Right-handed DNA Supercoiling by an Octameric Form of Histone-like Protein HU

MODULATION OF CELLULAR TRANSCRIPTION*

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In bacteria, the contribution of global nucleoid organization in determining cellular transcription programs is unclear. Using a mutant form of the most abundant nucleoid-associated protein HU, HUαE38K,V42L, we previously showed that nucleoid remodeling by the mutant protein re-organizes the global transcription pattern. Here, we demonstrate that, unlike the dimeric wild-type HU, HUαE38K,V42L is an octamer and wraps DNA around its surface. The formation of wrapped nucleoprotein complexes by HUαE38K,V42L leads to a high degree of DNA condensation. The DNA wrapping is right-handed, which restraints positive supercoils. In vivo, HUαE38K,V42L shows altered association and distribution patterns with the genetic loci whose transcription are differentially affected in the mutant strain.

Chromatin is defined as the complex conglomeration of chromosomal DNA and specific DNA-binding proteins. Its organization is dynamically attuned to various changes in transcriptional requirements in response to exogenous and endogenous cues. In eukaryotes, the basic unit of chromatin is the nucleosome, which consists of DNA wrapped in left-handed turns around a histone octamer (1). In prokaryotes, on the other hand, it is commonly believed that the chromosome (nucleoid) does not have a defined structure. Nevertheless, bacterial nucleoids are efficiently compacted by a disparate group of histone-like DNA-binding proteins with low molecular mass and high electrostatic charge. HU is one of the most conserved and abundant (50,000 dimers per cell during logarithmic phase) nucleoid-associated proteins identified in eubacteria (2). In most bacteria, HU exists as a 18-kDa homodimer. Only in Enterobacteriaceae, including *Escherichia coli*, HU is a heterodimer of two similar subunits, HUα and HUβ. HU has traditionally been accepted as the archetypal bacterial counterpart of eukaryotic histones and evolutionarily linked to histone H1 (3). HU is a nonspecific DNA-binding protein, which plays an architectural role in bending DNA (4, 9) and constraining negative supercoils (5). It also participates in specific control functions in DNA transactions like replication, transcription, recombination, and DNA repair (6–10). Earlier observations of compact nucleosome-like structures in DNA induced by HU binding (11, 12) were recently challenged by studies that suggested that HU might in fact counteract DNA compaction by antagonizing other DNA-condensing proteins like H-NS (13). There has also been conflicting reports about the exact contribution of HU toward global chromosomal superhelicity (14). Thus, despite HU being one of the most abundant nucleoid-associated proteins, the exact molecular mechanisms of HU action with regard to its role in chromosome architecture and function remain largely obscure and controversial.

We have recently identified a gain-of-function mutant form of HUα, HUαE38K,V42L, which caused a reconfiguration of the *E. coli* nucleoid from a loosely packed, dispersed structure into a densely condensed, globular conformation (15). The mutant HUα contains the amino acid changes E38K and V42L. The nucleoid compaction was accompanied by dramatic changes in the morphology, growth, and physiology of the *hupA* mutant; transcription of many inducible as well as constitutive genes expressed under laboratory conditions was silenced, whereas many cryptic virulence genes were activated. Thus, HUαE38K,V42L represented the *de novo* generation of a unique form of HU with characteristics and consequences distinctively diverse from that of the wild-type protein and offered a unique opportunity to explore the nexus between bacterial nucleoid organization and global gene expression. In this study, we analyzed the biochemical properties of HUαE38K,V42L to elucidate the molecular mechanism of its action with respect to DNA condensation and transcription control. The results show that the wild-type and mutant HU have contrasting functional and biochemical behaviors that are translated to their differential effects on global transcription pattern.

**EXPERIMENTAL PROCEDURES**

* Purification of Wild-type and Mutant HUα—The wild-type and mutant *hupA* genes were cloned in expression vector pET15b (Novagen) and transformed into BL21(DE3)/pLysS strains. The proteins were purified essentially as described previously (16). After nickel-nitrilotriacetic acid-agarose column purification, fractions containing pure HUα proteins were pooled and dialyzed against 2 mM HEPES (pH 7.9), 50 mM NaCl.

**Electrophoretic Mobility Shift Assays**—Mixtures of pUC19 DNA (6 nM) and increasing amounts (60, 120, 300, 600, and 1200 nM) of wild-type and mutant HUα proteins were incu-
bated for 25 min at 25 °C in 10 mM Tris (pH 8.0), 75 mM KCl. The products were separated by electrophoresis at 0.7 V/cm through an 0.8% agarose gel run in 0.5× TAE buffer and visualized by ethidium bromide staining. For proteinase K, EcoRI and DNase I digestions, the protein:DNA ratio used was 100:1.

