BIOCHEMISTRY
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Biochemistry **2010,** *49,* 4051–4059 4051
DOI: 10.1021/bi1003603

The C-Terminal Domain of Yeast High Mobility Group Protein HMO1 Mediates Lateral Protein Accretion and In-Phase DNA Bending†

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Received March 9, 2010; Revised Manuscript Received April 15, 2010

ABSTRACT: The *Saccharomyces cerevisiae* high mobility group protein HMO1 has two DNA binding domains, box A and box B, and a lysine-rich C-terminal extension. Among other functions, HMO1 has been implicated as a component of the RNA polymerase I transcription machinery. We report here that HMO1 promotes DNA apposition as evidenced by its stimulation of end-joining in the presence of T4 DNA ligase. Analysis of truncated HMO1 variants shows that enhanced DNA end-joining requires the C-terminal domain but that box A is dispensable. The efficiency of joining DNA ends with different nucleotide content parallels that of DNA ligase, and optimal ligation efficiency is attained when DNA is effectively saturated with protein, implying that HMO1 binds internal sites in preference to DNA ends. Removal of the C-terminal tail does not attenuate the self-association characteristic of HMO1 but alters the stoichiometry of binding and prevents intramolecular DNA cyclization. This suggests that the C-terminal domain mediates an accretion of HMO1 on DNA that causes in-phase DNA bending and that binding of HMO1 lacking the C-terminal domain results in out-of-phase bending. Taken together, our results show that HMO1 shares with mammalian HMGB proteins the ability to promote DNA association. Notably, the C-terminal domain mediates both DNA end-joining and an accretion of multiple HMO1 protomers on duplex DNA that produces in-phase DNA bending. This mode of binding is reminiscent of that proposed for the mammalian RNA polymerase I transcription factor UBF.

High mobility group (HMGB)[1] proteins are conserved, abundant proteins with multiple regulatory roles. HMGB proteins bend DNA, in the process creating DNA topologies that promote assembly of higher order nucleoprotein structures; in this architectural capacity, HMGB proteins have been reported to regulate processes such as transcription and chromatin remodeling (for review, see refs (*1*−*3*)). A corollary of DNA bending is the preferred binding to distorted DNA structures, and HMGB proteins have been shown to bind with high affinity to DNA repair intermediates, such as four-way junctions or DNA with cross-links or mismatches (*4*−*6*). Mammalian HMGB1 has also been found to stimulate DNA end-joining by bringing DNA duplexes into close proximity (*7*−*9*). Electron microscopy revealed that HMGB1 promotes DNA ligation through bending and looping linear DNA to form a compact DNA structure (*8*).

HMO1 is one of several HMGB proteins in *Saccharomyces cerevisiae*. The single HMG-box proteins NHP6A/B have, for example, been shown to regulate transcription by RNA polymerases II and III and to participate in chromatin remodeling by FACT (*10*−*14*). HMO1 and HMO2 are more similar to mammalian HMGB proteins by having two HMG-like domains, box A and box B, of which the box B domains correspond to box B of mammalian HMGB homologues, while the N-terminal box A domains have only weak similarity to consensus HMG-box domains. While the only documented function of HMO2 is to participate in DNA double strand break repair by binding DNA ends and by recruiting the DNA damage-associated chromatin remodeling complex INO80 to regions surrounding a DNA double strand break (*15*−*17*), numerous functions for HMO1 have been suggested. Most research has focused on its role in gene transcription by both RNA polymerases (pol) I and II and its role in rRNA processing and on its contribution to coordinating these activities in response to signaling by the target of rapamycin (TOR) kinase (*18*−*25*).

We show here that HMO1 shares with mammalian HMGB proteins the ability to promote DNA end-joining *in vitro*. Notably, our data show that the basic C-terminal extension of HMO1 is required not only for juxtaposition of distal of DNA sites but also for a lateral accretion of HMO1 protomers on DNA that results in in-phase DNA bending. This binding mode is akin to that inferred for the mammalian upstream binding factor (UBF), whose HMG domains promote a DNA topology that is important for RNA pol I transcription.

MATERIALS AND METHODS

Cloning and Purification of HMO1 Proteins. Cloning of HMO1, HMO1-boxA, and HMO1-boxAB into pET28b for expression with an N-terminal His_6 tag was reported

†Research was supported by the National Science Foundation (MCB-0744240 to A.G.) and the Louisiana Governor's Biotechnology Initiative and by a grant from the NIH-IMSD program to Louisiana State University.

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[1]Abbreviations: HMG, high mobility group; EMSA, electrophoretic mobility shift assay; pol, polymerase; UBF, upstream binding factor.

Published on Web 04/19/2010

previously (*26*, *27*). Fragments encoding HMO1-boxBC and HMO1-boxB were excised from previously constructed pET5a-HMO1-boxBC and pET5a-HMO1-boxB, respectively, using NdeI and subcloned into pET14b to create pET14b-HMO1-boxBC and pET14b-boxB in which the proteins are expressed with an N-terminal His_6 tag. DNA encoding HMO1-Tail was amplified using pET28b-HMO1 as template with primers Tail-fw (5′-CAGCATATGGCTGAACCCTC-3′, with NdeI site underlined) and Tail-rev (5′-ATCCGGATATAGTTCCTCCTT-3′; primer sequence matches that of pET28b downstream of HMO1, which was cloned using NdeI). The PCR product was ligated into pET14b following digestion with NdeI to create pET14b-HMO1-Tail. All of these constructs were sequenced for confirmation. Purification of these N-terminally His_6-tagged HMO1 variants was performed as described previously (*26*). All proteins were judged to be >95% pure by Coomassie staining of SDS−PAGE gels, as documented previously (*26*, *27*). Activity was confirmed by binding to plasmid pGEM5, as described (*26*, *27*).

Protein Cross-Linking. Proteins were cross-linked with 0.1% (v/v) glutaraldehyde in phosphate buffer (pH 7.0) in a total reaction volume of 10 μL at room temperature for 30 min. The reaction was terminated by addition of an equal volume of Laemmli sample buffer, and the cross-linked products were analyzed by SDS−PAGE followed by Coomassie Blue staining.

