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Intracellular Location of the Histonelike Protein HU in *Escherichia coli*

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Immunocytochemical labeling of thin sections of cryosubstituted, Lowicryl-embedded *Escherichia coli* cells with protein A-colloidal gold was used to study the structural organization of the bacterial nucleoid. We found that the histonelike protein HU was not associated with the bulk DNA in the nucleoid but was located in areas of the cell where metabolically active DNA is associated with ribosomes and where single-stranded DNA, RNA polymerase, and DNA topoisomerase I were also located. The resolution of the methods used did not allow us to decide whether HU was associated either with ribosomes or with transcriptionally active DNA, nor could we demonstrate interaction of HU with either.

In procaryotes, the circular chromosome is organized into approximately 50 discreet domains or loops (for reviews see references 11 and 51). A similar organization of chromosomal DNA is found in eucaryotes, where the boundaries of the loops are presumably defined by tethering of the chromatin to a nuclear scaffold or other structure (2, 18). The molecular interactions defining the domains of supercoiling in bacteria, as well as the structure of bacterial chromatin, remain elusive despite intensive investigation (11, 51).

The wrapping of the DNA around the core histones in nucleosomes could account for the negative superhelicity of DNA purified from eucaryotes (64; for discussion, see reference 68). A number of studies show that the bulk of eucaryotic DNA is relaxed in vivo and is not under torsional strain (61, 64). The situation in eubacteria, on the other hand, is different. Psoralen-binding experiments indicate that approximately 50% of the in vivo supercoils are unrestrained and are torsionally stressed (63, 64). Biochemical and genetic evidence supports a homeostatic mechanism for the control of the level of DNA supercoiling in procaryotes (9, 47, 52). Bacterial DNA gyrase introduces negative supercoils into the DNA at the expense of ATP, while DNA topoisomerase I relaxes negatively supercoiled DNA. DNA topology is important in a number of cellular processes, and the maintenance of a fairly limited range of DNA supercoiling appears to be essential for *Escherichia coli* cell viability.

A number of DNA-binding proteins have been identified in procaryotes that resemble eucaryotic histones (3, 5, 28, 29, 40–42, 46, 55–57, 62, 72; for reviews see references 11, 12, 22, and 51). A role for these proteins in a nucleosomelike organization of bacterial chromatin has been suggested to account for the presence of restrained supercoils in vivo (5, 63, 64). The most abundant of these proteins, HU, exists as HU1 and HU2 in *E. coli* and forms dimers and tetramers in solution. There is about one HU dimer per 200 base pairs of DNA in vivo, and DNA-HU protein complexes resembling nucleosomes are produced in vitro when the relative amount of HU is about 10 times higher (5, 12, 58). This body of data and the electron microscopy demonstration of labile beaded structures from freshly lysed bacterial cells (21) and the protection of 120-base-pair fragments during nuclease digestion of bacterial nucleoids (67) suggest that a labile nucleosomelike structure of bacterial chromatin may exist. Drlica and Rouvière-Yaniv (12) have proposed a model for DNA wrapped around HU that accounts for both nuclease-sensitive sites generated in DNA-HU complexes and the observed decrease in plasmid linking number by HU.

Several proteins with properties and structure highly homologous to those of HU protein have been isolated from other procaryotes or bacteriophages, e.g., HB protein from *Bacillus globigii* (29) and transcription factor TF1 from *Bacillus subtilis* phage SPO1 (20).

A specific DNA-protein complex that might be the basic unit of bacterial nucleoid structure, however, has yet to be isolated. Indeed, the argument has been made that beaded DNA structures can be generated with ethanol dehydration of naked DNA samples (14). In addition, nuclease-protected DNA fragments larger than 120 base pairs, which are typically observed with eucaryotic chromatin, have not been detected. Protein HU dissociates from double-stranded DNA (dsDNA) at much lower salt concentrations (5) than those found in vivo (1, 15). Therefore it has been suggested (5) that the HU-DNA complexes in vivo are very dynamic structures, resembling perhaps eucaryotic half-nucleosome structures (69).

Electron microscopy studies have shown that various non-eucaryotic DNA-containing plasmas are much more sensitive to cytological fixation and dehydration-induced aggregation than the interphase nuclei of eucaryotic cells (32, 37). This cytological evidence is also indicative of fundamental differences in the structural organization of eucaryotic and procaryotic DNA-containing plasmas. Particular types of fixation were necessary to maintain the fine-stranded, fibrillar appearance of aggregation-sensitive DNA-containing plasmas (60; reviewed in reference 33). These fixatives have been shown to produce gels when observed macroscopically (34).

It is known that typical electron microscopy fixatives, such as aldehydes and OsO_4, induce cellular ion leakage (70; M. L. J. Moncany, Thèse d'État, Université Paris VII, 1982), even when used under conditions that avoid solvent-induced aggregation. As a consequence of such changes in ionic conditions, the morphology of the nucleoid could be

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altered. It was recently found that cryosubstitution of cryofixed bacterial cells substantially reduced the solvent-induced aggregation of the DNA in comparison to that observed with standard methods at higher temperatures (35). In this procedure, growing cells are rapidly frozen (cryofixed), and the intracellular water, in the form of vitreous ice, is substituted with acetone containing glutaraldehyde at 185 K. The chemical fixative acts on the biological material at very low temperature in a virtually water-free medium. Rearrangements in the cell are strongly reduced by this procedure (26), as is evident when comparing thin sections of these cells with sections of frozen hydrated cells (13). The bacterial nucleoid, in thin sections of such cryosubstituted cells, is relatively dispersed throughout the cell and is filled with a very fine granular-fibrillar plasma, the aspect of which is quite different from the fibrillar one observed previously with chemical fixation (26).

The degree of dispersion of the bacterial chromatin within the cytoplasm might be the functional consequence of the absence of a nuclear membrane in bacteria, as transcriptional activity is coupled with translation (48). These considerations have been discussed previously (26, 71) and will be found in more detail in a forthcoming reviewing paper (E. Kellenberger, *in* K. W. Adolph, ed., *Chromosomes: Eukaryotic, Prokaryotic, and Viral*, in press).

The lack of information as to the chemical basis of the structural organization of the bacterial nucleoid prompted us to apply a combination of the two newly developed electron microscopy methods of cryosubstitution (16, 25) and immunolabeling with colloidal gold particles (54) to this problem. In two previous reports (4, 27), we have shown that different structural states of the *E. coli* DNA can be distinguished with specific antibodies. dsDNA is contained within the ribosome-free spaces, currently referred to as the bulk DNA or the DNA-plasma of the nucleoid. Single-stranded DNA was found located over the ribosomes along the periphery of the ribosome-free area (27).

By employing improved methods with a higher efficiency of labeling, we have continued our study of bacterial chromatin in an attempt to determine whether protein HU is a structural component of the nucleoid. In this paper, we show that this protein is found primarily over the ribosomal areas which are adjacent to the nucleoid and is not globally distributed throughout the DNA-plasma. The possible functional consequences of these results are discussed.

MATERIALS AND METHODS

Culture conditions. *E. coli* cells were grown in M9 minimal medium supplemented with 1% Casamino Acids, with aeration at 37°C, to a cell density of 2×10^8/ml and 5-ml cultures were harvested by filtration over a 0.22-μm Nucleopore filter. The cells were subsequently frozen as described in the cryofixation section.

