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Analysis of the human HMG and H1 proteins in chromatin

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in
Biology

By

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2011
The Thesis of Mai Tu Khuong is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
I dedicate this thesis to my mom and brother

for their continued love and support.
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ABSTRACT OF THE THESIS

Analysis of the human HMG and H1 proteins in chromatin

by

Mai Tu Khuong

Master of Science in Biology

University of California, San Diego, 2011

Professor James Kadonaga, Chair

The high mobility group (HMG) proteins are the most abundant non-histone chromosomal proteins within the nucleus. As architectural proteins, they are thought to play a large role in chromatin dynamics by interacting with the linker histone H1 at the nucleosome. The potential interplay among these proteins have been suggested by both biochemical studies and in vivo studies. Using a purified biochemical system, HMGB2 appears to be displaced at the nucleosome by H1.0, an H1 variant, and HMGN2 and H1.0...
can both bind at the nucleosome simultaneously. These findings may lead to a deeper understanding of chromatin dynamics and its effects on DNA-utilizing processes.
I.

Introduction
Chromatin has two critical roles within the eukaryotic cell. It packages the entire genome, which is often over a meter in length, into a nucleus that is several micrometers in diameter. It is also responsible for regulating DNA-utilizing biological processes such as transcription, replication, and DNA repair, through dynamic changes in chromatin structure (Chen and Li, 2010; Campos and Reinberg, 2009; Paranjape et al., 1994). Chromatin compaction hinders accessibility to nucleosomes repressing genomic activity, whereas chromatin decompaction promotes accessibility to nucleosomal DNA and activates transcription. These dynamic changes in structure are aided by many factors, including chromatin remodeling factors and architectural proteins that may alter the structure of chromatin.

Chromatin is a nucleoprotein complex composed of a 2:1 mass ratio of proteins to DNA and a 1:1 mass ratio of histones to DNA. It consists of the basic repeating unit called the nucleosome, which is composed of approximately 180 to 200 base pairs of DNA tightly wrapped around a histone core octamer with a molecule of linker histone H1 attached. The histone core octamer is composed of two molecules each of histones H2A, H2B, H3, and H4. The primary structure of chromatin, a 10 nm fiber also known as a having a “beads on a string” conformation, consists of nucleosomes connected by linker DNA. However, this structure is not seen in physiological conditions (low or no salt). The bulk of chromatin is seen in vivo as a condensed 30 nm fiber, and through further folding mediated by proteins whose functions are not well characterized, the 30 nm fiber is able to form a chromosome (Paranjape et al., 1994). It is clear that these structures exist, but the multitude of DNA and protein interactions involved to create these dynamic structures largely remain a mystery.
**Linker histone H1**

Most studies have focused mainly on the core histones and their role in chromatin dynamics, but there are also many chromosomal proteins, many of which have unknown functions. The most well-known of these proteins is the non-core, linker histone H1. It is highly abundant in chromatin, with roughly one molecule of H1 per nucleosome in higher eukaryotes (Bates and Thomas, 1981). Histone H1 consists of a main globular domain flanked by a lysine rich N-terminal and C-terminal tail. The precise location of histone H1 in chromatin is not known, but considerable data strongly suggests localization at the pseudodyad of the nucleosome (Thomas, 1999). It is thought that the lysine rich tails interact with the linker DNA, while the folded globular domain interacts with the DNA near the pseudodyad of the nucleosome (Allan et al., 1980; Crane-Robinson and Ptitsyn, 1989). Studies show that the incorporation of H1 is necessary to form the stabilized 30 nm chromatin fiber (van Holde and Zlatanova, 1996). H1 has also long been positively correlated with gene repression (Paranjape et al., 1994).

There are many subtypes of linker histone H1, which differ from the canonical H1 in tail length. Eleven human subtypes of H1 have been identified, one of which is histone H1.0 (Happel and Doenecke, 2009; Khochbin, 2001). Homologues of H1.0 have been found in many vertebrates as well as some invertebrates and it is not ubiquitously expressed in cells. Like the canonical H1, H1.0 is also positively correlated with gene repression (Happel and Doenecke, 2009).

**High Mobility Group Proteins**

The most abundant non-histone proteins in the nucleus are the high mobility group (HMG) proteins, whose functions in chromatin dynamics are not well understood.
The HMG proteins were first isolated and characterized in the 1970s as highly abundant, non-histone chromosomal proteins that were extractable at 0.35M NaCl, were soluble at 2% trichloroacetic acid, and exhibited high electrophoretic mobility (Goodwin et al., 1973). There are three families of HMG proteins: HMGA proteins, HMGB proteins, and HMGN proteins. Each family is defined by a characteristic structural motif. While proteins within the same family are related, the families themselves are not related to one another (Bianchi and Agresti, 2005). Studies suggest HMGs act as architectural proteins in chromatin and further studies into these proteins and their effects on chromatin dynamics could lead to a better understanding of chromatin compaction.

