DNA supercoiling with a twist

Edwin Kamau

Louisiana State University and Agricultural and Mechanical College, ekamau1@lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/999

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
DNA SUPERCOILING WITH A TWIST

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

By
Edwin Kamau
B.S. Horticulture, Egerton University, Kenya, 1996,
May, 2005
DEDICATIONS

I would like to dedicate this dissertation to my son, Ashford Kimani Kamau. Your age marks the life of my graduate school career. I have been a weekend dad, broke and with enormous financial responsibilities; it kills me to see how sometimes you cry your heart out on Sunday evening when our weekend time together is over. I miss you too every moment, you have encouraged me to be a better person, a better dad so that I can make you proud. At your tender age, I have learned a lot from you. We will soon spend all the time in the world we need together; travel, sports, music, pre-historic discoveries and adventures; the road is wide open. I will be there for you to guide you through unwavering tribulations and challenges of life and more important, I know we will make it, just the two of us, for if God is on our side, no one can be against us.
ACKNOWLEDGEMENTS

A journey of a thousand miles begins with a single step. At my first step, Dr. Anne Grove and Dr. John W. Fleeger were there for me, and with their support and kindness, I was able to transfer to the department of Biological sciences once I realized that what good is running when you're on the wrong road. To Dr. Anne Grove, thanks for your guidance, patience and dedication to my scientific nurturing and growth. To my wonderful and unconditionally dedicated committee members, Dr. David Donze, Dr. Patrick J. DiMario, Dr. Grove Waldrop and Dr. Graca Vicente, thank you for insightful discussions for completion of my research. Special thanks to Dr. David Donze and Dr. Patrick J. DiMario, I know I have taken plenty of your precious time and resources. To my lab mates; protein purification is like a frozen tear drop, you can never know its implication until you experience it. Kevin B, special thanks to you Mr. HMO. To Javel Bradley, I know Ashford is not easy. To my family, mum and dad, I have been away from home for a decade; I hope to see you on my graduation. To my brothers and sisters, I hope we will spend more holidays together; I have been the missing link. To my new career and new family the US army, I promise to serve this great country selflessly; the best is yet to come. To my friends, thanks. John J, thank you for your support and comic relieves, you life is a movie; I will consult a Hollywood director about that. And finally to my Fiancée, I know that it is not easy being more that we are more 10,000 miles apart, but we both know that good things happen to those who wait. Thanks for your support and unconditional love, I will love you always, Dec 17th 2005 is coming!
TABLE OF CONTENTS

DEDICATIONS..............................................................................................................ii

ACKNOWLEDGEMENTS...........................................................................................iii

LIST OF TABLES........................................................................................................vi

LIST OF FIGURES.....................................................................................................vii

ABSTRACT...................................................................................................................ix

CHAPTER 1. INTRODUCTION......................................................................................1
High-Mobility-Group (HMG) Proteins.................................................................1
Classification of HMGB Proteins.................................................................2
Structure of HMGB Proteins........................................................................3
Binding Targets of HMG Boxes.................................................................3
Determinants of Sequence-Specific Versus Non-Sequence Specific HMGB Proteins..4
The Role of HMG Box Basic Extensions......................................................6
HMGB1/2 Acidic C Terminal Tail...............................................................8
The Diverse Roles of HMGB1 in vivo.......................................................9
Extracellular HMGB1 Signals to Multiple Cellular Targets.................9
Regulated Expression and Subcellular Localization of HMGB1.............10
S. cerevisiae HMG-box Proteins.............................................................11
DNA Supercoiling by Vaccinia Topoisomerase I.......................................13
Classification of Topoisomerases.............................................................14
Cellular Roles of DNA Topoisomerases..................................................15
General Features of Type IA DNA Topoisomerase I...............................16
General Features of Type IB DNA Topoisomerase.................................17
Fluoroquinolone Compounds.................................................................18
References.........................................................................................................20

CHAPTER 2. THE SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 CONTAINS TWO FUNCTIONAL DNA BINDING DOMAINS
Introduction......................................................................................................27
Experimental Procedures.............................................................................30
Results.............................................................................................................37
Discussion.......................................................................................................51
References.......................................................................................................54

CHAPTER 3. A ROLE FOR THE SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 BEYOND DNA BINDING
Introduction....................................................................................................57
Experimental Procedures.............................................................................60
Results…………………………………………………………………………..66
Discussion……………………………………………………………………..69
References……………………………………………………………………..73

CHAPTER 4 PHOSPHORYLATION OF SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 BY CASEIN KINASE II AND THE ROLE OF THE C-TERMINAL DOMAIN IN DNA BINDING AND PROTEIN STABILITY
Introduction……………………………………………………………………...76
Experimental Procedures………………………………………………………79
Results………………………………………………………………………….83
Discussion……………………………………………………………………….91
References……………………………………………………………………….98

CHAPTER 5. FLUOROQUINOLONE-DEPENDENT DNA SUPERCOILING BY VACCINIA TOPOISOMERASE I
Introduction……………………………………………………………………...101
Experimental Procedures……………………………………………………103
Results…………………………………………………………………………107
Discussion………………………………………………………………………117
References……………………………………………………………………..120

CHAPTER 6. SUMMARY AND CONCLUSIONS
HMO1 Interaction with DNA…………………………………………………..123
Phosphorylation of HMO1……………………………………………………125
HMO1 Thermal Stability……………………………………………………125
HMO1 Cellular Localization………………………………………………..126
Vaccinia Topoisomerase I Supercoils DNA in the Presence of Fluoroquinolones……127
References……………………………………………………………………..127

APPENDIX..................................................................................129

VITA.............................................................................................131
LIST OF TABLES

Table 3.1 Yeast strains used in the experiments.................................61
Table 3.2 List of primers used.............................................................62
Table 3.3 Tetrad analysis.................................................................68
LIST OF FIGURES

Figure 1.1 Alignment of HMG-box helices I and II.........................................................5
Figure 1.2 Cocrystal structure of NHP6A and SRY with DNA........................................7
Figure 2.1 Alignment of HMG-box helices I and II.........................................................28
Figure 2.2 HMO1 and HMO1-BoxA have significant α-helical content.........................38
Figure 2.3 Electrophoretic analysis of 26 bp DNA titrated with HMO1.......................39
Figure 2.4 Binding of HMO1 and B. subtilis HU to 4-way junctions and linear duplex DNA..........................................................40
Figure 2.5 HMO1-BoxA binds DNA.............................................................................42
Figure 2.6 HMO1 has only modest preference for 4-way junction DNA..................44
Figure 2.7 Binding of HMO1 to supercoiled, relaxed and linear DNA..................46
Figure 2.8 (A) Binding of HMO1 to linear and circular 105 bp DNA..........................47
Figure 2.9 (A) Time-course ligation assay with 87 bp DNA......................................48
Figure 2.10 HMO1 does not supercoil relaxed DNA..................................................50
Figure 3.1 Strategy for generation of HMO1 or HMO2 knockout..........................63
Figure 3.2 Strategy for generation of HMO1-GFP or HMO1-boxAB-GFP fusion protein.................................................................65
Figure 3.3 Phenotype of various mutants from a tetrad dissection.........................67
Figure 3.4 In vivo localization of HMO1-GFP and HMO1-boxAB-GFP proteins........70
Figure 4.1 Thermal denaturation of HMO1 (A), HMO1-boxAB (B), HMO1-boxA......84
Figure 4.2 Binding of HMO1 (panel A) and HMO1-boxAB (panel B) to 4-way junction DNA.............................................................................86
Figure 4.3 Time-course ligation assay with 105 bp DNA..........................................88
Figure 4.4 (A) Phosphorylation of HMO1..................................................................90
Figure 4.5 Protein cross-linking ..............................................................................92
Figure 4.6 Digestion of HMO1 and HMO1-boxAB with BNPS-skatole………………..93
Figure 5.1 Structure of fluoroquinolones…………………………………………….....104
Figure 5.2 Inhibition of DNA relaxation by enrofloxacin.................................108
Figure 5.3 Supercoiling of relaxed DNA in the presence of enrofloxacin..............110
Figure 5.4 Structural specificity of fluoroquinolones........................................112
Figure 5.5. Suicide substrate assay.................................................................114
Figure 5.6 Enrofloxacin does not modulate DNA topology.............................116
The level of torsion in double-stranded DNA regulates base-pair stability and DNA conformation. It is important in initiation and regulation of specific DNA metabolic processes as well as chromatin assembly. High mobility group proteins (HMGB) are architectural proteins whose HMG DNA binding domains confer significant preference for distorted DNA, such as supercoiled DNA and 4-way junctions. HMGB proteins play a role in transiently regulating or conserving DNA torsion. Topoisomerases regulate DNA supercoiling, which has been argued to provide a coherent explanation for the main modes of transcriptional control - stringent control, growth-rate control and growth-phase control during normal cell growth.

In this study, we have shown that HMO1, a *Saccharomyces cerevisiae* HMGB protein which is required for normal growth, plasmid maintenance and regulating the susceptibility of yeast chromatin to nuclease, binds linear duplex DNA but has little preference for DNA with altered conformations. The divergent box A binds DNA and contributes structure-specific binding. Unlike most HMGB proteins, HMO1 does not supercoil relaxed DNA in the presence of topoisomerase. Casein Kinase II phosphorylates HMO1, altering its DNA binding properties. We have also shown that deletion of the highly basic C-terminal tail of HMO1 localizes this otherwise nuclear and cytoplasmic protein only to the cytoplasm. As the C-terminally truncated HMO1 has been reported to rescue the *hmo1* knockout phenotype, we conclude that the main function of HMO1 lies in the cytoplasm, and not in the nucleus.

Vaccinia topoisomerase I relaxes supercoiled DNA. We have shown that it interacts with enrofloxacin, a fluoroquinolone antibiotic which otherwise targets DNA
gyrase and topoisomerase IV. Enrofloxacin inhibits DNA relaxation by Vaccinia topoisomerase I. When presented with relaxed DNA, the enzyme executes the reverse reaction, supercoiling the DNA. Enrofloxacin does not interfere with the catalytic cleavage site of Vaccinia topoisomerase I or its ability to bind DNA. The mechanistic implication of these observations is that protein-DNA contacts downstream of the cleavage site must contribute to DNA supercoiling, contrary to the free rotation mechanism proposed for DNA relaxation.
CHAPTER 1
INTRODUCTION

DNA topology is critical in cellular processes such as replication, transcription, recombination, chromosome condensation and much more. DNA topology must therefore be fine-tuned to allow these critical processes to optimally proceed as deemed. High Mobility Group proteins (HMG) are DNA architectural proteins that have been shown to participate in these processes and have been shown to introduce supercoils into closed circular DNA in the presence of topoisomerase. Topoisomerases are proteins that remove/regulate DNA supercoiling, playing a critical role in the above mentioned cellular processes. In fact, drugs that interfere with the function of topoisomerases are in wide use both clinically and in research. We are describing HMO1, a *Saccharomyces cerevisiae* HMG protein that differs in its activity from previously characterized homologs in that it does not supercoil DNA. We also show unusual properties of Vaccinia topoisomerase I; this enzyme interacts with a fluoroquinolone drug enrofloxacin, which causes it to execute the reverse reaction, supercoiling DNA when presented with closed circular DNA, as opposed to relaxing supercoiled DNA.

High-Mobility-Group (HMG) Proteins

HMG are a family of ubiquitous proteins that constitute a significant fraction of non-histone nuclear proteins. They were first extracted from nuclei with 0.35 M NaCl by the British scientist H. M. Goodwin, and the name indicates that they are small proteins that run fast in SDS-polyacrylamide gels [1]. They consist of three subfamilies: HMGA, HMGB, and HMGN. HMG proteins are united by a common theme in that they alter the local conformation of DNA or nucleosomes, thus increasing their accessibility and
plasticity, and facilitating and enhancing various DNA-dependent activities such as transcription, replication, recombination and DNA repair [2-5]. The three subfamilies have a characteristic motif, such as the HMG-box in HMGBs, the AT-hook in HMGAs, and the nucleosomal binding domain in HMGNs. My dissertation will focus on the HMGB group of proteins to which HMO1, the protein of my dissertation research, belongs.

**Classification of HMGB Proteins**

HMGB proteins contain one or more homologous repeats of the ~80-amino acid sequence HMG box and are classified into two families based on their abundance, function and DNA specificity of this conserved region [2, 6-7]. The moderately sequence-specific family are transcription factors that usually contain a single HMG box and recognize dsDNA. They are typified by transcription factors such as sex-determining factor SRY and lymphoid enhancer factor LEF-1 [8, 9]. The non-sequence specific family binds DNA without sequence preference but instead structural preference. They are represented by the so-called architectural factors HMGB1/2, ubiquitous proteins in vertebrates, and the *Saccharomyces cerevisiae* non-histone chromosomal protein 6A and 6B (NHP6A/B) [10]. HMGB1/2 have two HMG boxes (A and B) and a long acidic tail containing aspartic or glutamic acid residues, linked to the HMG boxes by a region of basic residues. Most HMG-box proteins in organisms other than vertebrates have only a single HMG box which sometimes is accompanied by an acidic tail (e.g., HMG-D, HMG-Z [11,12]), but not always (e.g., NHP6A/B, [13]). NHP6A/B contains a highly basic amino acid region in front of the HMG box that has been shown to be important for elevated DNA binding affinity [14].
**Structure of HMGB Proteins**

Several structures of HMG boxes from both the non-sequence specific subfamily and the sequence specific subfamily of proteins have been determined in the presence and/or absence of DNA. Non-sequence specific HMG boxes for which structures have been determined in the absence of DNA include HMGB1 box A [15], HMGB1 box B [16, 17], HMG-D [18] and NHP6A [19]. Masse *et al.*, later determined the structure of NHP6A in the presence of DNA [20]. The structure of SOX4, a sequence specific HMG box protein was determined in the absence of DNA [21], while the structures of two sequence specific HMG proteins, SRY and LEF-1 were determined in the presence of their cognate DNA recognition sequence [22, 23].

The structures of both sequence specific and non-sequence specific homologs have been determined to have the same general global fold, characterized by a 3-dimensional, L-shaped structure with three α-helices (reviewed in [4, 24-25]). The long arm consists of the amino-terminal extended peptide and helix III, while the shorter arm comprises two central helices I and II. A hydrophobic core of conserved aromatic residues from the three α-helices is maintained at the apex of the L-shaped structure. This region has the most highly conserved amino acid residues in the HMG boxes. Outside the hydrophobic core, the conservation scores decline, getting lower gradually with procession toward the end of either arm of the protein. This trend probably reflects the relative importance of the various regions of the protein for adopting the HMGB fold.

**Binding Targets of HMG Boxes**

The structure of the sequence specific proteins show the DNA to be greatly distorted in the region of protein contact, with SRY introducing a bend of 80° [23] while
LEF-1 introduces a $120^\circ$ bend [9]. The HMG box binds to the minor groove of B-form DNA with its concave face and causes bending by partial insertion of hydrophobic residues close to the amino-terminus of helix I into the minor groove of the DNA. As a consequence, the DNA is severely underwound and widened, thus increasing the protein-DNA interface. The DNA is kinked towards the opposing major groove which is thereby highly compressed. Hydrophobic interactions as well as hydrogen bonds between the proteins and the DNA sequence mediate the specificity of this class of HMGB protein [20].

**Determinants of Sequence-Specific Versus Non-Sequence Specific HMGB Proteins**

Detailed structures of the non-sequence specific proteins have reiterated the main features of interaction but also reveal interesting and important differences. Extensive phylogenetic analysis by Masse et al., revealed determinants of sequence-specific versus non-sequence specific HMGB proteins [20]. Using NHP6A as a common reference frame, these authors noted that the residue in position 57 shows the most statistically significant correlation to DNA recognition type (Figure 1.1). For non-sequence specific HMGB proteins, this position is almost always negatively charged and most likely to be involved in stabilizing the fold of helix 2, as it is often surrounded by positively charged residues. Conversely, a variety of residues are present in this position in sequence specific HMGB proteins. Other negatively charged residues in helix 1 and helix 3 show correlation with non-sequence specificity, but not as significant as the residue in position 57. These authors speculated that these negative charges may be involved in restricting the path of the DNA on the protein through electrostatic repulsion, a function that sequence specific DNA-binding proteins can achieve by forming sequence specific
Figure 1.1 Alignment of HMG-box helices I and II. For non-sequence specific proteins, *S. cerevisiae* NHP6A along with box A of rat HMGB1, *Drosophila melanogaster* HMGD and box B of *S. cerevisiae* HMO1. Sequence specific proteins are mouse LEF-1, human SRY, and *Xenopus laevis* UBF box A. Residues corresponding to positions 29, 48 and 57 of NHP6A helices I and II, respectively, are in bold.
hydrogen bonds with DNA. Another important position that correlates to specificity of
the HMGB proteins is 48 which is almost always a bulky hydrophobic residue in non-
sequence specific proteins. In the structure of NHP6A and HMG-D, it has been shown
that it contributes to DNA bending by re-directing the trajectory of the DNA as it exits
the protein [20, 22]. This exit wedge has also been shown to be the critical determinan
t for selective binding by both HMGB1 Box A and NHP6A to pre-bent DNA resulting
from cisplatin modification or a bulge. In sequence specific proteins, this position usually
has a hydrophilic, often charged residue which makes specific hydrogen bonds to the
cognate DNA. These specific hydrogen bonds within the minor groove are believed to
direct the sequence specific HMGB proteins to specific DNA targets. The means by
which non-sequence specific HMGB proteins pick their B-DNA target seems different
from sequence specific DNA binding proteins. Looking at NHP6A and HMG-D
structures, it appears that Met29 is of primary importance for selecting a deformable
base-pair step in B-DNA. Clear and striking evidence of the difference in modes of
recognition between sequence specific and non- sequence specific homologs is seen
when the structures of NHP6A-DNA and SRY-DNA is compared (Figure 1.2). The two
proteins bind to the related DNA sequence in opposite orientations and 1 bp step shifted
[20, 23].

The Role of HMG Box Basic Extensions

Basic extensions found either C- or N-terminal to the HMG box are known to
increase the bending capacity and DNA-binding affinity of the HMG box. For example,
the 16 basic amino acids found in the N-terminus of the NHP6A HMG box is required for
stable complex formation on both linear DNA and DNA minicircles. At high
Figure 1.2 Cocrystal structure of NHP6A and SRY with DNA. Comparison of solution structures of NHP6A-DNA$^{\text{Sry}}$ and SRY-DNA (Protein data bank (PDB) accession numbers 1LWM and 1HRZ respectively) [20, 23]. DNA is shown in backbone model to clearly demonstrate that the two proteins (shown in ribbon form) bind related DNA sequence in opposite orientations.
concentrations, mutants lacking the N-terminus are able to promote minicircle formation and Hin invertasome assembly, but they are unable to form stable complexes with DNA, co-activate transcription, and complement the growth defect of Δnhp6a/b mutants [14]. Basic C-terminal extensions stabilize binding of HMG-D and LEF-1 by binding in the major groove opposite the distorted minor groove [22, 26].

**HMGB1/2 Acidic C Terminal Tail**

The HMGB1/2 acidic tail has been shown to lower the affinity of the HMG boxes for most DNA substrates, such as linear DNA, supercoiled DNA and four-way junctions. Interestingly, the tail is essential for structure-selective DNA-binding of the HMG boxes to DNA minicircles, and it has little effect on the affinity for DNA minicircles compared to other DNA substrates in vitro [27]. However, it was not established whether this is a consequence of interaction of the tail with the HMG box(es) or the basic extension region or simply charge repulsion. Thomsen et al. [28] showed that the interactions of the basic N-terminal and acidic C-terminal domains of the maize HMGB1 may contribute to the regulation of the DNA interactions of the protein. A similar phenomenon was also observed for the *Arabidopsis* HMGB1 and HMGB4 [28]. This led the authors to argue that the interaction of the variable terminal domains of the different plant HMGB proteins could serve as a molecular mechanism involved in the fine tuning of the functional properties of these architectural proteins depending on the structural requirements of specific nucleoprotein complexes.

Shirakawa *et al.* showed that the acidic tail may be involved in nuclear retention of HMGB2 [29]. In cells overexpressing HMGB1, the protein was shown to bind to a minichromosome derived from the transfected reporter plasmid, substituting for linker
histone H1, and to stimulate transcription from the reporter plasmid accompanied by decondensation of the minichromosome structure [30]. In addition, the acidic tail is required for stimulation of transcription [31] and it has recently also been demonstrated to stimulate nucleosome sliding by the chromatin remodeling factor ACF/CHRAC [32]. This study also showed that the HMGB1 acidic tail is a dominant factor involved in its binding to the nucleosome and is required for binding to nucleosome linker DNA.

