mechanism of *PHO5* activation by adding back various activities and cofactors to determine their effects on the transcription level from our well-characterized chromatin template. We have found that recombinant Pho4p and Pho2p can bind to both sets of *PHO5* promoter regulatory elements (UASps) on free DNA and assemble into chromatin. This finding reiterates the importance of regulation of Pho4p function through phosphorylation and nuclear localization (24, 30). Differences in nuclease protection patterns indicate these transcription factors bind their cognate sites and interact with each other differently on free DNA versus chromatin. For example, Pho4p produces enhanced cleavages downstream of the UASps in free DNA, but protects these regions in chromatin. In addition, while Pho2p cleavage protection at both binding sites is enhanced by Pho4p, the effect at the site upstream of UASp2 is much more apparent on chromatin than on free DNA. These findings are consistent with an important role for Pho4p-Pho2p interactions in *PHO5* activation and indicate that chromatin structure participates in these interactions.

In our system, Pho4p is sufficient for transcription activation on naked DNA, presumably through increased recruitment of the general transcriptional factors and RNA polymerase II. Pho4p physically interacts with TFIIB, TFIIEβ, and TBP (29). However, Pho4p alone could not counteract nucleosome repression.

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

The presence of Mg^{++} in the transcription buffer might be promoting compaction of the templates (19). However, while compaction of the DNA may be occurring and preventing core promoter access by the general transcription machinery, it does not prevent all access to the promoter, as the transcription factors Pho4p and Pho2p have access to the DNA at the same Mg^{++} concentrations used for *in vitro* transcription experiments (Figure 3.4).

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

Core histone tail acetylation, which primarily affects chromatin compaction, is a potential additional requirement for transcriptional activation (18). To test this idea, we

added acetyl CoA to the reactions and found that nucleosomal repression was relieved. Further, we determined the core histones, particularly H4, were highly acetylated under these conditions (Figure 3.7). The level of HAT activity in our WCE is quite high and was not dependent on recruitment. Consistent with this result, the histone acetylation state on the *PHO5* promoter *in vivo* has not been found to change significantly between the repressed and activated states (43). Since transcription is dependent on the presence of acetyl CoA and only the core histones are detectably acetylated, core histone acetylation is likely to be a prerequisite for transcription from the chromatin assembled *PHO5* promoter. A requirement for acetylation other substrates after transcriptional initiation, cannot be ruled out. However, the lack of acetyl CoA stimulation of transcription from free DNA templates argues against this idea.

The core histone acetylation specificity in the yeast WCE resembles that of the NuA4 complex (10). This acetylation pattern suggests that this complex is the most abundant or active HAT activity in the extract and may be primarily responsible for counteracting chromatin repression of basal and activated transcription in the presence of acetyl CoA. Supporting this idea, we find that addition of NuA4, but none of the other three complexes, stimulated transcription further (results not shown).

Although we have not shown directly that transcriptional activation requires chromatin remodeling, the simplest interpretation of our results is that maximal activation from the reconstituted *PHO5* promoter requires Pho4p, Pho2p, remodeling activity, and histone acetylation. Our results suggest that the role of histone acetylation and nucleosome remodeling lie downstream of Pho4p and Pho2p binding. Possibilities include allowing recruitment of the basal transcriptional machinery, initiation, and

elongation. Current experimentation in our laboratory is designed to differentiate between these possibilities.

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

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Figure 3.1. Micrococcal nuclease cleavage protection pattern on the reconstituted *PHO5* templates: nucleosome –1, nucleosome –2, and the enlarged linker DNA. (A) The primer mn5 binds to the lower DNA strand and extends downstream. The primers ml58, and mn2 bind the top DNA strand and extend upstream. The location of the recognition sites for the restriction enzymes used to generate probes are indicated below the promoter. The promoter elements are indicated above the promoter, open boxes for Pho2p binding sites, filled circles for Pho4p binding sites, a filled box for the TATA element, and bent arrows for the RNA start sites. The map units indicated below the figure are in base pairs with ATG designated as +1. (B, C, and D) Multi-round primer extension was carried out as described in Experimental Procedures with (B) primer mn5, (C) primer ml58, and (D) primer mn2. G, A, T, and C refer to the ddNTP included in the Sanger sequencing reactions resolved in these four gel tracks. Lane U is undigested DNA, lane D is digested free DNA, and lane C is digested chromatin. To the left of each figure are indicated locations in bp relative to the start codon (ATG) discussed in the text. To the right of each figure is a schematic map of the *PHO5* promoter indicating the locations of promoter elements and the inferred location of the nucleosomes. The bent arrows indicate the RNA start sites.

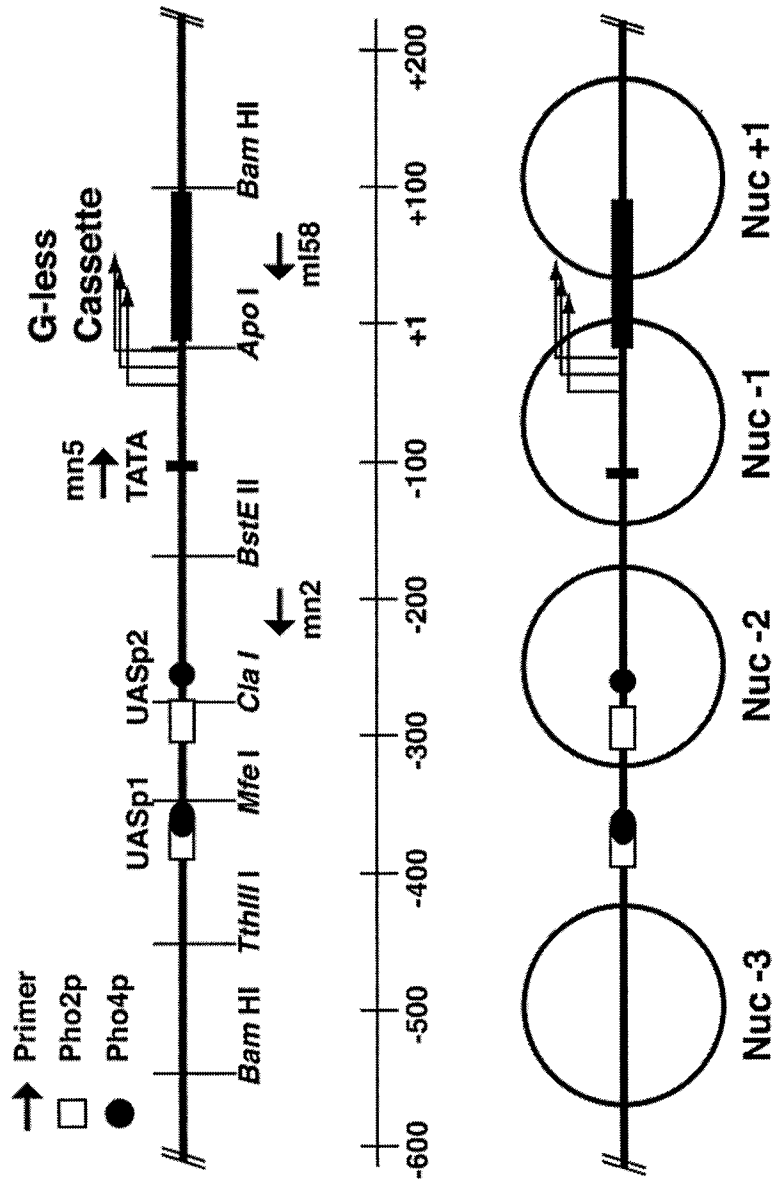

Figure 3.1A.

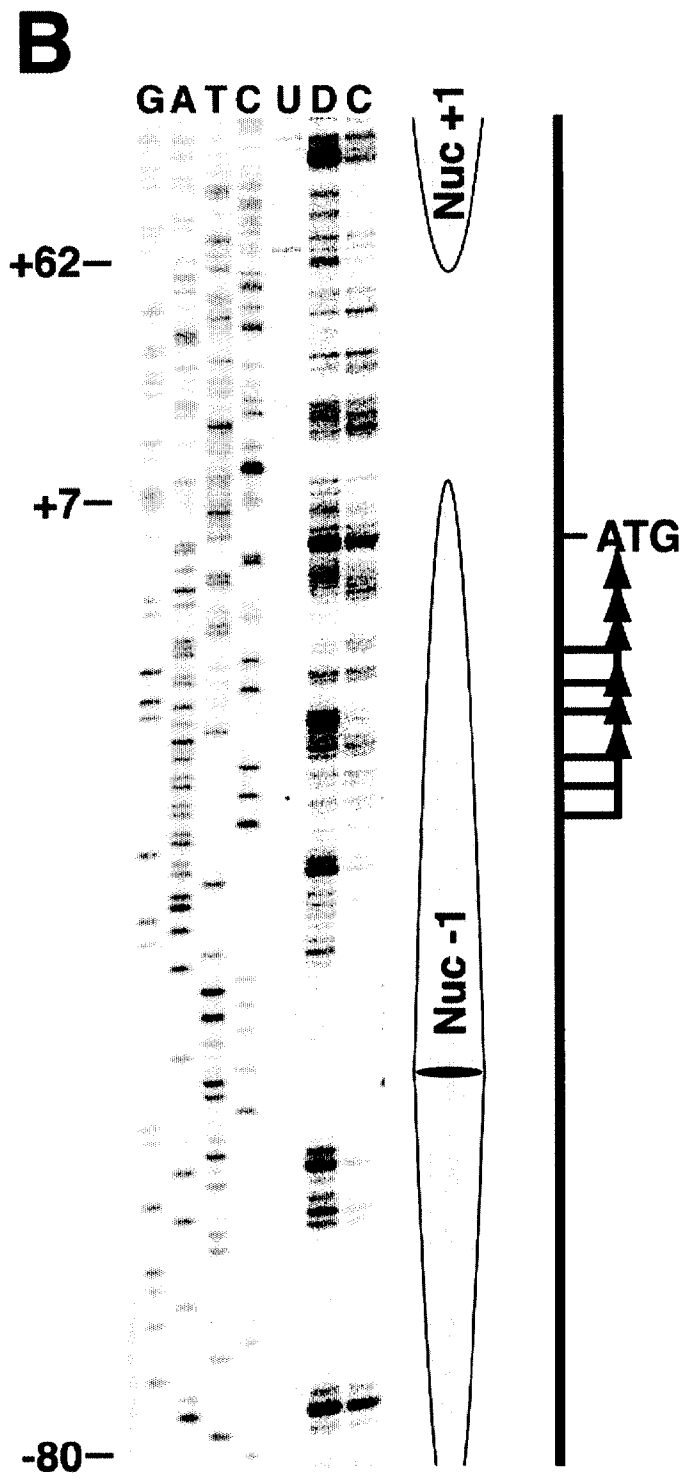

Figure 3.1B.

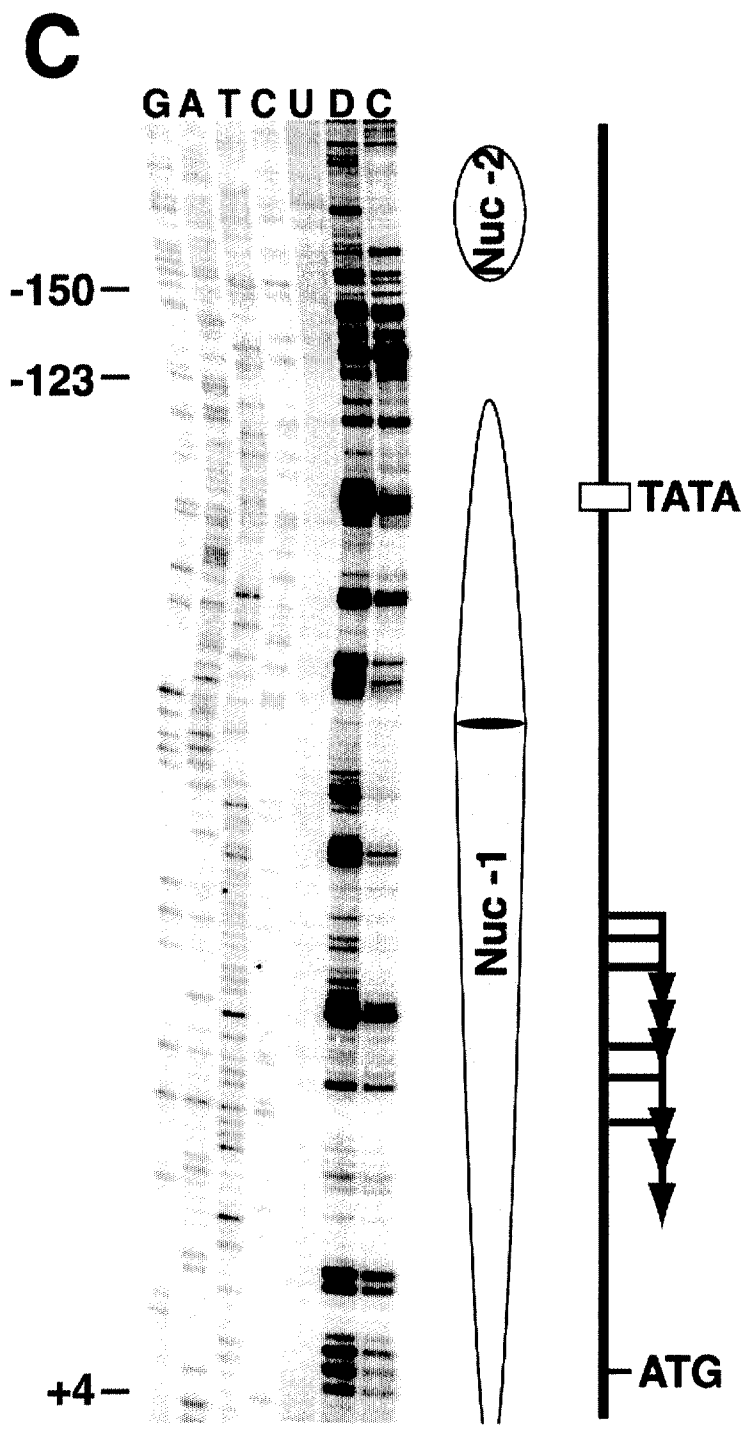

Figure 3.1C.

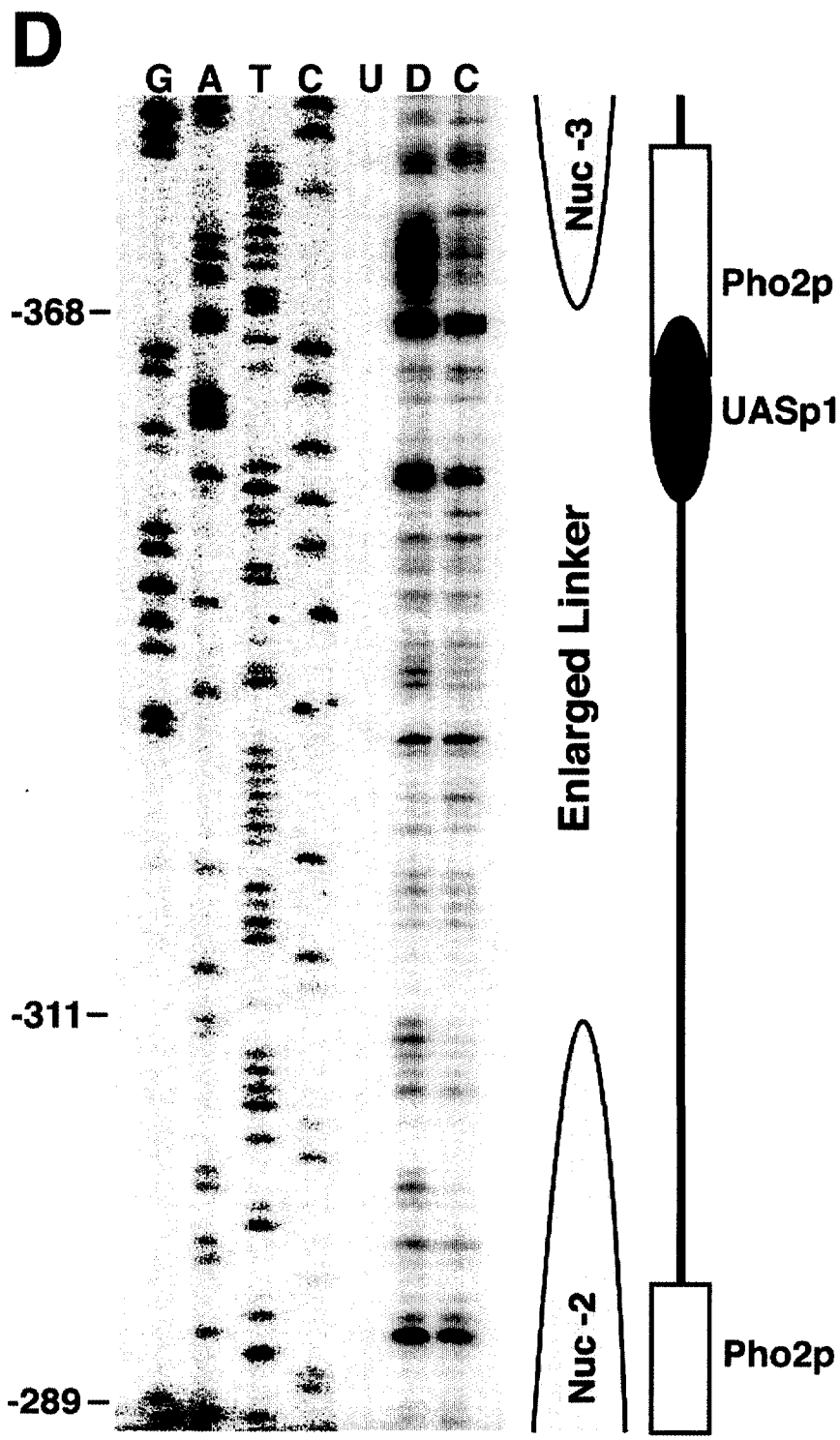

Figure 3.1D.

Figure 3.2. Strong repression of *PHO5* transcription by reconstituted nucleosomes. Plasmid templates, containing both UASs and the core promoter were reconstituted. (A) One-dimensional topological analysis. Plasmid DNA was reconstituted at the core histone to DNA ratio (w/w) indicated at the top, purified and resolved on 1% agarose gels in the absence (-Chl) or presence (+Chl) of chloroquine. Lanes S and R are supercoiled and relaxed markers, respectively. In the right margin, the positions of relaxed (Ir) and supercoiled (Is) closed circular DNA are indicated. (B) Micrococcal nuclease digestion analysis was used to verify that complete nucleosomes with uniform spacing were formed. Lane 1 contains undigested DNA. Free DNA (lanes 2, 3, and 4) and reconstituted chromatin (lanes 5, 6, and 7) were digested with increasing concentrations of nuclease and the products were resolved on an agarose gel. The digestion products corresponding to a mono-, di-, and trinucleosome are indicated as such in the right margin. Lanes M, are 100 bp DNA size markers. (C) Free DNA (lane 1) and chromatin templates reconstituted at the core histone to DNA ratio (w/w) indicated at the top (lanes 2 through 9) were transcribed using yeast whole cell extract. The *PHO5* transcripts are indicated at the left of the figure. These results were found to be reproducible through several repetitions of this experiment.

