Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10397–10401, December 1991
Biochemistry

DNA binding by the archaeal histone HMf results in positive supercoiling

(thermophile/Archaea/nucleosome/*Methanothermus*)

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Communicated by Carl R. Woese, August 26, 1991 (received for review May 2, 1991)

ABSTRACT **HMf, a histone from the hyperthermophilic archaeon *Methanothermus fervidus* binds double-stranded DNA molecules *in vitro*, forming compact structures that visibly resemble eukaryal nucleosomes. We show here that HMf binding increases the helical periodicity of DNA molecules to ≈11 base pairs (bp) per turn and that DNA molecules in these nucleosome-like structures are constrained in positive toroidal supercoils. Based on the mass of HMf needed to cause a change in linking number (ΔLk), the maximum ΔLk introduced into circular DNA molecules of known sizes, and electron microscopy, we estimate that each HMf–DNA structure contains between 90 and 150 bp of DNA wrapped in 1.5 positive toroidal supercoils around a core of four HMf molecules. A model and pathway for the formation of these structures *in vitro* are presented and the possible role of positive toroidal wrapping of the *M. fervidus* genome *in vivo* is discussed.**

Differences in the gross anatomy of the nuclear material, revealed by light microscopy, established the division of the biological world into eukaryotes and prokaryotes. Subsequent studies of the macromolecular organization of the nuclear material have reinforced this basic division. In eukaryal chromatin, the DNA molecule is wrapped around a protein core of histones producing nucleosomes (1, 2). This left-handed wrapping introduces negative toroidal supercoils. A compensatory accumulation of positive plectonemic supercoils in histone-free regions of the same DNA molecule is prevented by topoisomerase activity. DNA in eukaryal chromatin is therefore, overall, negatively supercoiled but not torsionally stressed. In contrast, DNA molecules in prokaryotes are under torsional stress and the very existence of nucleosome-like structures in prokaryotes is still a matter of conjecture (3–6). There are histone-like proteins and several descriptions of nucleosome-like particles but very little evidence for a defined DNA–protein structure found consistently in prokaryotic cells equivalent to the universal, eukaryal nucleosome. The extent of torsional stress in DNA molecules in prokaryotes is assumed to be the net result of DNA replication, transcription, toroidal wrapping, and the activities of topoisomerase and gyrase (3, 4, 7, 8).

Torsional stress plays a direct role in regulating gene expression in Bacteria (9) but this has yet to be addressed in the Archaea, a fundamentally different group of prokaryotes (10). There are both similarities and differences in DNA binding proteins from the Archaea when they are compared with the classical histone-like DNA binding proteins HU, H-NS, HLP1, and H from Bacteria (3, 4, 9, 11, 12). The archaeal histone-like protein, HTa, from *Thermoplasma acidophilum* is clearly related to the HU family of bacterial proteins (3, 6, 12, 13). These proteins bind double-stranded DNA molecules *in vitro* to form structures superficially similar to nucleosomes in which the DNA molecule is wrapped in a negative toroidal supercoil around a protein core (3, 4, 11, 12). In contrast, several archaeal DNA binding proteins have no detectable evolutionary relationship to known bacterial or eukaryal proteins and although some of these do form nucleosome-like structures, this is not always the case (12). Recently, we described a DNA binding protein, designated HMf, from the hyperthermophilic archaeon *Methanothermus fervidus*, which, on the basis of its primary sequence, is most closely related to histones and which binds to double-stranded DNA molecules to form structures very reminiscent of nucleosomes (14). We demonstrate here, however, that unlike the negative toroidal wrapping of DNA in nucleosomes and in HU–DNA complexes (1–4, 11, 12), the DNA molecules in HMf–DNA complexes are held in positive toroidal supercoils.

MATERIALS AND METHODS

Preparation of HMf. Cultures of *M. fervidus* were grown anaerobically at 83°C (15) and HMf was isolated from the resulting cell mass as described (14, 16).

