The non-histone proteins 6A/B (NHP6A/B) of Saccha-
romyces cerevisiae are high mobility group proteins that
bind and severely bend DNA of mixed sequence. They
exhibit high affinity for linear DNA and even higher
affinity for microcircumlinear DNA. The 16-amino acid
basic segment located N-terminal to the high mobility
group domain is required for stable complex formation on
both linear and microcircumlinear DNA. Although mutants lack-
ing the N terminus are able to promote microcircumlinear
formation and Hin invertasome assembly at high protein
concentrations, they are unable to form stable com-
plexes with DNA, co-activate transcription, and compli-
ment the growth defect of Δnhp6a/b mutants. A basic
patch between amino acids 13 and 16 is critical for these
activities, and a second basic patch between residues 8
and 10 is required for the formation of monomeric com-
plexes with linear DNA. Mutational analysis suggests that
proline 18 may direct the path of the N-terminal arm to facilitate DNA binding, whereas the conserved
proline at position 21, tyrosine 28, and phenylalanine 31
function to maintain the tertiary structure of the high
mobility group domain. Methionine 29, which may inter-
calate into DNA, is essential for NHP6A-induced micro-
circle formation of 75-bp but not 98-bp fragments in vitro,
and for full growth complementation of Δnhp6a/b
mutants in vivo.

The high mobility group (HMG)1 proteins are a family of
heterogeneous chromatin-associated DNA-binding proteins
in eukaryotic cells. They are the most abundant non-histone pro-
teins found in the nucleus and are divided into three classes:
HMG-I/Y, HMG14/17, and HMG1/2 (1, 2). These proteins were
classically recognized by their high electrophoretic mobility in
polyacrylamide gels and acid solubility. The HMG-I/Y class of
proteins function as accessory transcription factors, whereas
the HMG14/17 class are associated with nucleosomes. The
HMG1/2 class of proteins contain the HMG DNA binding do-
main and are present at a level of about 1 copy/2–3 nucleo-
somes (3). The HMG domain is a 70–80-amino acid region
consisting mainly of hydrophobic and charged residues with a
few highly conserved aromatic residues (4).

Members of the HMG1/2 class can be further divided into two
subfamilies based upon the number of HMG domains, their
DNA sequence specificity, and their evolutionary relationship
(5). The sequence-specific HMG1/2 proteins contain a single
HMG domain, which are usually restricted by cell type and
interact with relatively high affinity to a specific DNA se-
duence. These proteins include the human sex-determining
factor SRY (6, 7), the lymphoid enhancer-binding factor LEF-1
(8), and the T-cell factor TCF-1 (9). The other subfamily of
HMG1/2 proteins are ubiquitously expressed and bind to DNA
with structural specificity but little or no sequence specificity.

One group of the non-sequence-specific DNA-binding proteins
contain multiple HMG domains such as the abundant HMG1
and HMG2 proteins (10), human nucleolar transcription factor
hUBF (11), mitochondrial transcription factor mTF-1 (12), and
yeast ARS-binding protein ABF-2 (13). Other non-sequence-
specific HMG1/2 proteins found in yeast (14, 15), plants (16),
insects (17, 18), and protozoa (19, 20) contain only a single HMG
domain and, often, an accessory basic and/or acidic domain.

The HMG domain of all these proteins probably fold into an
L-shaped region of three α-helices, as shown by the NMR
structures of rat HMG1 domain A, HMG-D, and SOX4 (21–27).
The domain contains a primary hydrophobic core at the vertex of the L-shaped structure
formed by conserved aromatic residues from the three α-helices.
The NMR structures of the two sequence-specific HMG
proteins, SRY and LEF-1, include their DNA recognition se-
duence (23, 24). These structures show the DNA to be greatly
distorted in the region of protein contact, with an overall bend
of 80° and 120° for SRY and LEF-1, respectively. The DNA is
severely underwound, resulting in a widened and shallow mi-
or groove and a highly compressed major groove. In conjunc-
tion with the helical underwinding, large positive roll angles
are induced by numerous DNA-protein contacts, which include
a partial intercalation of an amino acid side chain into the
minor groove of the DNA.

The sequence-specific HMG structures provide a basis for understanding how the non-sequence-specific HMG1/2 pro-
teins interact with high specificity to distorted DNA containing
bends, cruciforms, or DNA kinked by cisplatin (28–32). Re-
cently, it has been demonstrated that a subgroup of non-se-
quence-specific HMG1/2 proteins, which contain only one HMG

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To whom correspondence should be addressed: Dept. of Biological
Chemistry, UCLA School of Medicine, Los Angeles, CA 90095-1737.
Tel.: 310-825-7800; Fax: 310-206-5272; E-mail: rjohnson@biochem.
medsch.ucla.edu.

1 The abbreviations used are: HMG, high mobility group; bp, base
pair(s); PCR, polymerase chain reaction; DTT, dithiothreitol; NLS, nu-
clear localization signal.
Properties of NHP6A Mutants

| Mutation | Oligonucleotide sequence (5’-3’) | Method of mutagenesis | E. coli expression plasmid | Yeast CEN plasmid |
|----------|----------------------------------|-----------------------|---------------------------|------------------|
| Δ(2–7)   | GCGCGGCGATATGGAAAGAATGGGCCCGTAGG | Direct PCR            | pRJ1338                   | pRJ1381          |
| Δ(2–12)  | GCGCGGCGATATGGAAAGAATGGGCCCGTAGG | Direct PCR            | pRJ1294                   | pRJ1370          |
| Δ(2–16)  | GCGCGGCGATATGGAAAGAATGGGCCCGTAGG | Direct PCR            | pRJ1295                   | pRJ1371          |
| P18A     | AACCACTAGAAAGAAGAATGGGCCCGTAGG   | Kunkel                | pRJ1335                   | pRJ1482          |
| P21A     | AACCACTAGAAAGAAGAATGGGCCCGTAGG   | Three-way ligation     | pRJ1294                   | pRJ1483          |
| HMK (C-term) | GCGCGGCGATATGGAAAGAATGGGCCCGTAGG | Kunkel                | pRJ1355                   | pRJ1484          |
| Y28D     | AGGGTTGTCGCCGCTCATTTGTCCTGAAAGAA | Kunkel                | pRJ1485                   |                  |
| Y28D     | AGGGTTGTCGCCGCTCATTTGTCCTGAAAGAA | Direct PCR            | pRJ1332                   |                  |
| M28AD    | CTGGTGGCGCTACAGC/GCATTTGCTCCCTAGC | Three-way ligation     | pRJ1293,1333              | pRJ1486          |
| F30V     | CTGGTGGCCTCTAGATCTGCTGACGAGAAGA  | Kunkel                | pRJ1357                   | pRJ1487          |
| F41VD    | CTGGTGGCCTCTAGATCTGCTGACGAGAAGA  | Kunkel                | pRJ1357                   | pRJ1372          |

* Underlined sequences indicate the mutated codon(s) except for the HMK primer, which represents the new coding strand at the C-terminal end of the NHP6A gene.