Atomic Force Microscopy—Solutions of supercoiled pUC19 DNA with and without wild-type HUα and HUαE38K,V42L were diluted with dilution buffer (10 mM Tris (pH 8.0), 75 mM KCl). Solutions of M13mp18 RF1 DNA (linearized with EcoRI) with and without wild-type HUα and HUαE38K,V42L were diluted with ligation buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl). Five μl of fresh solutions were adsorbed onto 11-mm diameter freshly cleaved mica disks (Ted Pella, Inc.) for 5–10 min. Each sample was washed with 200 μl of ultrapure water and dried with argon gas. In the case of the linear 444-bp DNA, all samples were prepared in 10 μM Tris (pH 8.0) and 50 mM KCl before being deposited on aminopropyl-silatrane-treated mica. The AP mica obviates the need for Mg2+ for DNA adsorption. Both PicoForce Multimode Atomic Force Microscopy (AFM)2 with Nanoscope IV controller and type E scanner head and the Bioscope with Nanoscope IIIa controller (Veeco/Digital Instruments) were used for imaging. All imaging was performed at room temperature and in air with tapping mode AFM. Oxide-sharpened silicon microcantilevers (Olympus America, Inc.) with nominal spring constants of 42 newton/m were used.

Analytical Ultracentrifugation—Equilibrium analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge. Self-associations of the wild-type HUα and HUαE38K,V42L were studied using 4-hole rotors at rotor speed of 25,000 rpm. All measurements were performed at 20 °C when binding equilibrium was reached. The buffers used for all samples were 50 mM KCl, 10 mM Tris (pH 8.0). Two sets of experiments were performed, with the wild-type HUα in the first and the mutant HUαE38K,V42L homodimer in the second set. For the wild-type HUα, two cells were loaded with 180 μl of 9 and 18 μM solutions of the wild-type protein. In the second experiment, two cells were loaded with 180 μl of 8 and 15 μM solutions of the HUαE38K,V42L protein. The second channel of each cell was filled with 185 μl of the buffer. Transmitted light intensities were collected at 230 nm because HU does not contain tryptophan, tyrosine, or cysteine residues. The mathematical model used to fit the data were,

\[ C_\mu(r) = C_\mu \exp[\Phi(r)] + C_\mu^h \exp[\ln K_{1,2} + 2\Phi(r)] + C_\mu^b \exp[\ln K_{1,4} + 4\Phi(r)] + e \]  

(Eq. 1)

where, \( \Phi(r) = A_{3,2}M_{1,2}(r^2 - b^2) \); b is the radius at the cell bottom, \( C_H = C_H(b) \) is the monomer mass at r = b, \( M_{1,2} \) is the molecular mass of the HU dimer (HU2), e is a baseline correction, and \( \ln K_{1,2} \) and \( \ln K_{1,4} \) are the natural logarithms of the equilibrium constants for HU2 – (HU2)2 and HU2 – (HU4)4 interactions, respectively; \( A_{3,2} = (1 - \gamma^2)\omega^2/2RT \) describes the centrifugal force, \( \gamma^2 \) being the partial specific volume of the solute, and \( \omega \), the rotational speed in rad/s.

DNA Supercoiling Assay—Supercoiled pUC19 plasmid DNA was partially relaxed by E. coli topoisomerase I (New England Biolabs). The partially relaxed DNA (8.6 nM) was incubated at 37 °C for 30 min with wild-type HUα and HUαE38K,V42L at increasing concentrations of 3.125, 6.25, 25, 75, 100, 125 μM, and 150 μM in a 10-μl reaction mixture containing 35 mM Tris–HCl (pH 8.0), 20 mM NaCl, and 5 mM dithiothreitol. Then calf thymus topoisomerase I (Invitrogen) was added and incubated at 37 °C for 2 h. Proteinase K (10 μg) was added and incubation continued for another 30 min. The DNA samples were analyzed by two-dimensional gel electrophoresis. The first dimension electrophoresis was run in 0.5× TBE at a constant voltage of 69 V for 16 h. The second dimension electrophoresis was run in 0.5× TBE containing 1 μg/ml chloroquine for 4 h at 77 V. For the analysis of in vivo plasmid topology, pGFPuv (Invitrogen) plasmid was transformed into MG1655 and SK3842 strains. The plasmids were separated on 1% agarose gel as well as two-dimensional agarose gel, as described above.

Chromatin Immunoprecipitation—Mid-log phase MG1655 and SK3842 cells were brought to equivalent colony forming units/ml (5 × 10^7 cells/ml) and treated with formaldehyde to a final concentration of 1%. After 30 min, cross-linking was quenched by the addition of glycine (final concentration 0.5 M). Cells were harvested by centrifugation and resuspended in 0.1× original volume of lysis buffer (10 mM Tris–HCl, pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, and 50 mg/ml lysozyme) for 10 min, followed by the addition of an equal volume of 2× immunoprecipitation buffer (100 mM Tris–HCl, pH 7.0, 300 mM NaCl, 2% Triton X-100, 0.2% sodium deoxycholate). Cellular DNA was sonicated to an average size of 500 bp. After centrifugation, the cleared supernatant was used as the “input” fraction for the chromatin immunoprecipitation (ChIP) assay. 5 μg of anti-HU antibody or 8 μg of anti-HNS antibody were added per ml of input fraction and incubated overnight at 4 °C. Complexes were incubated for 1 h with 30-μl bed volume of pre-equilibrated protein A-Sepharose CL-4B beads (Amersham Biosciences). Samples were then washed twice with IP buffer, once with IP buffer plus 500 mM NaCl, once with wash buffer (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and once with 10 mM Tris–HCl (pH 7.5), 1 mM EDTA. Immunoprecipitated complexes were eluted twice by incubation of beads with 30 μl of elution buffer (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 1% SDS) at 65 °C for 10 min. The two eluates were combined as the output fraction for PCR analysis. 3% of input fraction and 50% of output fraction were diluted with Tris–HCl (pH 8.0), containing 10 mg/ml RNase A, and heated overnight at 65 °C to reverse cross-linking. This was followed by treatment with 2 mg/ml Proteinase K for 2 h at 50 °C. Samples were ethanol precipitated and suspended in 30 μl of dH2O. 5 μl of the DNA samples were used in a 50-μl PCR reaction mixture. PCR was performed for 25 to 28 cycles and the PCR products were separated on 2% agarose gel. After staining with ethidium bromide, reverse images were photographed.