**The Diverse Roles of HMGB1 in vivo**

HMGB1 protein has been shown to interact with a diverse set of proteins. It plays an important role in transcription by interacting with several transcription factors, viral replication proteins, the RAG1 recombinase, and steroid receptors [33]. *Hmgb1* knockout mice die soon after birth with defects in the function of steroid receptors revealing that the gene is essential [34]. HMGB1 is the most mobile nuclear protein [35]. Manfredi and Bianchi (review in [36]) referred to HMGB1 as a chromatin chaperone that uses no energy besides Brownian motion, because it wanders where it is required within reasonable time, does its job and then leaves.

**Extracellular HMGB1 Signals to Multiple Cellular Targets**

Although HMGB1 was considered to be a nuclear protein, biochemical and immunological early studies performed by Bustin *et al.* suggested that its intracellular distribution is not restricted to the nucleus and that in certain cells, HMGB1 was also found in the cytoplasm [37]. It plays an important role in regulating cell migration, inflammation and metastasis. Wang *et al.* identified HMGB1 as a late mediator of endotoxin lethality in mice. These authors showed that monocytes/macrophages stimulated by lipopolysaccharide (LPS), tumour necrosis factor (TNF) or interleukin-1
(IL-1) secreted HMGB1 as a delayed response. In mice, injection of HMGB1 caused toxic shock whereas administration of anti-HMGB1 antibodies attenuated LPS-induced endotoxemia [38]. Sepsis is a systemic inflammatory response to bacterial products that can lead to multiple organ failure and death. Septic patients have shown increased serum level of HMGB1, which correlates with the severity of the infection. HMGB1 has also been shown to cause acute lung inflammation when administered intratracheally. Antibodies against HMGB1 decreased lung edema and neutrophile migration, whereas they did not reduce the levels of the other proinflammatory cytokines TNF-α, IL-1β or macrophage inflammatory protein-2 (MIP2). HMGB1 also participates in the regulation of neuroendocrine and immune responses to inflammatory processes [39]. Extracellular HMGB1 protein interacts with the extracellular matrix and the surrounding tissues and with several cell surface receptors, including the receptor for advanced glycation end products (RAGE) [40]. Necrosis is organ deterioration, whereas apoptosis is important for organ maintenance and differentiation. HMGB1 serves as a signal that determines whether cell death and injury will lead to necrosis or continue along a preintiated apoptotic pathway [35].

**Regulated Expression and Subcellular Localization of HMGB1**

In transformed cell lines, HMGB1 is highly expressed at $10^6$ molecules per cell, which is 10 times less than histones. However, in intact organs, its expression is developmentally regulated and/or responds to cues from the environment displaying an amazing variation in expression pattern, level of expression, and subcellular location. The *HMGB1* gene is transcriptionally controlled by steroid hormones, using various signaling pathways including the cytokine-activated JAK/STAT pathway [41]. It is also likely that
other expression control, such as post-transcriptional controls of mRNA stability or translation, and protein stability exist. Studies by Mosevitsky et al. looked at distribution of HMGB1 in different mammalian tissues and found that lymphoid tissues and testis had high HMGB1 levels in both nuclei and cytoplasm whereas brain and liver had low levels, mainly in the cytosol [42]. These authors suggested that [HMGB1] is low in differentiated cells, and high in undifferentiated cells. In intact mice, HMGB1 expression is tightly regulated in the central nervous system [43, 44, 45]. In tumour cells, HMGB1 protein is found in higher level than in normal tissue of counterpart cells [46, 47]. HMGB2 and HMGB3, which are structurally similar to HMGB1, have similar biochemical properties, but have a different expression pattern. These proteins are highly expressed in the embryo, and both are expressed at very low levels in the adults [48, 49]. Hmgb2−/− mice are viable, and only show a marked reduction in spermatogenesis [41]. Hmgb3 knock-outs have not been described yet.

**S. cerevisiae HMG-Box Proteins**

*S. cerevisiae* contains ten HMG-box proteins, including the HMGB1/2 homologs NHP6A/B, ~10 kDa proteins with a single HMG box, which have been shown to participate in the RNA polymerase II and III transcription systems [50-53]. HMO1 and HMO2 are ~25 kDa HMG-box proteins, also identified by homology-based motif-prediction programs as having only a single HMG-box, in a position corresponding to the HMG box B of mammalian HMGB. However, HMO1 also contains an N-terminal box A domain with weak similarity to consensus HMG-box domains. The relative abundance of NHP6A/B and HMO1/2 was recently determined as part of a global analysis of protein expression in yeast. All four proteins are moderately abundant, with an estimated 1.9 x
10^4 molecules of HMO1 per cell compared to ~4 x 10^3 for NHP6A/B and 1.8 x 10^3 for HMO2 [54]. Strains bearing *HMO1* or *HMO2* mutant alleles are viable, although HMO1 mutant strains have growth defects, compromised plasmid maintenance and nuclease sensitive chromatin, suggesting that HMO1 may play a role in stability of the chromatin structure [55]. HMO1 has been shown to interact genetically and physically with FKBP12 prolyl isomerase, a ubiquitous, highly conserved, abundant enzyme that catalyzes a rate-limiting step in protein folding. Dolinski and Heitman [56] found that mutations in *HMO1* and in the *FPR1* gene that encodes FKBP12 are synthetically lethal.

A recent study implicates HMO1 as part of the rRNA transcription apparatus, where it was proposed to function in a similar capacity as the Upstream Binding Factor (UBF), which is an auxiliary factor in the mammalian and amphibian RNA Polymerase I transcription systems; UBF has six HMG domains [57]. HMO1 has also recently been shown to participate in mutagenesis control, but its exact role remains to be elucidated.

The C terminus of HMO1 has a long run of basic residues not found in any other HMGB1/2 protein. *In vivo*, the C terminus is dispensible for function whereas HMG Box B is essential. As the basic C-terminus is predicted to harbor the nuclear localization signal, the implication of this observation is that the growth phenotype characteristic of the hmo1 knockout may be reversed by HMO1 mutant protein that is confined to the cytoplasm. Immuno-localization experiments have shown that full length HMO1 is present both in the nucleus and cytoplasm [55]. Although the biological significance of the presence of HMO1 in the cytoplasm has not been established, it may serve the same critical and diverse roles as does the extracellular HMGB1.
We show here that the divergent box A domain does participate in direct DNA contacts, and that it contributes modest structure-specific DNA binding to HMO1, while box B confers most of the DNA binding affinity. Interestingly, unlike many HMGB proteins that insert negative supercoils in the presence of topoisomerase I in topologically closed DNA [51, 55], HMO1 did not supercoil relaxed closed circular DNA. We have also shown that the HMO1 basic C-terminal tail participates in DNA binding, and that it reduces the stability of the protein. Phosphorylation of HMO1 by casein kinase II lowers its affinity for DNA, suggesting that HMO1 can be regulated by post-translational modification. In vivo studies showed that HMO2 knockout has a slower growth phenotype (contrary to a previous report [55]) which is only evident in the presence of an HMO1 knockout, indicative of an overlap in function of these proteins. Using green fluorescent protein (GFP), HMO1 was localized to the cytoplasm and the nucleus, while HMO1 with the C-terminal domain deleted (M210) was localized only to the cytoplasm. Previous studies [55] have shown that hmo1 knockouts transformed with the vector carrying the M210 gene fully recovers to show wild type phenotype. This shows that the essential and unique function of HMO1 protein is in the cytoplasm and not in the nucleus.

**DNA Supercoiling by Vaccinia Topoisomerase I**

DNA topoisomerases are ubiquitous enzymes that manage DNA topology in the cell. They participate in a variety of cellular processes by solving the DNA topological problems associated with replication, transcription, recombination, chromosome condensation, decatenation and unknotting of DNA (reviewed in [59, 60]). Topoisomerases fine-tune DNA topology to facilitate protein interactions with the DNA which can be negatively affected by too much or too little supercoiling. They alter DNA
topology by the concerted cleavage and religation of DNA strand(s). The nucleophilic attack of an active-site tyrosine on a phosphodiester DNA bond generates a phosphotyrosyl linkage, providing a transient protein-linked gate through which another DNA strand or duplex may pass. A second transesterification resolves the tyrosyl-DNA linkage to restore DNA integrity [59, 60, 61].

With the recognition that topoisomerases play an important role in controlling DNA topology, great interest has developed, especially due to the discovery of topoisomerase targeted compounds. Clinically important anticancer and antibacterial compounds that target topoisomerases are now in use. Camptothecin and its analogues have shown remarkable antitumor activity against pediatric and adult malignancies [62]. Fluoroquinolones are compounds that target topoisomerases and are in wide clinical use as antibacterial agents [63, 64, 65].

**Classification of Topoisomerases**

Topoisomerases are classified into subfamilies which are distinguished by common biochemical properties and similarity in structure. While DNA cleavage by all topoisomerases leads to formation of a transient covalent intermediate in which a tyrosine is linked to a broken DNA strand, type II topoisomerases cleave both strands, which results in DNA topoisomers differing in linking number in steps of two. They are further divided into two different subfamilies based on structural considerations; type IIA and IIB subfamilies. Type I topoisomerases relax supercoiled DNA, but unlike type II topoisomerases, type I enzymes cleave only one strand and the linking number changes in steps of one. They are also further classified as either type IA, if the protein link is to a 5′ phosphate or type IB, which form a covalent bond to a 3'-phosphate. Type I
Topoisomerases are found in all eukaryotes, where they relax both positive and negative supercoils [59, 60, 61].

**Cellular Roles of DNA Topoisomerases**

*Escherichia coli* has four DNA topoisomerases, two type IA (DNA topoisomerases I and III) and two type IIA enzymes (DNA gyrase and DNA topoisomerase IV). Genetically, functional overlap of each enzyme has been shown however, it appears that each enzyme has been optimized to carry out its own particular set of topological manipulations. DNA gyrase is the only known topoisomerase that generates negative supercoils by use of ATP as the energy source. As such, DNA gyrase plays an important role in chromosome condensation leading to proper chromosome partitioning at cell division [66, 67]. Any positive supercoils that are generated in the cell during processes like transcription or replication are resolved by DNA gyrase. Topoisomerase IV plays important roles in decatenation during replication [68] as well as relaxing negative supercoils in the cell [69], along with topoisomerase I [70] to prevent excessive negative supercoils generated by DNA gyrase or any other cellular processes like transcription and replication.

In *S. cerevisiae* and *Schizosaccharomyces pombe*, topoisomerase I (IB subfamily) is dispensable for growth, but topoisomerase IIA is required to decatenate linked chromosomes and prepare them for segregation at mitosis [71, 72]. Topoisomerase II relaxes supercoils of both signs and as such, it substitutes for topoisomerase I in its absence. Deletion of topoisomerase III (type IA subfamily) in *S. cerevisiae* results in slow growth exhibited by elevated levels of mitotic recombination. This deletion also results in sporulation failure due to a defect in meiotic recombination [73, 74].
In higher eukaryotes, topoisomerase I is indispensable during development and during cell division [75, 76]. Higher eukaryotes contain two isoforms of topoisomerase II and III termed α and β [59].

**General Features of Type IA DNA Topoisomerase I**

With the exception of the *Methanopyrus kandleri* encoded topoisomerase, all type IA topoisomerases are monomeric, and they require Mg(II) for the DNA relaxation activity. These proteins relax only negative supercoils, but the relaxation does not go to completion and they require an exposed single-stranded region within the substrate DNA [59, 77]. These enzymes have been shown to catalyze the knotting, unknotting, and interlinking of single-stranded circles as well as the knotting, unknotting, catenation, and decatenation of gapped or nicked duplex DNA circles (reviewed in [59]). Although considerable sequence diversity is observed in the type IA subfamily, *E. coli* topoisomerase I, a prototype of this subfamily that is well studied, represents some common features. The core cleavage/strand passage domain that is found at the N-terminal contains the active site tyrosine. Expression of this N-terminal fragment yields a protein that retains the ability to cleave a single-stranded oligonucleotide, but is unable to relax plasmid DNA [78]. This catalytic domain is followed by a Zn(II)-binding domain. Although dispensable for activity *in vitro*, the C-terminal domain is rich in basic amino acids and contributes to substrate binding with preference for a single-stranded DNA [79, 80, 81]. In comparison to other type IA topoisomerases, both *E. coli* and *S. cerevisiae* topoisomerase III lack a Zn(II) binding domain. Conversely, human topoisomerase IIIα and IIIβ have a Zn(II) binding domain. It seems, however, that type
IA topoisomerases possess a relatively basic C-terminal domain that although not conserved, contributes to DNA binding.

**General Features of Type IB DNA Topoisomerase**

The type IB subfamily of topoisomerases is made up of three classes which include eukaryotic topoisomerase I, poxvirus topoisomerase I and prokaryotic topoisomerase V from *M. kandleri* [82, 83]. These proteins share structural and functional properties with the tyrosine recombinases and some phage integrases [84]. Unlike type IA, type IB topoisomerases relax to completion both positive and negative supercoils and as such, there is no need for exposed single-stranded region within the substrate DNA. Type IB contains no bound metal ions, and DNA relaxation does not require Mg(II). Human DNA topoisomerase I has four distinct domains; 1) the N-terminal domain which has nuclear localization signals and sites for interaction with other proteins such as nucleolin and SV40, 2) the core domain which is highly conserved, binds DNA and contains most of the catalytic residues except for the active site tyrosine, 3) the linker domain which is poorly conserved and 4) the C-terminal domain which is conserved and contains the active site tyrosine. Human topoisomerase I is proposed to follow a “controlled rotation” mechanism for DNA relaxation, in which ionic interactions between DNA and protein regulate the DNA winding process [85, 86].

The poxvirus topoisomerases are considerably smaller than cellular topoisomerases, are of fairly uniform size (314-333), and probably constitute the minimal functional unit of a type IB topoisomerase. Vaccinia topoisomerase I is a 314-amino acid protein that binds to duplex DNA with stringent specificity for transesterification at DNA sites containing the sequence 5’-(C/T)CCTT↓ [87-91]. The 3’ phosphate of the incised
strand is linked to Tyr-274 of the enzyme. Vaccinia topoisomerase I consists of two structural domains joined through a trypsin-sensitive bridge. The N-terminal domain (amino-acids 1-80), which is highly conserved in poxvirus and vertebrate topoisomerases, contacts the-(C/T)CCTT DNA site in the major groove [92, 93]. The C-terminal domain (amino-acids 81-314) comprises an autonomous catalytic domain that performs the same repertoire of reactions as the full-sized topoisomerase I [94, 95]: relaxation of supercoiled DNA, site-specific DNA transesterification, and DNA strand transfer [94]. The structure of the catalytic domain is similar to that of human topoisomerase I except for a displacement of the active site tyrosine [71, 95]. The catalytic domain is also very similar to core domain III and a 19 amino acid region encompassing the active site of human topoisomerase I.

**Fluoroquinolone Compounds**

Since the first quinolone nalidixic acid was developed, the quinolones have undergone structural modifications, in particular the addition of a fluorine at position 6, to produce the fluoroquinolones (reviewed in [67]). This has seen their potency and pharmakokinetic profile greatly increase. Fluoroquinolones are topoisomerase II poisons known to target DNA gyrase and topoisomerase IV in bacterial cells [64, 65, 96]. The interaction of norfloxacin with the DNA-gyrase complex has been investigated in some detail. While binding of norfloxacin to gyrase was undetectable, it was shown to bind DNA directly and to exhibit a binding mode involving preferred recognition of single-stranded DNA. Drug binding was enhanced by the presence of gyrase, suggesting that DNA-binding revealed a cryptic norfloxacin binding site on the enzyme [97, 98]. The nature of the norfloxacin-DNA complex has been analyzed by several groups, with
somewhat disparate conclusions. Norfloxacin was reported to require magnesium ions for DNA binding, with Mg$^{2+}$ proposed to act as a bridge between the negatively charged DNA phosphates and the carbonyl and carboxyl moieties of norfloxacin [99]. However, more recent reports showed binding of norfloxacin to DNA in the absence of Mg$^{2+}$ and suggested a mode of DNA interaction that ruled out classical DNA intercalation or groove binding, based in part on the observation that the molecular plane of norfloxacin is nearly perpendicular to the helix axis [100-101]. These authors also found that DNA unwinding by norfloxacin is negligible and proposed instead that the compound may induce a bend in the DNA helix.

The inhibitory effect of fluoroquinolones is based on their ability to stimulate the forward rate of the topoisomerase II-mediated DNA scission; after binding the cleavable complex, the fluoroquinolone induces a conformational change in the enzyme that prevents religation [102-104]. The replication fork is blocked by the stabilization of these complexes, resulting in irreversible DNA-enzyme complexes, which probably leads to lethal release of double-stranded DNA breaks [63, 64, 105, 106].

The fluoroquinolone derivative ofloxacin was previously shown also to exhibit antiviral activity against Vaccinia virus, and the inhibitory activity shown to correlate with inhibition of topoisomerase I purified from Vaccinia virus cores [107]. Therefore, with wider screening of these fluoroquinolone compounds, it is likely to result in discovery of compounds with antiviral activity or compounds that can be optimized or act as a lead to more potent antiviral agents.

Here we describe a novel interaction of enrofloxacin with Vaccinia topoisomerase I \textit{in vitro}. Enrofloxacin, a fluoroquinolone antibiotic which targets DNA gyrase and
topoisomerase IV in bacterial cells inhibits DNA relaxation by Vaccinia topoisomerase I in a concentration dependent fashion. In the presence of relaxed DNA, however, enrofloxacin causes Vaccinia topoisomerase I to supercoil DNA, reversing its action as a DNA relaxing enzyme. Further characterization indicates that enrofloxacin does not interfere with the initial strand scission by Vaccinia topoisomerase I, nor does it interfere with enzyme’s ability to bind DNA. The mechanism with which Vaccinia topoisomerase I supercoils relaxed DNA, an energetically unfavorable, yet ATP-independent process, must entail protein-DNA contacts downstream of the cleavage site, as opposed to the free rotation mechanism proposed for DNA relaxation. In cell culture, enrofloxacin and several other fluoroquinolone compounds inhibited Vaccinia virus.

References


CHAPTER 2
THE SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 CONTAINS TWO FUNCTIONAL DNA BINDING DOMAINS

Introduction

High-mobility group (HMG) proteins constitute a significant proportion of non-histone proteins of eukaryotic chromatin. They are abundant proteins that are grouped into three major classes, in part based on their DNA-binding characteristics: HMGGA, HMGB, and HMGN [1-4]. HMGB proteins contain one or more homologous repeats of the ~80-amino acid sequence HMG box and are classified into two families based on the abundance, function and DNA specificity of this conserved region [1, 5, 6]. The moderately sequence-specific family is typified by transcription factors such as sex-determining factor SRY and lymphoid enhancer factor LEF-1 [7, 8], while the non-sequence-specific family is represented by so-called architectural factors HMGB1/2 and the Saccharomyces cerevisiae non-histone chromosomal proteins 6A and 6B (NHP6A/B) [9].

The tertiary structures of HMG boxes from sequence-specific and non-sequence-specific proteins have revealed an evolutionarily conserved, common global fold consisting of an L-shaped structure composed of three \( \alpha \)-helices [10-19]. The HMG DNA binding domain, which interacts with ~10 bp of duplex, binds to the minor groove of DNA by partial intercalation of one or two surface-exposed, conserved hydrophobic residues into the base pair stack. Consequently, the DNA is greatly distorted, resulting in a sharp bend and helical underwinding [4, 8, 14, 16, 20]. SRY and LEF-1 cause bending by insertion of helix I hydrophobic residues Ile and Met, respectively, into the base pair.
**Figure 2.1 Alignment of HMG-box helices I and II.** For non-sequence specific proteins, box A and box B of rat HMGB1 is shown along with *S. cerevisiae* NHP6A, *Drosophila melanogaster* HMGD and *S. cerevisiae* HMO1. The HMO1 box B insertion is indicated in lower case. Sequence specific proteins are human SRY, mouse LEF-1 and *Xenopus laevis* UBF box A. Residues corresponding to positions 16 and 37 of HMGB1 helices I and II, respectively, are in bold.
stack (Figure 1[8, 17]). HMGB1 contains tandem HMG box domains referred to as box A and box B; DNA-intercalating residues are located at positions 16 and 37 of helices I and II, respectively, and both can contribute to binding affinity [11, 13, 16, 19]. HMGB proteins recognize DNA with structural specificity, binding preferentially to distorted DNA such as 4-way junctions, mini-circles, and cisplatinated DNA, and they supercoil relaxed, topologically closed DNA in the presence of topoisomerase I [11, 16, 20-25]. HMGB proteins have been implicated in diverse biological processes such as transcription, recombination, replication and DNA repair by virtue of their ability to facilitate assembly of nucleoprotein complexes [1, 3, 5].