**EXPERIMENTAL PROCEDURES**

Construction of NHP6A Mutants—The NHP6A mutants were constructed by direct cloning of PCR products using mutant oligonucleotide primers or by site-directed mutagenesis using the method of Kunkel (51). The sequence of the oligonucleotides used to generate the mutations is given in Table I. In several cases (P21A, M29A, and M29D), mutant PCR products were cloned by a three-way ligation using internal restriction sites (Table I). pRJ1226 (pET11a-NHP6A; Ref. 33) was used as the template for PCR and the vector for reconstructing mutant NHP6A genes. pRJ1340 and pRJ1341 were generated by cloning into pBS KS+ and pBS KS-; respectively, a PCR product obtained using a 5’ NHP6A primer containing an EcoRI/Ndel site and a 3’ NHP6A primer containing a BamHI site. Single-stranded DNA for site-directed mutagenesis was prepared from pRJ1340 and pRJ1341 using Cl236 (dut, Ref. 52). The mutant genes were subsequently transferred using the Ndel and BamHI sites into pET11a for protein overexpression. Each mutant gene was sequenced in its entirety.

The NHP6A mutants were introduced into yeast as follows. The 300-bp control region upstream of the NHP6A gene was obtained by PCR of genomic S. cerevisiae DNA using primers containing XhoI and Ndel sites engineered at the 5’ and 3’ ends, respectively. After digestion with XhoI and Ndel1, the product was inserted into pRJ1340 to give pRJ1342, which links the NHP6A promoter to the NHP6A gene flanked by Ndel1 and BamHI restriction sites. This NHP6A region was then subcloned between the XhoI and BamHI sites into pRS314 (TRP1 CEN6 ARSH4; Ref. 53) to create pRJ1346. Different mutant NHP6A genes were placed in yeast as the wild-type gene using the unique Ndel1 and BamHI sites. In addition, HMG1 box B and box B’ were obtained by PCR of pET-RNMG1 (54) with Ndel1 and BamHI engineered ends. After digestion with Ndel1 and BamHI, these products were ligated into pRJ1364, positioning them downstream of the NHP6A promoter.

Protein Expression and Purification—Recombinant proteins were expressed from the pET11a-derivatives in Rj1878 (BL21 (DE3) hupA::cm hupB::km; Ref. 33). NHP6A synthesis was induced for 3 h at 37 °C in LB when the cells reached an A600 of 0.6 by the addition of 1 mM isopropyl-1-thio-β-D-galactoside. Two liters of cells were disrupted by sonication in 1/10 volume of 20 mm Tris-HCl (pH 7.5), 150 mm NaCl, 1 mm DTTO, 1 mm EDTA, and 1 mm phenylmethylsulfonyl fluoride. The extract was clarified by centrifugation at 30,000 × g, and the NaCl concentration was increased to 1 M. Polyelectrolyneine (Sigma) was added to 0.3%, and the nucleic acids were removed by centrifugation at 20,000 × g. Residual polyethyleneimine was removed by batch chromatography with 20% (v/v) cellulose phosphate P-11 (Whatman), and the supernatant was dialyzed overnight against 0.05 M buffer A (20 mM Tris-HCl, 1 mM DTT, 1 mM EDTA). The dialysate was passed through a 4-ml S-Sepharose (Pharmacia) column equilibrated in the same buffer, and the NHP6A protein was eluted in a 50-ml linear gradient from 0.05 M to 1.0 M NaCl. The mutant genes were subsequently transferred using the Ndel1 and BamHI sites into pET11a for protein overexpression. Each mutant gene was sequenced in its entirety.
and 50% glycerol) and dialyzed overnight in the same buffer. Wagner et al. (55) reported that trichloroacetic acid precipitation affected DNA binding by HMG1, but we have been unable to detect any difference between NHP6A purified using trichloroacetic acid precipitation or under entirely native conditions using multiple chromatography steps. Preparative gel mobility shift assays were performed by incubating four to eight individual NHP6A–DNA complexes from 98-bp DNA fragments with gel mobility shift and ligase-mediated circularization assays were performed as described in Ref. 33 and electrophoresed on 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide). Hin-catalyzed DNA inversion reactions were performed as described previously by using pMS551–83, which contains 83 bp between the centers of hisA1L and the proximal Fis binding site in the enhancer (56).

**Stoichiometry of NHP6A-DNA Complexes—**NHP6A-HMK, containing the heart muscle kinase recognition sequence (RRASV) fused to the C-terminal end of NHP6A, behaved identically to the wild-type in DNA binding assays (data not shown). 10 μg of NHP6A-HMK was labeled by incubating with 5 units of bovine heart muscle kinase (Sigma) and 10 mM of [γ-32P]ATP (>6000 mCi/mmol; Andotek) in 20 mM HEPES (pH 7.5), 100 mM NaCl, and 12 mM MgCl2 for 1 h at 37 °C. The reaction was quenched with 10 μl EDTA, precipitated with trichloroacetic acid as described previously (33). The products were then digested with 100 units of exonuclease III for 60 min, extracted with phenol/chloroform (1:1, v/v) and precipitated with ethanol resulting in >90% monomer circle. Gel mobility shift and ligase-mediated circularization assays were performed as described in Ref. 33 and electrophoresed on 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide). Hin-catalyzed DNA inversion reactions were performed as described previously by using pMS551–83, which contains 83 bp between the centers of hisA1L and the proximal Fis binding site in the enhancer (56).

