Lack of the H-NS Protein Results in Extended and Aberrantly Positioned DNA during Chromosome Replication and Segregation in *Escherichia coli*

Emily Helgesen,^a Solveig Fossum-Raunehaug,^{a,b} Kirsten Skarstad^{a,b}

Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital, Radiumhospitalet, Oslo, Norway^a; School of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway^b

ABSTRACT

The architectural protein H-NS binds nonspecifically to hundreds of sites throughout the chromosome and can multimerize to stiffen segments of DNA as well as to form DNA-protein-DNA bridges. H-NS has been suggested to contribute to the orderly folding of the *Escherichia coli* chromosome in the highly compacted nucleoid. In this study, we investigated the positioning and dynamics of the origins, the replisomes, and the SeqA structures trailing the replication forks in cells lacking the H-NS protein. In H-NS mutant cells, foci of SeqA, replisomes, and origins were irregularly positioned in the cell. Further analysis showed that the average distance between the SeqA structures and the replisome was increased by ~100 nm compared to that in wild-type cells, whereas the colocalization of SeqA-bound sister DNA behind replication forks was not affected. This result may suggest that H-NS contributes to the folding of DNA along adjacent segments. H-NS mutant cells were found to be incapable of adopting the distinct and condensed nucleoid structures characteristic of *E. coli* cells growing rapidly in rich medium. It appears as if H-NS mutant cells adopt a “slow-growth” type of chromosome organization under nutrient-rich conditions, which leads to a decreased cellular DNA content.

IMPORTANCE

It is not fully understood how and to what extent nucleoid-associated proteins contribute to chromosome folding and organization during replication and segregation in *Escherichia coli*. In this work, we find *in vivo* indications that cells lacking the nucleoid-associated protein H-NS have a lower degree of DNA condensation than wild-type cells. Our work suggests that H-NS is involved in condensing the DNA along adjacent segments on the chromosome and is not likely to tether newly replicated strands of sister DNA. We also find indications that H-NS is required for rapid growth with high DNA content and for the formation of a highly condensed nucleoid structure under such conditions.

Across all domains of life, it is crucial that genomes are structurally organized in a way that compacts DNA to fit inside the confined space of a cell and at the same time allows for interaction with key proteins performing replication, transcription, recombination, and repair (1–7). Unlike eukaryotic cells, bacterial cells do not possess an envelope-enclosed organelle for storage and handling of genomic DNA. The DNA is instead organized into compact bodies called nucleoids (3–5, 8). These nucleoids are highly complex, and the underlying organizational mechanisms appear to be remarkably similar to that of eukaryotic cells (3, 9). The nucleoid occupies the central part of the bacterial cell (8), and its shape is dependent on a variety of factors, such as environmental conditions or genetic mutations (7, 10–13). For example, significant nucleoid compaction occurs after exposure of *Escherichia coli* to UV light, due to a global reorganization in response to DNA damage and the activation of the SOS response (12, 13).

Certain types of proteins, called nucleoid-associated proteins (NAPs), are believed to have a great impact on nucleoid organization in bacteria (2–5, 14). Heat-unstable nucleoid protein (HU), factor for inversion stimulation (Fis), and histone-like nucleoid structuring protein (H-NS) are among the most intensively studied NAPs in cells of *E. coli* (1, 4, 15). HU is the most abundant NAP (16). Binding of HU to DNA is unspecific but increased at sites where there is a high density of supercoiled DNA (17) and single-strand breaks or gaps (18). HU exists as a homodimer or heterodimer (19), and it has been shown that HU interacts with to-

poisomerase I and influences nucleoid structure, gene expression, and recombination (20). Fis binds and bends AT-rich sites as a homodimer (21) and, similarly to HU, has an impact on nucleoid structure, transcription, and recombination (22). Moreover, Fis has been found to bind and bend *oriC* (23) to regulate the initiation of replication in an interplay between DnaA and other NAPs (24–26). H-NS was initially discovered because of its ability to modulate transcription *in vitro* (27) and was later found to form DNA-protein-DNA bridges by binding to AT-rich sequences as a hetero- or homodimer (28, 29). H-NS can also multimerize to “stiffen” segments of DNA, and a change in divalent cations drives a switch between the bridging and stiffening modes of the protein

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Address correspondence to Kirsten Skarstad, Kirsten.Skarstad@rr-research.no.

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in vitro (30–32). Because of these DNA-binding properties, H-NS acts as a global transcriptional repressor (33–35) and has also been reported to impact nucleoid structure (2, 6, 14, 36, 37). However, it has been difficult to elucidate its exact role and importance in this context, presumably due to its dual-purpose nature.

The SeqA protein can also be claimed to belong to the group of NAPs due to its ability to organize newly replicated DNA (10, 38–42). Additionally, it has been implicated as an important factor in the correct folding of the chromosome (43–45). SeqA binds specifically to hemimethylated GATC sites as a dimer and multimerizes to form a left-handed coil with DNA (43, 46–48). Fluorescently tagged SeqA structures can be seen as distinct foci in the cell, located mainly at center and quarter positions (38–40). Moreover, SeqA sequesters newly formed origins for one-third of the cell cycle (49) and contributes to ensure that no more than one initiation occurs per origin per cell cycle (50–52). Fluorescence imaging indicates that SeqA structures trail the replication forks at a considerable distance of ~200 to 300 nm, whereas the two sister SeqA structures behind the same fork are situated closer than 30 nm together (53).

It has been indicated that fluorescently tagged H-NS forms distinct foci in slowly growing cells of *E. coli* (37). A mutation in the N terminus of H-NS rendered the protein unable to multimerize, and the defined H-NS foci were replaced with fluorescent signals scattered throughout the cell (37). This confirmed the ability of H-NS to bind throughout the chromosome, found previously in *in vitro* studies (54, 55), and led to the theory that scaffolds of H-NS could function as global DNA-organizing centers in the cell (37, 56). A role of H-NS in the organization of DNA has also been supported by atomic force microscopy studies (14) and optical trap nucleoid studies (57). Conformation capture findings reported by Cagliero et al., however, contradicted the theory that H-NS tethers distantly spaced genetic elements in the nucleoid (10). Those authors did not find any significant interactions between H-NS-associated loci and suggested that chromosome rearrangements found in H-NS deletion strains arise due to indirect effects (10). Moreover, there are strong indications that the discrete H-NS foci reported previously (37) were formed due to the use of a “sticky” fluorescent tag (58).

