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DISSERTATION

MEDIATION OF GENE EXPRESSION VIA HISTONE-DNA INTERACTIONS: NUCLEOSOME POSITIONING ON THE *PHO5* GENE OF *SACCHAROMYCES CEREVISIAE*

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOHN LEONARD PILON III ENTITLED MEDIATION OF GENE EXPRESSION VIA HISTONE-DNA CONTACTS A STUDY IN NUCLEOSOME POSITIONING ON THE PHO5 GENE OF SACCHAROMYCES CEREVISIAE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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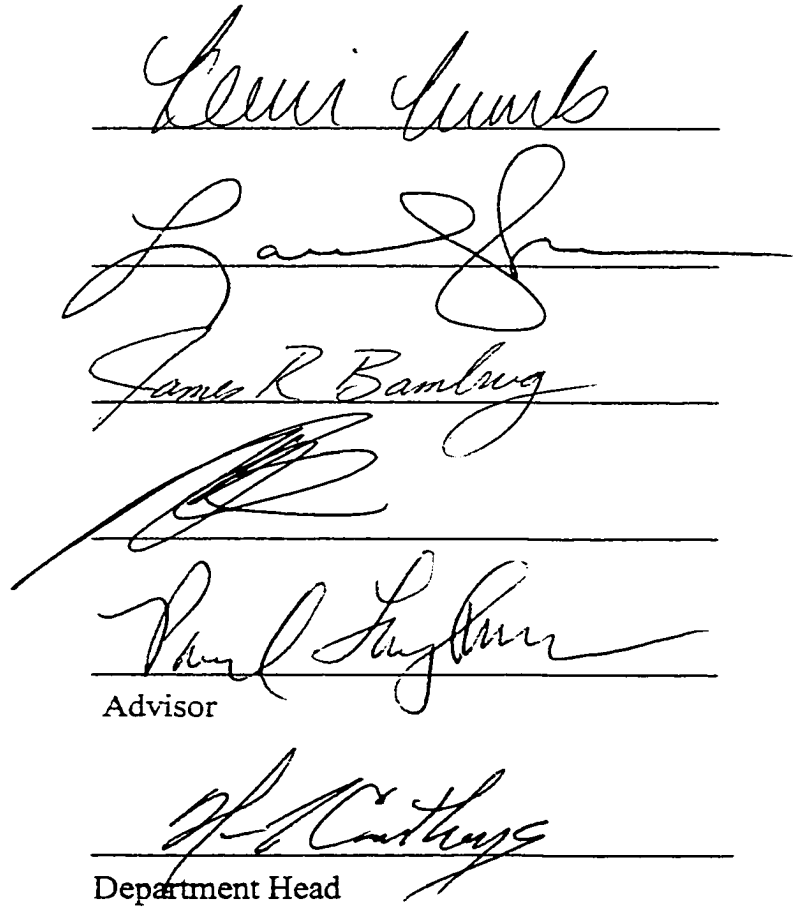

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MEDIATION OF GENE EXPRESSION VIA HISTONE-DNA INTERACTIONS: NUCLEOSOME POSITIONING ON THE *PHO5* GENE OF *SACCHAROMYCES CEREVISIAE*

ABSTRACT

The vast majority of genetic information in eukaryotic cells must be packaged in the highly repressive environment of chromatin to fit within the confines of the cell nucleus. To fully understand how genes are expressed the chromatin environment in which all transcription processes occur should not be overlooked. This study will focus on developing an understanding of how chromatin structure at the level of the nucleosome must become ordered to result in coordinated gene expression. The *PHO5* gene of the yeast *Saccharomyces cerevisiae* will be utilized for this research. The *PHO5* gene encodes a secreted acid phosphatase that is activated in conditions of low phosphate. In the repressed

secreted acid phosphatase that is activated in conditions of low phosphate. In the repressed state, four nucleosomes are positioned along the promoter region causing repression by blocking access of the basal transcription machinery to the minimal promoter element, while a critical nucleosome-free region is maintained to allow binding of the transcriptional activator Pho4p to the promoter region. Exactly how this nucleosome array is formed is not known. This document contains the development of an *in vitro* chromatin reconstitution system used to study the mechanism of gene activation and repression in the context of chromatin. This system, created entirely from the yeast *Saccharomyces cerevisiae*, was used to recreate the repressed state of the *PHO5* gene as demonstrated by nucleosome positioning and repression of transcription *in vitro*. The presence of proper nucleosome positioning in an *in vitro* setting show that intrinsic DNA properties are sufficient to properly position nucleosomes on the *PHO5* promoter. Moreover, it was demonstrated that variability in general isotropic DNA bending could largely account for nucleosome positioning on the *PHO5* promoter. This work reveals how histone-DNA contacts create an initial layer of gene regulation that we are now only beginning to understand.

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Chapter 1

Introduction and Research Aims

1.1A The nucleosome and histone proteins

The 46 chromosomes in a human somatic cell contain over a meter of DNA. This vast amount of genetic information must be constrained to fit within the nucleus, which measures approximately 5 microns in diameter (Walbot & Holder, 1987). This magnitude of packing is largely accomplished by the formation of protein-DNA complexes. This protein-DNA complex termed the nucleosome is the fundamental repeating unit of chromatin. The possibility that chromatin could be composed of a repeating unit was hinted at when chromatin was subject to digestion by nucleases. When the DNA fragments were resolved, a periodicity in length was shown to occur (Fig1). This periodicity, occurring throughout the

genome (80%), was subsequently shown to consist of a nuclease resistant core of 146 bp of DNA. This core could be isolated with its protein compliment, which was later identified to consist of four types of protein. These experiments eventually led to the nucleosome model proposed by Kornberg in 1974 (Kornberg, 1974).

The nucleosome is now known to contain 160-200 bp of DNA wrapped in 1.75 helical turns around an octamer of core histone proteins (Elgin, 1995). The nucleosome also has a linker region of DNA adjoining adjacent nuclease resistant nucleosome core particles (NCP). A linker histone called H1 is present in eukaroytes with the apparent exception of the yeast *Saccharomyces cerevisiae.* The amount of DNA associated with a nucleosome (repeat length) can vary in different organisms. For example, a repeat length of 166 bp is seen in yeast while higher eukaryotes show repeat lengths closer to 200 bp. However, the size of DNA associated with the core particle on more extensive nuclease digestion remains constant at 145 bp +/-2 bp across phylogeny. The path of the DNA as it enters and leaves the nucleosome occurs at the dyad axis. Noll (Noll, 1974) demonstrated a periodicity in DNA cleavage occurs when core particles are digested with the minor groove specific nuclease DNase-I. This occurs as the minor and major groove alter their orientation to the solvent. These early experiments confirmed that the DNA lies on the outside the NCP.

The histones proteins of the nucleosome are present in roughly 60 million copies per cell, which is nearly equal to the mass of the DNA itself(Van Holde, 1989). The histone

class of proteins consists of four core histones H2A, H2B, H3, H4 and the linker histone H1. Minor amounts of histone variants are also present in specialized areas of chromatin or during different phases of the cell cycle. These small basic proteins contain high ratios of lysine and arginine residues, resulting in net positive charge at physiological pH. This large net positive charge is mostly distributed in the N terminal region of the histones, and somewhat in the C terminus of histone H3 and H2A. The remainder of the core histone proteins contain similar amounts of acidic, basic and hydrophobic residues. Histone H1 is very lysine rich, with a molar percentage of 29.5 and a net charge of +58 at physiological pH (Bradbury *et al.*, 1981).

The histone proteins are highly conserved through evolution. Histones H2A and H2B are somewhat less conserved than their counter parts H3 and H4, while H1 is highly variable when compared to other proteins. Most of the variability of the histone proteins occurs in the N terminal tail domain of H2A, H2B and H1, whereas the core regions remain conserved. Histones H3 and H4 show nearly unparalleled sequence conservation. Histone H4 has only 9 different residues between calf, chicken, sea urchin, and yeast, with each protein being 102 residues long. Histone H3 also has a high degree of sequence homology across phlyogeny with calf, chicken, and sea urchin all being nearly identical (Van Holde, 1989). Moreover, of the over twenty H3 sequences available, only one does not contain 135 residues. The facts presented above demonstrate the critical role played by H3 and H4 in forming nucleosome

structure. Furthermore, due to the high degree of sequence conservation, it can be assumed that histone-DNA interactions are one of the most fundamental protein/DNA interactions occurring in the eukaryotic genome.

Core histone proteins can associate to form heterotypic dimers and tetramers in solutions containing high concentrations of salt. Isolated core histone proteins in low ionic strength are loosely coiled monomer's as the ionic strength of the solution is increased, the core histones fold into their familiar domains. D'anna and Isenberg studied the thermodynamics of the observed heterotypic histone interactions (D'Anna & Isenberg, 1972). They found that the strongest of these interactions is the formation of the H3/H4 tetramer and the H2B/H2A dimer. These hetrotypic interactions may stabilize secondary structure and are the typical state of the core histone proteins in solution at physiological pH and ionic strength. Further associations between the H3/H4 tetramer and the H2A/H2B dimer can occur to yield the core histone octamer. This can occur in concentrated solutions of core histones, in the presence of DNA, or in solutions with high ionic strength. This assembly is viewed as a two step process as shown below:

$$(H3/H4)_2 + (H2A/H2B) \longrightarrow (H3/H4)_2(H2A/H2B) \qquad \text{reaction 1.}$$

$$(H3/H4)_2(H2A/H2B) + (H2A/H2B) \longrightarrow (H3/H4)_2(H2A/H2B)_2 \qquad \text{reaction 2.}$$

The dominant force driving the formation of the octamer appears to be hydrogen bonding between histidine-tyrosine residues as shown by spectroscopic measurements. The data

collected show that of the 8 tyrosine residues present in the H2A/H2B dimer 5 become inaccessible to solvent in the octamer. Furthermore, the H3/H4 tetramer has 14 tyrosine residues of which all become solvent inaccessible in the octamer.

Histone proteins have been shown to undergo a variety of post-translational modifications. Of these modifications, the most well studied and understood is histone acetylation. The process of histone acetylation is carried out by histone acetyl transferases HATs. Two general types of HAT activity have been identified. Type B HATs can be isolated from the cytoplasm and catalyze the transfer of an acetyl moiety from acetyl-CoA to the ε-amino group of Lysine 12 of free histone H4 (Sternglanz, 1996). The role of type B HATs is to facilitate the formation of nascent chromatin from newly synthesized histones. Type A HATs are found within the nucleus and target histones in the form of nuclesomes (Kuo & Allis, 1998). HAT activity has been found associated with several transcriptional regulators. Histone deacetylases (HDAC) have also been identified and are associated with general repression of transcription (Pazin & Kadonoga, 1997). The role of these opposing enzymes will be discussed further in section 1.3B below.

1.1B Higher order chromatin structure

The packaging achieved by formation of the nucleosome is far from adequate to achieve the level of compaction needed to fit the DNA of a somatic cell within the nucleus.

Indeed, the wrapping of DNA into a nucleosome only achieves a five-fold compaction. How a linear array of nucleosomes folds to yield higher order structures is not fully understood. Early results by Thoma et al. showed that H1 plays a critical role in facilitating the folding of higher order chromatin fibers (Thoma *et al.*, 1979). The experiments utilizing electron microscopy showed that if H1 was present the chromatin fiber took on a zigzag type of appearance. Chromatin that had been stripped of H1 does not under go such a transition and appears unfolded in low ionic strength. These early experiments indicated the critical role for H1 in chromatin condensation, but *in vivo* relevance of some of the early EM experiments were questioned. The solution studies of Hansen et al. used analytical ultracentrifugation to study the effects of divalent ions on chromatin folding in the absence of H1 (Hansen *et al.*, 1989). The results showed that oligonuclesomes undergo three defined transitions as the Mg^{2+} concentration was increased. At Mg^{2+} concentration of <1mM a species that sediments at ~29S was observed. At Mg^{2+} concentrations of 1-2mM, sedimentation occurred in the 40-55S range. Although the populations were heterogeneous, these experiments indicate that folding of chromatin will occur in physiological relevant conditions in the absence of H1. Other experiments using scanning force microscopy show irregular open helical structures ~30 nm in diameter in low ionic strength (Woodcock *et al.*, 1993). How a chromatin fiber folds to yield the compaction ratios necessary for the placement of the genome in the nucleus is still unclear.

In the nucleus, one must consider the constraints placed on the formation of higher order chromatin structure, such as association of chromatin with nuclear laimins. Chromatin can associate strongly with the carboxy terminus of lamins with K_d=120-300 nM (Taniura *et al.*, 1995). Association of chromatin to the nuclear lamins occurs through the protein component of the NCP. This can effectively anchor chromatin to the nuclear envelope creating loop domains in the range of 30-50 kbp. These domains may provide a way to organize the interphase nucleus and provide for separating DNA into distinct transcriptional and topological domains. Evidence for transcriptionally active domains has been demonstrated in the chick lysozyme gene (Stief *et al.*, 1989). This domain is relatively sensitive to DNase-I digestion and covers ~24 kb. This region containing a 4 kbp transcript, is flanked by two scaffold attachment sites where the DNA is bound to the nuclear envelope. These nuclear attachment elements at the 5' and 3' ends of the chicken lysozyme gene transfected in cells conferred expression of the lysozyme gene independent of its chromosomal location.

1.1C Thermodynamics of nucleosome assembly

The thermodynamic parameters associated with the formation of the nucleosome are of great importance to the work presented herein. Tyrosine fluorescence and circular dichroism have been used to elucidate the energy associated with nucleosome formation.

Khrapunov et.al. used salt titration experiments to measure the K_d of the dissociation of H2A/H2B dimer from the nucleosome from DNA and K_t for the H3/H4 tetramer from the DNA (Khrapunov *et al.*, 1997). The change in fluorescence was measured as a function of ionic strength and dissociation constants calculated. These equations are shown below:

Equation 1: interaction of 2(H2A/H2B) dimers with H3/H4-DNA :

$$\log K_d = 5.9\text{-}16.2 \log[NaCl]$$

Equation 1B: interaction of 2(H2A/H2B) dimers with DNA

$$\log K_d = 1.8\text{-}7.1 \log [NaCl]$$

Equation 2: interaction of (H3/H4) tetramer with DNA:

$$\log K_t = 6.4\text{-}13.1 \log[NaCl]$$

Free energy change is then calculated from the relation $\Delta G = -RT\ln K$. With these equations the free energy change of the reaction can be calculated.

Equation 3:

$$\Delta G_{nuc} = \Delta G_{(H3H4)/DNA} + \Delta G_{2(H2A/H2B)/(H3H4\text{-}DNA)}$$

Thus, the resulting free energy change on the formation of a nucleosome at 1M NaCl is –17.9 kcal/mol. Association of the (H2A/H2B) dimer with the (H3/H4) DNA complex involves both interaction of the dimer with the DNA and the tetramer. The sum of these

interactions plus the contributions of mixing entropy (2.4 kcal/mol) are ~ -12.2 kcal/mol which is more than the experimentally measured value of -8.1 kcal/mol. The reason for this discrepancy can be accounted for in the energy required to bend the DNA around the (H2A/H2B) dimer.

Investigations into the sequence dependence of the energy of nucleosome formation have been conducted by Oohara and Wada using the same techniques employed in the above study. The results showed that the free energy of formation of a nucleosome fluctuates depending on the underlying DNA sequence (Oohara & Wada, 1987a; Oohara & Wada, 1987b). The stability of a nucleosome followed the order poly[(dA-dT)] > chicken erythrocyte DNA > poly[dG-dT)]. Calculations of the free energy of nucleosome formation from equations 2, 3 are as follows:

ΔGnuc poly[(dA-dT)] = -15.9 kcal/mol

ΔGnuc Chicken erythrocyte = -14.8 kcal/mol

ΔGnuc poly[(dG-dC)] = -13.4 kcal/mol

These results are slightly different then the value calculated above due to using a NaCl concentration that is the midpoint of the titration curve. It is evident from this set of experiments that the underlying DNA sequence can play a role in the free energy of nucleosome formation.

The real *in vivo* free energy of nucleosome formation is likely to be very different than the values calculated above. Indeed, the assumptions made in the above work are open to serious criticism (Drew, 1991). The free energy of nucleosome formation is likely to be related to post-translational modifications, ionic strength, and topological state of the DNA. Nonetheless, due to the impossibility of measuring the free energy of nucleosome formation *in vivo*, these experiments provide valuable insight into the thermodynamics of nucleosome assembly.

1.1D Crystal structures of the nucleosome

The crystal structure of the nucleosome has been solved to a low resolution of 7 angstroms by Richmond and co-workers (Richmond *et al.*, 1984). In this early work many previous observations were confirmed and new ones made. The nucleosome was confirmed to be disk shaped as suggested in early EM experiments. The second observation was that the path of DNA around a nucleosome is not uniform. It was found to bend tightly at defined locations while at other areas the path of DNA was strait. Eight years after this initial structure was solved, Arents and coworkers published a structure of the nucleosome to 3.1 angstroms. This study revealed a tripartite structure to the histone octamer in the nucleosome when viewed down the dyad axis. Also evident was the presence of the H3/H4 tetramer located in the central core of the nucleosome, while the H2A/H2B dimers appeared

as two smaller masses on either side of the H3/H4 tetramer. Interestingly, all of the core histone proteins were shown to be folded into similar domains. This helix-loop-helix motif, termed the histone fold, consists of a long central α helix bracketed by a loop and a shorter α helical region (Fig. 2). The dominate feature of the interaction between adjacent protein units was identified and termed the hand shake motif. This motif can be identified as the long helix passes under the same α helix of the neighboring protein. Publications quickly followed that identified the histone fold motif in the TBP associated factors (*Drosophila* TAF 42-TAF 62, human TAF 31-TAF15) and in the DNA packing protein HMfB of archaebacteria. It has been shown that, dTAF 42 (hTAF 31) share sequence homology with H3 hinting at the possibility of gene duplication of H3 to create dTAF 42 (Luger & Richmond, 1998; Xie *et al.*, 1996).

The most recent crystal structure solved by Luger et al. in 1997, resolved all of the core histones proteins as well as the DNA to 2.8 angstroms. This study confirmed the results of the above studies and elucidated in greater detail the path of the DNA around the nucleosome and the type of contacts that occur between histones and DNA. This work also furthered our understanding of how core histone tails may help in organizing chromatin structure.

To form high quality crystals it was necessary to use recombinant *Xenopus* core histones individually expressed in *E. coli,* and reconstituted with a palindromic repeat of

human α satellite DNA 146 bp in total length. The DNA in the crystal structure was found to contain exactly one base pair located at the dyad. This effectively split the DNA in contact with the NCP into 73 and 72 bp half's. The entry point of the DNA at the dyad was designated superhelix location (SHL) 0. As shown in Richmond's earlier work, the DNA is indeed sharply bent, having maximum curvature at SHL +/-1.5 and SHL +/-4.5 (Fig 2). B factor calculations show the DNA to be fixed at locations where the minor groove contacts the histone face while the opposing side of the DNA helix had higher degrees of motion. The DNA takes a path around the histone octamer that aligns the major and minor groove between each half of the NCP. This path results in channels being formed on the surface of the NCP.

Histone fold-DNA interactions occur every time the minor groove faces the protein surface. The types of interactions seen are summarized into five types. The N-termini of the α1 helix of H3, H4, H2B are used to bridge to individual phosphate groups through the positive charge generated by the helical dipole. A large number of protein-DNA interactions are through arginine side chains from the histone fold region. An arginine side chain projects into the minor groove 10 of the 14 times it faces the protein surface. Of the four remaining minor groove contact points an arginine residue from the N terminal tail region enters the minor groove. Non-polar interactions between residues and the deoxyribose group occur for example, at super helix 1.5, a proline residue in the first turn of α1 of H3, H4 contacts a

ribose group. Lastly, hydrogen bonds and salt links are frequently seen between the phosphate oxygen atoms and basic and hydroxyl residues of the core histones. Some of these interactions are shown in figure 3. These five types of interactions are seen at the three DNA binding sites within each individual histone. The three contact sites are two in the loop regions (L1, L2), and one from the N terminal α helix (α1). The α1 DNA binding sites have a smaller contact area than the L1,L2 sites. The integrity of the NCP was shown to be dependent on these L1,L2 contact sites.

A portion of the random coil tail regions of H3 and H2B were resolved in this structure. The tail region of these two histones project out between the channels made in the minor grooves from adjacent DNA gyres. This gives a periodicity such that a tail region extends out from the nucleosome every twenty base pairs. While the majority of the histone tail regions were unable to be resolved, the lysine rich H4 N terminal tails were shown to make interparticle contacts with H2A/H2B on the face of the juxtaposed nucleosome. The basic H4 tail region of K16 to N25 was shown to form salt bridges with an acidic patch on the H2A/H2B dimer to facilitate this interaction.