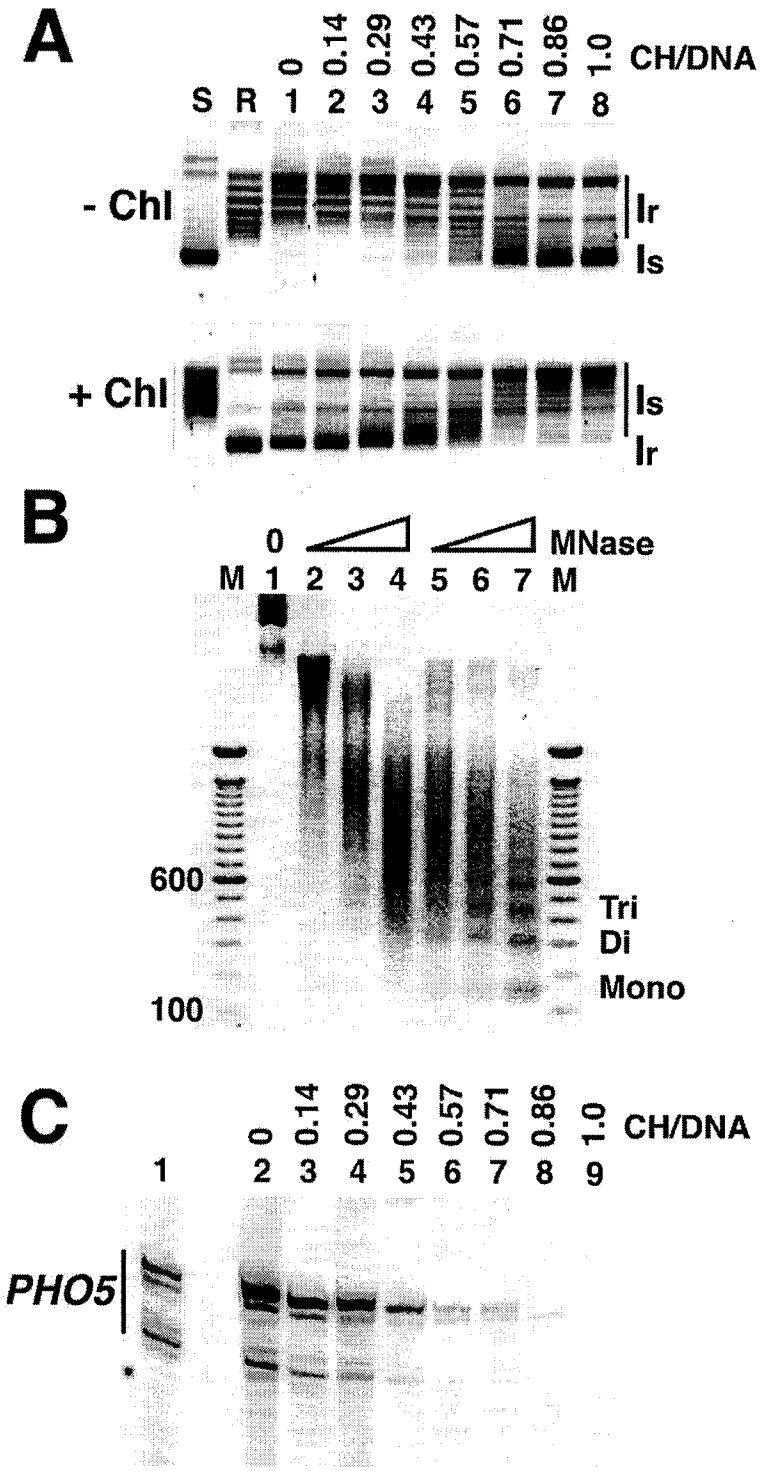

Figure 3.2A, 3.2B, and 3.2C.

Figure 3.3. Analysis of purified Pho4p and Pho2p proteins. (A) Coomassie stained gel (12%) of purified Pho4p and Pho2p. Marker sizes, in kDa, are indicated to the left of the gel. (B) Electrophoretic mobility shift assay (EMSA) showing Pho4p and Pho2p form a ternary complex on UASp1 of the *PHO5* promoter. The arrows indicate the Pho4p/DNA, the Pho2p/DNA complex, and the Pho4p/Pho2p/DNA complexes. All lanes contain 0.4 ng probe and 100 ng poly dI/dC competitor. The amounts of each protein in ng are indicated below the figure. (C) Competition EMSA demonstrating Pho2p binding specificity. Lanes 1 and 2 contain an additional 100 ng poly dI/dC, lanes 3 through 8 contain an additional 25, 50, 100, 200, 300, and 500 ng unlabeled wild type probe, respectively. Lanes 9 through 14 contain an additional 25, 50, 100, 200, 300, and 500 ng unlabeled mutant probe, respectively.

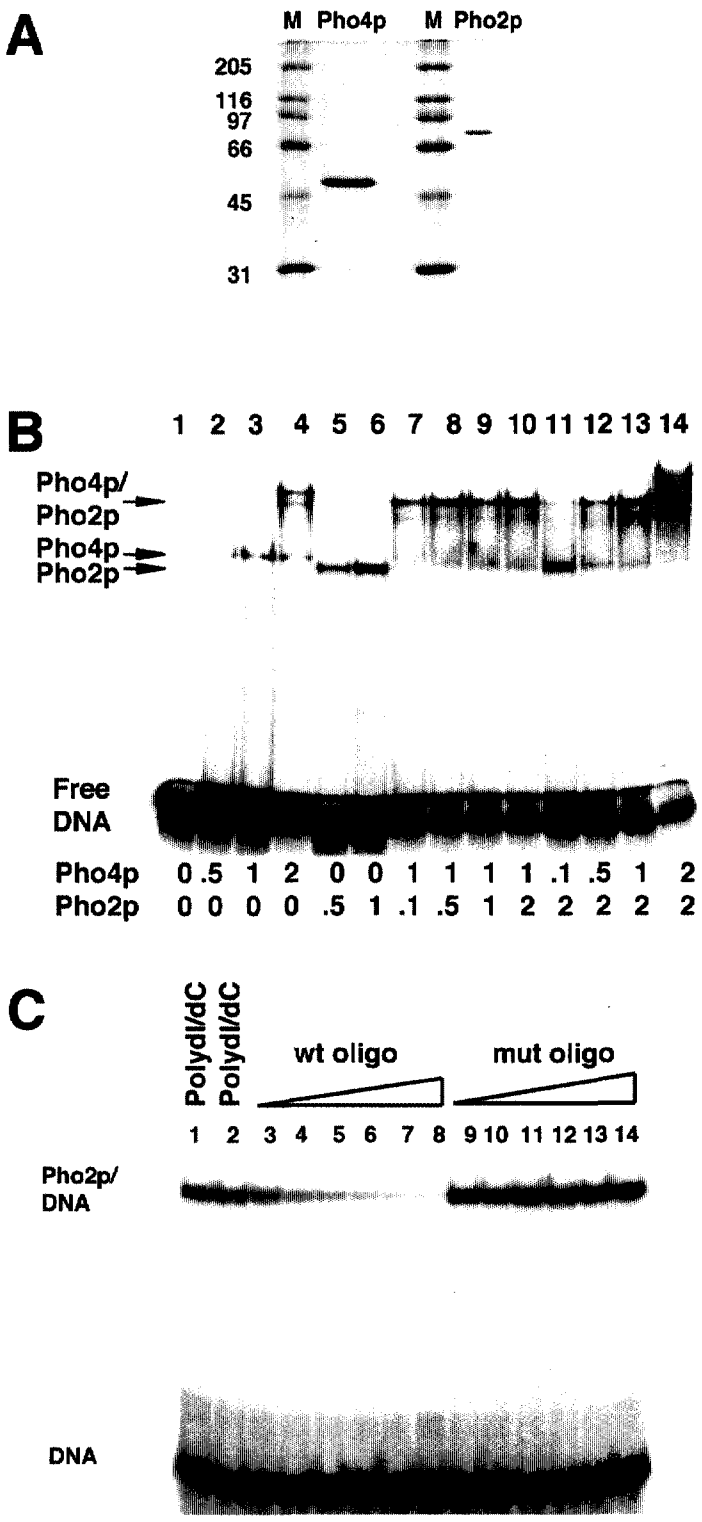

Figure 3.3A, 3.3B and 3.3C.

Figure 3.4. Primer extension footprinting of Pho4p and Pho2p binding on free and chromatin assembled *PHO5* promoter DNA. (A) Lanes 1 through 11 contain free DNA; lanes 12 through 22 contain chromatin. Lanes 1 and 12 are undigested samples (U). Lanes 11 and 22 contain no Pho2p or Pho4p (N). Lanes 2, 3, and 4 contain 250, 500, and 1000 ng Pho4p; lanes 5, 6, and 7 contain 90, 180, and 360 ng Pho2p; lanes 8, 9, and 10 contain 500 ng Pho4p and 90, 180, and 360 ng Pho2p, respectively. Lanes 13 through 21 contain identical amounts and combinations of Pho4p and Pho2p as lanes 2 through 10. The stars indicate sites of protection by Pho2p and the lines indicate the areas of protection by Pho4p. The G, A, T, and C lanes are dideoxy sequencing reactions run with the same primer as was used in the footprinting extension reactions. The ddNTP used for termination is indicated at the top of each lane. (B and C) Graphical representation of the propensity for cleavage on (B) free DNA (lanes 10 and 11) and (C) chromatin (lanes 21 and 22). A dashed line was used for digestions without Pho2p or Pho4p and a solid line was used for digestions with both Pho2p and Pho4p. Asterisks indicate cleavages reduced by Pho2p and lines indicate regions of cleavage reduced by Pho4p. The location of previously defined Pho2p and Pho4p binding sites are cartooned below the graphs (rectangles for Pho2p sites and ovals for Pho4p sites).

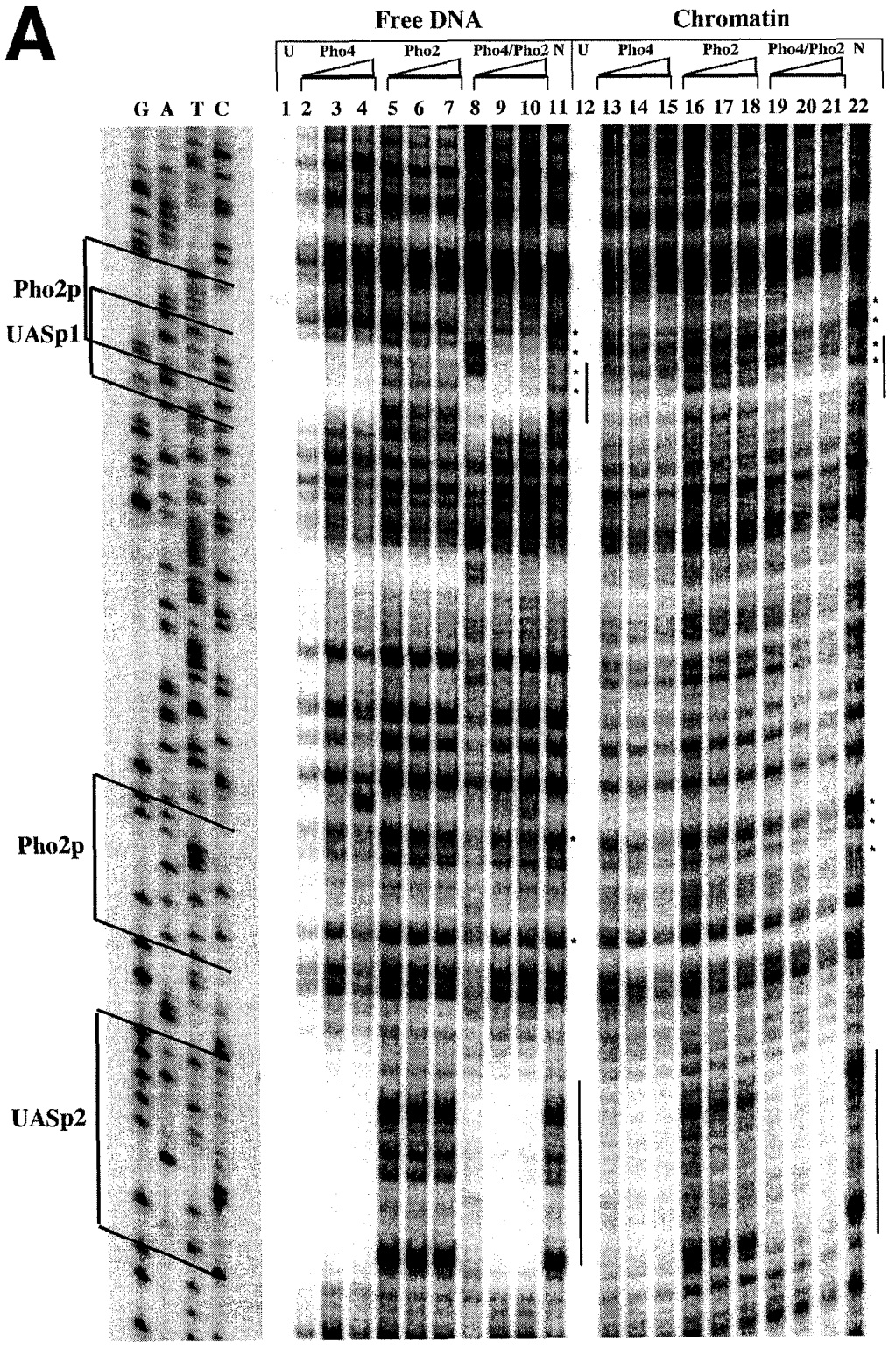

Figure 3.4A.

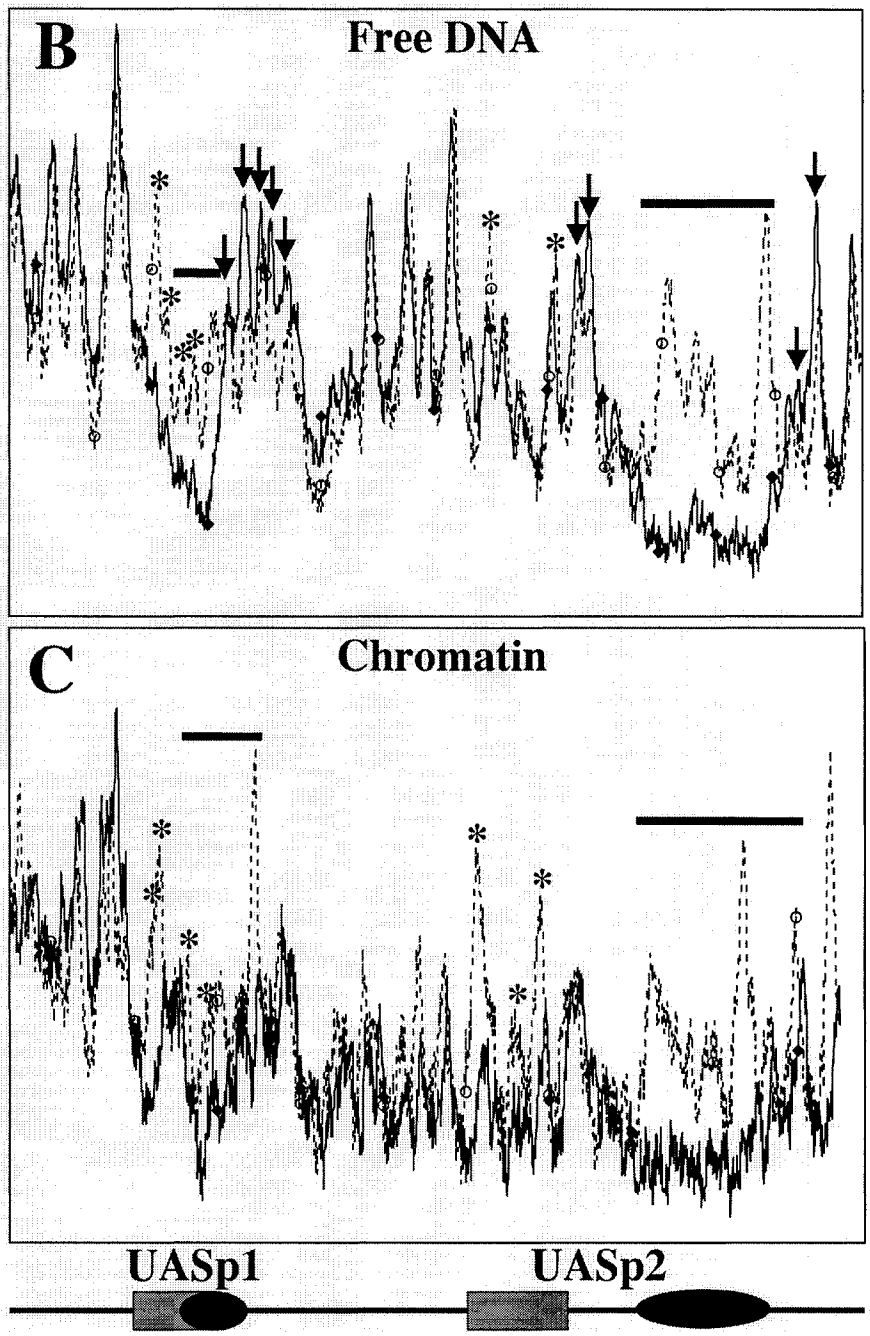

Figure 3.4B and 3.4C.

Figure 3.5. Nucleosome remodeling on reconstituted chromatin templates. All digestion reactions contained 1 μg DNA. Free DNA (lanes 1 to 3) was digested with 0.004, 0.016, and 0.032 U micrococcal nuclease (Mnase). Chromatin (lanes 4 to 18) was digested with 0.05, 0.25, and 0.5 U MNase. The digested chromatin samples contained chromatin alone (lanes 4 to 6); chromatin, the yeast nuclear extract 0.3 M SP-Sepharose fraction, and ATP (lanes 7 to 9); chromatin, 1 μg Pho4p, 450 ng Pho2p, and ATP (lanes 10 to 12); chromatin, nuclear extract 0.3 M SP-Sepharose fraction, 1 μg Pho4p, 450 ng Pho2p, and ATP (lanes 13 to 15 and lanes 16 to 18). (A) Southern blot analysis probing for nucleosomal protection over the UASp2 (nucleosome –2) and transcribed (nucleosome +1) regions. Following micrococcal nuclease digestion, purification and resolution on an agarose gel, the DNA was blotted to a membrane and probed with a fragment that binds the nucleosome –2 region (lanes 1 to 15) or the nucleosome +1 region (lanes 16 to 18). A ladder indicates no remodeling. A smear indicates remodeled chromatin. (B) Graphical representation of the distribution of counts in the lanes containing the most digested samples (lanes 3, 6, 9, 12, 15, and 18). A series of density peaks indicates no remodeling. A lack of peaks (a broad smear) indicates remodeled chromatin.