Preparation of DNA Substrates. Negatively supercoiled plasmid DNAs (form I) were isolated from *Escherichia coli* DH5α by alkaline lysis and CsCl/ethidium bromide equilibrium density-gradient centrifugation (17). Relaxed open circular DNAs (form II) were prepared by incubating form I DNA with wheat germ topoisomerase I (Promega) followed by deproteinization with proteinase K (type XI; Sigma) and phenol/chloroform extraction. *Eco*RI-linearized molecules of pUC19 DNA were end-labeled by using Sequenase (United States Biochemical) and [α-^{32}P]dATP. *Msp* I fragments of pBR322 DNA were dephosphorylated by treatment with calf thymus alkaline phosphatase (Boehringer Mannheim) and then end-labeled by using T4 DNA kinase and [γ-^{32}P]ATP (17).

Gel Electrophoresis. The numbers of plasmid topoisomers were determined by one-dimensional electrophoresis through 1.5% (wt/vol) agarose gels run at 1.5 V/cm in 90 mM Tris borate/2.5 mM EDTA, pH 8.3, for 16 h at room temperature. For two-dimensional agarose gel electrophoresis, the gels were incubated in 15 ng of ethidium bromide per ml for 1.5 h after electrophoresis in the first dimension and before electrophoresis in the second dimension at 3 V/cm for 5 h (18).

Fragments of pUC19 DNA generated by hydroxyl radical cleavage were separated by electrophoresis at 30 V/cm for 6 h through 8% (wt/vol) polyacrylamide gels containing 7 M urea.

Changes in the electrophoretic mobilities of *Msp* I fragments of pBR322 DNA when complexed with HMf were determined by electrophoresis through 6% nondenaturing polyacrylamide gels run at 2 V/cm for 30 h.

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Analysis of Topoisomers. Form II DNA molecules (0.5 μg) were incubated for 10 min at 37°C with HMf in a 20-μl reaction mixture containing 10 mM Tris·HCl (pH 8.0), 2 mM K_3PO_4, 1 mM EDTA, and 50 mM NaCl (TPEN). Wheat germ topoisomerase I (3 units) was added, and the reaction mixture was adjusted to 50 mM Tris·HCl (pH 8.0), 2 mM K_3PO_4, 2.5 mM EDTA, 50 mM NaCl, and 20% (vol/vol) glycerol and incubated at 37°C for 30 min. The reaction was stopped by mixing with 1/5th vol of agarose gel loading buffer (17) that contained 5% (wt/vol) SDS.

Hydroxyl Radical Footprinting. *Eco*RI-digested pUC19 DNA molecules (0.2 pmol) were ^{32}P-end-labeled and mixed with HMf in TPEN buffer. The hydroxyl radical cleavage reactions and electrophoretic analyses of the products were performed as described (2, 19, 20). Autoradiograms were quantitated by scanning densitometry using a Technology Resources Microscan 1000 gel analysis system (Nashville, TN).

RESULTS

Binding of HMf to Relaxed Circular DNA Molecules Results in Positive Supercoiling. Increasing amounts of HMf were bound to relaxed covalently closed, circular molecules of pUC19 DNA. Wheat germ topoisomerase I was then added to remove any plectonemic supercoils that formed in protein-free regions of the DNA and, following deproteinization, the resulting DNA molecules were subjected to one-dimensional and two-dimensional agarose gel electrophoresis. Conditions were used that resolved individual topoisomers and also separated negatively from positively supercoiled topoisomers (Fig. 1). At protein/DNA ratios <0.3:1 (all ratios are expressed as mass/mass relationships) HMf binding resulted in negatively supercoiled topoisomers. The DNA molecules in these HMf–DNA complexes must therefore have been underwound or constrained in negative toroidal supercoils. Previous electron microscopic studies revealed that severe kinks are introduced into DNA molecules by HMf binding at these low HMf/DNA ratios (14). At higher HMf/DNA ratios, the number and amounts of assayable negatively supercoiled topoisomers decreased, and at ratios >0.5:1 they were totally replaced by a larger number of positively supercoiled topoisomers (Fig. 1). The total number of assayable positive topoisomers decreased when the HMf/DNA ratio exceeded 0.8:1. This decrease probably reflected a shortcoming of the assay rather than an actual decrease in DNA wrapping. Topoisomerase I has difficulty accessing and relaxing plectonemic supercoils within very highly compacted DNA–protein complexes (11) and such compact structures do form at these high HMf/DNA ratios (14). As positively supercoiled DNA molecules resulted from HMf binding at ratios >0.5:1 (Fig. 1), the DNA molecules in these complexes must have been highly overwound or constrained in positive toroidal supercoils. Previous electron microscopic studies clearly demonstrated, at these higher HMf/DNA ratios, DNA molecules in tight circular configurations (14), and therefore we now conclude that these must result from positive toroidal wrapping of DNA around HMf.