1.1E Gene regulation in the context of chromatin

The packing of the DNA into the interphase nucleus creates obvious barriers to gene expression. How is it possible for the transcription machinery to gain access to their cognate

sites? How is it possible for the RNA polymerases to transcribe genes when the coding sequences are nucleosomal? Experiments with light microscopy in the 1930s, identified two general types of chromatin in the nucleus of higher eukaroytes. Chromatin that remainded in a highly condensed state was termed heterochromatin, while chromatin that was less condensed was termed euchromatin. Euchromatin was subsequently shown to be highly accessible to nuclease digestion and contain genes that are to be transcribed. This transcriptionally active chromatin was enriched from whole chromatin by chromatography and differences in solubility, and was found to contain histones with a high degree of acetylation as well as an absence of H1 (Allegra *et al.*, 1987; Komaiko, 1985). A plethora of research in the last decade has shown chromatin to be the substrate for a vast array of biochemical activities and processes. This has firmly established chromatin as an active participant in the regulation of gene expression.

Kim and colleagues developed a yeast strain (UKY403) that had the expression of histone H4 under the expression of the GAL promoter (Kim *et al.*, 1988). This construct allows expression of H4 to be turned off by switching the carbon source from glacatose to glucose resulting in nucleosome depletion. Durrin and Grunstein used nucleosome depletion to study changes in the expression of the regulated genes *PHO5, CUP1* and *HIS3* (Durrin *et al.*, 1992; Han *et al.*, 1988). The results showed that nucleosome loss caused increases in transcription even when the regulatory upstream activation sequences (UAS) had been

deleted. Moreover, when the promoters of *CUP1*, and *HIS3* had been fused to the *E coli* β – galactosidase gene (*Lac Z*), increases in transcription were seen upon nucleosome depletion.

In the above experiments nucleosomes appear to play a passive role in the regulation of genes by simply blocking access of the transcription apparatus to the DNA or creating unfavorable DNA topology for transcription. However, a more active role can be played by the core histone proteins, in particular the N-terminal regions of H3 and H4. Durrin (Durrin *et al.*, 1991) deleted the N-terminal tails of H4 and showed they were required for full activation of the GAL 4 regulated genes *GAL1*, *GAL7*, *GAL11* of *Sacharomyces cerevisiae* (1991). Activation of the *PHO5* gene was also reduced 4-5 fold by these deletions. When specific point mutations were made in the conserved lysine residues of histone H4 (5,8,12,16) to either arginine or glutamine, a decrease of 50 and 10 fold respectively in the level of *GAL 1* was observed induction (Wan *et al.*, 1995). Activation was decreased nearly 100 fold for the *PHO5* gene in the K (5,8,12,16)-R strain. In contrast to the role played in the activation of *GAL1* by the H4 tail, the H3 tail acts as a repressor of induction for *GAL1*. This was demonstrated when tail deletions of H3 showed hyperactivation under inducing conditions, while induction of *PHO5* appeared unaffected. The repression of the silent mating type loci in diploid cells of *S. cerevisiae* is also dependent on the amino terminal tails of H3 and H4 (Thompson. *et al.*, 1994).

S. cerevisiae has three cell types a and α in the haploid cell and a/α in diploid cells. The cell type is determined by the mating type genes present at the controlling *MAT* loci. In addition to the *MAT* loci there are two other loci, the silent mating type loci or *HM* loci, which contain repressed mating type genes. These telomere proximal genes must be repressed in the haploid cell to prevent an a/α phenotype and sterility. Genetic screens identified factors that were responsible for the repression at the *HML* loci, among these were *SIR2,3,4* (silent information regulator) proteins, histone H4, H3, and Rap1 (Johnson *et al.*, 1990). Rap1 was shown to interact with *Sir 3,4*, DNA in the telomere regions, and at the *HM* loci. *Sir 3 ,4* were also shown to interact directly with the N terminal tails of H3 (4-20) and H4 (16-29) (Elgin, 1995). It has been suggested that these proteins all interact to form quiescent heterochromatic regions that result in silencing from the *HM* loci. Rine and Hershkowitz also demonstrated in *sir 2,3,4* mutants that repression at the *HM* loci was lost (Rine & Herskowitz ,1987). Furthermore, this loss of silencing was associated with a similar level of histone acetylation when compared to other active genes (Braunstein *et al.*, 1993). These experiments show that the chromatin substrate is an active participant in the regulation of genes as diverse as mating type and metabolism.

The association between histone acetylation and transcription activity has long been known. What remained a mystery until recently was if histone acetylation was a consequence of transcription or a precursor to transcription. Genetic and biochemical

experiments have identified acetyl transferase activity associated with transcription factors, coactivators, and TAFs. Moreover, histone deacetylase activity has been found in repressors of transcription. The identification of acetyl transferase in activators of transcription would suggest that acetylation of the histone proteins is a necessary prerequisite to transciption.

The first well characterized HAT, p55, was cloned from *Tetrahymena thermophila* (Sternglanz *et al.*, 1991). Shortly thereafter, a homolog in yeast was identified to be Gcn5p, a transcriptional co-activator (Brownell *et al.*, 1996). When Gcn5p was purified it had the ability to acetylate lysine residues of free histone H3 at K14 and H4 at K 8, 16. This acetylation pattern was shown to be different from that associated with nucleosome assembly where K14 of H3 and K12 of H4 are targeted. Gcn5p can be associated with mediator complexes Ada and SAGA (Forsberg *et al.*, 1997). These complexes change the specificity of Gcn5p *in vitro* whereby histones in the nucleosome are now targeted for acetylation (Kuo & Allis, 1998; Pollard & Peterson, 1997). The human co-activators p300/CBP, known to interact with many transcription factors (p53, CREB, E1A), and which have been proposed to be part of mediator complexes, also contain histone acetyl transferase activity (Ogryzko *et al.*, 1996). In addition to the well studied p300/CBP, TAFs were also identified that are able to acetylate core histones (hTAF 250, dTAF230, yTAF 130) *in vitro* (Mizzen *et al.*, 1996).

Factors responsible for the removal of acetyl moieties from the ε-amino groups of lysine residues have also been isolated. One of these factors Rpd3 from yeast, was identified

as a negative regulator of certain genes in the early nineties (Taunton, *et al.*, 1996). Later Rpd3 was shown to contain histone deacetylase activity (Rundlett, *et al.*, 1996). A human homolog to Rpd3 was isolated based on its ability to interact with the deacetylase inhibitor trapoxin, and termed histone deacetylase 1 (HDAC1) (Taunton, *et al.*, 1996). Many of the HATs and HDACs are likely to contain activities that are redundant *in vivo*, since the genes encoding them are not always essential.

In addition to the HATs and HDACs, other factors that remodel chromatin structure are being discovered. The first of these to be well characterized is the SWI/SNF ATP-dependent chromatin remodeling complex. The components of this complex were identified originally through a genetic screen that reduced the expression of the mating type switching gene *HO*, and reduced the expression of the *SUC2* invertase gene (Peterson & Tamkun, 1995). The SWI/SNF (for switch and sucrose nonfermenting) complex containing the proteins Swi1p,, Swi2p, Swi3p, Snf5, Snf6 was eventually isolated as a stable macromolecular complex at an impressive 2MDa moleacular mass (Peterson, *et al.*, 1994; Peterson & Tamkun, 1995). Details into the molecular mechanism for the SWI/SNF complex came from genetic screens from which mutants were isolated that were able to suppress a *swi1* phenotype. Two swi-independent or sin mutants were isolated and characterized. *SIN1* was found to encode a protein related to HMG1/2, while *SIN2* was found to be histone H3 (Kruger, 1991). The mutations of H3 that give rise to the suppression

of a *swi*⁻ phenotype were located in the L2 domain connecting the central α2 helix with the terminal α3 helix. This is a critical area of protein-DNA contact in the nucleosome where the minor groove is fixed in place by the interaction with residues from the L2 domain (see figure 3) (Wolffe, 1995). Further evidence that supports the hypothesis that the molecular mechanism of SWI/SNF involves, at least in part, a breaking of protein-DNA interactions comes from experiments with tailless core histones. In these *in vitro* experiments, Guyon et al. were able to show chromatin remodeling did occur on chromatin templates without the presence of core histone tails (Guyon *et al.*, 1999). Other chromatin remodeling complexes have been isolated as well ACF, RSC, CHRAC and NURF all contain chromatin remodeling activities (Alexiadis *et al.*, 1998; Carins *et al.*, 1996; Orpahanides *et al.*, 1998).

Specifically positioned nucleosomes occur in many promoter regions, suggesting that specific nucleosome placement is necessary for proper gene regulation. The two types of nucleosome positioning that have been shown to occur are translational and rotational. Translational positioning refers to the location of a nucleosome or nucleosomal array on the DNA sequence. Rotational positioning refers to the orientation of the DNA helix on the octamer surface. Positioned nucleosomes have been shown to occur on the *PHO5* promoter of *S. cerevisiae*, 5S RNA, mouse mammary tumor virus long terminal repeat (MMTV-LTR) and the *Drosophila* ADH promoter (Jackson & Benyajati, 1993) (Simpson *et al.*, 1985) (Richard-Foy & Hager, 1987).

On the promoter region of the secreted acid phosphatase gene *PHO5*, a translationally positioned array of four nucleosomes organizes the first order of chromatin structure. This nucleosome array has translational positions that cover the core promoter and start site, while maintaining a nucleosome-free region that allows access to an up stream activiating sequence (UAS). The details of *PHO5* regulation will be discussed in section 1.3 below. The MMTV-LTR is an inducible viral promoter that is activated by glucocorticoids through interaction with activated glucocorticoid receptor (GR). The activated GR complex interacts cooperatively with the general transcription factors NF1 and OTF1 at the hormone responsive region (HRR) to initiate transcription. The chromatin structure of the MMTV-LTR has been mapped in several different contexts which show an array of six translationally positioned nucleosomes (Fragoso, *et al.*, 1995). High resolution footprint analysis show that nucleosomes A and B adopt a specific rotational position (Fragoso *et al.*, 1995). The rotational placement of nucleosome A appears to be of critical importance in allowing the binding of the GR to its site within nucleosome A (Archer *et al.*, 1991). Subsequent to GR binding, a structural change occurs in nucleosome A as measured by restriction enzyme accessibility and DNase-I/ micrococcal nuclease cleavage. This structural transition is followed by the loading of the transcription factors NF1 and OCT1 at their cognate sites resulting in transcription (Archer *et al.*, 1992; Wolffe, 1995).

One of the most well studied examples of a positioned nucleosome occurs on the 5S RNA gene. The expression of the 5S gene product is initiated through the binding of TFIIIA to an internal control region (ICR) located within a positioned nucleosome. The two copies (somatic and oocyte) of the 5S gene of *Xenopus* are developmentally regulated (Wolffe & Brown, 1985). These two types of 5S genes have nearly identical coding sequences with the major difference in DNA sequence occurring in the flanking sequence. The somatic type 5S gene is G+C rich while the oocyte type is A+T rich. The developmental regulatory pattern results in both the somatic and oocyte copy being expressed in the oocyte. Later in development, the expression of the oocyte copy ceases mediated by the repressive effects of increasing H1 production. Binding of TFIIIA occurs with similar affinity to both copies of this gene and binding of TFIIIA to its site can occur in the presence of a nucleosome. This pattern of developmental regulation was recently shown by Panetta et al. to be a consequence of differences in translational position on the two gene types (Panetta *et al.*, 1998). Using a single base pair mapping technique, Panetta and coworkers demonstrated that the translational positions present on the somatic type resulted in a higher frequency of productive interactions with TFIIIA. The oocyte copy had translational positions that resulted in a lower frequency of TFIIIA interaction. Furthermore, competition experiments with H1 demonstrated that on the oocyte copy H1 became bound, excluding TFIIIA binding, Whereas the somatic type retained the ability to bind TFIIIA and was refractory to H1

binding (Howe & Ausio, 1998; Panetta *et al.*, 1998). Considering gene regulation in the context of chromatin has shown how the developmental regulation of the two gene types occurs. As the production of histone H1 starts during development, the oocyte copy becomes repressed due to translational nucleosome positions that preferentially bind H1 instead of TFIIIA. The somatic copy continues to be expressed, the result of favorable TFIIIA binding sites on the nucleosome.

The above examples demonstrate how chromatin structure at the level of the nucleosome can play an integral role in gene expression. The *PHO5* gene has nucleosome positioning that allows for both repression and activation by organizing the promoter region. The MMTV-LTR has translational and rotational nucleosome positioning to permit factor binding and subsequent chromatin remodeling and gene expression. Developmental changes in the expression of 5S RNA gene products have been shown to be a result of differential nucleosome placement on the oocyte and somatic type 5S genes. These examples of nucleosome placement are all a result of intrinsic nucleosome positioning sequences in the DNA molecule within and juxtaposed to the NCP (the *PHO5* gene to be demonstrated in this document). The nature of these nucleosome positioning signals in DNA are complex, varied and sometimes controversial.

1.2 Isotropic and anisotropic DNA bending

1.2A B-DNA general structure

The structure of DNA solved by the X-ray crystallographic data of Rosylind Franklin and the model building of Watson and Crick is well known. This left handed helical structure is stabilized by the hydrogen bonding of complementary base pairing and the base stacking interactions between adjacent base pairs. The diameter of the double helix is 2 nm completing a full turn every 3.0-3.4 nm. Each turn contains on average 10.5 bp with each base being rotated 34° from the preceding base along the helix axis, thus the necessity of the helix. DNA appears relatively homogeneous in its physical characteristics. However, local variations can occur that result in macromolecular changes in DNA structure and properties. The DNA molecule must be flexible to allow for nucleosome formation and interaction with DNA binding proteins. For example, TATA binding protein (TBP) induces a sharp nearly 90° bend in the DNA upon binding (Kim *et al.*, 1993). Recently the formation of the TBP-DNA complex was demonstrated to be enhanced 100-fold by increasing the flexibility of the DNA at the sites of TBP induced bending. The orientation of TBP binding was also found to depend on DNA flexibility (Grove *et al.*, 1998). The flexibility of DNA is viewed as both anisotropic and isotropic. Anisotropy is having a preferred plane of bending, like a bicycle chain, isotropy means having an equal propensity to bend along all axis.

1.2B Isotropic DNA bending

It has been proposed that when considering DNA sequence dependent nucleosome positioning, rotational signals can arise from anisotropic DNA flexibility, while translational nucleosome positioning can be attributed to general isotropic bending parameters (Sivolob & Khrapunov, 1995). This discussion will focus initially on the latter. When considering isotropic flexibility, the DNA polymer is treated as a stiff elastic rod. The measurement of the variability in elasticity or overall rigidity of a given DNA sequence is calculated as the persistence length P shown in equation #4.

$$P = EI / kT$$

Where I is the surface moment of inertia, E is Young's modulus, k is Boltzmann's constant and T is the absolute temperature. Young's modulus E is a measure of the elasticity of any material, calculated as shown in equation #5.

$$E = {}^{F/A}\!\!\diagup\!\!_{\Delta L / Lo}$$

Where F is the applied force along the long axis, A is the cross sectional area, L is the length. Equation 4 and 5 give a general view of the variables that account for persistence length.

However, experiments that result in values for persistence length are generally conducted through ligation experiments of linear DNA to form circles.

Hogan and coworkers used methylene blue as a probe to measure the motion of synthetic DNA molecules (Hogan *et al.*, 1983). Triplet anisotropy decay was used to measure the flexibility of the DNA molecule, the rate of anisotropy decay is a direct measurement of molecular motion. Faster decay rates indicate a large degree of motion in the DNA molecule. The results of these experiments revealed that the Young's modulus for poly(dG)·poly(dC) was at least four-fold greater than poly(dA) poly(dT). Shear modulus was also demonstrated to be four-fold greater in poly(dG)·poly(dC) than poly(dA)·poly(dT).

Experiments were conducted into the biological relevance of these observations using the phage 434 repressor. The 434 repressor is known to bind the DNA helix as dimer. The central four base pairs of the binding site do not make contact with the repressor protein but must bend and twist to allow interaction between the two repressor protein units. Koudelka and coworkers generated mutant binding sites for the phage 434 repressor and then measured the binding constants of the mutant oligos (Koudelka *et al.*, 1987). The results showed that if the central base pairs were ACGT or AGCT the binding constants decreased fifty-fold. Moreover, the consensus ATAT or AATT binding constants were unchanged revealing that it was not the interaction between any prymindine/purine but required the interaction between the G/C neighbor pair, demonstrating the biological relevance for the

underlying flexibility of the DNA in mediating protein DNA interactions. The molecular origin for this discrepancy in flexibility of different DNA sequences stems from nearest neighbor interactions of base pairs. This results in favorable head to tail dipole-dipole interactions between G/C bases on the same DNA strand and maximum London-van der Walls interactions (Hogan & Austin, 1987). These interactions in addition to the three hydrogen bonds formed between GC base pairs of the DNA helix, contribute to the stiffness of the G/C base pair step.

Sivolob and Khrapunov used persistence lengths calculated by Hogan and Austin as well as the free energy of DNA bending derived by Bloomfield to develop an algorithm that predicts translational nucleosome positioning (Bloomfield *et al.*, 1974; Sivolob & Khrapunov, 1995). The free energy for DNA bending is shown in equation # 8:

$$G = (1/2)A\eta^2 L$$

Where A is the bending constant, η is the inverse of the radius and L is the length of the DNA molecule. A is related to persistence length (P) through the relation A=kTP or to Young's modulus by A=EI. The length (L) can be expressed in terms of the nucleosome as L=Nh, with N equaling the number of base pairs in the NCP (146bp), h is the distance

between base pairs (h = 0.34 nm). Thus, free energy of DNA bending (G) can be expressed as shown in equation 9.

$$G = \frac{kTPh}{2R^2} N$$

To determine the lowest free energy value for the deformation of the DNA molecule into nucleosome for a region of DNA a summation is substituted for the value of N. A free

energy value is calculated for each 146 base pair step as shown in equation #9A:

$$G_i = \frac{kTPh}{2R^2} \sum_{i=1}^{N} P_i$$

Sivolob and Kharpunov showed that translational nucleosome positioning on the 5S RNA gene was predicted well by equation. 9A. This algorithm was able to successfully predict the edge of a nucleosome to within +/- 10bp. The treatment of the DNA molecule as a homogenous rod with standard elastic properties, has allowed one explanation into the mechanism of certain DNA-protein interactions.

1.2C Anisotropic DNA bending

DNA anisotropy that results in DNA curvature or preferred plane of bending, can give rise to strong rotational nucleosome positioning signals. Some of the earliest experiments into nucleosome positioning were done with the 5S RNA gene by Drew and Callidine (Drew & Calladine, 1987). These experiments used the 5S RNA gene of *Xenopus* reconstituted into nucleosomes *in vitro* by salt gradient dialysis. The resulting reconstitutes were subject to digestion by micrococcal nuclease and DNase-I. Drew and Callidine observed a non-random distribution of base pairs in the nucleosome (Drew & Calladine, 1987). For example, the dinuclotides AA/TT or AT are found at higher frequencies where the minor groove faces the histone surface. In contrast the GC step is located most frequently where the minor groove faces the solvent. These finding were interpreted as AT steps are able to compress the minor groove while GC base pairs will compress the major groove. This type of anisotropy would favor nucleosome binding if GC and AT steps were placed out of phase with the DNA helix at 5 five base pair intervals. These findings led Shrader and Crothers to develop synthetic DNA sequences to test the free energy of formation relative to natural DNA sequences that position nucleosomes (Shrader & Crothers, 1989). The results demonstrate that a synthetic sequence $(A/T)_3NN(G/C)_3$ is able to form nucleosomes with greater affinities than the natural sequences studied. Moreover, hydroxyl-radical footprinting revealed the same non-random periodicity as first observered by Drew

and Callidine. The non-random placement of GC and AT base pairs in the nucleosome has allowed many research groups to develop algorithms that are able to predict rotational nucleosome positioning (Boffelli, *et al.*, 1991). In general, these algorithms place the AT and GC steps at five base pair intervals due to the ability to create bending into the minor and major grooves respectfully. The question remained, does a curvature exist that allows DNA to wrap efficiently around the nucleosome?