In Vitro End-Joining Assay. Supercoiled plasmid pGEM5 (3000 bp; Promega) was digested with appropriate restriction enzymes to generate sticky or blunt-ended linearized DNA. Increasing concentration of HMO1 variants was first incubated with 100 ng of linearized pGEM5 at room temperature for 30 min in end-joining reaction buffer (80 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, 10 mM DTT). Appropriate concentration of T4 ligase was added to the reactions, which were incubated at room temperature for 1 h. Ligation products were determined to be linear multimers by incubation with exonuclease III for 1 h. Reactions were terminated by adding stop buffer to final concentration of 5 mM EDTA, 1.1% glycerol, and 0.2 mg/mL proteinase K and incubated for another 1 h at room temperature. DNA was separated from proteins by adding SDS to a final concentration of 0.6% for 5 min before loading the entire sample on a 1% agarose gel in 0.5 × TBE (45 mM Tris−borate, 1 mM EDTA). Samples were electrophoresed at 50 V for 3 h and visualized by ethidium bromide staining.

Exonuclease III Sensitivity Assay. Plasmid pGEM5 was digested with SacII or NaeI to generate sticky or blunt-ended linearized DNA, respectively. HMO1 was dialyzed against 200 mM Tris, 50 mM NaCl, and 20% glycerol after purification to remove imidazole. One hundred nanograms of linearized pGEM5 was incubated with 1.2 μM HMO1 in 1 × exonuclease III buffer (pH 7.0) at room temperature for 1 h. One microliter of exonuclease III was added and incubated at room temperature for another hour. DNA was separated from proteins with 0.6% SDS for 5 min before loading the samples on a 1% agarose gel in 0.5 × TBE. Following electrophoresis at 50 V for 3 h, DNA was visualized by ethidium bromide staining. Experiments were performed at least in triplicate.

Electrophoretic Mobility Shift Assay. Oligonucleotides used to generate 26 bp DNA duplexes were purchased and purified by denaturing polyacrylamide gel electrophoresis. The top strand (5′-CGTGACTACTATAAATAGATGATCCG-3′) was ^{32}P-labeled at the 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP. To generate duplex DNA, a 2-fold excess of unlabeled, complementary oligonucleotide was mixed with labeled oligonucleotide, heated to 90 °C, and cooled slowly to room temperature (23 °C). An aliquot of ^{32}P-labeled duplex DNA was methylated by treatment with 0.5% dimethyl sulfate (DMS) for 10 min at room temperature in a total reaction volume of 10 μL. The reaction was stopped by addition of 2.5 μL of DMS stop solution (1.5 M sodium acetate and 1 M β-mercaptoethanol), and the DNA was recovered by ethanol precipitation. Electrophoretic mobility shift assays (EMSA) were performed using 8% polyacrylamide gels (39:1 (w/w) acrylamide:bisacrylamide) in 0.5 × TBE (45 mM Tris−borate (pH 8.3), 1 mM EDTA). Gels were prerun for 30 min at 175 V at room temperature before the samples were loaded with the power on. DNA and protein were mixed in a binding buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM dithiothreitol, and 0.05% Brij58. Each reaction contained 5 fmol of DNA in a total reaction volume of 10 μL. After electrophoresis, gels were dried, and protein−DNA complexes and free DNA were visualized by phosphorimaging. Experiments were performed at least in triplicate.

DNA Cyclization Assay. Plasmid pET5a was digested with BspHI to yield a 315 bp fragment, which was purified on a 2% agarose gel. The DNA fragment was ^{32}P-labeled at the 5′-ends with T4 polynucleotide kinase. Ligase-mediated DNA cyclization experiments were carried out with varying protein concentrations. Reactions were initiated by addition of T4 DNA ligase to a final volume of 10 μL. Reactions containing 1000 fmol of DNA and the desired concentration of HMO1 were incubated in 1 × binding buffer with 1 × ligase buffer at room temperature for 30 min. Formation of circular ligation product was confirmed by the addition of 1 μL of exonuclease III followed by a 30 min incubation at room temperature. Reactions were terminated using 3 μL of 10% SDS followed by phenol−chloroform extraction and ethanol precipitation. Reactions were analyzed on a 8% polyacrylamide gel (39:1 (w/w) acrylamide:bisacrylamide) with 0.5 × TBE as running buffer. After electrophoresis, gels were dried, and ligation products were visualized by phosphorimaging.

RESULTS

HMO1 Promotes DNA End-Joining by T4 DNA Ligase. Although the HMO1 box A domain bears little sequence similarity to consensus HMG box domains, it not only contributes to DNA binding but also confers the ability to bend DNA (*27*). In contrast, the box B domain contributes most of the DNA binding affinity but fails to bend linear DNA (*27*). In addition to these two domains, HMO1 has a C-terminal domain that is characterized by a stretch of basic amino acids (Figure 1). To determine if HMO1 shares with mammalian HMGB1 the ability to appose DNA ends for facilitated ligation by T4 DNA ligase, we carried out *in vitro* DNA end-joining with linearized plasmid pGEM5 DNA. Full-length HMO1 was assayed, as well as a series of truncated variants: HMO1-boxAB, lacking the lysine-rich C-terminal domain, a variant corresponding to the box A domain only, variants representing box B with or without the C-terminal domain, and the isolated C-terminal domain (Figure 1).

Ligation of linearized plasmid DNA with 2 nt 5′ overhangs by T4 DNA ligase was stimulated significantly by addition of HMO1, compared to a control ligation without HMO1 (Figure 2A). The efficiency of ligation increased with the concentration of HMO1

until a maximum was reached (0.4 μM when 0.2 unit of T4 ligase was used); further addition of HMO1 resulted in inhibition of DNA ligation. That a fraction of the DNA remains unligated at lower protein concentrations may reflect that HMO1 competes with ligase for binding to DNA ends. The ligation products were sensitive to exonuclease III digestion, indicating that they are not a result of DNA cyclization (data not shown). Assuming an ~20 bp site size for HMO1 (as reflected in its 26 nM affinity for 26 bp DNA but an inability to bind stably to 18 bp DNA (*26*)), 100 ng of pGEM5 would present ~780 nM internal sites but only 10.4 nM free DNA ends. With optimal ligation observed at 0.4 μM HMO1, when internal sites would be largely occupied, we therefore surmise that enhanced DNA end-joining is due to HMO1 primarily binding at internal DNA sites.

