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Interactions of the High Mobility Group Box Homolog 2 with distorted duplex DNA

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Interactions of the High Mobility Group Box Homolog 2 with distorted duplex DNA

A study of the role of HMO2-Box A residue valine in DNA strand intercalation

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3/22/2010

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Abstract

The functional roles of one of the HMG domains of the yeast homolog HMO2 were examined by identifying amino acid residues that are involved in DNA intercalation. Through site directed mutagenesis, a valine in the box A domain (site 19), predicted to intercalate between DNA base pairs, was substituted with alanine. A binding assay with linear DNA showed that the mutated protein has lower binding affinity compared to wild type HMO2, while both mutant and wild type proteins have similar affinity for supercoiled DNA. Binding assays involving distorted DNA showed that both wild-type and mutant protein bound with only modest preference to DNA with one loop and two loops that were seven base pairs apart. However, DNA with two loops that were nine base pairs apart and DNA with one loop and an abasic site proved to be the best substrates for both wild-type and mutant protein. HMO2 protein's preference for distortions that are nine base pairs apart over those that are seven base pairs apart suggests that distance between distortions is an important consideration in HMO2-DNA interactions. To determine what construct HMO2 prefers over DNA with two loops that are nine base pairs apart, a competition assay was run using supercoiled and linear DNA. Supercoiled DNA was able to out-compete DNA with 2 loops while linear was not. Moreover, results of a protection and an end-binding assay clearly indicate that HMO2 protein binds DNA ends as it effectively inhibits the actions of exonuclease and DNA ligase. Evidently, the substitution of the valine residue in HMO2 box A reduces the protein's ability to bind to linear DNA but has no significant effect on its binding to DNA ends or to DNA with distortions.

Introduction

Proteins serve as important structural and functional modulators of various cellular activities in living systems. How a protein interacts with important cellular components such as DNA is crucial to our understanding of its overall role. One such group of DNA-binding proteins belongs to the High Mobility Group Protein (HMG) superfamily. These are a relatively abundant collection of architectural nuclear proteins that are involved in transcription[1], replication, recombination and DNA repair (Kamau et al, 2004). Architectural proteins are known for their role in the folding, organization and compaction of genomic DNA-all of which alters chromatin[2] conformation. DNA lacking chromatin structure maintainers such as histones[3] are affected by these nuclear[4] proteins as they are easier to navigate.

High Mobility Group Proteins are classified according to their binding domain, or the part of the protein that physically binds DNA. There are essentially three main categories of HMG binding domains: AT-hook domains (HMGA), box domains (HMGB) and nucleosomal binding domains (HMGN) (Stros *et al*, 2004). Out of the three, the box domains are more characteristic of vertebrate HMG proteins and are therefore studied extensively. Yeast cells (*Saccharomyces cereviseae*) serve as a model for understanding vertebrate HMG protein functions because they possess HMG proteins with box domains that closely resemble those found in mammalian cells. Research into the roles of yeast homologue 1 (HMO1), one of the 10 homologues found in yeast, has shown that it is required for normal growth and plasmid[5]

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[1] RNA synthesis or the process of creating an equivalent RNA copy of a sequence of DNA

[2] complex combination of DNA and proteins that makes up chromosomes. It is found inside the nuclei of eukaryotic cells.

[3] strongly alkaline proteins found in eukaryotic cell nuclei, which package and order the DNA into structural units called nucleosomes

[4] pertaining to the nucleus

[5] an extra chromosomal DNA molecule separate from the chromosomal DNA which is capable of replicating independently from the chromosomal DNA. They are double stranded and in many cases, circular

maintenance and for regulating the susceptibility of yeast chromatin to nuclease[6] (Kamau *et al*, 2004).

Although expected to behave similarly to HMO1, the roles of HMO2 have not been explored extensively. Yeast HMO2 has been observed around double strand breaks in duplex[7] DNA and is suspected to play a specific role in repairing these defects in DNA structure (Ray and Grove, 2009). It consists of two boxes, A and B, and a long acidic C-terminal[8] domain. HMG boxes bind DNA without much sequence specificity and have a high affinity for bent or distorted DNA (Thomas, 2001). Each box domain is predicted to fold into three α helix[9] structures in an irregular array consisting of approximately 80 amino acids.

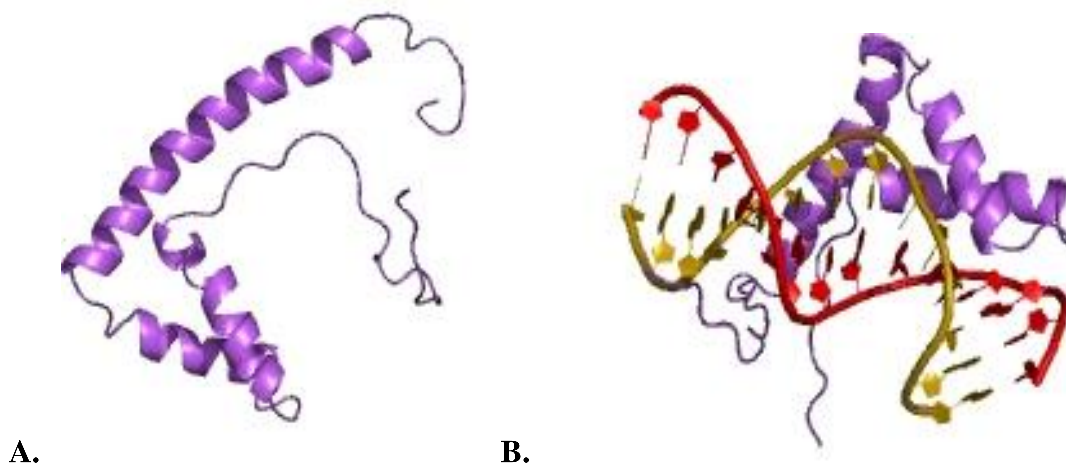


Figure 1. **(A)** Each box in HMO2 is predicted to fold into three α helices. **(B)** Ribbon diagram of the HMG-box helices binding within the minor groove of DNA where DNA damage has occurred (Stros *et al*, 2007).

---

[6] an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of DNA

[7] double-stranded

[8] Carboxyl-terminus of a protein or polypeptide is the end of the amino acid chain terminated by a free carboxyl group (-COOH).

[9] A common motif in the general three-dimensional form of each local segment of a protein

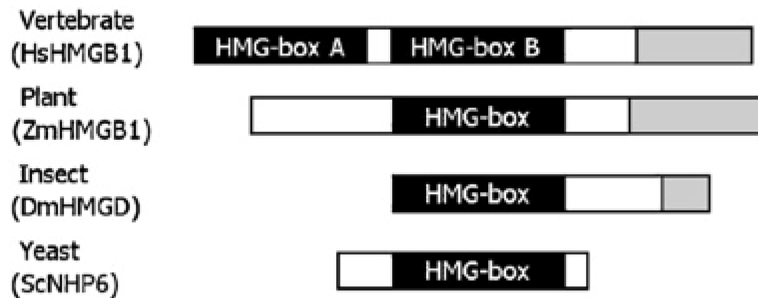


Figure 2. Schematic representation of structures of HMG-type proteins from various eukaryotes. Gray boxes indicate C terminal acidic domains. (Stros *et al*, 2007).

Structural and physical properties of DNA also provide important constraints on the binding sites formed on surfaces of DNA-binding proteins. Characteristics of such binding sites may be used for predicting DNA-binding sites from the structural and even sequence properties of unbound proteins (Zhou and Tjong, 2007). In the case of yeast HMO2, determining the structural defect(s) in the DNA duplex that allow for tighter DNA-protein binding can yield insight into the protein's recognition process in general. The ideal DNA substrate[10] for our protein would lead to a greater understanding of its conformation and its DNA repair capabilities.

