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DISSERTATION

DEVELOPMENT OF AN *IN VITRO* SYSTEM TO STUDY CHROMATIN REMODELING AND TRANSCRIPTION ACTIVATION OF THE *SACCHAROMYCES CEREVISIAE* *PHO5* GENE

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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Fall 1999

COLORADO STATE UNIVERSITY

October 4, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ANDREA RAE TERRELL ENTITLED DEVELOPMENT OF AN *IN VITRO* SYSTEM TO STUDY CHROMATIN REMODELING AND TRANSCRIPTION ACTIVATION OF THE *SACCHAROMYCES CEREVISIAE PHO5* GENE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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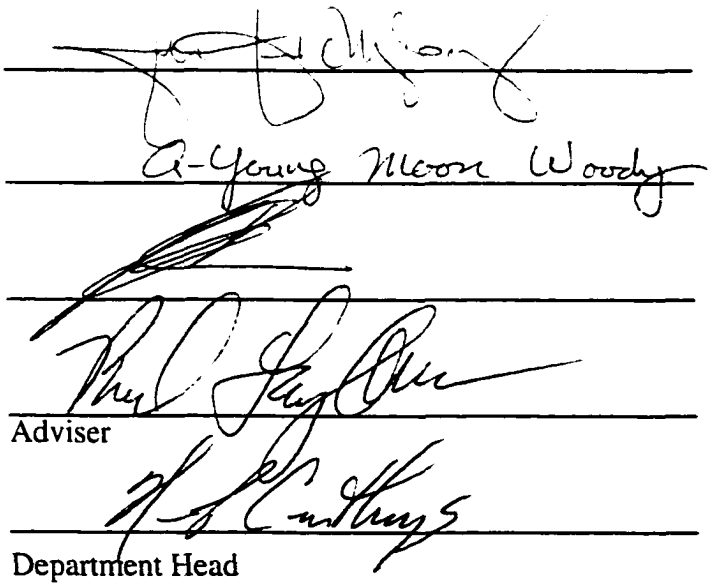

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ABSTRACT OF DISSERTATION

DEVELOPMENT OF AN *IN VITRO* SYSTEM TO STUDY CHROMATIN REMODELING AND TRANSCRIPTION ACTIVATION OF THE *SACCHAROMYCES CEREVISIAE PHO5* GENE

The *Saccharomyces cerevisiae PHO5* gene encodes the major secreted acid phosphatase in yeast. Expression of *PHO5* is regulated through its chromatin structure by phosphate availability. The gene is repressed when there is adequate phosphate in the environment and becomes activated upon phosphate starvation. There are four translationally positioned nucleosomes located over the *PHO5* promoter. All four nucleosomes become nuclease transparent upon transcription activation *via* the actions of Pho4p and Pho2p, two DNA binding transcription activators. To investigate the mechanism of activation of *PHO5*, an *in vitro* system was designed that utilizes purified yeast core histones and transcription activators, yeast extracts, and a plasmid template containing the *PHO5* promoter. This dissertation describes the use of this system to study the mechanisms of transcription activation and nucleosome remodeling on the *PHO5* promoter. Through *in vitro* transcription assays, I have determined that purified recombinant Pho4p activates transcription on naked DNA but is not sufficient to counteract the nucleosome repression of *PHO5*. Using primer extension footprinting on *in vitro* reconstituted chromatin, Pho4p and Pho2p were shown to bind the *PHO5* promoter regardless of whether it existed as naked or chromatin DNA. I have also used micrococcal nuclease digestion to determine that chromatin is being remodeled by the

same whole cell extract used for transcription assays, therefore the lack of derepression on chromatin templates is most likely a result of another unidentified barrier, possibly to elongation. I have also shown that fractionated nuclear extract can remodel *PHO5* chromatin and that the requirements for remodeling are different depending on the amount of extract added.

Unfortunately, the results presented in this dissertation fail to provide a clear understanding of nucleosome remodeling and transcription activation of *PHO5*. However, a great deal of time and effort has gone into this project and I believe this system, since it is now very well defined, will be a useful tool in future studies of gene regulation in the context of chromatin.

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ACKNOWLEDGEMENTS

First of all I would like to thank my advisor and friend Dr. Paul Laybourn for welcoming me into his lab and giving me the opportunity to do the work described in this dissertation. Paul gave me the freedom to pursue many different projects, some worked, some didn't, but I believe I learned more from the ones that were unsuccessful than from the ones that worked. In addition, I have gotten to work with some very good and interesting people; Dr. Dave Moss, Sriwan Wongwisansri (Apple), Amy Norton and countless other talented undergraduates, and especially John Pilon and Dr. Craig Martens, who in addition to being very intelligent scientists have also become my good friends. I also want to acknowledge my committee, Dr. Norm Curthoys, Dr. Jenny Nyborg, Dr. A-Young Woody, and Dr. Chris Wilcox for helpful suggestions during the course of my research, and for not making my life too difficult at committee meetings. I would especially like to thank Dr. Curthoys for his helpfulness during the last several months of my graduate career.

I want to thank my family for all the support they have given me, emotional and otherwise. They never hesitated to tell me how proud they were, which really meant a lot to me. I thank Abe, Bella, and Rose for never letting me forget that there is always time to play. And finally, I want to thank my best friend and fiancé John for being there and putting up with me during these past several years, and many years to come.

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Chapter 1

Background and Hypothesis

1.1 General Introduction

The regulation of gene expression is essential for the proper functioning of any organism. The work discussed in this dissertation is focused on gene expression at the level of transcription in the yeast *Saccharomyces cerevisiae*. However, unlike many studies on transcription, this work adds another level of complexity to the transcription regulation process, chromatin. Chromatin packages the DNA in the nucleus into very compact and highly ordered chromosomes. This ordered packaging is necessary both to get the DNA into a form that will fit inside the nucleus and also to allow the cell to respond immediately when it becomes necessary for a particular gene or set of genes to be expressed. Transcription regulation in general is becoming an increasingly important area of study as scientists are focusing more on the molecular basis of disease and are beginning to apply mechanisms elucidated in basic research labs for more applied research pursuits. Study of the regulation of transcription is important not only in the pursuit of general scientific knowledge but also for the ultimate improvement in our quality of life. Yeast and the RNA Polymerase II system are both ideal for studying gene expression for numerous reasons. Yeast cells are easily cultured and large quantities are readily obtained for biochemical investigations, genetic manipulation has become fairly simple especially since the entire yeast genome has been sequenced, and there is an

extensive amount of biochemical and genetic information available about the organism. The basic machinery of the RNA polymerase II system is already well understood and there are still a large number of research groups working on the fine details of the mechanisms of RNA polymerase II transcription. The research described in this dissertation was designed to contribute to the understanding of the role chromatin plays in the mechanisms that regulate the activity of a gene.

1.2 Yeast

Saccharomyces cerevisiae provides a good model for the study of chromatin. Yeast is a simple eukaryotic system and extensive genetic and biochemical information has been made available (51). Research using yeast as a model organism has been greatly facilitated since the sequence of the entire yeast genome was reported in the journal *Nature* in May of 1997, making it the first eukaryote for which the entire genomic sequence is known. Due to the similarity of many regulatory mechanisms and gene sequences, gene regulation by chromatin in yeast is predicted to be directly applicable to gene regulation in humans. The basic elements of cell structure and function are central to all eukaryotes. This has allowed yeast to emerge as a very important model organism because it is so easily manipulated, facilitating the study of complex mechanisms such as transcription. Yeast is also a major industrial tool so any effort to work out mechanisms and develop techniques, for example gene replacement techniques, can potentially be useful for industry.

1.3 Phosphate metabolism in yeast

Phosphate is required by all organisms primarily for nucleic acid and membrane biosynthesis. Phosphate metabolism involves a very large set of genes, including transporters, structural genes, and many regulatory genes. In yeast, phosphate is

imported into the cell through the cell wall. The preferred source of phosphate in yeast is inorganic phosphate (P_i) (51). In the absence of P_i, yeast can fulfill its phosphate requirements from organic compounds through cleavage of phosphoester bonds, catalyzed by phosphatases (89, 136). At least two systems for P_i uptake have been identified. Pho84p is a high affinity phosphate transporter (Km 8.2μM). Another transporter, the specific components of which have not yet been identified, has a much lower affinity (Km 770 μM) (112). *PHO84* expression is repressed about 60 fold by high P_i (120).

There are two types of phosphatases found in yeast cells, acid and alkaline, classified according to their pH optimum. Acid phosphatases function in the periplasm or are located in the cell wall and are optimized to work in the acidic environment outside the cell. Alkaline phosphatases function within the cell in vacuoles and in the cytoplasm and scavenge phosphate from internal cellular byproducts. The optimum pH for the acid phosphatases is 3 – 4 and for alkaline phosphatases is pH 8 (review: (88)). Both types of phosphatases are glycoproteins, having covalently linked sugars (14). There are four yeast acid phosphatases, *PHO5*, *PHO3*, *PHO10*, and *PHO11*. *PHO5* is the major acid phosphatase and produces the bulk of the acid phosphatase enzyme in the yeast cell (115). Within the acid phosphatase group, there are two types of regulation, repressible and constitutive (116). *PHO5*, *PHO10*, and *PHO11* are repressed by high P_i but *PHO3*, also known as thiamin phosphatase (93), is repressed by the *PHO5* gene product and exogenous thiamin (80), so it appears to be constitutively active when phosphate is adequate. During derepression, acid phosphatase activity increases by at least 100-fold, and when fully derepressed, the enzyme constitutes more than 1% of the total cellular protein (98).

All four acid phosphatase genes have extremely similar DNA sequences and quite likely share a single ancestral gene. *PHO3*, *PHO10*, and *PHO11* have 87%, 85%, and

86% DNA sequence homology, respectively, with *PHO5*, and the *PHO5* and *PHO3* genes are tightly linked on the right arm of chromosome II (76). Meyhack *et al.* cloned the tandem genes *PHO5* and *PHO3* and concluded that the phosphatase family most likely arose (like the *SUC* and *MAL* gene families in yeast) by duplication and dispersal of a single ancestral gene (76). Rogers *et al.* independently came to the same conclusion that the acid phosphatase genes arose from a single ancestral gene (98).

The acid phosphatase system, and especially the *PHO5* gene, in *Saccharomyces cerevisiae* makes an ideal system in which to study gene expression in the context of chromatin. *PHO5* is easily regulated by the presence or absence of P_i and as I will discuss in later sections, there are distinct differences in chromatin structure between the activated and repressed states of the gene.

1.4 Chromatin structure

Chromatin compacts and organizes the DNA, which in diploid human cells contains approximately 6 X 10^9 base pairs. If the DNA were to be stretched out as a linear molecule it would occupy about two meters, however, this genome must fit into a nucleus of approximately 5 μM in diameter, and still be unfolded and repackaged many times throughout a cell's lifetime (1). Over 80% of the DNA in a nucleus is incorporated into nucleosomes (81). In the nucleus, chromatin is primarily found as either euchromatin or heterochromatin. Euchromatin is generally associated with regions of the chromosome that are transcriptionally active and so the chromosome is in a less condensed form. Heterochromatin refers to regions of DNA that are highly condensed and transcriptionally inactive (32).

The fundamental unit of chromosomal DNA in the nucleus of eukaryotic cells is the nucleosome. The nucleosome consists of an octamer of two each of the four core histones; H2A, H2B, H3, and H4 (reviews: (124, 132)). Most eukaryotes have a fifth

histone, H1, which is termed the linker histone, and contributes to both the functional and structural properties of the nucleosome. A potential yeast H1 has been identified but not characterized (65), so for the purposes of this introduction, I will focus only on the four core histones. The nucleosome, protein plus DNA, has a mass of approximately 200,000 daltons (Da), with each histone having a mass of 11,000 to 16,000 Da. Around the outside of the octamer is wound 146 base pairs of DNA in one and three-quarter turns, although non-uniformly, to form the core particle (96). The DNA between the nucleosomes is designated as linker DNA and ranges in length from 20 bp to 100 bp (124). Nucleosomes in yeast typically have a 165 base pair repeated structure. The nucleosome forms first as a core particle with 120 base pairs (bp) of DNA wrapped around a tetramer of histones H3 and H4 (two each). Two heterodimers of H2A and H2B then associate with the H3/H4 tetramer incorporating an additional 26 base pairs of DNA, allowing an average linker of 20 base pairs, to complete the yeast nucleosome. The crystal structure of a synthetic nucleosome has recently been solved to 2.8Å resolution (Figure 1.1) (73).

The histones themselves are very well conserved among eukaryotic organisms (129). There are several common themes among core histone proteins including their highly basic nature, unstructured tails, and their evolutionary conservation. The histone proteins can also be highly modified by acetylation and phosphorylation, primarily on the N- or C-terminal tails. Acetylation of histones, both the mechanism and function, is the best understood histone post-translational modification at this time. Acetylated histones are primarily associated with active genes (review: (119)), although the understanding of this process and relevance in transcription regulation is still in its infancy. Modifications will be discussed in greater detail in a later section of this introduction.

Also associated with chromatin are what are known generally as non-histone chromosomal proteins. There are many categories of these proteins, including enzymes,

structural proteins, and functional proteins such as transcription factors. Unfortunately the study of this category of proteins is impeded by disruptive chromatin preparation techniques.

As previously mentioned, the DNA in a typical eukaryotic cell is about one meter long. By forming nucleosomes over the entire length of the DNA it would be compacted to about 15 to 20 cm, still much too large to fit into a nucleus. Higher order chromatin structure then becomes necessary to compact the DNA even further and to allow for accessibility when a particular region of the DNA is to become active (review: (132)). The first level of compaction beyond the basic nucleosome is the 30 nm fiber, referring to the diameter of the condensed nucleosome array. Because most chromatin preparation techniques disrupt higher order structure, the study of higher order chromatin structure is very much behind that of the basic nucleosome array. However, it is generally thought that the 30 nm fiber forms looped domains attached to a scaffold for organization in the nucleus. Fortunately, technology is currently progressing very rapidly, so this area of research should become one of much greater interest.

1.5 RNA Polymerase II Transcription

1.5a Transcription Initiation

RNA polymerase II is responsible for transcribing protein coding genes, and is the most complex of the eukaryotic RNA polymerases. Initiation of transcription by RNA polymerase II is a multi-step process (reviews: (137) (28) (18)). Figure 1.2 depicts the process of RNA polymerase II assembly on a promoter prior to transcription initiation. The earliest step in initiation is binding of the TBP containing subunit, TFIID, to the TATA box. Binding of TFIID to the TATA box is facilitated by TFIIA, which also binds to the DNA upstream of and adjacent to the TATA box. The next step in initiation is the

binding of TFIIB to TFIID, followed by binding of TFIIF to TFIID and TFIIB. TFIIB and TFIIF are also involved in binding to the polymerase and so function to bridge the interactions between the general transcription factors (TFIIA, B, D, F, E, and H) and the polymerase. The final step in initiation complex formation is the association of TFIIE and TFIIH with the polymerase. In Figure 1.2 TFIIJ is also indicated as another component of the pol II machinery. However, the current view is that TFIIJ is not a subunit (reviews: (18, 53). Once the initiation complex is assembled, TFIIH is believed to phosphorylate the C terminal domain of the polymerase (72) converting it from the promoter bound non-phosphorylated form to the phosphorylated form that can dissociated from the preinitiation complex to transcribe DNA (66). Upon initiation of transcription, TFIIA and TFIID remain bound to the DNA to allow for repeated rounds of transcription by recruitment of more pol II complexes while TFIIF stays associated with the polymerase. All of these steps are not absolute, and new components of the "holoenzyme" are constantly being identified, indicating that pol II transcription does not occur in the same way on every promoter.

It is widely accepted that at least some of the RNA polymerase II associates with TFIIB, TFIIF, TFIIH, and a variety of other proteins which serve multiple purposes, including mediating interactions with transcription factors and possibly modification of core histones. This complex exists in solution and is recruited as a "holoenzyme" to the DNA (23) (59). Many of the general transcription factors are multi-subunit complexes, including TFIIE, TFIIF, TFIIH, and TFIID. TFIID contains the largest number of components with TBP and multiple TAFs (TBP associated factors). TBP binds to the DNA and the TAFs are important in activation and possibly histone modifications. In

yeast however, TAFs are not required for basal levels of transcription (4), but two TAFs have been found to be essential genes and the protein products are required for activated transcription (95).

1.5b Activators

There are many types of DNA binding transcription activators, however in this dissertation I will focus only on two types, the homeodomain proteins and the basic helix-loop-helix proteins, both of which are involved in *PHO5* activation. The homeodomain proteins were originally identified as proteins involved in *Drosophila* development and expression of yeast mating type factors. The homeobox is the DNA sequence encoding the homeodomain of the transcription factor. The homeobox is an approximately 180 base pair region that is homologous in all homeobox containing genes (review: (38)). The homeodomain of the protein is involved in DNA binding. Pho2p, an activator of *PHO5* transcription, is a homeodomain protein and will be discussed in greater detail in a later section of this introduction.

Using X-ray crystallography and NMR techniques, the structures of several homeodomain containing proteins bound to DNA have been elucidated (reviews: (38) (61)). The common theme among homeodomain proteins is the C-terminal helix-turn-helix motif, which contacts the DNA in the major groove. The two helices are arranged perpendicular to each other and are separated by a β-turn. One helix lies across the major groove while the other helix, the recognition helix, makes sequence specific contacts with the DNA. Figure 1.3 is a cartoon diagram of the helix-turn-helix region of a homeodomain. Homeodomain proteins are somewhat promiscuous in their DNA

binding. The binding specificity of the homeodomain for specific promoters seems to be mediated by amino acids in the N-terminal arm of the protein (52).

The basic helix-loop-helix (bHLH) domain containing proteins, of which Pho4p is a member, is another family of DNA binding transcriptional activators. The HLH domain of these proteins consists of two amphipathic helices with the charged residues on one side of the helix. The helices are separated by a variable length loop (79). The HLH domain dimerizes allowing the basic region to bind to the DNA. In Pho4p for example, the protein must homodimerize prior to binding DNA at the upstream activating sequences in the *PHO5* promoter. Figure 1.4, generated with Rasmol, is the dimerized helix-loop-helix domain of Pho4p bound to UASp1 on the *PHO5* promoter (105).

The structure of transcription activators is often modular. In addition to a DNA binding domain, many activators also have a separate activation domain. Although this is not the case with homeodomain proteins such as Pho2p, Pho4p on the other hand does have an acidic activation domain responsible for interactions with other proteins. Acidic activation domains are widespread, including yeast Gal4p, the N-terminus of the glucocorticoid receptor, and the viral activator VP16. There is no strong sequence homology among these regions except for the fact that they contain a large percentage of acidic amino acids, and are primarily unstructured (reviews: (94) (43)). Acidic activators have been shown to stimulate formation of the transcription initiation complex by recruiting TFIIB to the promoter (24) (97). Activators also interact with TFIID in recruiting the transcription machinery to a promoter. Activators can directly interact with TBP or the interaction can be mediated by the TAFs (reviews: (39) (114)).

1.6 Chromatin and transcription

1.6a Repression and activation

On the majority of genomic DNA, nucleosomes are randomly placed. However, on some promoters, including *PHO5*, *GAL1*, and the mouse mammary tumor virus (MMTV), positioned nucleosomes occur and play a very important role in regulation of gene expression. From *in vitro* studies, it is clear that nucleosomes repress transcription (our unpublished results) and (27, 67). *In vivo* studies have shown that histone depletion derepresses transcription of the *CYC1*, *GAL1*, and *PHO5* genes (44, 45). This derepression occurs in the absence of the respective upstream activating sequences (UASs), which serve as transcription factor binding sites and are necessary for activated transcription, indicating that core histones function in the regulation of basal transcription from these promoters. *In vivo*, DNA becomes much more susceptible to nuclease cleavage upon activation (31) (35), indicating that chromatin is remodeled upon activation.

The *hsp* genes, or heat shock protein genes, are transcribed in response to extracellular stresses (reviews: (78) (90)). The inactive heat shock gene is bound by nucleosomes, however even in the repressed state of the gene, TFIID is bound upstream (118, 133). In mammalian and *Drosophila* cells upon heat shock, a transcription factor known as GAGA remains constitutively bound to the upstream promoter and mediates nucleosome remodeling by recruitment of ATP dependent chromatin remodeling machinery. This allows HSF (heat shock factor) to bind to the heat shock elements (HSEs) and activate transcription (118) (123). Another interesting event that occurs on the heat shock gene is the formation of a paused RNA pol II complex. The polymerase pauses after transcribing a short transcript of about 20 to 40 nucleotides (69). Upon binding of the heat shock factor, the paused polymerase releases to finish transcribing its transcript while another polymerase is recruited for initiation.

Another well-studied example of how chromatin functions in transcription repression and activation is the MMTV promoter, which is regulated with respect to chromatin in a slightly different manner than the heat shock genes. The promoter is fully nucleosomal in the absence of steroid hormones. The presence of six nucleosomes, A through F, defines the repressed state of the gene. In the presence of steroid hormone the receptor binds at multiple glucocorticoid response elements (GREs) and initiates the displacement of nucleosome B. This allows the transcription factor NF1 to immediately bind and recruit the general transcription machinery, which accesses the DNA and initiates transcription (117). On the MMTV promoter one factor, the steroid receptor, both mediates the remodeling of nucleosomes and immediately recruits NF1. In contrast the heat shock gene has separate factors for each task, GAGA maintains a remodeled chromatin structure until another stimuli activates HSF, which then binds to the promoter and recruits the transcription machinery. In the case of *PHO5*, the acidic activation domain of Pho4p both interacts with the general transcription machinery, although the interactions are not well characterized, (75, 134) and is also required for remodeling of nucleosomes prior to activation of transcription (111).

1.6b Transcription through nucleosomes

The role of nucleosomes as barriers to transcription elongation are being investigated using many approaches (25, 71, 86, 109, 110). T7 RNA polymerase is commonly used in experiments to study how RNA polymerases transcribe through a nucleosomal coding region. Using a 5S rRNA gene from sea urchin or the α-globin gene from mouse, the T7 polymerase was determined to transcribe through the nucleosomes without displacing them; however, elongation is hampered and accumulation of several shorter transcripts is seen (58, 83). Using an *in vitro* reconstituted mononucleosome with an SP6 promoter fragment ligated to the nucleosome following reconstitution, Lorch *et*

al. (71) showed that the SP6 polymerase, again a prokaryotic RNA polymerase, can transcribe through a nucleosome. In contrast with the T7 polymerase, transcription by the SP6 polymerase did result in displacement of the core histone octamer. Following transcription on a nucleosomal template with SP6 polymerase there is a greater density of nucleosomes upstream of the promoter than before transcription occurred, suggesting that as the polymerase transcribes, nucleosomes are being removed and re-deposited behind the polymerase (25, 109). The dissociation of the octamer appears not to be required as the polymerase proceeds, as transcription through nucleosomes can still be achieved by T7 polymerase when the histones have been crosslinked to each other (84). Prokaryotic RNA polymerases have greatly contributed to our knowledge of transcription through a nucleosome. However, it must be questioned whether this information is relevant to transcription in higher organisms since prokaryotic polymerases are small molecular weight (MW), single subunit enzymes and not large MW, multi-subunit complexes like eukaryotic RNA polymerases.

Although technically more challenging, a few groups have looked at transcription through nucleosomes by eukaryotic polymerases. It is necessary to form a paused polymerase complex on the template prior to reconstitution, or use chromatin remodeling machines, since nucleosomes on the promoter prohibit the transcription machinery from assembling. Although the fate of the nucleosomes are not known, it is assumed that nucleosomes are not a significant barrier to transcription, although elongation is much slower than on naked DNA (71) (49). Studies with RNA polymerase III have shown that the polymerase can transcribe through a small number of nucleosomes easily and can produce even longer transcripts, though less efficiently (36) (46).

Recently, a factor that has been named FACT (facilitates chromatin transcription), was identified to be required for transcription through *in vitro* assembled nucleosomes (assembled using the Drosophila S-190 extract (57)). In this system the RNA polymerase

II machinery could initiate transcription on chromatin templates, but could not elongate to produce the full length transcript even on remodeled templates. FACT activity was required to counteract the chromatin induced block to transcript elongation (86), possibly through direct disassembly of the nucleosome core particle (87). FACT is a novel human homolog of the *Saccharomyces cerevisiae* gene *SPT16/CDC68*, which is an essential gene involved in transcription regulation (87). The fate of nucleosomes on templates transcribed by eukaryotic polymerases is less defined and much more complex than on templates transcribed by prokaryotic polymerases. This is very much expected though, as the level of complexity at every stage of eukaryotic gene expression is much greater that in prokaryotic organisms.

1.7 Histone modifications and chromatin remodeling

Since the structure of the nucleosome, and how it functions to repress transcription, has been well studied, focus has shifted in the past decade to the process of activation in a chromatin context. Histone modification, especially acetylation, is well correlated with transcription activation. Chromatin remodeling of the promoter is required for activation and has also recently become a topic of keen interest.

1.7a Acetylation

Each of the four core histones contains three domains; a central structured domain, an amino terminal tail, and a carboxy terminal tail. Both tails are essentially unstructured. The hydrophilic amino terminal tail contains several positively charged residues, especially lysines. The tail undergoes extensive post-translational modifications, primarily acetylation. Acetylation of the amino groups of lysine residues removes their positive charge and may alter the interaction with other proteins and

nucleosomes. It has been proposed that acetylation disrupts internucleosomal histone-histone interactions in higher order chromatin structure (73).

It is widely accepted that acetylation of the core histones has a regulatory role in transcription. In yeast transcriptionally active chromatin contains hyperacetylated histones (26). In female mammals one of the two X chromosomes is transcriptionally inactive and contains a lack of H4 acetylation (50). It has been shown that by acetylating histones in a nucleosome that has a binding site for the transcription factor Gal4p, the binding of that factor to the DNA is facilitated, indicating a positive role for acetylation in transcription (68, 126).

Several histone acetyltransferases (HATs) have been identified, including a TAF and several transcriptional co-activators (review: (108)). Only one HAT, yeast Gcn5p, has been directly correlated with histone acetylation and transcription activation of specific genes (63, 128). In addition, only Gcn5p has been shown to acetylate core histones in chromatin (40). Gcn5p is found in both the ADA and SAGA complexes and must be associated with these complexes to acetylate histones within nucleosomes (40, 41). The Ada2 protein, a component of both the ADA and SAGA complexes, acts as an adapter that interacts with acidic activation domains (122) and TBP (8). Other putative HATs include PCAF (p300/CBP associated factor) (135), p300/CBP (85), and the $TAF_{II}250$ subunit of TFIID (77). Although all of these proteins have the ability to acetylate core histones *in vitro*, there is much work to be done to correlate their potential HAT activity with an actual *in vivo* function.

1.7b Deacetylation

Even less is known about the role of histone deacetylation in gene regulation. The first histone deacetylase (HDAC) identified was the yeast protein Rpd3p (review: (91)). Rpd3 is a member of a large multi-subunit complex that is involved in

transcription repression *via* the Sin3p co-repressor, a global repressor of transcription. Homologs of Rpd3 have been found in other organisms associated with DNA binding transcription repressors including the mammalian repressor proteins Mad (47, 64), and YY1 (135).

1.7c Remodeling

Transcription activation occurs through a hierarchical process. The initial step involves unfolding of chromatin, which allows access to the DNA by the transcription machinery. This initial remodeling of chromatin involves transcription factor binding and recruitment of chromatin remodeling machines. The Swi/Snf complex, the first chromatin remodeling complex to be identified, functions to counteract nucleosome repression of transcription by remodeling nucleosomes (20, 21, 106). Swi/Snf is a complex of non-DNA binding proteins containing a subunit with a DNA dependent ATPase domain, Swi2p. Swi/Snf remodels chromatin to facilitate transcription activation (92, 131). *In vitro*, purified Swi/Snf can alter histone/DNA interactions in a nucleosome (29). *In vivo*, Swi/Snf facilitates yeast transcription activator Gal4p binding to nucleosomal DNA (20).

A second chromatin remodeling complex, RSC, has also been isolated from yeast cells. RSC was shown to contain at least three Swi/Snf related components that have very similar activities with respect to their Swi/Snf counterparts, and in particular the ATPase function (22). Conflicting results indicate that Swi/Snf may or may not be associated with the pol II holoenzyme (22, 130). RSC is not associated with pol II (22). Many other complexes have been identified that remodel chromatin in an ATP-dependent fashion. They all contain a factor closely related to the yeast Swi2p in the Swi/Snf complex. The RSC complex contains Sth1p; Drosophila NURF, CHRAC, and ACF contain ISWI (for imitation of Swi2); and the brm ATPase is found in

Drosophila BRM and mammalian BRG1 (review: (30)). It is proposed that ATP-dependent chromatin remodeling machines use the energy of ATP hydrolysis to unwind the DNA from the core histone octamer (29).

1.8 *PHO5* regulation

1.8a Chromatin structure

PHO5 encodes a secreted acid phosphatase (5) which is secreted under conditions of phosphate starvation. In the repressed state when there is adequate phosphate in the environment, transcription is blocked by four nucleosomes positioned on the promoter. Upon activation of *PHO5* by phosphate starvation, all nucleosomes are displaced (3) allowing assembly of the transcription machinery and expression of *PHO5*. Figure 1.5 depicts the chromatin transition of the *PHO5* promoter upon phosphate starvation. The chromatin transition upon activation is DNA replication independent but does require glucose (100).

In the repressed state the nucleosomes are translationally positioned on the *PHO5* gene. Translational positioning refers to the region of DNA that is actually encompassed in the nucleosome, in contrast with rotational positioning, which describes whether a given piece of DNA is facing towards the solution or the histone octamer (132). On the *PHO5* promoter nucleosome –1 encompasses the TATA box and transcription start sites, preventing assembly of the transcription machinery. Nucleosome –2 is positioned so that a Pho4p (UASp2) and an adjacent Pho2p binding site are placed within it. Nucleosomes –2 and –3 are positioned to create an enlarged linker region (2). Located within this region is another upstream activation sequence (UASp1) that serves as a constitutively accessible binding site for the transcription activator Pho4p and another adjacent Pho2p binding site (7). This unusually large nucleosome free linker region is also required for nucleosome positioning (11). Nucleosome –4 is further upstream and plays no known

role in regulation. The chromatin structure of the *PHO5* promoter changes depending on the presence or absence of phosphate and these changes are reversible by removing or adding back P_i, which makes *PHO5* an excellent model gene for the study of gene regulation in the context of chromatin.

1.8b Pho4p

The Pho4 protein is composed of 309 amino acids and has a mass of 33,888 Da (70). Pho4p functions through its acidic activation domain and basic helix-loop-helix DNA binding domain; the first of this type of motif to be found in yeast (9). The HLH motif is located at the C terminus of the protein (9) and is required for homodimer formation prior to binding DNA. Shimizu *et al.* recently reported the crystal structure, to 2.8A, of the HLH domain of Pho4p bound to *PHO5* UASp2 (105) (Figure 1.4). The DNA binding sequence specificity of Pho4p is similar to that of the mammalian transcription activator *c-myc* (9). *PHO4* mutants are epistatic to all other *pho* regulatory gene mutations indicative of its central role in the regulation of phosphate metabolism (51). Pho4p is constitutively expressed at a low level (70).

Pho4p is regulated through its phosphorylation state (54) and regulates *PHO5* expression through its cellular localization (82). When phosphorylated, Pho4p is localized to the cytoplasm, which explains why Pho4p cannot bind to the *PHO5* UASs under repressing conditions (125). Pho4p has six potential sites of phosphorylation, all serines (82), that serve multiple purposes (60). Phosphorylation at two of the serines is required for binding to Msn5p, a transport protein that exports Pho4p from the nucleus in repressing conditions (55). Phosphorylation at another serine inhibits Pho4p nuclear import by disrupting the association of Pho4p with Pse1p, an import receptor responsible for the import of Pho4p into the nucleus in activating conditions (56). Phosphorylation at yet another serine inhibits the physical interaction of Pho4p and Pho2p (60).

It is likely that the acidic activation domain of Pho4p is responsible for recruitment of the pol II transcription machinery. It was determined that Pho4p physically interacts with TFIIB, TFIIEβ, and TBP (75). In addition, a TFIIB mutation was isolated that impairs induction of *PHO5* under activating conditions (134). A physical interaction was determined between wild type TFIIB and Pho4p but no interaction was seen between Pho4p and the mutant TFIIB. These results all support the hypothesis that Pho4p is the primary trigger for transcription activation of *PHO5* in the absence of P_i, through recruitment of the Pol II transcription machinery.

1.8c Pho2p

The gene product of *PHO2*, also known as Bas2p and Grf10p, is a 63,390 Da protein containing 559 amino acids (103). *PHO2* encodes a homeodomain protein, having 63% amino acid identity in the homeodomain with at least one of the vertebrate or *Drosophila* homeodomain proteins. The Pho2p homeodomain is located towards the N-terminus of the protein (19). Expression of *PHO2* is not regulated by phosphate and appears to be constitutive, although expressed at low levels (10). Interestingly, *pho2* null yeast are unable to sporulate (10). Pho2p is a very plieotropic activator and is involved in the expression of several metabolic and biosynthetic genes, including *ADE2* and *HIS4* (52, 113), *TRP4* (15), the yeast HO endonuclease gene, involved in mating type switching (17), and basal expression of *CYC1* (102).

The mechanism of DNA binding by Pho2p may be partially determined by its target site (52) since it can potentially bind to many different promoters at any given time. As with other homeodomain proteins the recognition helix of the Pho2p HD is positioned within the major groove of the DNA. Although Pho2p does not have a true activation domain it does bind TBP, TFIIB, and TFIIEβ, although with lesser affinity

than Pho4p (75). This interaction is most likely indirect and mediated by Pho4p, as the experiments were done from wild type whole cell extracts.

1.8d *PHO5* regulatory system

The members of the acid phosphatase gene family, including some of the regulatory factors, were originally identified in a standard mutant search for genes that affected phosphate metabolism (116). The four acid phosphatase genes and three of the regulatory genes, *PHO80*, *PHO81*, and *PHO85*, were identified. *PHO80* and *PHO85* are negative regulators and *PHO81* is a positive regulator of *PHO5* expression. In addition, *PHO84*, later shown to encode a phosphate transporter was obtained (120). Two years later *PHO4* was identified and characterized as a positive regulator of *PHO5* expression (121). Finally *PHO2* was determined to be required for transcription activation of the *PHO5* gene (62).

Pho80p and Pho85p interact to inhibit the function of Pho4p at high P_i by phosphorylating it (discussed earlier) (54). The expression of *PHO80* is independent of phosphate levels and its disruption causes a derepressed phenotype in the absence of phosphate. Overexpression of *PHO80* causes a reduction in acid phosphatase levels, even in derepressing conditions (74), suggesting that *PHO80* is required for the repressed state of *PHO5*. Through sequence analysis of *PHO80*, it was determined that the gene is homologous to two yeast cyclins, HCS26 and OrfD (54). *PHO85* was found to be a protein kinase related to $p34^{cdc2/CDC28}$, therefore it is accepted that the Pho80/85 complex acts as a cyclin/cyclin dependent kinase (CDK) complex (54). The mechanism of action of Pho80p/Pho85p is regulated through Pho81p by phosphate availability (116). Pho81p inhibits Pho80p/Pho85p kinase activity at low P_i, preventing phosphorylation of Pho4p. *PHO81* itself is negatively regulated at high P_i (101). Expression of yeast acid phosphatase is a very tightly controlled process, as are most metabolic pathways. The

regulation of *PHO5* has many parallels with other metabolic pathways, for example its regulation by a cyclin/CDK complex, which is an important feature in a model system.

1.8e Pho4p and Pho2p interactions

Through extensive deletion analysis of the promoter, three upstream activation sequences (UAS) were identified that when deleted, significantly diminished *PHO5* activation. (99). It was later determined that two protein factors, Pho4p and Pho2p, physically interact with the promoter at these UASs (33, 127) and are required for activation (136). As discussed earlier, Pho4p is the primary trigger for *PHO5* activation, as overexpression of *PHO4* in a *pho2* strain will partially compensate for the lack of Pho2p, however, the reverse is not true. Under physiological conditions though, both proteins are required for full expression of *PHO5* (33).

A functional interaction between Pho4p and Pho2p is necessary for full transcription activation (104). Pho2p and Pho4p form a DNA dependent, but phosphate independent complex *in vivo* (75); however, Pho4p still must be localized to the nucleus for the interaction to occur. *In vitro* findings also suggest a Pho4p/Pho2p physical interaction (7), as Pho4p and Pho2p form a ternary complex with DNA *in vitro* (6).

As discussed earlier, Pho2p is involved in expression of the *HO* gene. Pho2p has a similar role in expression of *PHO5* and *HO* as Pho2p stimulates Swi5, a transcription activator, to bind DNA but doesn't interact with Swi5p when not bound to DNA (16). Deletion of the basic region of Pho4p adjacent to the HLH domain gives Pho4p a Pho2p independent phenotype (104). The model proposed is that this basic region of Pho4p masks its activation domain and Pho2p must interact with this domain to unmask the activation domain of Pho4p. This model is similar to the interaction of Bas1p and Pho2p (Bas2p) on the *ADE2* promoter. Bas1p recruits Bas2p to the promoter and a complex

forms between the proteins, unmasking the Bas1p activation domain in an adenine regulated step (138).

There appear to be several possible roles for the Pho2p protein in chromatin remodeling and transcription activation on the *PHO5* promoter. At the Pho2p site adjacent to UASp1, the constitutively accessible Pho4p site, Pho2p binds DNA cooperatively with Pho4p (7). At UASp2 however, Pho2p is required for activation but not for Pho4p DNA binding (7). It is possible that at UASp1, Pho4p binds cooperatively with Pho2p and recruits a chromatin remodeling complex but does not necessarily participate in activation. Upon removal of at least nucleosome -2, Pho4p can bind to the higher affinity UASp2, have its activation domain unmasked by Pho2p, and proceed to activate *PHO5* transcription.