RESULTS

Mutant HUα Condenses Naked DNA in Vitro—Because the HUαE38K,V42L protein induced intense nucleoid condensation
DNA Wrapping by Octameric HU

FIGURE 1. DNA condensation by HUα/E38K,V42L in vitro. A, electrophoretic mobility shift assays with plasmid pUC19 DNA. The plasmid DNA was incubated without (lane 2) and with various amounts (protein:DNA molar ratios of 10:1, 20:1, 50:1, 100:1, and 200:1) of purified wild-type HUα (lanes 3–7) and HUα/E38K,V42L (lanes 8–12) proteins. B, deproteinization of HUα-DNA complexes. pUC19 DNA complexed with wild-type HUα (lanes 2 and 4) and HUα/E38K,V42L (lanes 3 and 5) proteins, at a protein:DNA molar ratio of 100:1, was treated with 50 μg/ml Proteinase K for 6 h (lanes 4 and 5). C, restriction endonuclease digestion of HUα-DNA complexes. pUC19 DNA complexed with wild-type HUα (lanes 3 and 4) or HUα/E38K,V42L (lanes 5 and 6) was treated with EcoRI (lanes 4 and 6). Lane 2, naked DNA. The DNA:protein ratio used in the reactions was 1:100. D, DNase I digestion of HUα-DNA complexes. pUC19 DNA complexed with wild-type HUα (lanes 2–6) and HUα/E38K,V42L (lanes 7–11) was treated with 1 unit of DNase I/100 ng of DNA for 0 s, 30 s, 1 min, 2 min, 3 min, and 5 min. Lane 1, naked DNA. The DNA:protein ratios used in the reactions was 1:100.

Next, we analyzed the nature of DNA-protein complexes formed by wild-type HUα and HUα/E38K,V42L by AFM imaging. AFM images of a 2,686-bp supercoiled plasmid DNA showed uniform structures with intertwined strands and smooth contours (Fig. 2A). The phase shift caused by the supercoiled DNA (Fig. 2A, inset) was smaller than that by the substrate mica itself. In the presence of wild-type HUα, at a protein:DNA molar ratio of 100:1, the DNA adopted a more relaxed appearance with protein molecules distributed randomly throughout the circle (Fig. 2B). Increasing protein concentrations did not generate any further change in the overall conformation of the HUα-bound DNA. This behavior of HUα homodimer is consistent with previous observations with wild-type HUα/HUB heterodimers (13) and reflects the ability of HU to restrain negative supercoils locally, without leading to any global DNA compaction. In the case of HUα/E38K,V42L homodimers, the conformational changes in the DNA at a lower protein:DNA ratio (20:1) were analogous to those generated by the wild-type protein (Fig. 2C), with portions of the intertwined DNA duplexes becoming more open. But, with increasing protein concentrations, some of the DNA molecules progressed to a different configuration with the formation of patches of dense, amorphous condensate having folds of DNA emanating from them (Fig. 2D). Finally, at a (HUα/E38K,V42L) 2:DNA molar ratio of 100:1, all the DNA molecules collapsed into compact globular particles (Fig. 2E). The condensates were 30–60 nm in diameter and 3–4 nm in height. In comparison, the mutant HUα protein itself was globular, but much smaller, appearing under the AFM as a particle with >10 nm diameter and a 1–1.5-nm height (Fig. 2F). This condensation of extended, unconstrained DNA molecules into globular particles is comparable with the coil-globule transition induced by neutral polymers and polycationic lipids. Supercoiled pUC12 DNA was shown to be transformed to 39–45-nm particles by cobalt hexamine chloride (17), which is very similar to the size of the plasmid DNA condensates that was obtained with HUα/E38K,V42L. Another striking characteristic of the DNA condensates was the significant phase shift in the AFM cantilever oscillation compared with that observed on the background mica or the non-condensed DNA (Fig. 2E, inset), indicating that the exterior of the condensed DNA has a significant charge redistribution compared with its free form. The minimum concentration of the mutant HUα required for complete condensation of the 2,686-bp supercoiled plasmid DNA...
was around 100 molecules of mutant HUα dimers per DNA molecule, with no more visible changes at higher protein concentrations. With a linear 7,249-bp DNA (Fig. 2G), wild-type HUα caused thickening of the DNA, as demonstrated by the considerable increase in thickness of the DNA strands along with some kinks at different positions of the DNA (Fig. 2H), similar to what has been reported previously (18). At lower concentrations of HUαE38K,V42L, some intermediate forms of DNA condensates were observed, where DNA strands projected out from the edges of partially condensed DNA molecules (Fig. 2I). At higher concentrations (protein:DNA molar ratio of 120:1), DNA was once again condensed into dense globular particles that were 50–150 nm in diameter and 10 nm in height (Fig. 2J). The phase shift observed with the supercoiled plasmids (Fig. 2E, inset) was also present here (Fig. 2J, inset). It appears that the compacting properties of HUαE38K,V42L are independent of size, sequence, and supercoiled status of the target DNA. Transmission electron micrographs of plasmid DNA-HUαE38K,V42L condensates revealed that the DNA was condensed into tightly coiled spheres with apparently little space in between (Fig. 2K).

