Multiple Nhp6 Molecules Are Required to Recruit Spt16-Pob3 to Form yFACT Complexes and to Reorganize Nucleosomes*

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The Saccharomyces cerevisiae Nhp6 protein contains a DNA-binding motif that is similar to those found in the high mobility group B family of chromatin proteins. Nhp6 binds to nucleosomes and made at least two changes in them: the nucleosomal DNA became more sensitive to DNase I at specific sites, and the nucleosomes became competent to bind Spt16-Pob3 to form yFACT-nucleosome complexes. Both changes occurred at similar concentrations of Nhp6, suggesting that they reflect the same structural reorganization of the nucleosome. Nucleosomes have multiple binding sites for Nhp6, and structural reorganization was associated with a concentration of Nhp6 about 10-fold higher than that needed for simple binding. We propose that the coordinated action of multiple Nhp6 molecules is required to convert nucleosomes to an alternative form as the first step in a two-step reorganization of nucleosomes with the second step being dependent on Spt16-Pob3. The presence of linker DNA had only subtle effects on these processes, indicating that both Nhp6 and yFACT act on core nucleosome structure rather than on the interaction between nucleosomes and adjacent DNA. These results suggest that Nhp6 and the related high mobility group B proteins may have a general role in promoting rearrangements of chromatin by initiating the destabilization of core nucleosomal structure.

Nhp6 is a DNA-binding protein that is encoded by two similar genes in Saccharomyces cerevisiae, NHP6A and NHP6B (1). Nhp6A and Nhp6B proteins (93 and 99 total residues, respectively) are 88% identical and functionally redundant (2, 3), so we refer here to both as Nhp6 protein. Nhp6 contains a single ~70-residue high mobility group (HMG) box motif of the type found in the HMGB family (4). HMG proteins are abundant constituents of chromatin that fall into several families with distinct DNA-binding motifs (5). HMG families are highly conserved and have been proposed to play many roles in chromatin function (5–7). Canonical HMG proteins contain two tandem copies of the same HMG box motif, so Nhp6 is not a true representative of this family (4). However, no yeast protein has the canonical HMGB structure, so Nhp6 is the closest relative of this conserved family in yeast and is likely to provide clues about the functions of the HMG box motif both in HMGB proteins and in other contexts.

Haploid yeast cells contain about 70,000 copies of Nhp6 (8)

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† The abbreviations used are: HMG, high mobility group; HMGB, high mobility group B; EMSA, electrophoretic mobility shift assay.

for a total nuclear concentration of about 30 μM, roughly equivalent to nucleosomes. While abundant and conserved, Nhp6 is not essential for viability (2). However, yeast cells lacking Nhp6 display severe defects including slow growth, temperature sensitivity, altered regulation of transcription, and an inability to tolerate mutations in a variety of transcription and replication factors (2, 8–12). Nhp6 is therefore non-essential, but it is important for normal growth.

Nhp6 supports the function of Spt16-Pob3, a heterodimer implicated in both DNA replication and regulation of transcription (10–12). Spt16 and Pob3 contain most of the sequences found in the components of the human FACT and frog DUF complexes (13, 14), but Pob3 lacks the HMG box motif found in the otherwise homologous FACT subunit SSRP1 (12, 15, 16). Nhp6 appears to provide HMGB function for Spt16-Pob3 both in vivo and in vitro as cells lacking Nhp6 cannot tolerate mutations in SPT16 or POB3 (11, 17), and Spt16-Pob3 can only bind to and reorganize nucleosomes in vitro if Nhp6 is present (11). Current data suggest that FACT family members, including Spt16-Pob3 with Nhp6 (SPN or yFACT), promote elongation by RNA and DNA polymerases by altering the structure of nucleosomes, making them less inhibitory to the passage of polymerases (14, 18).

