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Growth-phase-dependent synthesis of histones in the archaeon *Methanothermus fervidus*

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ABSTRACT **Histone preparations from *Methanothermus fervidus* (HMf) contain two small polypeptides, HMfA and HMfB, which in solution are dimers and compact DNA to form nucleosome-like structures. These archaeal nucleosome-like structures constrain positive DNA supercoils, in contrast to the negatively supercoiled DNA in eukaryal nucleosomes. HMfA has been found to make up as much as 80% of HMf preparations synthesized by *M. fervidus* cells during the exponential growth phase of batch cultures but to decrease to ≈50% as cultures enter the stationary phase. By using a nondenaturing polyacrylamide gel system at pH 6.1, we have demonstrated that HMf preparations contain HMfA homodimers, HMfB homodimers, and HMfA–HMfB heterodimers and that heating a mixture of recombinant HMfA and HMfB homodimers at 95°C for 5 min generates HMfA–HMfB heterodimers. Circular dichroism spectroscopy indicates that HMfA and HMfB have very similar secondary structures, but based on agarose gel electrophoretic mobility shifts, DNA topology assays, and electron microscopy, they have different DNA binding properties. HMfA binding to DNA could be detected at lower protein/DNA ratios than HMfB, but HMfB binding resulted in more extensive DNA compaction. The increased HMfB synthesized in cells approaching the stationary phase and the highly compacted state of HMfB-bound DNA are consistent with preparations for the impending period of limited genome activity.**

Methanothermus fervidus is a hyperthermophilic archaeon that grows optimally at 83°C but has genomic DNA that contains only 33 mol % G + C (1). Studies undertaken to determine whether DNA binding proteins help protect the *M. fervidus* genome from heat denaturation led to the identification of HMf (histone from *M. fervidus*), preparations of which contain two small polypeptides, designated HMfA and HMfB (molecular masses of 7468 and 7667 Da, respectively). These polypeptides have amino acid sequences that are 84% identical and are clearly related to the eukaryal nucleosome core histones, at the primary sequence level (2, 3) and in terms of their secondary structures (4). Consistent with this common evolutionary ancestry, HMf binding compacts DNA into nucleosome-like structures (NLSs); however, unlike the eukaryal paradigm, DNA molecules in these archaeal NLSs are constrained in positive rather than negative supercoils. Proteins with amino acid sequences and DNA binding properties very similar to HMf have now also been discovered in mesophilic methanogens and in nonmethanogenic Archaea (4, 5), indicating that HMf is not a hyperthermophily related novelty but, rather, the prototype of a widely distributed family of archaeal histones and, therefore, warrants detailed analysis. Early preparations of HMf contained HMfA and HMfB in approximately equal proportions (2), but recently we discovered that this ratio can vary substantially, indicating that HMf preparations could not be a homogeneous population of HMfA–HMfB heterodimers and could contain homodimers of HMfA and HMfB with different biologically relevant properties. Here we document that the composition of HMf preparations is growth-phase-dependent and that homodimers of HMfA and HMfB do have different DNA binding properties consistent with forming NLSs in active and inactive regions of the genome.

MATERIALS AND METHODS

Growth of Microorganisms. *M. fervidus* cultures were grown anaerobically at 83°C in a medium that contained (per liter) 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.3 g of $(NH_4)_2SO_4$, 0.6 g of NaCl, 5 g of $NaHCO_3$, 65 mg of $MgSO_4$, 50 mg of $CaCl_2$, 2.5 g of sodium acetate, 3.3 mg of $Na_2WO_4 \cdot H_2O$, 1 mg of resazurin, 2 g of yeast extract, 2 g of tryptone, and 10 ml of a trace metal solution (6). After sterilization, 0.5 g of $Na_2S \cdot 9H_2O$ and 0.5 g of cysteine hydrochloride were added and the pH was adjusted to 6.5. Cultures (20 liters) were grown in a MicroFerm model CMF-128S fermentor (New Brunswick Scientific) inoculated with 5% (vol/vol) of a *M. fervidus* culture grown to an OD_{580} of ≈1.0. The fermentor vessel was pressurized to 0.2 MPa (15 psi) with 4:1 (vol/vol) H_2/CO_2 and stirred slowly (impeller setting, 50 rpm) for 4 h. After the onset of exponential growth, the culture was sparged with 4:1 H_2/CO_2 at a flow rate of 1 liter/min and stirred at an impeller setting of 250 rpm. Cultures of *Escherichia coli* JM105, containing recombinant plasmids, were grown aerobically at 37°C in Luria–Bertani medium containing 20 μg of ampicillin per ml (7). The expression of the *hmfA* and *hmfB* genes, cloned downstream from the *tac* promoter in pKK223-3 (8), was induced by adding isopropyl β-D-thiogalactoside to 400 μM to cultures growing at an OD_{600} of ≈0.4. Synthesis of the encoded recombinant (r) HMfA or rHMfB was continued for ≈16 h by incubation of the cultures overnight at 37°C.

