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Chromosomal Structure of the Halophilic Archaebacterium *Halobacterium salinarium*

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The chromosomal structure of the extremely halophilic archaebacterium *Halobacterium salinarium* was examined. Sheared chromosomes prepared from the bacteria in the late exponential phase were separated into two peaks (peaks I and II) by sucrose gradient centrifugation, suggesting that the chromosomes consist of two parts differing in quality. The UV spectra of peaks I and II resembled those of DNA and eukaryotic chromatin, respectively. Electron microscopic observations revealed that the major component of peak I was protein-free DNA, while the major components of peak II were rugged thick fibers with a diameter of 17 to 20 nm. The rugged fibers basically consisted of bacterial nucleosome-like structures composed of DNA and protein, as demonstrated in experiments with proteinase and nuclease digestion. Whole-mount electron microscopic observations of the chromosomes directly spread onto a water surface revealed a configuration in which the above-described regions were localized on a continuous DNA fiber. From these results it is concluded that the *H. salinarium* chromosome is composed of regions of protein-free DNA and DNA associated with nucleosome-like structures. Peaks I and II were predominant in the early exponential phase and stationary phase, respectively; therefore, the transition of the chromosomal structure between non-protein-associated and protein-associated forms seems to be related to the bacterial growth phase.

A series of bacteria designated archaebacteria do not have any apparent nucleus surrounded by a nuclear membrane and so morphologically belong to the prokaryotes (5). However, many of their properties differ from those of most prokaryotes (39). Accordingly, Fox et al. (14) proposed that all cellular organisms be grouped into three primary kingdoms, eukaryotes, eubacteria, and archaebacteria, while Lake (21) proposed that all cellular organisms be classified into five primary groups. Recently, Woese et al. (38) proposed an improved classification in which all organisms are grouped in three domains, *Bacteria*, *Archaea*, and *Eukarya*.

In eukaryotes, or eukarya, chromosomal DNA exists in vivo in a protein-associated form, the nucleoprotein complex being called eukaryotic chromatin (3, 18). Eukaryotic chromatin is primarily composed of nucleosomes with a configuration comparable to that of "beads on a string" (25, 26). The nucleosome-core particle is about 11 nm and consists of 146 bp of DNA and a histone octamer (18, 28). The region of a string-like structure, namely, the linker fibers connecting the particles, is DNA (18, 28). Usually eukaryotic chromatin forms a thick fiber and further high-order structures, depending on physiological conditions (18).

In eubacteria, or bacteria, most regions of chromosomal DNA seem to exist in a protein-free form, although the bacteria do contain histone-like proteins (12, 13). Thus, this difference between protein-associated and non-protein-associated forms seems to be essential for eukaryotes and eubacteria, respectively.

On the other hand, in archaebacteria, or archaea, chromosomal DNA is assumed to exist in a protein-associated form, since histone-like proteins have been isolated from stable nucleoprotein complexes (8–11, 15, 19, 27, 33). Nucleosome-like structures were found associated to some extent with a portion of the chromosomal DNA in the acidothermophilic archaebacterium *Thermoplasma acidophilum* and the halophilic archaebacterium *Halobacterium salinarium* (34, 35). However, DNA binding proteins formed some structures that differed from nucleosomes in vitro in the acidothermophilic archaebacterium *Sulfolobus acidocaldarius* (22). Furthermore, the protein content in the nucleoprotein complexes is lower than that of eukaryotes (37). These findings suggest that the structure of the archaebacterial chromosome may differ from those of the eukaryotic and eubacterial chromosomes.

In the present study, we examined the structure of the whole chromosome of the halophilic archaebacterium *H. salinarium* and found that it consists of regions of both protein-associated DNA and protein-free DNA and that these regions are changeable depending on the growth phase. The former region was composed primarily of nucleosome-like structures and formed fibrous high-order structures.

MATERIALS AND METHODS

Materials. [^{3}H]thymidine (72 mCi/mmol) and [^{32}P]dCTP (3,000 Ci/mmol) were purchased from Amersham (Amersham, England) and New England Nuclear (Boston, Mass.), respectively. Proteinase K, micrococcal nuclease, and bovine pancreatic RNase were obtained from Boehringer Mannheim (Mannheim, Germany). Bovine pancreatic DNase I was obtained from Worthington (Freehold, N.J.). Restriction endonucleases *Hin*dIII and *Mlu*I were obtained from Nippon Gene (Toyama, Japan). A membrane filter (Molcut UFPITHK24) through which molecules smaller

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than 100 kDa can pass was obtained from Nihon Millipore Kogyo (Yonezawa, Japan) and used to exchange solvents and concentrate DNA or chromatin.

Bacterial strain and culture. An extremely halophilic archaebacterium, *H. salinarium* IAM130663, was obtained from the Institute of Applied Microbiology, University of Tokyo, and cultured as described previously (24). Bacterial cells in the late exponential phase were harvested and used for experiments. For labeling of chromosomal DNA, cells in the exponential phase were transferred to 10 ml of fresh medium containing 10 μCi of [^{3}H]thymidine and incubated for 12 h.

Sucrose gradient centrifugation. Bacterial cells labeled with [^{3}H]thymidine were harvested and washed three times with fresh medium by centrifugation at 2,500 × *g* for 10 min. The cells were suspended gently in 0.5 ml of buffer solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl and were allowed to lyse. The resulting viscous lysate was gently applied, with or without shearing, to 4.5 ml of a 5 to 20% sucrose gradient containing the above-described buffer solution. For shearing, the lysate was pipetted with a pipette tip until the lysate showed a reduced viscosity. Centrifugation was carried out at 105,000 × *g* for 1 h. After fractionation (0.2 ml each) and measurement of the A_{280}, 1.0 ml of 5% trichloroacetic acid was added to each fraction, and the mixture was passed through glass fiber filter paper (GF/C; Whatman, Maidstone, England) with 5% trichloroacetic acid and then ethanol. After the filter was dried, the radioactivity on the paper was measured in a liquid scintillation counter.