Curved DNA structures were identified by recent crystal structures that contain the GC step such as GGCC/CCGG and AGCT/TCGA. These crystal structures do show a bend at the GC step towards the minor groove (Goodsell *et al.*, 1993) (Godsell & Dickerson., 1994). This curvature can be rather dramatic up to 20° in some crystals. The data from crystal structures is supported by evidence from gel mobility assays conducted by Buckner (Buckner *et al.*, 1993). In these experiments the distance between AAAAA and GGCCC was altered and anomalous migration in polyacrylamide gels was observed. The findings showed that if the AAAAA and GGGCCC sequences were separated by integers of 4 to 6bp the relative mobility (R_L) increased to 1.50 to 1.75 for the large fragments of 168 to 210bp respectively. These results would suggest that the above sequences when, separated by half a turn of the DNA helix, are oriented in opposite directions.

The final well studied naturally occurring DNA sequence which may play a role in local alteration of DNA/chromatin structure and modulation of gene activity, is the homopolymeric (dA:dT) tract and phased A runs. The structure of this type of DNA sequence is known to be quite different from standard B-DNA. The helical repeat is shorter, (10.0 bp vs. 10.6 bp) the minor groove is narrower, and the structure is relatively rigid (Yoon *et al.*, 1988). The hompolymeric (dA:dT) tracts also have been demonstrated to form nucleosomes with a lower affinity when compared to other DNA sequences. Iyer and Struhl studied this motif in the transcriptional regulation of the *HIS3* gene(Vishwanath & Struhl, 1995). By increasing the length of the homopolymeric (dA:dT) tract in the promoter region, transcription increased four-fold. Moreover, it was demonstrated that the observed increase in transcription was a result of increased accessibility to chromatin structure and not in response to increased ability to bind transcription activators.. Hagerman tested the idea of phased A tracts and DNA bending by ligation of the sequences GA_3T_3C, $G_2A_3T_3C_2$, $G_3A_3T_3C_3$, to create in phase and out of phase A tract runs (Hagerman, 1985). The relative migration rates showed dramatic differences with the sequences $(GA_4T_4C)_n$ and $(G_2A_3T_3C_2)_n$ showing a migration of over twice the size expected for a DNA fragment of that length. This is in contrast to the out of phase A tract in the $G_3A_3T_3C_3$. GA_3T_3C sequences that have the expected mobility. These experiments have shown that helical orientation of A tracts greater than three base pairs, is important for modification of DNA structure.

The above examples show that DNA bending whether isotropic or aniostropic is of biological importance for binding of transcription factors and nucleosome placement. It is interesting to note a disagreement in the literature between the Patshne group and the Austin group, as to the origins of the reduced binding constant in the mutant phage 434 repressor. Austin's group attributes the fifty-fold reduction to the increased isotropic stiffness of the GC base pair step relative to the wild type TA step. Yet, Patshne's group concluded that the GC base pair step which bends into the minor groove, results in reduced binding (Koudelka et al., 1987). There is no evidence that suggests these two observations are mutually exclusive. How anisotropic and isotropic DNA bending contribute to nucleosome positioning may be unique to each bending mode. Anisotropic bending gives rise to rotational nucleosome positioning signals. These rotational signals appear on nearly every DNA sequence. The result is difficulty in assigning the translational location of a nucleosome on a long region of DNA based on anisotropic analysis alone. Treating the DNA polymer as an isotropic elastic rod with sequence-dependent differences in flexibility allows translational nucleosome positions to be attributed to DNA bending energy. Homopolymeric (dA:dT) tracts and phased A runs also can play a role in modulating gene expression and chromatin structure. The rigid poly(dA:dT) tract may act to destabilize nucleosomes as is evident by the early failure in attempts to reconstitute these sequences into nucleosomes. The presence of

poly(dA:dT) sequences in promoter regions of many *S. cerevisiae* genes suggest that these sequences function to counteract high levels of nucleosome mediated repression.

1.3 Model system: the *PHO5* gene

The regulated acid phosphatase gene of *S. cerevisiae PHO5* has many advantages in researching transcription regulation in the context of chromatin. This is due to the large body of work that has characterized the location of nucleosomes on the repressed promoter and the changes that occur to this chromatin structure upon activation. The *PHO5* gene encodes a secreted acid phosphatase that is repressed when phosphate is readily available. When the phosphate concentration becomes limiting the gene is activated. The *PHO5* gene is part of the acid phosphatase family, consisting of *PHO3, PHO10,* and *PHO11.* The *PHO5* gene is highly regulated and accounts for more than 90% of the observed phosphatase activity under conditions of phosphate starvation, while being completely inactive in high phosphate (Oshima, 1997). *PHO3* is repressed when *PHO5* is activated however, *PHO3* is constitutively expressed even in conditions of high phosphate. *PHO10* and *PHO11* are induced in conditions of low phosphate however, they are minor contributors to acid phosphatase activity. Positive regulators of the PHO genes have been identified. For *PHO5* these are *PHO4, PHO2,* and *PHO81.* Negative regulators of *PHO5* expression are *PHO 80* and *PHO85. PHO80* and *PHO85* form a cyclin, cyclin dependent kinase which

phosphorylates the positive regulator *PHO4*, to inhibit its activity by exporting the protein from the nucleus. *PHO81* functions as a positive regulator to by inhibiting the activity of the Pho80p/Pho85p complex (Svaren & Horz, 1997).

The *PHO5* gene chromatin structure has been well defined in both the activated and repressed states. The chromatin structure of the *PHO5* gene was first mapped by Almer and Horz in 1986 by indirect end-labeling and restriction enzyme accessibility assays. In this study, the presence of two hypersensitive sites at -370 and -920 bp upstream of *PHO5* were identified. This study also showed the presence of four translationally positioned nucleosomes across the promoter region (Almer & Horz, 1986). Almer and Horz further demonstrate that these four nucleosomes were lost or reconfigured in such a way as to make them nuclease transparent when phosphate was limiting (Almer *et al.*, 1986). Deletion analysis of the *PHO5* promoter region by Rudolph and Hinnen identified four regions upstream of the coding sequence responsible for activation of *PHO5* (Rudolph & Hinnen, 1987). Three of these regions were believed to be Upstream Activating Sequences (UAS) that bound *trans* -acting factors. The fourth site mapped in these experiments was the TATA box. One putative UAS is located at position –469, the exact region that corresponds to a hypersensitive site located between two positioned nucleosomes. While the TATA box and two UAS regions at –245, -185 appeared to be located within positioned nucleosomes. These results led to the hypothesis that positive regulatory elements, due to their location within

nucleosomes, were unable to bind *trans*- acting factors or TATA box binding protein (TBP). This idea was tested by Han et al. in histone depletion experiments(Han *et al.*, 1988). In these experiments expression of histone H4 was under the control of the *GAL1* promoter that allows expression of H4 to be shut off when cells were grown in the presence of glucose. When H4 expression ceased, nucleosome structure was lost over the promoter region and expression of *PHO5* increased. The *trans*- acting factors that bind to the UAS regions were shown to be the aforementioned positive regulators of *PHO5* expression, *PHO4* and *PHO2*, by Vogel et al. (Vogel *et al.*, 1989). The chromatin structure of the repressed *PHO5* promoter and the corresponding regulatory elements and *trans*- acting factors are shown in figure 4.

The *trans* – acting factors *PHO4* and *PHO2* are required for the chromatin transition and activation of *PHO5*. Pho4p is 312 amino acids long and has a basic-helix-loop-helix motif. Pho4p binds the DNA as a dimer and shares the CACGTG DNA binding motif with the proto-oncogene *c-myc* (Fisher *et al.*, 1991). Pho2p contains a homeobox sequence involved in DNA binding as well as acidic and glutamine rich regions implicated in transcriptional activation. Fascher and coworkers were able to show that both induction of *PHO5* and the chromatin transition were able to occur in a *pho2, pho4* strain if Pho4p was over expressed (Fascher *et al.*, 1990). Further, the transition to an active chromatin state occurred even under repressing conditions. Over expression of Pho2p did not generate the

chromatin transition or *PHO5* expression even in conditions of low phosphate. Of the two upstream *cis*–acting sequences for Pho4p in the*PHO5* promoter region, UASp1 was required for chromatin remodeling, UASp2 alone was not sufficient (Venter *et al.*, 1994). Moreover, a nucleosome was able to block Pho4p binding demonstrating the importance for maintaining a nucleosome-free region at UASp1. Maximal activation of the *PHO5* gene is a result of the cooperative binding of both Pho4 and Pho2p at their sites in the promoter region. Deleting out the TATA box has no effect on chromatin remodeling nor does activation require that cell division occur (Schmid *et al.*, 1992).

How nucleosomes are positioned on the *PHO5* promoter is not known. An episomal copy of the *PHO5* gene had identical nucleosome placement, indicating long range affects are not responsible for formation of chromatin structure. The possibility that Pho4p was able to position the nucleosome array was discounted when it was shown that deletion of UASp1 had no effect on positioning. Deletion analysis that replaced half of the DNA underlying nucleosome –1 had no effect on the position of nucleosome –2 (Fascher *et al.*, 1993). Nucleosome –1, even though half of the DNA was deleted, did not change positions but adopted a more "compact" positioned nucleosome. The authors interpreted this result as both boundary constraints and histone-DNA interactions as playing a role in nucleosome positioning. Histone-DNA interactions are critical in the regulation of the *PHO5* gene. This was eloquently shown through experiments conducted by Straka and Horz. In these

experiments the DNA underlying nucleosome –2 was replaced with green monkey α satellite DNA, pBR322 plasmid DNA, or deleted completely (Straka & Horz, 1991). The green monkey α satellite DNA is known to form stable nucleosomes *in vitro,* while pBR322 DNA forms relatively unstable nucleosomes. The green monkey α satellite DNA sequence prevented activation in low phosphate, while pBR322 plasmid sequence conferred weak constituitive expression. These results indicate that strengthening-histone-DNA interactions can very dramatically alter gene expression.

1.4 Research aims and document summary

The overall goal of the work presented here is to determine the mechanism of nucleosome positioning on the *PHO5* promoter region. To this end, a purified chromatin reconstitution system was developed which is described in chapter 2. This reconstitution system will allow the mechanism of transcription regulation in the context of chromatin to be studied. Chapter 3 will present the results of the mechanism of nucleosome positioning on the *PHO5* promoter region. Chapter 4 provides insights gained into the mediation of gene expression via histone-DNA interactions that resulted from this work. Future directions to further test how gene expression is mediated by chromatin at the level of the nucleosome are included.

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Figure 1.1 Chromatin ladder generated by digesting isolated nuclei from *S. cerevisiae* with micrococcal nuclesase. Notice the repeat length of ~160 base pairs. The far right hand lane is a 100 base pair DNA marker. (J. Pilon unpublished).

Figure 1.1

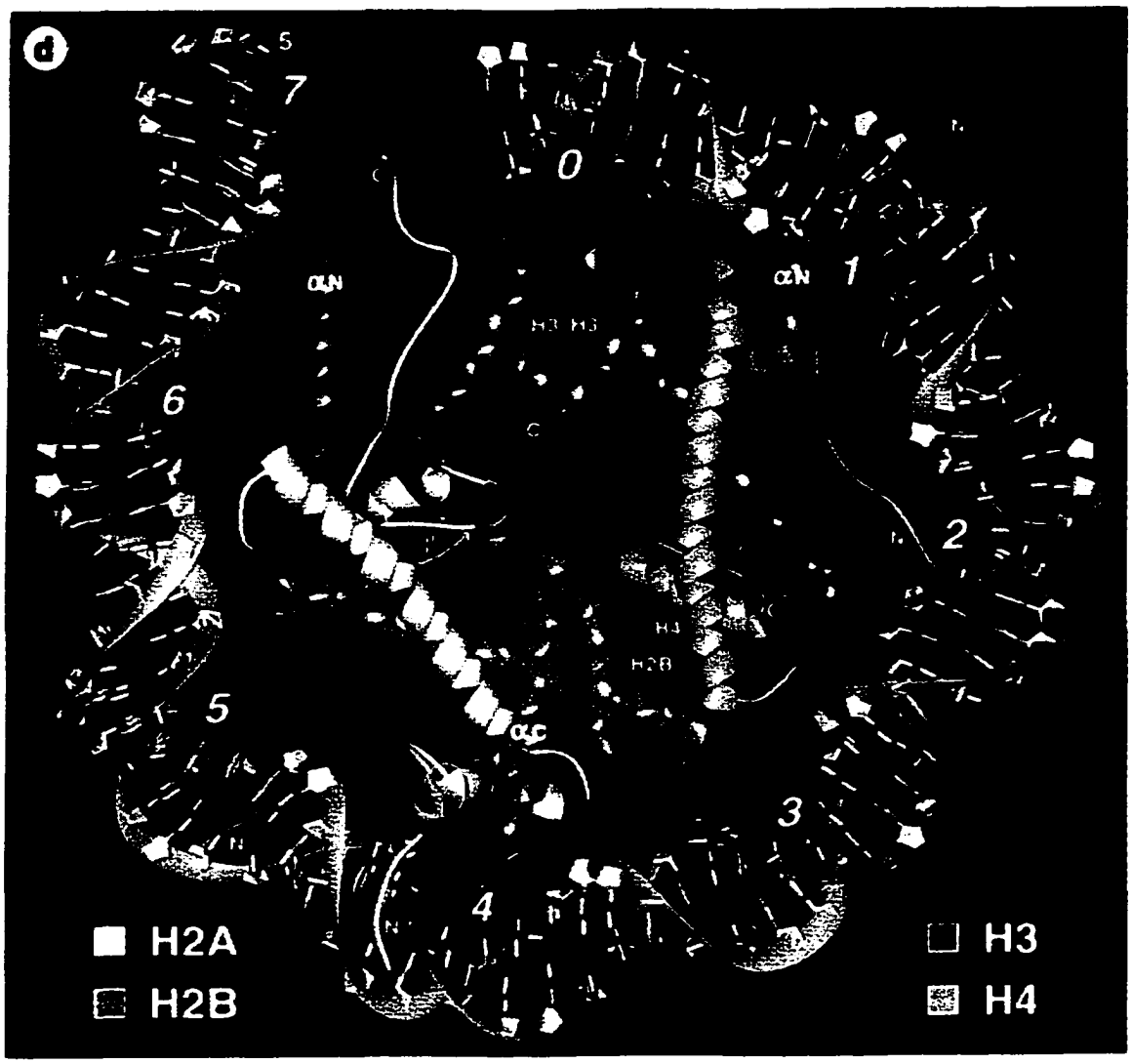

Figure 1.2 Crystal structure of the nucleosome. This represents one half of the nucleosome.Histone hand shake motif is readily appearent as the long α helix of one histone passes under its partner pair. Sharp bends are seen in the path of the DNA helix at SHL1.5 and 4.5. (Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-60).

Figure 1.2

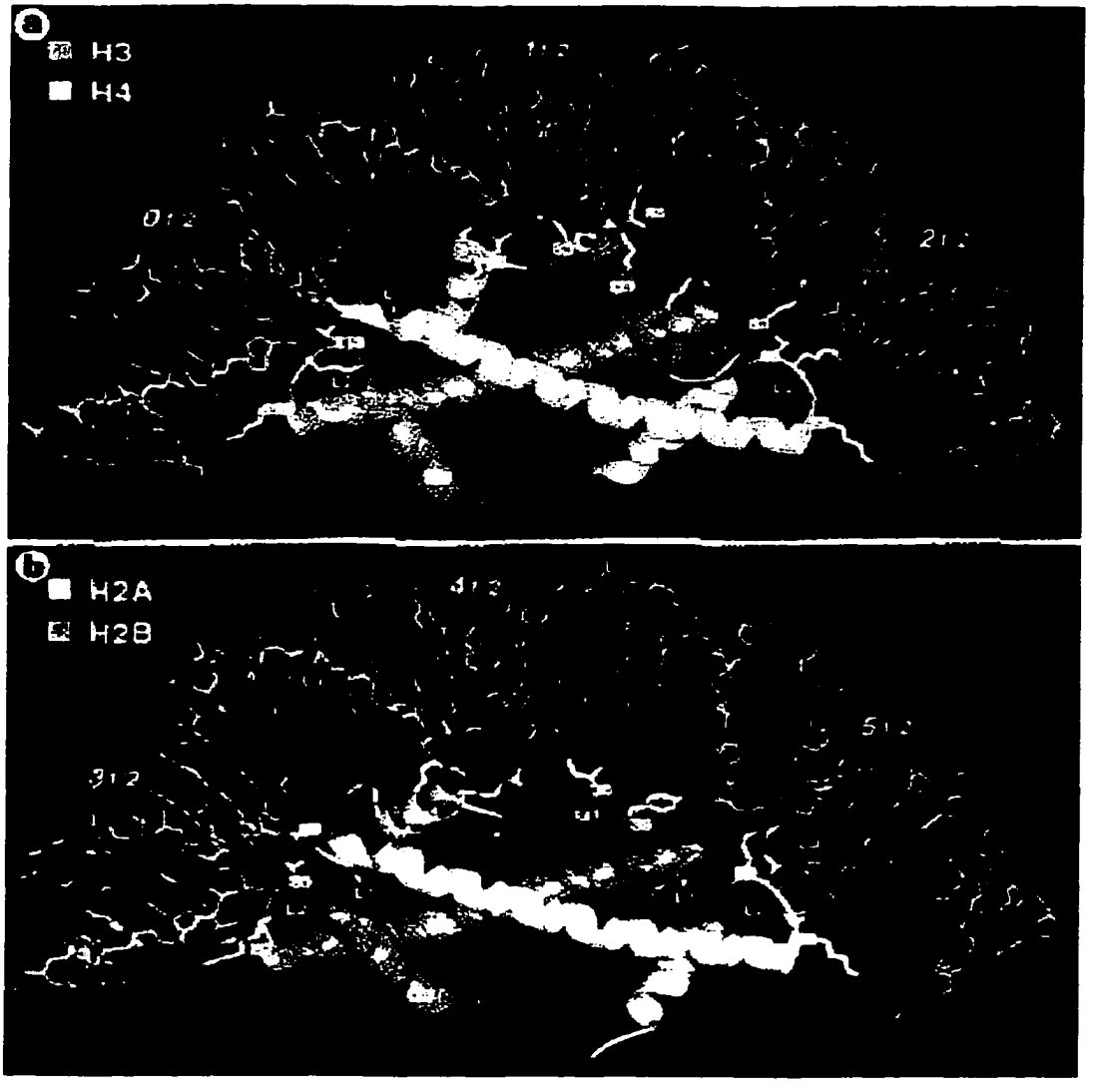

Figure 1.3 This figure shows the α1–L1-α2–L2-α3 structural element. Side chains that make hydrogen bonds, hydrophobic interactions and arginine residues projecting into the minor groove are shown (Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution [see comments]. Nature *389*, 251-60).

Figure 1.3

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 this document. For the preparation of this manuscript, I contributed to the process development, all of the figures with the exception of Figure 2.1 and Figure 2.6, the writing of the associated Materials and Methods and proofreading. The Associated reference is:

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2.1 Abstract

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 (Van Holde *et al.*, 1995; Wolffe, 1995). A nucleosome contains an octamer of two each of the four core histones; H2A, H2B, H3 and H4 (Bajwa *et al.*, 1984; Van Holde et al., 1995). 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 (Fisher & Felsenfeld, 1986; Forrester *et al.*, 1986). 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 (Benezra *et al.*, 1986; Fritton *et al.*, 1987; Richard-Foy & Hagar, 1986). 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 (Almer *et al.*, 1986; Bergman & Kramer, 1983; Fascher *et al.*, 1990; Straka & Hörz, 1991). 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 (Durrin *et al.*, 1991). 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 (Clark-Adams *et al.*, 1988; Han & Grunstein, 1988; Norris *et al.*, 1988). 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

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 (Scott & Schekman, 1980). Cells were grown to an A_{600} of 2 to 3 in 50 ml of M63 medium (Sambrook *et al.*, 1989). 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 (Scott &

Schekman, 1980). 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 (Nossal & Heppel, 1966). 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.

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 (Sherman, 1991) 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.* (Lue *et al.*, 1991) 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.

Yeast core histone purification - Purification of the core histones was based on the method of Simon and Felsenfeld (Simon & Felsenfeld, 1979) as modified by Laybourn and Kadonaga (Laybourn & Kadonaga, 1991). 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 (Laemmli, 1970) 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.

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 (Lennox & Cohen, 1989). Discontinuous SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (Laemmli, 1970). 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 (Lennox & Cohen, 1989). Gels were stained with Coomassie Brilliant Blue R-250.

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.

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.* (Goto *et al.*, 1983). 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 (Shimamura *et al.*, 1988) as modified by Shimamura *et al.* (Fukuma *et al.*, 1994) 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.

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.