1.9 *PHO5* nucleosome positioning and chromatin transition

Nucleosome loss by histone H4 depletion results in activation of the *PHO5* gene (45). This finding and the work of many others (11-13) indicate the major role chromatin plays in the repression and activation of the yeast *PHO5* gene.

1.9a Nucleosome positioning

Nucleosomes function to repress *PHO5* in high phosphate and must be positioned correctly to do so. The precise mechanisms of nucleosome positioning on *PHO5* are still being studied. However, we have unpublished data which indicate that intrinsic properties of the DNA are sufficient for positioning of nucleosomes –1 and –2, and maintenance of the enlarged linker region (See Chapter 4). It has been shown that the transcription activators Pho4p and Pho2p are not required for the nucleosome positioning on the *PHO5* promoter. The *PHO5* chromatin structure, as analyzed by low resolution indirect endlabeling, is identical in *pho2/pho4* double mutant strains and wild type strains

(33). In addition deletion of both the Pho2p and Pho4p binding sites at UASp1 does not disrupt the *PHO5* promoter chromatin structure (34). *Replacing the DNA in nucleosome –2 with other DNA, can deregulate PHO5 expression.* If *PHO5* nucleosome –2 is replaced with a fragment from African green monkey α-satellite DNA, which strongly positions nucleosomes, *PHO5* is unable to be activated in low phosphate conditions (107). If a fragment of pBR322 is used for the replacement, activation of *PHO5* wasn't affected but derepression in low phosphate occurred (107). These results indicate that disruption of nucleosome –2, which does not occur with the monkey DNA, is necessary for activation of *PHO5*, however the nucleosome must be fairly tightly positioned, as is not the case with the pBR322 DNA, so that repression of *PHO5* can occur in adequate phosphate.

1.9b Chromatin transition upon activation

Activation of *PHO5* is associated with a chromatin transition involving the displacement of four nucleosomes from the promoter. Nucleosome reconfiguration is not dependent on transcription, as deletion of the *PHO5* TATA box prevents *PHO5* expression but still allows the phosphate dependent chromatin transition to occur (34). The Pho4p activation domain is required for the chromatin transition and overexpression of just the DNA binding domain of Pho4p results in no chromatin transition upon phosphate depletion (111). Therefore, the activation domain most likely recruits a chromatin remodeling complex to *PHO5* in low phosphate conditions, following Pho4p binding to DNA. The histone modifications and nucleosome remodeling factors required for proper regulation of *PHO5* have not yet been determined. *GCN5*, encoding the histone acetyltransferase Gcn5p, likely has no physiological role in *PHO5* activation. *GCN5* only enhances *PHO5* expression when one UAS has been deleted (42). However, *GCN5* may be involved in basal *PHO5* expression as a *gcn5* null mutation decreases

constitutive *PHO5* transcription in high P_i medium. These results indicate that acetylation of *PHO5* nucleosomes by Gcn5p is not required for *PHO5* activation but may be involved in the low basal levels of transcription.

The Swi/Snf complex, an ATP dependent chromatin remodeling complex, is also not required for activation of the *PHO5* gene (37). Acid phosphatase levels reach about 70% of wild type in activating conditions and the chromatin transition is unaffected in *snf2* yeast cells. These findings suggest that the *PHO5* transcription activation machinery binds the promoter with an affinity great enough that there is no requirement for Swi/Snf complex recruitment to bring about chromatin remodeling. An equally plausible explanation is that chromatin remodeling on the *PHO5* promoter is performed by a yet to be identified chromatin remodeling complex that does not contain the Snf2p ATPase.

Nucleosome remodeling of the *PHO5* chromatin structure has been achieved *in vitro* on purified minichromosome templates. Remodeling required ATP, fractionated nuclear extract, Pho4p, and Pho2p, but not Snf6p, a component of SWI/SNF (48). As discussed earlier, overexpression of Pho4p in a *pho2* null background can partially compensate at the level of transcription but the chromatin transition is complete (33), indicating Pho2p is required for full expression of *PHO5* but only Pho4p is necessary to bring about the chromatin transition. The most plausible model at this time for transcription activation of *PHO5* would include Pho4p binding to UASp1 and recruiting an ATP dependent chromatin remodeling complex. This complex would carry out the chromatin transition allowing Pho4p to bind to UASp2 where it could recruit the RNA Polymerase II machinery to initiate transcription of *PHO5*.

1.10 Hypothesis

It is hypothesized that the *in vivo* chromatin structure and regulation of the *Saccharomyces cerevisiae PHO5* gene can be recreated *in vitro* using purified yeast core histones and a DNA template containing the *PHO5* promoter. Using this reconstituted chromatin template I will use purified transcription factors and various yeast extracts to attempt to mimic the process of nucleosome displacement and transcription activation observed on the *PHO5* promoter upon phosphate starvation. Because of the similarities in many pathways and mechanisms between yeast and higher eukaryotes, including the process of transcription, a clearer understanding of the role of chromatin structure in *PHO5* regulation will help to elucidate the role of chromatin in transcription in other eukaryotic systems.

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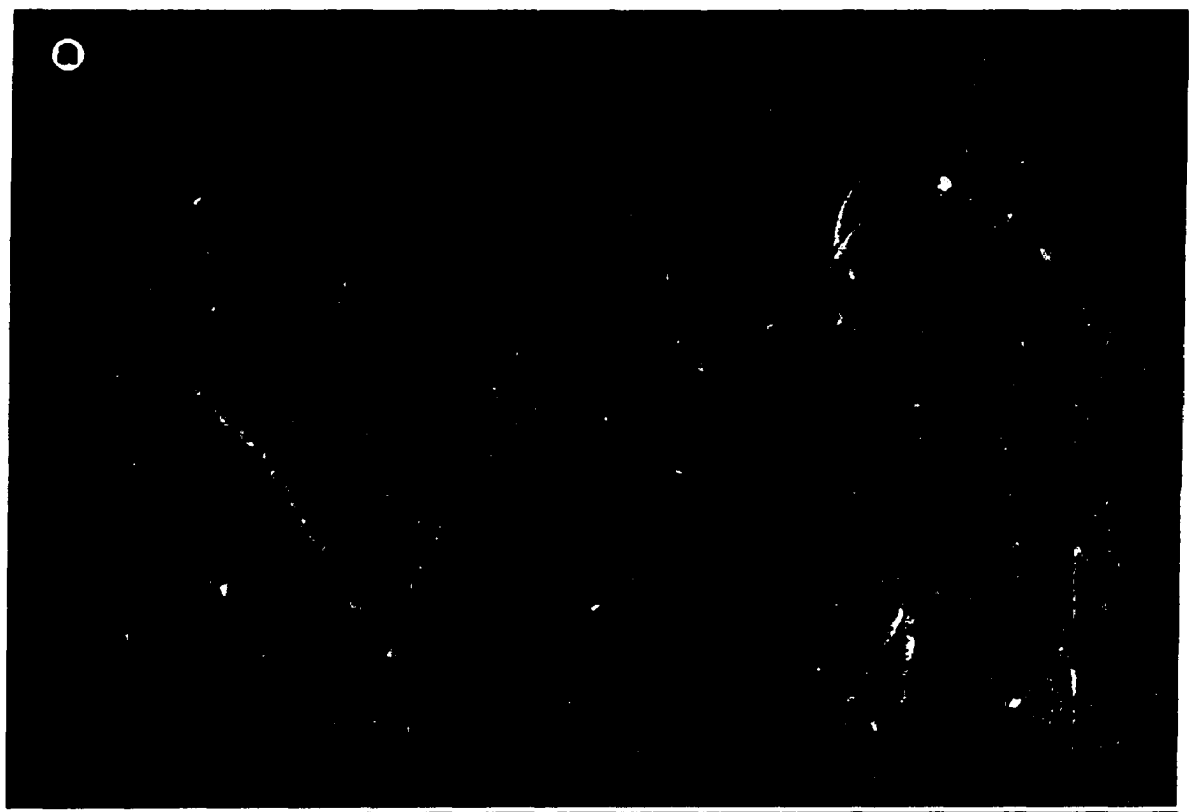

Figure 1.1. Crystal structure of the nucleosome at 2.8Å. The DNA is represented by the green and gold double helix. The histone proteins are represented as follows: yellow - H2A, red - H2B, blue - H3, and green - H4. This figure was adapted from Luger *et al.*, 1997.

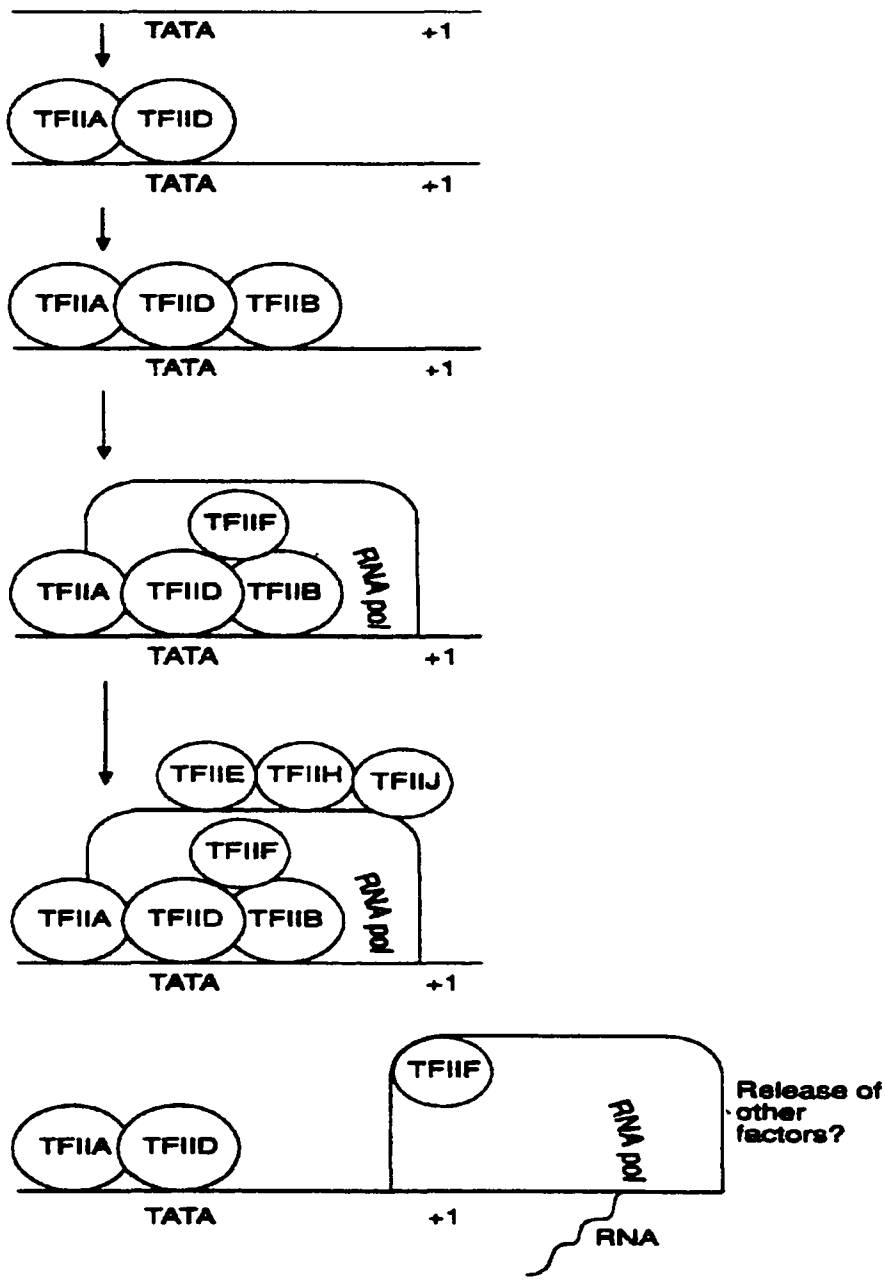

Figure 1.2. Assembly of the basal RNA polymerase II transcription machinery on a typical eukaryotic promoter. Reproduced from Figure 3.5 in the book "Eukaryotic Transcription Factors", Second Edition, David S. Latchman, 1995.

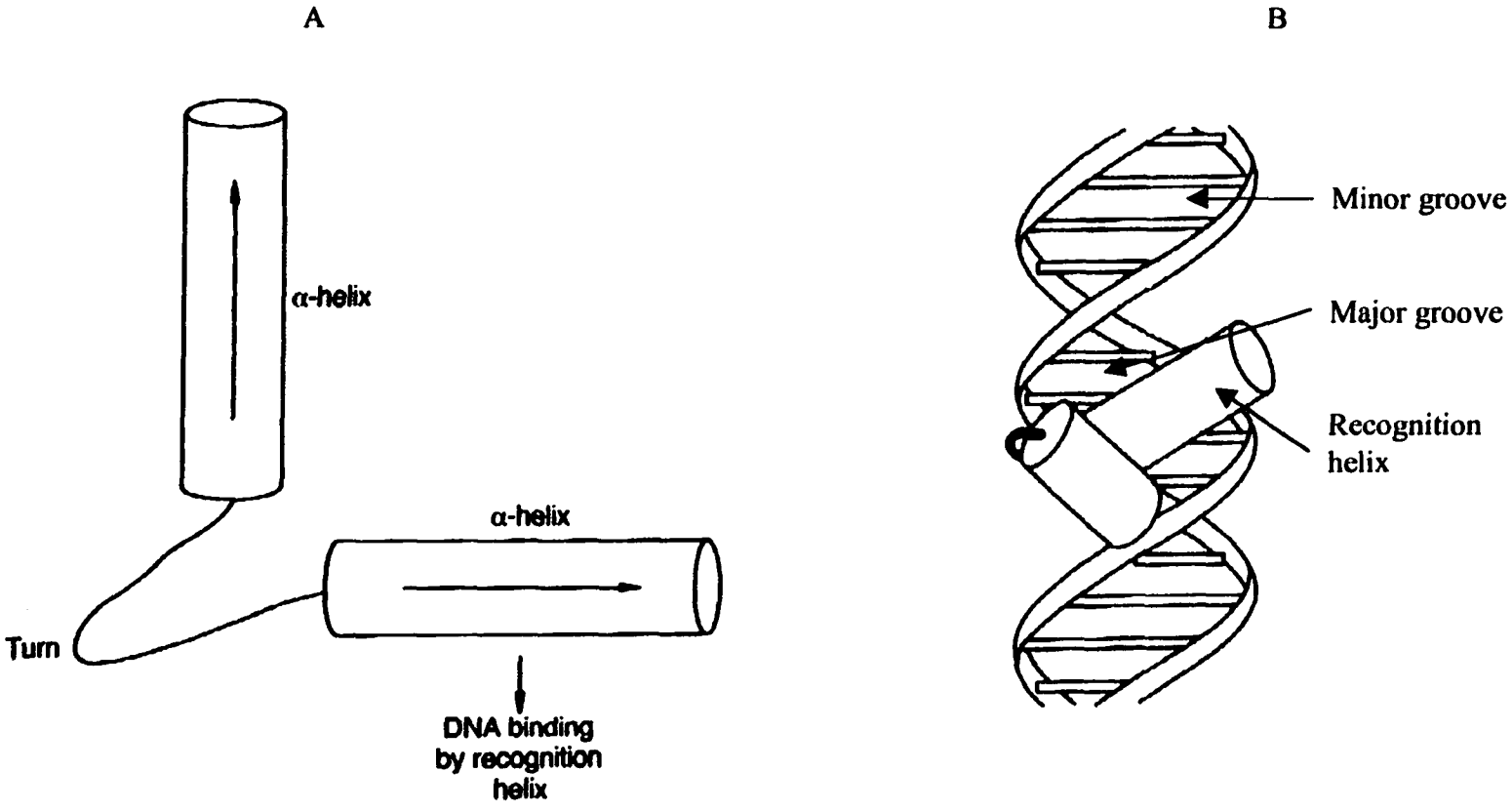

Figure 1.3. The helix-turn-helix motif of homeodomain proteins. A. A simplified view of the motif. B. A representation of the homeodomain bound to DNA. Reproduced from Figures 7.9 and 7.10 in the book "Gene Regulation A Eukaryotic Perspective", Second Edition, David S. Latchman, 1995.

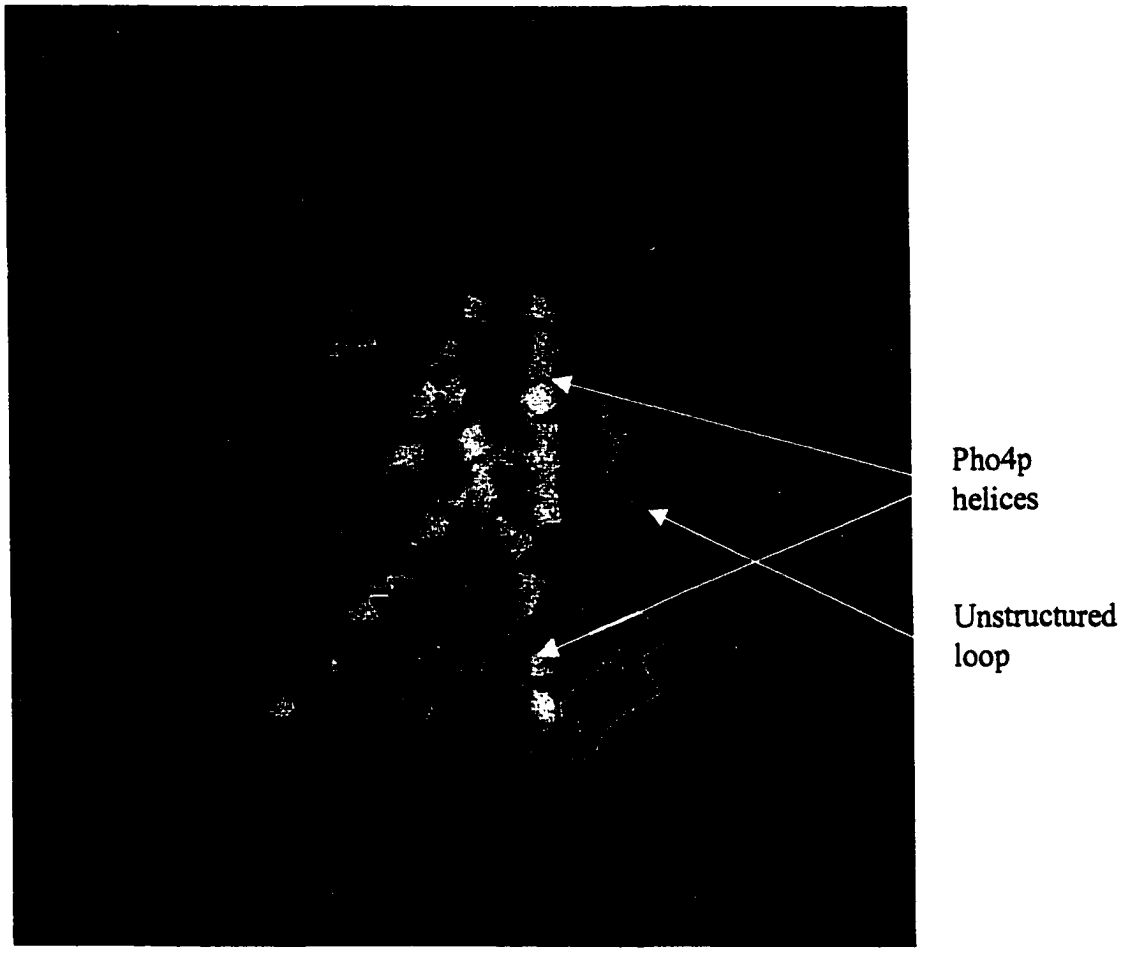

Figure 1.4. Dimerized Pho4p helix-loop-helix domain bound to UASp1 of the *Saccharomyces cerevisiae PHO5* promoter. The image was created using the program Rasmol with coordinates from the Swiss Protein Database. The DNA double helix is colored yellow and the protein is white. The two helices and the unstructured loop are indicated. The reference for the published data is (Shimizu *et al.* (1997)).

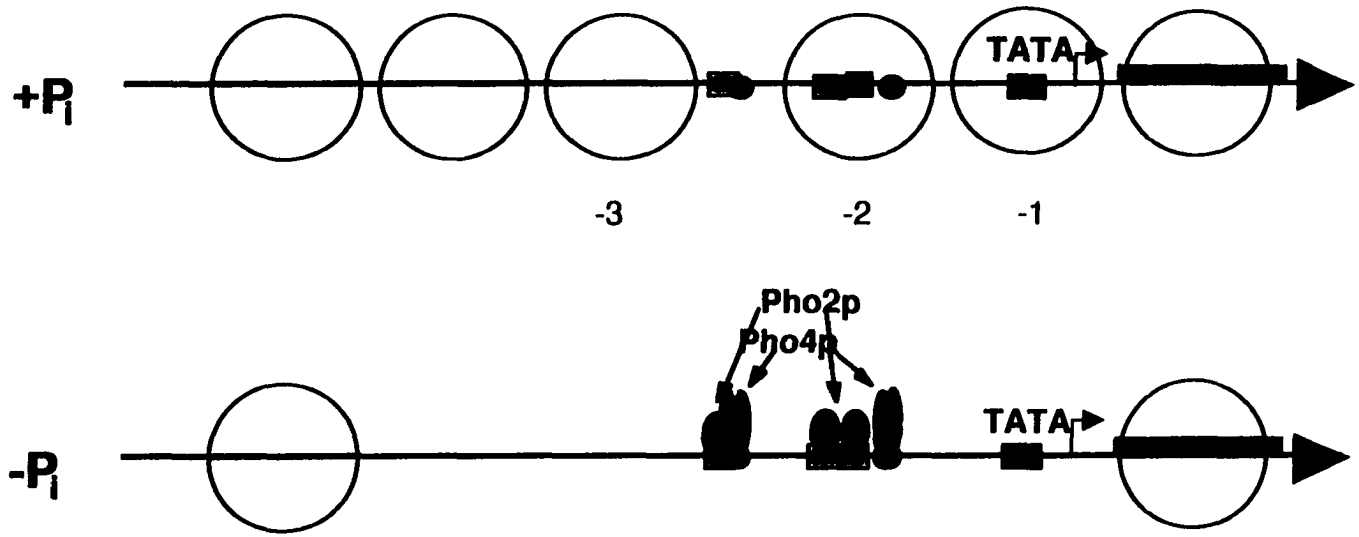

Figure 1.5. Transition of the *Saccharomyces cerevisiae PHO5* promoter from nucleosomal in high phosphate to non-nucleosomal in low phosphate conditions. The constitutively accessible Pho4p binding site, UASp2, and an adjacent Pho2p binding site are located between nucleosomes -2 and -3. The other Pho4p binding site, UASp1, and adjacent Pho2p binding sites are located in nucleosome -2. The TATA box and start sites, indicated by the bent arrow, are in nucleosome -1. This figure was adapted from two published papers. (Almer *et al.* (1986) and Barbaric *et al.* (1996)).

Chapter 2

Yeast Chromatin Reconstitution System Using Purified Yeast Core Histones and Yeast Nucleosome Assembly Protein-1

This chapter was published in *Protein Expression and Purification.* 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 experiment for Figure 2.6, participated in the writing of the corresponding Materials and Methods, and assisted in proofreading the manuscript. The reference for this chapter is:

> Pilon, J., Terrell, A., and Laybourn, P.J. 1997. Yeast Chromatin Reconstitution System Using Purified Yeast Core Histones and Yeast Nucleosome Assembly Protein-1. *PE&P. 10*: 132-140.

2.1 SUMMARY

Transcription regulation in the cell occurs in the context of chromatin. It follows that a thorough investigation of the mechanism of transcription regulation must take into account the role of chromatin structure. Through classical and molecular genetic experiments in yeast, great strides have been made in understanding the role of chromatin in eukaryotic gene regulation. To achieve a more detailed understanding of the biochemical mechanism of transcription regulation, a yeast chromatin reconstitution system is needed. This need drove us to develop a yeast core histone purification procedure for the reconstitution of these histones into chromatin templates using components wholly derived from yeast. We have purified native yeast core histones in milligram quantities and we have shown these histones to be competent for reconstitution of chromatin templates using yeast Nucleosome Assembly Protein-1. This accomplishment sets the stage for studies using the full power of yeast as an experimental organism to investigate the role of chromatin in transcription regulation.

2.2 INTRODUCTION

The DNA in all eukaryotic cells is packaged into chromatin, of which the fundamental unit is the nucleosome (1, 2). A nucleosome contains an octamer of two each of the four core histones; H2A, H2B, H3 and H4 (1, 3). One hundred and forty-six base pairs (bp) of DNA coil around the octamer in 1.75 left hand helical turns to form the core particle. In yeast the nucleosome consists of the core particle plus the intervening linker DNA alone. No bulk linker histone, for example histone H1, has been identified in yeast. The length of the DNA associated with a nucleosome in yeast, referred to as the repeat length, is 165 bp (1). In chromosomes the DNA is packaged into polynucleosome arrays

that further fold into higher order structures. Thus chromatin has a hierarchy of structural levels.

Chromatin functions in the regulation of gene expression through its hierarchical structure. In the first level, chromatin structure unfolds to allow access to the genes that are to be expressed in a specific tissue or cell (4, 5). In the next level of activation, transcription activators bind the gene promoter resulting in the displacement or reconfiguration of nucleosomes located over their DNA recognition elements (6, 7, 8). Once bound the activating factors also function to displace any nucleosomes located over the minimal promoter elements either directly or by assisting the basal transcription factors and the RNA polymerase II to do so. We have focused our research on this hierarchical regulation of transcription in chromatin. We have chosen yeast as model organism for this investigation. This choice was based primarily on the detailed understanding of the changes in chromatin structure that occur on several yeast promoters in the transition between the repressed and the activated states (9, 10, 11, 12). To date, nearly all of the experiments on the role of chromatin structure in yeast gene expression have been done *in vivo*. Biochemical analysis of this process using reconstituted chromatin templates is required to obtain a more detailed mechanistic understanding of transcription regulation on chromatin.

Activation of *PHO5* requires the histone H4 amino terminal tails (13). Substitution of arginine for lysines 5, 8, 12, and 16 mimics deacetylation by increasing the net positive charge. Deletion of the N-terminal tail or substitution of glutamines for the lysines mimic acetylation by decreasing the net positive charge. As one might predict, arginine substitution decreases activation. However, deletions and glutamine substitutions decrease activation, as well. This finding suggests that the H4 tail functions in more than nucleosome stability. Specifically, glutamines do not perfectly mimic acetylated lysines in the recognition by activators. Following this idea through, activation requires protein-protein interactions between the activator and the H4 tail, either directly or through

intermediary factors. These interactions could be yeast specific. Therefore, the reconstitution of yeast transcription regulation on chromatin templates is likely to require the use of components, including core histones, wholly derived from yeast.

Metazoan cells contain on the order of 100 copies of each core histone gene while haploid yeast cells contain only two copies. This biological fact and the ease of molecular genetic manipulation in yeast is the basis for the many elegant histone depletion and mutation experiments investigating the role of the core histones and their amino terminal tails in gene regulation (14, 15, 16; several others since). This fact allows the purification of homogeneous core histones containing deletions or point mutations in the amino terminal tail of an individual core histone, as well. The ability to use these various mutant yeast strains to produce mutant core histones provides another compelling reason to develop a yeast chromatin reconstitution procedure.

Here we describe the purification of yeast core histones in sufficient quantities for biochemical analyses. We have shown these histones to be competent for reconstitution of chromatin templates using yeast Nucleosome Assembly Protein-1 (Nap1p) as measured by two dimensional topological analysis and micrococcal nuclease digestion.

2.3 MATERIALS AND METHODS

2.3a *Production of lyticase and endoglucanase* - The production of yeast spheroplasting activity ("lyticase") from *Cellulomonas cellulans* (previously *Oerskovia xanthineolytica*, obtained from Randy Schekman, University of California, Berkeley) was based on the procedure of Scott and Schekman (17). Cells were grown to an A_{600} of 2 to 3 in 50 ml of M63 medium (18) containing 0.4% glucose at 30 °C while shaking. Fifty ml of this culture (a 1% volume) was used to inoculate five liters of yeast medium (M63 medium containing

autoclaved, washed Red Star yeast). These cultures were grown at 30 °C while shaking for 24 to 30 h until the yeast lytic activity had peaked. The bacteria and yeast were pelleted by centrifugation at 9,000 rpm for 15 min at 4 °C in a JA-10 rotor (Beckman). The lytic activity in the supernatant was concentrated 25- to 50-fold by using a Filtron Ultrasette (MWCO 10,000) as per the manufacturer's instructions. The activity was stored as a precipitate in 90% saturation ammonium sulfate at 4 °C. Typically two million units of "lyticase" activity is obtained from 5 liters of culture. One unit is defined as the amount of activity required to decrease the A_{600} of a yeast cell suspension by 10% in 30 min at 30°C in 50 mM Tris-HCl, pH 7.4, 40 mM β-mercaptoethanol (17). Before use in a large scale yeast core histone purification, the yeast lytic activity was pelleted by centrifugation at 10,000 rpm for 20 min at 4 °C in an SS-34 rotor (Sorval), resuspended in one tenth the volume spheroplasting buffer (see below). The yeast lytic activity was reassayed prior to use.

The *Cellumonas cellulans* β-1,3-glucanase (one component of lyticase) was expressed from the plasmid pUV5-G1S (a kind gift of S.-H. Shen) in *E. coli* strain DH5α as a pariplasmic protein. An overnight culture was diluted 50-fold into 1 liter LB medium containing 100 μg/ml ampicillin and the culture was grown at 37 °C with vigorous shaking to an A_{600} of 0.5. IPTG was added to 0.4 mM to induce expression for 5 h at 37 °C. Cells were harvested at 7,500 rpm for 10 min at 4 °C in a JA-10 rotor and the glucanase activity was extracted by osmotic shock according to the method of Nossal and Heppel (19). The glucanase activity in the supernatant was stored in 90% saturation ammonium sulfate at 4 °C. Prior to use the glucanase was pelleted and reassayed as described for the lyticase.

2.3b *Yeast cell growth and nuclei preparation* - Yeast cells (strain BJ926) were grown to log phase (5 to 8 x 10^7 cells per ml) in 50 liters of 2X SC media (20) by chemostat fermentation. Cells were harvested by centrifugation at 7,000 rpm at 4 °C for 10 min in a

JA-10 rotor. Approximately 1.25 kg of cells (wet weight) is obtained from 50 liters of medium. Cells have been stored frozen at -80 °C for up to one year with no discernible effect on the quality of the core histones.

Nuclei were prepared by a method based on Lue *et al.* (21) from 500 g of yeast cells (approximately 7 x 10^{12} cells). Cells were thawed and resuspended in 5 liters (10 ml/g cells) prespheroplasting buffer (100 mM Tris-HCl, pH 7.9, 60 mM β-mercaptoethanol) at room temperature and stirred for 15 min. Cells were then harvested as before and resuspended in 5 liters (10 ml/g cells) spheroplasting buffer (0.7 M sorbitol, 0.75% yeast extract, 1.5% peptone, 10 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol) at 30 °C and treated with 1 x 10^6 units of lyticase (2000 units per g of cells, see above) for 20 min. Cells were pelleted and resuspended in 2.5 liters (5 ml/g cells) spheroplasting buffer at 30 °C, and treated with 2 x 10^6 units β-1,3-endoglucanase (4000 units per g of cells, see above) at 30 °C while agitating slowly until the A_{600} of the cells diluted 100- to 200-fold in distilled water decreased to 20% to 30% of the starting A_{600}, or a maximum of 60 min. Spheroplasts were pelleted as before and washed once with 2.5 liters YPD (1% yeast extract, 2% peptone, 2% dextrose containing 1 M Sorbitol, 10 mM Tris-HCl, pH 6.8, 2 mM EDTA, 1 mM PMSF, 2 mM benzamidine, 2 mM sodium metabisulfite) at 30 °C and twice with 2.5 liters 1 M Sorbitol, 10 mM Tris-HCl, pH 6.8, 2 mM EDTA at 4 °C (containing the same protease inhibitors). Spheroplasts were then resuspended in 250 ml (0.5 ml/g cells) lysis buffer (18% Ficoll 400, 20 mM KH_2PO_4, pH 6.8, 0.25 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 3 mM DTT, 2 mM benzamidine, 2 mM sodium metabisulfite, 1 mM PMSF, 2 µM Pepstatin A, 0.6 µM Leupeptin, 2 x 10^{-4}% Chymostatin) at 4 °C. Spheroplasts were lysed by passing them through a Teflon-glass continuous flow homogenizer (Yamato LH-21) at 100 rpm three to six times at 4 °C. The nuclei were then purified by differential centrifugation. The homogenate was centrifuged at 4 °C two times at 6,500 rpm and three times at 5,500 rpm for 10 min in a JA-10 rotor. Between each spin the supernatants and looser pellet

components were transferred to new centrifuge bottles. The nuclei were then pelleted by centrifugation at 12,500 rpm for 30 min at 4 °C in a GSA rotor (Sorval). The nuclei were resuspended in 250 ml nuclei storage buffer (100 mM Tris-OAc, pH 7.9, 50 mM KOAc, 20% glycerol, 2 mM EDTA, 3 mM DTT, 2 mM benzamidine, 2 mM sodium metabisulfite, 1 mM PMSF, 2 µM Pepstatin A, 0.6 µM Leupeptin, 2 x 10^{-4} % Chymostatin) at 4 °C, then the nuclei were flash-frozen in liquid nitrogen and stored at -80 °C.

2.3c *Yeast core histone purification* - Purification of the core histones was based on the method of Simon and Felsenfeld (22) as modified by Laybourn and Kadonaga (23). Fifty grams of nuclei were digested with micrococcal nuclease (Worthington) at 6.8 units per ml in the nuclei storage buffer supplemented with 3 mM $CaCl_2$ at 37 °C for the time (usually 5 to 15 min, determined by a test digestion of a 1 ml sample under the same conditions). A digestion time that produces chromatin fragments with an average of 10 nucleosomes (1500 to 2000 bp) in length is used. Ribonuclease A (50 µl of approximately 10 mg/ml Worthington enzyme) was added during this incubation, as well. The digestion was stopped by addition of 0.5 M EDTA to 30 mM and the nuclei were removed by centrifugation at 16,000 rpm at 4 °C for 15 min in an SS-34 rotor (Sorval). The soluble nuclear components were then fractionated 50 ml at a time on a 1 liter Sephacryl S-300 HR column (Pharmacia) equilibrated with 100 mM Tris-OAc, pH 7.9, 50 mM KOAc, 10% glycerol, 2 mM EDTA, 3 mM DTT, 2 mM benzamidine, 2 mM sodium metabisulfite, and 1 mM PMSF and run at 5 ml/min. The chromatin was collected in the excluded peak fractions. The fractions containing chromatin were identified by 1% agarose gel electrophoresis followed by ethidium staining (DNA) and by 18% polyacrylamide-SDS gel electrophoresis (24) stained with Coomassie Brilliant Blue R-250 (histone proteins). The Sephacryl S-300 HR chromatin peak fractions were combined and the chromatin (DNA) concentration was determined by A_{260} after dilution in 1 M NaOH. The chromatin was then bound to a hydroxylapatite (Bio-Rad Bio-Gel HT) in a 5.0 cm diameter column at a

ratio of 2.5 mg chromatin DNA to 1 ml resin. The HT resin was pre-equilibrated at 0.3 M NaCl in 80 mM Na_2HPO_4, pH 6.8, 1 mM DTT, 0.1 mM PMSF, and 2 mM sodium metabisulfite. After binding to the column, the chromatin was washed with a 0.3 M to 0.5 M NaCl gradient over five column volumes followed by an additional five column volumes at 0.5 M NaCl. Core histones were then step eluted at 2.5 M NaCl, leaving the DNA bound to the resin. The core histone protein peak fractions were identified by 18% polyacrylamide-SDS gel electrophoresis and Coomassie staining as above.

2.3d *One dimensional and two dimensional denaturing gel electrophoresis of histone proteins* - Discontinuous Triton X-100-acid-urea (TAU) polyacrylamide gel electrophoresis was performed as per Lennox and Cohen (25). Discontinuous SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (24). The TAU gels contained 7.4 M urea, a 6% polyacrylamide stacking gel and a 15% polyacrylamide resolving gel. In the 2 dimensional gels the TAU dimension was the first dimension. The gel track from one sample was excised, equilibrated and electrophoresed in the SDS dimension according to Lennox and Cohen (25). Gels were stained with Coomassie Brilliant Blue R-250.

2.3e *Nucleosomal template reconstitution* - The yeast Nap1p (NAP-1, 26) was expressed in *E. coli* and purified according to Fujii-Nakata *et al.* (27) except that fractionation was carried out on a Q-Sepharose Fast Flow column (Pharmacia) prior to chromatography on a Mono Q column. Nucleosome reconstitution conditions were based on those described in that article. Purified yeast core histones and Nap1p are combined at a ratio of 1:1 (w/w) to a concentration of 0.25 mg/ml of each under the following conditions: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 100 µg/ml acetylated bovine serum albumen (New England Biolabs). The core histones and Nap1p were incubated at 37 °C for 15 min. The appropriate amount of core histones and Nap1p were then combined with 100 µg/ml 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.

