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A biochemical and biophysical study of nucleosome assembly by the Saccharomyces cerevisiae Nucleosome Assembly Protein 1

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A biochemical and biophysical study of nucleosome assembly by the *Saccharomyces cerevisiae* Nucleosome Assembly Protein 1

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry

by

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2006
The dissertation of Joon H. Huh is approved,
and it is acceptable in quality and form for publication on
microfilm:

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2006
For my family who have brought me this far
and to Li who will be with me forward.
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<tr>
<td>Asf1</td>
<td>Anti-silencing factor 1</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Caf1</td>
<td>Chromatin assembly factor 1</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-Cyclohexyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>Nap1</td>
<td>Nucleosome Assembly Protein 1</td>
</tr>
<tr>
<td>NCP</td>
<td>Nucleosome core particle</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>TAF1</td>
<td>Template activating factor 1</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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2002-2005 Molecular Biophysics Training Grant Fellow funded by the National Institute of Health.
ABSTRACT OF THE DISSERTATION

A biochemical and biophysical study of nucleosome assembly by the *Saccharomyces cerevisiae* Nucleosome Assembly Protein 1

by

Joon H. Huh

Doctor of Philosophy in Chemistry

University of California, San Diego, 2006

Professor Robert Dutnall, Chair
Professor Elizabeth A. Komives, Co-chair

Nucleosome Assembly Protein 1 (Nap1) is a highly conserved eukaryotic histone chaperone that preferentially binds histones H2A and H2B *in vivo* and can facilitate nucleosome assembly *in vitro*. To gain insight into the mechanism of Nap1-mediated nucleosome assembly we performed a detailed thermodynamic analysis of the Nap1-H2AH2B complex using recombinant *Saccharomyces cerevisiae* Nap1 (yNap1) and H2AH2B heterodimers. We find yNap1 is predominantly a dimer in solution and has a separable bipartite structure in which residues 81-150 (domain I) mediate dimerization and residues 172-372 (domain II) form a domain for binding yH2AH2B. Isothermal titration calorimetry reveals that a yNap1 dimer binds two yH2AH2B heterodimers via principal interaction sites in the two domain IIs, which
bind to the histone fold region of yH2AH2B with a $K_d < 10$ nM. Binding to additional yH2AH2B can occur via separate, lower affinity sites located in the acidic C-terminal tail of yNap1, which bind to the histone N-terminal tail regions with a $K_d \sim 110$ nM. Previous studies have demonstrated that the acidic C-terminal tail of yNap1 is dispensable for \textit{in vitro} nucleosome assembly activity, and therefore the minimum requirement for a functional Nap1-H2AH2B complex is a yNap1 dimer bound to two yH2AH2B. Analysis of the nucleosome assembly activity of full-length yNap1 and yNap1 truncation mutants suggests a concerted mechanism of H2AH2B deposition by Nap1 that rationalizes the rapid, high-fidelity assembly of nucleosomes \textit{in vitro} by Nap1. The proposed mechanism of nucleosome assembly is placed in context of recent publications.
I: Introduction
A. The chromatin context

All eukaryotes organize and package their nuclear DNA into protein-DNA complexes collectively referred to as chromatin (van Holde 1989). Chromatin has a profound influence on transcription, DNA replication and repair as well as providing a means for cells to compact their genetic material into the nucleus (Ehrenhofer-Murray 2004). The packaging of DNA into different chromatin structures can have a profound impact on access to the DNA. Highly condensed forms of chromatin called heterochromatin are refractory to the cell’s transcriptional machinery and can completely silence genes (Beaujean 2002). The more loosely packaged euchromatin contains the majority of the expressed genes in a given cell type. The less condensed structure of euchromatin is essential to the accessibility of the DNA to transcription factors and polymerases (Paranjape, Kamakaka et al. 1994). In addition to the regulation of access to DNA by structural occlusion, chromatin proteins can act as a scaffold that can be utilized as a means to recruit cellular factors for transcription and DNA repair (Strahl and Allis 2000).

Chromatin proteins can be modified by a myriad of post-translational modifications that can modulate both the biophysical properties of chromatin and can also act as signals for gene regulatory factors (Jenuwein and Allis 2001). The latter phenomenon has been well documented in recent years and has lead to the proposition of an epigenetic code (the “Histone code”) that appears to significantly influence genetic programs as well as other cellular processes. Therefore, the chromatin context of nuclear DNA is vitally important to processes that utilize genomic DNA.
There are several layers of chromatin organization. All eukaryotes organize their genomes into several large DNA molecules that are packaged into chromatin complexes called chromosomes. During most of the cell cycle the chromosomes are loosely organized into specific regions of the nucleus (except during mitosis when the chromosomes become highly condensed mitotic chromosomes). Although they are loosely organized, the DNA is still highly compacted relative to naked DNA of the same size. The organization is the result of the association of DNA with two groups of proteins: histone proteins and non-histone chromatin proteins. The histone proteins form the fundamental unit of chromatin, the nucleosome core particle while the non-histone chromatin proteins interact with DNA and/or the histone proteins to form more complex structures. The assembly of nucleosomes is the first and most fundamental step in \textit{in vivo} chromatin assembly that forms the context from which all of our genetic information is accessed. Therefore, a detailed study of the mechanisms of nucleosome assembly is crucial to understanding this fundamental cellular process.

\textbf{B. The core histone proteins and the nucleosome}

The fundamental unit of chromatin is the nucleosome core particle (NCP) composed of $\sim146$ bp of DNA wrapped 1.7 times around two copies each of the core histone proteins H2A, H2B, H3 and H4 (Figure 1.1) (Luger, Mader et al. 1997). The major core histone proteins are highly conserved in eukaryotes with a high degree of sequence identity for each histone gene (van Holde 1989). All of the core histone proteins contain a region of strong sequence similarity that folds into a three-helix bundle called the histone fold (Figure 1.2). All of the core histones exist in solution as specific non-nucleosomal histone complexes with intimate contacts made between
The nucleosome core particle is the fundamental unit of chromatin. It consists of one (H3H4)$_2$ tetramer flanked on either side by an H2AH2B dimer to form the histone core around which ~146 bp of DNA are wrapped. The DNA follows a left-handed super-helical ramp along the surface of the histone octamer ~1.7 super-helical turns. The assembly of nucleosomes onto a DNA molecule can reduce the linear length of the DNA molecule to 1/3 of its original length. This figure was generated from a molscript file from coordinates available on the PDB (1AOI).
Figure 1.2 The histone fold and histone complexes
Figure 1.2 shows a linear representation of a general histone protein and the specific histone folds of each of the major histone genes. Histones H2A and H2B form an obligate heterodimer with intimate associations involving a number of hydrophobic interactions. Histone H3 and H4 form an obligate heterotetramer. Two H3 histones interact with each other via a handshake motif utilizing their C-terminal helices. This figure was generated from a molscript file from coordinates available on the PDB (1AOI)
binding partners. Histones H2A and H2B form an obligate heterodimer while histones H3 and H4 form an obligate heterotetramer. In addition to the major histone proteins, most eukaryotes also produce a variety of H2A and H3 histone variants that are incorporated into nucleosomes. Evidence is mounting that the incorporation of variant histones can serve a specific cellular function (e.g., the exclusive packaging of macro-H2A on silent X-chromosomes) (Changolkar and Pehrson 2002; Fan, Gordon et al. 2002; Redon, Pilch et al. 2002).

High-resolution structural models of the histone octamer, the nucleosome core particle containing the major core histones, and the nucleosome core particle containing the histone variant H2A.Z have been published providing the molecular details of DNA packaging and histone-histone interactions in the nucleosome (Arents, Burlingame et al. 1991; Luger, Mader et al. 1997; Suto, Clarkson et al. 2000; Chantalat, Nicholson et al. 2003; Richmond and Davey 2003; Muthurajan, Bao et al. 2004). The nucleosome is composed of one (H3H4)2 tetramer flanked by two H2AH2B dimers. The histones form a disc like structure with a left handed super-helical ramp that binds DNA. The formation of the histone octamer, however, is not spontaneous under normal ionic strengths (Eickbush and Moudrianakis 1978). The histones are deposited onto DNA and form the core octamer structure in the process of forming the nucleosome core particle. The spatial orientation of the nucleosome as well as experimental data suggests that this is an ordered stepwise process. First, an (H3H4)2 tetramer is deposited onto DNA organizing ~80 bp of DNA followed by the addition of two H2AH2B dimers on either side of the (H3H4)2-DNA complex (Figure
Figure 1.3 Stepwise assembly

Figure 1.3 is a schematic representation of the steps of de novo nucleosome assembly in vivo. An (H3H4)$_2$ tetramer is first deposited onto DNA at the replication fork. This deposition allows (H3H4)$_2$ to organize ~80 bp of DNA around it to form a central core for assembly of the nucleosome. In vitro experiments suggest that (H3H4)$_2$-DNA complexes must be formed first for efficient, high fidelity nucleosome assembly in vitro. In a subsequent step, two H2AH2B dimers are added onto either side of the (H3H4)$_2$-DNA complex making specific contacts on the (H3H4)$_2$-DNA complex. Each of the H2AH2B dimers than organizes ~30 bp of DNA each to complete the nucleosome core particle.
1.3) (van Holde 1989). The deposition of each H2AH2B organizes approximately 30 bp of DNA per dimer to complete the nucleosome core particle.

C. Chromatin assembly

Chromatin assembly is an essential cellular process that packages nuclear DNA. The bulk of chromatin assembly occurs during S-phase to package replicated DNA but can occur independent of replication and at other stages of the cell cycle (Annunziato and Hansen 2000; Krude and Keller 2001; Smith 2002). The process of chromatin assembly begins with the assembly of nucleosomes. During replication, nucleosomes are assembled on daughter DNA duplexes via transfer of existing nucleosomes from ahead of the replication fork, and de novo assembly using newly synthesized histones. De novo nucleosome assembly in vivo occurs in a stepwise fashion: histones H3 and H4 bind to DNA first to form a (H3H4)$_2$-DNA complex, followed by the addition of two H2AH2B heterodimers.

Nucleosomes can be assembled in vitro using only DNA and histones, but this process is not spontaneous; typically, non-specific aggregates form if mixing occurs at physiological ionic strength (Tyler 2002). In the absence of any other macromolecules, nucleosome formation will only occur if histones and DNA are mixed at high salt concentration and the ionic strength lowered slowly by dialysis or dilution. Alternatively, simple polyanions, such as poly-glutamate or RNA, can assemble nucleosomes at physiological ionic strength if added to histones prior to mixing with DNA (Ito, Tyler et al. 1997). Rapid and efficient nucleosome assembly at physiological ionic strength, however, requires the addition of specific assembly factors. Nucleosome assembly factors are of two types; histone chaperones and ATP-
dependent nucleosome remodeling enzymes (Ito, Tyler et al. 1997; Tyler 2002; Loyola and Almouzni 2004). Addition of both of these types of factor will generate arrays of regularly spaced nucleosomes on linear or plasmid DNA. By themselves, histone chaperones can assemble nucleosomes in vitro although the nucleosomes do not possess regular inter-nucleosomal spacing.

Several different histone chaperones have been defined based on an ability to bind histones and facilitate nucleosome assembly. These include nucleoplasmin, chromatin assembly factor 1 (Caf1), anti-silencing factor 1 (Asf1), and nucleosome assembly protein 1 (Nap1). In general, each histone chaperone is conserved among eukaryotes but as a group they share little sequence similarity except for an acidic composition. They also display different histone binding preferences in vivo and can be categorized into those that bind histones H3 and H4 (e.g. CAF1, ASF1), or H2A and H2B (e.g. nucleoplasmin, Nap1). This is consistent with a two-step mechanism of nucleosome assembly. The newly synthesized H3 and H4 are probably bound by (H3H4)2 specific histone chaperones like CAF1 and imported into the nucleus where the complex is directed to replication forks. Once there, the chaperone may then deposit (H3H4)2 onto the newly synthesized DNA daughter strands. Histones H2A and H2B are bound by H2AH2B specific chaperones like Nap1 and imported into the nucleus. Once in the nucleus, the Nap1-H2AH2B complexes can be directed to (H3H4)2-DNA complexes for the full assembly of nucleosomes.

Although many histone chaperones have been characterized biochemically, the structural basis for nucleosome assembly activity by histone chaperones is poorly understood. At the outset of this study it was generally presumed that nucleosome
assembly by histone chaperones operated predominantly via an electrostatic screening mechanism preventing the misappropriate aggregation of histones and DNA (Dutnall 2004). A large charge difference could lead to rapid association kinetics consistent with the fast rate of chromatin assembly observed in vitro. Yet this simple mechanism ignores observations that suggest a more active role for histone chaperones in the assembly process. Many studies have demonstrated that removal of acidic regions from histone chaperones does not significantly impair the ability of the chaperone to bind histones or assemble nucleosomes as would be expected in a simple charge screening mechanism of nucleosome assembly (Fujii-Nakata, Ishimi et al. 1992; Umehara, Chimura et al. 2002). A recent study by Wagner et al (Wagner, Bancaud et al. 2005) has demonstrated that the histone chaperone Nap1 is more than three orders of magnitude more efficient in the assembly of nucleosomes than similarly sized poly-anions. Moreover, the high degree of conservation in histone chaperone families argues for the selection of specific histone-histone chaperone interactions that are necessary for nucleosome assembly in vivo (Kaufman and Botchan 1994). Taken together, these observations suggest that there is more to the mechanism of nucleosome assembly by histone chaperones than simple charge screening and that the structure and energetics of histone-histone chaperone complex play a key role in the assembly of nucleosomes. Therefore, understanding the mechanism of nucleosome assembly by histone chaperones will require knowledge of the structure and energetics of histone chaperone-histone complexes. In this study, I have focused on one of the best characterized and widely utilized histone chaperones Nap1.
D. Nap1/SET family

A wealth of information is available about Nap1 and recombinant Nap1 has been used extensively in chromatin assembly assays *in vitro*. Nap1 was first isolated from HeLa cell and mouse FM3a cell extracts as a 58/53 kD protein capable of binding all four-core histones and assembling nucleosomes *in vitro* (Ishimi, Hirosumi et al. 1984). All eukaryotes have at least 1 Nap1 protein with some higher eukaryotes, like humans and mice, possessing multiple Nap1 like genes (Figure 1.4). Knockout studies of Nap1 in mice, *Drosophila melanogaster* and *Caenorhabditis elegans* display lethal phenotypes, suggesting that some of the cellular functions of Nap1 are essential (Rogner, Spyropoulos et al. 2000; Lankenau, Barnickel et al. 2003).

In addition to Nap1, most eukaryotes also possess a related protein called SET/TAF1. The SET gene is frequently involved in a chromosomal translocation associated with leukemia. SET is identical to the template activating factor 1 (TAF1) protein, a human cellular factor required for replication and transcription from adenovirus genomic DNA. Together these two proteins form the Nap1/SET family of histone chaperones.

The Nap1 protein displays a high degree of sequence conservation with 30-50% sequence identity in the conserved core of the Nap1/SET family of proteins. Sequence alignments indicate that there are two regions of high sequence conservation (domains I and II) separated by a variable linker region. Domain I contains a conserved Nuclear Export Signal (NES) while domain II contains a conserved Nuclear Localization Signal (NLS). Several regions of largely acidic
Figure 1.4 Nucleosome Assembly Protein 1

Figure 1.4 shows a primary sequence alignment of the Nap1/SET family of proteins. There are two regions of high sequence conservation in the family. Domain I contains a conserved nuclear export signal (NES) while domain II contains a conserved nuclear localization signal (NLS). Domain I and II are linked together by a variable linker sequence with no obvious sequence conservation. Several acidic residue rich patches are distributed throughout the Nap1 primary sequence. A variable acidic C-terminal tail is a hallmark of the Nap1/SET family of proteins although it seems to be dispensable for assembly.
amino acid composition are distributed throughout Nap1 including a large acidic C-terminal tail, a hallmark of the Nap1/SET family of proteins.

In addition to its nucleosome assembly activity, Nap1 also plays a role in the nuclear import of H2AH2B heterodimers (Mosammaparast, Jackson et al. 2001; Mosammaparast, Ewart et al. 2002; Dong, Liu et al. 2005; Mosammaparast, Del Rosario et al. 2005). Several studies have demonstrated that Nap1 shuttles between the cytoplasm and the nucleus consistent with the presence of an NES and NLS in the conserved sequence of Nap1. Although histones H2A and H2B can enter the nucleus in the absence of Nap1 in *S. cerevisiae*, studies by Mosammaparast et al have demonstrated that Nap1 can interact specifically with histones H2A and H2B and the karyopherin Kap114 to aid in the transport of H2AH2B heterodimers into the nucleus through the Nuclear Pore Complex. This is consistent with an active role for Nap1 in the import and potential assembly of nucleosomes *in vivo*.

Nap1 has also been implicated as a modulator of gene transcription. A recent DNA microarray study demonstrated that in *S. cerevisiae* the deletion of yNap1 modulated the expression of approximately 10% of the yeast genome (Ohkuni, Shirahige et al. 2003). Other studies have demonstrated that Nap1 physically interacts with the histone acetyl transferase CBP/p300 and can augment the transcription of CBP/p300 dependent genes *in vivo* (Shikama, Chan et al. 2000). Nap1 and SET/TAF1 have also been shown to interact with sequence specific DNA-binding transcription factors (Rehtanz, Schmidt et al. 2004; Telese, Bruni et al. 2005). The exact mechanism of these observed effects is unclear but maybe related to alterations in chromatin structure brought about by Nap1 as recent studies have
demonstrated that Nap1 is capable of exchanging H2AH2B dimers for H2A.ZH2B dimers as well as assist in nucleosome sliding \textit{in vitro} as well as previous studies which have established a role for chaperones in the access of DNA targets (Walter, Owen-Hughes et al. 1995; Park, Chodaparambil et al. 2005).

