Functional Analysis of Nucleosome Assembly Protein, NAP-1
THE NEGATIVELY CHARGED COOH-TERMINAL REGION IS NOT NECESSARY FOR THE INTRINSIC ASSEMBLY ACTIVITY*

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A nucleosome assembly protein (NAP-1) of Saccharomyces cerevisiae facilitates the association of histones with DNA to form nucleosomes in vitro at physiological ionic conditions. The cloned gene was expressed in Escherichia coli using a T7 expression system, and the protein (417 amino acid residues) was purified by Mono Q column chromatography. Various deletion fragments of NAP-1 protein were also produced, and their nucleosome assembly activity was examined by supercoiling assay. The internal fragment containing the residues 43-365 was necessary and sufficient for the activity, and a long stretch of negatively charged region near the carboxyl terminus was dispensable. This minimal size fragment could form the 12 S NAP-1-histone complex as the whole protein could, whereas deleted fragments on either side could bind with core histones only to form aggregates.

In eukaryotes, DNA and histones are assembled in a repeating unit called a nucleosome. A nucleosome consists of two sets of the four core histones, H2A, H2B, H3, and H4, and approximately 145 base pairs of DNA (1, 2). Nucleosomes can be reconstituted by mixing DNA and histones dissolved in >1 M NaCl and 5 M urea and then dialyzing the mixture to remove salts and denaturant gradually (3). At a physiological ionic strength, DNA and histones form precipitates, and intact nucleosome structure can rarely be formed.

Nucleoplasmin isolated from the eggs of Xenopus laevis is the first example of such proteins that can promote nucleosome assembly in vitro at physiological conditions (4). N1/N2 were subsequently isolated in a form of complex with nucleoplasmin while histones H3 and H4 were associated with N1 and that both complexes were required for the maximal nucleosome assembly. Both nucleoplasmin and N1 contain clusters of negatively charged amino acids (7, 8). Recently, Kleinachmids et al. (9) showed that the deletion of the N1 acidic domain of N1 drastically reduced the histone binding. It was also reported that the negatively charged polyanions, such as polyglutamic acid or RNA, could assist nucleosome assembly in vitro (10, 11). These results suggested that such a long consecutive stretch, as many as 20 residues, of negatively charged amino acids might be directly involved in nucleosome assembly.

NAP-1 has the same activity as nucleoplasmin and N1/N2 and was first isolated from mammalian cells (12, 13) and found in most eukaryotic cells (14). The yeast NAP-1 gene was cloned, and the gene product expressed in Escherichia coli could assist nucleosome assembly in vitro (14). Deduced from the DNA sequence data, NAP-1 contains three negatively charged regions, and the longest one, with 15 glutamic and 13 aspartic residues out of 38 residues, is located near the COOH terminus. In this report, we constructed various deletion derivatives of the yeast NAP-1 gene, expressed them in E. coli, and determined the region essential for nucleosome assembly. We also showed the relation between the 12 S NAP-1-histone complex formation and the activity of nucleosome assembly.

MATERIALS AND METHODS

Construction of Expression Plasmids—An expression plasmid for NAP-1 was constructed as shown in Fig. 1. The Sau3AI-Apal fragment that codes for amino acid residues 1-42 and the Apal-HindIII fragment containing the residues 43-417 were inserted into the T7 expression vector pET3c (15) digested with BamHI and HindIII. The resulting plasmid pTN2 codes for an in-frame fusion product of the whole NAP-1 with the first 11 amino acids of the T7 gene 10 product at the NH2 terminus.

Each deletion derivative of NAP-1 was constructed as shown in Fig. 4. The COOH-terminal deletions were made by cleaving each site by an appropriate restriction enzyme and then inserting the SpeI linker which causes termination of translation by creating nonsense codons in all three reading frames. Both NH2-terminal and internal deletion derivatives were made by digesting pTN2 with restriction enzymes and inserting appropriate BamHI linkers to connect the remaining two DNA in-frame.

Expression in E. coli—Each expression plasmid was introduced into E. coli BL21 (DE3), a strain carrying the T7 RNA polymerase gene under the control of the lac UV5 promoter. When the A600 of the culture was 0.5, T7 RNA polymerase was induced by the addition of isopropyl-β-d-thiogalactopyranoside to the final concentration of 0.4 mM. The cells were harvested after 3 h of induction. 50 μg/ml ampicillin was included in all of the culture media (LB) to avoid the loss of recombinant plasmids. Every NAP-1 derivative was produced as the most abundant component in the E. coli crude extract.