Nhp6 is more abundant than Spt16-Pob3 (8, 17, 19), does not form a stable complex with them (11, 12), and nhp6Δ mutations cause phenotypes distinct from defects in SPT16 or POB3. These observations suggest that Nhp6 may function both within yFACT and in other pathways. HMG box proteins have been considered to act as “architectural” factors that bend DNA and induce juxtaposition of non-contiguous DNA sequences (5, 7). Alternatively they have been proposed to reduce the overall stiffness of DNA by repeatedly binding to DNA and releasing it in a bent form, allowing the DNA to access a range of shapes more rapidly (8, 20, 21). This “shape chaperone” activity has been proposed to assist the formation of structures containing bent DNA, including nucleosomes (20, 21). Here we report that the converse may also occur as Nhp6 binding appears to destabilize nucleosomes, promoting conversion to an alternative form.

The conservation of the HMG box motif among FACT members and the importance of Nhp6 in yFACT activity suggest that this motif plays an important role in FACT function. The distinct architecture of yFACT allowed us to examine the function of the HMG box motif separately from Spt16-Pob3 to ask how this module contributes to yFACT-mediated reorganization of nucleosomes. Here we show that increasing concentrations of Nhp6 made progressive changes in nucleosome structure. At an Nhp6 concentration about 10-fold higher than the concentration that produces Nhp6-nucleosome complexes, nucleosomes underwent a change that led to enhanced DNase I sensitivity at some sites and the ability to bind Spt16-Pob3. These results suggest a general role for Nhp6 and other HMG
proteins in destabilizing nucleosomes to promote formation of alternative chromatin structures.

EXPERIMENTAL PROCEDURES

Nucleosomes—DNA molecules containing the 146-bp sea urchin 5 S rDNA nucleosome positioning sequence (22) were produced by PCR amplification using a template with the positioning sequence inserted between the EcoRI and XbaI sites in pBlueScript KS+ (Stratagene, generously provided by J. Wittmeyer and B. Cairns). Primer sequences (available upon request) were designed that amplify the positioning sequence by PCR producing different flanking contexts as shown in Fig. 1. Products were end-labeled on one strand by digesting with an appropriate restriction endonuclease, treating with phosphatase, labeling with [γ-32P]ATP and polynucleotide kinase, and then digesting with a second endonuclease. Histone octamers were derived either from chicken erythrocytes (a gift from V. Ramakrishnan and V. Graziano, Ref. 23) or from bacteria expressing recombinant yeast histones (constructs generously provided by J. Wittmeyer and B. Cairns). Nucleosomes were assembled by slow dialysis from high salt solutions and purified by sucrose gradient centrifugation as described previously (11, 24).

Binding Reactions and DNase I Digestions—Spt16-Pob3 was purified as the intact heterodimer from yeast cells overexpressing both proteins as described previously (19). Nhp6 was purified from BL21-Codon-Plus(DE3)-RIL Escherichia coli (Stratagene) expressing NHP6A from plasmid pDR1228 (Ref. 25, a generous gift from R. Johnson). Cultures growing in rich medium at 37 °C were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 4 h, harvested by centrifugation, suspended in lysis buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl), and frozen. Cell suspensions were thawed, Nonden P-40 was added to a final concentration of 0.1% (v/v), and the cells were lysed by sonication at 0 °C. Debris were removed by centrifugation at 17,000 × g for 30 min. Trichloroacetic acid was added to the cleared supernatant with constant mixing to 2% (w/v). The precipitate was removed by centrifugation at 30,000 × g for 30 min, and then trichloroacetic acid was added to the supernatant to 10% (w/v). The precipitate was harvested by centrifugation at 39,000 × g for 30 min, rinsed with acetone, dried, and then dissolved in S buffer (20 mM Tris-Cl, pH 7.5, 2 mM Na2EDTA, 1 mM 2-mercaptoethanol, 10% (w/v) glycerol, 300 mM NaCl). After dialyzing twice against 1 liter of fresh S buffer, the sample was loaded onto a 5-ml HitTrap SP column (Amersham Biosciences), which was washed with S buffer and then eluted with a 25-ml gradient from 300 mM NaCl to 1 M NaCl in S buffer. Fractions containing Nhp6 were dialyzed against S buffer, concentrated, and stored at −70 °C. Working stocks of proteins were made by diluting concentrated stocks in 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM Na2EDTA, 1 mM 2-mercaptoethanol, and 10% (w/v) glycerol.