2.3f *Topological assay of nucleosome reconstitution* - Concomitant with the preincubation of purified yeast core histones and Nap1p, closed circular DNA (1 µg) was incubated with yeast topoisomerase I (5 units/ µg DNA) for 30 min at 30°C in 10 µl under the same reconstitution buffer conditions. Yeast topoisomerase I was purified according to Goto *et al.* (28). Appropriate amounts of the core histones plus Nap1p mixture were then combined with the relaxed DNA to a total of 20 µl in reconstitution buffer conditions and incubated for 60 min at 30 °C. The reaction was stopped by the addition of 100 µl 20 mM EDTA, pH 8.0, 200 mM NaCl, 1% SDS (w/v) and 250 µg/ml glycogen. Then 5 µl 2.5 mg/ml proteinase K (Sigma) was added and the mixture incubated at 37 °C for 15 min. Sodium acetate (300 µl at 0.3 M) was added, the DNA was then purified by extraction with 400 µl phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 1 ml 100% ethanol, and washed with 800 µl 75% ethanol. The pellets were then resuspended in loading buffer (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol, 5% glycerol) and 400 ng of the DNA was run on a 10 cm 1% agarose gel in 1X TBE (89 mM Tris, 89 mM borate, 2.5 mM EDTA) buffer at 100 volts in the absence or presence of chloroquine (1.8 µg/ml, 3.5 µM). The gel was stopped when the bromophenol blue dye had migrated 9.5 cm and was stained with ethidium bromide. Two dimensional topological gels were run according to Peck and Wang (29) as modified by Shimamura *et al.* (30) by electrophoresing 750 ng of DNA in the absence of chloroquine for 16 hours at 58 volts in the first dimension, equilibrating the gel in 1.8 µg/ml chloroquine 6 to 8 hours, turning the gel 90° and electrophoresing for 16 hours at 67 volts. Topological markers were generated by mixing DNA purified from chromatin reconstituted at histone to DNA ratios of 0, 0.4, 0.8, 1.0 and 1.2 (w/w). DNA was visualized by ethidium bromide staining.

2.3g *Micrococcal nuclease digestion of reconstituted chromatin* - Reconstituted chromatin templates (3 µg DNA) were digested in 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM $CaCl_2$ and 4.25 units per ml micrococcal nuclease (Worthington) at 21 °C in a total volume of 300 µl. Aliquots of 150 µl were removed at 2 min and 4 min into tubes containing 8 µl 0.5 M EDTA. To each aliquot 100 µl of stop/proteinase K digestion buffer (20 mM EDTA, pH 8.0, 200 mM NaCl, 1% SDS, 250 µg/ml glycogen) followed by 5 µl 2.5 mg/ml proteinase K (Sigma) was added and incubated at 37 °C for 15 min. To purify the DNA fragments, 300 µl 0.3 M sodium acetate was added and the samples were extracted, precipitated, washed and dried as before. The samples were resuspended and electrophoresed on a 10 cm 1.5% agarose gel in 1X TBE buffer at 100 volts at room temperature. The gel was stopped when the bromophenol blue marker dye had migrated 7.5 cm and stained with ethidium bromide.

2.3h *Sucrose gradient purification of reconstituted chromatin templates* - Chromatin templates (50 µg DNA) reconstituted at a ratio of 0.5 and 1.0 (w/w) yeast core histones to DNA were loaded onto linear 5% to 30% sucrose gradients (10 mM Tris-HCl, pH 7.8, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM Benzamidine, 13 ml final volume) and centrifuged at 40,000 rpm for 4 hours at 4 °C in Beckman SW41 rotor. Fractions (1.0 ml) were collected from the bottom and the DNA concentration was determined by measuring the A_{260}. The protein content in each fraction was determined by denaturing polyacrylamide gel electrophoresis in the presence of SDS as described above.

2.4 RESULTS

2.4a *Purification of yeast core histones* - We have succeeded in purifying yeast core histones in quantities (milligrams) sufficient for biochemical analysis (Figure 1.). It should

be noted that two other groups have published methods for the purification of yeast core histones previously (31, 32, 33). We feel our procedure offers advantages over these other methods for the following reasons. First, while similar to the purification protocol of Fukuma *et al.* (31), in our hands our procedure, using column rather than batch chromatography, consistently produces significantly purer histones that do not require concentration prior to use for nucleosome reconstitution. We have found that the concentration of yeast core histones results in their inactivation. Fukuma *et al.* determined that a ratio of 3 to 1 core histones to DNA is required for complete reconstitution, consistent with this inactivation. Second, unlike the hexahistidine tag strategy developed by Lorch and Kornberg (32), our protocol does not require any additional modification of the core histones aside from those being studied. Therefore, this protocol can be immediately applied to the purification of core histones from any yeast strain. In addition, the procedure as described produced a total of 80 µl of purified yeast core histones, which required about 5-fold concentration prior to use in the reconstitution of nucleosomes. Third, Davie *et al.* (33) using non-native methods (acid extraction) is not reasonably comparable with our core histone purification procedure. This approach is primarily an analytical one, not used for purifying core histones to be used for nucleosome reconstitution. We developed a purification procedure (see Materials and Methods) based on the yeast nuclear isolation method of Lue *et al.* (21) and the histone octamer purification protocol of Simon and Felsenfeld (22) as modified in Laybourn and Kadonaga (23). The major modifications were the inclusion of a ribonuclease A digestion of the RNA in the nuclear extract and the substitution of gel filtration chromatography in place of sucrose gradient ultracentrifugation for the partial purification of the chromatin fragments. The partially purified chromatin was bound to a hydroxylapatite column under conditions favoring DNA binding, but not protein binding. The bound chromatin was washed, and the core histones were eluted in a single salt step, leaving the DNA bound to the column matrix. When starting with 0.5 kg of yeast cells (wet weight) this method routinely

produced 5 to 10 milligrams of core histone proteins (a yield of 2.5% to 5%) that are greater than 95% pure as determined by SDS-PAGE.

2.4b *Electrophoretic analysis of purified yeast core histones on denaturing polyacrylamide gels.* - We have analyzed the purified yeast core histones in comparison with purified calf thymus core histones by one dimensional electrophoresis on both Triton-acid-urea (TAU) and SDS polyacrylamide gels as described in Experimental Procedures (Fig. 2.2A and 2.2B). Yeast core histones electrophoresed in the same order of mobility as those from calf thymus in both gel systems. All four yeast core histones migrated nearly identically on SDS-PAGE to those purified from calf thymus. However, in TAU gels the yeast core histones show quite different migratory properties than the calf thymus core histones, indicating differences which are not revealed in SDS-PAGE (Fig. 2.2A and 2.2B). The relative orientation of the core histone spots for each yeast histone in two-dimensional protein gel electrophoresis (Fig. 2.2D) was the same as that of the calf thymus histones (Fig. 2.2C). The lack of histone variants, except histone H2B, in yeast cells was also apparent. One should note the presence of two forms of H3 that were resolved in the SDS but not the TAU dimensions. Both the slower and faster migrating forms of H3 have been purified by gel electrophoresis and subjected to Edman sequencing analysis. This analysis verified that both spots are in fact histone H3 and that the faster migrating form (on SDS-PAGE) results from partial proteolysis of the amino terminal unstructured tail. The other core histones appear to be intact and none of the core histones show indications of multiple post-translational modification. The single major contaminant, migrating as an approximately 30 kDa protein in the SDS dimension, was also sequenced and determined to be the 60S ribosomal protein L7A-2.

2.4c *Reconstitution of chromatin templates using purified yeast core histones* - The Kikuchi laboratory identified a yeast protein, nucleosome assembly protein-1 (Nap1p, also

referred to as NAP-1), that will mediate the reconstitution of nucleosomes from purified core histones (26). Yeast core histone octamers were mixed with an equal mass of purified recombinant Nap1p (27). This mixture was then incubated with plasmid DNA at various ratios of core histones to DNA (w/w). For topological assays we relaxed the DNA with topoisomerase I before the addition of the yeast core histones - Nap1p mixture. The formation of a nucleosome will induce approximately one negative supercoil into the DNA. Under these conditions increasing ratios of histones to DNA produced increasing negative superhelicity indicative of increasing nucleosome density (see Fig. 2.3 and 2.4).

Two dimensional topological analysis (Fig. 2.4) allows determination of the number of supercoils formed. The same DNA samples run on the one dimensional gels (Fig. 2.3) were resolved further by electrophoresis in the absence of chloroquine in the first dimension and in the presence of chloroquine in the second (29). The samples, reconstituted at ratios or 0.8 and 1.2 core histones to DNA, respectively, were run down the left side of the gel in the first dimension. Topological markers were run to the right of the samples. The triangular pattern represents nearly all the topoisomers possible in this plasmid. To the right of the supercoiled markers is completely relaxed DNA. The midpoint of the distribution of topoisomers in the relaxed DNA was assumed to contain 0 supercoils. Through this analysis we determined that, at histone to DNA ratios of 0.8 and 1.2, an average of 16 to 17 nucleosomes (negative supercoils) and 18 to 19 nucleosomes were formed, respectively. The plasmid DNA is 3236 bp in length. This size plasmid allows the formation of a maximum of 19 to 20 nucleosomes per DNA molecule assuming 160 to 170 bp per nucleosome. This repeat length is typical of the close-packed nucleosomes formed in reconstituted chromatin, but also corresponds to the nucleosome repeat length in yeast of 165 bp ± 5 bp (34, 35).

Since histone $(H3/H4)_2$ tetramers alone are capable of inducing supercoiling in a DNA template, the topological assays were not fully diagnostic of complete nucleosome formation. In order to differentiate between tetramer and complete core particle formation

we digested the reconstituted chromatin templates with micrococcal nuclease. Micrococcal nuclease preferentially cleaves the DNA between the nucleosomes in the linker. Tetramers protect approximately 70 bp fragments and, under limiting digestion conditions, produce a ladder of DNA fragments with steps of this length. Core particles will protect 160 to 170 bp fragments to produce a ladder with steps of this length. The digestion pattern generated from micrococcal nuclease digestion of the reconstituted chromatin indicated an average nucleosome repeat length of 160 bp to 165 bp (see Fig. 2.5). This result clearly indicated that complete nucleosomes were formed. Sucrose gradient ultracentrifugation was used to fractionate reconstituted chromatin from free proteins. SDS-polyacrylamide gel electrophoresis of the sucrose gradient fractions demonstrated that each of the four core histones is present in equivalent stoichiometries (Fig. 2.6). This result strongly indicated that complete nucleosomes had been formed. In addition, sucrose gradient ultracentrifugation removes the bulk of the Nap1p. Taken together, these analyses conclusively demonstrated that the purified yeast core histones were competent for reconstitution of good quality chromatin.

2.5 DISCUSSION

We have developed a procedure for the purification of yeast core histones in quantities sufficient for biochemical analyses and have shown these histones to be fully competent for nucleosome reconstitution. Close packed chromatin templates with a spacing of 160 to 165 bp per nucleosome were formed. In yeast chromatin a nucleosome spacing of 165 bp ±5 bp was measured (34, 35). Thus the reconstituted chromatin templates reflect the nucleosomal packing seen in yeast cells. Finally, the ability to produce nucleosomal ladders with up to and beyond 5 steps is indicative of relatively even spacing and complete reconstitution.

We have assembled a homologous system in order to investigate RNA polymerase II transcription regulation in the context of chromatin. Specifically, we have begun to investigate the role of histones, in the form of nucleosomes, in the regulation of transcription initiation. For example, we wish to clarify the role of the histones in the repression of basal transcription initiation and the mechanism of nucleosome displacement or reconfiguration by transcription activating proteins. We have chosen the yeast *PHO5* promoter as a model for this investigation, based on the detailed understanding of the changes that occur in the chromatin structure on the *PHO5* promoter between the repressed and the activated states (9, 10, 11, 12). In preliminary experiments using the reconstituted chromatin templates we have observed strong repression of yeast RNA polymerase II transcription (not shown). It is also worth noting that Nap1p was found to be completely compatible with yeast *in vitro* transcription. The addition of Nap1p without core histones to *in vitro* transcription reactions does not inhibit basal transcription. We have attempted to form nucleosomes by the salt gradient dialysis procedure (36) using yeast core histones, but have not been successful. This same salt gradient dialysis procedure has been used successfully with calf thymus core histones several times in our laboratory. Therefore, it appears that the inability to use this procedure is a peculiarity of yeast core histones. It is probably worth noting that Morse *et al.* (37) reported similar difficulties and that both Fukuma et al. (31) and Lorch and Kornberg (32) also used alternative methods for reconstitution with yeast core histones.

The reconstitution of nucleosomes with yeast core histones on yeast promoters, representing the repressed state, will provide a basis for investigation of the mechanism of transcription activation on these promoters. This reconstitution system will also provide a means for identification of the mechanisms directing nucleosome positioning over a repressed promoter. We also have begun to reconstitute activated transcription conditions. Combining this activated transcription system with the reconstituted chromatin template will allow the investigation of the mechanism of chromatin remodeling by transcription

regulatory factors and to separate the role of these factors in counteracting nucleosome repression from there role in true transcription activation.

2.6 ACKNOWLEDGMENTS

We are grateful to Jim Kadonaga, Alan Wolffe, David Moss, and Brian Krett for helpful suggestions and advise during the course of this work; David Moss and Ryan Ogg for critical reading of this manuscript; to Tomoko Fujii-Nakata for generously providing the NAP-1 expression plasmid, pTN2; and to Shi-Hsiang Shen for the kind gift of the pUV5-G1S plasmid expressing glucanase.

This research was supported in part by Research Grant MCB-9505644 from the National Science Foundation, by a grant from The Council for Tobacco Research and a Faculty Research Grant from the Graduate School, Colorado State University. P.J.L. is a recipient of a Junior Faculty Research Award from the American Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

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Purification of Yeast Core Histones

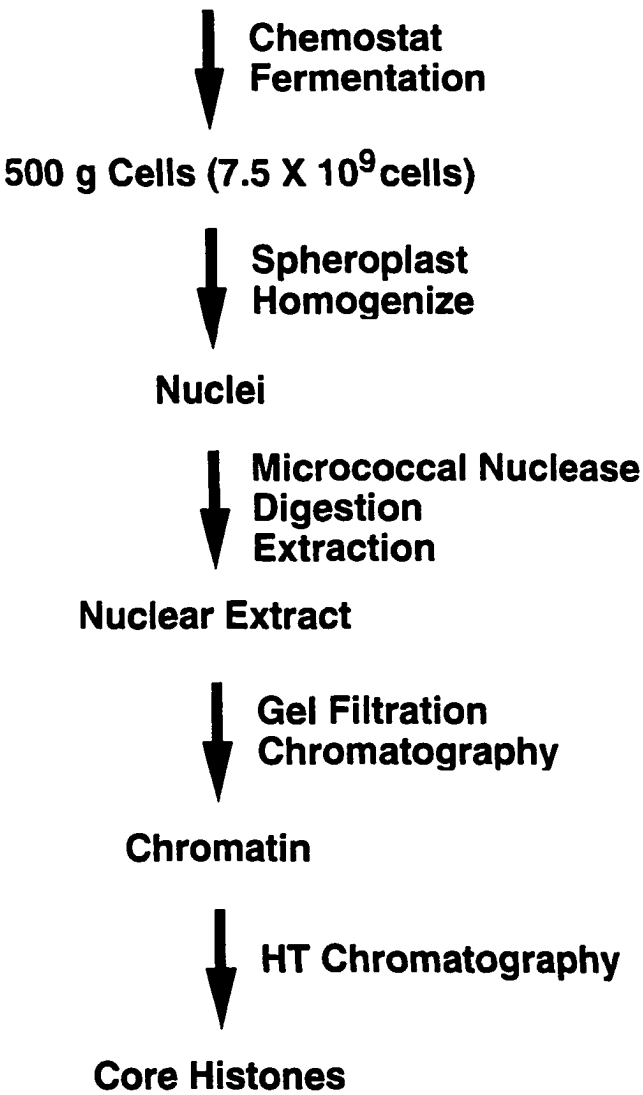

Figure 2.1. Schematic representation of the yeast core histone purification procedure.

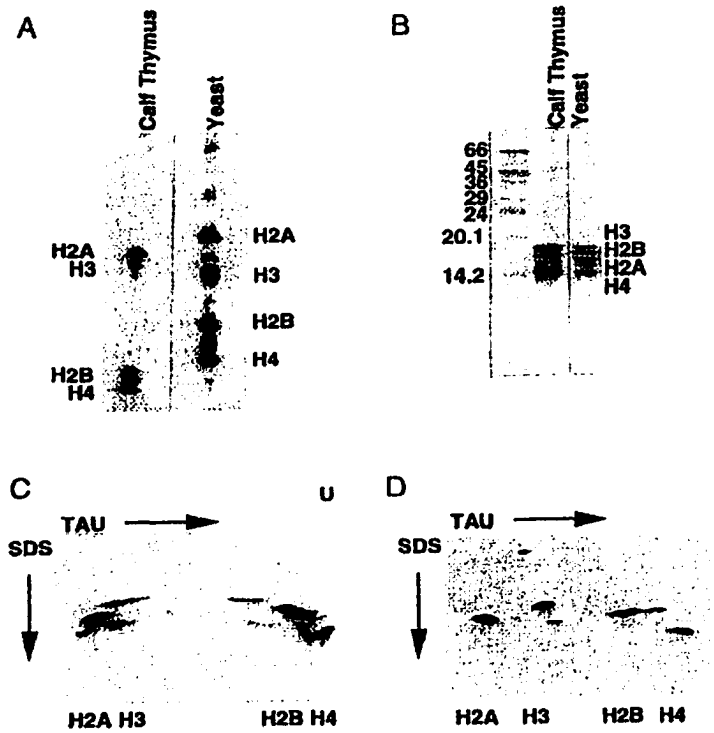

Figure 2.2. Denaturing gel electrophoresis of purified yeast core histones. One dimensional PAGE. Calf thymus (8.4 µg) and yeast (18 µg) core histones were resolved by (A) TAU (Triton-acid-urea) PAGE in a 15% polyacrylamide gel and by (B) SDS (sodium dodecyl sulfate) PAGE in an 18% polyacrylamide gel and visualized by Coomassie Blue staining. In the left and right margins of the TAU gel and the right margin of the SDS gel the identities of the core histone are indicated. In the left margin of the SDS gel the molecular mass of the protein size standards is indicated in kDa. Two dimensional PAGE. Gel tracks from the TAU gel containing calf thymus (C) and yeast (D) core histones (Fig. 1A) were excised, and electrophoresed in the second dimension in an SDS gel (18% polyacrylamide) and visualized by Coomassie Blue staining. The identities of the spots are indicated in the lower margins.

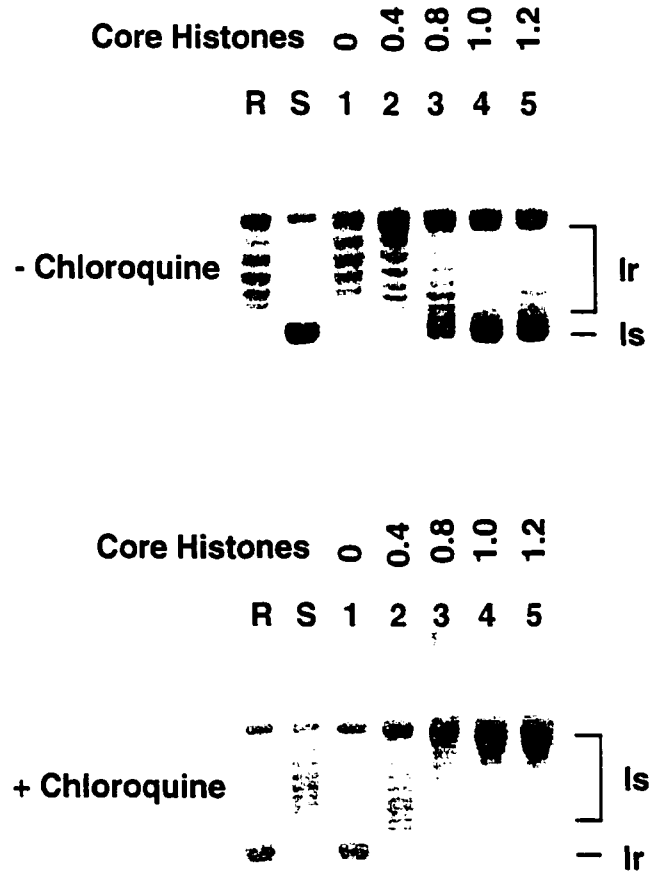

Figure 2.3. One dimensional topological analysis. Plasmid DNA (400 ng) purified from chromatin reconstituted at the core histone to DNA ratio (w/w) indicated at the top was resolved on 1% agarose gels run in the absence or presence of chloroquine as indicated in the left margin. Lanes S and R are supercoiled and relaxed markers, respectively. In the right margin the positions of relaxed (Ir) and supercoiled (Is) closed circular DNA are indicated for each gel condition.

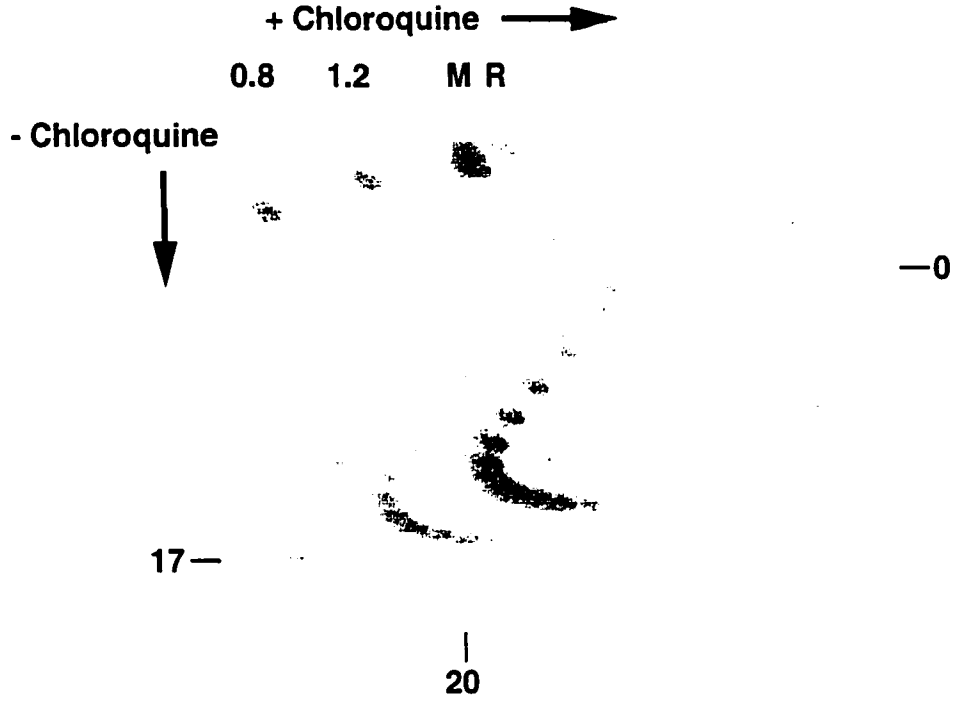

Figure 2.4. Two dimensional topological analysis. Plasmid DNA (750 ng) from chromatin reconstituted in the presence of topoisomerase I, purified and resolved on a 1% agarose gel. The first dimension is in the absence, the second in the presence of chloroquine. The samples, chromatin reconstituted with a ratio of 0.8 and 1.2 core histones to DNA (w/w) and supercoiled markers (M) and relaxed markers (R), are indicated in the upper margin.

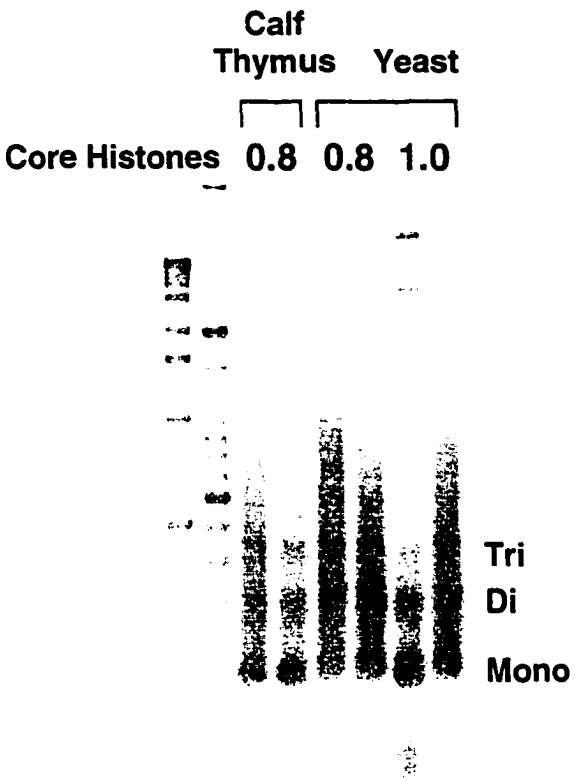

Figure 2.5. Micrococcal nuclease digestion. Reconstituted chromatin (3 μg DNA) was digested with micrococcal nuclease for 2 and 4 min as described in Materials and Methods and resolved on a 1.5% agarose gel. The chromatin was reconstituted with calf thymus and yeast core histones at the histone to DNA ratios (w/w) indicated in the upper margin. The outer most lanes are 1 kb and 100 bp DNA markers. The locations of mono-, di- and tri-nucleosome DNA fragments are indicated in the right margin.

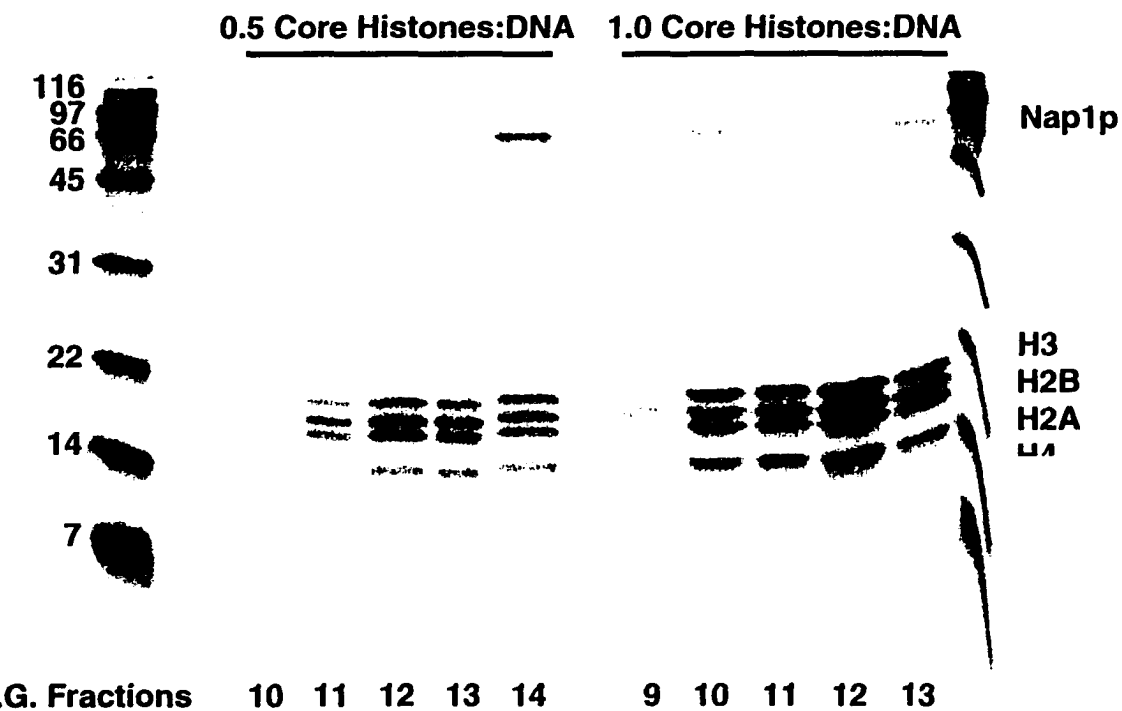

Figure 2.6. Purity and stoichiometry of yeast core histones on sucrose gradient purified chromatin templates. Chromatin templates were reconstituted, as describe in Materials and Methods, at a core histone to DNA ratio (w/w) of 0.5 and 1.0. The chromatin templates were purified by sedimentation on 5% to 30% sucrose gradients (see Materials and Methods). Gradient fractions were collected from the bottom and the proteins in the DNA-containing gradient fractions were resolved by 18% SDS-PAGE and stained with Coomassie Blue. The outermost lanes contain protein size standards, with the molecular weights indicated in the left margin. The gradient fraction run in each lane for chromatin templates reconstituted at 0.5 (left set) and 1.0 (right set) core histones to DNA is indicated below the gel. The identity of each protein in the sample lanes is indicated in the right margin.

Chapter 3

Nucleosome Remodeling on an I*n Vitro* Reconstituted Yeast *PHO5* Promoter

This chapter is written in the form of a manuscript to be submitted. Any experiments denoted "data not shown" in the text of the manuscript are either included at the end of this chapter as Supplemental Figures or in Chapter 4. Any Materials and Methods referred to elsewhere are described in detail in Chapter 5.

3.1 SUMMARY

The *PHO5* gene of *Saccharomyces cerevisiae* has become in important model with which to study gene expression in the context of chromatin. The *PHO5* gene encodes the major repressible acid phosphatase in *Saccharomyces cerevisiae*. The gene is repressed in high phosphate by the presence of four translationally positioned nucleosomes located over the promoter. Upon activation induced by low phosphate, the nucleosomes are reconfigured allowing expression of *PHO5*. In this study we have used templates reconstituted into chromatin with purified yeast core histones to investigate the process of nucleosome remodeling, a prerequisite for transcription activation, on the *PHO5* promoter. We show that the transcription factors Pho4p and Pho2p can bind to recognition sites on nucleosomal *PHO5*, but this alone is not sufficient for remodeling. We have also shown that nucleosomes can be remodeled using both fractionated nuclear extract and whole cell extract from yeast. The chromatin transition of the *PHO5* gene upon activation has been extensively studied *in vivo*, however, the mechanisms by which nucleosome displacement occurs have only been minimally investigated. We have developed an *in vitro* system that will serve as a useful tool for studying not only the process of nucleosome remodeling but also the mechanisms of transcription activation of *PHO5*.

3.2 INTRODUCTION

Chromatin functions to compact and organize the DNA in the nucleus of eukaryotic cells in a manner that will allow repeated access to genes for the process of transcription. The role of nucleosomes in transcription has become a major area of study. From *in vitro* studies, it is clear that nucleosomes repress transcription from genes transcribed by RNA polymerase II (19, 21, 27)(Chapter 4, this dissertation). In addition, depletion of histone H4 *in vivo*, resulting in nucleosome loss, derepresses transcription of

several RNA polymerase II transcribed yeast genes (11, 12), further confirmation that nucleosomes play a prominent role in gene regulation. Barring artificial nucleosome loss, chromatin must be remodeled prior to transcription activation. Recently many activities that remodel chromatin have been identified, including but not limited to the yeast SWI/SNF and RSC complexes, the *Drosophila* NURF, CHRAC, and ACF complexes, and the human SWI/SNF complex (reviewed in (18)). All of these machines contain a DNA dependent ATPase subunit, required for remodeling. We seek to elucidate the mechanisms of transcription activation of the yeast *PHO5* gene by determining how the process of nucleosome remodeling occurs. The machinery that remodels *PHO5* chromatin has not been determined, although SWI/SNF is not required (10, 14).

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 (3). Under repressive conditions, when there is adequate phosphate in the environment, the *PHO5* promoter is bound by specifically positioned nucleosomes (1). Activation of *PHO5* through phosphate starvation is accompanied by a loss of four nucleosomes from the promoter region (Figure 3.1) (2).

Activation is initiated through a signal transduction pathway which ultimately allows for dephosphorylation of the transcription factor Pho4p, allowing its transport into the nucleus (17, 23) where it can bind to two upstream activation sequences on the *PHO5* promoter, UASp1 and UASp2 (8, 31). Pho2p, a second transcription factor involved in *PHO5* activation, binds cooperatively with Pho4p *in vitro* at both UASs (4). Pho4p and Pho2p must physically interact *in vivo* for transcription activation to occur (29). This interaction can occur regardless of phosphate concentrations but does require the presence of DNA (22). Both transcription factors are required for full chromatin remodeling and activation of *PHO5* (33). However, Pho4p is the primary trigger for activation, as overexpression of *PHO4* in a *pho2* null strain is sufficient for the full

chromatin transition and provides some compensation for the loss of transcription activation (8). Activation of *PHO5* occurs in the absence of replication and does not require transcription (9, 28), suggesting activation of transcription and chromatin remodeling occur independently, although the chromatin transition must occur prior to activation. The activation domain of Pho4p is required for *PHO5* expression, indicating it probably recruits factors to the promoter for remodeling (30). 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 in activating conditions when *snf2* has been disrupted (10). Although the *trans*-acting factors and *cis*-acting sequences required for nucleosome remodeling and transcription activation of *PHO5* have been well characterized, the detailed mechanisms of the actual chromatin transition is not yet understood.

It has recently been shown that the chromatin of the *PHO5* promoter can be remodeled *in vitro* on an *in vivo* assembled minichromosome template (14). Remodeling of *PHO5* required Pho4p, Pho2p, ATP, and fractionated nuclear extract. This work has helped elucidate the mechanism of remodeling; however, it is not a truly *in vitro* system since the template was assembled into nucleosomes *in vivo* then subsequently isolated. To study the fine details of nucleosome remodeling a fully defined *in vitro* system should be used. We have used purified yeast core histones and recombinant Nap1p to reconstitute nucleosomes on a plasmid containing the *PHO5* promoter fused to a G-less cassette, to facilitate future transcription studies. This system is sufficient for correct positioning of nucleosomes on *PHO5* (27) (Chapter 4, this dissertation). We have used this system to further define the requirements for the *PHO5* chromatin transition.

3.3 MATERIALS AND METHODS

3.3a *Pho4p Purification.* Purification of Pho4p (expression vector T7-PHO4, a gift from the O'Shea lab) was adapted from Kaffman *et. al.* (16) 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. 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 KOAc), 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. For purification of Pho4p a 10 ml DEAE Sepharose FF (Pharmacia) column was equilibrated in RB0.1. The lysate was loaded onto the column at 0.7 ml/min and the column was washed with RB0.1. Proteins were eluted with RB1.0 (RB0.1 with 1 M KOAc) and the peak Pho4p fractions determined by SDS-PAGE and pooled. The pool was dialyzed into RB0.3 for six hours at 4°C then loaded onto a 5 ml SP Sepharose FF (Pharmacia) column equilibrated in RB0.3 at 0.4 ml/min. The column was washed with RB0.3 then the proteins were eluted with RB1.0. Peak fractions were determined as described above. 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 then proteins were eluted with a RB0.1 to RB1.0 gradient at 0.5 ml/min over 20 column volumes. Peak fractions were determined as described above, quantitated using the Coomassie Plus Protein Assay Kit (Pierce), and stored in liquid nitrogen.

3.3b *Pho2-His Purification.* Purification of Pho2-His was adapted from Brazas and Stillman (6) and is as follows. Pho2-His expression vector (M2025, a gift from David Stillman) was transformed into BL21 pLysS *E. coli*. 250 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 (Quiagen) 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 60 mM to 600 mM imidazole gradient in His binding buffer. The peak fractions were determined by SDS-PAGE then dialyzed against storage buffer (20 mM Hepes-KOH pH 7.9, 10 % glycerol, 0.5 mM EDTA, 0.5 mM DTT, 100 mM KOAc, 1 mM PMSF, 10 mM β-mercaptoethanol, and 1 mM benzamidine). Protein concentration was determined using the BCA Protein Assay Kit (Pierce).

3.3c *Whole Cell Extract Preparation.* Whole cell extracts 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.* (32).

3.3d *G-less cassette plasmid construction.* A 110 bp insert containing no guanines in the RNA like strand was ligated immediately downstream of the *PHO5* promoter in pMH313, creating pMHG-less. An *Mfe* I site was also inserted into the vector using PCR mutagenesis. The *Mfe* I site was created by changing a C to a T immediately downstream of UASp1.

3.3e *In vitro reconstitution of chromatin.* Performed as described in Chapter 4 of this dissertation.

3.3f *Primer extension footprinting.* Performed as described in Chapter 4 of this dissertation. The sequence of the primer is 5'ATTTGGCATGTGCGATCTCTT3'. The primer binds downstream of UASp2 and extends upstream.

3.3g *In vitro transcription.* Transcription reactions contained naked or reconstituted DNA (500 ng), 100 µg 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, .25 U RNase Inhibitor (5'/3'), 30 mM phosphocreatine (Sigma), .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. 120 µL RNase T1 buffer (10 mM Tris-Cl pH 7.5, 300 mM NaCl, 5 mM EDTA) and 50 U RNase T1 (Boehringer Mannheim) were added and incubated at 22°C for 15 min. Eight microliters 10% SDS, 17.5 µL 2.5 mg/mL Proteinase K (Sigma), and γ^{32}P-ATP end labeled recovery standard were added and incubated at 37°C for 20 min. Fifteen micrograms of glycogen was added and reactions were ethanol precipitated. Transcripts were pelleted, washed with 75% ethanol, resuspended in TBE-Formamide dye, and electrophoresed on a 8% polyacrylamide urea sequencing gel at 20 V/cm until the bromophenol blue ran through the gel. The gel was dried and exposed on a PhosphorImager screen.

3.3h *Nuclear Extract Preparation.* Performed exactly as described in Haswell and O'Shea (1999) using yeast strain YS18 (MATα; his3-11,15; leu2-3,112; ura3Δ5; canR) (a gift from Dr. B. Meyhack).