The HMO1 variant lacking the basic C-terminal tail (HMO1-boxAB) was unable to promote DNA ligation, even with concentrations up to 2.2 μM and 0.2 unit of T4 DNA ligase (Figure 2B). HMO1 variant HMO1-boxBC did stimulate DNA ligation, although with a lower efficiency compared to HMO1. Stimulation was not observed until the concentration of HMO1-boxBC reached 0.8 μM; however, effectively all DNA becomes ligated, suggesting that HMO1-boxBC, unlike full-length HMO1, cannot compete with DNA ligase for binding to DNA ends (Figure 2C). As for full-length HMO1, optimal enhancement of end-joining appears to occur at a protein concentration at which all internal sites may be occupied (the affinity of HMO1-boxBC is only 2-fold lower than that of full-length HMO1 (*27*)). HMO1-boxA, HMO1-boxB, and the C-terminal HMO1-Tail domain showed no enhancement of DNA ligation, even with higher concentration of proteins (Figure 2D− F). Removal of the C-terminal tail and/or box A was previously shown to impair the ability to introduce net DNA bending but to reduce DNA-binding affinity only modestly (*27*). These results therefore show that HMO1-mediated stimulation of DNA end-joining requires the basic C-terminal tail.

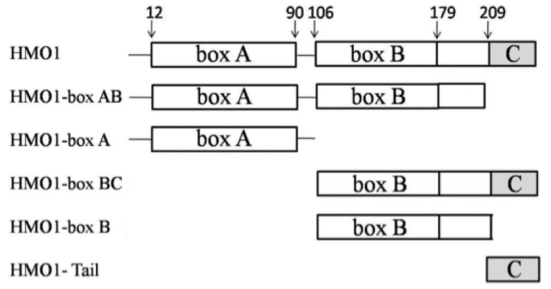

FIGURE 1: Schematic diagram of HMO1 domain organization. HMO1 is composed of HMG domains box A and box B and a highly basic C-terminal tail. Truncated HMO1 variants analyzed in this paper are indicated. All proteins were purified by nickel nitriloacetic acid chromatography to apparent homogeneity (*27*).

Dependence of HMO1-Mediated Ligation on the Type of DNA Ends. The above-described experiments used plasmid pGEM5 linearized with NdeI to generate DNA ends with 2 nt 5′ overhangs. To examine the effect of HMO1 on end-joining of DNA with different ends, pGEM5 was linearized with SacII, NotI, PstI, and NaeI to generate sticky ends with 2 nt 3′ overhangs, 4 nt 5′ overhangs, 4 nt 3′ overhangs, and blunt ends, respectively. As for DNA with 2 nt 5′ overhangs, HMO1 stimulates ligation of DNA with 2 nt 3′ overhangs, with a maximal effect at 0.4 μM concentration, with a further increase in HMO1 resulting in inhibition of the ligation (Figure 3Aa). NotI- and PstI-linearized substrates with 4 nt overhangs showed more robust ligation in that the ligation occurred with much lower concentration of T4 ligase (0.005 unit), likely reflecting the preference of T4 DNA ligase for 4 nt versus 2 nt overhangs. Enhanced ligation was seen at 0.1 μM, and it reached a maximum at 0.4 μM HMO1. Further increased [HMO1] caused inhibition of ligation, with only dimer formed at a concentration of 1.2 μM (Figure 3Ab,c). Under the same conditions, only a modest stimulation of ligation occurred at 0.2 and 0.4 μM HMO1 where only dimer was formed for NdeI-linearized pGEM5; a further increase in [HMO1] does not stimulate ligation (Figure 3Ad). DNA with blunt ends showed weak ligation in the presence of HMO1 by 0.2 unit of T4 DNA ligase; only dimer was formed at a

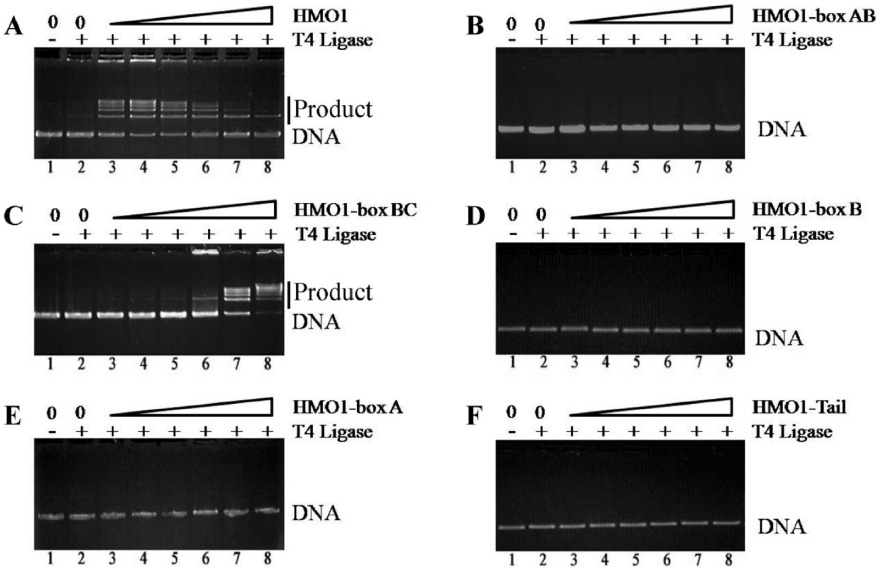

FIGURE 2: HMO1 protein enhances DNA end-joining by T4 DNA ligase. One hundred nanograms of pGEM5 linearized with NdeI was used as substrate. T4 DNA ligase (0.2 unit) was used per reaction. (A−F) Ligation in the presence of different HMO1 variants. Lanes 1, DNA only; lanes 2, DNA and T4 DNA ligase; lanes 3−8, (A) 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μM HMO1; (B) 0.3, 0.6, 1.0, 1.5, 1.8, and 2.2 μM HMO1-boxAB; (C) 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μM HMO1-boxBC; (D) 0.3, 0.6, 1.0, 1.5, 1.8, and 2.2 μM HMO1-boxB; (E) 0.3, 0.6, 1.0, 1.5, 1.8, and 2.2 μM HMO1-boxA; (F) 0.3, 0.6, 1.0, 1.5, 1.8, and 2.2 μM HMO1-Tail.