**FIGURE 2. Atomic force and electron microscopy of HUαE38K,V42L-mediated DNA condensation.** A, pUC19 DNA. B, plasmid DNA with wild-type HUα (DNA:protein ratio of 1:100). C–E, plasmid DNA with HUαE38K,V42L (1:20, 1:50, and 1:100 molar ratios, respectively). F, 1 mM solution of pure HUα mutant. G, linear M13mp18 RF1 DNA. H, linear DNA with wild-type HUα (1:120). I and J, partially and completely condensed linear DNA caused by HUαE38K,V42L (1:120 molar ratio). Image insets show phase with 10° z scale. Plot insets are section profiles along indicated white lines. A–E scan sizes are 750 nm, F is 650 nm, G is 2 μm, H and I are 1.7 μm, J is 2 μm, and z scale is 3 nm in all height images. A, D, E, and G–J are amplitude images, whereas B, C, and F are height images. K, transmission electron micrograph of pUC19 DNA complexed with HUαE38K,V42L.

HUαE38K,V42L Wraps DNA Around Its Core Surface—Next, we took a closer look at the architecture of individual complexes formed by HUαE38K,V42L on supercoiled and linear DNA. At a low protein:DNA ratio (10:1), there appeared small globular nucleoprotein structures at positions where the proteins were located. The DNA wrapped by HUαE38K,V42L-DNA complexes (Figs. 3A, i–ii) were quite uniform in dimensions, with heights that were 2–4 times the height of the naked DNA (height of naked DNA was ~0.6 nm, whereas heights of the DNA-protein looped complexes were 1.5–2.2 nm). The two simplest forms of HUαE38K,V42L binding to DNA would be: (a) HUαE38K,V42L binds to the DNA minor grooves by its β-sheet arms and induces local bends like the wild-type HU (19) or (b) HUαE38K,V42L wraps the DNA around itself in a nucleosome-like structure. A wrapping mode of binding would cause a significant reduction in the contour length of the DNA, whereas a simple DNA minor-groove binding mode would produce kinks but not much length reduction. Although the exact contour length of a supercoiled DNA is hard to measure accurately, formation of a single HUαE38K,V42L-mediated wrapped complex caused a significant reduction in the apparent length of the DNA. The contour length of unbound plasmid DNA was around 835 ± 20 nm. A reduction of roughly 50–60 nm was observed for a single wrapped complex (Fig. 3A, i and ii). The shortening of the DNA can most easily be explained by DNA wrapping around the protein core by more than 360°.
more positive) superhelical DNA conformations. The height of the protein core in single-wrapped complexes (Fig. 3A, i and ii) was slightly greater than the height of free HUαE38K,V42L molecules present in the same sample (~1.5–2 versus ~1.2–1.6 nm). At low protein:DNA ratios, individual wrapped complexes were clearly distinguishable. With increase in HUαE38K,V42L concentration, the number of wrapped protein-bound complexes per DNA molecule increased (Fig. 3A, iii and iv). Although, in a few cases the new complexes were formed at well separated locations, the assembly of new DNA-protein wrapped structures was favored predominantly in the vicinity of an existing wrapped complex. This implied that the formation of wrapped DNA-protein complexes on supercoiled DNA was cooperative in nature. The assembly of successive wrapped complexes gave the appearance of an overlap of contiguous loops, resulting in thick fibril-like segments of DNA. The diameter of these fibrils was consistent with successive 10-nm protein particles wrapped with DNA, as seen in the ~12 nm periodicity in the length-wise section profile in Fig. 3A, iv, inset. These closely spaced, multiple wrapped structures aggregated to form three-dimensional higher-order packing that appeared as patches of dense condensation on DNA molecules seen in Fig. 2, D and I. With an increase in protein concentration, in addition to a broadening and thickening of the DNA strands corresponding to the position of the wrapped DNA complexes, the plasmid assumed a more densely wound structure (Fig. 3A, v).