In the present study, we have investigated chromosome organization in cells lacking the H-NS protein by localization studies of fluorescently tagged SeqA protein, replisome, and origin regions in living cells. We found that the typical pattern of localization of SeqA, replisome, and origin foci in the cell was disrupted and that the average distance between the replisome and the SeqA structures trailing it was increased in H-NS mutant cells. Also, by visualizing nucleoids in fixed and living cells, we found that the nucleoids of H-NS mutant cells remained unchanged during growth under nutrient-poor versus nutrient-rich conditions, in contrast to wild-type cells. We suggest that H-NS, directly and/or indirectly, plays a significant role in maintaining the proper organization of DNA during replication and segregation.

MATERIALS AND METHODS

Bacterial strains. All strains used are derivatives of *E. coli* K-12 strain AB1157 (59) and are listed in Table S1 in the supplemental material. Localization studies of SeqA were done with cells containing yellow fluorescent protein (YFP) fused to the C-terminal end of SeqA and expressed from the endogenous chromosomal promoter. The YFP protein was described previously (60) and was connected to SeqA via a 4-amino-acid

linker (61). The *seqA-yfp* gene was transferred into AB1157 by P1 transduction (62) to obtain SF128 (see Table S1 in the supplemental material) (39). Characterization of SF128 by flow cytometry and Western blot analyses showed that the SeqA-YFP protein is functional in origin sequestration and that the cellular concentration of fluorescently tagged SeqA is about the same as that of wild-type SeqA (39).

The fluorescent-repressor-operator system (FROS) was used to study the localization of the origin region (63). The RRL215 strain (kindly provided by R. Reyes-Lamothe and D. J. Sherratt) (see Table S1 in the supplemental material) contained a *lac* operator array (240 copies) (from IL-01) (63, 64) and a *lacI*-mCherry construct (65). The *lac* operator array was located at the *attTn7* site (at 84.27 min) 15 kb counterclockwise from *oriC*. The *lacI*-mCherry construct replaced the *leuB* gene on the chromosome and was constitutively expressed from the *dnaA* promoters. RRL215 was constructed as described previously (65), except that the *lac* promoter was replaced with the *dnaA* promoters (R. Reyes-Lamothe, personal communication). The primers used for up-amplification of the *dnaA* promoters were as follows: PdnaA-F (5′-GTC ACA TGT AAT AAT TGT ACA CTC CG-3′ [PciI sequence, ACATGT]) and PdnaA-R (5′-AAG AAT TCT CCA CTC GAA CAA AAG TCG-3′ [EcoRI sequence, GAATTC]). For multiple insertions of modified genes, the chloramphenicol resistance gene with flanking Flp recognition target (*frt*) sites was removed from RRL215 with the aid of Flp recombinase from pCP20 (66) to yield strain SF143 (53) (see Table S1 in the supplemental material). The *seqA-yfp* gene was transferred into SF143 by P1 transduction (62) to yield strain SF148 (53) (see Table S1 in the supplemental material). The chloramphenicol resistance gene of *seqA-yfp* was removed with pCP20 to obtain strain EH01 (see Table S1 in the supplemental material).

For replisome localization studies, cyan fluorescent protein (CFP) fused to the C-terminal end of single-stranded binding protein (SSB) and inserted into the *lamB* site (kindly provided by A. Wright) was used. The *ssb-cfp* gene was transferred from strain GL224 (see Table S1 in the supplemental material) into EH01 by P1 transduction (62) to yield strain EH02 (see Table S1 in the supplemental material). EH02 cells contained the wild-type *ssb* gene on the chromosome in addition to the *ssb-cfp* fusion construct.

For nucleoid imaging, mCherry fused to the α -subunit of the HU protein and expressed from the endogenous chromosomal promoter was used (*hupA100::mCherry*) (kindly provided by S. Sandler) (67). *hupA100::mCherry* was transferred into AB1157 by P1 transduction (62) to obtain EH20 (see Table S1 in the supplemental material).

To obtain strain SF154 (see Table S1 in the supplemental material), the *hns-206::amp* gene was transferred from strain MOR242 (68) into strain EH02 by P1 transduction (62). The *hns-206* mutation has been shown to disrupt the *hns* gene so that expression is completely absent (69). The Δ *hns-746* mutation originated from strain JW1225-2 in the Keio Collection (70) and was verified to reproduce the phenotype of the strain with mutated *hns-206* by flow cytometry (see Fig. S3 in the supplemental material). For construction of the EH23 strain (see Table S1 in the supplemental material), the Δ *hns-746* mutation was transferred into EH20 by P1 transduction (62).

Cell growth. For all experiments, cells were grown at 28°C to an optical density (OD) of ~0.15 (early exponential phase) and prepared for flow cytometry or fluorescence microscopy analysis (see below). Different types of media were used. These media were glycerol medium (AB minimal medium [71] supplemented with 1 μ g ml⁻¹ thiamine, 80 μ g ml⁻¹ threonine, 100 μ g ml⁻¹ glutamine, 22 μ g ml⁻¹ histidine, 22 μ g ml⁻¹ arginine, 20 μ g ml⁻¹ leucine, 20 μ g ml⁻¹ proline, and 0.2% glycerol), glucose-CAA medium (AB minimal medium [71] supplemented with 1 μ g ml⁻¹ thiamine, 0.2% glucose, and 0.5% Casamino Acids), and LB medium (10 mg ml⁻¹ tryptone, 5 mg ml⁻¹ yeast extract, and 10 mg ml⁻¹ NaCl).