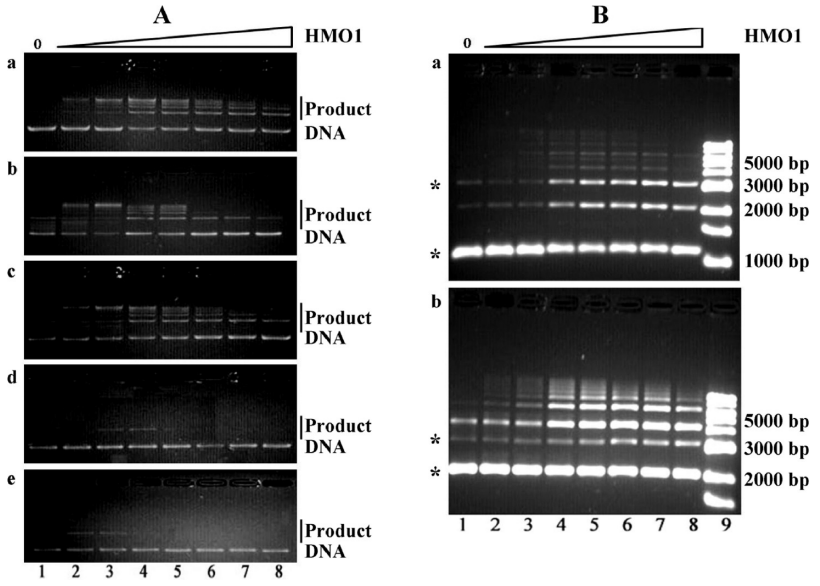

FIGURE 3: Effect of HMO1 on DNA ligation with different ends. Lane 1, DNA and T4 DNA ligase only. Lanes 2−8, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μM HMO1. (A) DNA ligation with complementary ends; 100 ng of linearized pGEM5 was used. (a) Two bp 5′ overhang ends generated by SacII digestion. T4 DNA ligase: 0.2 unit. (b) Four bp 3′ overhang ends generated by NotI digestion. T4 DNA ligase: 0.005 unit. (c) Four bp 5′ overhang ends generated by PstI digestion. T4 DNA ligase: 0.05 unit. (d) Two bp 3′ overhang ends generated by NdeI digestion. T4 DNA ligase: 0.05 unit. (e) Blunt ends generated by NaeI digestion. T4 DNA ligase: 0.2 unit. (B) DNA ligation with noncomplementary ends. One hundred nanograms of NotI-digested pGEM5 and 1000 or 2000 bp fragment of BspHI-digested pGEM5 were mixed as substrates. T4 DNA ligase: 0.005 unit. Lanes 9, 1 kb DNA ladder. (a) 1000 bp. (b) 2000 bp. Asterisks at the left indicate original DNA substrates.

concentration of 0.1 and 0.2 μM, and a further increase in [HMO1] did not stimulate ligation (Figure 3Ae). These results suggest that the stimulation of ligation by HMO1 depends on the length of DNA overhangs, with the most efficient stimulation seen for 4 nt overhangs. No difference was seen with 3′ or 5′ overhangs. Evidently, HMO1 promotes ligation of DNA at equivalent concentrations, regardless of the type of DNA end, arguing against preferred binding to either DNA with blunt or protruding ends; in all cases, however, a fraction of the DNA remains unligated at the optimal [HMO1], suggesting competition between HMO1 and T4 DNA ligase for binding to the DNA ends. Ligation of blunt-ended DNA is not stimulated significantly by HMO1, indicating that it does not work by simply recruiting the DNA ligase. That the efficiency of end-joining matches that of the ligase, combined with the HMO1 concentration dependence, suggests that the stimulation of end-joining is due to HMO1 binding internal sites, not DNA ends.

To substantiate this conclusion, we also examined the ability of HMO1 to stimulate ligation of DNA with noncomplementary ends (expected to be a very low efficiency reaction unless stimulated directly by HMO1). For this purpose, we mixed NotI-digested pGEM5 (4 nt 5′ overhangs) with a 1000 or 2000 bp fragment of BspHI-digested pGEM5 (4 nt 5′ overhangs) as DNA substrates to carry out the ligation experiment. A 5000 bp ligation product may be detected in reaction with 1000 and 3000 bp DNA as DNA substrates when 0.4−1.0 μM HMO1 was added, which could be generated by ligation of a dimer formed by two 1000 bp DNA fragments and the 3000 bp DNA substrate (Figure 3Ba). A 5000 bp ligation product is also formed with 2000 and 3000 bp DNA as substrates, although in very low abundance and with HMO1 optimum of 0.1−0.4 μM (Figure 3Bb). The low efficiency of joining incompatible ends is likewise consistent with reported properties of T4 DNA ligase (*28*).