3.3i *In vitro chromatin remodeling.* One microgram of reconstituted pMHG-less was digested with micrococcal nuclease (MNase, Worthington) in a 100 µl reaction volume following incubation with ATP, whole cell extract, Pho4p, Pho2-His, and/or fractionated nuclear extract (indicated in figure). Reactions contained 1 µg DNA, 6 mM $CaCl_2$, proteins and/or ATP as described in figure legends, and brought to volume with reconstitution buffer (27) (described in Chapter 4). 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 MNase. 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 and chloroform/IAA, and precipitated with ethanol. Eight hundred nanograms of DNA was run an a 10 cm minigel to verify MNase digestion and 200 ng was run on a 20 cm 1.5% agarose gel in 1 X TBE until a 100 bp DNA marker was approximately 2 cm from the end of the gel. The DNA was Southern blotted (Turboblotter, Schleicher and Schuell) and probed with a 169 bp Mfe I to BstE II fragment representing nucleosome –2.

3.3j *Electrophoretic mobility shift assays (EMSA).* Polyacrylamide gels were 5% 40:1 in 0.5 X TBE. EMSAs were run at 30 mA for 1.5 hrs (24 bp probe, Figure 3.2B) or 2.5 hrs (106 bp probe, data not shown). For the competition EMSA both wild type and mutant probes were 24 bp double stranded oligomers synthesized by MRF (Colorado State University). The sequences of the mutant oligos were 5'TTGCCTACGCCGGCGACTCCGCGA3' and 5'TGATTCGCGGAGTCGCCGGCGTAG3'. The sequences of the wild type probes were 5'TGATTAAAAGAGTTAATTGAATAG3' and 5'TTGCCTATTCAATTAACTCTTTTA3'. 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 Pho2-His, competitor as described in figure 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. Pho4p and Pho2-His ternary complex formation was shown by using 0.4 ng labeled probe and 100 ng poly dIdC in a 15μl reaction volume with transcription buffer.

3.4 RESULTS

3.4a Pho4p and Pho2-His are purified to near homogeneity and bind both naked and reconstituted DNA.

Pho4p and Pho2p are both required for the chromatin transition of *PHO5* upon phosphate depletion (8), therefore is was necessary to purify both proteins to study the chromatin transition *in vitro*. Recombinant Pho4p and Pho2-His were purified as described in Materials and Methods to near homogeneity as judged by Coomassie blue staining (Figure 3.2A). To verify that both proteins bind DNA, we showed by EMSA that Pho4p and Pho2-His bind a 106 base pair UASp1 probe (*Mfe* I to *TthIII* I) independently, and also form a ternary complex when combined (data not shown), as has already been shown (4). In addition since homeodomain proteins, of which Pho2p is a member, do not have well defined binding sites, we wanted to test the binding specificity of Pho2p to *PHO5* DNA. Pho2-His specifically binds to DNA sequence from the site adjacent to UASp2 on the *PHO5* promoter (Figure 3.2B). Binding can be competed in an EMSA only by unlabeled wild type probe (identical to labeled probe) but not by mutant probe in which the internal AT rich region has been randomized. The AT rich region was chosen for the mutation because Justice *et al.* (15) showed it as an important region for

homeodomain proteins to bind DNA. To further investigate the binding of Pho4p and Pho2-His to DNA we decided to use DNaseI cleavage and primer extension footprinting of both proteins on naked and *in vitro* reconstituted DNA (Figure 3.3). Pho4p and Pho2-His protect both naked DNA and chromatin from DNase I cleavage. Pho4p gives complete protection especially on naked DNA. The regions protected by Pho4p are indicated by vertical lines. The Pho2-His footprint, indicated by the stars, can only be seen when Pho4p is present; however, EMSAs indicate that Pho2-His can bind DNA in the absence of Pho4p. This implies that Pho2-His is binding the DNA but that DNase I does not readily cleave our template where Pho2-His binds. This also indicates that binding is cooperative since a footprint by Pho2-His is only seen when Pho4p is present. Footprints are observed on naked and chromatin DNA at the same amount of protein. However, the protection is greater on naked DNA compared with the protection seen on chromatin, suggesting the affinity of Pho4p and Pho2-His is less for reconstituted templates than for naked DNA. Increasing the amount of protein added to the footprinting reaction to attain more complete protection was not helpful as this caused reproducible alterations in the cleavage pattern, presumably due to aggregation of proteins on the DNA (Figure 3.3, see lane 4). The alteration of the cleavage pattern in lane 8 of Figure 3.3 appears to be an anomaly.

3.4b Pho4p does not counteract nucleosome repression of transcription.

Pho4p is the primary trigger for activation of *PHO5*. It is transported into the nucleus following a signal that phosphate is deficient (23). Pho4p most likely binds the constitutively accessible UASp1 to initiate the chromatin transition, a prerequisite for transcription activation. Since transcription activation, or counteraction of nucleosome repression, can be achieved on *in vitro* assembled templates (5, 7, 20) allowing the study of activities that remodel chromatin, we wanted to determine the transcription activation

potential of recombinant Pho4p in an *in vitro* transcription assay. Addition of Pho4p activates transcription approximately two fold on naked DNA (Figure 3.4, compare lanes 1 and 2). Since Pho4p presumably initiates the chromatin transition prior to activation, we wanted to determine whether Pho4p would counteract nucleosome repression of transcription. Formation of nucleosomes at a physiological concentration, as determined by one-dimensional topological analysis and micrococcal nuclease digestion (data not shown), represses *in vitro* transcription (Figure 3.4, compare lanes 1 and 3). By adding Pho4p to the transcription reaction containing reconstituted chromatin, no additional activation of transcription beyond that of naked DNA is seen. The small amount of activation seen by adding Pho4p to the chromatin templates, approximately two-fold, is not true counteraction of nucleosome repression, it is likely the result of activation on un-reconstituted templates. These results indicate that Pho4p cannot counteract nucleosome repression of transcription, although it is sufficient for activated transcription on naked DNA.

3.4c Yeast whole cell extract remodels *PHO5* chromatin structure in the absence of Pho4p, Pho2p, or additional ATP.

Since Pho4p cannot counteract nucleosome repression of *PHO5* transcription, we wanted to determine whether this was due to a lack of chromatin remodeling, which would prevent access to the DNA for the transcription machinery. Naked DNA (Figure 3.5, lane 1) or reconstituted chromatin (Figure 3.5, lane 2) was digested with micrococcal nuclease then probed with DNA representing nucleosome –2 of the *PHO5* promoter. A smear is seen in the naked DNA lanes and a nucleosome ladder of approximately 150 base pairs is seen with digested chromatin. When whole cell extract from strain YS27 (*pho4/pho2*) is added to the same weight:weight ratio (with respect to the amount of DNA) that is used in transcription reactions, a loss of the nucleosome ladder, indicating

chromatin remodeling, is observed (Figure 3.5, lane 3). Therefore, chromatin is being remodeled in our transcription reactions but this is not sufficient for Pho4p to counteract repression.

3.4d Fractionated nuclear extract, when added at limiting amounts, remodels *PHO5* chromatin and requires Pho4p, Pho2p, and ATP.

To further investigate the requirements for chromatin remodeling in a truly *in vitro* system, we decided to use fractionated nuclear extract from wild type yeast cells. This fraction has recently been found to remodel the *PHO5* chromatin structure of isolated minichromosomes (14). We show that by using a 7:1 ratio of extract to DNA (w:w), chromatin remodeling is dependent on the presence of Pho4p, Pho2p, and ATP (Figure 3.5, lane 7). These results are similar to those obtained by Haswell and O'Shea (14). Tentative results indicate that if a 20:1 ratio of fractionated nuclear extract to DNA is used, chromatin can be remodeled in the absence of exogenously added transcription activators or ATP (Figure 3.5, lane 4).

Taken together these results suggest multiple conditions in which chromatin remodeling of the *PHO5* promoter can occur. If remodeling complexes are present in high enough concentrations, as with YS27 whole cell extract, there is no requirement for exogenous ATP or recruitment by DNA binding transcription activators. If non-limiting amounts of fractionated nuclear extract are added, a similar result is obtained. However, this extract was prepared from wild type cells washed with sorbitol, which would initiate a phosphate deficient response, so Pho4p and Pho2p are likely present, but ATP is not. It is also possible that there is sufficient ATP in the extracts for chromatin remodeling complexes to be active, however this is doubtful as preparation of both extracts involves dialysis, using tubing with a molecular weight cut-off that would allow ATP to diffuse out. It is also possible that there exists a non-ATP dependent remodeling mechanism that

has yet to be identified. Finally, by limiting the amount of remodeling complexes, as with only a 7:1 ratio of fractionated nuclear extract:DNA, exogenously added ATP and transcription activators are both required for remodeling.

3.5 DISCUSSION

We have developed an *in vitro* system consisting of only closed circular DNA, purified yeast core histones, and Nap1p, a yeast nucleosome assembly protein, to study chromatin remodeling of the yeast *PHO5* promoter. Our *in vitro* reconstituted templates have been well characterized (26) and we have determined that nucleosomes are being translationally positioned *in vitro* in a manner very similar to their *in vivo* positions (27) (Chapter 4, this dissertation). This allows us to study the regulation of *PHO5* by adding back various extracts and/or purified factors to determine their effects on the transcription activation process in a much more defined environment.

We have shown that Pho4p and Pho2p can bind to *PHO5* DNA regardless of whether the DNA exists in a naked or chromatin conformation. This reiterates the importance of the necessity for upstream regulation of Pho4p (phosphorylation and nuclear localization) (16, 23). It is likely that the acidic activation domain of Pho4p is responsible for recruitment of the pol II transcription machinery. It was determined that Pho4p physically interacts with TFIIB, TFIIEβ, and TBP (22). In our system Pho4p is sufficient for transcription activation on naked DNA, presumably through increased recruitment of pol II, but it does not counteract nucleosome repression. However, the chromatin structure of the *PHO5* promoter is being remodeled by whole cell extract, which is present in the *in vitro* transcription reaction, suggesting that nucleosomes are not the barrier to transcription activation by Pho4p. The formation of nucleosomes does not render the DNA transcriptionally incompetent, as transcription from the reconstituted

template can be restored by proteinase K digestion and organic extraction of the DNA (data not shown).

We have shown that Pho4p and Pho2p can bind to the promoter and that at the very least nucleosomes –2 and +1 (discussed below) are being remodeled; however, activation is still not observed. This suggests another barrier to transcription in our system. It is possible that the presence of Mg^{++} in the transcription buffer is causing compaction of the templates and excluding the general transcription machinery. This scenario was heavily considered in light of the findings that initiation and elongation by RNA polymerase III are severely hampered by chromatin in the presence of Mg^{++} (13). Although compaction of the DNA may be occurring, it should not prevent initiation of transcription, as we show that the transcription factors Pho4p and Pho2p have access to the DNA in the same Mg^{++} concentrations used for *in vitro* transcription experiments.

Assuming that the RNA polymerase II machinery has access to the DNA through recruitment by Pho4p, a probable barrier to counteraction of nucleosome repression of transcription could be an inability of transcript elongation to occur, even following remodeling of *PHO5* chromatin. Elongation should not be prevented by the presence on a nucleosome in the coding region, as re-probing the experiments discussed above with a nucleosome +1 probe gives identical results, suggesting remodeling is also occurring to at least nucleosome +1 (data not shown). A protein factor named called FACT, isolated from HeLa cells, was recently identified that facilitates transcription elongation through nucleosomes *in vitro* (24, 25). It is possible that the yeast homolog of FACT, Spt16/Cdc68, is required for elongation through nucleosomes. If this activity is too dilute in our system, elongation of *PHO5* transcripts, even on a remodeled template, would be impeded.

We have also shown multiple conditions in which chromatin remodeling can occur. A Pho4p, Pho2p, and exogenous ATP independent condition, seen with YS27

whole cell extract; an exogenous ATP dependent mechanism, seen with non-limiting amounts of YS18 fractionated nuclear extract; and a mechanism requiring Pho4p, Pho2p, and ATP, seen with limiting amounts of YS18 fractionated nuclear extract.

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 remodeling in a very well defined environment. In addition, we have designed our system using a plasmid containing a G-less cassette to facilitate future transcription studies to combine with remodeling data, giving a more complete picture of the processes of nucleosome remodeling and transcription activation.

3.6 ACKNOWLEDGEMENTS

We would like to thank both Dr. David Stillman and Dr. Erin O'Shea for kind gifts of expression plasmids. Also, we wish to thank Dr. B. Meyhack and Dr. Wolfram Hörz for yeast strains. In addition we thank Dr. Norm Curthoys, Dr. Jennifer Nyborg, Dr. A-Young Woody, and Dr. Christine Wilcox for critical reading of this manuscript.

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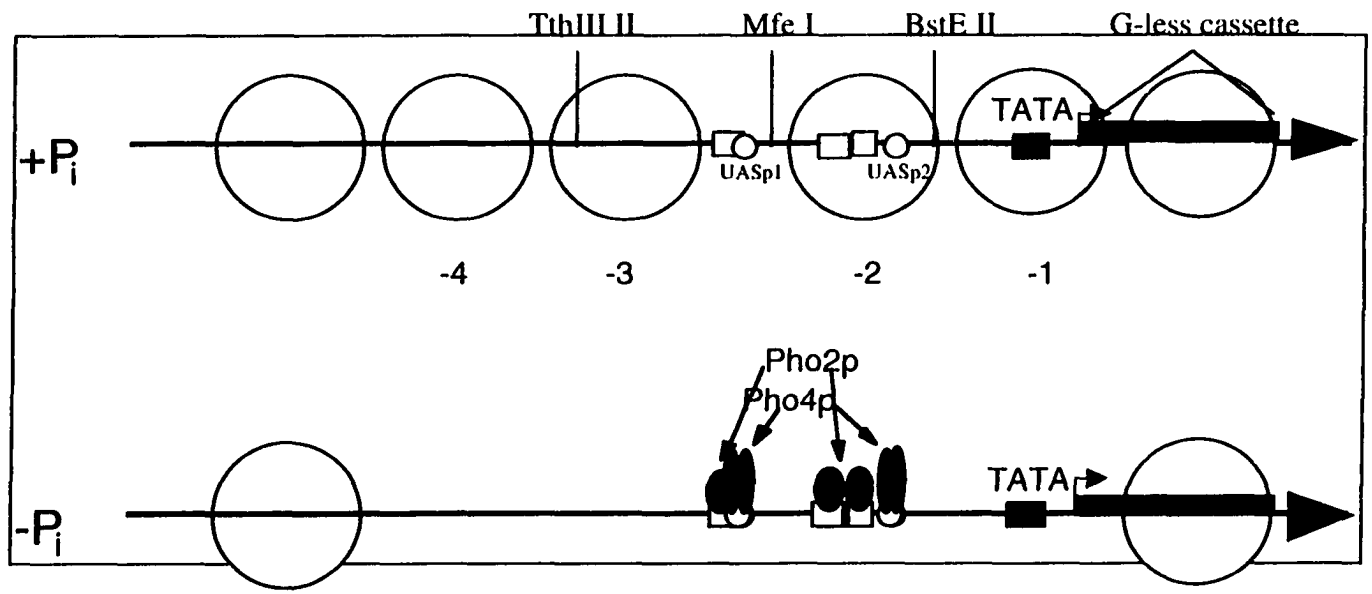

Figure 3.1. *PHO5* promoter chromatin structure. Top panel shows the repressed state of the promoter and the bottom panel shows the activated chromatin structure. The large open circles represent nucleosomes. All relevant features of the promoter are indicated.

Figure 3.2. Analysis of purified proteins and Pho2-His binding to naked DNA. A. Coomassie stained gel (12%) of purified Pho4p and Pho2-His. Marker sizes, in kDa, are indicated to the left of the gel. B. Competition EMSA with Pho2-His and cold wild type probe or mutated probe. Lanes 1 and 2 contain 100 ng poly dIdC as a competitor, lanes 3 through 8 contain 25, 50, 100, 200, 300, and 500 ng unlabeled wild type probe, respectively, as a competitor. Lanes 9 through 14 contain 25, 50, 100, 200, 300, and 500 ng unlabeled mutant probe, respectively, as a competitor.

Figure 3.3. Primer extension footprinting using Pho4p and Pho2-His on naked and *in vitro* reconstituted pMHG-less(mfe). Lanes 1 through 11 contain naked DNA, lanes 12 through 22 contain chromatin. Lanes 1 and 12 are undigested DNA (U). Lanes 11 and 22 contain no DNA binding proteins (N). Lanes 2, 3, and 4 contain 250, 500, and 1000 ng Pho4p; lanes 5, 6, and 7 contain 90, 180, and 360 ng Pho2-His; lanes 8, 9, and 10 contain 500 ng Pho4p and 90, 180, and 360 ng Pho2-His, respectively. Lanes 13 through 21 contain an identical amount and combination of protein as lanes 2 through 10 except that proteins were footprinted on reconstituted chromatin DNA rather than naked DNA as in lanes 2 through 10. The stars indicate sites of protection by Pho2-His and the lines indicate the area of protection by Pho4p The lanes furthest to the left are dideoxy sequencing reactions using the same primer as was used in the footprinting extension reactions. The ddNTP used for termination is indicated at the top of each lane.

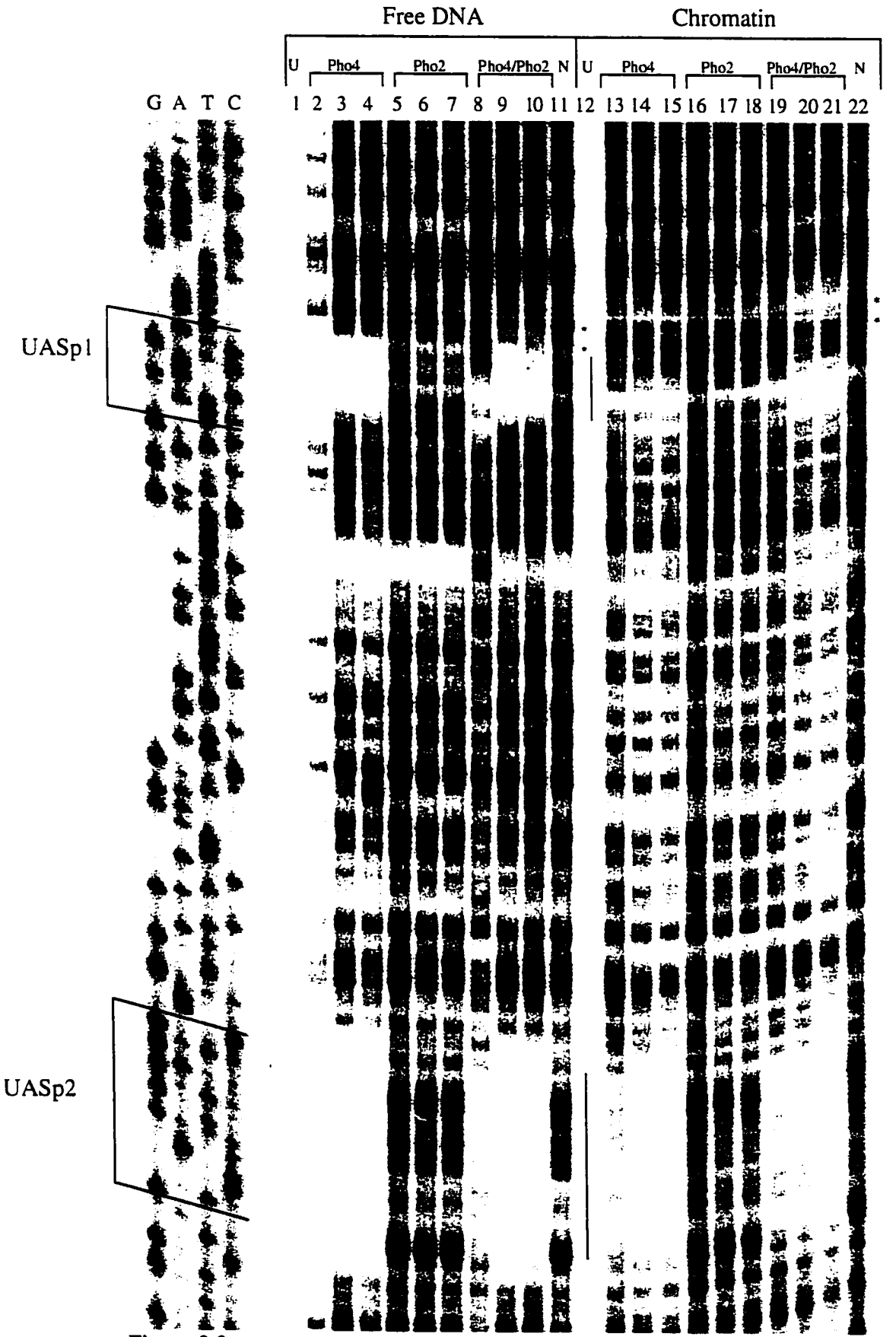

Figure 3.3.

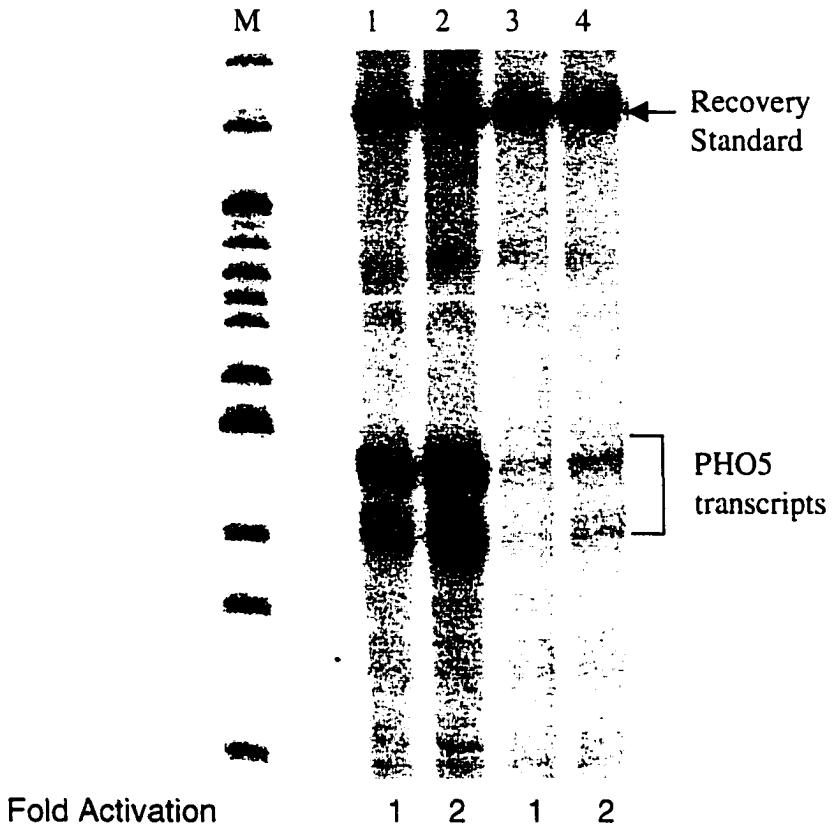

Figure 3.4. *In vitro* G-less cassette transcription. Lane M is radioactively labeled DNA marker from an Msp I digest of pBR322. Lane 1 is basal transcription from naked pMHG-less(mfe). Lane 2 is activated transcription after the addition of 250 ng Pho4p. Lane 3 shows transcripts from *in vitro* assembled chromatin templates. Lane 4 contains transcripts from chromatin after adding 250 ng Pho4p. The fold activation, with respect to the amount of transcripts from either naked or chromatin templates in the absence of activator, is indicated below the panel. Labeled recovery standard, the Pvu II to Pvu II fragment from pUC19, is indicated to the right. Fold activation was quantitated using Image Quant (Molecular Dynamics).

Figure 3.5. Southern blot of nucleosome remodeling on *in vitro* assembled chromatin templates. A. Gel lanes representing three different micrococcal nuclease digests of identical samples. All digestion reactions contained 1 µg pMHG-less(mfe) DNA. Lane 1, naked DNA digested with 0.004, 0.016, and 0.032 U MNase. Lanes 2 through 7, *in vitro* assembled DNA digested with 0.05, 0.25, and 0.5 U MNase. Lane 2, chromatin alone; lane 3, chromatin and 100 µg YS27 whole cell extract; lane 4, chromatin and a 20:1 ratio of fractionated nuclear extract:DNA (w:w) ; lane 5, chromatin and a 7:1 ratio of fractionated nuclear extract:DNA ; lane 6, chromatin, 1 µg Pho4p, and 450 ng Pho2-His; lane 7, chromatin, a 7:1 ratio of fractionated nuclear extract:DNA , 1 µg Pho4p, 450 ng Pho2-His, and ATP regeneration mix (0.2 µg/ml creatine kinase, 30 mM creatine phosphate, and 0.5 mM ATP). Following micrococcal nuclease digestion and separation on agarose gels, digests were Southern blotted then probed with a 169 base pair Mfe I to BstE II fragment representing nucleosome –2 of the *PHO5* promoter. A ladder represents intact chromatin, a smear represents either naked DNA or remodeled chromatin. B. NIH Image plots of the third lane in each set of digestions. The number is the top left corner of each plot corresponds to the number below each set of gel lanes in panel A.

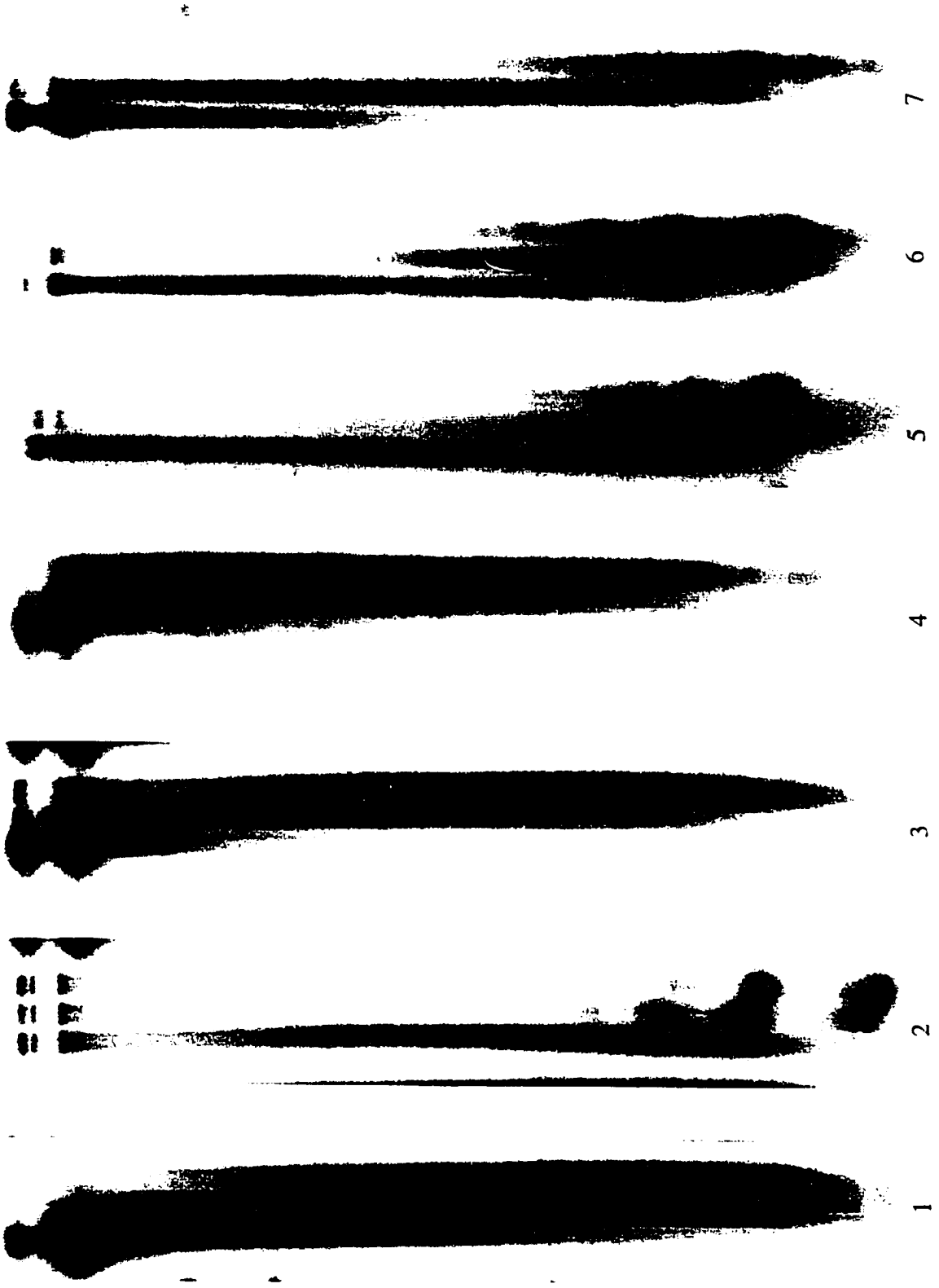

Figure 3.5A.

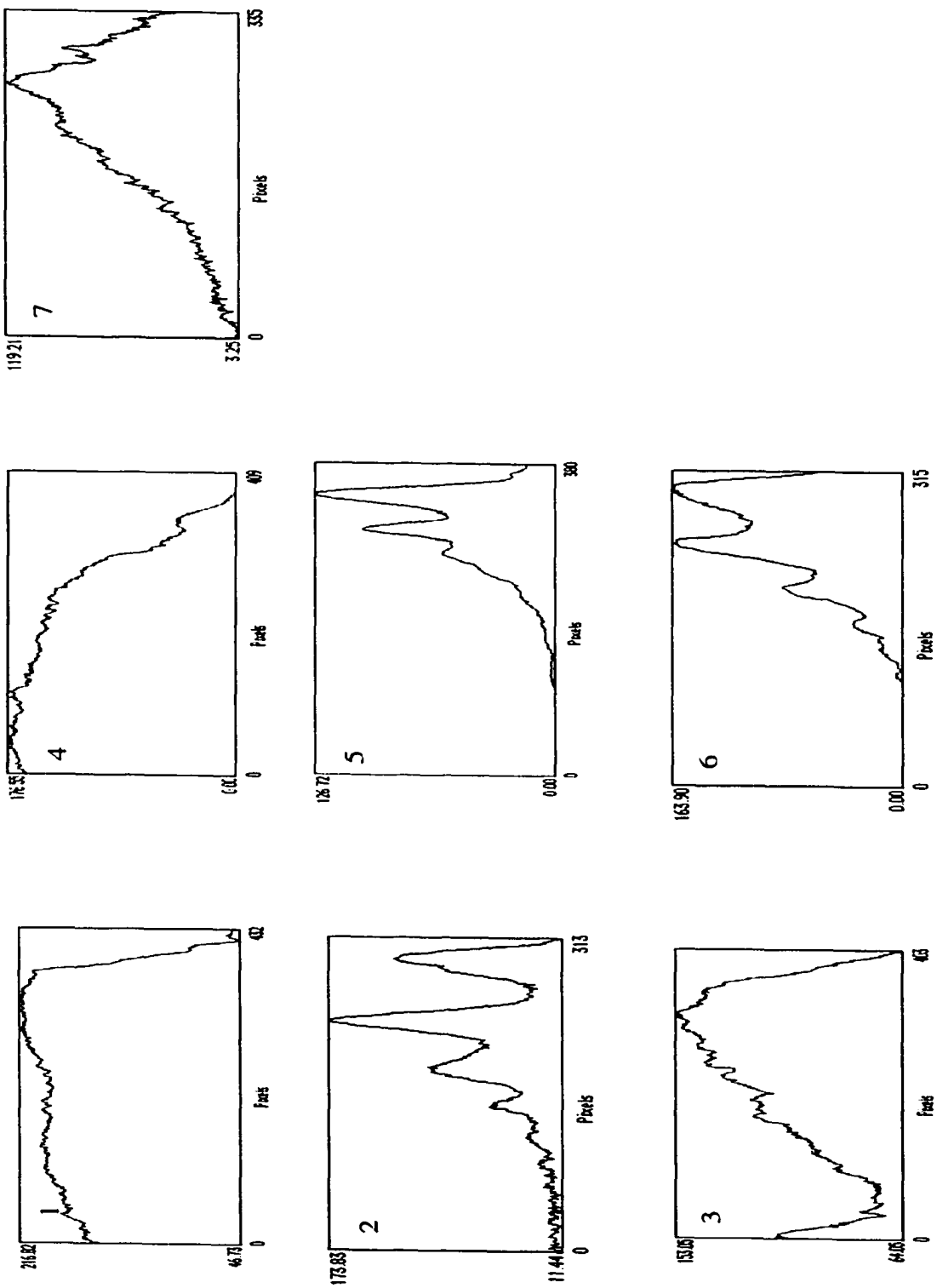

Figure 3.5B.

Supplementary Figures for Chapter 3

The following two figures were addressed in Chapter 3 as "data not shown."

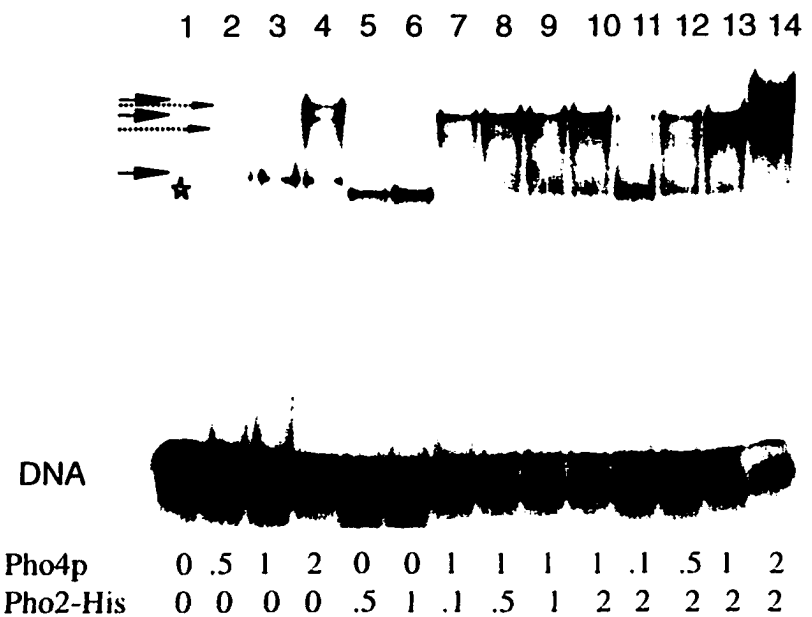

Figure 3.6. Pho4p and Pho2p form a ternary complex on UASp1 of the *PHO5* promoter. Short arrows are Pho4p/DNA complexes, star is Pho2-His/DNA complex, and long arrows are Pho4p/Pho2-His/DNA complexes. All lanes contain 0.4 ng probe and 100 ng poly dIdC as competitor. Amount of protein in nanograms is indicated below the figure.

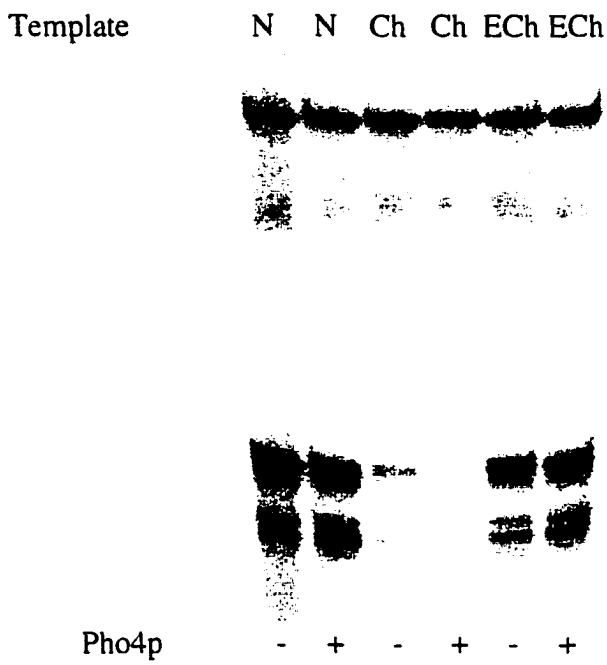

Figure 3.7. *In vitro* G-less cassette transcription. Following proteinase K digestion and extraction of chromatin templates, transcription can be recovered. Pho4p appears to be inactive, however the purpose of this experiment was to prove that templates were not being made transcriptionally incompetent by formation of nucleosomes.
N - naked DNA, Ch - reconstituted chromatin, ECh - reconstituted chromatin which was then extracted with 12.5µg proteinase K then extraction with phenol/chloroform/isoamyl alcohol. 500 ng of template was used and indicated reactions contained 500 ng Pho4p. YS25 whole cell extract (*pho4*) was used for transcription.

Chapter 4

Intrinsic DNA Properties Determine Nucleosome Positioning on the *PHO5* Promoter

This chapter has been submitted to *Journal of Molecular Biology*. The following text is identical to that which has been submitted. I have re-formatted the Bibliography and changed figure numbers to maintain consistency with the rest of the dissertation and to meet University guidelines. John Pilon and I are co-first authors on this manuscript. The experiments that I contributed are shown in Figures 4.1, 4.2, and 4.5. I also assisted in preparation of figures, writing Materials and Methods for the experiments I contributed, and editing of the manuscript.

Intrinsic DNA Properties Determine Nucleosome Positioning on the *PHO5* Promoter

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Running title: *Mechanism of PHO5 nucleosome positioning*

Key Words: *PHO5* promoter; nucleosome positioning; *in vitro* transcription; yeast core histones; chromatin templates

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4.1 Summary

To investigate the positioning mechanism of the nucleosomal array on the *PHO5* promoter, we reconstituted nucleosomes from purified yeast core histones on plasmid DNA. Indirect end-labeling and footprinting analysis show that intrinsic DNA properties are sufficient for translational positioning of nucleosomes –1 and –2 and for maintaining a nucleosome free region between nucleosomes –2 and –3, which approximates that seen *in vivo*. We predicted nucleosome translational positions that closely agree with those determined experimentally using an algorithm based on DNA flexibility (40), indicating that the nucleosome translational positions are largely determined by the free energy of DNA bending. These results indicate that the nucleosomal array is positioned predominantly through the energetics of sequence-dependent intrinsic properties of the promoter DNA.