In addition to structural considerations, the actual DNA repair process must also be examined in order to understand HMO2's function. A process of DNA intercalation has been shown to accompany DNA binding by HMGB proteins. Intercalation of DNA strands occurs when amino acid side chains of an appropriate size and chemical nature fit in between base pairs of DNA. Such behavior by HMO2 has led to the idea that it may bind preferentially to DNA repair intermediates. This is to say HMO2 would recognize and bind duplex DNA containing a variety of defects. Moreover, multiple studies concluding that the four-way junction DNA construct is the ideal substrate for HMGB proteins lends more credence to the claim that the box domain is directly involved in strand repair (Bianchi *et al*, 1998). The four-way junction is a

[10] a molecule acted upon by an enzyme

structure where two DNA double strands are held together by reciprocal exchange of two of the four strands, one strand each from the two original helices. The DNA repair process is ultimately made possible by the ATP-dependent chromatin-remodeling complex (INO80) that binds to double strand breaks in DNA. HMO2 is a component of IN080 and has been shown to be important for its recruitment to DNA double strand breaks. Such remodeling complexes have been evolutionarily conserved in mammalian cells and usually help DNA processing enzymes overcome accessibility difficulties brought about by the packaging of the eukaryotic genome into chromatin (Morrison *et al*, 2004). The yeast IN080 complex, which contains several actin[11] and actin-related proteins, is implicated in both transcription and DNA repair. The complex carries out its functions by using the energy of ATP hydrolysis[12] to move, destabilize or restructure nucleosomes (Clapier and Cairns, 2009).

The functional roles of the HMO2 homologue in the intercalation of strands can be observed by examining its binding ability after a mutation has been introduced. In bioinformatics, a sequence alignment is often used to arrange sequences of DNA, RNA, or protein and identify regions of similarity that may have resulted from a functional, structural, or evolutionary relationship between the sequences. Sequence alignment assays comparing HMO2 with HMO1 in *Saccharomyces cerevisae* were carried out in order to identify the amino acid residues of possible consequence to the protein's overall function. Since the Box A residues in both homologs failed to align properly, valine at site 19 and serine at site 47 were picked using educated guesses based on prior knowledge of HMG-Box A amino acid interactions. The alignment assay for Box B residues showed a leucine at site 102 and serine at site 117 at pivotal

[11] monomeric component of the cytoskeletal system that allows movement of cells and cellular processes

[12] the reaction by which chemical energy that has been stored and transported in the high-energy phosphoanhydridic bonds in ATP (Adenosine triphosphate) is released

regions of the HMO2 homolog. Mutations at these four sites will help us clue in on the amino acid residues that are the key players in this homolog.

A.

```
1    MSVEEKKRRL EELKDQNVVL GLAIQRSRLS VKRLKLEYGV LLERLESRIE
51   LDPELNCEDP LPTLASFKQE LLTKPFRKSK TKRHKVKERD PNMPKRPTNA
101  YLLYCEMNKE RIRQNGSLDV TRDLAEGWKN LNEQDRKPYY KLYSEDRERY
151  QMEMEIYNKK ISNIDADDDK EENEQKIKNN EEGSSTKVAD SKGGEDGSLV
201  SSN
```

B.

```
HMO1   93    KAVRRKI-ERDPNAPKKPLTVFFAYSAYVRQELREDRQKAGLPPLSSTEITQEISKKWKE  151
             K   R K+ ERDPN PK+P    +   Y     ++ +R++           S ++T+++++ WK
HMO2   80    KTKRHKVKERDPNMPKRPTNAYLLYCEMNKERIRQN---------GSLDVTRDLAEGWKN  130

HMO1   152   LSDNEKEKWKQAYNVELENYQREKSKY  178
             L++ +++ + + Y+ + E YQ E   Y
HMO2   131   LNEQDRKPYYKLYSEDRERYQMEMEIY  157
```

C.

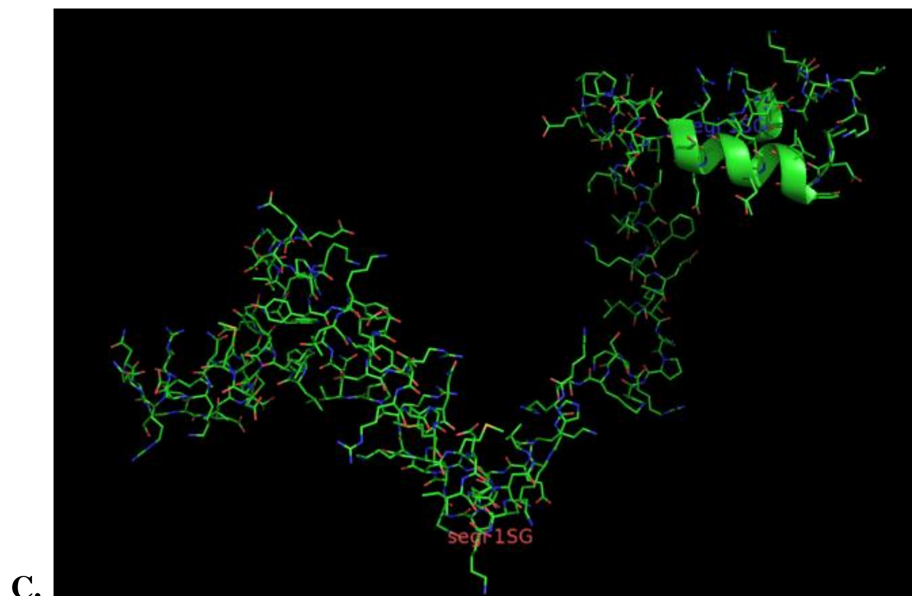
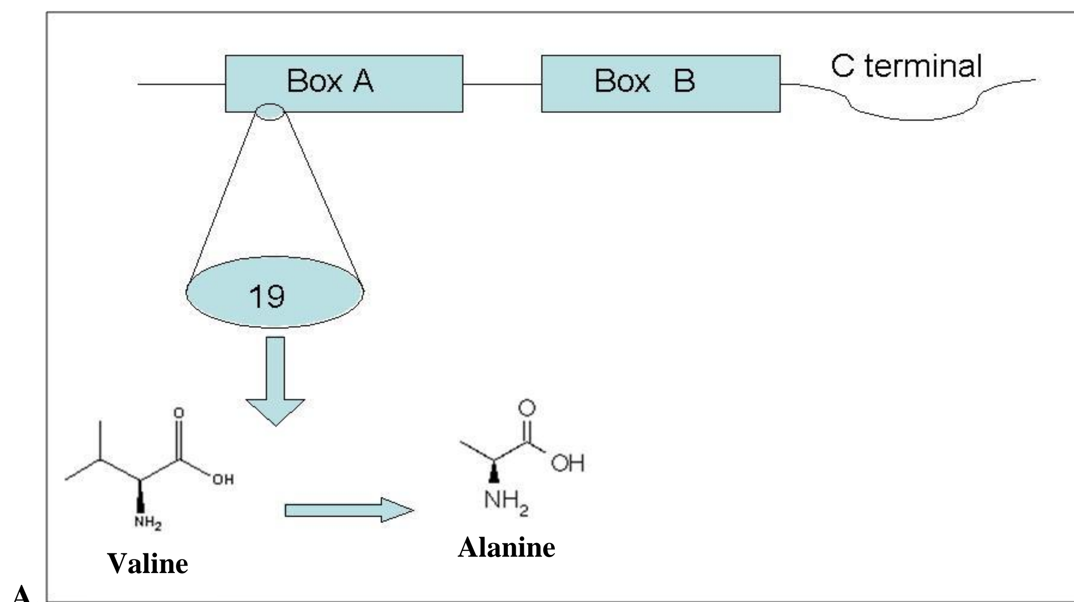


Figure 3. **(A)** Protein sequence of HMO2 with highlighted amino acids of interest: Valine (V) at site 19 and Serine (S) at site 47 from HMO2-Box A; Leucine (L) at site 102 and Serine (S) at site 117 from HMO2-Box B. Stretches of amino acids that make up the α helices are underlined (*Pubmed* FASTA). **(B)** Sequence alignment of HMO1 and HMO2 Box B (acquired from blasting protein sequences on *Pubmed*). Leucine at site 102 and Serine at site 117 have been highlighted in blue **(C)** Ribbon diagram of Helix III in the HMO2 structure (image generated by *Pymol).*

Experimental Procedures and Results

The gene encoding HMO2 was amplified from yeast genomic DNA and cloned into plasmid pET14b. Using the sequence of the wild type HMO2, we designed appropriate primers[13] that would carry out our first mutation of choice by substituting a valine residue at site 19 with an alanine. The shorter side chain of alanine would be expected to be less efficient at intercalating between DNA bases.



