Histone stoichiometry and DNA circularization in archaeal nucleosomes

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Received September 17, 1998; Revised and Accepted November 18, 1998

ABSTRACT

Recombinant (r)HMfB (archaeal histone B from *Methanothermobacter fervidus*) formed complexes with increasing stability with DNA molecules increasing in length from 52 to 100 bp, but not with a 39 bp molecule. By using ¹²⁵I-labeled rHMfB-YY (an rHMfB variant with I31Y and M35Y replacements) and ³²P-labeled 100 bp DNA, these complexes, designated archaeal nucleosomes, have been shown to contain an archaeal histone tetramer. Consistent with DNA bending and wrapping, addition of DNA ligase to archaeal nucleosomes assembled with 88 and 128 bp DNAs resulted in covalently-closed monomeric circular DNAs which, following histone removal, were positively supercoiled based on their electrophoretic mobilities in the presence of ethidium bromide before and after relaxation by calf thymus topoisomerase I. Ligase addition to mixtures of rHMfB with 53 or 30 bp DNA molecules also resulted in circular DNAs but these were circular dimers and trimers. These short DNA molecules apparently had to be ligated into longer linear multimers for assembly into archaeal nucleosomes and ligation into circles. rHMfB assembled into archaeal nucleosomes at lower histone to DNA ratios with the supercoiled, circular ligation product than with the original 88 bp linear version of this molecule. Archaeal histones are most similar to the globular histone fold region of eukaryal histone H4, and the results reported are consistent with archaeal nucleosomes resembling the structure formed by eukaryal histone (H3+H4)₂ tetramers.

INTRODUCTION

Members of the euryarchaeotal branch of the Archaea contain archaeal histones, proteins with primary sequences, the histone fold and dimer formation (1–4) in common with the globular regions of the eukaryal nucleosome core histones H2A, H2B, H3 and H4 (5,6). However, archaeal histones form both homodimers and heterodimers (7) and unlike their eukaryal relatives, they do not have N- and C-terminal amino acid sequences that extend beyond the histone fold. They assemble into DNA-containing complexes that visibly resemble eukaryal nucleosomes (1,8), but whereas eukaryal nucleosomes contain 146 bp of DNA wrapped in 1.65 circles around a histone octamer (9), archaeal histones form structures that protect only ~60 bp from micrococcal

nuclease (MN) digestion, and protein crosslinking studies have indicated that these structures, designated archaeal nucleosomes, contain a histone tetramer rather than an octamer (10,11). The histone content of the archaeal nucleosome has not, however, been determined directly and although electron microscopy revealed that the DNA is wrapped around a protein core (1,8), the length of DNA so circularized has not been established. The results reported here establish the archaeal histone content, the minimal length and topology of the DNA circularized by ligation in an archaeal nucleosome.

MATERIALS AND METHODS

Protein preparations

Recombinant (r)HMfB (histone B from *Methanothermobacter fervidus*) and rHMfB-YY, a variant of rHMfB constructed by K. Sandman using the Altered Sites kit (Promega Corp., Madison, WI) with tyrosine residues substituted for the isoleucine-31 (I31Y) and methionine-35 (M35Y) residues of rHMfB, were synthesized in *Escherichia coli* JM105 and purified as previously described (7,12). The tyrosine residues in rHMfB-YY facilitated ¹²⁵I-labeling, and ¹²⁵I-rHMfB-YY was generated by using IODO-BEADS with Na¹²⁵I as directed by the manufacturer (Pierce, Rockford, IL). rHMfB, rHMfB-YY and ¹²⁵I-rHMfB-YY preparations had identical DNA-binding activities based on electrophoretic mobility-shift assays (EMSA) and protein concentrations (stated throughout in terms of polypeptide monomers) determined by amino-acid analyses. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detection by autoradiography, the region of a gel containing a known amount of ¹²⁵I-rHMfB-YY was excised and solubilized by oxidation as described by Carey (13). The specific activity of the ¹²⁵I-rHMfB-YY preparation was then calculated based on liquid scintillation measurements of the ¹²⁵I content of the oxidized material.