Plasmid Constructions. The *hmfA* gene, cloned previously (9), was PCR-amplified by using primers 5′-TGGGATCCTGGAGGTGATAACGTGGGTG and 5′-CCCAAGCTTATTTGAACATTTTTCGAGC, which flank the coding sequence and contain added *Bam*HI and *Hin*dIII sites, respectively. After *Bam*HI and *Hin*dIII digestion, the amplified DNA fragment was ligated with *Bam*HI/*Hin*dIII-digested pUC18 (10), sequenced to confirm that no changes had been introduced during the amplification, and then subcloned as an *Eco*RI–*Hin*dIII fragment into the expression vector pKK223-3 (8). The *hmfB* gene, cloned previously (3), was subcloned as a 245-bp *Ssp* I fragment into *Sma* I-digested pUC19. The orientation of the subcloned fragment was determined by DNA sequencing and the *hmfB* gene was then transferred into pKK223-3 as an *Eco*RI–*Hin*dIII fragment.

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Abbreviations: HMf, histone preparation from *Methanothermus fervidus*; NLS, nucleosome-like structure; r, recombinant; EM, electron microscopy.

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Protein Purification. *M. fervidus* or *E. coli* cells, resuspended in a high salt buffer, were ruptured by passage through a French pressure cell at 138 MPa (20,000 psi), and cleared supernatants were prepared from the lysates as described (3). After dialysis of the supernatant overnight at 4°C against a low salt buffer (LS buffer = 100 mM NaCl/50 mM Tris·HCl, pH 8), $MgCl_2$ and phenylmethylsulfonyl fluoride (5 mM and 100 μM, respectively) and DNase I (20 μg/ml) were added, and the mixture was incubated at 37°C for 6–12 h. NaCl (3 M) was added and the mixture was heated at 95°C for 10 min. Proteins that denatured were removed by centrifugation and filtration through a 0.45-μm (pore size) Millipore membrane. The resulting solution was dialyzed against LS buffer. To obtain preparations of the native HMf from *M. fervidus* lysates, proteins in this solution were adsorbed to the matrix of a double-stranded DNA-cellulose column, washed with LS buffer, and eluted with 1 M NaCl/50 mM Tris·HCl, pH 8. To obtain preparations of rHMfA or rHMfB from *E. coli* lysates, proteins were adsorbed to the matrix of a Hi-Trap heparin-Sepharose column (Pharmacia) equilibrated with LS buffer, washed with LS buffer, and then eluted with a 0.1–1.5 M linear NaCl gradient in 50 mM Tris·HCl (pH 8). Fractions from the columns that contained HMf, rHMfA, or rHMfB were identified by SDS/PAGE.

HPLC. Protein preparations were diluted in 0.1% trifluoroacetic acid and adsorbed to a Microsorb-MV C_8 reverse-phase column (100 Å, pore size; 25 cm, length; Rainin Instruments) in a mobile phase that contained 5% solvent B (70% CH_3CN/0.085% trifluoroacetic acid) in solvent A (0.1% trifluoroacetic acid). HPLC, with a 55–75% gradient of solvent B in solvent A, resolved HMfA from HMfB and the peak assignments were verified by electrophoresis of fractions through acetic acid/urea polyacrylamide gels that contained 0.8% Triton X-100 (11); rHMfA and rHMfB were standards.

Circular Dichroism (CD) Spectroscopy. CD spectra of 50 μM rHMfA and 50 μM rHMfB in 200 mM NaCl/1 mM Tris·HCl, pH 7.5, were recorded on a Dichrograph CD6 spectropolarimeter (Instruments S.A., Jobin-Yvon, France) by using a 0.1-cm path-length cell. The precise concentrations of the protein solutions were established by quantitative amino acid analysis of the purified protein preparations. Spectra were signal-averaged 16 times and smoothed by using a nine-point sliding quadratic-cubic filter (12).