The Stimulatory Effect of HMO1 on DNA End-Joining Depends on the Concentration of Base Pairs but Not on the Relative Abundance of DNA Ends. We investigated further the stoichiometry of HMO1 with reference to the substrate DNA. When 25 ng of NdeI-digested pGEM5 substrate was used (2.3 nM free DNA ends), the ligation maximum was reached at 0.2 μM HMO1. Increasing the amount of substrate to 50 or 100 ng (i.e., increasing both the concentration of free DNA ends and total concentration of base pairs) does not significantly affect the concentration of HMO1 required for the most efficient ligation (0.2 μM). However, a notable difference is that a higher concentration of substrate results in ligation products being observed also at higher concentration of HMO1 (e.g., concentrations of HMO1 higher than 0.2 μM become inhibitory to ligation of 25 ng of DNA, concentrations at which ligation of 50−100 ng of DNA still occurs; Figure 4A). These results suggest the effect of HMO1 on ligation depends on the total amount of DNA substrate. Since the abundance of DNA ends is proportionally greater in reactions assembled with equivalent amounts of short compared to longer substrates, we also generated 1000 and 2000 bp size substrates by digesting pGEM5 with BspHI and carried out ligation reactions with 40 ng of each substrate. Under these circumstances, when the total concentration of base pairs is equivalent, but the concentration of free DNA ends is 2-fold greater in reactions assembled with 1000 bp DNA, greater ligation efficiency would be expected for 1000 bp DNA if HMO1 preferred binding to free DNA ends. This is not observed (Figure 4B). While 2000 bp DNA appears to be ligated more efficiently at lower protein concentrations, the maximal efficiency for both substrates is seen at equivalent [HMO1] (Figure 4B, lane 4). Taken together, these experiments suggest that HMO1 binds preferentially to internal sites.

HMO1 interacts with linearized DNA to facilitate ligation of DNA ends in a reaction that depends primarily on the total concentration of base pairs, which would argue against preferred binding of HMO1 to DNA ends. Consistent with this interpretation, we also note that HMO1 does not interfere with ligations

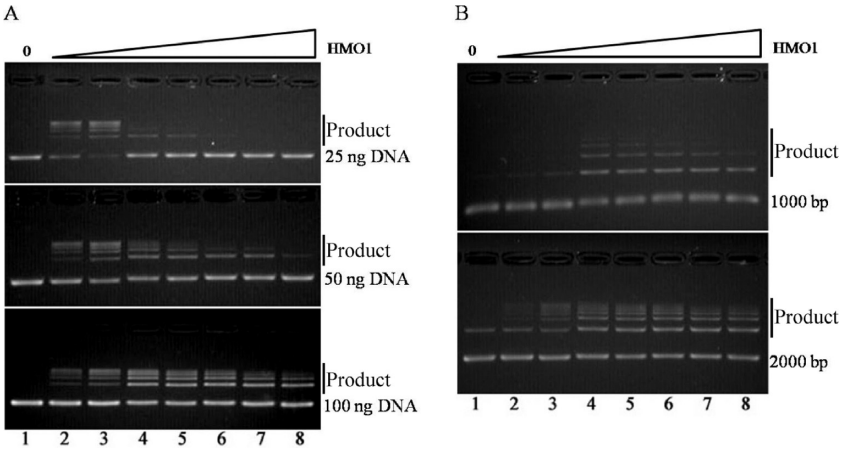

FIGURE 4: Effect of HMO1 on DNA ligation depends on the amount and the length of the DNA substrate. T4 DNA ligase: 0.02 unit. Lanes 2−8, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μM HMO1. (A) Different amounts of NdeI-digested pGEM5 were used as substrate. (B) 1000 and 2000 bp DNA fragments generated by digestion of pGEM5 with BspHI were used as substrates.

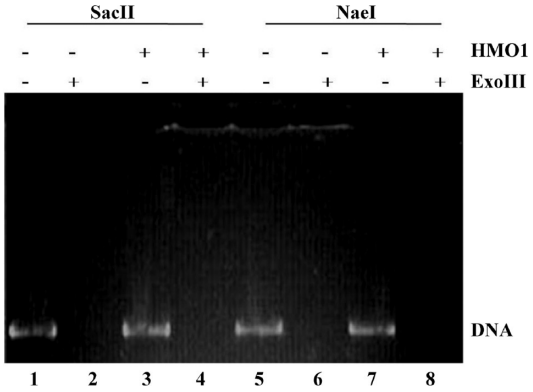

FIGURE 5: HMO1 does not protect DNA from exonuclease III digestion. Linearized DNA was treated with exonuclease III in the presence or absence of HMO1 (1.2 μM). Lanes 1−4, linearized DNA with sticky ends (digested with SacII). Lanes 5−8, linearized DNA with blunt ends (digested by NaeI). Lanes 1 and 5, DNA only. Lanes 2 and 6, DNA incubated with exonuclease III. Lanes 3 and 7, DNA incubated with HMO1. Lanes 5 and 8, DNA incubated with HMO1 and exonuclease III.

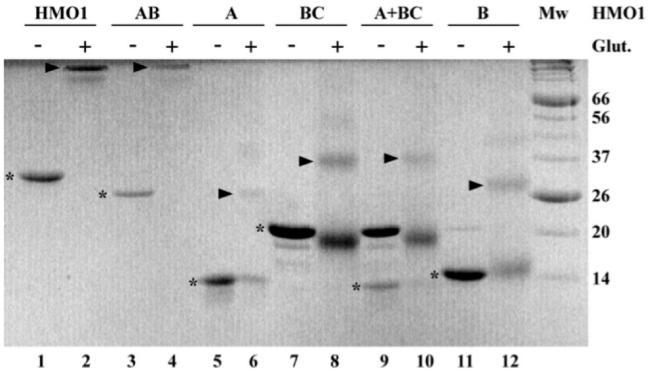

FIGURE 6: All HMO1 variants self-associate. SDS−PAGE gel stained with Coomassie Brilliant Blue showing HMO1 variants before and after cross-linking with 0.1% glutaraldehyde. Addition of glutaraldehyde (even-numbered lanes) is specified at the top, below the identification of individual HMO1 variants. Molecular weights are identified at the right. Asterisks identify the HMO1 variant, and arrowheads point to the predominant cross-linked species.

under conditions of excess ligase (data not shown). To assess whether HMO1 binds preferentially to DNA ends, we added exonuclease III to reactions in which HMO1 was incubated with either sticky or blunt-ended DNA. Regardless of the presence of HMO1, exonuclease III digests linearized DNA with sticky or blunt ends (Figure 5). We note that the failure to protect against exonucleolytic degradation by itself does not rule out end-binding, as the ability to protect effectively against exonuclease III also depends on the stability of the resulting complex; an unstable or very dynamic interaction between protein and DNA end would likely still permit efficient exonuclease III digestion of the DNA. However, an interpretation consistent with all data, not just the exonuclease III protection assay, is that HMO1 does not have a strong preference for DNA ends.