Flow cytometry and cell cycle analyses. Exponentially growing cells were immediately fixed in ethanol or treated with 300 μ g/ml rifampin and 10 μ g/ml cephalixin to inhibit replication initiation (72) and cell division

(73), respectively (run-out samples). For run-out samples, growth was continued for 3 to 4 generations after the drugs were added, and samples were fixed in ethanol. The cells ended up with an integral number of chromosomes (72), which represents the number of origins at the time of drug treatment. Flow cytometry was performed as previously described (74), using an LSR II flow cytometer (BD Biosciences) and FlowJo 7.2.5 software. Cell cycle parameters and numbers of origins and replication forks per cell were obtained by analysis of the DNA distributions obtained by flow cytometry by using a cell cycle simulation program as described previously (75).

Fluorescence microscopy. For fluorescence microscopy, exponentially growing cells were immobilized on a 17- by 28-mm agarose pad (1% agarose in phosphate-buffered saline) and covered with a no. 1.5 coverslip. For imaging of nucleoids in fixed cells, 10 μ l of ethanol-fixed cells was added to a microscopy slide and allowed to dry. Mounting medium containing 40% glycerol and the nucleic acid stain Hoechst 33258 at a final concentration of 5 μ g ml⁻¹ in 1× phosphate-buffered saline was added, and the sample was covered with a no. 1.5 coverslip. Both living and fixed cells were imaged directly after slide preparation.

Images were acquired with a Leica DM6000 microscope equipped with a Leica EL6000 metal halide lamp and a Leica DFC350 FX monochrome charge-coupled-device (CCD) camera. Differential interference contrast (DIC) images were acquired with an HCX PL APO 100×/1.46-numerical-aperture (NA) objective. Phase-contrast imaging was performed with an HCX PL APO 100×/1.40-NA objective. Narrow-band-pass filter sets (excitation [Ex] at band-pass [BP] 436/20 and emission [Em] at BP 480/40 for CFP, Ex at BP 510/20 and Em at BP 560/40 for YFP, and Ex at BP 545/30 and Em at BP 610/75 for Cy3) were used for fluorescence imaging.

During image acquisition, saturated pixels were avoided. The raw images were saved for further image processing (see below).

Image processing and analysis. Imaging adjustments (brightness and contrast) were performed with ImageJ or Fiji software. Analysis to obtain fluorescence intensity profiles (over the cell long axis) of the cells according to increasing cell length/age and fluorescence images of cells stacked horizontally according to cell length/age was done with the public-domain project Coli-Inspector, which is run under Fiji in combination with the ObjectJ plug-in (<http://simon.bio.uva.nl/objectj/>) (76). To obtain sufficient background for use of this plug-in, DIC images were processed in order to simulate phase-contrast images. This was done in ImageJ as follows: (i) images were converted to 8 bits (press Image/Type/8-bit in the ImageJ menu bar), (ii) a pseudo-flat-field filter with default settings was applied to correct for uneven lighting (Process/Filter/Pseudo Flatfield), (iii) shadows were applied to the DIC images to balance the inherent shadows that arise during DIC imaging (Process/Shadow/choose the direction opposite of the inherent ones), (iv) images were filtered by using the maximum filter (Process/Filter/Maximum) (images may be adjusted further by using thresholding [Image/Adjust/Threshold]), (v) images were inverted to simulate phase-contrast images (Edit/Invert), and (vi) images were converted back to 16 bits (Image/Type/16-bit) in order to allow merging with the fluorescence channels to make a composite image (this is required for running Coli-Inspector). This type of processing does not always give optimal results, so alternative approaches were used for some images/experiments. One of these approaches was to manually draw cell outlines by using the polygon selection tool in the ImageJ/Fiji toolbar. For each cell outline drawn, the selection was added to the ROI (region of interest) manager (by pressing T). When all cell outlines were added, the selections in the ROI manager were combined by selecting all ROIs in the ROI manager, pressing More/Or (combine), pressing T (this adds a new selection that contains all the single-cell ROIs), and deleting the single-cell regions. By marking the combined selection (in the ROI manager) and deleting the outside signal (Edit/Clear outside), everything but the cells is deleted. The selections can then be filled with white color (Edit/Fill), and the image can be inverted (Edit/Invert) in order to get black cells on a white background. However, since this protocol is quite tedious overall,

we have recently been using an automatic script to detect bacteria in DIC images, developed by Jan Brocher at BioVoxxel. The bacterium detection script runs under Fiji. Using this script, the cells will automatically be detected, and the cell outlines are transferred to the ROI manager. At this point, the process of combining outlines and deleting the outside signal can be done as explained above in order to simulate a phase-contrast image (for Coli-Inspector), and/or the single-cell ROIs/outlines can be saved and directly used for running our Python-based script for focus measurements (see below). The bacterium detection script is available upon request (see also <http://www.biovovxel.de/consulting/consulting-references/>). Note that none of the methods mentioned above (except manual drawing) are good at detecting cells that are in clusters. Fluorescence images for Coli-Inspector were processed as described below for the focus measurement script.

We have developed a Python-based script for automatic measurements of the distance between neighboring fluorescent spots/foci that are detected in two different fluorescence channels, as described previously (53). The script is run under Fiji and uses Find Maxima as a tool for detecting the center of mass of foci within regions of interest, i.e., within cells. It is possible to distinguish foci that are closer than the resolution limit of fluorescence microscopes when measuring the distance between spots in two different channels (53, 77, 78). Thus, in this study, we were able to present measurements of focus distances below the resolution limit of our microscopy system. The script is available online at the *Nucleic Acids Research* website (see the supplemental material in reference 53).

Image processing of fluorescence images for the focus measurement script was performed in ImageJ or Fiji by using the following tools: (i) background subtraction with the default rolling-disk diameter (10 pixels), (ii) deconvolution using the Richardson-Lucy algorithm (100 iterations), (iii) Median Filter, and (iv) Max Entropy. To identify each cell, single-cell ROIs (cell outlines) were generated from DIC images as explained above, and the script was run in Fiji on composite images (containing the two fluorescence channels) together with cell outlines from the ROI manager. (For a detailed protocol of image processing [of fluorescence images] prior to running of the focus measurement script, see the supplemental material in reference 53).