Stoichiometry of HMf–DNA Complexes. The maximum change in linking number (ΔLk) introduced as positive supercoils into form II molecules of pUC19 DNA [2686 base pairs (bp)], with increasing amounts of HMf, was 17 (Fig. 2). This is equivalent to one titratable positive supercoil introduced per ≈150 bp of DNA. The slope of the titration curve indicates that four additional HMf molecules were needed for each unit increase in ΔLk from 10 to 17 (Fig. 2). This calculation assumes that each HMf molecule contains two very similar polypeptide subunits (HMf-1 and HMf-2), both of which have molecular masses of ≈7700 Da (14). Experiments using the same protocol but with different plasmid DNAs, of different sizes, gave very similar values for the numbers of base pairs of DNA and molecules of HMf required per unit ΔLk. The value of ≈150 bp per ΔLk is, however, likely to be an overestimate of the number of base pairs per complex as other architectural constraints, in ad-

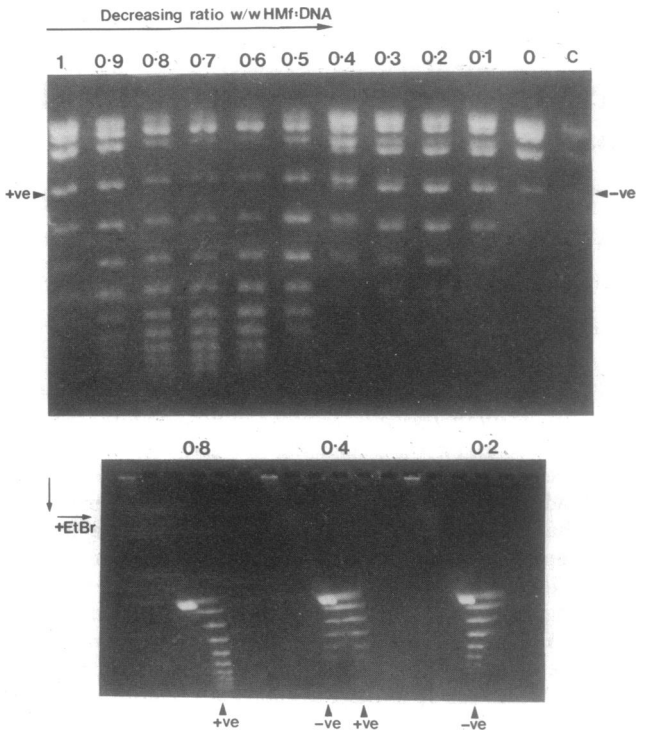

FIG. 1. One- and two-dimensional agarose gel electrophoretic separations of pUC19 topoisomers. Relaxed circular pUC19 DNA molecules (lane C) were mixed with HMf at the HMf/DNA ratios indicated above each lane and, after topoisomerase I treatment, the DNA molecules were deproteinized and subjected to agarose gel electrophoresis. The identification of bands as positive (+ve) and negative (−ve) topoisomers, after one-dimensional gel electrophoresis (*Upper*) was confirmed by two-dimensional gel electrophoresis (*Lower*). The presence of ethidium bromide (EtBr) during electrophoresis in the second dimension increased the mobility of positive topoisomers relative to negative topoisomers with the same superhelical density (18).