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

Purification of yeast core histones - We have succeeded in purifying yeast core histones in quantities (milligrams) sufficient for biochemical analysis (Figure 2.1.). It should be noted that two other groups have published methods for the purification of yeast core histones previously (Fukuma *et al* 1994, Lorch & Korenberg 1994, Davie *et al* 1991). 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.*(Fukuma *et al.*, 1994), 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 (Lorch & Kornberg, 1994), 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.* (Davie *et al* 1981) 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.* (1991) and the histone octamer purification protocol of Simon and Felsenfeld (1979) as modified in Laybourn and Kadonaga (1991). 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.

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.

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 (Ishimi & Kikuchi, 1991). Yeast core histone octamers were mixed with an equal mass of purified recombinant Nap1p (Fujii-Nakata *et al.*, 1992). 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 (Shimamura et al., 1988). 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 (Tatchell & Van Holde, 1977; Thomas & Furber, 1976).

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 (Tatchell & Van Holde, 1977; Thomas & Furber, 1976). 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 (Almer et al., 1986; Bergman & Kramer, 1983; Fascher et al., 1990; Straka & Hörz, 1991). 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 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.* (Morse *et al.*, 1987) reported similar difficulties and that both Fukuma *et al* (1979). and Lorch and Kornberg (1994) 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.

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Purification of Yeast Core Histones

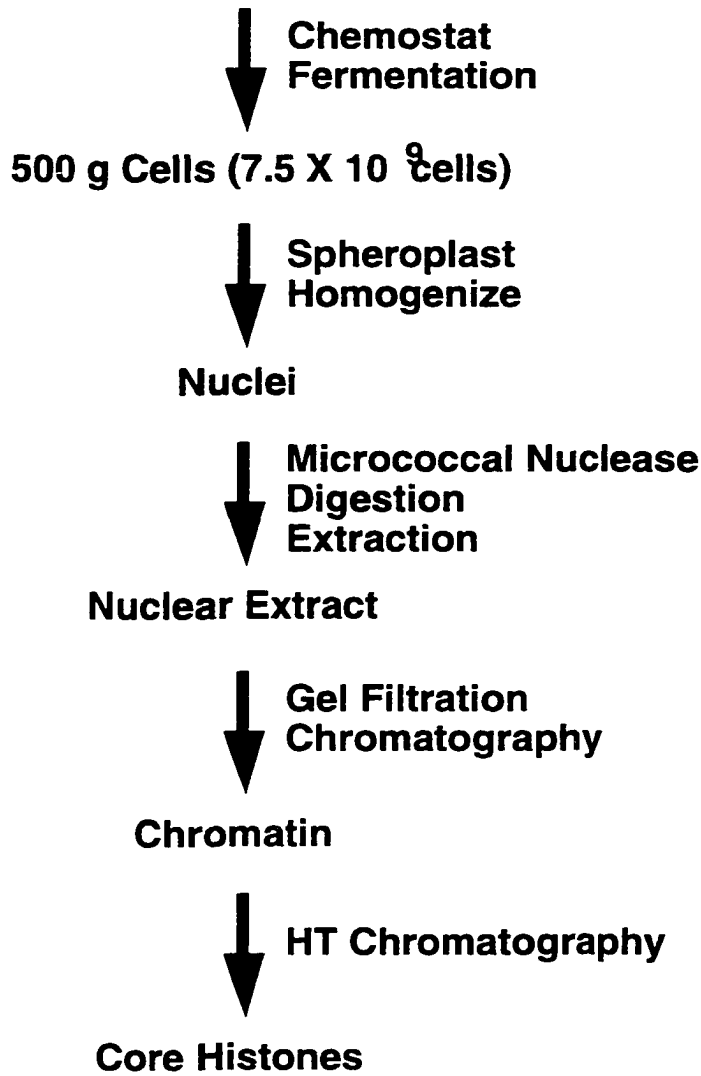

Figure 2.1. Schematic representation of the yeast core histone purification procedure.

Figure 2.1

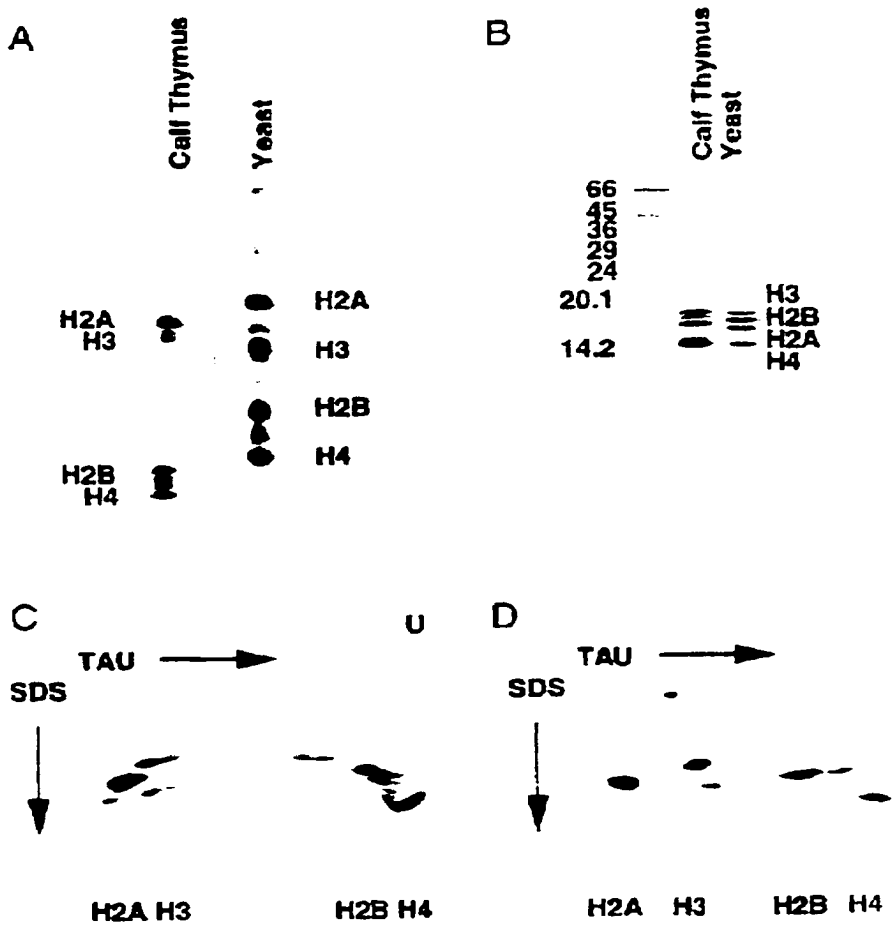

Figure 2.2. Denaturing gel electrophoresis of purified yeast core histones. One dimensional PAGE. Calf thymus (8.4 μg) and yeast (18 μg) core histones were resolved by (A) TAU (Triton-acid-urea) PAGE in a 15% polyacrylamide gel and by (B) SDS (sodium dodecyl sulfate) PAGE in an 18% polyacrylamide gel 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 2.2

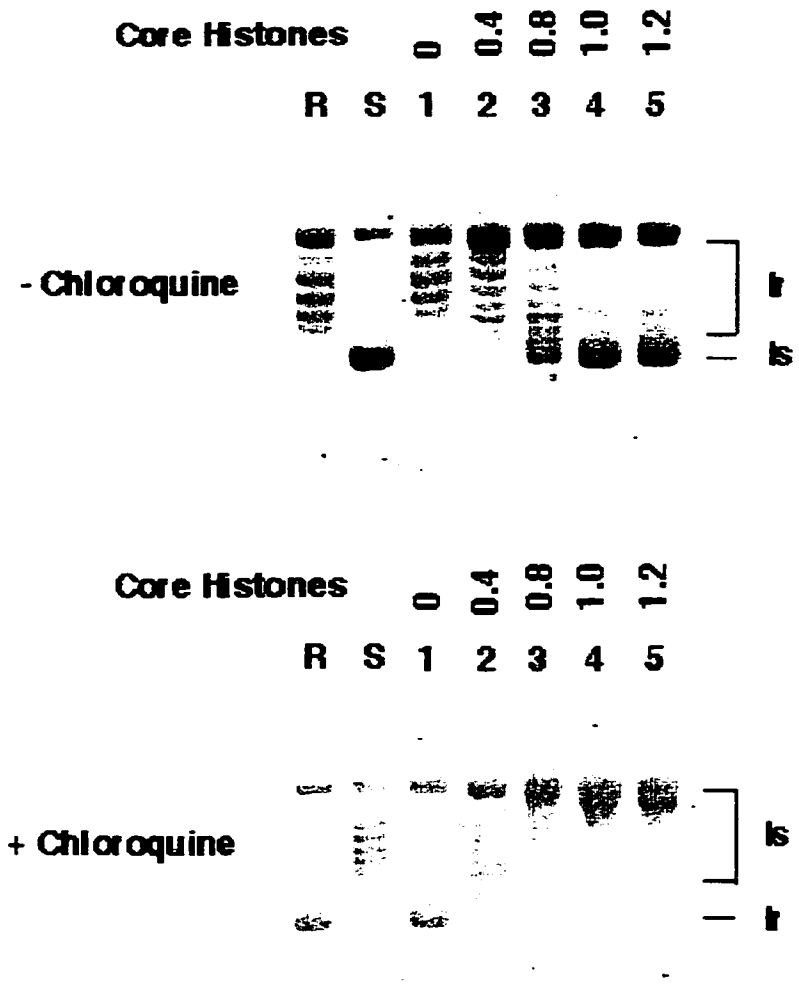

Figure 2.3. One dimensonal topological analysis. Plasmid DNA (400 ng) reconstituted at the core histone DNA ratio (w/w) indicated at the top, was resolved on 1% agarose gels run in the absence or presence of chlorquine as indicated in the left margin. Lane S and R are supercoiled and relaxd markers,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

Figure 2.3

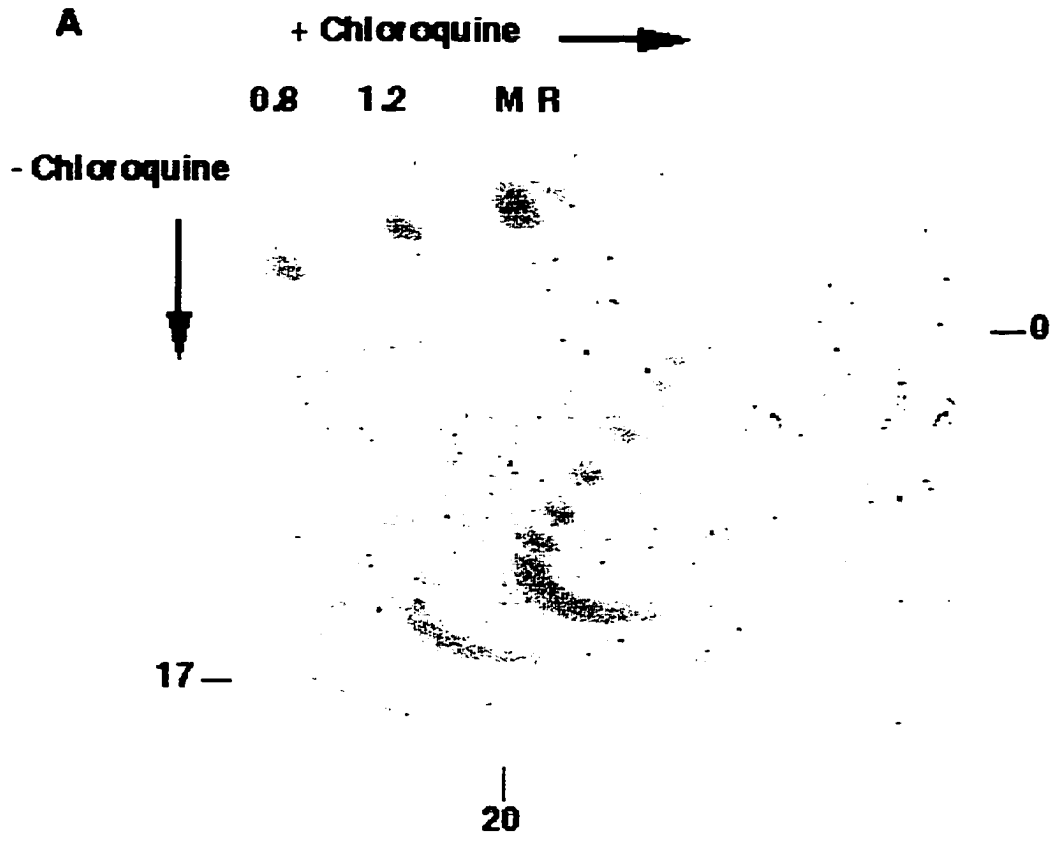

Figure 2.4. Two dimensonal topological analysis. Plasmid DNA (750ng) from chromatin reconstituted in the presence of topoisomerase I, purified and resolved on a1% agarosegel. The first dimension is the absence, the second in the presence of chloroquine. The samples, chromatin reconstituted with a ratio of 0.8 and 1.2 core histones to DNA (w/w) supercoiled markers (M) and relaxed markers (R), are indicated in the upper margin.

Figure 2.4

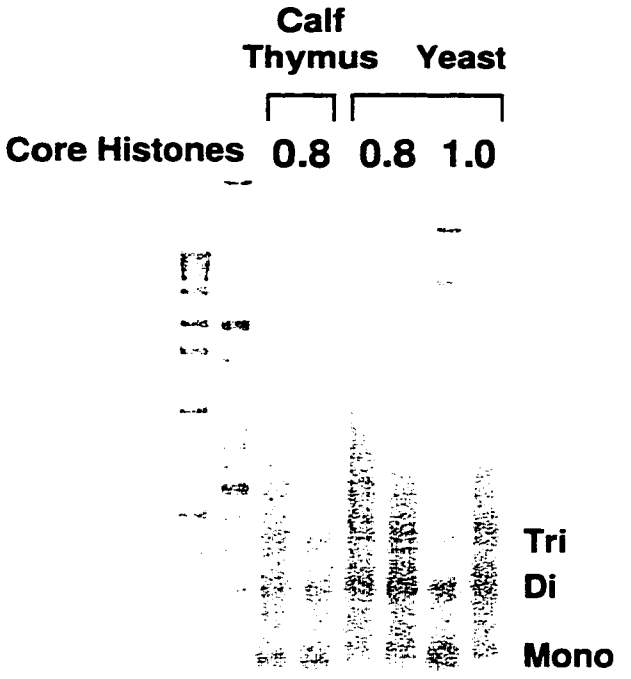

Figure 2.5. Micrococcal nuclease digestion. Reconstituted chromatin (3 μg DNA) was digested with micrococcal nuclease for 2 and 4 min as described in Materials and Methods and resolved on a 1.5% agarose gel. The chromatin was reconstituted with calf thymus and yeast core histones at the histone to DNA 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 2.5

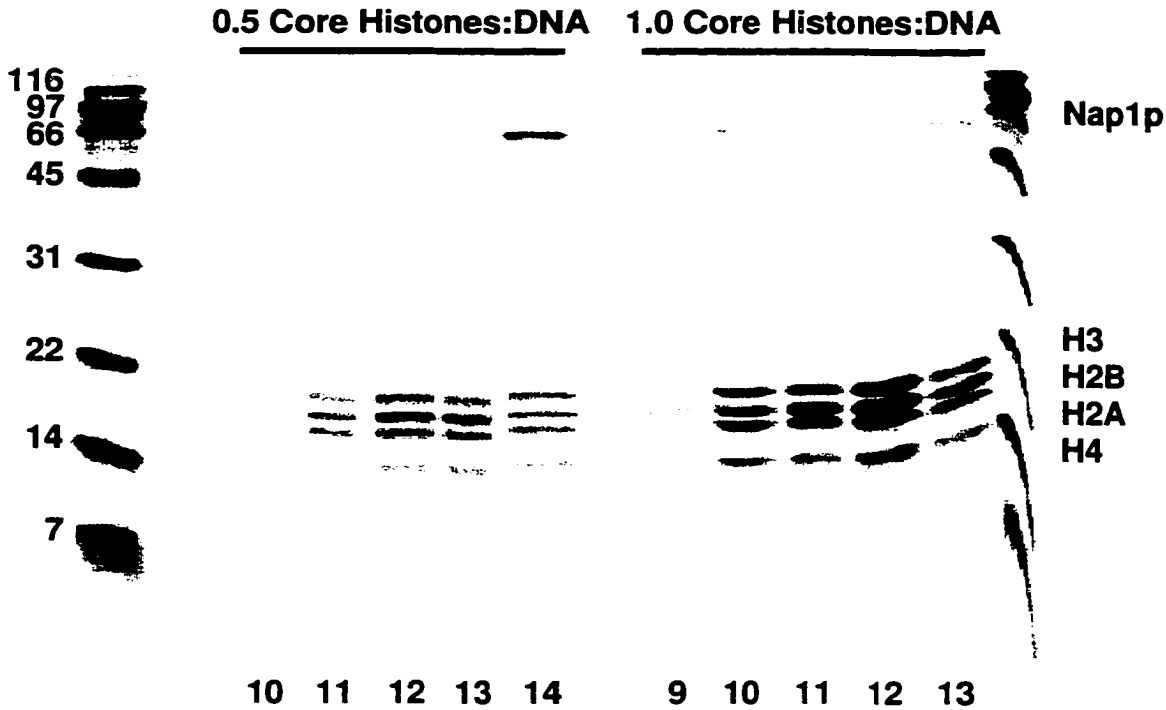

Figure 2.6. Purity and stoichiometry of yeast core histones on sucrose gradient purified chromatin templates. Chromatin templates were reconstituted, as describe in Materials and Methods, at a core histone to DNA ratio (w/w) of 0.5 and 1.0. The chromatin templates were purified by sedimentation on 5% 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.

Figure 2.6

Appendix to chapter 2
Miscellaneous experiments

2.1A Summary

In the process of developing the system presented in chapter 2, many assays were conducted that demonstrate the promise of this system in biochemical approaches to study transcription in the context of chromatin. First, purification of core histone octamer that have deletions of the N-terminal tails was achieved (Wan *et al*). One of these containing a deletion of the N-termini of histone H4 is competent for reconstitution into nucleosomes.

Second, due to the absence of a high degree of post-translational modifications, histone acetyl-transferases could be used to selective modify the histone octamer. These octamers could then be used in experiments to sort out in greater detail the effects of acetylation at the various lysine residues in the tail regions. Laura Tomky demonstrated that this approach could possibly be utilized. In these experiments with recombinant Gcn5 and

TAS HATS, she was able to specifically target histone H3 and resolve this modification via 2D-gel electrophoresis. Furthermore, in these experiments histone acetyl-transferase activity was shown to be present in fractions of purified core histones.

As mentioned above NAP-I is readily removed from our reconstitutes by sucrose gradient centrifugation. NAP-I also could be removed by chromatograpic proceedures. Interestingly in the HAT activity assays, NAP-I is acetylated *in vitro* by a factor present in the core histone fractions. Recent evidence has shown that NAP-I is phosphorylated by casein kinase (Li, *et al* 1999). If NAP-I is also a substrate for acetylation *in vivo* should be the focus of further experimentation.

Lastly, the nuclear extract which is chromatographically resolved in the purification procedure for yeast core histones contains many activities that could be purified further and utilized to study transcription in the context of chromatin. An example of this was the isolation of topoiosmerase-I and histone acetyl-transferase activity. It should be a simple matter to screen the fractions of the S-300HR gel filtration column via western blot analysis to locate transciption factors, chromatin remodeling activities and other nuclear proteins of interest.

This appendix will discuss the results of the work mentioned above that have been carried out to date.

2.2A Materials and Methods

Purification of core histones, 1D topological assays and 2D electrophoretic analysis was conducted as described in the preceeding chapter. The two yeast strains were PKY806 H4 del(4-23) containg pPK626 *hhf2 Δ4–23,* RMY 430 H3 del(4-30) containingpRM430 *(hht2Δ4–30).* Both strains are isogenic to strain RMY200 *MATa ade2-101(0ch) his3 Δ200lys2-801(amb)trp1Δ901 ura3-52 hht1.hhf1::LEU2hht2,hhf2::HIS3* pRM200*(CEN4 ARS1 TRP1 HHT2 HHF2)* . Prior to reconstitution with NAP-I purified core histones from yeast strain PKY806 (containingH4 N-terminal del 4-23) were precipitated with $(NH_4)_2SO_4$. Fractions were brought to 90% saturation, precipitated on ice 20', spun 40 minutes 40K Beckman 70ti rotor, and resuspended in reconstitution buffer (RCB) to give a protein concentration of 750ug/ml. This concentrated fraction was then combined with purified NAP-I in a 2:1 or 1:1 (NAP-I/core histone ratio w/w).