*S. cerevisiae* contains ten HMG-box proteins, including the HMGB1/2 homologs NHP6A/B, ~10 kDa proteins with a single HMG box, which have been shown to participate in the RNA polymerase II and III transcription systems [9, 26-28]. HMO1 and HMO2 are ~25 kDa HMG-box proteins, also identified by homology-based motif-prediction programs as having a only single HMG-box, in a position corresponding to box B of mammalian HMGB. However, HMO1 also contains an N-terminal box A domain with weak similarity to consensus HMG-box domains (Figure 1). The relative abundance of NHP6A/B and HMO1/2 was recently determined as part of a global analysis of protein expression in yeast. All four proteins are moderately abundant, with an estimated $1.9 \times 10^4$ molecules of HMO1 per cell compared to $\sim 4 \times 10^3$ for NHP6A/B and $1.8 \times 10^3$ for HMO2 [29]. Strains bearing *HMO1* or *HMO2* mutant alleles are viable, although HMO1 mutant strains have growth defects, compromised plasmid maintenance and nuclease sensitive chromatin, suggesting that HMO1 may play a role in stability of the chromatin structure [30]. HMO1 has been shown to interact genetically and
physically with FKBP12 prolyl isomerase, a ubiquitous, highly conserved, abundant enzyme that catalyzes a rate-limiting step in protein folding. These authors found that mutations in HMO1 and in the FPR1 gene that encodes FKBP12 are synthetically lethal [31]. A recent study implicates HMO1 as part of the rRNA transcription apparatus, where it was proposed to function in a similar capacity as the Upstream Binding Factor (UBF), which is an auxiliary factor in the mammalian and amphibian RNA Polymerase I transcription systems; UBF has six HMG domains [32]. HMO1 has also recently been shown to participate in mutagenesis control, but its exact role remains to be elucidated [33].

We show here that the divergent box A domain does participate in direct DNA contacts, and that it contributes modest structure-specific DNA binding to HMO1, while box B confers most of the DNA binding affinity. HMO1 displays a longer residence time on constrained DNA minicircles, consistent with its role in the maintenance of chromatin structure.

**Experimental Procedures**

**Cloning and Purification of Proteins**

The gene encoding HMO1 was amplified from yeast genomic DNA using primers modified to introduce NdeI sites at both ends of the PCR product; forward primer, 5′-GCCTGTCACCATATGACTACAG-3′, reverse primer, 5′-AGTAACGCATATGTCCGTCC-3′ (NdeI sites underlined). The HMO1 gene was cloned into the NdeI site of plasmid pET5a, and plasmid carrying the HMO1 gene was transformed into *E. coli* BL21(DE3)pLysS. HMO1 expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD<sub>600</sub> of 0.4 and the culture
incubated for 3 h. Cells were pelleted and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 5% glycerol, 5 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride (PMSF)). Cells were disrupted by sonication and nucleic acids digested by addition of DNase I followed by a one-hour incubation on ice. The extract was clarified by centrifugation at $5000 \times g$ for 20 min. The supernatant was dialyzed overnight against HA buffer pH 8.7 (20 mM Tris-HCL, 50 mM KCl, 10% glycerol, 1 mM EDTA, 4 mM 2-mercaptoethanol, and 1 mM PMSF). The dialysate was passed through tandem columns of DEAE-cellulose and CM-sepharose equilibrated with the same buffer. Proteins were eluted with a 120 ml linear gradient from 50 mM KCl (HA buffer) to 1 M KCl (HB buffer; 20 mM Tris-HCl, 1 M KCl, 10% glycerol, 1 mM EDTA, 4 mM 2-mercaptoethanol, 1 mM PMSF). Peak fractions were collected, dialyzed overnight in HA buffer pH 8.7, and passed through a Heparin column equilibrated with the same buffer and proteins were eluted and analyzed as described above. Peak fractions were collected and dialyzed overnight in HA buffer pH 7.0. The dialysate was passed through CM-sepharose equilibrated with the same buffer and proteins were eluted and analyzed as described above. Pure HMO1 fractions were concentrated and quantitated on Coomassie Blue stained SDS-PAGE gels using BSA as a standard.

The gene encoding HMO1 was also subcloned into pET28b for expression with an N-terminal His$_6$-tag. Using a PCR-based site-directed mutagenesis approach, a stop codon was inserted into the $HMO1$ gene in place of Pro91 to create the truncated protein HMO1-BoxA, lacking box B and the basic tail region, using primers forward, 5'-CGCTGCTTGAGTCAAGGCT-3', and reverse, 5'-ATAATAGCATCTTTATCATCATCAATAGGG-3'. Plasmids, HMO1-pET28b and
BoxA-pET28b, were transformed into *E. coli* Rosetta Blue. Cultures were grown to an OD$_{600}$ of 0.2 and expression was induced with 1mM IPTG for 3 h. Cells were pelleted, resuspended in a dialysis/lysis buffer pH 7 (50 mM Na$_3$H$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM PMSF), disrupted by sonication, and nucleic acids digested by addition of DNase I followed by a one-hour incubation on ice. The lysate was dialyzed overnight in dialysis/lysis buffer. The dialysate was passed through a Ni-NTA column equilibrated with dialysis/lysis buffer and washed with 5 column volumes of wash buffer pH 7 (50 mM Na$_3$H$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM PMSF). Proteins were eluted with a 50 ml linear gradient from 20 mM imidazole (wash buffer) to 250 mM imidazole (elution buffer: 50 mM Na$_3$H$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM PMSF) followed by 100 ml of elution buffer. Pure HMO1 and HMO1-BoxA fractions were quantitated on Coomassie Blue stained SDS-PAGE gels using BSA as a standard. *Bacillus subtilis* HU was cloned from *B. subtilis* genomic DNA and overexpressed in *E. coli*. Detailed procedures for its cloning and purification will be reported elsewhere.

**Circular Dichroism Spectroscopy**

CD spectra were recorded on an AVIV Model 202 CD spectrophotometer using a 1 cm pathlength sample cell. Wavelength scans from 200 nm to 240 nm were performed in triplicate, and data points were averaged and smoothed using standard methods. The wavelength scan for His$_6$-tagged HMO1 was recorded at 25ºC, with a protein concentration of 0.025 mg/ml in 2.5 mM Na$_3$H$_2$PO$_4$, pH 7.0, 0.5% glycerol, 10 mM NaCl. The wavelength scan for His$_6$-tagged HMO1-BoxA was recorded at 4ºC, using a
protein concentration of 0.05 mg/ml in 1 mM Tris, pH 7.0, 0.03% Tween 20, 10% glycerol, 2.8 mM KCl.

**Ligase-Mediated Circularization**

Ligation substrates were prepared by digestion of PCR amplified or plasmid DNA with appropriate endonucleases. pET5a was digested with *Bam*HI and *Bg*II to generate a 136 bp fragment while *Bsp*HI digestion generated a 105 bp fragment. An 87 bp fragment was generated by digestion of pcDNA3 with *Sac*I. A 75 bp DNA fragment was generated by PCR amplification of the 390-538 bp region of pUC18 using Taq polymerase and primers 5′-GCCAGTGCCAAGCTTGCATG-3′ and 5′-CTTTATGCTCTCGAGTCGTATGTT-3′, followed by digestion with *Sac*I. DNA fragments were purified on 7% (w/v) polyacrylamide gels and 5'-end labeled with T4 polynucleotide kinase and (γ-32P) ATP. Reactions were initiated by addition of 20 units of T4 DNA ligase to a final volume of 100 µl reaction buffer (20 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM MgCl2, 0.1 mM Na2EDTA, 1 mM DTT, 0.05% BRIJ58) containing 1200 fmol DNA, with or without HMO1. Time points were taken over 90 minutes. For each time point, 8 µl of the reaction mixture was quenched by addition of 5 µl stop buffer (75 mM EDTA, 15% glycerol, 0.1% bromophenol blue (BPB), 0.1% xylene cyanol, 6 µg/µl proteinase K). Samples were heated at 55°C for 15 minutes and resolved on prerun 7% (w/v) native polyacrylamide gels (39:1 acrylamide:bisacrylamide) gels at 4°C with 0.5X TBE (50 mM Tris-borate, 1 mM EDTA). Quantitation was performed on a Molecular Dynamics Storm Phosphorimager using software supplied by the manufacturer.
DNA Supercoiling

Reactions contained 0.2 µg relaxed closed circular pUC18 plasmid, prepared by adding 2.5 units of *Vaccinia* topoisomerase I (Epicenter) to supercoiled pUC18 in 50 mM Tris, pH 7.5, 2.5 mM MgCl₂, 100 mM NaCl and 0.1 mM EDTA. After 1.5 h at 37°C, varying amounts of HMO1 were added and the reactions incubated for 1 h at 37°C. Reactions were quenched with 5 µl termination buffer (5X TBE, 5% SDS, 15% sucrose, 0.1%BPB, 0.1%xylene cyanol, 2 µg/µl proteinase K) and incubated for 1 h at 37°C. Samples were loaded on 1% 1X TBE agarose gels and electrophoresed at 2 V/cm for 12 h in 1X TBE buffer.

Agarose Gel Retardation

Reactions were incubated at room temperature in 10 µl reaction buffer containing 0.2 µg linear, relaxed closed circular or supercoiled pUC18 and varying amounts of HMO1 or HMO1-BoxA. Complexes were resolved on 0.7% 1X TAE agarose gels and electrophoresed at 2.5 V/cm for 3 h in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide after electrophoresis.

Electrophoretic Mobility Shift Assays (EMSA) with DNA minicircles

The 105 bp fragment generated by *Bsp*HI digestion of pET5a DNA was 5'-end labeled with T4 polynucleotide kinase and (γ³²P) ATP. DNA minicircles were generated by intramolecular ligation of the ³²P-labeled 105 bp DNA fragment with 20 units of T4 DNA ligase in the presence of *Thermotoga maritima* HU for 2 h at room temperature (34). Samples were treated with Exonuclease III for 1 h at room temperature and the reactions quenched with stop buffer. The de-proteinised DNA was purified on a 6% (w/v) native polyacrylamide gel (39:1 acrylamide:bisacrylamide).
EMSA was performed with linear and circular 105 bp DNA. Reactions were incubated at room temperature in 10 µl reaction buffer containing 5 fmol DNA and varying amounts of HMO1. Where indicated, reactions were performed in the absence of MgCl₂. Samples were resolved on prerun 7% (w/v) native polyacrylamide gels (39:1 acrylamide:bisacrylamide) gels at 4°C with 0.5X TBE running buffer. Complexes were visualized by phosphorimaging. In DNA competition assays, 10 fmol of ³²P-labeled linear or circular 105 bp DNA was incubated with HMO1 for 30 min at room temperature to allow complex formation, followed by addition of varying amounts of linear pUC18 plasmid DNA. Samples were resolved as described above. For calculating fractional complex formation, the region on the gel from the slowest migrating complex to the free DNA was considered as complex.

EMSA with Four-Way DNA Junctions

Four-way junctions were constructed using a published protocol [35]; the sequence of individual strands was as described [36]. The 4-way junction was prepared by annealing the four strands, followed by purification of the junctions on native polyacrylamide gels. To ensure that all oligonucleotides were present, aliquots of the purified junctions were run on denaturing gels. EMSA were performed as described above with 5 fmol DNA and varying amounts of HMO1 or HMO1-BoxA; for experiments with HMO1-BoxA, the reaction buffer was modified to contain only 25 mM NaCl, MgCl₂ was omitted, and 0.5% Tween 20 was included. In competition assays, 20 or 1000-fold mass excess of unlabeled linear pUC18 DNA was added after the addition of HMO1. Complexes were resolved and quantified as described above.
EMSA with linear duplex DNA and DNA Containing Loops, Nicks, Gaps or Overhangs

Oligonucleotides were purchased and purified by denaturing gel electrophoresis. Oligonucleotides used to generate constructs with loops, nicks, gaps or overhangs included a common 37 nt top strand: 5′-CCTAGGCTACACCTACTCTTTTGAAGATTAGCTTC-3′. To generate 37 bp duplex DNA, complementary oligonucleotides were mixed at equimolar concentrations, heated at 90°C, and slowly cooled to room temperature. To generate looped DNA, a complementary strand with a pair of 2-nt mismatches with 11 nt spacing (3′-GGATCCGATGCATGAAACATAGTTAATCGAG-5′, with mismatched nucleotides indicated in bold) was annealed to the top strand. A spacing of 11 bp was chosen as it represents the spacing that yielded optimal complex formation with mammalian HMGB1 [37]. The 37 bp DNA with a central nick, gap, or a 3'-overhang were prepared as described [36]. The 18 bp duplex corresponded to the sequence of the 5' half of the 37 bp duplex. The sequence of the 26 bp duplex was: 5′-CGTGACTACTGTAAGTCGATGATCCG-3′. EMSA were performed as described above with 5 fmol DNA. The observed fraction of complex formation was corrected for dissociation during electrophoresis, and binding isotherms for HMO1 binding to 26 bp DNA was fitted as described [36]. All experiments were done at least in triplicate, and values are reported as the mean ± standard deviation.

Competition assays involving HMO1-BoxA were performed with 32P-labeled 26 bp DNA. Reactions were incubated at room temperature in 10 µL reaction buffer containing 10 fmol of DNA, 400 fmol B. subtilis HU or 4 pmol full-length HMO1, and varying amounts of HMO1-BoxA. B. subtilis HU or HMO1 was incubated with DNA at
room temperature for 5 min, followed by addition of HMO1-BoxA. Samples were resolved on prerun 7% native gels at 4°C with 0.5X TBE running buffer. Complexes were visualized by phosphorimaging.

**Results**

**DNA binding by HMO1 and HMO1-BoxA**

Recombinant HMO1 and His<sub>6</sub>-HMO1 were purified to apparent homogeneity, as judged by Coomassie Blue-staining of SDS-PAGE gels. For analysis of DNA binding by the divergent box A domain, N-terminally His<sub>6</sub>-tagged HMO1-BoxA was also prepared (Figure 2.2A). CD spectra of both full-length HMO1 and HMO1-BoxA are characterized by negative ellipticities at 208 and 222 nm, indicative of significant α-helical content (Figure 2.2B-C). The qualitatively comparable spectra suggest that HMO1-BoxA is an independently folded domain. The previously reported ability of HMO1 to self-associate [31], evident at higher protein concentrations, was also characteristic of the box A domain for which well-resolved CD spectra required the presence of detergent.

DNA binding was analyzed with Electrophoretic Mobility Shift Assays (EMSA). While no stable complex could be seen on incubation of 18 bp DNA with full-length HMO1 (data shown), a single complex formed with 26 bp DNA (Figure 2.3). No difference was seen between N-terminally His<sub>6</sub>-tagged and untagged HMO1 (data not shown; all subsequent experiments were performed with untagged HMO1). The apparent dissociation constant K<sub>d</sub> for HMO1 binding to 26 bp DNA is 39.5 ± 5.0 nM. HMO1 forms two complexes with 37 bp DNA (Figure 2.4A), as seen previously with the mammalian two-HMG box protein HMGB1 [37], suggesting similar site sizes. Half-maximal saturation is observed at 33.1±3.9 nM HMO1. For reactions performed in the
Figure 2.2 HMO1 and HMO1-BoxA have significant $\alpha$-helical content. (A) SDS-PAGE gels showing purified His$_6$-HMO1 (left) and His$_6$-HMO1-BoxA (right). (B)-(C) CD spectra of HMO1 and HMO1-BoxA, respectively.
Figure 2.3 Electrophoretic analysis of 26 bp DNA titrated with HMO1. (A) Reactions contain 5 fmol DNA. Reactions in lanes 2-14 contain 6, 12, 24, 48, 72, 96, 120, 144, 188, 192, 216, 240, and 264 nM HMO1. (B) Binding isotherm for HMO1 binding to 26 bp DNA. When error bars are not shown, they are smaller than the symbol size. Inset shows purified untagged HMO1 (lane 2). Molecular weight markers are identified at the left (in kDa).
Figure 2.4 Binding of HMO1 and *B. subtilis* HU to 4-way junctions and linear duplex DNA. (A) HMO1, (B) *B. subtilis* HU. Reactions contain 5 fmol DNA, and protein concentrations are 0, 1, 2, 4, 8, 16, 24, 28, 32, 36, 40 and 60 nM (identical for all panels).
absence of Mg$^{2+}$, the affinity is slightly higher with half-maximal saturation of 15.6±2.3 nM (data not shown). The lower affinity measured in the presence of magnesium ions may not be due to effects caused specifically by the divalent ions, but a general effect of raised ionic strength.

While no complex is detected upon incubation of 26 bp DNA with HMO1-BoxA, a competition experiment in which full-length HMO1 is incubated with 26 bp DNA in the presence of increasing concentrations of HMO1-BoxA shows a reduction in HMO1-DNA complex formation, suggesting that HMO1-BoxA competes for binding to the DNA (data not shown). However, since HMO1-BoxA self-associates, this experiment does not exclude the possibility that HMO1-DNA complex formation is impaired due to interactions between HMO1-BoxA and full-length protein. The competition experiment was therefore also performed using the unrelated architectural protein HU from *Bacillus subtilis* (Figure 2.5A). Consistent with DNA binding by HMO1-BoxA, HU-DNA complex formation is also reduced in the presence of HMO1-BoxA. Efficient competition requires µM concentration of HMO1-BoxA, suggesting low-affinity binding, consistent with HMO1-BoxA complexes with 26 bp DNA being too unstable to detect following electrophoresis.

**HMO1 and HMO1-BoxA have only limited preference for distorted DNA**

HMGB proteins have been shown to recognize distorted DNA structures selectively [21, 24, 35, 37, 38]. Compared to linear duplex DNA, however, HMO1 did not show binding preference for DNA with loops, nicks, gaps or overhangs (data not shown); by comparison, the 37 bp looped DNA construct served as a preferred substrate for mammalian HMGB1 [37]. Evidently, intrinsic DNA flexibility does not confer a
Figure 2.5 HMO1-BoxA binds DNA. (A) HMO1-BoxA competes with *B. subtilis* HU for binding to 26 bp DNA. Reactions contain 10 fmol DNA and 50 nM HU (lanes 2-8). Reactions in lanes 3-8 contain 0.2, 0.4, 0.9, 8.8, 17.6, and 26.4 µM HMO1-BoxA. (B) Electrophoretic analysis of HMO1-BoxA binding to 4-way junction DNA (5 fmol). Reactions in lanes 2-7 contain 2.2, 11, 22, 33, 44, and 66 µM HMO1-BoxA.
significant energetic advantage for HMO1 to bind DNA. Surprisingly, HMO1 also had only limited preference for 4-way junctions (Figure 2.4A). Half-maximal saturation is observed at 19.6±2.2 nM of HMO1 and for reactions performed in the absence of Mg$^{2+}$, half-maximal saturation is 6.1±1.1 nM. This is in distinct contrast to other HMGB proteins which were shown to bind 4-way junction DNA only in the open square conformation that is preferred in the absence of Mg$^{2+}$ [39]. For comparison, *B. subtilis* HU showed the expected preference for the 4-way junctions compared to linear DNA with a sequence that corresponds to the longest arms of the 4-way junctions, confirming integrity of the 4-way junction construct (Figure 2.4B). As shown in Figure 5B, HMO1-BoxA also exhibits a modest preference for 4-way junction DNA; consistent with self-association of HMO1-BoxA, stable complex formation is enhanced by the presence of detergent. Notably, the formation of detectable complexes with a migration pattern similar to that seen for full-length HMO1 confirms the ability of HMO1-BoxA to engage DNA directly.

When HMO1 was added to reaction mixtures containing both 4-way junction and linear duplex DNA, HMO1 bound with only modest preference to the 4-way junctions (Figure 2.6). In competition assays, EMSA was performed with either $^{32}$P-labeled 4-way junctions or $^{32}$P-labeled linear duplex DNA in the presence of unlabeled competitor linear or supercoiled plasmid DNA. HMO1 was competed off the linear and 4–way junction DNA equally efficiently by linear and supercoiled plasmid DNA (data not shown). These assays indicate that HMO1 does not have significant preference for supercoiled compared to linear plasmid DNA. To investigate the interaction of HMO1 with different DNA topologies further, agarose gel retardation assays were performed with negatively
Figure 2.6 HMO1 has only modest preference for 4-way junction DNA. Reactions contain linear duplex DNA and 4-way junctions. HMO1 was added at increasing concentrations, ranging from 10 nM to 120 nM.
supercoiled, relaxed and linear pUC18 plasmid. As shown in Figure 2.7, HMO1 has only modest preference for supercoiled DNA. Regardless of DNA topology, HMO1 causes the entire population of DNA to shift as a rather broad band, suggesting that HMO1 binds without cooperativity. Consistent with its low-affinity binding to 26 bp DNA, HMO1-BoxA does not form complexes with plasmid DNA that are detectable following electrophoresis (data not shown).