Investigation of aberrations and distortions in the optical system.

Since we measure distances that are similar to or less than the resolution of our optical system, it is important that the accuracy of the optical system is assessed carefully. We used Tetraspeck beads in addition to an Image Registration Target slide grid with 100-nm holes (Applied Precision, GE Healthcare) to investigate aberrations and distortions in our optical system (described in more detail in reference 53). The conclusions are that the optical system is very well calibrated and that the fluorescence channels are accurately aligned. The accuracy of localizing the center of mass of foci is 0 to 1 pixel (pixel size of 92 nm).

Cell cycle analysis of snapshot images of wild-type and H-NS mutant cells. Three separate experiments were performed, and care was taken to use the cognate cell cycle parameters (and not average values) when analyzing snapshot images for individual experiments (see Fig. 2 and 3 and Table 2). The numbers for distances between the replisome and the SeqA structures are given for all experiments in Table 3.

RESULTS

Cell cycle parameters and DNA content of H-NS mutant cells differ significantly from those of wild-type cells under three different growth conditions. In order to analyze images of cells containing fluorescent tags on chromosomal regions or on proteins related to replication and/or DNA organization, it is imperative to obtain information about cell cycle parameters. In contrast to eukaryotic cells, DNA replication in *E. coli* cells can span more than one generation (overlapping replication), and each chromosome may contain more than two replication forks (multifork replication). It has been shown that H-NS mutant cells (lacking the H-NS protein) have a simpler replication pattern (fewer replication

FIG 1 Cell cycle patterns of wild-type and H-NS mutant cells exponentially grown in glycerol, glucose-CAA, and LB media. Shown are cell cycle diagrams with parameters obtained by flow cytometry analysis of wild-type strain EH02 (SeqA-YFP, SSB-CFP, and *oriC*-mCherry) and H-NS mutant strain SF154 (*hns*-206, SeqA-YFP, SSB-CFP, and *oriC*-mCherry) cells, exponentially grown at 28°C in glycerol medium (A), glucose-CAA medium (B), and LB medium (C). The C-period (replication period) is indicated by a black line, and the D-period (postreplication period) is indicated by a dashed line. Average numbers for the doubling time, time of initiation, and length of the C-period are given in the diagrams, and standard errors of the means are included. Numbers represent results from at least three independent experiments. Schematic cartoons of replicating chromosomes are shown above the diagrams and indicate DNA content, numbers of origins (black dots), numbers of replication forks, and replication fork progression at different stages of the cell cycle. C, M, and G stand for current, mother, and grandmother generations, respectively. Flow cytometry histograms of exponential and run-out samples of cells, including those of background strain AB1157, can be found in Fig. S1 in the supplemental material.

forks and reduced DNA content) than that of wild-type cells under similar growth conditions (68, 79). Because of this, we grew cells in three different media to investigate which conditions would give more similar cell cycles and DNA/mass ratios between H-NS mutant and wild-type cells for subsequent fluorescence microscopy studies. The SF154 strain, containing SeqA-YFP, *oriC*-mCherry, SSB-CFP (representing the replisome), and mutant H-NS protein (*hns*-206), was grown at 28°C to early exponential phase (OD of ~0.15) alongside its wild-type version, EH02 (with fluorescent tags and the wild-type H-NS protein). The media used were minimal medium supplemented with glycerol (glycerol medium), minimal medium supplemented with glucose and Casamino Acids (glucose-CAA medium), and LB medium. Samples of exponentially growing cells and cells treated with rifampin and cephalixin to allow run-out of replication were prepared and subjected to flow cytometry analysis (see Materials and Methods). Schematic cartoons showing the cell cycles of the two strains are based on simulation of flow cytometry histograms (see Materials

and Methods) and can be found in Fig. 1 (see Fig. S1 in the supplemental material for flow cytometry histograms from exponentially growing cells and run-out samples).

Table 1 shows the relative amount of fluorescein isothiocyanate (FITC) fluorescence (representing mass), relative amount of Hoechst fluorescence (representing DNA content), and DNA/mass ratios, where the numbers are normalized against parameters for wild-type (EH02) cells grown in glucose-CAA medium. From these analyses, it became apparent that H-NS mutant cells had a reduced DNA/mass ratio compared to that of wild-type cells in all three media (Table 1) and significantly different growth rates and replication patterns (Fig. 1). Moreover, wild-type cells had on average nearly four-times-higher DNA contents in rich medium than in poor medium, while H-NS mutant cells showed a <2-fold increase in DNA content. Also, the generation time of the H-NS mutant cells in LB medium was about the same as that in glucose-CAA medium. In glucose-CAA medium, the C-period of H-NS mutant cells was significantly reduced compared to that of wild-

TABLE 1 Relative amounts of FITC and Hoechst fluorescence and DNA/mass ratios for wild-type (EH02) and H-NS mutant (SF154) cells grown in glycerol, glucose-CAA, and LB media

Strain	Medium	Relative amt of FITC ± SD ^a	Relative amt of Hoechst ± SD ^a	DNA/mass ratio
Wild type	Glycerol	0.57 ± 0.04	0.46 ± 0.006	0.80
H-NS mutant	Glycerol	0.74 ± 0.04	0.41 ± 0.050	0.55
Wild type	Glucose-CAA	1	1	1
H-NS mutant	Glucose-CAA	0.65 ± 0.01	0.51 ± 0.003	0.78
Wild type	LB	1.46 ± 0.16	1.76 ± 0.110	1.20
H-NS mutant	LB	0.73 ± 0.06	0.78 ± 0.040	1.07

^a Numbers were collected from three independent flow cytometry analysis experiments.