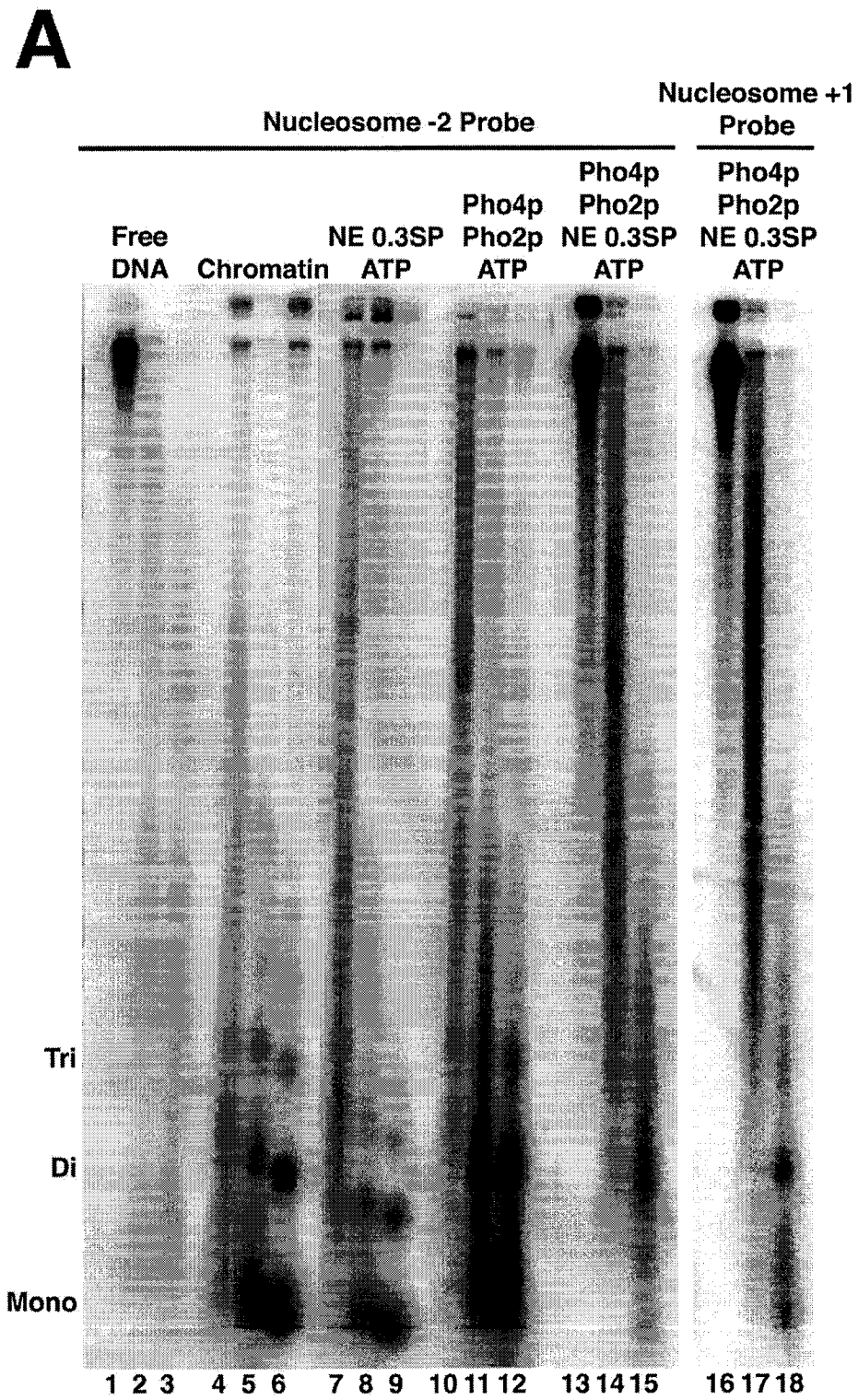

Figure 3.5A.

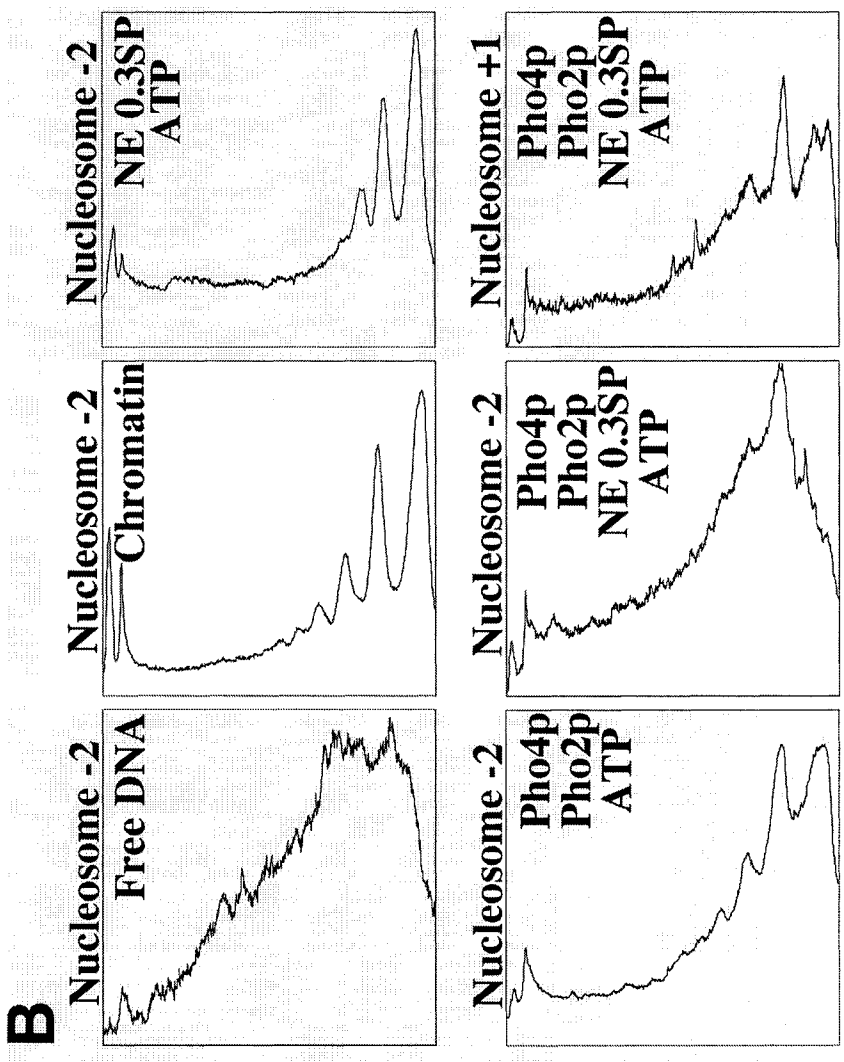

Figure 3.5B.

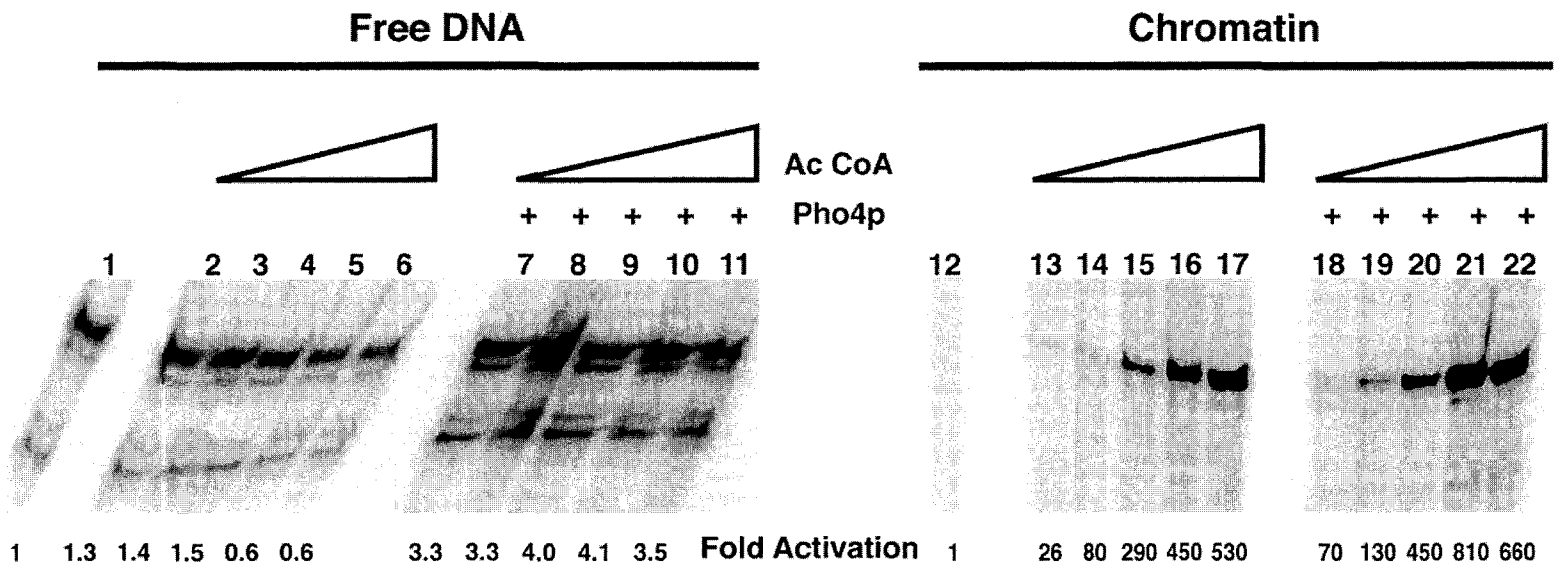

Figure 3.6. Chromatin Transcriptional repression is relieved with the addition of acetyl CoA. Acetyl CoA (0.05, 0.5, 2, 20, and 50 μM) was added to transcription reactions. All reactions contained a WCE from a *pho4* yeast strain (YS25). The addition of acetyl CoA stimulated transcription from chromatin but not free DNA independently from the effect of Pho4p addition (lanes 13 through 17 versus lanes 2 through 6). The addition of Pho4p (0.38 μg) stimulated transcription from both free DNA and chromatin (lanes 7 and 18). Fold activation is represented as transcript levels relative to those obtained from free DNA or chromatin templates in the absence of both acetyl CoA and Pho4p (lanes 2 and 13). These results were found to be reproducible through several repetitions of this experiment.

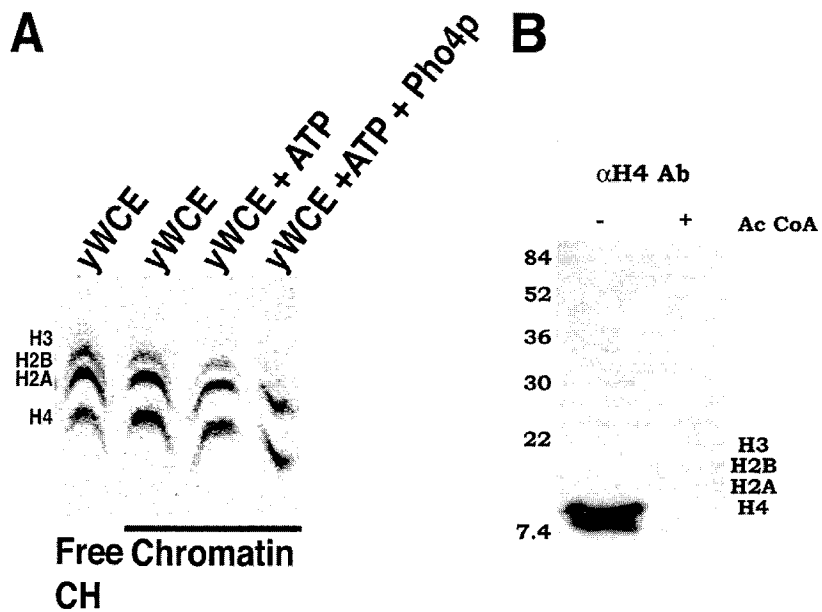

Figure 3.7. The addition of acetyl CoA to the WCE results in core histone acetylation. (A) Transfer of ^{14}C-acetyl to core histones. Free and chromatin assembled core histones were incubated with whole cell transcription extract in the presence of ^{14}C-acetyl CoA and the proteins were then resolved by SDS-PAGE. The effect of adding ATP and Pho4p to the yeast WCE on histone acetylation is shown in the two right-most lanes. (B) Western blot analysis of histone H4 acetylation. Chromatin templates were incubated with the WCE and acetyl CoA. The proteins were resolved by SDS-PAGE, transferred to a membrane, and blotted with anti-unacetylated H4 N-terminal tail antibodies (αH4-Ab). The same membrane was then blotted with anti-acetylated H4 N-terminal tail antibodies (αAcH4-Ab). The positions of protein size standards and purified yeast core histones run on the same gel and Coomassie blue stained are indicated.

Chapter 4

Histone Deacetylase Rpd3p Functions Directly and Indirectly in *PHO5* Regulation

This Chapter is written in the form of a manuscript to be submitted for publication in *Journal of Biological Chemistry*. The following text and references have been re-formatted to maintain consistency with the rest of the dissertation.

4.1 Summary

Histone deacetylase, Rpd3p, functions as a global transcriptional repressor of a diverse set of genes. Here we describe evidence supporting both direct and indirect roles for *RPD3* in the transcriptional regulation of *Saccharomyces cerevisiae PHO5*. We compared the regulation of *PHO5* expression in cells from *RPD3* wild type versus *RPD3* deletion *(rpd3Δ)* strains by measuring the level of encoded acid phosphatase activity and *PHO5* transcript. Both acid phosphatase activity and *PHO5* transcript levels displayed a two- to three-fold derepression in the *rpd3Δ* cells versus wild type cells grown under repressing conditions. However, *PHO2*, *PHO4*, *PHO81*, *PHO84* and *PHO86,* which function upstream of *PHO5* regulation, showed no transcriptional derepression in *rpd3Δ* strains. These findings suggest a direct role for Rpd3p in *PHO5* regulation through control of histone tail acetylation level on the *PHO5* promoter. Predictably, upon switching to activating conditions *PHO5* transcripts accumulated more rapidly in *rpd3Δ* relative to *RPD3* cells. Pho4p-mediated *PHO81, PHO84* and *PHO86* transcriptional activation is also potentiated in *rpd3Δ* cells. This more rapid response correlates with a defect in phosphate uptake and with premature recycling of Pho84p, the high affinity H^+/PO_4^{3-} symporter. These findings indicate Rpd3p also has an *indirect* role in *PHO5* regulation upstream of Pho4p transcriptional activation by affecting phosphate uptake through the timing of recycling of Pho84p out of the plasma membrane. Thus, *RPD3* participates in *PHO5* regulation both directly and indirectly.

4.2 Introduction

Large amounts of phosphate are required for cell growth and proliferation. In phosphate starved yeast *Saccharomyces cerevisiae* cells, phosphate is scavenged from covalently bound sources in the environment through the action of secreted phosphatases. The *PHO5* gene produces the bulk of the acid phosphatase and so its regulation is key to homeostasis of cellular phosphate. In addition, the *PHO5* gene serves as an key experimental model for transcription regulation in a chromatin context (11, 39). In the presence of abundant phosphate, the promoter is repressed through formation of four nucleosomes positioned over key promoter elements. When cells are starved for phosphate, the chromatin structure undergoes reconfiguration that renders the DNA in this region more accessible (1).

Many genes involved in phosphate scavenging (*PHO* genes) have been identified (19, 40, 41). Several genes in the *PHO* gene family participate in the regulation of *PHO5* expression (Figure 4.1). Transcriptional activators, Pho4p and Pho2p, are required to generate the active chromatin structure on the *PHO5* promoter and stimulate transcription (9). Pho80p-Pho85p is a cyclin/cyclin dependent kinase (CDK) pair that multiply phosphorylates Pho4p and negatively regulates Pho4p function. Phosphorylated Pho4p preferentially binds to Msn5p, a nuclear export protein. Pho4p phosphorylation also inhibits its interaction with the nuclear importer Pse1p/Kap21 and with the transcription factor Pho2p (17, 28). The net result is Pho4p cytoplasmic localization (nuclear exclusion). Pho81p inhibits Pho80p-Pho85p kinase activity during phosphate depletion and thus serves as a positive regulator of Pho4p activity.

Pho84p is a high affinity H^+/PO_4^{3-} symporter localized to the cytoplasmic membrane. *PHO84* functions genetically upstream of all other *PHO* genes save

PHO86 (5). Wykoff and O'Shea showed that Pho84p was the major contributor to phosphate uptake, of the five transporters identified to date (44). Mutation of the other four phosphate transporter genes had no effect on *PHO5* expression and cells retained both a high V_{max} and a low K_m for phosphate uptake. Lau *et al.*, identified Pho86p as an endoplasmic reticulum (ER) protein required for the exit of Pho84p out of the ER (20).

Reconfiguration of chromatin structure takes place on the *PHO5* promoter during activation, suggesting that chromatin remodeling activity is required (1, 2, 39). The results from in vivo experiments indicate the responsible chromatin remodeling complex contains Ino80p (8, 34). Biochemical and molecular genetic studies have indicated a role for histone modification in *PHO5* regulation, as well (39, 43). Transcription-related histone modifications include phosphorylation, methylation, acetylation, and ubiquitination. The reversible acetylation of lysine residues at the N-termini of core histones is generally linked to transcriptional activation. Histone acetylation is thought to weaken the interaction between nucleosomes, thus loosening the higher order structure and facilitating the access of the transcription machinery to the DNA (23). Interestingly, while *GCN5* and *ESA1* have been shown to affect the acetylation pattern on the *PHO5* promoter, they do not appear to be recruited by Pho4p and Pho2p (43).

The counterpart to acetylation is deacetylation. At least five genes encoding catalytic subunits of histone deacetylases have been identified in yeast. These include *RPD3*, *HDA1*, *HOS1*, *HOS2* and *HOS3* (6, 32, 42). *HDA1* and *RPD3* participate in determining the histone acetylation pattern on the *PHO5* promoter, also in a non-

recruited manner (43). Mutation of *RPD3* has been reported to have an impact on both activation and repression of *PHO5* expression (3, 32, 42, 43).