DNA molecules

³²P-labeled DNA molecules 39, 52, 62, 74 and 85 bp in length were generated from the polylinker region of pLITMUS28 [New England Biolabs (NEB), Beverly, MA] by digestions with *Xba*I and *Stu*I, *Bgl*II and *Eco*RV, *Spe*I and *Eco*RV, *Bam*HI and *Stu*I, and *Bam*HI and *Sna*BI, respectively, and filling in the single-stranded (ss) *Xba*I, *Bgl*II, *Spe*I and *Bam*HI half-sites using the Klenow fragment of DNA polymerase I with a dNTP mixture that contained [³²P]dATP (14). A 100 bp DNA molecule was obtained by PCR amplification of pRG101 DNA cloned into the *Eco*RV

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site of pLITMUS28 that resulted in clone #15 (11), together with 19 bp from the 5', and 20 bp from the 3' polylinker regions that flanked the 61 bp insert. This DNA molecule was ^{32}P -end-labeled using T4 polynucleotide kinase with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

DNA molecules with complementary, ss 5'-extensions (4 nt) flanking 53, 98 and 128 bp double stranded (ds) regions were obtained from the polylinker region of pLITMUS28 by digestion with *Bam*HI plus *Bgl*II, and from pLITMUS28 and pLITMUS28 containing 30 bp of pRG101 cloned into the *Eco*RV site [clone #26; (11)] by digestion with *Xba*I plus *Spe*I, respectively. Digestion of pLITMUS28 with *Eco*RV plus *Bsi*WI, and with *Eco*RV and *Eco*RI, followed by filling-in the *Bsi*WI and *Eco*RI half-sites, respectively, ligation and re-transformation into *E. coli* DH5 α resulted in plasmids with 23 (pLITMUS28 Δ 23) and 10 bp (pLITMUS28 Δ 10) deletions within the polylinker region. *Bam*HI plus *Bgl*II digestion of pLITMUS28 Δ 23 DNA and *Spe*I plus *Xba*I digestion of pLITMUS28 Δ 10 DNA generated molecules with 30 and 88 bp ds regions, respectively, with complementary ss 5'-extensions. The 30, 53, 88, 98 and 128 bp molecules labeled at only one terminus were obtained from pLITMUS28, pLITMUS28 Δ 23, pLITMUS28 Δ 10 and clone #26 DNA by digestion with *Xba*I or *Bgl*II, ^{32}P -end-labeling using T4 polynucleotide kinase, followed by digestion with *Spe*I or *Bam*HI. A 79 bp molecule ^{32}P -labeled at one end was obtained from the pLITMUS28 polylinker by *Eco*RV digestion, ^{32}P -end-labeling and then digestion with *Stu*I.

DNA concentrations and specific activities were determined by A_{260} measurements and liquid scintillation counting. All ^{32}P -labeled DNA molecules were gel purified before use.

EMSA

^{32}P -labeled DNA molecules were incubated with aliquots of rHMfB for 30 min at 25°C in 10 μl reaction mixtures that contained 100 mM KCl and 50 mM Tris-HCl (pH 7.5). The complexes formed were separated from unbound DNA by electrophoresis at 8 V/cm through non-denaturing polyacrylamide gels (8%T; 0.13%C) run in 90 mM Tris-borate (pH 8), 2 mM EDTA (TBE buffer) at 25°C, visualized by autoradiography, and the relative amounts of bound and unbound DNA were determined by laser scanning densitometry (LKB Instruments Inc., Rockville, MD).