A.

B.

<u>HMO2-BOX A primers</u>

val19ala fwd primer: AAT GTG **<u>GCT</u>** CTT GGA CTT GCA ATC Tm= 58.1°C

val19ala rev primer: CTG ATC TTT CAA TTC TTC AAG TCT GCG C Tm = 58 °C

Figure 4. (A) Schematic representation of site directed mutagenesis carried out in the HMO2 gene. **(B)** Primers designed to carry out site-directed mutagenesis in HMO2 gene. Optimal melting temperatures (Tm) are listed next to each primer.

[13] short, chemically synthesized fragments of DNA with a length of about twenty bases that hybridize to a target DNA, which is then copied by the polymerase

Site-Directed Mutagenesis[14] through Polymerase Chain Reaction (PCR)[15]

A reaction containing plasmid DNA (pET 14b containing HMO2 gene), forward and reverse primers, a polymerase used to elongate DNA strands (Taq) and a proofreading enzyme (Pfu) underwent repeated cycles of Polymerase Chain Reaction (PCR) in order introduce the designed mutation and amplify the entire plasmid.

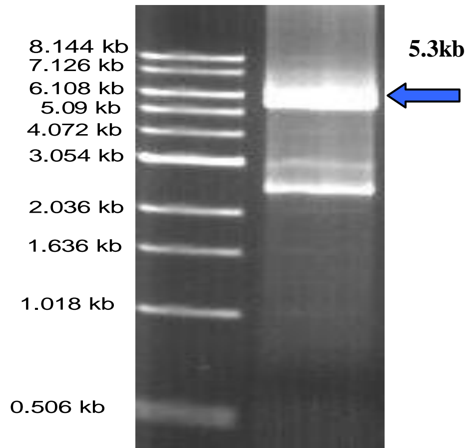


Figure 5. The PCR product (5µL) was run on a 1% agarose gel along with a standard 1kb marker. The blue arrow points to a thick band at 5.3 kb, which is the correct size of the plasmid.

Transformation into E. coli

The plasmid containing the mutated gene was then chemically transformed into Top10 cells. The antibiotic resistance conferred by the plasmid helped select for colonies containing the gene of interest on an agar plate. Colonies were grown overnight and plasmid prepped the following day. Samples that showed a 5.3 kb band when run on a 1% agarose gel were selected for overexpression in *E. coli* cells since they seemed to have taken up the correct plasmid. For confirmation, the plasmid DNA was sequenced.

[14] site-directed mutagenesis is a technique in which a mutation is created at a defined site in a DNA molecule. In general, the technique requires that the wild type gene sequence be known

[15] polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA

Purification and Overexpression of HMO2

Plasmids containing the gene of the correct size were then transformed in *E. coli* Rosetta Blue cells encoding chloramphenicol resistance. Expression of HMO2mut was induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hrs at an A_{600} of 0.4. Cells were pelleted and resuspended in Lysis Buffer containing 50mM Tris-HCl at pH 7.5 (10mM Imidazole). To that, a protease-inhibitor cocktail was added to protect the protein from serine proteases that may be released during cell lysis. Cell walls were then disrupted by sonication and its nucleic acid components degraded by adding DNase I, since we are only interested in the protein content of the cells. After an hour-long incubation on ice, the cell mixture was centrifuged and the supernatant was collected. To this protein solution, Nickel resin equilibrated in buffer was added and incubated for an hour. Our gene of interest was already His-tagged in the pET14b plasmid and was therefore attracted to the nickel beads. Imidazole is used to elute tagged proteins bound to Ni ions attached to the surface of beads in the chromatography column. This solution was then put into a column and continually flushed with Wash Buffer, which had a higher concentration of imidazole than the Lysis Buffer. Elution Buffer, with the highest concentration of imidazole among the buffers was finally added to free our protein of interest-the proteins that were bound strongest to the Nickel resin. Collected fractions were concentrated and quantitated on Coomassie Blue-stained SDS PAGE gels using bovine serum albumin as a standard.

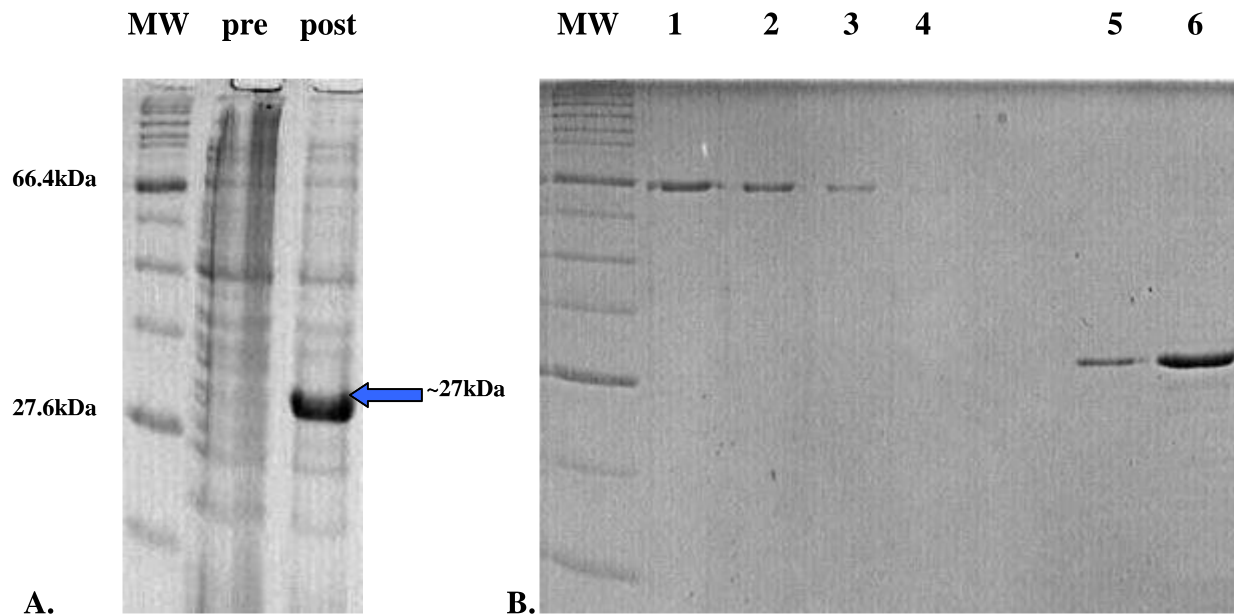


Figure 6. (A) SDS gel showing HMO2-mut-val19ala protein before (pre) and after (post) induction with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). The blue arrow points toward a dark band of molecular weight 27kDa that appears post-induction. **(B)** SDS gel quantifying the HMO2mut purified protein (Lanes 5 and 6) by comparing it with known Bovine Serum Albumin (BSA) standards (Lanes 1 through 4).

Binding assays

DNA binding assays were carried out on both wild-type and mutant protein to determine the change in binding ability of the protein after the amino acid substitution had taken place. It is expected that the HMO2 protein molecule should have less affinity for DNA after being mutated because it has undergone a conformational change. If the protein does bind, however, and subsequent DNA repair follows, we can conclude that the valine residue that has been replaced does not play a role in the intercalation of the DNA strands.