HMGA proteins are small (~11 kDa) proteins and are characterized by an AT hook domain, which binds in the minor groove of AT-rich sequences. In enhancersomes, HMGA proteins bend DNA to increase the affinity of specific transcription factors to their binding sites. They may also have a larger role in higher organizational levels of chromatin structure, by binding to AT-rich scaffold-/matrix- associated sequences that make up the nuclear matrix (Bianchi and Agresti, 2005). These HMGA proteins have been extensively studied, and their role in chromatin dynamics suggests that the other HMG proteins may also play an important role in chromatin structure and function.

HMGB proteins are the largest (~25 to 30 kDa) of the HMG proteins (Agresti and Bianchi, 2003). They are highly abundant in chromatin: in mammalian cells, there appears to be approximately 0.5 molecules of HMGB proteins per nucleosome (Kuehl et al., 1984). These proteins are also highly conserved among eukaryotes, with homologues in yeast, flies, and vertebrates. In humans, there are three HMGB homologues (HMGB1,
HMGB2, and HMGB3), which are approximately 80% identical to each other. The majority of the differences are located in the C-terminal domain.

HMGBs are characterized by the presence of at least one DNA binding domain called the HMG box and an acidic C-terminal tail consisting of only aspartic and glutamic acid residues. Mammalian HMGBs have two tandem HMG boxes, while the HMGB homologues HMG-D and Nhp6a/Nhp6b, from *Drosophila melanogaster* and *S. cerevisiae* respectively, have one HMG box (Thomas and Travers, 2001). The HMG box is found in both non-sequence specific DNA binding proteins as well as sequence-specific DNA binding proteins. HMG boxes are highly basic, which promotes interaction with the negatively charged sugar-phosphate backbone of DNA (Paull et al., 1993; Lnenicek-Allen et al., 1996).

The function of HMGB proteins in chromatin is currently uncharacterized. It has been proposed that they alter chromatin structure, like HMGA proteins, to promote or repress DNA-utilizing processes such as transcription, replication, and repair, but the mechanism for this has not been well studied. Several studies have identified a few biochemical properties of HMGBs suggesting they might be architectural proteins.

HMGB proteins are non-sequence specific DNA binding proteins. They are able to bind and bend linear DNA approximately 90 degrees and stabilize the bend (Paull et al., 1993; Lnenicek-Allen et al., 1996). They, however, have a higher affinity for distorted DNA structures such as a four way junction (Teo et al., 1995). HMGB proteins are suggested to bind at the pseudodyad, which is the entrance and exit for DNA on a nucleosome, and may resemble a four way junction. Studies have shown localization of
HMGB proteins to the pseudodyad through DNA footprinting and microccocal nuclease digestion (Nightingale et al., 1996; An et al., 1998).

There have been several studies that suggest possible interplay among the HMG proteins and H1. The dynamics between HMGB and H1 is of special interest because both proteins have been shown to bind at or near the pseudodyad of the nucleosome and to 4 way junction DNA, suggesting that there could be competition for binding to a nucleosome (Hill and Reeves, 1997). There are also examples of chromatin that lack H1 but contain HMGB proteins instead. In Drosophila, the HMGB homologue HMG-D is present in early embryonic chromatin, while H1 is absent; at the onset of zygotic transcription, HMG-D levels decrease dramatically while H1 levels increase (Ner and Travers, 1994). In vitro experiments have also shown that HMG-D bound to chromatin could be displaced by H1 (Ner et al., 2001). Based on these studies, it has been suggested that HMGB proteins bind at the nucleosome to produce a “primed” complex, in which the DNA becomes more accessible to promote DNA-utilizing processes such as transcription. This is in opposition to a less accessible conformation of the nucleosome, stabilized by H1, which would repress transcription (Travers, 2003). However, this proposed model has not been tested.

There have been several in vivo studies analyzing the function of HMGB proteins. Despite an 80% identity among the three mammalian HMGB proteins, each appears to have distinct roles. In mice, HMGB1 and HMGB2 are ubiquitously expressed during embryogenesis, but by adulthood, HMGB2 expression is limited to the brain and, in males, the testes (Calogero et al., 1999; Ronfani et al., 2001). HMGB3 expression is restricted to hematopoietic cells of the bone marrow. (Bianchi and Agresti, 2005).
Knockout studies in mice have shown that HMGB1 may have a more widespread role in development than HMGB2 and generally has been associated as a cytokine promoting inflammation (Scaffidi et al., 2002). HMGB1 knockouts were not embryonically lethal, but were hypoglycemic and did not survive past the first day unless fed with glucose (Calogero et al., 1999). HMGB2 knockouts survived, but showed reduced fertility in males, suggesting a role in spermatogenesis in adult males (Ronfani et al., 2001). HMGB3 knockouts were viable, but erythrocythemic (Nemeth et al., 2005). It is suggested that there is some redundancy due to single knockouts being viable. A double knockout for HMGB1 and HMGB2 is embryonically lethal, showing that these two proteins are needed during development (Bianchi and Agresti, 2005). It is necessary to first examine how these proteins take part in chromatin structure and function to begin to understand how these proteins play a role in vivo.