To further confirm the DNA wrapping ability of HUαE38K,V42L, we analyzed the contour length shortening of a linear PCR-generated 444-bp DNA fragment upon complex formation with HUαE38K,V42L. Representative images of the linear DNA complexed with wild-type and mutant HUα are shown in Fig. 3B. The protein-free 444-bp DNA had a contour length of 150 ± 4 nm (Fig. 3B, i). Wild-type HUα were found to form local bead-like structures. The height of the protein core in single-wrapped complexes (Fig. 3B, ii) was slightly greater than the height of free HUαE38K,V42L molecules present in the same sample (~1.5–2 versus ~1.2–1.6 nm). At low protein:DNA ratios, individual wrapped complexes were clearly distinguishable. With increase in HUαE38K,V42L concentration, the number of wrapped protein-bound complexes per DNA molecule increased (Fig. 3B, iii and iv). Although, in a few cases the new complexes were formed at well separated locations, the assembly of new DNA-protein wrapped structures was favored predominantly in the vicinity of an existing wrapped complex. This implied that the formation of wrapped DNA-protein complexes on supercoiled DNA was cooperative in nature. The assembly of successive wrapped complexes gave the appearance of an overlap of contiguous loops, resulting in thick fibril-like segments of DNA. The diameter of these fibrils was consistent with successive 10-nm protein particles wrapped with DNA, as seen in the ~12 nm periodicity in the length-wise section profile in Fig. 3B, iv, inset. These closely spaced, multiple wrapped structures aggregated to form three-dimensional higher-order packing that appeared as patches of dense condensation on DNA molecules seen in Fig. 2, D and I. With an increase in protein concentration, in addition to a broadening and thickening of the DNA strands corresponding to the position of the wrapped DNA complexes, the plasmid assumed a more densely wound structure (Fig. 3B, v). To further confirm the DNA wrapping ability of HUαE38K,V42L, we analyzed the contour length shortening of a linear PCR-generated 444-bp DNA fragment upon complex formation with HUαE38K,V42L. Representative images of the linear DNA complexed with wild-type and mutant HUα are shown in Fig. 3B. The protein-free 444-bp DNA had a contour length of 150 ± 4 nm (Fig. 3B, i). Wild-type HUα were found to form local bead-like structures.
structures on the DNA but the contour length of the HUα-bound DNA (Fig. 3B, ii and iii) was essentially the same as that of naked DNA. Surprisingly, we did not observe any sharp kinking or bending of the DNA upon HUα binding. HUαE38K,V42L formed large spherical structures and was bound both at the ends and internal positions along the DNA (Fig. 3B, iv–vii). Analysis of DNA contour lengths upon HUαE38K,V42L binding showed that the average length of the DNA with a single nucleoprotein complex was 132 ± 4 nm, amounting to a shortening by about 18 ± 4 nm (Fig. 3B, iv–vii). This represents an average DNA contour length reduction of 54 ± 11 bp as a result of HUαE38K,V42L binding. Although the path of DNA cannot be followed accurately in these images, the loss of this relatively large amount of DNA is consistent with the wrapping of the DNA around the HUαE38K,V42L protein core surface. With two nucleoprotein complexes, the length of the DNA was further reduced to 110 ± 5 nm (Fig. 3B, viii). This represented a contour length shortening by about 119 ± 14 bp that was roughly twice the reduction made by a single wrapped complex. Because HU is such a small protein, for a significant length of DNA to be wrapped around the protein core surface it is likely that a multimeric form of HUαE38K,V42L was involved (as shown in a later section). Thus, we conclude that HUαE38K,V42L wraps DNA in spherical, nucleosome-like structures.

HUαE38K,V42L Generates Positive Supercoiling in DNA—The DNA wrapping by HUαE38K,V42L is expected to change the writhe of a supercoiled plasmid DNA molecule. Intuitively, an initial unwinding of the supercoiled plasmid DNA by HUαE38K,V42L, as seen in the AFM images, followed by enhanced supercoiling of the plasmid DNA with increasing protein concentrations would suggest that HUαE38K,V42L introduces positive supercoils in a negatively supercoiled DNA. We investigated the topological changes generated in vitro in a closed circular DNA upon interaction with HUαE38K,V42L in comparison to wild-type HUα. Because HUα has relatively very low affinity for relaxed DNA (20), we used partially relaxed 2,686-bp plasmid DNA as substrate and incubated with increasing concentrations of the HUα and HUαE38K,V42L. After subsequent incubation with eukaryotic topoisomerase I to remove all unconstrained supercoils in the plasmid DNA followed by deproteinization, the reaction products were analyzed by two-dimensional agarose gel electrophoresis (Fig. 4). Increasing amounts of HUαE38K,V42L led to a progressive conversion of the negative topoisomers to completely positively supercoiled DNA species (Fig. 4A, lane 9). Protein-free DNA had an average of 8–9 negative topoisomers. With increase in HUαE38K,V42L concentrations, the average linking number changed from slightly negative (lane 2) to relaxed (lane 7) to positive (lane 9). The net change in average linking number was from −3 to +1. This change is most likely due to introduction of positive supercoils, which first neutralizes the existing negative supercoils and then appears as exclusive positive topoisomers. In the presence of calf thymus DNA topoisomerase 1 alone, under identical conditions, more than 90% of the negative topoisomers were converted to the relaxed form (Fig. 4B), indicating that the positive topoisomers were stabilized by HUαE38K,V42L in the DNA-protein complexes. Wild-type HUα at lower protein concentration increased the average linking number from −3 to −4 (Fig. 4C, lane 2). At higher protein concentrations, HUα seems to cause a slight reduction in the negative superhelicity of the plasmid DNA. We conclude that HUαE38K,V42L, at high concentrations, alters the topology of closed, circular DNA molecules by inducing positive supercoiling. To check whether HUαE38K,V42L can also generate positive supercoils in vivo, we analyzed the topology of the plasmid isolated from wild-type and hupA mutant strains. In agarose gels, plasmid isolated from the mutant strain showed two distinct populations: a faster-moving band that corresponded with the plasmid band isolated from the wild-type strain and a set of slightly slower moving bands, which were absent in the wild-type strain (Fig. 4D). In two-dimensional gel electrophoresis, the plasmid from the mutant strain showed that it indeed had two distinct populations of plasmid topoisomers. There was a band corresponding to negatively supercoiled topoisomers as well as a set of distinct positively supercoiled topoisomers (Fig. 4E). In contrast, the plasmid isolated from the wild-type strain was almost exclusively negatively supercoiled. This confirms that HUαE38K,V42L can generate positive supercoils in plasmid DNA both in vivo and in vitro. The reason why there are two populations of plasmid topoisomers would need further detailed investigations.
DNA Wrapping by Octameric HU