Electron microscopic observations. Sample solutions containing DNA or chromatin were concentrated about 10-fold, the solvents of the solutions were simultaneously replaced with 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA by use of a membrane (Molcut UFPITHK24), and the solutions were then spread onto a water surface with a platinum loop. DNA or chromatin floating on the water surface was picked up with carbon-coated grids and, for fixation, the grids were exposed to 5% neutralized formaldehyde for 10 min. After the grids were washed with distilled water, the preparations were stained with 1.0% uranyl acetate–acetone (1:3), air dried, and rotary shadowed with Pt-Pd (4:1) at an angle of $\tan^{-1}$ (1/10). For observation of negative staining, the process of rotary shadowing was omitted. For direct visualization of a whole chromosome, bacterial cells pelleted by centrifugation at 2,500 × *g* for 10 min were directly exposed to the water surface with a platinum loop. This procedure allows for bacterial lysis and spreading of the chromosome on the water surface. Floating chromosomes were prepared for electron microscopic observations as described above. Electron microscopic observations were carried out at 75 kV with a Hitachi H-600 electron microscope.

Enzyme treatments. Peak fractions from the sucrose gradient centrifugation were concentrated to about 10-fold, and the solvents of the fractions were replaced with suitable ones for enzyme treatments by use of a membrane (Molcut UFPITHK24). Proteinase K was added to the fractions at a final concentration of 0.005 U/0.35 ml in 20 mM Tris-HCl (pH 7.5)–1.0 mM EDTA at 30°C for an appropriate time. DNase I treatment (0.01 U/ml) was carried out with 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$ at 37°C. After incubation, the reaction was stopped by the addition of EDTA at a final concentration of 20 mM. After 60 min of exposure to proteinase K (as described above) and washing out of the proteinase K by filtration, the resulting products were further treated with restriction endonuclease *Hin*dIII at a final concentration of 200 U/ml in 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–100 mM NaCl at 37°C for 60 min. Micrococcal nuclease was added to the fractions at a final concentration of 50 U/ml in 20 mM Tris-HCl (pH 7.5)–2 mM $CaCl_2$–5 mM 2-mercaptoethanol at 37°C. After an appropriate time, the reaction was stopped by the addition of EDTA at a final concentration of 10 mM. Pancreatic RNase was added to the fractions at a final concentration of 0.01 μg/ml in 50 mM Tris-HCl (pH 7.5)–100 mM NaCl at 37°C for 60 min. As a control, mock treatment was carried out in each case with heat-treated (at 100°C for 10 min) enzyme. For RNase treatment, mock "digestion" was done by adding only a buffer solution and no enzyme. After each treatment, the preparations were observed by electron microscopy.

Agarose gel electrophoresis and Southern hybridization. Two hundred nanograms of DNA in peak I, peak II, or unfractionated chromosomes was digested with 4 U of *Mlu*I at 37°C for 4 h and subjected to 0.7% agarose gel electrophoresis at 25 V/14 cm for 15 h in Tris-borate buffer as described previously (23). After visualization of DNA bands with ethidium bromide staining (23), DNA was transferred to a filter membrane (Hybond-N; Amersham) and subjected to Southern hybridization at 65°C with a ^{32}P-labeled 16S rRNA gene or cDNA of rRNA as described previously (23).

Probes for Southern hybridization. Oligonucleotides corresponding to a 29-mer of the 5′ end of the 16S rRNA gene and to a 29-mer of the 3′ end of the anti-16S rRNA gene in *H. salinarium* (17) were prepared by use of a DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.). With these oligonucleotides as primers, the ^{32}P-labeled full-sized 16S rRNA gene was synthesized by the polymerase chain reaction (32) and used as a probe for hybridization. rRNA including 5S, 16S, and 26S rRNAs was isolated from the bacteria by use of an anion-exchange column tip (Qiagen-tip 5′; Diagen, Düsseldorf, Germany) in accordance with the manufacturer's instructions. With this rRNA as a template, ^{32}P-labeled cDNA of rRNA was synthesized by use of a cDNA synthesis kit with random primers (Amersham) in accordance with the manufacturer's instructions and used as a probe for hybridization.

Other methods. DNA was isolated from *H. salinarium* as described previously (23). Eukaryotic chromatin was prepared from rat liver as described previously (2). Protein and DNA contents were measured with bovine serum albumin and calf thymus DNA as standards by the methods of Bradford (4) and Kissane and Robins (20), respectively.

RESULTS

Sucrose gradient centrifugation of the chromosomes. The distribution of chromosomal DNA in sucrose gradient centrifugation was determined by measuring the radioactivity in the acid-insoluble materials of each fraction (Fig. 1). Unsheared chromosomal DNA sedimented rapidly (Fig. 1A), while sheared chromosomal DNA sedimented slowly and separated into two major peaks; of these peaks, the slow- and fast-sedimenting ones were designated peak I and peak II, respectively (Fig. 1B). Two peaks were also obtained by recentrifugation of the chromosomal DNA that sedimented to the bottom fractions in Fig. 1A after shearing by pipetting (data not shown). These results suggest that the bacterial chromosomes consisted of two major parts differing in quality.

Characterization of peaks I and II. (i) UV spectra of peaks I and II. Peak I in Fig. 1B contained considerable amounts of

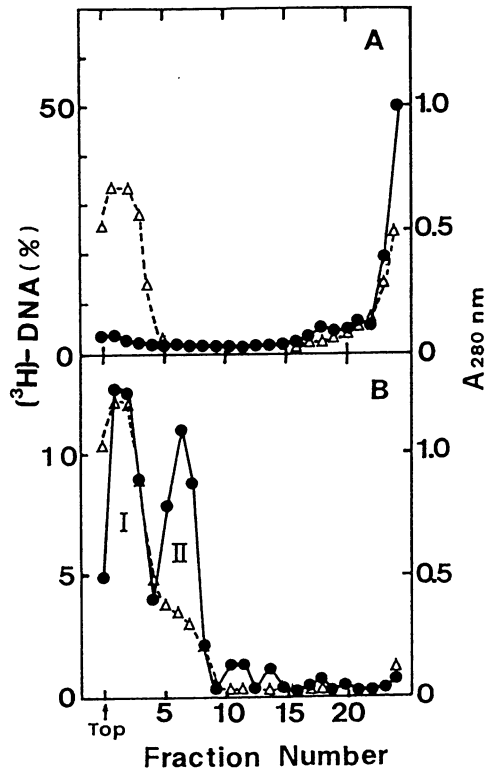


FIG. 1. Sucrose gradient centrifugation of bacterial chromosomes. A cell lysate containing ^{3}H-labeled chromosomal DNA was centrifuged in a sucrose gradient without shearing (A) or with shearing (B). The radioactivity in each fraction was expressed as a percentage of the total radioactivity (6,200 cpm and 5,900 cpm in A and B, respectively) applied. I and II, peaks I and II, respectively. Symbols: △, absorbance at 280 nm; ●, radioactivity.