Nap1 also has been implicated in modulating cell cycle progression (Kellogg, Kikuchi et al. 1995; Kellogg and Murray 1995; Altman and Kellogg 1997). yNap1 genetically interacts with the \textit{S. cerevisiae} B-type cyclin Clb2 and that yNap1 is required for the switch from polar to isotropic growth. yNap1 also interacts with Gin4 kinase, a protein required for normal progression through the cell cycle. yNap1 localizes to the presumptive bud neck along with other proteins prior to cell septation. These non-chromatin related activities of yNap1 are intriguing in that these cell cycle events could indicate a conserved role for Nap1 in signaling the end of chromatin assembly at the end of S-phase by direct influence on mitotic events. However, much further analysis is needed to understand Nap1’s role in any modulation of the cell cycle. A pictorial summary of Nap1/SET’s physical interactions is provided in Figure 1.5.

The best-characterized activity of Nap1 is its \textit{in vitro} nucleosome assembly activity. Nucleosome assembly activity is dependent upon Nap1’s ability to bind histones (Fujii-Nakata, Ishimi et al. 1992). \textit{in vitro} Nap1 can bind all four-core histones and the linker histone, however, \textit{in vivo} Nap1 only interacts with histones H2A and H2B as well as the histone H2A variant H2A.Z (Ishimi, Kojima et al. 1987; Rodriguez, Munroe et al. 1997; McQuibban, Commisso-Cappelli et al. 1998;
Figure 1.5 Nap1 interactions
A graphical summary of the known physical interactions of Nap1/SET family members and other cellular factors.
McBryant, Abernathy et al. 2003; Kepert, Mazurkiewicz et al. 2005; Saeki, Ohsumi et al. 2005; Shintomi, Iwabuchi et al. 2005). Nap1 cofractionates and co-immunoprecipitates with H2A and H2B from mouse cell extracts (Ishimi, Hirosumi et al. 1984). Similar experiments with *Drosophila* embryo extracts also demonstrate that Nap1 only immunoprecipitates with H2A and H2B and that H2A and H2B coprecipitate only the Nap1 histone chaperone (Ito, Bulger et al. 1996). Large-scale proteomics studies have identified interactions between Nap1 and histones H2A (and H2A.Z) (Uetz, Giot et al. 2000). The totality of the in vivo data suggests that the complex that is relevant for in vivo nucleosome assembly by Nap1 is the Nap1-H2AH2B complex.

**E. Focus of the study**

In this study I focused on a rigorous analysis of the Nap1-H2AH2B complex. I hypothesized that the interactions between Nap1 and H2AH2B heterodimers are more than just electrostatic and that stereospecific interactions between Nap1 and H2AH2B heterodimers directly influence Nap1’s nucleosome assembly activity. Some biochemical and biophysical characterization of Nap1 and Nap1-histone interactions had been performed prior to this work, yet some basic questions related to the molecular details remained unanswered such as what is the structure of Nap1 in solution, what is the stoichiometry of the Nap1-H2AH2B complex, and what are the energetics that govern the interaction of Nap1 and H2AH2B heterodimers?

I began my study with a detailed biophysical characterization of yNap1. In Chapter 2, I determined a domain structure for Nap1 as well as a stoichiometry for full-length yNap1 in solution. These data are correlated to a recent molecular model
of yNap1 based upon X-ray diffraction data of full-length yNap1 protein crystals (Park and Luger 2006). The data provide a picture of the Nap1 protein in solution in the absence of histone proteins providing firm footing for the interpretation of Nap1-H2AH2B binding studies detailed later in this study.

In order to study the interaction of yNap1 with H2AH2B heterodimers rigorously I must be able to obtain large quantities of homogenous yH2AH2B. In Chapter 3 I detail a novel method for the production of soluble recombinant histone proteins. The method provides a distinct advantage over other heterologous recombinant histone expression systems. These proteins were biochemically and biophysically characterized to insure faithful production of recombinant histones that are similar to those isolated from endogenous sources. In the course of this study, empirical molar extinction coefficients were determined to provide an accurate measure of protein concentration for the rigorous thermodynamic analysis that follows.

I applied a variety of biochemical and biophysical techniques in assessing the interaction between Nap1 and H2AH2B heterodimers as presented in Chapter 4. The rigorous techniques applied allowed me to firmly establish a stoichiometry for the yNap1-H2AH2B complex and equilibrium dissociation constants. These data were used to generate a model of the Nap1-H2AH2B interaction based upon analysis of full-length yNap1 as well as yNap1 truncation mutants.

Using the model of yNap1-H2AH2B interactions as a guide, I examined the ability of yNap1 to assemble nucleosomes in vitro. In Chapter 5 I determine the minimum Nap1-histone complexes that are capable of assembling nucleosomes in
vitro. A potential mechanism of assembly based upon these results and my model of
yNap1-H2AH2B interactions is provided. Recent studies of yNap1 nucleosome
assembly and histone exchange activity are also discussed in light of the proposed
mechanism of nucleosome assembly by yNap1. In the final chapter I shall put this
study of yNap1 in perspective with studies of other histone chaperones and discuss
the emerging picture of the active role of histone chaperones in the assembly of
chromatin.
II: Characterization of the solution structure of yNap1
A. Introduction

As a first step toward understanding the molecular mechanism of nucleosome assembly by Nap1, I undertook a study of the solution structure of the yNap1 protein to establish the domain structure and characteristics of yNap1 in solution. Although there were a few early hydrodynamic studies of the Nap1 protein, these studies failed to produce a clear picture of the oligomerization state of yNap1 in solution (Ishimi, Kojima et al. 1987; McBryant and Peersen 2004; Toth, Mazurkiewicz et al. 2005). Establishing a clear picture of the Nap1 protein is essential in interpreting the nature of Nap1-H2AH2B interactions that are detailed later in this study.

In this chapter, the characteristics of the yNap1 protein were analyzed using several biophysical and biochemical methods. Limited proteolysis of Nap1 proteins yielded a domain structure in solution from which truncation mutants were generated for biophysical and protein crystallography studies. During the purification of full-length yNap1 and yNap1 truncation mutants, gel filtration chromatography provided an estimate of molecular mass as full-length yNap1 and all yNap1 truncation mutants purified eluted as a single peak from gel filtration columns. Analytical ultracentrifugation sedimentation equilibrium experiments and chemical cross-linking studies of a subset of purified yNap1 proteins provided a more rigorous method for the determination of stoichiometry of yNap1 proteins. Consistent with previous reports and the molecular model of yNap1 determined from X-ray diffraction data of yNap1 protein crystals, full-length yNap1 is a dimer in solution. The examination of truncation mutants of yNap1 extends this analysis to demonstrate that domain I and
domain II can be separated and that domain I contains the majority of contacts involved in the dimerization of yNap1.

Finally this chapter will detail the protein crystallography studies of yNap1 protein.

**B. Methods and Materials**

**Expression and purification of recombinant Nap1**—Full-length recombinant yNap1 (Y1) was amplified from *S. cerevisiae* genomic DNA using the following primers: AAGCTTCATATGTCAGACCCTATCAGAAC (sense), and AAGCTTGGATCCTTATTATGACTGCTTGCATTCA (anti-sense), using standard methods. PCR products were cloned into pUC18 and sequenced. The ORF was then moved into pET29b (Novagen) and expressed by induction with 0.4 mM IPTG in *Escherichia coli* BL21 (DE3). Recombinant *Drosophila melanogaster* Nap1 (dNap1) was expressed in *E. coli* as described previously.

Truncation mutants of yNap1 were cloned using the PCR primer pairs summarized in Table 2.1. All truncation mutants were cloned into pET29b and expressed by induction with 0.4 mM IPTG in *E. coli* B834(DE3) pTrx (Y2) or BL21 (DE3) (Y10 and Y13).

yNap1, dNap1 and yNap1 truncation mutant proteins were purified using anion exchange, hydroxyapatite and size exclusion chromatography. Cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM Benzamidine, 1 mM PMSF, 6 mM β-Mercaptoethanol. Bacteria were lysed by adding lysozyme (Sigma, St. Louis) to a final concentration of 1 mg/mL re-suspended cell pellet volume and incubated for 1 hour on ice. The cell lysate was then homogenized by sonication and
Table 2.1 Table of primers used in the cloning of yNap1 and yNap1 truncation mutants in this study

<table>
<thead>
<tr>
<th></th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>AAGCTTCATATGTCAGACCCCTATCAGAAC</td>
<td>AAGCTGGATCTTATTAGTCTCCTCTCGAACCTCGAATTTC</td>
</tr>
<tr>
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<td>AAGCTGGATCTTATTAGTCTCCTCTCGAACCTCGAATTTC</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>AAGCTTCATATGTCAGACCCCTATCAGAAC</td>
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<tr>
<td>Y1</td>
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<tr>
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<td>AAGCTTCATATGTCAGACCCCTATCAGAAC</td>
<td>AAGCTGGATCTTATTAGTCTCCTCTCGAACCTCGAATTTC</td>
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clarified by high-speed centrifugation. The supernatant was applied to a Fractogel TMAE (EM Sciences) anion exchange column and protein eluted using a 0 - 1 M NaCl gradient in 20mM Tris-HCl (pH 8.0), 1 mM DTT and 1 mM EDTA. The fractions containing Nap1 were pooled, dialyzed extensively against 20 mM Na/K PO₄ (pH 7.0) and 1 mM DTT, and loaded onto a hydroxyapatite resin (BioRad) column and eluted using a linear gradient of 20 - 400 mM Na/K PO₄ pH 7.0 and 1 mM DTT. Fractions containing Nap1 were pooled and concentrated using an Amicon Stirred Concentration Cell (Millipore) using the appropriate ultrafiltration membrane and injected (2 mL injections) onto either Superdex 200 or Superdex 75 size exclusion column pre-equilibrated in 500 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA. Fractions containing yNap1 were pooled and dialyzed against 200 mM NaCl, 20 mM HEPES-NaOH pH 7.6, 2 mM DTT, 2 mM EGTA, and 3 mM MgCl₂. An amount of 50 % (v/v) glycerol was added until the final concentration of glycerol was 25 % (v/v) and the final purified protein was stored at –20 °C.

N-terminal sequencing and mass spectrometry were used to verify the faithful production of all recombinant proteins. Furthermore, the purity of the recombinant proteins was assessed by SDS-PAGE analysis and amino acid analysis (Y1, Y2 and Y10). The amino acid composition of the recombinant proteins matched the composition expected according to the published protein sequence suggesting a high degree of purity.

**Limited proteolytic digestion**—Purified recombinant yNap1 or dNap1 at 1 mg/mL was subjected to proteolysis by trypsin or chymotrypsin at a 1:100 mass ratio (protease:protein) at room temperature for 10 minutes. The limited digestion of
yNap1 was analyzed on a ThermoFinnigan corporation LCQ LC-MS (UCSD Biomolecular mass spectrometry facility).

**Determination of protein concentration**—Accurate protein concentrations were determined directly by amino acid analysis (UC-Davis Molecular Structure Facility) of at least two samples of proteins that had been previously dialyzed against 50 mM NaCl, 5 mM HEPES-NaOH pH 7.6 and 0.5 mM DTT. The protein concentrations determined by amino acid analysis were then correlated to absorbance measurements of identical samples at 280 nm and an extinction coefficient was calculated.

**EDC chemical cross-linking studies of full-length yNap1**—Full-length yNap1 protein at a concentration of 1 mg/mL was incubated with 1 mM EDC for 1 hour. The reaction was quenched with 10 mM β-Mercaptoethanol (β-Me) and SDS-PAGE sample buffer added. The samples were boiled and then analyzed on 8-20 % SDS-PAGE gel.

**Analytical ultracentrifugation sedimentation equilibrium experiments**—All analytical ultracentrifugation (AUC) experiments were carried out in a Beckman XL-A/XL-I analytical ultracentrifuge using a Beckman An60Ti 4-hole rotor at 20°C. Partial specific volumes for all Nap1 constructs were calculated from primary amino acid sequence using SEDNTERP (Johnson, Correia et al. 1981; Cole and Hansen 1999). Recombinant full-length yNap1 (Y1) was fractionated on a Superdex 200 size exclusion column in 150 mM NaCl, 5 mM HEPES pH 7.6 (NaOH titration), 0.5 mM DTT and eluted in a single peak. Fractions containing yNap1 were analyzed by AUC over a concentration range of 100 nM to 1 µM in a 6 sector charcoal-filled Epon centerpiece using quartz windows and absorbance optics at two different rotor speeds
(7000 and 10000 rpm). Prior to analysis, frozen Y10 protein stock was dialyzed extensively against 50 mM NaCl, 5 mM HEPES-NaOH pH 7.6, 0.5 mM DTT. A concentration range of 1 µM – 35 µM was analyzed as above at three different rotor speeds (12000, 20000, 28000 rpm). Y13 was analyzed on a Superdex 200 size exclusion column in 500 mM NaCl, 5 mM HEPES-NaOH pH 7.6, 0.5 mM DTT and eluted in a single peak. Fractions containing Y13 were then analyzed by AUC over a concentration range of 1 µM – 100 nM using two different rotor speeds (17000 and 24000 rpm). All data were analyzed using self-association models within Origin version 6.0 (Microcal and Beckman) fitting to an ideal single species. Molecular weight averages were determined from best-fit analysis with residual distributions.

Protein crystallization of yNap1—Full-length yNap1 as well as yNap1 truncation mutants were conducted using a hanging drop vaporization method. A sparse matrix screen from Hampton Research and rational screens of common protein crystallization reagents was used to initially screen the purified recombinant proteins for suitable crystallization conditions at both 4°C and 25°C. After setup, the crystal trays were examined 3 days, 1 week, 2 weeks, 3 weeks, and then monthly for protein crystals. Promising crystallization conditions were expanded in multiple dimensions with/and without additives.

C. Results

Purification and quantification of recombinant yNap1—Recombinant yNap1 and dNap1 were expressed and purified from E. coli to high degree of purity as described above (Figure 2.1). Samples of full-length yNap1 were analyzed for nucleosome assembly activity. Samples of yNap1 were submitted for amino acid analysis and
Figure 2.1 Purification of recombinant Nap1 proteins
A) An example summary gel of the purification of full-length recombinant yNap1. Lane 1 is a sample of the expression of yNap1 in E. coli. Lane 2 shows the soluble portion of yNap1 from E. coli. Lane 3 shows the insoluble portion. Lane 4 is after elution from an anion exchange chromatography step. Lane 5 is after elution from a hydroxyapatite chromatography step. Lane 6 is after a gel filtration chromatography step. The purified yNap1 protein runs approximately at the correct molecular mass relative to a molecular mass marker. B) A summary gel of the purification of recombinant dNap1 protein using a similar purification scheme to yNap1. C) A chromatin assembly assay utilizing recombinant full-length yNap1. This is a limited micrococal nuclease digestion following assembly of nucleosomes as described (Levenstein and Kadonaga 2002). (↓) indicate a ladder of digestion products produced from the limited digestion of a regularly spaced nucleosome array assembled on plasmid DNA.
mass spectrometry to insure the faithful production of recombinant yNap1 as well as determine an empirical molar extinction coefficient. In addition to full-length yNap1 (Y1), two yNap1 truncation mutants used in H2AH2B binding studies were also analyzed by amino acid analysis. Full-length yNap1 (Y1) was determined to have an extinction coefficient of 52160 M\(^{-1}\) cm\(^{-1}\). Y2 (residues 1-372) was determined to have an extinction coefficient of 72576 M\(^{-1}\) cm\(^{-1}\). Y10 (domain II) was determined to have an extinction coefficient of 36507 M\(^{-1}\) cm\(^{-1}\) (Table 2.2). These empirically determined extinction coefficients differ by ~30% from values predicted using the method described by Gill and von Hippel (Gill and von Hippel 1989). The difference between the empirical and predicted extinction coefficients may reflect the influence of structural and environmental factors on chromaphore absorption. For example, a UV absorbance wavelength scan of the Y2 Nap1 protein is significantly different from an absorbance wavelength scan of the full-length yNap1 protein suggesting a high degree of hyperchromaticity. The ability to predict extinction coefficients is highly dependent upon the amino acid composition of the protein. Deviations from “average composition” can lead to misleading predictions for extinction coefficients. Furthermore, comparison of the predicted concentration value of a Y2 stock solution from calculations based upon A\(_{280}\) vs. estimates based upon a BIORAD protein reagent assay suggest that the predicted concentration values for Y2 are inaccurate. Therefore, amino acid analysis of recombinant yNap1 proteins is the most reliable method for establishing absolute protein concentrations, a parameter that is vital to other aspects of the study presented here.
Table 2.2 Amino Acid Analysis of recombinant yNap1 proteins

<table>
<thead>
<tr>
<th></th>
<th>Y1</th>
<th>Y2</th>
<th>Y10</th>
</tr>
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<tbody>
<tr>
<td>predicted</td>
<td>36001 M⁻¹ cm⁻¹</td>
<td>35384 M⁻¹ cm⁻¹</td>
<td>26398 M⁻¹ cm⁻¹</td>
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<tr>
<td>actual</td>
<td>52160 M⁻¹ cm⁻¹</td>
<td>72576 M⁻¹ cm⁻¹</td>
<td>34271 M⁻¹ cm⁻¹</td>
</tr>
<tr>
<td>% difference</td>
<td>31%</td>
<td>51%</td>
<td>23%</td>
</tr>
</tbody>
</table>

* Calculations based upon accurate protein concentrations from Amino Acid Analysis of purified recombinant proteins. These concentrations were correlated to A₂₈₀ measurements of identical samples. Several A₂₈₀ measurements were taken to insure that the samples were measured in the linear range of the UV spectrophotometer used.
The Nap1 protein contains two stably-folded structural domains—The Nap1 protein is widely conserved in eukaryotes, sharing sequence similarity with a larger set of proteins called the NAP/SET family. The family contains two regions of high sequence conservation (30 – 50 % sequence identity) domain I and II (Figure 1.4). To investigate the domain structure of the Nap1 protein in solution, full-length recombinant yNap1 and dNap1 proteins were subjected to limited proteolytic digestion and the resulting fragments were identified by LC-MS. Limited tryptic digestion of yNap1 produced three major proteolytic fragments (Figure 2.2A and 2.2B). Similar fragments were produced upon tryptic or chymotryptic digestion of dNap1 (data not shown). The fragments correspond closely to domain I and II of the NAP/SET family of proteins suggesting that they are well fold and stable domains.