RPD3 was identified initially as a transcriptional repressor that functions along with *SIN3*. Subsequently, *RPD3* was shown to possess histone deacetylase activity through biochemical characterization of its mammalian homologue (38). Rpd3p is now known to function in a large complex that includes Sin3p, Sap30p and Pho23p (22). Rpd3p is recruited by the DNA binding protein Ume6p to the URS1 elements of *INO1* and *IME2* genes (15). This recruitment leads to localized histone deacetylation on approximately two nucleosomes adjacent to the URS1 element (16). However, there is no Ume6p binding site on the *PHO5* promoter. Vogelauer *et al.*, using chromatin immunoprecipitation (ChIP), showed that in an *rpd3Δ* strain the histones over the *PHO5* promoter are hyperacetylated (at H3 lysine 9 and H4 lysine12) compared to the wild type strain (43). This study also showed that histone acetyltransferases Gcn5p and Esa1p function together with histone deacetylases Rpd3p and Hda1p to maintain the "background" steady state of histone acetylation over the *PHO5* promoter. The histone acetylation patterns over the two other promoters and coding regions in the *PHO5* locus appear to be defined by global histone acetylation and deacetylation in the chromosomal region, as well. The core histone hyperacetylation on the *PHO5* promoter caused by *RPD3* deletion is thought to produce a more open chromatin structure, facilitating the accessibility for the general transcriptional machinery resulting in leaky basal transcription (derepression) in cells grown under *PHO5* repressing conditions (abundant phosphate). Therefore, *RPD3* functions directly in *PHO5* regulation through determining the histone acetylation

state on the promoter. However, we were interested in determining if *RPD3* also functions *indirectly* in *PHO5* regulation through the upstream regulators.

To identify potential indirect roles for Rpd3p in *PHO5* regulation, we analyzed the transcript levels from *PHO5* and several *PHO5* regulatory genes (*PHO2*, *PHO4*, *PHO81*, *PHO84*, and *PHO86)* in *RPD3* wild type versus *RPD3* deletion *(rpd3Δ)* yeast strains under repressed and activated conditions. Interestingly, we observed a more rapid *PHO5* induction by phosphate depletion in the *rpd3Δ* strain. Measurement of phosphate uptake rates suggests that rapid *PHO5* induction results from defective phosphate uptake. Disruption of *RPD3* had no significant effect on the transcript levels of *PHO2*, *PHO4*, *PHO81*, *PHO84*, and *PHO86* at high phosphate, but did result in more rapid induction of *PHO81*, *PHO84*, and *PHO86* at low phosphate. However, we do find that Pho84p is prematurely recycled in the *rpd3Δ* strain. Therefore, *RPD3* functions in *PHO5* regulation indirectly through an effect on retention of the high affinity phosphate transporter, Pho84p, in the cytoplasmic membrane during phosphate starvation.

4.3 Materials and Methods

4.3.1 Yeast Strains and Plasmids - The yeast strains used in this work are listed in Table 4.1. The original strains, YS18 and YS20, were kindly provided by Dr. Meyhack (Ciba-Geigy). YS20 was created by deletion of *PHO4* in the YS18 background (33). These strains were used to create *rpd3Δ* mutant (YS18R) and *pho4Δ rpd3Δ* double mutant (YS20PR by homologous recombination. The plasmid pMVL (32) containing a disrupted copy of the *RPD3* gene locus was digested with *Aat* II and *Bgl* II and the 3.5 kb fragment containing *rpd3*::*LEU2* was gel purified. The DNA

fragment was used to transform yeast cells by the conventional method. A Leu^+ prototroph was selected and the gene disruption was confirmed by Southern blotting. The strains were designated YS20PR and YS18R for *rpd3* mutation in *pho4* background (YS20) and *PHO4* background (YS18), respectively.

4.3.2 Acid Phosphatase Activity Assay - Synthetic complete media + /–phosphate was prepared as described previously (35). A yeast colony was picked from YPD plates of each strain and cultured in 5 ml synthetic complete medium (high phosphate) over night at 30 °C. The cultures were diluted one to fifty (to 250 ml) and grown to a density of 1 to 2 x 10^6. Yeast cells were centrifuged down, washed with sterile water, and resuspended in 50 ml high and no phosphate media. At 3, 6, and 9 h, 1 ml samples from each strain was collected and washed once with 10 ml water before resuspension in 1 ml water. The periplasmic acid phosphatase activity was measured in 0.5 ml containing 25 µl of 1M sodium acetate (pH 4.0), 5 µl of 0.45 mg/ml *p*-nitrophenylphosphate (substrate) and varying volumes of cell suspensions. The buffer and substrate were preincubated at 37 °C for 10 min, the cell suspensions were added, and the reactions were incubated for an additional 10 min at 37 °C. The reactions were stopped by addition of 0.12 ml of 25% trichloroacetic acid, and 0.6 ml saturated Na_2CO_3. Cells were pelleted and the absorbance of the supernatants at 405 nm was measured with a Beckman DU600 spectrophotometer. The units of acid phosphatase enzyme from each culture were interpolated from a standard curve, created by plotting known amount of commercial phosphatase vs. absorbance at 405 nm produced by that amount of phosphatase under the assay conditions. One unit of acid phosphatase

activity is defined as the amount of enzyme used to produce 1 μmol of *p*-nitrophenol in 1 min at 37°.

4.3.3 Phosphate Uptake Assays - Phosphate uptake assays were performed as described previously (21).

4.3.4 Preparation of RNA and cDNA Synthesis – Cells from the same cultures of each yeast strain used for the assay of acid phosphatase activity were also used for total RNA extraction. Approximately 5 x 10^7 cells were collected from cultures grown on phosphate-rich and no-phosphate media for 3, 6 and 9 h. RNA extractions were processed using the RNeasy mini kit (Qiagen). Samples of total RNA were quantified at OD 260/OD280 using a UV spectrophotometer (Beckman DU600). One micrograms of total RNA is subsequently treated with 1 unit of RNase-free DNase I (Roche) in the digestion buffer (20 mM Tris pH 7.5 and 10 mM $MgCl_2$) at 37° for 5 min. After ethanol precipitation, DNaseI-treated RNA was resuspended in 10 μl H_2O. Five μl was diluted to 400 μl in water and used in real-time PCR to check for DNA contamination. RNA in the other 5 μl was annealed with 25 picomol of oligo dT primers. The mixture is heated at 70 °C for 15 min to get rid of any secondary structure. cDNAs were synthesized in 15 μl reaction containing 1 mM dNTP, reverse transcriptase buffer and 200 unit of MMLV reverse transcriptase (Promega) at 42 ° for 1 hr. The final volume was adjusted with sterile H_2O to 400 μl and the amount to be used in real-time PCR was determined by titration.

4.3.5 Primer Design for Real-time PCR Analysis - Primer sets were designed for SYBR Green PCR analysis using web-based program "primerfinder version 0.06" (http://www.cellbiol.com/Tools.html#pick). DNA sequences from actin gene (YFL039C/ACT1), *PHO3* (YBR092C), *PHO5* (YBR093C), *PHO81* (YGR233C), *PHO84* (YML123C), and *PHO86* (YJL117W) gene were retrieved from *Saccharomyces* Genome Database (SGD). The sequences of primers used are listed in Table 4.2. Since the coding region of *PHO3* and *PHO5* are almost identical (87% identical), forward primers for *PHO3* and *PHO5* were designed to be complementary to the 5'UTR. Primers for the rest of genes examined were designed from coding sequence. The melting temperatures are in a range of 60° - 65° for all primer sets. The amplicon length was kept between 99-112 bp.

4.3.6 SYBR Green Real Time PCR - Real-time PCR experiments were performed using the iCycler iQ multi-color real time PCR detection system (BioRad). Several sets of cDNA templates were prepared from two sets of RNA to ensure the reproducibility of real time PCR. SYBR Green analysis-based PCRs were performed in duplicate in each experiment. The transcript levels of the genes of interest (*PHO2*, *PHO3*, *PHO4*, *PHO5*, *PHO81*, *PHO84*, and *PHO86*) were compared to that of the actin gene (*ACT1*). The total volume of each reaction is 25 µl. The PCR reaction contains 200 nM each of forward and reverse primer, 200 µM dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, SYBR Green diluted 50,000-fold from the manufacturer's stock solution (Molecular Probes), 1 nM Fluorescein (Biorad), 1 unit of JumpStart Taq DNA Polymerase (Sigma), and 5 to10 µl of cDNA template. All primer sets were tested with genomic DNA from YS18R strain to check for the

linearity of amplication and optimize PCR parameters. For all primer sets, thermocycler conditions were used as follows: stage1, initial denaturation at 95°, 3 min for 1 cycle; stage 2, denaturation at 95°, 30 s and annealing and elongation at 60°, 45 s for 40 cycles. After amplification, the PCR products were also checked for nonspecific amplification on 2% agarose gel electrophoresis and melt curve analysis.

4.3.7 Relative Level of Gene Expression - The fluorescent reporter dye SYBR Green is used to monitor the PCR reactions as it occurs in real time. SYBR Green fluoresces significantly when it binds to double-stranded DNA. The fluorescence increases as the PCR products increase in each successive round of amplification. SYBR Green binds DNA in a sequence-independent manner. After amplification, PCR products were checked for specificity by melting curve analysis and gel electrophoresis. The primers that yielded specific PCR products were used in this study. The cycle that the level of fluorescence rises appreciably above background is termed "threshold cycle" or Ct, which correspondingly reflect the starting amount of cDNA template in the sample. The relative level of gene expression is determined from the difference between reference gene Ct and gene of interest Ct. The relative levels of gene expression from *PHO2*, *PHO3*, *PHO4*, *PHO5*, *PHO81*, *PHO84*, and *PHO86* as compared with that from the actin gene (*ACT1)* under activated (no phosphate) and repressed (abundant phosphate) conditions was determined. The following equation was used for determination of the relative level of gene expression:

$$\text{Relative gene expression or fold change} = 2^{-\Delta(\Delta Ct)}$$

Where ΔCt = Ct target gene – Ct reference gene

And $\Delta(\Delta Ct) = \Delta Ct$ stimulated- ΔCt control

For fold activation,

$$\Delta(\Delta Ct) = \Delta Ct_{(-Pi)} - \Delta Ct_{(+Pi)}$$

For fold derepression,

$$\Delta(\Delta Ct) = \Delta Ct_{(+Pi,\ rpd3)} - \Delta Ct_{(+Pi,\ RPD3)}$$

The melt curve analysis from each set of primer pairs indicated that only specific PCR product was amplified (data not shown). Primer, $MgCl_2$ and SYBR Green concentration were optimized for real-time PCR.

4.3.8 Fluorescence Microscopy - Wild type and *rpd3Δ* cells containing the plasmid p*PHO84-GFP* were cultured in SC medium containing phosphate to mid log phase before switching to no phosphate medium. The expression of chimeric Pho84p-GFP was analyzed by fluorescence microscopy at 2, 4, 6 and 8 h. The plasmid p*PHO84-GFP* (EB0666), consisting of the intact *PHO84* locus fused with GFP coding sequence at C-terminus, was kindly provided by E. K. O'Shea (20).

4.4 Results

RPD3 disruption is generally thought to affect *PHO5* expression directly through the histone acetylation levels on the promoter. In addition, it may affect *PHO5* expression indirectly through Pho4p activity. To differentiate a direct effect from an indirect effect, four yeast strains (*PHO4 RPD3*, *pho4 RPD3*, *pho4 rpd3*, and *PHO4 rpd3*; Table 4.1) were compared with regard to expression levels of acid phosphatase activity and *PHO5* transcript. Since Pho4p is the ultimate regulator of *PHO5*, deletion of *PHO4* results in the loss of *PHO5* response to phosphate starvation. The *PHO5* expression level in the *PHO4 rpd3* strain versus the *pho4 rpd3* strain

grown in the presence of high phosphate is indicative of an *RPD3* function in *PHO5* repression through direct effects on promoter histone modification state. The *PHO5* activation pattern in the *PHO4 RPD3* strain versus the *PHO4 rpd3* strain grown in the absence of phosphate will indicate whether *RPD3* has any indirect role in *PHO5* regulation. Differences in the *PHO5* expression level or activation pattern will suggest a role for *RPD3* in the regulation of *PHO4* or upstream genes.

4.4.1 Acid Phosphatase Activity Expression is Derepressed in *rpd3Δ* Strains - The acid phosphatase activity from both *rpd3Δ* strains grown on high phosphate medium was two- to three-fold higher than that from the *RPD3* strains (derepressed) (Figure 4. 2A). The degree of derepression was comparable between *PHO4 rpd3* single mutant and the *pho4 rpd3* double mutant strains. These results support the idea that Rpd3p functions in *PHO5* repression directly through effects on the histone acetylation pattern on the *PHO5* promoter.

A time course of acid phosphatase expression levels upon phosphate removal in the *PHO4 RPD3* and *PHO4 rpd3* strains are not significantly different (Figure 4.2B). In addition, induction in the *pho4 RPD3* and *pho4 rpd3* strains is equally nonexistent. These results confirm that phosphate signaling functions through Pho4p

Derepression of acid phosphatase expression in the *pho4 rpd3* double deleted strain strongly supports a direct function of *RPD3* in repression of the *PHO5* promoter in cells growing in high phosphate. However, several steps lay between *PHO5* transcription and acid phosphatase expression. Therefore, the lack of an effect of *RPD3* disruption on acid phosphatase expression through Pho4p does not rule out a role for *RPD3* in the regulation of the other *PHO* genes involved in *PHO5*

transcriptional activation that may not be reflected in changes in acid phosphatase production. As a result, reverse transcriptase-real time PCR (RT-RT PCR) was used to quantify transcript levels. Specifically, to verify that *RPD3* only functions directly in transcriptional regulation we looked at *PHO5* and *PHO3* as compared with the actin gene (*ACT1*) under repressing (high phosphate) and activating (no phosphate) conditions in the same four yeast strains (Table 4.1). In addition, we determined the levels of *PHO2*, *PHO4*, *PHO81*, *PHO84*, and *PHO86* transcripts.

4.4.2 *PHO5* and *PHO3* Transcript Levels are Derepressed by *RPD3* Disruption - We quantified the level of *PHO5* mRNA in each of the four yeast strains grown in the presence of phosphate. In addition, since *PHO3* is located directly downstream of *PHO5* on the chromosome II we were interested in comparing the relative level of *PHO3* transcripts in the four strains as a measure of how localized the effect of *RPD3* disruption might be. *INO1* repression is known to be highly dependent on *RPD3*, and therefore served as a positive control. As shown in Figure 4.3, derepression is observed from *INO1* (4.6-fold), *PHO5* (2.7-fold) and *PHO3* (2.1-fold). *PHO5* derepression is comparable between the *PHO4* wild type and mutant backgrounds, in agreement with the results obtained using the acid phosphatase assay, again indicating that *RPD3* functions directly in repression of the *PHO5* and *PHO3* promoters. We then examined the effect of *RPD3* disruption on the transcriptional regulation of *PHO81*, *PHO84* and *PHO86*. Of these three genes, only *PHO86* is even slightly derepressed (1.5-fold).

4.4.3 *PHO5* Activation is Potentiated in *RPD3* Deletion Strains – In the *PHO4* strains under activating conditions (growth without phosphate), the *PHO5* transcript levels increase significantly (actin gene transcript levels are arbitrarily set to 1). However, *PHO3* transcript levels are significantly decreased. Thus, *PHO5* activation is highly phosphate and *PHO4*-dependent, while *PHO3* is not (Figure 4.4), consistent with results previously reported (30, 36).

Surprisingly, time course experiments with the *rpd3* mutant show that *PHO5* transcript level responds more quickly and to a higher level during phosphate depletion (Figure 4.4). This finding is consistent with the direct role of Rpd3p on the histone tail acetylation state on the *PHO5* locus. However, we could not rule out *RPD3* regulation of one or more of the other *PHO* genes in the *PHO5* regulatory cascade.

4.4.4 Activation of *PHO81*, *PHO84*, and *PHO86* is Potentiated by *RPD3* Disruption - We tested whether *RPD3* disruption affects the activation of transcript accumlation for *PHO81*, *PHO84*, and *PHO86*. All three genes are regulated by phosphate availability and have promoters containing Pho4p binding sites (5, 45). The *PHO81*, *PHO84*, and *PHO86* transcript levels were found to be phosphate and *PHO4*-dependent (Figure 4.5). The dependence of *PHO84* transcriptional activation is comparable to that of *PHO5*. Transcription of *PHO81* and *PHO86* are also activated by phosphate starvation, but to a lesser extent.

Interestingly, we observed a more rapid induction of *PHO81*, *PHO84* and *PHO86* in the *rpd3Δ* strain than in the wild type strain (Figure 4.5). However, only *PHO84* was activated to a higher level. Since these genes are not derepressed in

rpd3Δ strains, these findings indicate that *RPD3* affects the transcriptional regulation of *PHO81*, *PHO84* and *PHO86* indirectly through phosphate level signaling and Pho4p activity.

4.4.5 Transcriptional Regulation of *PHO2* and *PHO4* is *RPD3* Independent - Pho2p and Pho4p are the ultimate regulators of *PHO5* transcriptional activation. Expression of neither gene is affected by phosphate concentration (13). We find that *RPD3* disruption has no effect on transcription of either gene, as well (Figure 4.3).

In this study, we show that *RPD3* participates in both *PHO5* repression in the presence of abundant phosphate and activation during phosphate starvation. *RPD3* disruption directly derepresses *PHO5* expression as measured by acid phosphatase activity levels and transcript levels. In addition, *PHO5* activation is potentiated. This potentiation might be, at least in part, through a direct effect on histone acetylation state on the *PHO5* locus. However, *RPD3* disruption also affects the transcriptional activation of the other phosphate and Pho4p-dependent genes (*PHO81*, *PHO84* and *PHO86*), while having no effect on repression at high phosphate. Therefore, the results thus far suggest that *RPD3* also functions in *PHO5* regulation indirectly through signaling of the intracellular phosphate level. *PHO81* has a key regulatory function in determining Pho4p activity dependence on intracellular phosphate level. Since Pho84p and Pho86p are responsible for high affinity phosphate uptake and function upstream of *PHO81*, it seemed reasonable that *RPD3* disruption might affect phosphate uptake. Defective phosphate uptake would affect transcription of genes downstream in the regulatory pathway through the intracellular phosphate level.

4.4.6 *RPD3* Deletion Results in Compromised Phosphate Uptake - We tested the effect of *RPD3* disruption on phosphate uptake rate. As shown in Figure 4.6, the phosphate uptake rate in *rpd3Δ* cells is significantly compromised. It is worth noting that the initial uptake rates (zero to four h) in the *RPD3* wild type and deletion strains are quite similar and that the steady state rate (four to twelve hours) is what decreases by about three-fold in the *rpd3Δ* strain. The decreased uptake rate could be consistent with a decrease in transporter activity. A decrease in transporter activity could occur through a direct effect on protein level or an indirect effect through loss of proton motive force (pmf or Δp) across the cytoplasmic membrane.