E. coli cells infected with bacteriophage T4 mutants T4.43 (*B*am22) and T4.21 (*N*am90) were also prepared. Phage T4.43 (*B*am22) contains an amber mutation in gene 43, which codes for the phage DNA polymerase. No phage DNA is synthesized in cells infected with this mutant, although the bacterial chromosomal DNA is still degraded. In cells infected with phage T4.21 (*N*am90), containing a mutation in gene 21, the host DNA is degraded and phage DNA is synthesized. However, in the absence of gene product 21, aberrant phage proheads are assembled and the viral DNA is not packaged. As a result, huge pools of T4 DNA accumulate within the cell. Bacterial cell cultures were infected with phage T4.43 (*B*am22) at a multiplicity of infection (MOI) of 0.7. At 5 and 10 min postinfection (p.i.), the number of survivors was determined. At 10 min p.i., the cells were harvested by filtration as described above. In the case of phage T4.21 (*N*am90), a 5-ml culture was infected at an MOI of 5. Superinfection of the same culture at 8 min p.i. results in cell lysis inhibition and continued synthesis of phage precursor material. The infected cells were incubated for an additional 60 min and then harvested by filtration as described above.

E. coli cells were treated with chloramphenicol (25 μg/ml; purest grade, a gift from Parke Davis), incubated with aeration at 37°C for 60 min, and then harvested by filtration as described.

Cryofixation, cryosubstitution, and embedding. The essential parts of the methods used have been described previously (27): cells were harvested by filtration, collected on Job no. 807S cigarette paper, and immediately frozen either by slamming them on a polished copper block at 6 K (micrographs in Fig. 1, 5, and 8 [16, 27]) or immersion at 4 m/s into a 4:1 propane-isopentane mixture (30) at 77 K (micrographs in Fig. 2, 4, and 7).

Samples were substituted in acetone containing 3% (vol/vol) glutaraldehyde in the presence of a molecular sieve (0.4 nm; Perlform, Merck) at 185 K for 90 h. The temperature was then raised to 233 K for 6 h, and the samples were embedded in Lowicryl K4M or HM20 resins at 233 K by successive infiltration in the following mixtures: 100% acetone for 1 h; Lowicryl-acetone (1:1) for 2 h; Lowicryl-acetone (2:1) for 2 h; Lowicryl for 2 h; and overnight for 16 h in Lowicryl. The next day, the samples were infiltrated for 4 h with fresh Lowicryl and placed in gelatin capsules, and the resin was polymerized by indirect UV irradiation (360 nm) for 24 h at 233 K. This was followed by further hardening at room temperature for 3 days in UV light. For samples embedded in HM23, the procedure was identical except that resin infiltration and initial polymerization were done at 213 K. Thin sections for use in immunolabeling experiments were cut with a diamond knife on an LKB Ultramicrotome III.

Antisera. The HU antisera used in these experiments were prepared in collaboration with A. Kelus (Basel Institute for Immunology). A mixture of HU1 and HU2 protein was purified from *E. coli* as described by Imber et al. (29). The pure proteins were crosslinked with glutaraldehyde and injected into rabbits (53). Two polyclonal sera (12286 and 14585) were obtained after booster injections. The specificities of the sera were determined by immunoblot analysis of whole-cell extracts separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The rabbit *E. coli* DNA topoisomerase I antiserum was a kind gift of James C. Wang (Harvard University) and the goat anti-RNA polymerase serum was a kind gift of H. Heumann (Max Planck Institute für Biochemie, Martinsried b. Munich).

Protein A-colloidal gold complex preparation. Colloidal gold (approximately 14 nm in diameter) was prepared as described in Hobot et al. (27), according to the method of Frens (17), by reduction with sodium citrate. Tetrachlorogold III-acid trihydrate (Merck) was added to 80 ml of twice-distilled water to a final concentration of 0.01% and then boiled. Following the addition of 4 ml of a 1% (wt/vol) solution of trisodium citrate, the mixture was swirled gently and allowed to simmer until a reddish color developed (approximately 8 min). The mixture was allowed to cool to room temperature, and the pH was adjusted to 5.8 with 1 M HCl. One milliliter of a 0.5-mg/ml solution of protein A

(Sigma) was then added to the colloidal gold, with gentle stirring. After 3 min, 3,2 ml of 1% (wt/vol) polyethylene glycol (PEG) 20,000 was added, and the mixture was stirred for an additional 15 min. The protein A-colloidal gold complexes were centrifuged for 1 h at 60,000 × *g* (4°C) and suspended in 1.5 ml of 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 2 mg of PEG 20,000 per ml and 0.02% NaN_3. This concentrated solution was stored at 4°C.

Standard two-step immunolabeling procedure. The protein A-gold method described by Roth (54) was used to determine the localization of HU after nucleoid disruption (See Fig. 5). Following incubation for 10 min on a drop of ovalbumin solution (2.5% in PBS, pH 7.4) to saturate nonspecific protein A-binding sites, the grids with sections were then placed for 1 h on a drop of diluted antiserum (200× in PBS). The grids were washed with PBS and placed for 1 h on a drop of diluted protein A-gold (to an OD_{520} of 0.2 in PBS). The washing steps were done as jet washes of the section side only. The grids were shortly blotted on filter paper and stained on 2% aqueous uranyl acetate (30 min for bacterial specimens) and Millonig's lead citrate (1 min).

Precoupling of protein A-gold to IgG. The precoupling was performed as described (M. Dürrenberger, J. Electron Microsp. Tech., in press). Polyclonal rabbit serum (2 μl) was diluted 100-fold into 200 μl of PBS containing 1% bovine serum albumin (BSA; fraction V), and 25 μl of concentrated (OD_{520} = 4) protein A-gold (14 nm), prepared as described by Roth (54), was then added. The mixture was incubated for 2 h at 25°C, and the reddish color typical of colloidal gold was lost.

The small aggregates were then centrifuged for 2 min at 12,000 × *g*. The supernatant was discarded, and 100 μl of PBS–1% BSA was added to resuspend the pellet. This centrifugation and resuspension was repeated twice, each time in PBS–1% BSA. Following a third centrifugation step, the pellet was resuspended in 400 μl of PBS–1% BSA and sonicated at 35 kHz with a dip sonicator. After a few seconds of sonication, the reddish color reappeared. However, sonication was continued for 2 min to ensure optimal dispersion. Care was taken to avoid heating. The final solution was adjusted to an OD_{520} of about 0.1 with PBS–1% BSA and used immediately.

One-step immunolabeling procedure with precoupled antibodies. In immunolabeling experiments involving the use of precoupled antibodies (presented in Fig. 1, 2, 4, 7, and 8), the grids with sections were incubated for 10 min on a drop of ovalbumin (2.5% in PBS) as before and were then directly placed on a drop of freshly sonicated precoupled IgG-protein A-gold for 1 h. During the whole labeling procedure, the grids were kept dry on the side without thin section and the procedure was performed in a humid chamber. The grids were then jet washed with PBS and put on a drop of PBS containing 1% glutaraldehyde for 5 min to fix the complexes to the antigens and protect them from dissociation by low pH solutes (Dürrenberger, in press). An intensive jet wash with bidistilled water followed. The grids were blotted on filter paper and stained on 2% aqueous uranyl acetate (25 min for bacterial specimens) and Millonig's lead citrate (1 min).