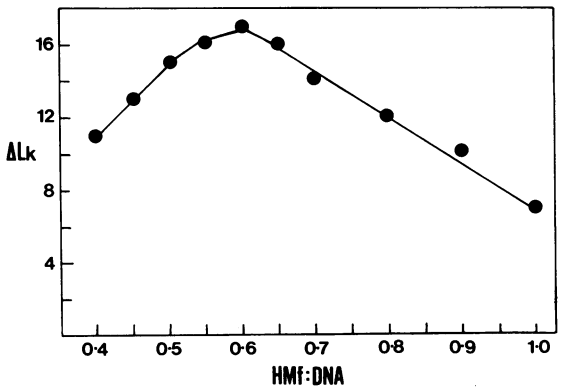

FIG. 2. Change in linking number (ΔLk) as a result of HMf binding to relaxed circular molecules of pUC19 DNA. The maximum number of positive topoisomers of pUC19 DNA detected after agarose gel electrophoresis (see Fig. 1) was plotted for each HMf/DNA ratio (wt/wt) investigated. The absolute maximum of 17 topoisomers formed by HMf binding indicates that 2686 ÷ 17—i.e., 158 bp—is needed per ΔL. The increased amount of HMf required to increase the maximum number of positive topoisomers from 10 to 17 indicates that four molecules of HMf were needed per unit increase in ΔL.

dition to the overall length of the available DNA, presumably must also limit the number of complexes formed. Only restriction fragments >90 bp long exhibited reduced mobilities through nondenaturing polyacrylamide gels, in the presence of HMf, using very demanding conditions of electrophoresis (Fig. 3) and this may be the minimal length of DNA needed to encircle an HMf core to produce a stable complex. Based on these arguments and the scrutiny of many electron micrographs, we conclude that the HMf–DNA complexes formed *in vitro* contain between 90 and 150 bp of DNA wrapped ≈1.5 times around a core of four HMf molecules.

Helical Periodicity of DNA Bound to HMf. The helical periodicity of DNA bound to proteins can be determined by hydroxyl radical cleavage (2, 19, 20). Unrestrained random DNA sequences have an average of 10.6 bp per helical turn (21), whereas DNA molecules traversing the surface of nucleosomes are overwound, with an average periodicity of 10.2 bp per helical turn (2). Fig. 4 demonstrates that DNA bound to HMf is underwound with a helical periodicity of ≈11 bp per turn. This increased number of base pairs per helical turn was found at both low and high HMf/DNA ratios. The efficacy of hydroxyl radical cleavage of DNA is dependent on the width of the minor groove, and short nucleotide sequences that bend DNA molecules (22), causing local distortions of the minor groove, can therefore be detected as regions of increased or decreased cleavage (2, 19). This can be seen in the cleavage pattern of the protein-free control DNA in Fig. 4. As previously demonstrated for histone binding to DNA molecules in nucleosomes (2), HMf binding exaggerates the already enhanced levels of cleavage that occur at some of these sites. The exaggerated cleavage of DNA molecules on the surface of nucleosomes, at sites inherently more sensitive to the hydroxyl radical, has been taken as evidence that sequence-dependent bending is maintained in nucleosomes. It has, in fact, been argued that bending actually determines the location at which nucleosomes assemble (2, 22, 23). Our results indicate that the same arguments could apply to the localization of HMf–DNA complexes.

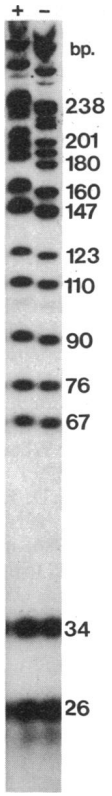

FIG. 3. Electrophoretic mobility of restriction fragments in the presence (lane +) or absence (lane −) of HMf. ^{32}P-end-labeled *Msp* I-generated restriction fragments of pBR322 DNA were mixed with HMf at a protein/DNA ratio of 1:1 and, after their separation by electrophoresis for 30 h through a 6% polyacrylamide gel run at 2 V/cm, were detected by autoradiography.