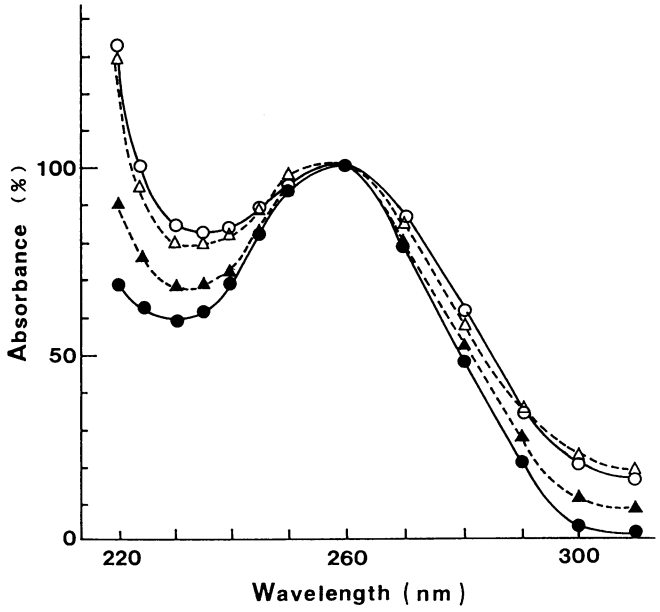
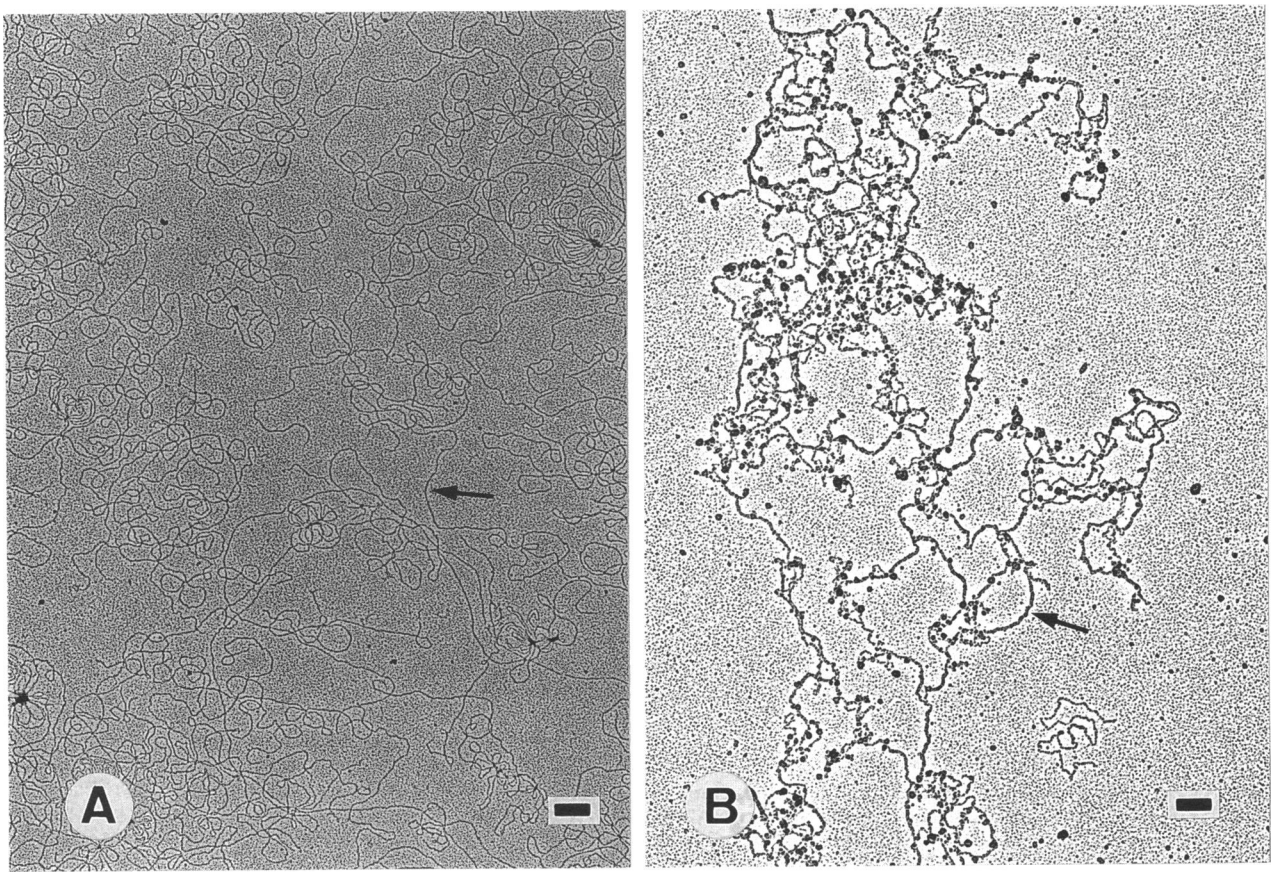


FIG. 2. UV spectra of peaks I and II. UV spectra of peak I (▲), peak II (△), isolated DNA (●), and rat chromatin (○) were measured in 20 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.2 M NaCl. Peak I was obtained as follows. The rapidly sedimented fractions of the radioactivity in Fig. 1A (fraction numbers 20 to 24) were suspended in 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.2 M NaCl and recentrifuged in a sucrose gradient after shearing as described in the text. The ^{3}H-labeled chromosomal DNA was separated by centrifugation into two peaks, and the profile was essentially the same as that obtained in Fig. 1B. Fraction number 7 in Fig. 1B was used as peak II. Isolated DNA and rat chromatin were prepared as described in the text. The absorbance at each wavelength was expressed as a percentage of that at 260 nm (0.4 in each case).

nonchromosomal subcellular components, as judged from the A_{280} in Fig. 1A and 1B. To obtain peak I free from nonchromosomal components, we recentrifuged the chromosomal fraction (bottom in Fig. 1A) after shearing. The resulting peak I and peak II (fraction number 7 in Fig. 1B) were subjected to measurements of UV spectra or protein content. Figure 2 shows the UV spectra of peak I, peak II, and controls. DNA isolated from the bacterium and chromatin isolated from rat liver showed typical UV spectra. The ratios of A_{230} to A_{260} were 0.58, 0.68, 0.70, and 0.86 in isolated DNA, peak I, peak II, and rat chromatin, respectively. The protein contents (milligrams) per milligram of DNA, measured by biochemical methods, were 0.17 ± 0.05 (n = 5) for peak I and 0.41 ± 0.04 (n = 5) for peak II. Thus, the major components in peak I and in peak II seem to be DNA with little contamination of protein and DNA together with protein, respectively.