**In Vitro Transcription—**Transcription reactions (25 μl) were performed using 130 μg of yeast nuclear extracts in the presence of the acidic activator GAL4-vp4, NHP6A, and 100 ng of G5E4T as the DNA template. mRNA levels were detected by primer extension. Reaction conditions, GAL4-vp4, and G5E4T have been described previously (41, 59).

**Physical Properties—**Circular dichroism spectra from 190 to 240 nm was measured with an Aviv 62ADS circular dichroism spectrophotometer with 20 iterations at 25 °C. Proteins were dialyzed into 10 mM sodium phosphate buffer (pH 7.4) and protein concentrations determined as above. Secondary structure characteristics were calculated by the self-consistent method using DICROPROT (60).2 Thermal denaturation curves were obtained under the same conditions at 222 nm. The temperature was varied from 10–90 °C with an incubation time of 5 min for every 5 °C.

**RESULTS**

**DNA Binding Properties of Wild-type NHP6A**

NHP6A forms stable complexes on DNA of varying sequences as revealed by polyacrylamide gel electrophoresis (Fig. 2A; Ref. 33). With increasing concentrations of NHP6A, a discrete set of higher order complexes are obtained. 32P-Labeled NHP6A was used to determine the stoichiometry of binding in the first and second complexes formed on a 98-bp DNA fragment as described under “Experimental Procedures.” Quantitation of four to eight individual NHP6A-DNA complexes from separate experiments gave average molar ratios of 0.92 ± 0.2 for the first retarded complex and 2.2 ± 0.4 for the second complex. Therefore, NHP6A binds to DNA as a monomer in a stepwise manner. The binding constant (Kb) for the initial complex is 100 nm, a value that is constant on DNA of different sequences (23–300 bp).

The ability of NHP6A to bind DNA is most directly demonstrated by ligase-mediated circularization assays where the wild-type protein can form up to 70% monomer circles on DNA substrates as short as 66 bp (33). By measuring the amount of wild-type NHP6A required to generate the half-maximum yield of 98-bp circles, we calculate a Kb of 1.5 × 10^11 M (Fig. 3B). NHP6A binds much more tightly to curved DNA of mixed sequence than to linear DNA. This is shown in Fig. 4A where binding to a 98-bp microcircle was assayed by polyacrylamide gel electrophoresis. Two discrete complexes are formed at low concentrations of NHP6A, followed by the formation of higher order species with increasing protein concentrations. Gel mobility shift assays on 75-bp microcircles also generates two high affinity complexes, but three high affinity complexes are formed on 66-bp microcircles (Fig. 4, F and G). The number of high affinity complexes was identical regardless of whether NHP6A was added to purified microcircles or whether the products of the NHP6A + DNA ligase reactions used to generate the microcircles were directly analyzed by native gel electrophoresis (data not shown). The binding constant for these curved DNA molecules is ~1.5 nM. Binding to the pre-bent substrates is extremely stable, as revealed by the resistance of NHP6A-microcircle DNA complexes to added competitor DNA (Fig. 4H). The addition of 2.5 mg/ml salmon sperm DNA (corresponding to a 2,500,000:1 w/w ratio of competitor to micro-circular DNA) was unable to remove NHP6A from the two high affinity sites on the 98-bp microcircle, but 5.0 μg/ml (500:1 w/w

2 DICROPROT software may be obtained via FTP (ftp.ibcp.fr).
ratio of competitor to linear DNA) was sufficient to dissociate most of the prebound NHP6A from a linear substrate.

**The N Terminus Is Necessary for Efficient Binding and Bending of DNA**

The 94-amino acid NHP6A protein contains a 16-amino acid region located N-terminal to the minimal HMG domain. This segment contains two blocks of basic residues: KKR between residues 2–6 and RKKK between 13 and 16 (Fig. 1). To determine the importance of these amino acids for DNA binding and NHP6A function, three different truncations of NHP6A were constructed: a deletion of the entire N terminus (2–16), a deletion of the first 12 amino acids (2–12), and a deletion that retains both blocks of basic residues (2–12–7). Deletion of the entire N terminus of NHP6A abolished the high affinity of the protein for linear DNA with no distinct DNA-protein complexes being formed (Fig. 2D). We estimate that this mutant binds approximately 600-fold poorer than for the wild-type protein by gel mobility shift assays. This resembles the low affinity association of HMG1 to linear DNA observed under similar assay conditions (29, 33, 61). NHP6A (2–12–7) retained both blocks of basic amino acids in the N terminus and exhibited completely normal DNA binding, although a weak functional association by the minimal HMG box of NHP6A represents a functional motif between residues 13 and 16, which are retained in this mutant, is the critical determinant within the N terminus for mediating high affinity DNA association. Although NHP6A (2–12) did not form individual complexes on linear DNA, it bound to 98-bp microcircles with similar affinity as the wild-type (Fig. 4B). In addition, the ability of (2–12) to form 98-bp microcircles was indistinguishable from wild-type (Fig. 3B).

Circular dichroism was used to assess the folding of wild-type NHP6A as compared with the N-terminal deletion mutant (Fig. 5A). According to the self-consistent method of Sreerama and Woody (62), wild-type NHP6A was predicted to contain 49% a-helix, <1% b-sheet, 22% turn, and 19% other structure. These values are slightly lower than the amount of a-helical structure predicted by circular dichroism data on HMG1 box B (31, 37, 63), although the C-terminal extension of box B was not included in their spectra. The spectrum of the NHP6A (2–16) mutant (Fig. 5A) showed that it was highly structured with approximately 67% a-helix, <1% b-sheet, 13% turn, and 11% other structure. These values are similar to the 75% estimate of a-helical content derived from NMR data on the minimal HMG1 box B (25, 26) and the HMG-D box (22). The increase in

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**FIG. 1. HMG domain proteins.** A, sequence alignment of five non-sequence-specific HMG box domain proteins and two site-specific HMG box proteins based upon homology model building (76). The position of the three a-helices that constitute the HMG box B domain is shown above the sequences (25, 26). The numbering scheme at the top of the figure refers to amino acid positions within NHP6A. B, mutations of NHP6A. C, 15% SDS-polyacrylamide gel electrophoresis of recombinant NHP6A proteins: wild-type (lane 1), D (lane 2), D (lane 3), D (lane 4), P18A (lane 5), P21A (lane 6), P18A/P21A (lane 7), M29A (lane 8), F30V (lane 9), and F31V (lane 10).
α-helical content in the NHP6A N-terminal deletion mutant as compared with wild-type is consistent with the N-terminal 16 amino acids of NHP6A being unstructured in solution. In addition, the $T_m$ of wild-type and NHP6A Δ2–16 were both found to be 39 °C (Fig. 5D), further indicating that the N terminus does not play a role in stabilizing the overall structure.