RESULTS

Nhp6 binds to DNA and to nucleosomes (11, 25). We have shown previously that Nhp6 promotes formation of complexes between Spt16-Pob3 and nucleosomes and that the enhanced sensitivity to DNase I associated with these yFACT nucleosome complexes is partially induced at a subset of sites by Nhp6 alone (11). This suggests that Nhp6 converts nucleosomes to an altered form in which the DNA is locally more accessible to DNase I or is a more suitable substrate for this nuclease and that unlike free nucleosomes this form is competent to bind Spt16-Pob3. DNA is dramatically bent by Nhp6 and disturb contacts at the entry-exit points (linkers are defined here as the DNA extending beyond the 146-bp sea urchin 5 S rDNA nucleosome positioning sequence, Ref. 22). Alternatively Nhp6 could bind directly to the core DNA associated with the histone octamer, forcing this DNA into an abnormally shape incompatible with normal stable nucleosomal structure. These models make distinct predictions concerning the amount of Nhp6 required to alter nucleosomes and the effect of changing the size of the linkers. We therefore constructed nucleosomes with different linker configurations and tested them for the ability to bind Nhp6 or yFACT as well as for changes in sensitivity to DNase I.

DNA and Nucleosomes Each Bind Multiple Monomers of Nhp6—Nhp6 bound to either 149- or 237-bp linear double-stranded DNA fragments in an electrophoretic mobility shift assay (EMSA) with an apparent $K_d$ of about 7 nM (Fig. 2A and Table I). As previously noted by the Johnson laboratory (8, 25), multiple binding forms were observed as the concentration of Nhp6 was increased (Fig. 2A). These intermediates have been shown to reflect the binding of integral numbers of Nhp6 monomers (28), showing that DNA molecules of this size contain multiple binding sites for Nhp6 that can be accessed simulta-

With modifications provided by J. Wittmeyer and B. Cairns, personal communication.
Multiple Nhp6 Molecules Are Needed for yFACT Complexes

Titrations were performed with various nucleosomes as shown in Fig. 2 and then the loss of the unbound form (affinity for Nhp6) or the accumulation of the yFACT:nucleosome form (yFACT:nucleosome formation) was determined as in Fig. 3 to determine the concentration producing half-maximal effects. Values from the number of independent experiments indicated (n) were averaged, and the S.D. was calculated. No linker includes nucleosomes of types 1-3 in Fig 1, and + linker includes types 4-8. nts, nucleotides; Yst, yeast; Chk, chicken; Nuc, nucleosome; SP, Spt16-Pob3.

### Table 1

| Condition                        | yFACT:Nuc formation (nM Nhp6) |
|----------------------------------|-------------------------------|
| Yst, no linker                   | 360 ± 160                     |
| Yst, + linker                    | 350 ± 120                     |
| Chk, no linker                   | 530 ± 120                     |
| Chk, + linker                    | 700 ± 100                     |
| Summary                          | 460 ± 180                     |

| Condition                        | Nucleosomes (nM Nhp6)         |
|----------------------------------|-------------------------------|
| Yst, no linker                   | 27 ± 13                      |
| Yst, + linker                    | 62 ± 46                      |
| Summary                          | 51 ± 42                      |

| Condition                        | Affinity for Nhp6 (nM Nhp6) |
|----------------------------------|-------------------------------|
| DNA (149 nts)                    | 10                            |
| DNA (237 nts)                    | 3                             |
| Summary                          | 13                            |

| Condition                        | Affinity for Nhp6 with SP (nM Nhp6) |
|----------------------------------|-------------------------------------|
| Chk ≥ linkers                    | 4                                   |
| Yst ≥ linkers                    | 9                                   |
| Summary                          | 13                                  |