**HMO1 exhibits a longer residence time on DNA minicircles**

DNA mini-circles are circularized DNA shorter than the persistence length of ~150 bp. Since the DNA is highly constrained, a diminished need for DNA distortion may lower the free energy of interaction. HMO1 bound 105 bp circular DNA comparably to 105 bp linear DNA (Figure 2.8A). However, in DNA competition assays with linear pUC18 plasmid, HMO1 was more efficiently competed off linear 105 bp DNA; at 400-fold excess plasmid DNA, HMO1 still bound 91% of the 105 bp circular DNA as opposed to only 13% of the linear 105 bp DNA (Figure 8B), suggesting a much longer residence time on the constrained minicircle.

**HMO1 bends DNA**

Ligase-mediated circularization assays were performed in which the efficiency with which T4 DNA ligase mediates ring closure of DNA fragments that are shorter than the persistence length is measured [20, 40]. The ability of HMO1 to enhance ligase-mediated DNA circularization was qualitatively assessed with 136, 105, 87, 75 and 65 bp duplex DNA. HMO1 facilitated formation of circles with DNA of 136, 105 and 87 bp, but not 75 or 65 bp (Figure 2.9).
Figure 2.7 Binding of HMO1 to supercoiled, relaxed and linear DNA. Lanes 1, 8 and 15 with no HMO1 and lanes 2-7, 9-14 and 16-21 with increasing amounts of HMO1 (1250 fmol-7500 fmol). Samples were analyzed on 0.7% agarose gels.
Figure 2.8 (A) Binding of HMO1 to linear and circular 105 bp DNA. EMSA with 10 fmol DNA. Lanes 2-6 and 8-12 contain 2.5, 10, 25, 50, 75 nM HMO1 respectively. (B) DNA competition assay. HMO1 (5 pmol) was incubated with 10 fmol linear (left panel) or circular (right panel) 105 bp DNA and titrated with unlabeled linear pUC18 DNA. Lanes 3, 4, 5 and 8, 9, 10 contain 4, 40 and 400-fold excess of pUC18, respectively.
Figure 2.9 (A) Time-course ligation assay with 87 bp DNA. 100 fmol 87 bp linear DNA was incubated without (left panel) or with HMO1 (250 nM, right panel) up to 96 minutes. Time points were taken at 6, 12, 24, 48, 72 and 96 min. Exonuclease III was added to reactions in lane 7 and 14 at 48 min time-points. Circular ligation products (lane 14) were resistant to digestion by Exonuclease III. (B) Quantitation of ligation assays for 136, 105 and 87 bp DNA. Effective concentrations of monomeric DNA differ for the three substrates, particularly for the 105 bp DNA for which formation of 210 bp dimer circles predominates. Initial rates therefore do not correlate with duplex length.
As expected, initial rates of cyclization are highest for the longer 136 bp DNA, however, the 87 bp DNA produced monomer circles most efficiently. Dimer circle formation and multimerization of the 87 bp DNA occurred in reactions with and without HMO1, although to a lesser extent compared to the other DNA constructs. The 105 bp DNA yielded the least efficient monomer circle formation, however, more than 50% of the DNA formed dimer circles even in the absence of the protein, lowering the effective concentration of monomeric DNA and causing the initial rate and net yield of monomer circles to be lower than that measured with 87 bp DNA. Secondly, the different cohesive termini of the DNA probes are recognized differentially by DNA ligase. By comparison, *S. cerevisiae* non-histone protein 6A (NHP6A) can form monomer circles with DNA substrates of 66 bp [20] while HMGB1/2 and HMG-D can form monomer circles with DNA substrates as short as 59 and 55 bp, respectively, at high protein concentrations [41, 42]. The protein concentrations used in our experiments are comparable to the concentrations used for other HMGB proteins; our data therefore indicate that HMO1 bends DNA, although not as effectively as other HMGB proteins.

**HMO1 does not supercoil relaxed DNA**

Many HMGB proteins insert negative supercoils in the presence of topoisomerase I in topologically closed DNA [25, 30, 43]. To assess the ability of HMO1 to supercoil DNA, relaxed closed circular pUC18 DNA was incubated with increasing amounts of HMO1 (Figure 2.10). There was no evidence of DNA supercoiling by HMO1, nor did HMO1-BoxA supercoil DNA (not shown). This contrasts with a previous publication by Lu *et al.* who, focusing on analysis of HMO1 purified from yeast, reported that HMO1 could indeed supercoil relaxed plasmid DNA [30].
Figure 2.10 HMO1 does not supercoil relaxed DNA. Lanes 1 and 2 contain supercoiled (S) and relaxed (R) pUC18 DNA, respectively. Increasing amounts of HMO1 (1250 fmol-7500 fmol) in lanes 3-6 and Bsu HU (1250 fmol-7500 fmol) in lanes 7-10 were added to relaxed circular DNA.
Discussion

Recent studies have shown that the difference between sequence-specific HMGB proteins and the non-sequence specific homologs depends on individual DNA intercalating residues and the global features of the HMG box, which determines the mode of DNA recognition. For the tandem HMG boxes of HMGB1, the DNA intercalating residues are at positions 16 or 37, located in helices I and II respectively, and both can contribute to the binding affinity of the HMG boxes. Box A has an alanine at position 16, which does not intercalate with the DNA but forms a hydrophobic contact, whereas phenylalanine at position 37 is used as a bending wedge (Figure 2.1). Box B has a phenylalanine at position 16 and isoleucine at position 37, both of which are potential intercalating residues. Sequence-specific HMGB proteins have polar residues at position 37, which participate in sequence-specific hydrogen-bond formation [8, 16, 42, 44]. For both box A and B of HMGB1, their ability to bend DNA is reflected in preferred binding to distorted DNA. Box B, with its two DNA-intercalating residues, introduces the greatest bend, whereas box A fails to bend DNA effectively [41, 45]. Box A has the greatest preference for distorted DNA due to stacking of the helix II bending wedge on an exposed base pair, whereas the affinity of box B for distorted DNA is only modestly greater than its affinity for duplex DNA [45, 46]. Accordingly, the presence of a hydrophobic DNA intercalating residue in position 16 of helix I appears to be important for bending, while a bending wedge in helix II may be required for selective recognition of distorted DNA.
We have found HMO1 to bend DNA but to have only modest preference for distorted DNA structures, including the 4-way junction. The HMO1 box A domain is highly divergent from the consensus, containing a five amino acid insertion in the middle of helix III, including a helix-breaking glycine that is likely to affect its structure and mode of DNA interaction. However, our data show that the box A domain has significant $\alpha$-helical content, consistent with an HMG-like fold, and that it is involved in direct DNA contacts (Figures 2.2 and 2.5). DNA bending by HMO1 would be consistent with the presence of hydrophobic residues in position 16 of both HMG boxes and the potential use of I37 of box A as an additional bending wedge. The limited preference of HMO1 for distorted DNA, combined with the low-affinity binding of HMO1-BoxA that is also only modestly enhanced by the presence of pre-bent DNA conformations, suggests that box A contributes the modest structure-specificity, while box B, which has no hydrophobic residue at position 37, confers most of the DNA binding affinity. The contribution of box A to DNA interactions is also consistent with the occluded site size for HMO1, which fails to form a stable complex on 18 bp DNA, but which forms two complexes on 37 bp DNA (Figure 2.4) as also seen for the mammalian two-HMG box protein HMGB1 [37].

Ligase-mediated circularization of small DNA fragments has been extensively utilized as a means of comparing the DNA bending activities of non-sequence specific DNA bending proteins. Both HMGB1/2 and NHP6A/B catalyze formation of 66 bp circles, although NHP6A/B are more efficient [13, 41, 47]. This high efficiency has been attributed to the relative stability of NHP6A/B-DNA complexes compared to HMGB1/2 and HU complexes [13, 41], but could also be due to a difference in the bend angle exerted by these proteins, with the angle exerted by NHP6A/B facilitating DNA strand
recognition by ligase [13]. HMO1 facilitated formation of circles only with DNA longer than 87 bp DNA (Figure 2.9), a lower efficiency that may correspond to either a reduced bend angle or a short residence time of HMO1 on linear DNA.

Work by Lu et al. [30] reports that HMO1 introduces negative supercoils into relaxed plasmid DNA, and that at high concentrations, HMO1 inhibits the unwinding reaction. We did not find evidence that HMO1 has the capability to introduce supercoils into relaxed plasmid DNA (Figure 2.10). In our work, we used recombinant HMO1, while Lu et al. purified HMO1 from yeast on the basis of its association with a DNA helicase activity, potentially resulting in isolation of posttranslationally modified protein [30]. SRY, which is a modestly sequence specific HMGB protein, also failed to supercoil relaxed DNA in the presence of topoisomerase I [45]. At position 37 of helix II where intercalating hydrophobic residues are located for non-sequence specific HMGB proteins, SRY has asparagine (Figure 2.1). Since the HMO1 box B and SRY both lack a hydrophobic residue at position 37, this may correlate with their intrinsic inability to supercoil relaxed DNA.

DNA minicircles most likely resemble possible distorted DNA targets, including features of chromatin structure, DNA topology during recombination, or bends introduced during transcription initiation. For HMO1, a much higher mass excess of plasmid DNA was required to compete for binding compared to complexes involving linear DNA (Figure 2.8). Presumably, the complex formed with the minicircle is less dynamic compared to complexes involving linear DNA. This increased residence time is probably a consequence of an optimized fit between the minicircle and HMO1. The more stable association of HMO1 with constrained DNA is consistent with its role in
maintaining the integrity of chromatin and in assembly of the rRNA preinitiation complex [30, 32].

References


CHAPTER 3
A ROLE FOR THE SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 BEYOND DNA BINDING

Introduction

High-Mobility-Group (HMG) proteins are a family of ubiquitous proteins that constitute a significant fraction of non-histone nuclear proteins and are grouped into three subfamilies, HMGA, HMGB, and HMGN [1-7]. These proteins are united by a common theme in that they bind DNA or nucleosomes, altering the local conformation, therefore increasing their accessibility and plasticity. Consequently, this facilitates and enhances various DNA-dependent activities such as transcription, replication, recombination and DNA repair (reviewed in [3, 6]). These three subfamilies have a unique protein signature and a characteristic functional motif, such as the HMG-box in HMGBs, the AT-hook in HMGAs, and the nucleosomal binding domain in HMGNs [2, 3, 6, 7].

HMGB proteins contain one or more homologous repeats of the ~80-amino acid sequence HMG box. They bind to DNA in the minor groove and bend DNA ~90° towards the major groove. HMGB proteins are classified into two families based on the abundance, function and DNA specificity of the HMG box [1, 3, 6, 8]. The moderately sequence specific family are transcription factors that usually contain a single HMG box, for example transcription factors such as sex-determining factor SRY and lymphoid enhancer factor LEF-1 [9, 10]. The non-sequence specific family binds DNA without sequence preference but instead structural preference. They are represented by the so-called architectural factors HMGB1/2, ubiquitous proteins in vertebrates, and the Saccharomyces cerevisiae non-histone chromosomal protein 6A and 6B (NHP6A/B) [11].
A recent global analysis of protein expression in yeast showed HMGB proteins NHP6A/B and HMO1/2 to be present in the nucleus at moderately abundant levels [12]. NHP6A contains only one HMG box, which is 45% identical to rat HMGB1 box B and 80% identical to NHP6B [13]. NHP6B is a functional paralog of NHP6A, and their biological function was determined using gene deletion experiments; a phenotype is only observed in the \textit{nhp6a/b} double knockout. Double mutant (\textit{nhp6\AA}) cells exhibit 50% reduction in growth rate at 30°C and cannot grow at 37°C in the absence of osmotic support [14]. NHP6 has multiple roles in transcription, including transcriptional initiation and elongation by Pol II and transcription by Pol III [15, 16]. For example, it has been shown that the \textit{nhp6\AA} growth defect can be suppressed by a multicopy plasmid with either \textit{SNR6} or \textit{BRF1} [15]; \textit{SNR6} encodes the U6 RNA required for mRNA splicing, and it is suggested that a deficiency in \textit{SNR6} RNA contributes to the temperature-sensitive growth defect seen in \textit{nhp6\AA}. BRF1 is the limiting component in TFIIIB, a factor required for Pol III transcription [17]. It is therefore likely that overexpression of BRF1 increases \textit{SNR6} expression which in turn facilitates the growth of \textit{nhp6\AA} at 37°C. Overexpression of TATA-binding protein (TBP) also suppresses the temperature-sensitive growth defect of \textit{nhp6\AA} [18]. TBP overexpression could suppress the \textit{nhp6\AA} growth defect by affecting either Pol II or Pol III transcription because in addition to its well-documented role in Pol II transcription, TBP is also a component of the RNA Pol III initiation factor TFIIIB[16, 19]. It has also been demonstrated that NHP6 is required for maximal transcription of the \textit{HO} endonuclease gene [20, 21]. For its role in elongation, NHP6 interacts genetically and biochemically with SPT16/POB3 [22, 23], the yeast equivalent of FACT (FAcilitates Chromatin Transcription, which is an accessory factor.
present in HeLa cell nuclear extract) that promotes elongation through chromatin templates [24]. NHP6 is required for SPT16/POB3 to bind to nucleosomes [23, 25].

HMO1 and HMO2 are ~25 kDa HMG-box proteins, identified by homology-based motif-prediction programs as having only a single HMG-box, in a position corresponding to box B of mammalian HMGB. However, HMO1 also contains an N-terminal box A domain with weak similarity to consensus HMG-box domains, which was recently shown to participate in DNA binding [26], and a long run of basic residues in the C-terminus, not found in any other HMGB proteins. Strains bearing \( \textit{HMO1} \) or \( \textit{HMO2} \) mutant alleles are viable, although HMO1 mutant strains have growth defects, compromised plasmid maintenance and nuclease sensitive chromatin, suggesting that HMO1 may play a role in stability of the chromatin structure. A double knock-out of \( \textit{HMO1} \sim \textit{HMO2} \) could not be rescued by addition of an osmotic stabilizer to the medium at 37°C as with \( \textit{nhp6} \Delta \Delta \), indicating that the mutant was not defective in osmotic tolerance [27]. These authors also did not find any apparent synthetic phenotype of a triple mutant \( \textit{nhp6a}, \textit{nhp6b}, \textit{hmo1} \) suggesting that there is no functional overlap of these proteins in the cells. \textit{In vivo}, the HMO1 C-terminus is dispensible for function whereas HMG Box B is essential [27]. As the basic C-terminus is predicted to harbor the nuclear localization signal, an important implication of this observation is that the growth phenotype characteristic of the \( \textit{hmo1} \) knockout may be reversed by HMO1 mutant protein that is confined to the cytoplasm. Immuno-localization experiments have shown that full length HMO1 is present both in the nucleus and cytoplasm [27].

Here we report generation of various combinations of \( \textit{NHP6}, \textit{HMO1} \) and \( \textit{HMO2} \) mutant strains. It is evident from the data obtained that certain \( \textit{HMO2} \) mutant strains have
a slower growth phenotype, contrary to the previous report [27]. Using fusions to green fluorescent protein (GFP), we show that a HMO1 mutant deleted for the C-terminal tail (HMO1-boxAB) was localized to the cytoplasm. In contrast, HMO1-GFP was distributed both in the nucleus and the cytoplasm, as previously reported [27, 28]. As also reported by Lu et al. [27], we find that growth rates of yeast strains expressing HMO1-GFP and HMO1-boxAB-GFP were the same. This is indicative of essential and unique functions of HMO1 protein in the cytoplasm, and not in the nucleus.

**Experimental Procedures**

**Strains, gene disruption and expression**

All the original strains used are listed in Table 3.1A and some of the generated strains are listed in Table 3.1B. Standard genetic methods were used for strain construction and gene disruption. Prototroph selection markers \textit{TRP1} and \textit{URA3} were amplified from pRS424 and pRS426 plasmids, previously constructed and published by Christianson et al. [29]. The of primers used for \textit{HMO1} and \textit{HMO2} gene disruptions are listed in Table 3.2A. To amplify the selection marker with homologous sequence (~80 nucleotides) upstream and downstream of \textit{HMO1} for gene disruption, HMO1F and HMO1R were used. For \textit{HMO2} gene disruption, HMO2F and HMO2R primers were used. Ten µg of PCR product was used to transform DDY3 haploid yeast cells selecting for tryptophan or uracil prototrophy. Transformants were assayed by genomic PCR with one primer specific for chromosomal sequence upstream of the HMO1 or HMO2 open reading frames and the second primer (universal) specific for the integrated selection marker, to determine whether the selection marker had integrated at the appropriate locus (Figure 3.1). The primer used to amplify the upstream region of \textit{HMO1} was HMO1UF
Table 3.1 Yeast strains used in the experiments. A. Original strains. B. Yeast strains generated in this work.

**A**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY3</td>
<td>$MATa$ ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 GAL can1-100</td>
</tr>
<tr>
<td>DDY587</td>
<td>$MATa$ ade2 can1 his3 leu2 LYS2 trp1 ura3 nhp6a:URA3 nhp6b:HIS3</td>
</tr>
<tr>
<td>DDY591</td>
<td>$MATa$ ade2 can1 his3 leu2 LYS2 trp1 ura3 nhp6a:KanMX nhp6b:ADE2</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY1295</td>
<td>$MATa$ ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmo1Δ::TRP1</td>
</tr>
<tr>
<td>DDY1301</td>
<td>$MATa$ ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmo2::URA3</td>
</tr>
<tr>
<td>DDY1329</td>
<td>$MATa$ ADE2 his3 leu2 LYS2 trp1 ura3 hmo1Δ::TRP1 nhp6b:HIS3</td>
</tr>
<tr>
<td>DDY1333</td>
<td>$MATa$ ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmo2::URA3 nhp6a:KanMX</td>
</tr>
<tr>
<td>DDY1385</td>
<td>$MATa$ ade2 can1 his3 leu2 LYS2 trp1 ura3 hmo1Δ::TRP1 hmo2Δ::URA3 nhp6a:KanMX nhp6b:HIS3</td>
</tr>
<tr>
<td>DDY1386</td>
<td>$MATa$ ADE2 his3 leu2 lys2Δ trp1 ura3 hmo1Δ::TRP1 hmo2Δ::URA3 nhp6a:KanMX nhp6b:HIS3</td>
</tr>
<tr>
<td>DDY1387</td>
<td>$MATa$ ADE2 his3 leu2 LYS2 trp1 ura3 hmo1Δ::TRP1 hmo2Δ::URA3 nhp6a:KanMX nhp6b:HIS3</td>
</tr>
<tr>
<td>DDY1429</td>
<td>$MATα$ ADE2 his3 leu2 lys2Δ trp1 ura3 hmo1Δ::TRP1 hmo2Δ::URA3</td>
</tr>
<tr>
<td>DDY1430</td>
<td>$MATa$ ADE2 his3 leu2 lys2Δ trp1 ura3 hmo1Δ::TRP1 hmo2Δ::URA3</td>
</tr>
</tbody>
</table>
Table 3.1 List of primers used. A. Primers used for to disrupt \textit{HMO1} and \textit{HMO2} genes in the original DDY3 strain. B. Primers used for generation of GFP-tagged HMO1 and HMO1-boxAB protein. Nucleotide sequence in upper case is the sequence homology to the desired chromosomal insertion site and the lower case is the sequence homology to the vector pFA6a-GFP.