4.4.7 Pho84p is Prematurely Recycled in *rpd3Δ* Cells - It has been shown that proton motive force plays an essential role to phosphate collection (21). In a genetic screen for genes in the phosphate-sensitive signal transduction pathway leading to *PHO5* expression, *PMA1*, encoding a plasma membrane ATPase Pma1p, was one of several genes identified. Pma1p functions as proton pump is essential for phosphate transport. Disruption of *PMA1* led to defective phosphate uptake, which in turn resulted in constitutive *PHO5* expression (21). The study showed that the defect in proton pumping could be rescued by providing acidic extracellular environment to restore the proton motive force across the plasma membrane (21). Thus, the reduced phosphate uptake rate observed in the *rpd3*Δ strain could result from indirect effects on Pma1p activity or expression. To test this hypothesis, we performed phosphate uptake assays at pH 3 and pH 4.5 with the *RPD3* wild type and *rpd3*Δ strains. If *RPD3* disruption affects phosphate uptake by decreasing proton motive force, the uptake defect should be relieved in the lower pH environment. As shown in Figure

4.7, increasing the proton gradient across plasma membrane by lowering the extracellular pH does not rescue the uptake defect. It should be noted that phosphate uptake in the *RPD3* wild type strain was decreased two-fold at pH 3 as compared to at pH 4.5 (802 vs. 1733 pmol/OD/ml/min). In addition, lowering the pH does not inhibit phosphate uptake further in the *rpd3Δ* strain. The optimum pH for the high affinity phosphate transporter Pho84p is 5.0 and for the low affinity phosphate transporter is 4.0 (37). A higher proton gradient does not reduce the phosphate uptake defect, suggesting that *RPD3* disruption affects phosphate uptake through some other means. These results are consistent with the phosphate uptake defect arising from a decrease in Pho84p levels.

Therefore, we investigated the impact of *RPD3* disruption on Pho84p protein level and cellular localization by following chimeric Pho84p-GFP expression using fluorescence microscopy. Both wild type and *rpd3*Δ strains containing the expression plasmid *PHO84-GFP* were cultured in phosphate-containing medium to mid log phase then switched to no phosphate medium. Cells were collected at 2, 4, 6 and 8 h of growth in the absence of phosphate. The efficiency of chimeric protein expression in both strains is comparable (80-90%). As shown in Figure 4.8A and 4.8B, *RPD3* wildtype and mutant strains harboring the p*PHO84-GFP* plasmid exhibited a fluorescent signal that was seen as early as 2 h of culture in no phosphate medium. Most of the fluorescent signal at this time point was associated with newly synthesized protein located in an intracellular compartment, although localization of some of the Pho84p-GFP to the plasma membrane can be seen. At 2 h, the percentage of cells exhibiting Pho84p-GFP to localization on the plasma membrane is higher in wild type (7.5 %) as compared to *rpd3Δ* cells (4 %). At 4 h, a stronger fluorescent signal was

observed and the fraction of cells in which Pho84p-GFP had localized to the plasma membrane increased in both strains (65.67 and 47.27 % in wildtype and *rpd3Δ* strains, respectively). Between 6 and 8 h, wild type cells reach a steady state of Pho84p-GFP plasma membrane localization (74.67% and 67.33%, respectively). In contrast, in *rpd3Δ* cells we observed a decreased proportion of cells with plasma membrane-localized Pho84p-GFP (45.67 % at 6 h and 35.67 % at 8 h) and the number of cells with plasma membrane-associated Pho84p-GFP never reach as high as that observed from wildtype. These results correlate well with the effect of *RPD3* disruption on phosphate uptake. At 4 h the phosphate uptake rate in the *rpd3Δ* cells is only slightly lower than that in wild type cells and the corresponding pattern of localization of Pho84p-GFP on plasma membrane from both *RPD3* wild type and mutant strains is observed (65.67 % in wild type vs. 47.27 % in mutant). While at 8 h both phosphate uptake and the number of cells with membrane-associated Pho84-GFP are reduced to less than half that of wild type cells (Figures 4.6 and 4.8B). These findings indicate that *RPD3* disruption affects the regulation of Pho84p recycling resulting in premature internalization in cells starved for phosphate.

4.5 Discussion

We set out to investigate a possible role for *RPD3* in *PHO5* regulation through indirect, upstream effects, in addition to the direct effects on histone acetylation levels on the *PHO5* locus. This was performed by measuring *PHO5* expression at the protein and RNA levels in yeast strains containing a disrupted *RPD3* gene (*rpd3*Δ). To separate direct effects on transcription through changes in the histone acetylation state from indirect effects through Pho4p regulation, we compared the level of *PHO5*

expression between *rpd3Δ PHO4* and *rpd3Δ pho4Δ* strains. We observed a 2- to 3-fold derepression of *PHO5* expression that was not dependent on *PHO4*. *PHO3*, which lies immediately downstream of *PHO5* was also derepressed. Vogelauer *et al.* showed that *RPD3* regulates the histone acetylation state over 4.25 Kb region of chromosome 2 including the *PHO5* promoter and coding region as well as *PHO3* (43). Therefore, the derepression that we observed from *PHO5* and *PHO3* in this study is likely the direct result of higher than normal histone acetylation on these promoters.

In addition to derepression, we observe a potentiation of *PHO5* activation. *PHO5* reaches half maximal activity after 1.5 h of phosphate starvation in the *rpd3Δ* strain compared to 3 h in the *RPD3* strain. On top of this, the maximum *PHO5* expression level in the *rpd3Δ* strain is more than 2 times greater. Interestingly, *PHO3* is not activated. In fact, *PHO3* is repressed normally by phosphate starvation, even in the absence of Pho4p and, therefore, *PHO5* activation. This result suggests that the mechanism of *PHO3* repression is independent of Rpd3p histone deacetylation and Pho4p inactivation. This also suggests that the mechanism of repression is dependent on some aspect of *PHO5* activation upstream of Pho4p activation and independent of *RPD3* function.

The potentiation of *PHO5* activation could, at least in part, be a direct result of increased steady state levels of histone acetylation over this locus. However, *PHO81*, *PHO84*, and *PHO86* activation during phosphate starvation was potentiated in the *rpd3Δ* strain, as well. None of these three genes is derepressed in the *rpd3Δ* strain grown in high phosphate medium. Thus, this potentiation by *RPD3* disruption is not due to any direct affect through histone acetylation on these loci. Indeed, our results suggest an indirect effect from upstream in the phosphate signaling pathway through

regulation of Pho4p activity. The growth rate for the *RPD3* and *rpd3Δ* strains on no-phosphate medium is the same (results not shown), thus potentiation of activation does not result from faster depletion of phosphate through more rapid growth. Another possibility is feed-forward activation acting through the derepression of *PHO81* expression. However, *PHO81* overexpression or constitutive hyperactivity alone has been shown not to cause significant *PHO5* derepression (7, 14, 18, 27). A third possibility is an effect on phosphate accumulation, which involves phosphate storage as polyphosphate in vacuoles and phosphate uptake (26).

RPD3 disruption does not affect the expression of the polyphosphate synthesis genes (3). In addition, disruption of these genes results in the plateauing of phosphate uptake in 5 to 10 minutes rather than the four hours we observe and a partial defect in polyphosphate synthesis has little to no affect on phosphate uptake rates (26). We do observe a phosphate uptake defect in the *rpd3Δ* strain. This defect is not a result of decreased *PHO84* or *PHO86* transcription. To the contrary, we see potentiation of the activation of these genes. This implies that the defect lies in a step downstream from *PHO84* and *PHO86* transcription.

PHO84 encodes the high affinity H^+/PO_4^{-2} symporter (4). Therefore, its function is dependent on the proton motive force (Δp) across the cytoplasmic membrane. For example, if the $-\Delta G$ of the Δp across the cytoplasmic membrane were reduced to half normal, one would expect a concomitant decrease in the maximum rate (but not the initial) rate of phosphate uptake when the $+\Delta G$ of pumping phosphate across the cytoplasmic membrane reaches equilibrium with the $-\Delta G$ of the proton electrochemical gradient. This idea is supported by the finding that extracellular pH affects phosphate uptake kinetics in yeast cells (25, 37). The proton motive force is

maintained by function of Pma1p, an ATP-dependent H^+ pump. In addition, it has been shown that *PMA1* is required for high-affinity phosphate uptake through Pho84p (21). We tested this possibility by performing phosphate uptake assays at lower than normal (pH 3.0) and normal (pH 4.5) extracellular pH with *RPD3* and *rpd3*Δ yeast cells. Reducing extracellular pH, to artificially provide a proton gradient across membrane, does not rescue the phosphate uptake defect in the *rpd3Δ* strain. This implies that the impact of Rpd3p on phosphate uptake system is not through disruption of proton motive force.

Pho84p transport activity is suboptimal at pH 3.5 while the other phosphate transporter activities are near their optimal pH of 4.0 (37). We observe a decrease in phosphate uptake rate in wild type, but not *rpd3Δ* cells to the level seen in the *rpd3Δ* cells. This observation further supports the idea that the difference in uptake rates between the wild type and *rpd3Δ* cells is due to a difference in Pho84p activity.

Expression, activity, and recycling of Pho84p are regulated (20, 24). Since *RPD3* disruption actually potentiates *PHO84* transcription, we tested whether the Pho84p or level cytoplasmic membrane localization is affected in *rpd3Δ* strains using the fusion protein Pho84p-GFP and fluorescence microscopy. Disruption of *RPD3* affects the rate of Pho84p-GFP recycling. In the *rpd3Δ* strain, localization of Pho84p-GFP to the plasma membrane peaks at 4 h after culture in the absence of phosphate. The number of cells with Pho84p-GFP localized on plasma membrane decrease to 45.67 % and 35.67 % at 6 and 8 h, respectively in the *rpd3Δ* cells versus 74.67 % and 67.33 % in the wild type cells. These findings correlate well to the defect we observed in the steady state phosphate uptake in *rpd3Δ* cells. The rate of phosphate transport is reduced more than two-fold from 4 h to 8 h after culture in no phosphate medium.

Therefore, we have narrowed down the possible step in phosphate transport affected by *RPD3* disruption to the regulation of cytoplasmic membrane localization of high affinity phosphate transporter, Pho84p. This regulation appears to involve the timing of the internalization process. Recycling has been shown to occur in late log phase growth and in response to high extracellular phosphate levels (20, 24). The recycling pathway for Pho84p has not been fully determined.

Membrane-associated Pho84p recycling initiates with its internalization and targeting to vacuole where it is degraded (this study and (20)). Recycling of other membrane-associated proteins in *Saccharomyces cerevisiae*, such as pheromone receptors and nutrient transporters, has been shown to involve ubiquitination and/or phosphorylation-related degradation processes (10, 12, 29). Ubiquitination of membrane proteins generally occurs in three steps (Reviewed in (31)). The enzymes E1, E2 and E3 function sequentially to add ubiquitin to proteins targeted for degradation in a vacuole. Chip array analysis of genes up-regulated by *RPD3* disruption identified three genes involved in ubiquitination-related degradation in vacuoles (3). These genes are *UBC1*, *UBP4/DOA4* and *PEP4/PRA1*, which encode the E2 enzyme, ubiquitin isopeptidase and vacuolar protease, respectively. Up regulation of these genes is likely to result in an increased turnover rate for the targeted proteins. Ubiquitin peptidase releases ubiquitin from the targeted protein before degradation to be recycled. Vacuolar protease catalyzes protein degradation in the vacuole. These two enzymes, therefore, are likely to function more generally. Ubc1p, the E2 enzyme, is thought to function more specifically on a subset of protein since it seems to bind selectively to E3 protein. The details of the pathway for Pho84p down regulation are not known. The finding that *RPD3* disruption affects the timing

of Pho84p recycling helps to direct future experimental avenues for establishing the Pho84p down regulation pathway.

Prior to this study *RPD3* was thought to function in *PHO5* regulation only through a direct effect on the histone acetylation state on the promoter. Our findings demonstrate that *RPD3* functions indirectly, as well, through regulation of the timing of Pho84p recycling. Therefore, Rpd3p not only functions to repress *PHO5* transcription in the presence of abundant phosphate, but also functions in the maintenance of Pho84p cytoplasmic membrane localization when free phosphate in limiting.

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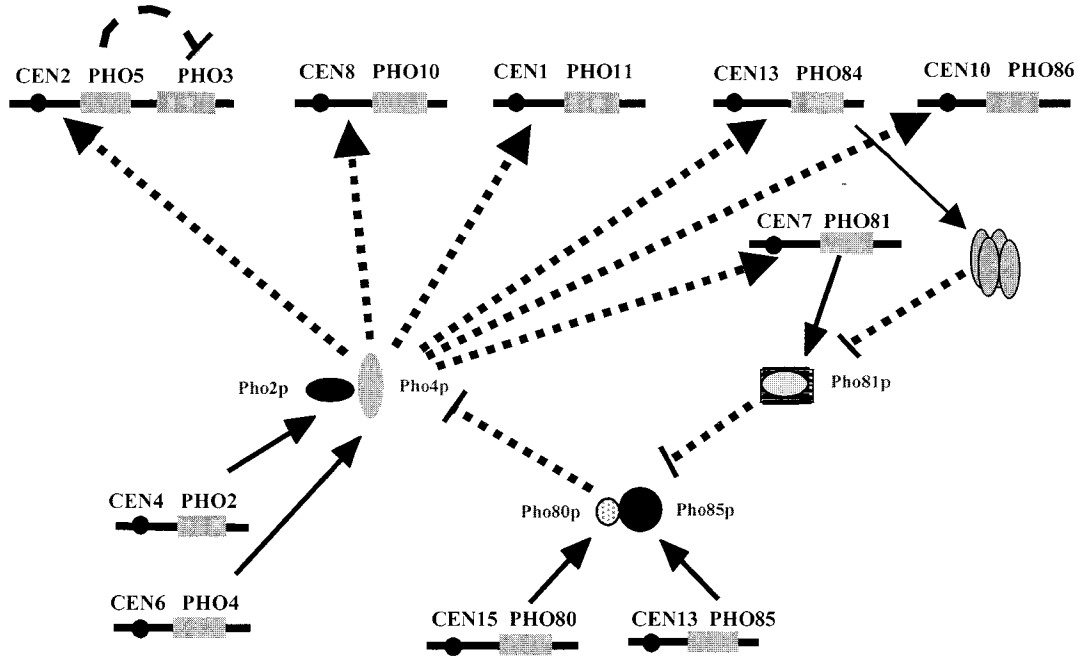

Figure 4.1. Schematic diagram of the *PHO5* regulatory cascade. Pho2p and Pho4p are sequence-specific DNA-binding transcription regulatory proteins. Binding sites for Pho4p are found on the *PHO5*, *PHO81*, *PHO84*, and *PHO86* promoters. All four of these genes are activated by phosphate starvation. High intracellular phosphate (Pi) represses Pho81p activity, which acts as an inhibitor of Pho80p/Pho85p Pho4p-kinase activity. Pho4p phosphorylation decreases its affinity for Pho2p and results in its cytoplasmic localization, thus inhibiting its ability to activate transcription. Phosphate depletion activates Pho81p resulting in Pho4p nuclear localization and transcriptional activation of responsive genes.

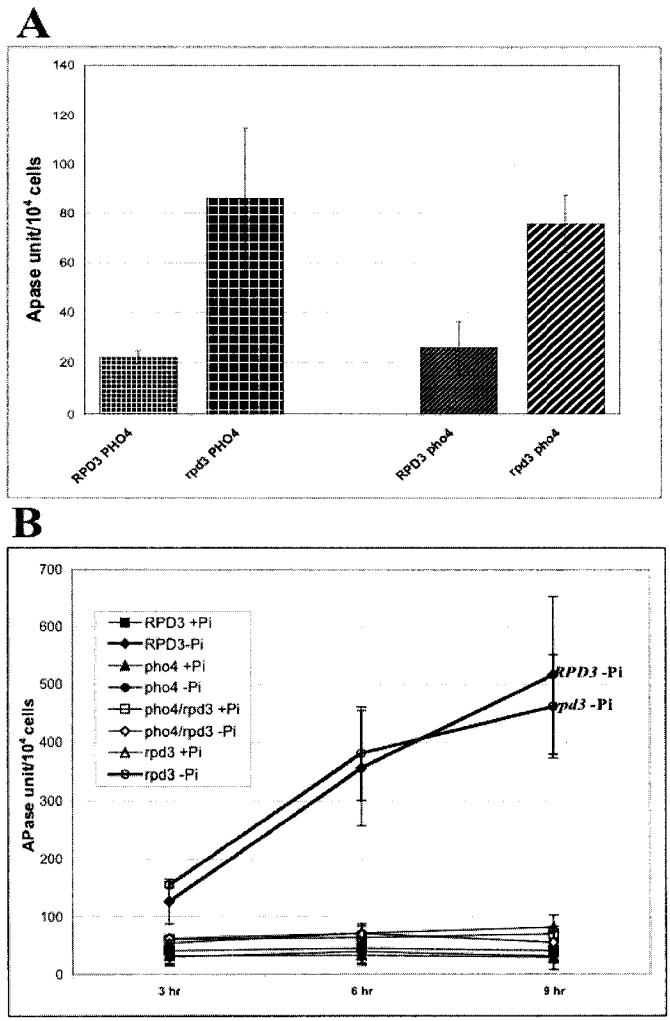

Figure 4.2. Expression of acid phosphatase activity is derepressed in *rpd3*Δ cells. Periplasmic acid phosphatase activity was measure on *RPD3 PHO4*, *rpd3*Δ *PHO4*, RPD3 *pho4*Δ, and *rpd3*Δ *pho4*Δ cells at 3, 6, and 9 h of growth in medium containing or lacking phosphate as described in Methods and Materials. (A) Acid phosphatase activity, plotted as units per 10^4 cells, on cells from each of the four yeast strains grown for 9 h on phosphate containing medium (+Pi). (B) Acid phosphatase activity on cells from each of the four yeast strains (*PHO4 RPD3* ν , *pho4 RPD3* σ , *PHO4 rpd3* λ , *pho4 rpd3* υ) grown in the presence (filled symbols) absence (open symbols) of phosphate (-Pi).