An electrophoretic[16] mobility shift assay or gel shift assay is a common affinity electrophoresis technique used to study protein-DNA or protein-RNA interactions. This procedure can determine if a protein or mixture of proteins is capable of binding to a given DNA

[16] motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. In this case, the fluid was a chemical buffer in which the gel was run and the DNA sample made up the dispersed particles

or RNA sequence. This procedure works on the principle that protein binding to DNA will cause it to increase in mass. On a gel, this increase in mass translates into a decrease in mobility of the DNA band. Thus, a gel shift in binding assays can constitute for a positive test for DNA-protein binding.

Linear Binding Assay

This binding assay involved plasmid DNA (PGEM 5) that was digested with the restriction endonuclease[17] Nde1. A significant gel shift is observed when HMO2-wild type protein is allowed to interact with linear DNA whereas no gel shift is observed in reactions involving linear DNA and HMO2-mutant protein.

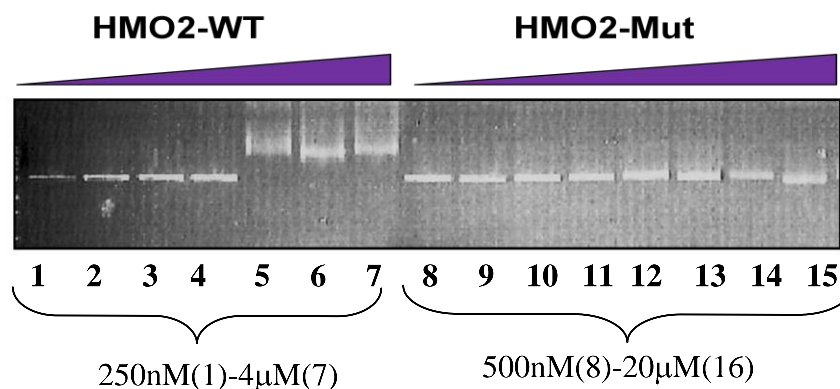


Figure 7. Increasing concentrations of wild type and mutant protein concentrations were added to 100 ng of linear DNA and incubated. Samples were run on a 1% agarose gel.

Supercoiled Binding Assay

A gel shift is observed in reactions where supercoiled plasmid DNA interacts with both mutant and wild-type HMO2 protein. The gel shift is more significant in reactions containing wild-type HMO2 than those containing mutant HMO2.

[17] enzymes that cleave the phosphodiester bond within a DNA strand

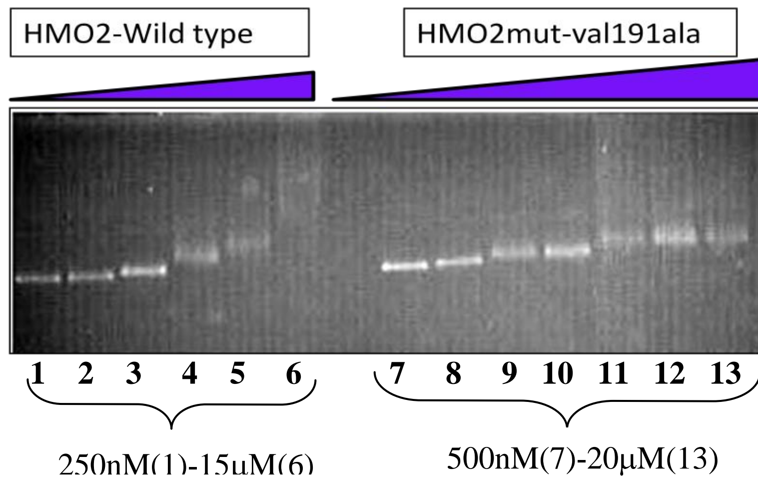


Figure 8. Increasing concentrations of wild type and mutant protein were added to 100ng of supercoiled plasmid DNA (PGEM5) and incubated. Samples were run on a 1% agarose gel.

Exonuclease Protection Assay

DNA	+	+	+	+	+	+
ExoIII	-	+	-	+	-	+
Wt	-	-	+	+	-	-
Mut	-	-	-	-	+	+

Figure 9. Linear DNA (100 ng of plasmid PGEM5 digested with Nde1), exonuclease III, dialyzed samples of Wild-type and of Mutant Protein were used in reactions and run on a 1% agarose gel

This assay determines the protective abilities of the mutant HMO2 against exonuclease. The lack of DNA band in lane 2 shows that exonuclease is effective in digesting the linear DNA. The presence of a DNA band in reactions containing DNA, exo III and HMO2 (lane 4 and 6) shows that both wild-type and mutant HMO2 inhibit exonuclease.

End Binding Assay

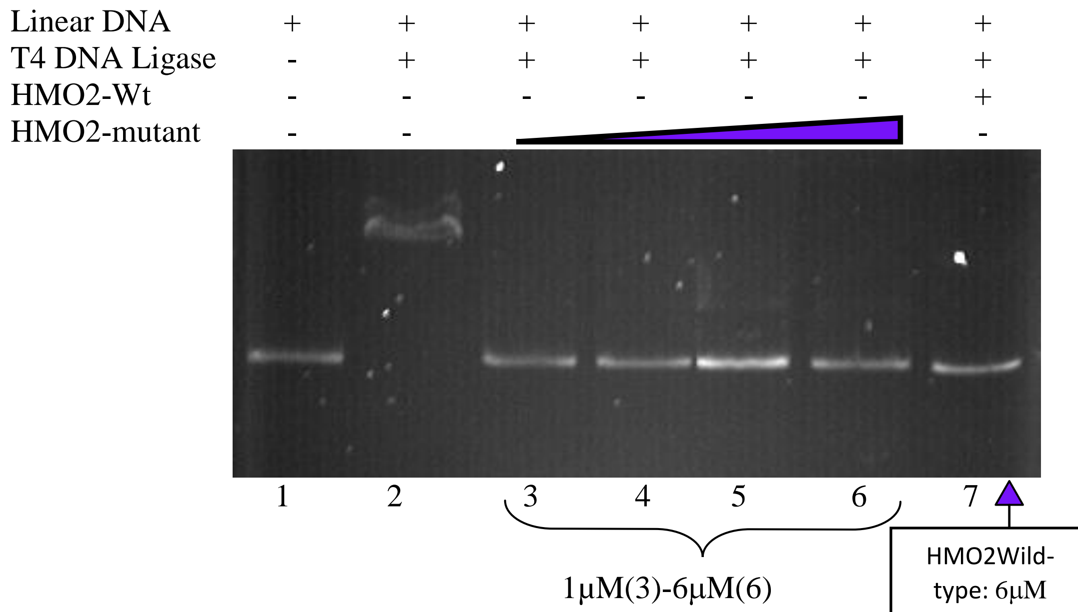


Figure 10. Linear DNA (100 ng of plasmid PGEM5 digested with Nde1), T4 DNA Ligase, HMO2-Mutant Protein, HMO2-Wild type protein and a mixture of Stop Buffer and 10% SDS were used in reactions and run on a 0.9% agarose gel

The end binding assay depicted in the above gel was carried out to determine whether the HMO2 mutant binds ends of linear DNA like wild-type HMO2 does. In Lane 2, a much slower moving band confirms that T4 DNA Ligase has effectively linked the ends of the linear DNA present together. The following lanes show no DNA ligase activity when increasing concentrations of HMO2-mutant is added. A reaction including the wild-type protein instead of the mutant protein is included to provide a basis of comparison for HMO2-mutant's interaction with DNA ligase. Evidently, the presence of HMO2 or the mutant protein prevents DNA ligase from ligating the DNA ends together.