Importance of Prolines at Position 18 and 21 for DNA Binding by NHP6A

NHP6A contains two prolines (positions 18 and 21) that are located within the N-terminal end of the HMG domain. The proline at position 21 of NHP6A, which shows remarkable conservation among all non-sequence-specific HMG1/2 proteins (4, 5), stabilizes a secondary hydrophobic core that associates the N- and C-terminal ends of the HMG domain (22). In LEF-1, proline 66, located at the C-terminal end of the HMG domain, appears to be important in directing its C-terminal basic extension that is essential for high affinity LEF-1-DNA binding (24). To determine whether the N-terminal prolines in the NHP6A HMG domain may be performing analogous roles, we created alanine substitutions at these positions and analyzed their DNA binding and folding properties.

NHP6A P18A displayed a 2.5-fold reduction in affinity for linear DNA whereas NHP6A P21A showed 4-fold reduced affinity (Fig. 2, E and F). The behavior of the double mutant P18A/P21A was identical to that of P21A (Fig. 2G). Binding to microcircular DNA was similarly reduced with a $K_D$ of 4 nM for P18A and 20 nM for both P21A and P18A/P21A (Fig. 4D). All three mutants were able to convert 70% of 98-bp linear DNA fragments into circles, although P18A required 2 times more protein than wild-type and both P21A and P18A/P21A required 4-fold more protein (Fig. 3B, Table II). Thus, the prolines at position 18 and 21 play a modest role in DNA binding.

CD analysis of the proline mutants indicated that P18A is folded identically to wild-type, whereas the P21A and the double mutant P18A/P21A displays a small (<10%) reduction in α-helical content compared with wild-type (Fig. 5B). Proline 21, therefore, contributes to the folding of the NHP6A HMG domain probably via hydrophobic interactions with residues of α-helix III (see Discussion). Proline 18 may facilitate positioning of the basic N-terminal segment upon DNA binding as also elaborated in the Discussion.

Importance of an Intercalating Side Chain for DNA Binding by NHP6A

DNA binding and bending by either LEF-1 or SRY is facilitated by a hydrophobic amino acid located near the N terminus of helix 1 that intercalates between base pairs via the minor groove (23, 24). Alignment of amino acid sequences as in Fig. 1A suggests that methionine 29 of NHP6A would be in the correct position to function as an intercalating side chain. To test whether methionine 29 of NHP6A is important for DNA binding, this residue was mutated to alanine. Surprisingly, M29A did not show any difference in its affinity to linear DNA as compared with wild-type (Fig. 2H). Even changing methionine 29 into a negatively charged aspartic acid resulted in less than a two-fold reduction affinity to linear DNA. Both M29A and M29D bound to 98-bp microcircles with a $K_D$ of 2.5 nM and formed 98-bp circles with similar efficiency as wild-type NHP6A (Fig. 3C).

The relatively modest effect on DNA binding by substitutions at position 29 led us to test whether adjacent amino acids could function in this capacity. Tyr-28, Phe-30, and Phe-31 were changed to valine or aspartic acid. F30V exhibited very little difference in affinity for linear DNA and 98-bp microcircles compared with wild-type and did not differ in its DNA bending properties. Y28D and F31D were strongly defective in binding to linear and circular DNA, but we show below that they are unfolded in solution. F31V was able to bind poorly to both linear DNA and microcircles (Figs. 2J & 4E); however, the ability to form microcircles was greatly reduced with a maximum of only 4% of the input DNA ligated into 98-bp circles when very high amounts of protein were added (Fig. 3B).

CD analysis demonstrated that Y28D and F31V were largely...
The formation of 75-bp microcircles are predicted to require the functional for 75-bp microcircle formation. Taken together, we conclude that Met-29 does play a role in NHP6A-induced DNA bending, but this is only revealed by assays that demand DNA bending near the maximum possible extent possible for wild-type NHP6A.

Ability of the NHP6A Mutants to Function in Biological Reactions

Growth Phenotypes—Using *Saccharomyces cerevisiae*, we are able to correlate the *in vitro* properties of DNA binding by NHP6A mutants to their biological functions. The double knockout mutant (Δnhp6a/b-RJY6012) exhibits a slow growth phenotype forming colonies of heterogeneous size, which are temperature- and cold-sensitive at 38 °C and 23 °C, respectively (33, 50). A yeast CEN shuttle vector containing the endogenous NHP6A promoter was used to express NHP6A and various mutants in the Δnhp6a/b cells. Western blotting confirmed that episcopal expression of NHP6A and most NHP6A mutants were similar to endogenous levels of chromosomal expression (Table III). The exceptions were Y28D and F31V, which are unfolded in solution and were present at <10% of wild-type levels. Interestingly, Δ(2–12) was present at 70% of the level of wild-type whereas Δ(2–16) was present at <20% of the wild-type level despite normal *in vitro* folding. The presence of episcopal NHP6A and all of the above mutants except for Δ(2–16), Y28D and F31V were able to reverse the temperature and cold sensitivity of Δnhp6a/b cells (data not shown).