Partial Purification of NAP-1—Cells producing NAP-1 and its derivatives in 0.5 liter of culture were harvested after induction and suspended in 5 ml of the buffer containing 100 mM Tris-HCl, pH 7.5, 20% sucrose, 0.5 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 200 μg/ml lysozyme. After 20 min at 0 °C, Brij 58 and KCl were added to the final concentration of 0.1% and 75 mM, respectively.

For the complete NAP-1 and its deleted forms that were soluble by this extraction method, the suspensions were centrifuged at 140,000 × g for 90 min (Beckman 60 Ti rotor) (16), and the supernatant was dialyzed against 25 mM Tris-HCl, pH 7.5, 2 mM DTT, 1 M NaCl, and 5 M urea and then dialyzing the mixture.

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1 The abbreviation used is: DTT, dithiothreitol.
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Construction of the expression plasmid of NAP-1. From clone 1 containing NAP-1 gene in λgt11 (14), a 719-base pair DNA fragment was isolated by digestion with EcoRI. It was further digested with Sau3AI and Apal, and the resulting 130-base pair fragment that encoded amino acids 1-42 of NAP-1 was obtained. From clone 3, a 2.0-kilobase pair DNA fragment that encoded amino acids 43-417 was isolated by digestion with Apal and HindIII. These two fragments were ligated into the T7 RNA polymerase-dependent expression vector solution containing peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) for the monoclonal antibody or peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for polyclonal antiserum. After the nitrocellulose membrane was washed, proteins that reacted with the anti-NAP-1 antibody were detected by chloronaphthol (Konica immunostain HRP kit). Polyclonal antibodies were raised against yeast NAP-1 prepared from E. coli using a T7 expression system. This polyclonal antiserum was found to be specific to yeast NAP-1 because it did not cross-react to human NAP-1 protein.

Binding of NAP-1 to the Core Histones—The NAP-1-histone binding reaction was carried out at 37 °C in the buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100. Core histones (6 μg) were incubated with NAP-1 (10 μg) for 30 min in 100 μl of the reaction mixture. These reaction mixtures were loaded onto a linear gradient of 10–30% sucrose containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% Triton X-100 and centrifuged at 45,000 rpm for 15.5 h (SW50.1, Beckman Instruments). Aliquots of the fractions were analyzed by SDS gel electrophoresis (15% acrylamide), and proteins were visualized by using a silver staining kit (Wako, Japan).

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Purification of Histones—Core histones were purified by the method of Simon and Felsenfeld (17). HeLa chromatin was applied to a hydroxyapatite column, and core histones were eluted from the column with 2 mM NaCl. To split core histones into two fractions, H2A + H2B and H3 + H4 were eluted from the column by stepwise elution with 0.93 and 2 mM NaCl, respectively.

Assay of Nucleosome Assembly—The reaction was carried out at 37 °C in the buffer (24 μl) containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 100 μg/ml bovine serum albumin. Cotransfer of linear circular DNA (0.2 μg) of pBR322 was preincubated with 500 units of topoisomerase I from HeLa cells (4 μl) for 10 min (14).

Core histones (0.6 μg), which had been prepared from HeLa cells, were preincubated with NAP-1 derivatives (1.0 μg) for 15 min in the same buffer solution (20 μl). Two reaction mixtures were combined and incubated further for 45 min. SDS and proteinase K were added to the final concentration of 0.2% and 100 μg/ml, respectively, and incubated for 15 min. DNA was purified by phenol/chloroform extraction and electrophoresed in 1% agarose gel in 89 mM Tris, 89 mM borate, 2.5 mM EDTA buffer system (TBE). Those nucleosomes that could form nucleosomes gain negative superhelical turns that failed to form nucleosomes remain as relaxed circles (28).

Immunoblot Analysis—Proteins were electrophoresed in 12.5% polyacrylamide gel containing 0.1% SDS according to the method of Laemmli (18). Proteins were stained with Coomassie Brilliant Blue or transferred electrophoretically to a nitrocellulose membrane. After the nitrocellulose membrane was incubated in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5% horse serum for 1 h, it was further incubated for 1 h at 37 °C with the spent medium of hybridoma cells (clone 4A8) that secrete a monoclonal antibody against HeLa NAP-1 (14, 19) or with the rabbit polyclonal antibodies against yeast NAP-1. The nitrocellulose membrane was washed with TBS containing 0.1% Triton X-100 and was soaked in the TBS solution containing peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) for the monoclonal antibody or peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for polyclonal antiserum. After the nitrocellulose membrane was washed, proteins that reacted with the anti-NAP-1 antibody were detected by chloronaphthol (Konica immunostain HRP kit). Polyclonal antibodies were raised against yeast NAP-1 prepared from E. coli using a T7 expression system. This polyclonal antiserum was found to be specific to yeast NAP-1 because it did not cross-react to human NAP-1 protein.