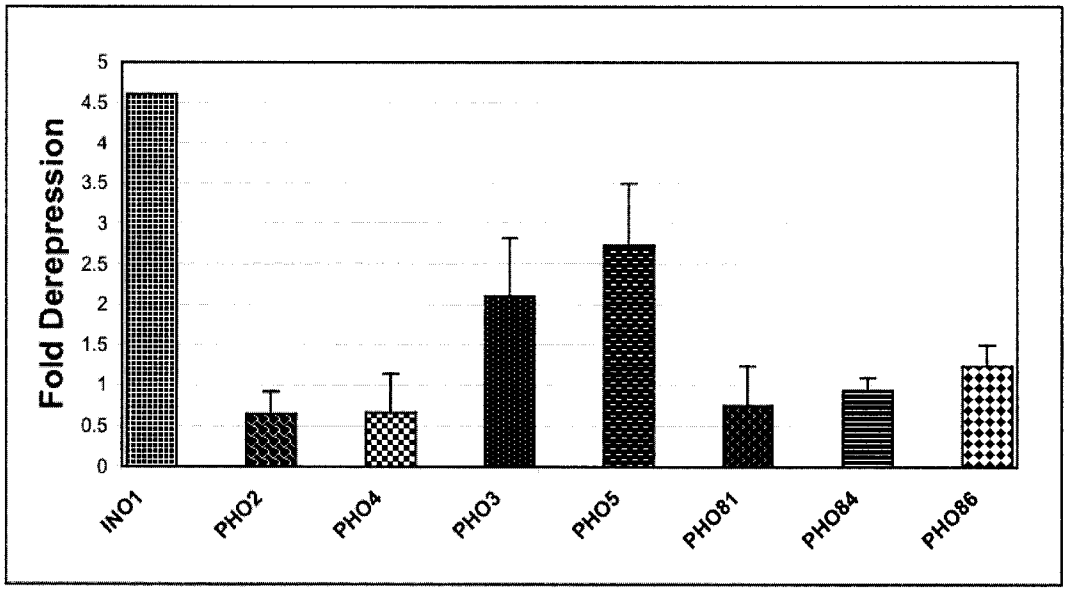

Figure 4.3. The *PHO5* and *PHO3* transcript levels are derepressed in *rpd3*Δ cells. Reverse transcription and real-time PCR were used to quantitate the transcript level of the following *PHO* genes; *PHO2*, *PHO3*, *PHO4*, *PHO5*, *PHO81*, *PHO84*, *PHO86* and *INO1*. The fold derepression is determined by transcipt level in *rpd3Δ* cells over that in wild type cells in the *PHO4* wild type background under repressing condition (+Pi). *INO1* serve as a positive control for derepression in *rpd3Δ* cells since transcription of *INO1* is known to be repressed by Rpd3p.

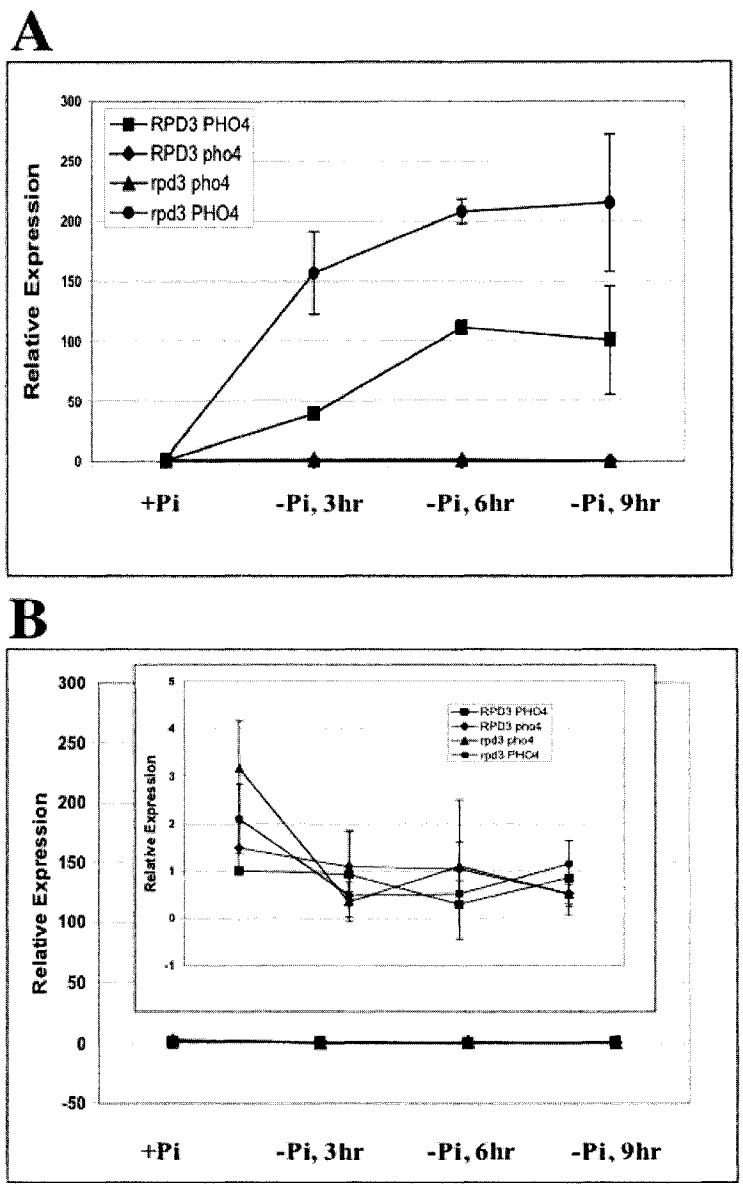

Figure 4.4. Activation of *PHO5* is potentiated in *rpd3*Δ cells. Reverse transcription and real-time PCR were used to quantitate the (A) *PHO5* and (B) *PHO3* mRNA levels in *RPD3 PHO4* (ν), *rpd3*Δ *PHO4* (λ) , *RPD3 pho4*Δ (υ) , and *rpd3*Δ *pho4*Δ (σ) cells grown in the absence of phosphate for 3, 6, and 9 h as described in Methods and Materials. Transcription of *PHO5* is potentiated in the *rpd3*Δ *PHO4* cells. However *PHO3* transcript levels from all 4 strains are shown to be repressed by phosphate starvation.

Figure 4.5. Activation of *PHO81*, *PHO84*, and *PHO86* is also potentiated in *rpd3*Δ cells. Reverse transcription and real-time PCR were used to quantitate the mRNA levels in *RPD3 PHO4*, *rpd3*Δ *PHO4*, *RPD3 pho4*Δ, and *rpd3*Δ *pho4*Δ cells grown in the absence of phosphate for 3, 6, and 9 h as described in Methods and Materials. Transcriptional potentation is also observed for *PHO81* (A), *PHO84* (B) and *PHO86* (C).

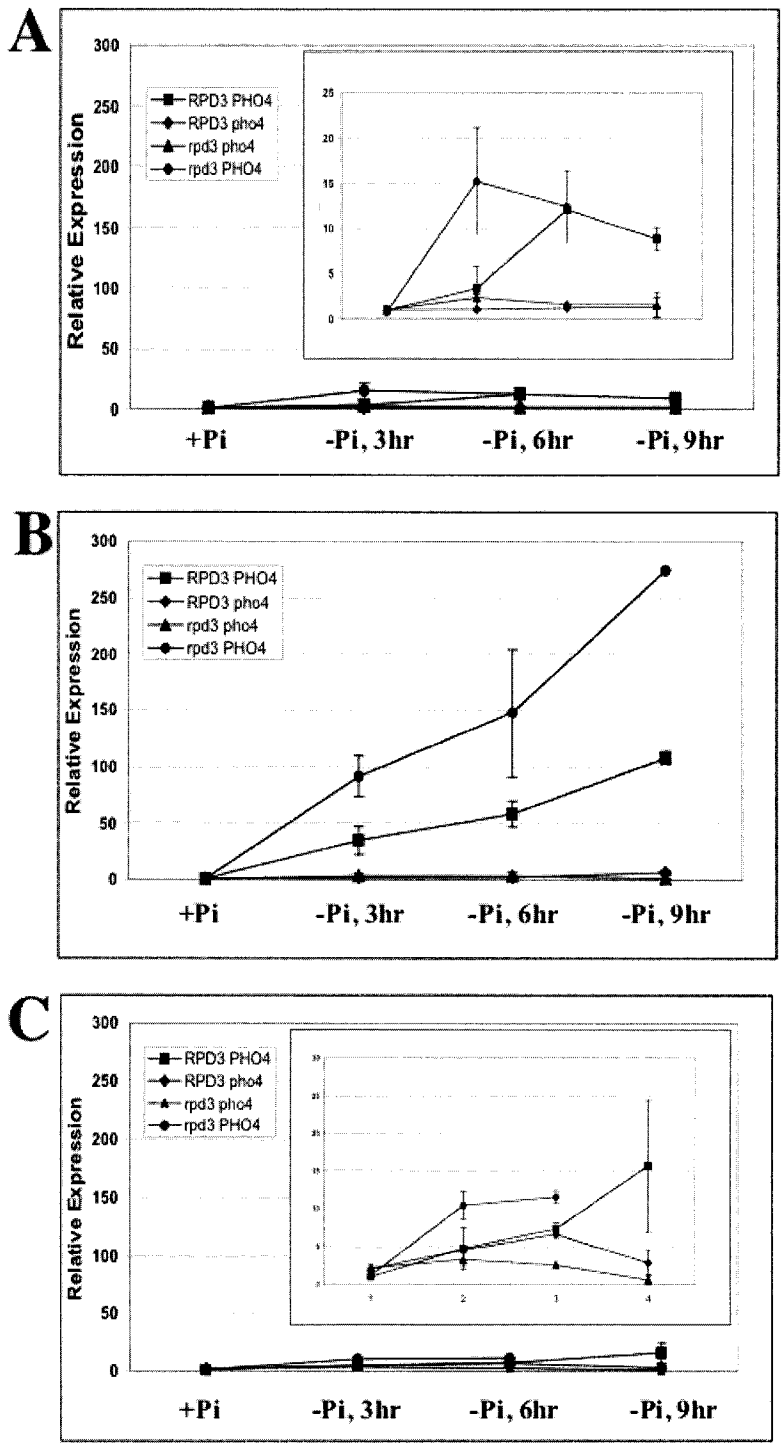

Figure 4.5A, 4.5B and 4.5C.

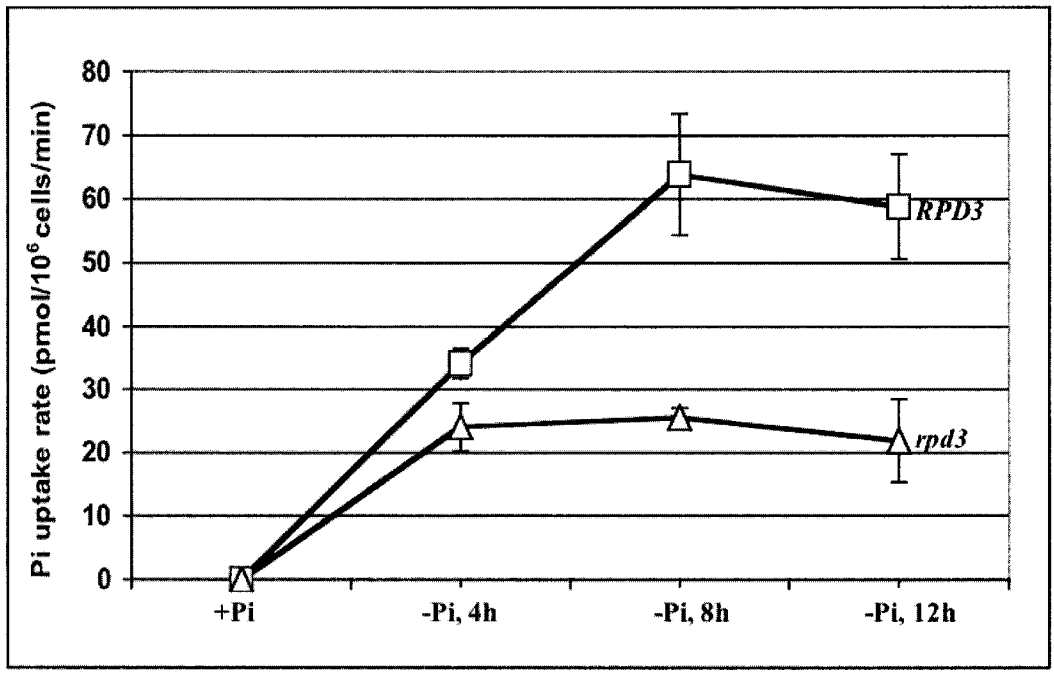

Figure 4.6. Phosphate uptake is defective in *rpd3Δ* cells. The rate of phosphate uptake into *RPD3 PHO4* (□), and *rpd3Δ PHO4* (△) cells grown in no phosphate medium for 4, 8, and 12 h was measured as described in Methods and Materials. The uptake rate is quite comparable in the first four hours. At 8 h and 12 h, however, the uptake rate observed in the *rpd3Δ* strain was over two-fold lower than that of wild type.

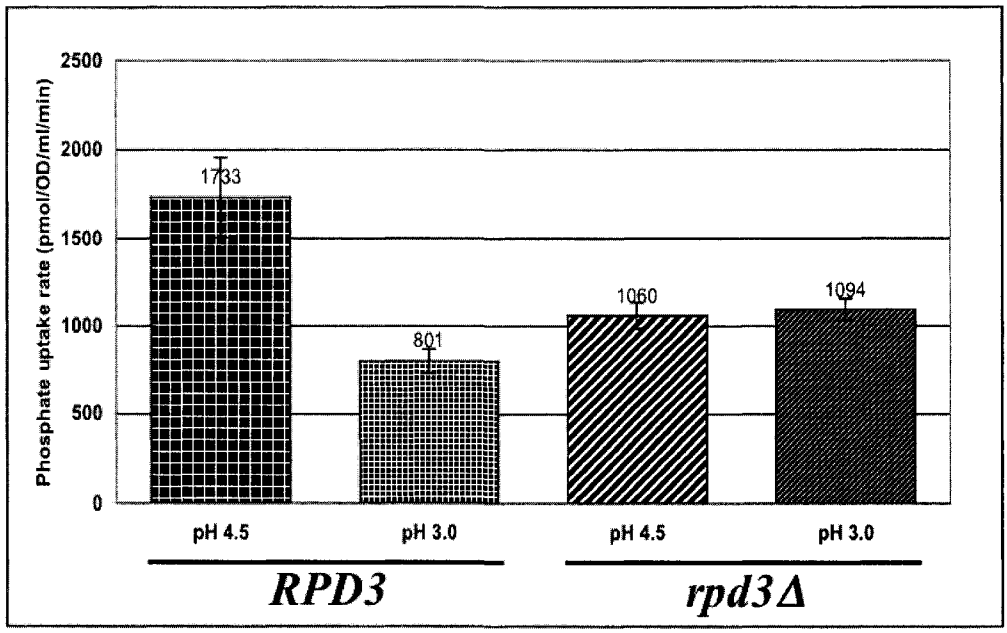

Figure 4.7. Phosphate uptake defect in *rpd3Δ* cells is not rescued by restoration of theproton motive force across plasma membrane. The phosphate uptake rate defect in the *rpd3Δ* cells cannot be rescued by lowering extracellular pH. At pH 4.5 (normal pH of culture in SC media), *rpd3Δ* cells exhibited a phosphate uptake rate almost two fold lower than that observed in wild type cells. Lower extracellular pH (pH 3), restores the proton gradient across plasma membrane, but does not help increase the phosphate uptake rate. However, the lower extracellular pH does decrease the phosphate uptake rate of the *RPD3* wild type cells to that of the *rpd3Δ* cells.

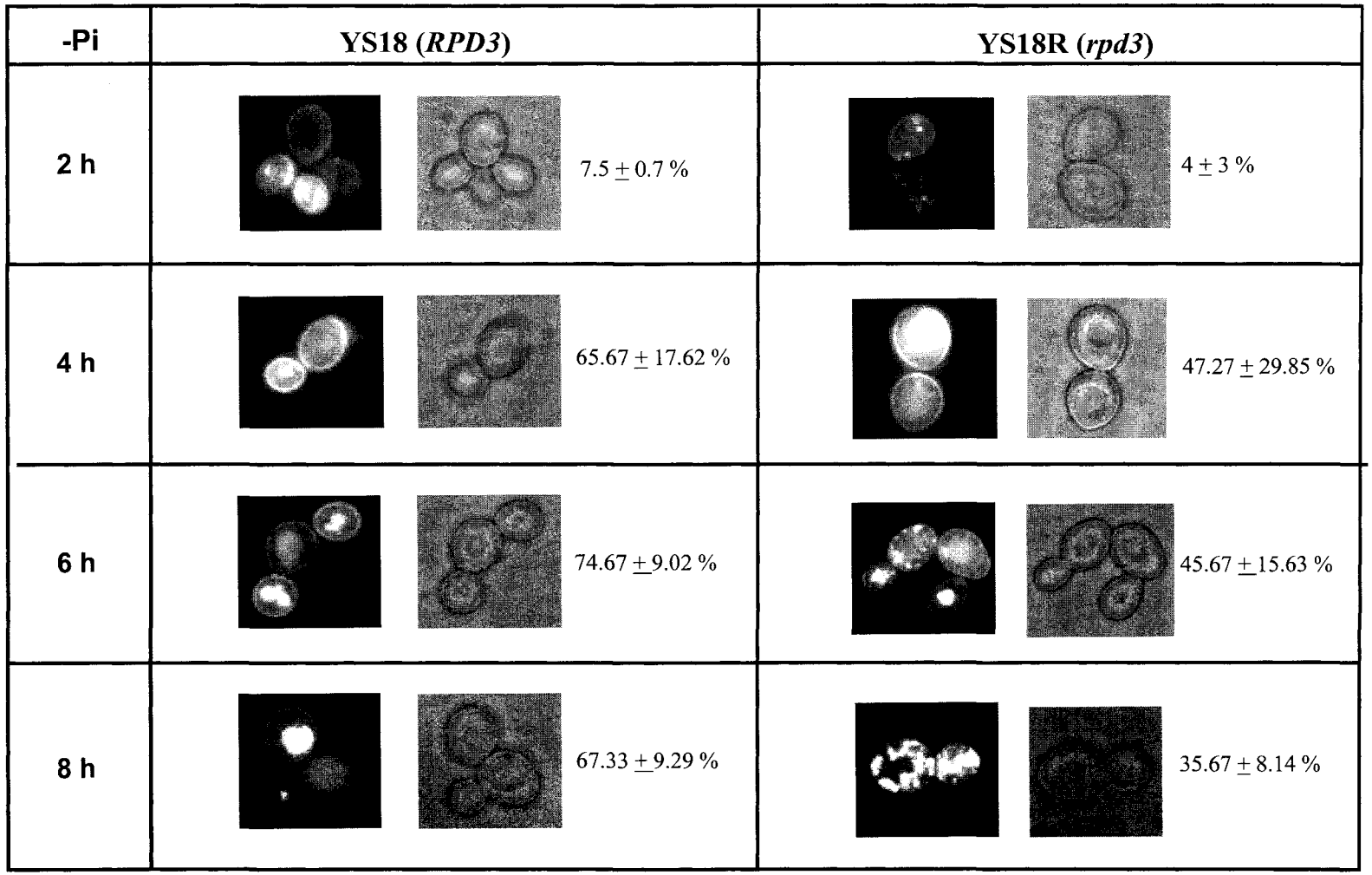

Figure 4.8A . Pho84p-GFP is prematurly recycled in *rpd3*Δ cells grown on no phosphate medium. Fluorescence microscopy of chimeric Pho84p-GFP was used to monitor Pho84p expression and cellular localization in *RPD3 PHO4* and *rpd3*Δ *PHO4* cells grown in –Pi medium for 2, 4, 6 and 8 h. The left panels are the fluorescence micrographs and the right panels are phase-contrast micrographs. The percentage of cells in which Pho84p-GFP is localized on plasma membrane is indicated for each strain to the right of the micrographs.