HMO1 Self-Association Is Not Sufficient To Mediate End-Joining. HMO1 has been previously reported to self-associate (*26*, *27*, *29*), a property consistent with the ability to bring DNA ends together. Extensive self-association at high concentrations of HMO1 is, for example, evidenced by its elution from a gel-filtration column as a large complex (data not shown). Notably, the failure of HMO1-boxAB to promote end-joining is not due to attenuated self-association, as indicated by the efficient cross-linking of both full-length and C-terminally truncated HMO1 in solution; for both proteins, addition of glutaraldehyde produces large cross-linked species that remain near the well of the gel (Figure 6, lanes 1−4). While cross-linking of HMO1-boxA (lanes 5 and 6), HMO1-boxB (lanes 11 and 12), and HMO1-boxBC (lanes 7 and 8) is also seen, reflecting self-association, these HMO1 variants primarily produce cross-linked species corresponding to dimers and do not form the higher order species observed with HMO1 and HMO1-boxAB. Notably, only HMO1-boxBC can promote apposition of DNA ends. Conversely, cross-linking of HMO1-boxA to HMO1-boxBC does not appear to be efficient, as no cross-linked species are seen that do not correspond to those seen with either HMO1-boxA or HMO1-boxBC (lanes 9 and 10). Cross-linking with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), which couples carboxyl groups to primary amines, also showed self-association of boxA and boxB but no evidence of boxes A and B cross-linking to each other (data not shown). Further, DNA bending by full-length HMO1 cannot be reproduced by combining HMO1-boxA and HMO1-boxBC; this is also consistent with (but not evidence of) lack of interaction (*27*). We therefore favor the interpretation that self-association of HMO1 is mediated by boxes A and B interacting with their counterparts in a

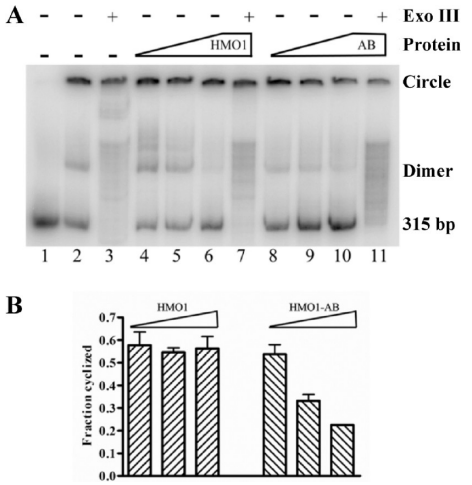

FIGURE 7: HMO1-boxAB prevents DNA cyclization. (A) Two hundred nanograms of 315 bp DNA (lane 1) was incubated with T4 DNA ligase (lanes 2−11) and the indicated HMO1 variant (for HMO1, 1, 4.8, and 12 μM; for HMO1-boxAB, 0.9, 4.5, and 9 μM). Reactions in lanes 3, 7, and 11 were also incubated with exonuclease III. Original 315 bp DNA, dimer, and cyclized product are identified at the right. (B) Fraction of cyclized product.

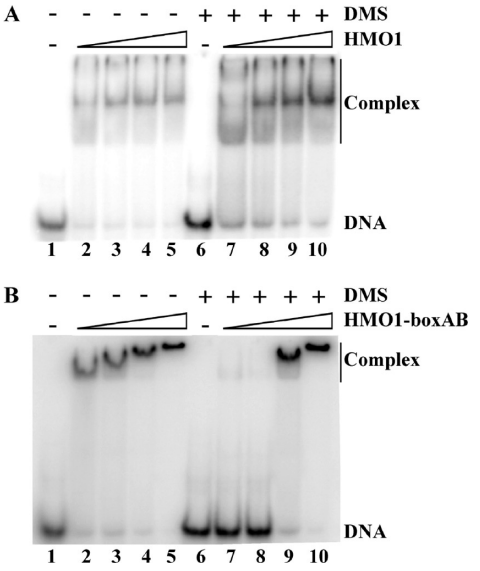

FIGURE 8: The lysine-rich C-terminal tail modulates interaction with short DNA. EMSA with 5 fmol of 26 bp DNA titrated with HMO1 (panel A) or HMO1-boxAB (panel B). Reactions in lanes 6−10 contain DNA methylated by incubation with DMS; reactions in lanes 1 and 6 contain no protein. Concentrations of HMO1 and HMO1-boxAB are 1.2−8 and 0.5−2.4 μM, respectively.

neighboring HMO1 (although we note that while the data support the occurrence of homomeric interactions, they do not exclude interactions between boxes A and B as such interactions may have occurred in solution but may have not been captured by this assay). The mechanism of HMO1 self-association notwithstanding, the significant conclusion is that protein self-association *per se* is insufficient for proper apposition of DNA ends for enhanced DNA ligation and that the mode of DNA binding by individual HMO1 variants must contribute to the observed effect on end-joining.

Removal of the C-Terminal Tail Produces a Binding Mode That Stiffens the DNA. A previously noted difference between HMO1 variants is the ability to introduce DNA bends, as evidenced by the ability to promote intramolecular cyclization of DNA shorter than the persistence length (*27*). The HMO1-mediated cyclization of 105 bp DNA was severely attenuated on removal of the C-terminal domain, indicating that it is required to achieve net DNA bending. To evaluate whether the failure to observe DNA bending by HMO1-boxAB may derive from multiple protomers producing out-of-phase bending, we performed the ring-closure experiment with 315 bp DNA that may cyclize in the absence of any DNA-bending protein (Figure 7). While cyclization of 315 bp DNA occurs in the presence of T4 DNA ligase, as evidenced by the formation of cyclized products that are resistant to exonuclease III (lanes 2 and 3), addition of HMO1-boxAB attenuates formation of cyclized product, while linear dimer may still be observed (lanes 8−10). In contrast, addition of full-length HMO1 initially promotes formation of linear multimers (lane 4), while at higher concentrations, cyclized product is formed in preference to linear multimers (lane 6). Both HMO1 and HMO1-boxAB bind DNA with positive cooperativity, predicting that both proteins would accumulate on the 315 bp DNA (*27*). That HMO1-boxAB, but not wild-type HMO1, prevents cyclization suggests that it stiffens the DNA, likely by accumulation of multiple protomers that cause out-of-phase bending.