(ii) Electron microscopic observations of peaks I and II. Electron microscopic observations revealed that the major components of peak I showed characteristic configurations of protein-free DNA with a diameter of 3 to 4 nm when shadowed (Fig. 3A). On the other hand, the major components of peak II were aggregates of fibrous structures, the diameter of which (approximately 25 to 30 nm when shadowed) was much larger than that of protein-free DNA (Fig. 3B). The thick fibers of these fibrous structures were rugged (Fig. 4A). Partially unwound regions of the rugged fibers represented DNA fibers associated with globular particles with a diameter of 12.2 ± 1.7 nm (n = 137) when shadowed (Fig. 4B). Figure 4C shows a similar region visualized by negative staining. Shown is a fine fiber of DNA with uniformly sized particles having a diameter of 8.1 ± 0.6 nm (n = 168) (without shadowing) and frequently associated in tandem. The distribution of the particles along the DNA fiber showed variable intervals (Fig. 4C). Thus, the rugged thick fibers in peak II seemed to be composed of DNA fibers and globular particles. At the region in which the particles were closely associated, there seemed to be a condensed thick fibrous structure, while in the region in which the particles were loosely associated, there was a beads-on-a-string-like structure, such as that seen with eukaryotic chromatin (25).

(iii) Analyses of peaks I and II by enzyme treatments. To characterize the material in peak I and peak II in more detail, we used several enzymes. RNase treatment of peak I and peak II led to no significant changes in the fibrous structures; hence, RNA is probably not a major component (data not shown). When peak I was treated with proteinase K, a fine fiber with a diameter of 3 to 4 nm (shadowed) remained undigested, whereas treatment with DNase I led to the disappearance of the fine fibers; we concluded that the fine fibers were DNA (data not shown). Proteinase K treatment of peak II led to an increased appearance of fine fibers with a diameter of 3 to 4 nm (shadowed) without particles,



concomitantly with the disappearance of the rugged fibers (Fig. 5A and B). Further treatment with DNase I or restriction endonuclease *Hin*dIII of proteinase K-treated peak II led to the disappearance of the fine fibers (data not shown) or to the appearance of fragmented fine fibers (Fig. 5C); therefore the fine fibers were indeed DNA. The rugged thick fibers in peak II seemed to be a complex of fine DNA fibers and proteins.

To search for a possible analogy between the rugged fibers and eukaryotic chromatin, we added DNase I or micrococcal nuclease, which generates nucleosome-core particles from eukaryotic chromatin (1, 36), to peak II. Limited digestion of peak II by DNase I yielded fragmented thick fibers containing dense particles and particles free from connecting DNA fibers (Fig. 5D). Micrococcal nuclease treatment generated free particles with a diameter of 12.0 ± 1.0 nm (n = 100) (shadowed), aggregates of the particles, and no fibrous structures (Fig. 5E). The generated particles seemed to be the same as those associated with the DNA fibers in Fig. 3, as judged from their diameter. Thus, the rugged fibers in peak II seemed to consist of DNA fibers and nucleosome-core-like particles, which were composed of DNA and proteins.

Estimation of the extent of the chromosomal region in which the nucleosome-like particles were found. The results of micrococcal nuclease digestion suggested that the nuclease can degrade the protein-free DNA region but not the region in which the DNA is associated with the nucleosome-like particles. Thus, one can estimate the proportions of regions of particle-associated DNA in the chromosome by measuring the amount of DNA resistant to micrococcal nuclease. Figure 6 shows the quantitative results of micrococcal nuclease digestion of peak I, peak II, and unfractionated whole chromosomes. Digestion by the nuclease left 20, 50, and 32% of the DNA undigested in peak I, peak II, and the whole chromosomes, respectively, while almost all of the isolated DNA was digested (Fig. 6). Levels of self-degradation during incubation with the mock nuclease treatment were about 11, 1, and 15% in peak I, peak II, and the whole chromosomes, respectively. Thus, the nuclease-resistant regions of the chromosomal DNA were estimated to be approximately 22, 51, and 38% of the total DNA in peak I, peak II, and the whole chromosomes, respectively.

Electron microscopic observations of whole chromosomes. For direct observation of whole chromosomes, the bacteria were lysed by contact with a water surface and a bacterial chromosome was spread out on the water surface; this spread-out chromosome was observed electron microscopically (Fig. 7). Figure 7A shows a typical chromosome that emerged from the bacterial envelope. It is obvious that it consisted of one continuous fibrous structure, although the three-dimensional structure was unclear (Fig. 7A). More than 95% of a continuous fiber is shown in Fig. 7A. The chromosome was composed of at least three regions distinguishable by their thicknesses and their association with particles: (i) a region of protein-free DNA with a diameter of