Nondenaturing Gel Electrophoresis. Samples of HMf, rHMfA, or rHMfB (2–4 μg) dissolved in 30 mM histidine/30 mM Mes, pH 6.1 (H-MES), that contained 200 mM NaCl were mixed with an equal vol of 2× H-MES that contained 20% (vol/vol) glycerol and 0.01% pyronin Y and electrophoresed (12 V/cm) through 16% T/3% C polyacrylamide gels (where T is the total gel concentration and C is the amount of crosslinker) that contained H-MES buffer and 10% glycerol. These proteins are positively charged at pH 6.1 and, therefore, migrated toward the negative electrode. They were visualized after electrophoresis by staining with Coomassie brilliant blue R-250 (Sigma).

Heterodimer Reconstitution. Aliquots (2 μg) of rHMfA and rHMfB, dissolved in H-MES containing 200 mM NaCl, were mixed, incubated at 95°C for 5 min, and then cooled slowly (≈30 min) to room temperature. The compositions of the resulting mixtures of rHMfA and rHMfB homodimers and rHMfA–rHMfB heterodimers were determined by nondenaturing gel electrophoresis by using the H-MES buffer system.

Electrophoretic Mobility-Shift Assays and DNA Topology Assays. Purified preparations of rHMfA and rHMfB were mixed with *Eco*RI-linearized pBR322. After a 15-min incubation at room temperature, 1/6 vol of 40% sucrose/0.25% bromophenol blue was added, and the DNA–protein complexes formed were electrophoresed (0.7 V/cm) through 0.8% agarose gels (Agarose I, Amresco, Euclid, OH) in 40 mM Tris acetate/2 mM EDTA (running buffer) (13). After staining with ethidium bromide, the complexes were photographed and the distances they had migrated through the gel were determined by measuring photographic enlargements.

Covalently closed relaxed pUC18 molecules were generated by incubation of supercoiled preparations of the plasmid DNA with human topoisomerase I (gift from M. Muller, The Ohio State University), and these pUC18 molecules were then used to determine the effects of rHMfA and rHMfB binding on DNA topologies as described (9).

Electron Microscopy (EM). The EM procedures used to visualize HMf–DNA complexes, after shadow casting and glutaraldehyde fixation (3), were used to visualize the complexes formed by rHMfA and rHMfB binding to relaxed circular pUC18 molecules.

RESULTS

Growth-Phase-Dependent Synthesis of HMfA and HMfB. Although HMfA and HMfB have amino acid sequences that are 84% identical, they can be separated by electrophoresis through polyacrylamide gels containing acetic acid, urea, and Triton X-100 (AUT gels) and by HPLC (2). Initially, to maximize the yield, *M. fervidus* cultures were grown to their maximum cell densities (and, therefore, into the stationary growth phase) before HMf preparations were isolated, and these HMf preparations contained approximately equal amounts of HMfA and HMfB (2). The growth of these *M. fervidus* cultures was not, however, optimal. Exponential growth occurred only at very low cell densities ($<10^8$ cells per ml) and growth was terminated at a final OD_{580} of ≈0.8. Improvements in the fermentation conditions have resulted in a sustained exponential growth phase, higher final cell densities (OD_{580} ≈1.4), and HMf preparations isolated at early growth stages that contained substantially more HMfA than HMfB, with some HMf preparations containing almost 80% HMfA. These variations remained when different purification protocols were tested (13). HPLC analyses of the crude cell extracts and at each step through the purification protocols demonstrated that these variations were not a result of the purification but rather existed *in vivo*, and therefore, a systematic study of this phenomenon was undertaken. HMfA was found to predominate in HMf preparations isolated from cultures in the exponential growth phase, but the relative amount of HMfB then increased as cultures approached the stationary phase, with HMfB eventually making up ≈50% of HMf preparations isolated from stationary-phase cultures (Fig. 1).