HMGN proteins are the smallest of the HMG proteins (~10 kDa). They are characterized by a nucleosome binding domain, which facilitates binding to nucleosomes at two high affinity binding sites (Albright et al., 1980; Alfonso et al., 1994; Mardian et al., 1980; Sandeen et al., 1980). A study has also identified a potential transcriptional enhancement domain, which may contribute to chromatin unfolding during transcriptional activation (Ding et al., 1997). HMGN proteins have only been observed in vertebrates and are highly abundant, with 0.5 molecules per nucleosome (Kuehl et al., 1984). Humans have four HMGN proteins (HMGN1-4), which are most similar to each other in the nuclear binding domain.

There have been many studies to analyze the function of HMGN proteins in chromatin dynamics. However, many of these studies are controversial. Recently,
HMGNs were found to be able to inhibit ATP-dependent chromatin remodeling (Rattner et al., 2009). This contradicts an older model, in which HMGN1 did not affect ATP-dependent chromatin remodeling by the SWI/SNF complex (Hill et al., 2005). There are also conflicting studies suggesting that HMGNs act as either a transcriptional repressor or transcriptional activator (Zhu and Hansen, 2010).

As HMGN proteins may activate transcription (Ding et al., 1994; Paranjape et al., 1995), it has been proposed that they may be able to counter repression by histone H1. Another study has observed residence time of H1 at the nucleosome decreases in the presence of HMGN proteins, and it has been speculated that HMGN is able to compete for binding sites with H1 (Catez et al., 2002).

In vivo studies suggest that HMGN proteins have a role during differentiation. HMGN1 and HMGN2 are found to be highly expressed in all mouse embryonic tissues. However, during embryogenesis, HMGN1 and HMGN2 genes are gradually down-regulated throughout the embryo, except in regions where cells are undergoing active differentiation. HMGN1 is known to bind at Sox9, a chondrocyte lineage master regulator, and in HMGN1 knockout mice, levels of HMGN2 are elevated at Sox9, further suggesting that HMGN proteins help regulate differentiation and that these proteins may have some levels of redundancy (Furusawa et al., 2006). HMGN1 knockout mice are also hypersensitive to UV and ionizing radiation (Birger et al., 2005). This suggests that HMGN1 proteins have a role in DNA repair as well.

HMGN proteins and HMGB proteins have been implicated in cellular processes such as differentiation, gene regulation, and DNA repair; thus, it is important to study the roles they play in chromatin dynamics. The interaction among the HMG proteins and
histone H1 is of special interest due to its observed dynamics in vivo, but it has not thoroughly been examined.
II.

Results
Purification of linker histone H1.0 and HMGB2 protein

Recombinant, flag-tagged linker histone H1.0 was expressed and purified from bacteria (Figure 1A). Flag-tag affinity purification yielded several impurities and therefore, was not used. The purification of H1.0 was redesigned from previously published protocols for purification of native H1 (Croston et al., 1991). Linker histone H1 and its variants, such as H1.0, are known to increase the nucleosome repeat length (Woodcock et al., 2006). Chromatin assembly reactions were performed in the absence and presence of recombinant H1.0, and analyzed by partial micrococcal nuclease digestion assay, which can show changes in the nucleosome repeat length. Chromatin that contained H1.0 showed an increase in nucleosome repeat length (Figure 1B), indicating that the recombinant H1.0 was active.

Recombinant HMGB2 was also expressed and purified from bacteria, using previously established protocols. A classic biochemical activity of HMGB proteins is the ability to bind to distorted DNA, such as the four-way junction DNA (Bianchi, 1988). The recombinant HMGB2 bound to 4-way junction DNA (Figure 1C); thus, it is also active.

Generation of antibodies specific against HMGB1 and HMGB2

Antibodies are a useful tool, and currently no antibodies exist that are specific between HMGB1 and HMGB2. Recombinant full-length HMGB1 and HMGB2 were previously used to generate antibodies. While they were specific to HMGB proteins, they were not specific between HMGB1 and HMGB2 (unpublished data). An alignment using ClustalW of the human HMGB1 (accession # CAG33144) and HMGB2 (accession # AAJ00020) sequences revealed that they are 81% identical (Figure 2A). Due to their high
similarity overall, it might be difficult to generate antibodies specific for either HMGB1 or HMGB2. However, most of the differences between HMGB1 and HMGB2 lay in the C-terminal region, and if only these parts of HMGB1 or HMGB2 are used, specific antibodies could be generated. The C-terminal regions of HMGB1 and HMGB2 were each cloned into pGEX-6P1 to synthesize GST-fusion proteins. These fusion proteins were purified by glutathione sepharose (Figure 2B and 2C) and used as antigens for polyclonal antibodies. Antibodies generated with these fusion proteins were specific between HMGB1 and HMGB2, but they also detected many other proteins in a whole cell extract (Figure 2D and 2E). These antibodies were affinity purified using CNBr-activated beads couple to recombinant full-length HMGB1 or HMGB2 proteins. Both low pH buffer (glycine-HCl, pH 2.5) and high pH buffer (triethylamine, pH 11.5) were equally effective in eluting antibodies (Figure 2F and 2G). In a whole cell extract, the purified HMGB1 antibodies only detected HMGB1, whereas the purified HMGB2 antibodies detected HMGB2 as well as another unidentified protein. These purified antibodies could be used for in vivo studies of HMGB1 and HMGB2.