A. PCR primers used for gene disruption

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMO1F</td>
<td>CCCAGCGGGCCCTCAGGGCTTACTGCCTTTTATATCTCTATTAGATGTACATCC</td>
</tr>
<tr>
<td></td>
<td>TACCACACCAACAAGCTGTCACACCCAGATGGTACATCC</td>
</tr>
<tr>
<td>HMO1R</td>
<td>TATATTTTATCCCTTTTTTTATTATTTATTTATTTGAAAGAACGATAGAGT</td>
</tr>
<tr>
<td></td>
<td>ATATAGTAACGAGTTTGTCCGTCCATCACTCCTTTACGCACTGTGCCG</td>
</tr>
<tr>
<td>HMO2F</td>
<td>GCACATATATATATCTGTTCTAGGGAATTGGATGAGAACGAGCACCCAGG</td>
</tr>
<tr>
<td></td>
<td>ATATAGTAGTAAATACGATAAGCAGATGAGTAGATGAGTAGGAGTAGCG</td>
</tr>
<tr>
<td>HMO2R</td>
<td>TTGAACAAATTTCTGGATAAAAGCGCTTAGAATGGTCAGATCTTCAAGAAA</td>
</tr>
<tr>
<td></td>
<td>ATAGAATAAATGGGATTTTATTTTCTCTTACGCACTGTGCCG</td>
</tr>
<tr>
<td>HMO1UF</td>
<td>ACCCGACTCGATTTATCTACC</td>
</tr>
<tr>
<td>HMO2UF</td>
<td>GTGCTAAGAATACTCTATGCAGG</td>
</tr>
<tr>
<td>UU</td>
<td>GACTCTCAGTAAATCTGC</td>
</tr>
<tr>
<td>HMO1DF</td>
<td>GGATATGAGTTTCTGGAAACAC</td>
</tr>
<tr>
<td>HMO2DF</td>
<td>TGGTAAATGAAAGCCACGTCTAG</td>
</tr>
<tr>
<td>DD</td>
<td>CCGCACAGATGCGTAAGGAG</td>
</tr>
</tbody>
</table>

B. PCR primers used for generation of GFP-tagged protein

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMO1-GFPF</td>
<td>GAAGAAGGATAAGAAGAAGGACAAATTCAACTCTTCTATTggtegacggatcccccggg</td>
</tr>
<tr>
<td>HMO1-GFPR</td>
<td>AGAAAGACAGTGAGAAAAATATAACGTGACGAGGTTCCTCCGTCAtcgatgaattcaggttttt</td>
</tr>
<tr>
<td>boxAB-GFPF</td>
<td>CCAACTCATGCGCCGGGTATCCAATTCTTTTACAGGCGACGCCggtegacggatccggg</td>
</tr>
</tbody>
</table>
**Figure 3.1 Strategy for generation of HMO1 or HMO2 knockout.** DDY 3 cells were transformed with PCR products containing the selectable marker gene and sequence homologous to the upstream and downstream of gene of interest (black region on the PCR product), HMO1 or HMO2. Through homologous recombination, HMO1 or HMO2 was knocked out and replaced with the integrated selectable marker. Transformants were assayed by genomic PCR with one primer specific for chromosomal sequence upstream of the HMO1 or HMO2 open reading frames and the second primer (universal) specific for the integrated selection marker, to determine whether the selection marker had integrated at the appropriate locus. The position and direction of the primers is shown as arrows.
and HMO2 was HMO2UF. The upstream universal reverse primer was UU. The sequence of the primer used to amplify the downstream region for HMO1 was HMO1DF and HMO2 was HMO2DF and the downstream universal reverse primer sequence DD (Table 3.2A). The hmo1Δ (DDY1295) and hmo2Δ (DDY1301) mutant strains were generated. DDY1329 was generated by mating DDY587 with DDY1295 and selecting for prototrophy tryptophan (HMO1) and histidine 3 (NHP6B). DDY1333 was generated by mating DDY591 with DDY1301 and selecting for uracil prototrophy (HMO2) and antibiotic Kanamycin antibiotic resistance (NHP6A). DDY1333 and DDY1329 cells were mated and grown in sporulation media for tetrad dissection.

Construction and analysis of GFP-tagged HMO1 and HMO1-boxAB

HMO1 was C-terminally tagged with GFP at its chromosomal location through oligonucleotide-directed homologous recombination (Figure 3.2). To generate HMO1-GFP, a pair of oligonucleotides were generated that had homology to the C-terminus of HMO1 (upper case in the primer sequence), which was the desired chromosomal insertion site at the 5' end of each primer and homology to vector pFA6a-GFP (F64L, S65T, R80Q, V163A)-His3MX6 (lower case in the primer sequence) containing the GFP tag and Histidine 3 as the auxotrophic marker at the 3' end. Vector pFA6a-GFP was kindly provided by Dr. Mark Longtine [30]. To generate HMO1-GFP, the forward primer used was HMO1-GFPF and the reverse primer used was HMO1-GFPR. To generate HMO1-boxAB, the forward primer was designed such that the sequence homology of the oligonucleotides deleted 36 amino acids from the C-terminus. The forward primer used was boxAB-GFPF and the same reverse primer as for HMO1 was used. The PCR products were used to transform DDY3 cells. Cells were grown in YMD-His media.
Figure 3.2 Strategy for generation of HMO1-GFP or HMO1-boxAB-GFP fusion protein. DDY cells were transformed with PCR products containing the GFP tag and HIS3 auxotrophic marker gene. The GFP was inserted at the C terminus of HMO1 or HMO1-boxAB through homologous recombination, yielding a C-terminally GFP-tagged protein.
(minimal media lacking histidine), to select for cells that had integrated the auxotrophic selection marker gene into their chromosome. Transformants were assayed by genomic PCR with one primer specific for the GFP tag and a second specific for the HMO1/HMO1-boxAB open reading frame, to determine whether the cassette had integrated at the appropriate locus. The PCR product obtained was sequenced to ensure that the gene cassette had been integrated at the appropriate locus in-frame. The cells were then analyzed by multiple wavelength fluorescence and visible light microscopy with digital imaging-capable using at x40 magnification.

Results

Characterization of mutant phenotypes

DDY1333 (hmo2, nhp6a) was mated with DDY1329 (hmo1, nhp6b), sporulated and subjected to tetrad analysis (Figure 3.3). Four viable spores were obtained showing that none of the four genes are essential for viability as previously shown [27]. Colonies were visually analyzed, looking at the sizes and light intensity. Table 3.3 shows the genes that were inactivated from each spore in Figure 3.3. Although the quadruple knockout was viable, it gave rise to the smallest colonies (A2, data not shown). Triple knockouts C1 (hmo1, hmo2, nhp6a) and A5 (hmo1, nhp6a, nhp6b) gave the second smallest colonies after the quadruple knockout. The Δnhp6a, Δnhp6b and Δhmo2 single knockouts did not show any phenotype (compare B2, C2, D3, A4, B5, C5 to the wildtype A1). Interestingly, when the HMO2 gene was deleted in the context of an HMO1 knockout, slightly smaller colonies with lower light intensity compared to Δhmo1 (D2, C3) were obtained (A3, D4, A6). It is also noteworthy to compare colony C1 to B4; the slightly
Figure 3.3 **Phenotype of various mutants from a tetrad dissection.** DDY1333 was mated with DDY1329, sporulated and subjected to tetrad analysis. Spores were germinated and grown on YPD plate for 3 days at 30°C. The genotype of each spore was scored using the standard method, by replica plating to minimal media lacking the nutrient corresponding to each marker gene (or containing G418 for the KanMX marker).
<table>
<thead>
<tr>
<th>Spore location in Figure 3.2</th>
<th>Genes Knocked out</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>None</td>
</tr>
<tr>
<td>B1</td>
<td>NHP6A, NHP6B, HMO2</td>
</tr>
<tr>
<td>C1</td>
<td>NHP6A, HMO1, HMO2</td>
</tr>
<tr>
<td>D1</td>
<td>HMO1, HMO2</td>
</tr>
<tr>
<td>A2</td>
<td>NHP6A, NHP6B, HMO1, HMO2</td>
</tr>
<tr>
<td>B2</td>
<td>NHP6A</td>
</tr>
<tr>
<td>C2</td>
<td>NHP6B, HMO2</td>
</tr>
<tr>
<td>D2</td>
<td>HMO1</td>
</tr>
<tr>
<td>A3</td>
<td>HMO1, HMO2</td>
</tr>
<tr>
<td>B3</td>
<td>NHP6A, NHP6B, HMO2</td>
</tr>
<tr>
<td>C3</td>
<td>HMO1</td>
</tr>
<tr>
<td>D3</td>
<td>NHP6A, HMO2</td>
</tr>
<tr>
<td>A4</td>
<td>NHP6B, HMO2</td>
</tr>
<tr>
<td>B4</td>
<td>NHP6A, HMO1</td>
</tr>
<tr>
<td>C4</td>
<td>NHP6A, NHP6B</td>
</tr>
<tr>
<td>D4</td>
<td>HMO1, HMO2</td>
</tr>
<tr>
<td>A5</td>
<td>NHP6A, NHP6B, HMO1</td>
</tr>
<tr>
<td>B5</td>
<td>HMO2</td>
</tr>
<tr>
<td>C5</td>
<td>NHP6A</td>
</tr>
<tr>
<td>D5</td>
<td>NHP6B, HMO1, HMO2</td>
</tr>
<tr>
<td>A6</td>
<td>HMO1, HMO2</td>
</tr>
<tr>
<td>B6</td>
<td>NHP6A</td>
</tr>
<tr>
<td>C6</td>
<td>HMO1, HMO2, NHP6B</td>
</tr>
<tr>
<td>D6</td>
<td>NHP6A, NHP6B</td>
</tr>
</tbody>
</table>
larger B4 colony is Δnhp6a Δhmo1, while C1 is Δnhp6a Δhmo1 Δhmo2, indicating that the difference in size is due to a Δhmo2 phenotype. Δhmo1 Δhmo2 (A3, D4, A6) have smaller colonies with less light intensity compared to ΔΔnhp6 (C4, D6) whereas Δhmo1 (D2, C3) compares to ΔΔnhp6. While the previously reported absence of a growth phenotype upon inactivation of hmo2 was inferred based on comparison to wild type cells, our data show that a growth phenotype may be detected in an hmo1 mutant background.

**Localization of HMO1-GFP and HMO1-boxAB-GFP in yeast cells**

HMO1 has previously been shown to be both a nuclear and cytoplasmic protein [27, 31]. In these experiments, we confirm the localization of HMO1-GFP to both the nucleus and the cytoplasm. However, HMO1-boxAB-GFP was localized only in the cytoplasm (Figure 3.4). Lu et al. [27] showed that a plasmid expressing the full length HMO1 or HMO1-boxAB complemented the hmo1 mutant phenotype, but a plasmid expressing HMO1 deleted for 30 amino acids of box B did not. This implies that the ability of HMO1 to rescue the hmo1 mutant phenotype is in the cytoplasm and not in the nucleus. This also confirms that the Nuclear Localization Signal (NLS) of HMO1 is located at the C-terminus as predicted by the NLS prediction program on the [www.expasy.org](http://www.expasy.org) website.

**Discussion**

**Growth phenotype**

HMO1 was first identified by its co-purification with a DNA helicase. Genetically, HMO1 was shown to be required for normal growth, plasmid maintenance and for regulating susceptibility of yeast chromatin to nuclease [27]. These authors
Figure 3.4 *In vivo* localization of HMO1-GFP and HMO1-boxAB-GFP proteins. A. HMO1-GFP is distributed both in the nucleus and the cytoplasm. The image on the right panel is the face contrast shot of the same cell. B. HMO1-boxAB-GFP is distributed in the cytoplasm. The image on the right panel is the face contrast shot of the same cell.
proposed that HMO1 binds to the small linker DNA of yeast, stabilizing a higher-order chromatin structure which is important for normal gene expression and/or DNA replication. Dolinski and Heitmain [31] showed that HMO1 interacts genetically and physically with FKBP12 Prolyl Isomerase. HMO1 has also recently been identified as a genuine RNA polymerase I, factor acting synergistically with Rpa49 (DNA-directed RNA polymerase I 49 kDa polypeptide, which is a non-essential but conserved subunit of RNA polymerase I corresponding to the animal RNA polymerase factor PAF53) during rDNA transcription [32]. In this study, we have shown that the unique functions of HMO1 are confined to the cytoplasm. Lu et al. [27] noted that HMO1 mutants were viable most likely because other proteins performed the essential functions of HMO1, although they might lack the abundance or binding properties to optimally replace loss of HMO1. In fact, a recent global analysis of protein expression in yeast revealed HMO1 as the most abundant HMGB protein in yeast [33]. By virtue of it abundance, HMO1 can likely protect the chromatin from nuclease digestion as observed for hmo1Δ [27]. These authors also showed that NHP6 had non-overlapping functions with HMO1 in the cell. Therefore, it is most likely that HMO2 would replace the function of HMO1 in the cell. In comparison to other HMGB proteins, both HMO proteins have a divergent A box where conserved and charged residues have been replaced by unrelated residues. The B box however, is highly conserved showing higher identity with the consensus. HMO2 lacks the unique highly basic C-terminal tail found in HMO1. Interestingly, we have observed a slight slow growth HMO2 phenotype that is only evident in hmo1 hmo2 mutant strains (Figure 3.2, Table 3.2). Δhmo2 alone does not show any apparent phenotype, most likely because the more abundantly expressed HMO1 takes over the
function of HMO2. In addition, the lack of a synthetic phenotype between *NHP6* and *HMO2* suggests that they have non-overlapping functions in the cell as well (Figure 3.2, compare spores B3 to D6), further confirming that HMO1 is the likely protein that replaces the function of HMO2 in ∆*hmo2* cells, and HMO2 replaces some of the functions of HMO1 in ∆*hmo1* cells. Overexpression of HMO2 in ∆*hmo1* strains would address this hypothesis.

**Cellular localization**

The C-terminus of HMO1 is dispensable for function *in vivo* [27]. These authors showed that a plasmid expressing the full length HMO1 or HMO1-boxAB complemented the mutant phenotype. Interestingly, we have localized HMO1-boxAB to the cytoplasm (Figure 3.4). This is a clear indication that the essential and unique functions of HMO1 are located in the cytoplasm and not in the nucleus. This data is supported by the fact that HMO1 genetically and physically interacts with cytoplasmically expressed FKBP12 Prolyl Isomerase, a product of the *FPR1* gene, which when mutated has been shown to be synthetic lethal with ∆*hmo1*[31]. We have previously shown that HMO1 has distinct DNA binding and bending properties from other HMGB proteins, consistent with its unique role *in vivo* [26]. Although HMO1 was speculated to play a role in organizing target structures for yeast RNA polymerase I-dependent transcription, direct interaction between HMO1 and Rpa49 or RNA polymerase I was not detected [32]. *S. cerevisiae* SPT16/CDC68 and POB3 (Polymerase one-binding) form a heterodimer, localizes to the nucleus and have been shown to affect DNA replication and transcription globally [23, 33, 34, 35]. *STP16* and *POB3* show strong genetic interactions with *NHP6* but only minor interactions with *HMO1* or *HMO2* [23, 27].
Lu et al.[27] described HMO1 as a likely homolog of HMGB1/2. HMGB1 has been considered as a ubiquitous nuclear protein with an architectural function, but it had also been described to be present in the cytoplasm [36]. Today, extranuclear and extracellular functions of HMGB1 have just started to come into focus. It participates in developmental and differentiation processes, triggers and modulates many of the inflammatory cascades in the body, and may even be involved in the metastatic invasion programme of cancer cells (reviewed in [37]). Similarly, our data indicate that HMO1 has extracellular functions which may or may not be similar to those of HMGB1. Notably, signaling by TOR (Target of Rapamycin) kinases is central to environmental stress responses in yeast. Suppression of TOR signaling, for example through inhibition of TOR kinases by interaction with FKBP12-rapamycin, leads to cell cycle arrest (reviewed in [38]). The reported interaction between HMO1 and FKBP12 suggest a possible role for HMO1 in such signaling pathways. Studies to characterize the extranuclear function of HMO1 will further expand signaling roles of HMGB proteins beyond HMGB1, which thus far is the only HMGB protein that has been shown to have signaling capabilities, expanding the importance of HMGB proteins across organisms.

References


CHAPTER 4

PHOSPHORYLATION OF SACCHAROMYCES CEREVISIAE HIGH MOBILITY GROUP BOX PROTEIN HMO1 BY CASEIN KINASE II AND THE ROLE OF THE C-TERMINAL DOMAIN IN DNA BINDING AND PROTEIN STABILITY

Introduction

High-Mobility-Group (HMG) proteins are relatively abundant non-histone nuclear proteins with one or more homologous DNA binding domains, the HMG box, each of ~80-amino acid residues. HMGB proteins are classified into two families based on their abundance, function and DNA specificity [1-5]. Some are transcription factors that bind DNA with sequence-specificity; they usually contain a single HMG box and are expressed in a few cell types [6]. They are exemplified by sex-determining factor SRY and lymphoid enhancer factor LEF-1 [7, 8]. The non-sequence-specific family binds DNA without sequence preference but instead structural preference. They are typified by HMGB1/2, ubiquitous, vertebrate, non-sequence specific HMGB proteins, and Saccharomyces cerevisiae non-histone chromosomal proteins 6A and 6B (NHP6A/B) [3, 9, 10]. The role of HMGB1 in regulation of transcription, either by remodeling chromatin and nucleosome structure or through direct interaction with transcription factors such as Hox, steroid hormone receptors, p53, NF-κB, and TBP is well documented (reviewed in [11]). The HMGB1 knockout mouse dies shortly after birth due to hypoglycemia from a defect in the transcriptional function of the glucocorticoid receptor [12]. Outside the cell, HMGB1 functions as a cytokine [13, 14] and is a ligand for a membrane receptor, RAGE [15, 16].

The structures of HMG boxes both from the non-sequence and the sequence specific subfamilies have been determined in the presence and/or absence of DNA,
revealing a general global fold, characterized by a 3-dimensional, L-shaped structure with three $\alpha$-helices (reviewed in [11, 12]). The HMG box binds to the DNA minor groove by partial intercalation of one or two conserved hydrophobic residues, causing the DNA to be highly distorted, resulting in a sharp bend and helical underwinding [8, 17, 18, 19, 20].

Posttranslational modification of HMGB proteins has been reported, but the functional significance of these modifications, except for the acetylation, is unknown [21]. Phosphorylation of HMG proteins appears to be a common posttranslational modification that is found in evolutionarily distant organisms [22]. For example, pulse-labeling studies on phosphorylation of the Chironomus HMGB1a and HMGB1b proteins showed that these proteins are phosphorylated by protein kinase C [23] and by Casein kinase II (CKII) [22]. Broccoli (Brassica oleracea) HMGB protein was found to co-purify with CKII with very high affinity and was efficiently phosphorylated even in the presence of an excess of exogenous substrates [24]. CKII$\alpha$ phosphorylates the acidic C-terminal domain of the HMGB1 and HMGB2/3 proteins [25]. HMG phosphorylation appears to alter the conformation, stability, and DNA binding properties of these proteins and is therefore essential for their function. For example, DNA supercoiling properties of HMGB1 were shown to be modified by phosphorylation [25, 26].

Basic extensions found either C- or N-terminal to the HMG box are known to increase the bending capacity and DNA-binding affinity of the HMG box. For example, the 16 basic amino acids found at the N-terminus of the NHP6A HMG box is required for stable complex formation on both linear DNA and DNA minicircles [20]. Basic C-terminal extensions stabilize binding of HMG-D and LEF-1 by binding in the major groove opposite the distorted minor groove [8, 27].
The HMGB1/2 acidic tail has been shown to lower the affinity of the HMG boxes for most DNA substrates, such as linear DNA, supercoiled DNA and 4-way junction (reviewed in [28]). Interestingly, the tail is not important for structure-selective DNA-binding of the HMG boxes to DNA minicircles since it has little effect on the affinity for DNA minicircles compared to other DNA substrates \textit{in vitro} [29]. However, it was not established whether this is a consequence of interaction of the tail with the HMG box(es) or the basic extension region or simply charge repulsion. Thomsen \textit{et al.} [30] showed that interactions of the basic N-terminal and acidic C-terminal domains of the maize HMGB1 may contribute to regulation of the DNA interactions of the protein. A similar phenomenon was also observed for \textit{Arabidopsis} HMGB1 and HMGB4 [30]. This led the authors to argue that the interaction of the variable terminal domains of the different plant HMGB proteins could serve as a molecular mechanism involved in the fine tuning of the functional properties of these architectural proteins, depending on the structural requirements of specific nucleoprotein complexes.

\textit{S. cerevisiae} contains several HMG-box proteins. For example, HMO1 is identified by homology-based motif-prediction programs as having only a single HMG-box, in a position corresponding to box B of mammalian HMGB1. However, HMO1 also contains an N-terminal box A domain with weak similarity to consensus HMG-box domains. \textit{HMO1} and \textit{HMO2} genes are non-essential, but deletion of \textit{HMO1} gene confers growth defects, impaired plasmid maintenance and nuclease sensitive chromatin [31]. HMO1 has also been shown to interact with FKBPI2, an abundant protein that interacts with a number of cellular proteins, including the target of Rapamycin (TOR) kinases that, for example, regulate rDNA transcription [32, 33]. A recent study includes HMO1
directly in the rRNA transcription apparatus, where it was proposed to function in a similar capacity as the Upstream Binding Factor (UBF), an auxiliary factor in the mammalian and amphibian RNA Polymerase I transcription systems; UBF has six HMG domains [34].