4.2 Introduction

In eukaryotes the genomic DNA is packaged into chromatin, which compacts and organizes the DNA (45). The basic unit of chromatin is the nucleosome, the X-ray structure of which has been solved to 2.8 Å resolution (25). The majority of nucleosomes are randomly placed throughout the genome. However, some nucleosomes adopt specific positions on the DNA (for a review see (49)). Positioned nucleosomes occur on 20 promoters and play an important role in transcriptional regulation (4). A nucleosome can adopt a *rotational* position, fixing the orientation of the major and minor groove at a given DNA sequence either toward the histone octamer or toward the solvent. In addition, a

nucleosome can adopt a *translational* position when it incorporates a specific DNA sequence.

Nucleosomes may be positioned through three mechanisms, intrinsic DNA properties, DNA-binding proteins, and silencing. *Anisotropic flexibility*, or the tendency to bend in a single direction, when periodically repeated results in DNA curvature and is associated with rotational positioning (36). Alternatively, DNA *isotropic flexibility*, or ability to bend in all directions, has been correlated with translational positioning (40). In the second mechanism, sequence-specific DNA-binding proteins can form a boundary or backstop, constraining the position of adjacent nucleosomes (50). Finally, silencing involves repressor proteins such as Ssn6p and Tup1p (or Cyc8p) that interact with sequence-specific DNA-binding factors and histone H4 to specifically place a nucleosome (33). An array of positioned nucleosomes might be defined through one or more of these mechanisms within a given DNA sequence.

Recent SELEX experiments with eukaryotic genomic DNA and with synthetic, random DNA have further refined the rules for nucleosome positioning by intrinsic DNA properties (24, 48). These rules describe DNA regions that more readily accommodate the deformations that result from nucleosome formation. In addition, DNA sequences that are less compatible with nucleosome formation, for example long runs of adenine nucleotides, could define an internucleosomal linker (9, 30, 35).

We have chosen the yeast *PHO5* promoter as a model to investigate the mechanisms of transcriptional regulation in the context of chromatin. This choice was based on the detailed understanding of the changes in chromatin structure that occur on the *PHO5* promoter between the repressed and the activated states (42). The *PHO5* gene, which encodes a secreted acid phosphatase, is activated in the absence of phosphate (3). Upon activation, four

nucleosomes are displaced or reconfigured to allow assembly of the transcriptional machinery (2). In the repressed state, the nucleosomes are specifically placed to create an enlarged linker region between nucleosome -2 and nucleosome -3 (2). Located within this region is UASp1 (upstream activating sequence), a binding site for the transcriptional activator Pho4p. Furthermore, in the repressed state nucleosome -1 is located over the core promoter (TATA-box and RNA start sites). This translational positioning implies that correct nucleosome placement on the *PHO5* promoter is required for proper regulation of *PHO5*. Determining the mechanistic details of nucleosome positioning on the *PHO5* gene will greatly enhance the understanding of transcriptional regulation in the context of chromatin.

Here we describe the reconstitution of chromatin templates possessing many important aspects of the repressed *PHO5* promoter. Reconstitution of nucleosomes to physiological nucleosomal density on the *PHO5* promoter strongly repressed transcription. Base-pair resolution analysis of the chromatin reconstituted with purified yeast core histones indicates that nucleosomes –1 and -2 are positioned very similarly to the *in vivo* nucleosome positions. In addition, an enlarged linker region is formed between nucleosomes –2 and –3. However, nucleosome –3 is positioned farther downstream on the reconstituted templates than *in vivo*. From these 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*. Since positioning occurs in the absence of a sequence-specific DNA-binding protein, we conclude that nucleosome positioning is not determined by a DNA-binding protein or through silencing. Using an algorithm based on the Gibbs free energy of DNA bending (40), we have determined the calculated nucleosome positioning across the *PHO5* promoter predicted by the sequence-dependent DNA flexibility. The

experimentally determined and predicted positions of nucleosomes –1 and -2 agree to within +/- 11 bp and 30 bp, respectively. This close agreement indicates that the isotropic elastic properties of the DNA are a dominant contributor to the translational position of nucleosomes on the *PHO5* promoter.

4.3 Results

4.3a 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 or pPHO5G-less in other chapters of this dissertation) and with 2.5 kb of the promoter and upstream sequences fused to a G-less cassette (pMH313-Gless or pMHG-less in other chapters of this dissertation). Nucleosomes were formed at the ratios (w/w) of 0, 0.125, 0.25, 0.5, 1.0 and 1.5 core histones to DNA. At the histone to DNA ratio of 1.0, nucleosomes were formed at physiological density as determined by topological assay of reconstituted pPHO5-Gless (Figure 4.1A) (29). The topological assay could not be done for the pMH313-Gless template due to the inability to resolve topoisomers of a such a large plasmid (14 kb). However, reconstitution of both plasmids was done side-by-side using the same core histone-Nap1p complex and reagents. Micrococcal nuclease analysis verified that complete nucleosomes were formed with uniform spacing (Figure 4.1B). The nuclease ladder indicates a nucleosome spacing of approximately 160 bp that is very uniform for a simple reconstitution system on non-repeat DNA. It should be noted that the smaller pPHO5-Gless plasmid produced a good mononucleosome and dinucleosome stop (29), but would not produce the six or

seven step ladders routinely seen with the much larger pMH313-Gless plasmid. Transcription from pMH313-Gless was repressed greater than 5-fold at a core histone to DNA ratio of 1.0 (Figure 4.1C). An identical level of repression was seen with the same degree of reconstitution of pPHO5-Gless. However, a 2-fold greater level of repression was observed on pMH313-Gless relative to pPHO5-Gless at core histone:DNA ratios of 0.25 and 0.5.

4.3b Indirect End-labeling Analysis of Nucleosome Positioning on the *PHO5* Promoter.

The level of transcriptional repression is greater than one would expect from random nucleosome placement (22). Thus, the *in vitro* transcription data suggested that some degree of nucleosome translational positioning was occurring on our reconstituted templates. To determine if positioning did occur, the micrococcal nuclease digestion pattern on the reconstituted pMH313-Gless template was analyzed at low resolution using indirect end-labeling to allow a direct comparison with the digestion pattern produced on the genomic *PHO5* gene in yeast nuclei (Figure 4.2A). Although less striking, the protection pattern seen *in vitro* is very similar to that seen *in vivo* with one notable exception. The strong cleavage sight located just downstream of the *Aat* II site is protected on our reconstituted templates but is accessible *in vivo*. This suggests that nucleosome –3 is positioned somewhat closer to nucleosome –2 than it is *in vivo*. A comparison of the cleavage pattern on the free DNA and on the *in vivo* assembled promoter clearly shows that much of the pattern is a result of the sequence preferences of the nuclease (Figure 4.2B). Cleavage is intrinsically weak where nucleosomes have been shown to position *in vivo* and intrinsically stronger in the linkers. In addition, much less DNA from the reconstituted templates than from the yeast genomic DNA must be run on the agarose gel to

obtain a reasonable signal on the Southern blot. As a result, the resolution for the reconstituted samples is much higher producing two and in some cases three bands where only one is seen in the *in vivo* samples. The similarity of the cleavage patterns between free DNA and chromatin is even more apparent when the results are displayed as aligned plots (Figure 4.2B). This high degree of simililarity was seen previously in higher-resolution indirect end-labeling of *in vivo* chromatin (Figure 3 (2)). The micrococcal nuclease cleavage pattern on the reconstituted *PHO5* promoter is consistent with nucleosomes –1 and –2 positioning quite similar to that seen *in vivo*. In addition, nucleosome –3 appears to be positioned, but somewhat further downstream *in vitro* than *in vivo*.

4.3c Primer Extension Footprinting Analysis of Nucleosome Positioning on the *PHO5* Promoter.

We reasoned that higher resolution analysis of the nuclease protection pattern might provide a clearer picture of the nucleosome positioning on our reconstituted templates. We used micrococcal nuclease digestion in conjunction with multi-round primer extension analysis (17, 34) to footprint the nucleosomes on the *PHO5* promoter. The primers used for this analysis are shown in Figure 4.3A.

Nucleosome –1. In the footprint analysis we observed cleavage protection over the TATA-box and RNA start sites (Figure 4.3B and 4.3C). The protection occurs between approximately +7 and -123 bp, relative to the ATG (translational start codon) and corresponds to nucleosome -1. Protection is defined as decreased band intensity in the chromatin lane (C) versus the free DNA lane (D). At the downstream end of nucleosome –1 strong protection ends at +7, with slight protection seen at +10 (Figure 4.3B). No cleavage protection or enhancement is seen from +19 to +55. However, there is some ambiguity in

where the protection ends due to the lack of cleavage between +10 and +19 on both the free DNA and chromatin samples. The upstream edge of nucleosome –1 was inferred from the protection at –123 (Figure 4.3C). From –129 through –156 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 the free DNA and the chromatin precludes defining the downstream edge of nucleosome –1 with greater precision. Thus, we place nucleosome –1 at +7 (± 5) bp to –123 (± 9) bp, with the dyad at approximately -58.

The clear footprint observed with primer FS and primer mn5 indicates a strong translational setting for nucleosome -1. In addition, the 130 bp length of DNA associated with nucleosome -1 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, 36 bp and 29 bp from the edge, respectively. Note the cleavage protection over 5 nt of the TATA element, indicating a lack of accessibility for TBP binding. Therefore, nucleosome –1 is well positioned on the *PHO5* promoter, as indicated by the protection from micrococcal cleavage.

A comparison of the nuclease cleavage patterns on the chromatin and free DNA templates in the region from +19 to +55 and -163 to -129 indicates a complete lack of protection. This pattern is consistent with these regions comprising a stretch of linker DNA 35 bp long between nucleosomes -1 and +1 and 36 bp long between nucleosomes -1 and -2.

Nucleosome –2. Protection from micrococcal nuclease cleavage is seen from -150 through -311, corresponding to nucleosome -2 (Figures 4.3C and 4.3D). The nuclease protection is nearly complete (Figure 4.3C, 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 4.3C).

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 4.3D). This protection covers a 161 bp region of the *PHO5* promoter containing 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 from –370 to -436 indicates that nucleosome -3 is positioned upstream of UASp1. In addition, we detect an enlarged linker region between -366 and –317, 49 bp in length. This linker is much larger that the 35 bp length of the other linkers and corresponds to the situation *in vivo* (2). Mapping the minimum length of the enlarged linker is based upon the unprotected cleavage sites from –365 to –316 (Figure 4.3D). A previous investigation mapped this enlarged linker (referred to as HS2) to a region from approximately -410 to -340 *in vivo* by standard indirect end-labeling and restriction endonuclease cleavage analysis (2). However, a closer inspection of the higher resolution indirect end-labeling analysis in this study indicates a downstream edge at approximately –315, consistent with our reconstituted chromatin (2). Importantly, an enlarged linker is formed that leaves the Pho4p binding site in UASp1 accessible. Nevertheless, our micrococcal nuclease protection indicates that nucleosome –3 is located approximately 40 bp farther downstream on the reconstituted templates than seen *in vivo*. This explains the cleavage protection downstream of the *Aat* II site seen with indirect end-labeling analysis (Figure 4.2).

In summary, nucleosomes -1 and -2 are positioned and an enlarged linker region between nucleosomes -2 and -3 is established on our chromatin templates reconstituted with purified yeast core histones using Nap1p. Thus, DNA-histone

interactions are sufficient for the translational positioning of nucleosomes -1 and –2 on the *PHO5* promoter. In addition, these interactions appear to be sufficient for formation of an enlarged linker containing UASp1.

4.3d UASp1 Is Accessible for Pho4p Binding on the Reconstituted *PHO5* Promoter.

The accessibility of UASp1 to Pho4p binding in the enlarged linker is thought to be constitutive, providing the "bridge head" for chromatin remodeling by Pho4p on the *PHO5* promoter (10, 11, 43, 47). Therefore, it was important to demonstrate the accessibility of this site on our reconstituted templates. For this we used DNAase I cleavage and primer extension analysis (Figure 4.4). A very clear "footprint" for Pho4p over UASp1 is seen in the nuclease cleavage pattern on the free DNA (DNA). Clear protection is seen on the reconstituted promoter (Chrom), as well. Therefore, UASp1 is accessible to Pho4p for binding on the reconstituted promoter, consistent with the formation of an enlarged linker through nucleosome positioning.

4.3e Thermodynamic Analysis of Nucleosome Formation on the *PHO5* Promoter

Although nearly any DNA sequence can be incorporated into a nucleosome certain sequences may be preferred (16, 20, 21, 31). "Rules" for DNA sequences that energetically favor nucleosome formation have been developed based on analyses of the DNA sequences found in natural core particles and of the sequence of naturally occurring nucleosome positioning DNA sequences (9, 23, 27, 35, 36, 38, 48). Algorithms have been developed that predict rotational positioning (7, 8). However, these algorithms are not designed to predict translational positioning.

To understand how histone-DNA interactions are sufficient to set translational nucleosome positions, we have focused on a model that predicts translational positioning based on the free energy of DNA bending. This model, developed by Sivolob and Khrapunov (40), uses nearest neighbor interactions between base pairs and the experimentally determined values of persistence length for dinucleotide steps. Summing these effects over 145 bp, the free energy of bending can be calculated for a DNA fragment of the length associated with a core particle. The algorithm treats the DNA as an isotropically flexible rod with sequence-dependent differences in flexibility along its length. Each dinucleotide step has an experimentally determined persistence length, with GG/CC being the "stiffest" and AT or TA steps being the most flexible. The algorithm scans through the DNA sequence in 145 bp blocks and calculates the free energy of bending for that block. The results are plotted as the center of the 145 bp fragment, corresponding to the nucleosome dyad. As shown in Figure 4.5A, our implementation of the algorithm successfully predicts the locations of the dyads for the nucleosome positions on the *Lytechinus variegatus* 5S rRNA gene mapped using site-directed cleavage (12, 40).

We have analyzed the free energy of bending of the *PHO5* promoter coming from both the upstream and downstream ends of a 972 base pair sequence from pMH313 (Figure 4.5B). Our primer extension footprinting mapped the nucleosomal dyads at positions –58, -231, and –438 ± 10 bp. Inspection of the DNA bending free energy profile in Figure 5B identifies free energy minima at positions –67, -250, and –437. These minima correspond well with our footprinting results.

Minima also occur from –360 to -333, which at first approximation highly favor formation of a nucleosome. On its own, a particular position may favor formation of a single nucleosome. However, in the context of an array of

nucleosomes, nucleosome positioning at a particular site may require that other nucleosomes form on positions with higher bending energies.

To predict the lowest overall free energy positions of an array of four nucleosomes on the *PHO5* promoter, we used Monte Carlo analysis (26). This analysis placed the nucleosomal dyads of nucleosomes –1, -2, and -3 at positions –67 (± 33), -268 (± 29) and –440 (± 23), respectively. This placement agrees with the experimental data and, importantly, excludes nucleosome placement in the free energy minima from –366 to -338. This result supports the hypothesis that these latter positions are not favored for nucleosome formation because the overall free energy of the nucleosomal array would be more positive. Overall, the close parity between the predicted and experimentally determined nucleosome positions indicates that the translational nucleosome positioning on the *PHO5* promoter is determined primarily by sequence-dependent isotropic DNA bending.

4.3f The Affinity of DNA Fragments Encompassing Nucleosomes –1 and –2 for Nucleosome Formation Is Much Greater Than for the Linker DNA Between Them.

Competition mobility shift assays under non-denaturing conditions were performed using three similar-sized fragments (approximately 200 bp) of the *PHO5* promoter corresponding to the DNA sequence incorporated into nucleosome –1 and nucleosome –2, and a DNA fragment centered on the linker DNA between these two nucleosomes (Figure 4.6). This latter fragment is predicted to be unfavorable to nucleosome formation by a peak in the bending free energy plot. The results of this assay indicate that nucleosomes have a similar propensity to form on the nucleosome –1 and –2 fragments, but have a much lower affinity for linker DNA between them. This finding is consistent

with this DNA sequence forming a boundary for the downstream edge of nucleosome –2 and the upstream edge of nucleosome –1. In addition, the results indicate that nucleosomes –1 and -2 form with a similar affinity to that of the *Lytechinus variagatus* 5S rRNA gene.

4.4 Discussion

The Hörz and Bergman laboratories independently identified an array of at least four translationally positioned nucleosomes on the repressed *PHO5* promoter (2, 6). These nucleosomes become nuclease transparent on the activated gene (2, 5, 10). Transcriptional regulation and nucleosome positioning were equivalent on a plasmid construct containing only the *Bam* HI to *Dra* I (-542 to +6) fragment of the *PHO5* promoter and on the genomic copy of the gene (41). Thus, this 548 bp fragment defines the minimal sequence requirements for correct regulation and nucleosome positioning. In addition, occupancy of UASp1 by transcription factor Pho4p is not required for nucleosome positioning (10, 47).

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* (28, 32). However, the strong repression of transcription from the *PHO5* promoter on pMH313-Gless at sub-saturating densities of nucleosomes suggests that the *PHO5* promoter, in this context, has a higher affinity for nucleosome formation, when compared with the

smaller pPHO5-Gless plasmid. The lower levels of repression seen at the sub-saturation nucleosome densities on pPHO5-Gless may result from less distinct positioning as indicated by poorly defined micrococcal nuclease digestion ladders and the inability to detect any level of nucleosome positioning by primer extension footprinting analysis (J. Pilon unpublished results). The difference in nucleosomal repression between pMH313-Gless and pPHO5-Gless indicates an important role for nucleosome positioning in *PHO5* repression.

We observed high levels of transcriptional repression, which correlated with increasing nucleosome density. The 5S rRNA gene is known to translationally position a nucleosome and to be strongly repressed by that nucleosome (44). Therefore, we hypothesized that nucleosomes are preferentially formed over the core promoter elements on our reconstituted *PHO5* templates, resulting in the strong transcriptional repression. To test this hypothesis, we determined the micrococcal nuclease cleavage pattern on chromatin and free DNA templates at low-resolution (for comparison with the *in vivo* pattern) and at high-resolution to better 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 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. The reconstituted chromatin templates were formed using purified yeast core histones and Nap1p. Hence, no other DNA-binding proteins are present and the nucleosome positioning on our reconstituted templates is directed primarily by sequence-dependent intrinsic flexibility of the

DNA. From the results of a series of deletion experiments Fascher et al. (1993) 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 nucleosome correctly *in vitro* (19). However, the *PHO5* promoter and the MMTV LTR promoter are the only single copy gene promoters determined to contain an array of nucleosomes that are translationally positioned primarily through histone-DNA interactions (46).

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* (2). *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 (2) suggests boundaries of –393 to –313 (80 bp). The enlarged linker on our reconstituted templates is formed from –366 to –317 (49 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 30 to 40 bp downstream *in vitro*. This is consistent with what is seen on partially purifed minichromosomes, suggesting that a positioning factor is being lost during preparation (Haswell and O'Shea, 1999).

Pho4p does not bind UASp1 under repressing conditions (47). In addition, the *PHO5* chromatin structure, as analyzed by low-resolution indirect end-labeling in *pho2* and in *pho4* cells, is identical to that of wild type cells (10). Finally, deletion of both the Pho2p and Pho4p binding sites at UASp1 does not disrupt the *PHO5* promoter chromatin structure (11). However, a 40 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 an unidentified protein can not be excluded at this time.

We have determined that sequence-dependent intrinsic properties of the DNA are primarily responsible for setting up the nucleosomal array on the *PHO5* promoter. This finding demonstrates that these interactions can have an integral role in creating the chromatin structure necessary for transcriptional repression and activation. In addition, our system provides an important tool for studying the intricacies of gene expression in the context of chromatin. To begin to determine the mechanism of nucleosome positioning, we have focused on the thermodynamics of nucleosome formation using an algorithm developed by Sivolob and Khrapunov (40). This algorithm treats the DNA as an isotropically

flexible rod with sequence-dependent differences in flexibility. According to the model, these free energy minima can predict the dyad of a translationally positioned nucleosome (Figure 4.5). Monte Carlo analysis was used to determine the free energy minima that provide the lowest overall free energy for an array of four nucleosomes. The results from this analysis are summarized by the arrowheads in Figure 4.7 (Calculated). The predicted dyads agree with the experimentally determined positions to a resolution of +/- 9 bp for nucleosome –1 and +/- 17 bp for nucleosome -2.

Our results suggest that nucleosomes –1, -2, and -3 are positioned by DNA isotropic flexibility alone. The differences between the free energy of bending of the valleys (sites nucleosome of positioning) and the peaks (sites where nucleosomes are not positioned) for all three nucleosomes is –5 to –10 kcal/mol, which is quite significant.

The situation for positioning of nucleosome -2 appears to be more complex than for nucleosomes –1 and -3, since there are free energy minima at positions –366 and -338 (Figure 5B). These positions may not be preferentially occupied for two reasons. First, Monte Carlo analysis of an array of nucleosomes on this DNA sequence predicted the use of the free energy minima at –440, -268, and –67, but not these other minima. The free energy of bending for the nucleosome -3 region suggests that formation of a nucleosome here is quite favorable. On dinucleosome size fragments from the MMTV-LTR promoter encompassing the nucleosomes B and A, histone octamers form nucleosome A first, setting the translational positioning for both (13). Therefore, the prior

formation of nucleosome -3 may be sufficient for precluding the formation of nucleosome -2 at –366 or -338.

A second reason that the Sivolob and Khrapunov model incorrectly predicts that positions –366 through -338 are preferentially occupied is that the model does not account for some sequence elements thought to produce kinks in DNA. Such kinks can arise from runs of A residues greater than 4 to 5 bp in length (14). Poly A sequences have a narrowed minor groove and are inflexible (1). Due to these properties, long poly A stretches disfavor nucleosome formation (30). A tract of seven A residues followed by two tracks of four As is located at the upstream edge of nucleosome -2 (-328 bp). These poly A tracts may function to maintain the nucleosome free region by keeping nucleosome -2 positioned farther downstream. Nucleosome A of the MMTV-LTR promoter was shown to be translationally positioned by $d(T)_6$ tracts (13). In addition, poly A elements have been shown to function in yeast gene regulation through effects on chromatin structure (18). Therefore, nucleosome positioning may, in part, be due to the poly A tract at the downstream edge of the enlarged linker region.

Nucleosome -2 is predicted by the calculated isotropic flexibility to be the least stable, perhaps aiding in its displacement upon activation. The free energy of bending required for the formation of nucleosome -2 is approximately 5 to 7 *kT* (approximately 2.8 to 3.9 kcal per mole) greater than that required for nucleosomes -1 and -3. While this difference is not nearly as large as between all three sites and the least flexible regions (see above), it is still significant. The conclusion that nucleosome –2 is less stable than nucleosomes –1 and -3 is

supported by the experiments of Straka and Hörz (41). The replacement of the DNA underlying nucleosome -2 with a sequence that strongly positions nucleosomes resulted in a large loss of activation. Replacing this sequence with random pBR322 DNA, unable to position a nucleosome, resulted in weakly constitutive activity. These results indicate the importance of intrinsic DNA properties affecting the propensity of nucleosomes to form in mediating gene activity.

While the bulk of nucleosomes are randomly placed throughout the genome, on many promoters nucleosomes are positioned. This nucleosomal positioning can allow for both the repression as well as the activation of expression of a given gene (49). The *PHO5* gene employs nucleosome positioning in both capacities by placing the TATA box and start sites within nucleosome –1 and by leaving the Pho4p binding site (UASp1) constitutively accessible between nucleosomes -2 and -3. The ability to reconstitute translationally positioned nucleosomes on the *PHO5* promoter provides an assay to determine the effect of DNA sequence mutations on nucleosome positioning.

We conclude that sequence-dependent intrinsic DNA flexibility is sufficient for much of the translational placement of the nucleosome array on the *PHO5* promoter. Moreover, this translational positioning can be defined by the isotropic flexibility of the DNA. Finally, our results clearly emphasize the a major role that sequence-dependent DNA structural properties play in the organization of chromatin structure, and therefore in transcriptional regulation.

4.5 Materials and Methods

4.5a *Plasmids.* The pPHO5-G-less plasmid was produced by subcloning the 526 bp *Bam* HI to *Apo* I fragment (-542 to -16) of pMH313 (15) 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.

4.5b *Nucleosomal template reconstitution.* Yeast core histones were purified as previously described (29). Purified yeast core histones and recombinant Nap1p were combined at a ratio of 1:2 (w/w). Acetylated bovine serum albumen (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. The core histones and Nap1p were incubated at 37°C for 15 min. The appropriate amount of core histones and Nap1p were then 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.

4.5c *One dimensional topological analysis.* To analyze the degree of reconstitution 5 µg DNA (pPHO5G-less) 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 volume. Following initial relaxation 10 U more Topoisomerase I was added. Reconstitution reactions contained 500 ng relaxed DNA, 3 mM $MgCl_2$ and octamer:Nap1p complex in reconstitution buffer in a reaction volume of 19 μl. Reconstitution was allowed to proceed for 2 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, then incubated at 37°C for 20 min. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by ethanol precipitation. The DNA was split in half and run on 1% TBE-agarose gels +/- 1.8 μg/ml chloroquine then stained with ethidium bromide.

4.5d *Micrococcal nuclease digestion of reconstituted templates.* Two micrograms DNA, either naked or reconstituted (CH:DNA ratio of 1:1) chromatin, 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. Digestion was stopped by the addition of 100 μl stop (described in topological analysis) plus 12.5 μg proteinase K and incubated at 37°C for 30 min. DNA was extracted, ethanol precipitated, and resuspended in H_2O. Samples were then resolved on a 10 cm 1 % agarose gel run in TBE buffer until the bromophenol blue dye had run to the bottom. The gel was then stained with ethidium bromide, destained, and the image digitally scanned.

4.5e *In vitro transcription with reconstituted templates.* 500 ng pMH313G-less (14,000 bp) or pPHO5G-less (3300 bp) were reconstituted to ratios of 0:1, 0.25:1, 0.5:1, 1:1, and 1.5:1 (w/w histone octamer:DNA) as determined by 1-D topological analysis (described above). Transcriptions reactions contained naked or reconstituted DNA (500 ng), 100 µg YS25 whole cell extract (51), 1% PEG 3350, 50 mM HEPES-KOH pH7.6, 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 α^{32}P-UTP in a 30 µL reaction volume. The reaction proceeded at 22 °C. After 30 min, 120 µL RNase T1 buffer (10 mM Tris-Cl pH 7.5, 300 mM NaCl, 5 mM EDTA) and 50 U RNase T1 (Boehringer Mannheim) were added and incubated at 22 °C, 15min. Then 8 µL 10% SDS, 17.5 µL 2.5 mg/mL Proteinase K (Sigma), and γ^{32}P-ATP end-labeled recovery standard (300 bp *Pvu* II fragment from pUC19) were added and incubated at 37°C for 20 min. Following addition of 15 µg glycogen the reaction was ethanol precipitated. Transcripts were pelleted, washed with 75% ethanol, resuspended in TBE-Formamide dye, and electrophoresed on a 8% polyacrylamide urea sequencing gel at 20V/cm until the bromophenol blue ran through the gel. The gel was dried and exposed on a PhosphorImager screen. Transcript levels were quantitated using NIH Image (http://rsb.info.nih.gov/nih-image/).

4.5f *Indirect Endlabeling.* For the preparation of genomic DNA one liter YS18 cells (kind gift of B. Meyhack, Ciba-Geigy Ltd., Basel) was grown to 2 X 10^7 cells/ml and harvested at 7000 rpm for 10 min at 4°C. Cells were resuspended in 200 ml

resuspension buffer (50 mM Tris-HCl pH 7.5, 30 mM DTT) and incubated at 30°C for 15 min then pelleted. Cells were resuspended in 10 ml endoglucanase buffer (1 X YP media, 1 M sorbitol, 5 mM DTT) and spheroplasted using recombinant β-glucanase (37). Spheroplasts were pelleted then resuspended in 200 ml 1X YPD plus 1 M sorbitol and incubated at 30°C for 10 min. Spheroplasts were washed twice in cold 1 M sorbitol then resuspended in 20 ml lysis buffer (18% Ficoll 400, 20 mM KH_2PO_4, 0.25 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, pH to 6.8, then add 3 mM DTT, 2 mM benzamidine, 2 mM $Na_2S_2O_5$, 1 mM PMSF, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 X 10^{-4}% chymostatin). Cells were lysed with 40 strokes in an all glass Dounce homogenizer using the loose pestle then 20 strokes using the tight pestle. Nuclei were pelleted at 15000 rpm for 30 min at 4°C in a Sorvall SS-34 rotor. Nuclei were resuspended in MNase digestion buffer (10 mM Tris-HCl pH 7.9, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 5 mM $CaCl_2$, and 1 mM PMSF). Aliquots of 400 μl were digested with 0.125, 0.25, 0.5, and 1 U MNase at 37°C for 10 min. Digestions were stopped by the addition of 100 μl stop (0.1 M EDTA, 5% SDS, and 25 μg/ml proteinase K) and incubated at 37°C for 30 min. DNA was extracted with phenol, phenol:chloroform:isoamyl alcohol, then chloroform:isoamyl alcohol and precipitated with ethanol. DNA was resuspended in 200 μl TE and 10 μg RNaseA was added and incubated at 37°C for 30 min, followed by extraction as before (excluding phenol alone) and two ethanol precipitations. The DNA was resuspended in 20 μl H_2O. For the preparation of *in vitro* samples see *Micrococcal nuclease digestion of reconstituted templates* above. For indirect end-labeling all samples were digested with *Apa* I and run on a 1.65% TBE-agarose gel with

markers prepared from pMHG-less. DNA was Southern blotted using a Turboblotter (Schleicher & Schuell) and probed with a 239 bp ApaI to SspI probe 5′ of the *PHO5* promoter. The blot was visualized using the PhosphorImager system.

4.5g *Micrococcal nuclease digestion of reconstituted chromatin and multi-round primer extension.* Reconstituted pMH313 chromatin templates (3 µg DNA) were digested at 37°C in 200 µl of 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. Naked DNA (3µg) was incubated in 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 removed into 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 as before. The DNA was resuspended in H_20 and the concentration determined by A_{260}. Seventy-five nmoles of ^{32}P end-labeled primers were added to 250 ng of digested samples or template alone. Multiple-round primer extension reactions were carried out in 50µl reaction volumes containing 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 10 mM Hepes-KOH pH 8.4, 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.

4.5h *Primer extension footprinting of Pho4p.* 500 ng naked or reconstituted chromatin DNA (pMH313-Gless), 100 ng poly dIdC, purified Pho4p (amount indicated in figure 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 (0.05 U for naked DNA, 0.4 U for chromatin) and DNA was digested at 30°C for 2 min. The reaction was stopped as described previously (1-D topological analysis) except the volume was brought to 500 µl prior to extraction. To verify reconstitution of chromatin templates 2 µg DNA (same template) were digested with 0.125, 0.25, and 0.5 U MNase (Worthington) at 30°C for 4 min in reconstitution buffer plus 5 mM $CaCl_2$. Samples were treated as described for DNaseI digestions. MNase digested DNA (1.8 µg) was run on a 1.2% TBE-agarose gel to verify a ~150 bp ladder. Primer extension reactions (50 µl reaction volume) contained 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'ATTTGGCATGTGCGATCTCTT3'). Twenty cycles of primer extension were done and the reaction was extracted with chloroform:isoamyl alcohol followed by the addition of 17 µl PCR quench (4 M NH_4OAc and 20 mM EDTA) and precipitation with ethanol. Products were resuspended in TBE-formamide dye and electrophoresed on a 5% polyacrylamide-urea gel (Long Ranger) next to sequence generated using the same primer and pPHO5G-less template, then dried and visualized using the PhosphorImager system.

4.5i *Prediction of nucleosome position.* Our program was written in C++ for the Macintosh PowerPC and is based on the calculations of Sivolob and Khrapunov (40). The Gibbs free energy of bending, G_n, was determined using:

$$G_n = (kTh/2R^2)\sum_{i=1}^{N} p_i .$$

R is the curvature radius equal to 4.3 nm, h is the distance between neighboring base-pairs equal to 0.34 nm, and p is the persistence length value for a given base-pair step at the ith position using set 1 parameters from Table 1 of Sivolob and Khrapunov (40). At each base-pair position, p is summed over the next 145 bp or base-pair steps of N= 144. G_n is presented in values of kT, where T is the absolute temperature, and k is Boltzmann's constant.

4.5j *Monte Carlo analysis.* To determine the equilibrium positions of nucleosomes on the *PHO5* promoter we used Metropolis Monte Carlo analysis (26). Four nucleosomes were randomly placed on non-overlapping sites corresponding to the G_n values. Then all the nucleosome positions were randomly changed and the difference between the new and old bending energies, ΔG, was calculated. If $\Delta G < 0$, then the new positions are accepted. If $\Delta G > 0$, then the new positions are not accepted unless the Monte Carlo condition that a random number generated between 0 and 1 is less than $\exp(-\Delta G/kT)$. If the new position is not accepted the old position is retained and counted. The routine was repeated for 2000 trials and then the mean position and the standard deviation were calculated for each nucleosome. Repeating the routine for a larger number of trials, for example 20,000 trials, made no significant difference in the standard deviations.

4.5k *Competitive mononucleosome reconstitution.* Probes were generated by cutting pPHO5 with *Mfe* I and *Hae* II (nucleosome –2), *Apo* I and *BspH* I (-2 to –1 linker), and *Hind* III and *BstE* II (nucleosome –1). The 5S rRNA probe was produced by cutting a single copy of the 207 bp *Lytechinus variegatus* 5S rRNA gene (Simpson et al. 1985) out of pUC19 with *Eco* RI and *Hind* III. The DNA fragments were purified by electrophoresis on an agarose gel. Fifty ng of DNA fragment was labeled with α^{32}P-dATP by end-filling. Three ng of labeled fragment was mixed with 1 ml of a 1 to 4 dilution of the Nap1p/core histone complex in the presence of 100, 200, and 400 ng competitor DNA (sheared yeast genomic DNA) and incubated at 30 °C for 45 min. Five µL 10% glycerol was added to each sample prior to running on a 5% polyacrylamide gel (1/3X TBE) and visualized by exposure to a PhosphorImager screen.

4.6 Acknowledgments

We are grateful to David Moss for helpful suggestions and advice during the course of this work, and to Norm Curthoys, Karolin Luger, Kevin Lumb, David Moss, Marv Paule, Laurie Stargell, and Sriwan Wongwisansri for critical reading of this manuscript. We thank Tony Long for help with the Monte Carlo analysis.

This research was supported in part by Research Grant MCB-9505644 from the National Science Foundation and a Faculty Research Grant from the Graduate School, Colorado State University. P.J.L. is a recipient of a Junior Faculty Research Award from the American Cancer Society.

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Figure 4.1. Strong repression of *PHO5* transcription by reconstituted nucleosomes. Plasmid templates, containing both UASs and the core promoter only or approximately 2000 bp of additional upsteam sequence were reconstituted at the core histone to DNA ratio (CH/DNA, w/w) indicated at the top of each lane. (A) One-dimensional topological analysis was performed as described previously (Pilon et al., 1997). Plasmid DNA was reconstituted at the core histone to DNA ratio indicated at the top, purified and resolved on 1% agarose gels in the absence or presence 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 fairly uniform spacing were formed on pMH313-Gless. Lane 1 contains undigested DNA. Free DNA (lanes 2, 3, and 4) and reconstituted chromatin (lanes 5, 6, and 7) was 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 in the right margin. Lanes M, are 100 bp DNA size markers. (C and D) Each reconstituted template (lanes 1 through 5, pPHO5-Gless; lanes 6 through 10, pMH313-Gless) were transcribed using yeast whole cell extract. The *PHO5* transcripts are indicated at the left of the figure. Lane M contains end-labeled DNA size markers, pBR322 cut with *Msp* I, with the length of selected fragments indicated to the left. Transcripts were quantitated relative to the recovery standard using NIH Image. The fold repression is indicated below each lane. These results were found to be reproducible through several repetitions of this experiment.