**Binding of H1.0 and HMGB2 to chromatin**

Several studies have suggested an interplay between H1.0 and HMGB2. The addition of HMGB proteins is observed to increase H1 mobility within the nucleus (Catez et al., 2002), but this study did not test the direct binding interaction of the two proteins in chromatin. Another study shows HMG-D, a Drosophila homologue of HMGB, being displaced by H1 in chromatin (Ner et al., 2001). However, no study has tested the competition for nucleosome binding between the human HMGB proteins and H1. To test this, a competition assay was designed, in which one protein was present during the start
chromatin assembly and the second protein was added 30 minutes later (Figure 3A). Following micrococcal nuclease digestion, samples were subjected by non-denaturing gel electrophoresis. DNA was detected by ethidium bromide staining, and proteins were detected by western blotting.

The addition of HMGB2 and/or H1.0 to the reactions resulted in shifts in the mononucleosome and dinucleosome bands, suggesting that HMGB2 and/or H1.0 was bound (Figure 3B and 3C). Analysis by western blotting indicated that H1.0 bound to both the mononucleosome and dinucleosome (Figure 3D; lanes 3, 4, and 5), whereas HMGB2 only bound to the mononucleosome (Figure 3E; lanes 2, 3, and 4). When HMGB2 and H1.0 were allowed to compete for nucleosome binding, there was no change in the mononucleosome or dinucleosome shift, when compared to reactions containing only HMGB2 or H1.0 (Figure 3C to 3E), suggesting that either HMGB2 or H1.0 is bound. Furthermore, both HMGB2 and H1.0 were detected at these shifted bands. Together, these findings suggest that binding of HMGB2 and H1.0 to the nucleosome is mutually exclusive and that they may be displacing each other from the nucleosome.

**Binding of H1.0 and HMGN2 to chromatin**

There is conflicting evidence regarding the relationship between HMGN proteins and H1, particularly in regards to their interaction in chromatin. To investigate the potential competition between H1.0 and HMGN2 in binding to chromatin, competition assays were performed similarly to those for HMGB2 and H1.0. Both HMGN2 (figure 4A; lane 2) and H1.0 (figure 4A; lane 5) were able to bind to the nucleosome and create a distinct shift. Regardless of whether HMGN2 or H1.0 is added first to chromatin, there was an increased shift of the mononucleosome and dinucleosome, relative to reactions
containing only H1.0 or HMGN2 (Figure 4A; lanes 3 and 4). Analysis by western blotting is underway to confirm that both H1.0 and HMGN are present at these nucleosomes.
Figure 1. Active H1.0 and HMGB2

A. Samples of eluted proteins were taken after 15S chromatography and run on an SDS-PAGE. (CL) indicates cleared lysate, (FT) indicates flowthrough, (M) Molecular weight marker, 123 bp ladder (Invitrogen).

B. Chromatin assembly reactions were carried out in the absence of presence (left lane) of two different amounts of H1.0 (middle and right lane). Each set of reactions were digested with two different concentrations of micrococcal nuclease. The star corresponds to the range of dinucleosomes; circle, range of trinucleosomes.

C. HMGB2 was added in increasing amounts to a constant amount of radiolabeled four way junction DNA and subjected to non-denaturing gel electrophoresis where binding activity was determined. (-) indicates no HMGB2 added to the DNA resulting in only free probe.
A. Coomassie stained SDS-Page

B. Micrococcal Nuclease Digestion Assay

C. Electrophoretic Mobility Shift Assay
Figure 2. Generation of antibodies specific against HMGB proteins
A. Schematic of HMGB1 and HMGB2’s C-terminal region from amino acids 166 – 215 and 166 – 209, respectively were cloned into pGEX-6P1 (top panel). The stars indicate the restriction enzymes that were used. The boxed C-terminal region of the protein alignment indicates main differences between HMGB1 and HMGB2 (bottom panel).
B–C. Coomassie stained SDS Page of GST-HMGB1 (B) and GST-HMGB2 fusion protein (C) purifications using glutathione sepharose chromatography
D–E. Serum was tested against hela whole cell extract, full length recombinant HMGB1, and full length recombinant HMGB2 protein to determine specificity of antibodies against their cognates. Rabbit #4900 was injected with the GST-B1 fusion protein (D) while #4940 was injected with the GST-B2 fusion protein (E).
F. Affinity purification of HMGB1 serum. All lanes were loaded with Hela whole cell extract and blotted with antibodies eluted from column. Antibodies were eluted using Glycine-HCl pH 2.5. (W) indicates a 10 mM Tris, pH 7.9 wash. (W2) indicates a 10mM Tris, pH 7.9, 50 mM NaCl wash.
G. Affinity purification of HMGB2 serum. All lanes were loaded with Hela whole cell extract and blotted with antibodies eluted from column. Antibodies were eluted using one of two methods: elution by glycine pH 2.5 or triethylamine pH 11.5
A.