Preparation and Separation of Homodimers of HMfA and HMfB. HMf is a dimer in solution, as determined by gel filtration (2) and by chemical crosslinking (R.A.G., unpublished results), and it was assumed previously that HMf existed solely as HMfA–HMfB heterodimers *in vivo*, as HMf preparations had HMfA/HMfB ratios of ≈1. The isolation of HMf preparations that clearly did not contain equal amounts of HMfA and HMfB argued for the presence of HMfA and HMfB homodimers, in addition possibly to HMfA–HMfB heterodimers. To confirm this, preparations of HMfA and HMfB homodimers were needed as standards, and a nondenaturing analytical system was needed to separate and identify HMfA homodimers, HMfB homodimers, and HMfA–HMfB heterodimers. Preparations of rHMfA and rHMfB were obtained by overexpression of the *hmfA* and *hmfB* genes separately in *E. coli*, and these were shown by chemical cross-linking to be homodimers in solution, and their identities were confirmed by N-terminal amino acid sequencing. At pH 6.1, homodimers of HMfA and HMfB were predicted to have positive, but different, net charges and a nondenaturing electrophoresis system was, therefore, devised to exploit this anticipated difference. As shown (Fig. 2*A*), this system clearly separated homodimers of rHMfA

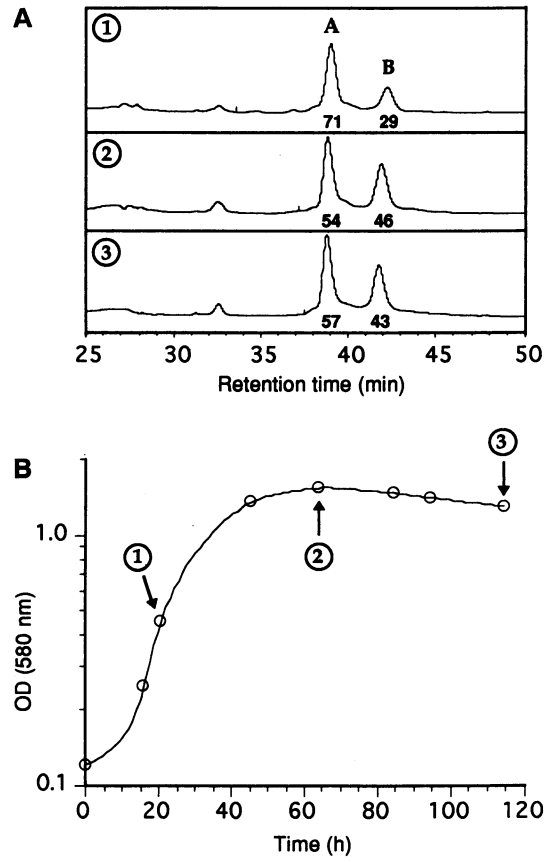

FIG. 1. Growth-phase-dependent synthesis of HMfA and HMfB. (*A*) Reverse-phase HPLC chromatogram of native HMf preparations isolated from *M. fervidus* cells taken from the culture at points 1–3 indicated in *B*. Peak heights on the chromatograms are proportional to A_{215}. The numbers under the peaks are the percent of the HMf preparation formed by that peak [HMfA (peak A) and HMfB (peak B)], calculated from the peak areas. (*B*) Time course of a *M. fervidus* fermentation, with the circled numbers indicating when the samples were taken that were used to generate the protein preparations analyzed in *A*.

(lane 1) from homodimers of rHMfB (lane 2) and demonstrated that preparations of native HMf from *M. fervidus* (lane 3) contained both HMfA and HMfB homodimers and HMfA–HMfB heterodimers. The composition of the heterodimer band was confirmed by two-dimensional electrophoresis using this nondenaturing system in the first dimension and a denaturing AUT gel in the second dimension. Reconstitution experiments also confirmed the composition of the heterodimer band. Incubating a mixture of rHMfA and rHMfB homodimers at 95°C for 5 min, followed by slow cooling to room temperature, resulted in rHMfA–rHMfB heterodimers (Fig. 2*A*, lanes 4 and 5) with the same electrophoretic mobility as the heterodimers present in native HMf preparations. Heterodimer formation was found to be temperature dependent, occurring at a barely detectable rate at room temperature, but at much higher rates at *M. fervidus* growth temperatures (80–90°C).

The net charges predicted for HMfA–HMfA, HMfA–HMfB, and HMfB–HMfB dimers, at pH 6.1, are +3.7, +5.7, +7.7, respectively, and as their molecular masses are very similar, an even distribution of electrophoretic mobilities was expected, but was not observed (Fig. 2*A*, lanes 3 and 5). The different monomer pairs must, therefore, have different conformations, or dimerization in one or more cases involves electrostatic interactions that influence electrophoretic mobility.