DNA Oligonucleotides[18] with built-in defects

The following binding assays **(Figures 11-16)** all involve increasing concentrations of mutant protein or both mutant and wild type protein added to 100ng of DNA constructs of different lengths and containing varying combinations of defects. All DNA olignoucleotides were first hybridized with a γ-32 P-labeled complementary strand. Reactions were incubated for an hour and run on 8% polyacrylamide gels. The complexes were visualized using phosphorimaging[19]. The gel pictures were then examined for presence of gel shifts, which served as positive indicators of DNA-protein binding

[18] a short DNA fragment

[19] We start with a radioactive sample- a blot with DNA bands that have hybridized with a labeled probe, for example. We place this sample in contact with a phosphorimager plate, which absorbs β-rays. These rays excite molecules on the plate, and these molecules remain in an excited state until the phosphoimager scans the plate with a laser. At that point, the β-ray energy trapped by the plate is released and monitored by a computerized detector. The computer converts the energy it detects to an image. On the image, black denotes the highest level of radioactivity

Binding Assay with 37bp DNA containing 1 CT loop

No gel shift is detected at even the highest HMO2-mutant protein concentrations.

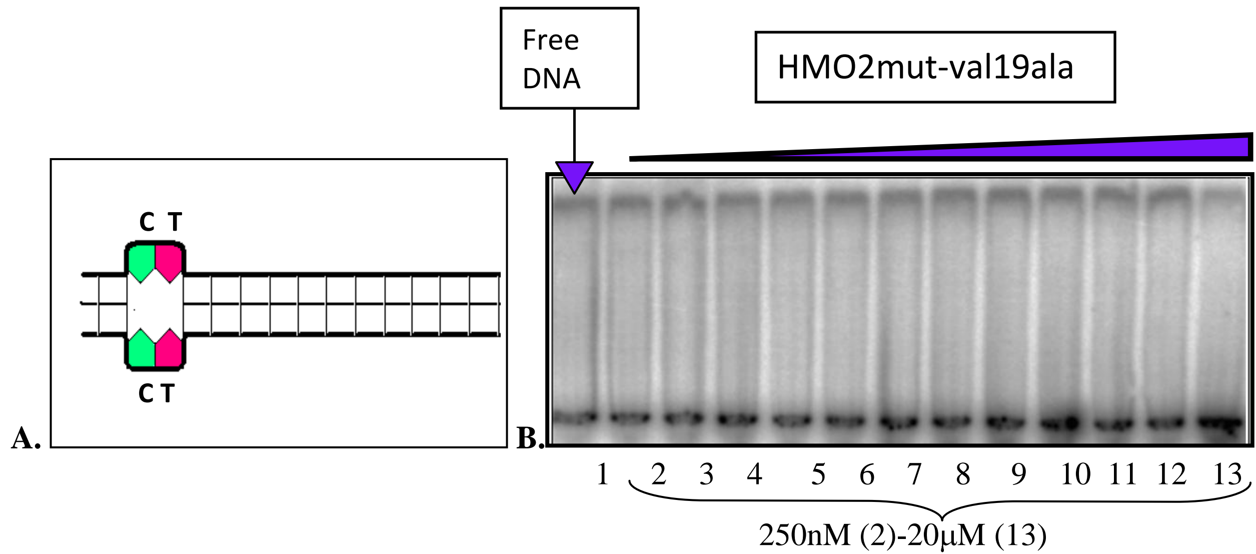


Figure 11. **(A)** Depiction of 37 base pair DNA construct containing 1 CT loop used in Binding Assay. **(B)** Increasing amounts of HMO2mut-val-19-ala incubated with 100 ng of DNA construct.

Binding Assay with 37 bp DNA containing 1 AA loop

No gel shift is detected at even the highest HMO2-mutant protein concentrations

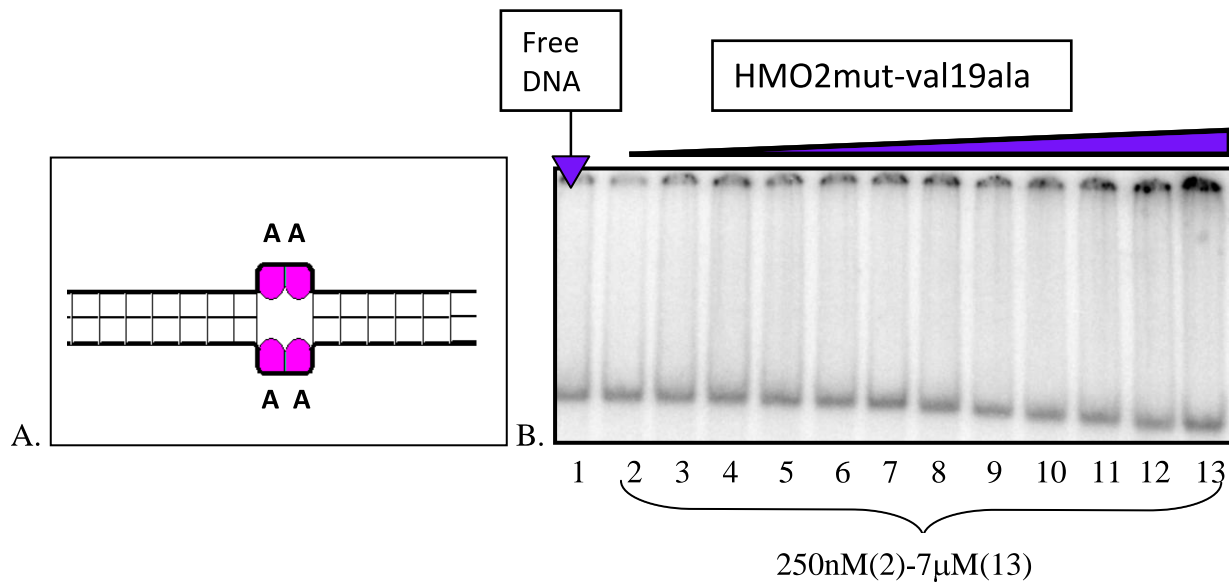


Figure 12. **(A)** Depiction of 37 base pair DNA construct containing 1 AA loop used in Binding Assay. **(B)** Increasing amounts of HMO2mut-val-19-ala incubated with 100 ng of DNA construct.

Binding Assay with 50bp DNA containing 1 CT loop and 1 abasic site

Complexes start to appear when about 1µM of wild-type HMO2 protein is added to this DNA construct. For mutant HMO2 protein, complexes start to appear when a slightly higher concentration (2µM) of protein is added.