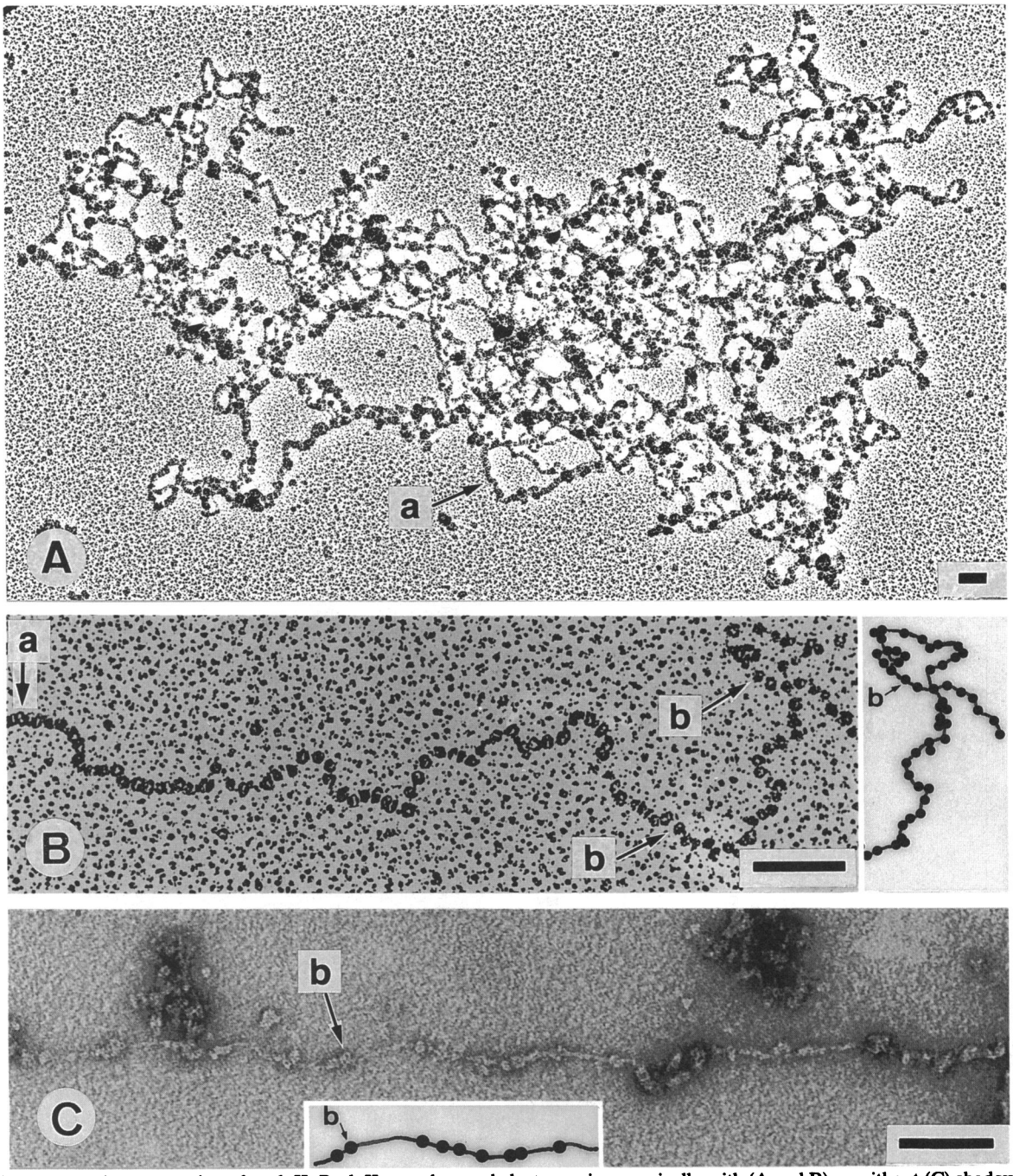


FIG. 4. Large-scale presentation of peak II. Peak II was observed electron microscopically with (A and B) or without (C) shadowing. (A) Aggregate of rugged thick fibers. (B and C) Nucleosome-like particle-associated regions of the chromosome. The insets in panels B and C are illustrations of a nucleosome-like particle-associated DNA fiber. a, rugged thick fiber; b, nucleosome-like particle. Bars, 100 nm.

3 to 4 nm (shadowed), (ii) a region of DNA fibers with particles at intervals and with a diameter of 12.0 ± 1.2 nm (n = 150) (shadowed), and (iii) a region of thick fibers with a diameter of 25 to 30 nm (shadowed). Figures 7B and C show that these regions were continuous. Figure 7D shows a region in which the particles stood tandemly along a DNA fiber, the configuration being similar to that seen in eukaryotic nucleosomes (25). The particles associated with the DNA fibers and the thick fibers were of the same size and morphology as those seen in the fractionated chromosomal DNA in Fig. 4B and 3B.

Changes in the chromosomal structure dependent on the growth phase. Exponential growth of the bacteria continued for 36 h, and the density of the bacteria, as determined by A_{520} measurements, increased to 0.8 from 0.1 at the start of the culture; the growth rate then decreased rapidly. At 48 h after the start of the culture, the density reached 1.0 and then remained constant.