Histone acetyltransferase assays were conducted by adding 5-50 micrograms histone protein to 5ul of either recombinant TAS or Gcn5P crude extract, 0.1uCi/uL tritiated acetyl-CoA (diluted into 10mM $NaHPO_4$ pH=5.0) in 50ul assay buffer. The assay buffer contained 50 mM Tris–Cl pH=8.0, 10% glycerol, 1mM DTT. 0.1mM PMSF. The reaction is incubated for 10 minutes at 27 C°. Aliquots of 10ul were then placed on P81 filter paper and washed 2x 15 minutes in $NaHCO_3$ pH=9.0. Filter paper was dried for one hour and counted in a

scintillation counter. The remainder of the reaction was Trichloroactetic acid precipitated and run on TAU gels followed by SDS-PAGE second dimension as described in chapter 2.

Removal of NAP-I from reconstituted chromatin by chromatography was conducted by injecting 1-5ug of reconstitutes over 4ml Sephacryl-CL4B column. The column was equilibrated in HEG buffer(Hepes-OH pH=8.0, 0.1mM EDTA, 5% glycerol) and run with a flow rate of 0.1ml/min, 50ul fractions were collected. Micrococcal nuclease digestion and SDS-PAGE was carried out as described in chapter 2.

Topoisomerase activity was assayed for from the gel filtration S300HR column. PUC DNA was added to 250ng/ul in reconstitution buffer with $MgCl_2$ added to a concentraion of 3mM. Relaxed DNA was run on 1% agarose gels and stained with EtBr.

2.2B Results

Purified core histones containing a deletion in the N-terminal (4-23) of were shown to be fully competent for reconstitution *in vitro*. Both the 2:1 and 1:1 NAP-I core histone ratios were shown to function in forming high quality reconstitutes. This is the first experiment to demonstrate yeast core histones (or any core histones) containing specific mutations can be reconstituted. In addition to the N-terminal H4 deletion, purified 806 cores show a proteolytic H3 product on gels. This protolytic product is from the N-terminal region of H3 as shown by Edman degradation amino acid sequencing (data not shown). This result

demonstrates that the core histone tails are not a necessary component for assembly of chromatin templates *in vitro*. These results are shown in figure 2A.1

Laura Tomky developed histone acetyl transferase assays using the recombinant HATS TAS and Gcn5p(1996). These assays showed that our purified yeast core histones could be acetylated using tritiated acetyl-CoA *in vitro* as measured by tritium incorporation (data not shown). Moreover, it was demonstrated that the quality of the HAT activity could be measured by 2D gel electrophoresis. The development of these assays set the stage for the ability to selectively modify specific lysine residues and study the effect of acetylation on both chromatin structure and transcription *in vitro*. An interesting result of this work was the presence of a contaminating HAT in some of our purified histone fractons themselves (data not shown). Lastly, in HAT assays conducted to determine the specificity of HAT activity on either free histone or in the form of nucleosomes it was shown that NAP-I was preferably acetylated by Gcn5p. This result was unexpected and may reflect a new substrate for HATS. However, NAP-I may not be a relevant substrate for acetyl-transferase activity *in vivo* maybe an artifact of the experimental conditions. These results are shown in figure 2A.2 and 2A.3

The presence of NAP-I as an assembly factor could present difficulties in the interpretation of data. This necessitated the development of methods to remove the high levels of NAP-I present in the assembly reactions. As discussed in chapter 2 above NAP-I could be removed by sucrose gradient centrifugation. However, due to the high level of skill and time required to run sucrose gradients, a chromatographic procedure was developed that

resulted in removal of the bulk of the NAP-I. The advantages of this approach is the time commitment with a run being completed in approximately 30 minutes, and the reproducibility of the elution profiles. These data are shown in figure 2A.4

Finally, a nuclear extract is partially fractionated in the process of purification of yeast core histones. It can be assumed that many transcription factors, chromatin remodeling activities and HAT/HDAC complexes are also present in this extract. A detailed biochemical study of the activities/factors present in these fractions has never been conducted. However, proof of the efficacy of this approach was demonstrated in the isolation of large quantities topoismerase –I activity. Moderately successful attempts were made to purify the transcription factor ABF-I and to assay for HAT activity that was present in this chromatographic process (data not shown). Utilizing some of the relevant activities present in the nuclear extract would greatly facilitate a biochemical approach to the study of transcription in the context of chromatin. These results are shown in figure 2A.5.

Reference:

Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, Y. S. & Allis, C. D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**(6), 843-851.

Li M, Strand D, Krehan A, Pyerin W, Heid H, Neumann B, Mechler BM (1999). Casein kinase 2 binds and phosphorylates the nucleosome assembly protein-I (NAP-I) in Drosohila melanogaster. *J. Mol. Biol.* **12**(5), 1067-84.

Wan, J. S., Mann, R. K. & Grunstein, M. (1995). Yeast histone H3 and H4 N termini function through different GAL1 regulatory elements to repress and activate transcription. *Proc. Natl. Acad. Sci. U S A* **92**(12), 5664-8.

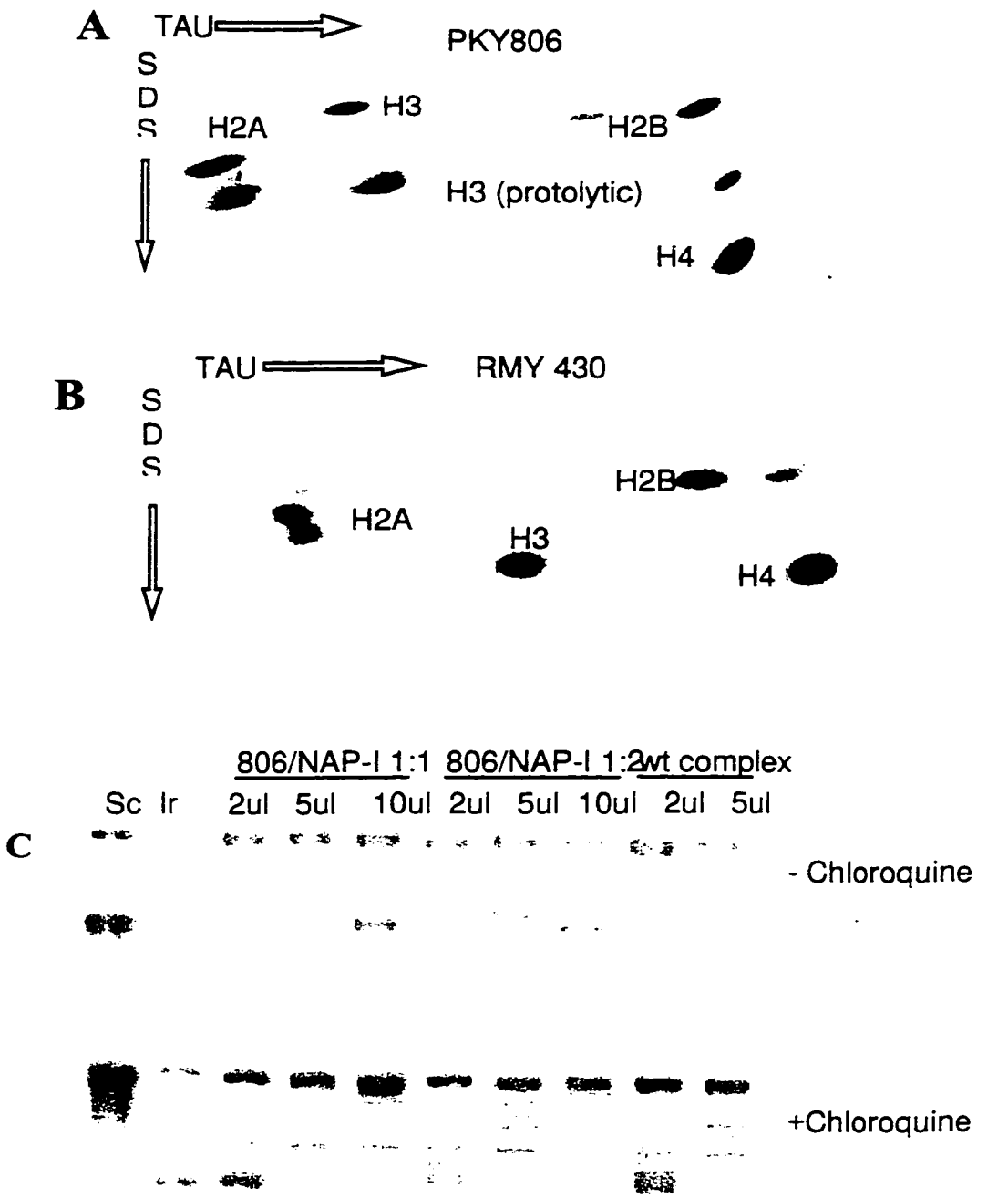

Figure 2A.1. Purification of mutant core histone proteins. Panel A shows the a 2D gel of purified core histones with a deletion of the N-terminal 4-23 residues of H4. B is the purification of core histones with a deletion of the N-terminal 4-30 residues. C Topological assay of histones reconstituted with core histones shown in A.

Figure 2A.1

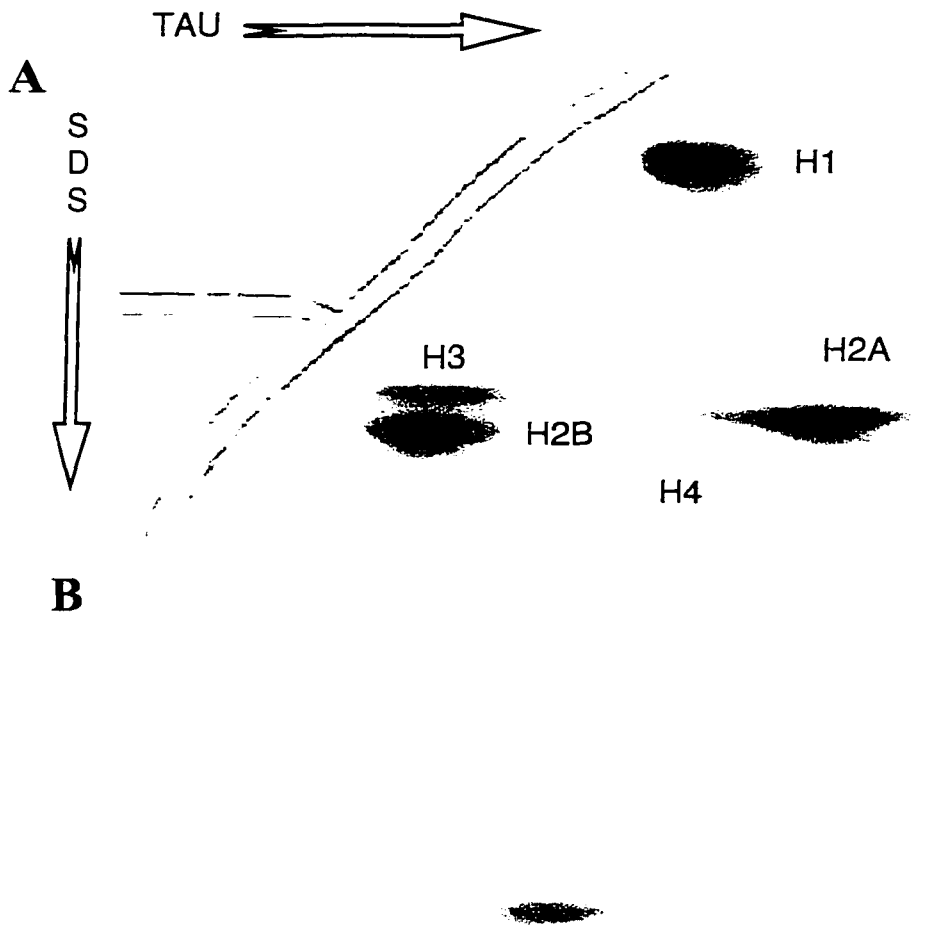

Figure 2A.2 A Histone acetylation as resolved by 2D gel electrophoresis.Calf Thymus core histone were acetlyated with recombinant Gcn5p and tritiated Acetyl-CoA. (A) Is the Coomassie stained gel. (B) Is the developed film of the gel shown in panel A demonstrating the specificity of the HAT reaction. (Laura Tomky unpublished results)

Figure 2A.2

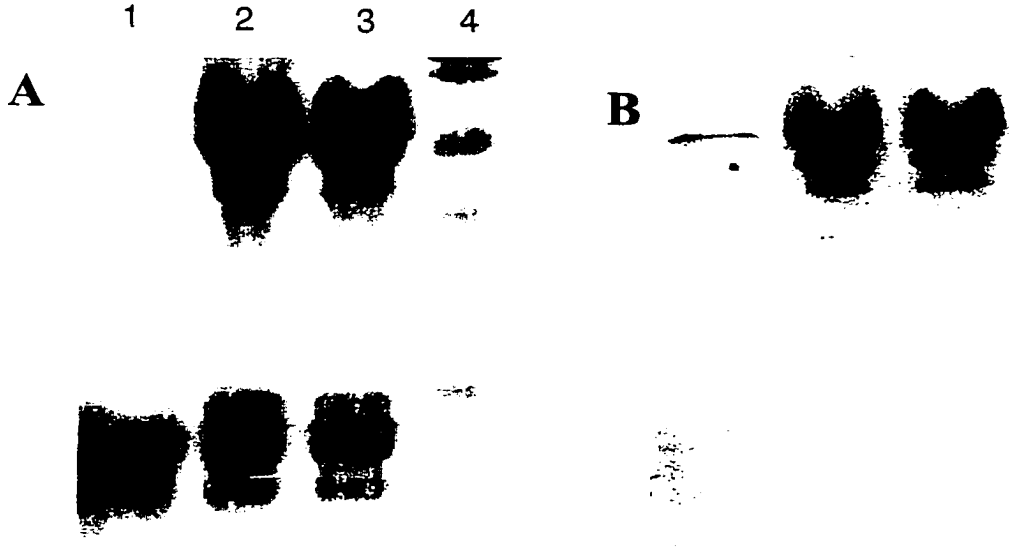

Figure 2A.3 NAP-I is acetylated by Gcn5p *in vitro*. A is the coomassie stained gel. B is an autorad of the gel shown in panel A. Lane 1 is free histone octamer, lane 2 has been reconstituted into a nucleosome, lane 3 is a NAP-I histone complex.

Figure 2A.3

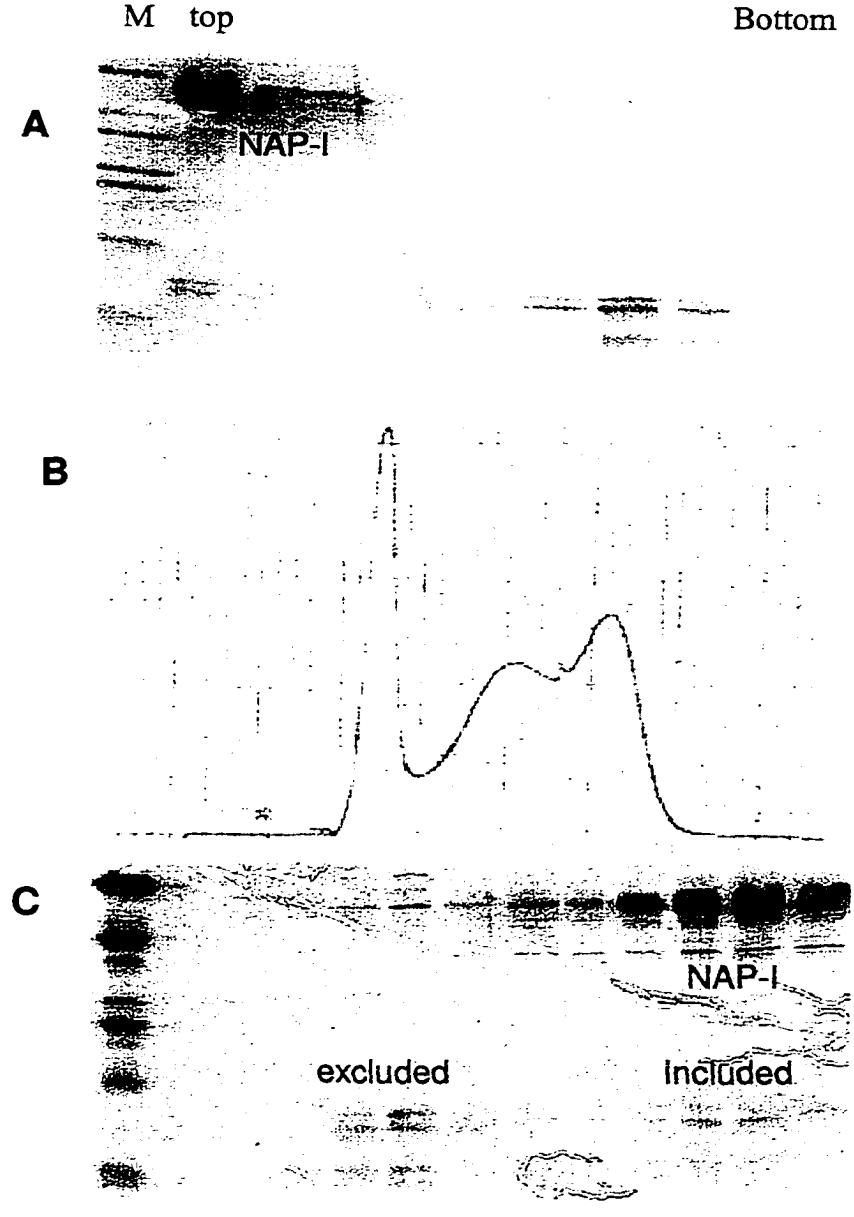

Figure 2A.4. Removal of NAP-I by various proceedures. (A) SDS-PAGE of a sucrose gradient left side of gel is the top of gradient . (B) chromatogram of the gel filtration column (C) SDS-PAGE of gel filtration column.

Figure 2A.4

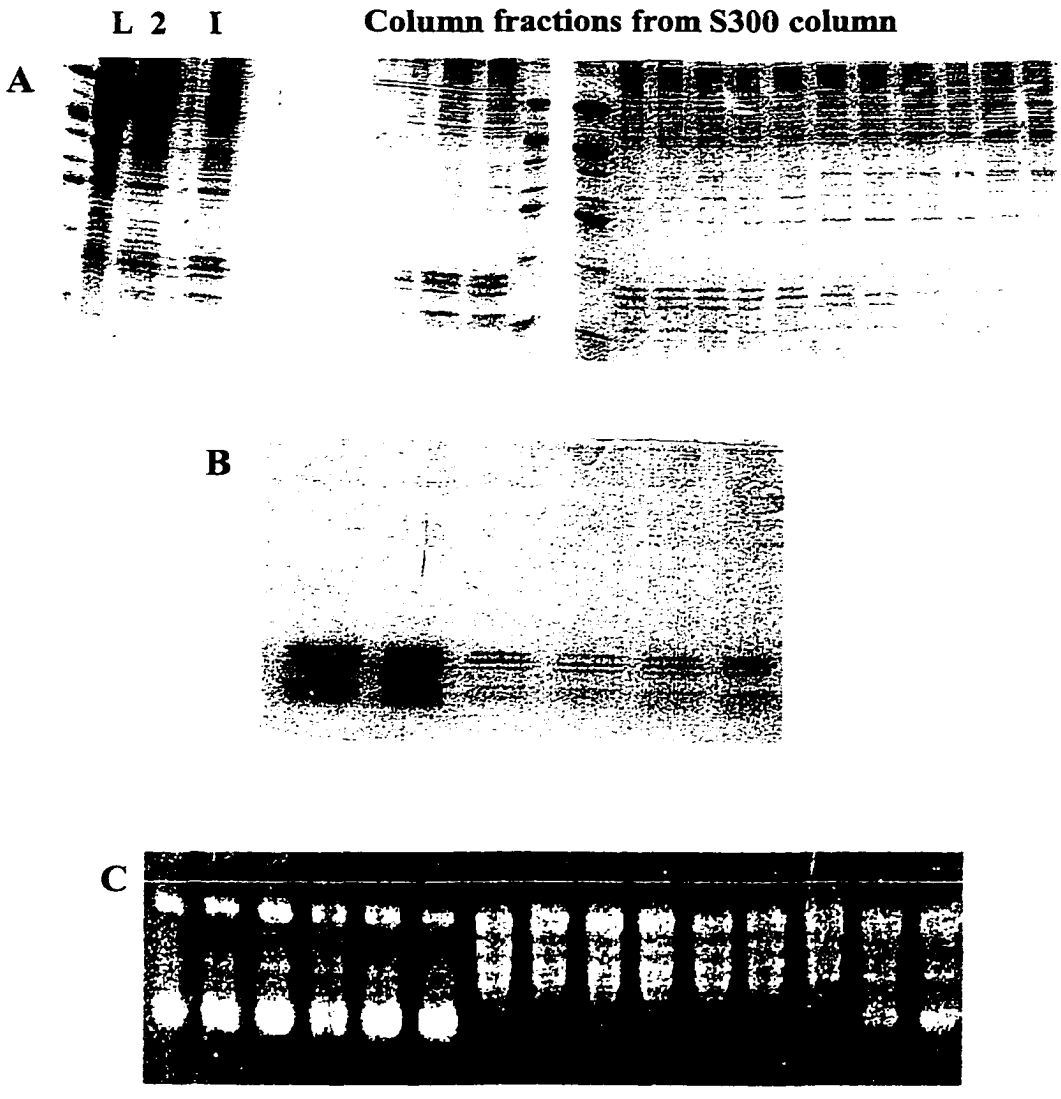

Figure 2A.5 Core histone purification and isolation of topoisomerase-I activity. (A) is an SDS-PAGE gel of the fractions off the S-300HR column. L is the lysis buffer, 2 is the second extract of nuclei, I is the input to the column. (B) shows fractions of the hydroxy-apatite column. (C) shows topoisomerase activity isolated from the S-300HR column.