| Condition                        | Nuc formation (nM Spt16-Pob3) |
|----------------------------------|-------------------------------|
| Chk, no linker                   | 360 ± 180                     |
| Yst, no linker                   | 350 ± 120                     |
| Chk, + linker                    | 530 ± 120                     |
| Summary                          | 460 ± 180                     |

| Condition                        | Summary (13)                  |
|----------------------------------|-------------------------------|
| Chk                              | 5                              |
| Yst                              | 18                             |
| Summary                          | 11                             |

### Figures

**Fig. 2. Characteristics of Nhp6 and yFACT binding revealed by EMSA.**

A. free DNA (149 bp, type 2 in Fig. 1) was incubated with or without 200 nM Spt16-Pob3 and with the concentration of Nhp6 indicated and then subjected to electrophoresis through a native polyacrylamide gel. Arrows indicate the positions of free DNA and DNA saturated with Nhp6 with other complexes migrating between them. B, nucleosomes (type 3 in Fig. 1, yeast histones) were incubated without or with 200 nM Spt16-Pob3 and 0, 12, 37, 110, 330, 1000, or 3000 nM Nhp6 and then analyzed by EMSA. Arrows indicate the positions of free DNA, free nucleosomes, nucleosomes saturated with Nhp6, and yFACT-nucleosomes. C, nucleosomes (type 1 in Fig. 1, chicken histones) were incubated with 200 nM Spt16-Pob3 (all lanes) and 0, 70, 140, 350, 600, or 1100 nM Nhp6 and then analyzed by EMSA. Labels are as in B. D, nucleosomes (type 2 in Fig. 1) were reconstituted using histone octamers purified from chicken erythrocytes (Chk) or recombinant yeast histones (Yst). Spt16-Pob3 was added to 0, 5, 10, 20, or 60 nM (triangle) or to 200 nM (+). Nhp6 was added to 10 μM (+) or 0, 64, 160, 400, or 1000 nM (triangle). Labels are as in B. At 200 nM Spt16-Pob3, 1000 nM Nhp6 the yFACT-nucleosome (Nuc) form is 44% of the total signal for the chicken nucleosomes and 75% for the yeast nucleosomes.

Nhp6 contacts 10–15 bp of DNA (27), so the 149-bp substrate shown in Fig. 2A should be able to bind 10–15 monomers of Nhp6. Very high concentrations of Nhp6 caused progressively slower migration of the DNA even after binding was saturated (Fig. 2A). This appears to be an artifact caused by Nhp6 molecules migrating in this region of the polyacrylamide gels independently of DNA (not shown; nucleosomes displayed the same effect, see Fig. 2, B and C). We estimated the affinity of Nhp6 for DNA or nucleosomes (Table I) by monitoring the loss of the unbound form, indicating binding by at least one monomer of Nhp6.

We observed previously that Spt16-Pob3 and Nhp6 form a complex that can be observed in native PAGE under the low stringency conditions used for the EMSA (11), but these proteins do not appear to form a stable complex under more physiological conditions (11, 12). Addition of Spt16-Pob3 to the Nhp6-DNA binding reactions caused a slight shift in the migration pattern of the DNA (Fig. 2A, arrows) toward a lower Nhp6 concentration. Spt16-Pob3 therefore causes a small decrease in the effective concentration of Nhp6 for binding DNA, consistent with the interpretation that Spt16-Pob3 and Nhp6 do not form a specific, independent, active complex analogous to human FACT.

A comparable ladder of binding intermediates was observed with nucleosomes (Fig. 2, B and C), suggesting that nucleosomes also have multiple binding sites for Nhp6. Due to the smaller proportional change in mass and differences in migration properties caused by different linker configurations, distinct ladders were not always observed, making it difficult in some cases to clearly discriminate the unbound form. This contributes to a high variance in measurements of the affinity of Nhp6 for nucleosomes, but we observed an average affinity with different types of nucleosomes of about 22 nM for Nhp6 (Table I). Nucleosomes and free DNA therefore appear to have a similar number of binding sites for Nhp6, and the affinity of the first binding event is about 3-fold lower for nucleosomes than for free DNA.