We have previously shown that the divergent HMO1 box A domain participates in DNA binding, and that it contributes modest structure-specific DNA binding to HMO1, while box B confers most of the DNA binding affinity [35]. In this study we show that HMO1 and HMO1 deleted of its C-terminal tail (HMO1-boxAB) get phosphorylated by CKII, yielding proteins with reduced affinity for DNA. These data suggest that HMO1 can be regulated by posttranslational modification. We have also analyzed the role of the HMO1 basic C-terminal tail in DNA binding. Circular Dichroism (CD) Spectroscopy measurements of protein melting temperatures revealed that HMO1-boxAB has a higher melting temperature than the wild type, suggesting that the C-terminal domain destabilizes the protein.

**Experimental Procedures**

**Cloning and Purification of Proteins**

The gene encoding HMO1 was amplified from yeast genomic DNA and subcloned into pET28b for expression with an N-terminal His<sub>6</sub>-tag. HMO1-boxA was generated as previously described [35]. To generate HMO1-boxAB, PCR-based site-directed mutagenesis was used to replace Ala210 with a stop codon. The sequence of the primers used are available upon request. Recombinant plasmids were used to transform E. coli Rosetta Blue strain (Novagen). Proteins were purified as previously described [35].
Circular Dichroism Spectroscopy

CD spectra were recorded on an AVIV Model 202 CD spectrophotometer using a 1 cm pathlength sample cell. Wavelength scans from 200 nm to 300 nm were performed in triplicate, and data points were averaged and smoothed using standard methods. The wavelength scan for His6-tagged HMO1 was recorded at 25°C, with a protein concentration of 0.025 mg/ml in 2.5 mM NaH₂PO₄, pH 7.0, 0.5% glycerol, 10 mM NaCl. The wavelength scan for His6-tagged HMO1-BoxA was recorded at 4°C, using a protein concentration of 0.05 mg/ml in 1 mM Tris, pH 7.0, 0.03% Tween 20, 10% glycerol, 2.8 mM KCl. For measurement of thermal stability, protein was diluted to 0.05 mg/ml in CD buffer (20 mM potassium phosphate (pH 7), 8 µM EDTA, 0.2% glycerol, 3 mM KCl), and measurements were made using a quartz cuvette with a 1-cm path length. Ellipticity readings from 300 to 200 nm (1-nm steps) were taken over the temperature range 19–70 °C, with steps of 3 °C (19–37 and 61–70 °C) or 1.5 °C (37–61 °C). Each sample also underwent a reverse scan from 67 to 19 °C. Three minutes were allowed for thermal equilibration after each step. Wavelength scans from 300 to 200 nm were performed at 19 and 70 °C to verify the native and denatured states of the protein, respectively. CD measurements of protein melting were performed in triplicate and corrected for buffer contributions to the signal. Calculation of the $T_m$ of the proteins was made based on ellipticity measurements from 230 to 220 nm. Temperature-dependent ellipticity values were plotted at each wavelength and fit to a two-state model for protein unfolding [36]. The $T_m$ is reported as the average ± S.D.
Electrophoretic Mobility Shift Assays (EMSA) with 26 bp duplex DNA and 4-way junction DNA

Four-way junctions were constructed as previously described; the sequence of individual strands are as described [35, 37]. Briefly, the 4-way junction was prepared by annealing the four strands, followed by purification of the junction on native polyacrylamide gels. To ensure that all oligonucleotides were present, aliquots of the purified junction were run on denaturing gels. EMSA was performed by incubating reactions at room temperature in 10 µl reaction buffer containing 5 fmol DNA and varying amounts of protein. Samples were resolved on prerun 7% (w/v) native polyacrylamide gels (39:1 acrylamide:bisacrylamide) gels at 4°C with 0.5X TBE (50 mM Tris-borate, 1 mM EDTA) running buffer. Complexes were visualized by phosphorimaging. Experiments were done at least in duplicate, and values are reported as the mean ± standard deviation. For calculating fractional complex formation, the region on the gel from the slowest migrating complex to the free DNA was considered as complex and the binding isotherms for protein binding were fitted as described [35].

Ligase-Mediated Circularization

Ligation substrates were prepared by digestion of pET5a with BspHI to generate a 105 bp fragment. DNA was 5'-end labeled with T4 polynucleotide kinase and (γ-32P) ATP. Reactions were initiated by addition of 20 units of T4 DNA ligase to a final volume of 100 µl reaction buffer (20 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM MgCl2, 0.1 mM Na2EDTA, 1 mM DTT, 0.05% BRIJ58) containing 1200 fmol DNA, with or without the protein. Time points were taken at intervals over 30 minutes. For each time point, 8 µl of the reaction mixture was quenched by addition of 5 µl stop buffer (75 mM EDTA, 15% glycerol, 0.1% bromophenol blue (BPB), 0.1% xylene cyanol, 6 µg/µl proteinase K).
Samples were heated at 55°C for 15 minutes and resolved on prerun 7% (w/v) native polyacrylamide gels (39:1 acrylamide:bisacrylamide) gels at 4°C with 0.5X TBE. Quantitation was performed on a Molecular Dynamics Storm Phosphorimager using software supplied by the manufacturer.

**Protein phosphorylation**

Proteins were phosphorylated using Casein Kinase II (CKII, New England Biolabs) as per the manufactures recommendation. One µg of protein was incubated with 1 unit of Casein Kinase II (CKII) in 1X CKII buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂) supplemented with ³²P-ATP with a final specific activity of 200 µCi/µmol in 10 µl final volume, and incubated at 30°C for one hour. Samples were run on a 15% SDS-PAGE and quantification was performed on Molecular Dynamics Storm Phosphorimager.

**Protein cross-linking**

Protein was incubated with 0.1% glutaraldehyde in 10 mM sodium phosphate (pH 7.0) at room temperature for 5 minutes. Samples were diluted 1:2 with SDS-sample buffer, and analyzed by electrophoresis on 17% SDS-PAGE followed by Coomassie blue staining.

**Proteolytic digestion with Enterokinase and BNPS-skatole**

For Enterokinase digestion, 2 µg protein was incubated at room temperature with 0.4 U Enterokinase (Novagen) in cleavage capture buffer (20 mM Tris pH 7.5, 50 mM NaCl, 2 mM CaCl₂), and aliquots taken after various times. The cross-linking reaction was quenched by addition of 10 µl SDS-sample buffer and analyzed on 17% SDS-PAGE gels. Proteins were detected by staining with Coomassie brilliant blue. For BPNS-skatole
(2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindoleline) (Sigma) digestion, 2 µg of protein were incubated with 40 µl BPNS-skatole solution (1 mg/ml in 80% acetic acid and 0.1% phenol) in 35 µl acetic acid. After 36 h at room temperature, the samples were lyophilized and analyzed as described above.

**DNA Supercoiling**

Reactions contained 0.2 µg relaxed closed circular pUC18 plasmid, prepared by adding 2.5 units of Vaccinia topoisomerase I (Epicentre) to supercoiled pUC18 in relaxation buffer (50 mM Tris, pH 7.5, 2.5 mM MgCl₂, 100 mM NaCl and 0.1 mM EDTA). After 1.5 h at 37°C, varying amounts of protein were added and the reactions incubated for 1 h at 37°C. Reactions were quenched with 5 µl termination buffer (5X TBE, 5% SDS, 15% sucrose, 0.1% BPB, 0.1% xylene cyanol, 2 µg/µl proteinase K) and incubated for 1 h at 37°C. Samples were loaded on 1% 1X TBE agarose gels and electrophoresed at 2 V/cm for 12 h in 1X TBE buffer.

**Results**

**CD spectra and Melting temperature**

Recombinant N-terminally His₆-tagged HMO1, HMO1-boxAB and HMO1-boxA were purified to apparent homogeneity, as judged by Coomassie Blue-staining of SDS-PAGE gels (Figure 4.1, panel D). Based on CD spectra, we have previously shown that HMO1 and HMO1-boxA have high α-helical content [35]. However, the CD spectrum of HMO1-boxAB displayed only a single negative peak ellipticity at 222 nm (data not shown). In fact, this peak was only achieved in the presence of high detergent (0.1%
Figure 4.1 Thermal denaturation of HMO1 (A), HMO1-boxAB (B), HMO1-boxA (C). Ellipticity measurements were recorded from 4 °C to 90 °C at wavelengths from 221-230 nm. D, SDS-PAGE showing purified His6-HMO1-boxA, His6-HMO1-boxAB and His6-HMO1 in that order.
Tween 20 compared to 0.03% for HMO1-boxA) and low protein concentrations (0.02 mg/ml compared to 0.05 mg/ml for HMO1-boxA, suggesting protein aggregation). Thermal denaturations of HMO1 and HMO1-boxAB were biphasic, a process consistent with a protein containing two distinct domains (Figure 4.1). HMO1 and HMO1-boxAB had melting temperatures of 23.5 ± 0.4°C and 57.6 ± 0.8°C, and 48.2 ± 2.0°C and 71.7 ± 0.7°C respectively. However, HMO1-boxA melting occurred in a single transition at 52.2 ± 0.2°C, indicative of unfolding of a single domain (Figure 4.1). HMO1-boxAB had a higher melting temperature than HMO1; in addition, HMO1-boxA had a relatively high melting temperature compared to HMO1. This is an indication that the C-terminal tail destabilizes the protein. However, it is also possible that with deletion of the C-terminal tail, one domain became more stable (melting temperature increased from 23.5°C to 71.7°C) while the other domain became less stable (melting temperature decreased from 57.6°C to 48.2°C). Arguments to support the likelihood that the first transition is melting of box A and the second transition is melting of box B is presented in the discussion.

HMO1-boxAB has lower affinity for DNA compared to the wild type protein

DNA binding was analyzed with Electrophoretic Mobility Shift Assays (EMSA). We have previously shown that HMO1 has only modest preference for DNA with altered conformations including the 4-way junction, which it binds with a half-maximal of 4.9 ± 1.5 nM (see figure 4 in [35]). HMO1-boxAB bound 4-way junction with a half-maximal saturation of 27.9 ± 1.4 nM (Figure 4.2). The half-maximal values implicate participation of C-terminal tail in DNA binding, directly or indirectly. Basic extension regions of HMGB proteins have been shown to contribute to DNA binding properties [28, 30, 39].
Figure 4.2 Binding of HMO1 (panel A) and HMO1boxAB (panel B) to 4-way junction DNA. Reactions contain 5 fmol DNA, and protein concentrations are 0, 5, 7.5, 10, 25, 30, 35, 40, 50, 100 and 120 nM. B, binding isotherms for HMO1 (solid line) and HMO1-boxAB (broken line) on the 4-way junction DNA. Reactions were done in duplicate; values are reported as the mean ± S.D.
HMO1-boxAB does not bend DNA

Ligase-mediated circularization assays were performed where the efficiency with which T4 DNA ligase mediates ring closure of DNA fragments that are shorter than the persistence length is measured [20, 40]. The ability of HMO1-boxAB to enhance ligase-mediated DNA circularization was qualitatively assessed with 105 duplex DNA. HMO1-boxAB did not facilitate formation of minicircles, unlike HMO1 (Figure 4.3, [35]). It has been shown that deletion of the basic N-terminus of NHP6A yields a protein with low affinity for linear DNA that retains the ability to convert 40% of a 98 bp DNA fragment into minicircles, compared to 70% for the wild type [20]. In contrast, HMO1-boxAB bound linear duplex DNA with an affinity close to that of the wild type protein (data not shown), but could not bend the DNA. This shows that HMO1 and NHP6A bind and bend DNA differently. Conversely, incubation of DNA with C-terminal truncated HMGB1/2 yielded DNA minicircles at lower concentrations, probably reflecting the high affinity for linear duplex DNA of these mutant proteins [28].

HMO1 and HMO1-boxAB are substrates for CKII

Using programs available for prediction of protein phosphorylation sites (www.expasy.com), HMO1 was predicted to have many Ser/Thr residues that can be phosphorylated by different kinases. CKII is a structurally and functionally conserved enzyme that is widely distributed among eukaryotic organisms and previously shown to phosphorylate HMGB1. S. cerevisiae has four essential genes that make up the protein kinase CKII complex, CKA1, CHA2, CKB1 and CKB2. We therefore chose to use CKII for analysis of HMO1. CKII is predicted to target six potential HMO1 phosphorylation
Figure 4.3 **Time-course ligation assay with 105 bp DNA.** 100 fmol 105 bp linear DNA was incubated with 1250 nM protein (HMO1 top panel and HMO1-boxAB lower panel) up to 30 minutes. Time points were taken at 0, 0.5, 1, 2.5, 5, 10, 20, and 30 min. Exonuclease III was added to reactions in the last lane at 30 min time-points. Circular ligation products (the last lane top panel) were resistant to digestion by Exonuclease III.
sites at positions, Ser11, Ser19, Ser33, Try54, Ser137 and Ser153. Equal amounts of HMO1, HMO1-boxA and HMO1-boxAB were reacted with CKII and $^{32}$P-ATP. The phosphorylated proteins were resolved on SDS-PAGE gel, and $^{32}$P incorporation was determined using phosphorimaging (Figure 4.4). HMO1 and HMO1-boxAB were phosphorylated, but HMO1-boxA was not. This data suggests that Ser137 and/or Ser153 are the phosphorylation sites for HMO1 by CKII, as these sites are located in the box B domain. CKII is a 130 kDa protein with $\alpha_2\beta_2$ tetrameric structure. The 44 kDa $\alpha$ subunit is catalytic and the 26 kDa $\beta$ subunit is thought to have regulatory properties, but it also gets autophosphorylated. HMO1-boxAB migrates at the same rate as the $\beta$ subunit; hence it was not possible to resolve its phosphorylation on this gel. EMSA was carried out to compare binding to 4-way junction DNA by HMO1 and HMO1-boxAB before and after phosphorylation. Phosphorylated proteins showed lower DNA binding affinity (Figure 4.4). To investigate if phosphorylation of HMO1 changed its DNA bending properties, ligase-mediated circularization assays were performed with phosphorylated HMO1 (data not shown). There was no discernable difference in minicircle formation between phosphorylated and non-phosphorylated HMO1.

**HMO1 forms oligomeric complex in solution**

Based on the molecular mass of HMO1 eluting from a sizing column, Lu et al. had predicted that HMO1 exists as an oligomeric complex in solution [31]. Cross-linking reagents can provide the means for capturing protein:protein complexes by covalently bonding them together as they interact. When HMO1 and HMO1-boxAB were incubated with 0.1% glutaraldehyde, they formed oligomeric complexes, as judged by SDS-PAGE.
However, HMO1-boxA formed oligomeric complexes, with dimers clearly forming (Figure 4.5).

Figure 4.4 (A) Phosphorylation of HMO1. Using $^{31}$P-ATP, HMO1 was phosphorylated with CKII, resolved on SDS-PAGE and scanned on a PhosphorImager. Lane 1 shows autophosphorylation of CKII. Lane 2 shows HMO1 phosphorylated. (B) DNA binding by phosphorylated HMO1 and HMO1-boxAB. HMO1 and HMO1-boxAb were phosphorylated and then used for EMSA with 4-way junction DNA. Lane 1 was the control with no protein. Increasing concentration (10, 20 or 40 nM) of phosphorylated or protein that has not been phosphorylated was used.
Figure 4.5 SDS-PAGE showing HMO1-boxA cross-linking. Protein was incubated with 0.1% glutaraldehyde and time points taken at 2.5, 5 and 7.5 minutes, Lanes 2-4. Lane 1 has no glutaraldehyde. Reactions were stopped by adding 10µl SDS-PAGE sample buffer and loaded on a 17% gel.
Protein cleavage analysis

Many proteins undergo significant conformational changes after binding to a ligand. Such changes can be assessed by cleaving the protein at different locations using peptide cleavage reagents. Using programs available for prediction of peptide cleavage sites (www.expasy.com), HMO1 was predicted to have 2 Enterokinase cleavage sites (Lys48, Lys84) and 2 BNPS-skatole [2-(2-nitrophenylsulfenyl)-3-methylindole] cleavage sites (Trp149, Trp160). Enterokinase is a serine protease that recognizes the amino acid sequence -Asp-Asp-Asp-Lys-\textendash-X and activates its natural substrate trypsinogen and releases trypsin by cleavage at the C-terminal end of this sequence. BNPS-skatole is a mild oxidant and brominating reagent that leads to polypeptide cleavage on the C-terminal side of tryptophan residues. It was evident from appearance of fast migrating protein fragments on SDS-PAGE gel, which corresponded to the cleaved protein fragments of appropriate sizes, that HMO1 and HMO1-boxAB were cleaved by both reagents as expected (Figure 4.6). However, addition of DNA to the reactions did not change the cleavage pattern. This shows that the binding of HMO1 and HMO1-boxAB to DNA substrate does not cause significant conformation change to alter the accessibility of the amino acids cleaved by these reagents.

Discussion

Interdomain interactions in HMO1

Based on thermal melting of HMGB1, Ramstein et al. [41] proposed that HMGB1 box A and B behave as independent domains in a protein truncated of the acidic C-terminus (A-B), and that the C-terminus interacts with one of the two domains.
Figure 4.6 Digestion of HMO1 and HMO1-boxAB with BNPS-skatole. The top panel is a cartoon representation of possible protein fragments sizes after digestion of HMO1 and HMO1-boxAB with BNPS-skatole which cleaves at Trp149 and Trp160. The lower panel shows protein digested with BNPS-skatole in the absence or presence of 4-way junction DNA after electrophoresis on a 17\% SDS-PAGE gel. Lanes 1 and 4 shows undigested protein. Lanes 2 and 5 shows protein digest in the absence of DNA. Lane 3 and 6 show protein digested in the presence of DNA. Cleaved protein fragments are identified at the right in the order of descending sizes as per the top panel, 1, 2, 3, 3b and 4b.
They showed the thermal melting of box A and B to be identical with transition temperatures of 43 °C and 41° C, respectively, and the melting temperature of A-B had nearly identical transitions of 40° C and 41° C. On the other hand, full length HMGB1 had biphasic transition values of 38° C and 55° C. The difference in the thermal melting temperature between A-B and HMGB1 indicated that the C-terminus interacted with one of the domains (as a result of increase of melting temperature from 41° C or 43°C to 55°C), stabilizing the protein. We have shown that HMO1 and HMO1-boxAB go through a biphasic thermal denaturation processes, indicative of two underlying melting processes. HMO1-boxA has a theoretical pI of 4.08, 15 negatively charged residues and 4 positively charged residues, whereas HMO1-boxB has a theoretical pI of 10.07, a total of 12 negatively charged residues and 15 positively charged residues. The C-terminal tail (the last 30 amino acids) has a theoretical pI of 10.07, 6 negatively charged residues and 14 positively charged residues. The high net positive charge of the C-terminus will interact with the high net negative charge of box A. It is also likely that the net positive charges in box B will interact with high net negative charges residues in box A. The amount of net charge helps argue for a stronger interaction of C-terminal tail with box A than with box B. Therefore, it is more likely that the larger change seen in melting temperature due to the removal of the C-terminal tail in HMO1-boxAB is due to severed interaction with box A. It is also likely that with the removal of the C-terminal tail, the interaction of box A and box B changes. It has been shown that removal of the basic region of HMGB protein increases the melting temperature of the protein [30]; an indication that interaction of the basic region with the protein domain lowers the melting temperature (destabilizes the protein). HMO1-boxA melts at 52.2 ± 0.2°C while HMO1-
boxAB melts at 48.2 ± 2.0°C and 71.7 ± 0.7°C. Interaction of box B with box A in HMO1-boxAB is likely to lower the melting temperature in box A compared to an intact box A in HMO1-boxA. This would then imply that the first transition seen in HMO1-boxAB represents melting of box A. Therefore, in full length HMO1, interaction of the C-terminal tail and box B with box A destabilizes box A, and in HMO1-boxAB, interaction of box B with box A destabilizes box A. We can therefore conclude that the second transitions seen both in HMO1 and HMO1-boxAB represent unfolding of box B. It is also unlikely that HMO1 box A and box B operate as independent domains since both domains interact with the C-terminal tail, unlike HMGB1 [41].