Statistical analysis. The micrographs were printed at a final magnification of 60,000×. A square network, corresponding to 25 lines/μm on the specimen, was placed over the prints, resulting in 625 squares per μm^2 and a resolution of 40 nm. Each of the single squares was judged to belong to one of the following classes: (i) gold particles present on bulk DNA, (ii) bulk DNA without gold particles, (iii) gold particles on the ribosomal area or cytoplasm and (iv) ribosomal area without gold particles. Gold particles were determined to be in a square only if the center of the particle laid within it. A number of equal-sized bacteria were evaluated in this manner, and average values ± the standard deviation (SD) were calculated.

For finer localization of the label with respect to structure, another statistical analysis was used in which 100 randomly oriented (sized) bacteria were chosen and the label belonging to one of the following classes was counted: (i) gold particles present on bulk DNA; (ii) gold particles over the "border zone," i.e., a 60-nm-wide zone over the ribosomes and contiguous to the bulk DNA of the nucleoid; and (iii) gold particles over the remaining ribosomal area, i.e., the cytoplasm. The number of counts in each category was then divided by 100.

RESULTS

Ultrarapid freezing and cryosubstitution of exponentially growing *E. coli* cells results in enhanced preservation of the bacterial nucleoid structure. The nucleoid exhibits a multicleft appearance and is dispersed throughout the ribosome-containing cytoplasm (4, 26, 27). These aspects of bacterial cell structure are more consistent with coupled transcription and translation in bacteria than the more confined nucleoid structure obtained when applying standard electron microscopy techniques to cells grown in medium containing 0.1 to 0.3 M salt (31; Kellenberger, in press). Immunolabeling of these sections with specific antibodies and colloidal gold particles (4, 27) identified and statistically verified the ribosome-free spaces as containing dsDNA and localized single-stranded DNA over the ribosomal areas contiguous to the bacterial nucleoid. Nascent RNA has previously been observed around the nucleoid periphery (59). Results obtained by immunolabeling thin sections of cryosubstituted exponentially growing *E. coli* cells with antibodies specific for protein HU are presented in Fig. 1. In Fig. 1a and b, two different polyclonal HU antibodies were used. The most electron-dense material (the darkest particles) represent colloidal gold particles associated with the IgG. By visual inspection of a limited number of representative micrographs, it was difficult to determine objectively the exact location of the label. Due to the high degree of dispersion of the nucleoid among the ribosomes, it was hard to clearly distinguish the location of label over the ribosomes or the ribosome-free space. In order to address these questions more objectively and present the data in a more readily interpretable form, we have made use of an unambiguous method for counting gold label on thin sections. A grid, which consists of a net of squares, each large enough to contain only 1 to 3 ribosomes, is laid over a micrograph, as shown in Fig. 2. In each cell section, the squares were categorized as containing bulk DNA (ribosome-free spaces) with gold particles, bulk DNA without gold particles, ribosomes with gold particles, or ribosomes without gold particles and were counted. In cases where a grid line bisected an individual gold particle, assignment to a specific square was based on the position of the center of the particle.

The data are shown in Table 1. About 80% of the squares contained ribosomes, and the remaining 20% were over the bulk DNA. Of the squares situated over this bulk DNA, only 6% contained gold. Of the squares situated over ribosomes, 37% contained gold. These results show that virtually no label is located over the bulk DNA, while 96% of the squares containing gold particles also contained ribosomes.

In another type of statistical analysis (see Table 3), gold label in numerous bacterial sections was categorized accord-

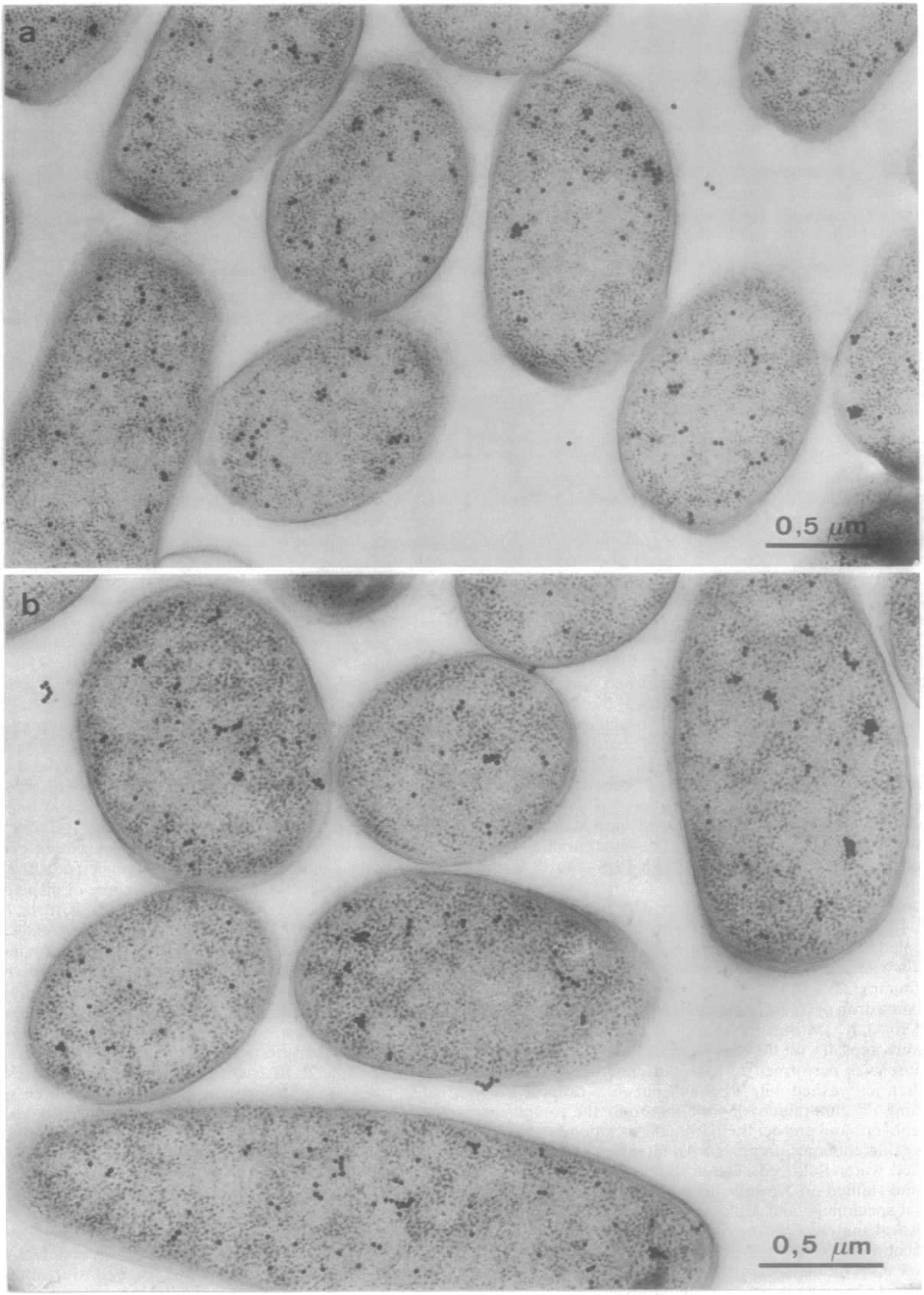

FIG. 1. Immunocytochemical localization of protein HU with serum 12286 (a) and serum 14585 (b) on *E. coli* B cells. (a) Bacteria were cryofixed, substituted, embedded in Lowicryl HM20 at −40°C, and labeled with the precoupled method. (b) Bacteria were cryofixed, substituted, embedded in Lowicryl HM23 at −60°C, and labeled with the precoupled antibodies. Label is localized over the ribosomal area. The background was lower than 0.1 counts per μm^2. For statistical evaluations, see Tables 1 and 3.