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Nucleosome assembly activity of this NAP-1 preparation
Partially purified NAP-1 derivatives were electrophoresed in SDS-polyacrylamide gel (12.5%) and stained with Coomassie Brilliant Blue or analyzed by immunoblotting using the monoclonal antibody against HeLa NAP-1 andards (Bio-Rad), bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor, which run at 80, 50, 33, and 28 kDa, respectively, were used for size markers. Phosphorylase a, bovine serum albumin, ovalbumin, carbonic anhydrase, and &beta;lactoglobulin (18 kDa) were used for size markers. The position of form I supercoiled DNA was examined (Fig. 3a). The position of form II supercoiled DNA was indicated that yeast NAP-1 expressed in E. coli using T7 expression vector had an activity of nucleosome assembly comparable to that of NAP-1 purified from mouse cells (13). Next, NAP-1 was mixed with either histones H2A+H2B or H3+H4 instead of core histones and each supercoiling activity was examined (Fig. 3b). When NAP-1 was reacted with only H2A+H2B histones, no superhelical turn was introduced at any amount. On the other hand, when NAP-1 was incubated with 0.3 or 0.6 &mu;g of H2A+H2B histones, superhelical turns were introduced into DNA. But no superhelical changes were observed in the presence of 0.9 &mu;g of histones H3+H4. In this case, the amount of NAP-1 becomes limiting over the excess amount of H3+H4 histones. Free histones tend to bind to DNA in a nonspecific manner to form an aberrant structure and inhibit the nucleosome assembly process. These results suggest that NAP-1 binds with H3 and H4 histones and transfers them to DNA to make the basal structure of nucleosomes.

**Analysis of Short Fragments of NAP-1**—To determine the regions necessary for nucleosome assembly, a part of DNA coding for NAP-1 was deleted as shown in Fig. 4. These deletion fragments were expressed in E. coli using a T7 expression vector, and each protein was partially purified by indicated that yeast NAP-1 expressed in E. coli using T7 expression vector had an activity of nucleosome assembly comparable to that of NAP-1 purified from mouse cells (13). Next, NAP-1 was mixed with either histones H2A+H2B or H3+H4 instead of core histones and each supercoiling activity was examined (Fig. 3b). When NAP-1 was reacted with only H2A+H2B histones, no superhelical turn was introduced at any amount. On the other hand, when NAP-1 was incubated with 0.3 or 0.6 &mu;g of H2A+H2B histones, superhelical turns were introduced into DNA. But no superhelical changes were observed in the presence of 0.9 &mu;g of histones H3+H4. In this case, the amount of NAP-1 becomes limiting over the excess amount of H3+H4 histones. Free histones tend to bind to DNA in a nonspecific manner to form an aberrant structure and inhibit the nucleosome assembly process. These results suggest that NAP-1 binds with H3 and H4 histones and transfers them to DNA to make the basal structure of nucleosomes.

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the portion of yeast NAP-1, which were prepared by immunizing rabbits with partially purified yeast NAP-1 product expressed in E. coli (Fig. 2a). The size of these proteins almost coincides with the value calculated from their sequence data. These proteins were all recognized by polyclonal antibodies against yeast NAP-1. But the shorter fragment lacking further 21 residues from the COOH terminus did not support the activity. Since the 52 residues from the COOH terminus were not essential, we constructed fragments depleted of both the NH2-terminal and the COOH-terminal regions. A deletion fragment that lacked the residues 1-42 and 366-417 had the nucleosome assembly activity, but the further deletion from the NH2 terminus, removing residues 43-126, abolished the activity. These results suggest that the region between residues 43 and 365 is necessary and sufficient for the nucleosome assembly activity.

To know the role of internal region, we constructed internal deletion fragments. As shown in Fig. 5, none of the internal deletion fragments (lanes 6-10) had the nucleosome assembly activity comparable with that of complete NAP-1, although a small amount of fully supercoiled DNA was formed under the conditions used (lanes 7 and 9). Some internal deletion fragments (lanes 6, 8, and 10) were inactive even though they carried all three negatively charged regions. These results (summarized in Fig. 4) suggest that the existence of negatively charged regions is not sufficient for the nucleosome assembly activity.