type cells, which was also reported previously (68, 79) (Fig. 1B). Although this was not the case in glycerol and LB media, the C-period represented a lower fraction of the generation time than that for wild-type cells in these media (0.64 and 0.73 in glycerol medium and LB medium, respectively, for H-NS cells and 0.87 and 1.07 in glycerol medium and LB medium, respectively, for wild-type cells). The conditions that produced comparable DNA/mass ratios and cell cycle patterns between the strains were growth in glycerol medium for wild-type cells (DNA/mass ratio of 0.80) and growth in glucose-CAA medium for H-NS mutant cells (DNA/mass ratio of 0.78) (Table 1 and Fig. 1). Note that the cell cycle of H-NS mutant cells is somewhat more variable than that of wild-type cells; i.e., the exponential DNA histograms are slightly broader. This means that there is somewhat greater cell-to-cell variability in this population (compare exponential flow cytometry histograms of EH02 and SF154 in Fig. S1 in the supplemental material). A few of the cells were filamentous (~1%; 1,265 cells counted), and these cells were removed in comparisons of fluorescence images of wild-type and H-NS mutant cells. No anucleate H-NS mutant cells were found.

Chaotic localization pattern of origin, SeqA, and replisome foci in H-NS mutant cells during the cell cycle. If H-NS is a major contributor to the highly organized packing of DNA in the cell, it should be expected that systematic cellular positioning of chromosomal loci, as well as of proteins associated with newly replicated DNA, is dependent upon a functional H-NS protein. We therefore set out to investigate and compare the positioning of origin, SeqA, and replisome foci simultaneously in wild-type (EH02) and H-NS mutant (SF154) cells. We used growth conditions that gave similar DNA contents and cell cycle patterns for wild-type and H-NS mutant cells (glycerol and glucose-CAA media, respectively), and cells were grown at 28°C to an OD of ~0.15 before samples were collected for flow cytometry and fluorescence microscopy analyses. Cells for microscopy studies were spread onto agarose pads (1% containing phosphate-buffered saline) mounted onto microscopy slides before images were acquired. Figure 2 shows the cell cycle of wild-type cells grown in glycerol medium (Fig. 2A), the cell cycle of H-NS mutant cells grown in glucose-CAA medium (Fig. 2D), representative fluorescence images of cells during the cell cycle (Fig. 2B and E), and the localization of foci throughout the cell cycle (Fig. 2C and F). We used the publicly available Coli-Inspector project (under the ObjectJ plug-in in Fiji software [76]) to stack fluorescence images of cells (in the horizontal position) according to cell length/relative age for each channel in Fig. 2C and F. Images showing a typical field of view for wild-type and H-NS mutant cells can be found in Fig. S2 in the supplemental material.

In wild-type cells, replication was initiated at two origins in the mother generation, at a relative age of ~0.9, and persisted into the current generation, where termination occurred at a relative age of ~0.7 ($\tau = 136$) (Fig. 2A). As shown in Fig. 2B and C, a newborn cell typically had one origin, one SeqA focus, and one replisome focus colocalized at the midcell. The origin focus split into two foci around the relative age of 0.3 to 0.5 and was gradually segregated to quarter positions before cell division. The SeqA and replisome foci were observed as one focus at the midcell or two closely spaced foci at the midcell during relative ages between ~0.2 and ~0.8. Most cells above the relative age of ~0.8 had SeqA and replisome foci colocalized at origin regions at quarter positions after the formation of the septum.

Replication in H-NS mutant cells had a similar pattern. Initiation of replication occurred at two origins in the mother generation at a relative age of ~0.9 and terminated in the current generation at a relative age of ~0.8 ($\tau = 88$) (Fig. 2D). In H-NS mutant cells, the placement of fluorescent foci seemed less systematic during the cell cycle than in wild-type cells (Fig. 2E and F). The youngest cells typically had origin, SeqA, and replisome foci near the midcell, and most old/dividing cells had foci close to quarter positions. However, at ages in between, we found a large variety of localizations of foci.

In order to get a better overview of the positioning of foci in wild-type and H-NS mutant cells, we used Coli-Inspector to plot fluorescence intensity profiles for each channel (along the cell long axis) in groups of increasing cell ages (Fig. 3A and B). For wild-type cells, the localization of foci followed a clear pattern during the cell cycle, as described above (Fig. 3A). In contrast, H-NS mutant cells showed a chaotic distribution of foci, especially for SeqA and the replisome (Fig. 3B). For example, at relative ages of 0 to 0.75, when wild-type cells showed a unison distribution of SeqA and replisome foci near the midcell, H-NS mutant cells displayed a wide variety of localizations of SeqA and replisome foci. This resulted in multiple intensity peaks within groups of cells. It can also be seen that the origin foci were more variable in localization than those in wild-type cells although not as extreme as those of the SeqA and replisome foci. After a relative age of ~0.75, most cells had origin foci near the quarter positions, but SeqA and replisome positions were again less systematic. Right before cell division, many of the cells appeared to contain SeqA foci close to the midcell instead of at the quarter positions. Taken together, these results indicate that origin, SeqA, and replisome foci are misplaced in the cell when the H-NS protein is missing.

The distance between SeqA and the replisome is increased by ~100 nm on average in H-NS mutant cells. We have previously shown that there is an average distance of 200 to 300 nm between the replisome and the large SeqA structure trailing it, which may correspond to several thousands of base pairs of DNA (53). Since H-NS is able to form hairpin loops on DNA *in vitro*, we wondered whether an increased distance between SeqA and the replisome could be observed in H-NS mutant cells *in vivo* as a result of decondensed DNA between the two structures. In order to investigate this, we used a Python-based script that automatically measures distances between the nearest neighboring foci in two separate channels (53), in our case between foci of SeqA and the replisome. Images of wild-type cells (EH02 in glycerol medium) and H-NS mutant cells (SF154 in glucose-CAA medium) were subjected to extensive analysis with this script. Cells from each strain were divided into five age groups (defined by relative cell length), and the average distance between SeqA and the replisome was found for each age group as well as for the total population (Table 2). From these data and from results of two equivalent experiments (Table 3), it can be seen that the total average distances between SeqA and the replisome were increased by 83, 105, and 87 nm in H-NS mutant cells compared to wild-type cells. The average distance between SeqA and the replisome for wild-type cells was similar to what was shown previously (200 to 300 nm) (53).