The generation time of the Δnhp6a/b cells during log phase is approximately 250 min, nearly 2.5 times slower than the NHP6A/B parent (Table III). The expression of normal NHP6A protein in RJY6012 restored the generation time of these cells to a near wild-type 130 min. The presence of Δ(2–16) had little stimulatory effect on the growth of the Δnhp6a/b mutant, but this may be primarily due to its low steady state expression levels. Δ(2–7), Δ(2–12), P18A, and F30V restored near normal growth rates, as expected from their *in vitro* properties. NHP6A M29A only partially rescued growth rates of Δnhp6a/b cells (190 min generation time), even though this mutant has normal *in vitro* DNA binding properties in most regards. Δnhp6a/b cells expressing P21A and P18A/P21A, which have a disruption of the secondary hydrophobic core, also grew significantly slower (175–180 min generation time). The addition of Y28D or F31V had no stimulatory effect on Δnhp6a/b cell growth, but as noted above, steady state levels of these proteins are very low.

HMG1 box B’ is able to efficiently complement the growth phenotype of Δnhp6a/b mutants (RJY6398, Table III), demonstrating the *in vivo* functional relationship between these homologous proteins. Significantly, HMG box B also requires a basic region to be active *in vivo* since the minimal HMG box B has no stimulatory effect on growth (RJY6271, Table III).

Activated Transcription at the CUP1 Locus in Vivo—The NHP6A mutants were also tested for their ability to specifically enhance activated transcription of the CUP1 promoter, one of a subset of genes whose activated expression is facilitated by the NH6P6A/B proteins (41). Following 2 h of exposure to 1 mM CuSO4 in minimal media, β-galactosidase activity from a CUP1-lacZ reporter construct was induced 40-fold in Δnhp6a/b cells expressing NHP6A from pRKJ1342, similar to the 47-fold induction measured in wild-type cells (Fig. 6A). The induced level of CUP1-LacZ expression in the Δnhp6a/b mutant cells was only 8-fold above basal level. Activated transcription of the CUP1 promoter by the different NHP6A mutants largely paralleled their effect on growth. Δ(2–16) had no activity, whereas Δ(2–12) displayed essentially wild-type CUP1 expression levels. CUP1 transcription in the presence of M29A was also

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Circularization of DNA fragments with NHP6A wild-type and mutants in ligation assays. A, a 32P-labeled 98-bp linear fragment with EcoRI ends was incubated with buffer alone (lane 1), NHP6A (lane 4), or NHP6A Δ(2–16) (lanes 5–15) and T4 DNA ligase for 10 min (lanes 5–15). DNA-protein molar ratios were 40:1 for NHP6A and ranged from 40:1 to 90,100:1 increasing by 2-fold increments for NHP6A Δ(2–16). exonuclease III was added to reactions in lanes 3–15, so the products that remain represent circular species only. B, NHP6A (lanes 1–4), NHP6A Δ(2–12) (lanes 5–8), and NHP6A P21A (lanes 9–12) all ranging from 20:1 to 160:1 increasing by 2-fold increments were added to identical ligation reaction conditions as in A. NHP6A F31V (lanes 13–16) ranging from 320:1 to 2560:1 was used. C, NHP6A M29A (lanes 1–4) was added to a 75-bp DNA fragment at molar ratios 20:1 to 160:1 increasing by 2-fold increments. In lanes 5–10, a 75-bp fragment with EcoRI ends was incubated: lane 5, NHP6A at 80:1; lanes 6–10, M29A ranging from 16:1 to 160,000:1, increasing by 10-fold increments.

The lack of any clear effect on DNA interactions by M29A, unfolded in solution, whereas M29A showed only a slight change in CD profile (Fig. 5C). The CD data, combined with the known HMG box structures, suggest that the Phe-31 and Tyr-28 side chains are oriented such that they are contributing to the primary hydrophobic core that stabilizes the HMG fold (22, 64). Since F31V forms complexes with DNA microcircles, interaction with the pre-bent DNA ligand is presumably stabilizing its folded structure. The HMG box structure in F31D, however, is probably completely disrupted since it is not capable of forming complexes with curved DNA.
Properties of NHP6A Mutants

| Protein | $K_d$ for linear DNA | Monomeric complexes | $K_d$ for curved DNA | $K_{circle}$ for 98-bp fragment |
|---------|----------------------|---------------------|----------------------|---------------------------------|
| NHP6A   | $1.0 \times 10^{-7}$ m | +                   | $1.5 \times 10^{-9}$ m | $1.5 \times 10^{-8}$ m (70%)    |
| NHP6A Δ(2–7) | $1.6 \times 10^{-7}$ m | +                   | $2.0 \times 10^{-9}$ m | $2.0 \times 10^{-8}$ m (70%)    |
| NHP6A Δ(2–12) | $1.8 \times 10^{-7}$ m | –                   | $2.0 \times 10^{-9}$ m | $2.0 \times 10^{-8}$ m (70%)    |
| NHP6A Δ(2–16) | $6.0 \times 10^{-8}$ m | +                   | $5.3 \times 10^{-9}$ m | $5.0 \times 10^{-8}$ m (40%)    |
| NHP6A P18A | $2.5 \times 10^{-7}$ m | +                   | $4.0 \times 10^{-9}$ m | $2.8 \times 10^{-8}$ m (70%)    |
| NHP6A P21A | $4.0 \times 10^{-7}$ m | +                   | $2.0 \times 10^{-8}$ m | $4.0 \times 10^{-8}$ m (70%)    |
| NHP6A P18A/P21A | $4.2 \times 10^{-7}$ m | +                   | $2.0 \times 10^{-8}$ m | $4.0 \times 10^{-8}$ m (70%)    |
| NHP6A Y28D | No relevant binding | –                   | No relevant binding | Does not form                  |
| NHP6A M29A | $1.2 \times 10^{-7}$ m | +                   | $2.5 \times 10^{-8}$ m | $2.0 \times 10^{-8}$ m (70%)    |
| NHP6A M29D | $1.5 \times 10^{-7}$ m | +                   | $2.8 \times 10^{-9}$ m | $2.0 \times 10^{-8}$ m (70%)    |
| NHP6A F30V | $1.7 \times 10^{-7}$ m | +                   | $5.0 \times 10^{-9}$ m | $2.0 \times 10^{-8}$ m (70%)    |
| NHP6A F31V | $3.7 \times 10^{-7}$ m | +                   | $5.0 \times 10^{-8}$ m | $<3.0 \times 10^{-7}$ m (<4%)    |
| NHP6A F31D | No relevant binding | –                   | No relevant binding | Does not form                  |

* Determined by the amount of protein required to shift 50% of the input 98-bp linear DNA into slower mobility complex(es) upon polyacrylamide gel electrophoresis.