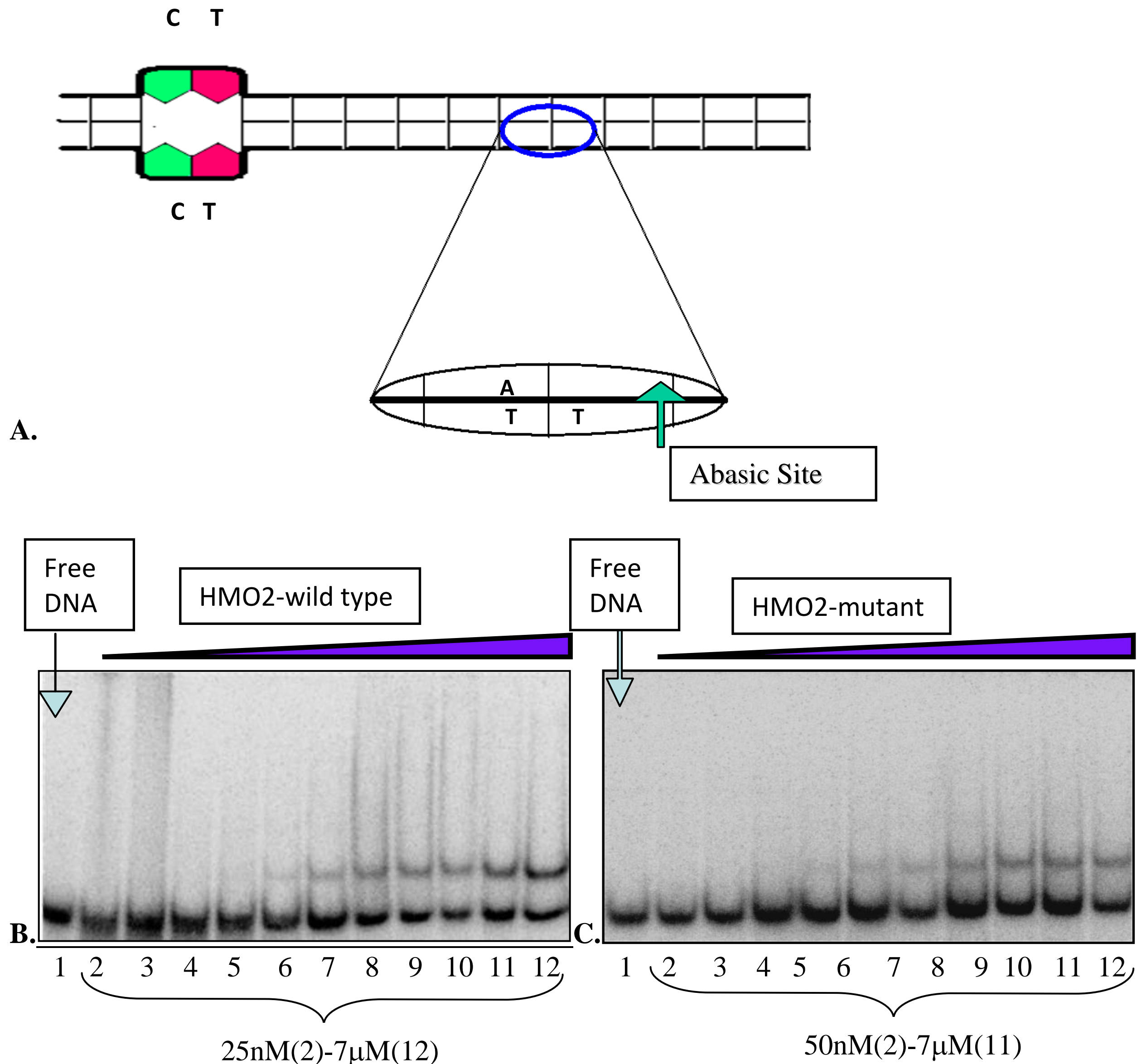


Figure 13. (A) Depiction of a 50-base-pair DNA construct containing 1 CT loop and an abasic site. An abasic site is one that is missing a base. **(B)** Increasing amounts of HMO2-wild type protein incubated with 1loop+abasic DNA construct. **(C)** Increasing amounts of HMO2mut-val-19-ala protein incubated with 1loop+abasic DNA construct.

Binding Assay with 50 bp DNA containing 2 loops 7 base pairs apart

Complexes do not form at any concentration of wild-type or mutant protein when incubated with this DNA construct

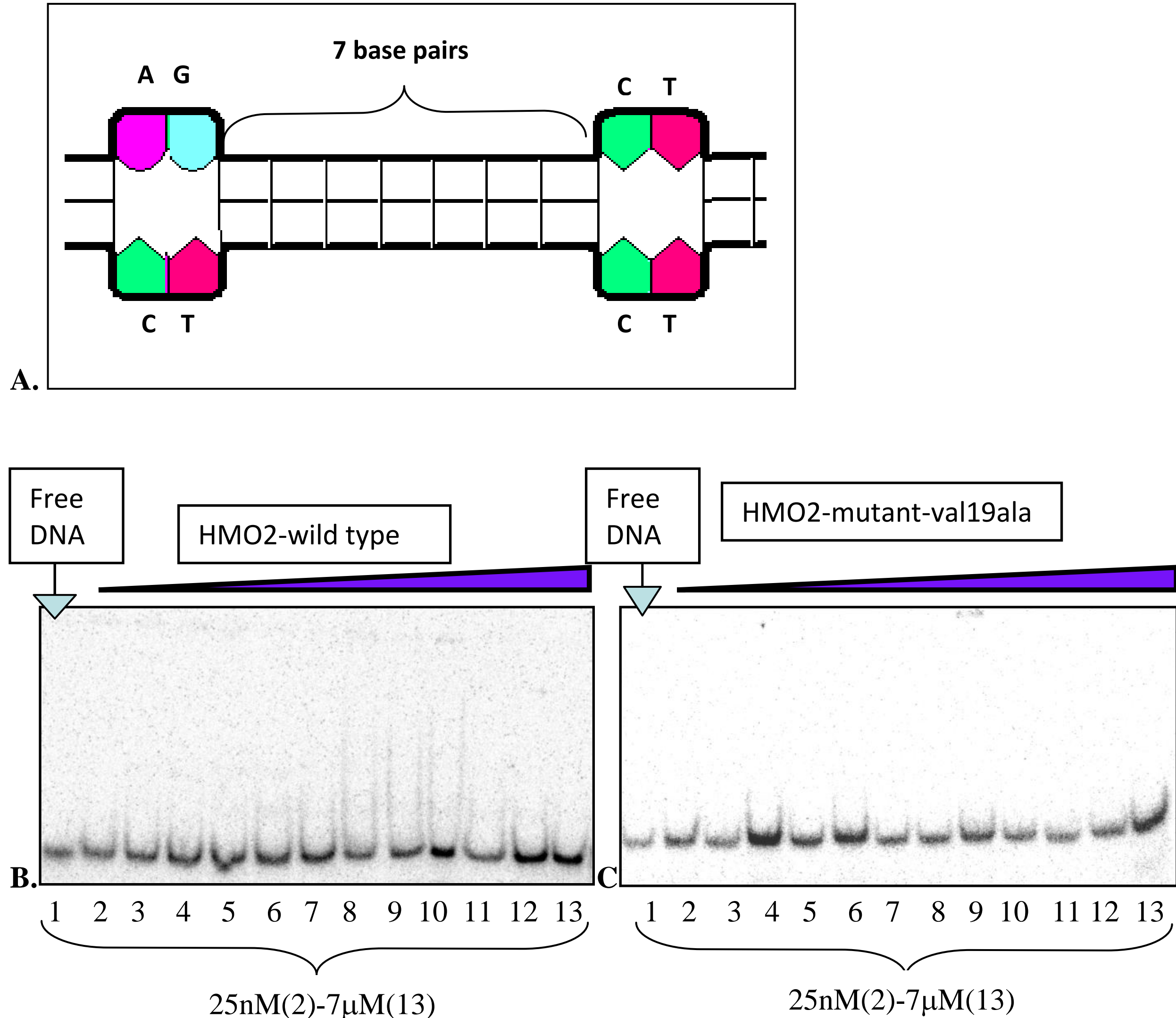


Figure 14. (A) Depiction of a 50-base-pair DNA construct containing 2 loops, 7 base pairs apart from each other. One loop is a CT loop while the other is a mismatch. **(B)** Increasing amounts of HMO2-wild type protein incubated with DNA construct containing loops separated by 7 base pairs. **(C)** Increasing amounts of HMO2-wild type protein incubated with DNA construct containing loops separated by 7 base pairs.

Binding Assay with 50bp DNA containing 2 loops 9 base pairs apart

Complexes start to appear when about 500nM of wild-type HMO2 protein is added to this construct. For mutant HMO2, complexes start to appear around when about 1μM protein is added.