**Binding of NAP-1 Derivatives with Core Histones**—We next examined whether the fragments that did not have the assembly activity could bind the core histones. These deletion derivatives of NAP-1, inactive for nucleosome assembly reaction, could bind the core histones as effectively as intact NAP-1 in the enzyme-linked immunosorbent assay (26) (data not shown). As reported previously (26), when native NAP-1 was mixed with the core histones under physiological conditions, a 12 S complex was formed that contained NAP-1 and four kinds of histones in equal amounts. When the fragment lacking both residues 1-42 and 366-417 (Fig. 5, lane 12) was reacted, large aggregates (>15 S) were formed in addition to the 12 S complex (Fig. 6a). As summarized in Table I, such an aggregate was not formed when the intact NAP-1 (Fig. 4, lane 1) and NAP-1 derivatives (lanes 5 and 11) were mixed with histones. Thus the fragments in lane 12 might be less
Fig. 6. Formation of histone-NAP-1 complexes. Histone-NAP-1 derivative complexes were fractionated by sucrose gradient centrifugation as described under “Materials and Methods.” A portion of each fraction (lanes 1–23) was subjected to SDS-polyacrylamide gel electrophoresis (15%) and stained with silver. The intensity of staining by this method was not proportional to the amount of protein present in the gel. Proteins recovered from the bottom of the tube were also electrophoresed in the left-end lane (P). Standard markers employed for the sedimentation values, catalase (11.3 S), and bovine serum albumin (4.4 S) were recovered in fractions 6 and 16, respectively, and those for the gel electrophoresis were ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa). a, NAP-1 fragment lacking both 1–42 and 366–417 (lane 12). b, NAP-1 fragment lacking both 1–126 and 366–417 (lane 13). c, NAP-1 fragment lacking 335–417 (lane 4). Each fragment was reacted with core histones.

### TABLE I

| NAP-1 derivatives | Calculated molecular mass of NAP-1 | S value of NAP-1-histone complex |
|-------------------|-----------------------------------|----------------------------------|
| 1                 | 49                                | 4                               |
| 2                 | 47                                | Not tested                       |
| 3                 | 44                                | 12                              |
| 4                 | 40                                | >15                             |
| 5                 | 31                                | Not tested                       |
| 6                 | 46                                | >15                             |
| 7                 | 41                                | >15                             |
| 8                 | 41                                | >15                             |
| 9                 | 43                                | >15                             |
| 10                | 40                                | >15                             |
| 11                | 44                                | 12                              |
| 12                | 39                                | 12 + >15                        |
| 13                | 29                                | >15                             |
| 14                | 22                                | >15                             |

Active than native NAP-1, although such a slight difference could not be detected by supercoiling assay. When each fragment lacking both residues 1–126 and 366–417 (lane 13) or a fragment lacking residues 335–417 (lane 4) was reacted (Fig. 6, b and c), only the aggregates were formed. Under the same condition, free core histones or the NAP-1 fragments sedimented at the 1–3 S position (data not shown). We tested the complex formation of the NAP-1 fragments as listed in Table I. The data indicate that all of the fragments that formed the 12 S complex had the nucleosome assembly activity comparable with the native NAP-1 and that all of the fragments that made aggregates did not have the nucleosome assembly activity. We concluded that the formation of the 12 S complex was essential for the activity and that the fragment between residues 43 and 365 was indispensable for the formation of the 12 S complex.

Epitope to Monoclonal Antibody—Although the fragment of NAP-1 missing residues 335–417 reacted with the monoclonal antibody against NAP-1 purified from HeLa cells, the deletion fragment missing the residue 257–417 did not. The internal deletion of NAP-1 missing the residue 257–334 did not react with monoclonal antibody either (Fig. 2b). This result indicate that the epitope against the monoclonal antibody is located between residues 257 and 334. This monoclonal antibody reacted with a 50–60-kDa protein in human, mouse, Xenopus, fruit fly, and yeast cell lysates (14). Therefore, a part of this region must be highly conserved among these species and is located in the region required for the NAP-1 function.

**DISCUSSION**

Clusters of negatively charged regions are found in N1, nucleoplasmin, and HMG-1, all of which could promote nucleosome assembly in vitro. Kleinschmidt et al. (9) suggested that the acidic domain in the center of protein N1 played a major role in binding histones. This is supported by the result that polyglutamic acid could promote nucleosome assembly at a physiological ionic condition.