Domain II contains a putative nuclear localization sequence (NLS) that was cleaved in both yNap1 and dNap1 proteins (residues 291 – 300 of yNap1). Classical NLS are typically surface exposed, lysine/arginine-rich loops that present ideal trypsin cleavage sites. In the molecular model of yNap1 determined from X-ray crystallography, the sequence containing the NLS was observed as a small surface exposed loop connecting the segments of an anti-parallel β-hairpin. Furthermore, functional analysis of yNap1 and other NAP/SET family members (Fujii-Nakata, Ishimi et al. 1992; Seo, McNamara et al. 2001; Shen, Huang et al. 2001) indicates that deletions that disrupt either of the proteolytic fragments identified in domain II (subdomain C and D from the X-ray crystal structure) reduce or abolish histone binding and nucleosome assembly activity, suggesting that the two proteolytic fragments belong to a single functional unit. Therefore the two proteolytic fragments
Figure 2.2 Domain structure of full-length yNap1
A) 1mg/mL recombinant yNap1 digested with trypsin or chymotrypsin for the indicated times.  B) Analysis of fragments from limited trypsin digestion of recombinant yNap1 at an early time point led to the identification of three distinct polypeptides by LC-MS. The peptides encompass the majority of the conserved regions of the Nap1/SET family of proteins.  C) Proteolytic cleavage sites were mapped out on to a recent X-ray crystal structure of yNap1. Black dots (●) indicate the major trypsin cleavage sites. The location of the variable linker region between domain I and II is indicated with an arrow (←) as is the conserved surface exposed NLS.
identified in domain II (residues 172 - 291 and 300 - 355 of yNap1) most likely belong to a single structural and functional domain as observed in the molecular model of yNap1. The proteolytic cleavage sites observed by limited proteolytic digestion map mainly to regions of the crystal structure that are either surface exposed or absent from the electron density map (Figure 2.2C).

The pattern of proteolytic cleavage, sequence conservation, functional analysis and the molecular model of yNap1 suggests that the Nap1 protein contains two stably-folded structural domains (domain I, residues 81-150 and domain II, residues 183-370). Further support for this conclusion came from biochemical and biophysical analysis of truncation mutants of yNap1 (see below). The segment between domain I and domain II is proteolytically sensitive, and is more variable in length and composition among NAP/SET family members. This suggests that domain I and domain II are connected by a linker segment of less defined structural conformation. Similarly, the N-terminal tail and C-terminal tail regions (residues 1-72 and 373 – 417, respectively, of yNap1), were rapidly removed by proteolysis, and are of low sequence complexity or similarity among NAP/SET family members, suggesting that they, too, are more flexible in solution. This flexibility may contribute to a larger stokes radius for the yNap1 dimer and may be the reason for the large observed molecular mass estimates for full-length yNap1 by gel filtration chromatography. These results are generally consistent with the electron density observed in the structure of yNap1 (Park and Luger 2006). The contribution of each domain to the function of yNap1 is detailed in the rest of this study.
Full-length Nap1 is a dimer in solution—During the purification of recombinant yNap1, gel filtration chromatography of full-length yNap1 suggested that Nap1 is a multimer in solution. *D. melanogaster* Nap1 also had an elution volume consistent with a multimer of dNap1. The removal of proteolytically sensitive N-terminal or C-terminal tails of yNap1 (Table 2.3) resulted in proteins that eluted from gel filtration chromatography in volumes more consistent with a dimer of Nap1. Chemical cross-linking experiments with full-length yNap1 using the contact cross-linker EDC resulted in the cross-link of yNap1 monomers into a band that is consistent with a dimer of yNap1 (~100 kD in mass) as assessed by SDS-PAGE analysis Figure 2.3A. Other higher order oligomers are also visible, but the predominant band is the 100 kD band of a yNap1 dimer. Analytical ultracentrifugation studies of full-length yNap1 also provided an estimate of molecular mass from sedimentation equilibrium experiments. Full-length yNap1 was analyzed via AUC under a range of concentrations and rotor speeds. Sedimentation equilibrium analysis showed that yNap1 is a stable dimer in solution (Figure 2.4) with a propensity to further oligomerize at high protein concentrations, as indicated by an increase in the average molecular weight and systematic deviations observed in the residuals. The species observed in chemical cross-linking studies of yNap1 with masses greater than 100 kD may be higher order oligomers of yNap1 and reflective the propensity of yNap1 to oligomerize into species beyond that of a dimer of yNap1.

Analysis of yNap1 truncation mutants that removed either the proteolytically sensitive N- or C-terminal tails yielded proteins that eluted from gel filtration chromatography with molecular mass estimates closer to that of a dimer of yNap1.
Table 2.3 Summary of constructs made along with gel filtration data

*Gel filtration mass estimates based upon at least 2 injections of each protein on either a Superdex 200 or Superdex 75 gel filtration column run at 1 mL/min.
Figure 2.3 The full-length yNap1 protein is a dimer
A) 1 mg/mL recombinant full-length yNap1 was chemically cross-linked with the zero length cross-linker EDC for 1 hour 25°C and then analyzed on 8-20 % SDS-PAGE. A species with a mass of ~100 kD appears in the presence of EDC consistent with a dimer of yNap1. B) Sedimentation equilibrium experiments of full-length yNap1 suggest that it is a dimer. The above fit is representative of data collected.
These data suggest that the large estimate of mass by gel filtration chromatography for full-length yNap1 is due to the presence of the N- and C-terminal tails of yNap1. The tails may contribute to a large stokes radius increasing the estimate of molecular mass or may interact with yNap1 to form higher order oligomers (beyond dimer). Binding studies with yH2AH2B suggests that the binding of histone proteins may abrogate higher order oligomers of yNap1 (See Chapter 4). This suggests that the yNap1 dimer may be the active form of yNap1.

**Domain I mediates dimerization of yNap1**—Sequence analysis of domain I and domain II of yNap1 by coiled-coil does not reveal any regions of yNap1 that appear to have a propensity for the formation of a coiled-coil structure, however, close inspection of domain I does reveal a section similar to SET/TAF1β where there are several hydrophobic residues in a region that looks like a portion of SET/TAF1β that is predicted to be a coiled-coil Figure 2.4. Using sequence alignments and the limited proteolysis data we extended the structural analysis of yNap1 by performing hydrodynamic analysis of yNap1 by examining truncation mutants that contain only domain I or domain II. The purified proteins were stable in solution and were both analyzed by size exclusion chromatography and AUC-sedimentation equilibrium experiments. The truncation mutant of yNap1 that contained only domain I (Y13) eluted during size exclusion chromatography with an apparent molecular mass of ~83 kD (Table 2.3) suggesting that it is a multimer in solution. Sedimentation equilibrium analysis of the domain I confirmed that it is a multimer, and yielded a molecular mass value generally consistent with formation of a dimer under the conditions examined. This suggests that domain I mediates dimerization of yNap1, consistent with results
**Figure 2.4 The coiled-coil like structure of yNap1**

Shaded positions indicated conserved hydrophobic residues spaced 3 or 4 residues apart. Asterisks indicate hSET/TAF1 residues shown by mutagenesis to be involved in dimer formation.

SET/TAF1

25-EKEQOEAEHDELQONEEDEQSEELKECKRLKLFO-65

yNap1

89-PKNVEKESQELKTIQSEEFEKEQVCFLKELYK-129
obtained in a previous study of the related SET/TAF1β protein (Miyaji-Yamaguchi, Okuwaki et al. 1999) and with the dimerization interface observed in the yNap1 crystal structure. The non-globular, coiled-coil like structure of domain I may contribute to the exaggerated estimate of molecular mass derived from size exclusion chromatography of the yNap1 domain I only mutant (Y13).

I also analyzed a truncation mutant that only contained domain II of yNap1 (Y10). Y10 eluted with an apparent molecular mass consistent with a monomer (Table 2.3). Sedimentation equilibrium analysis also indicated that domain II is predominately a monomer in solution. At high protein concentration (> 10 µM) Y10 displayed a propensity to self-associate or aggregate although it did not form a dimer or other distinct multimer under the conditions tested.

**Protein crystallography**—Full-length yNap1 as well as many of the yNap1 truncation mutants were analyzed for their ability to crystallize using both sparse matrix screens and rational screens of common precipitants. A large amount of effort was expended in the pursuit of protein crystals suitable for X-ray diffraction studies for the generation of a molecular model of yNap1. Representative pictures of protein crystals are presented in Figure 2.5.

**D. Discussion**

Analysis of the solution structure of yNap1 indicates that it contains two stably-folded domains, domain I and domain II, connected by a linker region, with flexible N-terminal and C-terminal tails (Fig. 5). Biochemical and biophysical analysis determined that yNap1 is an obligate dimer in solution with some propensity to further oligomerize at high protein concentrations, consistent with previous reports
Figure 2.5. Protein crystals of yNap1 truncation mutants
A) Y2 in PEG/LiCl; B) Y2(C200/272S) in PEG/Ammonium Sulfate; C) and D) Y2(3CS) in PEG/NiCl₂; E) Y4 in PEG/LiCl; F) Y5 in PEG/K Acetate; G) Y6 in PEG/Isopropanol; H) Y6 in PEG/Zn Acetate
Our analysis indicates that domain I is responsible for yNap1 dimer formation, as observed for the SET/TAF1β protein (Miyaji-Yamaguchi, Okuwaki et al. 1999) and in the recent crystal structure of yNap1. Domain I is capable of folding into a stable protein that is predominately a dimer in solution. This structural organization is consistent with sequence conservation among members of the NAP/SET family and reports that other members of the NAP/SET family also self-associate (Ito, Bulger et al. 1996; Shikama, Chan et al. 2000; Shimizu, Akashi et al. 2000). Domain II is also capable of folding into a stable compact protein. Domain II, in contrast to domain I, behaves like a monomer in solution at physiological ionic strengths and at moderate protein concentrations (< 10 µM). This bipartite solution structure is consistent with the molecular model of yNap1 constructed from X-ray diffraction data. Functional studies of yNap1 and the related SET/TAF1β protein suggest that both the ability of Nap1/SET to dimerize and an intact domain II are necessary for in vitro nucleosome assembly activity. The contribution of these domains to binding of histones and nucleosome assembly activity are investigated in the following chapters.

The finding of significant deviations from the predicted molar extinction coefficients was surprising and has major impact on the interpretation of equilibrium dissociation constants and stoichiometry of Nap1-H2AH2B complexes. The differences between the predicted values and the actual values may be due to hyperchromisity that is not accounted for in the prediction methods described by Gill and von Hipple. The empirical determination of protein concentration provides firm footing for the analysis that follows.
Part of the text of Chapter II is a reprint of materials used in this dissertation have been submitted as part of a manuscript “Thermondyanmic Analysis of the interaction of the histone chaperone Nap1 with histones H2A and H2B: Implications for a concerted mechanism of nucleosome assembly,” Huh JH, Bergqvist S, Hampton E, Schurter MA, and Dutnall RN for publication in Journal of Biological Chemistry. The author of this dissertation was the primary author and researcher of the material in the manuscript. Coauthors Bergqvist, Hampton and Schurter performed research support. Robert Dutnall directed and supervised the research.
III: Production of soluble recombinant histone proteins
A. Introduction

The nucleosome core particle (NCP) is composed of ~146 bp of DNA and a defined core of histone proteins (2 each of histones H2A, H2B, H3 and H4). This defined core of histone proteins forms a disc like octamer structure that DNA wraps around. The histone proteins have the distinction of being “one of the first groups of proteins to be recognized as a distinct class, with unique properties.” (van Holde 1989)  Histones are ubiquitous and are found in all somatic cells of all eukaryotes. The core histones have a high degree of sequence conservation across all eukaryotes. This conservation is consistent with the function of the histone proteins as the scaffold for the organization of the nucleosome core particle.

*In vivo* and *in vitro* histones form discreet non-nucleosomal sub-complexes. Rigorous biophysical and biochemical studies have identified the H2AH2B heterodimer and the (H3H4)$_2$ heterotetramer as the dominant non-nucleosomal histone sub-complexes. The formation of these sub-complexes appears to occur by a three-state folding mechanism where binding to an obligate partner mediates folding through a dimeric intermediate state to the native structure for H2AH2B and a more complicated involving an equilibrium between folded H3H4 dimers and (H3H4)$_2$ tetramers (Banks and Gloss 2004; Placek and Gloss 2005). In the absence of its obligate binding partner the individual histones display a tendency to aggregate, particularly at high protein concentrations. This is due mainly to the absence of the intimate hydrophobic interactions of the histone fold regions observed in either the H2AH2B dimer of the (H3H4)$_2$ tetramer.
In order to study histones and chromatin, large amounts of histones are needed. Histone proteins were first isolated from endogenous sources utilizing a variety of methods that either neutralized the acidic DNA phosphates or extracted the histones utilizing high ionic strength conditions. The isolation of histones from endogenous sources revealed the heterogeneity of the endogenous histone protein population. There are multiple histone variants as well as myriad of post-translational histone modifications (Jenuwein and Allis 2001). In order to study the biochemical and biophysical function of histone proteins and the nucleosome core particle, heterologous protein expression systems have been developed for the production of well-defined homogenous histone proteins (Luger, Rechsteiner et al. 1999). With these methods, milligrams of histones can be produced in bacteria that possess no post-translational modifications. These bacterial expressions systems also allow for the manipulation of histone DNA sequences and thus the ability to produce point mutations and deletion mutants such as the “tail-less histones”, histones lacking the proteolytically sensitive N-terminal tail segments.

A significant draw back of the established methods is that due to the absence of their obligate binding partner, expression of the individual histones in bacteria results in the formation of histone protein inclusion bodies. These methods therefore require that one first solubilize the expressed protein from inclusion bodies and then refold the histones in the presence of a stoichiometric amount of their obligate binding partner to form the appropriate histone complexes. Due to the laborious nature of these methods and the potential for modification by denaturants such as urea, the Dutnall lab developed an approach that produces soluble histone proteins.
from bacterial expression systems. Reasoning that the insolubility of singly expressed histones was due to the lack of the obligate binding partner, the lab has created novel co-expression methods to produce soluble H2AH2B heterodimers in bacteria as well as an optimized protocol for their rapid isolation. Similarly, histones H3 and H4 can also be co-expressed in bacteria, but are not completely soluble, possibly due to their higher intrinsic DNA-binding affinity. The Dutnall lab has also constructed an (H3H4)₂ co-expression plasmid (adapted from M. Levenstein) (Levenstein and Kadonaga 2002) that includes the histone chaperone Asf1 that provides a simple method to express and obtain soluble (H3H4)₂ heterotetramers. This chapter will detail the methods employed for the expression and purification of soluble H2AH2B heterodimers and (H3H4)₂ heterotetramers as well as the biophysical and biochemical characterization of these histone complexes. Finally, this chapter will detail the rigorous method employed to obtain an accurate measure of protein concentration for the purified recombinant histone proteins. A discussion of the utility and significance of these methods and findings is provided.

B. Materials and methods

Co-expression plasmids—Megan Anderson, Thien Ngo, Genaro Hernandez and Alice Ing constructed the co-expression plasmids for H2AH2B heterodimers and Asf1-(H3H4)₂ heterotetramers. The detail of the construction of these plasmids is in a manuscript that is in preparation for submission for publication. An example of a co-expression plasmid is presented in Figure 3.1.