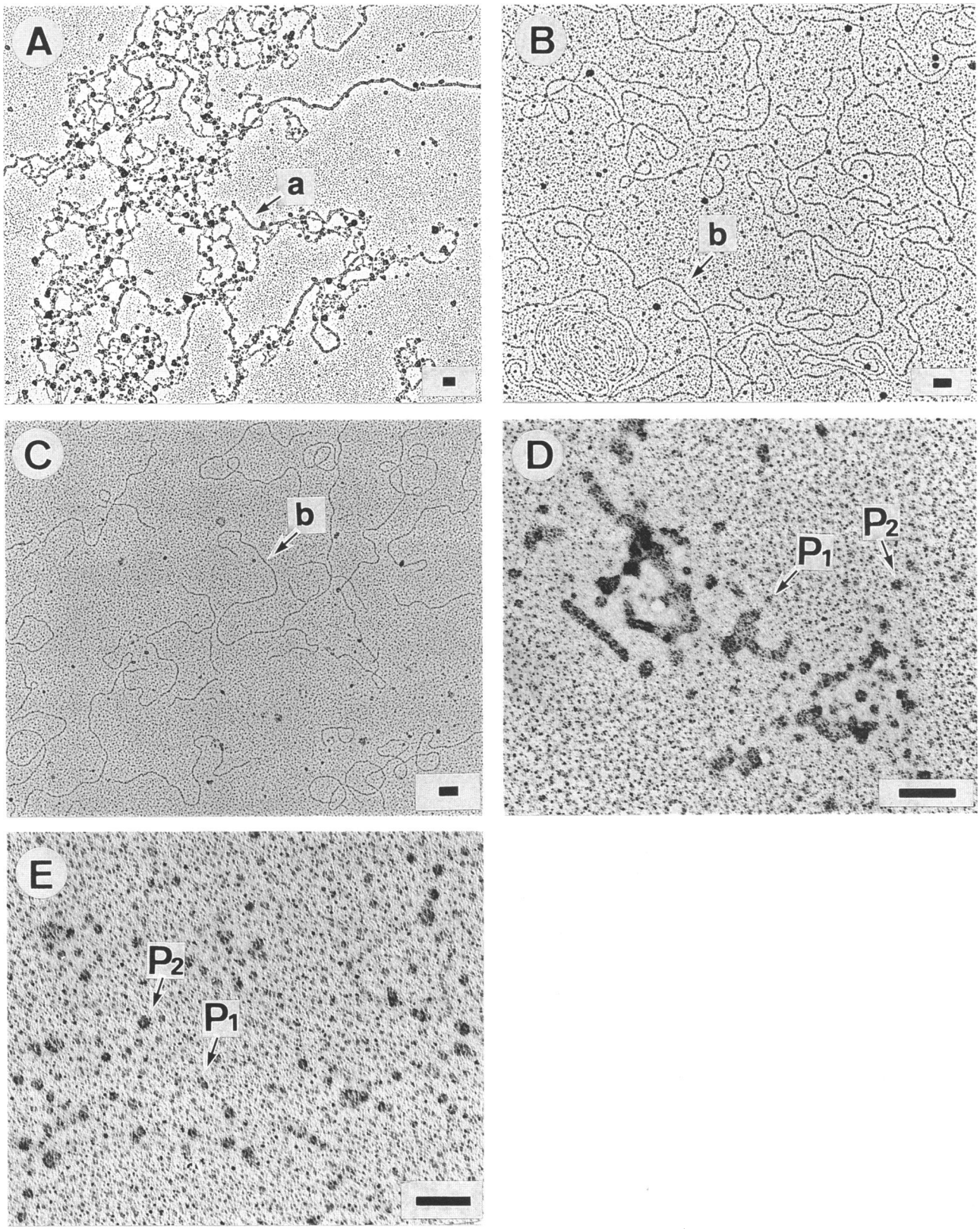


FIG. 5. Enzyme treatments of peak II. Peak II was treated with digestive enzymes and observed electron microscopically with shadowing. The enzyme treatments were as follows: A, control (proteinase K mock treated for 60 min); B, proteinase K treated for 60 min; C, *Hin*dIII treated for 60 min after proteinase K treatment; D, DNase I treated for 5 min; E, micrococcal nuclease treated for 60 min. a, b, p_1, and p_2 represent a typical rugged thick fiber, a protein-free DNA fiber, a nucleosome-like particle, and an aggregate of nucleosome-like particles, respectively. Bars, 100 nm.

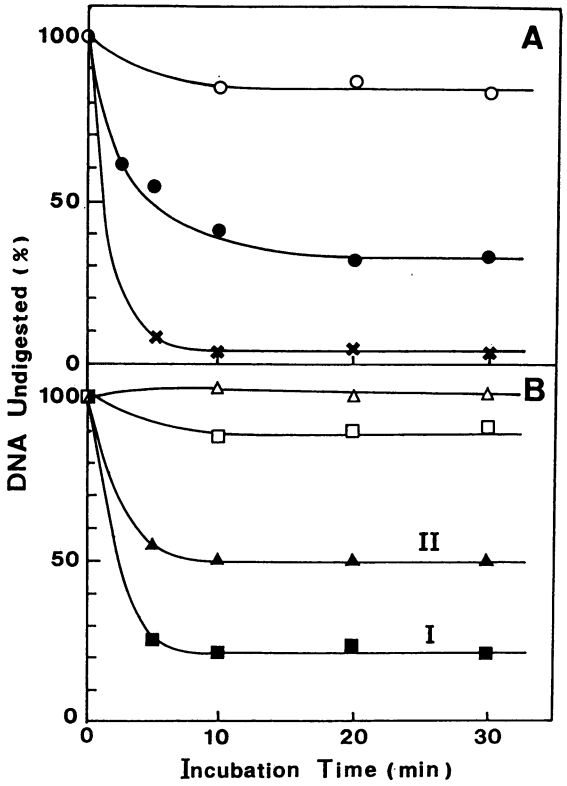


FIG. 6. Micrococcal nuclease treatment of peak I, peak II, and whole chromosomes. Peaks I and II and isolated DNA were prepared as described in the text. The cell lysate was pipetted and centrifuged at 1,000 × *g* for 5 min. The resulting supernatant was designated the whole chromosomes. These preparations, containing 1 μg of DNA each, were subjected to micrococcal nuclease digestion as described in the text. After incubation for the indicated times, each preparation was treated with ice-cold perchloric acid at a final concentration of 5% and centrifuged at 10,000 × *g* for 5 min. The DNA content in the precipitate (DNA undigested) was determined as described in the text. Symbols: ●, ■, ▲, and ×, nuclease-treated whole chromosomes, peak I, peak II, and isolated DNA, respectively; ○, □, and △, mock nuclease-treated whole chromosomes, peak I, and peak II, respectively.

The DNA contents in peak I and peak II in the bacteria in various growth phases were examined by sucrose gradient centrifugation (Fig. 8). About 77 and 23% of the DNA sedimented as peak I and peak II, respectively, in the early exponential phase (at 12 h after the start of the culture; A_{520}, 0.2) (Fig. 8A). About 40 and 60% of the DNA represented peak I and peak II, respectively, in the late exponential phase (at 36 h after the start of the culture; A_{520}, 0.8) (Fig. 8B). On the other hand, in the stationary phase (7 days after the start of the culture; A_{520}, 1.0), more than 90% of the DNA was included in peak II (Fig. 8C). Thus, it is evident that the largest amount of DNA is in the peak I form in the exponential phase and in the peak II form in the stationary phase and that the transition between these forms occurs in the late exponential phase.

Figure 9 (lanes 1 to 5) shows restriction patterns (*Mlu*I digestion) of DNA in peak I and peak II. Although it is difficult to identify each band, it is apparent that the patterns of the major DNA bands obtained for both peaks I and II in the early exponential phase and the stationary phase are quite similar to that obtained for the unfractionated whole chromosomal DNA. This result suggests that qualitatively, the major portion of chromosomal DNA can be present in peak I or peak II, depending on the growth phase.