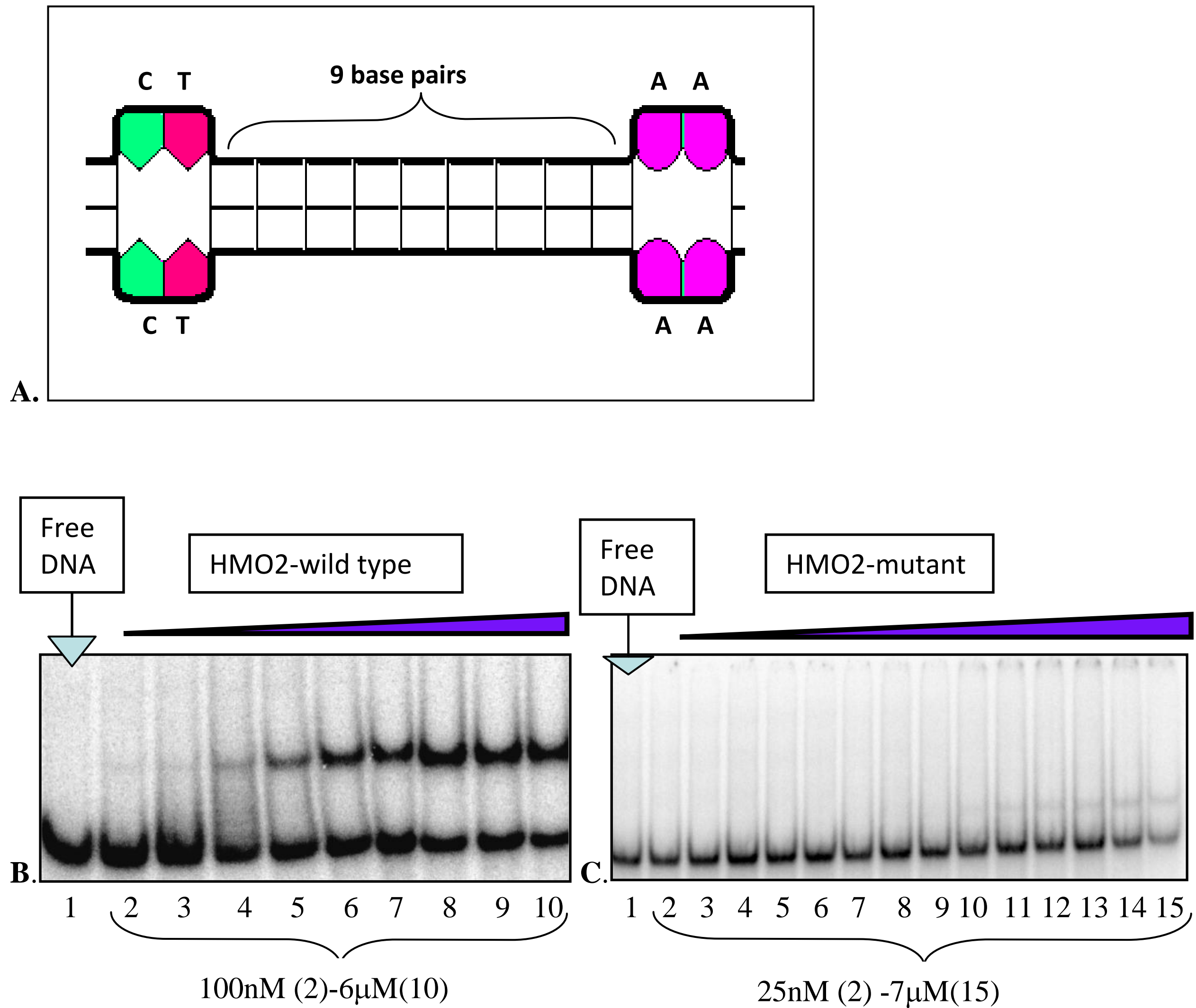


Figure 15. **(A)** Depiction of a 50-base-pair DNA construct containing 2 loops, separated by 9 base pairs. One loop is a CT loop while the other is an AA loop. **(B)** Increasing amounts of HMO2-wild type protein incubated with 100 ng of DNA construct. **(C)** Increasing amounts of HMO2mut-val-19-ala protein incubated with 100 ng of DNA construct.

<u>*Competition Assay*</u>

A competition assay was carried out to compare HMO2-mutant protein's affinity to linear DNA with its affinity to supercoiled DNA. The DNA construct containing 2 loops 9 base pairs apart was used as a constant in this assay because we have already established that HMO2-mutant protein has a strong preference for it. In Lane 2 of both assays, the reaction involving only 2-loop DNA and HMO2-mutant shows complex formation. In the first assay, this complex remains even as increasing concentrations of linear DNA are added in lanes 3 through 6. In the second assay, the complex disappears as increasing concentrations of supercoiled DNA is added.

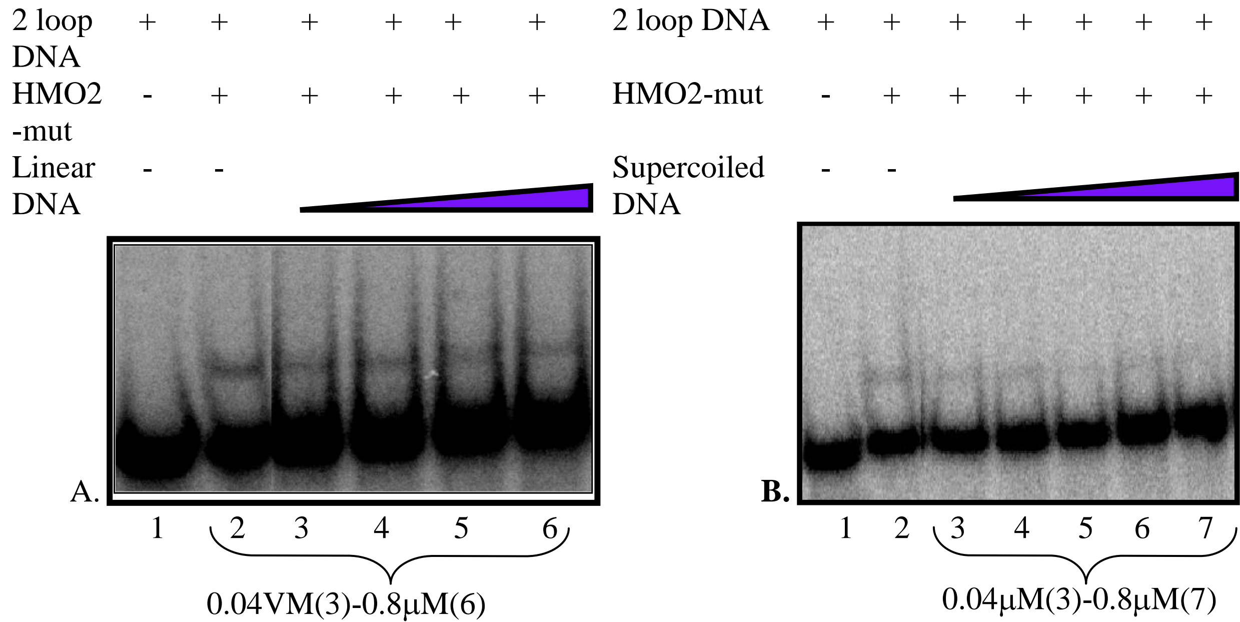


Figure 16. **(A)** 50bp DNA with two loops (9 base pairs apart) is first incubated with HMO2mut for an hour before increasing concentrations of linear DNA are added. **(B)** Increasing concentrations of supercoiled DNA are added in place of linear DNA in the first assay

Discussion

From the gel picture in **Figure 7**, we can observe a shift in the bands at the highest HMO2-wild type concentrations. This is not the case for the mutant protein, indicating that the mutation in HMO2 resulted in a loss of affinity for linear DNA. The gel shift in this assay is prominent at higher concentrations of wild-type protein.