^{125}I -rHMfB-YY + ^{32}P -DNA complex stoichiometry

Aliquots (76 nM) of the ^{32}P -labeled 100 bp DNA were mixed and incubated with 0.64 or 1.3 μM ^{125}I -rHMfB-YY for 30 min at 25°C. The complexes formed were separated from unbound DNA by electrophoresis and the regions of the gel that contained complexes and unbound DNA were identified by autoradiography, excised and solubilized by oxidation overnight in 17% HClO_4 , 21% H_2O_2 at 60°C (13). The rHMf-YY to DNA molar ratios of the complexes were determined by liquid scintillation counting (Beckman model 7500, Palo Alto, CA) of the oxidized material. Corrections were made for ^{125}I - ^{32}P channel spill-over and isotope decay. Background levels of unbound ^{125}I -rHMfB-YY and ^{32}P -DNA in the region of the gel containing complexes were determined by measuring the ^{125}I and ^{32}P contents of equal-sized gel fragments excised from adjacent tracks that were loaded with only ^{125}I -rHMfB-YY or ^{32}P -DNA.

Figure 1. EMSA of complex formation by rHMfB with DNA molecules of increasing length. Lanes 1–5 contained 10 nM of ^{32}P -labeled DNA molecules 39, 52, 62, 74 and 85 bp in length, respectively. Lanes 6–10 contained identical aliquots of these DNAs incubated with 2 μM of rHMfB for 30 min at 25°C. ^{32}P -labeled DNAs and complexes (indicated by the arrow) were visualized by autoradiography following electrophoresis through an 8% polyacrylamide gel.

DNA circularization and ligation product topology

Reaction mixtures (10 μl) containing a ^{32}P -labeled DNA molecule (8 nM) in ligase buffer [10 mM MgCl_2 , 10 mM dithiothreitol, 1 mM ATP, 50 mM Tris-HCl (pH 7.8) and 50 μg bovine serum albumin/ml (15–17)] and 0.06–6.7 μM rHMfB were incubated for 30 min at 25°C. T4 DNA ligase (80 U; NEB) was then added and incubation continued for 12 h at 16°C. Following incubation with 0.5% SDS and 2 μg proteinase K for 30 min at 37°C, the resulting deproteinized DNA molecules were separated by electrophoresis at 8 V/cm through non-denaturing polyacrylamide gels (8%T; 0.13%C) run in TBE buffer (14) and located by autoradiography. Ligation products, purified by excision and elution from the gel, were exposed to digestion by restriction enzymes, exonuclease III (exoIII) and/or calf thymus topoisomerase I (Gibco BRL Life Technologies, Gaithersburg, MD). The restriction fragments generated were separated by electrophoresis through polyacrylamide gels (8%T; 0.13%C) run in TBE (14), and exoIII and topoisomerase I reaction products by electrophoresis through polyacrylamide gels (8%T; 0.4%C) run in TBE containing 1 μg ethidium bromide (EtBr)/ml (18–22). ^{32}P -labeled DNA molecules were then visualized by autoradiography.

RESULTS

DNA length-dependent EMSA

Each histone dimer in the eukaryal nucleosome interacts through histone fold domains with 27–28 bp in 2.5 consecutive helical turns (9). At the center of the nucleosome, the histone fold domains of the (H3+H4) $_2$ tetramer make main-chain or side-chain hydrogen bonds with a region that extends over ~66 bp and consistent with (rHMfB) $_2$ and (H3+H4) dimers assembling into similar structures, rHMfB did not form complexes sufficiently stable for detection by EMSA with a 39 bp DNA molecule, but did so with molecules 52, 62, 74, 85 and 100 bp in length (Figs 1 and 2A). Under equimolar reaction conditions, the percentage of input DNA molecules assembled into such complexes increased substantially with increasing DNA length (Fig. 1), and only with DNA molecules ≥ 85 bp in length was 100% of the DNA incorporated into complexes.