Figure 2A.5

Chapter 3

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

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The contents of this chapter have been submitted to the *Journal of Molecular Biology*. The following has been reformatted to maintain consistency with the remainder of this document. Craig Marten contributed to this work by providing the computer analysis. Andrea Terrel contributed figure 3.1, 3.2 and 3.4. My contributions to this work are the purification of the proteins involved in the reconstitution experiments, figure 3.3 and 3.6, creative input into the origins of nucleosome positioning on the *PHO5* promoter the results of which are shown in Figure 3.5. I also assisted in the writing of the Materials and Methods for the experiments I contributed.

3.1 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 (Sivolob & Khrapunov, 1995), 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.

3.2 Introduction

In eukaryotes the genomic DNA is packaged into chromatin, which compacts and organizes the DNA (van Holde *et al.*, 1995). The basic unit of chromatin is the nucleosome, the X-ray structure of which has been solved to 2.8 Å resolution (Luger *et al.*, 1997). The majority of nucleosomes are randomly placed throughout the genome. However, some nucleosomes adopt specific positions on the DNA (for a review seeWolffe, 1995). Positioned

nucleosomes occur on 20 promoters and play an important role in transcriptional regulation (Beato & Eisfeld, 1997). 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 (Satchwell & Travers, 1989). Alternatively, DNA *isotropic flexibility,* or ability to bend in all directions, has been correlated with translational positioning (Sivolob & Khrapunov, 1995). In the second mechanism, sequence-specific DNA-binding proteins can form a boundary or backstop, constraining the position of adjacent nucleosomes (Wolffe, 1994). 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 (Roth, 1995). 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 (Lowary & Widom, 1997; Widlund *et al.*, 1997). 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 (Drew & Travers, 1985; Prunell, 1982; Satchwell *et al.*, 1986).

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 (Svaren & Horz, 1997). The *PHO5* gene, which encodes a secreted acid phosphatase, is activated in the absence of phosphate (Bajwa *et al.*, 1984). Upon activation, four nucleosomes are displaced or reconfigured to allow assembly of the transcriptional machinery (Almer *et al.*, 1986). In the repressed state, the nucleosomes are specifically placed to create an enlarged linker region between nucleosome -2 and nucleosome -3 (Almer et al., 1986). 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 (Sivolob & Khrapunov, 1995), 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.

3.3 Results

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) and with 2.5 kb of the promoter and upstream sequences fused to a G-less cassette (pMH313-Gless). 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 3.1A) (Pilon *et al.*, 1997). 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 3.1B). 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 (Pilon et al., 1997), 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 3.1C). 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.

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 (Laybourn & Kadonaga, 1991). 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 3.2A). 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 3.2B). 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 3.2B). This high degree of similarity was seen previously in higher-resolution indirect end-labeling of *in vivo* chromatin (Figure 3 Almer et al., 1986). 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*.

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 (Hull *et al.*, 1991; Roth & Simpson, 1991) to footprint the nucleosomes on the *PHO5* promoter. The primers used for this analysis are shown in Figure 3.3A.

Nucleosome –1. In the footprint analysis we observed cleavage protection over the TATA-box and RNA start sites (Figure 3.3B and 3.3C). 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 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 3.3C). 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 3.3C and 3.3D). The nuclease protection is nearly complete (Figure 3.3C, 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 3.3C). 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 3.3D). 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* (Almer et al., 1986). Mapping the minimum length of the enlarged linker is based upon the unprotected cleavage sites from –365 to –316 (Figure 3.3D). 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 (Almer et al., 1986). 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 (Almer et al., 1986). 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 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.

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 (Fascher *et al.*, 1990; Fascher *et al.*, 1993; Svaren *et al.*, 1994; Venter *et al.*, 1994). 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 3.4). 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.

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 (Hayes *et al.*, 1991; Jayasena & Behe, 1989a; Jayasena & Behe, 1989b; Puhl *et al.*, 1991). "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 (Drew & Travers, 1985; Lowary & Widom, 1998; Muyldermans & Travers, 1994; Satchwell et al., 1986; Satchwell & Travers, 1989; Shrader & Crothers, 1990; Widlund et al., 1997). Algorithms have been developed that predict rotational positioning (Calladine & Drew, 1986; Drew & Calladine, 1987). 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 (Sivolob & Khrapunov, 1995), 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 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 (Flaus *et al.*, 1996; Sivolob & Khrapunov, 1995).

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 3.5B). 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 3.5B 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 (Metropolis *et al.*, 1953). 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.

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 3.6). 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.

3.4 Discussion

The Hörz and Bergman laboratories independently identified an array of at least four translationally positioned nucleosomes on the repressed *PHO5* promoter (Almer et al., 1986; Bergman *et al.*, 1986). These nucleosomes become nuclease transparent on the activated gene (Almer et al., 1986; Bergman & Kramer, 1983; Fascher et al., 1990). 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 (Straka & Horz, 1991). 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 (Fascher et al., 1990; Venter et al., 1994).

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* (Paranjape *et al.*, 1994; Robinson & Kadonaga, 1998). 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 (Tremethick *et al.*, 1990). 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 (Simpson *et al.*, 1985). In addition, a 200 bp fragment from the Drosophila *Adh* promoter

will translationally position a nucleosome correctly *in vitro* (Jackson & Benyajati, 1993). 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 (Venditti *et al.*, 1998).

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* (Almer et al., 1986). *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 (Almer et al., 1986) 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 (Venter et al., 1994). 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 (Fascher et al., 1990). Finally, deletion of both the Pho2p and Pho4p binding sites at UASp1 does not disrupt the *PHO5* promoter chromatin structure (Fascher et al., 1993). 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 (Sivolob & Khrapunov, 1995). 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 3.5). 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 3.7 (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 3.5B). 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 (Flaus & Richmond, 1998). 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 (Hagerman, 1990). Poly A sequences have a narrowed minor groove and are inflexible (Alexeev *et al.*, 1987). Due to these properties, long poly A stretches disfavor nucleosome formation (Prunell, 1982). 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 (Flaus & Richmond, 1998). In addition, poly A elements have been shown to function in yeast gene regulation through

effects on chromatin structure (Iyer & Struhl, 1995). 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 (Straka and Hörz,1991). 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 (Wolffe, 1995). 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.

3.5 Materials and Methods

Plasmids. The pPHO5-G-less plasmid was produced by subcloning the 526 bp *Bam* HI to *Apo* I fragment (-542 to -16) of pMH313 (Han *et al.*, 1988) 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.

Nucleosomal template reconstitution. Yeast core histones were purified as previously described (Pilon et al., 1997). 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.

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.

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 Micrococcal nuclease. 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.

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 (Woontner *et al.*, 1991), 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/).

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 (Shen *et al.*, 1991). 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.

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.

Primer extension footprinting of Pho4p. 500 ng of 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.

Prediction of nucleosome position. Our program was written in C++ for the Macintosh PowerPC and is based on the calculations of Sivolob and Khrapunov (Sivolob & Khrapunov, 1995). 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 (Sivolob & Khrapunov, 1995). 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.

Monte Carlo analysis. To determine the equilibrium positions of nucleosomes on the *PHO5* promoter we used Metropolis Monte Carlo analysis (Metropolis et al., 1953). 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.

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.

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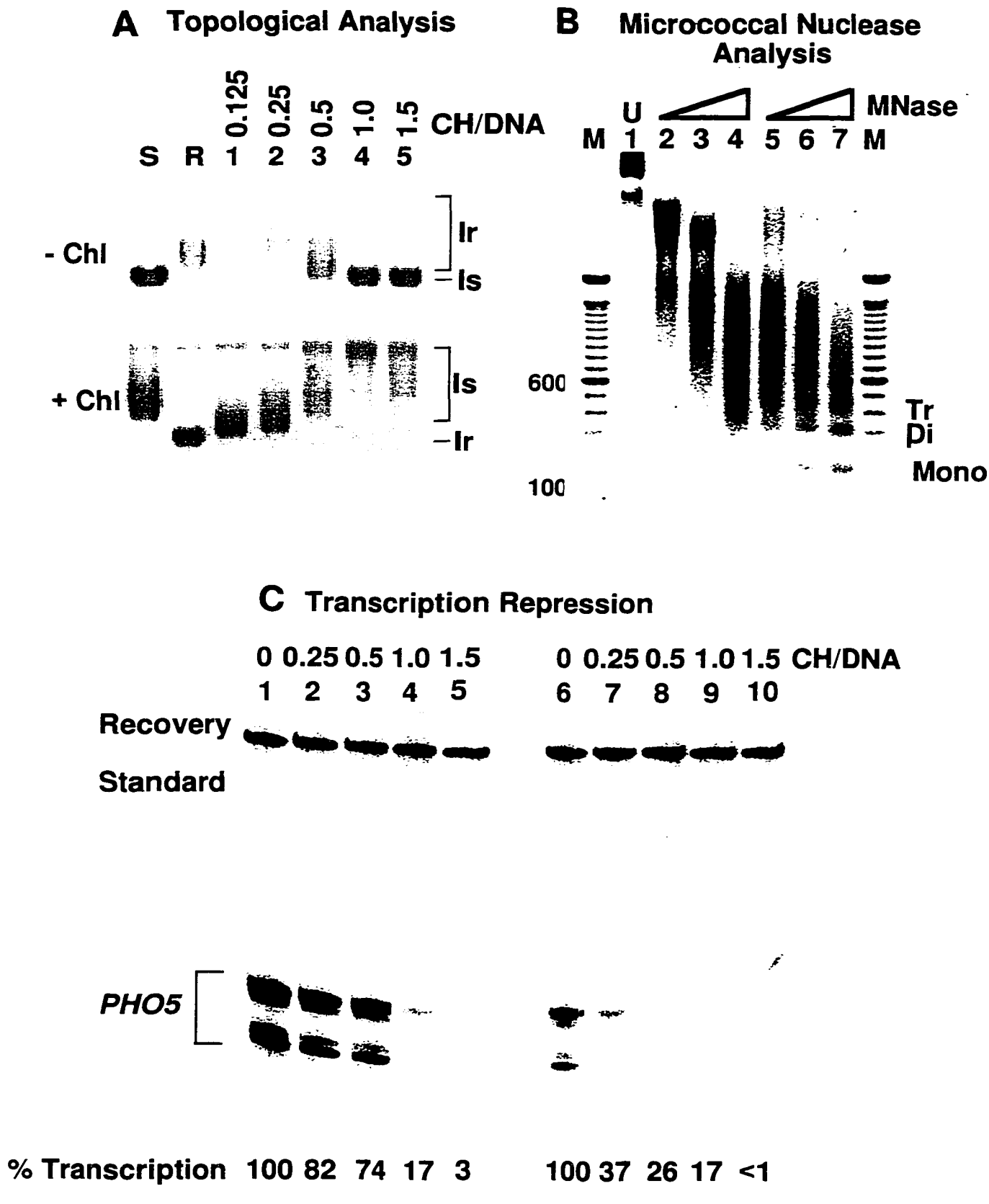

Figure 3.1

Figure 3.1. 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.

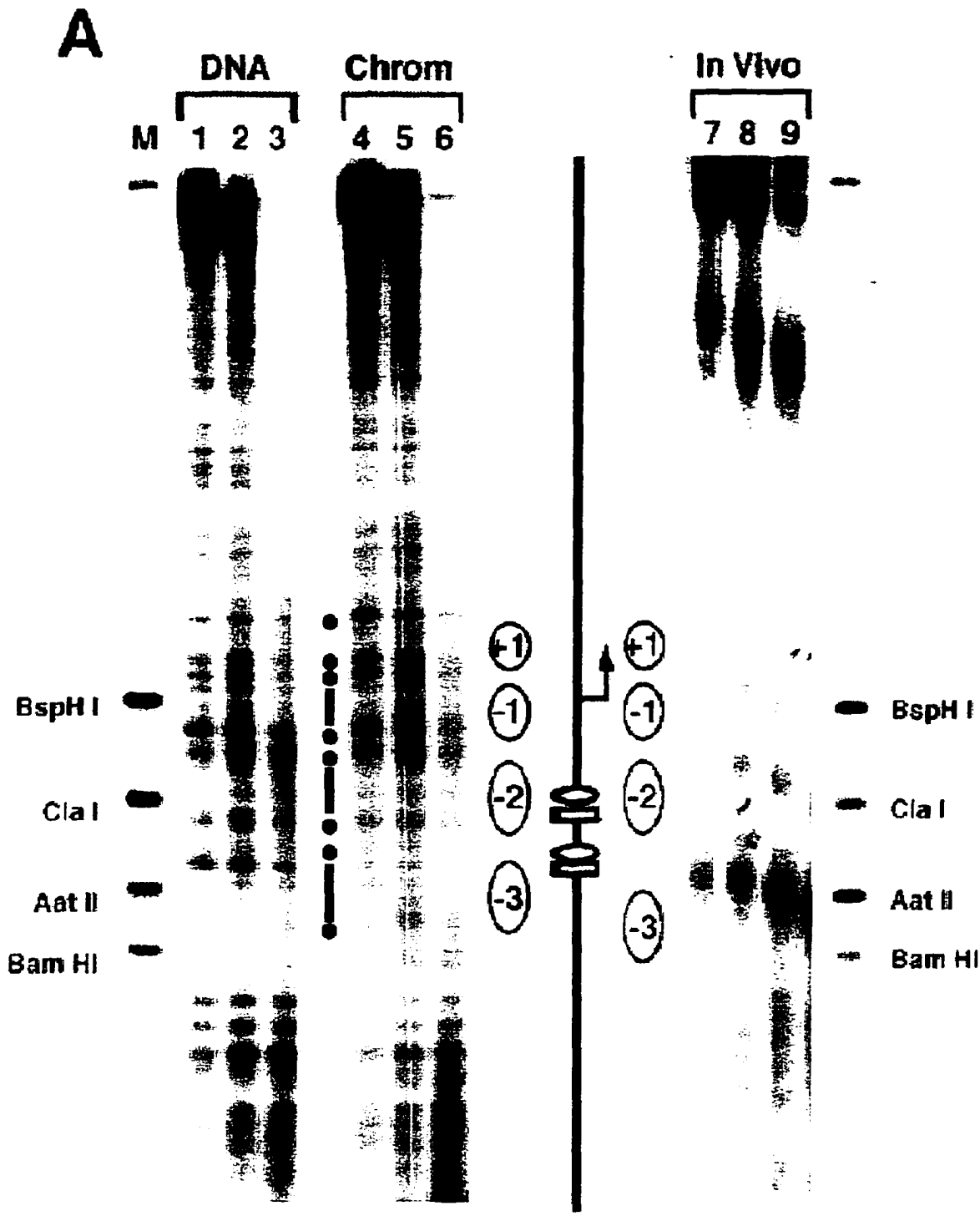

Figure 3.2A

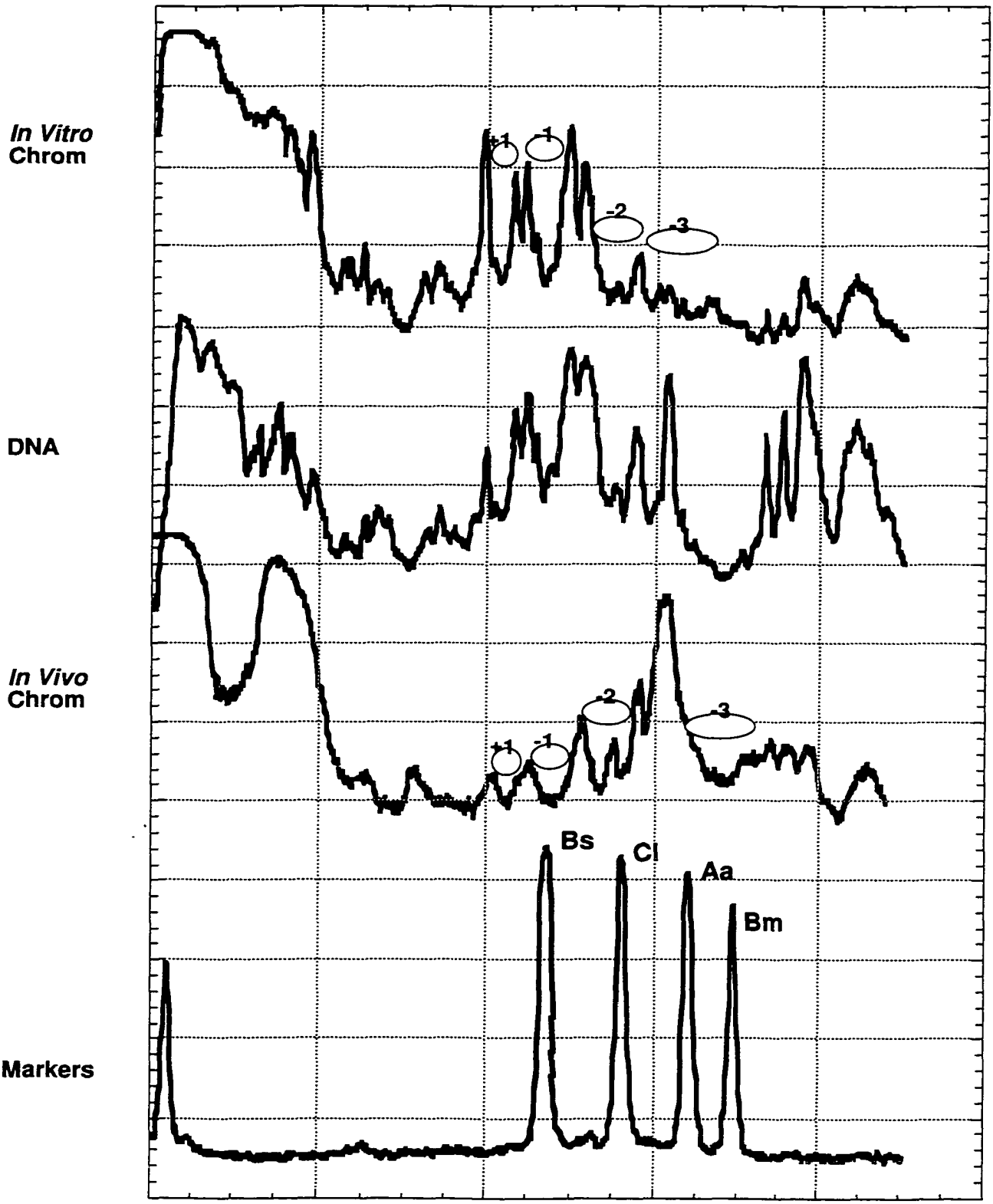

Figure 2B

Figure 3.2. 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.