**HUα**

**E38K,V42L** Self-associates to an Octamer Both in Vivo and in Vitro—To better correlate the DNA wrapping and condensing property of HUα E38K,V42L with its molecular structure, we investigated the oligomerization state of the mutant protein. Samples of His6-tagged wild-type HUα and HUα E38K,V42L were separated on 4–20% BisTris gels using non-denaturing sample buffer and omitting the sample boiling step prior to loading on the gels. There appeared to be clear differences in the oligomeric status of the wild-type and mutant HUα protein (Fig. 5A). In the case of wild-type HUα, there appeared to be two distinct bands, corresponding to the monomeric and dimeric forms of the protein. However, for the HUα E38K,V42L protein, there was a distinct higher molecular weight band in addition to the monomeric and dimeric forms. The molecular weight of this species corresponded to an octameric form of the mutant protein. The octamer formation was not sensitive to salt concentration. To eliminate the possibility that the observed self-association of HUα E38K,V42L was influenced by the histidine tags and establish that the octameric form was present under physiological conditions inside the cell, we performed Western blot analysis of the cell lysates from strains harboring the wild-type hupA gene and the mutant hupA gene under the same electrophoretic conditions as described above. As evident from Fig. 5B, in both log phase and stationary phase cultures, the mutant HUα E38K,V42L contained a strong octameric species in contrast to the wild-type HUα that had no detectable higher-order oligomers.

To further confirm the existence of the octameric state of HUα E38K,V42L, we used analytical equilibrium centrifugation to quantify the self-associations of wild-type HUα and HUα E38K,V42L proteins in solution, by fitting the absorbance data to assumed interaction models. We considered the following models. 1) No self-association of dimers. 2) Equilibrium association of dimers and tetramers. 3) Equilibrium association of dimers, tetramers, and octamers. 4) Equilibrium association of dimers, tetramers, and hexamers. 5) Equilibrium association of dimers and octamers. Wild-type HUα homodimers were found to associate weakly into tetramers (model 2) as shown in Fig. 5C. There were no detectable higher oligomers present. Global fit of the data to Equation 1 (described under “Experimental Procedures”) gave an association constant of \( K_{1,2} = 7.65 \) for wild-type HUα, which corresponds to a dissociation constant of \( K_{d} = 0.47 \) mM (dimer to tetramer). The free energy of association, \( \Delta G^0 \), was about \(-4.5\) kcal/mol. The mutant HUα E38K,V42L homodimers were also best fit by using Equation 1. However, at equilibrium, no tetramers were detected (Fig. 5D). Instead, it appeared that the protein formed a rather strong octamer (model 5), with an association constant of \( \ln(K_{1,2}) = -17.4\) kcal/mol of tetramer. The value of \( K_{d} \), which was about 16 \( \mu \)M for dimer to octamer formation, indicates a much stronger association than the wild-type HUα. This was consistent with the PAGE analysis described earlier, where HUα E38K,V42L octamers were clearly detectable under conditions where no visible tetramers of the wild-type HUα were present. These experiments clearly established that HUα E38K,V42L is present in simple dimer-octamer equilibrium and there are no additional heterodisperse, oligomeric species present. This self-association of the protein is independent of DNA binding.

**HUα** E38K,V42L Has Different Modes of Interaction with Different Genetic Loci—As mentioned in the Introduction, HUα E38K,V42L caused a profound shift in the global transcription pattern in the cell. Two of the representative genetic loci demonstrating this reprogramming of the transcription plan are gal (encoding enzymes for galactose metabolism) and hlyE (encoding the cytolytic protein hemolysin). The gal locus is super-repressed in the mutant hupA gene and the locus is tran-
Understanding the role of HU protein filamentation to turn off transcription. On the other hand, the hlyE gene expression was modulated by the mutant protein. These properties are all starkly different from the well studied characteristics of wild-type HU and forms the foundation for the sweeping changes in the nucleoid architecture and activity by virulence factors. Wild-type HU is functionally a dimer and has been well described as a DNA binding protein complex at the regulatory regions of genes whose transcription status in these two genetic loci, we used the ChIP technique to determine the degree and extent of association of HUα, with the regulatory regions gal and hlyE.