By looking at the different age groups of wild-type cells, it was clear that the youngest and the oldest cells had the shortest average distances between SeqA and the replisome (Table 2). The greatest distance between SeqA and the replisome was found for cells at the

FIG 2 Snapshot imaging of SeqA, replisome, and origin foci. (A and D) Cell cycle diagrams and schematic cartoons of replicating chromosomes of wild-type cells (EH02) grown in glycerol medium (A) and H-NS mutant cells (SF154) grown in glucose-CAA medium (D) obtained by flow cytometry analysis. The solid line indicates the C-period, whereas the dashed line indicates the D-period. The relative cell age is indicated from 0 to 1. (B and E) Snapshot images of representative wild-type (EH02) (B) and H-NS mutant (SF154) (E) cells showing the formation of discrete SeqA (pseudocolored green), replisome (pseudocolored cyan), and origin (pseudocolored red) foci. The cells are ordered from youngest (top) to oldest (bottom) based on relative cell length. (C and F) Integral fluorescence of each wild-type (EH02) (C) and H-NS mutant (SF154) (F) cell stacked and plotted as a function of cell length from youngest (top) to oldest (bottom), as determined by using Coli-Inspector. Each cell is displayed as a horizontal line. Approximately 300 cells from each strain from one independent experiment were analyzed. The experiment was reproduced twice.

relative age of 0.2 to 0.4, and this corresponds well to what was observed previously at time points when cells are about halfway through the replication period (53). In contrast, H-NS mutant cells showed higher average distances between SeqA and the replisome in all age groups than did wild-type cells. The standard deviations were also significantly increased, which indicates that the positioning of SeqA and the replisome relative to each other was

much more variable. As might be expected, it was difficult to find a pattern in distances between SeqA and the replisome in relation to cell cycle events. The distances between SeqA and the replisome simply increased with increasing cell length. For example, in the age group where initiation of replication is expected to occur (age of 0.8 to 1), we found the highest average distance between SeqA and the replisome for H-NS mutant cells.

FIG 3 Fluorescence intensity profiles of SeqA, replisome, and origin foci. (A and B) Fluorescence intensity profiles of SeqA (green lines), replisome (cyan lines), and origin (red lines) foci in wild-type cells grown in glycerol medium (A) and H-NS mutant cells grown in glucose-CAA medium (B), plotted according to position on the cell long axis and grouped into eight age groups (based on relative cell length) from youngest (top) to oldest (bottom), as determined by using Coli-Inspector. The relative cell age is indicated from 0 to 1 next to the intensity profile plots. The analyzed cells are the same as those shown in integral fluorescence plots in Fig. 2. (C) Number of SeqA foci per cell for wild-type and H-NS mutant cells at ages of 0 to 0.4 (containing two replication forks). Error bars represent standard errors of the means. Approximately 320 cells from each strain were analyzed.

TABLE 2 Average distances between SeqA and the replisome for wild-type (EH02) cells grown in glycerol medium and H-NS mutant (SF154) cells grown in glucose-CAA medium

Age	Avg distance between SeqA and replisome (nm) $\pm$ SD ^a	
	Wild type	H-NS mutant
0–0.2	178 $\pm$ 104	241 $\pm$ 202
0.2–0.4	271 $\pm$ 176	311 $\pm$ 277
0.4–0.6	234 $\pm$ 176	355 $\pm$ 335
0.6–0.8	267 $\pm$ 225	407 $\pm$ 345
0.8–1	206 $\pm$ 107	568 $\pm$ 414
Total population	239 $\pm$ 168	322 $\pm$ 292

^a Distances that exceed one-third of the cell length are not included. Standard deviations represent values from one experiment. Age groups are based on relative cell length. Approximately 200 cells from each strain were analyzed.

The close localization of sister SeqA complexes is not affected by the lack of H-NS. In previous work, we found that SeqA structures bound to newly replicated sister DNA molecules are situated close together (<30 nm apart) (53). We wanted to investigate whether this was also the case for H-NS mutant cells or if the H-NS protein could be involved in the close tethering of sister SeqA complexes. If so, the two sister SeqA complexes might be seen far apart in H-NS mutant cells. The number of SeqA foci per cell for wild-type and H-NS mutant cells was therefore counted (Fig. 3C). To ensure that only cells with two replication forks (one replicating chromosome) were included in the study, we counted the foci in young cells at the relative age of 0 to 0.4 and excluded the rest. We found that, similarly to wild-type cells, H-NS mutant cells contained mainly one or two SeqA foci in this period of the cell cycle (Fig. 3C). This means that SeqA structures on sister DNA are closer together than what can be resolved by microscopy. Thus, we do not find evidence that H-NS affects the colocalization of sister DNA molecules. Small differences (not detected here) in sister SeqA distances between wild-type and H-NS mutant cells cannot, however, be ruled out. H-NS mutant cells were found to have a higher percentage of cells with two foci than wild-type cells. This indicates that the two pairs of SeqA structures (following two replication forks) were not kept together to the same degree as in wild-type cells.

H-NS mutant cells do not exhibit distinct, condensed nucleoid shapes characteristic of growth in rich medium. From our results so far, it is reasonable to assume that nucleoids of H-NS mutant cells will appear different from those of wild-type cells. We

TABLE 3 Average distances between SeqA and the replisome obtained from three independent experiments

Strain	Medium	Avg distance between SeqA and SSB (nm) $\pm$ SD ^a		
		Expt 1	Expt 2	Expt 3
Wild type	Glycerol	239 $\pm$ 168	192 $\pm$ 196	212 $\pm$ 216
H-NS mutant	Glucose-CAA	322 $\pm$ 292	297 $\pm$ 290	299 $\pm$ 250

^a Standard deviations indicate values from one experiment. Numbers of cells included in the analyses were 198 and 147 wild-type and mutant cells, respectively, for experiment 1; 87 and 73 wild-type and mutant cells, respectively for experiment 2; and 185 and 170 wild-type and mutant cells, respectively, for experiment 3. The differences in the average distances between SeqA and the replisome in wild-type cells and those in H-NS mutant cells were 83 nm in experiment 1, 105 nm in experiment 2, and 87 nm in experiment 3.