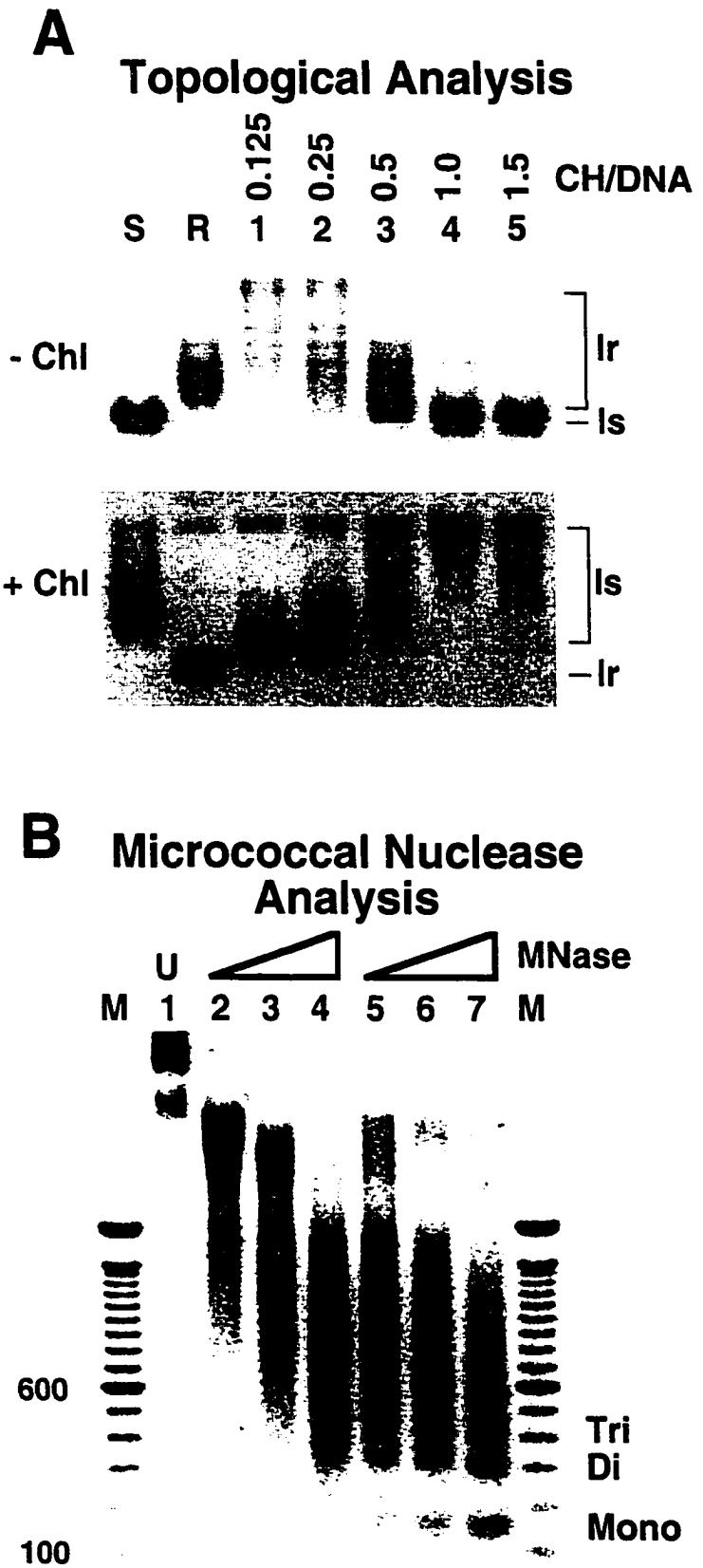

Figure 4.1A & B. Pilon et al.

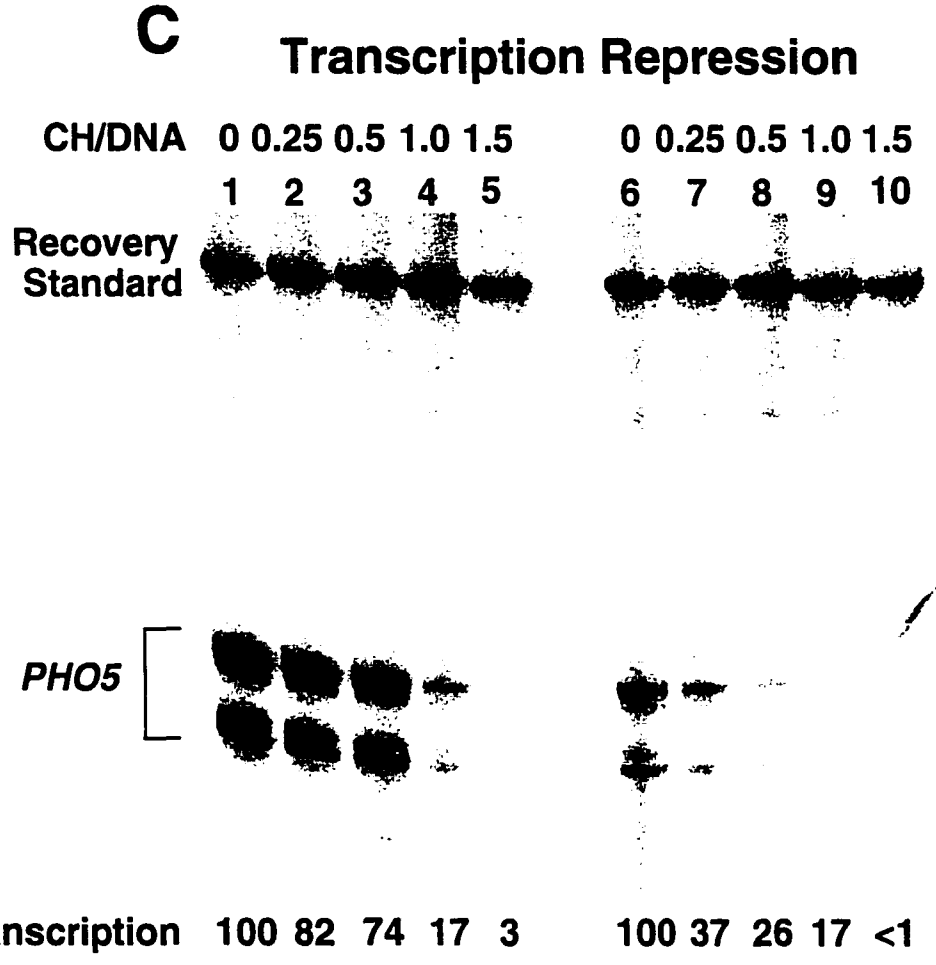

Figure 4.1C. Pilon et al.

Figure 4.2. Indirect end-labeling analysis of nucleosome positioning on the reconstituted and genomic *PHO5* promoter. (A) Free DNA (DNA), reconstituted pMH313-Gless (Chrom), and yeast nuclei were digested with increasing amounts of micrococcal nuclease, and the DNA was analyzed by indirect end-labeling. The second restriction endonuclease used to generate each DNA size marker is indicated in the left and right margins. Protected regions and accessible sites in the chromatin as compared to free DNA are indicted next to lane 4 by bars and filled circles, respectively. The solid vertical line between the *in vitro* and *in vivo* data represents the DNA strand containing the *PHO5* promoter with locations of Pho2p binding sites (rectangles), Pho4p binding sites (ovals), and the RNA start sites (bent arrow) indicated. The ovals numbered –3 through +1 represent the inferred positions of these nucleosomes on the *PHO5* promoter. (B) Plots of the relative number of counts down each lane from the top to the bottom of the gel. The plots for reconstituted chromatin (Chrom), free DNA (DNA), genomic chromatin (In Vivo), and size markers (Markers) are aligned to facilitate comparison.

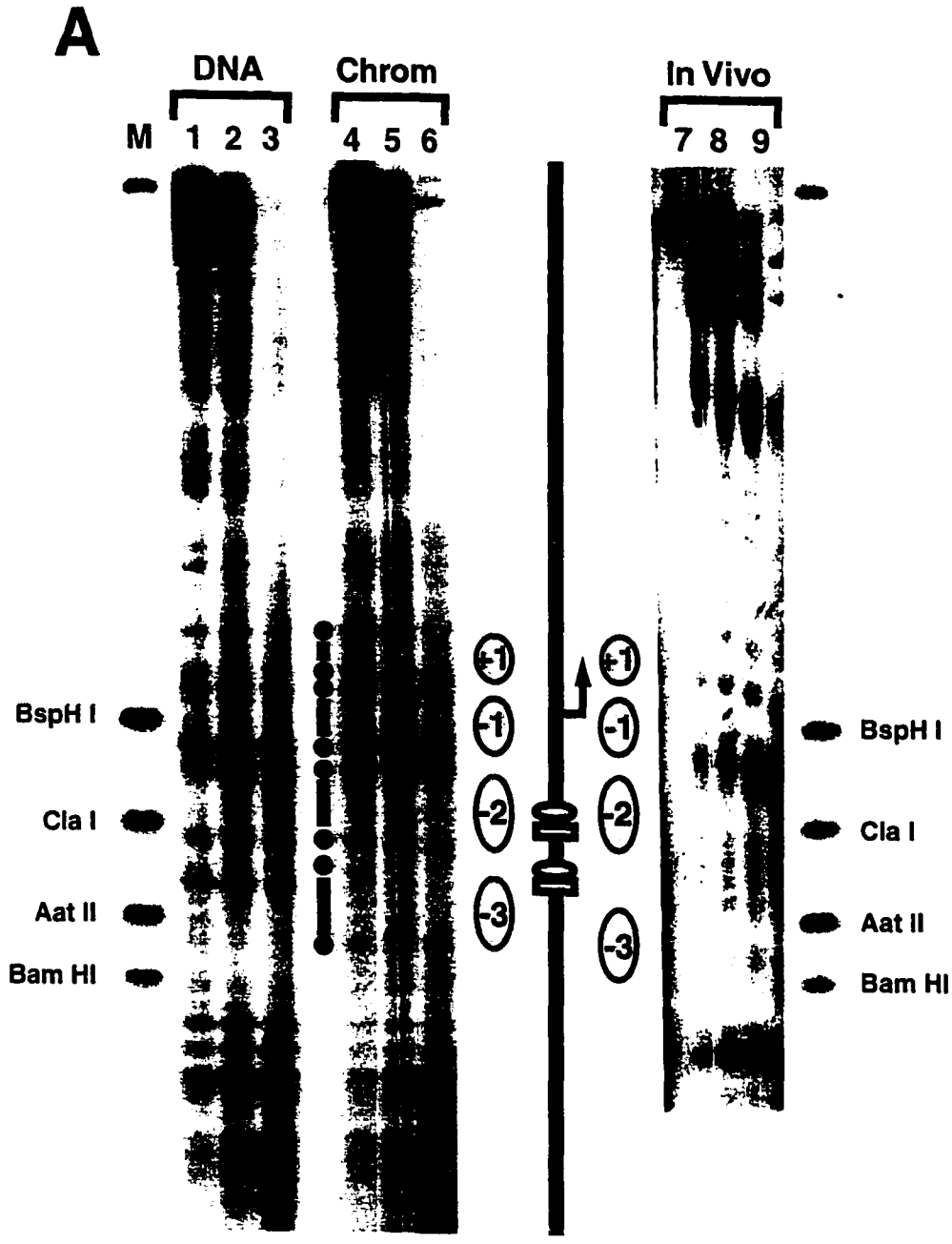

Figure 4.2A. Pilon et al.

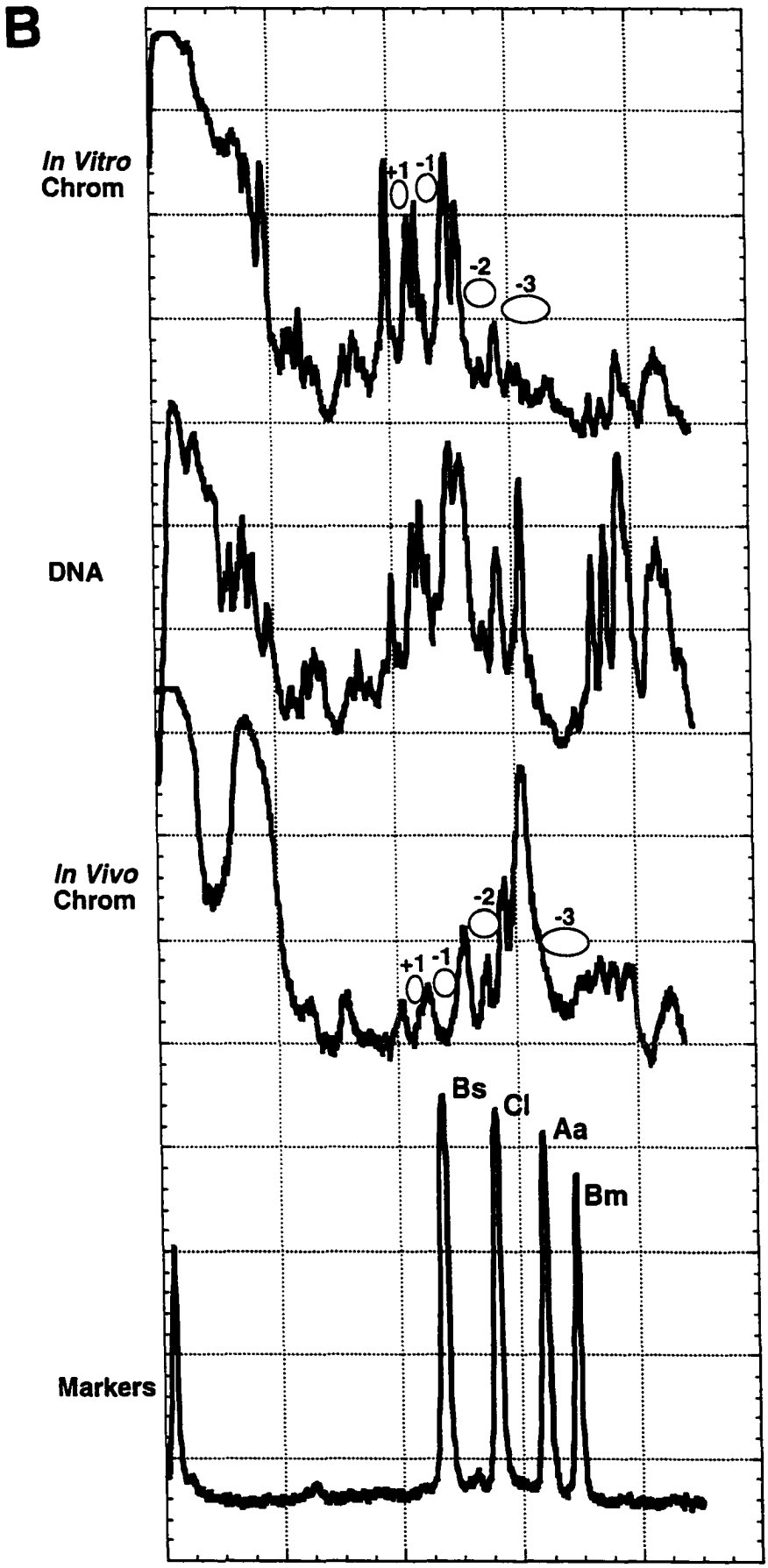

Figure 4.2B. Pilon et al.

Figure 4.3. Micrococcal nuclease cleavage protection pattern on the reconstituted *PHO5* templates: nucleosome –1, nucleosome –2, the enlarged linker DNA. (A) The primer mn5 binds to the lower DNA strand and extends downstream. The primers FS, mn7, and mn2 bind the top DNA strand and extend upstream. Recognition sites are indicated below the promoter as Bam for *Bam* HI, Cla for *Cla* I, and Bst for *BstE* II. 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 Materials and Methods. G, A, T, and C refer to the ddNTP included in the Sanger sequencing reactions resolved in these four gel tracks. Of the three samples, 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 the important 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 location of promoter elements and the inferred location of the nucleosomes. The bent arrows indicate the RNA start sites. The results of primer extension analysis using primer mn5 is shown in panel B, using primer FS is shown in panel C, and using primer mn2 is shown in panel D.

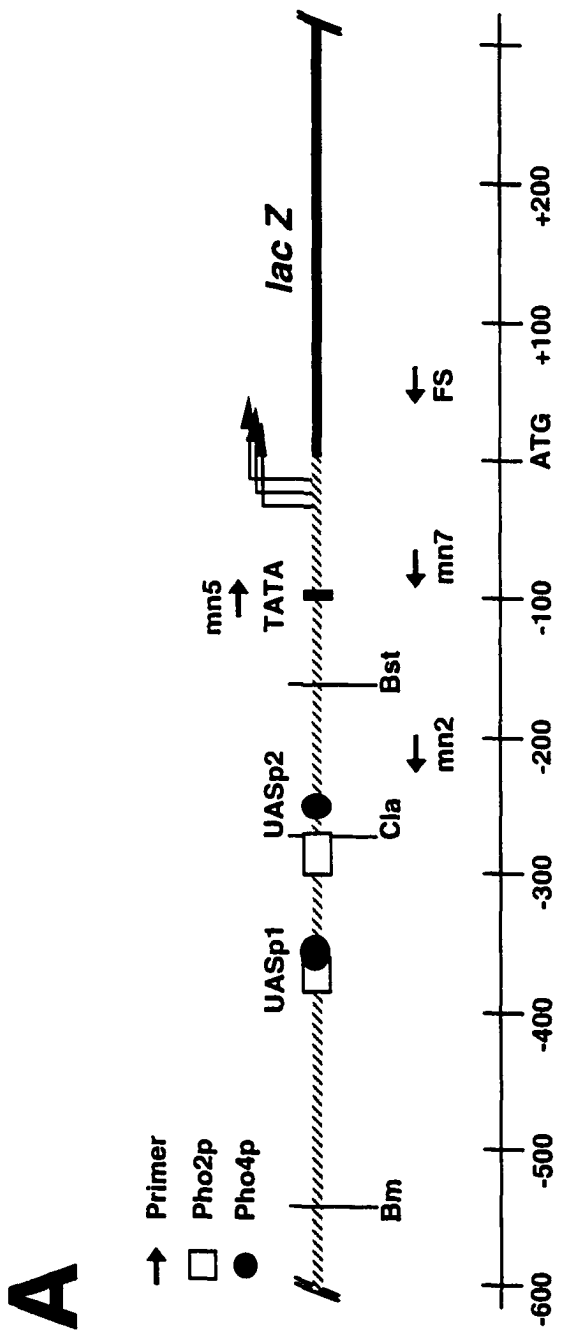

Figure 4.3A. Pilon et al.

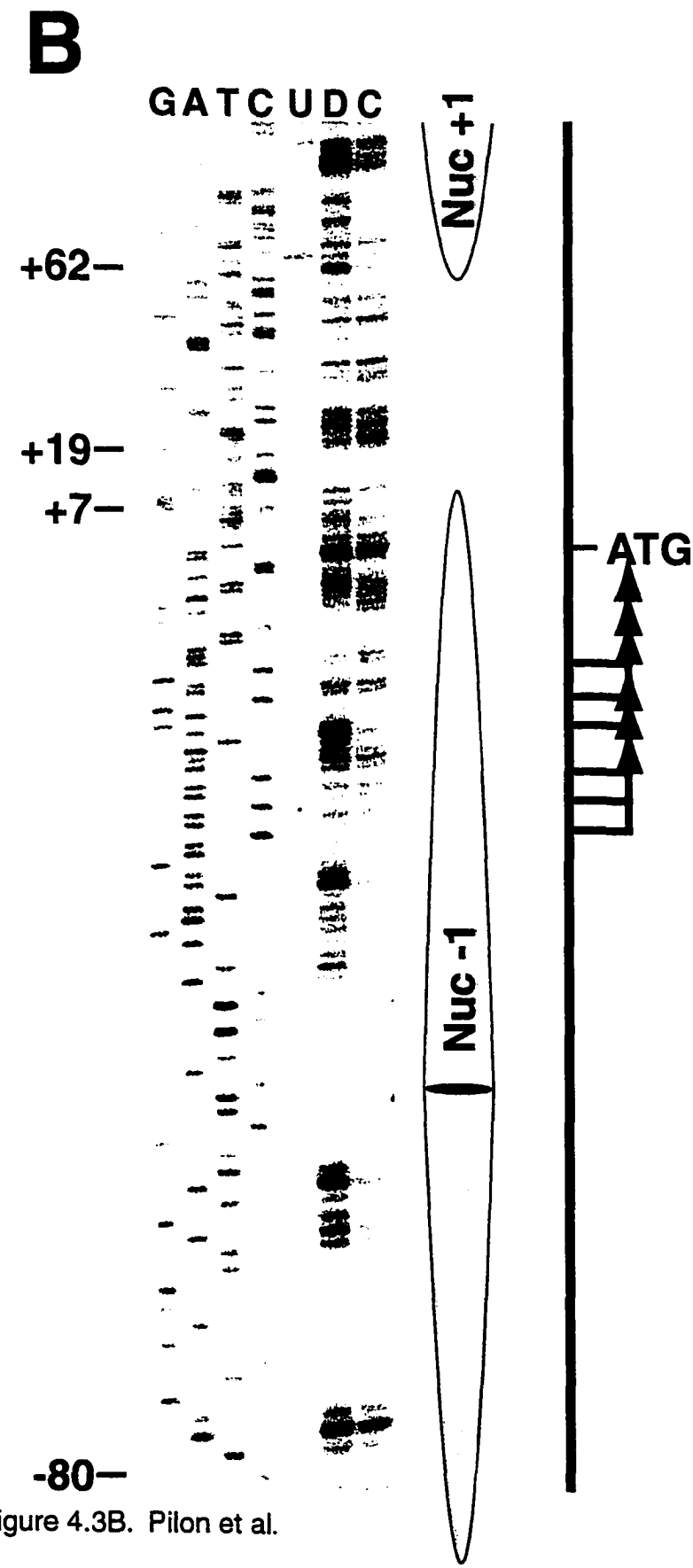

Figure 4.3B. Pilon et al.

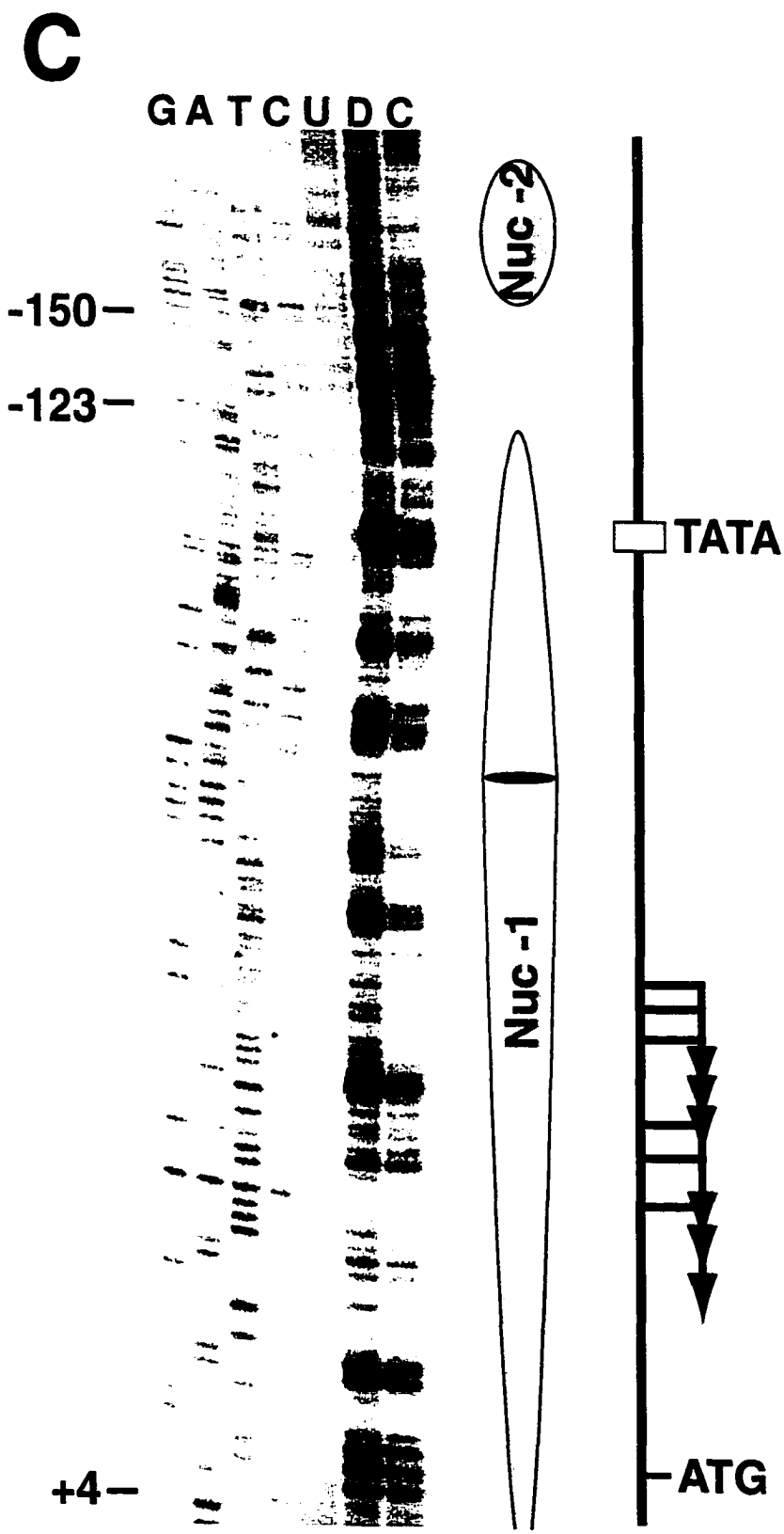

Figure 4.3C. Pilon et al.

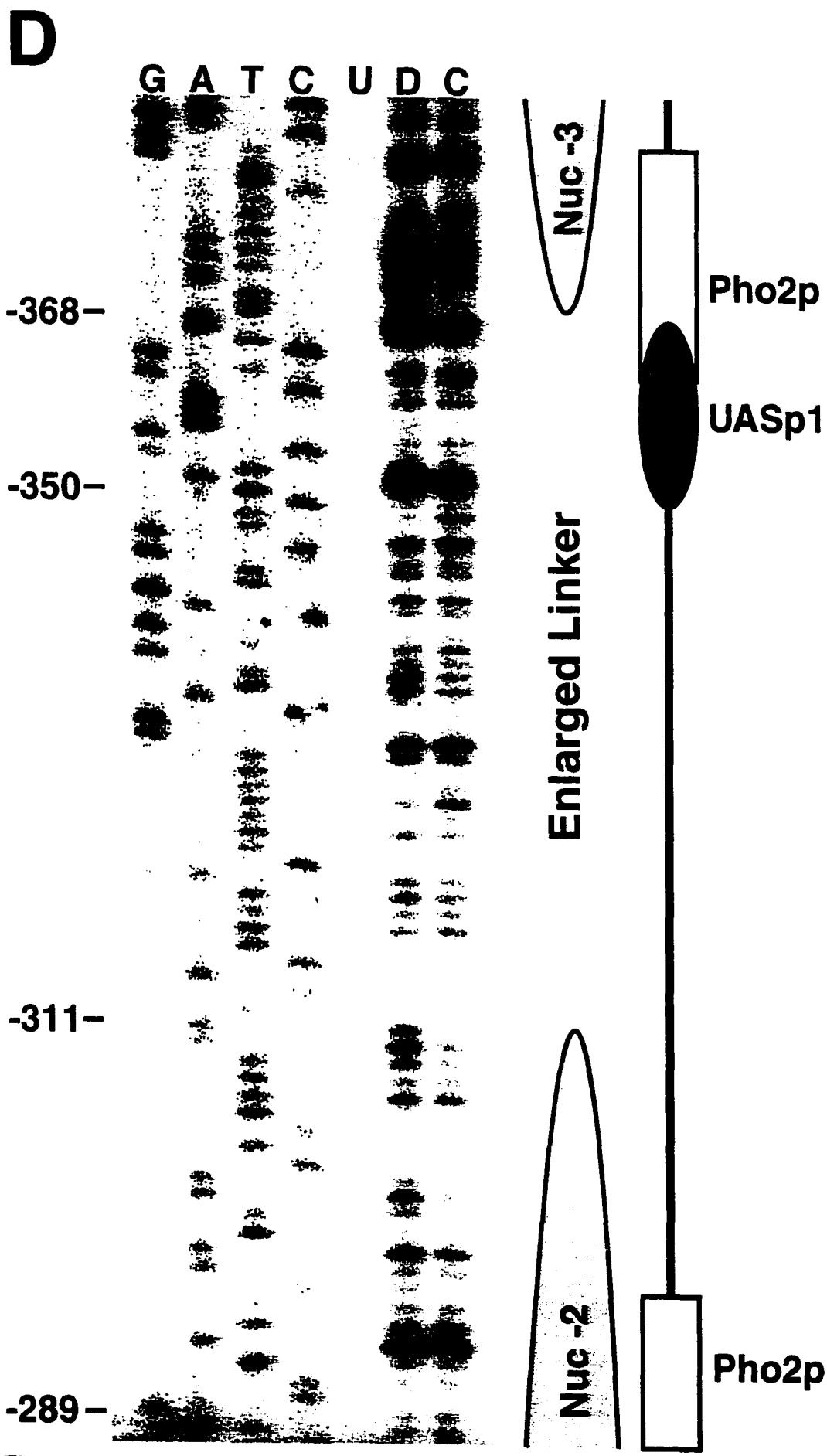

Figure 4.3D. Pilon et al.

Figure 4.4. DNAase I footprinting of Pho4p binding to free DNA and Chromatin. Nucleosomes were reconstitited on pMH313-Gless. This chromatin was then incubated with Pho4p and then digested with DNAase I. The digestion pattern on free DNA (DNA) and reconstitued templates (Chrom) was analyzed by primer extension. The reconstituted chromatin, in the absence of Pho4p, was digested with micrococcal nuclease (MNase I) and the digestion pattern analyzed using primer extension.

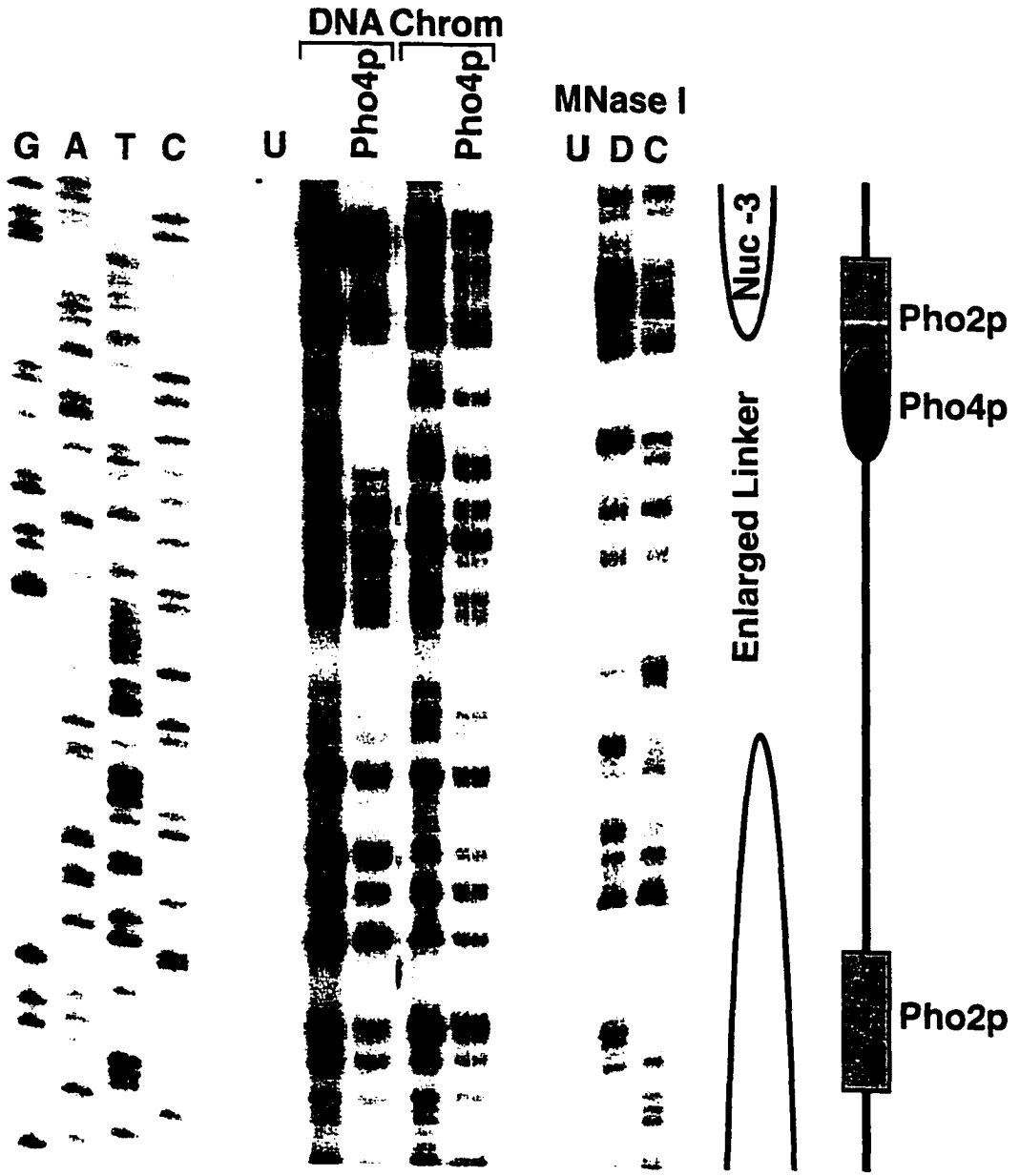

Figure 4.4. Pilon et al.

Figure 4.5. Sequence-dependent isotropic flexibility of the *PHO5* promoter. The Gibbs free energy of bending (G_n) summed over a 145 bp block of DNA sequence (Sivolob & Khrapunov, 1995) is plotted as *kT* or kcal/mol versus the dyad position in bp relative to the 5′ end of the 5S rRNA gene (A) and relative to the *PHO5* translational start codon (B). (A) *Lytechinus variegatus* 5S rRNA gene bending free energy plot. Below the figure the RNA start site (bent arrow) is shown. The filled triangles indicate the minima corresponding to the location of the dyad of the two nucleosome positions identified by site directed cleavage (Flaus et al., 1996). (B) *PHO5* promoter bending free energy plot. Below the plot is indicated the experimentally determined positions of nucleosome -1, -2 and -3. Also shown are the Pho2p binding sites (open rectangles), Pho4p binding sites (filled ovals), the A_7 tract (filled rectangle upstream of UASp2), the TATA element (filled rectangle downstream of UASp2), and the RNA start sites (bent arrows). Monte Carlo analysis of the *PHO5-lacZ* fragment was performed to determine the minima preferred by an array of nucleosomes. The open diamonds indicate the mean location of the dyads with error bars showing the standard deviation as predicted by Monte Carlo analysis. The filled triangles indicate the minima that correspond to the dyads for nucleosomal positions predicted by the Monte Carlo analysis and determined by indirect end-labeling and footprinting analysis.

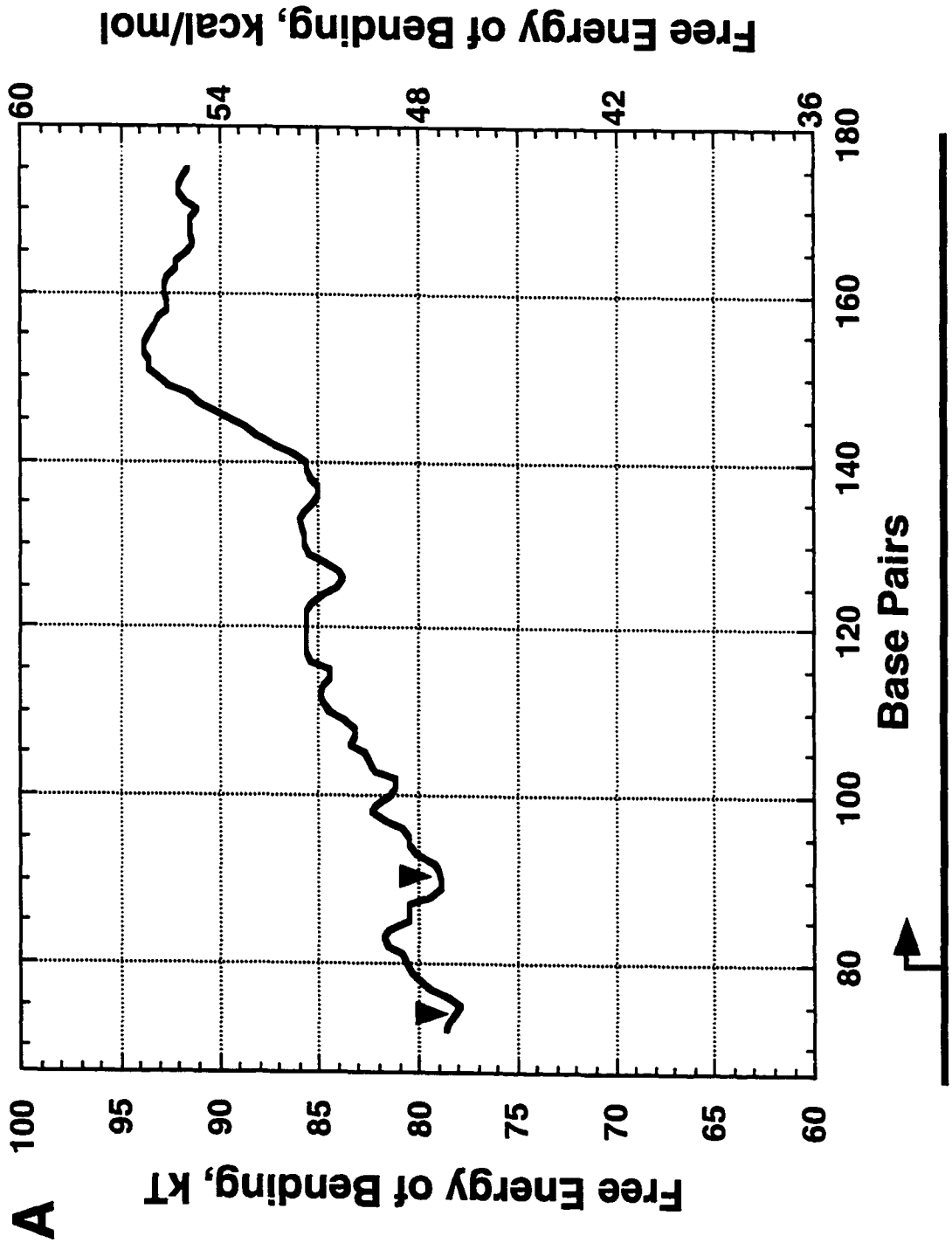

Figure 4.5A. Pilon et al.