To determine whether the results relate to the origin of the bacterial chromosome, we examined a particular site of the chromosome, since it is known that halophiles contain a variety of plasmids or bacteriophages (16, 29). Genomic DNA of *H. salinarium* has been shown to contain a single copy of an operon of an rRNA gene, including an rRNA gene cluster (17), and this gene cluster is expected to be located on the 6.3-kb DNA fragment digested with *Mlu*I (7). We therefore examined the location of the rRNA gene cluster by Southern hybridization with a ^{32}P-labeled 16S rRNA gene as a probe. Figure 9 (lanes 6 to 10) shows that a major band of 6.3 kb was present in peak I and peak II in the early exponential phase and the stationary phase, respectively. The same result was obtained when ^{32}P-labeled cDNA of rRNA was used as a probe (data not shown). Therefore, the site of the rRNA gene on the bacterial genome can be in peak I or peak II, depending on the growth phase.

DISCUSSION

On the basis of our observations of chromosomal structures in fractionated and directly spread chromosomes, we conclude that the chromosome of *H. salinarium* in the late exponential phase is composed of two regions: a protein-free DNA region and a protein-associated DNA region. The latter region seems to be formed basically of nucleosome-like particles for which we reported that the structure is native and not an artifact (35). The particles seem to be similar to eukaryotic nucleosome-core particles (18, 28), since they consist of protein and DNA and are liberated by micrococcal nuclease digestion (Fig. 5 and 6). These results are comparable to those obtained for eukaryotic nucleosomes (1, 36), although the size of the presently identified particles (8.1 nm) is smaller than that of the eukaryotic nucleosome-core particles (11 nm). Since the nucleosome-like particles were shown to cover considerable areas of the chromosomal DNA (Fig. 6), it is apparent that these particles are basic structures of the bacterial chromosome and may be necessary to maintain its structure. Similar globular particles of nucleoprotein have been found in the acidothermophilic archaebacterium *T. acidophilum*, and it was proposed that the particles contained 40 bp of DNA coiled around four molecules of protein (34). The presently identified particles seem to be similar to those found in *T. acidophilum*, since both are generated from chromosomes by micrococcal nuclease digestion. On the other hand, Lurz et al. (22) found that DNA binding proteins isolated from the acidothermophilic archaebacterium *S. acidocaldarius* formed specific structures with DNA in vitro. However, we found no similar morphology between these structures and the presently identified particles.

The major components of the rugged thick fibers with a diameter of 25 to 30 nm (shadowed) (Fig. 3 and 4) seem to be nucleosome-like particles (Fig. 4). The actual diameter of the rugged fibers is assumed to be about 17 to 20 nm, the value estimated by dividing 25 to 30 by 1.5, since the diameter of the nucleosome-like particles with shadowing (12.2 nm) is 1.5-fold that without shadowing (8.1 nm). It is known that eukaryotic nucleosomes form a high-order fibrous structure with a diameter of about 28 nm (18); therefore, the presently identified rugged thick fibers may be high-order forms of bacterial nucleosome-like structures.

Sucrose gradient centrifugation and agarose gel analysis

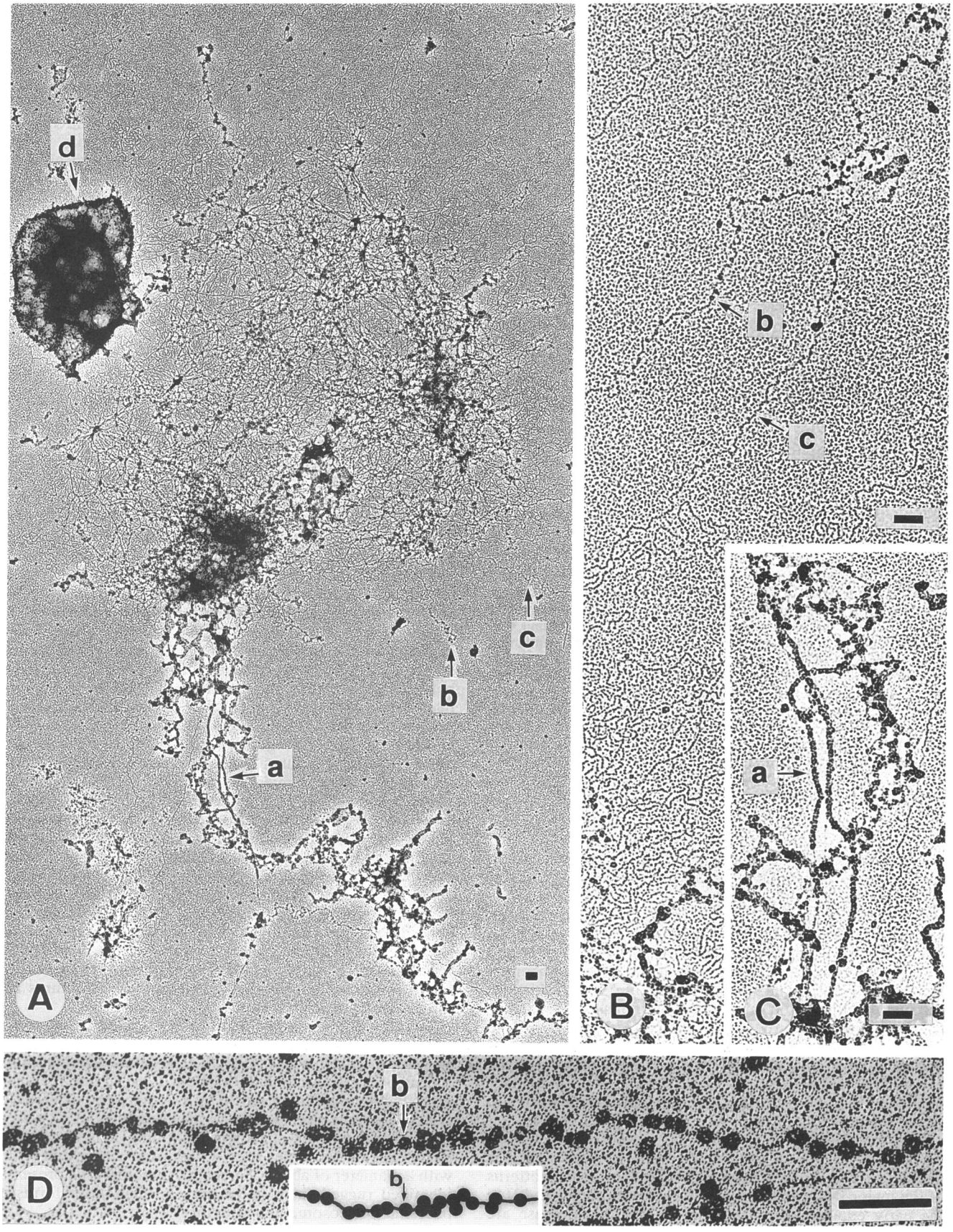

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FIG. 7. Direct visualization of a whole chromosome. Bacterial chromosomes were directly spread over a water surface and observed electron microscopically with shadowing. (A) Chromosome burst from the cell body. (B, C, and D) Large-scale presentations of a part of the chromosome. The inset in panel D represents an illustration of a nucleosome-like particle-associated DNA fiber. a, rugged thick fiber; b, nucleosome-like particle; c, protein-free DNA; d, ghost of a cell. Bars, 100 nm.