The C-Terminal Tail Alters Interaction with Short Duplexes. A single HMG domain interacts with ~10 bp of duplex (*5*, *30*−*32*). For HMO1, interaction of each box A/B domain with ~10 bp of duplex would predict association with an ~20 bp DNA site, a prediction corroborated by the inability of HMO1 to associate stably with 18 bp DNA (*26*) and formation of detectable complex with 22 bp DNA (data not shown) and 26 bp DNA (Figure 8A and ref *26*). Complexes with full-length HMO1, particularly complexes with lower mobility, are unstable and dissociate during electrophoresis. Notably, removal of the C-terminal tail to generate HMO1-boxAB results in the formation of complexes with 26 bp DNA with a distinct mobility (Figure 8B). Considering that HMO1-boxAB has lower M_w and significantly lower theoretical p*I* (8.43 for full-length HMO1 compared to 5.68 for HMO1-boxAB), a 1:1 complex of HMO1-boxAB with short DNA duplex (that would not be expected to migrate differently due to protein-mediated bending as the flanking duplex would be too short for such differences in mobility to be manifest) would be expected to migrate faster than an equivalent complex with full-length HMO1. However, we observe that HMO1-boxAB associates preferably with 26 bp DNA to produce a slow-migrating complex, suggesting that even this fastest migrating complex does not represent a 1:1 stoichiometry of protein to DNA. Association of HMO1 and HMO1-boxAB with 26 bp DNA was previously shown to be cooperative, with Hill coefficients of 1.6 ± 0.1 and 2.4 ± 0.2, respectively (*27*). While Hill coefficients only report on the stoichiometry of binding in the limiting case of infinite cooperativity, these data do suggest that more than two HMO1-boxAB molecules can bind to 26 bp DNA, an inference consistent with the pattern of complex migration. Taken together, our data therefore suggest that removal of the C-terminal tail promotes a closer apposition of protein molecules on the DNA. As 26 bp *B*-form DNA would present only two minor grooves for interaction with an HMG domain on one face of the helix, and since HMO1-boxAB contains two domains predicted to bind the DNA minor grooves, this DNA duplex is too short for two or more protomers binding on one face of the duplex. The formation of more stable complexes by multiple HMO1-boxAB protomers therefore suggests interaction on both faces of the duplex, an interpretation that is

consistent with the inference that HMO1-boxAB induces out-of-phase bending.

So far, our data suggest that the C-terminal extension of HMO1 is required to produce a binding mode of HMO1 in which multiple protomers bind in-phase with the helical repeat. However, while the primary binding interactions of HMGB proteins occur in the widened minor groove (*5*, *30*−*36*), basic extensions beyond the HMG-fold have been shown to lie in the major groove and have been proposed to promote DNA bending. An alternative interpretation could therefore be that the C-terminal extension of HMO1 binds in the DNA major groove to stabilize a DNA bend, resulting in bending by full-length HMO1 but not by HMO1-boxAB. To address whether this is so or, more generally, if extensions beyond or between box A and box B interact in the major groove, the 26 bp DNA was treated with dimethyl sulfate, which primarily methylates N7 of guanine in the major groove. Limited DNA methylation does affect complex formation by both HMO1 and HMO1-boxAB (Figure 8A-B); while complex formation by HMO1 is only marginally attenuated, consistent with the predominant interactions occurring in the DNA minor groove, complex formation by HMO1-boxAB is inhibited at low protein concentrations. A significant conclusion in the context of this work is that we do not see an effect of limited DNA methylation on DNA binding by full-length HMO1 that is attenuated on removal of the C-terminal domain, indicating that its function is not to engage the DNA major groove. Instead, we find the opposite effect, namely, that DNA binding by HMO1-boxAB is more severely affected by DNA methylation than full-length HMO1. Since full-length HMO1 does not appear to require significant interaction in the DNA major grooves, we infer that DNA methylation may hinder conformational changes in the DNA associated with HMO1-boxAB binding as opposed to preventing direct contacts.

DISCUSSION

The HMO1 C-Terminal Domain Is Required for DNA Apposition and Net DNA Bending. While both the box A and C-terminal domains of HMO1 must be present to observe net DNA bending (*27*), box A is dispensable for DNA end-joining; the more efficient stimulation of ligation by full-length HMO1 compared to HMO1-boxBC may be due to its higher affinity for a single DNA site (*27*). That HMO1-boxA is not required also suggests that apposition of DNA ends does not involve significant changes in DNA topology. Notably, we find that the lysine-rich C-terminal domain is essential for the ability of HMO1 to promote DNA end-joining (Figure 2). This is comparable to mammalian homologues where extensions beyond the HMG box domain have also been shown to be important for DNA end-joining; the acidic C-terminal flanking sequence of the mammalian HMGB1 box B domain was shown to be important for stimulation of DNA ligation, and the sequence preceding the box A domain has also been implicated, as acetylation of a unique lysine promotes end-joining at the expense of DNA bending (*9*).

HMO1 does not appear to bind with any preference to DNA ends, as evidenced by ligation efficiencies reflecting preferences of T4 DNA ligase and a concentration dependence that corresponds not to the concentration of free ends but to total DNA content. Indeed, optimal ligation efficiency occurs at a concentration of HMO1 that corresponds to occupancy of most internal sites. Second, protein self-association *per se* is insufficient to promote ligation of DNA ends (Figure 6). For mammalian HMGB1, Stros et al. (*7*) found that residues in the HMGB1 box B domain critical for its ability to stimulate ligation were also important for self-association of the box B domain on supercoiled DNA and the formation of large nucleoprotein complexes. While protein−protein interactions of HMO1 domains are undoubtedly involved in appositioning DNA ends, the absence of the C-terminal domain abolishes this effect, although it does not prevent self-association. As DNA-binding affinity is essentially unaffected by removal of the C-terminal domain from either full-length HMO1 or HMO1-boxBC (*27*), the C-terminal domain does not simply prevent a self-association that obscures the DNA-binding interface. We therefore surmise that the mode of DNA binding contributes to the observed effect on DNA end-joining.