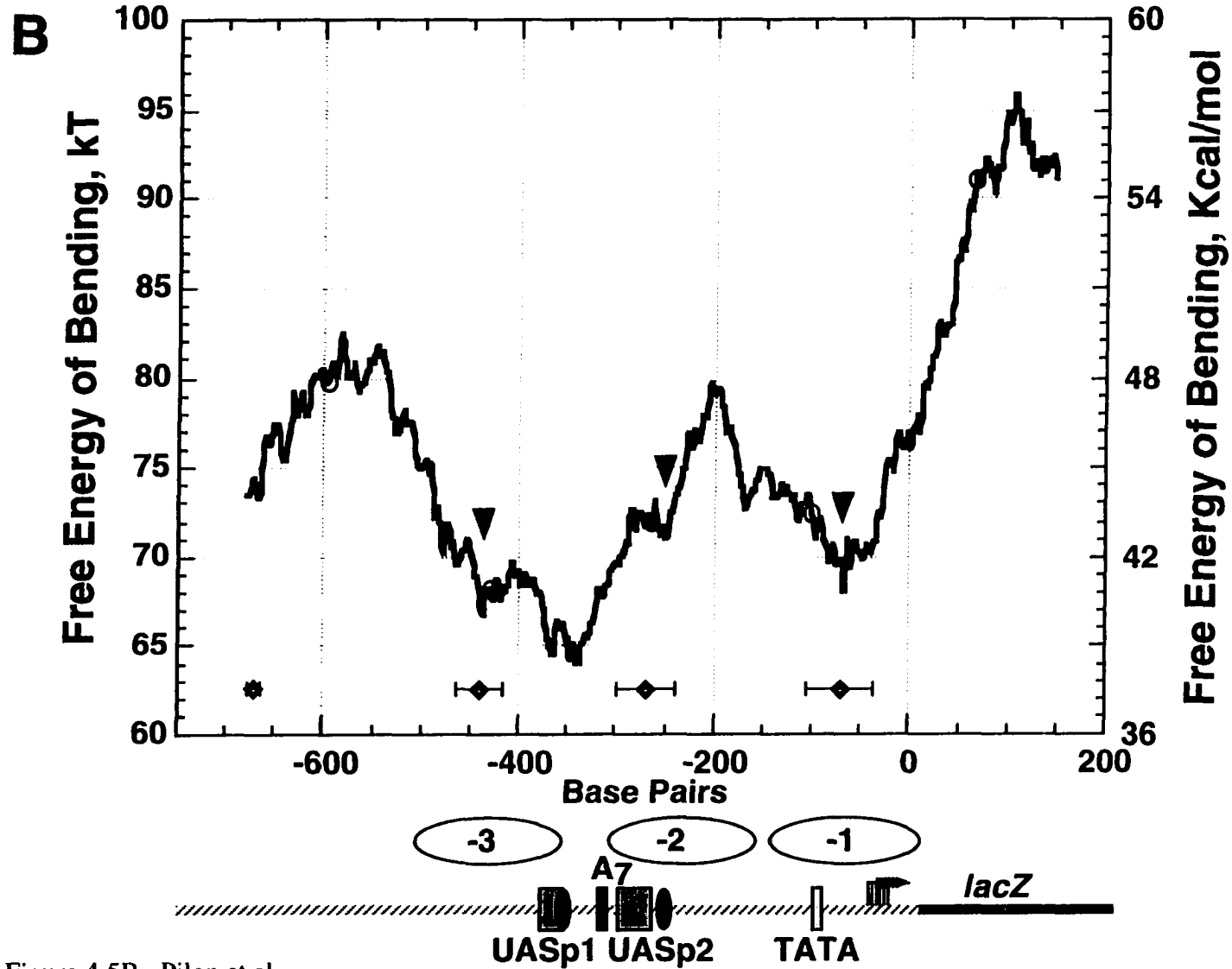

Figure 4.5B. Pilon et al.

Figure 4.6. Native gel electrophoretic analysis of competitive mononucleosome reconstitutions. Mononucleosomes were reconstituted onto short DNA fragment (approximately 200 bp) corresponding to nucleosome –1 and –2, a fragment centered on the linker DNA between these nucleosomes, and the 207 bp fragment of the *Lytechinus variegatus* 5S rRNA gene (Simpson et al., 1985). Reconstitution was done in the presence of 10-fold, 20-fold, and 40-fold excess of competitor DNA (fragmented yeast genomic DNA).

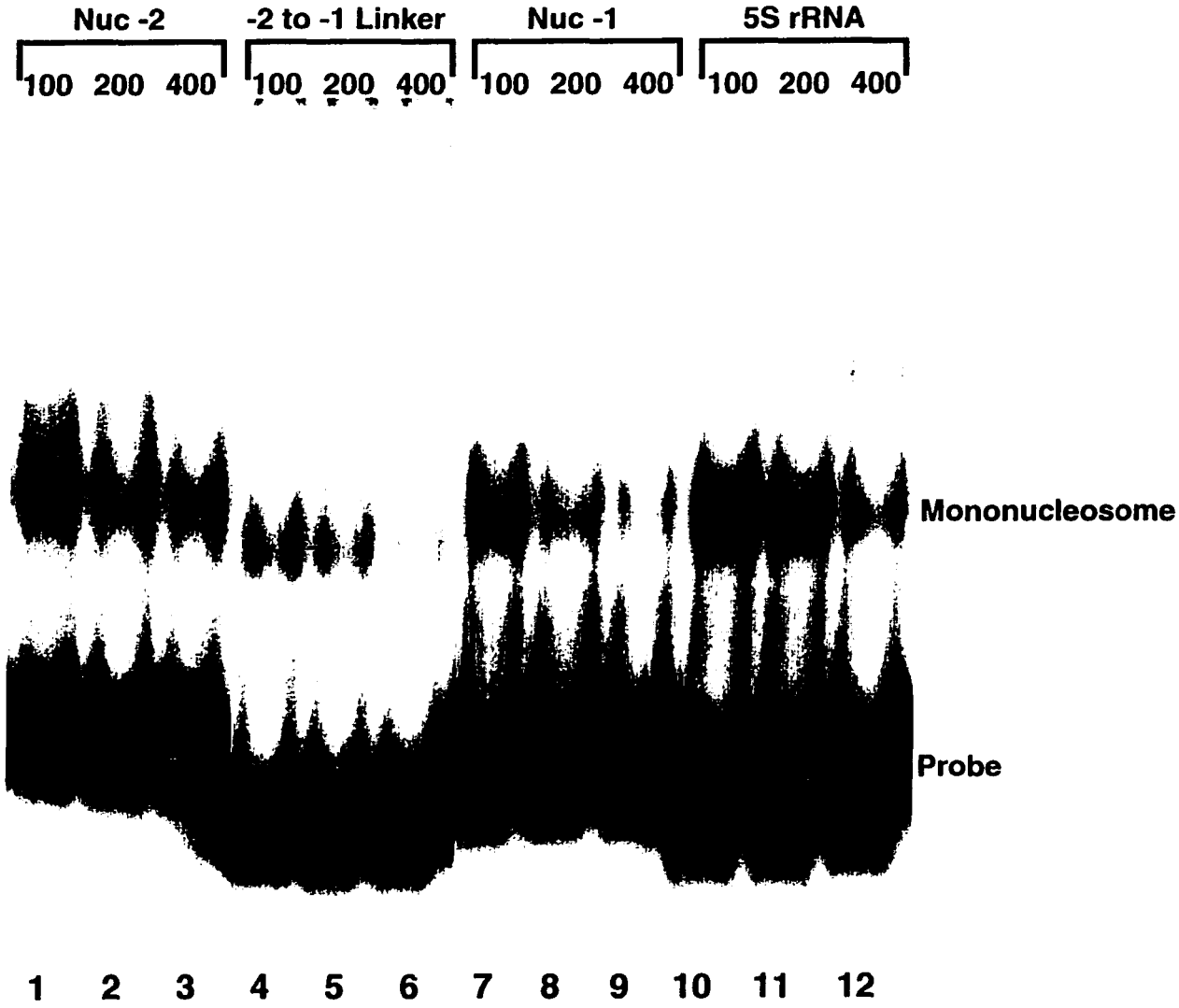

Figure 4.6. Pilon et al.

Figure 4.7. Reconstituted nucleosomes are translationally positioned on the *PHO5* promoter. The cleavage pattern on the *PHO5* promoter was determined by indirect end-labeling analysis and by primer extension footprinting analysis for the reconstituted templates (Figures 2 and 3) and by indirect end-labeling analysis *in vivo* (Almer et al., 1986; Straka & Horz, 1991). Open rectangles are Pho2p binding sites, filled ovals are Pho4p binding sites, the filled rectangle upstream of UASp2 is the A_7 tract, and the filled rectangle downstream of UASp2 is the TATA element. *In Vitro*, the locations of nuclease cleavage sites on the chromatin templates are indicated by vertical arrows. Regions of micrococcal nuclease protection are indicated by filled boxes. The filled boxes above the restriction endonuclease cleavage sites indicate cleavage protection in the chromatin templates. Calculated, the locations of the DNA bending free energy minima are indicated by arrowheads. The black arrowheads indicate minima predicted by Monte Carlo analysis to be used, the gray arrowheads indicate minima not predicted. *In Vivo*, the inferred location of nucleosomes on minichromosomes containing the *PHO5* promoter fused to the *lacZ* coding sequence in yeast nuclei in previous studies by other laboratories. The *in vitro* (experimental) and the theoretically predicted locations of nucleosomes –1, -2, and –3 correspond very closely. The *in vitro* and *in vivo* locations of nucleosome –1 and –2 are very similar, as well. However, the downstream edge of nucleosome –3 is located approximately 40 bp downstream *in vitro* than it is *in vivo*. This discrepancy suggests that a protein may bind upstream of UASp1 and shift nucleosome -3 farther upstream.

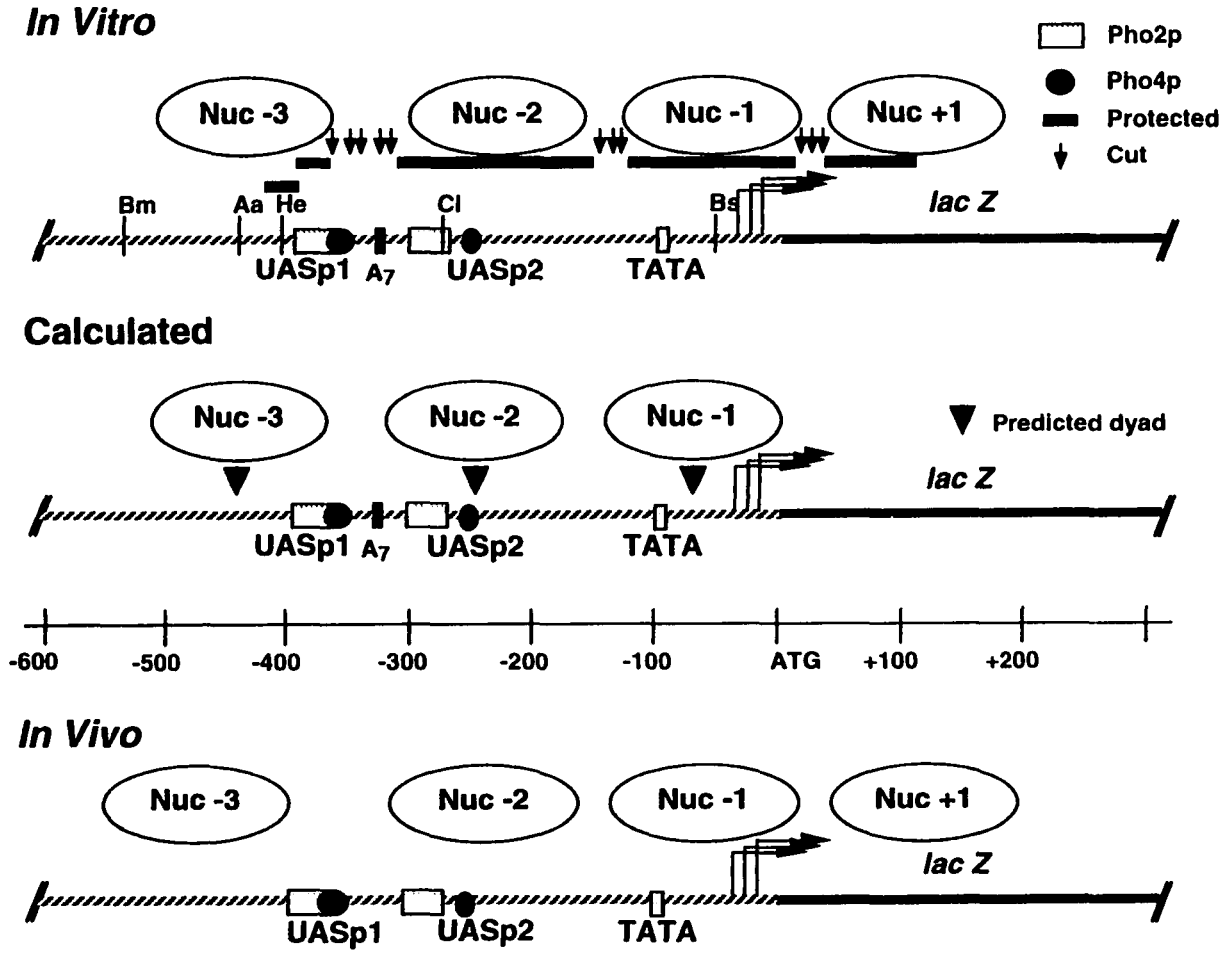

Figure 4.7. Pilon et al.

Chapter 5

Purification of Native Pho2p, Further Studies of Transcription Activation of *PHO5*, and Future Directions

This chapter includes non-published data and detailed methods that have been included in manuscripts but not in great detail, or only as a referenced method. I feel that this information may be useful to the researcher that continues with this project.

5.1 *In Vitro* Transcription System

Since a large portion of my dissertation research was to use *in vitro* transcription assays to study the role of purified Pho2p and Pho4p, I spent a great deal of time setting up an *in vitro* transcription system. This included characterization and preparation of yeast whole cell transcription extracts from wild type cells (BJ926), *pho4* cells (YS25), *pho2* cells (YS19), and *pho4/pho2* cells (YS27), which would allow me to study the role of Pho4p and Pho2p directly by adding back purified factors to a system devoid of these activators. Also included in this section is a full description of the plasmid templates used for transcription.

5.1a *Construction of pPHO5G-less.* Construction of this G-less template and the template described in the next section greatly facilitated our *in vitro* experiments. Prior to construction of these templates we used reverse transcriptase primer extension to visualize transcripts. The G-less cassette templates allowed direct visualization of the labeled transcripts. Dr. Laybourn initially began work on constructing pPHO5G-less. I received pUCC-less which had a 110 bp cassette, which in the proper orientation should be G-less with respect to the RNA like strand. The cassette was cut out of pUCC-less using Sal I and a linker was ligated onto one end so that the fragment could be put back into pUC directionally. The resulting fragment was re-ligated into pUC to create pUCG-less. I also received two pUC vectors containing a 331 bp fragment of the *PHO5* UAS region and a 216 bp fragment of the *PHO5* minimal promoter (TATA box and start sites – Dr. Laybourn had used PCR mutagenesis to change the start site region to remove any G nucleotides). The UAS fragment was ligated into pUCG-less using BamH I (upstream) and Apo I (downstream) to create pPHO5uasG-less. The minimal fragment was then ligated into the ApoI site of pPHO5uasG-less to create pPHO5G-less.

5.1b *Construction of pMHG-less(mfe).* The original template we used for *in vitro* transcription assays, pPHO5G-less, contained 550 base pairs of the *PHO5* promoter and a 110 bp G-less cassette cloned into pUC19. The resulting template was approximately 3300 base pairs. We assumed that this template would be sufficient for our studies of *in vitro PHO5* expression, since a plasmid construct containing only the BamH I to Dra I (-542 to +6) fragment of the *PHO5* promoter was equivalent to the genomic copy of the gene, with regard to regulation of expression and nucleosome positioning *in vivo* (15). Thus, this 548 bp fragment defines the minimal sequence requirements for correct positioning of nucleosomes. However, through *in vitro* footprinting studies, John Pilon determined that this plasmid did not position nucleosomes well, most likely due to steric constraints of forming nucleosomes. He then switched to plasmid pMH313, which contains the *PHO5* promoter and approximately 1800 base pairs of additional upstream *PHO5* sequence. This insert was cloned into a large yeast centromeric vector for a total size of 14000 base pairs. This plasmid also contained a *URA3* selection marker for growth in yeast, a yeast ARS, an ampicillin resistance gene for selection in *E. coli*, and an origin of replication for reproduction in *E. coli*. John found that this plasmid allowed nucleosomes to be positioned in a manner very similar to the *in vivo* positions (Chapter 4, this dissertation). The G-less cassette from pPHO5G-less was inserted into pMH313, to create pMHG-less, a transcription template. An Mfe I restriction site was also inserted into the promoter to facilitate mutational analysis of the enlarged linker region between nucleosomes –1 and –2. The plasmid with the Mfe I site shows no difference in nucleosome positioning or transcriptional competence from that of the wild type.

5.1c *Genotype verification of YS laboratory yeast strains.* All of the yeast strains used in my experiments were obtained from other laboratories. To verify that I had been sent the correct strains I used PCR to look at the *PHO4* and *PHO2* loci of the strains I would be

using in my work. This would allow me to be certain that the deletion strains were indeed different from the wild type strains. Unfortunately, I did not have enough information about the strain construction to predict the sizes of the PCR products from the knock out strains, so I assumed that if the products from the mutant strains were different from the wild type products, the disruptions had been made and the strains were correct. Primers were ordered that flank both the *PHO2* and *PHO4* genes (PHO2R, PHO2F, PHO4R, and PHO4F, from MRF). From the PHO2 primers I expected a 2164 bp product from the wild type strain and from the PHO4 primers I expected a 1554 bp product from the wild type strain. The PCR reactions contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, either 1.125 mM or 1.5 mM $MgCl_2$, 0.1 mM dA, dC, dT, and dGTP, 10 pmol of each primer, 2 U Taq, and a small scraping of cells directly from a YPD yeast plate. PCR cycles were done as follows: 1 cycle of 4 min at 98°C, 25 cycles of 2 min at 94°C, 2 min at 52°C, and 2 min at 72°C, and 1 cycle of 5 min at 72°C. Ten microliters of each of these reactions were used in a second round of PCR in the same conditions and the same cycles as before, except that the first cycle was 2 min at 94°C. Ten microliters of each of these reactions were run on an agarose gel then ethidium stained. The results shown in Figure 5.2 indicate that the correct amplification products for each set of primers are obtained for the wild type strain, YS18. Using the *PHO2* primers with the mutant strains I did not get any products, indicating that the disruption included one or both regions in which the primers would bind. I concluded from this that the *PHO2* locus was disrupted in strains YS19 and YS27. Using the *PHO4* primers with the mutant strains, I obtained products several hundred base pairs larger than products from the wild type strains. I concluded that part of the *PHO4* gene had been removed and the *URA3* selection marker had been inserted to create the *pho4* knockout.

Genotypes:

YS18 MATα; his3-11, 15; leu2-3, 112; ura3-432; canR

YS19 MATα; his3-11, 15; leu2-3, 112; ura3-432; pho2::ura3-432; canR

YS25 MATα; his3-11, 15; leu2-3, 112; ura3-432; pho4::ura3-432; canR

YS27 MATα; his3-11, 15; leu2-3, 112; ura3-432; pho4::ura3-432; pho2::ura3- 432; canR

5.1d *Whole cell extract preparation from BJ926, YS25, YS19, and YS27.* This protocol was derived from Woontner *et al* (17). The entire procedure is as follows. Cells were grown to mid log phase using continuous flow fermentation, washed twice in cold water (I have used 70 and 150 grams cells successfully), and pelleted for 10 minutes at 4°C and 7,000 rpm in a Sorvall GSA rotor. Unused cells were stored at -80°C. Cells were resuspended in 2 ml/gram breakage buffer (0.2 M Tris base, 0.39 M $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 20% glycerol, and 1 mM EDTA, pHd to 7.9 with glacial acetic acid, plus 1 mM DTT, 1 mM PMSF, 2 mM benzamidine, 0.5 µg/ml leupeptin, 2.5 µg/ml antipain, 0.35 µg/ml bestatin, and 0.4 µg/ml pepstatin A) then lysed in a bead beater with a cooled chamber by alternating 30 second bursts of grinding with two minutes of cooling. Lysis was monitored by microscope. When cells were sufficiently lysed (less than 30% of whole cells remaining (by eye)) the lysate was decanted from the beads by pouring through a metal mesh screen, small enough to catch the beads, and into a cooled graduated cylinder. The lysate was adjusted to 4.5 ml/gram with breakage buffer (poured over beads to wash). The lysate was then centrifuged at 4°C and 65,000 rpm for 75 minutes in a Beckman Ti70.1 rotor. The supernatant was adjusted to 2.94 M $(NH_4)_2SO_4$ by adding 337 mg solid per ml supernatant. The solution was neutralized by adding 10 µl 1 M KOH per gram $(NH_4)_2SO_4$. Proteins were stirred at 4°C for 30 minutes then pelleted at 40000 rpm for 30 minutes at 4°C in a Ti70.1 rotor. The resulting protein pellet was

resuspended in storage buffer (20 mM Hepes-KOH pH 7.5, 20% glycerol, 10 mM EGTA, 10 mM $MgSO_4$, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine, 0.5 µg/ml leupeptin, 2.5 µg/ml antipain, 0.35 µg/ml bestatin, and 0.4 µg/ml pepstatin A) to approximately 1-2 ml per 100 grams of cells. The extract was dialyzed against the same buffer with PMSF but no other protease inhibitors at 4°C until the conductivity of a 1:200 dilution dropped below 100 µS/cm. This took approximately six hours. The extract was then aliquoted and stored in liquid nitrogen. The amount used for transcription reactions was determined experimentally, but consistently worked best with 100 µg to 300 µg whole cell extract, as determined by Coomassie Plus Protein assay reagent (Pierce). Figure 5.3 shows *in vitro* G-less cassette transcription from pMHG-less(mfe) using all whole cell extracts that were prepared.

5.1e *Alpha-amanitin sensitivity assay.* One of the ways in which the three eukaryotic RNA polymerases can be distinguished is according to their sensitivity to the fungal toxin α-amanitin. RNA polymerase I is insensitive to the toxin and RNA polymerase III is only moderately sensitive. RNA polymerase II on the other hand is highly sensitive and is inhibited at toxin concentrations of 1 µg/ml (reviewed in (14)). This differential sensitivity can be utilized in *in vitro* transcription experiments to demonstrate that the RNA polymerase responsible for transcribing the template of interest is or is not RNA pol II. Since *PHO5* is a yeast protein coding gene and is transcribed by RNA pol II *in vivo*, I wanted to confirm that the same polymerase was transcribing our pPHO5G-less template *in vitro*. The transcription reactions contained pPHO5G-less (300 ng), YS25 whole cell extract (250 µg), and 0, 0.6, 2.3, 5.8, 11.7, and 29.2 µg/ml α-amanitin. Transcription is significantly inhibited at an α-amanitin concentration of higher than 5.8 µg/ml. This experiment confirms that RNA polymerase II is transcribing pPHO5G-less *in vitro* (Figure 5.4).

5.2 Purification of native Pho2p

Recent studies have shown that *in vivo*, Pho4p and Pho2p bind cooperatively to the *PHO5* promoter (1). Pho4p alone is able to activate transcription on a naked DNA template *in vitro*, however we have been unable to duplicate this activation on our *in vitro* reconstituted chromatin templates. Pho2p is also required for maximal activation of *PHO5* under physiological conditions; therefore, Pho2p is likely to be required for full activation *in vitro* in the context of chromatin.

Since one of my goals was to obtain transcription activation, or counteraction of nucleosome repression, on *in vitro* reconstituted chromatin templates, I decided to purify native Pho2p. There are expression vectors available containing truncated versions of the protein (4, 16) but no lab has been successful in expressing the entire *PHO2* gene product in *E. coli*. In addition, the recombinant form most likely lacks post-translational modifications that may or may not be necessary for proper *PHO5* expression. Because the truncated versions of Pho2p have not been shown to be active transcriptionally on the *PHO5* promoter, I decided to purify native Pho2p from a yeast whole cell extract using DNA affinity chromatography. This would allow us to study the role Pho2p plays in transcription activation of *PHO5 in vitro*. Since Pho2p is a constitutively expressed protein (2) and involved in expression of several biosynthesis genes (3, 5, 8, 16), I thought there would be sufficient quantities of Pho2p for purification from yeast.

Briefly, (detailed methods are described in the following paragraphs) wild type yeast strain BJ926 was grown using continuous flow fermentation and several hundred grams of cells were harvested. These cells were used to prepare an S-100 whole cell extract (10). The extract was passed over a Heparin Sepharose-CL4B column which binds most DNA binding proteins and allows other proteins to flow through. Fractions were assayed by electrophoretic mobility shift assay (EMSA) using an oligonucleotide

based on the Pho2p binding site adjacent to UASp1 on the *PHO5* promoter (Figure 5.5) and antibody supershift EMSAs with a polyclonal antibody directed against Pho2p (a gift from David Stillman's lab). The second step was done with a Mono-S column (Pharmacia). Mono-S columns are prepacked with a high binding capacity cation exchange resin. Finally, as a third step I constructed an oligonucleotide affinity column. The oligo used was the same as that used for the EMSAs. The column was constructed using the method of Kadonaga and Tjian (9). Figure 5.6 shows an EMSA of all the stages of purification including an antibody supershift with purified Pho2p. The Pho2p band was easily detectable by Western blot (Figure 5.7) but unfortunately not distinguishable on a silver stained gel due to the presence of many other positively staining bands.

5.2a *Construction of Heparin Sepharose CL-4B for native Pho2p purification.* 250 ml of Sepharose CL-4B (Pharmacia) was washed with one liter of H_2O using a coarse sintered glass funnel. The resin and 750 ml of 2M Na_2CO_3 were put in a 2 L plastic beaker and stirred slowly with a magnetic stirrer. Twenty-five grams of CNBr (Sigma) were dissolved in 12.5 ml acetonitrile then added to the resin. The mixture was stirred for two minutes then transferred to a coarse sintered glass funnel. The resin was washed quickly using a vacuum with 1 L 0.1 M $NaHCO_3$ (pH 9.5), 1 L H_2O, and 1 L 0.2 M $NaHCO_3$ (pH 8.5), all at 4°C. The resin was never allowed to dry out. All filtrates were combined and mixed with 20 g glycine and 20 g NaOH overnight to inactivate the CNBr. Three grams of heparin (500,000 U, Sigma) were dissolved in 250 ml 0.2 M $NaHCO_3$ (pH 8.5) and put in a 500 ml plastic bottle. The resin was transferred to the bottle and the mixture was incubated overnight at 4°C. The liquid was removed by filtering in a sintered glass funnel (save this filtrate) then resuspended in 400 ml 1 M ethanolamine-HCl, pH 8.0 and mixed at room temperature for four hours. The resin was then washed with 2 L 0.1 M

NaOAc (pH 4.0) plus 0.5 M NaCl, 2 L 2 M urea plus 0.5 M NaCl, 2 L 0.1 M $NaHCO_3$ (pH 10) plus 0.5 M NaCl, and finally with 2 L H_2O (save filtrates). The resin was then resuspended in 250 ml 10 mM Tris-HCl (pH 7.9) plus 0.04% NaN_3 and H_2O was added to a final volume of 500 ml. The Heparin-Sepharose CL-4B resin was stored at 4°C. A standard curve was generated using zero to 200 µl of 1.0 mg/ml heparin plus 0.15 M NaCl to 2 ml, 2 ml of Borate buffer (0.1 M H_3BO_3 and 0.15 M NaCl, pH to 8.5 with NaOH), and 1 ml 0.15 mg/ml Azure A (3:20 dilution of 1 mg/ml stock). The absorbance was measured at 500 nm. One hundred microliters of each filtrate was assayed and the amount of heparin bound was determined. Approximately 37% of the heparin bound in the preparation described above.

5.2b *Preparation of DNA affinity resin for native Pho2p purification.* The protocol followed was originally described by Kadonaga and Tjian (9). Complimentary oligonucleotides were designed based on the Pho2p binding site adjacent to UASp2 on the *PHO5* promoter. The oligos contained a 5' CTAG overhang for ligation. The sequence of the oligos were 5'CTAGTGAATAGGCAATCTCTAAAT3' and 5'CTAGATTTAGAGATTGCCTATTCA3'. The oligos were synthesized on the 1.0 µmol scale by MRF (Colorado State University). The oligos were run on a 16% polyacrylamide urea gel in 1 X TBE and visualized by UV shadowing. Approximately 1.5 mg of each oligo was electrophoresed. The gel slices containing the oligos were placed in 15 ml polypropylene conical tubes and 5 ml of TE was added to each gel slice. DNA was eluted by overnight incubation at 37°C with shaking. The acrylamide was filtered out through prerinsed glass wool in a Pasteur pipet and the DNA was concentrated to approximately 180 µl by 2-butanol extractions. The concentrated DNA was extracted once with diethyl ether and placed in a Speed-Vac until all ether had evaporated. TE was added to 200 µl and the DNA was precipitated on dry ice by

addition of 20 µl 3 M NaOAc, 2 µl 1 M $MgCl_2$, and 600 µl 100% ethanol. The DNA was then washed with 75% ethanol, dried, and resuspended in 100 µl TE. 500 µg of each oligo were combined and brought to 130 µl with TE. 20 µl T4 Kinase buffer (10X, New England Biolabs) was added and the oligos were annealed by heating at 88°C 2 min, 65°C 10 min, 37°C 10 min, and room temperature 5 min. The DNA was divided in half and to each 75 µl aliquot 15 µl 20 mM ATP, approximately 5 µCi γ^{32}PATP, and 100 U T4 Polynucleotide Kinase (NEB) was added and incubated for 2 hours at 37°C. The kinase was inactivated by addition of 50 µl 10 M NH_4OAc and 100 µl H_2O and heated to 65°C for 15 min then cooled to room temperature. The DNA was purified by precipitating with 750 µl 100% ethanol, resuspending in 225 µl TE, then extracting with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was re-precipitated with NaOAc and ethanol then resuspended in 65 µl H_2O. Ten microliters of ligase buffer (NEB), 20 µl 20 mM ATP, and 5 µl 600 U/ml T4 ligase was added and incubated for 2 hrs. at room temperature after which the ligation was monitored by agarose gel. If no ligation has occurred, the purification, beginning with phenol/chloroform/isoamyl alcohol extraction, should be done again. Once the average size of the oligomers is approximately a 10-mer, the DNA is extracted with phenol then with chloroform/IAA, precipitated with 33 µl 10 M NH_4OAc and 133 µl isopropanol, resuspended in 225 µl TE then re-precipitated with NaOAc and ethanol. The purified DNA was resuspended in 50 µl H_2O. Three grams of CNBr activated Sepharose CL-4B (Pharmacia) was hydrated with 10 ml fresh 1 mM HCl for one minute then transferred to a 60 ml coarse sintered glass funnel. The resin was washed by gradually pouring 500 ml 1 mM HCl over the resin using a vacuum, then with 100 ml H_2O and finally with 100 ml 10 mM potassium phosphate buffer, pH 8.0 (add 1 M K_2HPO_4 to 1 M KH_2PO_4 until the pH reaches 8.0). The resin was transferred to a 15 ml conical tube and 10 mM potassium phosphate buffer was added until the resin has the consistency of a thick slurry. Immediately the two 50 µl

aliquots of DNA were added and incubated on a rotating wheel overnight at room temperature. The resin was transferred to a 60 ml sintered glass funnel and washed twice with 100 ml H_2O and once with 100 mL 1 M ethanolamine-HCl, pH 8.0. Most of the radioactivity should remain in the resin. The resin was transferred to a 15 ml conical tube and 1 M ethanolamine-HCl, pH 8.0 was added until the mixture was a smooth slurry. DNA coupling to the resin proceeded for 2-4 hrs at room temperature on a rotating wheel. The resin was washed with 100 ml 10 mM potassium phosphate buffer (pH 8.0), 100 ml 1 M potassium phosphate buffer (pH 8.0), 100 ml 1 M KCl, 100 ml H_2O, and 100 ml storage buffer (TE plus 20% ethanol). The affinity resin was resuspended in 10 ml storage buffer and stored at 4°C.

5.2c *Preparation of S-100 extract for native Pho2p purification.* 150 grams of BJ926 cells were washed twice in cold H_2O then resuspended in 2 ml/g solubilization buffer (0.2 M Tris-HCl pH 8.0, 10% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 10 mM β-mercaptoethanol, and 1 mM PMSF). Cells were lysed using a cooled bead beater with eight to ten 30 sec. bursts followed by 2 to 3 minutes of cooling. Lysis was monitored by microscope. The lysate was decanted and PMSF was added to 1 mM and β-ME was added to 10 mM. Four molar $(NH_4)_2SO_4$, pH 7.9 was added to bring the supernatant to 0.4 M and proteins were precipitated by stirring on ice for 15 min then centrifuged at 33,000 rpm in a Ti50.2 rotor for one hour at 4°C. 0.35 g solid $(NH_4)_2SO_4$ per ml of supernatant was added and proteins were precipitated by stirring at 4°C for 30 min then spinning at 36,500 rpm for 30 min in a Ti50.2 at 4°C. Proteins were resuspended in a minimal volume of AN0 (20 mM Tris-HCl pH 8.0, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 2 mM benzamidine) and dialyzed 8 to 10 hours against AN100 (AN0 plus 100 mM NaCl). Proteins were flash frozen on liquid nitrogen and stored at -

80°C. Protein concentration was determined by Coomassie Plus Protein assay reagent (Pierce).

5.2d *Purification.* One gram of S-100 extract was run on a 125 ml heparin-sepharose column equilibrated with H100 buffer (20 mM Tris-HCl 7.9, 12% glycerol, 1 mM EDTA, 1 mM DTT, 10 mM β-ME, 1 mM PMSF, and 100 mM KCl). The column was washed with H100 until the A_{280} returned to baseline. Proteins were step eluted with H600 (H100 containing 600 mM KCl). The column was run on an FPLC using a Pharmacia XK26 column at 2 ml/min with the absorbance set at 2. Eight milliliter fractions were collected during the H600 elution. Protein containing fractions were assayed for DNA binding affinity by EMSA. EMSA reactions contained 0.2 ng α^{32}PdATP labeled 24-mer oligo (same as that used for construction of DNA affinity column), 100 ng poly dIdC, 4X EMSA buffer, (12% glycerol, 20 mM Tris-HCl pH 7.9, 25 mM KCl, 1 mM EDTA, 1 mM DTT, and 100 μg/ml BSA), and fractions from the column. The total reaction volume was 12 μl. Complexes were incubated on ice for 30 min then run on a 5% 49:1 polyacrylamide gel for 1.5 hrs at 30 mA. Gels were dried and exposed on a Phosphorimager screen (Molecular Dynamics). Protein containing fractions were pooled then precipitated by the addition of 0.35 g/ml $(NH_4)_2SO_4$, stirred at 4°C for 30 min, then spun at 36,500 rpm for 30 min in a Ti50.2 rotor. Proteins were resuspended in B buffer (12% glycerol, 20 mM Tris-HCl pH 7.9, 0.1 M KCl, 1 mM EDTA, 1 mM DTT, 10 mM β-ME, and 0.1% NP40). Competition EMSAs with sonicated herring sperm DNA were then done to determine the correct amount of competitor for affinity column purification. The amount of competitor to use for the affinity column is one fifth the amount used for the EMSA if the EMSA reaction was scaled up directly (9). Instead of running the affinity column immediately following the heparin sepharose column, I decided to use a Mono-S column first to further purify the

protein pool. A 0.1 to 1.0 M KCl gradient was run in B buffer, and Pho2p containing fractions were determined by conventional EMSA and supershift EMSA using 0.5 µl of a polyclonal α-Pho2p antibody (a gift from David Stillman) added halfway through incubation. Peak fractions were pooled, dialyzed into B buffer with 100 mM KCl, incubated with 13 µg/ml herring sperm DNA for 10 min on ice, and injected onto a 1 ml affinity column equilibrated with B buffer containing 100 mM KCl. The protein pool and resin were incubated for one hour at 4°C, then the column was washed with B100 until the A_{280} returned to baseline. The protein was eluted with a 100 mM to 1 M KCl gradient. The affinity column was equilibrated at 0.2 ml/min and the gradient was run at 0.4 ml/min. Fractions were assayed by EMSA and Western Blot (Figures 5.6 and 5.7).

5.2e *Western Blots.* 12% SDS-PAGE gels were run with potential Pho2p containing fractions then blotted to PVDF membrane (Millipore) using the Bio-Rad electroblotter system. The primary antibody was α-Pho2p polyclonal antibody (kindly provided by David Stillman). Goat anti-rabbit secondary antibody conjugated to perixidase was purchased from Calbiochem. Protein bands were detected using the Renaissance Chemiluminescence Reagents (NEN).

5.2f *Transcription with native Pho2p.* An *in vitro* transcription assay was done to determine whether the native Pho2p I had partially purified from yeast whole cell extract would contribute to transcription activation, either alone or in combination with Pho4p. I used Pho4p, native Pho2p, and Pho2-His (purification described in Chapter 3). The results of this experiment are shown in figure 5.8. As I have already shown, Pho4p activates transcription. Neither preparation of Pho2p activated transcription. Addition of native Pho2p eventually inhibited transcription and Pho2-His had no affect on transcription. Conclusions from this experiment are that the native Pho2p contains a

contaminant that inhibits transcription at higher amounts of protein, and no stimulatory transcription activity is observed at any amount of protein. Pho2-His, described in Chapter 3, also contained no transcription activation activity, although it does bind DNA. This indicates that the regions which were truncated for cloning of the gene are necessary for transcription activation. Therefore I still believe that purification of full length native Pho2p from yeast is important.