**DNA binding and bending properties of HMO1-boxAB**

Basic extensions found either C- or N-terminal to the HMG box are known to have a positive effect on bending capacity and DNA-binding affinity of the HMG box. This region stimulates binding to linear DNA and 4-way junctions, DNA supercoiling and circularization of short DNA fragments (reviewed in [28, 39]). For example in yeast, the basic N-terminus of the NHP6A HMG box is required for stable complex formation on both linear DNA and DNA minicircles. At high concentrations, mutants lacking the N-terminus are able to promote minicircle formation and Hin invertasome assembly, but they are unable to form stable complexes with DNA, co-activate transcription, and complement the growth defect of Δnhp6a/b mutants [20]. Basic C-terminal extensions stabilize binding of HMG-D and LEF-1 by binding in the major groove opposite the distorted minor groove [8, 27]. Severe negative effect are seen when the basic N-terminal domains of maize and rice HMGB1 are deleted [42, 43]. In this study, we show that deletion of the basic C-terminus of HMO1 has a negative effect on DNA affinity. It is
likely that the basic C-terminus contributes to DNA affinity of this protein by directly interacting with the DNA since it is highly charged. Thomsen et al. showed that the individual basic N-terminal domain of maize HMGB1 binds DNA [30]. Here, we have shown that the HMO1 C-terminus interacts with both box A and box B and as such, this interaction could modulate HMO1 affinity for DNA. A change in DNA binding could be due to a net change in available charges that facilitates DNA interactions, and/or a change in protein conformation, enabling a better DNA protein interaction. It has previously been shown that intramolecular interaction of the acidic tail and HMG-box domain(s) may modulate DNA interactions of HMGB proteins [28, 41, 44, 45]. It would be interesting to assess if the individual C-terminal tail would fold into an individual domain and if it would bind DNA. Although deletion of the HMO1 C-terminus does not severely affect HMO1 affinity for DNA, it completely abolishes its DNA bending properties (Figure 4.3). NHP6A and HMGB1 basic N-terminal domains have been shown to enhance the DNA bending properties of these proteins [20, 42], which correlates to these proteins DNA binding properties. It is apparent that changes in individual HMGB proteins in DNA binding properties correlate to changes in DNA bending properties.

**CKII phosphorylates HMO1**

CKII has previously been used in phosphorylation of HMGB serine residues [21, 22, 41]. CKII phosphorylates HMO1 (Figure 4.4, panel A) and HMO1-boxAB, which lowers the DNA affinity of these proteins, as shown using EMSA (Figure 4.4, panel B). Protein phosphorylation increases the negative net charge. In this study, we have shown that CKII phosphorylates HMO1 Ser137 and/or Ser153, both of which are located in box B. We have previously shown that box A binds DNA but with low affinity [35], hence
this strengthens the argument that box B confers most of the DNA binding affinity. Increasing the negative net charge in box B leads to reduction in DNA affinity. This phenomenon can be explained by the following possible scenarios; 1) electrostatic DNA repulsion, 2) increasing the negative net charge in box B leads to a stronger interaction with the C-terminus and as a result, the net positive charge available in box B and/or in the C-terminal tail that contributes to DNA binding is lowered, or 3) change in protein conformation and/or stability. The rate at which phosphorylation reduced HMO1 and HMO1-boxAB affinity for DNA looks similar (Figure 4.3), making the second scenario less likely since involvement (or lack thereof) of the C-terminal tail does not make a difference in effect that phosphorylation has in DNA binding of these proteins. It is interesting also to note that HMO1-boxAB does not bend DNA nor does it supercoil closed relaxed DNA, before or after phosphorylation (Figure 4.3, data not shown). We have also shown that protein cleavage with Enterokinase and BNPS-skatole does not confer a gross change of protein conformation in the presence of DNA or protein phosphorylation since cleavage patterns remain the same. Therefore, the conformational changes that HMO1 goes through upon DNA binding and/or after phosphorylation could not be detected by cleavage with Enterokinase or BNPS-skatole. It would be interesting to investigate the conformational changes using intrinsic fluorescence and thermal stability using CD spectroscopy of HMO1 before and after phosphorylation. This would be critical in trying to understand the role of HMO1 in vivo in its DNA interaction capacity, since this protein is most likely phosphorylated in vivo. The C-terminus of HMO1 has been shown to be dispensible for function in vivo [31]. We have shown HMO1-boxAB to be localized in the cytoplasm, unlike HMO1 which is localized both in
the nucleus and cytoplasm (unpublished data, [31, 34]). We therefore would like to propose that the majority functions of HMO1 are located in the cell cytoplasm and not in the nucleus. Recently, the extranuclear and extracellular functions of HMGB1 have just started to come into focus. It participates in developmental and differentiation processes, triggers and modulates many of the inflammatory cascades in the body, and may even be involved in the metastatic invasion programme of cancer cells (reviewed in [46]). As such, it is more likely that post-translational modification of HMO1 (phosphorylation) and HMGB1 plays more crucial role(s) in regulating the function(s) of these proteins in the cytoplasm than in the nucleus.

References


CHAPTER 5

FLUOROQUINOLONE-DEPENDENT DNA SUPERCOILING BY VACCINIA TOPOISOMERASE I

Introduction

DNA topoisomerases are ubiquitous enzymes that control DNA topology. While DNA cleavage by all topoisomerases leads to formation of a transient covalent intermediate in which a tyrosine is linked to a broken DNA strand, type I topoisomerases relax supercoiled DNA by cleaving only one DNA strand, causing the linking number to change in steps of one. The type IB enzymes, which form a covalent bond to a 3'-phosphate, are found in all eukaryotes, where they relax both positive and negative supercoils [1-3].

The type IB enzymes of poxviruses are the smallest topoisomerases known. Vaccinia topoisomerase I is a 314-amino acid protein that binds duplex DNA with stringent specificity for transesterification at 5'-(C/T)CCTT↓ sites, where the 3' phosphate of the incised strand becomes linked to Tyr-274 of the enzyme [3-7]. Vaccinia topoisomerase I consists of two structural modules; the N-terminal module contacts the cognate DNA site in the major groove, while the C-terminal module comprises an autonomous catalytic domain [8-11]. The structure of the catalytic domain is similar to that of human topoisomerase I, except for a displacement of the active site tyrosine [10-12].

Vaccinia encapsidates many enzymes that are needed for early viral transcription, including topoisomerase I. Inactivation of the gene encoding Vaccinia topoisomerase I
was recently shown to be associated with diminished infectivity in vitro due to reduced early transcription [13], suggesting that drugs that interfere with Vaccinia topoisomerase activity have the potential to be developed into effective antiviral agents. Vaccinia topoisomerase I displays unique pharmacological properties; it is resistant to camptothecin, otherwise a topoisomerase I poison, but sensitive to inhibitors of bacterial DNA gyrase such as novobiocin and coumermycin, both coumarin drugs that are used as antibiotics [14-19].

Fluoroquinolones are topoisomerase II poisons known to target DNA gyrase and topoisomerase IV in bacterial cells [20-22]. The interaction of norfloxacin with the DNA-gyrase complex has been investigated in some detail. While binding of norfloxacin to gyrase was undetectable, it was shown to bind DNA directly and to exhibit a binding mode involving preferred recognition of single-stranded DNA. Drug binding was enhanced by the presence of gyrase, suggesting that DNA-binding revealed a cryptic norfloxacin binding site on the enzyme [23-24]. The nature of the norfloxacin-DNA complex has been analyzed by several groups, with somewhat disparate conclusions. Norfloxacin was reported to require magnesium ions for DNA binding, with Mg\textsuperscript{2+} proposed to act as a bridge between the negatively charged DNA phosphates and the carbonyl and carboxyl moieties of norfloxacin [25]. However, more recent reports showed binding of norfloxacin to DNA in the absence of Mg\textsuperscript{2+} and suggested a mode of DNA interaction that ruled out classical DNA intercalation or groove binding, based in part on the observation that the molecular plane of norfloxacin is nearly perpendicular to the helix axis [26-27]. These authors also found that DNA unwinding by norfloxacin is negligible and proposed instead that the compound may induce a bend in the DNA helix.
The inhibitory effect of fluoroquinolones is based on their ability to stimulate the forward rate of the topoisomerase II-mediated DNA scission; after binding the cleavable complex, the fluoroquinolone induces a conformational change in the enzyme that prevents religation [28-30]. The fluoroquinolone derivative ofloxacin was previously shown also to exhibit antiviral activity against Vaccinia virus, and the inhibitory activity shown to correlate with inhibition of topoisomerase I purified from Vaccinia virus cores [31]. Here we describe a novel interaction of fluoroquinolones with Vaccinia topoisomerase I in vitro, focusing on enrofloxacin which is structurally closely related to ciprofloxacin (Figure 5.1). We show here that enrofloxacin inhibits DNA relaxation by Vaccinia topoisomerase I at lower concentrations than those required for inhibition by ofloxacin. In the presence of relaxed DNA, enrofloxacin causes Vaccinia topoisomerase I to supercoil DNA, reversing its action as a DNA relaxing enzyme. Notably, enrofloxacin does not induce human topoisomerase to supercoil relaxed DNA.

**Experimental Procedures**

Inhibition of DNA relaxation by fluoroquinolones.

Supercoiled closed circular pUC18 plasmid (0.2 µg) was incubated with 2.5 units of Vaccinia topoisomerase I (Epicentre; 10 units ~ 6 ng), wheat germ topoisomerase I (Promega) or human topoisomerase I (TopoGEN) and varying concentrations (2.5 µM to 1250 µM) of enrofloxacin, ofloxacin, norfloxacin, lomefloxacin (Sigma), ciprofloxacin or moxifloxacin (a generous gift from Bayer Pharmaceuticals) in topoisomerase reaction buffer (50 mM Tris, pH 7.5, 2.5 mM MgCl₂, 100 mM NaCl and 0.1 mM EDTA) for 2 hours at 37°C. Reactions were quenched with 5 µl termination buffer (250 mM Tris-borate, 5 mM EDTA (5X TBE), 5% SDS, 15% sucrose, 0.1% bromophenol blue (BPB),
Figure 5.1 Structure of fluoroquinolones.
0.1% xylene cyanol, 2 μg/μl proteinase K) and further incubated for 1 h at 37°C, except where noted. Samples were loaded on 1% 1X TBE agarose gels and electrophoresed at 2 V/cm for 16 h in 1X TBE buffer. Gels were stained with ethidium bromide (EtBr) and quantified using an Alpha Innotech digital imaging system.

**Supercoiling of relaxed DNA by vaccinia topoisomerase in the presence of fluoroquinolones**

Relaxed closed circular pUC18 plasmid was prepared by adding 2.5 units Vaccinia topoisomerase I to supercoiled pUC18 in topoisomerase reaction buffer for 2 h after which the DNA was phenol-chloroform extracted and ethanol precipitated. Supercoiling reactions contained 0.2 μg relaxed closed circular pUC18, 2.5 units of Vaccinia topoisomerase I and varying concentrations of fluoroquinolone. Reactions were incubated at 37°C for 2 h, quenched and electrophoresed as described above. To study the effects of coumermycin on supercoiling of relaxed DNA by Vaccinia topoisomerase I in the presence of enrofloxacin, varying concentrations of coumermycin (25 μM-500 μM) were incubated with topoisomerase for 10 minutes before addition of DNA and enrofloxacin. Reactions were analyzed as described above.

**Ligase assay with ethidium bromide and enrofloxacin**

Ligase-mediated reactions were initiated by addition of 1 unit of T4 DNA ligase to a final volume of 20 μl reaction buffer (20 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM MgCl₂, 0.1 mM Na₂EDTA, 1 mM DTT, 0.05% BRIJ58) containing 0.2 μg nicked DNA. (Nicked DNA was prepared by incubation of pUC18 DNA with N.BstNB I (NEB)). Increasing concentrations of EtBr or enrofloxacin were added, and reactions were incubated at room temperature for 3 h after which they were quenched by addition of 5 μl
of termination buffer and further incubated for 1 h at 37°C. Samples were analyzed as described above.

**Two-dimensional analysis of supercoiling**

DNA was supercoiled by incubating 0.2 µg relaxed closed circular pUC18, 2.5 units of Vaccinia topoisomerase I and 25 µM enrofloxacin at 37°C for 2 h followed by addition of termination buffer and a further incubation at 37°C for 1 h. To determine the direction of supercoils introduced by Vaccinia topoisomerase I in the presence of enrofloxacin, topoisomers were separated by electrophoresis at 2 V for 16 h in 1X TBE buffer. The gel was soaked in chloroquine (3 µg/ml) for 1 h, turned 90° and run at 4 V/cm for 8 h in 1X TBE buffer, then stained with EtBr and visualized as described above.

**Electrophoretic Mobility Shift Assays**

An 18/24 duplex DNA containing a single CCCTT motif was used in this analysis. The top strand (18-mer, either unmodified or 5'-CGTGTCGCCCTT*ATTCCG-3', with the asterisk indicating phosphorothioation) was 5'-end labeled with T4 polynucleotide kinase and [γ-32P] ATP and annealed to the bottom strand (24-mer, 5'-CACTATCGGAATAAGGGCGACACG-3'). Reactions were incubated at room temperature in 10 µl topoisomerase buffer (50 mM Tris-HCl pH 7.5) containing 25 nM DNA and varying amounts of Vaccinia topoisomerase I. Samples were adjusted to 5% glycerol and resolved on prerun 7% (w/v) native polyacrylamide (39:1 acrylamide:bisacrylamide) gels at 4°C with 0.5X TBE running buffer. Quantification was performed on a Molecular Dynamics Storm Phosphorimager using software supplied by the manufacturer.
Suicide substrate cleavage

The top strand of the non-phosphorothioated 18/24 duplex was 3’-end labeled with terminal transferase and [γ-32P] ATP and annealed to the bottom strand. DNA cleavage reactions were initiated by addition of 100 units of Vaccinia topoisomerase I to 130 µl of substrate cleavage buffer (10 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 2.5 mM MgCl₂, 2.5 mM CaCl₂) containing 500 nM DNA, with or without enrofloxacin. Time points were taken at 0.5, 1, 5 and 10 minutes. For each time point, 10 µl of the reaction mixture was quenched by addition of 5 µl termination buffer and further incubated for 1 h at 37°C. Samples were adjusted to 70% formamide, heated for 4 minutes at 95°C and resolved on prerun 20% (w/v) polyacrylamide (19:1 acrylamide: bisacrylamide) denaturing gels with 7M urea in 0.5X TBE. Gels were visualized and quantified on a Molecular Dynamics Storm Phosphorimaget as described above.

Results

Enrofloxacin inhibits DNA relaxation by topoisomerase I

Enrofloxacin inhibits relaxation of supercoiled plasmid DNA by Vaccinia topoisomerase I in a concentration-dependent fashion, with complete inhibition at ~1 mM drug (Figure 5.2). The concentration of enrofloxacin required to inhibit Vaccinia topoisomerase I is comparable to the effective concentration of the coumarin drugs novobiocin (~0.5 mM) and coumermycin (~0.2 mM) needed to inhibit DNA relaxation by Vaccinia topoisomerase I, but lower than the ~2 mM ofloxacin previously seen to elicit ~30% inhibition of topoisomerase I purified from virus cores [17, 31] Consistent with this observation, we also find that higher concentrations of ofloxacin are required to inhibit the enzyme compared to enrofloxacin (data not shown). Enrofloxacin also inhibits
### Figure 5.2 Inhibition of DNA relaxation by enrofloxacin

Reactions contained 0.2 µg supercoiled pUC18, 2.5 units Vaccinia topoisomerase I, wheat germ topoisomerase I or human topoisomerase I and increasing concentrations of enrofloxacin (enro): 2.5, 12.5, 62.5, 125, 250 and 1250 µM (lanes 3-9, 10-15, 17-22). Lanes 2, 9 and 16 are relaxation reactions with no drug added. Lane 1 is the supercoiled DNA. Relaxed (R) and supercoiled DNA (SC) is identified at the left.
relaxation of DNA by wheat germ topoisomerase I at equivalent concentrations, but does not produce any effects on human topoisomerase I within this concentration range (Figure 5.2).

**Vaccinia topoisomerase introduces negative supercoils into relaxed DNA in the presence of enrofloxacin**

Incubation of relaxed closed circular pUC18 DNA with Vaccinia topoisomerase I and increasing concentration of enrofloxacin resulted in the generation of a distribution of supercoiled DNA topoisomers (Figure 5.3A); the distribution differs from that seen when DNA is relaxed by topoisomerase I. The order in which reactants were added did not affect the efficiency with which Vaccinia topoisomerase I supercoiled relaxed DNA (Figure 5.3A, lanes 12 and 13). In the presence of enrofloxacin, wheat germ topoisomerase I also introduced supercoils into relaxed DNA, however, a lower superhelical density was generated. In contrast, enrofloxacin had no effect on human topoisomerase I (Figure 5.3B). Note that a distinct population of topoisomers is generated upon incubation of relaxed DNA with human topoisomerase I, each topoisomer interdigitating between topoisomers produced by wheat germ or Vaccinia topoisomerase I (Figure 5.3B, lanes 8 and 9).

To determine the direction of DNA superhelicity, a 2-dimensional agarose gel electrophoresis was performed. In the presence of chloroquine, negatively supercoiled DNA topoisomers, separated in the first dimension, lose superhelicity and migrate slower while positively supercoiled DNA gains superhelicity and migrates faster. Accordingly, negatively supercoiled topoisomers migrate with a left-handed curvature in the second dimension (e.g., Figure 5.3D), while positively supercoiled topoisomers generate a right-
Figure 5.3 Supercoiling of relaxed DNA in the presence of enrofloxacin. (A) Reactions contained 0.2 µg relaxed pUC18, 2.5 units Vaccinia topoisomerase I, increasing concentrations of enrofloxacin (enro): 12.5, 25, 50, 75, 125, 175 and 250 µM (lanes 3-9). In lanes 10 and 11, relaxed DNA was incubated with 30 or 60 µM enrofloxacin. In lane 12, relaxed DNA was incubated with Vaccinia topoisomerase I for 10 minutes, then 30 µM enrofloxacin was added and the reaction incubated further for 2 h. In lane 13, enrofloxacin was incubated with Vaccinia topoisomerase I for 10 minutes, after which DNA was added and the reaction incubated further for 2 h. (B) Reaction mixtures are as above, except with wheat germ topoisomerase I (left panel) and human topoisomerase I (right panel). Lanes 3-8 and 10-15 contain enrofloxacin (7.5 – 75 µM). Topoisomers are identified by a bracket, relaxed (R) and supercoiled DNA (SC) is identified at the left. (C-F) Two-dimensional analysis of supercoiling. The direction of electrophoresis is indicated. (C) Relaxation of negatively supercoiled DNA by Vaccinia topoisomerase I yields a distribution of positively supercoiled topoisomers. (D) Negative supercoils introduced by Vaccinia topoisomerase I in the presence of enrofloxacin. (E) Positive supercoils introduced by human topoisomerase I in the presence of enrofloxacin. (F) Positive supercoils introduced by human topoisomerase I in absence of enrofloxacin.
handed arc after electrophoresis in the second dimension (e.g., Figure 5.3C). While relaxation by Vaccinia topoisomerase I generates a population of positively supercoiled topoisomers, negative supercoils are introduced in the presence of enrofloxacin (Figure 5.3C-D). When a 2-dimensional agarose gel electrophoresis was performed on the population of topoisomers generated by human topoisomerase I in the presence or absence of enrofloxacin, positive supercoils were detected (Figure 5.3E-F). While ciprofloxacin and norfloxacin were as efficient as enrofloxacin at inducing the topoisomerase to produce DNA supercoiling, no effect was seen in the presence of equivalent concentrations of the structurally related compounds moxifloxacin and lomefloxacin (Figure 5.4), pointing to specific structural requirements for the observed interaction. Notably, both compounds found to be ineffective in modulating Vaccinia topoisomerase I activity contain a C8 substituent (a methoxy group in the case of moxifloxacin and fluorine for lomefloxacin (Figure 5.1)).

Effects of coumermycin and enrofloxacin on DNA binding by Vaccinia topoisomerase I

Coumermycin inhibits Vaccinia topoisomerase I by blocking its interaction with DNA [2, 17]. Coumermycin inhibited the enrofloxacin-mediated supercoiling of relaxed DNA by Vaccinia topoisomerase I at concentrations similar to those required for inhibition of DNA relaxation (data not shown), suggesting unhindered access of coumermycin to its binding site in the presence of enrofloxacin and prevention of enzyme-DNA complex formation.

Enrofloxacin does not modulate the Vaccinia topoisomerase I active site

Vaccinia topoisomerase I displays considerable DNA sequence specificity, binding and forming a covalent adduct at sites containing the sequence 5'-
Figure 5.4 Structural specificity of fluoroquinolones. Reaction mixtures are as described for Figure 3, except that Vaccinia topoisomerase I was incubated with (A) norfloxacin (0.25, 2.5, 12.5, 62.5, 125, and 250 µM in lanes 3-8), (B) moxifloxacin (2.5, 12.5, 25, 62.5, 125, 250, and 1,250 µM in lanes 3-9) or (C) lomefloxacin (concentrations as for panel B). Lanes 1 contain supercoiled DNA and lanes 2 relaxed DNA.
(C/T)CCTT↓ [3, 9]. Suicide substrates have been useful for analysis of DNA cleavage, containing a single CCCTT↓ cleavage site from which a non-covalently held 6-nt product is released in preference to religation (Figure 5.5A) [3, 32, 33]. Electrophoretic mobility shift assays (EMSA) in which Vaccinia topoisomerase I bound either the suicide substrate or a modified version with a phosphorothioate replacing the scissile phosphoester bond, thus trapping the covalent intermediate and preventing religation, showed no interference of enrofloxacin with DNA binding and formation of the covalent intermediate (data not shown). We also assayed whether enrofloxacin would modify the rate of Vaccinia topoisomerase I-mediated DNA cleavage under single-turnover conditions [33]. As determined from initial rates of cleavage product release, enrofloxacin does not modify the rate of Vaccinia topoisomerase I-mediated DNA cleavage (Figure 5.5B-C).