**Linkers Do Not Promote Nhp6 or yFACT Binding**—If linkers provide a more efficient binding site for Nhp6 than DNA within...
the nucleosomal core, then nucleosomes with linkers should have higher affinity for Nhp6 than nucleosomes without linkers. However, the presence or absence of linkers on nucleosomes made little, if any, change in their affinity for Nhp6 (Table I). If Nhp6 promotes γFACT-nucleosome complex formation by affecting nucleosomes specifically at entry/exit points, then linkers might promote complex formation without altering the overall affinity for Nhp6. We therefore compared the ability of Nhp6 to support the formation of γFACT complexes using nucleosomes with and without linkers. As shown in Table I, the presence of linkers did not enhance the formation of γFACT complexes. For example, nucleosomes formed with chicken histones formed half of the maximal level of complexes at 530 nM Nhp6 without linkers and 700 nM Nhp6 with linkers. Linkers are therefore either neutral, or they slightly inhibit the ability of nucleosomes either to bind Nhp6 or to form γFACT complexes.

**Formation of γFACT-Nucleosome Complexes Requires Multiple Nhp6 Molecules**—If binding of a single monomer of Nhp6 to a nucleosome is adequate to convert the nucleosome to a state competent for recruiting Spt16-Pob3, then formation of γFACT-nucleosome complexes should occur at the same concentration of Nhp6 as simple binding. Instead half-maximal formation of γFACT complexes required about 10-fold more Nhp6 than that needed for simple binding (Table I). Fig. 2 (B–D) shows that γFACT-nucleosome complexes only began to accumulate after the concentration of Nhp6 reached a level sufficient to drive essentially all nucleosomes into complexes with multiple Nhp6 molecules. As with free DNA, addition of Spt16-Pob3 caused a decrease in the effective concentration of Nhp6, so the apparent affinity of Nhp6 for nucleosomes in the presence of Spt16-Pob3 was about 50 nM Nhp6 (Table I). In contrast, the overall average for half-maximal formation of γFACT-nucleosome complexes in the same experiments was 460 nM Nhp6 (Table I). The average ratio of Nhp6 required for γFACT complex formation to simple binding in 16 independent experiments was 11 (Table I). It therefore appears that binding a single Nhp6 molecule is not adequate to support the formation of a γFACT-nucleosome complex.

Histone octamers purified from chicken erythrocytes may retain some of the covalent modifications that occur in vivo (6), and chicken histones do not have the same sequences as yeast histones (70% identity for H2A-H2B and 90% identity for H3-H4, Ref. 29). Mutations in γFACT subunits cause synthetic effects when combined with mutations in either histones or histone-modifying enzymes (9, 11, 30), so Nhp6 and γFACT activities could be affected by either histone modifications or altered histone sequences. As shown in Table I, nucleosomes assembled using endogenous chicken histone octamers required somewhat higher levels of Nhp6 to form γFACT-nucleosome complexes than nucleosomes assembled with unmodified recombinant yeast histone octamers. For example, nucleosomes with linkers formed γFACT-nucleosome complexes with a half-maximum of 350 nM Nhp6 when yeast histones were used and 700 nM with chicken histones. This effect was small but consistent; an example of a single experiment in which the same DNA was assembled into yeast or chicken nucleosomes for direct comparison is shown in Fig. 3A. Simple

![Graph A](image1.png)