Histone content of archaeal nucleosomes

As archaeal nucleosomes were known to protect ~60 bp from MN digestion (10), it seemed possible that two archaeal nucleosomes might assemble on DNA molecules ≥ 120 bp. Therefore, to avoid

Figure 2. Electrophoretic separation and isolation of rHMfB-YY-DNA complexes. (A) Complexes formed by incubating increasing amounts of rHMfB-YY (0.32, 0.64, 1.3, 3.9 and 5.2 μ M) for 30 min at 25°C with 76 nM aliquots of the 32 P-labeled 100 bp DNA molecule were separated from unbound DNA by electrophoresis through an 8% polyacrylamide gel and visualized by autoradiography. The control lane (0) contained histone-free DNA. (B) The 32 P-labeled 100 bp DNA was incubated, as in (A), with 0.64 and 1.3 μ M of 125 I-labeled rHMfB-YY and complexes formed separated from unbound DNA, as indicated, in lanes 1 and 2, respectively. An aliquot of the 32 P-labeled 100 bp DNA, and 0.64 and 1.3 μ M of 125 I-labeled rHMfB-YY, were in lanes 0, 3 and 4, respectively. The locations of the 125 I-rHMfB-YY + 32 P-DNA complexes were determined by autoradiography of the wet gel and with the adjacent regions from lanes 0, 3 and 4, were excised, oxidized (13), and 125 I and 32 P contents determined.

this, archaeal nucleosome stoichiometry experiments were undertaken using a 100 bp DNA molecule. Increasing amounts of rHMfB-YY were incubated with a fixed amount of the 32 P-labeled 100 bp DNA to determine conditions that resulted in ~50 and ~98% assembly of the DNA into archaeal nucleosomes (Fig. 2A). Archaeal nucleosome assembly was then repeated using these conditions with 125 I-labeled rHMfB-YY and 32 P-labeled DNA (Fig. 2B). Following electrophoresis and detection by autoradiography, the regions of the gel that contained nucleosomes were excised and the gel dissolved by oxidation to prevent isotope quenching by gel fragments (13). The 125 I and 32 P contents of the oxidized material were determined by scintillation counting and based on these measurements, the archaeal nucleosomes assembled under conditions where ~50 and ~98% of the DNA was bound by rHMfB contained 4.8 and 4.4, and 4.4 and 4.0 rHMfB monomers/DNA molecule, respectively, in two independent experiments. Therefore, under both assembly conditions, the archaeal nucleosomes assembled *in vitro* by rHMfB-YY with the 100 bp DNA contained a histone tetramer, consistent with the results of archaeal histone crosslinking in archaeal nucleosomes assembled *in vivo* (10).

DNA circularization

In the eukaryal nucleosome, 146 bp are wrapped 1.65 times around the histone octamer core, equivalent to 88 bp per circumference (9). Based on this value, and knowing that ~80 bp are circularized around the (H3+H4)₂ tetramer at the center of the nucleosome, it seemed likely that the increasing stability of rHMfB-DNA complexes with increasing DNA length (Fig. 1) reflected the ability of an (rHMfB)₄ tetramer to bend and maintain the DNA molecule in a circular configuration. This was investigated by adding DNA ligase after incubating rHMfB for 30 min with 32 P-labeled DNA molecules 30, 53, 79, 88, 98 and

Figure 3. Ligation of the 88 bp DNA molecule assembled into rHMfB-containing archaeal nucleosomes. The 88 bp 32 P-labeled DNA molecule was subjected to electrophoresis in lane 1 adjacent to a sample of the same DNA incubated with T4 DNA ligase for 12 h at 16°C before electrophoresis. Identical aliquots of the DNA (8 nM) were incubated with 0.06, 0.13, 0.335, 1.3 and 6.7 μ M of rHMfB for 30 min at 25°C, T4 DNA ligase added and following incubation for 12 h at 16°C, deproteinized and subjected to electrophoresis in lanes 4–8. The DNA in lane 3 was incubated with 6.7 μ M of rHMfB for 30 min at 25°C, and then incubated for 12 h at 16°C but without ligase added. The predominant ligation product formed in the presence (lanes 4–8) but not in the absence (lane 2) of rHMfB was identified, as indicated, as a 92 bp monomer circle (Fig. 4).