Expression and purification of H2AH2B heterodimers—The expression plasmids were transformed into E. coli B834 (DE3) pRARE and induced with 0.4 mM IPTG.
Figure 3.1 A schematic of co-expression plasmids for H2A H2B and H3H4-Asf1
A general schematic for the construction of histone co-expression constructs.
Cultures were induced for 3 hours at 37°C or overnight at 17°C. Histone H2AH2B heterodimers and (H3H4)$_2$ heterotetramers were purified using cation exchange chromatography and hydroxyapatite resin chromatography. Briefly, the cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM Benzamidine, 1 mM PMSF, 6 mM β-Mercaptoethanol. Bacteria were lysed by adding lysozyme (Sigma, St. Louis) to a final concentration of 0.5 mg/mL re-suspended cell pellet volume and incubated for 1 hour on ice. The cell lysate was then homogenized by sonication. After homogenization, 1 volume of 1 M HCl is added to the cell lysate and incubated on ice for 30 minutes and then clarified by high-speed centrifugation. The supernatant is then neutralized by the addition of 0.25 volumes of 2 M Tris base and then diluted 5 fold. The supernatant was then applied to a Fractogel-SO$_3$ (EM Sciences) cation exchange column. The column is washed for 2 column volumes with 20 mM Tris-HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA. The protein is then eluted using a 0 - 2 M NaCl gradient in 20 mM Tris-HCl (pH 8.0) and 1 mM DTT. Fractions containing H2AH2B or (H3H4)$_2$ were pooled together and diluted 5 fold in 20 mM Na/K PO$_4$ pH 7.0 and applied to a hydroxyapatite resin (BioRad) column and eluted using a linear gradient of 0-2 M NaCl and 1 mM DTT. H2AH2B heterodimers elute at a NaCl concentration of ~ 1 M NaCl while (H3H4)$_2$ heterotetramers elute from hydroxyapatite chromatography at a NaCl concentration of ~1.2 M NaCl. Histones were then concentrated while exchanging buffers for storage at -20°C. Briefly, fractions containing histones were then pooled and concentrated using an Amicon Stirred Concentration Cell (Millipore) using the appropriate ultrafiltration membrane to a volume of ~10 mL. 12 mL of 40 mM HEPES pH 7.6, 2 mM DTT,
and 2 mM EDTA was added to H2AH2B and 2 mL of 40 mM HEPES pH 7.6, 2 mM DTT, and 2 mM EDTA was added to (H3H4)2. The concentration of NaCl was estimated at ~400 mM for H2AH2B and ~800 mM for (H3H4)2. The histones were then concentrated to ~1-2 mL volumes in an Amicon Stirred Concentration Cell. For H2AH2B, 10 mL of 400 mM NaCl, 40 mM HEPES pH 7.6, 2 mM DTT and 2 mM EDTA was added and the solution concentrated to ~1-2 mL. Another 10 mL of 400 mM NaCl, 40 mM HEPES pH 7.6, 2 mM DTT and 2 mM EDTA was added and the solution concentrated to a final H2AH2B concentration of ~10 mg/mL. A similar procedure was used for (H3H4)2 with the change of 800 mM NaCl instead of 400 mM NaCl. A 50 % glycerol solution was added to the ~10 mg/mL histone solutions and the proteins stored at –20°C.

Chemical cross-linking—1 mg/mL histone solutions (~ 30 mM histones) were chemically cross-linked with 8 mM glutaraldehyde in 150 mM NaCl, 10 mM HEPES pH 7.6, 1 mM EDTA, 1 mM DTT. Chemical cross-linking proceeds for 1 minute and the reactions are quenched by 1 volume of SDS-PAGE sample loading buffer and boiled at 95°C. Samples are analyzed on 8-20 % SDS-PAGE and stained with coomassie brilliant blue.

Gel filtration chromatography—2 mL of ~4-10 mg/mL histone solutions were analyzed on a Superdex 75 column in 500 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT at a flow rate of 1 ml/min.

Salt dialysis mononucleosome assembly—A 204 bp fragment of DNA was obtained from digestion of a plasmid harboring a 12 copy array of a fragment of the 5S RNA gene of *Xenopus laevis* by Aval/BsoBI digestion. The 204 bp DNA fragment
contains a strong nucleosome positioning sequence that can form one nucleosome at a single translational position under optimal conditions. The DNA was purified by gel electrophoresis on a 1% agarose gel run in 1 X TBE. The fragment was cut out and purified using a Qiagen Gel Extraction Kit (Valencia, CA). DNA and recombinant S. cerevisiae and D. melanogaster histones were combined in 2 M NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 5 mM β-Me, and 0.05 % NP-40. The mixtures are placed in a siliconized 1.8 mL tube and a dialysis membrane stretched across the top. These mixtures are then placed in 600 mL of 2 M NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 5 mM β-Me, and 0.05 % NP-40 for 45 minutes. The mixtures are dialyzed using a gradient dialysis method against 50 mM NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, and 5 mM β-Me. as described by Luger et al. Nucleosomes are analyzed on 0.7 % agarose gels in 0.25 X TBE and run for 1 hour at 4°C at 20 mA. Heat shifted material was incubated at 55°C overnight in a PCR thermalcycler.

Amino acid analysis—Purified histone proteins were extensively dialyzed against 50 mM NaCl, 5 mM HEPES pH 7.6, 0.5 mM DTT. A dilution series of dialyzed protein was analyzed by Biorad protein assay analysis standardized against a known quantity of BSA utilizing a spectrophotometer reading A600. A dilution series of dialyzed protein was also analyzed for absorbance at A280. Duplicate samples containing ~25 µg of purified protein (based upon estimates of molar extinction coefficient predicted by a method described by Gill and von Hippel) was sent to the Molecular Structure Facility at UC-Davis for amino acid analysis. Direct measurement of amino acid content of the samples was used to calculate an empirical concentration for the
samples. The empirical concentrations were then correlated to measurements made of identical samples to calculate a molar extinction coefficient.

C. Results

Solubility and purification of H2AH2B heterodimers produced by co-expression—Co-expression of histone H2A with H2B leads to production of histones in a soluble form, suggesting that they correctly associate and fold to form H2AH2B heterodimers. The fraction of histone in the soluble fraction varies with the species source. For yH2A/yH2B, typically greater than 50% of the expressed protein is present in the soluble fraction. For cH2A/cH2B all of the detectable histone is present in the soluble fraction (Figure 3.2). The fraction of soluble dH2A and His6-dH2B is much lower but the acid extraction procedure used in the purification of H2AH2B detailed in the materials and methods greatly increases the protein yield.

The soluble H2AH2B heterodimers can be purified by a combination of cation exchange, hydroxyapatite chromatography. Figure 3.3 shows an example of such a procedure for yH2AH2B. The cleared acid extracted cell lysate is initially applied to a cation exchange column with 1 mM EDTA. The bound material is then eluted with a NaCl gradient without 1 mM EDTA. yH2A and yH2B co-elute in a single peak at around 1 M NaCl. Histone containing fractions are then combined and diluted 5 fold by phosphate buffer and applied to a hydroxyapatite column. The dilution of fractions containing H2AH2B avoids a dialysis step that can lead to decreased protein yields due to loss of the proteins by protein absorption by the dialysis membrane. yH2A and yH2B again co-elute in a single, sharp peak at approx. 800 mM NaCl (figure 3.3b). The behavior of the co-expressed recombinant H2A and
Figure 3.2 The solubility of co-expressed H2AH2B heterodimers
Example solubility analysis for full-length yH2AH2B and cH2AH2B.
Figure 3.3 Purification of H2AH2B heterodimers
H2B is very similar to that observed for elution of histones H2A and H2B from endogenous sources (Simon and Felsenfeld 1979). SDS-PAGE analysis reveals that the purity of the histones is very high following hydroxyapatite chromatography and suitable for most biochemical applications. The histones may be stored at -20°C for up to 1 year with no appreciable degradation or loss.

**Solubility and purification of (H3H4)2 heterotetramers by co-expression with the histone chaperone Asf1**—d(H3H4)2 tetramers were co-expressed with Asf1 and produced soluble dH3 and dH4. Using this co-expression method, stoichiometric amounts of H3 and H4 can be produced in *E. coli*. Greater than 80% of the dH3 and dH4 produced is in the soluble fraction (Figure 3.4). Initially, a Ni-NTA purification was attempted to isolate Asf1-(H3H4)2 complexes. This strategy, however, did not yield a significant amount of the complex. In order to rapidly purify dH3 and dH4 the clarified cell lysate was applied to a cation exchange column followed by a hydroxyapatite column as described for the isolation of H2A2B heterodimers. The purification scheme resulted in the rapid isolation of (H3H4)2 tetramers. The method provides a convenient means for the production of soluble (H3H4)2 tetramers.

Similar techniques were also attempted for y(H3H4)2. Co-expression plasmids have been constructed, however, the solubility of y(H3H4)2 have not been examined in this co-expression system. y(H3H4)2 were expressed and purified from co-expression plasmids containing only H3 and H4 by extraction with HCl as described above.

**Chemical cross-linking analysis of purified histones**—Chemical cross-linking of H2AH2B heterodimers and (H3H4)2 heterotetramers resulted in patterns of chemical cross-linking consistent with those observed for histones isolated from endogenous
Figure 3.4: The solubility of H3H4 by co-expression of the histone chaperone dAsf1.

(→) indicate the position of either His6-dAsf1, dH3 or dH4. The majority of dH3 and dH4 are soluble and can be purified utilizing established chromatography strategies.
sources (Figure 3.5). H2AH2B heterodimers cross-link into a single species ~25 kD in mass (by comparison to a molecular mass marker on SDS-PAGE). Purified recombinant (H3H4)$_2$ chemically cross-link into a species that are consistent with the complex chemical cross-linking pattern observed for (H3H4)$_2$ heterotetramers purified from endogenous sources (Thomas 1989).

Nucleosome assembly—Purified recombinant S. cerevisiae and D. melanogaster histones were assembled into mono-nucleosomes on a 204 bp fragment of DNA from the 5S RNA gene of X. laevis using a gradient salt dialysis method. The purified recombinant histones were first combined in various ratios and analyzed on SDS-PAGE in order to obtain an equimolar histone solution. The histone solutions were then combined with DNA at a final NaCl concentration of 2 M and dialyzed as described. Three bands were detected on a 0.7 % agarose gel in 0.25 X TBE (Figure 3.6). The lowest band was free 204 bp DNA fragment. The middle band contained both histones and DNA. The upper band also contained histones and DNA. Heat treatment of the entire sample lead to the disappearance of the middle band suggesting that the middle band may be the “mispositionned” nucleosome observed by Luger et al (Luger, Rechsteiner et al. 1999). Digestion with micrococal nuclease of the presumptive mononucleosomes resulted in a fragment of DNA approximately 146 bp in size suggesting that the upper band is indeed a mononucleosome.

Amino acid analysis—The data received from MSF-UC-Davis suggests that the molar extinction coefficients predicted from the sequence of the histone proteins is inaccurate. Predicted molar extinction coefficients and actual molar extinction
Figure 3.5 Chemical cross-linking of recombinant histone proteins
Recombinant yeast histones cross-link into specific histone complexes with the cross-linking reagent glutaraldehyde as described by (Thomas 1989)
Figure 3.6 The assembly mononucleosome containing recombinant histones

Mononucleosomes were assembled using a gradient salt dialysis method described by Luger et al. Two different mixtures of recombinant *Drosophila* histones were made (using estimates of protein concentration determined by SDS-PAGE) and used in the assembly. Two translational positions for mononucleosomes were observed. Upon incubation at 55°C overnight only the more stable nucleosome translational position was observed.
coefficients for *S. cerevisiae* histones are summarized in Table 3.1 at the end of this chapter. The large deviations are discussed below.

**D. Discussion**

The production and purification of recombinant histone proteins has had provided a means for obtaining a homogenous product suitable for rigorous biochemical and biophysical examination. The absence of heterogeneity was key to the production of histones used in the nucleosome core particle crystals used to create a high-resolution model of the nucleosome core particle from X-ray diffraction data. The methods detailed above build upon previous heterologous protein expression systems to produce soluble H2AH2B heterodimers and (H3H4)2 heterotetramers. Overcoming solubility issues encountered in the production of single recombinant histones in bacteria allows for the rapid and facile isolation of histones from bacterial sources without any denaturing/refolding steps. The improved efficiency produces histones that are similar to those previously produced by recombinant methods as well as histones purified from endogenous sources.

One of the most striking findings of the study has been the observation of the large discrepancy between the estimated value of the molar extinction coefficient and the empirical value calculated from amino acid analysis of recombinant histone proteins. The method described for the prediction of molar extinction coefficient by absorbance of light at 280 nm assumes a “normal” amino acid composition. Studies by Pace et al. (Pace, Vajdos et al. 1995) have already demonstrated that proteins that are deficient/or lack tryptophan residues are particularly susceptible to deviations from predicted values. The lack of empirical molar extinction coefficients for the
histones is surprising considering their composition. For most studies the difference observed between the predicted and the actual molar extinction coefficient may be unimportant, however, in trying to determine a stoichiometry and thermodynamic parameters, absolute protein concentrations are vital for the interpretation of data. As with the determination of accurate protein concentrations for yNap1, the empirical molar extinction coefficients determined in this study provide firm footing for the analysis presented in the following chapter.

Part of the text of Chapter III is a reprint of materials used in this dissertation have been submitted as part of a manuscript “Thermodynamic Analysis of the interaction of the histone chaperone Nap1 with histones H2A and H2B: Implications for a concerted mechanism of nucleosome assembly,” Huh JH, Bergqvist S, Hampton E, Schurter MA, and Dutnall RN for publication in Journal of Biological Chemistry. The author of this dissertation was the primary author and researcher of the material in the manuscript. Coauthors Bergqvist, Hampton and Schurter performed research support. Robert Dutnall directed and supervised the research.
Table 3.1 Amino Acid Analysis of recombinant yeast histone proteins

<table>
<thead>
<tr>
<th></th>
<th>yH2AH2B</th>
<th>yH2AH2BΔT</th>
<th>y(H3H4)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>predicted</td>
<td>11387 M$^{-1}$cm$^{-1}$</td>
<td>10023 M$^{-1}$cm$^{-1}$</td>
<td>24000 M$^{-1}$cm$^{-1}$</td>
</tr>
<tr>
<td>actual</td>
<td>18188 M$^{-1}$cm$^{-1}$</td>
<td>12946 M$^{-1}$cm$^{-1}$</td>
<td>47472 M$^{-1}$cm$^{-1}$</td>
</tr>
<tr>
<td>% difference</td>
<td>37%</td>
<td>23%</td>
<td>49%</td>
</tr>
</tbody>
</table>

* Calculations based upon accurate protein concentrations from Amino Acid Analysis of purified recombinant proteins. These concentrations were correlated to $A_{280}$ measurements of identical samples. Several $A_{280}$ measurements were taken to insure that the samples were measured in the linear range of the UV spectrophotometer used.
IV: The Characterization of Nap1-H2AH2B complexes
A. Introduction

Though many histone chaperones have been characterized biochemically, the structural basis for nucleosome assembly activity by histone chaperones is poorly understood. Determining the mechanism of nucleosome assembly by histone chaperones requires knowledge of the structure and energetics of histone chaperone-histone complexes. A wealth of information is available about yNap1 and recombinant yNap1 has been used extensively in chromatin assembly assays in vitro (Fujii-Nakata, Ishimi et al. 1992; Pilon, Terrell et al. 1997; Wongwisansri and Laybourn 2004). Nap1 can bind all four core histones and the linker histone in vitro (Ishimi, Kojima et al. 1987; Rodriguez, Munroe et al. 1997; McQuibban, Commissocappelli et al. 1998; McBryant, Abernathy et al. 2003; Kepert, Mazurkiewicz et al. 2005; Saeki, Ohsumi et al. 2005; Shintomi, Iwabuchi et al. 2005) but in vivo, Nap1 is primarily associated with histones H2A, H2B and the H2A variant, H2AZ (Ito, Bulger et al. 1996; Chang, Loranger et al. 1997; Uetz, Giot et al. 2000; Mosammaparast, Jackson et al. 2001; Gavin, Bosche et al. 2002; Mosammaparast, Ewart et al. 2002; Mizuguchi, Shen et al. 2004; Mosammaparast, Del Rosario et al. 2005). In order to understand the mechanism by which Nap1 facilitates nucleosome assembly in vitro the nature of the Nap1-H2AH2B complex must be elucidated, specifically the stoichiometry of the complex and the thermodynamic parameters of the interaction. Having established that full-length yNap1 is predominantly a dimer in solution (concentrations less than 10µM) a study of the interaction of the yNap1 dimer with yH2AH2B dimers in solution should yield information about the
stoichiometry and thermodynamic parameters governing the interaction. Several papers have attempted to analyze Nap1-H2AH2B complexes utilizing several different biochemical and biophysical methods.

In Ishimi et al, the authors analyzed Nap1-histone complexes by sedimentation velocity experiments. The authors concluded that two Nap1 is able to bind the core histones into specific complexes. A 7S complex of Nap1 and H2AH2B with at 2 : 1 mass ratio of Nap1 to histone suggests that the binding stoichiometry maybe 2 to 1. A 8S Nap1 complex with (H3H4)2 tetramers was formed. The actual stoichiometry of the complexes could not be determined from the experiments preformed, but demonstrated that Nap1 bound to histones in a specific fashion.

In a recent study published by McBryant et al. an Electrophoretic Mobility Shift Assay (EMSA) was used to investigate the interaction of yNap1 with yH2AH2B with the authors concluding that a 1 yNap1 dimer binds 1 yH2AH2B heterodimers. In this study, the EMSA analysis never extends substantially beyond a ratio of 1 yNap1 dimer to 1 yH2AH2B heterodimer. In the study presented in this chapter, similar EMSA analysis that extends beyond 1 yNap1 dimer to 1 yH2AH2B heterodimer will clearly demonstrate that the yNap1 dimer is capable of binding more than 1 yH2AH2B heterodimer. In Toth et al. (Toth, Mazurkiewicz et al. 2005) Nap1-H2AH2B complexes Were analyzed using a fixed concentration of labeled H2AH2B and a molar ratio of 2 Nap1 per H2AH2B was needed for full shift of the labeled H2AH2B into a distinct complex. This complex may be saturated with respect to H2AH2B, but it does not demonstrate saturation of binding sites on Nap1. These studies ignore the ability of the yNap1 dimer to bind more than 1 yH2AH2B
heterodimer and therefore present and incomplete picture of the association of yNap1 with yH2AH2B. The study presented here clearly demonstrates that yNap1 is capable of binding 1 yH2AH2B heterodimer per yNap1 monomer (or two yH2AH2B heterodimers per yNap1 dimer) to a principal A site and another yH2AH2B heterodimer to a secondary B site. Thus the maximum number of H2AH2B heterodimers a Nap1 dimer can bind is 4 H2AH2B heterodimers.