FIG 4 Snapshot imaging of nucleoids in fixed and living wild-type and H-NS mutant cells grown in glycerol, glucose-CAA, and LB media. Shown is snapshot fluorescence imaging of nucleoids in ethanol-fixed wild-type (EH02) and H-NS mutant (SF154) cells stained with Hoechst dye (pseudocolored magenta) and in living wild-type (EH20) and H-NS mutant (EH23) cells (pseudocolored red) containing an HU-mCherry tag. The cells were grown exponentially in glycerol (top rows), glucose-CAA (middle rows), and LB (bottom rows) media.

therefore visualized the nucleoids of H-NS mutant and wild-type cells under the microscope. Cells were grown to an OD of ~ 0.15 and prepared as explained above. Images of representative fixed and living cells grown in glycerol, glucose-CAA, and LB media can be found in Fig. 4.

Fixed cells from strains EH02 (wild type) and SF154 (H-NS mutant) were stained with Hoechst dye (Fig. 4, magenta). In glycerol medium, the Hoechst-stained nucleoids had a rod-like and extended shape, for both wild-type and H-NS mutant cells. In some cells, the H-NS mutant nucleoids appeared slightly spiraled compared to those of wild-type cells, but except for this, we did not observe any significant differences. In glucose-CAA and LB media, however, it was striking that the nucleoids of wild-type cells became structurally more distinct and condensed. This was especially prominent in LB medium, where the cells contained 2 to 4 round or oval-shaped nucleoids. The change in nucleoid characteristics from slow to rapid growth was also reported previously (reviewed in reference 80) but was not observed for H-NS mutant cells. The appearance of H-NS mutant nucleoids remained similar during growth under nutrient-poor and nutrient-rich conditions. We also investigated whether the nucleoids of EH02 cells were different from those of the background strain AB1157 but did not find any differences (see Fig. S4 in the supplemental material).

To check that fixation and Hoechst staining of cells did not produce artificial nucleoid features, we additionally investigated

nucleoid characteristics in living cells containing fluorescently tagged HU protein (HupA-mCherry). The strains utilized were EH20 (wild type) and EH23 (H-NS mutant). Flow cytometry histograms of EH20 and EH23 cells were similar to those of EH02 and SF154 cells, respectively, and can be found in Fig. S3 in the supplemental material (compare Fig. S1 and S3 in the supplemental material). The nucleoid shapes found in living cells were similar to those found in fixed cells (Fig. 4, red). H-NS mutant cells displayed rod-shaped or slightly spiraled nucleoids in all three media, whereas the shape of the wild-type nucleoids became more condensed and characteristic under nutrient-rich conditions, as described above for fixed cells.

Taken together, our results indicate that there are no major differences between wild-type and H-NS mutant nucleoids under growth conditions where cells from the two strains contain comparable DNA concentrations and exhibit similar cell cycle patterns (wild-type cells in glycerol medium versus H-NS mutant cells in glucose-CAA medium). However, H-NS mutant cells were not capable of adopting the distinct nucleoid shape characteristic of wild-type cells growing in rich medium. Thus, the results confirm that the three-dimensional organization of nucleoids is normally very different under nutrient-rich and nutrient-poor conditions and indicate that H-NS mutant cells retain a type of “poor-nutrient-like” DNA organization with a low DNA content in rich medium.

DISCUSSION

H-NS might be needed for handling physiological concentrations of DNA during rapid growth. Here we have shown that cells of an H-NS mutant strain have a low DNA content compared to that of wild-type cells in three different media (Table 1 and Fig. 1). Moreover, H-NS mutant cells were not able to increase their growth rate above that found in glucose-CAA medium, even when nutrients were more abundant (Fig. 1). These effects may indicate that cells of the H-NS mutant are not capable of containing physiological levels of DNA during growth under nutrient-rich conditions.

From the data shown in Fig. 4 and reported in previous studies (reviewed in reference 80), we have seen that the appearance of nucleoids is different in slowly and rapidly growing wild-type cells. Nucleoids of rapidly growing cells are typically condensed into distinct shapes, while slowly growing cells show a more extended and diffusive configuration of the DNA. This means that in spite of increased amounts of DNA during rapid growth, the nucleoids occupy a relatively small spatial volume and are thus very highly condensed. Moreover, the DNA seems to adopt specific three-dimensional conformations throughout the cell cycle. Since H-NS mutant cells do not change the shape of their nucleoids from nutrient-poor to nutrient-rich conditions, this leads us to speculate that H-NS may be more prominently involved in the architecture of nucleoids during rapid growth and that there are greater requirements for DNA condensation and organization under such conditions. A previous study showed that *hns* transcription is dependent upon ongoing DNA synthesis, in order to maintain a relatively constant H-NS/DNA ratio (81). This indicates that there is a demand for H-NS during exponential growth and, consequently, that this demand is higher in rapidly growing cells with high DNA content. If we think about H-NS as a DNA-organizing protein, it is not hard to imagine why cells may reduce their DNA content and adapt to a more “simple” organization of the nucleoid if H-NS is missing. From our nucleoid images, this organization appeared similar to the organization seen in wild-type cells during slow growth in glycerol medium (Fig. 4). However, structural details are difficult to spot due to limited optical resolution. Moreover, image analysis of origin, replisome, and SeqA structures reveals that this is not the case (see below).