**Enrofloxacin does not supercoil DNA**

Although fluoroquinolones have been reported not to associate with DNA in a classical intercalative or groove-binding mode that leads to DNA unwinding [26, 27], several experiments were performed to rule out the possibility that the fluoroquinolones modulate DNA topology. First, relaxed DNA was incubated with increasing concentrations of enrofloxacin in the absence of the enzyme. At concentrations up to 60 µM, enrofloxacin had no effect on DNA migration, arguing against an intercalative mode of interaction with the DNA (Figure 5.3, lanes 10 and 11). Secondly, if enrofloxacin were merely causing DNA unwinding, then negative supercoils would also be expected to result from incubation with human topoisomerase I, and that is not observed (Figure 5.3).
Figure 5.5. **Suicide substrate assay.** (A) An 18-nt CCCTT-containing oligonucleotide hybridized to a 24-nt oligonucleotide. (B) Reaction in lane 1 contains substrate DNA with no enzyme. Lanes 2-6 time-course reactions with 2.5 units of Vaccinia topoisomerase; reactions were terminated after 0.5, 1, 5 and 10 minutes. Lanes 7-10 are similar to 2-6, but contain 25 µM enrofloxacin. (C) The fraction of cleaved DNA as a function of time of incubation.
Note, however, that the reactions presented in Figures 5.2-5.4 were terminated with buffer containing SDS, which would be expected to disrupt interactions and release bound drug. Therefore, if the fluoroquinolone had induced DNA unwinding, and if the topoisomerase had relaxed the resulting positive DNA supercoils, then removal of bound drug should cause the DNA to rewind and to form negative supercoils. We tested this experimentally, noting that Vaccinia topoisomerase I was previously found to relax positive DNA supercoils preferentially (Figure 5.6) [34]. As expected, incubation of Vaccinia topoisomerase I with relaxed DNA in the presence of EtBr, which unwinds DNA ~26° upon binding [35], leads to a distribution of positively supercoiled topoisomers when reactions are terminated without disruption of the drug-DNA interactions (Figure 5.6A). Exposure of the reactions to SDS (not shown) or phenol-extraction leads to the expected removal of EtBr and formation of negatively supercoiled topoisomers (Figure 5.6B). By contrast, negatively supercoiled topoisomers are formed on incubation of Vaccinia topoisomerase I with relaxed DNA in the presence of enrofloxacin, whether reactions are terminated by treatment with SDS (Figure 5.3), phenol-extraction (Figure 5.6D) or without disruption of the drug-DNA interaction (Figure 5.6C), indicating that the observed DNA supercoiling is not due to rewinding of DNA upon extraction of the drug. Finally, nicked pUC18 was religated with T4 DNA ligase in the presence of increasing concentrations of EtBr or enrofloxacin (Figure 5.6E-F). In the presence of EtBr, religation produced different DNA topoisomers compared to the control, and as expected, at higher EtBr concentration, the DNA was fully supercoiled. When religated in the presence of enrofloxacin, the topology of the covalently closed DNA was not altered.
Figure 5.6 Enrofloxacin does not modulate DNA topology. (A-D) Two-dimensional analysis of DNA supercoiling. (A-B) Vaccinia topoisomerase I incubated with relaxed DNA and EtBr, with reactions terminated without SDS, yielding a distribution of positively supercoiled DNA topoisomers, as seen also for relaxation of negatively supercoiled DNA (Figure 3C) (A), or by phenol-extraction, resulting in negatively supercoiled DNA (B). (C-D) Vaccinia topoisomerase I incubated with relaxed DNA and enrofloxacin, with reactions terminated without SDS (C) or by phenol-extraction (D), in both cases yielding negatively supercoiled DNA. The direction of electrophoresis is indicated. (E) Ligation of nicked DNA in the presence of ethidium bromide (EtBr) or enrofloxacin. Lane 1 is nicked DNA and lane 2 is religated DNA. Lanes 3-6 are ligation of nicked DNA with 0.5, 1, 2 and 4 µM EtBr, lanes 7-10 are religation of nicked DNA with 25, 37.5, 75 and 125 µM enrofloxacin. Topoisomers are identified by a bracket. (F) Two-dimensional analysis of DNA topoisomers formed by ligation of nicked DNA in the presence of EtBr.
Discussion

To relax DNA, Vaccinia topoisomerase I binds non-covalently to duplex DNA and cleaves one DNA strand with concomitant formation of a transient covalent DNA-protein intermediate, followed by DNA topological changes and religation. The data presented here show that in the presence of specific fluoroquinolones, Vaccinia topoisomerase I reverses its role as a DNA relaxing enzyme and introduces DNA supercoils. As demonstrated for norfloxacin [26, 27], enrofloxacin would also be expected to bind DNA without inducing significant unwinding (Figure 6). Since the rate of formation of the covalent topoisomerase-DNA intermediate is equivalent in the presence or absence of enrofloxacin (Figure 5), we find it less likely that the topoisomerase binds a preformed DNA-fluoroquinolone complex, and propose instead that the compound may bind preferentially to DNA that has undergone structural distortions due to enzyme binding (as suggested for targeting of fluoroquinolones to a DNA-gyrase complex) [23, 24]. Our data also suggest that Vaccinia topoisomerase I would bind and cleave its cognate DNA sequence in the presence of the fluoroquinolone, with steps beyond formation of the transient covalent intermediate modulated by the drug.

Vaccinia topoisomerase I, which removes an average of five superhelical turns per cleavage and religation event, was proposed to do so by a free rotation mechanism in which the non-covalently held DNA swivels in an energetically favorable process to relieve superhelical tension [32]. In contrast, type II topoisomerases are ATP-dependent and are believed to operate as molecular clamps, forming an enzyme-operated gate in one
double-stranded DNA segment and passing the other DNA segment through this gate [36, 39]. DNA gyrase introduces negative supercoils into DNA, a feature that requires the enzyme to dictate the directionality of strand passage. The mechanism of fluoroquinolone-mediated supercoiling of DNA by Vaccinia topoisomerase I cannot involve free rotation because first, supercoiling of DNA is not energetically favorable, yet this process is ATP-independent, and second, introduced supercoils are negative, indicating that the enzyme dictates directionality. As the fluoroquinolones have been shown not to bind DNA in a classical mode that results in DNA underwinding (Figure 6) [26, 27], and since the observed effect is not a general property of all fluoroquinolones (Figure 4), we suggest that topological changes in the DNA are driven by protein interactions.

The N-terminal domain of Vaccinia topoisomerase I interacts with the DNA major groove while the catalytic C-terminal domain interacts with the minor groove on the face of the helix that contains the scissile phosphodiester [40]. DNA binding triggers a conformational change at the interdomain linker, causing Vaccinia topoisomerase I to bind DNA circumferentially as a C-shaped protein clamp [8-10, 40]. It is possible that the fluoroquinolone interferes with these conformational changes; the enzyme may adopt a conformation that does not alter its catalytic activity, yet introduces structural changes that enables it to wrap the DNA in a toroidal supercoil. The mechanism of free rotation of DNA to release superhelical tension was proposed in part based on the observation that a 2-6 nt 3′-fragment dissociates in preference to religation and that only 2 bp 3′ to the cleavage site is required for cleavage [4, 41]. However, the enzyme does protect at least
13 bp downstream of its cleavage site [5]. Whereas these interactions may not be required for cleavage, our data are consistent with their role in topological changes or religation.

Enrofloxacin does not mediate DNA supercoiling by human topoisomerase I (Figure 3B). In contrast to Vaccinia topoisomerase I, human topoisomerase I is proposed to follow a “controlled rotation” mechanism for DNA relaxation, in which ionic interactions between DNA and protein regulate the DNA winding process [12, 42]. In our relaxation assays, we obtained the typical Gaussian distribution of positively supercoiled topoisomers that did not change in the presence of enrofloxacin (Figure 3). The formation of positively supercoiled topoisomers is consistent with a controlled rotation of DNA in which contacts to the enzyme, possibly downstream of the cleavage site, serve to orient the DNA. A difference in mechanism of DNA relaxation between Vaccinia topoisomerase I and human topoisomerase I may contribute to the differential effects of enrofloxacin on these two enzymes. Alternatively, the binding site for fluoroquinolones may be obscured or poorly conserved in the human enzyme. Distinct pharmacological properties are indeed characteristic of Vaccinia topoisomerase I, which is resistant to classical topoisomerase I poisons, but sensitive to other gyrase inhibitors such as coumermycin [2, 17].

The recent observation that inactivation of the gene encoding Vaccinia topoisomerase I causes decreased infectivity suggests that compounds that target this enzyme have therapeutic potential [13]. Our data suggest that fluoroquinolones may be structurally optimized to target poxvirus topoisomerases and to serve as antiviral agents. This notion is corroborated by a previous report showing that the fluoroquinolone ofloxacin has selective, antiviral activity against Vaccinia virus in cultured mammalian
cells [31]. The potential for optimizing fluoroquinolone compounds as antiviral agents against poxviruses is particularly significant in the face of the threat associated with the use of smallpox as a biological weapon.

References


Orchestration of DNA topology is critical in diverse cellular processes and its manipulation has been shown to be one of the central roles played by HMGB proteins and topoisomerases. For example, it has been shown that the chromosome of *Escherichia coli* is maintained in a negatively supercoiled state, and supercoiling levels are affected by growth phase and a variety of environmental stimuli [1]. A recent study measured the transcriptional response to a loss of supercoiling caused either by genetic impairment of a topoisomerase or addition of specific topoisomerase inhibitors during log-phase growth in *E. coli* and identified genes whose changes are statistically significant. Transcription of 7% of the genome (306 genes) was rapidly and reproducibly affected by changes in the level of supercoiling [2]. Thus, the global control of transcription throughout the life cycle of an organism can be formalized as an interacting network of gene products and effectors that control RNA polymerase selectivity and effective superhelicity. These effectors can be proteins, including the HMGB proteins or compounds such as fluoroquinolones. To better understand this complex puzzle, we characterized HMO1, a *Saccharomyces cerevisiae* HMGB protein. We looked at its DNA binding and bending properties as well its posttranslational modification. We also looked at the effect fluoroquinolone drugs have in influencing Vaccinia topoisomerase I ability to change DNA superhelicity.

**HMO1 Interaction with DNA**

Lu J *et al.* [3] initially characterized HMO1, indicating that it is required for normal growth, plasmid maintenance and for regulating the susceptibility of yeast
chromatin to nuclease. In this work, we show that HMO1 binds linear duplex DNA in a non-sequence specific manner, and that its divergent box A domain participates in DNA interactions. While the box A domain contributes modest structure-specific binding, the box B domain is required for high-affinity binding. HMO1 has only modest preference for DNA with altered conformations, including DNA with nicks, gaps, overhangs or loops, as well as for 4-way junction structures and supercoiled DNA. HMO1 binds 4-way junctions with half-maximal saturation of 19.6±2.2 nM, with only a modest increase in affinity in the absence of magnesium ions (half-maximal saturation 6.1±1.1 nM). When HMO1 was phosphorylated with Casein Kinase II (CKII), its DNA binding affinity was slightly reduced in 4-way junction DNA. Lack of preference for the 4-way junction was also seen by Yen Y et al. [4] in the non-histone protein 6A (NHP6A). However, binding to the 4-way junctions DNA is considered to be a common property of the HMGB proteins (reviewed in [5]).

Ligase-mediated circularization of small DNA fragments has been extensively utilized as a means of comparing the DNA bending activities of non-sequence specific DNA bending proteins. Both HMGB1/2 and NHP6A/B catalyze formation of 66 bp circles, NHP6A/B being more efficient, which has been attributed to the relative stability of NHP6A/B-DNA complexes compared to HMGB1/2 and HU complexes [6, 7]. However, this could also be due to a difference in the bend angle exerted by these proteins, with the angle exerted by NHP6A/B facilitating DNA strand recognition by ligase [6]. HMO1 facilitated formation of circles only with DNA longer than 87 bp DNA, a lower efficiency that may correspond to either a reduced bend angle or a short residence time of HMO1 on linear DNA.
Work by Lu J et al. [3] reported that HMO1 introduces negative supercoils into relaxed plasmid DNA, and that at high concentrations, HMO1 inhibited the unwinding reaction. We did not find evidence that HMO1, either phosphorylated or non-phosphorylated, have the capability to introduce supercoils into relaxed plasmid DNA. This can be explained by the lack of an important intercalating hydrophobic residue found in many HMGB proteins, but missing in HMO1 box B and SRY (an HMGB protein that also does not supercoil DNA).

**Phosphorylation of HMO1**

Casein kinase II (CKII) has previously been used in phosphorylation of HMGB serine residues [8, 9, 10]. We have shown in our study that CKII phosphorylates HMO1 and HMO1-boxAB but not HMO1-boxA. Protein phosphorylation increases the negative net charge, altering the conformation, stability and DNA binding properties of proteins. Phosphorylated HMO1 and HMO1-boxAB had reduced affinity for DNA.

**HMO1 Thermal Stability**

Thermal stability of HMO1 and HMO1-boxAB was measured by CD spectroscopy. We have shown that HMO1 and HMO1-boxAB goes though a biphasic thermal denaturation process, but that HMO1-boxA has a single transition. Our data strongly suggests that the C-terminal tail interacts with both box A and box B, lowering the melting temperature of these domains. This phenomenon, that the basic extensions of an HMGB protein cause a reduction of melting temperature, has been reported elsewhere [11]. We have also concluded from our data that the first transition seen in both HMO1 and HMO1-boxAB is melting of box A and the second transition is melting of box B. However, purification and characterization of box B would address this interpretation.
Phosphorylation of HMGB proteins has shown that phosphates contribute to stability of the protein [12]. It would also be interesting to look at the effect of phosphorylation on stability of HMO1 and HMO1-boxAB.

**HMO1 Cellular Localization**

The C-terminus of HMO1 is dispensible for function *in vivo* [3]. We have localized HMO1 without the C-terminus (HMO1-boxAB) to the cytoplasm. This is a clear indication that the essential and unique functions of HMO1 are located in the cytoplasm and not in the nucleus, and especially since HMO1 has weak DNA binding and bending properties compared to other HMGB proteins. This data is supported by the fact that HMO1 genetically and physically interacts with cytoplasmically expressed FKBPI2 Prolyl Isomerase, a product of the *FPR1* gene, which when mutated has been shown to be synthetic lethal with ∆hmo1[13].

HMGB1 is a nuclear, extranuclear and extracellular protein that participates in many signaling processes in the cell. (reviewed in [14]). Interestingly, our data implicate HMO1 to function in the extranuclear maybe at similar capacity as HMGB1. Notably, signaling by TOR (Target of Rapamycin) kinases is central to environmental stress responses in yeast. Suppression of TOR signaling, for example through inhibition of TOR kinases by interaction with FKBPI2-rapamycin, leads to cell cycle arrest (reviewed in [15]). The reported interaction between HMO1 and FKBPI2 suggest a possible role for HMO1 in such signaling pathways. Studies to characterize the extranuclear function of HMO1 will further expand signalling roles of HMGB proteins beyond HMGB1, which thus far is the only HMGB protein that has been shown to have signaling capabilities, expanding the importance of HMGB proteins across organisms.
**Vaccinia Topoisomerase I Supercoils DNA in the Presence of Fluoroquinolones**

Vaccinia topoisomerase I is a site-specific DNA strand transferase that acts through a DNA-(3'-phosphotyrosyl)-enzyme intermediate, resulting in relaxation of supercoiled DNA. Although Vaccinia topoisomerase I is not an essential enzyme, its role in early transcription makes it a potential antiviral target. We have shown that Vaccinia topoisomerase I interact with enrofloxacin and a few other fluoroquinolone compounds, and that these compounds inhibit DNA relaxation. When Vaccinia topoisomerase I is presented with relaxed DNA in the presence of enrofloxacin, it executes the reverse reaction, supercoiling the DNA. However, the function does not interfere with the catalytic cleavage site, nor does it interfere with enzyme’s ability to bind DNA. The mechanism with which Vaccinia topoisomerase I supercoils relaxed DNA, an energetically unfavorable, yet ATP-independent process, must entail protein-DNA contacts downstream of the cleavage site, as opposed to the free rotation mechanism proposed for DNA relaxation. These characteristics of Enrofloxacin-Vaccinia topoisomerase I interactions presents a tool to study conformational dynamics of topoisomerase I.

**References**


Copyright Permission Policy

**ASBMB Journals**
- *Journal of Biological Chemistry*
- *Molecular and Cellular Proteomics*
- *Journal of Lipid Research*
- *Biochemistry and Molecular Biology Education*
- *ASBMB Today*

**ASBMB does not charge for and grants use without requiring your copyright permission request for:**

- Original authors wanting to reproduce portions of their own work; or to republish their material in not-for-profit formats or venues.
- **Students wanting to reproduce or republish their work for educational purposes.**
- **Students using other authors' material for their theses.**
- Reproduction or republication of abstracts only.
- Photocopying up to 5 copies for personal use.
- Non-profit educational institutions making multiple photocopies of articles for classroom use; all such reproduction must utilize institutionally owned equipment for this purpose.

*Use of copyrighted material requires proper citation.*
As an author, you retain rights for large number of author uses, including use by your employing institute or company. These rights are retained and permitted without the need to obtain specific permission from Elsevier. These include:

- the right to make copies of the article for their own personal use, including for their own classroom teaching use;
- the right to make copies and distribute copies (including through e-mail) of the article to research colleagues, for the personal use by such colleagues (but not commercially or systematically, e.g. via an e-mail list or list serve);
- the right to post a pre-print version of the article on Internet web sites including electronic pre-print servers, and to retain indefinitely such version on such servers or sites (see also our information on electronic preprints for a more detailed discussion on these points.);
- the right to post a revised personal version of the text of the final article (to reflect changes made in the peer review and editing process) on the author's personal or institutional web site or server, with a link to the journal home page (on elsevier.com);
- the right to present the article at a meeting or conference and to distribute copies of such paper or article to the delegates attending the meeting;
- for the author’s employer, if the article is a ‘work for hire’, made within the scope of the author’s employment, the right to use all or part of the information in (any version of) the article for other intra-company use (e.g. training);
- patent and trademark rights and rights to any process or procedure described in the article;
- the right to include the article in full or in part in a thesis or dissertation (provided that this is not to be published commercially);
- the right to use the article or any part thereof in a printed compilation of works of the author, such as collected writings or lecture notes (subsequent to publication of the article in the journal); and
- the right to prepare other derivative works, to extend the article into book-length form, or to otherwise re-use portions or excerpts in other works, with full acknowledgement of its original publication in the journal.

Other uses by authors should be authorized by Elsevier through the Global Rights Department (for addresses see Obtaining Permissions), and authors are encouraged to let Elsevier know of any particular needs or requirements.
VITA

Edwin Kamau was born in Nakuru, Kenya, attended Hyrax Primary school which is located on Hyrax hill, one of the richest archeological and palaeontological site and home to early form of man. He attended Rongai High school, a Christian missionary school. He joined Egerton University and in 1996, he was awarded a bachelors degree in Horticulture. For a short while, he attended Treasure Valley Community College in Ontario, Oregon, where he majored in music. From 1997-1999, he held various positions in different greenhouse/nursery companies as a diseases and pest control manager. In 1999, he enrolled in the Master Program in the Entomology department at Louisiana State University. In fall 2001, he switched programs and enrolled in the doctoral program in the Department of Biological Sciences. There, he studied DNA binding and bending properties of HMO1, a *Saccharomyces cerevisiae* HMGB protein. He also studied interaction of Vaccinia topoisomerase I with a fluoroquinolone drug enrofloxacin, which causes it to execute the reverse reaction, supercoiling DNA when presented with closed circular DNA, as opposed to relaxing supercoiled DNA. As a graduate student he taught introductory biology and advanced biochemistry lab courses. He will complete the requirements for the Doctor of Philosophy degree in biochemistry in May 2005. Edwin Kamau signed to join the army in Dec 2004. In June 2005, he is moving to Texas for Officer Basic Course. Thereafter, he has been posted at the Walter Reed Army Institute of Research, Washington DC, where he is planning to join the Experimental therapeutic department. His initial assignment is for 36 months.