128 bp in length. In the presence of rHMfB, ligation of the 88 (Fig. 3), 98 and 128 bp (data not shown) DNA molecules generated one predominant product that did not co-migrate with any of the linear oligomers generated by ligation of these molecules in the absence of rHMfB. Restriction enzyme digestions and resistance to *exoIII* confirmed that these ligation products were covalently-closed, circular monomers 92, 102 and 132 bp in length, formed by annealing and ligation of the 4 nt, complementary ss ends of the 88 (Figs 4C and 5), 98 and 128 bp linear molecules (data not shown). A small amount of an *exoIII* resistant molecule with an electrophoretic mobility consistent with a circular monomer was also generated when rHMfB complexes formed with the 79 bp DNA molecule were incubated with DNA ligase, but there was no evidence for circular monomer formation with the 53 and 30 bp molecules. Ligation of these shorter molecules in the presence of rHMfB did result in circular molecules, based on their *exoIII* resistance (17,20), but restriction enzyme digestions revealed that these were circular dimers and trimers. Presumably, the 53 and 30 bp molecules were ligated in solution into linear dimers and trimers that were then sufficiently long for assembly into archaeal nucleosomes and ligation into circles.

Topology of the ligated circles

The superhelical topologies of the monomer circles generated by ligation of the 88 and 128 bp linear molecules assembled into rHMfB-containing archaeal nucleosomes were determined, after rHMfB removal, by comparing their electrophoretic mobilities in the presence of EtBr, before and after relaxation by topoisomerase I. As expected for circular DNAs <250 bp, ligation resulted in only one topoisomer (18,21). Relaxation of these molecules resulted in *exoIII* resistant molecules that migrated more slowly than the original ligation products in the presence of a saturating concentration of EtBr (Fig. 5), indicating that the ligation products were positively supercoiled circular DNAs (18–22).

Figure 4. Restriction map of the 88 bp DNA molecule, and restriction enzyme analysis of the circular ligation product. (A) Restriction map of the linear 88 bp DNA molecule with complementary, 4 nt ss 5'-extensions (*Xba*I and *Spe*I half-sites) 32 P-labeled (●) at one terminus. (B) Structure predicted for the 92 bp circular ligation product. (C) The 88 bp DNA molecule, 32 P-labeled at the *Xba*I terminus (●) (lanes 1 and 5) and the 32 P-labeled *Hind*III (22 bp; lanes 2) and *Bsi*WI (63 bp; lane 6) fragments generated from this molecule detected by autoradiography following electrophoresis through an 8% polyacrylamide gel. The major product generated by ligation of the 88 bp 32 P-labeled DNA molecule in the presence of rHMfB (Fig. 3) was subjected to electrophoresis without restriction enzyme digestion (lanes 3 and 7) and following digestion with *Hind*III (lane 4) and with *Bsi*WI (lane 8). *Hind*III and *Bsi*WI digestions of this ligation product generated molecules with the same electrophoretic mobility as the 88 bp linear DNA, consistent with the ligation product being a monomer 92 bp circle.

rHMfB assembly on linear and positively supercoiled DNA

Using EMSA, archaeal nucleosome assembly with the linear 88 bp DNA molecule was compared with assembly using the 92 bp circular version of this molecule. rHMfB assembled into archaeal nucleosomes at lower histone to DNA ratios with the circular DNA than with the linear DNA. Less than 10% of the linear DNA was incorporated into archaeal nucleosomes at the histone to DNA ratio that resulted in 100% incorporation of the circular, supercoiled DNA (Fig. 6).

DISCUSSION

Based on their primary sequences, archaeal histones and eukaryal nucleosome core histones have evolved from a common ancestor (2), and based on the conservation of the histone fold (3,4), they

Figure 5. Superhelical topology and exoIII resistance of 92 and 132 bp circular ligation products. (A) The 88 bp (Fig. 4A) and (B) 128 bp linear molecules subjected to electrophoresis in the presence of EtBr (lanes 1) adjacent to the same molecules ligated in the presence of rHMfB (lanes 2). Lanes 3 contained the ligated molecules incubated for 1 h at 37°C with 60 U calf thymus topoisomerase I. Samples of the molecules in lanes 1–3 were incubated with exoIII (0.5 U) for 30 min at 37°C and subjected to electrophoresis in lanes 4–6, respectively. Topoisomerase I generated relaxed (R) molecules that migrated more slowly in the presence of EtBr than did the ligation products, indicating that ligation resulted in positively supercoiled molecules (S; 18–22). The topoisomerase I substrate and product were resistant to exoIII digestion, consistent with covalently-closed circular DNA molecules (17,20), whereas the linear 88 and 128 bp molecules and molecules generated in the ligation reactions with mobilities consistent with circles with a single-strand nick (N), were digested by exoIII.