![Diagram](image)

B.

![Image](image)

C.

![Image](image)
Figure 2 continued. Generation of antibodies specific against HMGB proteins
Figure 2 continued.
Figure 3. HMGB2 and H1.0 bind at the mononucleosome
A. Schematic of incorporation of HMGB2 and H1.0 during chromatin assembly. Either (protein 1) HMGB2 or H1.0 is added along with the core histones and Nap1 prior to assembly. Once 30 minutes of assembly has passed, either no protein is added or the second protein is added and the reaction goes on for another 30 minutes.
B. Non-denaturing gel stained with ethidium bromide, in which a possible of combinations with HMGB2 and H1.0 were added either at the start of chromatin assembly or 30 minutes into assembly.
C. Western blot of non-denaturing gel. Polyclonal antibodies against H2A-H2B were used to determine the position of the nucleosomes.
D. Western blot of non-denaturing gel. Polyclonal antibodies against Flag were used to determine the position of Flag-H1.0.
E. Western blot of non-denaturing gel. Polyclonal antibodies against HMGB2 were used to determine the position of HMGB2.
**Figure 4. HMGN2 and H1.0 bind at the mono- and dinucleosome**
Non-denaturing gel stained with ethidium bromide, in which a possible of combinations of HMGN2 and H1.0 were added either at the start of chromatin assembly or 30 minutes into assembly.
Protein 1 - HMGN2 H1.0 HMGN2 H1.0
Protein 2 - - HMGN2 H1.0 -

- Shifted Dinucleosome
- Dinucleosome
- Shifted Mononucleosome
- Mononucleosome

*Ethidium Bromide Stained*
III.

Discussion
Several studies suggest interplay among HMG proteins and the linker histone H1 (Catez et al., 2002; Ding et al., 1997; Hill and Reeves, 1997; Ner and Travers, 1994; Nightingale et al., 1996). Gel shifts were performed in conjunction with western blots to determine whether this proposed interplay existed. The gel shifts were able to provide further supporting evidence that there is indeed interplay among these HMG proteins and H1.0.

HMGB2 and H1.0 appear to compete for binding to the mononucleosome. Regardless of the order of addition of each protein during chromatin assembly, the mononucleosome shift migrated the same distance as a mononucleosome with only H1.0. This suggests H1.0 may displace HMGB2 from binding at the nucleosome. However, western blots confirm the presence of both HMGB2 and H1.0 at the shifted mononucleosome. This may mean both HMGB2 and H1.0 are binding to the nucleosome, altering the conformation of the nucleosome so that it migrates the same distance as a H1.0 bound nucleosome. However, examples of chromatin that lack H1, but have HMGB proteins suggest a non-simultaneous binding of HMGB proteins and H1.0 (Ner and Travers, 1994; Jackson et al., 1979). From these studies, a more likely possibility is that they can displace each other at the nucleosome.

Further analysis is needed to understand this potential competition between HMGB2 and H1.0. If H1 does displace HMGB2 from chromatin, increased amounts of H1.0 would more effectively remove HMGB2 from the nucleosomes. Furthermore, additional improvements will be made to the experiments. Fluorescent antibodies could overcome the loss of antigen during membrane stripping and would allow for
simultaneous detection of several proteins. Generation of antibodies specific against H1.0 is also currently underway, as the Flag antibodies also detect ACF.

Analysis of the HMGN2 and H1.0 competition suggest that these two proteins can actually bind simultaneously to the nucleosome. However, confirmation of both HMGN2 and H1.0 at the nucleosomes has not been completed yet, due to loss of proteins during membrane stripping. The ability of both proteins binding simultaneously to the nucleosome would contradict a previous study, in which HMGN proteins were proposed to compete for H1.0 binding sites by an observed increase in H1 mobility within the nucleus when HMG proteins were added (Catez et al., 2002). However, this previous study was done in vivo and other factors may be involved that could facilitate the displacement of H1.0 to the nucleosome. Factors could be recruited at the presence of HMGN proteins at the nucleosome, but with a purified in vitro system, these factors do not come into play.

Genome Wide Localization of HMGB proteins

In this study, HMGB1 and HMGB2 specific antibodies have been successfully generated and purified. These antibodies will be useful in the study of the genome-wide localization of HMGB proteins. The genome-wide localization of these proteins in mammalian cells is not known, and a study on this will likely reveal crucial targets of the HMGB proteins possibly giving insight as to how these proteins play a role in cellular processes such as gene regulation.