* Ability to form discrete protein-DNA complexes of increasing complexity beginning with a monomer of NHP6A.

* Determined by the amount of protein required to shift 50% of the input 98-bp microcircular DNA into slower mobility complex(es) upon polyacrylamide gel electrophoresis.

* $K_{circle}$ is defined as the amount of protein needed to make 50% of the maximal amount of monomer circles from input DNA in ligase-mediated circularization reactions. The maximal percentage of monomer circle for each mutant is given in parentheses.

* Up to 25,600:1 molar ratio of protein to DNA was used.

* Most of the binding probably represents nonspecific interactions.

Fig. 4. Gel mobility shift assays on 32P-microcircle DNA. A, 32P-labeled 98-bp microcircles were incubated with buffer alone or NHP6A. B, NHP6A Δ(2–12) protein. C, NHP6A Δ(2–16). D, NHP6A P18A/P21A. E, NHP6A F31V. F, a 66-bp microcircle was incubated with buffer alone or NHP6A. G, a 75-bp microcircle incubated with buffer alone or NHP6A. H, competition assays. In lanes 1–5, a 98-bp linear fragment incubated with buffer alone (lane 1) or 32 ng of NHP6A (lanes 2–5) for 20 min, in which 10–1000 ng of salmon sperm competitor DNA was added in 10-fold increments (lanes 3–5) and the samples loaded after 10 min. In lanes 6–10, 98-bp microcircles were incubated with buffer alone (lane 6) or 1 ng of NHP6A (lanes 7–10) for 20 min. 10, 20, and 50 μg of competitor DNA were then added (lanes 8–10) and the sample loaded after 30 min.

notably reduced.

Stimulation of In Vitro Transcription—The G₅E₄T template containing five GAL4 binding sites upstream of the adenoviral E₄ promoter was used as a model substrate to test the ability of the mutants to facilitate activated transcription in vitro. Transcription in yeast nuclear extracts activated by GAL4-VP4 was stimulated 2–5-fold by increasing amounts of exogenous NHP6A (Fig. 6B). A similar increase in transcript levels was observed by the addition of bovine HMG1 or HMG2 (Fig. 6C), but Hu, an unrelated Escherichia coli DNA binding protein, had no effect on this reaction (data not shown). NHP6A (Fig. 6D) or HMG1/2 (data not shown) had no effect on basal transcription from this promoter, similar to that observed for the GAL1 promoter (41).

Most of the mutants stimulated transcription at G₅E₄T to a similar extent as wild-type (Fig. 6C and data not shown). NHP6A Δ(2–16) was completely defective even when large amounts of protein (5–2 μg) were added, but Δ(2–12) behaved indistinguishably from wild-type. Transcription in the presence of M29A approached wild-type levels, but was significantly reduced in the presence of M29D. As expected, the unfolded mutants Y28D and F31V had little or no stimulatory activity.

* Hin-catalyzed Site-specific DNA Inversion—When the recombinational enhancer is located within 100 bp of a recombination site, Hu or an HMG1/2 protein is needed to assemble...
Properties of NHP6A Mutants

The formation of stable complexes of defined numbers of NHP6A protomers on microcircular DNA of varying lengths and cHMG1a proteins also do not display this property (17, 66). Even though NHP6A binding is presumably targeted to the distorted structures present on microcircular DNA, it does not bind preferentially to four-way junctions (data not shown). High affinity binding to four-way junctions is a property of a member of a subclass of HMG1/2 proteins that contain a HMG-D (30, 65). Even though NHP6A binding is presumably targeted to the distorted structures present on microcircular DNA, it does not bind preferentially to four-way junctions (data not shown). High affinity binding to four-way junctions is a feature of many HMG proteins, although the related HMG-D and cHMG1a proteins also do not display this property (17, 66).

The formation of stable complexes of defined numbers of NHP6A protomers on microcircular DNA of varying lengths can be used to estimate the degree of DNA bending introduced upon NHP6A binding. The measured persistence length of

tasome assembly with the exception of the unfolded proteins Y28D and F31V, which bind and bend DNA poorly at all tested concentrations. NHP6A Δ(2–16) displayed surprisingly high activity in this assay (Fig. 7B), particularly in comparison to its complete inactivity in stimulating transcription in vitro. The proficiency of Δ(2–16) in promoting invertasome formation at moderately high concentrations of protein probably reflects its ability to promote microcircular formation at very high protein concentrations.

**DISCUSSION**

**DNA Binding and Bending by Wild-type NHP6A—NHP6A is a member of a subclass of HMG1/2 proteins that contain a single HMG box and bind nonspecifically to linear DNA with relatively high affinity. NHP6A binds equivalently to DNA varying in sequence and length and forms distinct complexes upon polyacrylamide electrophoresis with a *K_D* of 100 nM. We show in this report that each complex represents a monomer of NHP6A bound to DNA. Although the complexes formed on linear DNA are stable during long term electrophoresis in a gel matrix, they readily dissociate in the presence of excess DNA. NHP6A binds with >50-fold higher affinity to microcircular DNA. The complexes formed on microcircular DNA are extremely stable; NHP6A remains bound to the microcircles in the presence of 1000-fold excess linear DNA. Preferential binding to microcircular DNA has also been reported for HMG1 and HMG-D (30, 65). Even though NHP6A binding is presumably targeted to the distorted structures present on microcircular DNA, it does not bind preferentially to four-way junctions (data not shown). High affinity binding to four-way junctions is a feature of many HMG proteins, although the related HMG-D and cHMG1a proteins also do not display this property (17, 66).**