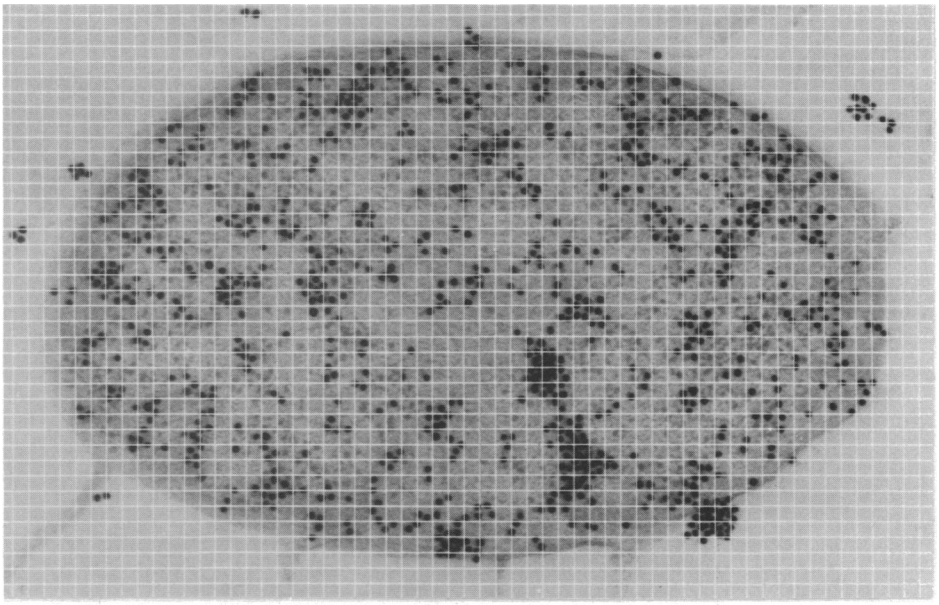

FIG. 2. Network of 25 lines per μm was put over bacterial sections for statistical evaluation. Each square was judged to belong to one of the following categories: gold present on bulk DNA; bulk DNA without gold; gold with ribosomes; or ribosomes without gold.

ing to its location over the bulk DNA, over the ribosomes in the border region (of about 60 nm width), or over ribosomal areas further away from the DNA plasma. The results demonstrate that most of the HU label was in the border region.

While the methods employed in these experiments resulted in better preservation of cellular structures (4, 26, 27, 35), the possibility of other artifacts remains. For example, some of the HU epitopes recognized by our polyclonal antibodies might reside in protein domains that are altered during sample preparation. As a result, the distribution of gold particles might be representative of only a subpopulation of the HU protein found within the cell. Two different polyclonal antibodies that recognized only protein HU in immunoblots of whole-cell extracts (Fig. 3) were used in Fig. 1. Although the use of polyclonal IgG maximized the number of epitopes that might be recognized and ultrarapid freezing increased structural preservation, we cannot exclude the possibility of conformational changes of HU.

An additional consideration is that binding of the HU protein to dsDNA in a nucleosomelike fashion may prevent the IgG from recognizing the HU protein, because the HU epitopes would be covered by the DNA, while free HU or HU bound to single-stranded nucleic acids or to ribosomes might be detected. These questions were addressed in the following two sets of experiments. In the first we attempted

TABLE 1. Gold particle counts of HU-immunolabeled (serum 14585) *E. coli* B cells

Cell no.	No. of squares (% of total)			
	Gold on bulk DNA	Bulk DNA without gold	Gold on ribosomal area	Ribosomal area without gold
1	6 (3.1)	42 (22.3)	69 (36.7)	71 (37.7)
2	1 (0.5)	25 (13.8)	66 (36.6)	88 (48.8)
3	3 (1.5)	46 (23.0)	63 (31.5)	88 (44.0)
4	5 (1.3)	80 (21.8)	137 (37.4)	144 (39.3)
5	4 (1.8)	39 (18.1)	70 (32.5)	102 (47.4)
6	2 (1.1)	25 (14.2)	55 (31.4)	93 (53.1)
7	2 (1.0)	49 (25.2)	48 (24.7)	95 (48.9)
8	3 (1.4)	36 (17.1)	50 (23.8)	121 (57.6)
9	3 (1.4)	40 (19.2)	67 (32.2)	98 (47.1)
10	1 (0.5)	29 (13.4)	57 (26.5)	128 (59.5)
11	2 (1.0)	38 (20.3)	50 (26.7)	97 (51.8)
12	4 (2.2)	40 (22.9)	41 (23.5)	89 (51.1)
13	1 (0.4)	43 (18.6)	77 (33.4)	109 (47.4)
14	0	30 (15.2)	65 (32.9)	102 (51.7)
15	1 (0.4)	31 (12.8)	66 (27.2)	144 (59.5)
16	0	35 (18.2)	48 (25.0)	109 (56.7)
17	5 (2.3)	36 (17.2)	63 (30.1)	105 (50.2)
18	1 (0.5)	32 (18.0)	53 (29.9)	91 (51.4)
19	0	94 (20.3)	132 (28.6)	235 (50.9)
20	1 (0.6)	44 (24.7)	50 (28.0)	83 (46.6)
Avg % ± SD	1.23 ± 0.77	18.81 ± 3.7	29.93 ± 4.2	50.04 ± 5.8

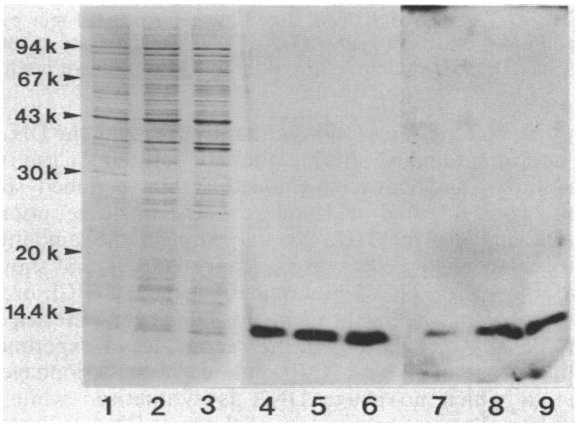

FIG. 3. SDS-PAGE of bacterial lysates together with their immunoblots with the two HU antisera (see text). Lanes 1, 4, and 7 are lysates of *E. coli* K-12; lanes 2, 5, and 8 are lysates of *E. coli* B; and lanes 3, 6, and 9 are lysates of *E. coli* B infected with T4. The corresponding anti-HU blots were performed with serum 14585 (lanes 4 to 6) and serum 12286 (lanes 7 to 9). Lanes 1 to 3 are Coomassie blue stained. Sizes are shown in kilodaltons.