The location of each of the interaction sites is also elucidated from deletion analysis by both EMSA and isothermal titration calorimetry. Site A is located in domain II while site B is contained in the acidic C-terminal tail of yNap1. Moreover, site A of yNap1 interacts predominantly with the histone fold region of yH2AH2B heterodimers while site B interacts with the basic N-terminal tail regions of yH2AH2B. A model of the binding of yH2AH2B heterodimers by yNap1 is purposed based upon these interaction studies.

**B. Methods and Materials**

**Electrophoretic Mobility Shift Assays**—The components for EMSA analysis were extensively dialyzed into 175 mM NaCl, 20 mM MOPS pH 7.2 (NaOH titration), 1 mM MgCl₂, and 1 mM DTT. EMSAs were performed by incubating 5 µM yNap1 (Y1 or Y2) with increasing amounts of recombinant yH2AH2B, or yH2AH2BΔT, at the indicated molar ratios at 25 °C for 1 hour in a 20 µL reaction that contained 3 % (v/v) glycerol. Half of the reactions were then analyzed by 5 % (w/v) Native PAGE in 0.25 X TBE. Gels were fixed and stained with Coomassie brilliant blue as described. Y10-yH2AH2B and Y10-yH2AH2BΔT interactions were analyzed in a similar manner except that the concentration of Y10 was 10 µM to compensate for the
smaller molecular mass of Y10, which impacts the visualization of complexes via staining with Coomassie brilliant blue.

**Chemical cross-linking**—Nap1-H2AH2B complexes were chemically cross-linked at 25°C for 5 hours or overnight using 10 mM 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide, EDC, (Sigma-Aldrich). Samples were removed after 5 hours of cross-linking and overnight and quenched with 100 mM β-Mercaptoethanol prior to analysis by 8% SDS-PAGE. Proteins were visualized by staining with Coomassie brilliant blue.

**Intrinsic Trp Fluorescence Spectroscopy**— All fluorescence measurements were carried out in a Fluoromax-3 fluorimeter with magnetic stirrer attachment. Reaction components were dialyzed extensively into a buffer containing 150 mM NaCl, 10 mM HEPES-NaOH pH 7.6, 1 mM DTT, 1 mM MgCl2. yNap1 was placed into a 4 mL quartz cuvette with a 10 mm path-length at a concentration of 140 nM for Y10 and 69 nM for Y1. Concentrated yH2AH2B was directly added to the cuvette (negligible volume. < 0.5% total volume per addition) and allowed to mix for 5 minutes at 25 °C. Trp residues in yNap1 were excited using 295 nm light and an emissions wavelength scan was taken from 300 nm – 400 nm. Prior to analysis the emission spectrum of a solution of histones alone (at the appropriate concentration) was subtracted from that of the respective Nap1-histone mixture. The fraction of complex formed, Fc, was then determined from the intensity of fluorescence using an average from a window of emissions wavelengths (330 – 340 nm) to compensate for a blue shift in the emissions spectra. Fc is defined as: (FH – Fo)/(FS – Fo), where FH is the fluorescence intensity in the presence of histone concentration H, FS is the
fluorescence intensity at saturation, and F0 is the fluorescence intensity in the absence of histones. The fraction complex was plotted as a function of yH2AH2B concentration and estimates for the dissociation constant and n, the number of binding sites, were obtained by non-linear regression analysis using Microsoft Excel software using the following equation:

\[
F_c = ([L]_t + K_d + n [P]_t) - \\
\frac{([L]_t + K_d + n[P]_t)^2 - 4 n [L]_t [P]_t}{2 [P]_t}
\]

where \([L]_t\) and \([P]_t\) are the total concentration of ligand, H2AH2B, and protein (yNap1), respectively.

**Isothermal Titration Calorimetry**—Prior to analysis, components were extensively dialyzed against 175 mM NaCl, 20 mM MOPS-NaOH pH 7.2, 1 mM MgCl2, and 1 mM DTT. All data were collected on a MCS-ITC instrument (Microcal Inc.). Y10 at 5.4 \(\mu\)M was titrated with yH2AH2B at 64 \(\mu\)M using 15.5 \(\mu\)L injections with a 250 second time delay between injections at 20 °C. Data were analyzed using a non-linear least squares fit analysis in the Origin software program (provided by Microcal Inc.). Identical machine reaction parameters were used for the analysis of Y10-yH2AH2B interactions with Y10 at a concentration of 5.4 \(\mu\)M and yH2AH2B at a concentration of 74 \(\mu\)M. ITC analysis of full-length yNap1, as well as the C-terminal acidic tail deletion construct Y2, required that yNap1 be placed in the reservoir since at least two binding sites per Nap1 dimer was anticipated. All other reaction parameters were identical to Y10-yH2AH2B analysis. For Y1-yH2AH2B interactions Y1 was at a concentration of 4.4 \(\mu\)M in the reservoir with 43 \(\mu\)M yH2AH2B titrated in 15.5 \(\mu\)L
injections. For Y1-yH2AH2BΔT, Y1 was at a concentration of 5.63 µM in the reservoir with yH2AH2BΔT at 56.3 µM titrated in 15.5 µL injections. Y2-yH2AH2B interactions used Y2 at 5.3 µM and yH2AH2B at 43 µM. Y2-yH2AH2BΔT interactions used Y2 at 5.3 µM and yH2AH2BΔT at 56.3 µM.

C. Results

Domain II binds H2AH2B with high affinity—I analyzed the ability of each of the domains of yNap1 to bind H2AH2B. Previous studies have determined that H2AH2B is an obligate heterodimer with a dissociation constant of ~2 nM. The EMSA analysis as well as the other thermodynamic techniques employed in this study are well above the dissociation constant of H2AH2B and therefore, H2AH2B can be regarded as a stable species. I assayed the ability of domain I to bind to H2AH2B using an electrophoretic mobility shift assay (EMSA). The yNap1 domain I truncation mutant (Y13) migrated as a single species during native gel polyacrylamide gel electrophoresis. Incubation of Y13 with yH2AH2B produced no significant change in its electrophoretic mobility until high concentrations were reached (> 10 µM), whereupon the intensity of the domain I band decreased but no defined complex was observed. The loss of some protein at these concentrations may be due to non-specific protein interactions or otherwise may indicate that any interaction between the isolated domain I of yNap1 and yH2AH2B is extremely weak (Kd > 10 µM) which is inconsistent with the observed affinity of full-length yNap1 for yH2AH2B.

I next analyzed the ability of domain II to bind H2AH2B. EMSA using the isolated yNap1 domain II mutant (Y10) indicated that it can bind yH2AH2B. As
Figure 4.1 EMSA and EDC chemical cross-linking analysis of Y10 with H2AH2B
A) A truncation mutant that only contains domain II of yNap1 (Y10) binds H2AH2B heterodimers regardless of the presence or absence of the basic N-terminal tails of yH2AH2B.  
B) EDC chemical cross-linking of Y10 to yH2AH2B heterodimers previously cross-linked by gluteraldehyde reveals that Y10 can bind 1 yH2AH2B heterodimer.
shown in Figure 4.1A, a single shifted complex with distinct electrophoretic mobility was observed upon addition of increasing amounts of yH2AH2B to Y10. When an N-terminal tail deletion mutant of yH2AH2B was used, no significant difference in binding was observed (Figure 4.1A), suggesting that the N-terminal histone tails are not required for interaction with domain II. To ascertain the stoichiometry of the Y10-yH2AH2B complex I used a chemical cross-linking approach, using EDC to cross-link Y10 to yH2AH2B that had previously been covalently linked quantitatively using glutaraldehyde. This revealed a single product with a mass of ~51 kDa (Figure 4.1B) consistent with a complex containing a single yH2AH2B heterodimer (~28 kDa) and a single yNap1 domain II (~23 kDa). Since the yH2AH2B heterodimer is inherently stable, and lacks any significant dissociation under these conditions (Placek and Gloss 2002; Placek, Harrison et al. 2005), this can be regarded as a simple, bimolecular complex. The binding of yNap1 domain II to yH2AH2B was further characterized by an intrinsic fluorescence perturbation assay and isothermal titration calorimetry (ITC) to determine the thermodynamic parameters describing the interaction. Since EMSA analysis indicated that the N-terminal tail regions of H2A and H2B are not required for binding, I initially focused on interactions with yH2AH2BΔT.

The yNap1 domain II construct Y10 contains three evenly spaced tryptophan residues and fluoresces with an emission maxima at ~340 nm (Figure 4.2A). The yeast histones H2A and H2B contain no tryptophan residues and do not possess significant intrinsic fluorescence with excitation at 295 nm. Incubation of increasing amounts of yH2AH2BΔT with the domain II produced a proportional increase in the
Figure 4.2 A Trp fluorescence perturbation assay for Y10 with yH2AH2BΔT
(A), Representative steady-state fluorescence emission spectra of the yNap1 domain II (140 nM) alone, and following addition of yH2AH2BΔT (at the concentrations indicated). Addition of H2AH2B to Nap1 increases intrinsic Trp fluorescence of yNap1. An excitation wavelength of 295 nm was used and measurements were taken at 5 minute intervals with mixing using a Fluoromax-3 spectrofluorimeter. (B), Binding data derived from fluorescence data (see Experimental Procedures for details of analysis) and fitted curve using non-linear regression analysis which reveals a simple 1:1 interaction \( n = 1.02 \) with \( K_d = 107.6 \) nM.
intrinsic tryptophan fluorescence of Y10 indicative of intermolecular association. The increase in fluorescence was concomitant with a blue shift in the emission maxima, consistent with one or more tryptophan residues moving into a more solvent excluded environment upon binding yH2AH2BΔT. The increase in fluorescence converged to a saturation point indicating that all of the binding sites for yH2AH2BΔT were occupied on Y10. Using non-linear regression techniques (Brown 2001; Wilkinson 2004) the fractional saturation could be fit well (R² = 0.87) by a rectangular hyperbola assuming a simple binding equilibrium (Figure 4.2B). Based on this analysis the yNap1 domain II contains a single binding site for yH2AH2BΔT with an equilibrium dissociation constant (Kd) of 107.6 nM.

I used isothermal titration calorimetry to provide an independent measure of the interaction strength and further thermodynamic parameters. Analysis of Y10 with yH2AH2BΔT gave a 1:1 stoichiometry and a Kd of 139 nM +/- 57 nM, in good agreement with the values obtained from the fluorescence-based assay (Figure 4.3A). The association is driven mainly by favorable enthalpy with a small unfavorable entropy component (Table 4.1). I also analyzed the interaction of Y10 with full-length yH2AH2B (Figure 4.3B). As anticipated from EMSA, the stoichiometry and interaction strength were similar to those for yH2AH2BΔT (Table 4.1), but the thermodynamic signature of the association event is different since there are both favorable enthalpy and entropy gains made by the interaction of Y10 with full-length yH2AH2B. This may be the result of decreased binding surface on yH2AH2B offset by entropy contributions related to the conformation of the N-terminal tails of yH2AH2B (Placek and Gloss 2002). It should also be noted that the N-terminal
Figure 4.3 ITC analysis of Y10 interaction with H2AH2B
A) 74 μM Y10 was titrated into 1.337 mL of 6.4 μM yH2AH2B using 15.5 μL injections at 20 °C. A single binding event is evident ($K_d$, $\Delta G$, $\Delta H$, and $T\Delta S$ are summarized in Table 1). B) 64 μM yH2AH2BΔT titrated into 1.337 mL of 5.4 μM Y10 using 15.5 μL injections at 20 °C. A single binding event is evident ($K_d$, $\Delta G$, $\Delta H$, and $T\Delta S$ are summarized in Table 1).
deletion mutants remove a region based on proteolytic sensitivity, rather than a structurally defined region, and this is known to affect H2AH2B stability (Placek and Gloss 2002). These results are consistent with those I obtained from EMSA that showed that the affinity of the domain II for H2AH2B does not depend upon the presence of the N-terminal tail regions. Having established that domain II of the yNap1 monomer can bind a single H2AH2B dimer, I next analyzed full-length dimeric yNap1.

A dimer of yNap1 binds two H2AH2B via the domain IIs of yNap1 with high affinity and two additional H2AH2B via the acidic C-terminal tail—The ability of full-length yNap1 to bind yH2AH2B heterodimers was initially analyzed by EMSA. The incubation of a fixed amount of yNap1 (5 µM monomer concentration) with increasing amounts of yH2AH2B resulted in the appearance of an electrophoretically distinct primary species that migrated slower than free yNap1 at a molar ratio of 1 yNap1 monomer to 0.6 yH2AH2B heterodimer (Figure 4.4A, ⊲). This species continued to interact with yH2AH2B and shifted into a second species (Figure 4.4A, ⊱) at a 1 : 1.2 molar ratio. This species also continued to associate with yH2AH2B heterodimers and super-shifted into a species that did not enter the gel at a molar ratio of 1 : 1.8 (Figure 4.4A, indicated by the arrow ⊲). This pattern of yNap1-yH2AH2B associations is reproducible and shows that multiple complexes can be formed between yNap1 and yH2AH2B heterodimers in vitro. The data suggest that the Nap1 dimer binds initially to one H2AH2B heterodimer followed by the binding of a second H2AH2B heterodimer (Figure 4.4A, ⊲ and ⊱, respectively). The additional interaction of yNap1 with yH2AH2B cannot be visualized due to the fact that these
Figure 4.4 EMSA analysis of full-length yNap1 (Y1)
A) Nap1-H2AH2B complexes were analyzed using an electrophoretic mobility shift assay. 5 μM full-length yNap1 was incubated with increasing amounts of yH2AH2B and then analyzed on a 5 % Native PAGE in 0.25 x TBE. A primary complex (↓) is observed at a molar ratio of 1 yNap1 to 0.6 yH2AH2B. A second complex (↑) appears at a molar ratio of 1 yNap1 to 1.2 yH2AH2B concomitant with the disappearance of the primary complex. The second complex super-shifts further into a species that does not enter the gel (↔). B) Analysis of full-length yNap1 with yH2AH2BΔT shows a distinct complex that saturates at a molar ratio of 1:1.2 and does not appreciably further associate with histones.
complexes do not enter the gel as species with distinct electrophoretic mobility, perhaps due to charge neutralization or the formation of complexes too large to enter the gel matrix.

In order to analyze the composition of the Nap1-H2AH2B complexes observed by EMSA, Nap1-H2AH2B complexes were chemically cross-linked using 10 mM EDC for 5 hours or overnight at 25°C and analyzed by 8% SDS-PAGE. yNap1 cross-links into a dimer in the presence of EDC (Figure 4.5). H2AH2B heterodimers are cross-linked to Nap1 by EDC. At a molar ratio of 1 yNap1 monomer : 0.5 H2AH2B heterodimers, a cross-linked band of approximately 120kD appears (Figure 4.5, ◊) which is consistent with a dimer of yNap1 plus 1 H2AH2B. At a molar ratio of 1 yNap1 monomer : 1 H2AH2B the major cross-linked protein band appears to be approximately 150kD in mass (Figure 4.5, ▼) consistent with a dimer of yNap1 plus 2 H2AH2B. At a molar ratio of 1 yNap1 monomer : 2 H2AH2B a band of approximately 200kD appears (Figure 4.5, ▵) consistent with 1 dimer of yNap1 and 4 H2AH2B. These complexes correspond to complexes observed by EMSA of yNap1-yH2AH2B complexes at similar molar ratios (Figure 4.4A).

At extended chemical cross-linking times, yNap1 can cross-link into higher molecular weight oligomers (Figure 4.5, *). The higher molecular weigh oligomers captured by chemical cross-linking may reflect the propensity of yNap1 to further oligomerize at high protein concentrations as observed in AUC experiments of yNap1. The addition of H2AH2B to yNap1 abrogates the propensity of yNap1 dimers to further oligomerize as seen by the disappearance of the higher molecular weight oligomers of yNap1 concomitant with the appearance of cross-linked Nap1-H2AH2B
Figure 4.5 EDC chemical cross-linking of full-length yNap1 to yH2AH2B
EDC chemical cross-linking of Nap1-H2AH2B complexes analyzed on an 8% SDS-PAGE gel. 2 µM full-length yNap1 was incubated with the indicated molar ratio of yH2AH2B and cross-linked overnight with 10 mM EDC. The addition of yH2AH2B to yNap1 produced bands consistent with 1 yNap1 dimer plus 1 yH2AH2B (▼), 2 yH2AH2B (▼) and 4 yH2AH2B (↔). A high molecular weight species (*) present in Free Nap1 disappears as more H2AH2B is added.
complexes. The higher molecular weight oligomers are completely absent when a molar ratio of 1 yNap1 monomer to 2 H2AH2B dimers is reached (Figure 4.5, C).

Analysis of EDC chemical cross-linking of Nap1-H2AH2B complexes at an earlier 5-hour time-point reveals that only the dimer of yNap1 is cross-linked. The overall pattern of Nap1-H2AH2B complexes is similar to the pattern observed with overnight cross-linking by EDC, however, the bands are not as distinct owing to the inefficiency of EDC chemical-crosslinking and the heterogeneity of the cross-linked species (Figure 4.5).