Implicated roles of HMG proteins in transcriptional regulation

Although transcription is mainly regulated by chromatin-remodeling factors and gene specific transcription factors, it is thought that architectural proteins, HMGB and
HMGN, also play a regulatory and interactive role with linker histone H1. H1 has long been positively correlated with gene repression (Paranjape et al., 1994) while HMGB and HMGN have both been implicated in gene activation and repression.

Biochemical studies have implicated HMGB proteins as activators of transcription evidenced by an H1 to HMGB exchange at the nucleosome (Ju et al., 1996) and increasing the affinity of chromatin remodeling factors to the nucleosome (Bonaldi et al., 2002). An in vivo study demonstrates the overexpression of HMGB1 results in a less compact chromatin structure as well as the enhanced transcription of a reporter gene (Ogawa et al., 1995). However, HMGB proteins have also been implicated as repressors of transcription. One biochemical study shows DSP1, a *Drosophila* HMG homologue, as part of a transcriptional repressing complex (Lehming et al., 1998). In cultured cells, both HMGB1 and H1 are shown to bind to the silencing promoter of TNF-α, a proinflammatory gene (El Gazzar et al., 2009). Of these studies, only one examines HMGB proteins in the context of chromatin and only alludes to an activator role in transcription (Ju et al., 1996).

To determine the transcriptional role of HMGB2 in chromatin, HMGB2 effects will be analyzed on target genes that will have been identified using genome-wide sequencing. An increase in levels will suggest HMGB2 as an activator while a decrease would suggest a repressor. To determine whether HMGB2 addition can affect H1-mediated transcriptional repression, HMGB2 will be added to chromatin following H1 incorporation and transcription performed to determine whether there the levels of transcription change.
HMGN proteins have also been observed as activators and repressors of transcription (Bianchi and Agresti, 2005; Ding et al., 1994, Paranjape et al., 1995 or 1995). Biochemical studies have shown HMGN proteins to increase the rate of transcription (Ding et al., 1997; Paranjape et al., 1995) as well as repress transcription at the Sox9 gene (Furusawa et al., 2006). It would be insightful to further investigate whether HMGN proteins act more as regulators of transcription and whether there is potential interplay between HMGN2 and H1.0. This would be performed similarly to the HMGB study.

Further study of how HMG proteins and H1 interact with one another in chromatin dynamics may prove fundamental to understanding how chromatin structures arise and how they regulate the DNA-utilizing processes.
IV.

Materials and Methods
Expression and purification of recombinant human HMGB2

pET24HMGB2 was created by Samuel Pitak by cloning the HMGB2 sequence into the pET24 vector using the NcoI and NotI restriction sites. BL21-Rosetta cells (Novagen) were transformed according to manufacturer protocol (34 ug/mL chloramphenicol + 30 ug/mL kanamycin) with pET24HMGB2. Cultures were incubated at 37°C until OD$_{600}$ = 0.6 where induction of expression by 1mM isopropyl b-D-thiogalactopyranoside (IPTG), at 16°C for 16½ to 17 hours. Cells were resuspended in HMGB purification buffer (HPB) (10 mM HEPES (K$^+$) pH 7.6, 10 mM KCl, 0.1 mM EDTA, 10% Glycerol, 0.01% NP-40, 0.2 mM PMSF, 1 mM DTT, 10 mM β-glycerophosphate) containing 200 mM NaCl, lysed by sonication, and cleared by centrifugation in a Sorvall SS-34 rotor at 11,000 rpm at 4°C for 15 min. The cleared lysate was loaded onto Q Sepharose Fast Flow column (diameter = 1 cm; column volume = 10 mL), and proteins were eluted with a linear gradient of 200 mM to 800 mM NaCl in HPB over 20 column volumes. Fractions containing HMGB2 were dialyzed against HPB + 0M NaCl until conductivity was equivalent to HPB + 100 mM NaCl, and the sample was subjected to Source 15S chromatography (diameter=0.5 cm; column volume=2 mL). Protein was eluted with a linear gradient of 100 mM to 700 mM NaCl in HPB over 20 column volumes. HMGB2 typically elutes when conductivity is just above 10 ms/cm. Peak fractions were pooled and dialyzed against HPB + 200 mM NaCl, until sample conductivity was equivalent to buffer, and then subjected to Source 15Q chromatography (diameter=0.5 cm; volume=2mL). Proteins eluted with a linear gradient of 200mM to 1 M NaCl in HPB over 20 column volumes. Peak fractions with HMGB2 were pooled and
dialyzed against HMGB storage buffer (HSB) (10 mM Hepes (K⁺) pH 7.6, 50 mM KCl, 0.1 mM EDTA, 10% Glycerol, 0.01% NP-40, and 1mM DTT).

**HMGB2 binding assay**

The HMGB2 binding assay was based on a DNA binding assay, previously described (Bianchi, 1988). The following oligonucleotides were used in the construction of the four way junction as mentioned in the paper.