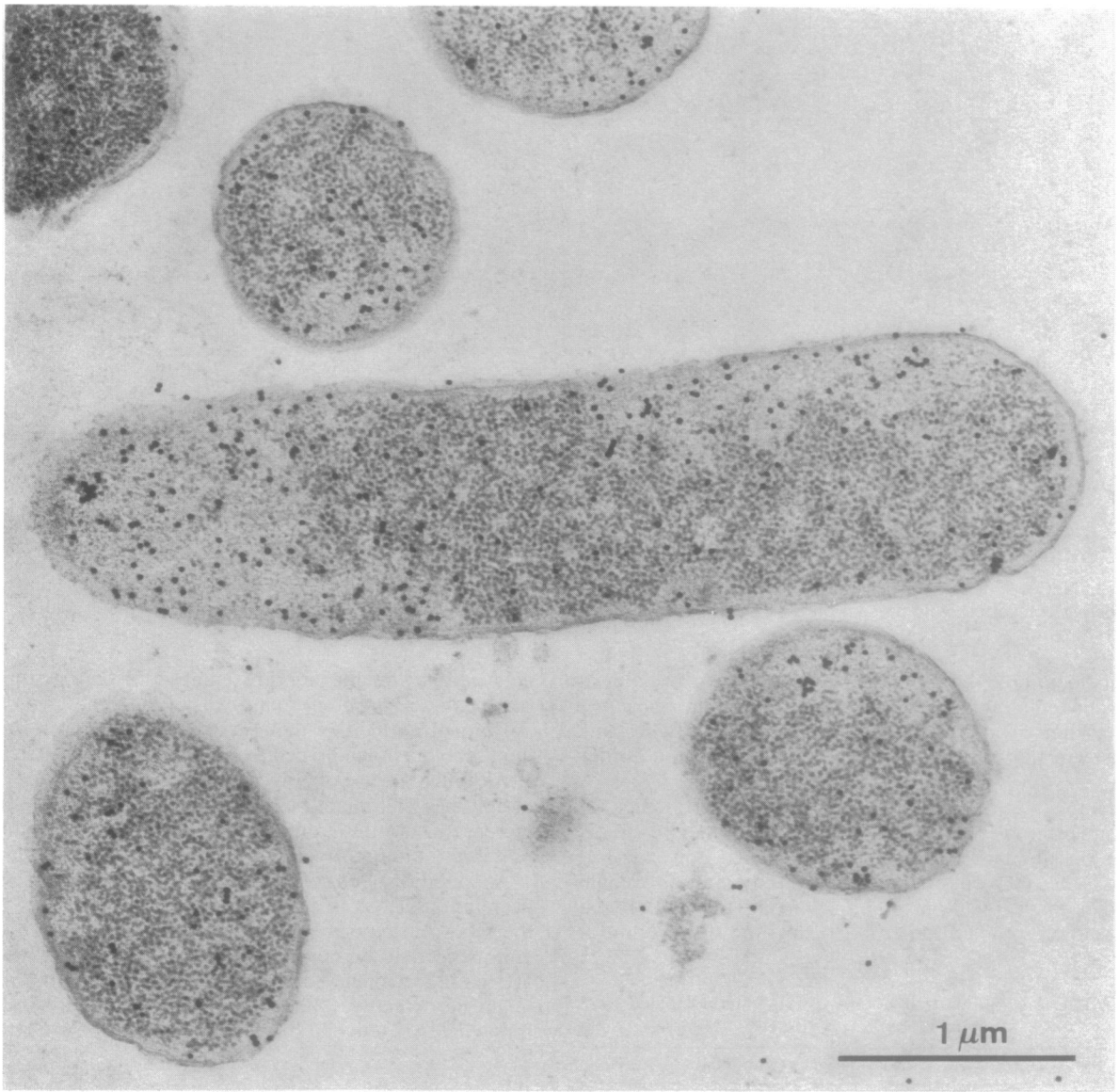

FIG. 4. T4-infected *E. coli* B cells were frozen 10 min p.i. and labeled against HU protein. The marginal vacuoles, containing the breakdown products of the host DNA, had a label density of 180 counts per μm^2, whereas the ribosomal area had a label density of 30 counts per μm^2. The HU protein followed the DNA breakdown products rather than binding to the ribosomes.

to reveal epitopes on sections either by digesting the DNA or by conformationally altering the HU protein. Cryofixed, substituted, and Lowicryl-embedded thin-sectioned specimens were digested with micrococcal nuclease prior to immunolabeling for HU. No increase in the amount or distribution of gold particles was detected (data not shown). Also, incubation on 1% glutaraldehyde, 0.01% HCl, or 8 M urea prior to labeling brought no significant difference in label density or location. In the second set of experiments we made use of two facts: (i) mutants of bacteriophage T4 exist in which no phage DNA is synthetised while the complete chemical breakdown of the host DNA still occurs and (ii) Rouvière-Yaniv and co-workers have shown that protein HU is still present in *E. coli* cells infected with bacteriophage T4 (Rouvière-Yaniv, personal communication). The results of Fig. 3 confirm that HU was detectable in immunoblots of whole-cell extracts of T4-infected cells.

Before discussing these experiments, we have to follow the reproductive cycle of bacteriophage T4. Infection leads to a breakdown of the nucleoid and to chemical degradation of the host DNA (24; reviewed in references 39 and 65). Within 3 to 6 min after infection, the breakdown products of the host DNA accumulate in marginal vacuoles (Fig. 4), which later disappear (38, 45, 49). On thin sections the newly synthesized phage DNA becomes visible some 15 to 20 min after infection as a "pool" which is morphologically indistinguishable from the nucleoid of an uninfected cell (38). By using a T4 mutant in which DNA packaging into proheads is inhibited, the pool of vegetative (replicating) phage DNA becomes very strongly enlarged.

When thin sections of T4-infected cells were immunolabeled with precoupled IgG-protein A-gold complexes, we observed the following: 6 min p.i., HU protein was localized together with the breakdown products of the host DNA in the marginal vacuoles (Fig. 4). There were approximately 180 gold particles per μm^2 over the marginal vacuoles and 34

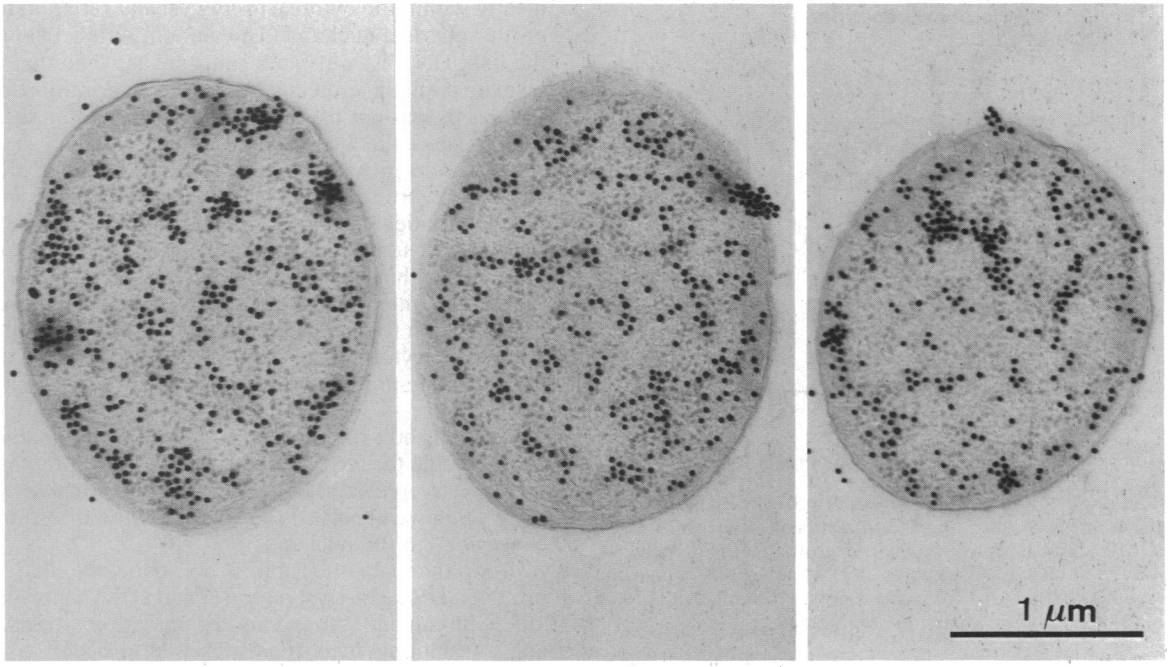

FIG. 5. Intracellular localization of the HU protein on the ribosomal area 60 min after infection of *E. coli* B with T4.21(*N*am90). After T4 infection, the protein HU was not degraded and was found at the same location as in uninfected cells: The DNA pool of vegetative phage (ribosome-free spaces) was not labeled. The statistical evaluation is given in Table 2.

gold particles per μm^2 over the ribosomal area. Thus, it seems that fragmentation of the host DNA results in a large increase of its HU-binding capacity. This result also demonstrates that most of the HU is not bound to 70S ribosomes. At this stage of infection only a few of the ribosomes were already involved in translation.