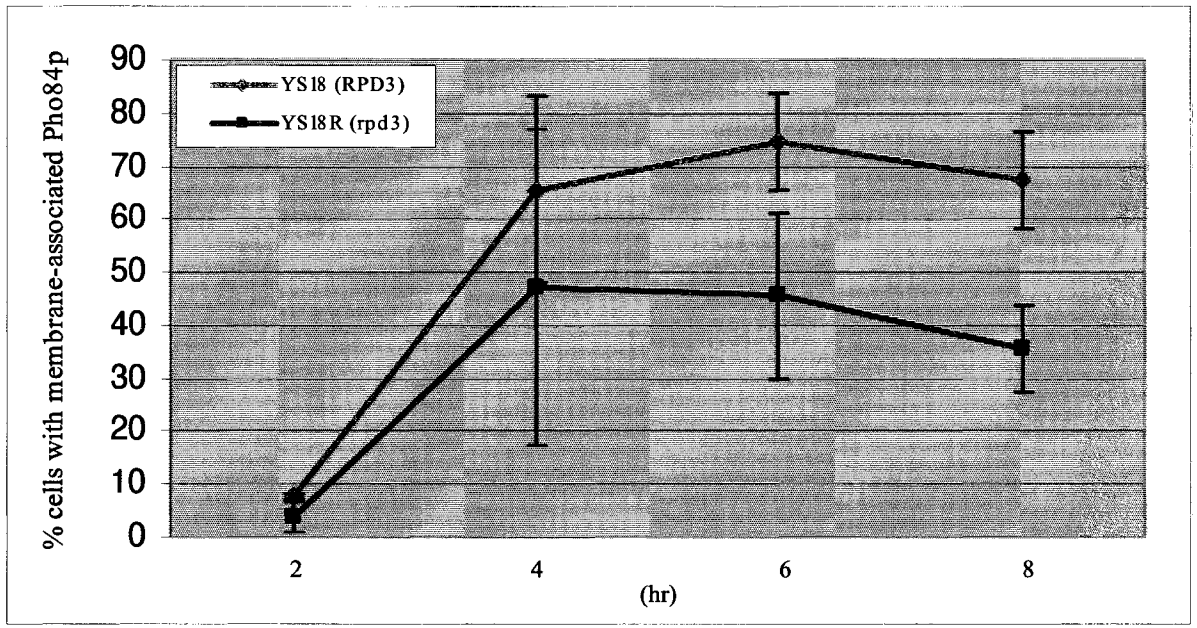

Figure 4.8B. Percent cells with membrane-associated Pho84p-GFP at 2, 4 , 6 and 8 hr cultured in –Pi medium.

From YS18 (*RPD3*) at 2 (7.5 ± 0.7), 4 (65.67 ± 17.62), 6 (74.67 ±9.02) and 8 (67.33 ±9.29) hr.

From YS18R (*rpd3*) at 2 (4 ± 3), 4 (47.27 ± 29.85), 6 (45.67 ±15.63) and 8 (35.67 ± 8.14) hr.

TABLE 4.1

Yeast strains and plasmids

Strain	Genotype
YS18	(MATα his3-11,15; leu2-3,112; ura3Δ5; canR)
YS18R	(MATα; his3-11,15; leu2-3,112; ura3Δ5; **rpd3::LEU2**; canR)
YS20	(MATα; his3-11,15; leu2-3,112; ura3Δ5; **pho4::URA3;** canR)
YS20PR	(MATα; his3-11,15; leu2-3,112; ura3Δ5; **pho4::URA3; rpd3::LEU2** canR)

TABLE 4.2

Primer sequences for SYBR Green real-time PCR analysis

Amplified gene	sequence 5'-3'	Product size (bp)
Actin	F, CTGTCGAGAGATTTCTCTTTTACC	108
	R, GCCCCTATTTATTCCAATAATATCG	
PHO2	F, CGTCAGAATTTGGTTTCAGAACAG	112
	R, GATCGTAATCGTTGGCAATATCAC	
PHO3	F, GTAAAGAAAGGGCCATTCCAAATTACC	99
	R, CTCTCCGAGGGGAATTGTACCTG	
PHO4	F, AGAGCAGCATTCTTGATAAAGTCGG	91
	R, CTCGTCGTTTTGCTCGTTGAAG	
PHO5	F, GCAAGCAAATTCGAGATTACCAATG	99
	R, GCTAGTTTGCCTAAGGGAATGGTACC	
PHO81	F, CCATTCCCACGCTAAAGGCTAG	103
	R, GGCAGCTTTATTTTCTTGCAGTCTC	
PHO84	F, TGATGTCCTACGTTTACTGGCACG	103
	R, ACCAAAACCAAATTGACCAATAACAG	
PHO86	F, ACCACTTGATATTGATGCTCCTCC	103
	R, GTTCTTGTCTGATGAAATCCGC	
INO1	F, ATATTGCTCCAATCACCTCCG	256
	R GTGCTTATTCGCCAATACCG	

Chapter 5

Summary of *PHO5* Regulation, Development of Chromatin Assembly on a Solid Phase and Future Directions

5.1 Summary of *PHO5* Regulation

A summary of what we know about *PHO5* is divided into two parts. Part one involves signaling and the *PHO* pathway that modulates *PHO5* transcription. Part two constitutes the function of transcription co-activators and chromatin reconfiguration activities that are required for *PHO5* expression.

5.1.1 *PHO* Gene Regulatory Pathway

Numerous studies in the past few years have provided insights into several aspects of the *PHO* gene regulatory pathway. These include phosphate signaling to regulate the pathway leading to transcription of phosphatase encoding genes. Under phosphate starvation, several phosphatase encoding genes (acid, alkaline and substrate-specific phosphatases) are activated. Acid phosphatase, primarily expressed from *PHO5* catalyzes liberation of inorganic phosphate from extracellular sources. Signaling through the *PHO* pathway also activates several phosphate transporter genes. The high affinity

phosphate transporter Pho84p is mainly responsible for phosphate uptake (18). Consistent with this, expression and phosphate transport activity of Pho84p are modulated by extracellular phosphate concentration (9, 10). *PHO86*, identified as an endoplasmic reticulum (ER) resident protein that facilitates ER exit of Pho84p, is also up-regulated during phosphate depletion (12, 19). *PHO81* is up-regulated during phosphate starvation, as well (12). Up-regulation of *PHO5*, *PHO81*, *PHO84* and *PHO86* during phosphate starvation is consistent with the results obtained in this study as shown by reverse transcriptase real time PCR (Chapter 4). In addition to the group of phosphatase and phosphate permease genes, genome wide analysis also demonstrated that a new set of genes in the *PHO* pathway was up regulated under phosphate depletion. These genes encode proteins involved in polyphosphate accumulation (12). During growth in high phosphate medium, phosphate accumulation in vacuole occurs through polyphosphate synthesis. Polyphosphate formation serves as a "phosphate sink" so preventing short-term saturation of cellular phosphate accumulation. Consistent with this finding, Neef and Kladde (11) also demonstrated that decreased polyphosphate level activated mitotic induction of *PHO5* in a Swi/Snf and Gcn5p-dependent manner. Addition of phosphate also repressed mitotic gene expression and increased accumulation of polyphosphate. The level of polyphosphate fluctuated inversely with the level of *PHO5* mRNA. Defects in polyphosphate synthesis induced early *PHO5* expression and also increased the rate, degree and duration of expression. Taken together, this suggests that phosphate level regulates both mitotic gene expression of *PHO5* and activation of *PHO5* under phosphate depletion of asynchronized cells. Most importantly, phosphate sensor(s) or receptor(s)

are likely to be inside cells since intracellular phosphate seems to be the key control of *PHO* gene expression.

5.1.2 Transcription Regulation of *PHO5*

Several transcriptional co-activators with chromatin remodeling activities that regulate *PHO5* transcription have been identified. Under activating conditions in asynchronous yeast cells, transcription of *PHO5* appears to be mostly Swi/Snf and Gcn5 independent (5, 6). In contrast, *PHO8* transcription, despite regulation by the same transcription activator Pho4p, depends on both activities (7). This raises an interesting question as to how Pho4p manages to activate transcription through different coactivators on the two genes. Pho4p has been shown to be able to bind its recognition site on the *PHO5* promoter within a nucleosome (14) and *PHO8* in the absence of remodeling activities (7) suggesting that Pho4p is capable of binding without help from remodeling activity and that the remodeling factors function in a step after Pho4p binding. Pho4p has been shown to bind and induce conformational changes in transcription factor TFIIB (17). Recruitment of the SAGA complex to the *PHO5* promoter is Pho4p-dependent and Spt3p, a SAGA subunit, is required for *PHO5* activation through its function of TBP recruitment (1). Thus, Pho4p is likely to function through recruitment of the transcriptional coactivator, SAGA, and transcription factor TFIIB leading to preinitiation complex formation.

Even though *PHO5* expression is Swi/Snf and Gcn5 independent, in mitosis *PHO5* requires both activities to achieve full induction (8, 11). This reflects the more global role of these chromatin remodeling activities in transcription in addition to their

gene-specific recruitment by transcription activators. Consistent with a global function in chromatin remodeling, histone acetylation is also found to function similarly (16). Global acetylation has widespread effects over a large chromosomal region, including *PHO5*. A higher acetylation state over the promoter region causes *PHO5* to be readily transcribed after switching cells to no phosphate media. The acetylation status over the *PHO5* promoter is modulated by balancing the activities of HATs (Gcn5 and Esa1p) and HDACs (Rpd3p and Hda1p) (16). Deletion of *RPD3* then potentiates transcription of *PHO5*. That also explains the role of Gcn5p in the kinetics of *PHO5* expression as deletion of *GCN5* caused a delay in activation (2). The direct role of Rpd3p on the *PHO5* promoter is consistent with what we observed in Chapter 4. In addition, we showed that Rpd3p functions indirectly to modulate the turn over rate of the phosphate transporter Pho84p, in turn affecting phosphate uptake. The details of the mechanism underlying Rpd3-mediated regulation of Pho84p turn over rate are still an open question.

In addition, the INO80 complex, a new complex containing ATP-dependent remodeling activity, has been shown to function in *PHO5* transcription (3, 13). However, how this complex functions in *PHO5* regulation is not well understood.

5.2 Development of Solid Phase-Based Chromatin System

The *in vitro* chromatin reconstitution system from yeast has been developed in the Laybourn laboratory. This system provides an excellent tool to study the mechanism of transcription regulation in the chromatin context since most components are purified proteins. As we have shown that transcription-related activities can be reconstituted *in*

vitro on the assembled chromatin, this system is ready to be used in dissecting more details in transcription regulation.

So far experiments were performed on circular plasmid DNA containing the *PHO5* promoter. Even though it has proved to be useful, it has limitations for some studies. Using a solid-state (bead-bound) system, several aspects of chromatin impact on transcription regulation can be studied more readily. These include protein-protein or protein-DNA interactions between chromatin modifying-related activities. Therefore, development of solid-state chromatin template will provide insights into the stepwise mechanism of *PHO5* regulation.

I have developed a solid-state chromatin template using the *Bam* HI- *Bam* HI DNA fragment containing *PHO5* promoter and the yeast reconstitution system. The restriction map of pPHO5 G-less between the two *Bam* HI sites is shown in Figure 5.1. The *Bam* HI- *Bam* HI DNA fragment from pPHO5 G-less cassette was amplified using PCR with one primer biotinylated at 5' end to allow the DNA to be attached to streptavidin-conjugated beads. The sequence of primers is as follows; PUCFS (5' CGC CAG GGT TTT CCC AGT CAC GAC 3') and PUCRS (Biotynylated 5' GCG GAT AAC AAT TTC ACA CAG GAA 3'). The size of the amplicon is 792 bp. PCR amplified *Bam* HI- *Bam* HI DNA fragment was gel purified and assembled into chromatin. Nucleosome formation was verified by micrococcal nuclease digestion and restriction enzyme accessibility assays. Chromatin was bound to streaptavidin–agarose beads. The details of each step are described in the following sections.

5.2.1 PCR Amplification of *Bam* HI- *Bam* HI DNA Fragment from the plasmid pPHO5 G-Less Cassette and Gel Purified by QIAEX II DNA Extraction Kit (Qiagen)

A 50 μl PCR reaction contained 0.5 ng plasmid DNA pPHO5 G-less, 200 μM dNTP, 200 nM each primer, 1 X PCR buffer (commercial available) and 1 unit of Taq polymerase (Fisher). Thermal cycler was programmed for 35 cycles. Denaturation temperature was set at 95 °C for 4 min in the first cycle and 1 min for the rest of the cycle in the amplification process. Annealing temperature was set at 55 °C for 1 min. Elongation temperature was set at 72 °C for 2 min and the last cycle for 10 min. The PCR product was resolved on a 1.2% agarose gel and the corresponding 792 bp band was gel purified using QIAEX II DNA extraction kit (Qiagen). DNA was dissolved in water and concentration determined by gel electrophoresis and spectrophotometer.

5.2.2 Chromatin Assembly with the Gel Purified DNA Fragment

Chromatin was assembled with different mass ratio of yeast core histones and DNA. The assembly reactions were similar to those performed on a plasmid DNA. GST-yeast Nap1p was incubated with the recombinant yeast core histones at a mass ratio of 8:1 for 30 min at 4 °C followed by addition of assembly buffer (described in chapter 2) and gel-purified DNA. The reactions were incubated at 30 °C for 4 hr. Micrococcal nuclease digestion was performed after incubation to check the quality of the reconstituted chromatin. The protocol for micrococcal nuclease digestion is as described in chapter 2. As shown in Figure 5.2, chromatin assembled at mass ratio of core histones to DNA at 4:1 yielded complete assembly. At the lower ratio (with the higher amount of

DNA than core histones available) the assembly was incomplete and resulted in degradation of free DNA and the smear pattern below the mononucleosome band.

5.2.3 Restriction Enzyme Accessibility Assay

Restriction enzyme accessibility assay can be used to verify nucleosome formation and roughly indicates the position of nucleosomes on the DNA template. Unlike free DNA, restriction sites in the nucleosomes are partially protected from digestion. On the *PHO5* promoter (Figure 5.1), *Mfe* I and *Bst*E II restriction sites are located in the nucleosome free region. Therefore, percent accessibility is not different between naked DNA and chromatin. However, *Cla* I and *Hae* II sites are located in the nucleosomes, thus, percent accessibility is reduced in chromatin as compared to naked DNA.

After chromatin assembly, 175 ng of free DNA and chromatin were incubated with 5 units of restriction enzymes (mentioned above) for 1 hr at room temperature in 20 μl reaction volume. Reactions were stopped by addition of 100 μl of stop solution (20 mM EDTA pH 8.0, 0.2 M NaCl, 1% SDS, 0.25 mg/ml glycogen) and 5 μl of 2.5 mg/ml proteinase K. The proteinase K digestion was incubated for 20 min at 37 ^{o} C and followed by phenol chloroform extraction, and ethanol precipitation. DNA was dissolved in water and resolved on 2% agarose gel (Figure 5.3).

As shown in Figure 5.3, the accessibility for restriction enzymes is different between the free DNA and chromatin. Percent protection for free DNA and chromatin is calculated from dividing the band intensity of uncut DNA products (left over of 792 bp DNA band) by the total of band intensity from every band in each lane. The approximate

nucleosome positions are as shown in Figure 5.1 in this chapter. *Cla* I site is in nucleosome –2 and *Hae* II is in nucleosomes -1 and -3. Therefore, both sites are presumably protected from digestion when exposed to the corresponding restriction enzymes. Both *Mfe* I and *Bst*E II sites are in the linker DNA, therefore, restriction enzyme digestion patterns from both free DNA and chromatin supposedly are similar. As expected, *Cla* I restriction enzyme digestion pattern suggested some protection in chromatin (34%) as compared to free DNA (24%). However, *Hae* II digestion showed almost equally protection in chromatin (13%) and free DNA (15%) suggesting that accessibility are equally the same between DNA and chromatin. There are two *Hae* II restriction sites on this DNA fragment. Digestion only one site yields two DNA products of either 215 bp and 580 bp or 270 bp and 525 bp (Figure 5.1). Further digestion results in three DNA products if digestion is complete (215 bp, 310 bp and 270 bp) or five DNA products if digestion is not complete (Figure 5.1). The band intensity of two DNA fragments, 580 bp and 525bp, from free DNA was less than that from chromatin suggesting further digestion (cut more than once) (Figure 5.3). Even though percent protection suggests the same accessibility for free DNA and chromatin (which means *Hae* II sites is in nucleosomes free region), the reduced band intensity of 580 bp and 525 bp DNA suggests that chromatin might provide protection for further cut (which means *Hae* II is also in the nucleosomes). In addition, the restriction digestion from *Mfe* I and *Bst*E II showed partially protection from chromatin suggesting the position of nucleosome over the restriction sites. These two sites, however, is in the nucleosome free region *in vivo*. The protection that we observed *in vitro* probably resulted from *in vitro* chromatin dynamics or the presence of heterogeneous species of assembled nucleosome.

These preliminary results obtained from the restriction enzyme accessibility assay are not conclusive. A few experimental factors need to be justified. The unit of each restriction enzymes used in the assay should be adjusted accordingly. The DNA staining solutions or staining processes should be optimized to reflect the actual amount of each DNA band. Using the spacing factors such as yeast Isw1p might assist the production of more homogeneous species of reconstituted chromatin.

5.2.4 Streptavidin Agarose Preparation and Binding Reaction with Assembled Chromatin

After nucleosome assembly, the next step was binding of chromatin to the streptavidin agarose beads. Forty microliters of 50 % slurry bead suspension was centrifuged for 30 sec and the supernatant was removed. One hundred microliters biding buffer (10 mM HEPES pH 7.6, 50 mM KCl, 5 mM DTT, 5 mMPMSF, 5 mM $MgCl_2$, 0.25 mg/ml BSA and 5% glycerol) was added to wash and equilibrate streptavidin-conjugated agarose beads. After the last equilibration, binding buffer was removed and the chromatin solution was added to agarose beads. The binding reaction was carried out at 30 ^{o}C for 1 hr with agitation. After binding, the reaction was centrifuged and supernatant was removed. Agarose beads were washed once and resuspended in binding buffer. Percent recovery after binding was determined using restriction enzyme *Hind* III digestion. There is one *Hind* III site at the 5' end of 792 bp DNA fragment (Figure 5.1). To check the efficiency of binding to agarose beads, the bead-bound free DNA and chromatin were digested with *Hind* III digestion to release DNA from binding. After deproteinization, DNA was resolved on 1.2 % agarose gel. The efficiency of binding was

compared between the *Hind* III digested free DNA band (U) and bead-bound DNA and chromatin bands (Figure 5.4).

The preliminary results show that the development of chromatin on a solid phase is progressing. Even though binding efficiency suggests that a lot of chromatin does not bind to streptavidin agarose beads, this low efficiency could probably be improved by increasing the amount of agarose beads in the binding reaction. If the binding efficiency is reproducible with the increasing amount of streptavidin agarose beads, then the low efficiency of binding can be disregarded. The agarose bead could also be replaced with the magnetic bead system, which seems to be easier to handle and is likely to give less background than agarose-based system.