ChIP experiments define DNA domains that are associated with a particular protein, with the location of the peak corresponding to the actual binding site of the protein and the spread of signal on either side determining the level of association. The width of the distribution pattern is proportional to the number of protein molecules bound at the locus. We used primer pairs to probe 200 bp of the promoter regions of gal and hlyE and two upstream and downstream regions whose distances were 500 bp from the 5′ and 3′ ends, respectively, of the promoter-specific fragment (Fig. 6D). Immunoprecipitation with anti-HU antibody showed that, in the presence of galactose, wild-type HUα had little or no association with the promoter, the promoter-upstream or the promoter-downstream regions probed in the gal regulatory region (Fig. 6A). Conversely, under the same conditions, HUαE38K,V42L associated robustly not only with the gal promoter region but showed almost identical levels of association with both the upstream and downstream region probes. Such a spread of the mutant protein over a large DNA segment indicated that multiple molecules of HUαE38K,V42L were bound to this region, probably in the form of nucleoprotein filaments, to turn off transcription. On the other hand, the hlyE locus presented a much different picture (Fig. 6B). Here, HUαE38K,V42L was found to be associated with the promoter region but the association dropped sharply on either side of this region, showing that, unlike in gal, HUαE38K,V42L binding to the hlyE promoter region did not spread. The wild-type HUα was sometimes found to be weakly associated with the promoter-upstream region but was not consistently detectable in all samples. The hlyE locus is under the repressive control of H-NS and has two H-NS binding sites, located on either side of the transcription start site (21). We examined the association of H-NS with the hlyE locus in the presence of HUαE38K,V42L (Fig. 6C). Chromatin immunoprecipitation with anti-H-NS antibody in the wild-type background revealed that H-NS was bound strongly to the promoter region. However, the signal was sharply diminished in the mutant stain expressing HUαE38K,V42L. These results revealed that binding of HUαE38K,V42L interfered with the formation of the H-NS nucleoprotein complex at the hlyE promoter.

If gal and hlyE represented the new targets of HUαE38K,V42L for which wild-type HUα had little or no affinity, the hupA gene itself represented a location where the normal association of wild-type HUα was reversed for HUαE38K,V42L. Fig. 6E shows that HUα was strongly associated with the hupA regulatory region, as has been reported before. However, there was almost no immunoprecipitable hupA DNA in the case of HUαE38K,V42L. Taken together, these experiments demonstrate that HUαE38K,V42L has an altered map of distribution on the bacterial chromosome and has at least two different modes of interaction with the regulatory loci of the genes whose expression are altered by this protein.

**DISCUSSION**

The bacterial nucleoid is a dynamic entity whose structure is maintained by a delicate balance between the histone-like proteins, condensins like MukB, global superhelicity, and general transcription status of the cell. An alteration in any one of the key players in this inter-connected web is expected to shift the balance to a different state of nucleoid equilibrium. These kinds of changes can be extremely detrimental to cell physiology and result in an unsustainable condition. We have isolated a mutant form of bacterial HU protein, HUαE38K,V42L, which caused major changes in the nucleoid architecture and activity by virtue of its radically different biochemical and functional properties. Wild-type HU is functionally a dimer and has been well characterized as a DNA-binding protein that constrains negative supercoils. HUαE38K,V42L forms octamers in solution and is capable of inducing extremely high levels of DNA condensation. It wraps DNA around itself to form nucleosome-like structures that restrain positive supercoils. Finally, the mutant HUα protein shows a major change in the distribution pattern within the chromosome and has at least two different kinds of association patterns in the regulatory regions of genes whose expression was modulated by the mutant protein. These properties are all starkly different from the well studied characteristics of wild-type HU and forms the foundation for the sweeping morphological and physiological changes encountered in cells harboring the mutant hupA gene in the chromosome (15).

Unlike H-NS, where genuine evidence for its DNA-condensing properties are abundant, the exact role of HU in nucleoid compaction is murky at best. Overproduction of HU does not
increase chromosome condensation or impede cellular transcription (22). AFM results show that HU-bound DNA assumes a more open conformation (13). One of the current theories about the role of HU in nucleoid organization is that HU, in fact, acts as a foil for H-NS action by counteracting DNA condensation and the nucleoid structure is determined by the opposing roles of these two histone-like proteins. Given the high degree of DNA condensation by HUα<sup>E38K,V42L</sup>, both in vivo and in vitro, it initially appears extraordinary that the bacterial nucleoid is not only able to withstand this degree of powerful compaction but exhibit a robust, albeit a very different, transcription profile. Interestingly, there have been reports of HU from two different thermophilic bacteria that can induce strong DNA condensation in biochemical experiments (23, 24), similar to what was observed with HUα<sup>E38K,V42L</sup>. There have also been reports of HU-like proteins from chloroplasts and mitochondria that caused a high degree of nucleoid compaction in E. coli without being detrimental to cell growth and survival (25–27). The in vivo concentration of HUα<sup>E38K,V42L</sup> molecules (1 dimer per 100 bp), which has been shown to be similar to that of wild-type HU, would be far less than the amount needed for complete condensation of the nucleoid (1 dimer per 26 bp). Using large bacterial artificial chromosomal DNA at a protein:DNA ratio of 1 dimer per 100 bp, we can show that HUα<sup>E38K,V42L</sup> causes localized patches of dense condensation on the chromosome, interspersed with regions of HUα<sup>E38K,V42L</sup>-free DNA. The DNA condensation mechanism of HUα<sup>E38K,V42L</sup> is probably related primarily to its DNA wrapping property. In this scenario, successive toroidal DNA wrappings by HUα<sup>E38K,V42L</sup> would help promote DNA condensation by cooperative protein-protein interaction between adjacent wrapped DNA-bound proteins, similar to eukaryotic and archaeal histones.