**Fig. 3.** Determination of half-maximal binding concentrations. Conversion of nucleosomes to bound forms was detected by EMSA as in Fig. 2 and quantitated by phosphorimaging. The amount of γFACT-nucleosome (Nuc) form as a percentage of the total signal in each lane was determined. Values were normalized to the highest value obtained for that nucleosome form in each experiment (% Max) and plotted as a function of the concentration of Nhp6 or Spt16-Pob3. A, nucleosomes without linkers (type 2 in Fig. 1) assembled with either yeast or chicken histones were incubated with 200 nM Spt16-Pob3 and varying concentrations of Nhp6 up to 10 μM Nhp6 (not shown). B, as in A except Nhp6 was constant at 10 μM, and Spt16-Pob3 was varied up to 200 nM. No linker indicates type 2 nucleosomes, and + Linker indicates type 8 nucleosomes (Fig. 1). C, as in B with 1 or 10 μM Nhp6 except + Linker indicates type 4 nucleosomes (Fig. 1, yeast histones).
binding of Nhp6 was similar for yeast and chicken nucleosomes, but yeast nucleosomes required 7-fold more Nhp6 to produce γFACT complexes, whereas chicken nucleosomes required 20-fold more (Table I). This suggests that increasing the concentration of Nhp6 causes progressive changes in nucleosomes, and the threshold level required to allow binding of Spt16-Pob3 is partly dependent on either the sequence of the histones or the presence of modifications.

**Spt16-Pob3 Binds with High Affinity**—Spt16-Pob3 formed γFACT-nucleosome complexes in the presence of saturating amounts of Nhp6 with an overall apparent $K_d$ of about 7 nM (Fig. 2D and Table I). Either the presence of linkers or the use of chicken histones caused a requirement for somewhat higher concentrations of Spt16-Pob3 relative to yeast nucleosomes without linkers (Table I and Fig. 3, B and C). The effects were small but reproducible within individual direct comparisons (Fig. 3B). We also noted that a smaller proportion of the nucleosomes formed with chicken histones were converted to the γFACT-nucleosome form even at saturation (Figs. 2D and 3B and not shown). This suggests that this population of nucleosomes is heterogeneous with only some members suitable for reorganization by γFACT. Spt16-Pob3 does not bind to DNA (19), and increasing the concentration of Nhp6 10-fold did not relieve the inhibition caused by linkers (Fig. 3C). It is therefore unlikely that the inhibition is due to sequestration of either Spt16-Pob3 or Nhp6 to non-productive linker sites. Linkers may make nucleosomes slightly more stable to reorganization by γFACT, and a portion of the endogenous nucleosomes may resist binding by Spt16-Pob3 because of either modified histones or suboptimal histone sequences.

**EMSA and DNase I Assays Appear to Detect the Same Nucleosomal Reorganization**—Electrophoretic mobility changes can have diverse causes, so it is important to ask whether the observed shifts reflect the same reorganization of nucleosomes detected as enhanced sensitivity to DNase I (11). If so, then each should display the same responses to titration of Spt16-Pob3 and Nhp6. As described below, the dose responses of the EMSA and DNase I assays were very similar for Spt16-Pob3 and for a subset of the sites affected by Nhp6 alone. However, analysis of the effects of Nhp6 revealed a more complex picture in which specific sites displayed distinct sets of characteristics, consistent with progressive effects of Nhp6 with increasing concentration.
levels of Spt16-Pob3 (Figs. 5 and 6). In all cases, enhanced DNase I sensitivity was induced at 6–10-fold higher concentrations of Nhp6 than was simple binding, but individual sites displayed one of three distinct responses to further increases in Nhp6 or the addition of Spt16-Pob3 (Fig. 5 and Table II). The first class is represented by T64 and B71 (Fig. 5). Even very high levels of Nhp6 caused very little change in DNase I sensitivity at these sites in the absence of Spt16-Pob3, and in its presence sensitivity was induced at levels of Nhp6 similar to those associated with yFACT complex formation by EMSA (Table II). For these sites, induction of DNase I sensitivity was dependent on yFACT. The second class is represented by T41 and B113 (Fig. 5). At these sites, Nhp6 alone caused a significant enhancement of DNase I sensitivity in the absence of Spt16-Pob3. As with the first class, this effect occurred at concentrations of Nhp6 associated with the ability to recruit Spt16-Pob3 (Table II). Addition of Spt16-Pob3 either had no effect on DNase sensitivity at these sites or mildly inhibited the effect of Nhp6 alone. The third class is represented by B86 and B100 (Fig. 5). Nhp6 alone also enhanced DNase I sensitivity at these sites, but this effect was significantly inhibited by further addition of Spt16-Pob3, especially at low concentrations of Nhp6. This inhibition occurred at the same concentration of Spt16-Pob3 as formation of yFACT complexes (Fig. 5). These three classes of responses indicate that Nhp6 and yFACT each cause specific changes in nucleosome structure and can be interpreted in terms of a two-step model for nucleosomal reorganization as described under “Discussion.”