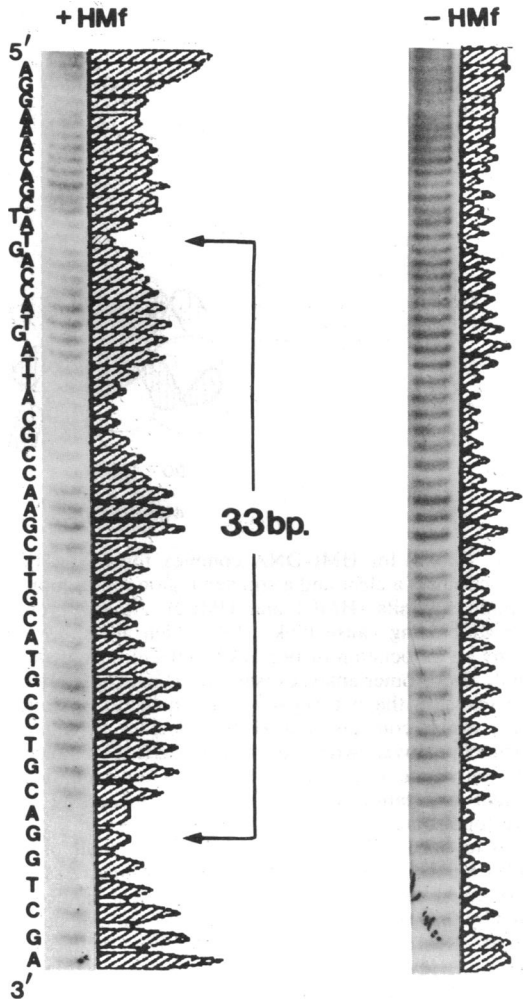

FIG. 4. Hydroxyl radical footprints of pUC19 DNA. Plasmid DNA was linearized by *Eco*RI digestion, ^{32}P-end-labeled, and bound to HMf (+ HMf). Autoradiograms obtained after hydroxyl radical cleavage, electrophoresis, and autoradiography were quantitated by scanning densitometry and the resulting intensity profiles are shown adjacent to the corresponding regions of the autoradiogram. The results presented are from an experiment in which the HMf/DNA ratio was 1:1. An ≈11-bp helical periodicity was, however, observed in the hydroxyl radical cleavage patterns at all HMf/DNA ratios investigated, from 0.1:1 to 1:1. The sequence of the region of pUC19 used in the experiment is shown aligned with the corresponding bands in the autoradiograms. Decreased hydroxyl radical cleavage of protein-free DNA molecules has been reported to occur at A+T-rich sequences and [pyrimidine (5′–3′) purine] sequences (2, 19, 20) and some variability is detectable in the cleavage patterns obtained with the HMf-free pUC19 control DNA (− HMf).

DISCUSSION

Model for the HMf–DNA Complexes Formed *in Vitro* (Fig. 5). HMf binding at low protein/DNA ratios causes kinks in the DNA (14) but, assuming that these kinks occur with equal frequency in both the positive and negative directions, they will have little or no net effect on superhelicity (Fig. 5). The negative topoisomers that do result from HMf binding at