Unlike the previous assay with linear DNA, we can observe a shift at the higher concentrations of both mutant and wild-type HMO2 in **Figure 8**. However, the shift is not as significant in the case of the mutant HMO2 thereby suggesting that the mutation results in a decrease in affinity for supercoiled DNA. This is in keeping with our expectations of HMO2. Supercoiled DNA can be considered to be a type of distorted DNA due to its irregular topography. These results are in line with previous research where HMO2 has exhibited a strong affinity for distorted DNA. That the mutant protein is still able to bind supercoiled DNA, albeit not as well as wild-type, further delineates that supercoiled DNA is indeed distorted. Exonuclease III (ExoIII) is an enzyme that belongs to the exonuclease family. ExoIII catalyzes the stepwise removal of mononucleotides from 3´-hydroxyl end of duplex DNA. Linear DNA serves as the substrate for Exo III and provides free ends on both sides. This is evident from the lack of a DNA band in Lane 2 (**Figure 9)**, which represents a reaction involving only DNA and Exo III. When HMO2 protein is added to the mix (Lane 4 and 6), the DNA band is still visible on the gel indicating that HMO2 is able to protect free DNA ends from Exo III. Comparing the DNA band in lane 4 (Wt) to the one in lane 6 (Mut), we can observe that the DNA band containing HMO2-Wt is more prominent than the one containing HMO2-Mut. This leads us conclude that wild-type HMO2 protects DNA ends slightly better than the mutant does. Thus, the

substitution does not affect the preferred binding to DNA ends characteristic of HMO2 as binding is still observed in both instances.

The results of the end binding assay carried out with HMO2 protein is portrayed in **Figure 10**. This assay utilizes T4 DNA ligase, an enzyme that can link together two DNA strands that have double-strand breaks or a break in both complementary strands of DNA. Since our plasmid DNA has been linearized with Nde1, it provides the perfect double-strand break for the DNA Ligase to bind to. It is evident that the DNA ligase in this reaction has successfully linked the linear DNA ends together because a heavier DNA band appears in Lane 2. To subsequent reactions in this assay, increasing amounts of HMO2-mutant protein is added. The single DNA bands that appear in Lanes 3 through 6 are identical to the free DNA band in Lane 1. This points to the fact that our HMO2 protein binds DNA double strand break ends and prevents DNA ligase from binding and carrying out its activity.

The lack of gel shifts in all the reactions in **Figure 11B** with mutant HMO2 suggests that protein had no affinity for the DNA in this assay. This DNA construct seems to not have been distorted enough for HMO2 to bind to it.

To test whether the type of loop had an effect on the affinity of HMO2 mutant to DNA, an assay was run using a 37 base pair oligonucleotide containing 1 AA loop (**Figure 12B**). While the length of oligonucleotide was kept constant, the type of loop was changed. Phosphorimaging of the gel showed no signs of gel shifts at even the highest concentrations of protein. These results show that the type of loop is not a factor to consider in DNA-HMO2 protein interactions. The lack of changes in binding affinity between the two shows that the mutation was not of any consequence in this assay.

The DNA construct containing one CT loop and 1 abasic site used in the next binding assay is depicted in **Figure 13A**. In both **Figure 13B** and **13C,** we can observe complex formations, or fractions of the DNA in a particular reaction exhibiting gel shifts.
These results indicate that a higher concentration of HMO2 is needed to bind to this distorted segment of DNA. Since complex formation start around the same concentrations for both wild-type and mutant HMO2 (around 1μM for wild-type HMO2 and 2μM for mutant HMO2), we can conclude that the mutation does not make much of a difference in the affinity for this particular combination of DNA distortions. Evidently, HMO2 protein finds this DNA construct suitable for binding.

In this assay, the DNA construct represented by **Figure 14A,** contains two loops that are 7 base pairs apart. Complex formation is not observed at any concentration of neither the wild-type nor the mutant HMO2 protein (**Figure 14B and 14C**). This assay goes against our hypothesis that some binding would occur due to the presence of two distortions in the DNA construct. Since we know the distance between the two loops, this finding leads us to question of whether the space between distortions in DNA is a factor in HMO2 binding.
Building off what we learned in the previous assay, we decided to try a construct with a little more space between the distortions. This construct, depicted in **Figure 15A**, contains 2 loops that are 9 base pairs apart. In this assay, the additional distance between the two loops on the DNA construct seems to have benefited protein binding. We can observe complex formation at protein concentrations as low as 1μM in both wild-type and mutant (**Figure 15B and 15C**). This finding tells us that HMO2 protein has a strong preference for distorted DNA with loops that are nine base pairs apart. It is also an important clue to HMO2's protein configuration and its binding site

conformation. The distance of two base pairs between distortions evidently makes all the difference to the HMO2 protein because its binding site is able to fit better.

Since HMO2 had such a strong preference for the DNA construct containing two loops separated by nine base pairs, we chose to use it in competition assays. The linear and supercoiled binding assays have already shown us that wild-type protein binds to both types of DNA better than the mutant protein does. However, these results do not tell us whether the HMO2 protein prefers one type of DNA over the other. The competition assays use the DNA with two loops as a constant and allows it to bind to our mutant protein first. The only variable in these assays is the linear or supercoiled DNA, which is later added in increasing concentrations. Since complex formation is expected in the reaction containing only the two loop DNA and protein, we will observe whether this complex remains when the variable DNA is added to the mix.

In the first competition assay represented in **Figure 16A**, we can observe some DNA-protein complexes in Lane 2, which only contains HMO2 mutant and 2 loop DNA. These complexes remain in the next four lanes even though increasing concentrations of linear DNA have been added to the mix. From this assay, it is evident that the addition of linear DNA had no affect on the binding affinity of HMO2 mutant protein as some of it has remained bound to DNA with 2 loops throughout all the reactions.

We also observe a DNA-protein complex in Lane 2 of the second assay (**Figure 16B**). This complex denotes an HMO2-mutant bound to DNA with 2 loops. However, unlike the previous assay with linear DNA, protein-DNA complexes disappear as more supercoiled PGEM5 DNA is added to the reactions. Supercoiled DNA is thus able to compete for HMO2's binding site as it separates HMO2 from DNA with two loops.

Conclusion

The DNA binding assays carried out with HMO2 shed more light on the protein's overall function in yeast cells. Assay results were examined to verify whether the mutation had made a difference in the protein's binding ability and whether the DNA construct had any structural advantages that increased HMO2's binding affinity. As is to be expected with HMO2, complex formation was observed in DNA constructs with more than one defect in almost every case. The introduced mutation reduced HMO2's affinity for linear DNA but had no significant effect on its affinity for supercoiled DNA. The ability of the mutant HMO2 to protect free DNA ends from exonuclease digestion was determined; both wild-type protein and the mutated protein were able to protect the DNA from exonuclease activity. The end binding assay further proves that HMO2 binds double-stranded breaks in DNA or linear DNA ends. The observation that DNA ligase is unable to act on DNA ends in the presence of both HMO2 wild-type and mutant protein leads us to conclude that HMO2 preferably binds DNA ends. Moreover, the results of the exonuclease protection assay and the end binding assay suggests that the mutation does not affect the preferred binding to DNA ends characteristic of HMO2. In binding assays with distorted oligonucleotides, both wild-type and mutant HMO2 protein bound with only modest preference to DNA with one loop (tandem mismatches) and two loops that were seven base pairs apart. However, both bound with significant preference to DNA with two loops that were nine base pairs apart and to DNA with one loop and one abasic site. The results of the binding assay involving DNA with two loops, nine base pairs apart, gives us insight into the conformation of the binding domain of HMO2. In comparison to HMO2 binding assays involving DNA with two loops seven base pairs apart, those involving DNA with two loops nine base pairs apart are far more successful due to the two additional base pairs between distortions. The distance between

loops can be of importance to the binding affinity of HMO2 due to various reasons. For example, each box in HMO2 may need a loop to bind. Moreover, research showing that HMO2 binds 50 base pair DNA better than it does 37 base pair DNA suggests that HMO2 may bind as a dimer (Ray and Grove, 2009). In order to determine whether HMO2-mutant prefers binding to linear or supercoiled DNA, competition assays were carried out using DNA with two loops nine base pairs apart as a constant. Supercoiled was able to compete out DNA with two loops while linear was not. Evidently, the valine in HMO2 box A is important for the protein's ability to bind to linear DNA, while its substitution does not alter the preferred binding of HMO2 to DNA ends or to DNA with distortions. Mutating the other residues speculated to be involved in DNA intercalation may show which part of HMO2 is performing this role.

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