Unlike the responses in the presence of Spt16-Pob3, many of the effects with Nhp6 alone failed to saturate with increasing Nhp6 concentration. Several sites displayed sensitivity to DNase I in the absence of Spt16-Pob3 only in the lane with 10 μM Nhp6 (T53 in Fig. 6, for example). The concentration of Nhp6 in a nucleus is about 30 μM, but much of this is likely to be bound to DNA so it is not clear that these effects are physiologically relevant. However, they do indicate that Nhp6

### Table II

| Size (nts) | Half-maximal effect (μM Nhp6) |
|-----------|-----------------------------|
| No Spt16-Pob3 | +Spt16-Pob3 |
| T115 | 800 | 400 |
| T11 | 6500 | 380 |
| T105,106 | 5000 | 600 |
| T85-88 | 5000 | 560 |
| T74 | 5500 | 320 |
| T64 | 4000 | 350 |
| T53 | 600 | 400 |
| T48 | 4600 | 560 |
| T41 | 600 | 800 |
| Binding (EMSA) | 20 | 60 (Nhp6), 385 (yFACT) |
| B59 | No change | 420 |
| B71 | 4000 | 420 |
| B76,77 | 2000 | 800 |
| B86 | 5000 | 850 |
| B89 | 450 | 3200 |
| B97 | No change | 4700 |
| B100 | 600 | 4200 |
| B111 | No change | 470 |
| B113 | 3400 | 3500 |
| B116 | 600 | 2800 |
| B119 | 2000 | 3800 |
| Binding (EMSA) | 80 | 60 (Nhp6), 600 (yFACT) |
Nhp6 has a single HMG box motif related to the HMGB family of abundant, conserved chromatin factors (1, 4). Nhp6 binds to DNA and to nucleosomes (11, 25), and both enhance sensitivity to DNase I at specific sites in nucleosomes and also allow formation of yFACT-nucleosome complexes (11). Here we have shown that both of these effects occurred at similar concentrations of Nhp6, suggesting that both result from the same alteration of nucleosome structure induced by Nhp6. Nucleosomes have multiple binding sites for Nhp6, and we show that nucleosome reorganization required the action of several Nhp6 molecules. yFACT makes use of the independent Nhp6 protein, but FACT complexes from higher eukaryotes contain a single HMG box motif within the Pob3-like subunit. These results therefore both suggest a general role for HMG box proteins in promoting rearrangements of chromatin by altering nucleosomes, and they raise questions about how HMG box motifs function within individual members of the FACT family.

**Multiple Nhp6 Molecules Are Required to Alter Nucleosomal Structure**—Nhp6 binds within the minor groove of DNA (27) and could therefore bind to a nucleosome each of the 14 times this feature faces the surface, consistent with the multiple binding intermediates observed (Fig. 2). Linker DNA could provide additional binding sites. Enhanced DNase I sensitivity and the ability to bind Spt16-Pob3 both occurred at a concentration of Nhp6 that was 6–20 times greater than the concentration that was sufficient to form Nhp6/nucleosome complexes (Table I). At this concentration, at least several Nhp6 monomers were associated with each nucleosome, and many nucleosomes were saturated with Nhp6 (Fig. 2). Simple binding of a single Nhp6 molecule therefore does not appear to be sufficient to induce the change in structure associated with altered DNase I sensitivity and competence to form yFACT complexes. Further, the sigmoidal response to Nhp6 titration (Fig. 3A) also suggests that the action of multiple Nhp6 molecules is required to promote nucleosome reorganization.