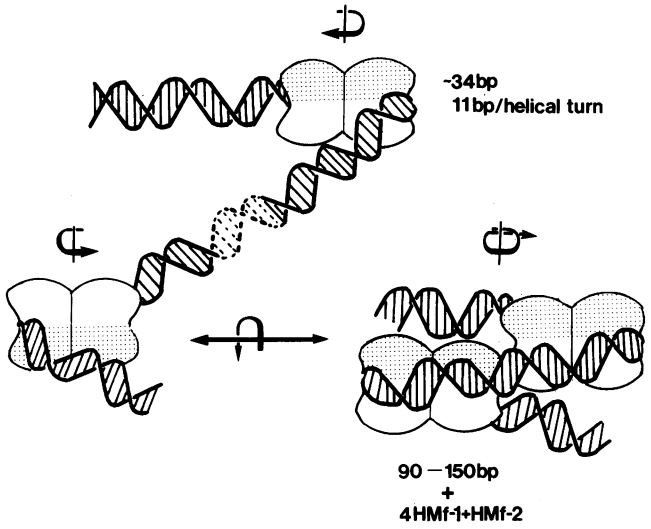

FIG. 5. Model for HMf–DNA complex formation. Each HMf molecule contains a clear and a stippled region representing the two polypeptide subunits (HMf-1 and HMf-2). At low protein/DNA ratios HMf binding cause kinks (14), which are shown as being formed by an association of two HMf molecules (four polypeptide subunits). Topoisomer analyses (see Fig. 1) indicate only one intermediate (causing the net negative superhelicity) en route to the complex, which contains four HMf molecules (8 subunits), and 90–150 bp of positively wrapped DNA. If single HMf molecules bind initially, only one intermediate has been detected during their subsequent association into tetramers. The increasing number of negative topoisomers detected after HMf binding at protein/DNA ratios up to 0.2:1 (Fig. 1) is explained as a result of the DNA molecules kinked around HMf being underwound. Assuming that there are equal numbers of left-handed and right-handed kinks, as indicated by the arrows above the complexes, kinking per se should have no net effect on superhelicity. At HMf/DNA ratios >0.2:1 HMf molecules already bound to DNA interact and by rotation of the DNA molecule direct the formation of complexes that contain only positive toroidal supercoils.

ratios <0.2–1 are explained as a consequence of the DNA molecules in kinks being underwound (Fig. 4). Based on the number of HMf molecules needed at low HMf/DNA ratios to cause a ΔLk of 1 and an 11-bp helical periodicity, we can calculate that each kink must contain ≈34 bp. As the number of HMf molecules per DNA molecule is increased, protein–protein interactions must occur. These direct the formation of complexes that we calculate contain between 90 and 150 bp of DNA wrapped in ≈1.5 positive toroidal supercoils around a core of four HMf molecules [Figs. 2 and 3 (14)]. HMf binding to DNA and the HMf–HMf protein interactions appear to be sequential and reversible events. Adding protein-free DNA to HMf–DNA complexes containing only positively supercoiled DNA results in a rapid redistribution of HMf protein (16) and the reappearance of titratable negative superhelicity.

Role of HMf *in Vivo*. The mass ratio of HMf/DNA in growing *M. fervidus* cells is ≈0.3:1 (refs. 14 and 16; D. Stroup, personal communication), a ratio that *in vitro* results in both positive and negative supercoiling (Fig. 1). This is, however, an average value and, assuming that HMf does bind to the genomic DNA *in vivo* (5, 6), it seems likely that there are HMf-free regions and genomic regions constrained in positive toroidal supercoils. HMf binding is rapidly reversible (16), which should facilitate HMf migration away from DNA replication forks and sites of active transcription to sites of inactivity. Intergenic regions in methanogens are unusually A+T rich and frequently contain several oligo(A) sequences (24), which presumably cause localized bending of the DNA (22) and have been implicated in promoter activity (25). Such localized DNA bending could also direct or deter HMf binding, providing a mechanism by which HMf could play a direct role in regulating gene expression.

The cytoplasm of *M. fervidus* contains ≈950 mM K^+ and 300 mM 2′,3′-cyclic diphosphoglycerate (26). This unusual salt protects *M. fervidus* enzymes from heat denaturation *in vitro* (26) and its presence, at such a high concentration *in vivo*, must also counteract the tendency of the *M. fervidus* genome to unwind and denature at growth temperatures above 80°C. HMf may, in fact, be needed to ensure that at this high intracellular salt concentration the DNA strands of the *M. fervidus* genome do not become inseparable. Having regions of the *M. fervidus* genome wrapped around HMf in positive toroidal supercoils will generate negative superhelicity in the remainder of the genome, which should counteract the stabilizing effect of a very high intracellular salt concentration. *M. fervidus* cells also contain reverse gyrase (18, 27), an enzyme that can introduce positive supercoils into circular DNA molecules and that presumably also participates in balancing the intracellular superhelicity of the *M. fervidus* genome. Reverse gyrase and HMf binding should have opposite effects on the superhelicity of protein-free regions of the *M. fervidus* genome. We expected therefore that these opposing activities would always both be present or both be absent, but this does not appear to be the case. We have purified and characterized a DNA binding protein (HMt) from *Methanobacterium thermoautotrophicum* ΔH (unpublished results) that has virtually identical properties to HMf, but this thermophilic methanogen does not contain reverse gyrase (27).

We thank C. Daniels, M. Frank-Kamenetskii, M. Muller, and our colleagues in the Reeve Laboratory for their constructive comments and input into the development of the model presented. This work was supported by Contract NOOO14-86-K-0211 from the Office of Naval Research.

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