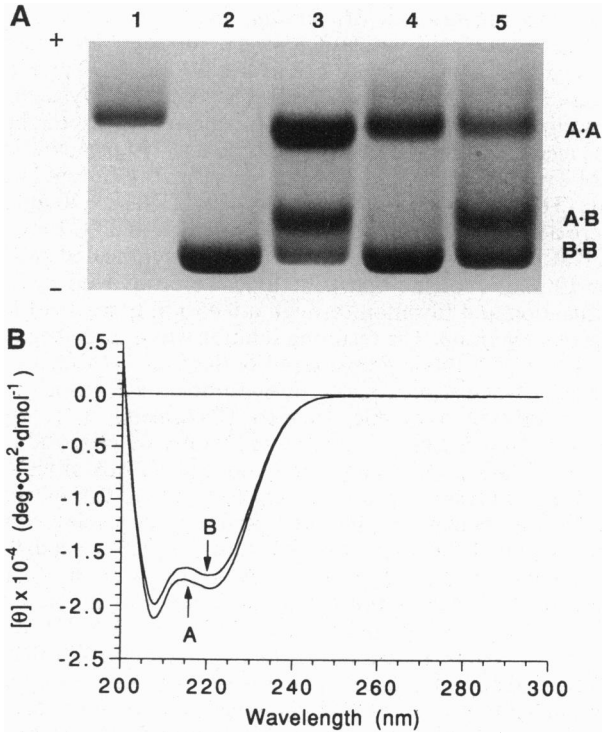

FIG. 2. Comparison of HMfA and HMfB. (*A*) HMf proteins separated by nondenaturing PAGE at pH 6.1. Lanes: 1, rHMfA; 2, rHMfB; 3, native HMf (isolated at point 1 of Fig. 1*B*); 4, a mixture of rHMfA and rHMfB; 5, same mixture as in lane 4, but incubated at 95°C for 5 min before electrophoresis. The bands formed by the HMfA homodimers (A·A), HMfB homodimers (B·B), and the HMfA–HMfB heterodimers (A·B) and the positive (+) and negative (−) electrodes are identified. (*B*) CD spectra of 50 μM rHMfA (spectrum A) and 50 μM rHMfB (spectrum B). Molar ellipticities (θ) were calculated based on rHMfA and rHMfB having molecular masses of 7468 and 7667 Da, respectively. The transition wavelength (≈200 nm) and the negative ellipticities at 208 nm and 222 nm predict ≈60% α-helical content for both proteins (14).

CD Spectra of rHMfA and rHMfB. Having demonstrated that HMf preparations contain HMfA and HMfB homodimers, structural and functional differences were sought in these homodimers. The primary sequences of HMfA and HMfB predict that both polypeptides contain predominantly three α-helical regions (4) and the CD spectra obtained, consistent with this prediction, indicate that ≈60% of amino acid residues in both polypeptides are contained in α-helices and ≈40% are in random chain configurations (Fig. 2*B*). As the CD spectra of rHMfA and rHMfB are essentially identical, the differences in their DNA binding properties (see below) must result from differences in their primary and/or tertiary structures or from differences in their secondary structures so subtle that they are not apparent in the CD data.

DNA Binding and Compaction by rHMfA and rHMfB Homodimers. The electrophoretic mobility of linear DNA fragments (>2 kbp) through agarose gels is increased by HMf binding (3, 13), and using this as the basis for an electrophoretic mobility shift assay revealed differences in rHMfA and rHMfB. The mobility of linear pBR322 molecules was increased by rHMfA and rHMfB binding, but a higher mass ratio of rHMfB to DNA was needed to obtain the maximum increase in DNA mobility (Fig. 3*A*) and at this point the rHMfB–DNA complexes formed migrated farther through the gels than the rHMfA–DNA complexes. At higher protein/DNA ratios, rHMfB binding apparently generated complexes that were more compacted, and this was confirmed by topology assays and EM. The effects of binding increasing amounts of rHMfA or rHMfB on DNA topology were