Nhp6 displayed a small preference for binding to free DNA, suggesting that linker DNA could act as a loading site for Nhp6 to interact with nucleosomes. However, addition of linkers did not enhance the affinity of Nhp6 for nucleosomes nor did linkers improve the ability of Nhp6 to induce DNase I sensitivity or to promote binding of Spt16-Pob3 (Table I). Linkers therefore do not appear to play an important role in reorganization of nucleosomes by Nhp6 or yFACT.

Reorganization of nucleosomes reconstituted with endogenous chicken histones required somewhat more Nhp6 than reorganization of nucleosomes formed with recombinant yeast histones. The source of histones did not significantly affect the simple binding affinity for Nhp6; instead the level needed to induce DNase I sensitivity and to promote yFACT complex formation was altered (Table I and Fig. 3). Very high levels of Nhp6 (500-fold higher than the level associated with simple binding) increased the number of sites displaying enhanced DNase I sensitivity in the absence of Spt16-Pob3. Taken together, these observations suggest that increasing concentrations of Nhp6 cause progressive changes in nucleosome structure. At a threshold level about 10-fold above the concentration needed for simple binding, Nhp6 induced a change in the nucleosome that was detected as enhanced localized sensitivity to DNase I and competence to bind Spt16-Pob3. The threshold value was influenced by the properties of the nucleosome, perhaps by differences in the inherent stability of the nucleosome or by the effects of histone modifications or sequence variation on the activity of Spt16-Pob3 (9, 11, 30, 31). Further increases in Nhp6 concentration caused additional sites to become sensitive to DNase I, but the pattern remained distinct from that of free DNA indicating that this represents a further specific disturbance of the nucleosome, not disruption of the structure.

**Nhp6 Promotes the First Stage of a Two-step Reorganization of Nucleosomes by yFACT**—The patterns of altered DNase I sensitivity induced by Nhp6 alone and by yFACT suggest that nucleosomes can adopt at least three configurations: the free state, an Nhp6-altered state, and a yFACT-reorganized state. Nhp6 alone promoted the first change, which is reflected by enhanced sensitivity to DNase I at sites such as T41, B89, B100, and B113 (Fig. 5) and competence to bind Spt16-Pob3. The second change required both Nhp6 and Spt16-Pob3 and is therefore yFACT-dependent (see T64 and B71 in Fig. 5). The yFACT-dependent state does not appear to be simply an enhanced or stabilized version of the Nhp6-induced state but instead seems to be a distinct third state. First, the effects at B89 and B100 caused by Nhp6 alone were largely reversed by Spt16-Pob3, indicating that sites that are exposed to DNase I in the Nhp6-induced form can return to an occluded state in the yFACT form (Figs. 5 and 6). Second, sensitivity to DNase I at T64 and B71 was not significantly enhanced by Nhp6 alone even at very high concentrations (Figs. 5 and 6), so not all changes can be accomplished by Nhp6 alone. DNase I activity is affected both by structure and sequence, so other DNA sequences and additional probes of structure will be needed to further characterize these altered nucleosomal forms. However, these results establish that Nhp6 and yFACT promote distinct but related reorganizations of nucleosomes.

We find that Nhp6 alone directly affects nucleosomal structure in a specific, localized way. By inference, related proteins such as the HMGB family may also act to facilitate transitions between chromatin states by initiating destabilization of nucleosomes. Further investigation will be required to characterize the nature of the structural changes that occur when Nhp6 and yFACT reorganize nucleosomes and to determine how HMG box motifs contribute to these changes either as multiple separate proteins as in yFACT or as the single tethered motif found in other FACT family members.

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