**TABLE III**

*S. cerevisiae* strain generation times and in vivo levels of mutant proteins

| Strain* | Generation time* | NHP6A/B expression* |
|---------|------------------|---------------------|
| RJY5372 (wild-type + pRS313) | min | 107 ± 8 | + + + |
| RJY5374 (Δnhp6a + pRS313) | 118 ± 8 | + + + |
| RJY5376 (Δnhp6b + pRS313) | 121 ± 1 | + + + |
| RJY6261 (Δnhp6a/b + pRS313) | 252 ± 24 | - |
| RJY6263 (Δnhp6a/b + NHP6A) | 131 ± 1 | + + + |
| RJY6430 (Δnhp6a/b + NHP6A Δ(2–7)) | 127 ± 12 | + + + |
| RJY6425 (Δnhp6a/b + NHP6A Δ(2–12)) | 158 ± 3 | + + + |
| RJY6417 (Δnhp6a/b + NHP6A Δ(2–16)) | 224 ± 10 | + |
| RJY6432 (Δnhp6a/b + NHP6A P18A) | 146 ± 1 | + + + |
| RJY6434 (Δnhp6a/b + NHP6A P21A) | 180 ± 7 | + + + |
| RJY6436 (Δnhp6a/b + NHP6A P18A/P21A) | 175 ± 6 | + + + |
| RJY6438 (Δnhp6a/b + NHP6A Y28D) | 255 ± 8 | +/– |
| RJY6440 (Δnhp6a/b + NHP6A M29A) | 192 ± 13 | + + + |
| RJY6442 (Δnhp6a/b + NHP6A F31V) | 143 ± 6 | + + + |
| RJY6629 (Δnhp6a/b + NHP6A F31V) | 250 ± 10 | +/- |
| RJY6271 (Δnhp6a/b + HMG1 box A) | 299 ± 25 | ND |
| RJY6298 (Δnhp6a/b + HMG1 box B’) | 158 ± 9 | ND |

*Yeast strains were wild-type, Δnhp6a, Δnhp6b, or Δnhp6a/b containing a plasmid with no insert, NHP6A, NHP6A mutant, or HMG1 genes. All strains were grown in minimal media under plasmid selection.

**Generation times were measured during the exponential growth phase.

- NHP6A/B expression was determined by Western blotting. + + + represents wild-type levels, + + represents about 70% of wild-type, + <20%, +/- 10%, and ND is not determined.

the catalytically competent invertasome (35, 38, 56). In this reaction, the auxiliary DNA bending protein is believed to function strictly as a DNA architectural factor to facilitate DNA looping of the enhancer segment. In Fig. 7A, the amount of NHP6A mutant added to the DNA inversion reaction was adjusted to compensate for defects in DNA binding. Under these conditions, all the mutants are able to stimulate inver-
DNA (67) gives an average intrinsic flexibility of about 2.4°/bp (360°/150 bp). Thus, DNA lengths of 66, 75, and 98 bp can generate 158°, 180°, and 235° of curvature. Based on the number of NHP6A protomers bound to preformed microcircles (Fig. 3) or upon formation of microcircles (data not shown) of lengths 98 bp (two complexes), a 75 bp (two complexes), or 66 bp (three complexes), the minimal amount of protein-induced bending can be calculated. To create a 98-bp microcircle, each of the two NHP6A-induced bends required to complete the DNA circle would be approximately 60°, which corresponds to a 120° DNA bending angle by each NHP6A protomer relative to linear DNA. A 75-bp microcircle requires a 45° bend in the DNA from each of the two bound NHP6A protomers resulting in a 135° bending angle. Since the 66-bp microcircle has three bound NHP6A protomers, an equilateral triangle can be used to estimate the induced bending of the DNA to be 60°, which corresponds to a bending angle of 120°. Therefore, from these experiments we estimate NHP6A induces bend angles between 120 and 135°. These values are in the range of the angles observed with LEF-1 by NMR (120°; Ref. 24) and cHMG1a by fluorescence resonance energy transfer (150°; Ref. 68). However, they are greater than the 80° observed in the SRY-DNA complex by NMR (23), or the 60° estimated for DNA binding by HMG-D by a ligase-mediated circularization assay (30).

High Affinity DNA Binding by NHP6A Requires Its Unique N Terminus—The 16-amino acid segment located N-terminal of the minimal HMG domain of NHP6A is essential for its unusually stable DNA interaction. Removal of this region abolishes the ability of the protein to form discrete complexes on both linear and microcircular DNA and eliminates most of its biological activities. The remaining minimal HMG box is capable of poorly binding to DNA in vitro, as evidenced by its ability to form microcircles, promote Hin invertasome formation, and induce DNA supercoiling in the presence of topoisomerase I (data not shown) at high protein concentrations. These activities provide strong evidence that the minimal HMG box remains capable of an authentic, albeit weak, HMG-DNA interaction. Circular dichroism and thermal stability data indicate that the N-terminal segment is unstructured in solution and has no effect on the integrity of the folded HMG domain. Upon NHP6A-DNA interaction, the basic N-terminal arm presumably associates with the DNA to anchor the complex. We imagine that the N-terminal arm may cross over the phosphate backbone and protrude into the major groove (Fig. 8A), as does the C-terminal arm of LEF-1 (24), or possibly continue along the minor groove as schematically drawn in Fig. 8B.

Properties of NHP6A Mutants

FIG. 6. Effect of NHP6A mutants in transcription. A, Activated transcription at CUP1 in vivo. Levels represent -fold induction of β-galactosidase at the CUP1 locus following 2.5 h with CuSO4. Values represent the average and standard deviation obtained from two to four individual transformants. B, in vitro transcription reactions on the G₆E₅T template were performed using yeast nuclear extract supplemented with NHP6A in the absence (lanes 1–4) or presence of 146 ng of GAL4-VP4 (lanes 5–8). C, in vitro transcription reactions with 1 μg of NHP6A, various mutants or purified calf thymus HMG2 (lanes 2–10). Basal (lane 1) indicates no activator or NHP6A added.
shift assays. The deficiency in stable complex formation on linear, but not microcircular DNA, does not seem to affect any of its biological activities that were measured. The first 7 amino acids of the N-terminal arm play no detectable role in DNA binding or biological properties of NHP6A.