5.2g *Discussion and Future Directions.* Native Pho2p can be significantly purified using DNA affinity chromatography. I was able to purify the protein enough to get a single band on a gel shift and this band had slower mobility when Pho2p antibody was added. This Pho2p contains no ability to activate transcription on its own, or to augment Pho4p activation. I feel that purification of this protein is important and would suggest an alternate method. It should be possible to construct a yeast expression vector with a full length Pho2p that has a tag. Using the mega primer method, *PHO2* can be amplified out of yeast genomic DNA and a 6-His tag attached. Expression of Pho2-His would be under the control of its own promoter. The construct would be inserted into a 2 μM plasmid (10 – 40 copies/yeast cell) and transformed into strain YS19 (*pho2*). A whole cell extract would be prepared from the transformed cells and Pho2p-His could then be purified using Ni-NTA resin. This project is currently being undertaken by undergraduate students in the lab.

5.3 Mononucleosome Reconstitutions

Using mononucleosomes has become a very popular method to study remodeling and protein factor binding to chromatin. We decided that mononucleosomes might be an ideal way to look at Pho4p binding and nucleosome remodeling. The following three

sections describe three different methods I have used for reconstituting mononucleosomes. All reconstitutions were done on the same 169 bp Mfe I to BstE II fragment from the *PHO5* promoter.

5.3a *Octamer transfer reconstitution.* This method was adapted from a chapter in Methods in Enzymology contributed by Dr. Jerry Workman. Twenty nanograms of γ^{32}PATP labeled probe was combined with three micrograms of bulk chromatin from the S-300 step of our yeast core histone purification (13) in a reaction volume of 12 µl containing 1 M NaCl, 50 mM Hepes-KOH (pH 7.5), and 1 mM EDTA. This was incubated at 30°C for 20 min. The reaction was diluted to 0.85 M NaCl, to 0.65 M, to 0.5 M, and finally to 0.3 M NaCl using buffer containing no salt. After each dilution the mixture was incubated at 30°C for 30 min. The reconstitution was then diluted to 0.1 M NaCl by the addition of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% NP40, 5 mM DTT, 0.5 mM PMSF, 20% glycerol, and 100 µg/ml BSA then incubated at 30°C for 30 min. Figure 5.9, lanes 2 through 7 is an EMSA of reconstituted mononucleosomes using octamer transfer. With all three bulk chromatin fractions used (F37, 38, and 39) there are three different shifted complexes indicating three different translational positions on the DNA fragment. This is a very common phenomenon and the complexes are the same as those obtained using Nap1p mediated reconstitution (Figure 5.9, lanes 8 and 9), discussed in the next section.

5.3b *Nap1p mediated assembly.* Yeast core histones and GST-Nap1p were combined in a ratio of 1:2 (w/w) respectively and incubated at 37°C for 15 min. This complex was added to the α^{32}PdATP labeled nucleosome probe in a ratio of 2.5:1 (complex:DNA) and incubated at 30°C for 5 - 6 hrs. To deplete the mononucleosome of Nap1p, a since determined unnecessary step, the assembly reaction was loaded onto a 0.5 ml Glutathione

Sepharose 4B (Pharmacia) column equilibrated in PBS plus 0.3 M NaCl, allowed to bind at room temperature, then eluted with the same buffer while manually collecting one drop fractions. Fractions were assayed for nucleosome assembly by EMSA. For all mononucleosome EMSAs approximately 0.5 ng DNA (assembled) was loaded on a 4.5 % 29:1 polyacrylamide gel (0.5 X TBE) and electrophoresed at 200 V for 6 hrs. The gel was dried and exposed on a PhosphorImager screen.

5.3c *Salt gradient reconstitution.* In a 100 µl volume 250 ng α^{32}PdATP labeled probe, 5 µg calf thymus DNA cut with Hha I (as a competitor), 2.5 or 5 µg purified yeast core histones, 2 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA were combined and dialyzed for 30 min. at 4°C in a microdialysis unit using 3500 MWCO dialysis tubing. Over 36 hours the reconstitution was dialyzed down to 0.5 M NaCl at 0.2 mL/min and then over 12 hrs the reconstitution was dialyzed into TE plus 1 mM DTT. Figure 5.10 shows an EMSA of salt gradient reconstituted mononucleosomes either immediately following reconstitution or after heating at 37°C for two hours which should allow the mononucleosomes to slide into a homogeneous position on the DNA and produce a single band on the EMSA. This was not the actual result. Upon heating, a small amount of the mononucleosomes fell apart, indicated by the increase of free DNA, and the multiple complexes did not consolidate into one band.

5.3d *Conclusions.* Based on the similar results obtained from all three mononucleosome assembly techniques, I can conclude that Nap1p mediated reconstitution works as well as the other methods and is much easier. Mononucleosomes are obviously required for structural studies (11) however I believe their usefulness is limited in the study of transcription activation and nucleosome remodeling. More information can be gathered about proteins binding to DNA through direct footprinting of a reconstituted plasmid

template using primer extension. In addition since remodeling and activation *in vivo* occurs on a large array of nucleosomes, using a reconstituted plasmid mimics the *in vivo* conditions more closely than a single nucleosome.

5.4 *In vitro* transcription with fractionated nuclear extract

As I showed in Chapter 3, fractionated nuclear extract, either alone in non-limiting amounts or in limiting amounts in addition to Pho4p, Pho2p, and ATP, can remodel chromatin. I also showed that whole cell extract can remodel chromatin but will not counteract repression of transcription. The next logical step was to test the effect of fractionated nuclear extract in an *in vitro* transcription assay. I found that fractionated nuclear extract could not counteract nucleosome repression of transcription (Figure 5.11), although it does remodel chromatin (Chapter 3). So, even though the promoter becomes nuclease transparent, counteraction of nucleosome repression does not occur. These results are an indication that activation and remodeling are two independent events. There are most likely additional requirements for transcription activation which were addressed in Chapter 3. One possible argument that was not discussed in Chapter 3 was that Pho2p, which I did not add to transcription reactions, may have been required. Although no exogenous Pho2-His was added to the transcription reaction, the fractionated nuclear extract was prepared from strain YS18 and the whole cell extract was prepared from strain YS25. Both strains are wild type at the *PHO2* locus, therefore endogenous Pho2p should be present.

5.5 Conclusions and Future Directions

We have succeeded in reconstituting nucleosomes on a closed circular plasmid template. The translational positions of the *in vitro* assembled nucleosomes closely

resemble the *in vivo* positions on the *PHO5* promoter. Our system is unique because it uses only purified components for the chromatin reconstitution. This work is described in Chapter 4 of this dissertation.

Using our *in vitro* reconstitution system, I have set up a system to study chromatin remodeling and transcription activation of *PHO5 in vitro*. Developing this system involved construction of a G-less cassette plasmid on which nucleosomes are correctly positioned with respect to the *PHO5* promoter, preparation of transcription extracts from wild type, *pho4*, *pho2*, and *pho4/pho2* cells, and purification of the transcription factors involved in *PHO5* transcription.

I have shown that nucleosomes can be remodeled on our templates in several different situations. Remodeling can occur with YS25 whole cell extract alone, with non-limiting amounts of fractionated nuclear extract from YS18 cells, or with limiting quantities of fractionated nuclear extract plus Pho4p, Pho2-His, and ATP. Pho4p can activate transcription from the naked *PHO5* promoter however, no activation, or relief of nucleosome repression, can be observed under any circumstances, even on remodeled chromatin templates.

The activity that remodels *PHO5* chromatin *in vitro* could potentially be isolated by further fractionation of the nuclear extract. It would also be interesting to purify the SWI/SNF complex from yeast (6) and test it in our system. Although this complex does not remodel *PHO5* chromatin *in vivo*, it would likely be a useful positive control for our *in vitro* system. I was able to partially purify native Pho2p, but this protein actually repressed *in vitro* transcription. Since the truncated His-tagged version of Pho2p also did not activate transcription *in vitro*, I believe it is important to continue with purification of native Pho2p, since it is obviously required for full *PHO5* expression *in vivo*. Finally, I believe that it might be worth the significant effort to use the minichromosome isolation protocol developed by the O'Shea lab (7) for some *in vitro* transcription experiments.

The plasmid pMHG-less(mfe) is a yeast 2 μM vector and could be transformed into any of our YS laboratory strains. The plasmid contains a *URA3* selection marker and the YS strains require uracil for growth. Non-histone chromosomal proteins possibly required for transcription on a nucleosomal template might be associated with these *in vivo* assembled templates. In addition, since preparation of crude FACT activity can be performed by running a single phosphocellulose column with HeLa nuclear extract (12), it would be interesting to purify this activity in anticipation that it might relieve the barrier to transcription activation on our templates.

In conclusion, although I have been unsuccessful in elucidating the mechanisms of transcription activation on a chromatin template, I was able to observe nucleosome remodeling. I believe the most important outcome of my project was the development of a very well defined system for further study of the mechanisms of gene expression in the context of chromatin.

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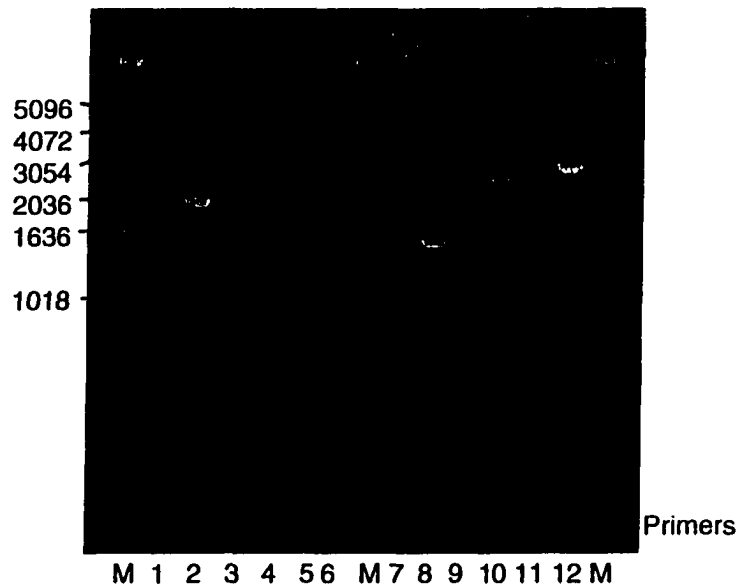

Figure 5.1. Ethidium stained gel of PCR products. M lanes are marker lanes with the sizes in base pairs indicated to the Left of the gel. Lanes 1 through 6 contain products amplified using PHO2 primers. Lanes 7 through 12 contain products Amplified using PHO4 primers. Expected product sizes and specific reaction conditions are included in the text.

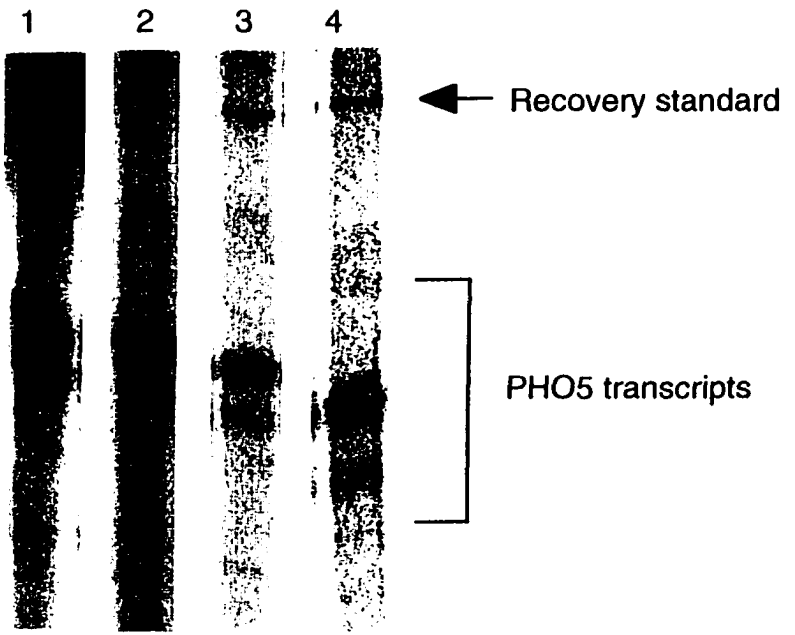

Figure 5.2. Transcription using wild type (BJ926) in lane 1, YS25 in lane 2, YS27 in lane 3, and YS19 in lane 4. All reactions contained 500 ng pMHG-less(mfe) and 250 micrograms BJ926 extract, 150 micrograms YS25 extract, Or 100 micrograms YS27 and YS19 extracts. Transcripts are 110 to 120 bases in length due to multiple start Sites of PHO5.

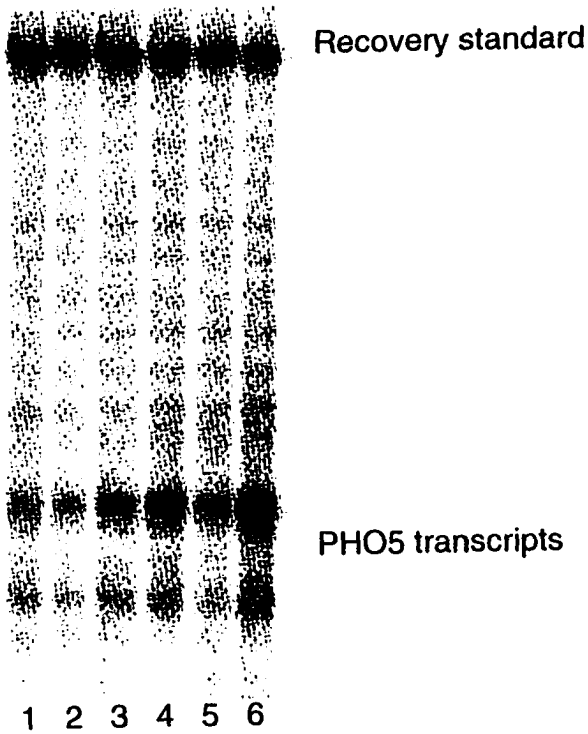

Figure 5.3. Alpha amanitin sensitivity assay. Transcription using 300 ng pPHO5G-less and YS25 whole cell extract. Lane 1 contains 875 ng alpha amanitin; lane 2, 350 ng; lane 3, 175 ng; lane 4, 70 ng; lane 5, 17.5 ng; and lane 6 contains no alpha amanitin. Results verify that RNA polymerase II transcribes from the PHO5 promoter *in vitro*.

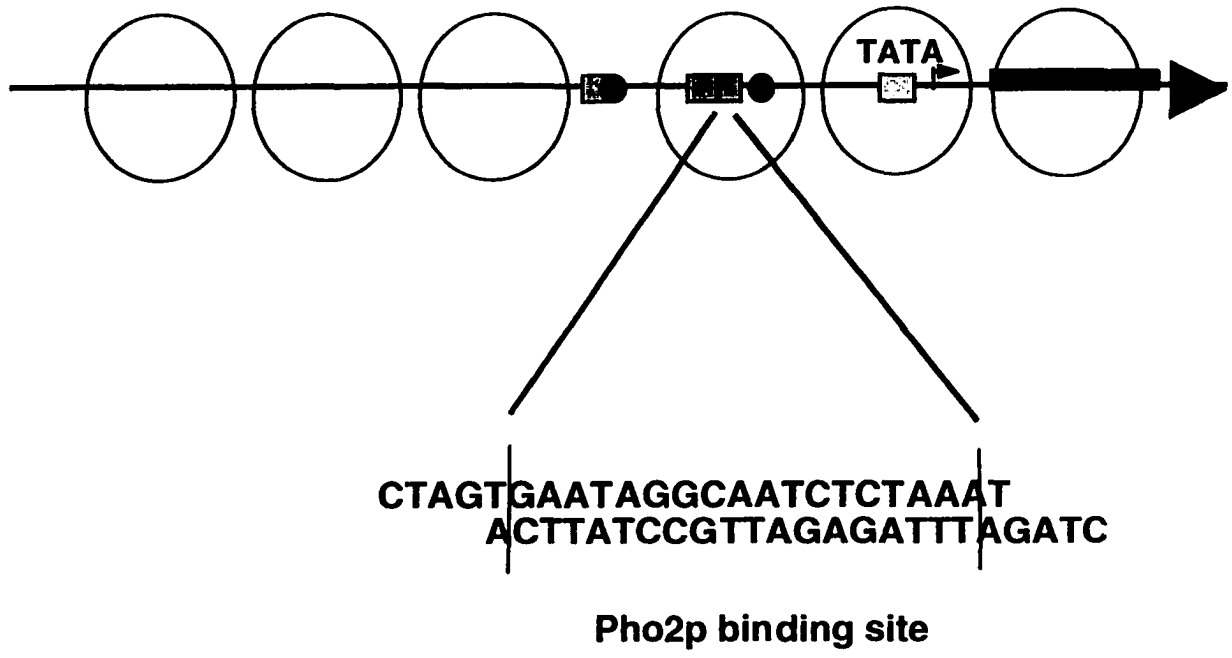

Figure 5.4. Oligonucleotide sequences used for construction of DNA affinity column and for EMSAs during native Pho2p purification.

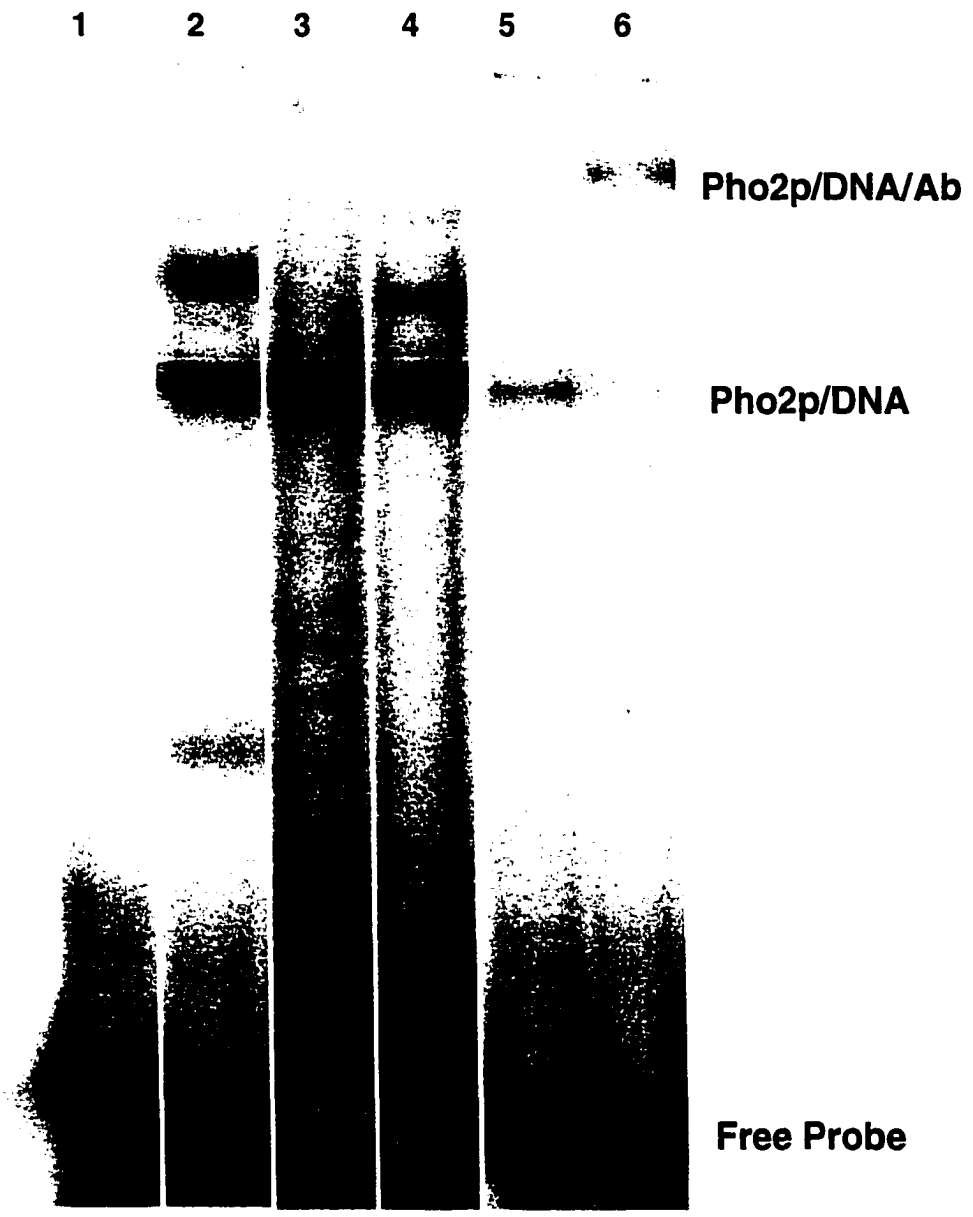

Figure 5.5. EMSA showing stages of native Pho2p purification. Lane 1 contains free probe, lane 2 is the S-100 extract, lane 3 is the heparin sepharose pool, lane 4 contains the peak Pho2p containing fraction from the Mono-S column, lane 5 contains the peak fraction from the DNA affinity column, and lane 6 contains the affinity column fraction plus α-Pho2p antibody. Lanes 1 through 4 contain 100 ng of poly dIdC DNA as a competitor, lanes 5 and 6 contain no competitor.

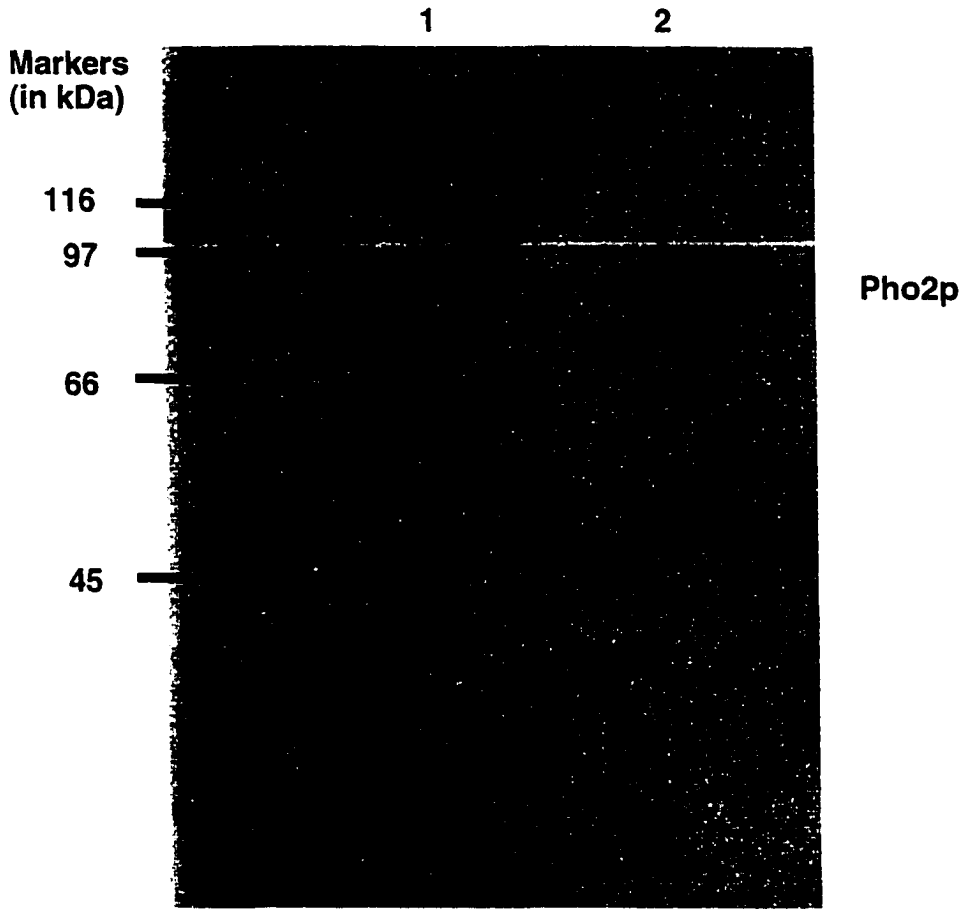

Figure 5.6. Western blot of purified native Pho2p. Lane 1 contains 10 microliters and lane 2 contains 30 microliters of the peak Pho2p binding fractions from a single round of affinity column purification. Prior to the affinity column, an S-100 whole cellextract was purified on a heparin-sepharose column followed by a Mono-S column. The primary antibody is rabbit anti-Pho2p (a gift from David Stillman's lab) and the secondary antibody is goat anto-rabbit conjugated to HRP (Calbiochem). Detection was done using the Renaissance Chemiluminescence Reagent (NEN). Pho2p is an 83 kilodalton protein.

Figure 5.7. Transcription with 250 ng pMHG-less(mfe) and 100 µg YS27 whole cell extract (*pho4/pho2*). Lane M is a marker lane and contains an end labeled MspI digest of pBR322. Lanes 2 and 3 contain Pho4p alone which activates transcription (and at higher amounts inhibits, lane 4). Lanes 5, 6, and 7 contain Pho2-His which does not activate transcription on its own. Lanes 8 and 9 contain Pho4p and Pho2-His. There is no additional activation beyond that of Pho4p alone (in lane 10 there is inhibition by Pho4p again). Lanes 11, 12, and 13 contain purified native Pho2p alone which does not activate transcription. Lanes 14, 15, and 16 contain native Pho2p and Pho4p, again there is no additional activation beyond that seen with Pho4p (native Pho2p strongly inhibits transcription at higher amounts, lanes 13 and 16).

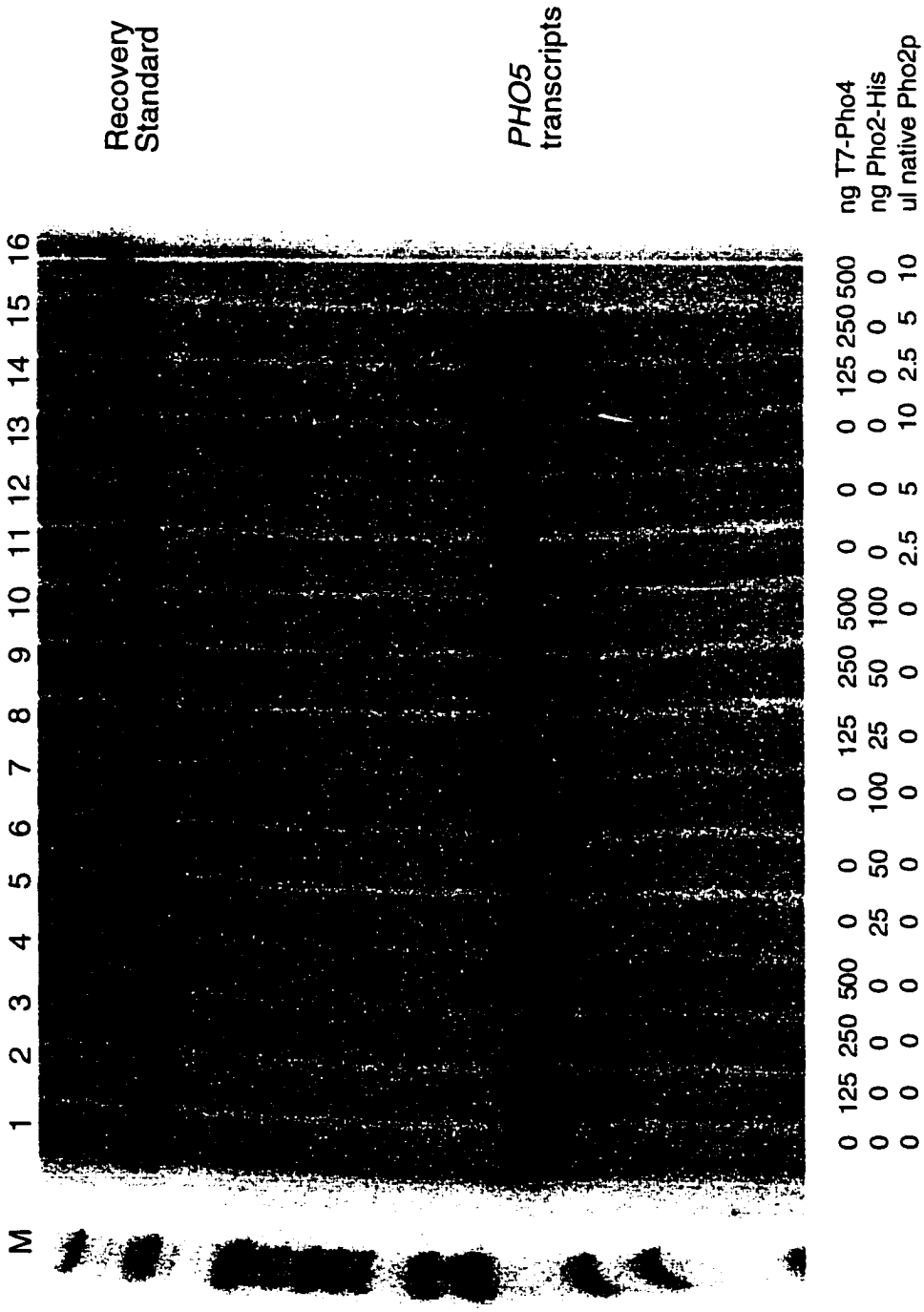

Figure 5.7

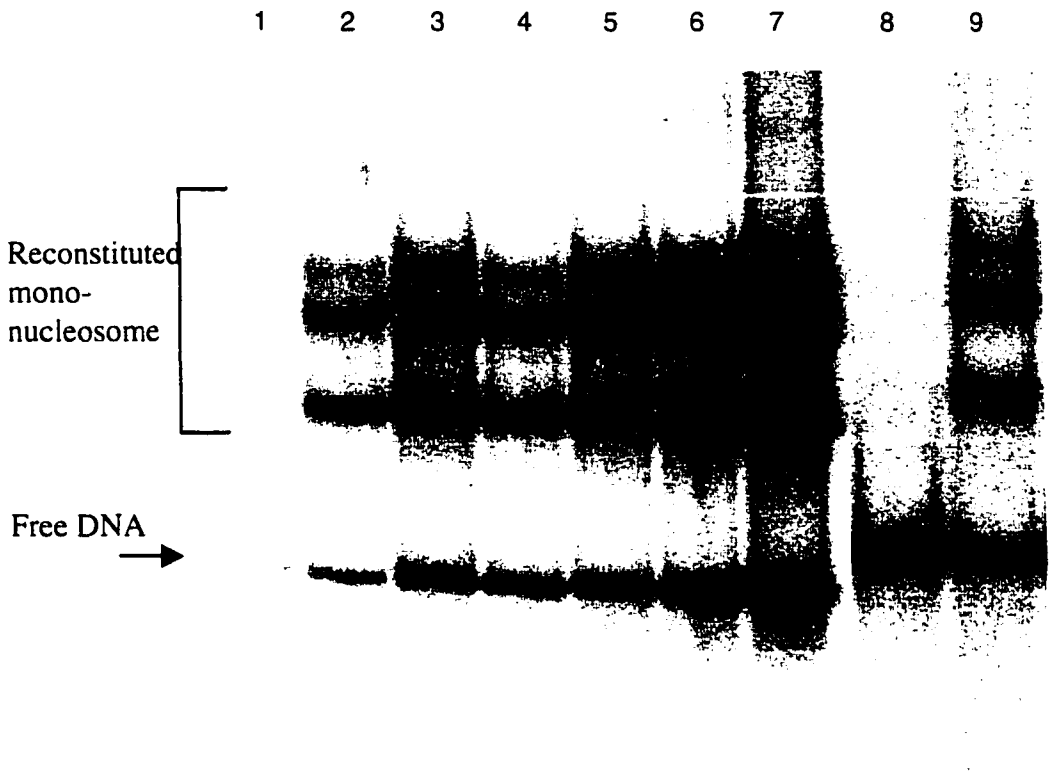

Figure 5.8. Reconstitution of mononucleosomes using octamer transfer reconstitution and Nap1p mediated reconstitution. Lanes 2 through 7 contain octamer transfer reconstituted mononucleosomes. Lane 9 contains Nap1p mediated reconstituted mononucleosomes. Lanes 1 and 8 contain free probe, a 169 base pair *Mfe* I to *BstE* II DNA fragment from pMHG-less(mfe). Lanes 2 and 3 contain 6 µl and 12 µl of the reconstitution reaction, respectively, using fraction 37 from the S-300 column. Lanes 4 and 5 contain 6 µl and 12 µl of the reconstitution using fraction 38. Lanes 6 and 7 contain 6 µl and 12 µl of the reconstitution using fraction 39. Reconstitutions contained a 150:1 ratio of bulk chromatin to DNA (w:w). Lane 9 contains reconstituted mononucleosomes using Nap1p mediated reconstitution with purified yeast core histones. Nap1p/core histone complex was formed using a 2:1 Nap1p to core histones ratio, and nucleosomes were formed using a 10:1 complex to DNA ratio (all w:w).

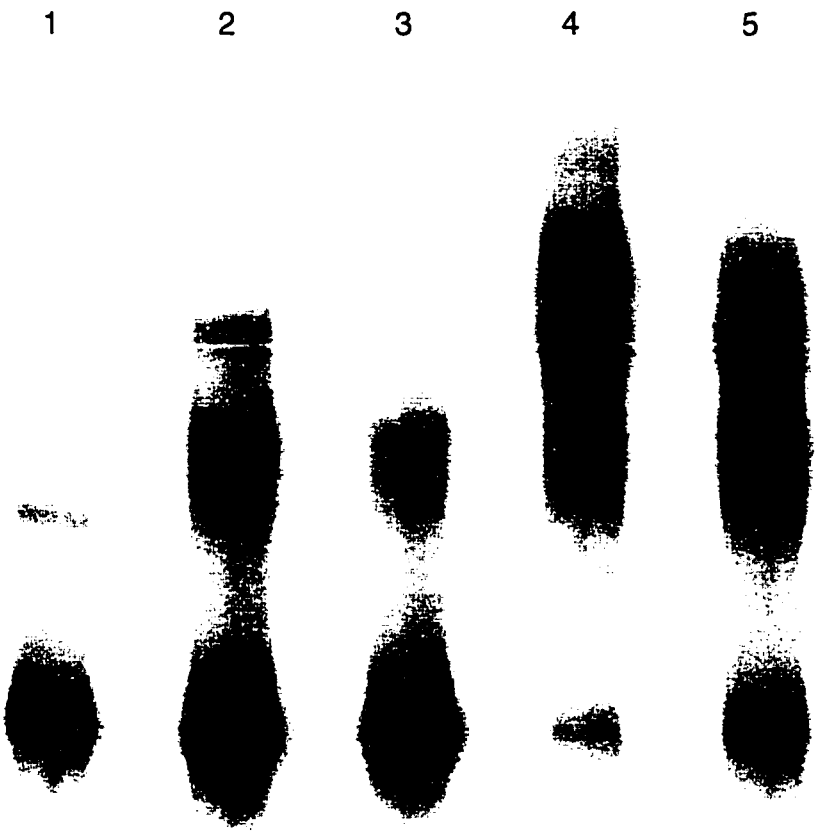

Figure 5.9. Salt gradient reconstitution of mononucleosomes using a labeled 169 base pair Mfe I to BstEII DNA probe and calf thymus DNA as the unlabeled competitor. Lane 1 contains free probe. Lane 2 contains reconstituted mononucleosomes at a ratio of 0.5:1 (w:w) with respect to the amount of calf thymus DNA. Core histones are purified yeast core histones. Lane 3 is identical to lane two except that the reconstitution reaction was heated to 37°C prior to loading on the gel. Lane 4 contains reconstituted mononucleosomes at a ratio of 1:1 with respect to calf thymus DNA. Lane 5 is identical to Lane 4 except that reaction was heated to 37°C for two hours prior to loading on gel.

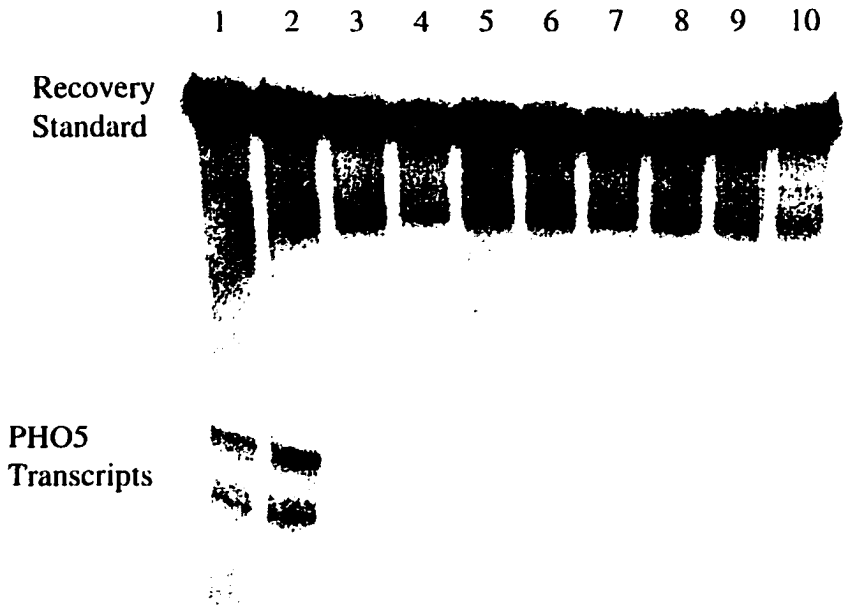

Figure 5.10 *In vitro* transcription with fractionated nuclear extract. Lanes 1 and 2, naked DNA; all other lanes, reconstituted chromatin. All reactions contain 500 ng pMHG-less(mfe) and even numbered lanes contain 500 ng T7-Pho4p. Lanes 3 and 4, extract; lanes 5 and 6, 5.3 µg extract, lanes 7 and 8, 10.5 µg extract, and lanes 9 and 10, 16.6 µg extract.