DNA staining solutions (SYBR Gold, Molecular Probe) and staining processes significantly affect DNA band intensity. For the restriction enzyme accessibility and binding experiments (Figure 5.3 and 5.4), different DNA staining solutions should also be tested to justify the interpretations.

According to the *in vivo* experiments (1, 11), SAGA histone acetyltransferase have been shown to be recruited to the *PHO5* promoter under activating conditions. This finding suggests the interaction between Pho4p (or the possible ternary complex of Pho2p-Pho4p-DNA that probably form proper docking site for those activities) and SAGA subunits. The solid phase-based chromatin assembly system can be used to demonstrate these interactions between those proteins.

5.3 Future Directions: Identification of Histone Acetyltransferase and ATP-Utilizing Chromatin Remodeling Activities that Function on the *PHO5* Promoter *In Vitro*

The minichromosome reconstituted on the plasmid DNA containing the *PHO5* promoter is primarily shown to posses several aspects of transcription related-activities (chapter 3). This promising chromatin assembly system should be used to study *PHO5* transcriptional regulation in more detail.

5.3.1 Identification of the HAT Activities that Function in Activation of *PHO5 In Vitro*

In vitro transcription on the *PHO5* promoter is demonstrated to be HAT-dependent (14). However, the entities containing HAT activity that contributed to transcriptional activation are unknown. The transcription whole cell extract (WCE) contains a fair amount of endogenous HAT activities (Figure 3.6 and 3.7, Chapter 3). To identify HAT complex(s) that function in *PHO5* activation *in vitro*, HAT-free transcription WCE should be used in transcription reactions. To create the HAT-free transcription WCE, the Ni^{2+}-NTA agarose, used in purification of yeast HAT complexes (4), was used to deplete HAT activity from transcription WCE. After removal of intrinsic HAT activity from the transcription WCE, the purified HAT complex(s) will be added to the transcription reaction and the complex(s) involving in *PHO5* activation will be identify.

I have attempted to deplete HAT activity from transcription WCE several times. The amount of Ni^{2+}-NTA agarose beads was adjusted based on protocol from Eberhater *et al.* (4). Surprisingly, HAT activity in the transcription WCE was increased rather than

depleted (Figure 5.5). Increasing amounts of Ni^{2+}-NTA agarose beads resulted in more and more HAT activity after depletion. In addition to HATs, Ni^{2+}-NTA agarose beads probably remove HAT inhibitor(s) from the transcription WCE. Since the HAT-depleted transcription WCE will be needed for transcription, this activity was also examined. Unfortunately, transcription activity was significantly reduced after HAT depletion (data not shown). This is probably because some HAT activities are tightly associated with transcription machinery or are an integral part of machinery (for example, TAF_{II} 250). The findings that HAT-depleted WCE yielded better HAT activity and reduced transcription activity make it less possible to use this method to identify HAT(s) that function in *PHO5* transcription *in vitro*. The alternative is to separate HAT activities from transcription WCE through standard purification.

5.3.2 Functional Investigation of HAT and ATP-Dependent Chromatin Remodeling Activities in *PHO5* Chromatin Dynamics *In Vitro*

Restriction enzyme accessibility assay has served as an effective way to determine nucleosome positioning and verify chromatin dynamics on the *PHO5* promoter *in vivo* (2, 5, 6). I have performed a few experiments with the restriction enzyme accessibility assay using minichromosomes. The results suggested that each remodeling activity differentially affects individual positioned nucleosome on the *PHO5* promoter. However, the result is not reproducible, which is likely because the assembled nucleosome on plasmid DNA is heterogeneous. To obtain the more homogeneous chromatin, sucrose gradient ultracentrifugation should be used after chromatin reconstitution to prepare chromatin for restriction enzyme accessibility assay. If this

experiment is set up successfully, it can be used to investigate function of several remodeling complexes on the chromatin dynamics on the *PHO5* promoter.

One thing that would be interesting to do is using yeast Isw1p in the chromatin assembly reaction. Isw1p was shown to contain nucleosome spacing activity and produce the DNA ladder of about 175 bp from micrococcal nuclease digestion (15). With the nucleosome spacing activity, yIsw1p probably facilitates formation of the more homogeneous nucleosome.

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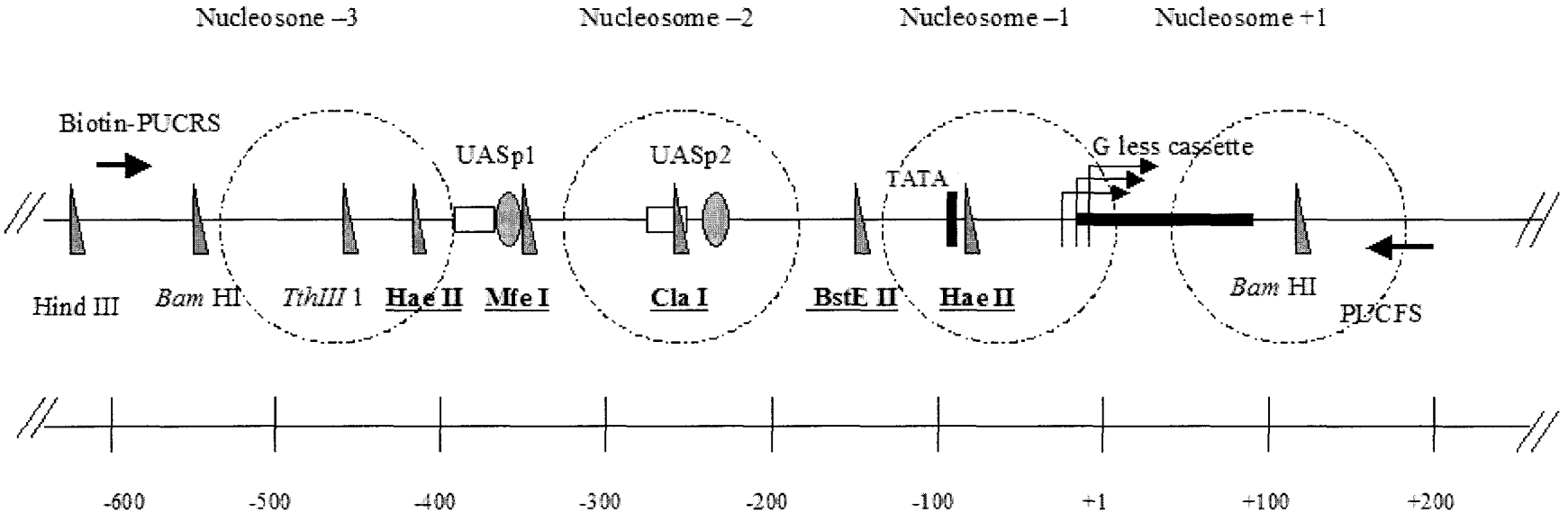

Figure 5.1. Restriction map and *in vitro* nucleosome positions on the *PHO5* promoter of *Bam* HI-*Bam* HI DNA fragment. The restriction enzyme in bold character was used in restriction enzyme accessibility assay. Biotin-PUCRS and PUCFS were primers that were used in PCR reaction to amplify the 792 bp DNA fragment containing *PHO5* promoter and G less cassette.

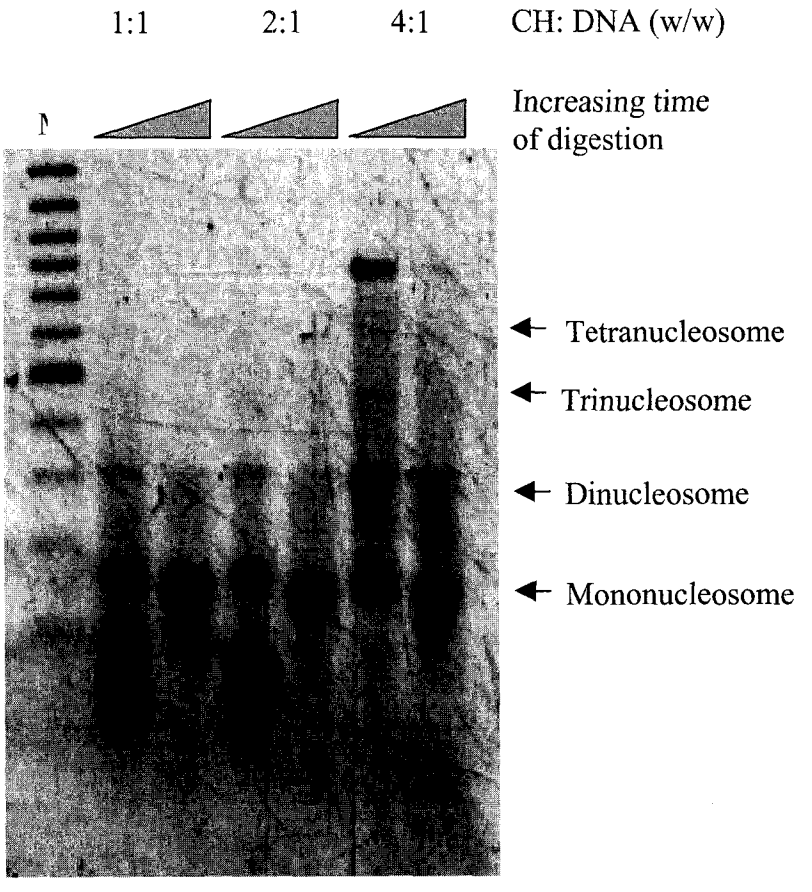

Figure 5.2. Micrococcal nuclease digestion of reconstituted chromatin . Chromatin was assembled with different ratio of core histones to DNA (792 bp, PCR amplified *Bam* HI-*Bam* HI DNA fragment containing the *PHO5* promoter). Four nucleosomes were formed as shown by the presence of DNA ladder up to four bands. The smear patterns below mononucleosome band indicated incomplete chromatin assembly, which meant more DNA in the assembly reaction than the available core histone, thus resulted in degradation of free DNA.

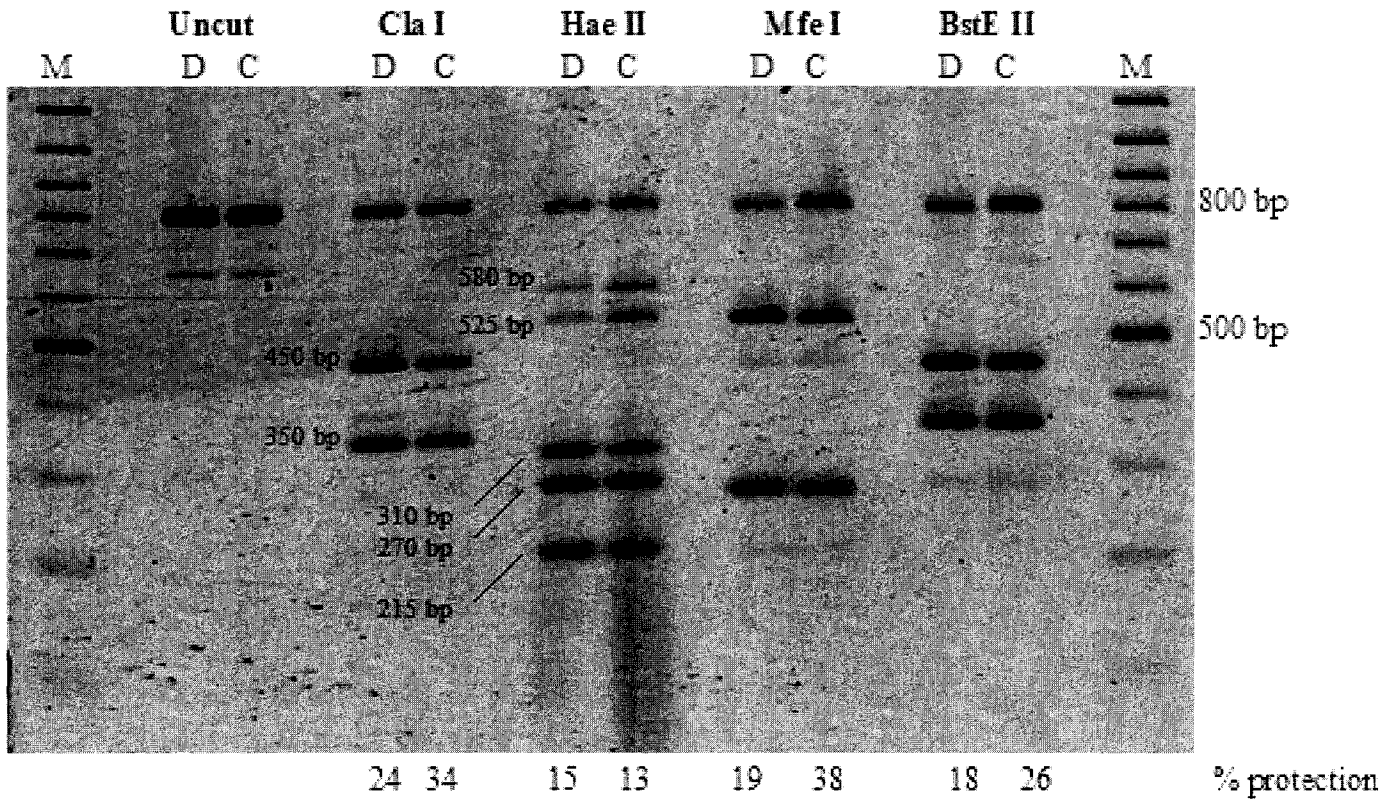

Figure 5.3. Restriction enzyme accessibility assay on assembled chromatin. Chromatin was reconstituted with linear DNA containing *PHO5* promoter, GST-yNap1p and recombinant yeast CHs. Both free DNA (D) and chromatin (C) are then subjected to restriction enzyme digestion (*Cla* I, *Hae* II, *Mfe* I and *Bst*E II). Restriction map is shown in Figure 1. DNA Marker (M) is indicated at the upper end of figure. Calculation of % accessibility is described in section 5.2.3.

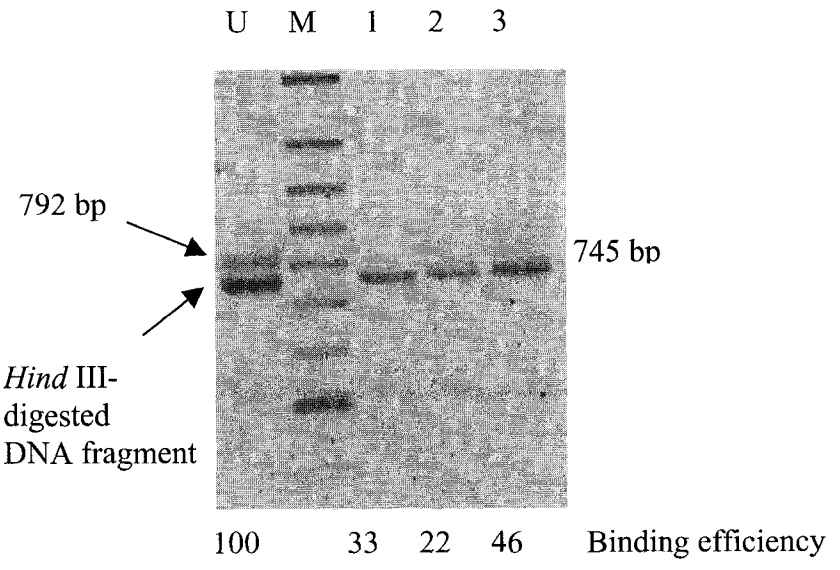

Figure 5.4. Binding efficiency of DNA and chromatin to streptavidin agarose beads. The samples, unbound and *Hind* III-digested free DNA (U), beads-bound free DNA (1), beads bound chromatin 1:4 (CH:GST-yNap1p) (2), beads bound chromatin 1:8 (CH:GST-yNap1p) (3), and DNA markers (M) are indicated.

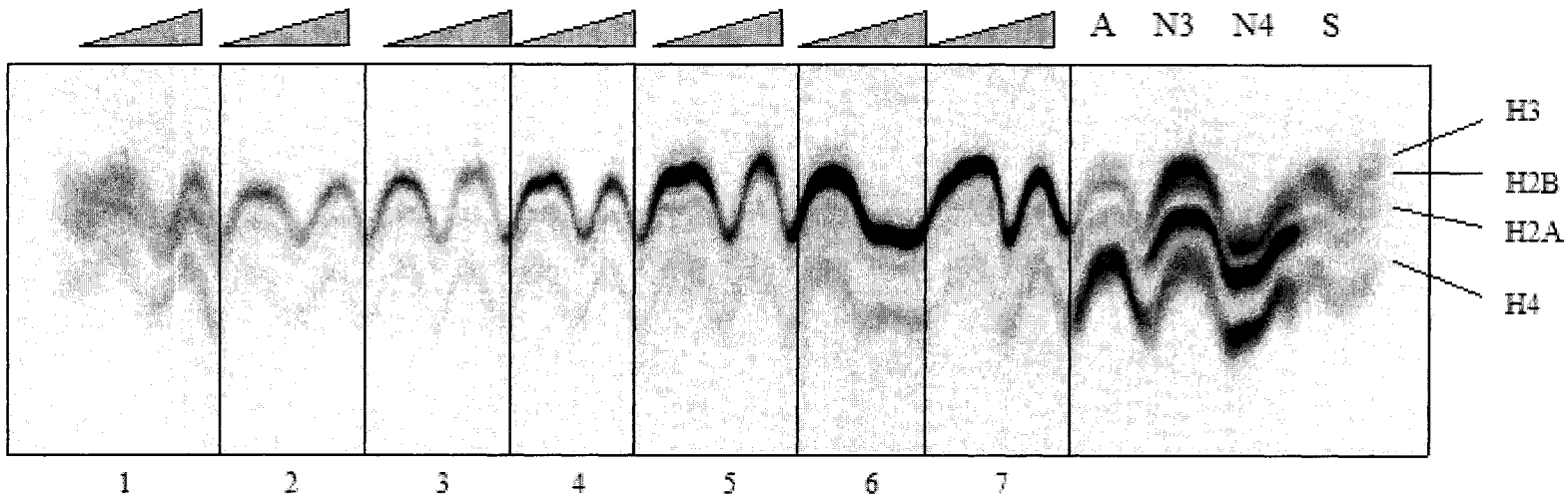

Figure 5.5. HAT assay using recombinant yeast CH to check for the remaining HAT activity in the HAT-depleted transcription WCE. Purified, fractionated yeast HAT complexes, ADA (A), NuA3 (N3), NuA4 (N4), and SAGA (S) are used as a control. Sample number 1 showed HAT activity in the untreated transcription WCE. Sample number 2-7 showed HAT activity in the Ni $^{2+}$-NTA-treated transcription WCE. Sample number 2 treated with the lowest amount and number 7 with the highest amount of Ni $^{2+}$-NTA.

Increasing amount of transcription WCE