We find it significant that NHP6A $\Delta$(2–16) is not capable of potentiating transcription in vitro, even in reactions containing a large excess of protein. Moreover, this mutant was unable to form promoter complexes together with TBP and TFIIA, unlike wild-type NHP6A or the NHP6A $\Delta$(2–12) mutant (Ref. 41; data not shown). By contrast, NHP6A $\Delta$(2–16) is still able to promote Hin invertasome assembly at moderately high concentrations. These differences may indicate that the NHP6A protein is performing a specific function in transcription rather than functioning merely in an architectural role, as is probably the case for the recombination reaction.

Steady state cellular levels of NHP6A $\Delta$(2–16) are <20% of wild-type. This difference suggests that the mutant protein may be rapidly turned over in the yeast cells even though it is well structured. The apparent instability of NHP6A $\Delta$(2–16) could be due to its weak DNA binding properties that result in poor nuclear retention and thereby rapid degradation. A lack of retention in the nucleus may also result from the removal of a nuclear localization signal (NLS) present in the N-terminal arm. The amino acid sequence of NHP6A between amino acids 8 and 16 (KKRTTRKKK) matches an NLS motif (69). Other NLS sequences have been identified in the HMG domain of mUBF, SRY, SOX9, and LEF-1 (70–73). Cellular levels of NHP6A $\Delta$(2–12) are also reduced to about 70% of wild-type, but NHP6A $\Delta$(2–7) levels are normal. This provides further support for the importance of residues 8–16 for protein stability, and implies that the residues between 13 and 16 are more important than the residues between 7 and 12. Further experiments will be needed to confirm that the basic N terminus contains a nuclear localization signal in addition to being required for high affinity DNA binding.

Other HMG proteins also require a basic region adjacent to the HMG domain. Teo et al. (37) could not form microcircles with HMG1 box A or B but could form them with box B', which contains a patch of basic amino acids at its C terminus. This observation is likely to be related to our finding that HMG1 box B' is able to largely complement the growth defect of $\Delta$nhp6A/B mutants, whereas HMG box B is ineffective (although an NLS in the box B' basic region may also contribute to this difference). HMG-D also contains a positively charged patch of amino acids at the C-terminal end of its HMG domain, which are required for efficient DNA binding on linear DNA in vitro (30). However, in contrast to the NHP6A $\Delta$(2–16) mutant, the minimal HMG-D domain can form stable complexes on microcircles. A short basic patch adjacent to the C terminus of LEF-1 was found to be required for high affinity and bending by this sequence-specific HMG protein (36).

Role of Prolines at the N Terminus of the NHP6A HMG Domain—NHP6A contains two prolines at positions 18 and 21, which are predicted to be located near the top of the L-shaped HMG domain adjacent to the N-terminal arm (see Fig. 5) based upon the structures of box A and B from HMG1 (21, 22, 25, 26).
When proline 18 in NHP6A was changed to an alanine, a 2–3-fold decrease in DNA binding to linear and microring DNA was measured. CD analysis did not reveal a significant difference between wild-type NHP6A and NHP6A P18A. We postulate that proline 18 may direct the peptide backbone to facilitate positioning of the N-terminal arm. In the absence of this proline, the N terminus is still able to interact with DNA, although not quite as effectively. In all in vitro reactions tested, NHP6A P18A is very active, provided additional protein is added to compensate for the modest effect on binding affinity. This role for proline 18 of NHP6A is analogous to the function of proline 66 of LEF-1, which directs its C-terminal arm into the major groove where extensive DNA contacts are made (24). A proline to alanine substitution at the same relative position in HGMI box A as our NHP6A P18A mutant has also been analyzed, but it was not shown to have any effect on DNA binding (64).

The proline at position 21 of NHP6A is conserved among all non-sequence-specific HGMI/2 proteins but corresponds to a valine or isoleucine in sequence-specific HMG proteins. The importance of this residue for NHP6A is shown by a 4- to over 10-fold reduction in binding affinity of P21A to linear and microring DNA, respectively. NHP6A P21A is partially defective in its ability to complement Δhmp6A/B mutants for growth and CUP1 expression, but increased levels of protein largely compensate for reduced activity in reactions in vitro. CD analysis indicates that the P21A mutation causes a 10% loss of α-helicality. Based upon the structures of HMG-D, HGMI box A and B, and evidence from cHMG1a (21, 22, 25, 26, 34), the conserved proline at position 21 is probably involved in stabilizing a secondary hydrophobic pocket between helix III and the extended peptide chain between the N terminus and the start of helix 1 (Fig. 8). Thus, the reduction in DNA binding affinity of this mutant is attributed to a structural disruption of the N terminus of the HMG domain and consequently the N-terminal arm.

The Hydrophobic Core of NHP6A—Mutations at Tyr-28 and Phe-31 lead to an unfolded protein, as determined by CD analysis, and an unstable protein in vitro. The aromatic side chains of these conserved amino acids are predicted to be directed into the primary hydrophobic core that stabilizes the three-helix fold (Fig. 8), as observed for other HMG box proteins (23, 74, 75). Methionine 29 is located at the analogous position in NHP6A by sequence alignment (Figs. 1A and 8). However, NHP6A M29A and even M29D binding to linear DNA is nearly indistinguishable from wild-type, and the Met-29 mutants are proficient in Hin inversion, and inducing DNA supercoiling (data not shown) in vitro. On the other hand, NHP6A M29A only restored about 50% of the growth rate defect that results from deleting NHP6A/B. Moreover, activated transcription of CUP1 in the presence of M29A in vitro is significantly reduced, as well as activated transcription of GmP4T in vitro in the presence of M29D (and M29A to a small extent). Thus, Met-29 appears to be important for some activities, including co-activation of transcription, but not other HMGI1-promoted functions. The different behavior of Met-29 mutants in these reactions may be related to their ability to form microcirles of varying lengths."
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Determinants of DNA Binding and Bending by the *Saccharomyces cerevisiae* High Mobility Group Protein NHP6A That Are Important for Its Biological Activities: ROLE OF THE UNIQUE N TERMINUS AND PUTATIVE INTERCALATING METHIONINE

Yi-Meng Yen, Ben Wong and Reid C. Johnson

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