1: CCCTATAACCCCTGCATTGAATTCCAGTCTGATAA
2: GTAGTCGTGATAGGTGCAGGGGTTATAGGG
3: AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACTA
4: TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAAGAGCTACTGT

At 2.5 uM each, the four oligonucleotides were mixed together with 5x annealing buffer (5x TE, 100 mM KCl, and 2.5 mM MgCl₂) and heated to 85°C before being cooled to room temperature overnight. Each reaction composed of 4x binding buffer (40 mM K-Hepes, 40 mM KCl, 200 mM NaCl, 0.4 mM EDTA, 1 mg/mL BSA, 12% glycerol, 0.8 mM PMSF, and 4 mM DTT), varying amounts of HMGB2, and labeled fork DNA. Each reaction (20 uL) was incubated for 30 minutes on ice before subjected to a 6% non-denaturing polyacrylamide gel (30:0.8 acrylamide:bisacrylamide, 4% glycerol, 0.5x TBE) which was pre-run in 0.5x TBE for 30 minutes at 150V at 4°C. The gel was run in fresh 0.5x TBE for 3 hours at 150V. Bands were detected using phosphorimaging.

**Cloning, expression, and purification of GST-B1 and GST-B2 peptides**

Overlap extension PCR was performed to amplify the sequences for amino acids 166-215 in HMGB1 and 166-209 in HMGB2 and also to include restriction enzymes BamHI and XhoI. The following oligonucleotides were used:
BamHI (B1): CAGGGGCCCTGGGATCCGGAAAGCCTGATGCAGCAA
XhoI (B1): ACGATGCGCCGCTCGAGCTATTATTCATCATCATTCTTCTTC
BamHI (B2): CAGGGGCCCTGGGATCCGGCAAAAGTGAAGCAGGAAA
XhoI (B2): ACGATGCGCCGCTCGAGCTATTCTTCATCTTCATCCTCTTCC

These amplified sequences were inserted and ligated into the pGEX-6P-1 vector (GE) at the BamHI and XhoI sites. Plasmids were renamed pGST-B1pep, which expresses the C-terminal domain of HMGB1 fused to a GST tag and pGST-B2pep, which expresses the C-terminal domain of HMGB2 fused to a GST tag. Expression of these proteins follows the same procedure as for HMGB2 expression. Proteins were resuspended in GST wash buffer (GWB) (1x PBS, pH 7.3, 10% glycerol, 1mM DTT, 0.2mM PMSF, and 10 mM β-glycerophosphate), lysed by sonication, and centrifugation in a Sorvall SS-34 rotor at 11,000 rpm for 15 min. The cleared lysate was added to Glutathione Sepharose 4B resin and incubated for 1.5 hours at 4°C with rotation and the resin slurry was loaded onto an Econo-pac column (20mL). The resin was washed with 25 mL of GWB four times and the proteins were eluted with 10 mL of GST elution buffer (50mM Tris-HCl, pH 7.9, 2.29uM reduced glutathione, 10% glycerol, 0.2 mM PMSF, 1 mM DTT, 10 mM β-glycerophosphate). Proteins were dialyzed for an hour at 4°C in HSB.

**Generation and purification of antibodies against HMGB1 and HMGB2**

The generation of polyclonal antibodies was done by Covance immunological services using purified GST-B1 and GST-B2 peptides. Antibodies were affinity purified using CNBr-Sepharose activated beads coupled to recombinant full length HMGB1 or HMGB2. To 0.5 mL of CnBr-Sepharose, 0.5 mg of protein was coupled and the resin was washed with 0.5 mg of BSA for 1 hour with rotation. Antiserum was incubated with
the resin for 10 minutes at room temperature with rotation, and the resin slurry was loaded onto an Econo-tube column (20mL). The resin was washed with 20 bed volumes of 10mM Tris, pH 7.9, followed by 20 bed volumes of 500 mM NaCl, 10 mM Tris, pH 7.9. Antibodies were eluted with 2 mL of either 100 mM glycine-HCl, pH 2.5 or 100mM triethylamine, pH 11.5, and 2M Tris, pH 7.9 was added to neutralize the eluents. Eluted antibodies were analyzed by western blotting.