revealed that the major portion of the chromosomal DNA, including a region of the rRNA gene, can be in the non-protein-associated form or in the protein-associated form, depending on the growth phase (Fig. 8 and 9). This result suggests that the non-protein-associated and protein-associated forms of chromosomal DNA observed as peak I and peak II, respectively, are native and are not artifacts produced by cell lysis and that the transition between these forms occurs reciprocally in relation to the growth phase. On the basis of the results (Fig. 8 and 9), the chromosomal structures of the bacteria in the late exponential phase (Fig. 1 to 7) seem to represent a form of chromosome that is in transit from the non-protein-associated form to the protein-associated form.

Histone-like proteins have been isolated from eubacteria (30). An *Escherichia coli* histone-like protein, HU, binds to DNA and is able to form nucleosome-like particles in vitro (31). Eubacterial chromosomes are frequently assumed also to exist in vivo in a protein-associated form, as is the case with eukaryotic chromosomes. However, no such structure has been found in chromosomes in *E. coli* (12). Broyles and Pettijohn (6) assumed that the HU-DNA complex is dynamic and unstable in vivo. Nevertheless, recent investigations showed that HU is distributed in ribosomal areas that are adjacent to the nucleoid and is not distributed globally throughout all the DNA plasm; the protein probably participates in gene expression (13). Eubacterial chromosomes seem to be essentially nucleosome free and contain no long region of protein-associated DNA. On the other hand, eukaryotic chromosomes characteristically are primarily composed of nucleosomes and contain no long region of protein-free DNA (18). Thus, the non-protein-associated and protein-associated forms of chromosomal DNA seem to be essential for eubacteria and eukaryotes, respectively. It seems unusual that the presently studied bacterial chromosome can occur in both forms; however, this characteristic of the chromosome may be essential for archaebacterial chromosomes, considering that an ancestor of archaebacteria may be phylogenically between those of eubacteria and eukaryotes (38), and the transition of the chromosome from the non-protein-associated form to the protein-associated form in archaebacteria may reflect processes that occurred when the eukaryotic type (protein-associated type) of chromosome came into being. Further studies of archaebacterial chromosomes are needed to elucidate the evolution of chromosomal structures.

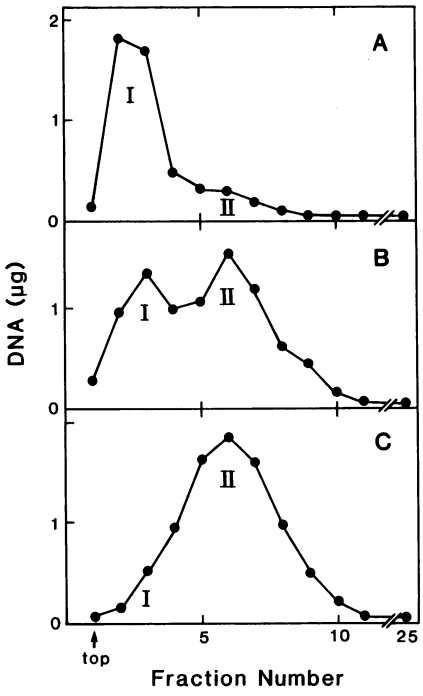

FIG. 8. Changes in the chromosomal structure in relation to the growth phase as analyzed by sucrose gradient centrifugation. Cell lysates prepared from the bacteria in various growth phases were centrifuged in a sucrose gradient after shearing, and the DNA content in each fraction was determined as described in the text. The growth phases and cultures of the bacteria used were as follows: A, early exponential phase (10 ml of culture; A_{520}, 0.2); B, late exponential phase (5 ml of culture; A_{520}, 0.8); C, stationary phase (5 ml of culture; A_{520}, 1.0).

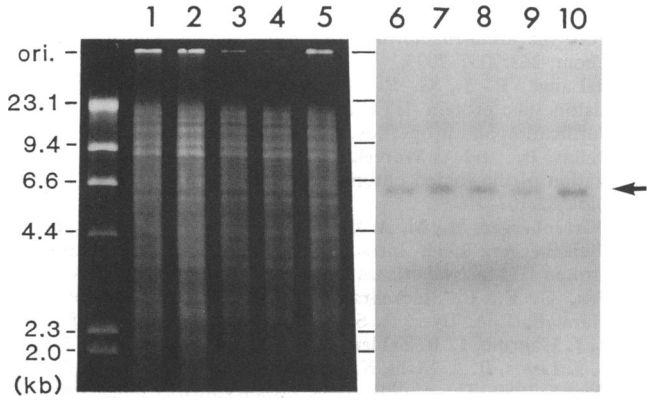

FIG. 9. Restriction patterns of DNA and location of the rRNA gene in peak I and peak II. The DNA in peak I and peak II in Fig. 8 was isolated and subjected to agarose gel electrophoresis after digestion with *Mlu*I and then subjected to Southern hybridization with a ^{32}P-labeled synthetic 16S rRNA gene as a probe. Lanes: 1 to 5, restriction patterns of DNA visualized by ethidium bromide staining; 6 to 10, autoradiography of Southern hybridization. The growth phases of the bacteria and the DNA peaks used were as follows: lanes 1 and 6, stationary phase and unfractionated whole DNA; lanes 2 and 7, early exponential phase and peak I; lanes 3 and 8, early exponential phase and peak II; lanes 4 and 9, stationary phase and peak I; lanes 5 and 10, stationary phase and peak II. The lane to the left of lane 1 represents marker DNAs (λ DNA digested with *Hin*dIII), and the numbers to the left of this lane represent the sizes of the DNA in kilobases. The arrow shows the position of a restriction fragment on which an rRNA gene cluster was located. ori., origin.

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