Later on in infection, HU protein is again localized in the transcriptionally and translationally active region, i.e., associated with the ribosomes contiguous to the pool of DNA. More than 98% of the gold particles were associated with ribosomal areas (Fig. 5 and Table 2). Again, the HU label was not located over the pool of phage DNA.

T4 phages containing a mutation in the phage-encoded DNA polymerase (the product of gene 43) no longer synthesize viral DNA, although degradation of the host DNA proceeds normally. By infecting *E. coli* cells with such a T4 mutant, it is possible to produce cells that are virtually devoid of DNA. The DNA of the infecting phage(s) corresponds to less than a tenth of a bacterial genome. Since protein HU is still present in these cells, the majority of the protein (if bound to dsDNA in uninfected cells) would no longer be bound to dsDNA in these phage-infected cells. Therefore, if HU epitopes were hidden by binding to dsDNA, then the level of HU label in T4.43$^-$-infected cells should be up to 10-fold higher than in uninfected cells. In order to obtain a specimen which contained both infected and uninfected cells, we infected *E. coli* B with T4.43$^-$ at an MOI of only 0.7. About half of this population stayed uninfected. Such a specimen was frozen 10 min after infection, and thin sections were labeled for HU protein. The number of gold particles was counted only on cross sections of the cells. Such sections are all about equal in size and provide numbers representative of the intracellular concentration of labeled protein HU. By their morphology, infected and uninfected cells are indistinguishable. We therefore plotted the gold particle counts in the form of a histogram (Fig. 6). If there was a difference in the amount of detectable HU in the cells, it would be manifested in two distinct maxima in the number of particles per section, differing by a factor of much more than 2. However, only one peak could be found, demonstrating that no significant number of HU complexes exists of which the epitopes are hidden by DNA. This result definitively excludes the hypothesis that the bulk DNA of the nucleoid contains HU which would not be detectable by immunolabel.

Upon arrest of protein synthesis, the bacterial nucleoid assumes a specific shape which is approximated by a hollow sphere with a core. Cultivation of cells in the absence of a required amino acid (6) or treatment of cells with sublethal doses of aureomycin, chloramphenicol (37), and puromycin (Kellenberger, unpublished results) leads to the acquisition of this particular nucleoid morphology. This is probably due to the uncoupling of DNA transcription and RNA translation

TABLE 2. Gold particle counts of HU-immunolabeled (serum 12286) T4 21$^-$-infected *E. coli* B cells 60 min p.i.

Cell no.	No. of squares (% of total)			
	Gold on bulk DNA	Bulk DNA without gold	Gold on ribosomal area	Ribosomal area without gold
1	6 (0.6)	130 (12.9)	370 (36.6)	504 (49.9)
2	4 (0.5)	111 (14.5)	263 (34.3)	389 (50.7)
3	2 (0.3)	71 (12.3)	221 (38.4)	282 (48.9)
4	1 (0.2)	60 (13.9)	156 (36.0)	216 (49.8)
5	2 (0.3)	77 (12.2)	210 (33.5)	338 (53.9)
6	4 (0.6)	93 (13.4)	253 (36.5)	343 (49.5)
7	5 (0.4)	121 (10.3)	420 (35.7)	630 (53.5)
8	3 (0.3)	175 (18.6)	370 (39.4)	391 (41.6)
9	5 (1.0)	63 (13.0)	173 (35.7)	243 (50.2)
10	2 (0.3)	93 (17.2)	186 (34.5)	257 (47.8)
Avg % ± SD	0.5 ± 0.02	13.83 ± 2.4	36.06 ± 1.8	49.58 ± 3.4

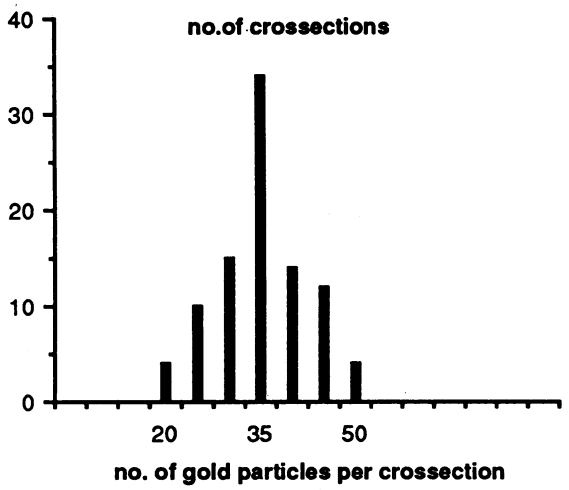

FIG. 6. Frequency of gold particles per cross section. Data are for *E. coli* B infected with T4.43 (*B*am22) at an MOI of 0.7 (50% surviving cells) and labeled against the protein HU. If antigenic epitopes were hidden by binding to dsDNA, two distinguishable maxima should be found. In our experiments, no significant (2 to 10 times higher) second maximum could be detected.

on the ribosomes. It is of some importance to note here that by chloramphenicol treatment the cells are not rendered leaky for K^+ and Mg^{2+} (Moncany, thesis), in contrast to stationary-phase cells, which leak the intracellular cations (15). When thin sections of chloramphenicol-treated *E. coli* cells were immunolabeled for HU protein, it was found mostly within the cytoplasm (Fig. 7) and rarely in the center of the spherical nucleoid. The amount of label counted over the bulk DNA was insignificantly higher than that observed in exponentially growing cells. The distribution of HU in these cells was not markedly different from that in untreated cells, despite the altered nucleoid morphology.

The results presented above for the labeling of protein HU strongly favored its association with transcriptional-translational activities. A crude estimation of the number of transcriptional "forks" (some 2,000 per actively growing cell [50]) was within the theoretically calculated limits of detection by the immunolabeling methods used (36). The number of replication forks was less than 10 per cell and thus would not be observable by our methods. We therefore continued to investigate proteins presumed to be involved in transcription.

Figure 8 shows micrographs of cells immunolabeled with IgGs specific for RNA polymerase and DNA topoisomerase I, respectively. Table 3 gives the statistical evaluations. Here again we observed the label over the ribosome regions contiguous to the nucleoid.

From the data in Table 3 we conclude that all three proteins, HU, RNA polymerase, and DNA topoisomerase I, are predominantly found in regions where transcriptional and translational activity is supposed to occur.

