Histone stoichiometry and DNA circularization in archaeal nucleosomes

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ABSTRACT

Recombinant (r)HMIB (archaeal histone B from \textit{Methanothrix 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 \textsuperscript{125}I-labeled rHMIB-YY (an rHMIB variant with I31Y and M35Y replacements) and \textsuperscript{32}P-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 rHMIB 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. rHMIB 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)\textsubscript{2} tetramers.

INTRODUCTION

Members of the euryarchaeotal branch of the Archaea contain archaeal histones, proteins with primary homodimers, the histone fold and dimer formation \textsuperscript{(1–4)} in common with the globular regions of the eukaryal nucleosome core histones H2A, H2B, H3 and H4 \textsuperscript{(5,6)}. However, archaeal histones form both homodimers and heterodimers \textsuperscript{(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 \textsuperscript{(1,8)}, but whereas eukaryal nucleosomes contain 146 bp of DNA wrapped in 1.65 circles around a histone octamer \textsuperscript{(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 \textsuperscript{(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 \textsuperscript{(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)HMIB (histone B from \textit{Methanothrix fervidus}) and rHMIB-YY, a variant of rHMIB 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 rHMIB, were synthesized in \textit{Escherichia coli} JM105 and purified as previously described \textsuperscript{(7,12)}. The tyrosine residues in rHMIB-YY facilitated \textsuperscript{125}I-labeling, and \textsuperscript{125}I-rHMIB-YY was generated by using IODO-BEADS with Na\textsuperscript{125}I as directed by the manufacturer (Pierce, Rockford, IL). rHMIB, rHMIB-YY and \textsuperscript{125}I-rHMIB-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 \textsuperscript{125}I-rHMIB-YY was excised and solubilized by oxidation as described by Carey \textsuperscript{(13)}. The specific activity of the \textsuperscript{125}I-rHMIB-YY preparation was then calculated based on liquid scintillation measurements of the \textsuperscript{125}I content of the oxidized material.

DNA molecules

\textsuperscript{32}P-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\textsubscript{I} and Stu\textsubscript{I}, Bgl\textsubscript{II} and EcoRV, SpeI and EcoRV, BamHI and StuI, and BamHI and SnaBI, respectively, and filling in the single-stranded (ss) XbaI, BglII, SpeI and BamHI half-sites using the Klenow fragment of DNA polymerase I with a dNTP mixture that contained [\textsuperscript{32}P]dATP \textsuperscript{(14)}. A 100 bp DNA molecule was obtained by PCR amplification of pRG101 DNA cloned into the EcoRV

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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 32P-end-labeled using T4 polynucleotide kinase with [γ-32P]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 BamHI plus BglII, and from pLITMUS28 and pLITMUS28 containing 30 bp of pRG101 cloned into the EcoRV site [clone #26; (11)] by digestion with XhoI plus SpeI, respectively. Digestion of pLITMUS28 with EcoRV plus BstWI, and with EcoRV and EcoRI, followed by filling-in of BstWI and EcoRI half-sites, respectively, ligation and re-transformation into E.coli DH5α resulted in plasmids with 23 (pLITMUS28AΔ23) and 10 bp (pLITMUS28AΔ10) deletions within the polylinker region. BamHI plus BglII digestion of pLITMUS28Δ23 DNA and SpeI plus XhoI digestion of pLITMUS28Δ10 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, pLITMUS28Δ23, pLITMUS28Δ10 and clone #26 DNA by digestion with XhoI or BglII. 32P-end-labeling using T4 polynucleotide kinase, followed by digestion with SpeI or BamHI. A 79 bp molecule 32P-labeled at one end was obtained from the pLITMUS28 polylinker by EcoRV digestion, 32P-end-labeling and then digestion with StuI.

DNA concentrations and specific activities were determined by A260 measurements and liquid scintillation counting. All 32P-labeled DNA molecules were gel purified before use.

EMSA

32P-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%; 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).

125I-rHMfB-YY + 32P-DNA complex stoichiometry

Aliquots (76 nM) of the 32P-labeled 100 bp DNA were mixed and incubated with 0.64 or 1.3 µM 125I-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% HClO4, 21% H2O2 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 125I-32P channel spill-over and isotope decay. Background levels of unbound 125I-rHMfB-YY and 32P-DNA in the region of the gel containing complexes were determined by measuring the 125I and 32P contents of equal-sized gel fragments excised from adjacent tracks that were loaded with only 125I-rHMfB-YY or 32P-DNA.

RESULTS

DNA length-dependent EMSA

Each histone dimer in the eukaryal nucleosome interacts through histone fold domains with 27–28 bp in length. Therefore, to avoid

Figure 1. EMSA of complex formation by rHMfB with DNA molecules of increasing length. Lanes 1–5 contained 10 nM of 32P-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. 32P-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 32P-labeled DNA molecule (8 nM) in ligase buffer [10 mM MgCl2, 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.

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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 archaenal nucleosomes

As archaenal nucleosomes are known to protect ~60 bp from MN digestion (10), it seemed possible that two archaenal nucleosomes might assemble on DNA molecules ≥120 bp. Therefore, to avoid
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 32P-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 125I-labeled rHMfB-YY and complexes formed separated from unbound DNA, as indicated, in lanes 1 and 2, respectively. An aliquot of the 32P-labeled 100 bp DNA, and 0.64 and 1.3 µM of 125I-labeled rHMfB-YY, were in lanes 0, 3 and 4, respectively. The locations of the 125I-rHMfB-YY + 32P-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 125I and 32P contents determined.

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 nM) for 30 min at 25 °C with 76 nM aliquots of the 32P-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 32P-labeled 100 bp DNA was incubated, as in (A), with 0.64 and 1.3 µM of 125I-labeled rHMfB-YY and complexes formed separated from unbound DNA, as indicated, in lanes 1 and 2, respectively. An aliquot of the 32P-labeled 100 bp DNA, and 0.64 and 1.3 µM of 125I-labeled rHMfB-YY, were in lanes 0, 3 and 4, respectively. The locations of the 125I-rHMfB-YY + 32P-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 125I and 32P contents determined.

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 32P-labeled DNA molecules 30, 53, 79, 88, 98 and 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.

Figure 3. Ligation of the 88 bp DNA molecule assembled into rHMfB-containing archaeal nucleosomes. The 88 bp 32P-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 µM 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 µM 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).

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).
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 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, nucleosome requires the addition of two (H2A+H2B) dimers, archaeal nucleosomes similarly contain an archaeal histone (H3+H4) 2 tetramer-containing structure. Specifically, (H3+H4) 2 tetramers protect nearly 73 bp from MN digestion, circularize ~80 bp, and 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 ≥280 bp in a configuration that facilitates circularization (Fig. 3), and assemble on negatively 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 DNA molecules were apparently also over-wound 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 superhyelicity 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 32P-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, (H3+H4)2 tetramers may also constrain DNA in a positive toroidal supercoil (39,40), and if (H3+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 archaean nucleosome, and a strong candidate for the antecedent of the eukaryal nucleosome (41).

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