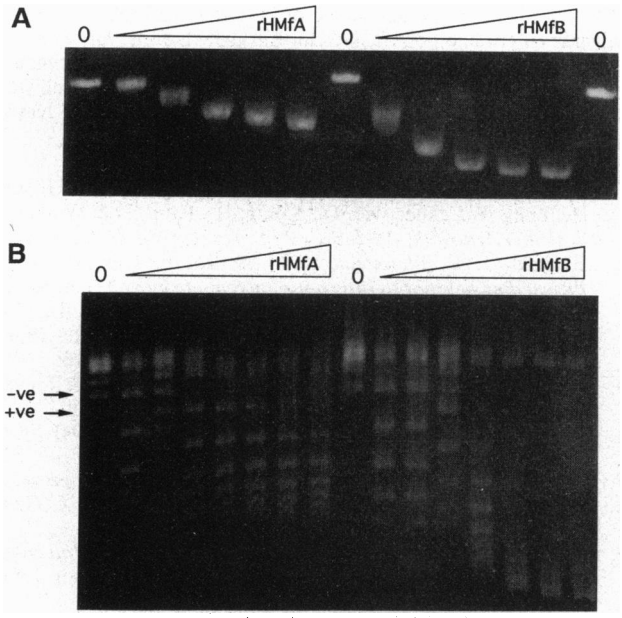

FIG. 3. Agarose gel electrophoresis of DNA–protein complexes. (*A*) Electrophoresis of linear pBR322 molecules mixed with rHMfA at protein/DNA mass ratios of 0.3, 0.6, 0.9, 1.2, and 1.5, and with rHMfB at protein/DNA mass ratios of 0.8, 1.6, 2.4, 3.2, and 4.0. Control lanes with no protein added are indicated by 0. (*B*) Electrophoretic separation of topoisomers of pUC18 generated by binding with rHMfA or rHMfB at protein/DNA mass ratios of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4. Control lanes with no protein added are indicated by 0. Positively (+ve) and negatively (−ve) supercoiled molecules with the same linking number are identified.

assayed as the introduction of topoisomers into relaxed circular pUC18 molecules. Both proteins increased the negative superhelicity of the pUC18 DNA at low but increasing protein/DNA ratios, and then at higher protein/DNA ratios, positively supercoiled topoisomers were generated by toroidal wrapping (15). The shift from the generation of negative to positive topoisomers occurred at a 0.4:1 protein/DNA mass ratio for rHMfA, but at a 0.6:1 ratio for rHMfB, and whereas rHMfA binding introduced a maximum of only 6 positive supercoils per pUC18 molecule, rHMfB binding resulted in molecules with >15 positive supercoils (Fig. 3*B*). Consistent therefore with the electrophoretic mobility shift assay, topoisomer formation demonstrated that rHMfA bound more effectively than rHMfB to pUC18 molecules at low protein/DNA ratios, but rHMfB binding at higher protein/DNA ratios resulted in protein–DNA complexes that contained many more toroidal supercoils and, therefore, must have been more compacted. When visualized by EM at a protein/DNA mass ratio that resulted in 6 and >15 positive supercoils in pUC18 molecules bound by rHMfA and rHMfB, respectively (Fig. 3*B*), every pUC18 molecule bound by rHMfB clearly contained several NLSs (Fig. 4*d*), whereas, overall, there were fewer NLSs detectable in the rHMfA bound molecules and some molecules lacked detectable NLSs (Fig. 4*b*). The negative superhelicity introduced into DNA molecules by the binding of native HMf (15) or rHMfA or rHMfB at low protein/DNA ratios (Fig. 3*B*) apparently results from a slight unwinding of the DNA helix (15), and at these ratios "kinks" but not NLSs are seen in the protein–DNA complexes (Fig. 4*c*).