seem likely to interact and form similar structures with DNA (6). Archaeal histones are most similar to eukaryal histone H4, the most conserved eukaryal histone (23), and although completion of the nucleosome requires the addition of two (H2A+H2B) dimers, histone (H3+H4)₂ tetramers alone form stable structures with DNA, and recognize and respond to nucleosome positioning signals (24–29). The results reported here, together with earlier crosslinking and MN protection results (10), indicate that archaeal nucleosomes resemble (H3+H4)₂ tetramer-containing structures. Specifically, (H3+H4)₂ tetramers protect ~73 bp from MN digestion, circularize ~80 bp, constrain the DNA in an overwound configuration and assemble readily on positively or negatively supercoiled DNAs (9,28–31). Archaeal nucleosomes similarly contain an archaeal histone tetramer, protect ~60 bp from MN digestion (10), wrap molecules ≥80 bp in a configuration that facilitates circularization (Fig. 3), and assemble on negatively (1) or positively supercoiled DNAs (Fig. 6). As the circular DNAs so generated from 88 and 128 bp linear DNA molecules were positively supercoiled after archaeal histone removal, these linear DNAs were apparently also overwound when held in the archaeal nucleosomes (Fig. 5). Ligation of these molecules into positively supercoiled circles is also consistent with positive toroidal wrapping of the DNA around the archaeal histone core (32,33), although helical twist rather than writhe is expected to be the dominant feature in determining the overall superhelicity of such very small circular DNAs (18,21).

It has been suggested that all four eukaryal histones were needed for nucleosome development (34); however, the ancestral histone, like contemporary archaeal histones, must have formed homodimers and here we have documented that archaeal histone homotetramers do form nucleosome-like structures. With the

Figure 6. EMSA of rHMFb assembly with linear and positively-supercoiled, circular DNA. Aliquots (4 nM) of the ^{32}P -labeled linear 88 bp DNA (upper gel) and of the 92 bp positively supercoiled circular molecule (Fig. 4) were subjected to electrophoresis without (0) and after incubation with 13, 64, 128 and 322 nM rHMFb for 30 min at 25°C. The labeled DNA and complexes formed were detected by autoradiography.

divergence into H3, H4, H2A and H2B, the eukaryal histones became locked into specific heterodimer partnerships and with the evolution of N- and C-terminal extensions gained regulatory roles and the ability to assemble higher-order chromatin (9,34–36). As the same heterodimer partnerships and expanded histone structures and functions appear to exist throughout the Eukarya (23), these features must have evolved before the divergence of the Eukarya, although the N- and C-terminal extensions are still not essential for nucleosome assembly, positioning or maintenance (28,29,37,38). In the nucleosome crystal structure, these extensions appear to stabilize the helical protein ramp formed by the histone fold domains that determines the pathway of negative toroidal DNA wrapping (9). However, $(\text{H3}+\text{H4})_2$ tetramers may also constrain DNA in a positive toroidal supercoil (39,40), and if $(\text{H3}+\text{H4})_2$ tetramers lacking their N- and C-terminal extensions similarly form a structure that can constrain DNA in either a positive or negative supercoil, the structure formed would be essentially an archaeal nucleosome, and a strong candidate for the antecedent of the eukaryal nucleosome (41).

ACKNOWLEDGEMENTS

We thank K. Sandman for the gift of rHMFb-YY, and gratefully acknowledge the continued critical interest and intellectual support of our colleagues. Research was supported at Ohio State University by NIH grant GM53185, and studies undertaken by C.S.C. at Massachusetts Institute of Technology were supported by grant CA34992 awarded to S. J. Lippard.

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