I also analyzed the binding of a yH2AH2B truncation mutant, lacking the basic N-terminal histone tail regions, yH2AH2BΔT. The EMSA analysis of full-length yNap1 interactions with yH2AH2BΔT, under identical conditions to those used for full-length yH2AH2B, presented a simplified association reaction in which yH2AH2BΔT only shifted full-length yNap1 into a single species (Figure 4.4B) that saturated at a molar ratio of one yNap1 monomer to one yH2AH2BΔT heterodimer (i.e. one dimer of yNap1 to two yH2AH2BΔT heterodimers). This indicates that the N-terminal tails of histones H2A and H2B are not required for the interaction of yNap1 with yH2AH2B. Presumably the complex observed in Figure 4.4B is similar to complex (W) in Figure 4.4A for full-length yNap1 and yH2AH2B. Chemical cross-linking analysis with EDC of this complex revealed that yNap1 does bind two yH2AH2BΔT heterodimers.

I speculated that the N-terminal tails of yH2AH2B interact with the long unstructured acidic C-terminal tail of yNap1, I therefore analyzed a truncation mutant of yNap1 lacking the acidic C-terminal tail (Y2; Table 2.3) by EMSA analysis with
both yH2AH2B and yH2AH2BΔT. The EMSA were qualitatively similar to the EMSA pattern seen for full-length yNap1 with yH2AH2BΔT (Figure 4.4B). This suggests that the removal of the C-terminal tail of yNap1 has a similar effect to the removal of the N-terminal tails of yH2AH2B. This suggests that the primary and secondary complexes (Figure 4.4A, ▲ and ▼, respectively) observed in EMSA of yNap1 with yH2AH2B are mediated by domain II of yNap1. Further evidence for this conclusion is provided by ITC analysis (see below).

Having established that the yNap1 dimer can bind multiple yH2AH2B heterodimers, I analyzed the interaction using the intrinsic fluorescence perturbation assay and ITC methods that were used for analyze the interactions of the isolated domain II construct Y10 with H2AH2B. Incubation of full-length yNap1 with yH2AH2B produced a blue-shift of the emission maxima, and an increase in the intensity of intrinsic tryptophan fluorescence of the yNap1 protein in a manner qualitatively similar to that observed for the Y10-yH2AH2BΔT interaction, indicative of an intermolecular interaction. However, the data could not be reliably fit by a non-linear regression analysis. Examination of the data revealed that the data points did not produce the expected rectangular hyperbola but more closely resembled two intersecting lines. This suggests that the concentration of yNap1 (69 nM) utilized in the assay is far above the $K_d$ (> 100 X $K_d$; (Wilkinson 2004) and therefore that yH2AH2B binds to full-length yNap1 with very high affinity (< 1 nM). Although a more accurate estimate of $K_d$ could not be obtained under these conditions, the intersection point of the two lines indicated a binding stoichiometry of 1 : 1 (yNap1 monomer: yH2AH2B heterodimer). This is in agreement with EMSA results for full-
length yNap1 and yH2AH2BΔT that suggested a 1 : 1 stoichiometry. The absence of a signal indicating binding of yH2AH2B to the acidic C-terminal tail of yNap1 in the fluorescence perturbation assay is due to a lack of tryptophan residues within the acidic C-terminal tail that could be influenced by the binding of yH2AH2B heterodimers. I therefore chose to analyze the interaction by ITC, a method that could reveal both binding sites as well as provide thermodynamic parameters.

ITC analysis of the interaction of full-length yNap1 with yH2AH2B also indicated that the affinity of yNap1 for yH2AH2B is very high ($K_d < 10$ nM). Furthermore, the binding isotherm (Figure 4.6A) revealed a sequential binding reaction involving two classes of binding site: an A site and B site. The primary site (A site) is occupied first by yH2AH2B with a stoichiometry of binding of 2 yH2AH2B to 1 yNap1 dimer. High affinity binding to the A sites ($K_d < 10$ nM) is driven mainly by a favorable entropy component overcoming an unfavorable enthalpy component (Table 4.1). Significant binding to the second class of binding site (B sites) occurred only after the A sites had been completely occupied by yH2AH2B, in an independent, identical fashion, and with a different thermodynamic signature. Here, a favorable enthalpy component is the driving force behind the association with a weaker affinity ($K_d = 110$ nM). These data show that a dimer of yNap1 can bind two yH2AH2B to the two A sites and subsequently bind two more yH2AH2B to the B sites.

ITC analysis of the interaction of full-length yNap1 with yH2AH2BΔT showed that removal of the N-terminal histone tails abrogates the B site binding event observed with full-length yH2AH2B (Figure 4.6B). This, again suggests that the B
Figure 4.6 ITC analysis of Y1 and Y2

A) 43 µM yH2AH2B is titrated into 1.337 mL of 4.4 µM Y1 using 15.5 µL injections at 20 °C. A two-site model is used to fit the binding isotherm. The A site has a 1:1 stoichiometry (yNap1 monomer: H2AH2B dimer) as does the B site. The A site binds with a mechanism that is driven by favorable entropy. The B site is driven by favorable enthalpy ($K_d$, $\Delta G$, $\Delta H$, and $T\Delta S$ are summarized in Table 4.1). B) 56.3 µM yH2AH2BΔT is titrated into 1.337 mL of 5.63 µM Y1 using 15.5 µL injections at 20 °C. A single binding event is observed ($K_d$, $\Delta G$, $\Delta H$, and $T\Delta S$ are summarized in Table 4.1). Removal of the N-terminal tails of yH2AH2B completely abrogates binding of H2AH2B to the B site of full-length yNap1 consistent with observations made in EMSA analysis. C) 49 µM yH2AH2B is titrated into 1.337 mL of 5.3 µM Y2 using 15.5 µL injections at 20 °C. Deletion of the long acidic C-terminal tail of yNap1 abrogates binding of H2AH2B dimers to the B site of yNap1. Binding to the A site is similar to full-length yNap1 though the binding affinity decreases ~8 fold ($K_d$, $\Delta G$, $\Delta H$, and $T\Delta S$ are summarized in Table 4.1). The large positive injection enthalpies (top panel Fig. 4.6C) may be the source of the affinity decrease observed for Y2. A two-site model was also fit to Y2-H2AH2B data and yielded no large difference in fitted parameters ($K_d$, $n$ or $\Delta H$) when compared to the fit from a one binding site model.
site interaction between yNap1 and yH2AH2B is mediated by the N-terminal tails of yH2AH2B. The binding of yH2AH2BΔT to the A site in full length yNap1 decreased by at least 10 fold (Table 4.1) suggesting that interactions with the N-terminal tails of yH2AH2B also play a role in the binding of yH2AH2B to the A site of yNap1. The loss of binding affinity observed by ITC due to loss of the N-terminal tails of yH2AH2B was not observed in EMSA analysis of full-length yNap1 with yH2AH2BΔT because the reactions were carried out at concentrations far above the $K_d$ which masked the effects. The loss in affinity for the A-site appears to come primarily from a decrease in $TΔS$ which negates a modest favorable enthalpy gain compared to the interaction in the presence of the N-terminal tails (Table 4.1). The N-terminal tail regions of H2A and/or H2B therefore contribute to the overall binding affinity of yNap1 for yH2AH2B in addition to serving as a site for interaction with the B site on full-length yNap1.

ITC analysis of truncation mutant Y2 with either yH2AH2B or yH2AH2BΔT revealed only a single binding event, with a similar thermodynamic signature to the A site of full-length yNap1 (Figure 4.6B, Figure 4.6C). This protein lacks the acidic C-terminal tail region of yNap1, demonstrating that this is the location of the B site interaction. Binding to the A site in Y2 was worse than for full-length yNap1 irrespective of the presence or absence of the N-terminal histone tail regions: $K_d \sim 157$ nM for yH2AH2B, $K_d \sim 80$ nM for yH2AH2BΔT (Table 4.1). This suggests that the C-terminal tail can not only interact with the N-terminal tails of H2A and/or H2B but also contributes to binding to the A site. Comparison of binding with or without the yNap1 C-terminal tail reveals a consistent trend that the observed enthalpy change
Table 4.1 Summary of thermodynamic parameters for yNap1-H2AH2B interactions.

<table>
<thead>
<tr>
<th>Interacting proteins</th>
<th>$n$* (Site)</th>
<th>$K_d$ nM</th>
<th>$\Delta G$ kcal/mol</th>
<th>$\Delta H$ kcal/mol</th>
<th>$T\Delta S$ kcal/mol</th>
</tr>
</thead>
</table>
| Y1 + yH2AH2B         | (A) 0.87 +/- 0.06  
                        (B) 1.08 +/- 0.15 | < 10.00  
                        110 +/- 77.0 | -11.60 +/-0.96  
                        -9.46 +/- 0.40 | 4.78 +/- 0.75  
                        -5.51 +/- 0.76 | 16.40 +/-1.61  
                        3.95 +/-1.14 |
| Y1 + y(H2AyH2B)$\Delta T$ | (A) 1.28 +/- 0.07  
                        (B) 0 | 96.3 +/- 43.0 | -9.48 +/- 0.24  
                        2.96 +/- 1.30 | 12.40 +/-1.06 |
| Y2 + yH2AH2B         | (A) 0.76 +/- 0.02  
                        (B) 0 | 157 +/- 87.0 | -9.21 +/- 0.34  
                        10.90 +/- 0.88 | 20.20 +/-0.54 |
| Y2 + yH2AH2B$\Delta T$ | (A) 1.09 +/- 0.28  
                        (B) 0 | 80 +/- 17.0 | -9.56 +/- 0.13  
                        5.94 +/- 0.77 | 15.50 +/-0.74 |
| Y10 + yH2AH2B        | 0.95 +/- 0.05  
                        102 +/- 16.0 | -9.41 +/- 0.09  
                        -5.36 +/- 0.54 | 4.06 +/-0.63 |
| Y10 + yH2AH2B$\Delta T$ | 1.03 +/- 0.07  
                        139 +/- 57.0 | -9.25 +/- 0.25  
                        -11.00 +/-1.04 | -1.76 +/-0.79 |

* $n$ is the number of H2AH2B dimers associated with a binding site (A site or B site) on yNap1. Y1 and Y2 were both analyzed using a two-site binding model (two binding sites per yNap1 polypeptide, therefore 4 total binding sites for a dimer of full-length yNap1).

All ITC experiments were performed at least in duplicate with standard deviations reported for each measurement.
is more unfavorable whereas the entropy change is more favorable in the absence of the tail (Table 4.1). Combined with the analysis of interactions in the presence or absence of the N-terminal tails of H2A and H2B with full length yNap1, it appears that interactions between the C-terminal tail of yNap1 and the N-terminal tails of H2A and/or H2B contribute to the mechanism of binding to the A site. This contribution may come in the form of electrostatic steering (Janin 1997) and/or other energetically favorable mechanisms such as conformational entropy compensation.

D. Discussion

The study presented shows that the yNap1 domain II is sufficient to bind H2AH2B with high affinity, forming a 1:1 complex. This is consistent with previous studies of several NAP/SET family members (Fujii-Nakata, Ishimi et al. 1992; Shikama, Chan et al. 2000; Seo, McNamara et al. 2001; Miyaji-Yamaguchi, Kato et al. 2003). This interaction does not require the N-terminal tail regions of the histones suggesting that the interaction occurs primarily with the globular, histone-fold region. However, contributions from the C-terminal tail region of histone H2A have not yet been explored. The observations therefore lead to a general schematic model for the Nap1 protein in which domain I and domain II make separable contributions to dimer formation and binding H2AH2B respectively (Figure 4.7).

My analysis demonstrates that the full-length yNap1 dimer contains two high affinity yH2AH2B-binding sites (A sites) and two, lower affinity B sites. The A site is partly, or wholly, situated in domain II of yNap1 whereas the B site is located in the acidic C-terminal tail region. The affinity of yH2AH2B for the isolated, monomeric domain II is significantly weaker than the A site in the context of the
Figure 4.7 A cartoon representation of Nap1 binding H2AH2B heterodimers
Biophysical and biochemical analysis of yNap1 and yNap1-H2AH2B interactions demonstrates that yNap1 can bind two H2AH2B heterodimers.
yNap1 dimer. This suggests that the A site includes parts of the yNap1 protein beyond the limits of the domain II construct (residues 173–372) used in this study, that binding to the A sites in the yNap1 dimer occurs cooperatively, or that some combination of these factors is responsible for the high affinity binding. In addition, the yNap1 C-terminal tail region appears to contribute to binding to the A-site (see below).

My data shows that the pair of high affinity A sites is first fully occupied by two copies of H2AH2B, before significant binding to the lower affinity B sites in the C-terminal tails occurs. Previous studies have demonstrated that deletions that remove part or all of the domain II (A site) drastically reduce nucleosome assembly activity, whereas the acidic C-terminal tail (B site) of yNap1 is not required for in vitro assembly activity (Fujii-Nakata, Ishimi et al. 1992). This indicates that H2AH2B-binding to the C-terminal tail is not necessary for assembly activity. Interestingly, the aforementioned study also showed that deletion constructs corresponding closely to the isolated domain II studied here lack nucleosome assembly activity (Fujii-Nakata, Ishimi et al. 1992), indicating that dimerization mediated by the domain I is required for assembly activity. Similarly, dimer formation of the SET/TAF1β protein is required for its chromatin remodeling ability as an adenovirus core DNA template activating factor (Miyaji-Yamaguchi, Okuwaki et al. 1999). Taken together, these observations imply that the minimum complex required for robust yNap1 nucleosome assembly activity is a yNap1 dimer that can bind two copies of H2AH2B (via the two domain IIs).
My results indicate that the N-terminal tail regions of H2A and H2B are not required but do have an influence on binding to the A site, and are required for binding to the B site. In the context of the yNap1 dimer, removal of the N-terminal tails reduces affinity for the A site and this appears to be largely due to loss of favorable entropy, suggesting that they primarily contribute to the binding energy via conformational mechanisms, rather than by direct interactions. The affinity of H2AH2B-binding to the A site is also reduced when the yNap1 C-terminal tail is removed. This is due to a more unfavorable enthalpic contribution to binding energy, which is offset to some extent by a greater favorable entropy gain (Table 4.1). In the absence of more precise structural information of the complex, caution must be exercised in interpreting these data. Interaction of the C-terminal tail of yNap1 with the N-terminal tails of yH2AH2B may promote binding to the A site via an electrostatic steering mechanism (Janin 1997), provide a transient docking site for H2AH2B prior to engagement with the high affinity A site, or influence the conformation of the N-terminal tails to favor binding.

My conclusions regarding the stoichiometry of the yNap1-H2AH2B complex are in conflict with previous reports (McBryant, Abernathy et al. 2003; Toth, Mazurkiewicz et al. 2005). I note that our EMSA analysis of full-length yNap1 with full-length yH2AH2B is similar to a study by McBryant et al. (McBryant, Abernathy et al. 2003), but extends to higher concentrations of H2AH2B and clearly demonstrates that Nap1-H2AH2B interactions do not fully saturate at a molar ratio of 1 yNap1 dimer to 1 yH2AH2B. The discrepancy in results may be attributable to the manner in which Nap1-H2AH2B complexes were assembled. In McBryant et al. the
complexes were assembled at 4 °C using extended incubation times (16 hours), yet typical in vitro nucleosome assembly reactions only require that Nap1 is incubated with histones for periods of only minutes up to ~1-2 hours at room temperature for robust assembly activity (Fujii-Nakata, Ishimi et al. 1992; McQuibban, Commissio-Cappelli et al. 1998; Wongwisansri and Laybourn 2004). Furthermore, our ITC analysis indicates that the binding of the first yH2AH2B to yNap1 is predominantly driven by favorable entropy gains and incubation at 4 °C may therefore perturb the association of Nap1 with H2AH2B and lead to misleading results. In Toth et al. (Toth, Mazurkiewicz et al. 2005) Nap1-H2AH2B complexes were analyzed using a fixed concentration of labeled H2AH2B and a molar ratio of 2 Nap1 per H2AH2B was needed for full shift of the labeled H2AH2B into a distinct complex. This complex may be saturated with respect to H2AH2B, but it does not demonstrate saturation of binding sites on Nap1. Our analysis shows that a molar ratio of 1 yNap1 to 0.5 mole of yH2AH2B is not a saturated yNap1-H2AH2B complex. The sedimentation velocity experiments presented by Toth et al. are therefore studies of a non-saturated yNap1-H2AH2B complex (with respect to yNap1). Furthermore, sedimentation velocity studies rely upon mass and hydrodynamic shape. yNap1 and dNap1 (Ito, Bulger et al. 1996) elute with anomalously large apparent mass values when analyzed by size exclusion chromatography (a hydrodynamic technique) and similar anomalies may therefore occur in sedimentation velocity experiments. The data I have presented from two solution equilibrium techniques (fluorescence spectroscopy and ITC) are independent of hydrodynamic shape and provide a
thermodynamically rigorous method for obtaining both binding affinity and stoichiometry.

The data presented clearly demonstrate that Nap1 can bind more than one H2AH2B heterodimer and that the principal sites of interaction between Nap1 and H2AH2B are in domain II of Nap1 and the globular domains of H2AH2B respectively.