DISCUSSION

M. fervidus cells have been shown to contain HMfA and HMfB homodimers, and HMfA–HMfB heterodimers and the

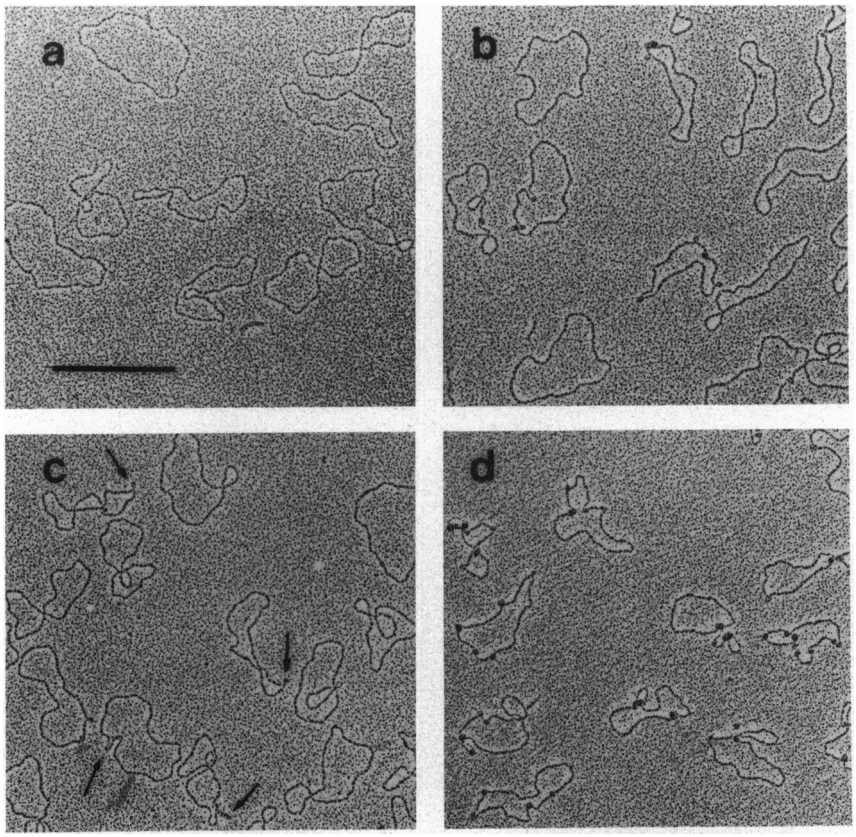

FIG. 4. Electron micrographs of relaxed pUC18 molecules bound by rHMfA or rHMfB. (*a*) Control pUC18 molecules with no protein added. (*b*) pUC18 molecules bound by rHMfA at a 1:1 protein/DNA ratio. (*c* and *d*) pUC18 molecules bound by rHMfB at protein/DNA ratios of 0.2:1 and 1:1, respectively. The arrows in *c* indicate kinks introduced by rHMfB. (Bar in *a* = 1.0 kbp.) (×42,500.)

HMfA/HMfB ratio have been shown to be growth phase regulated. Formation of heterodimers from homodimers has been demonstrated to be rapid *in vitro* at the growth temperature of *M. fervidus* (83°C), suggesting that the relative amount of homodimers and heterodimers *in vivo* is also likely to be growth phase dependent. Similar growth-phase-dependent synthesis of DNA binding proteins has been documented in Bacteria. For example, in *E. coli*, there is an increase in the nucleoid-associated DNA binding proteins H-NS and IHF as cells enter the stationary growth phase (16, 17), and synthesis of the FIS protein increases after dilution of stationary-phase cells in growth medium but then decreases when growth begins (18).

Since the relative amount of HMfB increases as *M. fervidus* cultures enter stationary phase, these cells must have an increased proportion of HMfB homodimers and probably also of HMfA–HMfB heterodimers. Cells at this growth stage must be adjusting their genomes for limited activity, and the DNA binding properties of HMfB appear appropriate for this situation. In contrast to HMfA, binding by HMfB generates more compact structures (Fig. 3*A*), can introduce more NLSs into closed circular DNA molecules (Figs. 3*B* and 4), and could, therefore, archive more of the *M. fervidus* genome in NLSs. The "active" genome of exponentially growing cells must also be compacted to accommodate space constraints but still must be readily accessible for transcription and replication. As HMfA homodimers are prevalent during the exponential growth phase, they presumably play a role in these processes. Positive but limited wrapping of the *M. fervidus* genome by HMfA could provide adequate compaction and generate increased negative superhelicity in the HMfA-free remainder of the genome (15) to facilitate both transcription and replication. The overall mass ratio of HMf to DNA *in vivo* is ≈0.25:1 (19) but, locally, this ratio could vary substantially if HMf binds preferentially *in vivo* to different domains, topological structures, or sequences within the *M. fervidus* genome. *In vitro* HMf assembles NLSs preferentially at inherently bent DNA sequences (20) and phased tracts of poly[d(A-T)], that are likely to cause inherent bending and, therefore, HMf binding, are very common in the A+T-rich *M. fervidus* genome. Protein binding to specific regulatory sequences, protein-induced bending, and long-range changes in the superhelicity of upstream regions induced by "distant" protein binding regulate the expression of genes in Bacteria and Eukarya (21, 22), and HMf binding could contribute to all of these local and global regulatory functions in the archaeon *M. fervidus*. The different DNA binding properties of HMfA and HMfB, coupled with their synthesis under different growth conditions, may then reflect the fine tuning of these HMf regulatory functions.

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