It is not unexpected that some label was always found over ribosomal areas which are relatively distant from the bulk DNA. We have to take into account that thin sections are about 40 to 60 nm thick. Of this thickness, some 20 to 30 nm could consist of a lobe or "cleft" of the nucleoid penetrating into an extended ribosomal area. It is thus easy to imagine that part of such a lobe might be together with

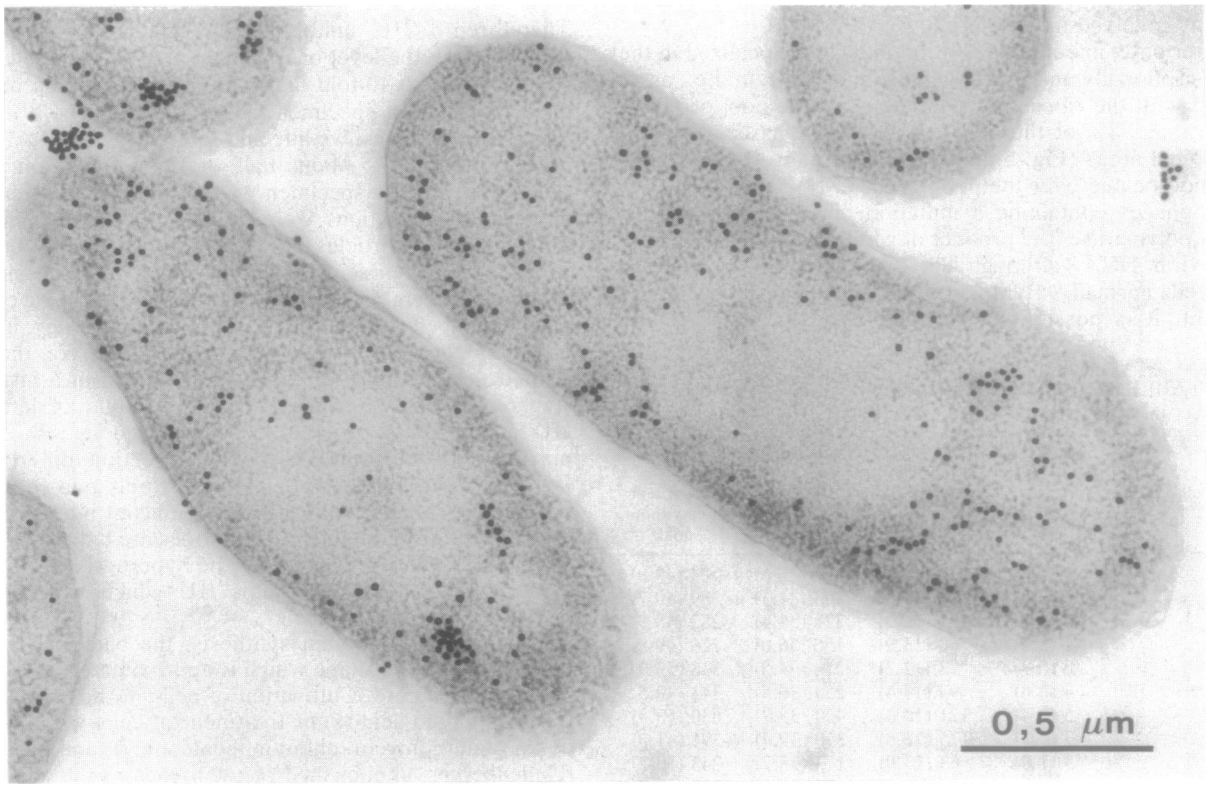

FIG. 7. Intracellular localization of the HU protein in *E. coli* B cells treated for 60 min with chloramphenicol (25 μg/ml). Label with anti-HU was found over the ribosomal areas and possibly some in the center of the ring-shaped section of the nucleoid.

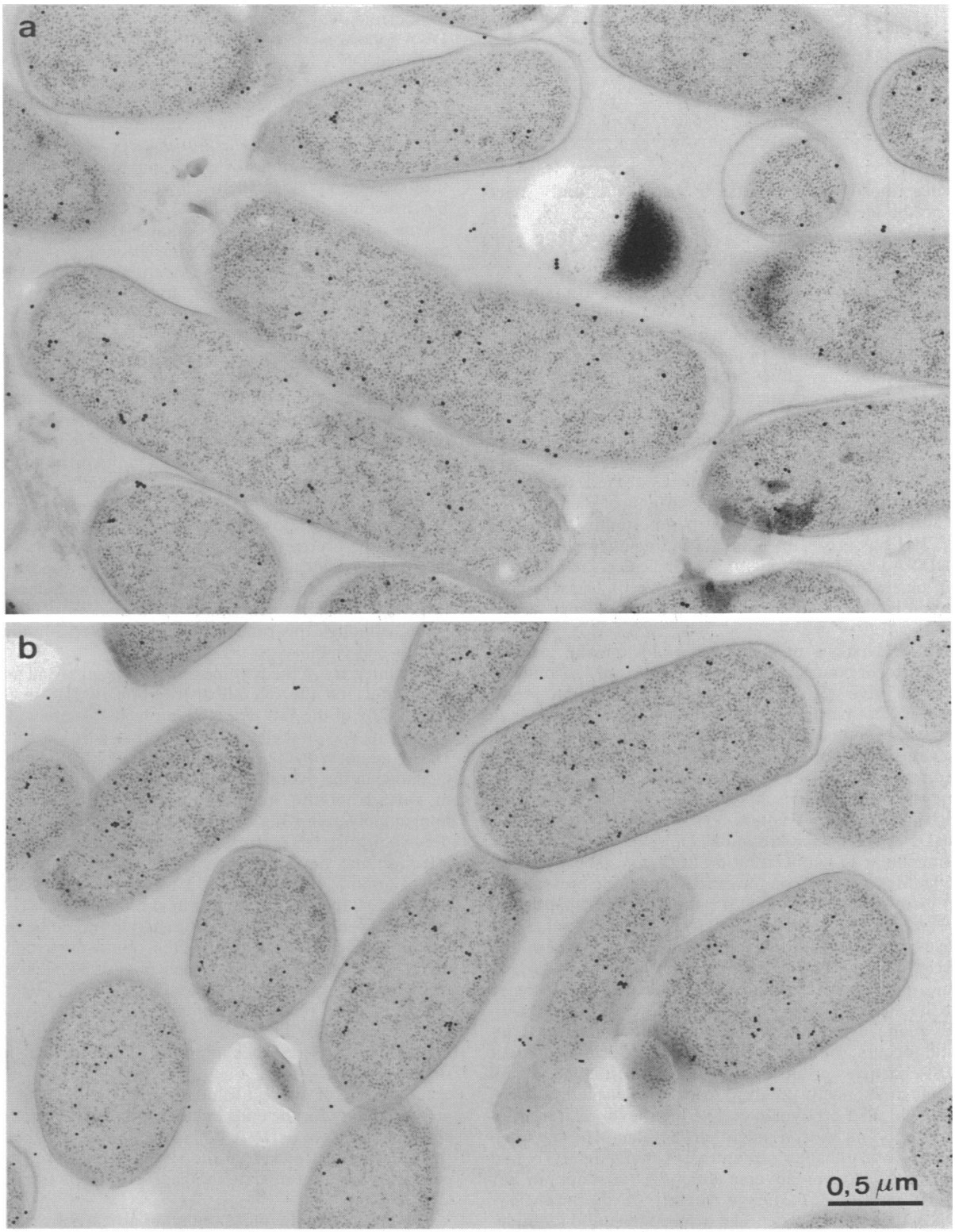

FIG. 8. Immunolabel against *E. coli* RNA polymerase (a) and *E. coli* DNA topoisomerase I (b) on sections of *E. coli* cells prepared as described in the legend to Fig. 1a. The statistical evaluation is given in Table 3.