Part of the text of Chapter IV is a reprint of materials used in this dissertation have been submitted as part of a manuscript “Thermondyanmic Analysis of the interaction of the histone chaperone Nap1 with histones H2A and H2B: Implications for a concerted mechanism of nucleosome assembly,” Huh JH, Bergqvist S, Hampton E, Schurter MA, and Dutnall RN for publication in Journal of Biological Chemistry. The author of this dissertation was the primary author and researcher of the material in the manuscript. Coauthors Bergqvist, Hampton and Schurter performed research support. Robert Dutnall directed and supervised the research.
V: Characterization of \textit{in vitro} nucleosome assembly by yNap1
A. Introduction

Nap1-mediated nucleosome assembly in vitro is a process that proceeds with great speed and fidelity. A recent study by Wagner et al. (Wagner, Bancaud et al. 2005) demonstrated that Nap1 is more than 1000 fold more efficient in the assembly of nucleosomes in vitro than simple polyanions, suggesting that the mechanism of Nap1-mediated assembly involves more than simple screening of the charges of the histone proteins. The assembly process is stepwise, with (H3H4)2 deposited first onto DNA. An H2AH2B heterodimer must then be added onto either side of the (H3H4)2-DNA complex. This could occur in sequential manner, with each H2AH2B being added independently to the (H3H4)2-DNA complex or could occur via a mechanism that deposits both H2AH2B heterodimers onto an (H3H4)2-DNA complex in a single concerted event. The ability of the yNap1 dimer to bind two copies of H2AH2B to a principal interaction site (A site) with high affinity suggests that the yNap1 dimer may be capable of delivering two H2AH2B dimers simultaneously to an (H3H4)2-DNA. This may represent the most efficient mechanism of nucleosome assembly by yNap1.

In order to evaluate this intriguing possibility, full-length yNap1 (Y1) and several yNap1 truncation mutants were analyzed for their ability to assemble nucleosomes in vitro. Having already established that Y1 and Y2 dimers can bind at least two H2AH2B heterodimers, that Y10 can bind one H2AH2B heterodimer, and that Y13 dimer cannot bind H2AH2B, I evaluated their nucleosome assembly activity using two different assays. A well-established DNA super-coiling assay was used to determine what portions and Nap1 were required for nucleosome assembly. In this
assay, the introduction of positive DNA supercoils into a relaxed DNA plasmid by the formation of nucleosomes is indicative of nucleosome assembly. Similar analysis was then carried out on a mononucleosome assembly assay I developed to more thoroughly probe the products of the nucleosome assembly process. In this assay, the formation of protein-DNA complex of defined electrophoretic mobility is indicative of nucleosome assembly. I then extended this analysis by examining the nucleosome assembly activity of full-length yNap1 more extensively in an attempt to shed light upon the mechanism of nucleosome assembly.

B. Methods and materials

DNA supercoiling assays—Were performed by Debra Urwin of the Kadonaga Lab as described in Methods of Enzymology Vol 371 pgs. 499-515.

Mononucleosome assembly assay using yNap1 truncation mutants—Equimolar amounts of full-length yNap1 and yNap1 truncation mutants were pre-incubated with 10 µM purified chicken erythrocyte histones in 150 mM NaCl, 25 mM HEPES pH 7.6 (KOH titration), 1 mM DTT, 1 mM MgCl₂, 3 % glycerol, for 2 hours at 25°C. A purified 204 bp fragment of the X. laevis 5S RNA gene was added to the Nap1-histone mixtures to a final concentration of 18 ng/µL. The mixture was incubated at 25°C for 2 hours. The assembly reactions were then directly loaded into a 0.7% agarose gel run in 0.25 X TBE at 15 mA for 1 hour at 4°C. The gels were then stained in 0.5 µg/mL EtBr solution for 20 minutes and destained extensively with deionized H₂O. Gels were visualized on a UV transilluminator with images captured using an Alpha Imager system (San Leandro, CA).
Electrophoretic mobility shift assay—1 µM purified D. melanogaster histones were incubated with the indicated molar ratio of yNap1 overnight at 4°C in 175 mM NaCl, 10 mM MOPS pH 7.2 (NaOH titration), 1 mM DTT and 5% glycerol. ~ 2 µg of purified D. melanogaster histones were loaded into each sample well of a 5% native polyacrylamide slab gel run in 0.25 X TBE. The gel was run at 70 V/cm for 3 hours at 4°C. The EMSA was visualized by staining with Coomassie brilliant blue as described.

Mononucleosome assembly using different ratios of full-length yNap1—1 µM purified D. melanogaster histones were pre-incubated with yNap1 at the indicated molar ratios in 175 mM NaCl, 10 mM MOPS pH 7.2 (NaOH titration), 1 mM DTT, and 5% glycerol as above. 4µL of the Nap1-histone mixtures were then added to 16µL of the same buffer containing 200 ng of a 204 bp DNA fragment the 5S RNA gene of X. laevis. The reactions were incubated at 25°C for the indicated amount of time. The reactions were then directly loaded into a 0.6% agarose gel run in 0.5 X TBE at 10 mA for 3 hours at 4°C. The gels were then stained and visualized as described above.

C. Results

Nucleosome assembly by yNap1 requires a dimer of yNap1 capable of binding two H2AH2B heterodimers—Initial evaluation of full-length yNap1 and yNap1 truncation mutants by Debra Urwin, a post-doctoral fellow in the Kadonaga lab, demonstrated that recombinant yNap1 has robust nucleosome assembly activity (Figure 5.1). Under equimolar conditions, the yNap1 truncation mutants were not nearly as efficient as full-length yNap1 in the assembly of nucleosomes with some truncation
Figure 5.1 Chromatin assembly activity of yNap1 and yNap1 truncation mutants
Debra Urwin, a post-doctoral fellow in the Kadonaga lab performed DNA super-coiling assays to analyze full-length yNap1 (Y1) and the Nap1 truncation mutants Y2, Y10 and Y13. In the absence of Nap1, there is little to no DNA super-coiling. Both Y1 and Y2 can assemble nucleosomes that introduce positive super-coils into the relaxed plasmid DNA used in this assay. The yNap1 truncation mutants that only contain domain I (Y13) or domain II (Y10) cannot introduce positive DNA super-coils even at extended incubation times suggesting that they are incapable of assembling nucleosomes.
mutants wholly unable to assemble nucleosomes as indicated by a lack of positive super coiling relative to Nap1 controls and full-length yNap1. The acidic C-terminal tail truncation mutant Y2 is able to assemble nucleosomes onto plasmid DNA, but not nearly as efficiently as full-length yNap1 as the extent of DNA super coiling is not as great as full-length at any time point. Analysis of truncation mutants that contain only domain I or domain II of Nap1 (Y10 and Y13 respectively) showed that these truncation mutants were completely unable to assemble nucleosomes onto plasmid DNA as the amount of supercoiling was the same as a no Nap1 control at all time points. These results are consistent with previous deletion studies of Nap1.

In order to analyze the quality of nucleosome assembly reactions I developed a mononucleosome assembly assay from components used in the salt dialysis method of nucleosome assembly. Previous studies had demonstrated that pre-incubation of all four-core histones with Nap1 allows Nap1 to associate with the histones and facilitate their assembly into nucleosomes onto plasmid DNA. Reasoning that a smaller fragment of DNA should also be a viable target for nucleosome assembly, I pre-incubated recombinant full-length yNap1 with purified chicken erythrocyte histones (a kind gift from the lab of V. Ramakrishna) at molar ratios previously described in other studies. The addition of these yNap1-histone mixtures to a 204 bp DNA fragment of the 5S RNA gene of *X. laevis* produced electrophoretically distinct species on agarose gel electrophoresis with electrophoretic mobilities similar to mononucleosomes produced from salt dialysis as observed in Chapter 3 (Figure 5.2). In the absence of yNap1, the histones and DNA form aggregates that do not enter the gel. An advantage of this mononucleosome assembly assay is that the extent of
Figure 5.2 Mononucleosome assembly by yNap1 and yNap1 truncation mutants
Core histones were added directly to DNA or following incubation with Nap1 constructs as indicated. Y2* is a cysteine/serine mutant (with all three cysteines in this construct swapped for serines). All Nap1 constructs were at approximately at equal mole quantities. Y1, Y2, and Y2* display robust mononucleosome assembly activity. Y9 and Y12 appear unable to assemble nucleosomes. Y10 appears as though it may be capable of assembling nucleosomes, but this activity is irreproducible.
assembly can be more directly quantified from the intensities of free DNA and nucleosomal DNA, although an absolute quantification of DNA amounts in nucleosome bands is hindered by a decrease in EtBr binding efficiency. Confident that yNap1 is capable of assembling nucleosomes onto small linear fragments of DNA, I then tested a subset of the yNap1 truncation mutants.

At equal molar ratios of Nap1 to histones, all of the truncation mutants examined were not as efficient in the assembly of mononucleosomes as full-length yNap1 (Figure 5.2). The most conservative truncation mutant (Y2) lacking only the unstructured acidic C-terminal tail of yNap1 was ~20-30% less efficient in the assembly of mononucleosomes (as assessed from the amount of free DNA observed). The Y9 truncation mutant, containing domain II of yNap1 and the long acidic C-terminal tail of yNap1, did not assemble mononucleosomes efficiently. The Y10 truncation mutant (monomer of yNap1 domain II), while capable of binding H2AH2B heterodimers, also is unable to assemble mononucleosomes efficiently and produces a slower migrating species that is not consistent with any observed mononucleosome products observed from gradient salt dialysis methods of assembling mononucleosomes. Y12, a yNap1 truncation mutant containing domain I of yNap1, which does not bind H2AH2B, also was deficient in the assembly of nucleosomes. Overall, these results are consistent with the plasmid based DNA super coiling assays and previous studies suggesting that the minimum Nap1 species required for nucleosome assembly is a dimer of Nap1 capable of binding two H2AH2B heterodimers.
The ratio of yNap1 to histone determines the mechanism of assembly—Having established that full-length yNap1 is capable of assembling mononucleosomes, I set about determining the minimum amount of yNap1 needed for robust nucleosome assembly activity. I reasoned that the ability of yNap1 dimers to bind multiple H2AH2B heterodimers suggests that the ratio of Nap1 to histones in nucleosome assembly assays may influence the ability of Nap1 to bind histones and therefore to assemble nucleosomes. A wide range of yNap1 to histones ratios have been used to assemble mononucleosomes in vitro, so I began by examining the nature of the Nap1-histone complexes typically used in chromatin assembly assays by EMSA.

EMSA of Nap1-histone complexes revealed that even at a ratio of one Nap1 dimer to one histone octamer, all of the purified core histone proteins are bound by yNap1 and migrate toward the positive electrode (Figure 5.3). The complexes at the 1:1 ratio did not form a discreet band but rather a smear, possibly indicating some degree of heterogeneity and/or a dynamic complex structure. The association of an octamer of core histones with a dimer of yNap1 is consistent with an earlier hydrodynamic study that described a yNap1 dimer capable of binding an octamer of histone proteins. These complexes may be similar to the complexes observed in EMSA of Y1 with yH2AH2B at molar ratios of one yNap1 dimer to four yH2AH2B heterodimers (this ratio being an equivalent amount of histone dimer species, two H2AH2B heterodimers and one (H3H4)2 heterotetramer). In this complex, yNap1 may bind the two H2AH2B dimers to their high affinity A site and bind the (H3H4)2 tetramer with the long acidic C-terminal tails (B sites). At a molar ratio two Nap1 dimers to one histone octamer equivalent (as under these salt conditions the histones
Figure 5.3 EMSA of Nap1 histone complexes
EMSA analysis of Nap1 : histone complexes at the indicated ratios on slab-PAGE in 0.25 X TBE. In the absence of Nap1 the core histones migrate as a single band toward the negative electrode. The in the presence of one Nap1 dimer to one histone octamer equivalent, all of the histones are bound by yNap1 and now migrate toward the positive electrode, although there is no distinct band visible. The subsequent addition of more Nap1 to a fixed amount of histones shows the formation of discreet Nap1-histone complexes as well as the appearance of free Nap1 at molar ratios of 3 : 1 and above.
exist as H2AH2B heterodimers and (H3H4)2 tetramers) distinct bands are observable in the EMSA. Beyond a ratio of two Nap1 dimers to one core histone octamer an appreciable amount of free Nap1 is observed, which run faster than Nap1-histone complex bands. This suggests that the one Nap1 dimer is sufficient to bind all for core histones, but that two Nap1 dimers are needed to bind the histones into discreet Nap1-histone complexes. The two Nap1 dimer to one histone octamer equivalent ratio is the ratio expected if one Nap1 dimer binds two H2AH2B heterodimers to its principal A sites and another Nap1 dimer binds the (H3H4)2 tetramer (i.e. two H3H4 heterodimers) to its principal A sites and/or B sites.

I next evaluated the ability of different Nap1-histone mixtures to assemble mononucleosomes. I first examined if there was any difference in the extent of nucleosome assembly at different ratios of Nap1 by examining three different ratios of Nap1 dimer to histone octamer equivalent (1:1, 2:1 and 4:1) Figure 5.4. The one Nap1 dimer to one octamer ratio did not assemble nucleosomes efficiently (lane 3). At a ratio of two Nap1 dimers to one histone octamer the majority of the DNA is assembled into mononucleosomes (lane 4). At a ratio of four Nap1 dimers to one histone octamer (lane 5) mononucleosomes are also assembled but in addition to the mononucleosome, another electrophoretically distinct species appears on the gel running between free DNA and mononucleosomes (lane 5). A study published recently (Mazurkiewicz, Kepert et al. 2006) has suggested that this lower band is a hexasome (1 (H3H4)2 tetramer and 1 H2AH2B dimer). Quantification of both free DNA and the nucleosome bands suggests that lane 4 contains more assembled nucleosomes than lane 5. The quantification must be taken with a grain of salt since
Figure 5.4 The ratio of Nap1 to histones can influence the products of assembly
Different ratios of Nap1 to core histone proteins can produce markedly different results under conditions where all other variables are the same. At a ratio of two Nap1 dimers per one histone octamer equivalent, most of the small linear DNA fragment is assembled into mononucleosomes. At ratios below 2:1 or significantly above 2:1 different results are observed.
the binding of EtBr by DNA is greatly affected by its assembly into nucleosomes. Even with this limitation, it is clear that mononucleosomes can be assembled by Nap1 at a molar ratio of two Nap1 dimers to one histone octamer while a ratio of one Nap1 dimer to one histone octamer is insufficient for robust nucleosome assembly under these conditions.

In order to confirm this observation, I expanded this analysis to include intermediate Nap1 : histone octamer ratios to verify that two Nap1 dimers to one histone octamer equivalent was the minimal unit needed for efficient assembly. Similar results were obtained to the above experiment. At ratios below two Nap1 dimers to one histone octamer equivalent, the assembly of mononucleosomes was inefficient. Even at extend time periods when all of the DNA is assembled into mononucleosomes by ratios of Nap1 dimer to histone octamer at or above 2 : 1, the reactions below two Nap1 dimers to one histone octamer equivalent do not make mononucleosomes. At the 2 : 1 ratio at 30 minutes a significant proportion of the DNA has been assembled into nucleosomes. A ratios above two Nap1 dimers to one histone octamer mononucleosomes are also assembled, however, at high ratios of Nap1 dimer to histone octamer the lower “hexasome” band appears at the 30-minute time point. Extended incubation time of these reactions leads to the disappearance of the “hexasome” band with a concomitant increase in the intensity of the mononucleosome band, suggesting that this may be an intermediate in the assembly of nucleosomes at these ratios of Nap1 to histone or that they are aberrant products that are resolved over time, perhaps by the histone exchange activities of yNap1.
Figure 5.5 A ratio of two Nap1 dimers to one histone octamer assembles mononucleosomes efficiently

The ratio of Nap1 to core histones directly influences the products mononucleosome assembly products observed. At a ratio of two Nap1 dimers to one equivalent of a histone octamer Nap1-histone complex readily form mononucleosomes. Below this ratio the rate of assembly decreases dramatically. At ratios well above 2:1 excess Nap1 may influence the mechanism of nucleosome assembly by a mass action effect possibly leading to the formation of a “hexasome” intermediate.
D. Discussion

The assembly of nucleosomes by histone chaperones does not merely involve the electrostatic screening of charges but rather discreet histone-histone chaperone complexes. The analysis of yNap1 truncation mutants demonstrates that for Nap1 proteins a dimer of Nap1 capable of binding two H2AH2B dimers is required for nucleosome assembly activity. The ability to bind four H2AH2B dimers (two by the A sites and two by the B sites) does not appear to be absolutely required for \textit{in vitro} nucleosome assembly, consistent with previous studies of Nap1 deletion mutants. The function of the long acidic C-terminal tail of yNap1 (B-site) is unclear although it clearly contributes to some facet of nucleosome assembly by yNap1 as its removal decreases the efficiency of assembly.

Perhaps the long acidic C-terminal tail of Nap1 (B site) interacts with the tails of histones H3 and H4. Previous studies have demonstrated that yNap1 has a higher affinity for H3 and H4 N-terminal tails than those of H2A and H2B and that this preference depends on the C-terminal tail (24; E. Gamache and R.N.D., unpublished results). Moreover, the ability of the related SET/TAF1β protein to inhibit acetylation of histone H3 and H4 also requires the presence of the acidic C-terminal tail, presumably to bind the N-terminal tails of H3 and H4 (45). These data suggest that the acidic C-terminal tail of yNap1 interacts with the basic N-terminal tails of histones H3 and H4. We speculate that in the \textit{in vivo} assembly reaction, these interactions contribute to an electrostatic steering mechanism that more effectively targets the Nap1-H2AH2B complex to (H3H4)2-DNA complexes. The absence of this electrostatic steering may not completely abrogate nucleosome assembly but may
decrease the rate of assembly. It is clear, however that the core-conserved regions of Nap1 must be intact for Nap1 to retain nucleosome assembly activity. Removal of either domain I (dimerization) or domain II (H2AH2B binding domain) completely abrogates nucleosome assembly activity.