Distinct modes of DNA binding induced by the presence of the lysine-rich C-terminal domain are also evidenced by its role in DNA bending. HMO1-boxAB does not promote cyclization of 105 bp DNA (rather, reactions with HMO1-boxAB are seen to produce linear dimer at concentrations where formation of circular product is inhibited, indicating that it does not function by merely inhibiting DNA ligase (*27*)). Instead, HMO1-boxAB prevents the cyclization of 315 bp DNA that occurs in its absence (Figure 7). In contrast, full-length HMO1 efficiently promotes cyclization of short DNA duplexes and does not inhibit ligase-mediated cyclization of 315 bp DNA. Since both proteins bind DNA with positive cooperativity (*27*), accretion of full-length HMO1 on the DNA evidently results in in-phase DNA bending, while accumulation of HMO1-boxAB likely causes out-of-phase bending, as evidenced by the stiffening of 315 bp DNA. This interpretation is supported by the mobility of complexes with 26 bp DNA that suggest the ability of more than two HMO1-boxAB molecules to bind simultaneously; as each boxA/B domain is predicted to bind DNA minor grooves, such binding would predict association on opposite faces of the DNA duplex with out-of-phase bending occurring as a result.

DNA Apposition and In-Phase Bending May Promote DNA Looping. For several HMGB proteins, it has been suggested that basic extensions beyond the HMG box contribute to DNA bending by occupying a compressed major groove; for example, NHP6A has a basic N-terminal extension whose Arg and Lys residues contact the phosphate backbone on the opposite face of the expanded minor groove that is occupied by the HMG box, and LEF1 has a basic C-terminal extension that is similarly directed toward the major groove by a proline-mediated kink (*30*−*36*). For HMO1, the marginal sensitivity to limited methylation argues against significant interactions in the major groove (Figure 8). By inference, the inhibition of DNA binding by HMO1-boxAB on DNA methylation therefore points to conformational changes that are hindered by methylation and implying an altered binding mode associated with elimination of the lysine-rich tail.

Considering that boxA is essential for DNA bending (*27*), our data suggest that multiple HMO1 molecules bind with their box A domains in-phase with the helical repeat, perhaps in consecutive minor grooves, and that the C-terminal extensions block access to the opposing minor grooves. In analogy with other HMGB proteins, HMO1 likely also unwinds its DNA site as evidenced by the observation that its ability to constrain DNA supercoils is salt-dependent and the observation that 87 bp DNA is cyclized more efficiently than 105 bp DNA (*27*); the former would constitute 8.3 turns and the latter 10.0 turns assuming *B*-form DNA, implying a change in the helical twist on

HMO1-binding to create compatible DNA ends for T4 DNA ligase. Such binding mode is consistent with the formation of unstable complexes of full-length HMO1 with 26 bp DNA, not quite long enough to accommodate two HMO1 molecules with their boxA/B domains binding in consecutive minor grooves (Figure 8). In contrast, not only does removal of the C-terminus abrogate DNA apposition, but our data suggest that it permits an association of HMO1-boxAB such that its box A domains bind out-of-phase, that is, on opposite faces of the DNA duplex. Such binding might allow a DNA segment to be contacted simultaneously by two HMO1-boxAB molecules, thus creating a more stable complex. On longer DNA, HMO1-boxAB may therefore form filaments whose DNA ends cannot be brought into alignment for T4 DNA ligase to work.

The structure of an HMGB didomain bound to DNA suggests that 16 bp DNA may be the minimum duplex length capable of affording the necessary contacts. The structure of the HMGB didomain composed of SRY and mammalian HMGB1 box B revealed that both HMG boxes bind the minor groove, as expected, and that they are oriented in a head-to-head mode covering a total of 16 bp, of which the middle segment is contacted by both HMG domains. The DNA is locally underwound and features a widened minor groove and a compressed major groove. Intercalating residues from each HMG box is separated by 9 bp, yielding in-phase bending (*32*). A binding mode in which HMO1 accumulates on duplex DNA with its boxA/B domains in-phase with the helical repeat, combined with its ability to juxtapose distant DNA sites, predicts the formation of DNA loops. Indeed, HMO1 was recently proposed to stabilize a looped or other topologically constrained DNA conformation during S phase transition (*37*).

A Role for HMO1 in DNA Looping? In *S. cerevisiae*, rRNA (rRNA) genes are encoded in a single array consisting of 150−200 head-to-tail repeats. Yeast RNA pol I is brought to its promoters by upstream activating factor (UAF), for which no mammalian homologue has been identified, core factor (CF), equivalent to mammalian SL1, TATA binding protein, and Rrn3, which is equivalent to mammalian TIF-IA (for review, see ref *38*). Mammalian RNA pol I also requires upstream binding factor UBF, which has been suggested to produce an enhancesome structure consisting of UBF dimers that fold the DNA into tight loops. Such loop formation is brought about by the in-phase DNA bending produced by three sequential HMG domains of each UBF monomer (*39*, *40*). UBF has been implicated in both initiation and elongation by mammalian RNA polymerase I (*41*). Yeast encodes no homologue of UBF; instead, HMO1 has been shown to be important for maximal pol I transcription, but the molecular basis for its involvement remains unclear (*18*). However, an intriguing similarity between mammalian UBF and yeast HMO1 is that both proteins have been reported to localize throughout the transcribed rDNA region (*19*, *20*, *22*, *42*, *43*). It is also noteworthy that HMO1 overexpression suppresses an *rpa49*Δ mutation, as RPA49 corresponds to the mammalian PAF53, which interacts directly with UBF (*19*, *41*, *44*). It is therefore tempting to speculate that HMO1 contributes to yeast pol I transcription through its ability to constrain DNA topologies akin to the looping inferred for UBF.

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