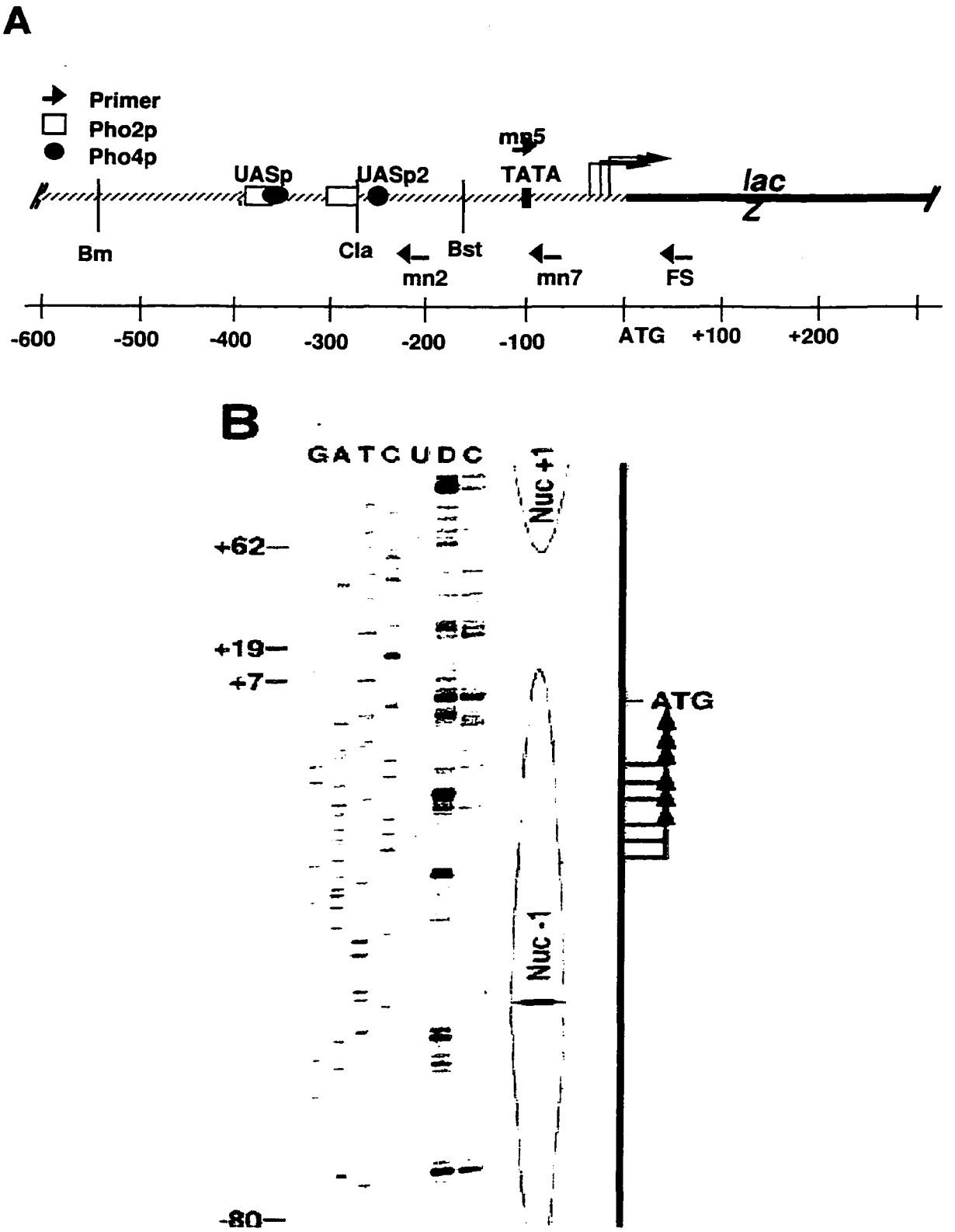

Figure 3.3 A&B

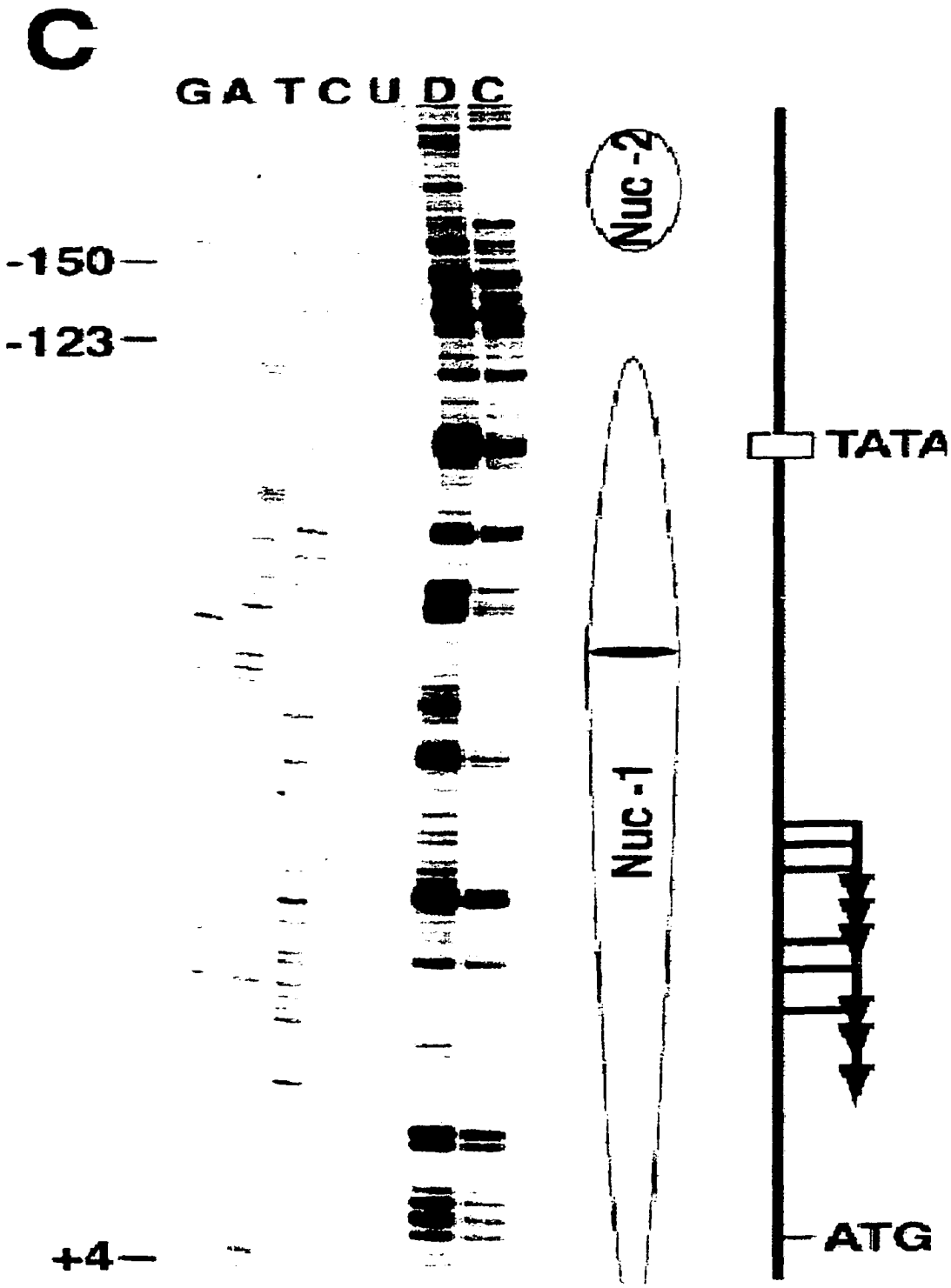

Figure 3.3C

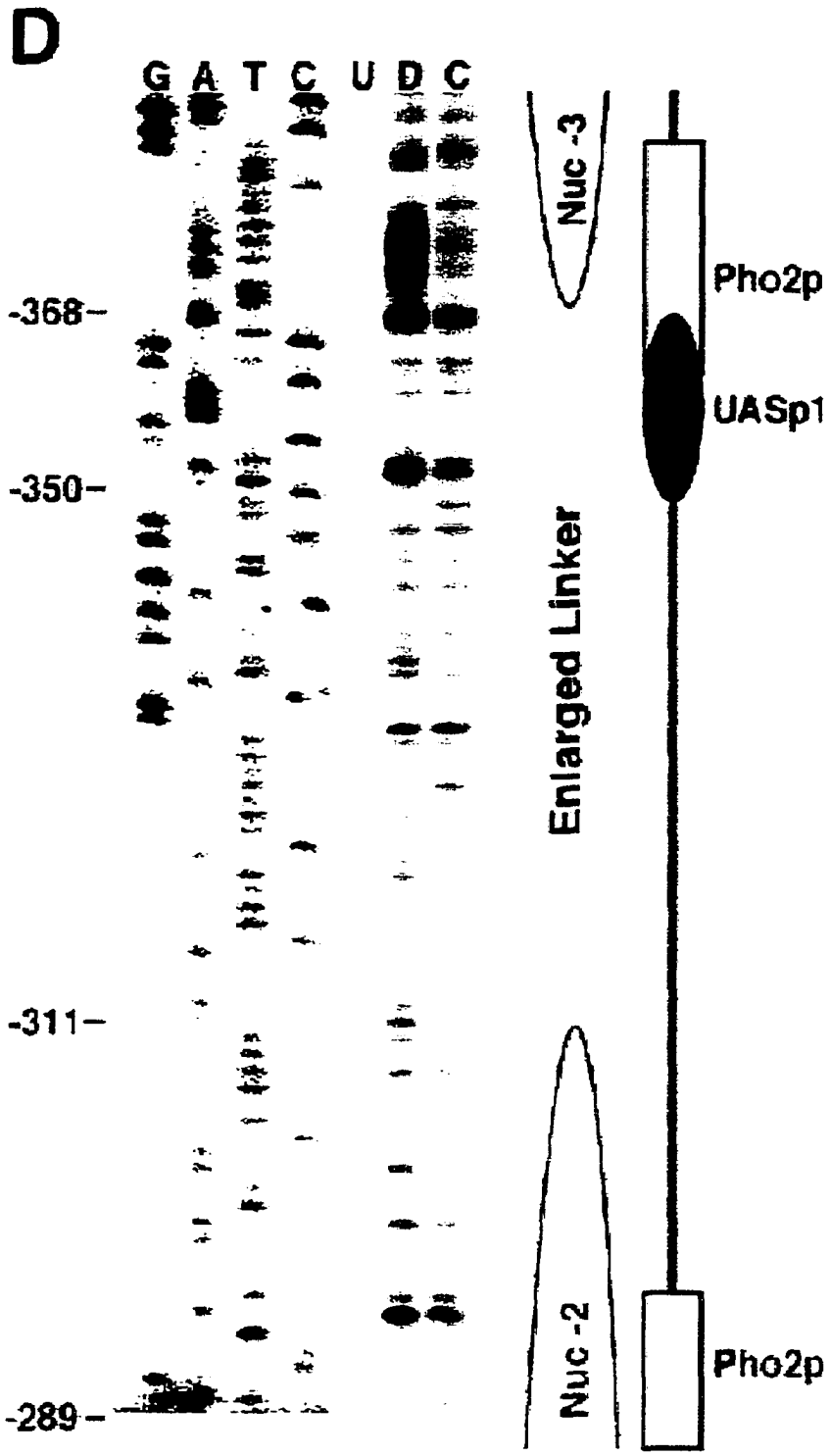

Figure 3.3 D

Figure 3.3. 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.

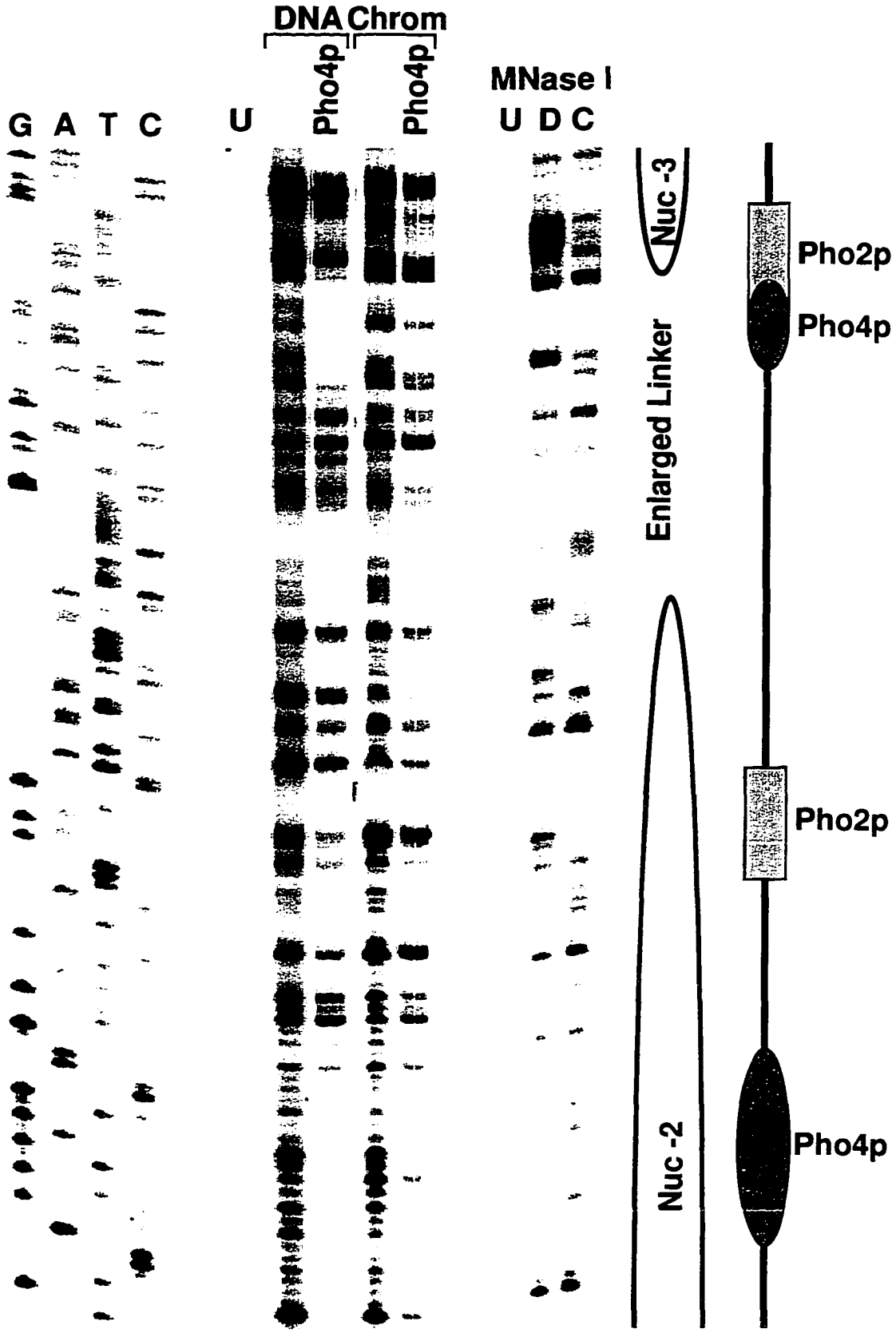

Figure 4

Figure 3.4. 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.

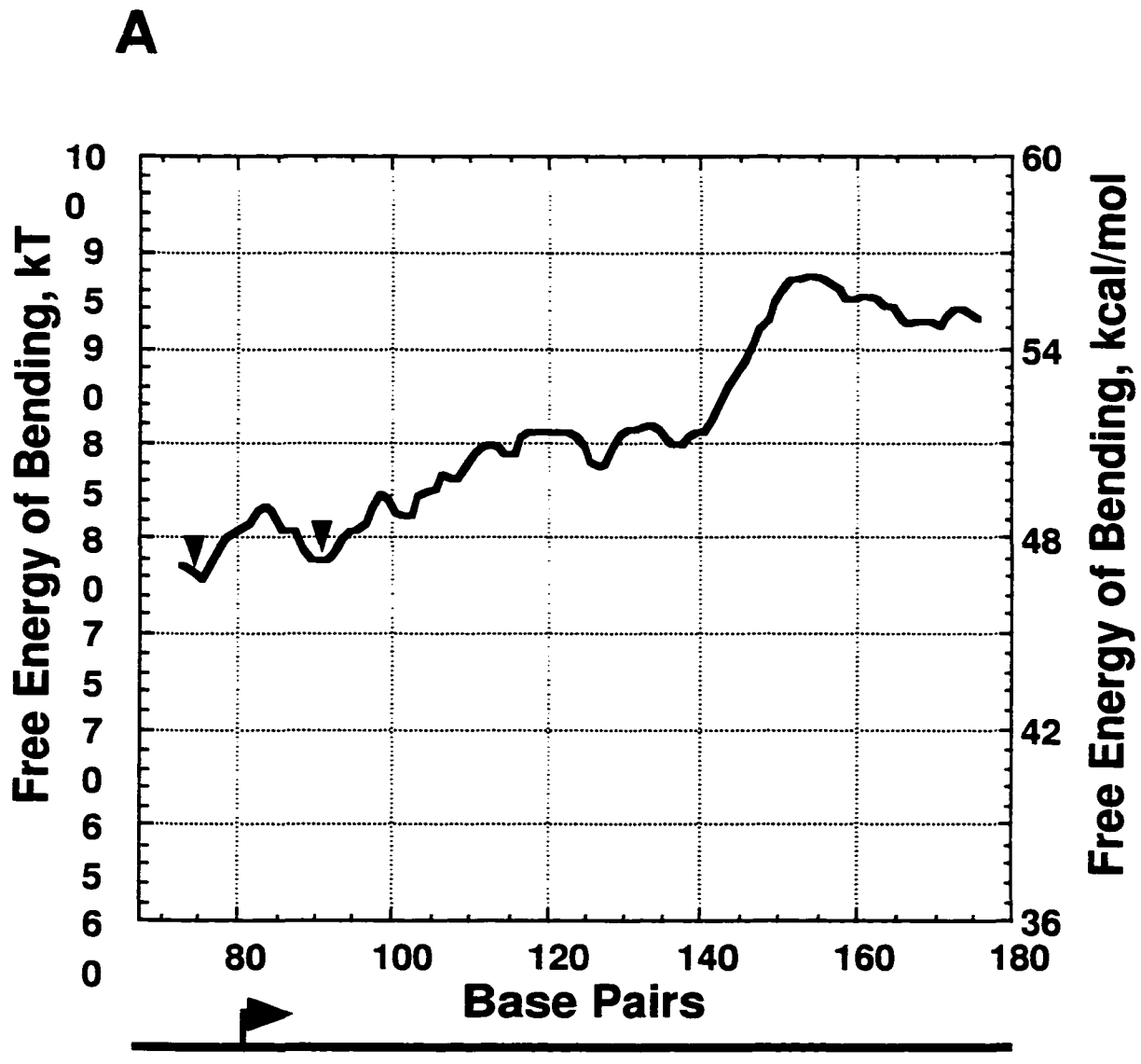

Figure 3.5A

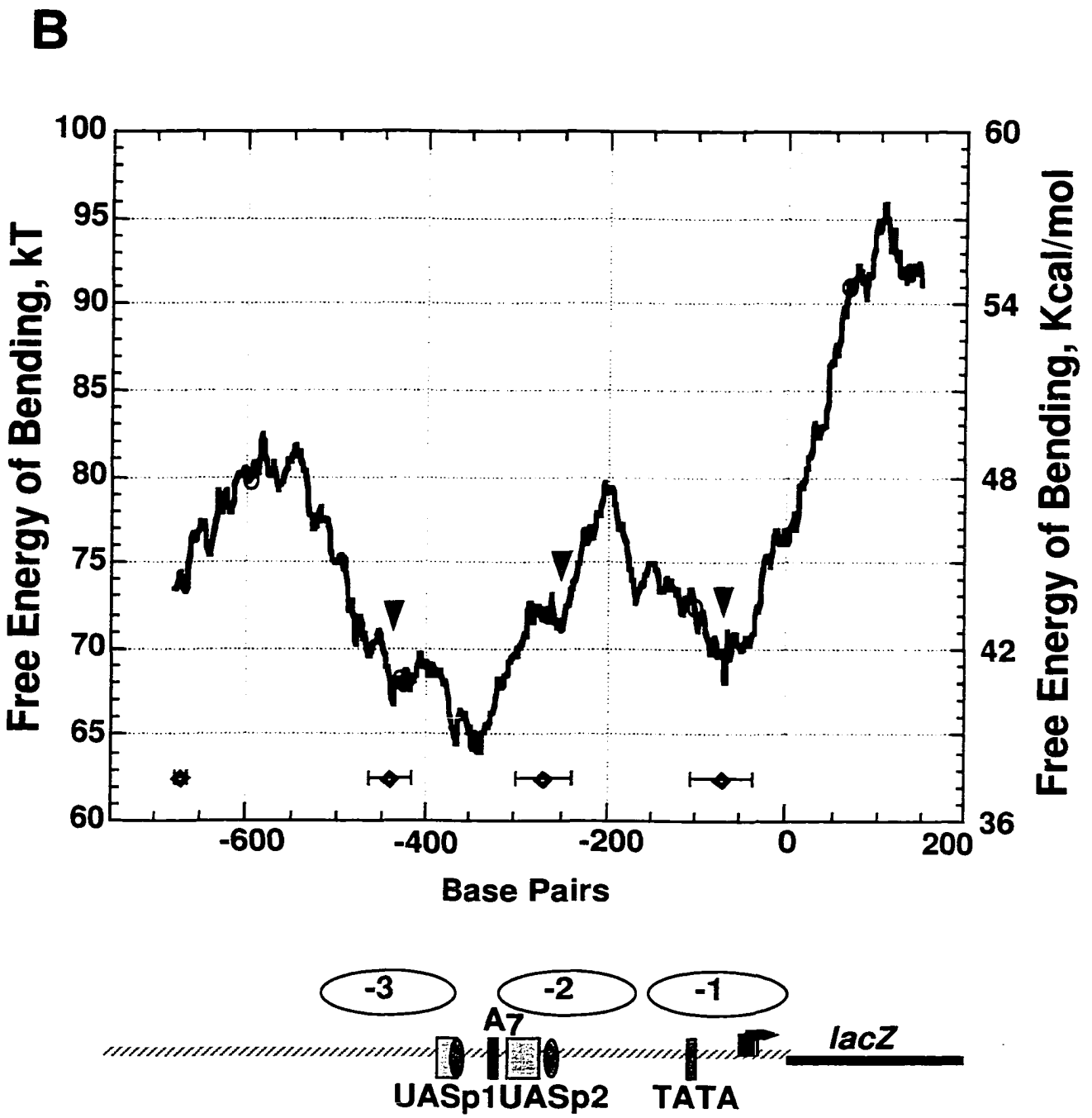

Figure 3.5B

Figure 3.5. Sequence-dependent isotropic flexibility of the *PHO5* promoter. The Gibbs free energy of bending (*Gn*) 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 A7 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.