H-NS contributes to proper organization of DNA during replication and segregation. As shown in Fig. 2 and 3, the positioning of SeqA, replisome, and origin foci was aberrant in H-NS mutant cells compared to that in wild-type cells with similar replication patterns and DNA contents. SeqA and replisome foci appeared particularly disorganized, with localization patterns scattered throughout large parts of the relative cell length (Fig. 3). However, origin regions also displayed variable positioning, particularly in cells at relative ages between 0.3 and 0.75, which is perhaps more easily noticed by comparing cell stacks of fluorescent origin foci in Fig. 2C and F.

Although foci of SeqA and replisome structures do not represent specific locations on the chromosome, they tell us something about the organization of new DNA during replication. In wild-type cells, SeqA is mainly located at the midcell and at the quarter positions and trails the replication forks with a distance of 200 to 300 nm. As mentioned above, this clear pattern was not observed in H-NS mutant cells, in which the distances were increased by ~100 nm. Not only does this emphasize that newly replicated

FIG 5 Simplified model of the organization of the stretch of DNA between SeqA and the replisome in wild-type and H-NS mutant cells. Shown is a hypothetical illustration of the two replication forks of one replicating chromosome for wild-type (top) and H-NS mutant (bottom) cells, including SeqA and replisome structures. Newly replicated DNA is shown in gray, and old/unreplicated DNA is shown in black. The average distance between SeqA and the replisome is indicated in the illustration. Example cells from wild-type (EH02) and H-NS mutant (SF154) strains are included.

DNA is irregularly placed in the cell, it also leads us to hypothesize that H-NS is involved in organizing the stretch of DNA between SeqA and the replisome, possibly by bridging the DNA helices into loops (Fig. 5). Since H-NS binds all over the chromosome, the stretch of DNA between SeqA and the replisome could be representative of a more global situation of DNA organization by H-NS (i.e., that H-NS organizes DNA in a similar manner throughout the chromosome). This result provides *in vivo* evidence for the idea that H-NS forms “hairpin DNA” and contributes to the folding of DNA along adjacent segments.

Although H-NS affects the distance between the replisome and the SeqA structures trailing it, it does not seem to affect the close localization of SeqA structures located on sister DNA molecules (Fig. 3C). Thus, although H-NS may in theory be able to bridge sister DNA together, it cannot have a primary role in keeping sisters close on the stretch of DNA between the replisome and the SeqA structures. In other words, we do not find evidence for bridging of newly replicated sister DNA by H-NS.

Despite the fact that H-NS mutant cells have a chaotic localization of foci, it appears as if the regulation of cell division is properly maintained. We found no anucleate cells in the population, and as pointed out above, the origins eventually seem to find their quarter positions before the cells divide. The observation that H-NS mutant cells are somewhat more variable in size upon cell division emphasizes this, as it indicates that cell division may be delayed in some cells due to trouble with DNA organization and partitioning.

Complexity in interpreting the effect of the lack of H-NS. In addition to, or as a consequence of, modulating DNA topology, H-NS is also a global repressor of gene expression. This dual property of H-NS is intriguing since H-NS binds and condenses DNA (seemingly) quite nonspecifically but regulates gene expression in a specific manner. However, it also makes it difficult to elucidate what the “true,” or primary, effect of an H-NS deletion is. We have

presented results that indicate trouble in physiology and DNA organization when H-NS is missing. However, it may also be that this is a consequence of skewed gene expression or of a redistribution of other DNA-binding proteins.

Factors involved in replication initiation or DNA synthesis might be directly or indirectly up- or downregulated as an effect of the lack of H-NS. Recently, it was reported that a large number of genes are indirectly downregulated in strains lacking the H-NS protein, and interestingly, these were the genes which tended to have higher expression levels than others in wild-type cells (82). We and others (68, 79) find that the C-period represents a lower fraction of the cell cycle in H-NS mutant cells (Fig. 1). A logical explanation for this could be reduced expression of the DnaA protein, which leads to initiation at a higher mass. However, a previous study showed that transcription of the *dnaA* gene is in fact not downregulated when H-NS is missing (79).

One example of genes that have been reported to be upregulated upon deletion of H-NS is genes encoding ribonucleotide reductase (RNR) (*nrdA* and *nrdB*) (83). We therefore speculated whether the increased level of RNRs could be involved in the shortening of the C-period by facilitating a higher rate of deoxyribonucleotide formation. However, as a first step in this investigation, we found, in contrast to the results reported by Cendra et al. (83), that the levels of RNR were not increased (measured by Western blotting [data not shown]). Thus, RNR is not involved in the shortening of the C-period in H-NS mutant cells. It could be that DNA that is less organized/condensed is faster to replicate, since there are fewer obstacles (i.e., H-NS nucleoprotein complexes) that need to be removed in front of the replication fork. However, it should be pointed out that replication was indeed not fast for H-NS mutant cells in glycerol and LB media (compared to wild-type cells in glycerol and LB media), although the C-period represented a smaller fraction of the cell cycle. It may be that increased replication rates can be seen only when wild-type and H-NS mutant cells have a more similar growth rate. In any case, H-NS mutant cells spend less of their cell cycle on replication, regardless of the medium.

One probable effect of the loss of H-NS may be that RNA polymerase (RNAP) gains access to binding sites normally bound by H-NS and is consequently redistributed. Transcription may have a significant effect on chromosome organization, and genetic loci with high transcriptional activity form distinct foci with RNAP in rapidly growing cells (84, 85). Also, it has been shown that transcriptionally silent regions on the chromosome overlap those bound by NAPs, while transcriptionally active regions overlap binding sites for RNAP (86). Taken together, this indicates that genes are partitioned into distinct subregions of the cell depending on their transcriptional activity. Thus, it may be that the redistribution of RNAP and changes in transcriptional activity play a role in disrupting the positioning of DNA in H-NS mutant cells. Alternatively, it may be that impairment of DNA condensation (by loss of H-NS) is enough for DNA positioning defects to occur.

Although the alterations in DNA organization and cell physiology found for H-NS mutant cells may be difficult to validate as direct effects of the loss of the H-NS protein, we suggest that H-NS, directly and/or indirectly, plays a significant role in maintaining the proper organization of DNA, especially for supporting rapid growth with high DNA concentrations.

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