HUα<sup>E38K,V42L</sup>, at high concentrations in vitro, introduced positive supercoils in plasmid DNA in the presence of topoisomerase I. Plasmid isolated from the hupA mutant strain also had a population of positively supercoiled topoisomers along with the negatively supercoiled plasmid species. From these results, we propose that the mutant HU generates positive supercoils at chromosomal segments where it binds as octamers, creating regions of positively supercoiled domains in the chromosome. The generation of positive supercoils would suggest that the HUα<sup>E38K,V42L</sup> wraps the DNA in a right-handed fashion. The supercoiling status in the cell is a principal determinant of both gene expression patterns as well as the compaction state of the nucleoid (28). Negative supercoiling provides the energy for DNA melting required for most DNA transactions, whereas positive supercoiling stabilizes the double helix. Intuitively, the DNA regions bound and condensed by multiple, closely spaced HUα<sup>E38K,V42L</sup> would be expected to be transcriptionally inactive due to steric hindrance. This was supported by the chromatin immunoprecipitation assays that showed that inside the cell, under inducing conditions, the HUα<sup>E38K,V42L</sup> occupied a very large segment of the gal regulatory region while the wild-type HUα had almost no association with this region. The formation of this kind of repressive nucleoprotein complex is similar to those formed by H-NS at the loci that under its control. But, whereas H-NS functions exclusively as a global repressor by forming these silencing complexes, HUα<sup>E38K,V42L</sup> displays a locus-specific binding pattern distinct that is correlated to the transcription status of that locus. The hlyE locus, which is a phenotypically silent gene in E. coli, is activated in the presence of HUα<sup>E38K,V42L</sup>. ChIP assays showed, unlike in the gal region, the HUα<sup>E38K,V42L</sup> has only limited association with the hlyE regulatory region, covering a discrete part of the region that was probed. This suggests that, at active transcription loci, binding of HUα<sup>E38K,V42L</sup> in the promoter region is restricted. The binding of HUα<sup>E38K,V42L</sup> to the upstream region of hlyE also negatively affected the binding of H-NS that is the typical regulator bound to this region. We hypothesize that the binding of a restricted number of HUα<sup>E38K,V42L</sup> to the hlyE promoter modulates the DNA topology unfavorably for H-NS binding and favorably for RNA polymerase recruitment. Interestingly, many of the genes activated by HUα<sup>E38K,V42L</sup> like hlyE, proU, and csg are under the negative regulation of H-NS. Factors that relieve H-NS-mediated gene silencing are usually sequence-specific transcription activators like IHF, CRP, Cfa-D, and VirF. If a promiscuous DNA-binding protein like HU is transformed into a dominant H-NS antagonist, the effect on the basal gene expression program would be far more widespread. We also showed that, not only does HUα<sup>E38K,V42L</sup> have non-conventional DNA binding targets, it is also absent from some of its traditional sites like the hupA regulatory region. The DNA structural specificity and molecular mechanism that determines the mode of interaction that HUα<sup>E38K,V42L</sup> would follow at any particular genetic locus would require further detailed analysis.

The technical inability to detect an ordered structure of bacterial nucleoid, the promiscuous ground state of global transcription, and the association of nucleoid condensation to general shut-down of cellular functions have long fostered the belief that bacteria do not have the sophisticated hierarchy of chromosome organization like that found in Archaea and Eukarya. The finding that a mutant form of HUα can assemble toroidal-wrapped nucleoprotein complexes locally, which is translated to a global change in nucleoid architecture and transcription profile, indicates that the correlation between chromosome organization and transcription pattern is more complex in bacteria than commonly believed. Any factor that has the potential to alter the level of nucleoid compaction must have an inherent effect of regulating DNA accessibility and consequently, global transcription. So far, almost all studies have linked increased chromosomal compaction in bacteria to general downshifts in genomic activity. HUα<sup>E38K,V42L</sup> reveals unexpected functional links between bacterial chromosome condensation and defined transcription pattern changes. Like in eukaryotes, bacterial chromosome re-organization may provide the structural basis for whole genome transcription changes necessary for adaptation under certain stressful environmental conditions. Additional biochemical and physiological studies of HUα<sup>E38K,V42L</sup> can provide valuable insights into the mechanism and dynamics of nucleoid organization and its functional outputs.

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3 S. Kar and S. Adhya, unpublished results.
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