TABLE 3. Localization of the immunolabel of HU, RNA polymerase, and topoisomerase

Serum	No. of counts averaged over 100 cells			
	Background/ μm^2	Over bulk DNA	Over border zone	Over cytoplasm
Anti-HU	1.4	0.5	23.6	10.7
Anti-RNA polymerase	<0.1	0.6	18.7	9.1
Antitopoisomerase	<0.1	0.05	6.8	0.7

overlying ribosomes such that a "ribosome-free" space could not be identified.

DISCUSSION

Addition in vitro of protein HU facilitates a number of cellular processes, including *dnaA*-mediated *oriC* DNA replication in *E. coli* (10) and phage Mu DNA transposition (reviewed in reference 8). In addition, HU has been shown to have a stimulatory effect on DNA transcription in vitro (56) and is found associated with the purified 30S native subunit of *E. coli* ribosomes but not with the 50S subunit or 70S ribosomes (66). In every instance, some single-stranded or cleaved nucleic acid is either generated in the particular process, such as double-stranded breaks in the DNA during Mu transposition, or involved in the structure, such as RNA in the 30S ribosomal subunit. It was reported by some that protein HU has a greater affinity for single-stranded DNA than dsDNA (19). Interestingly, the translation factor 1 of *B. subtilis* phage SPO1 acts as an inhibitor of transcription, although it has nearly the same amino acid sequence as HU (20).

Protein HU is one of a number of histonelike proteins postulated to organize bacterial DNA into nucleosomelike particles in vivo (5, 12, 51). Such a suggestion seems plausible given that up to 50% of the negative supercoils in the bacterial genome are restrained in some structure (63). The demonstration of nucleosomelike particles in vitro with purified simian virus 40 DNA, protein HU, and nick-closing enzyme provide further support (5, 12, 58). However, the binding of HU to DNA is not very strong: HU dissociates from dsDNA columns at around 200 mM NaCl (5, 72), while the intracellular K^+ concentration in *E. coli* is between 350 and 650 mM, depending on the external osmotic pressure (1, 15, 50). Broyles and Pettijohn (5) estimate that the half-life of HU-DNA complexes in 50 mM NaCl is 0.6 min, a value that decreases at higher salt concentrations. They suggest that the HU-containing nucleosomelike particles in vivo are dynamic, perhaps resembling eucaryotic half-nucleosomes (69) or the altered nucleosomes in yeast cells obtained with strongly reduced amounts of histone H2B in which transcription still occurs, but not replication (23). The model of HU-DNA particles proposed by Drlica and Rouvière-Yaniv (12), while dramatically different from the eucaryotic nucleosome, would also be dynamic. The high level of transcriptional activity in *E. coli* might necessitate that bacterial chromatin be a dynamic structure, as more tightly bound proteins might have an adverse effect on transcription. For example, Lorch et al. (43) have recently shown for eucaryotic DNA that the presence of nucleosomes inhibits initiation of transcription in vitro, while elongation of the transcript proceeds with histone displacement.

Our experimental results rule out the possible role of protein HU in a nucleosomic organization of procaryotic DNA. HU was not found distributed throughout the DNA plasma, as would be expected for a procaryotic histone. Instead, more than 94% of the HU was located over the ribosomes, which are situated near the ribosome-free space or nucleoid. Our results also show that RNA polymerase and DNA topoisomerase I are located in this area, as is single-stranded DNA (27). Presumably, transcription and translation occur in this region, possibly on small DNA loops that enter the ribosomal areas. However, the resolution of our experiments does not allow us to determine whether HU is involved in these metabolic processes or whether it is simply bound to the ribosomes or exists in a nucleosomelike structure specifically along the nucleoid boundary, as suggested by Drlica and Rouvière-Yaniv (12).

One intriguing possibility is that HU plays a role in the coupling of transcription and translation in procaryotes. As HU has been determined to be identical to the 30S ribosomal subunit-associated protein NS (40, 46), we suggest that HU might target this subunit to transcriptional complexes on the DNA, either through direct binding of the HU protein to the DNA or through specific interactions with HU proteins associated with the transcriptional complex itself. The 30S subunit would then form the preinitiation complex with the nascent mRNA, initiation factors, and $tRNA^{Met}$. The HU previously bound to the subunit would be released and either bind to other free 30S ribosomal subunits or be recruited to other DNA structures along the nucleoid periphery. The genes encoding both forms of HU in *E. coli* have recently been cloned (12); however, it has not yet been determined whether the proteins are essential for cell viability (see note added in revision). Thus, the function we propose for HU may facilitate initiation of translation in vivo by specifically targeting ribosome subunits to transcriptionally active genes, although this process might not be absolutely essential for cell viability.

The integrity of the chromosomal DNA also had a tremendous effect on the overall distribution of HU in the cell. About 80% of the HU detected in phage T4-infected cells at 7 min p.i. was associated with the partially degraded host DNA and not with the cytoplasm as in uninfected cells. Such a change in the overall distribution of HU might also result, in part, from the inhibition of host DNA transcription immediately following T4 infection. This occurs independently of host DNA degradation (65). After T-even phage infection, no new ribosomes are synthesized. Either the dissociation of 70S ribosomes is transiently inhibited or, if it does occur, the 30S ribosomes might have their HU-binding ability transiently abolished. Both actions could be mediated by a phage-encoded protein. One could even consider a phage-encoded HU analog which might specifically recognize and bind to the hydroxymethylate T-even phage DNA, which, at short times after infection, is not yet available in large amounts. With this assumption, one would have to explain why HU resumes its normal location later in infection.

It could be of interest to determine whether HU exhibits greater affinity for the ends of cleaved dsDNA than for intact single-stranded or dsDNA. This might have some bearing on the requirement for HU in the strand transfer reaction that generates the intermediate in phage MU DNA transposition (7).

The absence of the most abundant procaryotic histonelike protein in the DNA plasma makes the suggested nucleosomelike organization of the *E. coli* genome less likely. A nucleosomic organization of the bulk DNA might involve other proteins, but there are few likely candidates. Other types of compactosomes similar to these induced by ethanol

treatment cannot be excluded (14). It may be that the bulk of bacterial DNA is not neutralized by basic proteins but rather in the form of an Mg-polyamine salt (4, 50). This could explain why bacterial chromatin, in contrast to eucaryotic chromatin, cannot be gelled by many fixatives that react mainly with proteins and precipitates into coarse aggregates during ethanol dehydration (34; Kellenberger, in press). The possibility might be considered that processes such as RNA translation and DNA transcription, recombination, and replication, particularly the DNA-protein interactions associated with them, are of prime importance in the structural organization of the bacterial nucleoid and not the presence of a specific DNA-protein particle.

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ADDENDUM

At a meeting at Calloway Gardens, Pine Mountains, Georgia, on the bacterial chromosome (22–25 May 1988, organized by M. Riley and K. Drlica), the results of this paper were reported. The personal communication by J. Rouvière-Yaniv about the persistence of HU after infection will be published together with new information in a forthcoming paper by J. Rouvière-Yaniv, A. Jacq, U. Hibner, and E. Brody (submitted).

Two groups (Rouvière-Yaniv, France, and Iamamoto, Japan) had independently produced mutants that abolished functions of HU1, HU2, or both. Only the double mutants show a substantially reduced growth rate. Our group has started to study the morphological and immunocytochemical consequences of these mutations.

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