We have already shown that three negatively charged re-
Nucleotide sequence of clone 734-2 and its translation are shown in Table I. The NAP-1 fragment (lanes 3 and 4) missing the largest negatively charged region of 38 amino acids at the COOH terminus retained the nucleosome assembly activity. The fragments (Fig. 4, lane 4) lacking the region with a helix-turn-helix motif (residues 305–320) did not have the activity. These results suggest that a long stretch of negatively charged regions might be dispensable for the nucleosome assembly but other structural features might be important. Since the fragment lacking the COOH-terminal negatively charged region is still acidic (pI = 4.9), it is possible that the acidity of the protein as a whole is one of the important factors for the nucleosome assembly.

We also found that the fragments active in the nucleosome assembly could precedently form a 12 S histone-NAP-1 complex. Other inactive derivatives formed large aggregates, when mixed with core histones, which sedimented at the bottom of sucrose gradient. These results indicate that the formation of the 12 S complex by NAP-1 and core histones is correlated with the nucleosome assembly. It has been shown that nucleosomes are formed when this 12 S complex is mixed with DNA (13).

Some deleted fragments were not soluble by the lysozyme/Brij extraction method. We solubilized these proteins in urea to purify them. These proteins were inactive or less active for nucleosome assembly. We cannot exclude the possibility that treatment with urea changes the proper conformation of the proteins and renders them inactive, although the treatment of purified NAP-1 protein with urea does not affect the activity at all.

In vivo studies have demonstrated that during DNA replication, histones H3+H4 become associated with newly replicated DNA before assembly of histones H2A+H2B (20–24). N1/N2 bind H3+H4 histones, and this complex can induce supercoiling of DNA (25). So, we examined whether NAP-1 could induce superhelical turns when it was reacted with only H3+H4 histones (Fig. 3b). With this combination, negative supercoils were introduced to the same extent as in the reaction containing all four core histones. Ishimi et al. (26) showed that an 8 S complex was formed by mixing H3+H4 histones with mouse NAP-1. These results suggest that NAP-1 binds with H3+H4 histones and transfers them to DNA to form the backbone structure of nucleosomes with an arginine-rich kernel (29). When deletion fragments of NAP-1 were reacted with only histones H3+H4, those that could promote nucleosome assembly with core histones could also introduce supercoiling of DNA in this system (data not shown). These results suggest that supercoiling of DNA depends on the interaction of NAP-1 with histones H3+H4. Kleinschmidt et al. (25) indicated that histones H3+H4 bound to N1/N2 could introduce supercoiling of DNA at a histone-DNA ratio of 2:1, but could not at a histone-DNA ratio of 6:1. Our results in Fig. 3 seem to coincide with the results of Kleinschmidt, et al. (25) and suggest that the ratio of histones to NAP-1 in the reaction mixture is important for nucleosome assembly. Zucker and Worcel (27) indicated that DNA supercoiling induced by the histones H3+H4/N1 complex was caused by the subnucleosomal particles containing histones H3+H4 and 65 base pairs of DNA. We do not know whether supercoiling induced by histones H3+H4 and NAP-1 complex is caused by the subnucleosomal particles.

An intriguing observation is that polyclonal antibodies raised against yeast protein recognize NAP-1 from yeast origin but not from any other organisms. Since the monoclonal antibody raised against HeLa NAP-1 cross-reacted with yeast NAP-1 as well as that of Xenopus and Drosophila, its epitope must be in the very conserved region. In the course of screening the Xgt11 cDNA library of mouse spermatocyte by using the monoclonal antibody, we picked up one false clone in addition to the authentic mouse NAP-1 clone. From the sequence analysis of the false clone, it was found that oligopeptide (13 residues as shown in Table II, clone 734-2) was fused to the end of β-galactosidase in the clone, which gave an extremely strong positive signal to its fusion product by immunoblotting analysis (better than any other positive clones). In Table II, the sequence of this fusion peptide is compared with the epitope regions of yeast NAP-1 and the corresponding mouse NAP-1 sequence. Apparently, FNF sequence is common to all the three clones, suggesting that this stretch of a few amino acids may be the epitope of monoclonal antibody.

Comparing mouse NAP-1 sequence with that of yeast, the putative epitope sequence and another KGIPFELWLT sequence (14) are strictly conserved (8 residues out of 9 in both circumstances), while other parts are considerably diverted (homology of amino acid sequences is less than 30%). This may explain the failure of polyclonal antibodies to cross-react with NAP-1 from any other organisms than of yeast origin.

The NAP-1 protein could be produced and purified in quantity, which is very useful to reconstitute chromosomes in vitro. These chromosomes can serve as a template for transcription or replication in vitro. Since the chromosomal structure in the initiation site of replication or transcription is very important, the reconstruction of chromosomes with or without initiator proteins could greatly accelerate the future studies of regulation of transcription and replication of chromosomes.

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