**Expression and purification of Flag-H1.0**

Rosetta (DE3) (Novagen) were transformed according to manufacturer protocol (34 ug/mL chloramphenicol) with pET11d-FlagH1.0, which was given as a gift from Woojin An. Culture were grown at 37°C until the O.D. at 600 nm was approximately 0.6. Expression of H1.0 was induced by IPTG (1mM) for 1 hr at 37°C. The purification of H1.0 was based on the purification of native H1, as previously described (Croston et al., 1991). Cells were resuspended in lysis buffer [(20 mL/500 mL pellet) 500mM NaCl, 20mM Tris, pH 7.9, 0.2mM EDTA, 10% glycerol, 0.2mM PMSF, 10mM β-mercaptoethanol, 1 ug/uL pepstatin, 1 ug/uL leupeptin, 1 ug/uL aprotinin], sonicated, and cleared by centrifugation at 11,000 rpm at 4°C. Pulverized ammonium sulfate was added to 2.1M and insoluble proteins were pelleted by centrifugation in a Sorvall SS-34 rotor at 15,000 rpm at 4°C. The cleared lysate was subjected to Phenyl Sepharose CL-4B chromatography (diameter=1.6 cm; column volume, 20 mL) and H1.0 was eluted with a linear gradient of 2.1 to 0.1M ammonium sulfate in HEMG (25 mM Hepes pH 7.6, 12.5 mM MgCl2, 0.1 mM EDTA, 10% Glycerol, 1 mM DTT, 0.5 mM PMSF) over 7.5 column volumes. Flag-H1.0 fractions were dialyzed against 100 mM KCl in HEMG until conductivity was equivalent to 150 mM KCl in HEMG. Nonidet P-40 (1/1000 volume of
35

a 10% v/v, solution) was added to the final concentration of 0.01% (v/v) and then diluted
to 100 mM KCl in HEMG where it was loaded onto a Source 15S column (diameter, 0.5
cm; volume, 1 mL) Proteins were dialyzed into 100 mM KCl in HEMG.

Chromatin Assembly Protein Purification

Bacterially-synthesized D. melanogaster His6-tagged NAP1 ('NAP1') was
purified essentially as described (Lusser et al., 2005), except that a P11 phosphocellulose
(Whatman) cation exchange chromatography step was added prior to the final Source
15Q (GE Healthcare) anion exchange chromatography. D. melanogaster ACF and
topoisomerase I (ND423 N-terminally truncated form containing the catalytic domain)
were purified as described (Fyodorov and Kadonaga, 2003). Native D. melanogaster core
histones were purified from embryos (Fyodorov and Levenstein, 2002).

Chromatin Assembly

Chromatin assembly reactions were performed as previously described (Fyodorov
and Kadonaga, 2003) except that 5% polyethylene glycol/5% polyvinyl alcohol was
excluded and 16 molecules of HMGB2 per nucleosome and/or 0.85 molecules of H1.0
per nucleosome were added either prior to assembly or after 30 minutes of assembly.
Standard reactions contained relaxed circular plasmid DNA (0.353 ug, cesium chloride
prepped pGIE-0), core histones (0.35 µg), NAP1 (1.4 µg), ACF (10 nM), ATP (3 mM),
and topoisomerase I (1 nM) in a final volume of 70 µL. The buffer composition of the
final reaction mixture was as follows: 14.5 mM Hepes (K⁺), pH 7.6, 3 mM Tris, 100 mM
KCl, 5 mM NaCl, 5.5 mM MgCl2, 0.1 mM EDTA, 6.3% (v/v) glycerol, 0.01 % NP-40,
1mM DTT, 20 µg/mL bovine serum albumin, and 20 µg/mL human insulin. Reactions
with the addition of HMGB2 and/or H1.0 contained varied slightly from the standard
reaction (0.441 ug, pGIE-0; buffer composition 14 mM Hepes (K\(^+\)), pH 7.6, 6.2% glycerol). The reaction products were analyzed by DNA supercoiling (Fyodorov and Kadonaga, 2003) and electrophoretic mobility gel shifts assays.

**Non-denaturing gel electrophoresis**

Assembled chromatin was digested with micrococcal nuclease. Reactions contained chromatin (32 uL of a chromatin assembly reaction, CaCl\(_2\) (1.86 mM), and micrococcal nuclease (0.025 – 0.033 units) in a total volume of 43 uL. Digests were carried out for exactly 10 minutes and stopped by addition of EDTA to 60.5 mM (5.2 uL of 0.5M EDTA). Reaction products (20 uL) were loaded onto a 7% Native Page [(30:1) Acrylamide: Bisacrylamide, 0.5x TBE, 4% glycerol], which was pre-run in 0.5x TBE for 1 hour at 50V at 4°C. The gel was run in fresh 0.5x TBE for 16.5 hours at 50V at 4°C.

**Western Blots**

Prior to transfer, the non-denaturing gel was soaked for 30 minutes in 50 mL transfer buffer (47.9 mM Tris, 38.6 mM Glycine, 0.037% SDS, 20% methanol) and proteins were transferred to a polyvinylidene fluoride membrane. Following transfer, the membrane blocked to 5% milk in TBST. Primary and secondary (donkey anti-rabbit HRP; JacksonImmuno) antibody incubations were performed in 1% milk in TBST, and detection of antibodies was done with the ECL Plus Western Blotting Detection System (GE). To strip the blots, membranes were incubated for 30 min at 50°C in stripping buffer (100 mM \(\beta\)-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.8) with gentle agitation, followed by washes with TBST. Membranes were stored in TBST at 4°C following stripping.
References


Ner SS, Travers AA. (1994) HMG-D, the Drosophila melanogaster homologue of HMG 1 protein, is associated with early embryonic chromatin in the absence of histone H1. EMBO J. 13, 1817-1822.