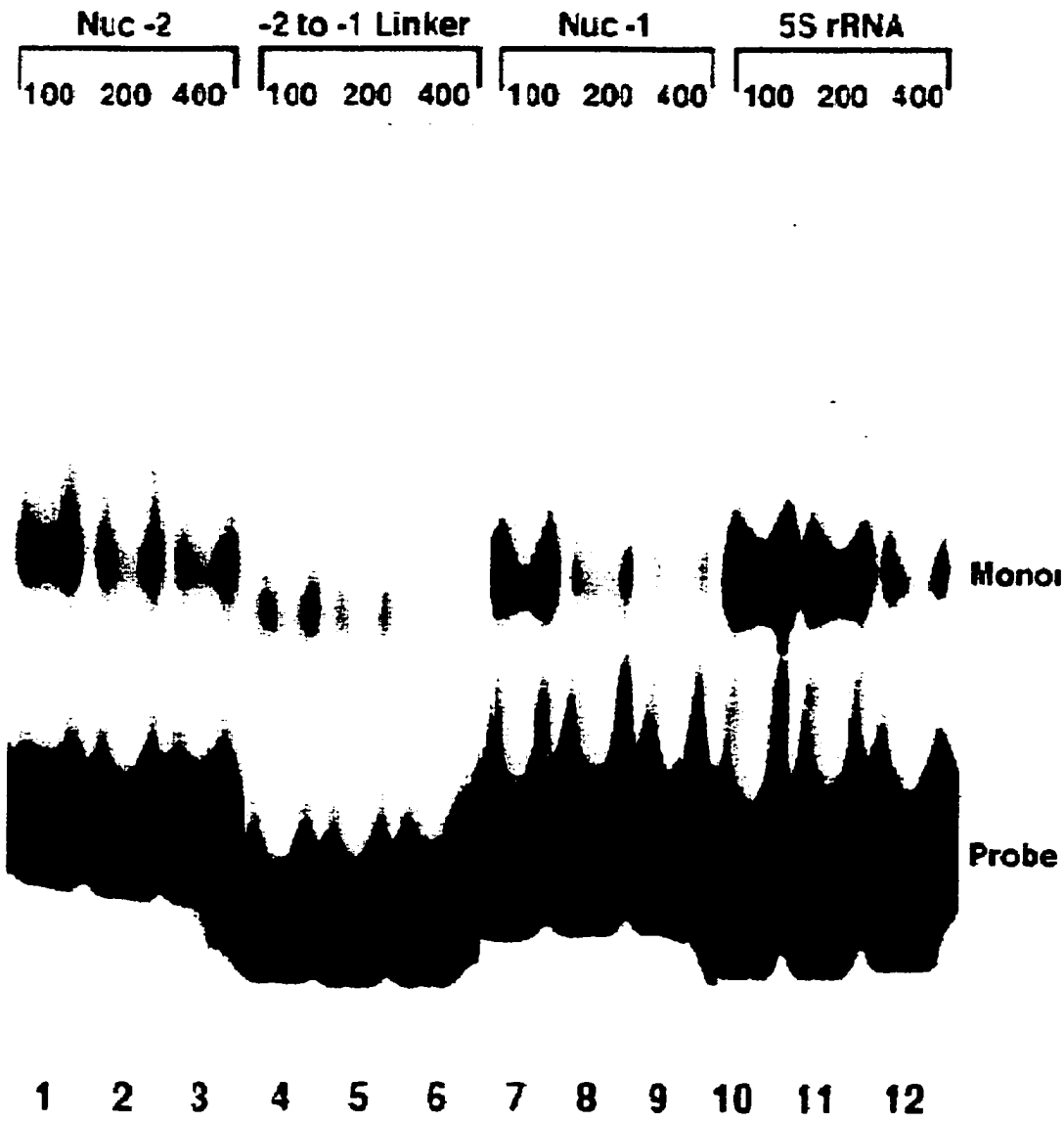

Figure 3.6

Figure 3.6. 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).

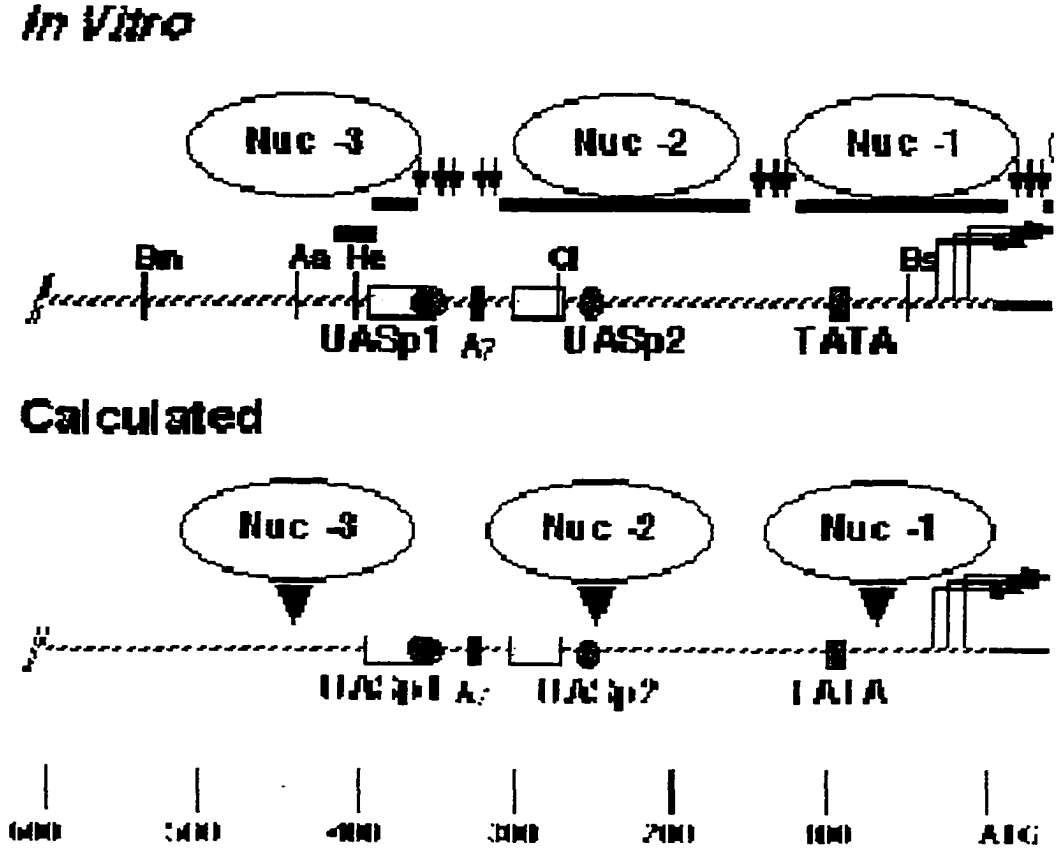

Figure 3.7

Figure 3.7. 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 A7 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.

Chapter 4

Mediation of Gene Expression by Histone-DNA interactions

4.1 Introduction

As discussed above, sequence dependent DNA properties can function to position nucleosomes and regulate gene expression. Translational nucleosome positioning on the 5S gene is of critical importance to the developmental regulation of gene expression. Experiments on the *PHO5* and MMTV-LTR promoters demonstrate how nucleosome positioning is of critical importance to gene activation and repression. In addition to the importance of positioned nucleosomes in gene regulation, histone-DNA contacts can also play a role in repression of gene expression. Genetic evidence from *sin*$^-$ mutants has shown histone-DNA contacts are an integral part in the regulation of gene expression. *Sin*$^-$ mutants

(switch-independent) transcribe genes in the absence of the chromatin remodeling activities of the ATP dependent SWI/SNF complex (Kruger *et al.*, 1995). The evolution of energy requiring chromatin remodeling complexes whose function is to loosen histone-DNA interactions, demonstrates the fundamental importance of histone-histone and histone-DNA interactions in the regulation of gene expression.

4.2 Histone-DNA interactions and the regulation of *PHO5*

Wechser and coworkers identified a single point mutation in histone H4 that was able to suppress a *swi* phenotype (Wechser *et al.*, 1995). In these experiments, the R45H mutation caused decreased levels of supercoiling and increased accessibility to nucleosomal DNA. When the plasmid pMH313 was used in $\beta-$ galactosidase assays for transcription of the *PHO5* gene, a 5.6-fold increase in trascription was observed under the repressing conditions of low phosphate in the R45H mutant. Interestingly, the mutated R residue is located at superhelix 0.5 on the NCP, where the minor groove of the DNA is fixed in place by interjection of the arginine side chain moiety. This implicates a functional requirement for some type of chromatin remodeling activity in the full activation of the *PHO5* gene. However, experiments have shown that the SWI/SNF complex does not play a role in *PHO5* expression (Gaudreau *et al.*, 1997).

Deletion analysis of the *PHO5* promoter by Rudolph and Hinnen identified regulatory sequences in the *PHO5* promoter (Rudolph & Hinnen, 1987). These regulatory elements were subsequently shown to be upstream activating sequences, which are bound by transcriptional activators Pho4p and Pho2p (Vogel *et al.*, 1989). However, one of the proposed regulatory sequences does not act as *cis*- acting sequence (Δ20 construct in fig 1). In the Δ20 construct, gene activity returns to normal as the size of the deleted region is increased, indicating this region is not a *cis*-acting activation sequence UAS. It can be hypothesized that in the Δ20 construct the repressive effects of nucleosome –2 is some how increased. This can occur either through alteration of a rotational frame which prevents efficient binding with the transcriptional activators Pho4p and Pho2p to their sites, or nucleosome –2 obtains a more stable translational position. In the deletion constructs an EcoRI linker was ligated to the BAL 31 digested fragments. This would move nucleosome –2 roughly 5bp from its original position, if it had a unique rotational frame. Future unambiguous nucleosome mapping work needs to be conducted to determine if nucleosome –2 obtains a unique rotational frame.

Replacement of the phased A tract located in the hypersensitive region between nucleosome –2 and –3 with a "random" DNA sequence, also had an effect on regulation of the *PHO5* gene. In these experiments, plasmid pMH313 was used in β–galactosidase assays. The results of these experiments were increased activation under low phosphate conditions

(John L. Pilon unpublished results). How and if chromatin structure is changed by this mutation is needed to understand this finding.

4.3 Future directions

Many questions remain about what role histone-DNA interactions play in gene expression. By elucidating the mechanism of nucleosome positioning on the *PHO5* promoter, many insights were gained as to the broad implications of being able to alter levels of gene expression by changing histone-DNA interactions. Future work could focus on how different DNA sequences can alter gene expression by changing chromatin structure.

As indicated by deletion analysis of the *PHO5* promoter region, rotational positioning of nucleosome –2 maybe important for expression of *PHO5*. To elucidate if indeed nucleosome –2 has a unique rotational frame, dyad mapping as employed by Panetta *et al.* would need to be utilized (Flaus *et al.*, 1996; Panetta *et al.*, 1998). To this end, primers have been designed to introduce a cysteine residue in place of Ser 47 on histone H4. The engineered cysteine residue would be derivatized to form a cytaminyl-EDTA moiety. Fenton chemistry is then used to cleave H_2O_2 to generate an OH radical, which results in single base pair cut at the dyad. This system could also be used to improve on the mapping presented in chapter three.

The linker region between nucleosomes –1 and –2 was shown to be partially refractory to nucleosome formation *in vitro* (Figure 3.6). This sequence could be sub-cloned such that the sequence would be incorporated into the different positioned nucleosomes in the promoter region of *PHO5*. It could be hypothesized that this would act as a "transcriptional activating sequence" and would cause a derepression of gene expression by destabilizing nucleosome structure. This result would be similar to the result obtained by Vishwanath and Struhl (Vishwanath & Struhl, 1995) which showed that homopolymeric dA:dT sequences can activate transcription by destabilizing chromatin structure.

More work could be done to improve the algorithm used to calculate translational nucleosome positioning in chapter three (Sivolob & Khrapunov, 1995). Specifically, an obvious improvement could be made with respect to incorporating variations in persistence length due to the presence of DNA structures extending beyond nearest neighbor interactions. This may help resolve the question as to why the most flexible region in the *PHO5* promoter is nucleosome free as determined by equation 9A in chapter one. The hypersensitve site between nucleosome –2 and –3 contains phased A tracts which has been shown to be a strait inflexible sequence (Haran & Corthers, 1989). Measurements of persistence length could be obtained by ligation experiments with constructs containing phased A tracts. The result of these experiments could then be accounted for in the summation thus improving prediction of nucleosome positions.

This improved algorithm could be used to predict nucleosome positions on other promoters to determine if translational nucleosome positioning occurs via sequence dependent changes in DNA flexibility.

4.4 Summary and conclusions

This dissertation contains the development of an experimental system to study gene regulation in the context of chromatin. The appendix to Chapter 2 shows the possibility that the system could hold into a biochemical approach to chromatin and its role in gene expression, if used properly. The failure of our lab to achieve transctiptional activation on chromatin templates using purified Pho4p and Pho2p alone shows that *in vitro* at least, more factors and activities are required (Andrea Terrell Dissertation). These activities are most likely chromatin remodeling activities such as those present in the SWI/SNF complex. Chapter three has shown that translational nucleosome positioning on the *PHO5* gene is a result of sequence dependent differences in isotropic DNA flexibility. This work and much literature cited in this document provide strong evidence of a central role for histone-DNA interactions in the regulation of gene expression.

It is my firm belief that evidence for this type of gene regulation will be present genome wide. One can speculate that transcription activating sequences and transcription repressing sequences, *which act by increasing or decreasing the repressive effects of*

chromatin, are relevant features of gene expression. Transcription activating sequences or transcription repressing sequences can have a mode of action that may result from anisotropic, iosotopic DNA bending. Interesting patterns of gene distribution have been shown that hint at the possibility of such a mechanism. These patterns show that AT rich regions of the human genome contain far fewer genes than GC rich regions (Gardiner, 1996). This gene distribution pattern is shown in table 1. Do GC rich regions function as transcriptional activating sequences thus resulting in the observed gene distribution? Much speculation can be made as to the genome wide effects of histone-DNA interactions and mediation of gene expression. If this form of gene regulation becomes recognized as important and is rigorously studied, much may be learned about perplexing questions of gene regulation.

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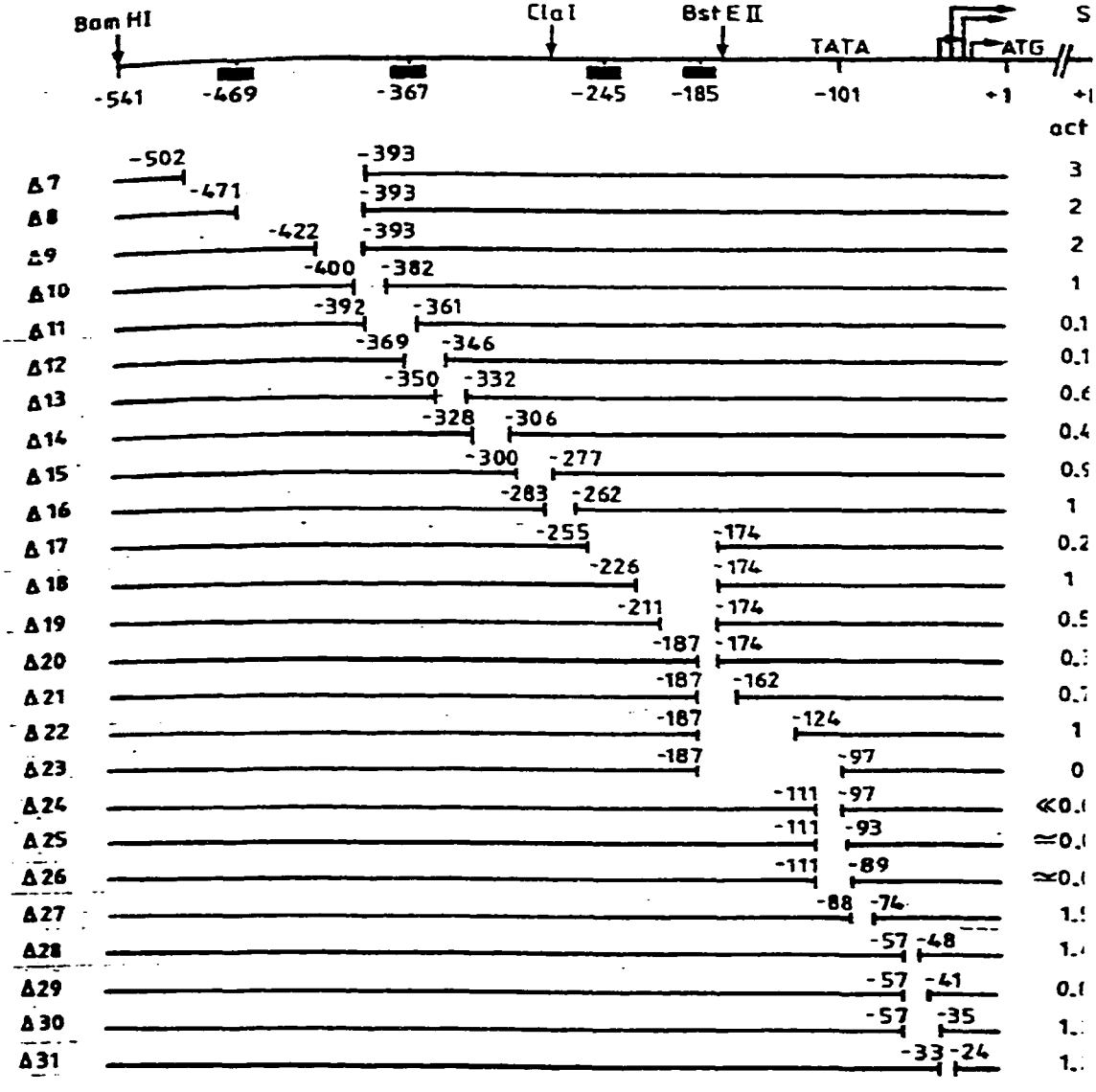

Figure 4.1 *PHO5* promoter deletions. Gene activity is expressed relative to wild type levels. Notice deletion construct 20 which results in a decrease to only 30% of wild type. Rudolph, H. & Hinnen, A. (1987). The yeast PHO5 promoter: Phosphate-control elements and sequences mediating mRNA start-site selection. *Proceedings of the National Academy of Sciences* **84**, 1340-1344.

Figure 4.1

Tabel 1
Base compostion and gene distribution

Isochore class	GC %	Genome%	Genes%	Mb DNA	Gene density
L1	38	30	34	1860	1/50kb
L2	41	32			
H1	44	21	38	190	1/25kb
H2	49	10			
H3	53	3	39	90	1/3kb

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