The ratio of Nap1 to core-histones plays a vital role in determining whether yNap1 can assemble nucleosomes. Even though one yNap1 dimer is sufficient to bind all four core histones and neutralize the positive charges on the histones, these complexes are not capable of rapid and efficient nucleosome assembly on mononucleosome size pieces of DNA. It is only at ratios of two Nap1 dimers per histone octamer that the rapid and high fidelity assembly of nucleosomes is observed. At ratios beyond 2:1, a significant amount of free Nap1 dimers are observed which suggests that two Nap1 dimers are sufficient to bind a histone octamer. These data are consistent with findings in the early work of Ishimi et al. Furthermore, analysis of the nucleosome assembly activity of yNap1 at ratios higher than 2:1, resulted in the appearance of a lower “hexasome” product from assembly reactions. Given sufficient time, these products resolved themselves into nucleosomes suggesting that they are either intermediates in the process of nucleosome assembly at these ratios or misassembled nucleosomes that are resolved given enough time.

The assembly reactions observed for Nap1 dimer : histone octamer ratios above two to one in these experiments are similar to observations made by Mazurkiewicz et al in a recent JBC paper (Mazurkiewicz, Kepert et al. 2006). The authors concluded that Nap1 dimers (at a molar ratio of four Nap1 dimers to one histone octamer equivalent reported) assemble nucleosomes via a hexasome
intermediate, however, the authors never vary the amount of Nap1 to examine if nucleosome assembly occurs at other molar ratios thereby missing the assembly activity of Nap1 at a two Nap1 dimers to one histone octamer ratio. These results are therefore incomplete and in fact may only be analyzing one possible mechanism of nucleosome assembly by Nap1 proteins. Moreover, the authors’ use of predicted molar extinction coefficients leads to erroneous protein concentrations. Recalculation of protein concentrations based upon the empirically derived molar extinction coefficients suggests that the authors are more likely at molar ratio of 6 : 1 (Nap1 dimer to histone octamer equivalent). Therefore, the conclusions the authors draw must be viewed with these caveats in place. This does not completely discount their findings, but rather suggests that there may be two different mechanisms of nucleosome assembly wholly dependent upon the relative concentration of Nap1 to histone.

The two possible deposition mechanisms for H2AH2B introduced earlier may both be used by Nap1 under specific conditions. At sufficient histone concentrations Nap1 dimers may be have fully loaded A sites capable of a concerted H2AH2B dimer deposition mechanism. Under conditions where there are locally high concentrations of Nap1 or low concentrations of free H2AH2B heterodimer, the Nap1 dimer may bind to only one H2AH2B heterodimer thereby necessitating nucleosome assembly/disassembly proceed via a sequential mechanism. The EMSA analysis contained in this study has already demonstrated that sub-saturated Nap1-H2AH2B complexes can be formed at molar ratio of one Nap1 dimer to one H2AH2B dimer consistent with this type of a mechanism. Perhaps this is what is observed at molar
ratios of Nap1 dimer to histone octamer beyond 4 : 1 used in Mazurkiewicz et al. Here Nap1 dimers could interact with H2AH2B heterodimers to form sub-saturated Nap1-H2AH2B complexes. Since these complexes only contain a single H2AH2B dimer, the assembly of the nucleosome must occur via sequential delivery of H2AH2B by Nap1.

The existence of two possible mechanisms of nucleosome assembly by Nap1 that depends upon the relative abundance of Nap1 and histones may be advantageous for the cell. The delivery of two H2AH2B dimers to an (H3H4)2-DNA complex seems to be the most efficient means to assemble a nucleosome relying upon one binding event to get the contents of the nucleosome in close spatial proximity. This may be the mechanism employed by the cell during S-phase as large amounts of replicated DNA are assembled into nucleosomes with large of amounts of new histones that are produced coordinately in S-phase. A Nap1 dimer, which is also up regulated at S-phase, can bind two new H2AH2B in the cytosol and help import them into the nucleus where they are used in the rapid assembly of nucleosomes with high fidelity.

The second mechanism which may go through a sub-saturated (the A sites half occupied so a molar ratio of one Nap1 dimer to one H2AH2B dimer is observed) Nap1-H2AH2B complex maybe relevant for nucleosome assembly events such as H2AH2B exchange or the passage of polymerases through a chromatin template where partial disruption of nucleosomes have been observed. Here, targeting of Nap1 to specific loci may increase the local concentration of Nap1 dimer to such a degree that it can bind only one H2AH2B dimer.
These possibilities certainly warrant examination, however, the current nucleosome assembly assays are not well defined for such analysis due to the fact that there are both Nap1-H2AH2B interactions and Nap1-(H3H4)_2 interactions. Ideally, one would like to create a system in which stable (H3H4)_2-DNA complexes can be produced to which defined Nap1-H2AH2B complexes can be added to evaluate just the contribution of Nap1 interaction to the H2AH2B deposition step. As a first attempt at such a study I have been able to assemble mononucleosomes onto a small linear fragment of DNA by a step wise addition of (H3H4)_2 to DNA with the subsequent addition of defined Nap1-H2AH2B complexes (Figure 5.6). The results are promising, however a good degree of caution must be exercised. Stable (H3H4)_2-DNA complexes are not readily evident under the gel electrophoresis analysis presented here. Perhaps future experiments will allow for the careful analysis of H2AH2B deposition by Nap1 in such a system.
Figure 5.6 Stepwise mononucleosome assembly

(H3H4)$_2$-DNA complexes were incubated for 2 hours at 30°C with either buffer (lane 2), Nap1 (lane 1), or increasing amounts of H2AH2B or Nap1-H2AH2B complexes and then analyzed on a 0.7% agarose gel in 0.25 X TBE. Lane 11 contains a mononucleosome marker made by gradient salt dialysis. Reactions contained 50 pM DNA, 40 pM (H3H4)$_2$ and in lanes 4-6 or 7-9 H2AH2B : (H3H4)$_2$ molar ratios of 1.2, 1.6 or 2.0.
VI: Perspectives
A. The Nap1 dimer can bind multiple copies of H2AH2B and assembles nucleosomes using a concerted mechanism

The yNap1 protein has been used extensively in *in vitro* chromatin assembly assays, yet, at the outset of this study, the mechanism of nucleosome assembly by yNap1 and in fact any histone chaperone was poorly understood. For years many had assumed that histone chaperones simply acted as molecular salt, simply neutralizing the highly basic histone proteins preventing their improper aggregation onto DNA. This assumption completely ignored several lines of evidence that suggested that histone chaperones play a more active role in the assembly of nucleosomes. The existence of histone chaperones suggests that polyanions such as RNA or polyglutamate, although capable of *in vitro* nucleosome assembly, are insufficient for assembly of nucleosomes *in vivo*. Furthermore, the high degree of sequence conservation within chaperone families indicates that evolutionary processes have selected these sequences for a specific purpose. These families of histone chaperones display distinct histone binding preferences *in vivo*. The partition of (H3H4)$_2$ heterotetramers and H2AH2B heterodimers into distinct complexes is consistent with the stepwise assembly of nucleosomes *in vivo* where each specific histone-histone chaperone complex contributes to the assembly of nucleosomes *in vivo*. Finally, a recent kinetic study has demonstrated that H2AH2B specific chaperone Nap1 is capable of assembling nucleosomes 3 orders of magnitude more efficiently than similar sized polyanions providing experimental data for the hypothesis presented in this study, namely that the three dimensional structure of histone-histone chaperone
complexes directly influences the ability of histone chaperones to assemble nucleosomes.

The goal of this work was to understand the molecular mechanisms of nucleosome assembly by the *S. cerevisiae* Nap1 protein. In order to achieve this goal, I determined the predominant solution structure of yNap1, examined the interaction of yNap1 with yH2AH2B heterodimers and analyzed the *in vitro* nucleosome assembly activity of yNap1. This work was carried out in parallel with efforts to obtain protein crystals of yNap1 and/or yNap1 truncation mutants that were of sufficient quality to obtain high-resolution X-ray diffraction data for the generation of a molecular model of yNap1. Although I never obtained protein crystals of sufficient quality, the rigorous biochemical and biophysical analysis of the yNap1 and the yNap1-H2AH2B complex contributes a great deal to our collective understanding of nucleosome assembly by histone chaperones.

In Chapter 2 I present a biophysical and biochemical study of the yNap1 protein in solution. The domain analysis by limited proteolytic digestion of purified recombinant yNap1 and dNap1 suggests that the Nap1 protein contains two well-folded structural domains. These domains contain two regions of high sequence conservation in the Nap1/SET family of proteins. Mapping out these digestion sites on a recently published X-ray crystal structure rationalizes the digestion pattern and suggests that there are two well-folded domains in yNap1 in solution. A panel of truncation mutants was generated using recombinant DNA technology based upon the domain analysis from limited proteolytic digestion. Rigorous biophysical analysis of full-length yNap1 and a subset of the truncation mutant panel revealed that the Nap1
protein is predominantly a dimer in solution. The majority of the contacts necessary for dimer formation are contained in the first region of high sequence conservation in the Nap1/SET family of proteins as a truncation mutant that only contained domain I of yNap1 eluted as a multimer from gel filtration chromatography and appeared to be a dimer in sedimentation equilibrium experiments. Domain II contains a stable protein fold that behaves as a monomer in solution.

In Chapter 3 I presented a novel method for the production of soluble recombinant histone proteins. This method has distinct advantages over the published methods of heterologous histone protein production. The proteins produced by this system fold in *E. coli* and are at the correct stoichiometric ratios. Since the proteins are soluble, denaturants such as urea are unnecessary thereby circumventing labor-intensive methods and potential modifications from denaturing-refolding methods. The finding that the published molar extinction coefficients for histone proteins based upon methods published by Gil and von Hippel are appreciably different from actual empirical molar extinction coefficients is a significant finding. It is surprising that this error has not been corrected given the fact that histones were among the first proteins to be isolated and classified. Obtaining an accurate measure of protein concentration is essential for the interpretation of thermodynamic data presented in Chapter 4.

In Chapter 4 I presented the first truly rigorous examination of the Nap1-H2AH2B complex. Using EMSA, I was able to determine that domain II of yNap1 was sufficient to bind H2AH2B heterodimers in a one to one ratio. Armed with this data and the knowledge that full-length yNap1 is a dimer I fully expected that the
full-length yNap1 dimer would be capable of binding more than one H2AH2B dimer. EMSA and ITC analysis of full-length yNap1 clearly demonstrates that this is indeed the case. The full-length yNap1 dimer is capable of binding up to four H2AH2B heterodimers. Two H2AH2B dimers are bound to a principal high affinity interaction site (A site) followed by binding of two more H2AH2B dimers to a lower affinity interaction site (B site). The binding to the A site appears to involve the globular domains of H2AH2B as removal of the N-terminal tails of H2AH2B do not effect binding to the A site. The removal of the N-terminal tails of H2AH2B does however affect binding of H2AH2B to the lower affinity B site as the absence of the N-terminal tails of H2AH2B abrogates binding of H2AH2B to the B site of full-length yNap1. The analysis of deletion mutants of yNap1 revealed that the B site is contained within the long acidic C-terminal tail of yNap1. Therefore, the C-terminal tail of yNap1 appears to interact with the flexible N-terminal tails of yH2AH2B. A model of Nap1-H2AH2B interactions was generated based upon this binding data.

In Chapter 5 I analyzed the nucleosome assembly activity of yNap1 and yNap1 truncation mutants. Utilizing both an established chromatin assembly assay and a mononucleosome assembly assay that I developed, I evaluated the ability of full-length recombinant yNap1 and a subset of truncation mutants to assemble nucleosomes. The ability of yNap1 to assemble nucleosomes appears to be dependent upon the nature of the Nap1-histone complexes. In the absence of H2AH2B binding, a dimer of domain I of yNap1 is unable to assemble nucleosomes. A truncation mutant of yNap1 that only contains domain II, although capable of binding H2AH2B with high affinity, is also unable to assemble nucleosomes. Only
yNap1 molecules that are capable of forming a dimer and possess the ability to bind two H2AH2B heterodimers are able to assemble nucleosomes from purified D. melanogaster histones. Preliminary analysis of the mechanism of nucleosome assembly by full-length yNap1 suggests that there may be two different modes of assembly that are wholly dependent upon the relative ratio of Nap1 to histone. However, I was able to establish that the minimum amount of yNap1 required for robust nucleosome assembly is one Nap1 dimer per two histone dimer equivalents (two H2AH2B heterodimers or one (H3H4)2 heterotetramer).

The weight of the data presented in this study suggests that the nucleosome assembly activity of Nap1 is wholly dependent upon the nature of the Nap1-histone complexes available for assembly. The Nap1 protein must be a dimer that has the ability to bind two H2AH2B heterodimers for the Nap1 protein to possess any significant nucleosome assembly activity. The most efficient assembly of nucleosomes by Nap1-histone complexes occurs when the ratio of Nap1 dimer to histone octamer is 2 : 1 suggesting that one Nap1 dimer binds two H2AH2B dimers and another Nap1 dimer binds an (H3H4)2 tetramer. These data suggest that specific Nap1-histone complexes are involved in the assembly of nucleosomes in vitro and presumably in vivo. These requirements provide evidence that mechanism of nucleosome assembly by histone chaperones is not merely a simple electrostatic screening of charges but mediated by specific histone-histone chaperone complexes.

B. Model of assembly

The study presented provides direct evidence that the nature of histone-histone chaperone complexes can play a direct role in the assembly of nucleosomes. This
may be a general principal in the function of histone chaperones. The Nap1 dimer can bind two H2AH2B heterodimers to two high affinity A sites (domain II of Nap1). This allows Nap1 to bring two H2AH2B dimers to an (H3H4)_2-DNA complex for the rapid assembly of a nucleosome in a single encounter rather than waiting for the sequential deposition of two H2AH2B heterodimers in independent encounter events. But perhaps there is more to this story than just bringing the right number of parts to complete the puzzle. Comparison of the Nap1 structure with the structure of H2AH2B heterodimers in the nucleosome suggests the intriguing possibility that Nap1 may organize the H2AH2B dimers in a fashion similar to that in the nucleosome, allowing for the deposition of a preformed H2AH2B_2 tetramer-like structure directly onto an (H3H4)_2-DNA structure (Figure 6.1). Such a mechanism could rapidly facilitate the assembly of nucleosomes by forming a histone octamer core that can be wrapped immediately by DNA. These types of histone-histone chaperone interactions may also allow histones to make the intimate contacts that are observed in the nucleosome. In doing so, the formation of these specific histone-histone chaperone complexes may allow for the pre-screening of H2A variants by H2A-H2A interactions and the incorporation of two copies of the same H2A variant into a single nucleosome. Although this is mere speculation, other histone chaperones appear to have similar potential mechanisms.

Akey and colleagues (Akey and Luger 2003) have suggested that the X. laevis protein Nucleoplasmin is a decamer in solution (two pentameric Nucleoplasmin molecules stacked on top of each other). The orientation of the Nucleoplasmin monomers in the decamer structure would put two histone-binding units directly
Figure 6.1 A model of concerted nucleosome assembly by Nap1
A schematic representation of Nap1 interacting with two H2AH2B dimers. The Nap1 dimer may organize the H2AH2B dimers in a fashion similar to their structure in the nucleosome. Such organization may allow Nap1 to prescreen H2AH2B interactions (i.e. distinguish between H2A variants) and allow for the rapid assembly of the nucleosome by simply docking a preformed H2AH2B complex onto the nucleosome.
across from each other in the decamer providing 5 similar histone-binding sites in the decamer. Each of these sites is thought to interact with two H2AH2B heterodimers. This stereospecific binding of H2AH2B by Nucleoplasmin may enable the rapid deposition of two H2AH2B dimers onto (H3H4)_2-DNA complexes during the packing of vast amounts of chromatin during development. More rigorous analysis of Nucleoplasmin and Nap1 are needed to explore these potential facets of histone-histone chaperone interactions.

C. Future directions

The most fruitful future direction of this project would be to obtain Nap1-H2AH2B protein crystals for X-ray diffraction studies. With high resolution structural models of both Nap1 and H2AH2B heterodimers phase angles may be obtained from molecular replacement approaches all one needs are high quality Nap1-H2AH2B protein crystals. A detailed molecular model of Nap1-H2AH2B interactions can be used to interpret the thermodynamic data obtained in this study and provide more of the molecular details of the interaction. These details can then be compared to the molecular interactions observed for H2AH2B dimers in the nucleosome core particle structures as a starting point for developing a model for the mechanism of transfer of two H2AH2B heterodimers from a Nap1 dimer to an (H3H4)_2-DNA complex. In conjunction with these efforts, the EDC chemical cross-links between full-length yNap1 and yH2AH2B could also be mapped out using a limited proteolysis approach coupled to LC-MS. The EDC chemical cross-links are zero-length cross-links between acidic residues and basic residues that are in close proximity. Mapping out these contacts and superimposing the known structure of
H2AH2B heterodimers onto the structure of yNap1 may provide a reasonable estimate of the structure of the Nap1-H2AH2B complex.

In addition to these structural studies, more mechanistic studies of yNap1 truncation mutants along with histone truncation mutants by more defined step wise assembly assays, as presented at end of Chapter V, may provide answers to the function of the long acidic C-terminal tail of yNap1. Future research may build upon these analyses toward the overall goal of understanding the molecular mechanism of nucleosome assembly by histone chaperones.
References


